METHODS IN MOLECULAR MEDICINE™

Cancer Cell Culture

Methods and Protocols

Edited by

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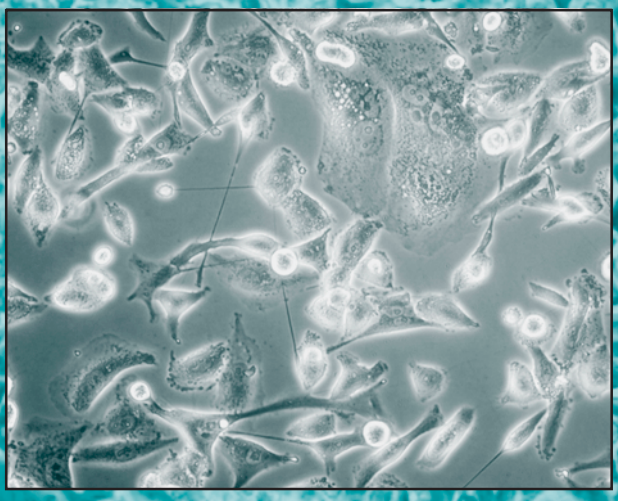

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Basic Principles of Cancer Cell Culture

Simon P. Langdon

1. Introduction

Cell culture is practiced extensively throughout the world today. The techniques required to allow cells to grow and be maintained outside the body have been developed throughout the 20th century. In the 50 years since the publication of the first human cancer cell line, HeLa ***(1)***, thousands of cell lines representing most of the spectrum of human cancer have been derived. These have provided tools to study in depth the biochemistry and molecular biology associated with individual cancer types and have helped enormously in our understanding of normal as well as cancer cell physiology. Although some caution is required in interpreting data obtained by studying cells in vitro, it has allowed investigation of a complex disease such as cancer to be simplified to its component parts. The aim of this chapter is to introduce some of the basic concepts involved in the practice of cell culture.

2. Evolution of Cancer Cell Culture

The science of cell and tissue culture has evolved steadily throughout the last century and its origins can be traced back to 1885 (*see* **Table 1**).

In that year, Wilhelm Roux reported that the medullary plate of a chick embryo could be maintained in saline solution for several days. Many of the early experiments used material derived from amphibians as it was cold blooded and often demonstrated tissue regeneration. In 1887, Arnold demonstrated that frog lymphocytes could migrate and survive in saline. Soon after, in 1898, the first experiment using human tissue was reported when Ljunggren showed that human skin could survive in vitro if placed in ascitic fluid. With the turn of the century, longer culture experiments were attempted and in 1903, Jolly was able to maintain salamander leukocytes in vitro for a month. However, despite these early experiments, it is Ross Harrison who is generally regarded as the "father" of tissue culture. Harrison explanted tissue from frog embryos into frog lymph clots and the fragments of tissues not only survived but nerve fibres grew from the cells ***(2)***. These experiments were fundamental in showing continuation of function in vitro and also in establishing a general technique of tissue culture. This technique

From: *Methods in Molecular Medicine, vol. 88: Cancer Cell Culture: Methods and Protocols*
Edited by: S. P. Langdon © Humana Press Inc., Totowa, NJ

Table 1
Early Milestones in Cancer Cell Culture

Date	Event	Investigator
1885	First tissue (chicken embryo) maintained in vitro (for several days)	Wilhem Roux
1898	First human tissue (skin) maintained in vitro (in ascitic fluid)	Ljunggren
1903	First tissue (salamander leucocytes) to be maintained for 1 mo	Jolly
1907	First functional experiment (frog nerve fibre growth) and first general technique (use of lymph clot)	Ross Harrison
1911	First investigations of factors in medium required for growth and survival	Warren Lewis
1922	First culture of epithelial cells	Albert Ebeling
1943	First continuous rodent cell line	Wilton Earle, George Gey
1951	First continuous human cancer cell line (HeLa)	George Gey
1955	Systematic definition of nutritional needs of animal cells in culture	Harry Eagle
1961	Normal cells (fibroblasts) have a finite lifespan in culture	Hayflick/Moorhead
1965	First defined serum-free medium	Ham
1965–present	Development and use of large numbers of cell lines	Multiple

was developed further by Montrose Burrows, who replaced lymph clot with plasma clot, and by Alexis Carrell who showed that embryo extracts had useful growth promoting activities and could aid growth within culture ***(3,4)***.

In 1911, Warren Lewis began studies to identify factors required for growth in culture, and by 1914 Losee and Ebeling were culturing cancer cells. The first continuous rodent line was generated by Wilton Earle in 1943 at the National Cancer Institute and this investigator is credited with being the first to grow cells on glass and from single cells ***(5)***. In 1951, George Gey developed the first human cancer continuous cell line, HeLa, and this cell line is still used extensively today ***(1,6)***. The 1950s and 1960s were marked by detailed studies by a host of investigators, including Eagle, Fischer, Parker, Healy, Morgan, White, and Waymouth, defining the nutritional requirements of cells in culture leading to the development of the media in current use. In the 1960s, Ham designed a fully defined serum-free medium ***(7,8)***, and in the 1970s, Sato and his colleagues optimized the addition of hormones and growth factors to serum-free media ***(9)***. Since the 1970s there has been the continuous development of thousands of cancer cell lines providing large numbers of models for most forms of cancer.

Table 2
Definition of Cell Culture Terms

Term	Definition
Cell culture	Maintenance of dissociated cells in culture
Tissue culture	Maintenance of tissue explants in culture
Cell line	A culture that is subcultured beyond the initial primary culture phase
Finite cell line	A cell line with a limited lifespan that eventually undergoes senescence
Continuous cell line	A cell line that is essentially immortal and continues indefinitely
Primary culture	The initial culture derived from in vivo material
Clone	The progeny isolated from a single cell
Immortalization	Enabling of cells to extend their life in culture
Lag phase of growth	Initial phase of growth when cells are subcultured
Log phase of growth	Most rapid growth phase when culture shows exponential growth
Plateau phase of growth	Phase when cell become confluent
Population doubling time	Time for cell number to double
Cell banks	Repositories of cancer cell lines and related materials
Substrate	The matrix on which a culture is grown
Passage	Subculture of cells from one container to another
Confluent	Situation wherein cells completely cover the substrate

3. Cell Culture Definitions and General Germs

Cell culture, like many other areas of technology, has developed its own language. Some of the more commonly used definitions are listed in **Table 2**.

The term "cell culture" refers to the culture of disaggregated cells while "organ culture" describes the use of nondispersed tissue, both encompassed by the description "tissue culture". The initial culture taken directly from an individual is referred to as the "primary culture" and when diluted and transferred into further containers (a process referred to as "subculture" or "passage"), it becomes a "cell line." Cell lines may be categorized as either "continuous" lines, which have the potential for indefinite population expansion, or "finite," lines, which undergo a limited number of population doublings before "senescence." Becoming a continuous cell line requires "transformation" and this necessitates either the presence of cells that are already transformed at the initiation of the culture or undergoing transformation in the early generations.

Cell lines may exist either as adherent cultures or they may grow in "suspension." Most cell types will adhere to a "substrate" such as plastic or glass and proliferate as a monolayer, while suspension cultures do not attach to a substrate and will grow floating in medium.

Table 3
Components of Media

Components of Eagle's basal medium (BME)			
Amino acids	Vitamins	Inorganic salts	Other
Arginine	Biotin	$CaCl_2$	D-Glucose
Cystine	D-Ca pantothenate	KCl	Phenol red
Glutamine	Choline	$MgSO_4$	
Histidine	Folic acid	NaCl	
Isoleucine	i-Inositol	$NaHCO_3$	
Leucine	Nicotinamide	NaH_2PO_4	
Lysine	Pyridoxal HCl		
Methionine	Riboflavine		
Phenylalanine	Thiamine HCl		
Threonine			
Tryptophan			
Tyrosine			
Valine			
Additional components added in other media			
Amino acids	Vitamins	Inorganic salts	Other
Alanine	Ascorbic acid	$Fe(NO_3)_3$	HEPES
Asparagine	Biotin	KH_2PO_4	Hypoxanthine
Aspartic acid	Cholesterol	$MgCl_2$	Linoleic acid
Cysteine	Niacin	Na_2SeO_3	Putrescine
Glutamic acid	p-aminobenzoic acid	$CuSO_4$	Pyruvate
Glycine	Nicotinic acid	$FeSO_4$	
Hydroxyproline	Pyridoxine	$ZnSO_4$	
Proline		$Ca(NO_3)_2$	
Serine		KNO_3	

4. Basic Requirements of Cells in Culture

For cells to thrive in culture, a variety of conditions must be met. As for the in vivo setting, nutritional and environmental conditions are essential for cell health. Nutrition is provided by media with or without the addition of serum. For adherent cells, attachment to a substrate (now generally plastic) is important while carbon dioxide and oxygen levels have to be maintained within certain limits.

4.1. Media

The synthetic media currently in use today were developed in the 1950s. These basal media contain amino acids, carbohydrates, vitamins, and salts (*see* **Table 3** and Appendix 1).

Serum or further components, such as growth factors and hormones, are added to the basal media to provide the complete mix necessary for growth. The composition of

media range from those containing the most essential ingredients, for example, Eagle's basal medium (BME) to those containing a much broader range of components, such as Medium 199. The medium must also be buffered to allow a stable pH, ideally around pH 7.4.

One of the first media to be developed was BME. This emerged from Harry Eagle's studies that sought to identify a medium that would support a wide variety of both normal and malignant cell types ***(10,11)***. This medium was subsequently modified to produce a number of popular media. Increasing the amounts of individual amino acids gave Eagle's minimum essential medium (MEM) ***(11),*** while Dulbecco's modified Eagle's medium (DMEM) contained a fourfold increased concentration of amino acids and vitamins ***(12)*** with a further change increasing the glucose content 4.5-fold. Iscove's modified Dulbecco medium (IMDM) was developed to support hemopoietic precursors and is a modification of DMEM, containing selenium, additional amino acids and vitamins, sodium pyruvate, and HEPES buffer ***(13)***. Glasgow minimum essential medium (GMEM) was a variation of Eagle's medium that was used to study factors affecting cell competence. This contains a twofold increased concentration of individual amino acids and vitamins ***(14)***.

In 1950, Morgan and colleagues described a medium that could support the growth of explanted tissue ***(15)***. This became known as Medium 199 and contained many more components than found in Eagle's medium and had broad applicability for the culture of many cell types. A less complex version of Medium 199 is CMRL 1066 developed at the Connaught Medical Research Institute ***(16)***. Initially designed as a serum-free medium, it can be supplemented with serum to support the growth of many cell types. Another medium developed to address serum-free growth was Waymouth's medium, designed as a totally serum-free medium for cultivation of mouse L929 cells but proven useful for other cell lines also ***(17)***.

Ham's Nutrient Mixtures F10 and F12 were originally developed for the growth of Chinese Hamster Ovary (CHO) cells either with or without serum supplementation ***(7,8)***. The combination of F12 and DMEM as a 1/1 mix has found widespread use in serum-free formulations combining the richness of F12 with its trace elements and increased vitamins with the nutrient potency of DMEM. Ham and coworkers went on to develop the MCDB series of media which were developed for serum-free growth of individual cell lines using supplements or low levels of fetal bovine serum protein. In 1959, McCoy described a basic formulation that was subsequently modified to create a medium supporting the growth of a wide variety of primary cultures ***(18)***. RPMI 1640, developed by Moore and colleagues at Roswell Park Memorial Institute ***(19),*** is a medium extensively used for supporting the growth of many types of cell culture. This has become the medium of choice for many tissue culture laboratories.

Media is available from commercial sources in several formats—normal strength medium (shelf life, 9–12 mo), concentrates that are diluted down (generally 10X) (shelf life, 12–24 mo) and powdered medium that has a long shelf life (2–3 yr) and can be made when needed. Concentrates and powdered medium require the addition of sterile water.

4.2. Serum

Although media contains many of the essential nutrients required for growth, additional key elements are provided by serum. Serum supports the survival and growth of cells in culture to the extent that it is capable of replacing many of the in vivo hormonal, nutritional, and stromal elements present in the in vivo cell environment. Serum proteins include hormones, growth factors, lipids, transport (binding) proteins, enzyme cofactors, and attachment factors ***(9)***. The concentrations of individual components of serum will vary with the age and health status of the animals of origin and for this reason fetal and newborn calf sera are extensively employed for most cancer culture studies though human and equine sera are also used. Typically, concentrations of 5–20% serum in media are considered optimal depending on cell type. Higher percentages generally add little benefit for increased cost. The levels of serum proteins will vary to some degree from batch to batch of sera and individual cell cultures will have differing requirements for these components. It is generally recommended that a batch of serum is bought that can last from 1–2 yr stored at –20°C. Since batches of sera from individual suppliers differ to some degree in their composition, several small amounts should be obtained from a range of suppliers and then a number of cell lines used by the laboratory should be tested. The parameters to be assessed will generally include growth rates and attachment efficiency. Generally, some information on the biochemical analysis together with certain growth and microbiological tests is available on the Certificate of Analysis.

4.3. Serum-Free Media

The use of serum has permitted the growth of many cell types in culture, however there are a number of drawbacks associated with its use. First, although the ingredients of media are clearly defined, sera will vary in its composition dependent on its batch. These differences in composition may produce some changes in a number of parameters including growth rate, attachment, and other functional endpoints. Some components in serum are in fact growth inhibitory although further supplementation may still be required where some components are present at insufficient levels. For certain purposes, the presence of proteins at undefined levels may also prove a complication, for instance where measurements of a cell secreted protein are being made or the effects of media conditioned by cells in a bioassay necessitate a fully defined background. Finally, although sera are now routinely checked for the presence of contaminants, viruses in particular have frequently been present in the past.

For these reasons, together with considerations of cost and greater reproducibility, there has been a move to the use of totally defined media. In the 1960s and 1970s, two strategies were developed to define media that did not require the addition of serum. Sato and colleagues pioneered the addition of specific supplements to existing basal media while Ham and coworkers increased the concentrations of components of the basal medium until they could support growth. The key categories of additives include hormones, binding proteins, lipids, trace elements, and attachment factors.

Sato's experiments in supplementing medium identified several factors that appeared to have widespread value in maintaining the growth of many cell types in

media alone ***(9,20)***. These included insulin, transferrin, and selenite (ITS) and often epidermal growth factor and hydrocortisone (HITES). Serum albumin and fibronectin are also widely used. The media selected is frequently a 1/1 mixture of DMEM and Ham's F12 though other media such as RPMI 1640, IMDM, and the various MCDB media are also commonly used. Other additives that can have great value for specific cell lines include fibroblast growth factor, estrogen, glucagon, prostaglandins, and triiodothyronine ***(9)***.

Ham's laboratory developed the MCDB series of media to provide a defined and optimally balanced environment to promote growth of specific cell types. For example, fibroblasts grow in MCDB 110, keratinocytes in MCDB 201, and 1551 and CHO cells in MCDB 302 media ***(21)***.

When transferring from serum-containing medium to serum-free it is advisable to reduce the serum content gradually to aid adaptation to a reduced nutritional environment. Transferring to a serum-free medium requires more care when trypsinization is undertaken. Generally, serum will rapidly inactivate trypsin, however if serum is not being used, then a trypsin inhibitor can be added to neutralize the trypsin. If attachment to the substrate is poor, precoating with a collagen or fibronectin solution may help.

4.4. The Substrate

For growth and differentiation in culture almost all types of cancer cells require interaction with a substrate. Most cancer cell types will grow as monolayers on plastic or glass. The major exceptions are hematopoietic cell lines and most small cell lung cancer cell lines that prefer to grow in suspension as single cells and as clusters of cells respectively. Polystyrene is the most widely used plastic, though other substrates such as polycarbonate, polytetrafluoroethylene and polyvinyl are also available. These plastics require treatment with irradiation or chemicals to produce a charged surface. Cell adhesion can be greatly increased by coating the substrate with extracellular matrix (ECM) components. Widely used ECM proteins include collagen, fibronectin, and laminin. Alternatively, substrates can be precoated with serum or with medium conditioned by cells which produce ECM molecules.

Although monolayer culture is often the simplest and most convenient mode of culture, more complex systems can provide a greater level of information. Growth within an ECM such as collagen or Matrigel in three dimensions, rather than on two, can provide more complete morphological and biochemical differentiation ***(22,23)***. The use of dual cultures wherein two populations of cells, e.g., carcinoma cells and fibroblasts, are separated by a filter, allows study of paracrine interactions and the roles of diffusible factors (*see* Chapters 28 and 29).

4.5. Physical Environment

Maintenance of the physical environment conditions within certain limits is required for optimal cell growth. For most mammalian cultures, the preferred temperature is 36.5° ± 1°C and although cells can still grow at lower temperatures, they will die rapidly at temperatures above 40°C. Culture media contain buffering systems that generally require a CO_2 atmosphere and frequently 5% CO_2 is preferred. Most cancer cells

require a pH value that is around 7.2–7.4 and media should be changed regularly as cell growth produces respiratory byproducts that acidify the media. The pH indicator commonly added to medium is phenol red which is yellow at pH 6.5, orange at pH 7.0, red at pH 7.4, and purple at pH 7.8, thus providing a simple visual indication of the pH status of the culture.

Finally, humidity levels are important as evaporation of water from the medium will concentrate salt levels which could eventually cause cell lysis. For optimal growth, the osmolality of the media should be kept within relatively narrow limits.

5. Primary Cell Culture

Primary culture, i.e., the initial culture established from an individual, represents the situation most closely related to the original tissue. The primary material used may be either a fragment, for example an explant, that can be made to attach to the substrate wherein cells can migrate and grow directly from the fragment, or tumor material that can be broken up by mechanical or enzymatic means into single cells or clusters of cells.

The source of the material can have an impact on the efficiency of this process, with cultures being more easily established from primary ascitic or pleural effusions already containing cells in suspension than from solid tumors. Enzymes routinely used for disaggregation include trypsin and collagenase.

Many of the cell types within the initial cell mix may not adhere to a substrate readily or grow under the culture conditions, and the balance of cell types in culture may change rapidly with time as the fast-growing cells outgrow the slower or nonproliferating cell types. This loss of heterogeneity has both advantages and disadvantages. With selection to produce a more homogeneous cell population, if the predominant emerging cell type obtained is the one of interest then this might be considered helpful and desirable. For the development of cell lines, this is necessary to allow a pure population to emerge. The disadvantage of selective growth is that the heterogeneity and diversity of the multicellular tumor is lost with the subsequent absence of key intracellular interactions.

6. Cancer Cell Lines

The development of a culture beyond the primary culture results in a "cell line." The importance of a cell line lies in its ability to provide a renewable source of cell material for repeat studies. Cell line models should reflect the properties of their original cancers, e.g., maintenance of histopathology when transplanted into immunodeficient mice, genotypic and phenotypic characteristics, gene expression and drug sensitivity ***(24)***. However, as it is frequently fast growing cell lines from poorly differentiated tumors that are generally selected for growth in vitro, the cell lines in widespread use may not necessarily always reflect those found in the majority of the clinical disease ***(24)***.

Virtually all types of cancer cells can now be grown in culture. The classical studies of Hayflick and Moorhead in the early 1960s demonstrated that diploid human fibroblasts could undergo a limited number of divisions (approx 50) in culture before enter-

ing "crisis" and senescence ***(25)***. Most cancer cell lines will undergo indefinite numbers of divisions, and immortal cell lines can extend to thousands of divisions. For a cell line to become "continuous" (rather than "finite") cells must be present at low levels in the initial culture that have the ability to divide indefinitely or cells have to undergo "transformation." This transformation can be produced via chemical or viral means (*see* Chapter 26).

Once a new cell line has been established, it should be characterized and confirmed to be free of contamination. These aspects are covered in Chapters 4 and 5 and 31.

As cell lines undergo increasing numbers of passages, they may lose certain features such as differentiation characteristics; however they may also demonstrate greater homogeneity as the most rapidly growing subclones will emerge. Stocks of the cell lines should periodically be frozen at a variety of passage numbers to provide a renewable resource.

As model systems, cell lines possess a number of advantages over primary cultures. As mentioned above, their predominant strength is the ability to repeat studies with a well characterized culture system that can be used in multiple laboratories. With continued culturing a relatively homogeneous cell population will arise unlike the primary culture that may contain many types of stromal and infiltrating cell types potentially complicating the interpretation of data.

6.1. General Growth Characteristics of Cell Lines

When cell lines are subcultured, they will pass through several well-defined stages of growth. Monolayer cells when initially subcultured will take time to adhere to the substrate and, if they have been disaggregated by proteolytic enzymes (like trypsin), need time to repair the damage caused by these enzymes. At this point the culture will grow relatively slowly and this stage is referred to as the "lag phase." As the culture starts growing, paracrine exchange of growth factors will help accelerate the growth rate and an increasing percentage of cells will undergo cell division. The initial cell density is a factor here and the higher the cell density, the more rapidly the culture will grow. The culture will demonstrate its most rapid phase of growth, often exponential and therefore called the "log phase." Finally, as the monolayer fills the available substrate area with cells in close contact with each other (confluency) and relatively high use of sera and media components, the growth rate will slow down to a "plateau" at a particular "saturation density" that is characteristic for a cell line (for a particular set of conditions) and this is referred to as the "plateau phase." It is generally recognized that the longer the cells are in the plateau phase before subculture, the longer they remain in the lag phase after subculture.

6.2. Cancer Cell Collections

Cancer cell lines are widely available through a number of large cell banks. The largest of these are listed in **Table 4** and in addition there are many other national collections that are often government sponsored and nonprofit making. The World Federation for Culture Collection has 469 culture collections in 62 countries (http://www.wfcc.info), although not all hold cancer cell cultures. The number of more spe-

Table 4
Major Cell Line Banks

Bank	Web Address	Postal Address	Further Information
American Type Culture Collection (ATCC)	http://www.atcc.org	10801 University Blvd., Manassas, VA, 20108-1549, USA	Contains over 4000 cell lines, incl. 950 (700 human) cancer cell lines
Corriell Cell Repository	http://locus.umdnj.edu/ccr	401 Haddon Ave., Camden, NJ 08103, USA	Contains cell lines covering over 2000 genetic diseases
Deutsche Sammlung Von Mikroorganismen Und Zellkulturen GmbH (DSMZ)	http://www.dsmz.de	Mascheroder Weg 1b, D-38124 Braunscweig, Germany	Contains over 500 human and animal cell lines
European Collection of Animal Cell Culture (ECACC)	http://camr.org.uk	CAMR, Salisbury, Wilts, SP4 OJG, UK	Contains over 900 human and animal cell lines
Interlab Cell line Collection (ICLC)	http://www.iclc.it	L.Go Rosanna Benzi 10, 16132, Genova, Italy	Contains over 200 human and animal cell lines
Japanese Collection of Research Bioresources (JCRB)	http://cellbank.nihs.go.jp	1-1-43 Hoen-Zaka, Chu-Ku,Osaka 540, Japan	Contains over 1000 human and animal cell lines
RIKEN Gene Bank	http://www.rtc.riken.go.jp	3-1-1 Koyadai, Tsukuba, Science City, Ibaraki 305, Japan	Contains over 1000 human and animal cell lines

cialized banks concentrating on specific cancer types is also expanding rapidly and these are most easily identified through worldwide web searches.

It is recognized that if a cell line can be obtained from a reputable bank then that is the best source. These provide guarantees of authentication and freedom from contamination that may not be the case when transferring between laboratories. The latter transfer often spreads microbial contamination (especially mycoplasma) and increases the opportunities for mix-ups. Sometimes, however, an academic laboratory may be the only source of a unique cell line.

6.3. Development of New Cell Lines or Use of Existing Lines?

There are now very large numbers of cell line panels available in the National Cell Banks. It is therefore worth reconsidering the merit of developing new cell lines if cell lines are readily available. The advantages of using existing cell lines include their instant availability and, at least for some cell types, the hundreds of models available within the cell banks (*see* Appendix 2)

Many lines are well characterized and novel data generated may be related to the existing literature on that model. Time does not have to be spent on developing new models but can be devoted to using them. However, in many situations, cell lines are not available and may need to be derived. A great deal of time and effort can be spent in creating and characterizing new cancer cell lines; however, there are several situations where this is desirable. First, there may not be any or only a few established cell lines available for the tumor type under study. Cell lines with particular characteristics, e.g., derived resistance to a new drug, may also be unavailable. Finally, there will be novelty in deriving the new cell line that has to balanced against the effort required to characterize it.

7. Basic Laboratory Design and Selection of Equipment

Although the design and selection of equipment for a cell culture facility will depend on the scale of the operation, several requirements are fundamental to even the smallest of laboratories. Housed within the space dedicated to cell culture will be the microbiological safety cabinet(s), inverted microscope(s), sink, centrifuge, and specific small apparatus such as tissue culture flasks, sterile glassware, and plastics. Ideally, CO_2 incubators should also be in the same room to minimize movement of cells around the laboratory. Storage facilities such as liquid nitrogen tanks or fridges/freezers holding tissue culture media or serum do not necessarily need to be in the same room, and similarly sterilization equipment and autoclaves may reside nearby. Within this area, human traffic should be minimized especially in the vicinity of the tissue culture cabinets.

All cell culture manipulations should be undertaken in microbiological safety cabinets designed to protect the laboratory worker from exposure to aerosols from cell culture. Such cabinets operate on the principle that air from the vicinity of the culture is filtered through a HEPA (high efficiency particulate air) filter before exiting the cabinet. They are classified at levels I, II, and III. Class I cabinets are the simplest and easiest to maintain but offer least sterile protection to the cell culture. Class II cabinets

are probably the most widely used for cell culture work and offer good protection to both the operator and cell cultures since air passing over the working area is HEPA filtered. Class III cabinets are completely sealed units and are used for more hazardous types of work. For all classes of cabinet, quality of airflow and filter integrity should be tested routinely every 6–12 mo. Before use, cabinets should be switched on for 10–20 min and surfaces wiped with ethanol both before and after use. Cabinets may also be equipped with a UV light that can be used to sterilize the surfaces of the cabinet.

8. Safety

A number of biohazards are associated with cell culture and the new user should be made aware of these. Potential hazards may be harbored by cancer cells both within cultures or within sera ***(26)***. Cancer cells may carry viruses while serum may carry not only viruses but other microorganisms as well. Clinical material should always be regarded as potentially infectious until proven otherwise and treated as such. Blood borne pathogens such as hepatitis B virus (HBV) and the human immunodeficiency virus (HIV) are perhaps the most common risk although cells transformed with viral agents, such as SV-40, Epstein-Barr virus (EBV), and HBV should also be treated with caution. Viral infection is also possible from the culture of animal material ***(27)***. A reported incident of cancer developing from a needle-stick injury highlights the risk of possible transfer and infection but good practice should make this risk extremely low ***(28)***.

With careful technique and appropriate caution, the risks of working within a general cell culture facility undertaking studies with cancer cell lines or primary cultures should be no greater that working in any other area of a cell or molecular biology laboratory.

References

1. Gey, G. O., Coffman, W. D. and Kubicek, M.T. (1952) Tissue culture studies of the proliferative capacity of cervical carcinoma and normal epithelium. *Cancer Res.* **12,** 364–365.
2. Harrison, R. G. (1907) Observations on the living developing nerve fiber. *Proc. Soc. Exp. Biol. Med.* **4,** 140–143.
3. Burrows, M. T. (1910) The cultivation of tissues of the chicken embryo outside the body. *JAMA* **55,** 2057.
4. Carrel, A. (1912) The permanent life of tissue outside of the organism. *J. Exp. Med.* **15,** 516.
5. Earle, W. R., Schilling, E. L., Stark, T. H., Straus, N. P., Brown, M. F. and Shelton, E. (1943) Production of malignancy in vitro. IV. The mouse fibroblast cultures and changes seen in the living cells. *J. Natl. Cancer Inst.* **4,** 165–212.
6. Masters, J. R. (2002) HeLa cells 50 yr on: the good, the bad and the ugly. *Nature Rev.* **2,** 315–318.
7. Ham, R. G. (1963) An improved nutrient solution for diploid Chinese hamster and human cell lines. *Exp. Cell. Res.* **29,** 515–526.
8. Ham, R. G. (1965) Clonal growth of mammalian cells in a chemically defined, synthetic medium. *Proc. Natl. Acad. Sci. USA*, **53,** 288–293.
9. Barnes, D. and Sato, G. (1980) Serum-free culture: a unifying approach. *Cell* **22,** 649–655.
10. Eagle, H. (1955) Amino acid metabolism in mammalian cell cultures. *Science* **130,** 432–437.

11. Eagle, H. (1955) Nutrition needs of mammalian cells in culture. *Science* **122,** 501.
12. Dulbecco, R. and Freeman, G. (1959) Plaque formation by the polyoma virus. *Virology* **8,** 396–397.
13. Iscove, N. N., Guilbert, L. W. and Weyman, C. (1980) Complete replacement of serum in primary culture of erythropoietin-dependent red cell precursors (CFU-E) by albumin, transferrin, iron, unsaturated fatty acid, lecithin and cholesterol. *Exp. Cell Res.* **126,** 121–126.
14. MacPherson, I. and Stoker, M. (1961) Polyoma transformation of hamster clones—an investigation of genetic factors affecting cell competence. *Virology* **16,** 147–151.
15. Morgan, J. F., Morton, H. J. and Parker, R. C. (1950) The nutrition of animal cells in tissue culture. Initial studies on a synthetic medium. *Proc. Soc. Exp. Biol. Med.* **73,** 1–8.
16. Parker, R. C. (1957) Altered cell strains in continuous culture: a general survey. In *Special Publications of the New York Academy of Sciences*: 5 (Whitelock, O., ed). NY Acad. Sci., New York, 303–313.
17. Waymouth, C. (1959) Proliferation of sublines of NCTC clone 929 (strain L) mouse cells in a simple chemically defined medium (MB752/1). *J. Natl. Cancer Inst.* **22,** 1003.
18. McCoy, T. A., Maxwell, M. and Kruse, P. F. (1959) Amino acid requirements of the Novikoff hepatoma in vitro. *Proc. Soc. Exp. Biol. Med.* **100,** 115–118.
19. Moore, G. E., Gerner, R. E. and Franklin, H. A. (1967) Culture of normal human leukocytes. *JAMA* **199,** 519–524.
20. Barnes, D. and Sato, G. (1980) Methods for growth of cultured cells in serum-free medium. *Anal. Biochem.* **102,** 255–270.
21. Ham, R. G. and McKeehan, W. L. (1978) Development of improved media and culture conditions for clonal growth of normal diploid cells. *In Vitro* **14,** 11–22.
22. Weaver, V. M., Fischer, A. H., Peterson, O. W., and Bissell, M. J. (1996) The importance of the microenvironment in breast cancer progression: recapitulation of mammary tumorigenesis using a unique human mammary epithelial cell model and a three-dimensional culture assay. *Biochem. Cell Biol.* **74,** 833–851.
23. Weaver, V. M. and Bissell, M. J. (1999) Functional culture models to study mechanisms governing apoptosis in normal and malignant mammary epithelial cells.*J. Mammary Gland Biol. Neopl.* **4,** 193–201.
24. Masters, J. R. W. (2000) Human cancer cell lines: fact and fantasy. *Nat. Rev.* **1,** 233–236.
25. Hayflick, L. and Moorhead, P. S. (1961) The serial cultivation of human diploid cell strains. *Exp. Cell Res.* **25,** 585–621.
26. UKCCCR Guidelines for the use of cell lines in cancer research. *Brit. J. Cancer* **82,** 1495–1509.
27. Lloyd, G. and Jones, N. (1984) Infection of laboratory workers with Hantavirus acquired from immunocytomas propagated in laboratory rats. *J. Infect.* **12,** 117–125.
28. Gugel, E. A. and Saunders, M. E. (1986) Needle-stick transmission of human colonic adenocarcinoma. *New Engl. J. Med.* **315,** 1487.

2

Essential Techniques of Cancer Cell Culture

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1. Introduction

Cell culture utilizes a number of core techniques, and although there can be marked diversity in how these procedures are practiced, there are elements and features that are universally applied. This chapter describes some of the essential techniques and provides typical protocols. It is assumed that aseptic technique will be used for all these procedures unless mentioned otherwise.

1.1. Primary Culture

A primary cell culture is the initial culture set up directly from a body tissue. Primary cancer cultures can be initiated and derived from a variety of tissue types such as solid tumor fragments (primary or metastatic) or cell suspensions, for example, aspirates, including peritoneal ascites or pleural effusions. Cell suspensions can be particularly convenient for developing cell lines as they are already growing as single cells or clusters, avoiding the need for mechanical or enzymatic dispersion. The cellular composition of primary cultures is often very variable with hematopoietic and stromal cell types contributing to the cellular mix. Fibroblasts, in particular, can be problematic as they attach readily to matrices and often outgrow the cancer cell population. Cancer cells differ from most normal cell types in their ability to grow in suspension, for example, in agar, but generally cultures are initiated by allowing cells to adhere to a substrate before proliferating. A number of strategies have been developed to help disperse fragments of tissue and these include mechanical and enzymatic methods *(see* **Subheading 1.2.)**.

1.2. Routine Feeding and Maintenance

Cell cultures should be examined regularly and routinely (preferably daily) both macroscopically and microscopically. The cell morphology and cell density should be checked by microscope and the presence of contaminants such as fungus and bacteria should be evaluated. Macroscopically, the color and turbidity of the medium should be monitored. Media (plus serum and other additives) should be changed regu-

From: *Methods in Molecular Medicine, vol. 88: Cancer Cell Culture: Methods and Protocols*
Edited by: S. P. Langdon © Humana Press Inc., Totowa, NJ

larly and not allowed to become depleted, depriving cells of specific nutrients, or becoming acidic. The pH of the medium is most easily monitored by the addition of color indicators such as phenol red. The frequency of media renewal will be dependent on the growth rate of the culture with more rapidly growing cultures requiring more regular changes.

1.3. Subculture of Cells

When a culture has occupied the complete surface of a flask (for a monolayer culture) or has grown to a point where media has been depleted of nutrients (for a suspension culture), then it requires "subculture" (also described as "passaging" or "splitting") to maintain healthy growth. This process reduces the cell density back to a level where the cells will grow optimally again and not exhaust the medium of nutrients too rapidly. The process of detachment of the adherent monolayer to give a single cell suspension is often referred to as "harvesting." The use of proteolytic enzymes such as trypsin, breaks the cell-cell and cell-substrate links and creates a single-cell suspension. Subculture of a primary culture into a secondary culture produces a "cell line." Once a cell-line is created, not only should it be given a designation, but a record of the number of subcultures or passages should be kept to provide a general indication of the lifetime of the culture. The cell line should be characterized and this process is described in Part II.

1.4. Cloning

A clone is the population of cells derived from an individual cell and cloning is the process of isolating this individual cell and developing its progeny. Cloning will thus produce cultures that are genetically homogeneous at the outset. The colony forming efficiency (CFE) is a measure of the ability of a culture or cell line to produce colonies and represents the number of colonies produced/number of individual cells cultured. Primary cultures tend to have low CFE values, typically <1% while cell lines generally have much higher values, typically varying from 10–100%. Methods describing the cloning of cells on plastic and within agar are described.

1.5. Cell Counting

The determination of cell number is a key measurement both for setting up experiments with cancer cell lines as well as monitoring cell responses under experimental conditions. Two protocols provided here have advantages depending on the application, for example, the scale of the experiment or the number of cell lines to be counted. The simplest protocol involves the use of a hemocytometer (Improved-Neubauer) and is appropriate when only a small number of samples are to be counted. This is the least expensive approach because minimal outlay on equipment or reagents is required. A hemocytometer is an etched glass chamber that will hold a quartz cover slip exactly 0.1 mm above the chamber floor.The counting chamber is precisely etched in a total surface area of 9 mm^2. Calculation of cell number is based on counting the number of cells within a defined area underneath the cover slip. The second method is automated and involves the use of an electronic counter such as the Z2 from Beckman Coulter. This approach allows rapid and accurate counting of large numbers of cultured cells

and is widely used within the biomedical sciences but involves a greater initial outlay for the equipment.

1.6. Cryopreservation

The preservation of cell stocks at temperatures below –130°C has allowed the long-term storage of cells for periods of at least 2–3 decades. Several features are important for optimizing the viability of cells. The use of cryoprotective agents that prevent ice crystals forming and the fragmenting of membranes is essential. The most commonly used cryoprotective agent is dimethylsulfoxide (DMSO), but glycerol is an alternative. The rates of freezing and thawing also influence viability, and a freezing rate of approx 1°C/min is considered optimal. In contrast, thawing should be rapid and this is most easily achieved by placing ampules in a water bath at 37°C. Cells are generally stored in liquid nitrogen at –196°C, but can remain viable for short periods of time at –80°C.

1.7. Troubleshooting

A number of simple problems are routinely encountered in cell culture studies. Some of the more common issues are listed in **Table 1** with potential causes and their associated solutions.

2. Materials

2.1. Primary Culture

1. Specimen collection containers, for example, sterile Universal containers.
2. Cell disaggregation enzymes.
3. Sterile scissors.
4. Sterile forceps.
5. Sterile scalpels.
6. Tissue sieve.
7. Phosphate buffered saline (PBS).
8. Cell culture medium, for example, Dulbecco's modified Eagle's medium (DMEM), + 10% fetal calf serum (FCS), penicillin (100 U/mL)/streptomycin (100 mg/mL).

2.2. Routine Maintenance and Feeding

1. Cell culture media.
2. Inverted microscopes.
3. Trypsin-EDTA (for adherent cultures).
4. Phosphate-buffered saline (PBS) for adherent cultures.
5. Tissue culture plastics.

2.3. Subculture

1. Any adherent cell culture, for example, SKOV-3 ovarian cancer cell line.
2. Tissue culture flasks.
3. Complete cell culture medium, for example, RPMI 1640 containing 10% heat inactivated fetal calf serum (FCS).
4. Trypsin–EDTA (Gibco BRL).
5. Dulbecco's PBS (Oxoid, Unipath Ltd, Basingstoke, England).

Table 1
Common Problems Associated With Cell Culture

Problem	Possible cause	Potential solution
Cells difficult to remove from plastic	Enzyme solution too weak	Higher concentration needed
	Inhibitor present in medium (for example, serum)	Cells require more careful washing
	Cells too confluent and enzyme cannot access cell-substrate interface	Cells require trypsinisation at lower cell density
Cells not adhering readily to plastic	Cells too heavily treated with trypsin	Use less trypsin or treat for less time
	Insufficient serum or attachment factors	Add more
	Dissociating agent (for example, not inactivated fully)	Add serum or specific inhibitors
	Mycoplasma contamination	Discard if infected
Suspension cells clumping together	Mycoplasma contamination	Discard if infected
	DNA from lysed cells sticking cells together	Add DNAse
Poor growth in culture	Absence or lower than normal levels of certain additives	Add missing components
	Contamination by bacteria, mycoplasma or fungi	Discard if infected
	Cell density too low	Increase density
Cell death/low viability	Incorrect pH	Correct pH
	Faulty media	Correct preparation
Too acidic pH	CO_2 content too high	Modify
	Contamination	If infected, discard
Too basic pH	Insufficient CO2	Caps too tight
	Too few cells	Increase cell density

2.4. Cloning

1. Cells.
2. Cell culture medium.
3. Trypsin-EDTA.
4. Hemocytometer or cell counter.
5. Petri dishes.
6. Agar.

2.5. Cell Counting

2.5.1. Hemocytometer Counting

1. Cell line of interest.
2. PBS.
3. Trypsin-EDTA.
4. Tissue culture media containing 10% FCS.
5. Tally Counter.
6. Syringe and needles or Pasteur pipets.
7. Microscope.
8. Hemocytometer (Improved Neubauer).
9. 0.4% Trypan blue in PBS (optional).
10. 10% Formalin (optional).

2.5.2. Electronic Counting

1. As above but with electronic counter, for example, Beckmann Coulter Z2.
2. Counting pots.
3. Fixed volume dispenser (optional).
4. Ice tray.

2.6. For Cryopreservation

1. Cells requiring freezing.
2. Cell culture medium: Medium plus 10% FCS.
3. Freezing mixture: 10% DMSO/20% serum/70% medium or 10% DMSO/90% serum.
4. Trypsin-EDTA.
5. Hemocytometer or cell counter.
6. Liquid nitrogen freezer.
7. Cryotubes (freezing ampules).
8. Pipets.
9. Gloves and face mask.

3. Methods

3.1. Primary Culture

3.1.1. Initial Establishment from Solid Primary Tumor or Solid Metastasis

1. Obtain cancer material at surgery (*see* **Note 1**). Place fragments of the material into tissue culture medium, (e.g., RPMI 1640 or DMEM) in sterile plastic containers, for example, a Universal container. Keep material cold (on ice) and transfer to the tissue culture suite as rapidly as possible.

2. Within a sterile environment, for example, a Class II hood, select the most viable tissue and discard any necrotic tissue. Wash the tissue initially either with medium, PBS, or Hank's balanced salt solution (HBSS) to remove blood.
3. Two options are then available for tumor disaggregation—mechanical or enzymatic dispersion.
4. For mechanical disaggregation, cut tumor fragments into small pieces (1–2 mm diameter) by the use of crossed scalpels. This is most easily done on a Petri dish. Add a small amount of cell culture medium. Using a sterile pipet or pastette, transfer the fragments to a 25-cm^2 culture flask. If a sterilized metal sieve is available, this can be used to remove everything other than clusters of cells or single cells. To help attachment, it is often easiest to use the minimum volume of culture medium initially (2–3 mL) (*see* **Note 2**) and then add additional medium for a total volume of 5–10 mL. After several days, explants and clusters will attach to the plastic and cells will grow out from the sites of adherence.
5. Alternatively, small fragments can be broken up by the use of proteolytic enzymes. Trypsin, collagenase, hyaluronidase, elastase, dispase, and papain are all useful enzymes for this purpose. Dependent on the tissue, optimization of the enzymes and their working concentrations are required to obtain the best dissociation without excessive destruction. Sometimes, enzyme cocktails have been used overnight on ice and these may have a gentler effect (*see* **Note 3**).
6. If trypsin is used, the fragment is still chopped initially into relatively small pieces, for example, 2–3 mm diameter. The small fragments are then added to a trypsin solution (0.25%) at a density of 1 g tissue/10 mL and stirred at 37°C for 30 min. The supernatant is collected and centrifuged at 600*g* for 5 min to collect a cell pellet. This cell pellet is resuspended in full culture medium (containing serum which will inactivate the trypsin). The fragments will not necessarily disaggregate fully after a single 30-min step and the process can be repeated until most cells have become suspended in the supernatant. A modification of this process is to soak the initial fragments overnight at 4°C in the trypsin solution, allowing effective tissue penetration, and a single 30-min treatment at 37°C will be more effective. The cell suspension obtained is then centrifuged and cell culture medium (containing serum) added.
7. The other enzyme widely used for this step is collagenase as collagen is one of the major extracellular matrix proteins present in many stroma. Collagenase is added to culture medium (containing serum) at 200–1000 U/mL and can be left for 2–5 d.
8. The choice of medium to be used varies and is generally dependent on the media used in a particular laboratory. Ideally, several culture flasks should be set up using a variety of conditions as cells may grow differentially dependent on the media type. Popular media include DMEM, RPMI 1640, F12, McCoy's either alone, or in various 1/1 combinations of these. Serum at a percentage of 10% is also included. Higher concentrations of serum (up to 20%) are sometimes used and although this may be beneficial to certain cancer cell types, it may be even more beneficial to noncancer cells, such as fibroblasts, within the culture and help promote their overgrowth (*see* **Note 4**).
9. If the cells were growing within a fluid, e.g., an ascites or effusion, this can be spun down and the supernatant added at a level of 10–20% to the medium. Similarly, as the primary culture grows, the conditioned medium, i.e., medium that has been "conditioned" by the secretion of cell components, can be removed periodically and added back to fresh medium at a percentage of 10%.
10. Cultures should be monitored regularly to check which cell types adhere and grow.
11. Once there is sufficient material to maintain and expand the culture, it is essential to cryopreserve samples. Cell cultures should also be tested for the presence of mycoplasma.

3.1.2. Initial Establishment from Cell Suspensions

If the clinical tissue is a fluid such as an ascites, aspirate, or effusion the following method can be used.

1. Collect freshly obtained clinical fluids, for example, ovarian ascites, from the patient and transfer to a sterile environment. For an ovarian ascites, the volume is typically of the order of 1 L.
2. Centrifuge the fluids for 20 min at 3000*g* and 4°C to produce a cell pellet.
3. Discard the fluid and resuspend the cell pellet in PBS.
4. If the sample contains a particularly high number of red blood cells it is beneficial to remove the majority of these by centrifuging through Histopaque (or Ficoll-Paque). The tumor cell pellet is suspended in PBS or HBSS (10 mL) and placed onto Histopaque (10 mL) in a Universal container.
5. Tubes are centrifuged at 1000*g* for 20–30 min.
6. Cells at the interface of the buffer and the Histopaque are collected by pastette or pipet, resuspended in buffer, and centrifuged at 600*g* for 5 min. This wash is repeated.
7. Cells are then resuspended in cell culture medium and placed in a tissue culture flask (*see* **Notes 5**,**6**).

3.2. Routine Feeding and Maintenance

3.2.1. Adherent Monolayer

1. For monolayer cultures that are not being subcultured, remove spent media with a sterile pipet and add an equal volume of fresh media (plus serum plus any additives).
2. The periodicity of feeding depends on the growth rate of the primary culture or cell line. In general, feeding 2–3 times/wk is recommended.

3.2.2. Suspension Culture

1. For suspension cultures, medium containing cells must be centrifuged in a sterile manner, at 600*g* for 5 min in a Universal container.
2. The cell pellet is resuspended in fresh medium.
3. Often, if media is being depleted as a result of growth, then the culture can simply be divided 1/5–1/10 into fresh complete-culture medium without the necessity to spin cells down.

3.3. Subculture of Cells

3.3.1. Monolayer Cells

Adherent cells that are in late log phase require subculturing to maintain optimal growth (*see* **Note 7**).

1. Check the culture to ensure cells are in late log/early plateau phase (80–90% of the surface area is covered) and confirm that the cells are healthy and free of contamination.
2. Remove the cell culture medium by pipet and discard.
3. Wash cells twice with PBS to remove traces of serum that will inactivate trypsin and discard.
4. Add 1–2 mL trypsin-EDTA to a 25-mL flask (and scale up accordingly for larger sized flasks). Swirl the solution across the monolayer to ensure the trypsin reaches all cells. Return the flask to the incubator for 5–10 min (*see* **Note 8**).
5. Check the detachment of the cells at intervals. This is best done using a microscope, but with experience the monolayer sheet can be seen to disperse macroscopically. The cells

should not be left in trypsin for long periods once detached. Fresh culture medium containing serum is then added to inactivate the trypsin in the cell suspension. Pipeting or syringing this suspension will then help break up any cell clusters into single cells.

6. The cell suspension can then either be counted if an accurate cell density is required at subculture, or the suspension can then be "split" into an appropriate ratio. For example, it is often convenient to split the cells 1/5 or 1/10 depending on growth rates.
7. For a 1/10 ratio, a 1/10 aliquot of the cell suspension would be placed into a new flask with the full amount of cell culture medium required for that flask size. Subculturing from primary cultures should involve relative small split ratios, e.g., 1/2 or 1/3. Cell culture flasks are then returned to the CO_2 incubator. After 24 h, the culture should be checked to ensure that cells are reattaching and the pH of the medium is approx pH 7.4 (*see* **Note 9**).
8. Medium is then changed as necessary until the next subculture.

3.3.2. Suspension Culture

For cells growing in suspension, subculturing does not require the trypsinization steps needed for harvesting adherent cells.

1. The cell suspension is first checked to ensure cells are in late log/early plateau phase and to confirm that the cells are healthy and free of contamination (*see* **Note 10**).
2. The cell suspension can then either be counted if an accurate cell density is required at subculture, or the suspension can then be "split" into an appropriate ratio. For example, it is often convenient to split the cells 1/5 or 1/10 depending on growth rates. For a 1/10 ratio, a 1/10 aliquot of the cell suspension would be placed into a new flask with the full amount of cell culture medium required for that flask size.
3. Cell culture flasks are then returned to the CO_2 incubator. After 24 h, the culture should be checked to ensure that the pH of the medium is approx pH 7.4 (*see* **Note 9**).

3.4. Cloning

3.4.1. Dilution Cloning on Plastic

For monolayer cultures, trypsinize and harvest cells to prepare a cell suspension as described in **Subheading 3.3.1.** This step is unnecessary for cells growing in suspension. Pass cells through a pipet, pastette, or needle to produce a single cell suspension.

1. Dilute cells to a range of low concentrations, e.g., 100, 30, and 10 cells/mL.
2. Place cell suspensions into individual wells of a 24–well plate (1 mL/well).
3. Allow colonies to grow. Typically this will take 3–4 wk. Change media as growth commences.
4. Inspect each well to identify wells in which a single colony has grown. It may be necessary to repeat the range of cell concentrations plated to encompass either a higher or lower range.
5. Wells in which a single colony is present are then expanded by subculture as described above (*see* **Subheading 3.3.**). They should be designated carefully to indicate a link with the parental culture.

3.4.2. Cloning in Agar

Cancer cells can conveniently be cloned in agar as they demonstrate anchorage independent growth. This provides one the means of allowing the malignant cells to

grow in the presence of nontransformed cells that will generally not grow under these conditions.

1. Prepare agar. Agar solutions can be prepared prior to use. A 5% solution of agar in distilled water is dispensed into glass Universal containers. Autoclave for 15 min. The agar solution should be kept at 45°C or higher to prevent it from forming a gel.
2. For monolayer cultures, trypsinize and harvest cells to prepare a cell suspension as described in **Subheading 3.3.1.** This step is unnecessary for cells growing in suspension. Passage cells through a pipet, pastette, or needle to produce a single cell suspension. Count cells. Prepare a cell suspension at 2.5X final density.
3. Cell culture media (for example, Ham's F12 plus 10% FCS) is warmed to 37°C.
4. Using a pipet or pastette, add 2 mL of 5% agar to 18 mL cell culture medium in a prewarmed glass-Universal and mix thoroughly.
5. Add 3.6 mL of this 0.5% agar solution to 2.4 mL of the cell suspension in individual tubes (with caps that allow diffusion) and mix thoroughly.
6. Place tubes on ice for 5 min and close caps. Then place tubes into a CO_2 incubator at 37°C.
7. After 1 wk, 1 mL fresh medium plus serum can be added to each tube. The tube is re-fed weekly until colony size is greater than 50 cells.
8. Periodically, tubes are inspected to determine whether colonies have formed. A tube is selected, liquid medium removed from the tube, and the agar plug is placed into an empty Petri dish. The plug is pressed down to allow viewing through an inverted microscope.

3.4.3. Estimation of Colony Forming Efficiency (CFE) on Plastic

1. Monolayer cells are trypsinized as described in **Subheading 3.3.1.** and a cell count is taken of the cell suspension (*see* **Subheading 3.5.**).
2. Cell dilutions are prepared with differing numbers of cells (e.g., 10,000, 3000, 1000, 300, and 100 cells/2 mL).
3. Dispense 2 mL aliquots into Petri dishes or individual wells of a 6-well plate.
4. Allow cells to attach.
5. Replace full culture medium 2–3 times/wk.
6. When sufficient time has elapsed to allow colonies (>50 cells) to form (approx several weeks) and dependent on the growth rate of the cell line, colonies are counted.
7. It is generally convenient to count wells containing approx 100–200 colonies. At higher densities, colonies will start to merge and it will be unclear as to whether colonies developed from single cells or have merged. Colonies can also be stained with dyes such as hematoxylin or crystal violet to aid counting.

3.5. Cell Counting

3.5.1. Hemocytometer Counting

1. Trypsinize monolayer cells until they are detached from the plastic (*see* **Subheading 3.3.**) and then add medium containing FCS to inhibit cell damage by over-trypsinization. This step is not necessary for suspension cultures.
2. Ensure that the cells are in single-cell suspension. This should be done by repeatedly drawing the cells into a pastette or a syringe and checking the appearance of a drop of cells under the microscope (*see* **Note 11**).
3. Prepare the hemocytometer and cover slip. These should be clean and wiped with 70% alcohol (*see* **Note 12**). Take care not to scratch the silvered surface.
4. Slightly moisten the hemocytometer and cover slip (*see* **Note 13**) and place the cover slip over the grid. Gently move the cover slip back and forth resulting in its attachment to the

hemocytometer and the appearance of Newton's rings (rainbow colors like those formed by oil on water).

5. The hemocytometer is now ready to be filled. Place a pipet filled with a well-suspended mix of cells at the edge of the coverslip and then by slowly expelling some contents, draw the fluid into the chamber by capillary action.
6. Obtain the cell concentration by counting cells in the grid area. Several choices are available depending on the density of the cells:
   a. Count the 25 squares within the large middle square. Total cell count in 25 squares × 10,000 = number of cells/mL.
   b. Count the number of cells in the 4 outer squares. Total cell count in 4 squares × 2500 = number of cells/mL. The choice of methods is dependent on the cell concentration. The accuracy of the procedure depends upon the number of cells counted.
7. Each hemocytometer normally has two grid areas and it is good practice to count both and use the mean count to calculate cell number.
8. One advantage of using the hemocytometer method is that it allows for a variation of technique involving the use of Trypan blue dye to enable differentiation between dead/damaged cells and the healthy viable cell population.

3.5.2. Viable Cell Counting Using Trypan Blue

Trypan blue is a dye that does not interact with the cell unless the cell membrane is damaged. Healthy undamaged cells exclude the dye, but it is readily absorbed by damaged cells and renders them clearly visible (blue) under the microscope.

1. A 0.5-mL aliquot of cell suspension, obtained as previously described, is incubated with 0.5 mL of 0.4% Trypan blue dye (5 min at room temperature).
2. Cells are counted using the normal hemocytometer protocol and the percentage of dead or damaged cells can be established.

3.5.3. Electronic Counting

Detailed operating manuals and training are provided by Beckman Coulter. The following is a brief operating summary:

1. Switch on instrument 30 min prior to use. To avoid build up of debris in the pipe-work the instrument is routinely emptied after each use (daily) and refilled with Coulter Clenz. This is removed and the empty instrument refilled with saline. This is done automatically by making selections from the menu. Prepare counting pots by adding the required volume of PBS (typically 9.8 mL, allowing samples to be added in 0.2 mL increments). The use of an automatic dispensor makes this task much easier.
2. Instrument parameters are set on the set up page. These include sample volume particle size range (upper and lower thresholds) and the number of counts per sample.
3. Cells should be harvested and trypsin inactivated by adding an equal volume of FCS-containing media. Where necessary cells are agitated to give a single cell suspension (*see* **Note 11**). Cell samples contained in 24 well trays may be stabilized for longer by placing them on an ice tray.
4. Measure 0.2 mL of cell suspension into each counting pot just prior to counting. Mix gently by rolling or inverting. Do not shake vigorously as this will create air bubbles which may be counted by the instrument.

5. Flush aperture by selecting this option from the start-up menu. Check background by counting a blank sample. If this is unacceptably high repeat the flushing step.
6. Count the sample. Repeat process for further samples. It is not necessary to flush the aperture between replicate samples.
7. The results may be directed to a printer or to a computer.

3.6. Cryopreservation

3.6.1. Freezing

1. Cells that are typically in log phase of growth should be used, and ideally medium should be replaced 24 h prior to freezing.
2. For monolayers, cells should first be washed with PBS (after removal of culture medium) and then treated with trypsin-EDTA (5 mL/75 cm^2 flask) to detach the monolayer.
3. After confirmation of detachment by observation through a microscope, trypsin should be inactivated by addition of culture medium (containing 10% serum).
4. An aliquot of the cell suspension is then removed and a cell count taken using either a hemocytometer or cell counter.
5. Cells are then pelleted by centrifugation (5 min at 600*g*) and resuspended in freezing mix (kept cold on ice)(*see* **Note 14**) at a density of approx 10^7 cells/mL (*see* **Note 15**).
6. The cell suspension is then aliquoted into freezing ampules, e.g., cryotubes or other appropriate freezing vials (*see* **Note 16**). These vials should be labeled with the essential information, including at least the cell name, passage number, date, and the name of the individual storing the vials
7. Ampules are then placed into the freezing chamber of a programmable freezer if this is available. A simple alternative is to place ampules into a polystyrene container and then put this into a –70°C/-80°C freezer overnight. This achieves a cooling rate that approximates 1°C/min. Once ampules are at –70°C to –80°C, they can be transferred directly to liquid nitrogen (*see* **Note 17**).

3.6.2. Recovery and Thawing

1. When thawing, cells should be warmed rapidly by removing an ampule from the liquid nitrogen and placing it into a 37°C water bath (*see* **Note 18**). Ampules should be washed with 70% ethanol before opening.
2. Once thawed the freezing solution should be placed into 10% serum/90% medium (1 mL freezing solution into 20 mL serum/medium) and spun down at 600*g* for 5 min. This may be repeated and this will effectively remove the DMSO from the cells (*see* **Note 19**).

4. Notes

1. If sufficient material is available, it is desirable to store some of the primary material in liquid nitrogen. This is useful for later characterization.
2. The addition of antibiotics in media added to primary cultures is also a useful precaution and combinations such as penicillin/streptomycin are widely used.
3. Various cocktails of digestive enzymes are used. For example, the cocktail suggested in Chapter 8 for brain tissues include 0.002% DNase type I, 0.01% hyaluronidase type V, and 0.1% collagenase type IV in RPMI 1640 and tissue is treated at 37°C for 1–3 h.
4. A number of strategies are used to help isolate and selectively aid the growth of cancer cells compared to stromal (especially fibroblastic) cells when initially in culture. If the

fibroblasts rapidly adhere but clusters of cancer cells are more loosely attached, these clusters can be made to detach by tapping the flask. The floating clusters are then collected by centrifugation of the medium and resuspended in a new flask. Similarly, trypsinization can be used to separate rapidly attaching/detaching cells from more adherent cell types. Finally, if clear cancer colonies are viewed by microscope in a "sea" of fibroblasts, then after marking the positions of colonies with a marker pen on the base of the flask, use a scraper to mechanically remove the fibroblast overgrowth.

5. Cells from suspensions can be cryopreserved at the outset to allow culture to be set up at a later time.
6. The use of a higher percentage of serum (15–20%) can be helpful at the outset. Depending on the source of the culture, "conditioned medium " that the cells were growing in, e.g., ascitic or pleural effusion fluid, may provide some benefit and can be added at a level of 10% final volume.
7. Subculturing is best performed when cells are still in log phase and at their healthiest.
8. The cells should be trypsinized for the minimum period necessary. This will vary from cell-line to cell-line and may vary from 1 min to longer than 20 min. Tapping the flask when cells have rounded up will also help detachment.
9. Different cell lines will take varying amounts of time to reattach after trypsinization. Many cell lines will have reattached almost completely within 24 h but others take longer. As the cell density is relatively low after subculturing it is important to ensure that the media is well gassed; otherwise, it will become alkaline as the pH increases.
10. When growing in suspension live cells are typically bright when viewed under phase contrast. Viability can be assessed by use of a vital dye such as Trypan blue (*see* **Subheading 3.5.2.**).
11. Most cell lines will readily form single-cell suspension by repeated agitation with a pastette. If this is not sufficient then a 10-mL syringe with an attached needle should be used. Begin with a wide-bore needle and then try smaller-bore needles until a satisfactory suspension is achieved. If a number of samples are to be counted, for example, from a 24-well tray, then it is best to prepare only a few samples at a time as some cells may reclump if left sitting for a prolonged period.
12. If the hemocytometer technique is routinely used, the counter and cover slips may be stored in a small volume of 70% ethanol after cleaning, in readiness for next use.
13. When moistening the counter and cover slip do not overwet them. It is usually sufficient to breathe on the counter or the cover slip prior to bringing them into contact. If this does not work, then cooling the counter by running cold water over it may help. Staining of cells often facilitates visualization and counting. Cells should be treated with 10% formalin (time/conditions) and then stained with Trypan blue or other stain to increase visibility of the cells.
14. The freezing mixture can vary in composition. The key determinant is for the DMSO to be present at 10%. Serum percentages can then vary from 20% to 90% with medium making up the difference. Glycerol can be used instead of DMSO.
15. Cells are frozen at high cell densities and also recovered at high density.
16. Either plastic or glass ampules can be used. Plastic ampules have the advantage of being presterilized and are easy to label; glass ampules, when sealed, are more likely to exclude nitrogen.
17. Cells can be preserved in liquid nitrogen either in the liquid phase (–196°C) or in the vapor phase (–120°C to –156°C).

18. Care should be taken on thawing, as liquid nitrogen may cause ampules to explode. Liquid nitrogen is hazardous and gloves and protective face equipment should be used when handling it. DMSO can penetrate skin and carry dissolved products across the skin barrier, so it should be handled with caution.
19. When a batch of cells is stored away, it is good practice to thaw one of the ampules to confirm viability.
20. General safety considerations include the following:
    a. All cell culture work should be undertaken in a microbiological safety cabinet, preferably Class II.
    b. Aseptic technique should be used at all times in this cabinet.
    c. The work surfaces should be sprayed with alcohol (or similar decontaminant) before and after use.
    d. All biological waste should be autoclaved and liquids or media treated with bleach.
    e. Mouth pipeting should be avoided.

3

Characterization and Authentication of Cancer Cell Lines

An Overview

Simon P. Langdon

1. Introduction

With over 3000 cancer cell lines described in the literature and thousands in regular use, it has become essential to characterize and authenticate cell-line models. Characterization of the properties of cell lines is important for a number of reasons. First, the relationship of the cell line to the cells of origin should be established to confirm that the cell line is derived from and is representative of its tissue of origin. If the cell line is to have any value as a model it should reflect the properties of the cell type from which it was derived. For example, for a cell line established from a breast carcinoma it is helpful to show that the cell line has characteristics consistent with breast and epithelial origin. Although the genetic profile should remain constant, expression may change and features such as differentiation characteristics may be lost over time in culture. Similarly, as the culture develops, certain clones may emerge with selection and predominate. Particularly important is the need to check for purity and potential cross-contamination with other cell lines. The history of cell culture indicates that cross-contamination between cell lines is widely prevalent and continues to be an ongoing problem ***(1–3)***. During the 1970s and 1980s, multiple studies initiated by Stanley Gartler and Walter Nelson-Rees demonstrated that one in three cell lines were either contaminated or even totally replaced by other cell lines ***(4–8)***. The most frequent contaminant was the HeLa cervical carcinoma cell line which had been established in 1951 ***(9)*** and had been widely distributed to many research laboratories. As a result of its rapid growth rate, once mixed with other cell lines it would generally outgrow them. Unfortunately this problem has not disappeared, and contamination continues to be widespread ***(1,2,10)***. This area is covered in more detail in Chapter 30.

From: *Methods in Molecular Medicine, vol. 88: Cancer Cell Culture: Methods and Protocols*
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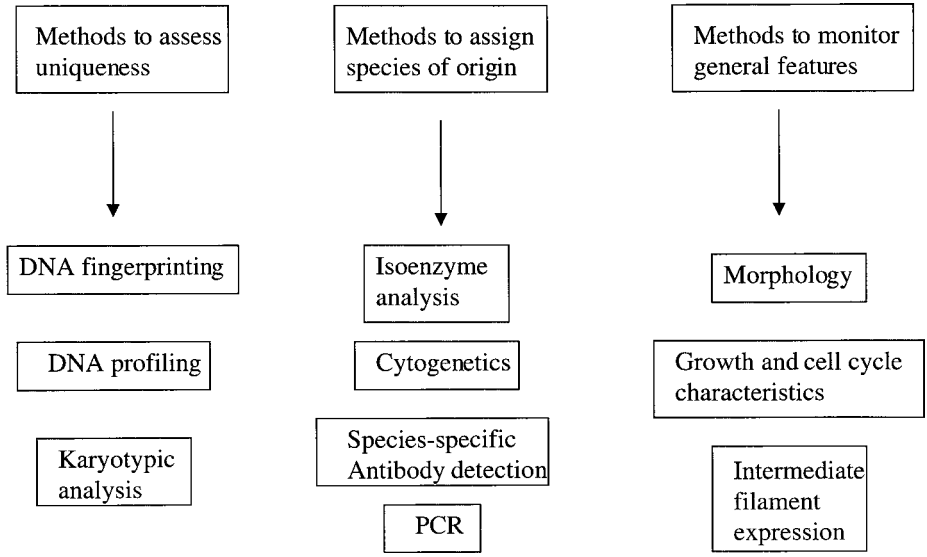


Fig. 1. Methods of characterization for cancer cell lines.

Cross-contamination between cell lines of the same species is not the only issue since contamination with cell lines of other species is also frequent. Therefore methods are required that not only define and confirm the unique nature of a cell line but also identify its species of origin **(Fig. 1)**.

Definitive methods that characterize and authenticate the individuality and identity of a cell line include DNA fingerprinting/profiling and cytogenetic analysis and these are covered in detail in Chapters 4 and 5.

Techniques to verify the species from which the cell line was obtained have been developed and these are valuable not only to confirm the proposed origin of the cell line but to check for the presence of contamination with other cell lines. Other levels of characterization include the monitoring of the general features of the cell line and these include observation of morphology, growth rates, colony-forming efficiency, antigen expression, ploidy, and cell-cycle characteristics.

2. Authentication and Characterization of New Cancer Cell Lines

When a new cell line is being developed, consideration should be given to a number of issues *(11)*. First, the origin of the cell line should be clearly established. This is generally not too great an issue for cell lines being developed from primary tumors but is more relevant if the surgical material is ascites, some form of effusion, or even a metastatic deposit that is not fully validated. The latter is exemplified by the recent redesignation of the SW626 cell line as likely to be of colon origin rather than ovarian cancer origin based on cytokeratin profiles *(12)* although it had been regarded as

an ovarian cancer cell line since 1974. Clearly, the diagnosis of disease should not be in dispute, and as much clinical and pathological information as possible should be collected. Key data include histology, stage and grade of differentiation of the tumor, treatment information (e.g., radiotherapy, chemotherapy), and whether there was a response to treatment. It is essential that material from the origin of the cell line should be stored. This is useful both for pathological verification of the diagnosis (and to ensure that it was representative of the rest of the tumor) and also to provide material for DNA fingerprinting/profiling and/or cytogenetic characterization for comparison with the newly developed cell line.

As the cell line is expanding in culture, it is valuable and important to keep full records of the development of the newly cultured line, in particular, the passage number. It is important to characterize cell lines at an early stage in culture, preferably after only a few passages. Some expansion is needed for two reasons. First, enough material is required to bank away samples of the cell line, maintain it in culture, and for characterization. Second, the primary and early secondary cultures will generally contain a mixture of cell types, for example, a new carcinoma culture in addition to containing epithelial cells may contain other cell types such as fibroblasts or macrophages, and some culturing is needed to allow these to be removed and a pure population to emerge. Next, the new cell line should be tested for the presence of mycoplasma.

Recommendations have been made as to how the cell line might be designated ***(11)***. The key considerations are that its name should be unique (so that it will not become confused with other cell lines) and that it should remain anonymous with respect to the patient. The first derived human cell line, HeLa, was named after the patient donating the cells, Henrietta Lacks; however, in an attempt to preserve some degree of confidentiality the donor was said to be Helen Lane or Helen Larson ***(13)***. This approach is best avoided. Many designations attempt to indicate both the institute where cell line was derived and also its type, for example, SKOV-3 (Sloan Kettering Ovarian Cancer). This is a helpful strategy. If the cell line is to be used extensively, it is important to provide as much information as possible in the first publication of the cell line. Consideration should also be given to whether the cell line should be placed into one of the major cell culture banks.

3. DNA Fingerprinting and DNA Profiling

DNA fingerprinting has proven a valuable approach to authenticating and characterizing cell lines ***(14–16)***. The technique was developed in 1985 by Alec Jeffries and has since found extensive use in forensic DNA typing ***(17,18)***. This technique exploits the variability found within the "noncoding" regions of the human genome, which represent approx 90% of DNA bases. In the 10% of the genome that encodes genes, variation between individuals is very limited. However, within the remaining 90% of bases there is marked diversity between individuals, the assumed result of such regions being of lesser importance for survival. Moreover, it has become evident that large regions of this noncoding DNA are organized into repeat sequences called variable number of tandem repeats (VNTR). Two types of repeat sequences have been defined: minisatellites (repeat lengths of 10–100 bp) and microsatellites or short tan-

dem repeats (STRs) (repeat lengths of 2–5 bp). Many of the initially identified VNTR regions had large numbers of base pairs (20–50) per repeat and the number of repeats could extend from 50 to several hundred. Such a region could therefore vary from 1000 bp to over 10,000. The number of repeats and the length of the VNTR is therefore very characteristic, and in a variable VNTR locus over 95% of the population will have alleles of different lengths (i.e., are heterozogous at this locus). The probability of two unrelated individuals having the same combination of allelic lengths at a specified VNTR locus is much less than 1%.

Several strategies have been developed to fingerprint or profile these variable regions. These strategies can be divided into multilocus and single-locus approaches. Multilocus probes interact with a range of loci throughout the genome, producing "fingerprints" on Southern blot analysis. Analysis of single loci produces "profiles" of single or double bands. Classical DNA profiling has used the large VNTR regions and subjected these to restriction fragment length polymorphism (RFLP) analysis. After digestion with a restriction enzyme, a VNTR region will usually produce restriction fragments of two different lengths, one obtained from the paternal and the other from the maternal chromosome. These are separated by electrophoresis and identified by Southern blot analysis using targeted probes. Autoradiography can then be used to detect the positions of the fragments. Having identified band positions by autoradiography, the membrane can be re-used and, once the first probe is removed, further probes investigated. Analysis of from four to six VNTR loci will then produce a highly specific DNA profile. Alternatively, membranes can be treated with a mixture of probes to produce a "DNA fingerprint." The probes are used at lower stringencies and detect polymorphisms at multiple loci, yielding more information. The major advantage of this method is the exploitation of the high degree of variability in the VNTR regions so the likelihood of two individuals possessing the same DNA profile is extremely low. The limitation of the method is that the restriction fragments may not be clearly separated and it can be difficult to distinguish fragments of similar but different lengths.

Current DNA profiling tends to analyze STR regions in conjunction with polymerase chain reaction (PCR) amplification of DNA. STRs are abundant polymorphic loci and are dispersed throughout the genome. Each STR locus consists of a number of core repeats of 2–5 bp in length, and in practice tetranucleotide repeats have been shown to be very robust for PCR typing. This approach is both faster and less labor intensive than RFLP analysis as many of the steps are automated. While RFLP analyses typically require 20 nanograms of DNA, 1 nanogram is often sufficient for PCR-based analysis. After amplification of the DNA using primers targeted to the STRs, the size of the STR alleles are determined by electrophoresis of the PCR products. Multiple STR loci are analyzed to obtain the same discriminatory power as single locus systems. A recent report has proposed the use of STR profiling for producing an international reference standard for human cell lines ***(19)***. Six unlinked autosomal and one X-linked STR loci were used to determine individual genotypes. This method was authenticated in 253 human cell lines, is relatively inexpensive <$200 per test, and is commercially available.

Cell banks have used a number of DNA fingerprinting techniques. Investigators at the European Collection of Animal Cell Culture (ECACC) have used the probes developed by Jeffreys (33.6 and 33.15) to confirm identity and differentiate between closely related cell lines ***(15,16)***. Investigators at the DSMZ (German Collection of Microorganisms and Cell Cultures) utilize a PCR technique to amplify minisatellite loci (ampFL) in conjunction with RFLP analysis using (GTG)5 multilocus fingerprinting. This provides a sequential use of both fingerprinting approaches. This method is described in Chapter 4.

4. Cytogenetic Analysis

Cytogenetic analysis has proven a useful method to distinguish individual cell lines over the past two decades. Even with the advent of DNA profiling, which is technically more feasible for many laboratories, cytogenetic analysis retains a useful complementary approach to defining and characterizing an individual cancer cell line or culture. It possesses some important advantages over profiling. First, the observation of specific chromosomal changes provides potential clues as to the changed biology in the disease under examination, e.g., the role of the Philadelphia chromosome in chronic myeloid leukemia ***(20)***. Microscopic observation allows the monitoring of diverging subgroups that may not be detected by biochemical sampling of whole populations. The development of fluorescence *in situ* hybridization (FISH) has also made the technique available to the nonexpert cytogeneticist.

Conventional cytogenetics exploits several chromosome staining techniques to help identify chromosomes and their modifications. These include trypsin Giemsa (G) ***(21)***, quinacrine fluorescent (Q)***(22)***, constitutive heterochromatin (C) ***(23)***, and reverse Giemsa (R) staining ***(24)***. These stains produce distinct banding patterns which provide more detailed resolution of the chromosomes. The G-banding technique is in widespread application and uses trypsin to first digest certain chromosomal proteins producing strong staining bands after treatment with Giemsa. Quinacrine (mustard or dihydrochloride) intercalates DNA and generates bands resulting from differential quenching of the fluorescence and produces a pattern different to that of G-staining. C-band staining emphasizes heterochromatin present at the centromeres while R-banding provides a banding pattern different but as informative as G staining. The development of FISH has provided the ability to probe at all levels from whole chromosomes down to individual genes. FISH has several major advantages over traditional cytogenetic techniques. First, the resolution of FISH is often superior to that of classical banding analysis. Second, it can be undertaken independently of the cell cycle since signals can be visualized in interphase nuclei. With the use of different fluorochromes, FISH can simultaneously detect multiple targets. This has led to the development of a number of multicolor applications ***(25)***, including multiplex-FISH (M-FISH) ***(26)***, spectral karyotyping (SKY) ***(27)***, color-changing karyotyping ***(28)***, and combined binary ratio labeling (COBRA) ***(29)***. These applications have provided a means of dealing with the very complex karyotypes found in some tumors and their cell lines. Coincident with the development of these techniques has been the requirement to develop imaging instrumentation and software to visualize (and help interpret) these images.

5. Methods to Verify Species of Origin

A variety of techniques are available to confirm the species of origin of cell lines. These include isoenzyme profiling, cytogenetic analysis, species-specific antibody staining, and PCR methods.

5.1. Isoenzyme Analysis

Certain enzymes exist as isoforms with varying structural configurations (isoenzymes), and the separate isoforms possess different electrophoretic mobilities. Each species has a distinct isoenzyme profile, and this is reflected in characteristic migration patterns when analyzed by gel electrophoresis. Several enzymes have proven particularly useful for the purpose of distinguishing individual cell cultures ***(30-32)*** and identifying the species of origin and they include glucose-6-phosphate dehydrogenase (G6PD), lactate dehydrogenase (LDH), nucleoside phosphorylase (NP), malate dehydrogenase, mannose phosphate isomerase, peptidase B, and aspartate aminotransferase. Cell lysates are first subjected to electrophoresis on an agarose gel and then allowed to react with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) (MTT) in the presence of phenazine methosulfate and specific substrate to form a purple formazan band. Migration ratios of the bands can then be compared against standards or compared with expected ratio values. At least three enzymes are routinely assessed and frequently these are G6PD, LDH, and NP. This technique generally claims to detect a contaminant if present at a level of 10% or above ***(32)*** and has been used by the cell collection banks to authenticate cell lines ***(33)***.

5.2. Cytogenetic Analysis

Cytogenetic techniques can readily identify interspecies contamination in cell lines. Often, the appearances of chromosomes of differing species are sufficiently different that simple microscopic observation of metaphase chromosomes indicates intermixing or an incorrect species designation. A number of approaches have been useful for differentiating between human and mouse chromosomes and have been particularly useful in studies of human/mouse interspecies somatic cell hybrids. These include C-banding and use of the Hoechst 33258 stain, which stains strongly at centromeric regions ***(34)***. Another technique is the alkaline Giemsa staining, which uses color to help differentiate chromosomes from separate species ***(35)***. Simple ploidy, as detected by fluorescence-activated cell scanning (FACS) analysis can also be a simple indicator as different species possess varying DNA contents.

5.3. Fluorescent Antibody Staining

This method involves the use of a species-specific rabbit antiserum to bind to target cells that is then coupled to a fluorescently labeled anti-rabbit globulin. Labeled cells are then observed by use of fluorescence microscopy. The dye fluorescein isothiocyanate (FITC) is routinely used for this method. This technique claims to have the ability to detect the presence of a single contaminating cell in a population of 10,000 ***(36)***.

5.4. PCR

With the increasing availability of PCR in most laboratories, the use of selected genomic targets provides a convenient approach to verifying species of origin ***(37,38)***. The DSMZ uses this approach to verify the species of origin of cell lines (http://www.dsmz.de/mutz/mutzpcra.htm), utilizing primer sets that amplify alu, β-globin, and rDNA sequences. Following the PCR amplification of isolated genomic DNA, agarose gel electrophoresis separates the amplified DNA segments according to size and allows discrimination between cell lines originating from different species. This can be verified further by restriction analysis using specific restriction enzymes to cut the amplified DNA segments to varing lengths.

6. General Characteristics of Cancer Cell Lines

There are a number of simple parameters that are worth monitoring to ensure that cancer cell lines are behaving in a reproducible manner. These are not generally sensitive indicators and are unlikely to reveal subtle changes; however, their alteration should warn that either the cell line is changing, something is amiss in the culture environment, or that contamination (microbial or cellular) has occurred.

6.1. Morphology

Probably the most immediately apparent feature of a cell line is its microscopic appearance. Many cell lines have very characteristic appearances in terms of cell size, shape, and population growth patterns although these may be dependent on culture conditions. For example, cells may have an epithelioid (rounded) or fibroblastic (elongated) morphology that will be dependent on adhesion. Some cell lines grow in very tight colonies while others scatter very readily. Some cell types, such as small-cell lung cancer, will grow as suspension colonies with relatively few adherent cells. Adhesion to the substrate varies enormously and there is a large variation in the rate that cells will either attach to tissue or culture plastics and also detach on trypsinization. Mycoplasma infection can influence adherence, and cultures demonstrating a change in adhesion should be checked for the presence of contamination.

6.2. Cell Growth Rates and Colony Forming Efficiencies

Under regular growth conditions where the same sources of tissue culture reagents are in use, cell lines have relatively constant growth rates and saturation densities. Marked deviation from the regular values is an indication that something may be wrong. Characteristic of a cell line under defined conditions is the percentage of cells that are able to produce colonies, either in a matrix such as agar or on plastic.

6.3. Expression Profiles

Certain proteins can be particularly informative in helping to characterize cells and their origins, and these can be measured by a wide variety of techniques such as Western blotting, FACS analysis, or immunocytochemistry. Intermediate filaments are particularly helpful, including cytokerations (epithelial cells), vimentin (mesenchymal cells), desmin (myogenic cells), neurofilament protein (neurons), and glial fibrillar

acidic protein (glial cells) ***(39,40)***. In addition to the above more general proteins, many cell lines are strongly characterized by the presence of particular proteins. For example, the MCF-7 breast cancer cell line is widely used for expression of high levels of the estrogen receptor and demonstrated responses to it. Similarly the A431 vulval carcinoma line markedly overexpresses the epidermal growth factor receptor and this feature has led to its extensive use.

6.4. Ploidy and Cell Cycle Characteristics

Many laboratories now possess flow cytometers, which can be used to monitor two simple parameters of the cell line, namely DNA content and cell cycle distribution.

Analysis of nuclear DNA content by staining with DNA-interacting agents, such as propidium iodide can indicate first the ploidy content of the cell and secondly the percentage of cells in the differing stages of the cell cycle (G0,G1,S,G2/M).

6.5. Summary

The above general features of cell lines are too nonspecific to be used to define a cell line but are valuable as simple monitors to indicate that a cell line is behaving in a reproducible and defined manner. Sudden changes in these characteristics should alert the tissue culturist to potential problems that may cause unwanted effects and may indicate that more detailed characterization is necessary. Often, if a problem is not persistent, it is easiest to discontinue a culture and obtain fresh material from liquid nitrogen. Similarly, if a cell line is maintained for only a limited period of time in culture (for example, 10 passages) before replacement with fresh stocks, then phenotypic drift should be minimal.

References

1. O'Brien, S. J. (2000) Cell culture forensics. *Proc. Natl. Acad. Sci. USA* **98,** 7656–7658.
2. Stacey, G. N., Masters, J. R. W., Hay, R. J., Drexler, H. G., Macleod, R. A. F., and Freshney, R. I. (2000) Cell contamination leads to inaccurate data: we must take action now. *Nature* **403,** 356.
3. Macleod, R. A. F., Dirks, W. G., and Drexler, H. G. (2002) Persistent use of misidentified cell lines and its prevention. *Genes Chromosomes Cancer* **33,** 103–105.
4. Gartler, S. M. (1968) Apparent HeLa contamination of human heterodiploid cell lines. *Nature* **217,** 750–751.
5. Nelson-Rees, W. A., Flandermeyer, R. R., and Hawthorne P. K. (1974) Banded marker chromosomes as indicators of intraspecies cellular contamination. *Science* **184,** 1093–1096.
6. Nelson-Rees, W. A., and Flandermeyer, R. R. (1976) HeLa cultures defined. *Science* **191,** 96–98.
7. Nelson-Rees, W. A., Daniels, D. W., and Flandermeyer, R. R. (1981) Cross-contamination of cells in culture. *Science* **212,** 446–452.
8. Nelson-Rees, W. A. and Flandermeyer, R. R. (1977). Inter- and intraspecies contamination of human breast tumor cell lines HBC and BrCa5 and other cell cultures. *Science* **195,** 1343–1344.
9. Gey, G. O., Coffman, W. D., and Kubicek, M. T. (1952) Tissue culture studies of the proliferative capacity of cervical carcinoma and normal epithelium. *Cancer Res.* **12,** 264–265.

10. MacLeod, R. A. F., Dirks, W. G., Matsuo, Y., Kaufmann, M., Milch, H., and Drexler, H. G. (1999) Widespread intraspecies cross-contamination of human tumor cell lines arising at source. *Int. J. Cancer* **83,** 555–563.
11. UKCCCR (2000) UKCCCR guidelines for the use of cell lines in cancer research. *Brit. J. Cancer* **82,** 1495–15091.
12. Furlong, M. T., Hough, C. D., Sherman-Baust, C. A., Pizer, E. S., and Morin, P. J. (1999) Evidence for the colonic origin of ovarian cancer cell line SW626. *J. Natl. Cancer Inst.* **91,** 1327–1328.
13. Masters, J. R. (2002) HeLa cells 50 years on: the good, the bad and the ugly. *Nat. Rev. Cancer* **2,** 315–319.
14. Gilbert, D. A., Reid, Y. A., Gail, M. H., Pee, D., White, C., Hay, R. J., et al. Application of DNA fingerprinting for cell-line individualization. *Am. J. Hum. Genet.* **47,** 499–514.
15. Stacey, G., Bolton, B., Doyle, A., and Griffiths, B. (1992) DNA fingerprinting—a valuable new technique for the characterization of cell lines. *Cytotechnol* **9,** 211–216.
16. Stacey, G. N., Bolton, B. J., and Doyle, A. (1991) The quality control of cell banks using DNA fingerprinting. *EXS* **58,** 361–370.
17. Jeffreys, A. J., Wilson, V., and Thein, S. L. (1985) Hypervariable "minisatellite" regions in human DNA. *Nature* **314,** 67–73.
18. Jeffreys, A. J., Wilson, V., and Thein, S. L. (1985) Individual-specific "fingerprints" of human DNA. *Nature* **314,** 76–79.
19. Masters, J. R., Thomson, J. A., Daly-Burns, B. et al., (2001) Short tandem repeat profiling provides an international reference standard for human cell lines. *Proc. Natl. Acad. Sci. USA* **98,** 8012–8017.
20. Rowley, J. D. (1973) A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining. *Nature* **243,** 290–293.
21. Seabright, M. (1973) Improvement of trypsin method for banding chromosomes. *Lancet***1,** 1249–1250.
22. Caspersson, T., Zech, L., and Johansson, C. (1970) Differential binding of alkylating fluorochromes in human chromosomes. *Exp. Cell Res.* **60,** 315–319.
23. Pardue, M. L. and Gall, J. G. (1970) Chromosome localization of mouse satellite DNA. *Science* **168,** 1356–1358.
24. Dutrillaux, B., Finaz, C., de Grouchy, J. and Lejeune, J. (1972) Comparison of banding patterns of human chromosomes obtained with heating, fluorescence, and proteolytic digestion. *Cytogenetics* **11,** 113–116.
25. Lichter, P. (1997) Multicolor FISHing: what's the catch. *Trends Genet* **13,** 475–479.
26. Speicher, M. R., Ballard, S. G., and Ward, D. C. (1996) Karyotyping human chromosomes by combinatorial multi-fluor FISH. *Nature Genet.* **12,** 368–375.
27. Schrock, E., du Manoir, S., Veldman, T. et al. (1996) Multicolor spectral karyotyping of human chromosomes. *Science* **273,** 494–497.
28. Henegariu, O., Heerema, N. A., Bray-Ward, P. et al. (1999) Colour-changing Karyotyping: an alternative to M-FISH/SKY. *Nat. Genet.* **23,** 263–264.
29. Tanke, H. J., Wiegant, J., van Gijlswijk, R. P. M. et al. (1999) New strategy for multicolour fluorescence *in situ* hybridisation: COBRA: combined binary ratio labeling. *Eur. J. Hum. Genet*. **7,** 2–11.
30. O'Brien, S. J., Kleiner, G., Olson, R., and Shannon, J. E. (1977) Enzyme polymorphisms as genetic signatures in human cell cultures. *Science* **195,** 1345–1348.

31. O'Brien, S. J., Shannon, J. E., and Gail, M. H. (1980) A molecular approach to the identification and individualization of human and animal cells in culture: isozyme and allozyme genetic signatures. *In Vitro* **16,** 119–135.
32. Nims, R. W., Shoemaker, A. P., Bauernschub, M. A., Rec, L. J., and Harbell, J. W. (1998) Sensitivity of isoenzyme analysis for the detection of interspecies cell line cross-32. contamination. *In Vitro Cell. Dev. Biol. Anim.* **34,** 35–39.
33. Steube, K. G., Grunicke, D., and Drexler, H. G. (1995) Isoenzyme analysis as a rapid method for the examination of the species identity of cell cultures. *In Vitro Cell. Dev. Biol. Anim.* **31,** 115–119.
34. Lin, M. A., Latt, S. A., and Davidson, R. L. (1974) Identification of human and mouse chromosomes in human-mouse hybrids by centromere fluorescence. *Exp. Cell Res.* **87,** 429–433.
35. Friend, K. K., Dorman, B. P., Kucherlapati, R. S., and Ruddle, F. H. (1976) Detection of interspecies translocations in mouse-human hybrids by alkaline Giemsa staining. *Exp. Cell Res.* **99,** 31–36.
36. Stulberg, C. S. (1973) Extrinsic cell contamination of tissue culture. In: Fogh J (ed). *Contamination in Cell Cultures,* Academic Press, New York, pp. 1–23.
37. Thacker, J. (1993) Fingerprinting of mammalian cell lines with a single PCR primer. *Biotechniques* **16,** 252–253.
38. Nelson, D. L., Ledbetter, S. A., Corbo, L. et al. (1989) Alu polymerase chain reaction: A method for rapid isolation of human-specific sequences from complex DNA sources. *Proc. Natl. Acad. Sci. USA* **86,** 6686–6690.
39. Puts J. J., Vooijs G. P., Huysmans A. et al. (1986) Cytoskeletal proteins as tissue-specific markers in cytopathology. *Exp. Cell Biol.* **54,** 73–79.
40. Quentmeier H., Osborn M., Reinhardt J., et al. (2001) Immunocytochemical analysis of cell lines derived from solid tumors. *J. Histochem. Cytochem.* **49,** 1369–1378

4

Authentication of Cancer Cell Lines by DNA Fingerprinting

Wilhelm G. Dirks and Hans G. Drexler

1. Introduction

1.1. History of Cell Line Identification

The requirement for authentication of cell lines has a history almost as long as cell culturing itself, presumably beginning when more then one cell line could be cultured continuously. The application of specific species markers, including cell-surface antigens and chromosomes, showed that interspecies misidentification was a widespread problem ***(1,2)***. Subsequently, it was shown that intraspecies contamination of human cell cultures was also a serious problem that could be monitored by the innovation of isoenzymatic analysis ***(3).*** After extending this approach to multiple polymorphic isoenzymes, the persistence of specific marker chromosomes in long-term-passaged cell lines demonstrated the unique power of cytogenetics ***(4)***. Based on the detection of chromosomal markers, it was convincingly demonstrated that multiple cell lines under active investigations were actually derived from one source, namely the HeLa cell line ***(5)***. Furthermore, cross-contamination among established cell lines occurred at frequencies as high as 16–35% in the late 1970s ***(6)***. Recently, our department demonstrated an incidence of 18% of false human cell lines ***(7–9)***, indicating intraspecies cross-contaminations as a chronic problem and highlighting the badly neglected need for intensive quality controls regarding cell line authenticity.

1.2. DNA Fingerprinting Technologies

Compared to polymorphic isoenzymes or marker chromosomes, a much higher resolution in discrimination among human cell lines was achieved using restriction fragment length polymorphism (RFLP) of simple trinucleotide repetitive sequences ***(10)***, subsequently leading to the concept of "DNA fingerprinting" ***(11)***. The principle of "DNA fingerprinting" is based on the phenomenon that genomes of higher organisms harbor multiple variable number of tandem repeat (VNTR) regions, show-

From: *Methods in Molecular Medicine, vol. 88: Cancer Cell Culture: Methods and Protocols*
Edited by: S. P. Langdon © Humana Press Inc., Totowa, NJ

Table 1
Amplifiable Human Fragment Length Polymorphism Loci

Designation	Status	Synonym	Chromosomal location	Repeat length (bp)	Product length (bp)	Heterozygosity (%)
Minisatelite VNTR						
Apo-B1	SLS	—	2p23-p24	15	522–909	80[a]
Col2A1	SLS	—	12q12-q13.1	31–34	600–850	81[b]
D1S80	SLS	MCT118	1	16	400–940	85[c]
D17S5	SLS	YNZ22	17p13.3	70	168–1080	78[d]
D2S44	SLS	YNH24	2pter	31	600 to >5000	97[e]
PAH	SLS	—	12q22-q24.2	30	370–760	78[f]
Microsatelite STR						
(GTG)N	MLS	—	Chromos. ends	3	10 to >15000	>99.9[g]

VNTR, variable number of tandem repeats; STR, short tandem repeats; SLS, single-locus system; MLS, multiple-locus system.

[a-g]With regard to heterozygosity, *see* Dirks et al. ***(9)***.

ing multi-allelic variation among individuals ***(12)***. Sequence analysis demonstrated that the structural basis for polymorphism of these regions is the presence of tandem-repetitive, nearly identical DNA elements which are inherited in a Mendelian way. Depending on the length of the repeats, VNTRs are classified into minisatellites, consisting of 9 to >70-bp core sequences and microsatellites, which include all short tandem repeats (STRs) with core sizes from 1–6 bp (*see* **Table 1**).

Both categories of repeats can be governed by one definite locus or are spread all over the genome and belong to the single-locus system (SLS) or multiple-locus system (MLS), respectively. Using SLS fingerprinting, a few loci used in sequential combination can distinguish between two individuals who are not identical twins. MLS fingerprints using hundreds of relevant polymorphic loci in a single step have generally a higher resolution potential but also a restriction to classical restriction fragment length polymorphism (RFLP) analyses. With the advent of the polymerase chain reaction (PCR) technology and availability of complete sequence information of the human genome, DNA fingerprinting has been revolutionized. The number of accurately mapped mini- and microsatellite markers available are greater by 100-fold for each chromosome. The primer sequences for amplification of specific STRs or VNTRs, as well as the information on the PCR product sizes and estimated heterozygosity, are available from a number of genome databases within the World Wide Web (*see* **Note 1**).

1.3. Tasks of DNA Fingerprinting in Scientific Cell Culture

Most facilities culturing cells use multiple cell lines simultaneously. Because of the complexity of experimental designs today and because of the fact that the broad use of

cell lines in science and biotechnology continues to increase, the possibility of inadvertent mixture of cell lines during the course of day-to-day cell culture is always present. Based on the reputation of a laboratory, the information on an exchanged cell line within a scientific cooperation is normally thought to be correct. This is the main reason for the increasing problem of cross-contaminated cell cultures all over the world. However, cross-contaminations of cell cultures seem to appear as the "peccadillo" of scientists, since publication of alerts on misidentified or cross-contaminated cell lines are most often simply ignored by the scientific community. Routine identification and early detection of contamination of a given cell line with another should prevent mistaken interpretation of experimental results.

A typical modern laboratory applying molecular biology and cell culture techniques is normally not equipped with expensive robots and kits for forensic DNA profiling. Therefore, the purpose of this chapter is to describe a rapid, practical, inexpensive, and reliable method with a high discrimination potential for students, technicians, and scientists. The fingerprinting protocols below are based on standard techniques including PCR and enable the following features:

1. Authentication of a newly generated and immortalized cell line if DNA of the respective patient/donor is available (*see* **Note 2**).
2. Confirmation of cell line identity between different passages of an intensively used cell line, e. g., the human embryonic kidney cell line 293 (HEK 293; *see* **Note 3**).
3. Evaluation of "purity" of a given cell line and detection of the percentage of cell mixture in an early stage of contamination.
4. Characterization of somatic hybrid cell lines involving human cells.
5. Mapping of loss of heterozygosity of chromosomal regions, for example, to detect tumor suppressor genes.

The following subheadings present the technique of multiplexed PCR amplification fragment length polymorphism (AmpFLP) of six prominent and highly polymorphic minisatellite VNTR loci (*see* **Table 1**) and one additional locus for sex determination using the detection of the SRY gene on the Y chromosome. The combination of six VNTRs increases the exclusion rate to a sufficient extent and allows discrimination of one human cell line from another at the level of 10^6. In order to rule out any false positives, it is highly recommended that suspicious cell lines are tested further using the multi-locus fingerprint system if they reveal identical or similar DNA profiles based on AmpFLP VNTR. The combination of rapidly generated DNA profiles based on single-locus VNTR loci and confirmation of duplicate AmpFLP banding patterns using multi-locus fingerprints, constitutes a highly reliable and robust method independent of the quantity of individual cell lines examined.

2. Materials

2.1. DNA Extraction

1. Phosphate-buffered saline (PBS): 140 m*M* NaCl, 27 m*M* KCl, 7.2 m*M* $Na_2HPO_4 \cdot 12\ H_2O$, 14.7 m*M* KH_2PO_4, pH 7.2; autoclave.
2. Absolute isopropanol and absolute ethanol.
3. TE 10/1: 10 m*M* Tris-HCL, 1 m*M* EDTA, pH 8.0, prewarmed to 50°C.

Table 2
Primer Sequences of Highly Polymorphic Human VNTR Loci

Primer designation	Primer sequences
ApoB1-F	5'-ATGGAAACGGAGAAATTATGGAGGG-3'
ApoB1-R	5'-CCTTCTCACTTGGCAAATACAATTCC-3'
D1S80-F	5'-GAAACTGGCCTCCAAACACTGCCCGCCG-3'
D1S80-R	5'-GTCTTGTTGGAGATGCACGTGCCCCTTGC-3'
D17S5-F	5'-AAACTGCAGAGAGAAAGGTCGAAGAGTGAAGTG3'
D17S5-R	5'-AAAGGATCCCCCACATCCGCTCCCCAAGTT-3'
D2S44-F	5'-AGCAGTGAGGGAGGGGTGAGTTCAAGAG-3'
D2S44-R	5'-GAAAACACTTCAGTGTATCTCCTACTCC-3'
COL2A1-F	5'-CCAGGTTAAGGTTGACAGCT-3'
COL2A1-R	5'-GTCATGAACTAGCTCTGGTG-3'
PAH-F	5'-GTTATGTGATGGATATGCTAATTAC-3'
PAH-R	5'-GTGGTGTATATATATGTGTGCAATAC-3'
SRY-F	5'-CTCTTCCTTCCTTTGCACTG-3'
SRY-R	5'-CCACTGGTATCCCAGCTGC-3'

4. High Pure PCR Template Preparation Kit (Roche) (*see* **Note 4**).
5. Water bath prewarmed to 72°C.
6. Standard tabletop microcentrifuge capable of 13,000*g* centrifugal force.

2.2 Multiplex PCR Fingerprinting

1. Thermal cycler: Perkin Elmer Cetus 480 (*see* **Note 5**).
2. *Taq* DNA polymerase (Qiagen); 10X PCR reaction buffer (Qiagen); 5X Q-solution (Qiagen).
3. 6X Loading buffer: 0.01% (w/v) bromophenol blue, 0.01% (w/v) xylene cyanol, 60% glycerol (v/v), 60 m*M* EDTA.
4. Primer (any supplier): (*see* **Table 2**). The primers should be concentrated at 100 µ*M* in TE (10/1) as stock solutions and stored at –20°C, while working solutions should be aliquoted at 10 µ*M* in small amounts (approx 25–50-µL aliquots) and stored frozen at –20°C.

2.3. Multi-locus DNA Fingerprinting

2.3.1. Restriction Endonuclease Digestion

1. 2-mL reaction tubes.
2. Multiblock heater.
3. Microcentrifuge.
4. Disposable pipets.
5. Restriction endonuclease *Hin*f I (high concentration 50 U/µL).
6. 10X *Hin*f I restriction buffer.
7. 5X gel loading buffer.
8. Bidistilled water

9. Absolute isopropanol (–20°C).
10. 70% (v/v) ethanol (–20°C).

2.3.2. Agarose Gel Electrophoresis.

1. 500-mL Erlenmeyer flask.
2. 1X Tris-Acetate-EDTA (TAE)-buffer: (40X TAE stock solution: 1.6 *M* Trizma base, 0.8 *M* Na-Acetate, 40 m*M* EDTA. Adjust pH to 7.2 with glacial acetic acid).
3. Ultra Pure agarose.
4. Microwave oven.
5. Electrophoresis system consisting of gel tray and comb, electrophoresis chamber and power supply, digoxigenin-labeled molecular weight DNA marker II (Roche), ethidium bromide (5 mg/mL), and UV-transillumination screen.

2.3.3. Blotting and Hybridization

1. Whatman paper.
2. Nylon membrane positively charged (Roche).
3. Parafilm, 2 glass plates (30 cm × 30 cm), paper towels, 500-g weight, 0.4 *M* NaOH.
4. Oven capable of 120°C temperature for DNA fixation.
5. Rotatable hybridization oven, plastic wrap.
6. 6X SSC: 0.9 *M* NaCl, sodium citrate, pH 7.0), 0.4% (v/w) blocking reagent (Roche) in 6X SSC, pH 7.0.
7. Prehybridization solution: 28 mL bidistilled water, 16 mL 25% (v/w) dextran sulfate, 4 mL 10% (v/w) SDS, 2.32 g NaCl, 100–200 µg/mL of heat-denatured E. coli DNA.
8. Hybridization solution: 10 mL of prehybridization solution complemented with 100-130 pmol of the digoxigenin-labeled oligonucleotide $(GTG)_5$ for 150 cm^2 of membrane.
9. Stringency washes: wash buffer I : 3 X SSC (pH 7.5) ; wash buffer II : 3 X SSC (pH7.5), 0.1% SDS.

2.3.4. Chemiluminescent Detection of Digoxigenin-Labeled DNA

1. Maleic acid buffer: 0.1 *M* maleic acid, 0.9 *M* NaCl; adjust pH to 7.5 with conc. NaOH, autoclave.
2. 10% (v/w) blocking reagent in maleic acid buffer.
3. Wash buffer A: 0.3 % (v/v) Tween-20 in maleic acid buffer.
4. Activation buffer: 0.1 *M* Tris-HCl, 0.9 *M* NaCl, 50 m*M* $MgCl_2$; adjust pH to 9.5 with HCl.
5. Substrate solution: CSPD (Roche) 1/100 dilution in activation buffer; X-ray developer, X-ray fixer, X-ray film, X-ray film cassettes.

3. Methods

3.1. Preparation of High-Molecular-Weight DNA

The principle of this assay is that cells are lysed during a short incubation time with proteinase K in the presence of a chaotropic salt (guanidinium-hydrochloride), which immediately inactivates all nucleases. Nucleic acids bind selectively to glass fibers prepacked in the filter tube. Bound genomic DNA is purified in a series of rapid washing and spinning steps to remove inhibiting cellular components. Finally, low salt elution releases the DNA from the glass fiber cushion.

1. In order to prevent isolation of DNA fragments from apoptotic cells take the cell lines to be tested for identity from cell cultures with viabilities over 80%.

2. Centrifuge the cell culture suspension containing 3-5 × 10^6 diploid cells in a 15-mL tube at 2000*g* for 4 min. Remove the supernatant with a disposable pipet and discard. Resuspend the remaining pellet carefully in 5 mL PBS using a pipet and centrifuge again.
3. After the washing step, resuspend the pellet in 200 µL PBS by vortexing. Make sure that tiny clumps of cells are carefully resuspended. Prewarm the waterbath to 72°C.
4. To isolate the genomic DNA, the commercially available DNA extraction kit from Roche is applied. Add 200 µL of solution I (guanidinium-hydrochloride), well mixed, to the sample solution and mix by pipeting.
5. Immediately add 40 µL proteinase K, mix well using a vortex, and incubate at 72°C for 10 min.
6. Add 100 µL of isopropanol to the sample, mix well, and apply the whole mixture to a filter tube. Centrifuge for 1 min at 6000*g*.
7. Discard the flow-through, add 500 µL of inhibitor removal buffer, and centrifuge again for 1 min at 6000*g*.
8. Discard the flow-through, add 500 µL of wash buffer, and centrifuge again for 1 min at 6000*g*.
9. Repeat **step 8.**
10. Add a new tube and 200 µL of elution buffer preheated to 72°C and centrifuge for 1 min at 6000*g*. For maximum yield repeat the elution step using 100 µL elution buffer. The purified genomic DNA concentration should be approx 10 ng/mL per sample, depending on the ploidy status of the cell line used. The genomic DNA should be stored at 4°C.

3.2. Hotstart Multiplex PCR Fingerprinting

The amplification procedure and the parameters described here are optimized for the application in 0.5-mL reaction tubes in a Perkin Elmer DNA Thermal Cycler 480 (*see* **Note 5**). An adjustment for any other equipment may be necessary. Follow strictly general rules to avoid DNA carryover contaminations.

1. Carry out DNA extraction using equipment (pipets, microcentrifuge, etc.) that is independent from the PCR set up. Optimally, separate the laboratory used for this procedure from rooms where the PCR reaction is set up or the PCR products are analyzed.
2. Use reagents that have been stored in small aliquots to provide a constant source of uncontaminated reagents. Test new aliquot batches and compare for quality prior to use.
3. Never carry out reamplifications.
4. If possible, set up the reactions in a PCR working station or a hood that allows for irradiation of used pipets, tips, and tubes by UV-light.
5. It is highly recommended that gloves be worn during the whole procedure.

Furthermore, it is also fundamental to integrate the appropriate positive and negative controls, e.g., HeLa DNA and H_2O, respectively). Prepare a premaster mix calculated for 50 µL per reaction of each sample, plus 1 additional reaction according to **Table 3.**We recommend using colored tubes for aliquots of primer stocks as well as for the premaster mixtures and PCR reactions for the individual loci.

1. Transfer 40 µL of the premaster mix to 0.5 mL PCR reaction tubes according to the sample number. Add 1 µL dH_2O to the water control reaction.
2. Prepare the *Taq* DNA polymerase mix (10 µL per reaction, plus 1 additional reaction) containing 1X PCR buffer and 1 U *Taq* polymerase per reaction. Keep the mixture on ice.

Table 3
Preparation of Premaster Mixtures for Printing Individual Single-Locus Spots

Stock-solution	Apo-B1 (blue)	D17S5 (red)	D1S80 (yellow)	D2S44 (green)	Col2A1 (white)	PAH (orange)	SRY (pink)
10X PCR buffer	4	4	4	4	4	4	4
5X Q-solution	—	10	10	10	10	—	10
dNTP (2 µM)	1	1	1	1	1	1	1
Forward/reverse primer	1	1	0.5	2	1	1	1
Bidest. H_20	33	23	24	18	23	33	23
DNA (10–20 ng/µL)	1	1	0.5	5	1	1	1

3. Store all reagents used for the preparation of the master mix and take out the samples of DNA to be tested. Do not handle the reagents and samples simultaneously or with the same gloves. Add 1 µL of the DNA preparation (approx 10–20 ng) to the reaction solutions.
4. Transfer the reaction mixtures of **step 1** without *Taq* polymerase to the thermal cycler, add carefully a drop of oil (if necessary) and start one cycle with the following parameters:

   Cycle step 1: 5 min at 95°C
   Cycle step 2: 3 min at 75°C
   Cycle step 3: 2 min at 55°C (or 62°C for D2S44 only, *see* below)
   Cycle step 4: 5 min at 72°C

   During cycle step 2, open the thermal lid and add 10 µL of the *Taq* polymerase mix to each tube to perform a hot start PCR. For many samples, the duration of this step can be prolonged. Open and close each reaction tube separately to prevent evaporation of the samples when no oil is used. Allow at least 30 s after closing the lid of the thermal cycler to equilibrate the temperature within the tubes and to remove condensate from the lid before continuing to the next cycle step.
5. After this initial cycle, perform 35 thermal cycles with the following parameters:

Common program	*D2S44 only*
Cycle step 1: 4 s at 95°C	4 s at 95°C
Cycle step 2: 1 min at 55°C	2 min at 62°C
Cycle step 3: 1 min at 72°C	2 min at 72°C

   plus 1 s of extension time during each cycle.

6. The reaction is finished by a final amplification step at 72°C for 10 min and the samples are then cooled to room temperature.
7. Prepare a 1.2% agarose-TAE gel containing 0.1 µg/mL of ethidium bromide. Submerge the gel in 1 X TAE and add 10 µL of the amplification product (8 µL reaction mix plus 2 µL of 6X loading buffer to each well) and run the gel at 10 V/cm. Visualize the specific products on a UV screen and save the image for documentation (*see* **Fig. 1**).
8. With regard to precise fingerprinting, we recommend using a gel-analyzing software program (any supplier) capable of fragment length determination and of saving the DNA profiles into a database.

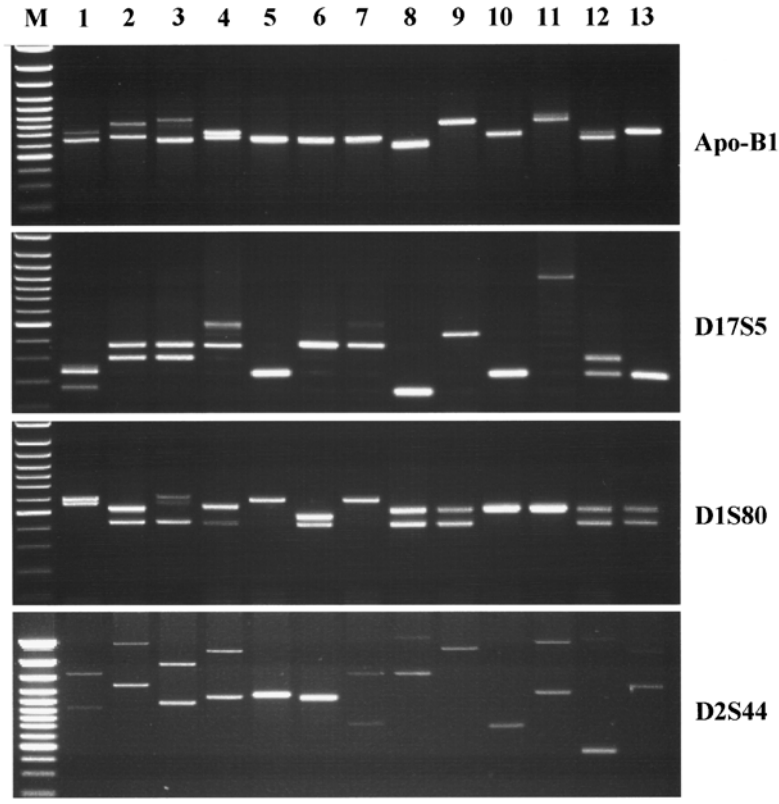


Fig. 1. AmpFLP VNTR DNA profiles of prominent human cross-contaminating cell lines. Genomic DNA of cell lines was used to amplify alleles from Apo-B1, D17S5, D1S80, and D2S44. After size determination of the amplicons from each loci on a separate gel using gel analyzing software, the data are entered in a database, creating a specific DNA profile for each cell line. Lanes 1–13: 1, HeLa, human cervix carcinoma; 2, CCRF-CEM, human T-cell leukemia; 3, CTV-1, human acute myeloid leukemia; 4, NALM-6, human B cell precursor leukemia; 5, HEL, human erythroleukemia; 6, SCLC-21H, human small cell lung carcinoma; 7, DU-145, human prostate carcinoma; 8, T-24, human urinary bladder carcinoma; 9, COLO-800, human melanoma; 10, U-937, human histiocytic lymphoma; 11, HT-29, human colon adenocarcinoma; 12, SK-HEP-1, human liver adenocarcinoma; 13, K-562, human chronic myeloid leukemia in blast crisis.

3.3. High Resolution Multi-locus DNA Fingerprinting

DNA profiles generated by the use of multi-locus probes result in a banding pattern harboring 30–50 bands in each lane. It is important to place suspicious cell lines in neighboring lanes, if they have revealed identical or similar DNA profiles based on the AmpFLP VNTRs technique.

3.3.1. Restriction Endonuclease Digestion of Genomic DNA

1. Adjust every DNA sample containing 25 µg genomic DNA to 800 µL of volume using TE (10/1) buffer. (Do not vortex the DNA solution prior to restriction endonuclease digestion, as this may cause random shearing.) Set-up the following reaction in a 2.0 mL sterile reaction tube:

10X Enzyme buffer:	100 µL
Restriction enzyme:	150 U
25 µg DNA:	800 µL
Adjust with TE buffer (10/1):	1000 µL

2. After adding all components, flick tubes briefly and spin the tubes in a microcentrifuge for a few seconds. Incubate in a 37°C multiblock heater or water bath for 4 h on a slow shaking platform (*see* **Note 6**).
3. Add 800 µL of ice-cold isopropanol and flick tubes to mix.
4. Centrifuge at 13,000g for 30 min. Remove isopropanol immediately using a stretched glass pipet.
5. Add 200 µL of ice-cold 70% ethanol; vortex the tubes to wash DNA (may be stored overnight or longer at –70°C). Remove as much as possible of the ethanol and allow to air dry for about 10 min. (To prevent difficulties on resuspension, do not over-dry DNA pellets in a speed-vac.)
6. Resuspend DNA pellet in 20 µL of TE buffer. Pipeting up and down a few times accelerates the resuspension process. Add 10 µL of gel loading buffer, vortex briefly, and microcentrifuge for a few seconds before loading the gel.

3.3.2. Agarose Gel Electrophoresis

1. To prepare a 0.7 % agarose solution, add 1.75 g of agarose to 250 mL 1X TAE buffer in a 500 mL Erlenmeyer flask.
2. Dissolve the agarose solution by heating in a microwave oven. When the agarose is completely dissolved, replace the water lost from evaporation with double-distilled water and mix gently.
3. Allow the agarose solution to cool down to 55–60°C before pouring into the gel mold.
4. While the agarose is cooling, prepare the gel mold by applying two pieces of autoclave tape across the open ends. Place the gel mold on a level surface. Pour the cooled agarose solution over the entire gel mold. Break any bubbles that may be present and immediately insert the toothcomb (1-mm thickness recommended).
5. Allow the agarose to solidify for 30 min and another 30 min at 4°C.
6. Remove the tapes from each end of the gel mold and place it into the electrophoresis apparatus. (If possible, the gel run should be carried out at 4°C, otherwise cool down the running buffer on ice.)
7. Fill the electrophoresis chamber with 1X TAE so that the buffer is about 5 mm above the surface of the gel.
8. Load samples and markers (digoxigenin-labeled analytical markers and 1 kb ladder) into the designated wells.
9. Electrophorese, with circulating buffer, at 40 V (constant voltage) for 19 h or until the bromophenol blue dye has migrated to the bottom edge of the gel.
10. Stain DNA in the gel with ethidium bromide solution for 20 min and destain with two rinses of double distilled water.

11, Photo documentation: Carefully slide the gel onto the transilluminator and photograph the gel with an autoradiogram ruler placed adjacent to the marker lane (1 kb ladder) for orientation.
12. Trim excess of agarose from the gel, and prepare for Southern transfer.

3.3.3. Southern Blotting and DNA Fixation

The objective is to set up a flow of buffer from the reservoir through the gel and the membrane, so that the DNA fragments are eluted from the gel and transferred onto the membrane, where they will be fixed.

1. A box (22 × 22 cm) should be wrapped with 3MM Whatman paper and placed in the middle of the buffer tray or dish.
2. Apply a plastic frame with a window (for example, Parafilm stripes) that is 1 cm shorter in length and width than the size of the gel and place it onto the wick. The Parafilm serves as a barrier to prevent transfer buffer from bypassing the gel (short circuiting).
3. Fill the buffer tray with 0.4 *M* NaOH (at least 500 mL) until the solution is 3 cm underneath the gel. Carefully place the gel, wells facing down, on the wick in the middle of the plastic frame and ensure that the edges of the gel and the frame have a 0.5-cm overlap at each site. Avoid scratching the back of the gel!
4. Center the nylon membrane on top of the gel and wet the membrane with 0.4 *M* NaOH. Remove all bubbles between the membrane, gel, and wick by rolling smoothly over the surface of the membrane using a 10-mL pipet. Do not apply too much pressure to the gel as this may cause distortion.
5. Wet two pieces of Whatman 3MM paper of the same size as the gel in 0.4 *M* NaOH and place them on top of the membrane. Remove air bubbles between the membrane and the Whatman 3MM paper.
6. Layer on a 3-in stack of paper towels, place the glass plate and then the 500-g weight on top of the stack, and ensure that the weight and paper towels cannot tilt.
7. Allow the transfer to proceed for 8–20 h.
8. Remove paper towels and mark the position of the comb slots and the outline of the gel onthe membrane with a fine-up marker.
9. Place the damp membrane on a piece of Whatman 3MM paper and bake in an oven at 120°C for 30 min.
10. Remove the membrane from the oven. The membrane can be used immediately for prehybridization/hybridization or stored dry at 4°C.

3.3.4. Preblocking, Prehybridization, and Hybridization

Prehybridization prepares the membrane for probe hybridization by blocking nonspecific nucleic acid binding sites to reduce background activity. It is recommended that a rotating hybridization oven be used; otherwise carry out the following coating procedure of the membrane using a plastic bag and a waterbath.

1. Place the membrane in a plastic bag with 10 mL 0.5% blocking reagent buffer and shake the membrane slowly for 1 h on a rotating platform at room temperature.
2. Incubate the membrane in 5–10 mL prehybridization solution prewarmed to 42°C in a rotating hybridization oven for 4 h.
3. Discard the prehybridization solution and replace it with 5–10 mL of prewarmed hybridization solution containing 10–20 pmol/mL of digoxigenin-labeled oligonucleotide GTG_5 (*see* **Note 7**).

4. After incubation overnight with rotating, discard the hybridization solution and replace with prewarmed wash buffer I. Wash the membrane for 25 min at 42°C and repeat this step.
5. Wash membrane twice using prewarmed wash buffer II for 10 min. Proceed immediately to the detection procedure or store the membrane in wash buffer I at 4°C.

3.3.5. Chemiluminescent Detection of Digoxigenin-Labeled DNA

Chemiluminescent detection is a three-step process whereby the membrane is first treated with a milk powder solution to prevent nonspecific attraction of the antibody to the membrane. Second, the membrane is incubated with a dilution of antidigoxigenin Fab fragments, and finally incubated with a chemiluminescent solution, which is the substrate for generation of photons recorded on a X-ray film. Keep the membrane wet throughout the following procedure:

1. Block the membrane in 150 mL of 2% blocking buffer for 30 min at room temperature using a clean dish. Centrifuge the antibody at 15,700*g* for 1 min in order to reduce background activity. Dilute the antidigoxigenin alkaline phosphatase Fab fragments 1/10,000 in a volume of 20 mL (approx 150 mU/mL).
2. Place the membrane in a heat-sealable plastic bag which has one or two unsealed sites, pipet the antibody solution, and remove all air-bubbles.
3. Heat-seal the plastic bag and incubate the membrane for 30 min on a rotating platform at room temperature.
4. Discard the antibody solution. Using a clean dish, wash the membrane twice for 15 min at room temperature using 200 mL of wash buffer A.
5. Pour off the wash buffer and incubate for 2 min in 100 mL of activation buffer.
6. Place the membrane in a plastic bag and add 10 mL of 1/100 dilution of CSPD chemiluminescent for 2 min. Ensure that the solution is completely distributed over the membrane.
7. Place the membrane between two sheets of Whatman 3MM filter paper and wipe to remove excess CSPD solution.
8. Heat-seal the wet membrane into a new plastic bag or protect the membrane with a cling film and expose the membrane to an X-ray film for various time periods (5, 15, and 30 min). Select the exposure time for optimal signal intensities.
9. Take a picture of the autoradiograph and save the image for documentation (*see* **Fig. 2**). We recommend using a gel-analyzing software (any supplier) capable of fragment length determination and saving the DNA profiles in a database.

4. Notes

1. A good starting point for searching for microsatellite markers on a specific chromosome or for information on given markers with regard to PCR product sizes, allelic frequencies, or heterozygosity is the home page of the National Human Genome Research Institute at the National Institutes of Health, Bethesda, USA (http://nhgri.nih.gov/).
2. The establishment of cell lines from specific tumors should be carried out according to the guidelines of Drexler et al. ***(13)***. With regard to an unequivocal authentication procedure, nonpathogenic tissue material should be taken at the same time the tumor material is taken from the donor. It is convenient to prepare genomic DNA from blood lymphocytes. In general, 10 mL of blood will provide sufficient DNA for thousands of PCR assays.
3. The main reason for the still increasing frequency of cross-contaminated cell cultures is the uncontrolled and blind-faith exchange between scientists. It is imperative that scien-

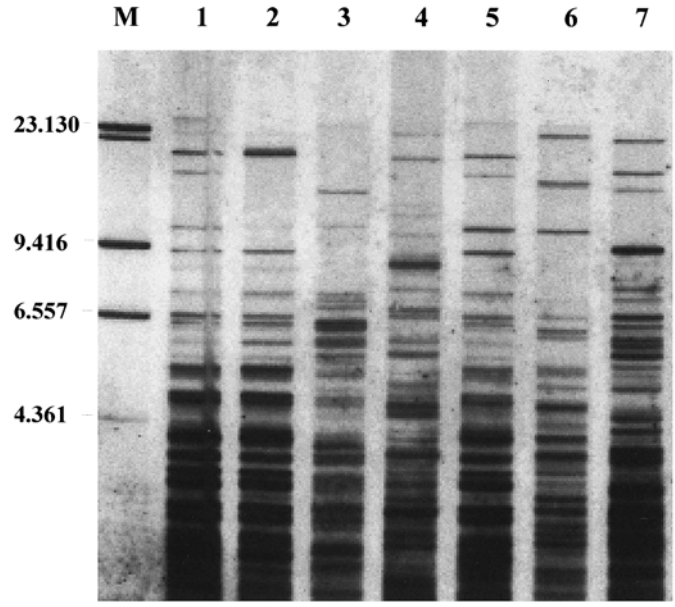


Fig. 2. Multi-locus $(GTG)_5$ DNA profiles of human cell lines.The autoradiograph shows a multi-locus DNA fingerprint using $(GTG)_5$ oligomers as a probe. 10 µg of DNA of indicated cell lines were digested to completion, size-separated on a 0.7% agarose gel, and blotted onto a nylon membrane. After hybridization with $(GTG)_5$ and washing procedures, a chemiluminescent detection of the probe was carried out as described in **Subheading 3.3.** The blot was exposed for 30 min to an X-ray film. Lanes M and 1–7: BEWO, human choriocarcinoma; JAR, human choriocarcinoma (derivative of BEWO); JVM-2, human chronic B cell leukemia; NK-92, human natural killer lymphoma; MOLT-14, human T cell leukemia; 5637, human urinary bladder carcinoma; PEER, human T-cell leukemia.

tists obtain the relevant cell cultures from reputable sources like cell banks, which routinely verify the quality and authenticity of the material (ATCC in USA: www.atcc.org; DSMZ in Europe: www.dsmz.de).

4. The use of other kits capable of isolating genomic DNA is possible but has to be optimized in order to avoid the presence of inhibitory substances in the DNA preparations. Generally, DNA can be safely stored at 4°C temperature for several months. We recommend freezing aliquots of genomic DNA for long-term storage, but repeated freeze-thawing will cause shearing of the high molecular weight DNA.
5. The use of thermal cyclers other than the Perkin Elmer Cetus 480 might require some modifications in the amplification parameters, for example, duration of the cycling steps, which are shorter in comparison to other applications. Also Mg^{2+}, primer, or dNTP concentrations might need to be altered. The same is true if other kinds of *Taq* polymerase are used or obtained from different suppliers; for example, we found that the parameters described were not transferable to HotStarTaq with a prolonged denaturation step (Qiagen).

6. Because of the viscosity of high molecular weight DNA we recommend placing the DNA samples on a shaking platform with 80 rpm during restriction endonuclease digestion.
7. Nearly all suppliers of oligonucleotides offer the possibility of labeling the primers chemically with digoxigenin. We recommend placing a single digoxigenin label at the 5'-position of the primer.

References

1. Rothfels, F. H., Axelrad, A. A., and Simonovitch, L. (1959) The origin of altered cell lines from mouse, monkey, and man as indicated by chromosomes and transplantation studies. *Proc. Can. Cancer Res. Conf.* **3,** 189–214.
2. Simpson, W. F., and Stuhlberg, C.S. (1963) Species identification of animal cell strains by immunofluorescence. *Nature* **199,** 616–617.
3. Gartler, S. M. (1968) Apparent HeLa contamination of human heterodiploid cell lines. *Nature* **217,** 750–751.
4. Miller, O. J., Miller, D. A., and Allerdice, P. W. (1971) Quinacrine fluorescent karyotypes of human diploid and heteroploid cell lines. *Cytogenetics* **10,** 338–341.
5. Nelson-Rees, W. A., and Flandermeyer, R. R. (1976) HeLa cultures defined. *Science* **191,** 96–98.
6. Nelson-Rees, W. A. (1978) The identification and monitoring of cell line specificity. P*rog. Clin. Biol. Res.* **26,** 25–79.
7. MacLeod, R. A. F., Dirks, W. G., Matsuo, Y., Kaufmann, M., Milch, H., and Drexler, H. G. (1999) Widespread intraspecies cross-contamination of human tumor cell lines arising at source. *Int. J. Cancer* **83,** 555–563.
8. Drexler, H. G., Dirks, W. G., and MacLeod, R. A. F. (1999) False human hematopoietic cell lines: cross-contaminations and misinterpretations. *Leukemia* **13,** 1601–1607.
9. Dirks, W. G., MacLeod, R. A., Jaeger, K., Milch, H., and Drexler, H. G. (1999) First searchable database for DNA profiles of human cell lines: sequential use of fingerprint techniques for authentication. *Cell. Mol. Biol.* **5,** 841–853.
10. Epplen, J. T., McCarrey, J. R., Sutou, S., and Ohno, S. (1982) Base sequence of a cloned snake W chromosome DNA fragment and identification of a male-specific putative mRNA in the mouse. *Proc. Natl. Acad. Sci. USA* **79,** 3798–3802.
11. Jeffreys, A. J., Wilson, V., and Thein, S. L. (1985) Hypervariable minisatellite regions in human DNA. *Nature* 1985, **314,** 67–73.
12. Nakamura, Y., Leppert, M., O'Connell, P., et al. (1987) Variable number of tandem repeat (VNTR) markers for human gene mapping. *Science* **235,** 1616–1622.
13. Drexler H. G., and Matsuo Y. (1999) Guidelines for the characterization and publication of human malignant hematopoietic cell lines. *Leukemia* **13,** 835–842.

5

Cytogenetic Characterization of Tumor Cell Lines

Roderick A. F. MacLeod and Hans G. Drexler

1. Introduction

1.1. Background: The Utility of Cytogenetic Characterization

The total number of human tumor cell lines is probably too great to count accurately, but given the established sum from hematopoietic tumors alone, over 1000 known samples ***(1)***, the grand total may well exceed 10,000. Because only a minority of these cell lines are in regular use, individuals describing new cell lines should justify their establishment during characterization.

There are several reasons why cytogenetic data have come to define a core element of tumor cell line characterization; the principal reason is the unique key they provide to their neoplastic origins. A wide range of tumors have been shown to display consistent and often distinct types of chromosome rearrangement that allow the neoplastic descent of cell lines carrying such changes to be inferred ***(2)***. Data documenting a myriad of cytogenetic correlations with the various malignant diseases they are associated with are compiled and continually updated, principally by Felix Mitelman and his colleagues, and are now accessible online at http://cgap.nci.nih.gov/Chromosomes/Mitelman.

Recurrent chromosome changes provide a portal to underlying mutations at the DNA level in cancer, and any cell lines bearing these changes provide ideal territory for mining them. It seems that the phenomenon of recurrent chromosome changes in various types of neoplasia reflects developmentally programmed patterns of gene expression and responsiveness within diverse cell lineages ***(3)***. Dysregulation of sensitive genes facilitates evasion of existing antineoplastic controls, including those mediated by cell cycle checkpoints or apoptosis. The tendency of cells to produce certain types of neoplastic mutations via chromosomal mechanisms, principally translocations, duplications, and deletions renders these changes microscopically visible, thereby facilitating cancer diagnosis by chromosome analysis. Arguably, of all neoplastic changes those effected chromosomally combine the greatest informational content with the least likelihood of reversal. This is particularly true of the primary cytogenetic changes that play

From: *Methods in Molecular Medicine, vol. 88: Cancer Cell Culture: Methods and Protocols*
Edited by: S. P. Langdon © Humana Press Inc., Totowa, NJ

key roles in neoplastic transformation and whereby the neoplastic phenotype and cell proliferation ultimately depend. Therefore, all cell lines established from patients with chronic myeloid leukemia (CML) with t(9;22)(q34;q11) causing fusion of *BCR* at Chr 22q11 with *ABL* at Chr 9q34, which is known to be the primary change in this disease, retain this change in vitro ***(4)***.

Multiple karyotypic changes in different subclones of HeLa cells (enabling HeLa contamination to be detected at a glance) have been conserved despite separate culture for over half a century, even though none have been shown to play a specific pathological role in the tumor of origin (cervical carcinoma). This suggests that secondary chromosome changes may also be stable in vitro. Nevertheless, it is not yet possible to classify certain important types of tumors cytogenetically, and disappointingly few specific recurrent changes have been described in the various epithelial tumors. Hence, the usefulness of karyotype analysis for the characterization of cell lines lies principally among those derived from tumors with stronger associations with specific chromosome rearrangements, i.e., hematopoietic ***(5)***, mesenchymal, and neuronal ***(6)***, rather than epithelial tumors.

Microscopic, unlike biochemical, methods facilitate direct observations performed at the single cell level enabling detection of intercellular differences. Accordingly, a second virtue of cytogenetic data is in the detection of distinct subclones (present in many cell lines) and the monitoring of stability therein. With the exception of doublings in their modal chromosome number (tetraploidization), human tumor cell lines appear to be more stable than commonly supposed ***(6,7)***. Although not to deny the existence of aneuploidy and structural cytogenetic alterations arising in vitro, data directly documenting chromosome instability remain scarce. In particular, few or no longitudinal studies of long-term cytogenetic stability have been published using material cloned to exclude outgrowth of cryptic subclones, a plausible alternative explanation. Indeed, one of very few studies of long-term stability documented by cytogenetic methods sensitive enough to allow detection of subtle cytogenetic changes failed to detect any chromosome instability in a cytogenetically rearranged human myeloid leukemia cell line (GF-D8) over long-term passage ***(8)***. Similarly, we have compared the detailed karyotypic structures of three cell lines independently established from Hodgkin's lymphoma (HDLM-1/2/3). Despite highly complex karyotypes, each cell line carries over 50 different chromosomal breakpoints and these cell lines have remained almost identical karyotypically in vitro ***(7,9)***. It has been suggested that chromosomal rearrangement may reach peak intensity in vivo, where these rearrangements are subject to physiological controls. These physiological controls are known to occur in lymphocyte development during the germinal center reaction, of which formation of DNA double-strand breaks is an essential part ***(10,11)***. An unexpected corollary would be that cell lines, in some cases, may be more stable than their precursor cells.

A third virtue of cytogenetic data is in cell line authentication. We have found that at least one-sixth of new human tumor cell lines have been cross-contaminated by older, mainly "classic" cell lines ***(12,13)***. This problem, first publicized over 30 years ago thanks to the pioneering work of Walter Nelson-Rees ***(14)*** but neglected of late ***(15, 16)***, poses an insidious threat to experimental cancer research ***(17)***. This problem

is most harmful where contaminants and their targets differ significantly in character or where the range of available cell lines is limited and, therefore, sensitive to distortion. Ideally, authentication should be documented at the time of first publication by demonstrating concordant DNA profiling of tumor and derived cell line alike (*see* Chapter 4).

As a result of apathy among investigators and journals alike, it seems, the establishment of lamentably few cell lines has been documented in this way. Therefore, for want of reference data, the vast majority of users wishing to authenticate tumor cell lines may be forced to relinquish DNA profiling in favor of the only practical alternative, cytogenetics. This avenue is closed to users of nontumor cell lines, for which karyotypes are generally too nondescript to be of any value in identification. Whether in pictorial (karyogram) or written (karyotype) forms, cytogenetic particulars are frequently documented in publications describing new tumor cell lines to serve as useful points of reference "downstream." In addition, for those establishing cell lines from tumors, comparison of their respective cytogenetic features provides an alternative to DNA profiling for confirming identity, at least among cases displaying significant degrees of chromosomal alteration.

1.2. Cytogenetic Methodology

Cancer cytogenetics has steadily gained in importance over the last three decades as a result of a series of advances, both technical and informational. It first became routine to distinguish and identify each of the 24 different human chromosomes (referred to as numbers 1 to 22, X and Y) when methods for recognizing their substructures (bands) were described in the early 1970s, principally quinacrine-banding (Q-banding) ***(18)*** and giemsa-banding (G-banding) ***(19)***. A further modification, trypsin G-banding ***(20)***, has gained wide currency since its introduction in 1973 as a result of its relative speed and simplicity. Soon thereafter, banding techniques were instrumental in the identification of the "Philadelphia chromosome" (Ph) marker in CML and its origin via a reciprocal translocation, t(9;22)(q34;q11) ***(21)***, a mechanism not known when the Ph was first observed more than a decade earlier ***(22)***. This observation marked the birth of our current picture of neoplasia as a disease of gene alteration. The advent of computer-aided image analysis in the early 1990s gave G-banding a further boost in handling complex tumor karyotypes by improving speed, sensitivity, and accuracy.

The second major advance, universally referred to as FISH (fluorescence in situ hybridization), took seed during the late 1980s ***(2,24)*** and has continued to flourish. Similar to conventional isotopic in situ hybridization (isotopic-ISH), which had become by then an established, though troublesome and time-consuming technique, FISH exploits the stability and specificity of DNA-DNA hybrids formed on metaphase chromosomes after exposure to homologous DNA under renaturating conditions. Isotopic-ISH was rapidly superseded by FISH following the availability of non isotopically labeled deoxynucleotides combined with a straightforward method for their efficient incorporation into DNA by nick translation. This led to suitable probes becoming commercially available. FISH serves to bridge the gap between classical cytogenetics and molecular biology. The range of FISH is particularly impressive, enabling

analysis of entire chromosomes, segments thereof ("chromosome painting"), or single genes, using probes comprising several megabases, or several kilobases or less of DNA respectively ("single-locus probes"). Tumor karyotypes are, nevertheless, in some cases simply too complex for straightforward analysis, even when augmented by chromosome painting. Complex karyotypes must be tackled using multicolor-FISH (M-FISH) probes, whereby each of the 24 human chromosomes is represented by a unique mixture of up to five or more differently colored probes ***(25)***. M-FISH analysis requires use of extra filters to distinguish typically six colors (to include the chromosomal counter stain) or, in the case of the analogous system, spectral karyotyping (SKY), a spectrophotometer. Although these various permutations of versatility and sensitivity impose corresponding demands on instrumentation, all FISH systems are basically similar and require broad-spectrum illumination (by ultraviolet or xenon light) and sensitive cameras to detect weaker signals, particularly those generated by short probes. All systems require special software to merge the different color channels, to improve signal/noise ratios and contrast, for example, and to generate pseudocolored images suitable for documentation.

The most recent advances are informational and come from sequence/mapping data of the human genome project. Accurately mapped and sequenced bacterial/P1 artificial chromosome (BAC/PAC) clones made available as a result of these efforts allow suitably equipped investigators to map chromosome rearrangements in cancer cells at the level of single genes and beyond, thus shortlisting plausible candidates for dysregulation. This probe resource together with that of well characterized tumor cell lines comprise a formidable tool for investigating the genetic basis of cancer, and, indeed, several important cancer genes have been discovered this way.

For those planning de novo cytogenetic analysis of tumor cell lines it is convenient to split the task into the following steps: harvesting (*see* **Subheadings 2.1.** and **3.1.**), G-banding (*see* **Subheadings 2.2., 2.3.,** and **3.2.**), and FISH (*see* **Subheadings 2.3., 2.4.,** and **3.3.**).

2. Materials

Chemical reagents may be stored up to 4 wk at 4°C, unless otherwise stated.

2.1. Harvesting

1. Cell culture(s) maintained in logarithmic phase.
2. *N*-Deacetyl-*N*-methylcolchicine (colcemid): (Invitrogen, Karlsruhe, Germany) 4 µg/mL stock solution (100X).
3. 100X Stock solution: 25 µg/mL of 5-Fluoro-2'-deoxyuridine (FUDR) (Sigma, Taufkirchen, Germany) and 1 mg/mL 1-β-D-Ribofuranosyluracil (uridine) (Sigma), 1 part/3 parts.
4. 100X 1-(2-Deoxy-β-D-ribofuranosyl)-5-methyluracil (thymidine) (Sigma): dissolve 50 mg in 100 mL. Filter sterilize through 0.22 µm filter.
5. 0.5 g/L Trypsin, 0.2 g/L EDTA (Invitrogen) for removal and dispersal of adherent cells. Store at –20°C).
6. Stock hypotonic solutions: 5.59 g/L KCl; or 9.0 g/L NaCitrate. Working hypotonic solutions: mix KCl and NaCitrate, e.g., 20/1, 10/1, 1/1, 1/10, 1/20 shortly before use, allowing time to reach desired temperature.

7. Fixative: absolute methanol and glacial acetic acid (3/1). Use fresh but may be stored up to 4 h at 4°C.

2.2. G-Banding Only

1. Slides (frosted-ends for annotation): wash mechanically overnight in warm ion-free detergent, rinse twice in deionized water, oven-dry, and leave overnight in ethanol (70%). Slides should then be polished using a lint-free cloth (or nonshredding tissue) and stored wrapped in aluminum foil at –20°C until use.
2. Phosphate buffered saline (PBS): adjust to pH 6.8 (Giemsa solution) or pH 7.2 (trypsin).
3. 140X Trypsin stock solution: dissolve 17.5 mg trypsin 1/250 (Difco, Hamburg, Germany) in PBS, pH 6.8. Store 500-µL aliquots at –20°C for up to 6 mo.
4. Giemsa stain, (Merck, Darmstadt, Germany, cat. no. 1.09204.0500). Dissolve 5 mL in 100 mL PBS, pH 7.2 and filter before use.
5. Routine microscope with phase contrast (PC) illuminator and the following objectives: 10× (phase-contrast), 40× (phase-contrast), and 50× (brightfield-dry) for slide evaluation and preliminary analysis.

2.3. G-Banding and FISH

1. Image analysis system for G-banding and FISH (*see* **Note 1**).
2. Laboratory oven for slide aging (G-banding) or slide drying (FISH).
3. 100-mL Coplin jars (glass) for staining and washing.
4. 4X SSC: 35.1 g NaCl, 17.7 g NaCitrate, made up to 1 L. Adjust to pH 7.2.
5. 0.5X SSC, 2X SSC, etc.; for example dilute from 4X SSC stock but monitor pH.

2.4. FISH Only

1. Ethanol: absolute, 90%, 70%. May be used twice and then discarded.
2. Pepsin stock solution: dissolve 250 mg pepsin (Sigma P7012) in 12.5 mL deionized H_2O. Freeze 500-mL aliquots (–20°C) and store for up to 6 mo.
3. Pepsin working solution: dilute 500 mL stock solution in 100 mL deionized H_2O containing 1 mL 1 N HCl.
4. Formaldehyde solution: 1% formaldehyde in PBS, pH 7.2, containing 50 mM $MgCl_2$.
5. Acetone, for use in mild pretreatment.
6. Hybridization buffer: Hybrizol VII (Qbiogene, Heidelberg, Germany). Store at room temperature (contains formamide).
7. Cold competitor DNA for prehybridization with probes containing repeat sequences: 1 mg/mL Cot-1 DNA (Roche, Mannheim, Germany); store at –20°C.
8. Nail varnish (clear).
9. Rubber cement.
10. Hybridization chamber: sealed container with an internal shelf to separate slides (above) from humidifier, for example, water-impregnated towels.
11. Hybridization bed: prewarmed freezer block kept in incubator at 37°C. Use during application of probes to slides.
12. Wash solution: 4X SSC with 0.1% Tween-20, molecular biology grade (Sigma). Slides may be placed in wash solution between any steps to prevent drying out.
13. Plastic cover slips for probe detection (Qbiogene).
14. Mounting medium: dissolve 50 ng/mL 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) in Vectashield antifade mounting medium (Alexis, Grünberg, Germany).
15. Cover slips: glass, grade 0, 22 × 60 mm.

16. Chromosome painting probes: store at –20°C unless otherwise stated. (*see* **Note 2**).
17. Research microscope with the following brightfield objectives with numerical apertures as high as budgetary limitations permit 10×, 63× (fluorescence), 63× (Planapochromatic). Ideally, a cytogenetics research microscope should be equipped with an automatic filter wheel and configured to an appropriate FISH imaging system (*see* **Note 1**).

3. Methods

3.1. Harvesting and Slide Preparation

Human cells in continuous culture divide typically every one-to-three days, though exceptions at both ends are not uncommon. The mitotic metaphase is the only stage throughout the cell cycle at which chromosomes are clearly visible. This phase lasts but an hour or less, severely reducing the number of cells open to conventional cytogenetic analysis. The metaphase fraction must be enriched accordingly by exposure of the growing cultures to colcemid or, more rarely, to some other mitotic blocking agent for a few hours, or longer in the case of slow growing cells (*see* **Note 3**). It is therefore important to ensure that cell cultures are in their logarithmic growth phase by feeding and, if necessary diluting/seeding out. Neglect of this simple precaution is a common cause of failed harvests.

It is difficult to overemphasize just how crucial initial harvesting and slide preparation are to subsequent success with both G-banding (*see* **Subheading 3.2.**) and FISH (*see* **Subheading 3.3.** and **Note 4**). Harvesting is often the step least rewarded by success. For unknown reasons, some acute leukemia cell line derivates resist successful harvesting. Furthermore, a hypotonic treatment that consistently yields good preparations with one cell line may be totally unsuitable for another cell line of similar derivation. This inconvenient problem precludes use of standard harvesting protocols applicable to all cell lines, unlike DNA preparation. It is therefore necessary to ascertain empirically which harvesting procedure is optimal for each cell line by trial and error. This is achieved by harvesting in parallel cell-aliquots that have been exposed to a range of hypotonic conditions, for example, by varying buffers, the duration of incubation, and temperatures. Continuous cell lines permit the luxury of repetition, but we must take full advantage of it. Cytogenetic harvesting is exquisitely sensitive to the biological variability inherent in living systems and must often be repeated several times until satisfactory results are achieved.

Fixation, on the other hand, is amenable to standardization. Once fixed, cells may be stored for a few years at –20°C until required for G-banding or FISH. Immediately prior to slide making, cell suspensions should be washed in fixative. Slide making is performed by dropping suspension onto ice-cold, "squeaky-clean" slides held at a slight incline atop a prefrozen (–20°C) freezer cold-block. Two drops aimed at the slide region immediately under the frosted zone and at the lower-middle, respectively, should result in figure-eight spreading patterns that facilitate both G-banding and FISH. Once completed, slides may be stored for a few years (at –80°C), aged briefly at room temperature for FISH, or aged overnight at 60°C for G-banding.

1. Add colcemid to growing cultures for 2–4 h.
2. Optional: incubate overnight with FUDR to improve chromosome morphology (*see* **Note 5**).

3. *Suspension culture:*
   Aliquot (for example, 4× in 10-mL tubes), centrifuge (5 min at 400g), and discard supernatant.

   Adherent culture:
   Shake vigorously to remove mitoses and retain supernatant in centrifuge tube (50 mL). Meanwhile, rinse remaining adherent cells with serum free medium or PBS and discard wash. Add sufficient trypsin/EDTA to cover the cells and incubate briefly (5–15 min) with intermittent light agitation. When cells are ready (i.e., "rounded up") shake vigorously and remove cells by rinsing with supernate from the centrifuge tube. Then centrifuge aliquots as with suspension cultures. (The serum present in the culture medium will act to inactivate residual trypsin activity.)
4. Resuspend centrifugates gently by manual agitation. Add 5–20 vol from various working hypotonic solutions (for example, 20/1, 1/1). Incubate paired aliquots at room temperature (initially) for 1 min and 7 min, respectively (**Table 1**).
5. Centrifuge and discard supernate. Resuspend gently and carefully add ice-cold fixative, at first dropwise, then increase amount to almost fill tube.
6. Store refrigated for >1 h.
7. Equilibrate to room temperature (to minimize clumping), then centrifuge (5 min at 400*g*). Repeat.
8. Store overnight at 4°C.
9. Next day, equilibrate to room temperature, then centrifuge (5 min at 400*g*). Repeat twice.
10. Resuspend in sufficient fixative, adjusting the volume to yield a lightly opaque suspension.
11. Remove four precleaned slides (one per harvest tube) from storage at (–20°C) and place on a plastic-covered freezer-block held at a slight incline away from operator (by insertion of a pipet, for example).
12. Locally humidify by breathing heavily on slides.
13. From a height of 30 cm place two drops of suspension onto each slide, the first immediately below the frosted zone, the second about two-thirds along the slide. Do not flood with fixative (*see* **Notes 6** and **7**).
14. Lift slides in pairs for speed. Breathe on slide again to maximize spreading.
15. Optional: to improve spreading, gently ignite residual fixative with a camping stove or Bunsen burner. Do not allow slide to get hot as this may spoil subsequent G-banding and FISH.
16. Label and air dry: allow slides to stand almost vertical until dry.
17. Examine slides by phase-contrast microscopy and assess each hypotonic treatment individually (*see* **Note 3**).
18. Prepare slides from successful treatments, mixing suspensions if more than one is deemed adequate. Label.
19. Store unused suspension at (–20°C).

3.2. Trypsin G-Banding (GTG-Banding)

Although a number of banding methods are available, the most popular is G-banding by trypsin pretreatment ***(20)***. This method selectively depletes the chromatin of certain proteins to produce strong lateral bands after staining with Giemsa (**Fig. 1A**).

Analysis of chromosomes harvested using the technique described in **Subheading 3.1.** should typically reveal approx 300 bands, although with stretched or submaximally condensed (prometaphase) chromosome preparations, over 1000 bands may be

Table 1
Data Sheet for Determining Optimal Hypotonic Conditions for Harvesting Cell Lines

Cell Line		*RS4;11 (DSMZ ACC 508)*												
Harvest		**Hypotonic Treatment**					**Results** [4.]			**Quantities of Slides and Suspensions** [5.]				
Tube [1.]	**Col.** [2.]	**KCl** [2.]	**NaCit.** [2.]	**Other** [2.]	**Temp** [3.]	**Time** [3.]	**MI**	**Spr.**	**Qual.**	**Use tube?**	**GTG**	**Giemsa**	**FISH**	**Store** [6.]
Harvest #1 [1.]	*15 january 2002*													
-a	*3h*	*20*	*1*	*-*	*RT*	*7′*	*A*	*A/AA*	*B+*	*mix*	*6*	*1*	*4*	*yes*
-b	*ditto*	*20*	*1*	*-*	*ditto*	*1′*	*A*	*A/AA*	*B+*	*mix*				
-c	*ditto*	*1*	*1*	*-*	*ditto*	*7′*	*A*	*A/AA*	*B+*	*mix*				
-d	*ditto*	*1*	*1*	*-*	*ditto*	*1′*	*A*	*B*	*BC*	*discard*				
Action [7.]	*Harvest #1 satisfactory ® discard culture.*													

Data are those of an actual experiment, the harvesting of cells from the RS4;11 cell line, processed to prepare the G-banding and FISH slides images shown in **Fig. 1**.

Abbreviations: Col., colcemid; MI, mitotic index; Spr., spreading; Qual., quality.

Explanations:

1. *Tube/Harvest:* to avoid subsequent confusion, it is essential to identify each harvest that in turn is prepared by mixing labeled tubes yielding acceptable preparations.
2. Both the time of exposure to colcemid, and the concentrations of colcemid and hypotonic buffers (if nonstandard) should be noted for future reference.
3. Both the temperature and duration of hypotonic treatments are crucial and should be recorded.
4. *Results:* to assess the efficacy of harvest conditions, it is necessary to compare their relative efficiencies in yielding metaphases (MI), which are well spread without excessive breakage (Spr.) and in which chromosome morphology is satisfactory (Qual.).
5. *Quantities of slides and suspensions:* indicates what should be done with suspensions from each harvest tube (e.g., mixing for slide-making and/or storage) and how many slides are to be prepared for G-banding (GTG), solid staining (Giemsa), and FISH.
6. *Store:* here may be indicated whether any of the harvest tubes/mixtures are suitable for storage as suspensions at (–20°C).
7. *Action:* decisions regarding the need for repeating the harvest and, if so, how may be written in this box. In the case of RS4;11, the first harvest was deemed adequate.

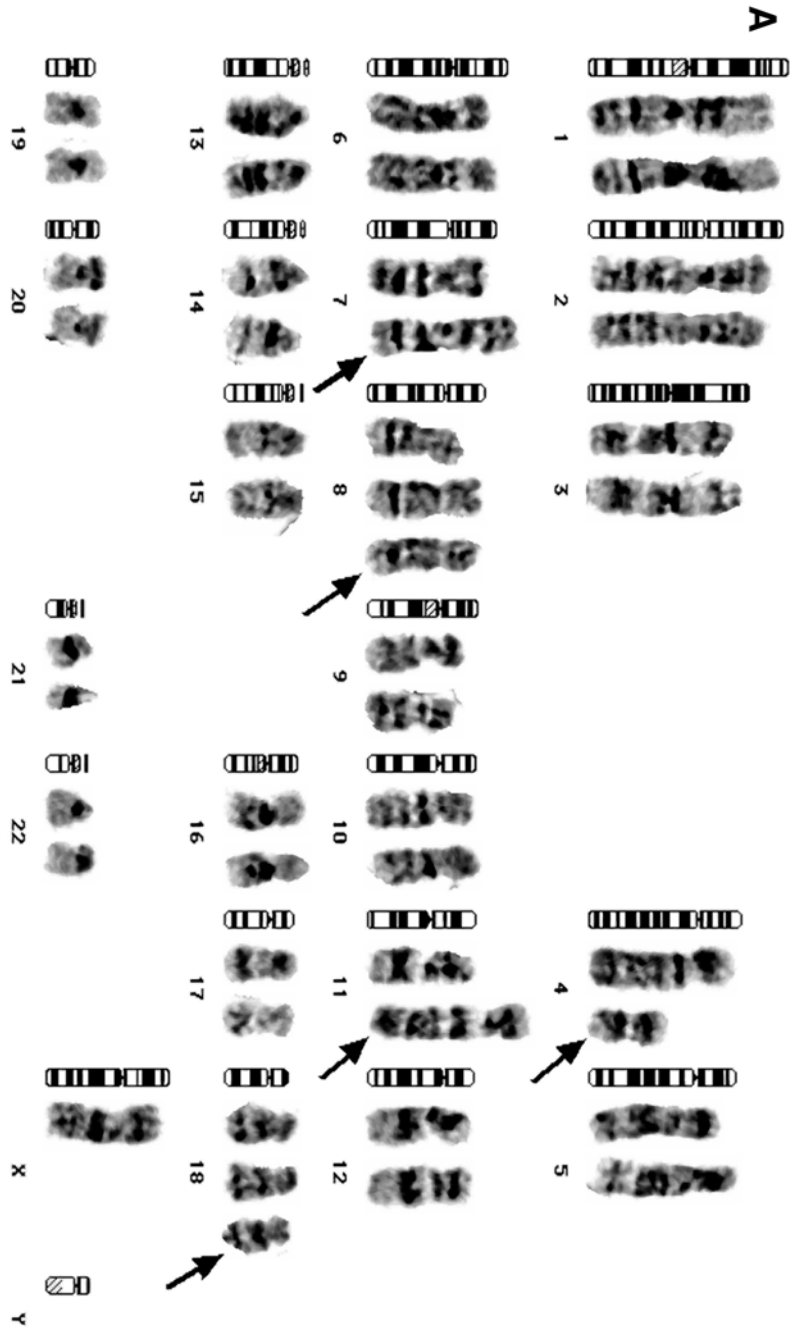
observed. With the advent of FISH, the need for high resolution banding is less obvious, at least in cancer cytogenetics where analysis with BAC clones is simpler and more informative. In fact, by obscuring their salient points of recognition, high resolution banding may even hamper analysis, unless performed by experts. As a rule of thumb, with G-banding alone, it is possible to detect, if not fully characterize, most chromosome rearrangements present in cell lines carrying up to about a half-dozen separate changes. Where unbalanced translocations predominate, as is the case among epithelial solid tumors, all but superficial analysis will most probably require additional chromosome painting.

1. Fresh slides are unsuitable for immediate G-banding. Slides must be first aged by baking overnight at 60°C (*see* **Note 8**). Approximately six to eight slides that contain a sufficient supply of well-spread metaphases with adequate chromosome morphology should be prepared for each cell line.
2. Prepare in advance three Coplin jars containing: 500 µL trypsin in 70 mL PBS, pH 7.2, ice-cold PBS, pH 6.8, to stop enzymic activity, and 5% Giemsa in PBS, pH 6.8.
3. The Coplin jar, containing trypsin in PBS should be placed in a water bath at 37°C beforehand and not used until the internal temperature has equilibrated.
4. To determine optimal trypsin incubation times, dip the first slide halfway into the trypsin for 10 seconds (s) and the whole slide for the remaining 10 s to test, in this case, for 10- and 20-s trypsin times simultaneously on one slide.
5. Immediatedly stop trypsin activity by immersion in cold PBS for a few seconds.
6. Stain in Giemsa solution for 15 min.
7. Rinse briefly in de-ionized H_2O and carefully blot dry using paper towels (as used for Southern blotting).
8. Examine microscopically (*see* **Note 9**). Scan for likely metaphases at low power. Examine those selected at higher power using the Epiplan dry objective. From the chromosome banding quality, decide whether the suitable trypsin time lies within the 10- to 20-s range spanned by the test slide. If satisfactory, repeat **Subheading 3.2., steps 1–7**. If unsatisfactory, repeat **steps 1–8** using longer (30–45 s) or shorter (3–6 s) typsin test times, as appropriate until the optimal incubation time becomes apparent.

3.3. FISH

Chromosome painting aptly describes the FISH application using libraries of probes that hybridize to the entire length or large regions of specific chromosomes (*see* **Fig. 1B**).

In contrast to single-locus probes, painting probes comprise DNA sequences from multiple loci, none of which need be specified. Painting probes may be used singly or in combinations—the latter maximizing the informational possibilities, for example, by confirming a translocation inferred by G-banding. Whichever probe combination is adopted, it is usually necessary to counterstain the chromosomes. The standard counterstain is 4',6'-diamidino-2-phenylindole hydrochloride (DAPI), which yields a deep blue color more intense at the centromeric heterochromatin, in particular that of chromosomes 1, 9, and 16 and in the terminal long-arm region of the Y-chromosome. In better preparations, DAPI generates negative G-bands, which with the aid of most image analysis programs may be readily converted into G-bands, albeit rather faint ones. (Although propidium iodide which stains chromosomes an intense red is sometimes used as a counterstain, it has been largely replaced by DAPI for this reason.)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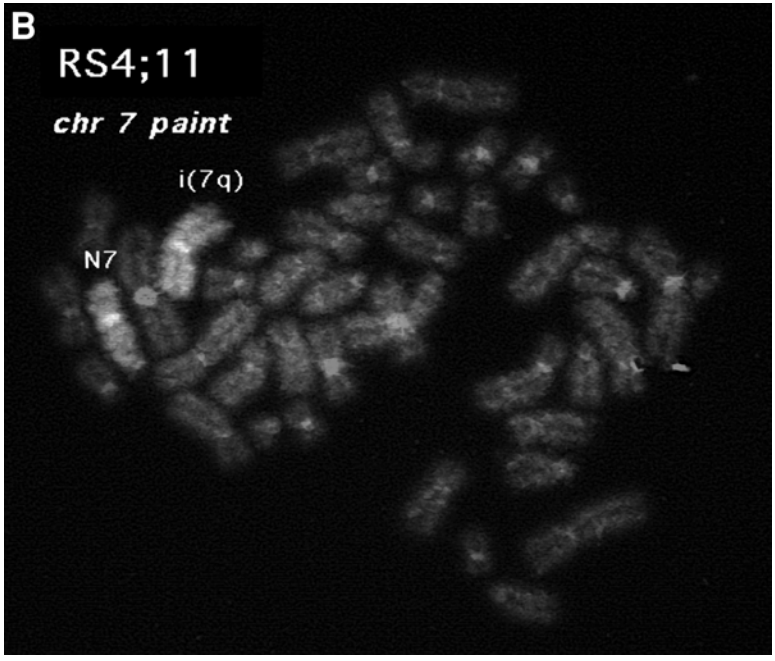

Fig. 1. Cytogenetic characterization of a human leukemia cell line (RS 4;11). G-banding karyogram and FISH analysis of a cell line (RS4;11) established from a 32-year-old female patient with acute lymphoblastic leukemia (ALL) at relapse ***(26)***.

The ISCN karyotype ***(27)*** of the cell depicted in the karyogram **(A)** was as follows: 47/48<2n>X/XX,t(4;11)(q21;q23),i(7)(q10),+8,+18, the rearranged chromosomes being indicated by arrows. In this case G-banding revealed the presence of an unambiguous primary change known to be recurrent in ALL, a balanced, reciprocal translocation, t(4;11), whereby most of the long-arm of chromosome 4 (breakpoint at band 4q21) is exchanged with the subterminal long-arm region of chromosome 11 (breakpoint at 11q23). This rearrangement is associated with poor prognosis ***(2)*** and may in some cased be induced by treatment for a previous malignancy. The accompanying structural change, an isochromosome for the long arm of chromosome 7, i(7q), was described at early passage ***(26)*** and probably arose in vivo, being a common secondary change in ALL ***(2)***. The numerical changes, additional copies of chromosomes 8 and 18, may have arisen in vivo or be in vitro artefacts. Interestingly, another t(4;11) cell line, MV4;11, also carries additional copies of chr. 8 and 18 ***(28)***, an otherwise rare combination, suggesting either that cells with this translocation may be predisposed to both trisomies, or that this combination favors growth in vitro. The cell depicted represents one of two major subclones, the one that lacks one of two X chromosomes. Loss of the second X chromosome (Y in males) is rather common in tumor cell lines and is also known as an age-related change in vivo. With the excepton of the trisomies (chr. 8 and 18), this karyotype is almost identical to that described in the original report.

The FISH images **(B,C)** show metaphase chromosome preparations hybridized with painting and single-locus probes, respectively. In both cases, preparations were counterstained with DAPI, which generates intense signals at all centromeric regions as a result of the presence of satellite DNA that preferentially binds DAPI. *(Continued on next page.)*

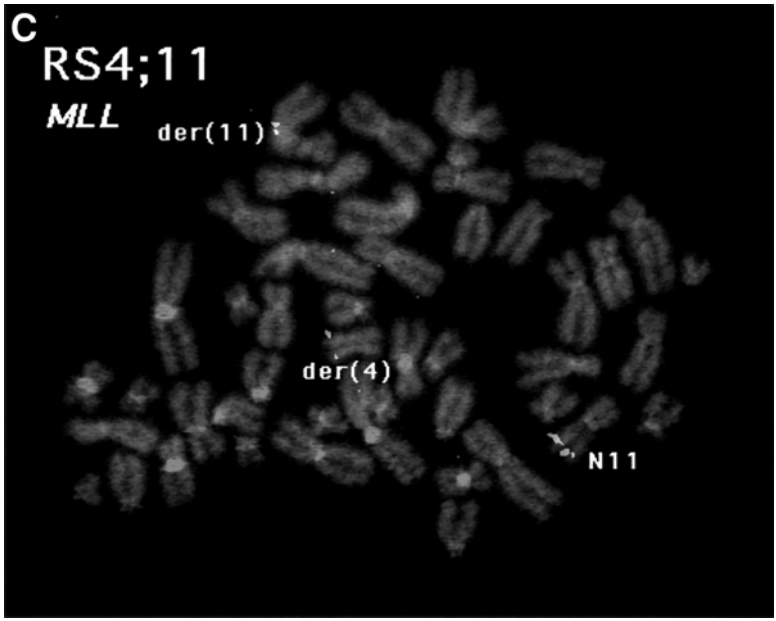

Fig. 1. *(Continued from previous page.)* The FISH image (B) depicts chromosome painting with a chr. 7 library probe directly labeled with red fluor (Qbiogene). Note the intense signals from the centromeric satellite sequences present on both the normal 7 (N7) and i(7q). Isochromosomes serve to increase relative copy numbers of long-arm regions at the expense of short-arm regions, or vice versa.

The FISH image (C) shows the results of hybridizing metaphase cells from RS4;11 with a commercial DNA probe (Qbiogene) designed to detect translocations occurring within the consensus breakpoint region of the *MLL* gene at chromosome 11q23. The *MLL* probe is labeled with digoxigenin-11-dUTP and detected by one round of incubation with antidigoxigenin-FITC (Roche). Note the strong signal yielded by the unrearranged MLL present on the normal 11, while both der(4) and der(11) partners display weaker signals resulting from splitting.

In this case, the FISH data are consistent with that of the G-banding and confirm that the *MLL* gene is a target of the t(4;11). These data support the conclusions that RS4;11 is both authentic and an appropriate model for ALL with t(4;11) and would, therefore, be a suitable resource for studying molecular changes therein, for example, breakpoint cloning and sequencing. *MLL* is a promiscuous gene, not only partnering *AF4* at 4q21, as exemplified by RS4;11, but several other genes as well, including *AF9* at 9p22 in t(9;11)(p22;q23) found in acute myeloid leukemia (AML-M5a) and represented by several cell lines, e.g., Mono-Mac-6 ***(29)*** and in cryptic form in MOLM-13/14 ***(30)***. In each case, gene fusion results in formation of a chimeric mRNA that is translated into a novel protein. In addition to providing in vitro models for their respective tumors of origin, cell lines with recurrent translocations provide infinitely renewable resources of DNA to be used as positive controls for PCR diagnosis.

Painting probes may be produced by PCR amplification of human chromosomal material retained by monochromosomal human/rodent-hybrid cell lines. By exploiting human specific repeat sequences (for example, Alu) as primer targets it is possible to amplify human DNA selectively. Such probes inevitably include significant amounts of human repeat DNA, hybridizing indiscriminately across the genome, and must be suppressed. This is achieved by preincubating probe material together with unlabeled ("cold") human DNA enriched for repetitive sequences by a two-step denaturation-renaturation process. During renaturation the most highly repetitive sequences (Cot-1 DNA) are the first to reanneal, allowing more complex, slower reannealing DNA to be digested away using single-strand-specific DNase. For this reason, most commercial painting probes include Cot-1 DNA.

Single-locus probes may be produced by labeling large insert clones and are available commercially for a variety of neoplastic loci. FISH, using a commercial probe covering the MLL locus at chromosome 11q23 is depicted in **Fig. 1C**.

However, such probes are costly and limited in coverage. New probes may be identified via genome browsers (for example, Ensembl) and ordered from a suitable resource center. An important class of single locus probes hybridize to chromosome-arm specific sequences present in the subtelomeric chromosome regions, which are favored sites of translocation and may be targets for instability, "jumping translocation" ***(9)***. Unlike single-locus cDNA probes prepared by reverse transcription of specific mRNA containing no repeat sequences, large insert clones invariably contain repeat sequences requiring suppression by prehybridization with Cot-1 DNA.

The critical step in FISH is the post-hybridization stringency wash that may be performed at either low temperatures using formamide, which lowers the stability of the DNA double-helix, or at higher temperatures using low SSC concentrations alone. Stringency washing allows the operator to control the balance of probe signal intensity against background. The stability of DNA-DNA hybrids on FISH slides allows repeated cycles of stringency washing. When starting with untested FISH probes, it is feasible to start off using a less stringent wash which, if yielding unacceptable background levels, may be repeated at higher stringencies (i.e., at lower SSC concentrations) (*see* **Notes 10** and **11**).

The following steps describe a basic but flexible FISH protocol, applicable to a wide variety of probes and, therefore, useful for those intending to combine probes from different sources, i.e., for experiments with the highest informational value. FISH experiments performed with single commercial probes should typically be performed according to manufacturers' protocols, although they are unlikely to differ substantially from that presented here (*see* **Notes 12–17**)

Indirectly labeled probes, e.g., with digoxogenin or biotin, require additional detection steps that may be plugged into the following protocol.

1. Slides: use either fresh (1- to 7-d-old) or archival slides stored at –80°C.
2. Optional: to reduce background signal, preincubate in pepsin solution for 2 min at 37°C (*see* **Note 10**).
3. Slide dehydration: pass slides through an alcohol series, 2 min in 70% (×2), 90% (×2), and 100% ethanol in Coplin jars.

4. Dry overnight at 42°C.
5. Deproteinize in acetone for 10 min (to minimize background autofluorescence).
6. Slide denaturation: 2 min at 72°C in 30 mL 2X SSC plus 70 mL formamide. The temperature of this step is critical. Therefore, avoid denaturing too many slides simultaneously. If a high throughput is desired, slides should be prewarmed. Quench in prechilled (–20°C) 70% ethanol for 2 min.
7. Repeat **step 3** (alcohol series).
8. Varnish slide label (to prevent subsequent eradication).
9. Place slide on prewarmed block at 37°C.
10 Remove probe from freezer, noting the concentration of labeled DNA. Add excess Cot-1 DNA (20–50X probe).
11. Probe denaturation: pipet desired volume of probe into microfuge tube (sterile) and incubate in a "floater" for 5 min at 72°C in waterbath. (NB: If recommended by manufacturer, omit probe denaturation.)
12. Probe prehybridization: collect probe by brief centrifugation, then incubate for 15–60 min at 37°C in a second water bath.
13. Probe application: using shortened micropipet tips (sterile) carefully drop 8–12 µL of probe (making up the volume with Hybrizol, if necessary) onto each slide-half. Two hybridizations may be performed on each slide (separated by a drop of Hybrizol, to inhibit mixing). Cover slides carefully with glass coverslips, tapping out any bubbles, and seal with rubber cement.
14. Hybridization: place slides carefully in moistened and sealed hybridization chamber. Leave overnight (or for up to 72 h) in incubator (preferably humidified) at 37°C.
15. After hybridization carefully remove rubber cement and coverslips in 2X SSC using forceps.
16. Stringency washing: wash slides for 5 min at 72°C in 0.5X SSC (*see* **Notes 10** and **11**).
17. Optional, for use with digoxigenin labeled probes: briefly prewash in wash solution at room temperature and shake the excess wash free. Important: Do not allow slides to dry out until dehydration **step 18.** To each slide apply 40 µL antidigoxigenin antibody haptenized with fluorescein-isothiocyanate (FITC) (Qbiogene, Heidelberg, Germany) and cover with plastic coverslip. Incubate for 15–30 min at 37°C in hybridization chamber. Wash for 5 min (×3) in wash solution at room temperature in subdued light.
18. Dehydration (alcohol series): as in **step 3** but performed in subdued light.
19. Mounting and sealing: using abbreviated micropipet tips, carefully place 3 drops DAPI/Vectashield mountant, with each drop containing approx 30 µL, along the slide. Apply coverslip and tap out any large bubbles using the blunt end of a pencil or equivalent. Seal with nail varnish. Allow varnish to dry.
20. Visualization: slides should be visualized at high power under oil immersion with a ×63 objective with a high numerical aperture. Although Zeiss supplies an immersion oil specially designed for fluorescent microscopy (518F), its propensity to floculate spontaneously and at low temperatures renders it unsuitable for routine application to slides stored at 4°C.

4. Notes

1. Performing image analysis onscreen greatly facilitates both speed and accuracy. The ability to position homologs side-by-side and to flip them at a mouse-click assists dissection of unresolved (marker) chromosomes. Karyograms may be subsequently printed with comparable ease, eliminating the need for laborious cut and paste routines. FISH imaging

systems are available from several manufacturers, based either on PC or Macintosh platforms. For further information consult the website of Applied Imaging (http://www.aicorp.com/), which supplies a variety of such systems. Imaging systems confer significant benefits, including amplification of weak signals, merging of differently colored signals together with fiduciary superimposition (if necessary), enhancement of contrast, reduction of background, generation of pseudo G-bands from DAPI counterstain, and rapid documentation and printing.

2. It is seldom possible to resolve all rearrangements present in cancer cell lines, particularly smaller markers lacking recognizable banding patterns. This is where chromosome painting should be used to maximize detail and accuracy. The choice of painting probes is a complicated question. We have had consistently good results with painting and satellite-DNA probes obtained from larger manufacturers. For those using untested probes, it is useful first to calibrate these using normal chromosomes. This effort is usually well invested. Some probes generate unnecessarily bright signals. Knowing this beforehand allows such probes to be "stretched" by dilution with Hybrizol. All too often, probes arrive that yield inadequate or inappropriate signals. Timely identification of such problems by pretesting not only facilitates refund or replacement but may prevent the pursuit of false trails inspired by probes which hybridize to more than one region.
3. Sometimes insufficient metaphases are present on slide preparations. Such slides, though of possible use for G-banding in critical cases, are useless for FISH where probe costs are often critical. For slowly dividing cell lines (doubling times >48 h), colcemid times may be lengthened first to 6 h, then to 17 h (overnight), simultaneously reducing colcemid concentrations by a half to minimize toxicity. However, paucity of metaphases is usually a result of depletion by overly harsh hypotonic treatments. Contrary to most published protocols, we find that reducing hypotonic exposures to 1 min and, if necessary, performing this step in microfuge tubes to facilitate speedy centrifugation to reduce total hypotonic times still further, is often effective.
4. The ideal slides for analysis should fulfill three criteria: a sufficiency of metaphases to enable analysis without wasteful repetition, adequate chromosome spreading—sufficient to minimize overlapping without undue breakage, and good morphology, i.e., large but undistended chromatids lying in parallel. To document progress in harvesting procedures and aid evidence-based searches for their improvement, we use a standard data sheet that records progress towards these ideals. An actual example is shown in **Table 1**, which presents harvesting data for the cell line RS4;11, the subsequent G-banding and FISH analysis, which are presented in **Figs. 1 A–C**. In this case, reasonable preparations were obtained at the first attempt using the standard protocol (**Subheading 3.1., step 4**). Although all four hypotonic combinations yielded adequate numbers of metaphases (A), only tubes a–c yielded satisfactory spreading, i.e., a mixture of well-spread and overspread chromosomes (A/AA), and chromosome morphology (B+), and were subsequently mixed prior to slide preparation. A total of 11 slides were prepared: 6 for G-banding, 1 for Giemsa staining alone (to check for presence of small chromosomal elements which G-banding sometimes render invisible), and 4 for FISH. In addition, remaining cell suspension in fixative was stored (–20°C) for future use.
5. Chromosome morphology is often correlated with spreading and improvements may be effected using the same methods. As a general rule, the best morphologies are produced by hypotonics containing 50% or less NaCitrate. Excessive amounts of the latter tend to yield fuzzy irrregular morphologies that produce disappointing results with G-banding and FISH alike. Some types of cell, and derived cell lines alike, consistently yield short

stubby chromosomes that appear refractory to all attempts at improvement, for example, acute lymphoblastic leukemia. In such cases, it may be helpful to try FUDR pretreatment. Accordingly, treat cultures overnight with FUDR/uridine. Next morning, resuspend in fresh medium with added thymidine to reverse the blockade and harvest 7 to 9 h later.

6. Insufficient spreading results in tight metaphases with an excess of overlapping chromosomes: such cells may be amenable to FISH but are useless for G-banding. In such cases spreading may be improved by harsher hypotonic treatment, whether by increasing the proportion of KCl to 100%, by increasing the hypotonic time up to 15 min, or by performing the latter at 37°C instead of room temperature (RT).
7. However, paradoxically, many cell lines yield their best spreading at 1 min indicating how little we understand the underlying biological processes involved. Gentle flaming often assists spreading and has minimal effect on G-banding or FISH. In our experience, "dropping from a height" yields only minor improvement in spreading, though heavy breathing, performed both immediately before and after dropping, is beneficial, presumably by increasing local humidity levels.
8. Excessive spreading, on the other hand, is often cured by reducing the proportion of KCl, or by reducing hypotonic treatment times, or by retaining more of the original medium from the first centrifugation (**Subheading 3.1., step 3**).
9. As a general rule, good chromosomes yield good G-banding results. Exceptions include chromosomes which are too "young" (puffed up or faint banding) or "over the top" (poor contrast or dark banding). Artificial aging by baking overnight at 60°C speeds up results and eliminates variations in optimal trypsin times resulting from climatic or seasonal variations in temperature or humidity.
10. Accurate chromosome analysis based on G-banding lies at the center of cancer cytogenetics. The ability to recognize each of the 24 normal human chromosome homologues must precede analysis of rearrangements. Because the majority of human cancer cell lines carry chromosome rearrangements, the choice of cell lines for learning purposes is critical. Learning should be performed using either primary cultures of normal unaffected individuals, e.g., lymphocyte cultures or B-lymphoblastoid cell lines known to have retained their diploid character. Individuals intent on acquiring the ability to perform karyotyping are strongly advised to spend some time in a laboratory where such skills are practised daily, for example, a routine diagnostic laboratory.
11. The main problem commonly encountered when using FISH probes is weak signal intensity. This may arise because the probe itself is inherently weak, the wash too stringent, or the chromosomes insufficiently denatured. To test for these scenarios, repeat the stringency wash (**Subheading 3.3, step 16**) but with either 2X or 1X SSC in the wash buffer. In parallel, repeat the slide denaturation (**Subheading 3.3., step 6**) increasing the denaturation time to 4 min. When neither alteration brings improvement, and the probe is new and untested or old and infrequently used, it is likely that the probe is weak. For those equipped with advanced imaging systems incorporating a camera of high sensitivity, it is often possible to capture images from probe signals invisible to the naked eye. In the case of new commercial probes, the supplier should be contacted.
12. On the other hand, FISH experiments are sometimes plagued by high background signal, or "noise". Commercial probes are usually, relatively free from this problem. Increasing the wash stringency (**Subheading 3.3., step 16**) by reducing the SSC concentration to 0.1X may help. Alternately, adding Cot-1 DNA to the hybridization mix may help to reduce hybridization noise. Among non-commercial probes, excessive noise may be cured by reducing the probe concentration. Normal DNA concentrations for single-locus probes

should range from 2–6 ng / μL up to 10–20 ng / μL for painting probes. Nonspecific noise caused by either autofluorescence or protein-protein binding after antibody staining may be reduced by additional slide pretreatment by incubation in pepsin solution.(**Subheading 3.3., step 2**). Incubate slides for 2 min in acidified pepsin solution at 37°C. Rinse in PBS (pH 7.2) for 3 min at RT. Postfix slides, held flat, in 1% formaldehyde solution for 10 min at RT using plastic cover slips. Rinse in PBS (pH 7.2) for 3 min at RT. Continue with **Subheading 3.3., step 3**)

13. Probes with larger targets often cross-hybridize to similar DNA sequences present on other chromosomes. It is important first to identify patterns of cross-hybridization by FISH onto normal chromosomes to avoid confusing the latter as rearrangements. Some resource centers, notably BAC/PAC Resources, list cross-hybridization patterns for their FISH probes.
14. The first aim of FISH performed on cancer cell lines is to characterize those rearrangements of interest that resist analysis by G-banding. This requires both intuition and luck. Clearly, the need for the latter is reduced where G-banding is optimized. The most difficult rearrangements to resolve involve unbalanced-multiple chromosomes. Sometimes, however, originally reciprocal translocations appear unbalanced as a result of loss or additional rearrangement of one partner. In such cases, the identity of the "missing partner" may be determined from among those chromosomes where one or more homologues appear to be missing.
15. Having identified the chromosomal constituents of cryptic rearrangements, the next task is to reconcile FISH with G-banding data enabling breakpoint identification. In cases where chromosome segments are short, or their banding patterns nondescript, this ideal may be difficult. The International System for Chromosome Nomenclature (ISCN) enables almost all rearrangements to be described with minimal ambiguity ***(27)***.
16. Quo vadis? Having successfully completed cytogenetic analysis of a tumor cell line to the point of ISCN karyotyping the question arises, what to do with the data? The first question to be addressed is identity: has the cell line in question been karyotyped previously and, if so, does the observed karyotype correspond with that previously reported? In our experience, complete correspondence between cell lines analyzed is rare, even where their identity has been confirmed by DNA fingerprinting. First, among complex karyotypes complete resolution may be unnecessary and is, indeed, rarely achieved. This leaves significant scope for uncertainty and differences in interpretation. ISCN karyotypes are inferior to karyogram images in this regard. Wherever possible consult the original journal or reprint, as photocopies seldom permit reproduction of intermediate tones that are the "devil in the detail" of G-banding. Second, a significant minority of cell lines display the propensity to evolve karyotypically during culture in vitro, .i.e., "instability". Instability may effect numerical or structural changes. Thus, for reasons that remain obscure, B-lymphoblastoid cell lines often become tetraploid at later passages. Although the effects of tetraploidization may initially appear dramatic, the phenomenon generates few or no genetic imbalances. More insidious are cases where cell lines displaying normal, or near normal, karyotypes at early passage evolve into distinct subclones after separation. Such a cell line is CCRF-CEM, derived from a patient with T-cell acute lymphocytic leukemia (ALL), which has spawned a multitude of subclones—all cytogenetically distinct ***(12)***; and, sometimes following cross-contamination events, masquerading under aliases. Those wishing to compare their karyotypes with those derived at the DSMZ may consult either the DSMZ descriptive catalogue ***(28)*** or website which features an interactive database facilitating searches (http://www.dsmz.de/).

17. The second and more important question concerns the neoplastic significance of cytogenetic changes observed in tumor cell lines. Investigators should naturally familiarize themselves with any recurrent changes previously observed in similar tumors. In addition to books ***(2)*** and specialist journals (e.g., *Blood, Cancer Genetics and Cytogenetics, Cancer Research, Genes Chromosomes Cancer, International Journal of Cancer, Leukemia*) regularly publishing such data, a number of websites are available allowing interactive searches, notably Mitelman's Database (http://cgap.nci.nih.gov/Chromosomes/Mitelman) The Atlas of Chromosomes in Cancer (http://www.infobiogen.fr/services/chromcancer/), and The Cancer Genome Anatomy Project (http://www.ncbi.nlm.nih.gov/ncicgap/), provide useful background information. For those confronted by new or only putatively recurrent cytogenetic alterations the question arises whether the change(s) observed in the cell lines are merely stochastic or genuinely neoplastic. For neoplastic candidates, for example, those ascertained by analogy with clinical data, the cell line carrying it provides ideal material for determining the breakpoints at the genomic level by hybridizing with clones chosen from the databases listing them, e.g., BAC-PAC Resources (http://www.chori.org/bacpac/) or Ensembl (http://www.ensembl.org/Homo_sapiens/). Precise breakpoint data are the first steps towards identifying genes targeted for dysregulation by cytogenetic changes in cancer.
18. Finally, a word to the wise: the literature is littered by all-too-many reports describing recurrent chromosome changes first ascertained in "independent" cell lines which on closer examination turn out to be subclones ***(16)***. Always check that cell lines purporting to come from different patients display distinct DNA fingerprints.

Acknowledgments

We wish to thank our colleagues, Maren Kaufmann—several of whose suggestions are silently incorporated in the foregoing protocols—for her expert technical work, and Dr. Stefan Nagel for his critical reading of the manuscript.

References

1. Drexler, H. G. (2001) *The Leukemia-Lymphoma Cell Line Facts Book.* Academic Press, San Diego, CA, p. 2.
2. Heim, S., and Mitelman, F. (1995) *Cancer Cytogenetics (2nd ed.).* Wiley-Liss, New York, NY.
3. Reya, T., Morrison, S. J., Clarke, M. F., and Weissman, I.L. (2001) Stem cells, cancer, and cancer stem cells. *Nature* **414,** 105–111.
4. Drexler, H. G., MacLeod, R. A. F., and Uphoff, C. C. (1999) Leukemia cell lines: in vitro models for the study of Philadelphia chromosome-positive leukemia. *Leuk. Res.* **23,** 207–215.
5. Drexler, H. G., MacLeod, R. A. F., Borkhardt, A., and Janssen, J. W. G. (1995) Recurrent chromosomal translocations and fusion genes in leukemia-lymphoma cell lines. *Leukemia* **9,** 480–500.
6. Marini, P., MacLeod, R. A. F., Treuner, C. et al. (1999) SiMa, a new neuroblastoma cell line combining poor prognostic cytogenetic markers with high adrenergic differentiation. *Cancer Genet. Cytogenet.* **112,** 161–164.
7. Drexler, H. G., Matsuo, Y., and MacLeod, R. A. F. (2000) Continuous hematopoietic cell lines as model systems for leukemia-lymphoma research. *Leuk. Res.* **24,** 881–911.
8. Tosi, S., Giudici, G., Rambaldi, A. et al. (1999) Characterization of the human myeloid leukemia-derived cell line GF-D8 by multiplex fluorescence in situ hybridization,

subtelomeric probes, and comparative genomic hybridization. *Genes Chromosomes Cancer* **24,** 213–221.

9. MacLeod, R. A. F., Spitzer, D., Bar-Am, I., et al. (2000) Karyotypic dissection of Hodgkin's disease cell lines reveals ectopic subtelomeres and ribosomal DNA at sites of multiple jumping translocations and genomic amplification. *Leukemia* **14,** 1803–1814.
10. Vanasse, G. J., Concannon, P., and Willerford, D. M. (1999) Regulated genomic instability and neoplasia in the lymphoid lineage. *Blood* **94,** 3997–4010.
11. Küppers, R. and Dalla-Favera, R. (2001) Mechanisms of chromosomal translocations in B cell lymphomas. *Oncogene* **20,** 5580–5594.
12. MacLeod, R. A. F., Dirks, W. G., Matsuo, Y., Kaufmann, M., Milch, H., and Drexler, H. G. (1999) Widespread intraspecies cross-contamination of human tumor cell lines arising at source. *Int. J. Cancer* **83,** 555–563.
13. Drexler, H. G., Dirks, W. G., and MacLeod, R. A. F. (1999) False human hematopoietic cell lines: cross-contaminations and misinterpretations. *Leukemia* **13,** 1601–1607.
14. Nelson-Rees, W. A., Daniels, D. W., and Flandermeyer, R. R. (1981) Cross-contamination of cells in culture. *Science* **212,** 446–452.
15. Markovic, O., and Markovic, N. (1998) Cell cross-contamination in cell cultures: the silent and neglected danger. *In Vitro Cell Dev. Biol. Anim* **34,** 1–8.
16. MacLeod, R. A. F., Dirks, W. G., and Drexler, H. G. (2002) Persistent use of misidentified cell lines and its prevention. *Genes Chromosomes Cancer* **33,** 103–105.
17. Stacey, G. N., Masters, J. R. W., Hay, R. J., Drexler, H. G., MacLeod, R. A. F., and Freshney, R. I. (2000) Cell contamination leads to inaccurate data: We must take action now. *Nature* **403,** 356.
18. Caspersson, T., Zech, L., and Johansson, C. (1970) Differential binding of alkylating fluorochromes in human chromosomes. *Exp. Cell Res* **60,** 315–319.
19. Sumner, A. T., Evans, H. J., and Buckland, R. A. (1971) New technique for distinguishing between human chromosomes. *Nat. New Biol* **232,** 31–32.
20. Seabright, M. (1973) Improvement of trypsin method for banding chromosomes. *Lancet* **1,** 1249–1250.
21. Rowley, J. D. (1973) A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining. *Nature* **243,** 290–293.
22. Nowell, P. C. and Hungerford, D. A. (1960) A minute chromosome in human granulocytic leukemia. *Science* **132,** 1497.
23. Cremer, T., Lichter, P., Borden, J., Ward, D. C., and Manuelidis, L. (1988) Detection of chromosome aberrations in metaphase and interphase tumor cells by in situ hybridization using chromosome-specific library probes. *Hum. Genet.* **80,** 235–246.
24. Lichter, P., Cremer, T., Borden, J., Manuelidis, L., and Ward, D. C. (1988) Delineation of individual human chromosomes in metaphase and interphase cells by in situ suppression hybridization using recombinant DNA libraries. *Hum. Genet.* **80,** 224–234.
25. Lichter, P. (1997) Multicolor FISHing: what's the catch? *Trends Genet.* **12,** 475–479.
26. Stong, R. C., Korsmeyer, S. J., Parkin, J. L., Arthur, D. C., and Kersey, J. H. (1985) Human acute leukemia cell line with the t(4;11) chromosomal rearrangement exhibits B lineage and monocytic characteristics. *Blood* **65,** 21–31.
27. ISCN (1985) An International System for Human Cytogenetic Nomenclature: Report of the Standing Committee on Human Cytogenetic Nomenclature. *Birth Defects Orig. Artic. Ser.* **21,** 1–117.

28. Drexler, H. G., Dirks, W., MacLeod, R. A. F., Quentmeier, H., Steube, K. G., and Uphoff, C. C. (2001) *DSMZ Catalogue of Human and Animal Cell Lines (8th ed.).* Braunschweig, Germany.
29. MacLeod, R. A. F., Voges, M., and Drexler, H. G. (1993) Mono Mac 6: a mature monoblastic leukemia cell line with t(9;11)(p21;q23). *Blood* **82,** 3221–3222.
30. Matsuo, Y., MacLeod, R. A. F., Uphoff, C. C., et al. (1997) Two acute monocytic leukemia (AML-M5a) cell lines (MOLM-13 and MOLM-14) with interclonal phenotypic heterogeneity showing MLL-AF9 fusion resulting from an occult chromosome insertion, ins(11;9)(q23;p22p23). *Leukemia* **11,** 1469–1477.

6

Isolation and Culture of Colon Cancer Cell Lines

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1. Introduction

Colorectal cancer is the second leading cause of cancer-related death in the United States, and an estimated 148,300 cases will occur in 2002, representing approx 11.5% of all new cancers. In men and women, colorectal cancer incidence declined between the mid-1980s and the mid-1990s and stabilized thereafter ***(1)***. In contrast, the incidence in Japan, Korea, and Singapore is rising rapidly, probably resulting from the acquisition of a Western lifestyle ***(2)***. The incidence of colorectal cancer increases with advancing age, with the peak incidence at 60–79 yr. Colorectal carcinoma is rare before the age of 40 yr except in individuals with genetic predisposition or a preexisting condition such as inflammatory bowel disease ***(3)***.

Multiple genetic alterations involving the activation of several oncogenes and the loss of two or more cancer-suppressor genes are essential in the development of various human cancers. The genes related to colorectal carcinogenesis can be divided into two groups on the basis of their presumed function. The first group is involved in cellular signaling pathways, and includes genes such as *APC*, *K-ras*, and *DCC*. The second is involved in maintaining genomic stability, and includes genes such as *p53* and mismatch repair genes. Mutations in *APC*, a tumor suppressor gene, is an early event in sporadic colorectal cancers and underlies familial adenomatous polyposis (FAP). The alterations of mismatch repair genes such as *hMSH2*, *hMLH1*, *hPMS1*, *hPMS2*, and *hMSH6* are responsible for the tumors of hereditary nonpolyposis colorectal cancers (HNPCC) patients and some sporadic colorectal cancers with microsatellite instability ***(4–6)***.

Cell lines from human colorectal cancers are useful tools in the study of cell biology and in the development and testing of new therapeutic modalities. A large bank of well-characterized cell lines should reflect the diversity of tumor phenotypes and provide adequate models for tumor heterogeneity. In general, colorectal cancer cell lines are relatively easy to establish. Colorectal carcinoma cell lines can be developed from ascitic effusions, metastatic tissues (regional lymph nodes and distant metastatic sites, such as liver), and primary tumors. The establishment of cell lines from ascitic effu-

From: *Methods in Molecular Medicine, vol. 88: Cancer Cell Culture: Methods and Protocols*
Edited by: S. P. Langdon © Humana Press Inc., Totowa, NJ

sions has proven to be more efficient than establishment from primary tumors because the cancer cells are rich, free floating, and primed for in vitro growth. However, effusions usually contain mesothelial cells and lymphocytes. Mesothelial cells can more readily attach to flasks compared to cancer cells and generally survive for more than one year ***(7–11)***. Lymphocytes can inhibit the growth of cancer cells. Enzymatic digestion of solid primary tumor tissues may result in poor recovery of viable cells and overgrowth of cancer cells with contaminating stromal cells. The faster growing stromal fibroblast-like cells easily outgrow and overgrow the cancer cells. The mechanical spill-out method provides a simpler, faster, and less traumatic method of obtaining cells for culture ***(12)***. This method allows the minimization of stromal cell contamination because stromal cells are not easily detached from the tissue matrix by mechanical means. Rutzky and Moyer ***(13)*** summarized the following features of key relevance for successful culture: (1) nonenzymatic or minimal dissociation of tumor tissue, (2) seeding of cultures as explant and at high cell densities, (3) removal of contaminating fibroblasts usually after they have aided culture initiation, and (4) delaying passage until high cell densities have been achieved and plating cells at high density.

In this section, we describe the culture of primary colorectal cancer cell lines, and include detailed protocols relating to establishment from ascitic effusions and primary tumor tissues using mechanical spill-out, the isolation of pure cancer cells, and the maintenance, propagation, and preservation of cancer cells. Our laboratory has extensive experience with serum-containing and fully defined media for the establishment of continuous cell lines derived from colon cancers. Most of our colon cancer cells were initially cultured in ACL-4 medium supplemented with 5% heat-inactivated (fetal bovine serum) FBS (AR5). ACL-4 is a fully defined medium formulated for the selective growth of human lung adenocarcinoma cells and has proved useful in the establishment of colorectal cancer cell lines ***(7,11,14,15)***. ACL-4 is a complex medium, consisting of four of the five HITES additives. HITES medium is used for the selective growth of small cell lung cancer (SCLC) and contains insulin, transferrin, hydrocortisone, estradiol, and selenium added to a basal medium, RPMI-1640. The ACL-4 medium lacks the estradiol component of the HITES medium. Epidermal growth factor (EGF) is also present. BSA helps to compensate for some of the high molecular weight proteins present in serum, and aids growth in semisolid media. Triiodothyronine (a selective mitogen), ethanolamine, and phosphorylethanolamine (precursors of membrane lipids), HEPES buffer (to compensate for the loss of buffering properties of serum), glutamine, and sodium pyruvate (added empirically) are also added ***(12,14,15)***.

2. Materials

2.1. Initiation Media for Primary Cell Culture

1. RPMI 1640 medium: RPMI 1640 powder, 25 m*M* HEPES buffer, 20 m*M* sodium bicarbonate, antibiotics (streptomycin-penicillin, usually 100 U/mL), filter through 0.22 µm bottle top filter.
2. ACL-4 medium: RPMI 1640 as basal medium, 20 µg/mL insulin, 10 µg/mL transferrin, 25 n*M* sodium selenite, 50 n*M* hydrocortisone, 1 ng/mL epidermal growth factor, 10 µ*M*

ethanolamine, 10 μ*M* phosphorylethanolamine, 100 p*M* triiodothyronine, 2 mg/mL bovine serum albumin (BSA), 2 m*M* glutamine, and 0.5 m*M* sodium pyruvate.
3. Initiation and growth medium (AR-5 medium): ACL-4 medium supplemented with 5% heat-inactivated FBS.

2.2. Reagents and Apparatus

1. Sterile phosphate buffered saline (PBS), pH 7.0.
2. Sterile 100 mm Petri dishes.
3. Sterile scissors.
4. Sterile forceps.
5. 10-mL pipets.
6. Pasteur pipets.
7. Ficoll gradient.
8. Culture flasks (25 cm^2) coated with attachment factors such as collagen and laminin.

3. Methods

3.1. Procurement of Tumor Specimens

Solid tumors (primary tumors and tumors in lymph nodes) must be excised carefully and aseptically from pathologically proven colon cancer samples and transferred to a cell culture laboratory in RPMI 1640 medium (*see* **Note 1**). The transporting RPMI 1640 medium should contain antibiotics, such as penicillin-streptomycin, to prevent bacterial contamination. Ascitic effusions should be collected in sterile glass bottles or syringes. Heparin (5 μg/mL) may be added to the collected fluids to prevent clotting, if the effusions contain many erythrocytes.

3.2. Primary Cell Culture

3.2.1. Procedure for Solid Tumors

1. Transfer tumor tissues to fresh sterile PBS in a dish and rinse.
2. Transfer tumor tissues to a new dish and dissect off necrotic areas, fatty tissues, blood clots, and connective tissues with forceps and scissors.
3. Finely mince the tumor tissues with sterile scissors.
4. Disassociate tissue pieces into small aggregates by vigorous pipeting with 10 mL of PBS.
5. Allow heavier pieces to sediment by gravity.
6. Carefully harvest the tumor cell aggregates that contain washes by pipet and transfer to a 15-mL sterile centrifuge tube.
7. Centrifuge and remove the supernatant.
8. Wash by resuspending in PBS, centrifuge, and again remove the supernatant.
9. Resuspend the pieces in 5 mL of AR-5 initiation medium.
10. Transfer the pieces to a 25-cm^2 flasks.
11. Harvest the remaining large pieces, place in a 15-mL sterile centrifuge tube, centrifuge, remove the supernatant, and culture immediately.
12. Cap the flask and maintain in a humidified incubator at 37°C in an atmosphere of 5% CO_2 and 95% air.

3.2.2. Procedure for Ascitic Effusions

The processing of ascitic effusions depends on the volume and the cellular content (erythrocytes, mesothelial cells, monocytes, and tumor cells). For specimens with few

or no erythrocytes, the cells are centrifuged, resuspended in RPMI 1640 medium, and seeded into 25-cm^2 flasks. For specimens with large numbers of erythrocytes, the cells are centrifuged and resuspended in RPMI 1640 medium, and cancer cells are isolated by Ficoll gradient.

1. Add 7 mL Ficoll gradient to a 15-mL sterile centrifuge tube.
2. To the 7 mL Ficoll gradient add (carefully layering on top of Ficoll gradient) 7 mL of diluted ascitic fluid using a sterile Pasteur pipet with its tip touching the side of the tube.
3. Centrifuge at 400*g* for 30–40 min in a tabletop centrifuge at room temperature.
4. Carefully aspirate the upper layer using a clean Pasteur pipet, leaving the cancer cell layer undisturbed at the interface.
5. Using a clean Pasteur pipet, transfer the cancer cell layer to a clean centrifuge tube.
6. The pellets from the gradient usually contain a significant number of tumor aggregates. After a single wash with PBS, resuspend the pellets in PBS and leave standing in a rack at room temperature, to allow heavier cell aggregates to sediment by gravity. After aspirating off the supernatant, which contains most of the erythrocytes, collect the cell aggregates.
7. Add at least 3 vol (6 mL) of PBS to the cancer cells in the centrifuge tube.
8. Suspend the cells by gently drawing them in and out of a Pasteur pipet.
9. Spin the cells at 400*g* for 10 min, decant the supernatant from the cell pellet, add 10 mL of RPMI 1640, resuspend the cells with a Pasteur pipet, and then spin at 400*g* for 10 min. Repeat this washing step.
10. Decant the medium and resuspend the cell pellet in 5 mL of AR-5 initiation medium.
11. Seed the cell pellet into 25-cm^2 flasks.
12. Cap the flask and maintain the culture in a humidified incubator at 37°C in an atmosphere of 5% CO_2 and 95% air.

3.3. Isolation of Cancer Cells

Cancer cells can grow as floating aggregates, firmly or loosely adherent colonies, or as both adherent and floating subpopulations (*see* **Note 2**). Isolation of the cancer cells is performed when heavy tumor-cell growth is observed. Several methods can be employed to isolate pure cancer cells.

3.3.1. Floating Aggregates

If floating aggregated cancer cells are grown in suspension, pure cancer cells can be easily obtained because most of the fibroblasts are attached to the surface of the culture flask.

1. Harvest and transfer the floating aggregates to the sterile centrifuge tube.
2. Spin the cells at 400*g* for 5 min, decant two-thirds vol of supernatant from the cell pellets, and add 5 mL of ACL-4 medium supplemented with heat-inactivated 5% FBS.
3. Seed the cell pellets into 25-cm^2 flasks.
4. Cap the flasks and maintain the culture in a humidified incubator at 37°C in an atmosphere of 5% CO_2 and 95% air.

3.3.2. Adherent Colonies

RAPPING METHOD:

This method is used when cancer cells are loosely attached to the surface of the culture flask and grow as adherent colonies among the stromal fibroblast cells. Of

these cancer cells, some cancer cells or whole colonies are detached from the colonies and grow in suspension.

1. Gently tap the flasks.
2. Harvest the individual cancer cells and colonies.
3. Transfer the floating aggregates to a sterile centrifuge tube.
4. Spin the cells at 400*g* for 5 min, decant two-thirds vol of supernatant from the pellets and add 5 mL of ACL-4 medium supplemented with 5% FBS.
5. Seed the cell pellet into 25-cm^2 flasks.
6. Cap the flask and maintain the culture in a humidified incubator at 37°C in an atmosphere of 5% CO_2 and 95% air.

Scraping Method:

Usually, firmly adherent colonies surrounded by stromal fibroblasts form large densely packed colonies that continue to grow laterally, slowly infiltrating and displacing the adjacent fibroblasts or mesothelial cells (*see* **Fig. 1**).

Eventually, the entire flask may be taken over by these tumor cells. In this case, cancer cells can be isolated by scraping and differential trypsinization ***(12)***. In our experiences, when large adherent cancer cell colonies are less impeded by surrounding fibroblasts, scraping is the best way of isolating the cancer cells. The cells are less damaged and stromal contamination is minimized ***(7,11,12,16,17)***.

1. Maintain the culture until the adherent colonies form well isolated large colonies (5–10 mm in diameter). This may take several months.
2. Select colonies and mark with marker pen on the flask base.
3. Decant the medium and add 1 mL of growth medium.
4. Detach the adherent colonies by scraping with a policeman or cell scraper, to allow them to float freely in the medium.
5. Add 4 mL of growth medium and harvest the detached colonies with a Pasteur pipet.
6. Transfer the cancer cells into 25 cm^2 flasks.
7. Maintain cultures in humidified incubator at 37°C in an atmosphere of 5% CO_2 and 95% air.

Differential Trypsinization Method:

Some cancer cells grow among stromal fibroblasts and produce small colonies. Usually these cancer cells do not overgrow the fibroblasts (*see* **Fig. 2**).

In these cases, the differential trypsinization method is preferred. The method depends on differences in cell susceptibility to trypsinization ***(12)***. Some cancer cells are more susceptible to trypsinization than fibroblasts and vice versa.

1. Decant the medium, rinse with PBS, and remove the PBS by aspirating.
2. Add 1.5 mL of diluted trypsin (0.025%, 0.05%, or 0.1% trypsin)
3. Place into incubator at 37°C for approx 3 min.
4. Observe the flasks under the inverted microscope when they are done incubating, and determine whether the cancer cells or fibroblasts are detached. If both cancer cells and stromal fibroblasts remain attached, incubate the culture flasks until either the cancer cells or fibroblasts detach.
5. If cancer cells have detached and the fibroblasts remain attached, add 5 mL of growth medium containing serum. Harvest the detached cancer cells. If, on the other hand, can-

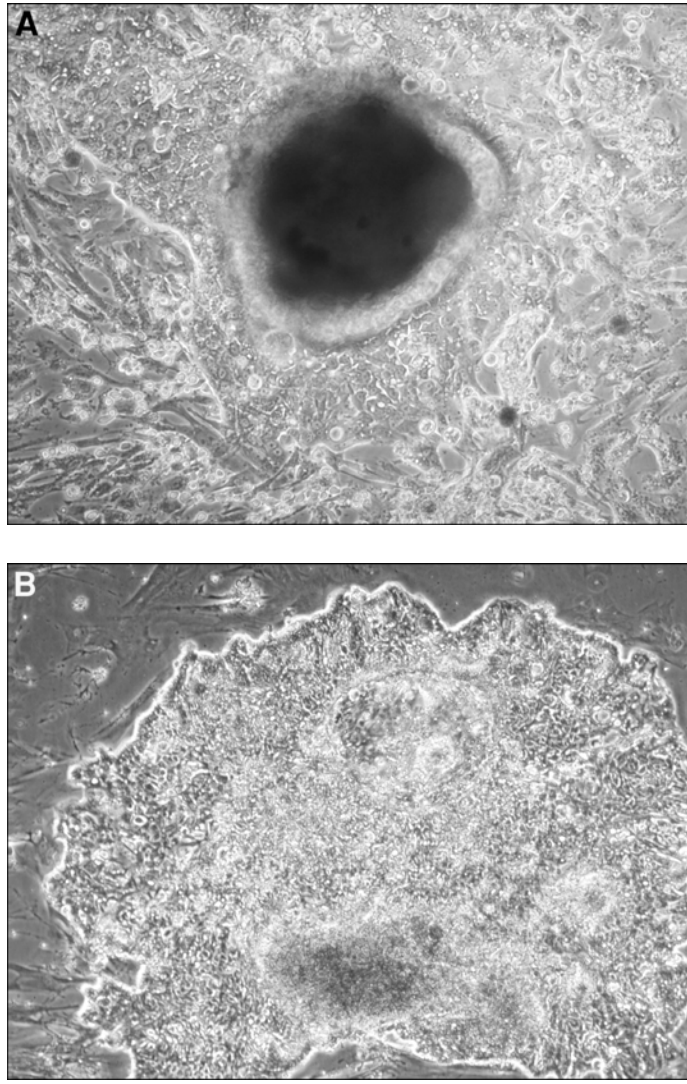

Fig. 1. Primary cell culture of primary tumor from colorectal cancer patients.

cer cells remain attached and the fibroblasts have detached, remove the medium, rinse with the growth medium, and then add 5 mL of growth medium.

6. Centrifuge the harvested cancer cells, remove the supernatant, and add 5 mL of AR-5 growth medium.
7. Transfer the cancer cells into 25-cm^2 flasks.
8. Maintain cultures in a humidified incubator at 37°C in an atmosphere of 5% CO_2 and 95% air.

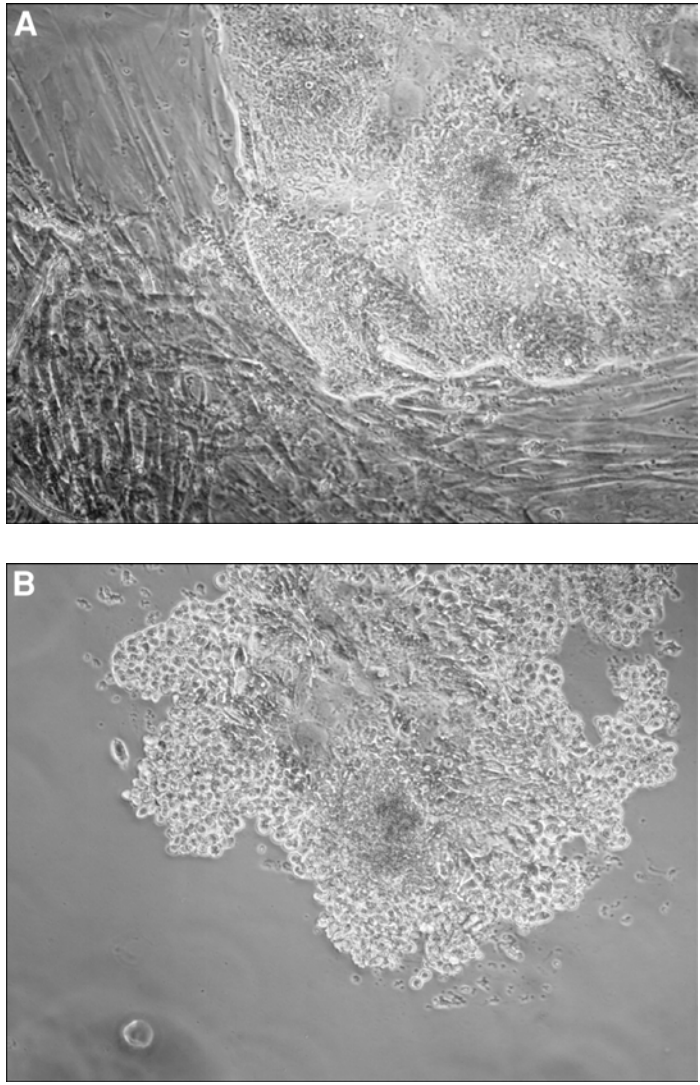

Fig. 2. **(A)** Before trypsinization. **(B)** After trypsinization and 1 d later.

3.4. Propagation and Preservation of Cancer Cells

After establishing cancer cell lines, initial passages are performed when heavy tumor-cell growth and large colonies are observed. Subsequent passages are performed every week or two. The ACL-4 medium ***(14,15)*** supplemented with heat-inactivated 5% FBS (used in primary cell culture) can be used also for the propagation of colorectal cancer cell lines ***(7,8,10,11)***. This medium must be used until the isolated

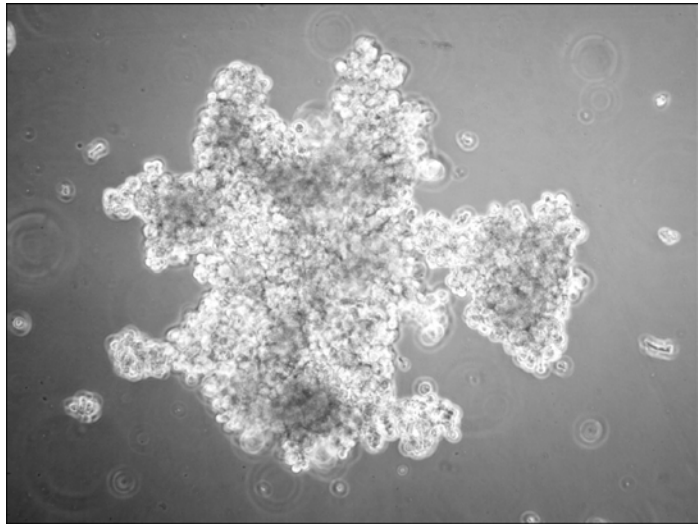

Fig. 3. SNU-769A colorectal carcinoma cell line. Cancer cells grow as loosely attached and freely floating cell aggregates. Individual cells are round, and no attached cells are noted.

cancer cells are considered a cell line (*see* **Note 3**). After the final establishment of cell lines, AR-5 medium can be replaced with RPMI 1640 medium supplemented with 10% heat inactivated FBS ***(11)***.

3.4.1. Floating Aggregates

Floating cell aggregates grow as loosely attached cell aggregates, in ball-like tightly packed clumps, etc. Loosely attached cell aggregates are subpassaged after dissociation by pipeting **(Fig. 3)**.

3.4.2. Tightly Packed Clumps **(Fig. 4)**

1. Collect the clumps by pipet and transfer to a sterile centrifuge tube.
2. Spin cells at 400*g* for 5 min, discard the supernatant from pellets, rinse with sterile PBS, centrifuge, and again remove the supernatant.
3. Add 2 mL of trypsin-EDTA (usually 0.05%) in PBS and incubate at 37°C for approx 3 min.
4. Add 5 mL of RPMI 1640 medium supplemented with 10% FBS and centrifuge.
5. Decant the supernatant and redisperse the cancer cells by pipeting with the growth medium.
6. Seed the cell pellet into 25-cm^2 flasks
7. Cap the flask and maintain the cultures in humidified incubators at 37°C in an atmosphere of 5% CO_2 and 95% air.

In this case, some cancer cells can be grown as adherent colonies and/or as a monolayer, and other cells can be grown as floating cell aggregates.

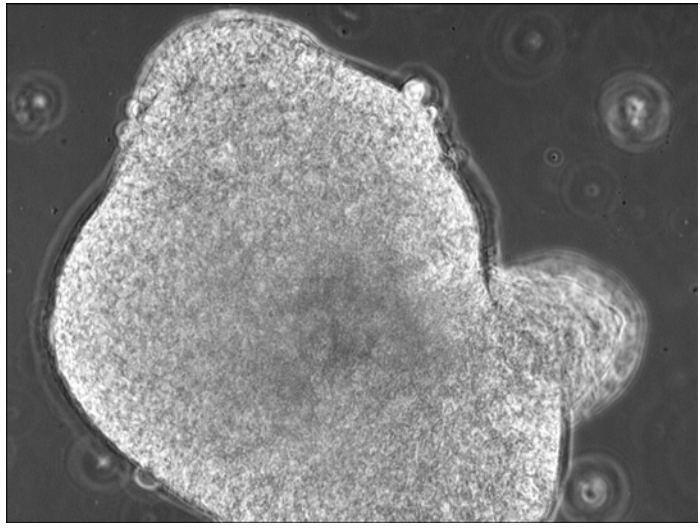

Fig. 4. SNU-1047 colorectal carcinoma cell line. Cancer cells grow as tightly packed clumps.

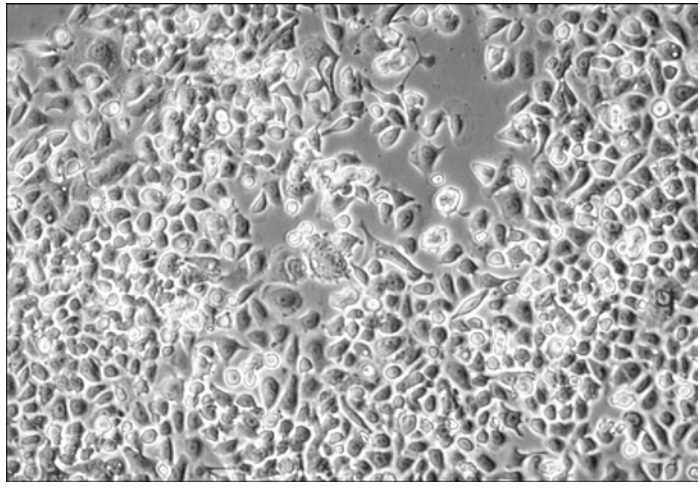

Fig. 5. SNU-407 colorectal carcinoma cell line. Cancer cells are polygonal in shape and grow as adherent cells.

3.4.3. Adherent Cells **(Fig. 5)**

1. Remove the supernatant and rinse with sterile PBS.
2. Add 2 mL of trypsin-EDTA (usually 0.05%) in PBS and incubate at 37°C for approx 3 min.
3. Add 5 mL of RPMI 1640 medium supplemented with 10% FBS and centrifuge.
4. Decant the supernatant and redisperse the cancer cells by pipeting them with the AR-5 growth medium.

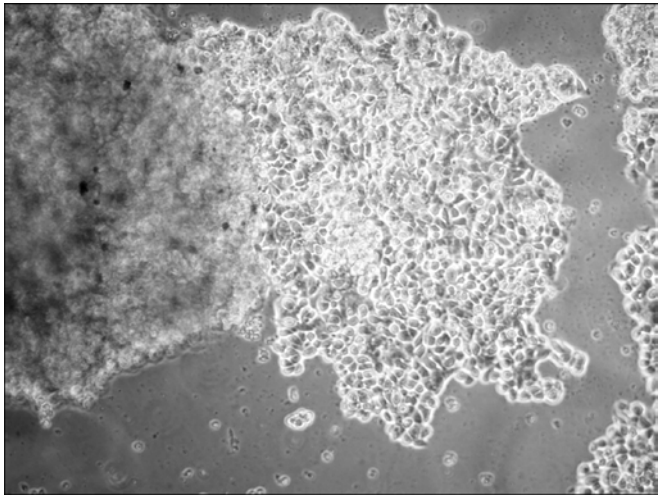

Fig. 6. SNU-175 colorectal carcinoma cell line. Cancer cells grow as both adherent cells *(right)* and floating cell aggregates *(left)*.

5. Seed the cell pellet into new 25-cm^2 flasks
6. Cap the flasks and maintain the cultures in humidified incubators at 37°C in an atmosphere of 5% CO_2 and 95% air. If the adherent cancer cells are not detached in the flasks, the concentrations of trypsin-EDTA and the incubation times can be increased.

3.4.4. Both Adherent and Floating Subpopulations **(Fig. 6)**

3.4.4.1. Floating Subpopulations

1. Collect the floating cell aggregates and transfer to sterile centrifuge tubes.
2. Remove the supernatant, and resuspend the cells in the growth medium.

3.4.4.2. Adherent Subpopulations

1. Add 2 mL of trypsin-EDTA (usually 0.05%) in PBS and incubate at 37°C for approx 3 min.
2. Add 5 mL of ACL-4 medium supplemented with 5% FBS and centrifuge.
3. Decant the supernatant, and combine the cells with the floating subpopulations.
4. Seed the cell pellet into new 25-cm^2 flasks.
5. Cap the flask and maintain the culture in a humidified incubator at 37°C in an atmosphere of 5% CO_2 and 95% air.

3.5. Representative Characteristics of Cultured Colon Cancer Cell Lines

The SNU-769A cell line was derived from a mucinous adenocarcinoma signet ring cell type (*see* **Fig. 3**). The original tumor was composed of singly scattered tumor cells with eccentrically located nuclei and abundant cytoplasmic mucin **(Fig. 7)**.

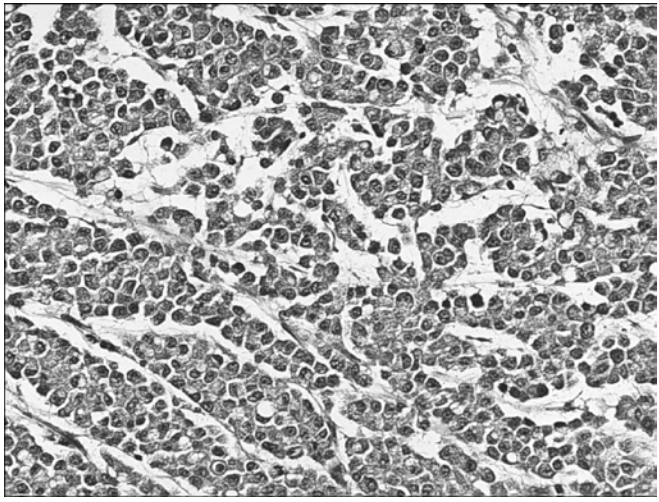

Fig. 7. Primary tumor (mucinous adenocarcinoma of signet ring cell type) of SNU-769A colorectal carcinoma cell line.

The tumor cells did not form glands or acini and showed solid growth pattern with minimal desmoplasia. Among the tumor cells, extracellular mucin pool was also noted. Cells of SNU-769A cell lines grow as loosely attached and freely floating cell aggregates. Individual cells are round, and no attached cells are noted (*see* **Fig. 3**).

The SNU-1033 cell line was derived from a poorly differentiated adenocarcinoma. The original tumor was characterized by solid pattern of growth with focal glandular differentiation. The majority of tumor cells were cuboidal and showed considerable nuclear pleomorphism (**Fig. 8A**).

Tumor cells of SNU-1033 demonstrate the attached epithelioid morphology and prominent dome formation (**Fig. 8B**).

The SNU-1040 cell line was derived from a well-differentiated adenocarcinoma. The original tumor was characteristically composed of well-formed glands. The tumor cells were columnar to cuboidal and maintained nuclear polarity (**Fig. 9A**).

Cultured tumor cells of SNU-1040 demonstrate the prominent adherent epithelioid cells (**Fig. 9B**).

4. Notes

1. Whenever possible, invasive areas from the serosal surface should be selected for primary tumor cultures to reduce the possibility of microbial contamination.
2. If the cancer cells are adherent or grow as floating aggregates, the medium is changed weekly until a substantial outgrowth of cells is observed.
3. It is recommended that early passages, whenever passaged, are frozen in cryoprotective medium to prevent loss of the cell lines by contamination or other laboratory accidents.

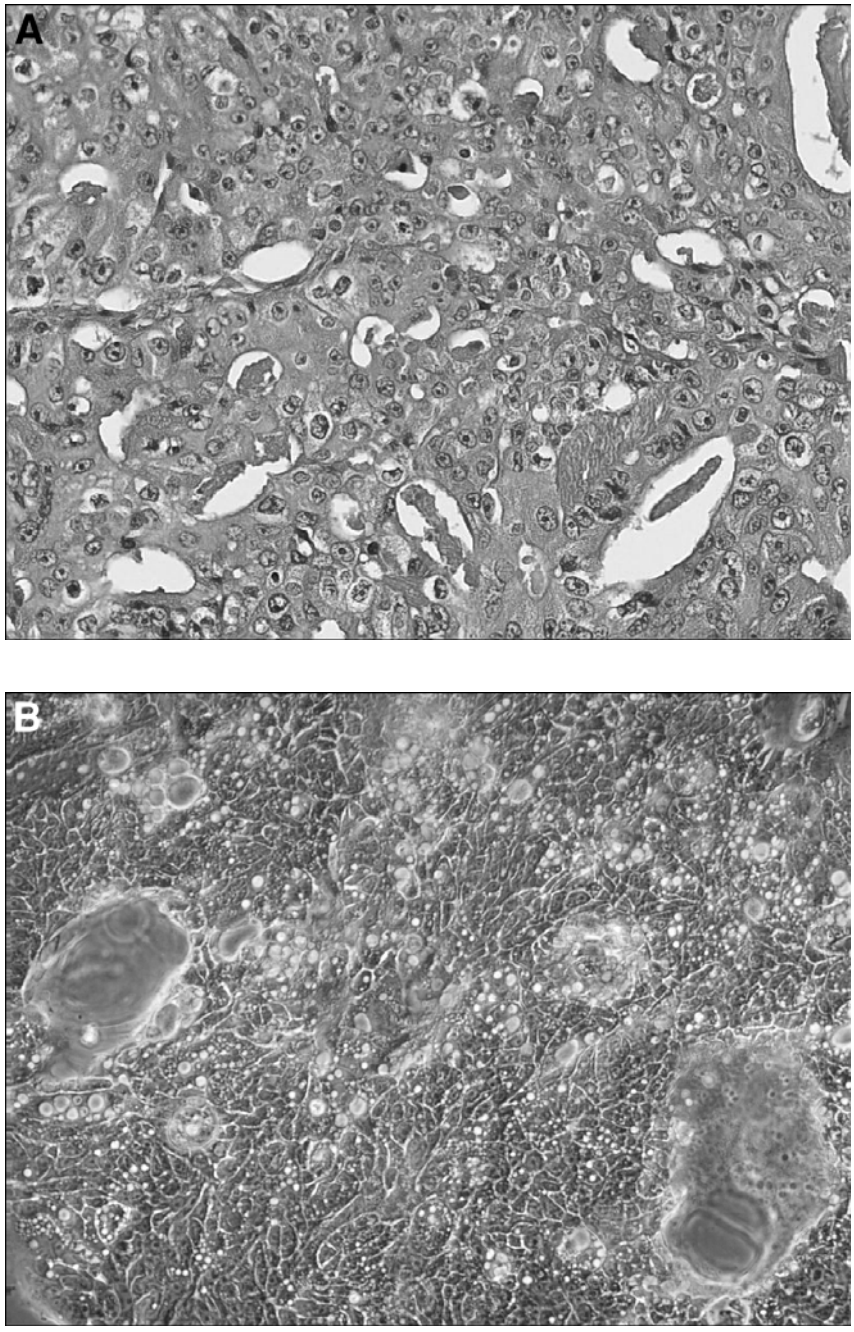

Fig. 8. **(A)** Primary tumor (poorly differentiated adenocarcinoma) of SNU-1033 colorectal carcinoma cell line. **(B)** Phase contrast microscopy of SNU-1033 colorectal carcinoma cell line.

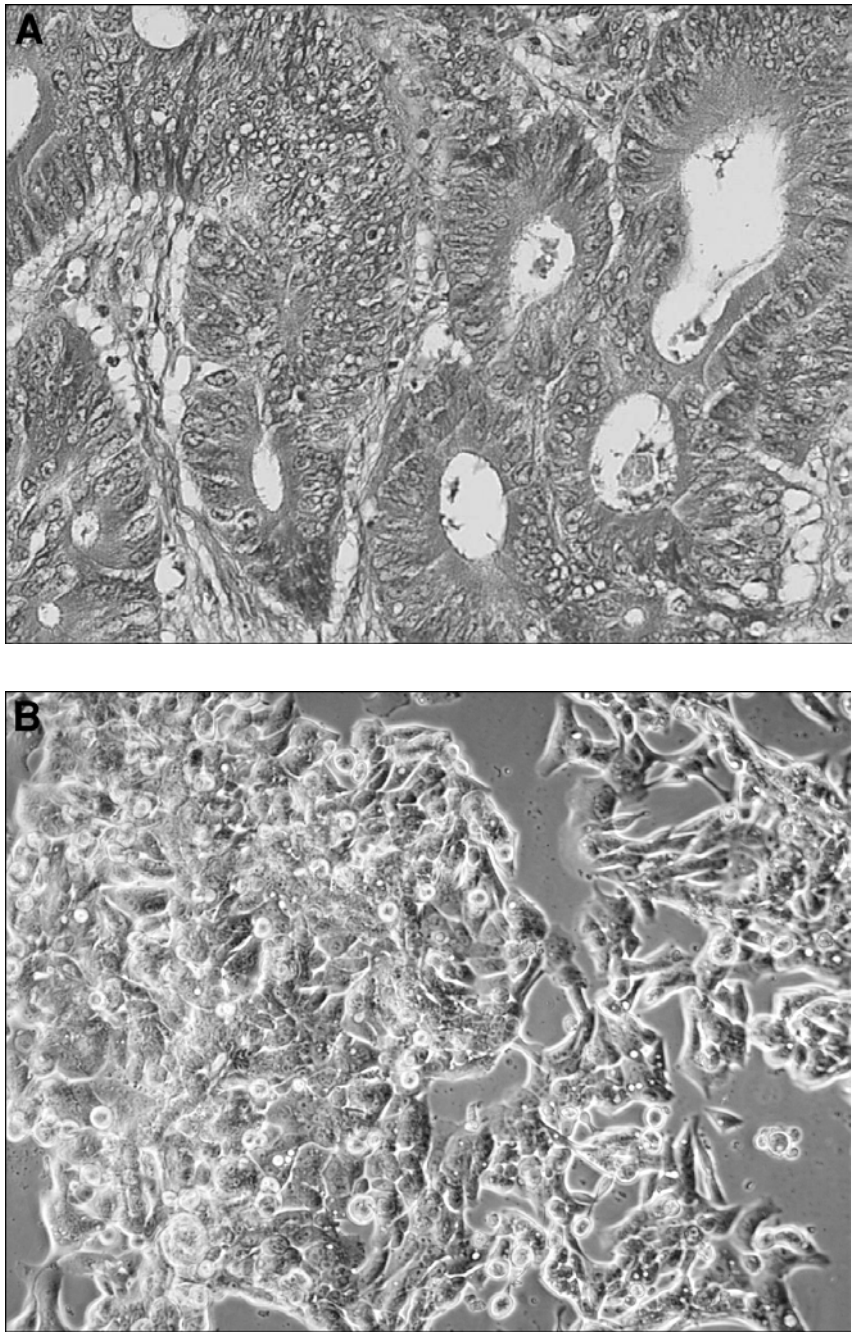

Fig. 9. **(A)** Primary tumor (well-differentiated adenocarcinoma) of SNU-1040 colorectal carcinoma cell line. **(B)** Phase contrast microscopy of SNU-1040 colorectal carcinoma cell line.

References

1. Jemal A., Thomas, A., Murray, T., and Thun, M. (2002) Cancer statistics. *CA Cancer J. Clin.* **52**, 23–47.
2. Honda, T., Kai, I., and Ohi, G. (1999) Fat and dietary fiber intake and colon cancer mortality: a chronological comparison between Japan and the United States. *Nutr. Cancer* **33**, 95–99.
3. Hamilton, S. R., Volgelstein, B., Kudo, S., Riboli, E., Nakamura, S., and Hainaut, P. (2000) Carcinoma of the colon and rectum. In: Hamilton, S. R., Aaltonen, L. (eds.) *Pathology and Genetics of Tumors of the Digestive System,* IARC Press, Lyon, pp. 105–119.
4. Smith, K. J., Johnson, K. A., Bryan, T. M., et al. (1993) The *APC* gene product in normal and tumor cells. *Proc. Nat. Acad. Sci. USA* **90,** 2846–2850.
5. Fishel, R., Lescoe, M. K., Rao, M. R. S., et al. (1993) The human mutator gene homolog *MSH2* and its association with hereditary nonpolyposis colon cancer. *Cell* **75,** 1027–1038.
6. Kane, M. F., Loda, M., Gaida, G. M., et al. (1997) Methylation of the hMLH1 promoter correlates with lack of expression of hMLH1 in sporadic colon tumors and mismatch repair-defective human tumor cell lines. *Cancer Res.* **57,** 808–811.
7. Park, J. G., Oie, H. K., Sugarbaker, P. H., et al. (1987) Characteristics of cell lines established from human colorectal carcinoma. *Cancer Res.* **47,** 6710–6718.
8. Park, J. G., Yang, H. K., and Hay, R. J. (1994) Colorectal cancer cell lines. In: Hay, R. J., Park, J. G. and Gazdar, A. (eds.) *Atlas of Human Cancer Cell Lines*, Academic Press, San Diego, pp. 317–341.
9. Sekiguchi, M., and Suzuki, T. (1994) Gastric tumor cell lines. In: *Atlas of Human Cancer Cell Lines*. Hay, R.J., Park, J. G., and Gazdar, A. (eds.) Academic Press, San Diego, pp. 287–316.
10. Park, J. G., and Gazdar, A. F. (1996) Biology of colorectal and gastric cancer cell lines. *J. Cell. Biochem.* **24 (suppl),** 131–141.
11. Oh, J. H., Ku, J. L., Yoon, K. A., et al. (1999) Establishment and characterization of colorectal-carcinoma cell lines. *Int. J. Cancer* **81,** 902–910.
12. Oie, H. K., Russell, E. K., Carney, D. N., and Gazdar, A. F. (1996) Cell culture methods for the establishment of the NCI series of lung cancer cell lines. *J. Cell. Biochem.* **24(suppl.),** 24–31.
13. Rutzky, L. P., and Moyer, M. P. (1990) Human cell lines in colon cancer research. In: Moyer, M. P. (ed.) *Colon Cancer Cells,*Academic Press, London, pp. 155–202.
14. Gazdar, A. F., and Oie, H. K. (1986) Growth of cell lines and clinical specimens of human nonsmall cell lung cancer in a serum-free defined medium. *Cancer Res.* **46,** 6011–6012.
15. Gazdar, A. F., and Oie, H. K. (1986) Cell culture methods for human lung cancer. *Cancer Genet. Cytogenet.* **19,** 5–10.
16. Park, J. G., Frucht, H., LaRocca, R. V., et al. (1990) Characteristics of cell lines established from human gastric carcinoma. *Cancer Res.* **50,** 2773–2780.
17. Park, J. G., Yang, H. K., Kim, W. H., et al. (1997) Establishment and characterization of human gastric carcinoma cell lines. *Int. J. Cancer* **70,** 443–449.

7

Isolation and Culture of Melanoma Cell Lines

Adam I. Riker

1. Introduction

Since the dawn of mankind, there has been cancer. From so simple a beginning, cancer, in all of its forms and complexities, has become the second leading cause of death in the United States. It is responsible for one out of four deaths, and totaled 549,838 (23% of all deaths) for the year 1999 ***(1)***. It is estimated that it will surpass cardiovascular disease as the number one killer by the year 2015. For melanoma, the incidence is increasing at an alarming rate. For the year 2002, new melanoma cases will be diagnosed in 53,600 individuals, claiming 7,400 lives ***(1)***. We have had limited success in the treatment of advanced stage melanoma with most patients succumbing to death within the first year of diagnosis.

There is a renewed hope and effort for the development of successful treatment options for patients with stage IV disease. Over the last decade, researchers and clinicians worldwide continue to make progress in understanding the immunobiology of melanoma, embracing new ideas and insights into the complex interactions that occur within the tumor milieu. It is within this tumor microenvironment where many of the answers to our questions can be found. Thus, to reach this goal, we have developed a very successful method of obtaining and establishing tumor cell lines for in vitro expansion and characterization. This has resulted in a stable supply of reagents for in vitro use that, in turn, can reflect the host/tumor interactions within the in vivo tumor microenvironment. Improved tumor models, an autologous system of reagents, and the establishment of a melanoma tumor (cell line) bank have advanced our knowledge about tumor escape mechanisms, immune cell activation, and the malignant transformation of cells.

The ultimate in vitro tumor model would completely reflect the in vivo tumor microenvironment in function and mechanism. It would become the genetic fingerprint for each patient, thus allowing the design of an optimal therapeutic vaccination strategy. It would be a true representation of antigen expression, cell surface receptor density, and genotypic makeup. Unfortunately, such a model does not currently exist.

From: *Methods in Molecular Medicine, vol. 88: Cancer Cell Culture: Methods and Protocols*
Edited by: S. P. Langdon © Humana Press Inc., Totowa, NJ

Clearly, as the tumor nodule grows, the tumor cells within it exist as a heterogeneous population of cells that are oligoclonal in nature at both the genetic and phenotypic level ***(2)***. Each cell, and indeed, each metastatic tumor nodule must be treated as a separate entity when envisioning the development of a vaccination strategy that is to have 100% lethality to *all* metastatic disease. To overcome this problem, the development of autologous cell lines in vitro provides a small glimpse into the cellular and immunological events that have taken place in vivo. A novel and somewhat unique approach involves the development and characterization of melanoma cell lines established from samples obtained by fine needle aspiration biopsy (FNAB) of subcutaneous lesions in patients with advanced melanoma.

There are many melanoma cell lines that are commercially available for any number of in vitro studies. Some are characterized in certain respects, but most only provide a guarantee that they are pure melanoma cell lines without contamination with other cells, such as fibroblasts. Characterization, functional analysis, and clonality of such cell lines are not guaranteed and are usually not addressed. The establishment and analysis of cell lines by techniques such as tumor cell digestion also have inherent faults as a result of the underlying question of whether these cell lines are a true representation of the cell population from which they originated in vivo. Furthermore, contaminating fibroblasts, macrophages, dendritic cells, T cells, and NK cells have all been shown to be present in various numbers in melanoma cell cultures established from tumor cell digests (*see* **Notes 1–5**). Fibroblast contamination remains a formidable hurdle even today. The in vitro data obtained from such "integration" between tumor cells and fibroblasts must be looked at with an eye of suspicion.

The utility of FNAB as a novel approach to obtain melanoma samples from patients with advanced disease has provided us with a wealth of patient-specific information. Furthermore, the samples obtained can be grown to large numbers in vitro for further characterization and functional analysis ***(3)***. The tumor nodule(s) remain for further prospective analysis by serial FNAB before, during, and after therapy. Excisional biopsy of tumors and subsequent expansion of tumor cell lines has been the traditional tool utilized for the analysis of tumor/host interactions. The obvious drawback of this method is that it allows for the evaluation of only a single lesion at a specific time point. The removal of the tumor excludes comparative analysis of the same lesion over time, thus severely limiting our understanding of the natural progression of the neoplastic process and its activity secondary to the immune pressure placed upon it. FNAB of multiple synchronous lesions results in the vital comparison of metastatic nodules from the same patient, characterizing differences in melanoma antigen (MA) and HLA expression, genetic alterations, receptor density, cytokine profile, and other molecular differences.

2. Materials

2.1. Cell Culture

1. Complete media (CM): Iscove's media (Biofluids Inc., Rockville, MD) supplemented with 10 m*M* HEPES buffer, 100 U/mL penicillin-streptomycin (Biofluids), 10 g/mL ciprofloxacin (Bayer West Haven, CT) 0.03% L-glutamine (Biofluids), and 0.5 mg/mL of

amphotericin B (Mediatech, Cellgro, VA) with 10% heat-inactivated human AB serum (Gemini Bioproducts, Calabasas, CA) (*see* **Note 6**).
2. Standard cell culture supplies should be readily available (pipets, culture flasks of all sizes, prepared complete media). The serial expansion of cells will initially require 48-well and 24-well flat bottom culture plates (Costar Inc., Corning, Calabasas, NY), T-25, T-75, T-175 culture plates (Costar Inc.).

2.2. Other Reagents

1. Diff-Quik stain.

3. Methods

3.1. Sample Collection and Culture from FNAB

3.1.1. Procedures and In Vitro Expansion of Melanoma Cells Obtained by FNAB

1. The most important part of collecting samples by FNAB is gaining the necessary experience through supervision by a qualified pathologist or cytopathologist with an expertise in the technique of FNAB. There is a learning curve associated with accurately collecting samples by FNAB, and one is advised to seek the guidance and support of those trained in this area.
2. A 23-gage needle is attached to a 10-mL syringe that rests within a specialized holder (Cameco Medical Limited, London) to facilitate operation with one hand while the other hand stabilizes the lesion to be aspirated. Cleanse the nodule and surrounding areas with alcohol and sterile gloves in order to obtain a sterile sample.
3. If a local anesthetic is to be used, a 1% lidocaine solution (without epinephrine) can be utilized to infiltrate the skin, creating a small wheal subcutaneously. Be careful to not infiltrate the tumor nodule itself, the wheal should be raised on the skin overlying the tumor nodule. In most circumstances, a local anesthetic is not necessary. Patients tolerate FNAB with a 23-gage needle extremely well with minimal discomfort. Remember, by using a local anesthetic and raising a wheal, the anatomy and tissue planes become distorted, making it difficult to localize the needle within the tumor nodule.
4. Once ready to aspirate, stabilize the lesion with the thumb and index finger prior to entering it with the needle. Enter the subcutaneous lesion, ensuring that the bevel of the needle is within the tumor mass. Apply a small amount of suction.
5. Remove and collect the cells while applying continous slight suction to the syringe with the cells remaining within the needle shaft and hub. Collect the cells with a back and forth motion of the syringe, gently changing the angle by 5 to 10 degrees with each forward motion. This will ensure an adequate collection of cells from different areas of the nodule. Be sure to release any suction within the syringe prior to removal of the needle; otherwise, most of the sample is aspirated into the syringe and sprayed on its walls.
6. Place a small sample on several slides and Diff-Quik stain. At the bedside, the sample is evaluated under the microscope to ascertain cellular morphology, degree of cellularity, and adequacy of the sample. An experienced cytopathologist can usually confirm the diagnosis at the bedside.
7. Once an adequate sample is obtained for diagnosis and immunostaining, a second pass is then collected under sterile conditions for in vitro culture. Place the sample into cell culture media at the bedside and take to the laboratory for in vitro culturing and expansion (*see* **Notes 1–7**).

3.1.2. In Vitro Culturing and Cellular Expansion

1. A total cell count is performed, including all nucleated and red blood cells (RBC), with final plating into a 24- or 48-well plate at 2–4 $\times$ 10^6 cells/well. ACK Lysing Buffer (Biofluids) can be used safely in order to lyse the RBCs prior to cell plating. Working without contaminating RBCs seems to be beneficial and there is no evidence that metabolites of lysed RBCs are beneficial for growth of tumor cells .
2. Cultures are maintained by changing media every 3 days (d). Remove half of the media and replace it with fresh media by carefully suctioning off the top portion of the undisturbed cell culture in the 24- or 48-well plate. As the cells grow and expand, they are adherent to the bottom and are trypsinized and split, based on the rapidity of their growth. It is absolutely necessary to keep the cells together within close proximity to each other for optimal cellular interaction and growth (*see* **Note 7**).
3. Once the tumor cell line has been properly expanded (meaning at least 10 T-175 flasks), final cellular characterization can then be performed by electron microscopy, karyotyping, receptor and tumor antigen analysis, fluorescence-activated cell scanning (FACS) analysis, and immunocytochemistry (ICC). The remaining flasks are frozen down and placed in liquid nitrogen for later use.

3.1.3. Immunocytochemistry

The following monoclonal antibodies are used: W6/32 (Sera Labs, Westbury, NY) for HLA class I; IVA-12 (American Type Culture Collection, Rockville, MD [ATCC]) for HLA class II; KS-I for HLA-A2, fluorescein isothiocyanate (FITC) antihuman CD8 and FITC antihuman CD4 (Pharmingen, San Diego, CA), antiMART-1/Melan-A murine IgG2b (M2-7C10 and antigp100 mAbHMB-45 (Enzo Diagnostics, Farmingdale, NY). The secondary antibody is goat antimouse IgG (FITC). ICC is performed on cytospin preparations from the original fine needle aspirate (FNA) sample and on subsequent established cell lines. The cytospins are fixed in acetone and stained with the same mAbs used for the FACS analysis (Biogenex, San Ramon, CA). For secondary staining, biotinylated goat-antimouse IgG (Kirkergaard & Perry Laboratories, Gaithersburg, MD) is used, followed by avidin-biotin-peroxidase (Vectasin Elite Kit, Vector Laboratories, Burlingame, CA). ICC was performed on cytospin preparations from the original FNA sample and on subsequent established cell lines.

3.1.4. Success Rate of Establishing Melanoma Cell Lines in Culture

The overall success rate of establishing melanoma cell lines is in the range of 70–90%. This is a result of meticulous attention to detail, both in terms of obtaining an adequate specimen and in cell culturing techniques. Several cell lines were established from synchronous lesions from the same patient. Each nodule must be treated as a separate entity and cell line. Almost all cell lines grew adherent to the flask bottom. Occasionally, there was evidence of fibroblast overgrowth by 48 h. In general, if the fibroblasts do not grow within the first 48–72 h, serial expansion by tumor cells will eventually lead to a pure melanoma cell culture, with little or no contamination by fibroblasts or other cells. It is important to realize that the established melanoma cell line is probably originating from the clone that has gained a survival advantage, restricting less robust clones from growing. Characterization and functional analysis

of the daughter cell lines have been performed for direct comparison to the in vivo tumor microenvironment of the original lesion (*see* **Notes 8–13**).

4. Notes

1. The successful establishment and characterization of melanoma cell lines in vitro has been met with a number of difficulties, highlighting the need for improved culture techniques. Traditionally, standard techniques of in vitro cell culture involved obtaining a tissue sample from an excisional biopsy or wide local excision, followed by a variety of processing techniques. Tumor samples are often digested with collagenase and other enzymes, then plated in enriched media. This technique has resulted in a rate of successful establishment of melanoma cell lines of approx 30%, and less for other cell types such as colon and breast cancer.
2. In previous studies, there has been limited success in growing primary uveal and conjunctival melanoma cell lines. Various regimens of processing tumor explants involve the mincing of tumor into 1–3-mm^3 chunks followed by placement into enzyme (collagenase, type IV, DNAase, type IV, hyaluronidase, type V)-containing media. Most frequently, the tumor "chunks" are allowed to stir overnight followed by filtering of the tumor cell suspension through a metal mesh filter with plating of the cell suspension in various supplemented media preparations. The growth media for tumor samples of ocular melanoma are highly variable, some utilizing growth factors such as EGF and IGF as well as other components such as phorbol esters and cholera toxin.
3. The most consistent problem with techniques involving a whole specimen is the variety of cells within the sample. Basically, a chunk of tumor contains tumor cells, but it also contains a host of other cells and tissue types as well. Often these tissues outnumber the primary tumor specimen by more than a thousandfold. Fibroblasts and stromal tissue cells are difficult to filter away from the tumor cells, resulting in overgrowth of the primary cell culture and rendering the sample unusable for further analysis.
4. Utilizing a culturing method of straining tumor material through a linen cloth with continuous irrigation with DMEM media, Luyten et al. reported the successful establishment of 2 of 16 (13%) primary uveal cell lines and 3 of 4 (75%) metastatic cell lines ***(4)***. McNamara et al. describe a method for the establishment of short term (4–13 wk) conjunctival melanoma cell cultures that allows for the immediate preparation of cell cultures, with cells dividing in vitro within 24 h of receipt of the enucleation specimen ***(5)***.
5. Behm et al. showed that multiple aspirations of benign lymph nodes can be performed without scar formation or significant architecture disruption that would affect histologic interpretation ***(6)***. Ljung et al. revealed that malignant epithelial cells, in this case, breast cancer cells, are almost exclusively collected ***(7)***. Their hypothesis is that tumor cells are less cohesive and, therefore, are easier to dislodge than adjacent stromal cells and benign breast epithelia. Of interest, they further comment that blood elements and lymphocytes may "contaminate" the tumor specimens.
6. Most complete media consists of fetal calf serum (FCS) of various percentages, with others adding horse serum (3%) because of its described ability to support the growth of skin melanocytes ***(5)***. However, problems can arise with unwanted antigenic responses against the FCS, thus obviating the use of human AB sera instead.
7. Meticulous cell culture techniques are required to achieve this success rate. Sterile methods must be utilized at every step of growth and cell expansion, to ensure that the media and other reagents, such as IL-2, are changed at least every third day. Complacency with cell culture will result in contamination by fungus, bacteria, or fibroblasts.

8. A major assumption of excisional biopsy specimens placed in vitro for cell culturing is that the tumor represents a homogeneous population of cells representative of other metastatic lesions in vivo from the same patient. It is now well documented that in metastatic melanoma, synchronous lesions are remarkably heterogeneous in their expression of melanoma-associated antigens (MAA) and HLA ***(8-12)***. Thus, the evaluation of a single lesion may not represent the full spectrum of synchronous metastatic lesions in terms of functionality and response to therapy.
9. Melanoma cell cultures originating from excisional biopsies can provide useful information regarding tumor/host interactions at only a single time point. Removal of the entire lesion precludes further prospective analysis, and subsequent data obtained from this specimen is thus limited to a retrospective analysis only. Characterization of a lesion from a single point greatly diminishes the potentially crucial information gained by the sequential collection and evaluation of FNA samples of the same lesion over time prior to, during, and after various treatment regimens.
10. It is important to analyze the daughter cell line obtained from the original FNAB in order to determine the similarity between the in vitro antigen expression with that obtained from the original in vivo sample. This can be done by immunocytochemistry (ICC) with measurement of the levels of expression of various tumor antigens, such as gp100, MART-1, and tyrosinase.
11. The daughter cell lines, in the vast majority of cases, can be compared to the original FNAB sample by immunological and molecular methods. In general, there is a strong correlation in melanoma antigen expression between fresh tumor explants and the cultured daughter cell lines. Cell lines generated from metastases with high expression of gp100 were found to have high levels of gp100 by FACS analysis compared to the expression of the same antigens as detected by ICC on freshly explanted tumor cells ***(3)***.
12. Occasionally, it was noted that if the fresh tumor explant was positive for gp100, subsequent in vitro culturing of this sample resulted in the loss of this MAA by FACS analysis. This selective pressure is known to occur in vitro, since the loss of these tumor antigens is not essential for the survival of the tumor cell. It is important to remember that MA expression is of little importance to the tumor cell, and bears no relationship to the neoplastic process. Thus, their expression in melanoma cells is a remnant of the melanocytic origin, and its loss of expression may occur without significant repercussions upon cancer cell survival. Therefore, these antigens could be particularly sensitive to immune selection during disease progression, under the effects of antigen specific immunization, and, of course, in vitro culturing. The exact incidence of this occurrence is unknown.
13. It is possible that a selection bias occurs whenever a population of tumor cells is placed in vitro for further expansion and characterization. It is unknown which cellular dynamics are actually occurring in vitro, but it may be that the most robust subpopulation of cells become the dominant cell line, overgrowing the smaller cell populations. It is not known whether such long-term cultures continue to converge in a clonal nature, or develop intracultural differences detectable only at the level of the genome. Regardless, there will always be some bias built into any such tumor model, and the overall level of tumor heterogeneity within the established cell culture does not seem to be any higher than that seen in vivo.
14. In conclusion, the isolation and culture of melanoma cells utilizing FNAB is a fast, efficient and highly reproducible method of obtaining human melanoma samples for subsequent in vitro expansion and characterization. Upon successful expansion, the cells provide the foundation for all subsequent functional analysis and tumor/T-cell interac-

tions. For the most part, the results obtained can be translated to the in vivo tumor microenvironment, providing a powerful method for the prospective analysis and immunosurveillance of the response to vaccination trials in patients with advanced disease. From a single FNAB of a melanoma lesion, "contaminating" cells such as CD4 (+), CD8 (+) T cells, dendritic cells, and NK cells provide a further level of complexity of what is thought to be necessary for a successful immune response to occur.

References

1. Smith, R. A., Cokkinides, V., von Eschenbach, A. C. (2002) Cancer Statistics, 2002: Progress or cause for concern? *CA Cancer J. Clin.* **52,** 6–8.
2. Cormier, J. N., Hijazi, Y. M., Abati, A., et al. (1998) Heterogeneous expression of melanoma-associated antigens (MAA) and HLA-A2 in metastatic melanoma in vivo. *Int. J. Cancer* **75,** 517–524.
3. Riker, A. I., Panelli, M. C., Kammula, U. S., et al. (1999) Development and characterization of melanoma cell lines established by fine-needle aspiration biopsy: Advances in the monitoring of patients with metastatic melanoma. *Cancer Det. and Prev.* **23,** 387–396.
4. Luyten, G. P., Naus, N. C., Mooy, C. M., et al. (1996) Establishment and characterization of primary and metastatic uveal melanoma cell lines. *Int. J. Cancer* **66,** 380–387.
5. McNamara, M., Kennedy S. M. (1997) Successful establishment of uveal and conjunctival melanoma in vitro.*In vitro Cell Dev. Biol. Anim.* **33,** 236–239.
6. Behm, F. G., O'Dowd, G. J., Frable, W. J. (1984) Fine-needle aspiration effects on benign lymph node histology. *Am. J. Clin. Pathol.* **82,** 195–198.
7. Ljung, B. M., Chew, K., Deng, G., Matsumura, K., Waldman, F. M., and Smith, H. (1994) Fine needle aspiration techniques for the characterization of breast cancers. *Cancer* **74,** 1000–1005.
8. Cormier, J. N., Panelli, M. C., Hackett, J. A., et al. (1999) Natural variation of the expression of HLA and endogenous antigen modulates CTL recognition in an in vitro melanoma model. *Int. J. Cancer* **80,** 781–790.
9. Marincola, F. M., Hijazi, Y. M., Fetsch, P., et al. (1996) Analysis of expression of the melanoma-associated MART-1 and gp100 in metastatic melanoma cell lines and in in-situ lesions. *J. Immunother.* **19,** 192–205.
10. Scheibenbogen, C., Weyers, I., Ruiter, D. J., Willhauck, M., Bittinger, A., Keilholz, U. (1996) Expression of gp100 in melanoma metastases resected before and after treatment with IFN-α and IL-2. *J. Immunother.* **19,** 375–380.
11. de Vries, T. J., Fourkour, A., Wobbes, T., Verkroost, G., Ruiter, D. J., and van Muijen, G. N. Heterogeneous expression of immunotherapy candidate proteins gp100, MART-1, and tyrosinase in human melanoma cell lines and in human melanocytic lesions. *Cancer Res.* **57,** 3223–3229.
12. Lehmann, F., Marchand, M., Hainaut, P., et al. (1995) Differences in the antigens recognized by cytolytic T cells on two successive metastases of a melanoma patient are consistent with immune selection. *Eur. J. Immunol.* **25,** 340–347.

8

Isolation and Culture of Human Brain Tumor Cells

German G. Gomez and Carol A. Kruse

1. Introduction

Sixty percent of all intracranial neoplasms are of glial origin. As a result of their infiltrative growth and heterogeneity, standard treatment (surgery, chemotherapy, and radiotherapy) is noncurative ***(1,2)***. Patient survival is poor and has not significantly improved over the past 20 years. It is a common belief in the neuro-oncology field that development of combination therapies for malignant brain tumors will be essential if improvement in survival is to occur ***(3,4)***. To test experimental single and multimodal therapies, animal and in vitro models of brain tumors are necessary.

Successful isolation and culture of primary human malignant glioma cell lines gives researchers in vitro models for studying brain tumor progression and genetics, mechanisms of resistance to chemo-, radio-, and immunotherapies, tumor invasion, and new therapeutic agents ***(5-12)***. We describe the materials needed and procedures used to isolate human brain tumor cells from surgical tissue specimens and from cavitational ultrasonic surgical aspirates (CUSA; **Fig. 1**) ***(13)***.

We also specify the media and conditions required to establish and maintain human brain tumor cells in culture. Finally, we detail ways to characterize the cells.

2. Materials

2.1. Transport Vessels and Fluids

1. Sterile 80 mL specimen collection containers (Starplex Scientific, Etobicoke, Ontario, CA) containing 10 mL of RPMI 1640 medium. To avoid loss of small biopsy specimens, they can be placed between Telfa dressing (Kendall Company Ltd, UK) moistened with sterile saline.
2. The tissue collection filter from the specimen trap contains tissue fragments in sterile saline irrigation fluid from the CUSA.
3. Erythrocyte lysis buffer components: 8.3 g/L ammonium chloride, 0.02 g/L disodium ethylenediaminetetraacetic acid (EDTA) and 1.0 g/L sodium bicarbonate.

From: *Methods in Molecular Medicine, vol. 88: Cancer Cell Culture: Methods and Protocols*
Edited by: S. P. Langdon © Humana Press Inc., Totowa, NJ

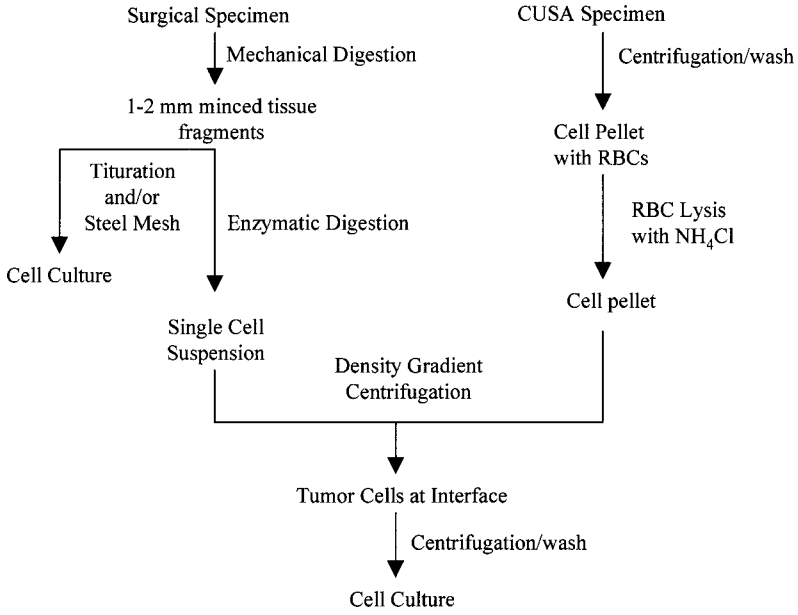

Fig. 1. Methods used to isolate brain tumor cells for culture from primary surgical or CUSA specimens.

2.2. Cell Digestion

1. DNase type I (Sigma).
2. Hyaluronidase type V (Sigma).
3. Collagenase type IV (Sigma).
4. Cellector tissue sieves with 100 mesh screens (VWR Scientific Products, www.vwrsp.com).
5. Ficoll-Paque density 1.083 g/mL gradient medium (Pharmacia, Piscataway, NJ).

2.3. Cell Growth Media, Supplements, Buffers, and Reagents

1. Culture medium: Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Grand Island, NY) + 10% fetal bovine serum (FBS; Gemini Bio-Products Woodland, CA), 100 U/mL penicillin/100 µg/mL streptomycin (Fisher Scientific, Pittsburgh, PA), 40 mg/mL gentamicin (Elkins-Sinn, Inc., NJ), and 200 m*M* L-glutamine (Life Technologies).
2. Roswell Park Memorial Institute (RPMI) 1640, Ham's F10 (F10), or F12 (F12) media.
3. Hank's balanced salt solution without calcium and magnesium (HBSS; Life Technologies).
4. Phosphate buffered saline (PBS; Life Technologies) with Versene (1 m*M* EDTA).
5. 0.025% Trypsin in PBS.

3. Methods

3.1. Acquisition of Tissue

With compliance issues at today's forefront, it is generally accepted that Institutional Review Boards (IRB) have knowledge of tissue being banked and/or cultured and that human consent is required. Hospitals and associated academic institutions

generally find it less cumbersome to ask for consent on the hospital's surgical consent form.

To obtain tissue, RPMI 1640 medium is placed in sterile specimen collection containers at 4°C until needed. In the operating room, and after the surgical pathologist deems that adequate material is available for diagnosis, resected remnant tissue is placed directly into the RPMI 1640 medium and transported immediately to the research laboratory for further manipulation. In some instances, tissue fragments are also obtained from the specimen trap attached to the CUSA. The specimen trap containing the specimen filter is a suitable container for transport to the laboratory, where further manipulations ensue in the biosafety hood and adequate protective labwear and practices are used (*see* **Note 1**).

3.2. Tissue Manipulations

3.2.1. Mechanical Dispersion of Tissue

1. If the tissue is bloody, 1–2 rinses of the tissue with 5–10 mL of HBSS should follow with aspiration of the fluid into a trap with hypochlorite (bleach) or other disinfectant.
2. If enzymatic digestion is planned, leave the tissue within the specimen container for further manipulation. Alternatively, with sterile forceps transfer brain tumor tissue to a glass Petri dish. Add 5–10 mL RPMI 1640 medium and finely mince the tissue (1–2 mm) with sterile scissors. At this point you may proceed with purely mechanical dispersion of the minced tissue fragments, or proceed with enzymatic digestion (*see* **Subheading 3.2.2.**).
3. Mincing is followed by repeated tituration with a tissue culture pipet. At this point transfer the cell suspension into a 15-mL conical centrifuge tube and allow several minutes for the large fragments to settle to the bottom of the tube. Place the supernate containing single cells and cell fragments directly into culture (*see* **Subheading 3.4.**). Transfer the larger fragments that settle to a tissue sieve with a stainless steel mesh. Using a blunt ended glass rod, force the tissue through the screen, rinse the screen with RPMI 1640, and then place the cell suspension into culture (*see* **Subheading 3.4.**)

3.2.2. Enzymatic Digestion of Minced Tissue

1. Common practice is to enzymatically digest the minced tissue into a single cell suspension. Add concentrated enzymes to the specimen container at final concentrations of 0.002% DNase type I, 0.01% hyaluronidase type V, and 0.1% collagenase type IV.
2. Add a magnetic flea to the suspension and then place the container at a low setting providing gentle agitation on a magnetic stirrer. Place the tissue at 37°C for 1–3 h (*see* **Note 2**). It can be determined visually when the clumps disintegrate into a single cell suspension.
3. To remove enzymes, transfer the resulting the cell suspension to a centrifuge tube, maximize the volume in the tube with HBSS, and centrifuge at 200*g* for 10 min. Aspirate the supernatant and process the cell pellet for density gradient centrifugation (*see* **Subheading 3.3.**).

3.2.3. Fragmented Tissue Isolated from CUSA Specimen Traps

1. The specimen trap and tissue collection filter **(Fig. 2)** from the CUSA (Valley Labs, Model 200, Boulder, CO) is nonsterile when shipped. It must be sterilized before use. It withstands STERRAD gas sterilization (Advanced Sterilization Products, Irvine, CA; www.sterrad.com) involving heat exposure up to 70°C. After tissue collection, seal the specimen trap in the operating room for transport to the research laboratory.

Fig. 2. Specimen collection filter from CUSA specimen trap.

2. To gain access to the filter for removal of tissue, set the filter on a sterile flat metal tray and use scissors to slit the bag open at the top end. Recover the fluid inside the bag and place in 50-mL centrifuge tubes. Then lay the bag flat on the tray and slit it down the middle. Scrape any tissue adherent to the walls of the trap with a flat spatula and transfer into the same centrifuge tubes. Pipet HBSS sparingly to the inside of the trap, then recover, and add this cell suspension to the scraped cells. Add HBSS to the maximum volume of the tubes and obtain cell pellets by centrifuging at 200*g* for 10 min at room temperature. The supernatant is then aspirated and discarded.
3. Next, remove red blood cell contamination present in the pellet. Resuspend the pellet in approx 2–5 mL of HBSS. Then add erythrocyte lysing buffer to the resuspended cell pellet at the maximum tube volume. Perform this procedure at room temperature, or warm the contents of the tube slightly by agitating it in a 37°C water bath. Generally, 5–10 min is sufficient. When hemolysis is noted (*see* **Note 3**), centrifuge the tubes once again at 200*g* for 15 min. The supernatant is aspirated and the cell pellet washed by centrifugation several more times with HBSS. After the final wash, the cell pellet is resuspended in HBSS and prepared for density gradient centrifugation (*see* **Subheading 3.3.**).

3.3. Density Gradient Centrifugation of Single Cell Suspensions

1. Resuspend the cell pellet in two vol of HBSS and carefully layer over one vol of Ficoll-Paque density gradient medium (*see* **Note 4**). Centrifuge the tubes at 400*g* for 20 min.
2. Collect the viable cells at the buffer/Ficoll-Paque interface (*see* **Note 5**). Wash the cells isolated at the interface twice with HBSS by centrifugation.

3.4. Culture and Maintenance

3.4.1. Culture Initiation

1. With minced tissue, place aliquots of the cell aggregates into multiple tissue culture flasks. Add a maximum of 3–4 mL of tissue culture medium to 25 cm^2 vented flasks (or loosely capped nonvented flasks) so the tissue fragments can settle and adhere to the flask surface. Place the cultures in the incubator and leave undisturbed for 2–3 days (d) so that outgrowth from the attached tissue occurs. After this does occur, increase the volume to 5 mL.
2. With cells isolated from the Ficoll-Paque gradients, resuspend the cell pellets in 1–2 mL of DMEM growth medium with supplements for aliquoting into a variety of growth media.

To maximize the chances of recovering all cell types within the heterogeneous cell populations, a variety of growth media is used if sufficient numbers are available for culture (*see* **Note 6**). All growth media is supplemented with 10% FBS (v/v), 2 m*M* L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 50 µg/mL gentamicin, pH 7.2.

3. Dispense 9 mL of growth medium and 1 mL of tumor cell suspension into a 75 cm^2 culture flask (or 4.5 mL of medium to 0.5 mL of tumor cell suspension in a 25 cm^2 culture flask). The number of flasks initiated depends upon the volume of tumor tissue available. Place the flasks in an incubator with a humidified chamber at 37°C in 5% CO_2.
4. Viable tumor cells attach within the first several days of culture, whereas the majority of glia do not. Other normal brain cells, such as microglia/macrophage, fibroblasts, endothelial, or trapped glia may initially attach; however, their numbers will decline rapidly and eventually they will be eliminated from culture because of their slow growth ***(9)***. Initially, every 4–5 d about 50% of the growth medium along with cells in suspension culture are removed and replaced with fresh growth medium until the predominant cell type is the attached cell. The cultures are monitored and fed with fresh growth medium at least once a week.

3.4.2. Passaging and Cryopreservation

1. When cells become confluent (1–4 wk), save the conditioned cell culture medium and transfer to a centrifuge tube. Gently rinse the attached cells with Versene in PBS and aspirate. Then add 2–4 mL of 0.025% trypsin in PBS and return the flask to the incubator and monitor every 5 min.
2. When the cells detach from the culture flask (*see* **Note 7**), quench trypsin activity by adding cell culture medium with supplements. Pellet the cells by centrifugation, remove the supernatant, and replace with several milliliters of fresh culture medium and resuspend for cell counting.
3. Using a hemocytometer, determine cell counts and viability by trypan blue dye exclusion.
4. At this point, transfer the cell suspension to a larger culture flask, or divide the cells equally among multiple culture flasks. Generally, cells from a confluent monolayer in one flask are split among four or five equally-sized flasks. Culture cells in fresh culture medium containing 20% of the conditioned medium from which the cells were grown prior to passage.
5. Additionally, cryopreserve a percentage of the low-passage cells to avoid potential loss resulting from microbial contamination or unforeseen incidents, such as incubator failure. Cells resuspended in growth medium are mixed with an equal volume of ice-cold, heat-inactivated fetal calf serum (FCS) containing 15% dimethlysulfoxide (DMSO). Freeze in cryogenic vials using a Cryomatic Cellevator (L.A.O. Enterprises, Gaithersburg, MD) suspended above the liquid nitrogen in a tank to lower the temperature at 1°C/min. Long-term storage of vials is accomplished by transferring the vials into liquid nitrogen or into a –80°C freezer (*see* **Note 8**).

3.4.3. Recovery and Expansion of Cryopreserved Cells

1. Quick-thaw cells by placing the freezing vial(s) in a 37°C water bath. Once thawed, immediately dilute the cells into 10 mL HBSS and wash twice by centrifugation (*see* **Note 9**).
2. Once the cells are washed, resuspend them in the appropriate growth medium. After a cell count and viability determination, plate the cells at 0.5–1.5 million viable cells per 75 cm^2 flask. Dependent on cell viability, density at which cells are plated, and length of time the cells are cryopreserved, it may take up to 2 wk for cells to recover and begin expansion.

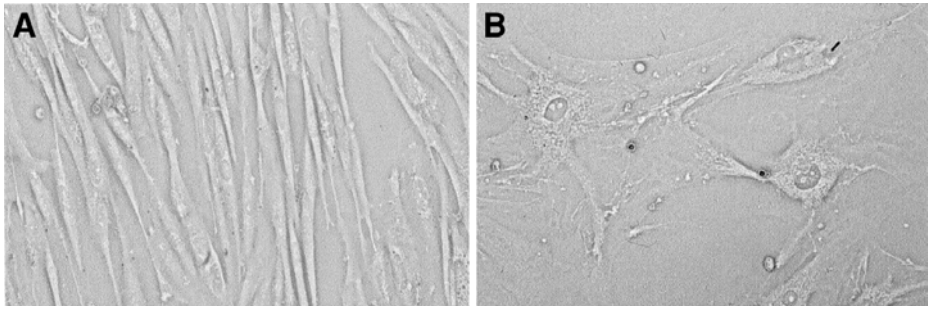

Fig. 3. Morphologic shapes of cultured glioma cells displaying **(A)** a fibroblastic morphology: slender, elongated, spindle-shaped cells with oval nuclei, or **(B)** an epithelioid morphology: large, vesiculated, flat polygonal cells with abundant cytoplasm and interconnecting processes.

3.5. Characterization of Brain Tumor Cells

1. Brain tumor cells acquire multiple morphologies when in confluent monolayer ***(5)***. Phenotypically, four growth patterns are described as fibroblastic, fasicular, epithelioid, and glial. Two morphologic types that we and others report as common **(Fig. 3)** appear as either long, slender spindle-shapes or as larger cells with flat, vesiculated epithelioid shapes ***(6,10,11,14;*** *see* **Note 10**). We have success in getting 40–50% of cell specimens to plate in culture; however, those that make it to a cell line designation (≥passage 20) may only amount to 10–15% of those that initially plate. Others stop growing, or grow so slowly that they are less useful as a cell culture model. If cells are planned for use in vitro models, verify mycoplasma negativity of the cultured cells and monitor for it routinely (*see* **Note 11**).
2. Demonstrate the glial origin of the brain tumor cells at early passage. This is commonly done by immunohistochemical staining of the cells for markers ***(11)***. Cells of astrocytic lineage generally are glial fibrillary acidic protein (GFAP) positive. This particular marker may be lost upon repeated passage. Many astrocytic tumors also express vimentin, platelet derived growth factor (PDGF), neuron specific enolase (NSE), and S100 protein. Oligodendrocytic cells generally express galactocerebroside C. Neurofilament is used as a neuronal marker. If contaminating fibroblasts are present, fibronectin and vimentin will be expressed on them.
3. Once lineage is confirmed, then perform characterization of the cells for karyotype, tumorigenicity, genetic, and molecular characteristics. Other sections of this volume will address the methods for these assays, which include among others, proliferation assays, plating efficiency, tumorigenicity assays, mobility, and invasion assays. Neuropathologists are finding that genetic and molecular techniques may play a role in distinguishing astrocytomas from oligodendrogliomas, or mixed oligoastrocytomas. Diagnostically, this carries great significance, as the latter two types may respond to chemotherapeutics, whereas astrocytomas and glioblastomas do not ***(15)***. As such, fluorescence *in situ* hybridization for chromosomes 1p and 19q deletions, or assays for loss of heterozygosity at chromosome 10 may eventually be routinely used to help confirm diagnoses made by histologic criteria ***(16,17)***. Furthermore, the degree of malignancy of astrocytomas may also be defined by molecular

criteria ***(18,19)***. High-grade astrocytomas also frequently display chromosome 7 amplification, resulting in epidermal growth factor receptor (EGFR) overexpression ***(20,21)***. The loss of the chromosome 17 *p53* tumor suppressor gene, or mutations of it are also noted in high frequency in astrocytic tumors ***(14,17,22)***.

4. Notes

1. If sufficient sample is available (≥1 mL) for further characterization, it is good practice to obtain small pieces of tissue that are snap frozen with liquid nitrogen after wrapping in foil or placing in Tissue Tek OCT embedding compound (Catalog #2583, Sakura Finetek, Torrance, CA); these can be stored at –80°C. This complements the tissue taken by the surgical pathologist, the bulk of which is generally formalin-fixed and available in paraffin blocks. When only very small biopsy specimens are available this cannot be done, and generally the mechanical dispersion method is chosen to minimize loss of cells that occur by other more extended processing methods involving more manipulation.
2. At times it is convenient to enzymatically digest the minced tissue overnight. If so, the digestion occurs at room temperature.
3. Hemolysis is observed by noting the change from a turbid suspension to a red transparent (hemolytic) color at the interface of the fluid and tube. Although it is important to minimize cell exposure to ammonium chloride, if hemolysis is incomplete, this step may be repeated.
4. In 15-mL conical tubes, generally overlay 4 mL of Ficoll-Paque with 8–10 mL of resuspended cell pellet volume. In 50-mL tubes place 12 mL of Ficoll-Paque with 24–36 mL of resuspended cell pellet. Sharper, more easily visible bands are generally obtained with gradients in the 15-mL tubes.
5. Aspirate fluid from the top to within several milliliters of the band visible at the interface. Cells are collected at the interface. Red blood cells and necrotic cells are at the bottom of the gradient.
6. Brain tumor cells typically grow well in either F10 or F12 media mixed with DMEM at 1/1 (v/v). If cells are not available in the amounts needed for multiple flasks, F12/DMEM is the medium of preference. DMEM, RPMI 1640 media, and a mixture of RPMI and DMEM at a 2/1 (v/v) are also used.
7. Some cells may not detach easily with trypsin. Physical collision of the flask against a gloved hand may help to dislodge the cells from the surface. Only minimal use of cell scrapers (Fisher Scientific, Pittsburgh, PA) is recommended, as continued use of scrapers promotes cell aggregation on subsequent passaging.
8. A cryogenic controlled rate freezing container (Fisher Scientific) with an isopropyl alcohol bath also may be used. These containers generally handle larger numbers of vials. Maintenance of cell viabilities is better when storage is in liquid nitrogen, rather than at –80°C.
9. Cells need to be quickly thawed and the DMSO diluted, as the latter is toxic to cells and can affect cell viability in culture.
10. Our experience is that the latter larger, vesiculated cells do not grow as rapidly and are often lost over time in a heterogeneous culture. The vesiculated cells initially may grow well (10–15 passages), but do not continue to do so after repeated passage. Therefore, establishment of this type of morphologic cell as a cell line is rare.
11. Hoechst dye can be used for mycoplasma detection. Many companies offer detection kits (MTC/NI Rapid Detection System, Gen-Probe Inc., San Diego, CA). If positive, the cells can be treated with a course of antibiotics, such as BM-cyclin (Roche Pharmaceuticals, Mannheim, Germany).

References

1. Cavanee, W. K., Bigner, D. D., Newcomb, E. W., Paulus, W., and Kleihues, P. (1997) Diffuse astrocytomas. In: Kleihues, P. and Cavenee, W. K. (eds.) *Pathology and Genetics of Tumours of the Nervous System*, International Agency for Research on Cancer, Lyon, France, pp. 2–9.
2. Laws, E. R., Jr. (1998) Central nervous system tumors: what have we learned and where are we heading? *Ca Cancer J. Clin.* **48,** 327–329.
3. Paul, D. B. and Kruse, C. A. (2001) Immunologic approaches to therapy for brain tumors. *Curr. Neurol. Neurosci. Rep.* **1,** 238–244.
4. Virasch, N. and Kruse, C. A. (2001) Strategies using the immune system for therapy of brain tumors. In: Liang, B. C. (ed.) *Hematology/Oncology Clinics of North America*, Vol. 15, Saunders, Philadelphia, PA, pp. 1053–1071,.
5. Bigner, D. D., Bigner, S. H., Ponten, J., et al. (1981) Heterogeneity of genotypic and phenotypic characteristics of fifteen permanent cell lines derived from human gliomas. *J. Neuropathol. Exp. Neurol.* **40,** 201–229.
6. Bakir, A., Gezen, F., Yildiz, O., et al. (1998) Establishment and characterization of a human glioblastoma multiforme cell line. *Cancer Genet. Cytogenet* **103**, 46–51.
7. Helseth, E., Dalen, A., Unsgaard, G., Vik, R., and Helseth, A. (1989) Overexpression of the epidermal growth factor receptor gene in a human carcinoma cell line derived from a brain metastasis. *J. Neuro-oncol.* **7,** 81–88.
8. Joy, A., Panicker, S., and Shapiro J. R. (2000) Altered nuclear localization of bax protein in BCNU-resistant glioma cells. *J. Neuro-oncol.* **49,** 117–129.
9. Silbergeld, D. L., and Chicoine, M. R. (1997) Isolation and characterization of human malignant glioma cells from histologically normal brain. *J. Neurosurg.* **86,** 525–531.
10. Kruse, C. A., Mitchell, D. H., Kleinschmidt-DeMasters, B. K., et al. (1992) Characterization of a continuous human glioma cell line DBTRG-05MG: growth kinetics, karyotype, receptor expression and tumor suppressor gene analysis. *In Vitro Cell. Dev. Biol.* **28A,** 609–614.
11. Kruse, C. A., Varella-Garcia, M., Kleinschmidt-DeMasters, B. K., et al. (1998) Receptor expression, cytogenetic, and molecular analysis of six continuous human glioma cell lines. *In Vitro Cell. Dev. Biol.* **34,** 455–462.
12. Westphal, M. and Meissner, H. (1998) Establishing human glioma-derived cell lines. *Methods in Cell Biol.* **57,** 147–165.
13. Oakes, W., Friedman, H. S., Bigner, S. H., Bullock, N. H., and Bigner, D. D. (1990) Successful laboratory growth and analysis of CUSA-obtained medulloblastoma samples, *J. Neurosurg.* **72,** 821–823.
14. Gjerset, R. A., Fakhrai, H., Shawler, D. L., et al. (1995) Characterization of a new human glioblastoma cell line that expresses mutant p53 and lacks activation of the PDGF pathway. *In Vitro Cell. Dev. Biol. Animal* **31,** 207–214.
15. Dropcho, E. J. (2001) Novel chemotherapeutic approaches to brain tumors. In: Liang, B. C. (ed), *Hematology/Oncology Clinics of North America,* Vol. 15, Saunders, Philadelphia, PA, pp. 1027–1052.
16. Fuller, C. and Perry, A. (2002) Fluorescence in situ hybridization (FISH) in diagnostic and investigative neuropathology. *Brain Pathol.* **12,** 67–86.
17. Meyer-Puttlitz, B., Hayashi, Y., Waha, A., et al. (1997) Molecular genetic analysis of giant cell glioblastomas. *Am. J. Pathol.* **151,** 853–857.
18. Shapiro, J. R. (2001) Genetics of nervous system tumors. In: Liang, B. C. (ed.) *Hematology/Oncology Clinics of North America,* Vol. 16, Saunders, Philadelphia, PA, pp. 961–977.

19. Rempel, S. A. (2001) Molecular biology of nervous system tumors. In: Liang, B. C. (ed.) *Hematology/Oncology Clinics of North America,* Vol. 15, Saunders, Philadelphia, PA, pp. 979–1006.
20. Malden, L. T., Novak, U., Kaye, A. H., and Burgess, A. W. (1988) Selective amplification of the cytoplasmic domain of the epidermal growth factor receptor gene in glioblastoma multiforme. *Cancer Res.* **48,** 2711–2714.
21. von Deimling, A., Louis, D. N., von Ammon, K., et al. (1992) Association of epidermal growth factor receptor gene amplification with loss of chromosome 10 in human glioblastoma multiforme, *J. Neurosurg.* **77,** 295–301.
22. Fults, D., Brockmeyer, D., Tullous, M. W., Pedone, C. A., and Cathon, R. M. (1992) p53 mutation and loss of heterozygosity on chromosome 17 and 10 during human astrocytoma progression. *Cancer Res.* **52,** 674–679.

9

Isolation and Culture of Renal Cancer Cell Lines

Jae-Gahb Park, Ja-Lok Ku, and So-Yeon Park

1. Introduction

Renal cell carcinoma (RCC) represents approximately 2% of all cancers and accounts for 85% of renal cancers in adults. There are more than 30,000 new cases per year and in excess of 12,000 deaths in the United States ***(1)***. RCC is rare in the first two decades of life, comprising only 2% of pediatric tumors. The incidence increases with advancing age, peaking in the sixth decade of life.

Until recently, RCC was regarded as a single entity with a wide variety of gross and histologic appearances and variable clinical course. However, new studies have indicated that RCC is not a single tumor, but rather a group of tumors with distinct histologic features, cytogenetic abnormalities, and genetic alterations. Accordingly, a new classification of RCC has recently been introduced and the major types of RCC are as follows: conventional (clear cell) renal carcinoma, papillary renal carcinoma, chromophobe renal carcinoma, and renal cell carcinoma, unclassifiable ***(2)***.

Cytogenetic studies of clear cell RCC have shown that most tumors exhibit a deletion or unbalanced translocation resulting in loss of genetic material in chromosome 3p harboring *VHL* gene (3p25.3) ***(3)***. A second nondeleted allele of the *VHL* gene shows somatic mutation in 50% and hypermethylation-induced inactivation in an additional 10–20% of these tumors. Papillary RCC is characterized by trisomies (chromosome 7, 16, and 17) and loss of the Y chromosome ***(4)***. In addition, a translocation between chromosome X and 1 has been reported ***(5)***. Recently, germline mutations of *c-met* oncogene at 7q31 have been detected in families with hereditary papillary renal cell carcinoma ***(6)***.

Tumors grown in cell culture or in athymic nude mice provide more homogenous tumor materials for study of morphological, biochemical, and molecular changes associated with initiation or progression and for use in identification of renal cancer genes. ***(7)***. RCC cell lines have been cultured in vitro by many laboratories during the past three decades, and many human RCC cell lines have been reported recently ***(7–11)***. A large bank of well-characterized, human RCC cell lines should reflect the diversity of tumor phenotypes and provide adequate models for tumor heterogeneity.

From: *Methods in Molecular Medicine, vol. 88: Cancer Cell Culture: Methods and Protocols*
Edited by: S. P. Langdon © Humana Press Inc., Totowa, NJ

In general, RCC cell lines are relatively easy to develop and can be established from metastatic tissues and primary tumors (success rate, 9.5–49%). Solid renal cancers are composed of a mixture of tumor cells of various histological types as well as normal cells such as lymphocytes and connective tissue cells, which complicates their molecular analysis ***(12)***. Lymphocytes can inhibit the growth of cancer cells. Enzymatic digestion of solid primary tumor tissues may result in poor recovery of viable cells and overgrowth of cancer cells with contaminating stromal cells. The faster growing stromal fibroblast-like cells easily outgrow and overgrow the cancer cells. The mechanical spillout method provides a simpler, faster, and less traumatic method of obtaining cells for culture ***(13)***. This method allows the minimization of stromal cell contamination because stromal cells are not easily detached from the tissue matrix by mechanical means. Rutzky and Moyer ***(14)*** summarized the features of key relevance for successful culture: nonenzymatic or minimal dissociation of tumor tissue, seeding cultures as explant and at high cell densities, removal of contaminating fibroblasts usually after they have aided culture initiation, and delaying passage until high cell densities have been achieved and plating cells at high density.

In this chapter, we describe the culture of primary renal cell carcinoma cell lines, and include the detailed protocols relating to the establishment from metastatic and primary tumor tissues using mechanical spillout, the isolation of pure cancer cells, and the maintenance, propagation and preservation of cancer cells.

In primary cell culture, dispersed tumor cells can be plated in plastic culture flasks with a basal medium such as RPMI 1640, or Dulbecco's minimum essential medium (DMEM) supplemented with 10–20% heat-inactivated fetal bovine serum (FBS) ***(7,8,10)***. Our laboratory has extensive experience with serum-containing and fully defined media for the establishment of continuous cell lines derived from human cancers. Our RCC cell lines ***(11)*** were initially cultured in ACL-4 medium supplemented with 5% heat-inactivated FBS (AR5) ***(15,16)***. ACL-4 is a fully defined medium formulated for the selective growth of human lung adenocarcinoma cells ***(15,16)*** and has proved useful in the establishment of hepatocellular, laryngeal, and colorectal cancer cell lines ***(17-19***). ACL-4 is a complex medium, consisting of 12 additives to basal medium ***(13,15,16).***

2. Materials

2.1. Initiation Media for Primary Cell Culture

1. RPMI 1640 medium: RPMI 1640 powder, 25 m*M* HEPES buffer, 20 m*M* sodium bicarbonate, antibiotics (streptomycin-penicillin, usually 100 U/mL); filter through 0.22 µm bottle top filter.
2. ACL-4 medium: RPMI 1640 as basal medium, 20 µg/mL insulin, 10 µg/mL transferrin, 25 n*M* sodium selenite, 50 n*M* hydrocortisone, 1 ng/mL epidermal growth factor, 10 µ*M* ethanolamine, 10 µ*M* phosphorylethanolamine, 100 p*M* triiodothyronine, 2 mg/mL bovine serum albumin (BSA), 2 m*M* glutamine, 0.5 m*M* sodium pyruvate.
3. Initiation and growth medium (AR-5 medium): ACL-4 medium supplemented with 5% heat-inactivated FBS.

2.2. Reagents and Apparatus

1. Sterile PBS, pH 7.0.

2. Sterile 100-mm Petri dishes.
3. Sterile scissors.
4. Sterile forceps.
5. 5- and 10-mL pipets.
6. Ficoll gradient.
7. Culture flasks (25 cm^2) coated with attachment factors such as collagen and laminin.

3. Methods

3.1. Procurement of Tumor Specimens

Solid tumors (metastatic and primary tumors) must be carefully and aseptically excised from pathologically proven renal cancer samples and transferred to a cell culture laboratory in RPMI 1640 medium (*see* **Note 1**). The transporting RPMI 1640 medium should contain antibiotics, such as penicillin-streptomycin, to prevent bacterial contamination.

3.2. Primary Cell Culture

3.2.1. Procedure for Solid Tumors

1. Transfer tumor tissues to fresh sterile PBS in a dish and rinse.
2. Transfer tumor tissues to a new dish and dissect off necrotic areas, fatty tissues, blood clots, and connective tissues with forceps and scissors.
3. Finely mince the tumor tissues into very small pieces with sterile scissors.
4. Disassociate tissue pieces into small aggregates by vigorous pipeting with 10 mL of PBS.
5. Allow heavier pieces to sediment by gravity.
6. Carefully harvest the tumor cell aggregates containing washes by pipet and transfer to a 15 mL sterile centrifuge tube.
7. Centrifuge and remove the supernatant.
8. Wash by resuspending in PBS, centrifuge, and again remove the supernatant.
9. Resuspend the pieces in 5 mL of AR-5 initiation medium.
10. Transfer the pieces to a 25-cm^2 flasks.
11. Harvest the remaining large pieces, place in a 15-mL sterile centrifuge tube,centrifuge, remove the supernatant, and culture immediately.
12. Cap the flask and maintain in a humidified incubator at 37°C in an atmosphere of 5% CO_2 and 95% air.

3.3. Isolation of Cancer Cells

Most renal cancer cells can grow as either firmly or loosely adherent colonies. If the cancer cells are adherent, the medium is changed weekly until a substantial outgrowth of cells is observed. Isolation of the cancer cells is performed when heavy tumor-cell growth is observed. Several methods can be employed to isolate pure cancer cells.

3.3.1. Rapping Method

This method is used when cancer cells are loosely attached to the surface of the culture flask and grow as adherent colonies among the stromal fibroblast cells. Of these cancer cells, some cancer cells or whole colonies are detached from the colonies and grow in suspension.

1. Gently tap the flasks.
2. Harvest the individual cancer cell and colonies.
3. Transfer the floating aggregates to a sterile centrifuge tube.
4. Spin the cells at 400*g* for 5 min, decant two-thirds volumes of supernatant from the pellets and add 5 mL of ACL-4 medium supplemented with 5% FBS.
5. Seed the cell pellet into 25-cm^2 flasks.
6. Cap the flask and maintain the culture in a humidified incubator at 37°C in an atmosphere of 5% CO_2 and 95% air.

3.3.2. Scraping Method

Usually, firmly adherent colonies surrounded by stromal fibroblasts form large densely packed colonies that continue to grow laterally, slowly infiltrating, and displacing the adjacent fibroblasts or mesothelial cells. Eventually, the entire flask may be taken over by these tumor cells. In this case, cancer cells can be isolated by scraping and differential trypsinization ***(13)***. In our experience, when large adherent cancer cell colonies are less impeded by surrounding fibroblasts, scraping is the best way of isolating the cancer cells; the cells are less damaged and stromal contamination is minimized ***(11,17–19)***.

1. Maintain the culture until the adherent colonies form well-isolated large colonies (5–10 mm in diameter). This may take several months.
2. Select colonies and mark with marker pen on the flask base.
3. Decant the medium and add 1 mL of growth medium.
4. Detach the adherent colonies by scraping with a policeman or cell scraper, to allow them to float freely in the medium.
5. Add 4 mL of growth medium and harvest the detached colonies with a pipet.
6. Transfer the cancer cells into 25-cm^2 flasks.
7. Maintain cultures in humidified incubators at 37°C in an atmosphere of 5% CO_2 and 95% air.

3.3.3. Differential Trypsinization Method

Some cancer cells grow among stromal fibroblasts and produce small colonies. Usually these cancer cells do not overgrow the fibroblasts. In these cases, the differential trypsinization method is preferred. The method depends on differences in cell susceptibility to trypsinization ***(13)***. Some cancer cells are more susceptible to trypsinization than the fibroblasts and vice versa.

1. Decant the medium, rinse with PBS, and remove the PBS by aspirating.
2. Add 1.5 mL of diluted trypsin (0.025% or 0.05% or 0.1% trypsin).
3. Place into incubator at 37°C for approx 3 min.
4. Observe the flasks under the inverted microscope through incubating, and determine whether cancer cells or fibroblasts are detached. If both cancer cells and stromal fibroblasts remain attached, incubate the culture flasks until either the cancer cells or fibroblasts detach.
5. If cancer cells have detached and fibroblasts remain attached, add 5 mL of growth medium containing serum and harvest the detached cancer cells. If, on the other hand cancer cells remain attached and the fibroblasts have detached, remove the medium, rinse with the growth medium, and then add 5mL of growth medium.

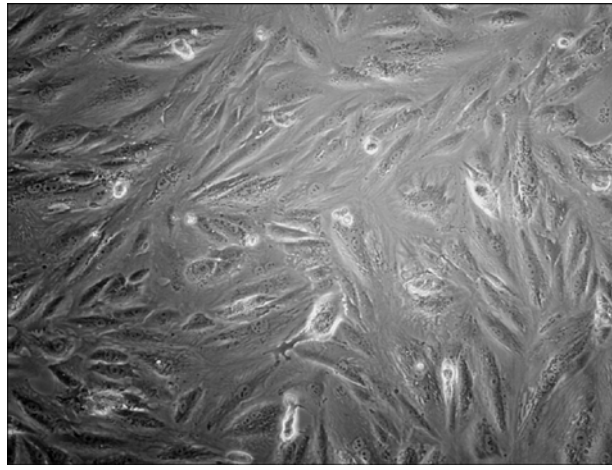

Fig. 1. Phase contrast microscopy of SNU-1272 renal cell carcinoma cell line.

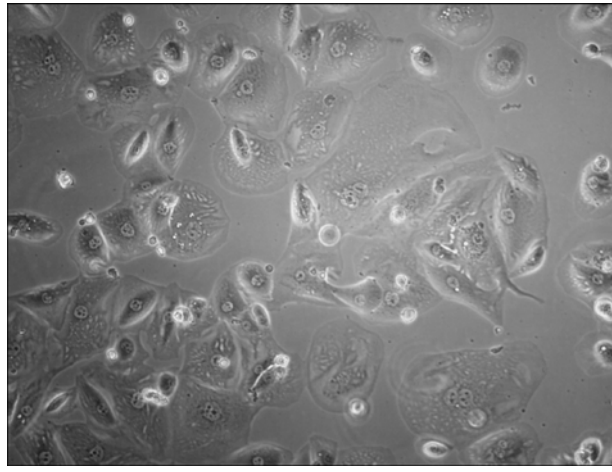

Fig. 2. Phase contrast microscopy of SNU-328 renal cell carcinoma cell line.

6. Centrifuge the harvested cancer cells, remove the supernatant, and add 5 mL of AR-5 growth medium.
7. Transfer the cancer cells into 25-cm^2 flasks.
8. Maintain cultures in a humidified incubator at 37°C in an atmosphere of 5% CO_2 and 95% air.

3.4. Propagation and Preservation of Cancer Cells

After establishing cancer cell lines (*see* **Figs. 1** and **2**), initial passages are performed when heavy tumor-cell growth and large colonies are observed. Subsequent passages are performed every week or two.

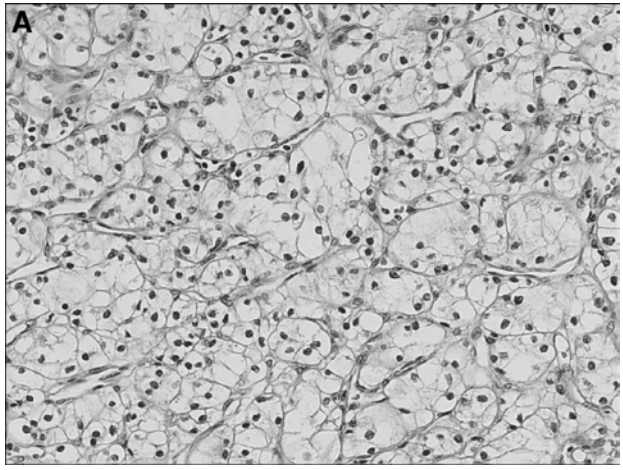

Fig. 3. **(A)** Primary tumor (clear cell type) of SNU-349 renal cell carcinoma cell line.

The ACL-4 medium supplemented with heat inactivated 5% FBS, which is used in primary cell culture, can be also used for the propagation of renal cancer cell lines ***(11)***. This medium must be used until the isolated cancer cells are considered as a cell line. After establishment of cell lines, AR-5 medium can be replaced with RPMI 1640 medium supplemented with 10% heat inactivated FBS ***(11)*** (*see* **Note 2**).

1. Remove the supernatant and rinse with sterile PBS.
2. Add 2 mL of trypsin-EDTA (usually 0.05%) in PBS and incubate at 37°C for approx 3 min.
3. Add 5 mL of RPMI 1640 medium supplemented with 10% FBS and centrifuge.
4. Decant the supernatant and redisperse the cancer cells by pipeting with the AR-5 growth medium.
5. Seed the cell pellet into new 25-cm^2 flasks.
6. Cap the flasks and maintain the cultures in a humidified incubator at 37°C in an atmosphere of 5% CO_2 and 95% air.

If the adherent cancer cells are not detached in the flasks, the concentrations of trypsin-EDTA and the incubation times can be increased.

3.5. Representative Characteristics of Cultured Renal Cell Carcinoma Cell Lines

The SNU-349 cell line was derived from a renal cell carcinoma, clear cell type. The original tumor of SNU-349 cell line was characterized by optically clear cytoplasm of tumor cells in acinar or alveolar arrangement (**Fig. 3A**). The nuclei were round and uniform. The nucleoli were absent or small nucleoli were occasionally seen. SNU-349 cancer cells grow as polygonal cells with flattened cytoplasm and a strong adherence

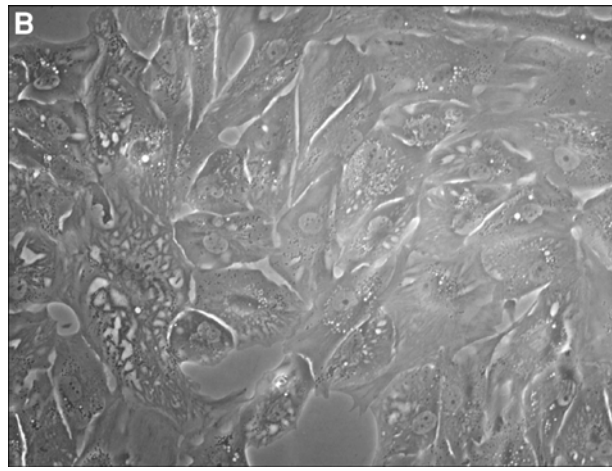

Fig. 3. **(B)** Phase contrast microscopy of SNU-349 renal cell carcinoma cell line. Tumor cells demonstrate the polygonal morphology.

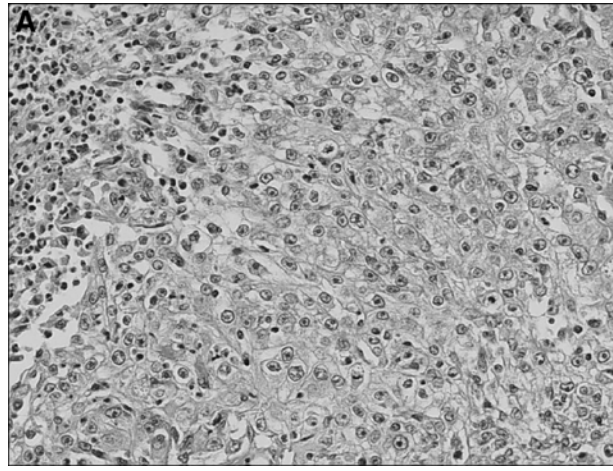

Fig. 4. **(A)** Primary tumor (granular variant of clear cell type) of SNU-482 renal cell carcinoma cell line. *(Continued on next page.)*

to the plastic flask containing the culture (**Fig. 3B**). The SNU-482 cell line was derived from granular variants of clear cell renal cell carcinoma. The original tumor of SNU-482 was aggressive; it invaded into the renal artery and vein, and metastasized to the regional lymph nodes (**Fig. 4A**). The cytoplasm of tumor cells was variably eosinophilic and granular. The nuclei were large and irregular and had prominent nucleoli.

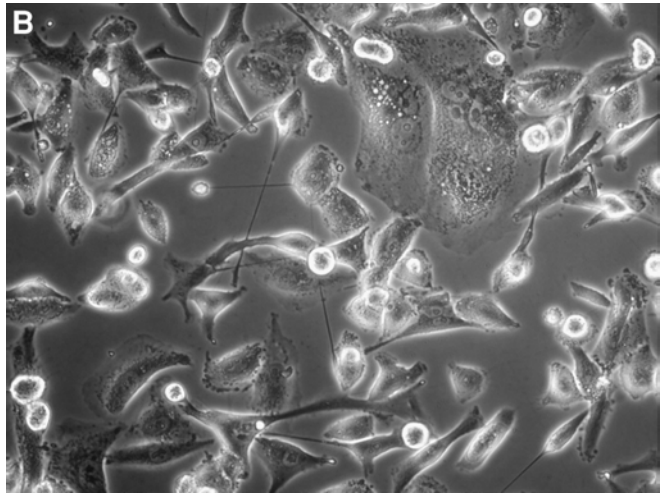

Fig. 4. *(Continued from previous page.)* **(B)** Phase contrast microscopy of SNU-482 renal cell carcinoma cell line.

The tumor cells were arranged in solid acinar or tubular pattern with multifocal geographic necrosis. The cells of SNU-482 were stellate (**Fig. 4B**) with the cytoplasmic processes frequently overriding the adjacent cells, resulting in a 3D configuration. Some of the cells were round or oval with minimal adherence to the plastic surface. The nuclei of these round cells appeared smaller than those in the flattened cells, an apparent example of nuclear pleomorphism.

4. Notes

1. Whenever possible, invasive areas from the serosal surface should be selected for primary tumor cultures, to reduce the possibility of microbial contamination.
2. It is recommended that early passages are frozen in cryoprotective medium to prevent loss of the cell lines by contamination or other laboratory accidents.

References

1. Mortzer, R. J., Bander, N. H., and Nanus, D. M. (1996) Renal-cell carcinoma. *N. Eng. J. Med.* **335,** 865–875.
2. Storkel, S., Eble, J. N., Adlakha, K., et al. (1997) Classification of renal cell carcinoma *Cancer* **80,** 987–989.
3. Neumann, H. P. H., and Zbar, B. (1997) Renal cyst, renal cancer and von Hippel Lindau disease. *Kidney Int.* **51,** 164.
4. Kovacs, G. (1989) Papillary renal cell carcinoma: a morphologic and cytogenetic study of 11 cases. Am. J. Pathol. **134,** 27–34.
5. Sidhar, S. K., Clark, J., Gill, S., et al. (1996) The t(X;1)(p11.2;q21.2) translocation in papillary renal cell carcinoma fuses a novel gene to the TEF3 transcription factor gene. *Hum. Mol. Genet.* **5,** 1333–1338.

6. Schmidt, L., Duh, F. M., Chen, F., et al. (1997) Germline and somatic mutations in the tyrosine kinase domain of the MET proto-oncogene in papillary renal carcinomas. *Nat. Genet.* **16,** 68–73.
7. Anglard, P., Trahan, E., Liu, S., et al. (1992) Molecular and cellular characterization of human renal cell carcinoma cell lines. *Cancer Res.* **52,** 348–356.
8. Grossman, H. B., Wedemeyer, G., and Ren, L. (1985) Human renal carcinoma: characterization of five new cell lines. *J. Surg. Oncol.* **28,** 237–244.
9. Ebert, T., Bander, N. H., Finstad, C. L., Ramsawak, R. D., and Old, L. J. (1990) Establishment and characterization of human renal cancer and normal kidney cell lines. *Cancer Res.* **50,** 5531–5536.
10. Gerharz, C. D., Ramp, D., Olert, J., et al. (1994) Cytomorphological, cytogenetic, and molecular biological characterization of four new human renal carcinoma cell lines of the clear cell type. *Virchows Arch.* **424,** 403–409.
11. Shin, K. H., Ku, J. L., Kim, W. H., et al. (2000) Establishment and characterization of seven human renal cell carcinoma cell lines. *BJU Int.* **85,** 130–137.
12. Linehan, W. M., Miller, E., Anglard, P., Merino, M., and Zbar, B. (1989) Improved detection of allele loss in renal cell carcinomas after removal of leukocytes by immunologic selection. *J. Natl. Cancer Inst.* **81,** 287–290.
13. Oie, H. K, Russell, E. K., Carney, D. N., and Gazdar, A. F. (1996) Cell culture methods for the establishment of the NCI series of lung cancer cell lines. *J. Cell. Biochem.* **24 (suppl),** 24–31.
14. Rutzky, L. P. and Moyer, M. P. (1990) Human cell lines in colon cancer research. In: Moyer, M. P. (ed.) *Colon Cancer Cells*, Academic Press, London, pp. 155–202,.
15. Gazdar, A. F. and Oie, H. K. (1986) Growth of cell lines and clinical specimens of human non-small cell lung cancer in a serum-free defined medium. *Cancer Res.* **46,** 6011–6012.
16. Gazdar, A. F. and Oie, H. K. (1986) Cell culture methods for human lung cancer. *Cancer Genet. Cytogenet.* **19,** 5–10.
17. Park, J. G., Lee, J. H., Kang, M. S., et al. (1995) Characterization of cell lines established from human hepatocellular carcinoma. *Int. J. Cancer* **62,** 276–282.
18. Ku, J. L., Kim, W. H., Lee, J. H., et al. (1999) Establishment and characterization of human laryngeal squamous cell carcinoma cell lines. *Laryngoscope* **109,** 976–982.
19. Oh, J. W., Ku, J. L., Yoon, K. A., et al. (1999) Establishment and characterization of colorectal-carcinoma cell lines. *Int. J. Cancer* **81,** 902–910.

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Isolation and Culture of Prostate Cancer Cell Lines

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1. Introduction

The ability to establish cell cultures from primary tumors and metastases of prostate cancer in a reliable and consistent manner is a valuable tool for studying the biology of these tumors and evaluating the effectiveness of novel therapies. A procedure to propagate human prostatic epithelial cells in vitro for a limited number of cell generations was developed by others ***(1,2)*** and was extended to prostate cancer cells from primary tumors ***(1,3)***. However, reproducible methods to propagate prostatic cancer cells from more advanced tumors have not been established. This may reflect the fact that prostate cancer cells derived from primary tumors are thought to have nutritional requirements similar to normal prostatic epithelial cells ***(1,3)***, whereas more advanced tumors may have different growth requirements, depending on the specific genetic alterations acquired during disease progression. It is therefore understandable why a single protocol may not result in optimal growth of all cancer cells in vitro (which would open up possibilities for long-term culture), that the establishment of human prostate cancer cell lines is very difficult, and that there are few established cell lines ***(4)***. During the past five years, we have processed 100 prostate cancer specimens in attempts to determine their optimal growth conditions and to establish long-term cultures, cell lines, and xenografts. We have processed different types of tissue samples (tissue fragments, needle biopsy samples, and fine-needle aspiration biopsy samples) from prostate cancer samples obtained at radical prostatectomy and samples from different metastatic sites (bone, lymph node, liver, skin, adrenal gland, brain, and lung). As a result we have established two human prostate cancer cells lines ***(5)*** and several prostate cancer xenografts. In this chapter, we will describe our experience.

1. Materials

1. Culture media:
   a. RPMI 1640 ***(6)*** with 5 µg/mL insulin, 30 n*M* sodium selenite, 10 n*M* hydrocortisone.
   b. BRFF-HPC1 ***(7)***, a modification of PFMR-4 ***(8,9)*** basal medium ***(10)*** without phenol red, with less calcium (0.2 m*M*), 15 m*M* HEPES buffer, 5 ng/mL epidermal

From: *Methods in Molecular Medicine, vol. 88: Cancer Cell Culture: Methods and Protocols*
Edited by: S. P. Langdon © Humana Press Inc., Totowa, NJ

growth factor (EGF), 100 µg/mL bovine pituitary extract (BPE), 0.28 µ*M* hydrocortisone, 5 µg/mL insulin, 0.1 n*M* dihydrotestosterone, 250 µg/mL bovine serum albumin, 25 ng/mL cholera toxin, 5 µ*M* phosphoethanolamine, and 10 µg/mL trypsin inhibitors ***(7)***.

c. F12K ***(11)*** with 5 µg/mL insulin, 30 n*M* sodium selenite, 250 n*M* hydrocortisone, 25 ng/mL cholera toxin, 5 ng/mL EGF, and 100 µg/mL BPE.

2. Collagen coated dishes: dishes coated with Vitrogen (Cohesion, Palo Alto, CA) in 12 m*M* HCl (1/4 v/v) or commercially available tissue culture dishes coated with collagen type I.
3. Hank's balanced salt solution (HBSS) without calcium chloride, magnesium chloride, or magnesium sulfate and with 10 m*M* HEPES.
4. 10X Red blood cell (RBC) lysis buffer: 1% Potassium bicarbonate, 8.26% ammonium chloride, and 1 m*M* ethylenediaminetetraacetic acid (EDTA). Sterile filter and store at 4°C for up to 5 d.
5. Collagenase digestion medium: 10 mL growth medium plus 40–100 U/mL collagenase, sterile filtered, and used fresh.
6. Trypsin/EDTA solution: 0.2% trypsin/0.02% EDTA in HBSS.
7. Freezing medium: growth medium plus 10% fetal bovine serum (FBS) and 10% dimethyl sulfoxide.
8. Six- to 8-wk-old male athymic (nude) or mice with immunodeficiency disease (SCID).

3. Methods

3.1. Tissue Collection

The selection and sampling of cases is best performed in collaboration with a pathologist, urologist, and/or oncologist (*see* **Note 1**). It is essential to minimize the time between sample acquisition and processing. It is also important to incorporate the clinical information (for example, tumor response to therapy) into the protocol for processing and propagation of the tissue specimen.

1. Dissect small sections of prostate cancer tissue from different areas of the tumor.
2. Collect the tissue sample in a sterile vessel containing HBSS with HEPES and store at 4°C for processing within a few hours.
3. To optimize the use of prostate cancer specimens and because prostate cancer xenografts can be expanded in sufficient quantities to allow biochemical and molecular studies, it is also important, whenever possible, to set aside tissue for direct injection into immunosuppressed mice.
4. Subject the mirror-image tissue sample from each sample obtained for tissue culture to routine tissue processing (formalin fixation and paraffin embedding) and histopathologic analysis of H&E-stained sections to assess the tumor cells in the dissected material. Perform the procedures under sterile conditions. Whenever possible, freeze a small amount of blood or normal tissue for comparative purposes (for example, analysis of loss of heterozygosity). These samples can also be used to confirm the origin from a particular patient if necessary.

3.2. Tissue Processing

3.2.1. Tissue Preparation for Tissue Culture

The purpose of this step is to obtain clumps of epithelial cells and microexplants of viable neoplastic cells. There is no single recommended procedure for all specimens.

The procedure must be adjusted for the source of the material (primary tumor or metastasis) and the findings at macroscopic examination (*see* **Note 2**). An initial gross examination of the specimen will help to determine the appropriate steps to follow. The quality of the tumor sample is the most important factor determining the rate of success in obtaining initial growth of tissue explants. Unfortunately, metastatic tumors with the highest growth potential usually have extensive necrosis that interferes with the growth of epithelial and stromal cells. To reduce the effect of tumor necrosis and to increase the rate of success in establishing cell lines of metastatic prostate carcinoma we currently use tissue samples from several areas of each tumor and increase the total number of samples processed.

1. Place the tissue specimen in a sterile 35- to 60-mm dish and gently wash it several times with HBSS, to remove blood and any contaminant from the initial processing.
2. Using sterile forceps and scissors, remove fat and necrotic tissue.
3. Mince the tissue sample into pieces of approx 1 mm^3 by using scalpels and/or sharp scissors so that minimal cell damage is done.
4. At this time, note whether single cells are easily released from the tissue by the pressure of the instruments and how firm the tissue is; this will suggest whether the tissue specimen must be enzymatically digested to obtain cell clumps and microexplants. If the tissue is easily disaggregated into cell clumps, then no enzymatic digestion is necessary. Otherwise, add collagenase (40 U/mL) to the growth medium.
5. Monitor the digestion every 2 h (by gently shaking the tube with the digestion mixture), and periodically check cell viability in an aliquot under the microscope (by the trypan blue exclusion assay). Usually tissue specimens from primary tumors require overnight digestion and tissue specimens from metastasis require approx 1–4 h. The duration of digestion can be modified by changing the collagenase concentration. Manipulation of tissue should be minimal and the cells placed in the optimal growth conditions as soon as possible.
6. Collagenase digestion is less harmful to epithelial cells than is digestion with other enzymes (for example, trypsin) but leads to incomplete digestion, so after the enzymatic digestion, digested tissue should be mechanically disaggregated by pipeting up and down with a 10- or 25-mL pipet to obtain small clumps of epithelial cells and microexplants.
7. Although we have obtained better results with microexplants and clumps than with single cells, single cells released during digestion can be collected by allowing clusters to settle.
8. Wash the digestion mixture of the cells, cell clumps, and microexplants by centrifugation (50–100*g* for 20 seconds [s]) and resuspend the pellet in HBSS. Repeat this process two more times, and then resuspend the cell pellet in growth medium plus 10% FBS.
9. As RBCs in the digested tissue usually interfere with tumor cell survival and attachment, if many RBCs are present, then resuspend the digested tissue in HBSS, and add four volumes (vol) of 1X RBC lysis buffer, incubate it 10 min at room temperature, and centrifuge at 100*g* for 20 s. Repeat this procedure as necessary, and then resuspend the pellet in HBSS, and repeat the centrifugation and resuspension twice. Finally, resuspend the cell pellet in growth medium plus 10% FBS.
10. Seed the resuspended cells on collagen-coated dishes in growth medium with 10% FBS, at a high cell density, because epithelial cells grow more efficiently when plated at high density and because it is expected that only a low proportion of the cells will grow in vitro.

Samples derived from bone marrow aspiration or biologic fluids (for example, ascites) should be centrifuged (50–100*g* for 20 s) and the pellet obtained resuspended

in HBSS. Repeat this process two more times, and then resuspend the cell pellet in growth medium plus 10% FBS. The sample should be then processed as detailed in **steps 9–10**.

3.2.2. Tissue Preparation for Injection in Immunodeficient Mice

The purpose of this step is to obtain tissue samples of viable neoplastic cells of approx 3–5 mm^3. The procedure is as follows:

1. Place the tissue specimen in a sterile 35- to 60-mm dish and gently wash it several times with HBSS, to remove blood and any contaminant from the initial processing.
2. Using sterile forceps and scissors, remove fat and necrotic tissue.
3. Cut the tissue sample into pieces of approx 3–5 mm^3 by using scalpels and/or sharp scissors so that minimal cell damage is done.

Samples derived from fine needle aspiration or biologic fluids (for examples, ascites) should be centrifuged (50–100*g* for 20 s) and the pellet resuspended in HBSS. Repeat this process two more times, and if many RBCs are present the sample should be then processed as detailed in **Subheading 3.3.2., step 6**. Finally the cell pellet should be resuspended in a minimal volume of growth medium.

3.3. Cell Culture

3.3.1. Background

In total, we have processed tissue samples derived from normal prostate, primary prostate cancer of various histologic grades, and prostate cancer tissue from different metastatic sites. Tissues were obtained from surgical specimens or excisional biopsies of distant metastatic sites (32 specimens), incisional biopsies (17 specimens), bone marrow aspiration specimens (12 specimens), ascites (5 specimens), and pleural effusions (2 specimens). Although serum free conditions have been developed to propagate prostate epithelial cells from primary tumors ***(1–3)***, in our current tissue-processing protocol, growth and attachment of most cultures derived from metastatic sites require the presence of serum. We grew all specimens in medium supplemented with 10% FBS, monitored the cultures daily, and adjusted the serum concentrations accordingly as detailed in following paragraph, this subheading.

In our attempts to grow prostate cancer cells in vitro, we processed each tissue specimen as described in **Subheading 3.2.**, and if enough material was available, we tried several different growth media per specimen. Nine of the surgical specimens or excisional biopsies initially attached and grew, some remained viable for up to four passages, and two human prostate cancer cells lines, MDA PCa 2a and MDA PCa 2b, were established ***(5)***. There was no difference in the initial cell growth obtained in samples from primary tumors or metastatic sites, however it must be emphasized that most primary tumors used are androgen independent and have failed multimodality therapy and therefore cannot be compared with success rates using therapy-naive primary tumors. Most tissues derived from biopsies and fine needle aspirations did not render cell growth. The tissue-culture media that most efficiently supported growth of the prostatic cells were RPMI 1640 with additives and BRFF-HPC1 ***(7)***.

The MDA PCa 2a and MDA PCa 2b lines were derived from a bone metastasis of a prostate cancer that was undergoing androgen independent growth. The processed tissue from which MDA PCa 2a was derived was initially seeded in collagen-coated dishes and grown in RPMI 1640 with additives and 10% FBS. Tissue microexplants attached, but epithelial cells arising from the microexplants formed clumps that remained viable in suspension. After 1 mo, we collected the clumps in suspension and placed them in a collagen-coated dish with BRFF-HPC1 plus 10% FBS. The MDA PCa 2a cells subsequently grow as monolayers.

The processed tissue from which MDA PCa 2b was derived was initially seeded in collagen-coated dishes and grown in BRFF-HPC1 supplemented with 10% FBS. The cells grew in monolayer. Normal fibroblasts were also growing as undesired contaminants, so we reduced the FBS to 2% until the fibroblasts died (after about 4 mo) and then increased the FBS back to 10%. For MDA PCa 2a and MDA PCa 2b, using collagen-coated dishes and serum was absolutely necessary for cell growth at the early phases of establishment.

For the contents of BRFF-HPC1 *see* **Subheading 2., item 1a.** The basic nutrient medium PFMR-4 evolved by modifications of Ham's F12 and differs from F12K mainly by the addition of HEPES, replacement of cysteine with cystine, and reduction in the osmolarity to 280 mOsm/kg ***(8,11)***. PFMR-4 basal medium was demonstrated to be better at supporting growth of prostate cancer cells than other types of basal media ***(8,9)***. Previous reports have demonstrated that the addition of insulin, cholera toxin, and EGF improves growth of prostate cancer cells in vitro ***(8,9)***, although those results were density dependent. Subsequent studies demonstrated that addition of bovine pituitary extract and hydrocortisone to the growth medium substantially improved prostate cell growth in vitro ***(3)***. Similar growth requirements were described by McKeehan et al. ***(12,13)*** for epithelial cells derived from normal and tumor tissue from the rat prostate ***(12,13)***.

Hydrocortisone, insulin, transferrin, estradiol, and selenium (HITES) is a modification of RPMI 1640 ***(6,14)*** that was originally developed as a selective agent for growing small cell lung cancer cells. We have used a modified version of RPMI 1640 with insulin, sodium selenite, and hydrocortisone alone. MDA PCa 2a cells ***(5)*** were established with this combination and the LNCaP prostate cancer cell line ***(15)*** was established with RPMI 1640. This suggests that this basal medium formulation favors the growth of prostate cancer cells from metastatic sites.

3.3.2. Recommended Procedures for Cell Growth

1. If enough tissue is available, try several tissue-culture conditions for each specimen. We recommend using BRFF-HPC1, RPMI-1640 with additives, and F12K with supplements, all supplemented with 10% FBS.
2. After seeding, allow the cells to attach for 3–4 d without any further manipulation.
3. Then, examine cell cultures by phase contrast microscopy to assess cell viability, attachment of cell clumps and microexplants to the dishes, and outgrowth of epithelial cells and/or fibroblast-like cells (*see* **Note 3**). Also, as we have observed that some prostate cancer cells survive for long periods of time in suspension, look for viable cells in the culture medium. The following outcomes can be observed:

a. Microexplants with outgrowths of epithelial cells (**Fig. 1A**). When this is observed, the culture conditions in that particular dish are probably appropriate, the medium should be changed with fresh medium every 4–6 d, and the culture should be monitored every 3–4 d. If cell colonies with fibroblast-like morphology appear (**Fig. 1B**), reduce the concentration of FBS to 2–5% and closely monitor the epithelial cells for viability.
b. Microexplants attach to the dish, and there is no outgrowth of epithelial cells, but clumps of epithelial cells are released to the medium (**Fig. 1C**)**.** These clumps appear round and shiny under the phase contrast microscopy, which indicates they are viable **(Fig. 1D).** The culture should be collected, centrifuged, resuspended in growth medium plus 10% FBS, and placed back in the same dish, which will avoid excessive dilution of cells. If the number of floating clumps increases after several changes of medium, then seed the floating clumps into another dish with a different growth medium plus 10% FBS. Closely monitor the cells to ensure there is no lose of viability. If enough cells are available, seed viable cells in three different growth media with 10% FBS.
c. Cell clumps derived from microexplants or directly from digested tissue attach and show signs of mitosis **(Fig. 1E** and **1F).** Add fresh medium every 4–6 d and closely monitor the culture. If cell colonies with fibroblast-like morphology appear **(Fig. 1B)**, reduce the concentration of FBS to 2–5% and closely monitor the epithelial cells for cell viability.

4. If these procedures do not render cell growth or attachment, try combinations of the methods described in **steps 1–3** and additions or subtraction of various factors (for example, hydrocortisone) to obtain cell growth. Monitor the cultures daily to determine course of action.
5. Allow the cells to grow until semiconfluent before they are subcultured or frozen. The split ratio after subculture will depend on the doubling time of the cell line; in general, low-passage cell lines have a long doubling time, and therefore a ratio of 1/2 is recommended. If the cells grow very slowly and more than one dish is available, we suggest splitting one dish 1/1 before the culture is subconfluent. This will prevent microbial contamination (which is likely to occur when a culture is maintained for a long period of time in the same dish), and may also stimulate cell growth. Routinely document cell morphology and manipulation of cultures, and freeze cells at each passage.
6. For subculture, use trypsin/EDTA and the following standard procedure ***(6)***.

a. Add enough trypsin/EDTA to cover the culture dish.
b. Leave it for 15–30 s.
c. Withdraw the excess trypsin/EDTA (make sure the cells have not detached).
d. Incubate the dish at room temperature, and monitor cells rounding up by microscopy.
e. Add growth medium plus 10% FBS and resuspend the cells by repeatedly pipeting over the surface of the culture dish.
f. Transfer the resuspended cells to a centrifuge tube, rinse the dish with HBSS, and add the rinse to centrifuge tube.
g. Centrifuge the cells (50–100*g* for 20 s) and resuspend the pellet in HBSS. Repeat the process two more times, and then resuspend cell pellets in growth medium plus 10% FBS and transfer it to collagen-coated dishes.

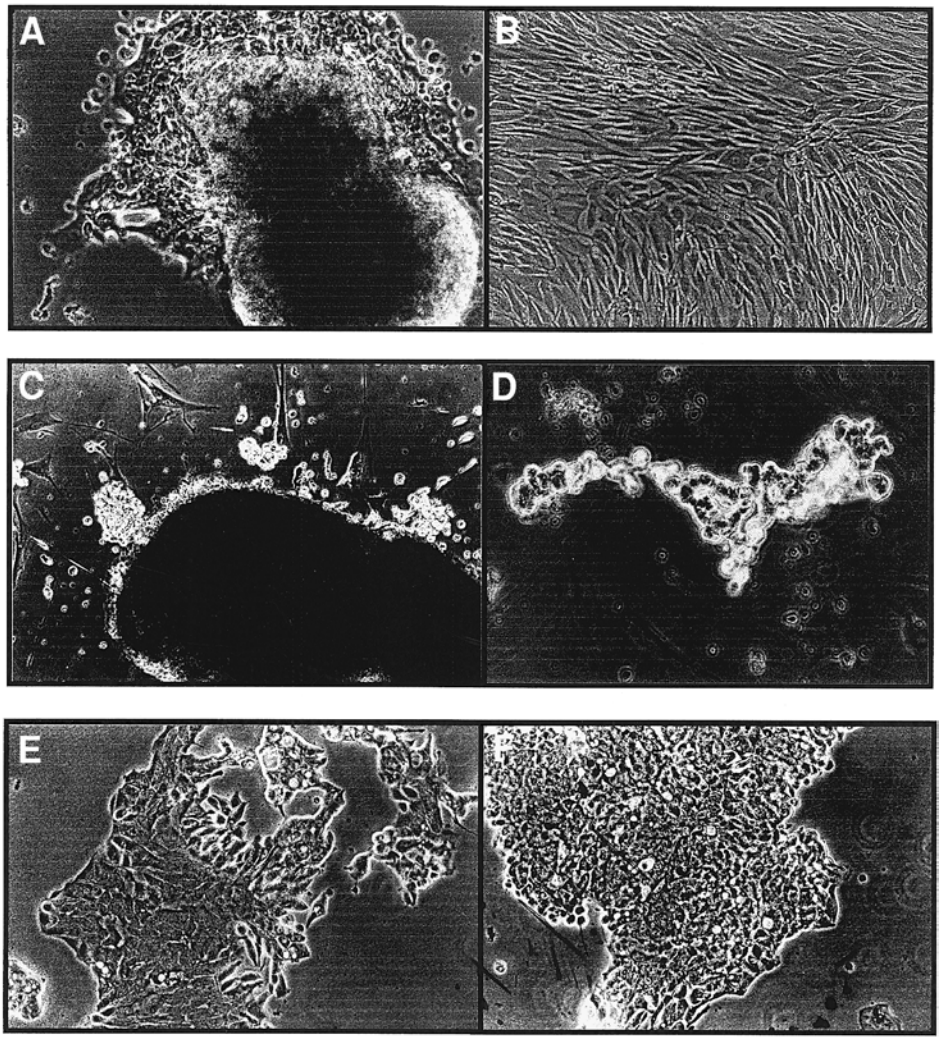

Fig. 1. Phase-contrast micrographs of cell cultures derived from human prostate cancers. **(A)** Outgrowth of prostate cancer epithelial cells after 2 wk of incubation of a primary microexplant. The undisaggregated tissue fragment (primary microexplant) is seen as a dense area in the center. Original magnification ×120. **(B)** A relatively pure fibroblastic area in a human prostate cancer culture at passage 4. Original magnification ×200. **(C)** Primary microexplant from human prostate cancer (a dense area in the center), with human prostate cancer cell clumps (asterisk) arising from the microexplant and remaining in suspension. Fibroblast-like cells are also seen growing in the dish. Original magnification ×100. **(D)** Human prostate cancer cell clumps in suspension. Note that the cells are shiny, indicating that they are viable. Original magnification ×200. **(E,F)** Early passages of human prostate cancer cells derived from metastatic sites. Original magnification ×100.

3.3.3. Experimental Alternatives to Improve Prostate Cancer Cell Growth In Vitro

Classic research in tumor biology has focused on the cancer cells directly, but over the last years, it has become increasingly clear that the cellular microenvironment also has an important role in tumor progression ***(16)***. Fibroblasts from normal breasts have been found to inhibit the growth of nontumorigenic mammary epithelial cells, although fibroblasts from cancerous breasts stimulated proliferation ***(17)***; similar results have been found in coculture experiments with prostate cells ***(16,17)***. The metastatic dissemination pattern of prostate cancer is unique in that it has a predilection for bone in about 80% of cases—with a tendency to develop bone metastases as the only site of progression. This suggests that the bone microenvironment may provide growth stimulating factors for prostate cancer cells ***(18,19)***. In agreement with this hypothesis, previous work by L. W. Chung and colleagues showed that prostate- and bone-derived fibroblasts (nontumorigenic fibroblasts from an osteogenic sarcoma) accelerated the growth of prostate cancer cells in vivo and were mitogenic for prostate cancer cells in vitro ***(20)***. The use of prostate- and bone-derived fibroblasts in coculture with prostate cancer cells may therefore be of use to increase the growth potential of prostate cancer cells in vitro.

Prostate cancer is also unique in that it is the only malignancy that consistently produces osteoblastic bone metastases ***(18,19)***, suggesting an interaction between prostate cancer cells and cells of the osteoblast linage. We have established an in vitro model of bone metastases using MDA PCa 2a and MDA PCa 2b cell lines ***(21)***. The model consists of a bicompartmental coculture system of primary mouse osteoblasts and human prostate cancer cells. In this system the cells share medium but are not in physical contact. Using this system, we found that bone-derived MDA PCa 2a and MDA PCa 2b prostate cancer cells induce osteoblast growth and differentiation ***(21)***, we also found that DNA synthesis was increased in these prostate cancer cells in coculture with primary mouse osteoblasts (unpublished results). These findings suggest the existence of a paracrine loop between bone forming prostate cancer cells and primary mouse osteoblasts. This may also be a suitable approach to optimize prostate cancer growth in vitro.

3.4. Human Prostate Cancer Xenografts

3.4.1. Background

We have processed 68 tissue specimens derived from prostate cancer and have injected samples of the processed specimens into athymic (nude) and SCID mice to obtain in vivo tumor growth. We have processed 24 surgical specimens or excisional biopsies of distant metastatic sites, 23 incisional biopsies, 11 bone marrow aspirates, 4 ascites, and 6 pleural effusions. We obtained in vivo tumor growth of metastases in 12 of the 24 surgical specimens or excisional biopsies, 3 of 23 excisional biopsies, none of the bone marrow aspirates, one from the ascites, and one from pleural effusions (*see* **Note 4**).

No difference in the initial in vitro cell growth or in vivo propagation was found in specimens derived from therapy resistant primary tumors or metastases. There was no

difference in the in vivo cell growth obtained with samples from primary tumors or the different metastatic sites. Most primary tumors that are used have failed therapy and therefore cannot be compared with the success rate of therapy-naive primary tumors (*see* **Note 5**). These results indicate that biopsies and fine needle aspirations do not yield significant cell growth in vivo, and therefore surgical specimens or excisional biopsies should be used as a source of tissue whenever possible.We have used nude and SCID mice and found no difference in tumor yield under our protocol.

3.4.2. Recommended Procedure for In Vivo Cell Growth

1. Anesthetize immunodeficient mice (100 mg/kg ketamine + 2.5 mg/kg acepromazine intraperitoneal).
2. Using sterile scalpel make a small incision in the right axilla area.
3. Using sterile forceps make a small subcutaneous pocket through the incision.
4. Mix tumor pieces with Matrigel (1/1, v/v) and place it in the subcutaneous pocket.
5. Close subcutaneous incision with wound clips and monitor mice until they are able to flee or defend themselves.
6. Monitor mice twice weekly for tumor bulk.

Whenever a cell suspension is available for injection, mix it with Matrigel (1/1, v/v), aspirate mixture into a syringe, and inject up to 100 µL of cell suspension subcutaneously into the axilla area.

3.5. Alternatives for Cell Culture

The basal medium PFMR-4A with supplements has been used as a serum free medium for propagating normal and cancer cells from primary tumors ***(1)***. PFMR-4A is also a modification of PFMR-4 containing 10 times more isoleucine, leucine, threonine, glutamine, inositol, thymidine, and methionine, and no trace elements ***(1,22)***. The supplements used are similar to those used with BRFF-HPC1 but the hydrocortisone concentration is a 100-fold less, there is no dihydrotestosterone, and α-tocopherol, and retinoic acid are included ***(1)***.

McKeehan and colleagues ***(12,13,24)*** developed the nutrient medium WAJC 401, which contains insulin, EGF, cholera toxin, BPE, prolactin, and dexametasone and supports rapid proliferation of normal and tumor epithelial cells from rat prostate. WAJC 401 is a modification of MCDB 151 ***(24)*** and differs from PFMR-4A mainly in its lower calcium concentration ***(1)***.

3.6. Pitfalls

A recent publication reported that two cell lines thought to be from prostatic cancer (TSU-Pr1 and Jca-1) may in fact be derivatives of T24 bladder carcinoma cells ***(25)***. Moreover, it has also been suggested that other prostate cancer cell lines share common origins ***(26,27)***. The discovery of cross-contamination of many cell lines with HeLa cells ***(28,29)*** was the first clear evidence that cell lines with very short doubling times can contaminate other cell lines. This is a particular concern for low-passage cultures with long doubling times, because a small number of cells can inadvertently be transferred from a rapidly growing cell line into the slower culture and easily overgrow it. Accidental transfer can occur if a pipet touches the neck of a

bottle of medium or by the formation of aerosols in the tissue-culture hood when flasks are open.

To avoid cross-contamination, dedicate a tissue-culture hood to handling primary cultures and low-passage cell lines. If this is not possible, then, in addition to the standard procedures of good practice in a tissue-culture facility ***(6)***, the following guidelines should be enforced: only one cell line should be used in a tissue-culture hood at any one time, the hood should be cleaned before the introduction of another cell line, bottles or aliquots of medium should be dedicated for use with only one cell line, and fast-growing cell lines should be handled at the end of the day.

3.7. Assessment of Cell Type and Prostate-Specific Markers, Authentication of Origin, and Characterization of a New Cell Line

The epithelial origin of the cells can be verified by their morphology and keratin expression. Expression of specific keratins is often altered by cell culture; therefore, we recommend using pan-keratin antibodies that recognize many cytokeratins. Cultured cells can be immunostained by using the avidin-biotin-peroxidase complex technique. The cell cultures also should be further characterized by immunological analysis of the tissue-specific markers prostate specific antigen and prostatic acid phosphatase.

Because of the potential cross-contamination, it is important to store samples of the normal tissue or DNA from the cell line source to allow for future authentication by DNA fingerprinting. It is also desirable to obtain a DNA fingerprint or profile of the cell line before it is used.

The cultures should also be characterized cytogenetically (by G-banding or fluorescence *in situ* hybridization), and their population doubling time, colony-forming efficiency, morphology under phase-contrast microscopy, and tumorigenic potential in immune-deficient mice should be determined.

Finally, screening for mycoplasma contamination is essential and should be performed routinely.

4. Notes

1. A tissue culture facility to propagate prostate cancer specimens must be based on a coordinated, multidisciplinary effort to obtain fresh tissue.
2. At the time the specimen is received, sampling and selection must be implemented through histopathological assessment of viable cells in the fresh specimen. Close interaction with the attending physician helps to incorporate the benefit of clinical observations into the culture conditions.
3. Two frequent contaminants have to be considered. One is normal epithelial cells, which (under serum-free conditions) may be selected for when a primary tumor is being cultured. Primary tumors frequently are surrounded by normal prostate tissue and the culture conditions do not select against normal prostate epithelial cells. The normal epithelial cells can overgrow the cancer cells in culture. The best way to avoid this is to use a tissue sample in which tumor cells predominante. At present, there are no markers for identifying prostate cancer cells in vitro, but molecular cytogenetics methods can be used to assess whether genetic gains or losses present in the original tumor are present in the cell culture. In any case, the final proof of the origin of an epithelial cell culture is that only cancer-derived epithelial cells will become immortal (grown for more than 20 passages in vitro).

A second major contaminant is stromal cells. Prostate cancer is multifocal and is frequently surrounded by fibroblasts and/or smooth muscle cells. When the tumor is from a metastatic site, the most common contaminants are fibroblasts, which can usually be easily identified by the expert eye on phase-contrast microscopy **(Fig. 1B)**. We described some techniques earlier to select for the epithelial component. The most important technique is to monitor the culture daily and try alternative procedures such as reducing the serum concentration, the selective adhesion technique (after 2–4 h of subculture, when the fibroblasts will have attached, harvest the epithelial cells by gently washing the culture with HBSS, and transfer the epithelial cells to a new dish), and the selective detachment technique (briefly expose the culture to trypsin, which will release fibroblasts more readily than epithelial cells, and then gently wash the culture with HBSS and transfer to a new dish).

3. In our experience fine needle aspirations do not constitute a suitable source of cells to obtain growth in vitro or in vivo.
4. No difference in the initial in vitro cell growth or in vivo propagation was found in specimens derived from therapy-resistant primary tumors or metastases.

References

1. Peehl, D. M. (1992) Culture of human prostatic epithelial cells. In: Freshney, R. A. (ed.) *Culture of Epithelial Cells,* Wiley-Liss, New York, pp. 159–180.
2. Chaproniere, D. M. and McKeehan, W. L. (1986) Serial culture of single adult human prostatic epithelial cells in serum-free medium containing low calcium and a new growth factor from bovine brain. *Cancer Res.* **46,** 819–824.
3. Peehl, D. M. and Stamey, T. A. (1986) Growth response of normal, benign hyperplastic, and malignant human prostatic epithelial cells in vitro to cholera toxin, pituitary extract, and hydrocortisone. *The Prostate* **8,** 51–61.
4. Navone, N. M., Logothetis, C. J., von Eschenbach, A. C., and Troncoso, P. (1999) Model systems of prostate cancer: uses and limitations. *Cancer Met. Rev.* **17,** 361–371.
5. Navone, N. M., Olive, M., Ozen, M., et al. (1997) Establishment of two human prostate cancer cell lines derived from a single bone metastasis. *Clin. Cancer Res.* **3,** 2493–2500.
6. Freshney, R. I. (ed.) (1994) *Culture of Animals Cells: A Manual of Basic Techniques*, 3rd ed. Wiley-Liss, Inc., New York, pp. 51–59, 86–88, 153–156, 353.
7. Iype, P. T., Iype, L. E., Verma, M., and Kaighn, E. (1998). Establishment and characterization of immortalized human cell lines from prostatic carcinoma and benign prostatic hyperplasia. *Int. J. Oncol.* **12,** 257–263.
8. Kaighn, M. E., Kirk, D., Szalay, M. and Lechner, J. F. (1981) Growth control of prostatic carcinoma cells in serum free media: interrelationship of hormone response, cell density and nutrient media. *Proc. Natl. Acad. Sci. USA* **78,** 5673–5676.
9. Peehl, D. M. and Stamey, T. A. (1984) Serial propagation of adult human prostatic epithelial cells with cholera toxin. *In vitro* **20,** 981–986.
10. Lechner, J. F., Babcook, M. S., Marnell, M., Shankar Narayan, K., and Kaighn, M. E. (1980) Normal human prostate epithelial cell culture. In: Harris, C. C., Trump, B. F., Stoner, G. D. (eds.) *Normal Human Tissue and Cell Culture. B. Endocrine, Urogenital, and Gastrointestinal Systems.* Methods in Cell Biology, vol. 21B, Academic Press, London, pp.195–225.
11. Kaighn, M. E. (1973) Human liver cells. In: Kruse, P. F. and Patterson, M. D. (eds.) *Tissue Culture Methods and Applications*. pp. 54–58.

12. McKeehan, W. L., Adams, P. and Fast, D. (1987) Different hormonal requirements for androgen independent growth of normal and tumor epithelial cells from rat prostate. *In Vitro Cell Dev. Biol.* **23,** 147–152.
13. McKeehan, W. L., Adams, P. S. and Rosser, M. P. (1984) Direct mitogenic effects of insulin, epidermal growth factor, glucocorticoid, cholera toxin, unknown pituitary factors and possibly prolactin, but not androgen, on normal rat prostate epithelial cells in serum-free, primary cell culture. *Cancer Res***. 44,** 1998–2010.
14. Carney, D. N., Bunn, P. A. Jr., Gazdar, A. F., Pagan, J. A., and Minna, J. D. (1981) Selective growth in serum-free hormone-supplemented medium of tumor cells obtained by biopsy from patients with small cell carcinoma of the lung. *Proc. Natl. Acad. Sci. USA* **78,** 3185–3189.
15. Horoszewicz, J. S., Leong, S. S., Kawinski, E., et al. (1983) LNCaP model of human prostatic carcinoma. *Cancer Res* **43,** 1809–1818.
16. Radisky, D., Hagios, C., and Bissell, M. J. (2001) Tumors are unique organs defined by abnormal signaling and context. *Semin. Cancer Biol.* **11,** 87–95.
17. Olumi, A. F., Grossfeld, G. D., Hayward, S. W., et al. (1999) Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium. *Cancer Res.* **59,** 5002–5011.
18. Cook, G. B. and Watson, F. R. (1968) Events in the natural history of prostate cancer: using salvage curves, mean age distributions, and contingency coefficients. *J. Urol.* **96,** 87–96.
19. Boyce, B. F., Yoneda T., and Guise T. A. (1999) Factors regulating the growth of metastatic cancer in bone. *Endocr. Relat. Cancer* **6,** 333–347.
20. Gleave, M., Hsieh, J. T., Gao, C., von Eschenbach, A. C. and Chung, L. W. K. (1991) Acceleration of human prostate cancer growth in vivo by factors produced by prostate and bone fibroblasts. *Cancer Res.* **51,** 3753–3761.
21. Yang, J., Fizazi, K., Peleg, S., et al. (2001) Prostate cancer cells induce osteoblast differentiation through a Cbfa1-dependent pathway. *Cancer Res.* **61,** 5652–5659.
22. Peehl, D. M. and Stamey, T. A. (1986) Serum-free growth of adult human prostatic epithelial cells. *In vitro Cell Devel. Biol.* **22,** 82–90.
23. McKeehan, W. L., Adams, P. S. and Rosser, M. P. (1981) Modified nutrient medium MCDB 151, defined growth factors, cholera toxin, pituitary factors, and horse serum support epithelial cell and suppress fibroblast proliferation in primary cultures of rat ventral prostate cells. *In vitro* **18,** 87–91
24. Peehl, D. M. and Ham, R. G. (1980) Clonal growth of human keratinocytes with small amounts of dialyzed serum. *In vitro* **16,** 526–538.
25. van Bokhoven, A., Varella-Garcia, M., Korch, C. and Miller, G. J. (2001) TSU-Pr1 and JCA-1 cells are derivatives of T24 bladder carcinoma cells and are not of prostatic origin. *Cancer Res.* **61,** 6340–6344.
26. van Bokhoven, A., Varella-Garcia, M., Korch, C., Hessels, D. and Miller, G. J. (2001) Widely used prostate carcinoma cell lines share common origins. *Prostate* **47,** 36–51.
27. Varella-Garcia M., Boomer, T. and Miller, G. J. (2001) Karyotypic similarity identified by multiplex-FISH relates four prostate adenocarcinoma cell lines: PC-3, PPC-1, ALVA-31, and ALVA-41. *Genes Chromosomes Cancer* **31,** 303–315.
28. Gartler, S. M. (1967) Genetic markers as tracers in cell culture. Second Bicennial Review Conference on Cell Tissue and Organ Culture. NCI Monographs, pp. 167–195.
29. Nelson-Rees, W. A. and Flandermeyer, R. R. (1977) Inter- and intraspecies contamination of human breast tumor cell lines HBC and BrCa5 and other cell cultures. *Science* **195,** 1343–1344.

11

Isolation and Culture of Ovarian Cancer Cell Lines

Simon P. Langdon

1. Introduction

Ovarian cancer is the leading cause of death from cancer of the female reproductive system with 1 in every 70 women developing the disease. In the United States for the year 2000, there were projected to be approx 23,000 diagnoses and 14,000 deaths from ovarian cancer ***(1)***. Most patients present with disease that has advanced beyond the ovaries, and although the majority of patients will show an initial response to chemotherapy, virtually all patients with advanced disease at presentation will die.

Human ovarian cancer cell lines have provided valuable model systems to study a variety of tumor characteristics, including cellular biology, genetics, and chemosensitivity. A large number of ovarian cancer cell lines have been established and many of these are in widespread use **(Table 1; *2–18*)**. Some examples are illustrated in **Fig. 1**. These models reflect the diverse characteristics found in clinical specimens such as a varying response to chemotherapy and differing p53 status.

Although ovarian cancer cell lines have been obtained from primary tumors, solid metastatic deposits, and pleural effusions **(Table 1)**, most have been derived from the peritoneal ascites of ovarian cancer patients. Ascitic fluid provides a convenient source of tumor cells and may be more readily available than primary or metastatic material as it is routinely drained to alleviate discomfort. Furthermore, these cells are more likely to be primed for in vitro culture, as they are already surviving as single cells or small clusters, thus avoiding the need for mechanical or enzymatic disaggregation. The cell mixture contains not only malignant carcinoma cells but also lymphocytes, macrophages, red blood cells, mesothelial cells, and fibroblasts. The use of differential centrifugation and trypsinization techniques allow relatively pure populations of carcinoma cells to be selected within several passages. These new cell lines can then be characterized to verify epithelial origin and the degree of contamination by non-epithelial cells can be assessed by the use of antibodies targeted to lymphocytes and fibroblasts.

From: *Methods in Molecular Medicine, vol. 88: Cancer Cell Culture: Methods and Protocols*
Edited by: S. P. Langdon © Humana Press Inc., Totowa, NJ

Table 1
Properties of Established Ovarian Carcinoma Cell Lines

Cell Line	Histology	Source	Prior Treatment	Ref
41M	Adenoca	Ascites	None	***2***
59M	Endometr adenoca	Ascites	None	***3***
138D	Serous adenoca	Ascites	Carb	***2***
180D	Adenoca	Ascites	P	***2***
200D	Serous adenoca	Solid	None	***2***
253D	Serous adenoca	Ascites	Cy/MPA	***2***
A2780	Carcinoma	—	None	***4***
CAOV-3	Adenoca	Tumor	Cy/Adr/FU	***5***
CAOV-4	Adenoca	Fallopian met	—	—
CH1	Papillary adenoca	Ascites	P/Carb	***3***
COLO 110	Serous adenoca	Sol met	None	***6***
COLO 316	Serous adenoca	Pleural	None	***6***
COLO 319	Serous adenoca	Ascites	None	***6***
COLO 330	Serous adenoca	Ascites	Mel/Radiother	***6***
DO-s	WD mucinous adenoca	Ascites	—	***7***
EFO-21	Dediff serous adenoca	Ascites	—	***8***
EFO-27	Mucinous papillary adenoca	Omentum	—	***8***
ES-2	PD clear cell ca	Primary	None	***9***
HOC-1	WD serous adenoca	Ascites	None	***5***
HOC-7	WD serous adenoca	Ascites	None	***5***
HTOA	WD serous adenoca	Primary	None	***10***
IGROV1	Adenoca	Primary	None	***11***
OAW 28	Adenoca	Ascites	P/Mel	***3***
OAW 42	Serous adenoca	Ascites	P	***3***
OV-90	Serous papillary adeno	Ascites	—	***12***
OV-1063	Papillary adenoca	Ascites	Cy/Adr/P/HMM	***13***
OvBH-1	Clear cell ca	Ascites	None	***14***
OVCAR-2	Adenoca	Ascites	P/Cy	***15***
OVCAR-3	PD papillary adenoca	Ascites	P/Cy/Adr	***16***
OVCAR-4	Adenoca	Ascites	P/Cy/Adr	***17***
OVCAR-5	Adenoca	Ascites	None	***18***
PE01	PD serous adenoca	Ascites	P/FU/CHL	***19***
PE04	PD serous adenoca	Ascites	P/FU/CHL	***19***
PE06	PD serous adenoca	Ascites	P/FU/CHL	***20***
PE014	WD serous adenoca	Ascites	None	***20***
PE016	PD serous adenoca	Ascites	Radioth	***20***
PE023	WD serous adenoca	Ascites	P/CHL	***20***
PEA1	PD adenoca	Pleural	None	***20***
PEA2	PD adenoca	Ascites	P/Pred	***20***
SKOV-3	Adenoca	Ascites	T	***21***
SMOV-2	Clear cell adenoca	Primary	—	***22***
T014	WD serous adenoca	Sol met	None	***20***
TOV-112D	Endometr adenoca	Primary	—	***12***
TOV-21G	Clear cell ca	Primary	—	***12***

Adenoca, adenocarcinoma; Adr, adriamycin; Carb, carboplatin; CHL, chlorambucil; Cy, cyclophosphamide; FU, 5-fluorouracil; HMM, hexamethylmelamine; Mel, melphalan; met, metastasis; MPA, medroxyprogesterone actetate; P, cisplatin; PD, poorly differentiated; pleural, pleural effusion; Pred, prednimustine; Radioth, radiotherapy; Sol met, solid metastasis; T, thiotepa; WD, well differentiated.

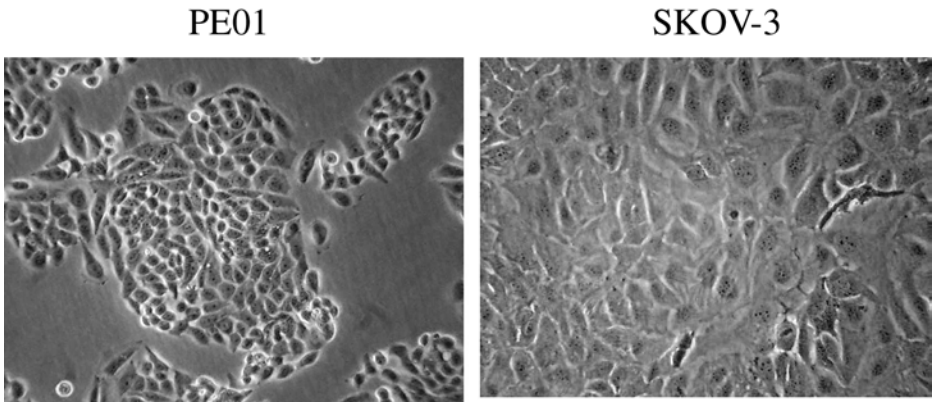

Fig. 1. Phase contrast micrographs of the PE01 and SKOV-3 ovarian cancer cell lines.

Once established, many ovarian cancer cell lines can be grown in fully defined media allowing detailed analysis of the influences of regulatory molecules, e.g., the effects of hormones, growth factors, cytokines, or the impact of therapeutic molecules, for example, cisplatin among the cytotoxic drugs.

2. Materials

1. Culture medium: RPMI 1640 medium (Life Technologies, Paisley, Scotland), 10% fetal calf serum (FCS; Life Technologies), 100 U/mL penicillin/100 µg/mL streptomycin (Gibco-BRL, Life Technologies).
2. 2 m*M* sodium pyruvate (Sigma).
3. 2.5 µg/mL insulin (Sigma).
4. Phosphate buffered saline (PBS), pH 7.4.
5. Histopaque (Sigma).
6. Trypsin (0.05% w/v)/EDTA (0.02% w/v) in PBS.

3. Methods

3.1. Initial Culturing from Ascites

1. Transfer freshly obtained ascitic fluid drained from the peritoneum of an ovarian cancer patient (at the time of paracentesis or staging laparotomy) to a sterile environment , for example, class II hood (*see* **Notes 1** and **2**). The average volume collected from any individual patient is approx 1 L.
2. Centrifuge the fluid for 20 min at 2000*g* and 4°C to produce a cell pellet.
3. Discard the fluid and resuspend the cell pellet in 20 mL PBS.
4. Red blood cells are removed by Histopaque (Sigma). Place the 10 mL tumor cell suspension carefully onto Histopaque (10 mL) in a Universal container and centrifuge at 2000*g* for 30 min at room temperature.The tumor cells remain at the interface of the Histopaque. Remove by pastette and transfer to a new Universal container.
5. Resuspend the cell pellet in 10 mL PBS and centrifuge at 1000*g* for 10 min. Repeat this wash once.
6. Resuspend the cells in tissue culture medium (*see* **Notes 3–6**).

7. Take a cell count. Culture aliquots of 10^5 cells/mL (10 mL/25 cm^2-tissue culture flask) at 37°C, 100% humidity and 5% CO_2 in a tissue culture incubator. Attachment of cells to the plastic substrate occurs and eventually a monolayer will form.
8. At this initial phase of establishment, cells require careful monitoring (by microscope) since growth is variable. For example, during the establishment of the PE01 and PE04 cell lines, subculturing took place after 21 and 4 wk respectively ***(1)***. Tissue culture medium should be replaced 2–3 times/wk

3.2. Initial Culturing from Solid Primary Tumor or Metastatic Deposit

1. Obtain primary or secondary tumor material at the time of surgery. Transfer fragments of the tumor as rapidly as possible into a sterile Universal container containing RPMI 1640 and place on ice to maintain viability.
2. Transfer the Universal container to a sterile environment (for example, a class II hood). Dissociate tumor fragments either mechanically using crossed scalpel blades (Swann-Morton, Sheffield, England) or enzymatically (*see* **Note 7**). After either mechanical or enzymatic disassociation, filter cell suspensions through a sterile gauze to remove clumps.
3. The cell suspension is then processed according to **Subheading 3.1., steps 6–8**.

3.3 Maintenance and Development of Cell Lines

After initial culturing, careful monitoring is needed to determine the level of contamination by fibroblasts. Although fibroblasts will cease proliferating after a number of generations, they may outgrow the carcinoma cells if the initial percentage of fibroblasts is too high.

3.3.1. Removal of Fibroblasts

1. Selective trypsinization can remove most of the fibroblast population. Fibroblasts detach rapidly from plastic after trypsinization and a short treatment (<2 min) with 0.05% (w/v) trypsin/0.02% (w/v) EDTA will remove many of the fibroblasts before the carcinoma cells detach. Fresh media containing serum will then rapidly inactivate remaining trypsin.
2. Fibroblasts attach to plastic more rapidly than the epithelial cells and those cells that attach within the first 2–3 h are predominantly fibroblasts. If the cell suspension is transferred to a new flask after this period, many of the fibroblasts will remain in the original flask, which can then be discarded.
3, Monoclonal antibodies targeted to fibroblast-specific antigens (for example, Thy-1) can allow selective removal of the fibroblasts when in cell suspension.
4. If sufficient numbers of clusters of carcinoma cells initially adhere to the plastic substrate then the surrounding fibroblasts can be scraped off with a plastic scraper. Colonies can be indicated by use of a marker pen on the base of the flask.

3.3.2. Removal of Mesothelial Cells

Mesothelial cells may also be present and these can be removed as follows. Mesothelial cells but not epithelial cells attach to fibrin meshes that are produced on initial culturing. A short exposure to trypsin/EDTA (2 min) produces complete detachment of the mesothelial cell sheet attached to the fibrin mesh without removing epithelial islands.

3.4. Subculturing and Characterization of Cultures

1. When the cell culture approaches confluence, it should be subcultured. Remove tissue culture medium and wash cells with 20 mL PBS.

2. Add Trypsin/EDTA to the flask for 5–15 min to detach cells.
3. Once cells are in suspension, place into fresh serum/medium and transfer to another flask (*see* **Note 8**).
4. After several passages, the fibroblasts, lymphocytes, and mesothelial cells should have disappeared and only carcinoma cells remain. At this stage it is appropriate to confirm the epithelial nature of these cells. Place a small aliquot of cells onto a multispot slide and stain by standard immunocytochemical methods. There are many monoclonal antibodies that can be used for the purpose of identifying specific cell types and we have found the following antibodies to be useful: E29 (Dako, Cambridge, England) targeting epithelial membrane antigen will identify epithelial cells; 2B11 (Dako) targeting leucocyte common antigen (CD45) will identify lymphocytes; 5B5 (Dako) targeting the β subunit of prolyl-4-hydroxylase and the disulphide isomerase will identify fibroblasts.
5. More detailed assessments of antigen expression may be valuable. Expression of specific cytokeratins and other epithelial markers including human milk fat globulin-2 (HMFG2) and OC 125 (detected by CA125) are often measured.
6. Other useful characterization procedures include DNA profiling and karyotyping.
7. Regular testing for mycoplasma contamination is also advised, e.g., by PCR identification or by staining with Hoechst 33528 dye and viewing under a fluorescent microscope, or by standard microbiological techniques.

4. Notes

1. All ascitic samples should be treated with care and all procedures carried out within class II containment facilities.
2. Heparin has also been widely used to prevent cell aggregation and can be added directly to the initial ascites fluid (10,000 U/L ascites).
3. The choice of specific medium has varied widely among different laboratories. RPMI 1640, DMEM, Ham's F-12 and α-MEM have all been used as the basic medium and the choice depends mainly on the medium in use within the laboratory establishing the cell line. The use of Ham's F12 has also enabled cell lines to develop which otherwise were destined to die ***(2)***.
4. The use of additives has also varied widely among different laboratories. In addition to the presence of serum and medium, antibiotics are routinely added and most popular are penicillin and streptomycin, although 50 µg/mL gentamicin and 2.5 µg/mL amphotericin have also been used. Other standard additives for early cultures include insulin and pyruvate. Historically, extra glutamine was often added to the media but formulations in current use are more stable.
5. The portion of FCS used has commonly been 10%. Some cell lines have been recorded as initially growing in 10% but then deteriorating and this effect was prevented by a reduction to 5% serum ***(2)***.
6. The use of autologous human ascitic filtrate may help the establishment and growth of the cell line. This can be added to the media at a level of 10%.
7. Disaggregation of solid tumors can be accomplished either mechanically or enzymatically. The simple use of crossed scalpels is easy and if adequate numbers of small clusters of cells are produced then this is sufficient to generate a cell line. Enzymatic digestion will typically yield a two- to sevenfold greater yield of single viable cells. A number of "enzyme cocktails" have been described and the following have all been used to establish ovarian cancer cell lines: 0.8 g/100 mL collagenase II (Sigma; EC 3.4.23.3), 0.002 g/100 mL DNase I (Sigma; EC 3.1.21.1), and 500 U/mL pronase (Boehringer Mannheim).

8. Subculture in the early stages should involve a split ratio of 1/2 or 1/3; eventually this can be increased to 1/5 to 1/10.

References

1. American Cancer Society: Statistics for 2001: Cancer Facts and Figures 2001. http://www.cancer.org.
2. Wilson, A. P., Dent, M., Pelovic, T., Hubbold, L., and Radford, H. (1996) Characterisation of seven human ovarian tumour cell lines. *Br. J. Cancer* **74,** 722–727.
3. Hills, C. A., Kelland, L. R., Abel, G., Siracky, J., Wilson, A. P. and Harrap, K. R. (1989) Biological properties of ten human ovarian carcinoma cell lines: calibration in vitro against four platinum complexes. *Br. J. Cancer* **59,** 527–534.
4. Behrens, B. C., Hamilton, T. C., Masuda, H., et al. (1987) Characterization of a cis-diamminedichloroplatinum(II)-resistant human ovarian cancer cell line and its use evaluation of platinum analogues. *Cancer Res.* **47,** 2414–2418.
5. Buick, R. N., Pullano, R., and Trent, J. M. (1985) Comparative properties of five human ovarian adenocarcinoma cell lines. *Cancer Res.* **45,** 3668–3676.
6. Woods, L. K., Morgan, R. T., Quinn, L. A., Moore, G. E., Semple, T. U., and Stedman, K. E. (1979) Comparison of four new cell lines from patients with adenocarcinoma of the ovary. *Cancer Res.* **39,** 4449–4459.
7. Briers, T. W., Stroobants, P., Vandeputte, T. M., et al. (1989) Establishment and characterization of a human ovarian neoplastic cell line DO-s. *Cancer Res.* **49,** 5153–5161.
8. Simon, W. E., Albrecht, M., Hansel. M., Dietel, M., and Holzel, F. (1982) Cell lines derived from human ovarian carcinomas: growth stimulation by gonadotropic and steroid hormones. *J. Natl. Cancer Inst.* **70,** 839–845.
9. Lau, D. H., Lewis, A. D., Ehsan, M. N., and Sikic, B. I. (1991) Multifactorial mechanisms associated with broad cross-resistance of ovarian carcinoma cells selected by cyanomorpholino doxorubicin. *Cancer Res.* **51,** 5181–5187.
10. Ishiwata, I., Ishiwata, C., Soma, M., Nozawa, S., and Ishikawa, H. (1987) Characterization of newly established human ovarian carcinoma cell line—special reference of the effects of cis-platinum on cellular proliferation and release of CA125. *Gynecol. Oncology* **26,** 340–354.
11. Benard, J., Da Silva, J., De Bois, M-C., Boyer, P., et al. (1985) Characterization of a human ovarian adenocarcinoma line, IGROV1, in tissue culture and in nude mice. *Cancer Res.* **45,** 4970–4979.
12. Provencher, D. M., Lounis, H., Champoux, L., et al. (2000) Characterization of four epithelial cancer cell lines. *In vitro Cell. Dev. Biol. Anim.* **36,** 357–361.
13. Horowitz, A. T., Treves, A. J., Voss, R., et al. (1985). A new human ovarian carcinoma cell line: establishment and analysis of tumor-associated markers. *Oncolology* **42,** 332–337.
14. Bar, J. K. and Harlozinska, A. (2000) Morphological and phenotypic characterization of a newly established ovarian carcinoma cell line (OvBH-1). *Anticancer Res.* **20,** 2975–2980.
15. Pirker, R., FitzGerald, D. J., Hamilton, T. C., et al. (1985) Characterization of immunotoxins active against ovarian cancer cell lines. *J. Clin. Invest.* **76,** 1261–1267.
16. Hamilton, T. C., Young, R. C., McKoy, W. M., et al. (1983) Characterization of a human ovarian carcinoma cell line (NIH:OVCAR-3) with androgen and estrogen receptors. *Cancer Res.* **43,** 5379–5389.
17. Louie, K. G., Behrens, B. C., Kinsella, T. J., et al. (1985). Radiation survival parameters of anti-neoplastic drug-sensitive and -resistant human ovarian cancer cell lines and their modification by buthionine sulfoximine. *Cancer Res.* **45,** 2110–2115.

18. Louie, K. G., Hamilton, T. C., Winker, M. A., et al. (1986) Adriamycin accumulation and metabolism in adriamycin-sensitive and -resistant human ovarian cancer cell lines. *Biochem. Pharmacol.* **35,** 467–472.
19. Wolf, C. R., Hayward, I. P., Lawrie, S. S., et al. (1987). Cellular heterogeneity and drug resistance in two ovarian adenocarcinoma cell lines derived from a single patient. *Int. J. Cancer* **39,** 695–702.
20. Langdon, S. P., Lawrie, S. S., Hay, F. G., et al. (1988) Characterization and properties of nine human ovarian adenocarcinoma cell lines. *Cancer Res.* **48,** 6166–6172.
21. Fogh, J., Fogh, J. M., and Orfeo, T. (1977) One hundred and twenty seven cultured tumour cell lines producing tumours in nude mice. *J. Natl. Cancer Inst.* **59,** 221–226.
22. Yonamine, K., Hayashi, K., and Iida, T. (1999) Establishment and characterization of human ovarian clear cell adenocarcinoma cell line (SMOV-2) and its cytotoxicity by anti-cancer agents. *Hum. Cell* **12,** 139–148.

12

Isolation and Culture of Leukemia Cell Lines

Hans G. Drexler

1. Introduction

1.1. Importance of Leukemia Cell Lines

The availability of continuous human leukemia cell lines as a rich resource of abundant, accessible, and manipulable living cells has contributed significantly to a better understanding of the pathophysiology of hematopoietic tumors ***(1)***. The first malignant hematopoietic cell lines, i.e., Burkitt's lymphoma-derived lines, were established in 1963 ***(2)***. Since then, large numbers of cell lines have been described, although not all of them have been characterized in full detail ***(3)***. The major advantages and common characteristics of leukemia cell lines are listed in **Table 1**. The spectrum of malignant hematopoietic cell lines is not restricted to various types of leukemia but also includes the lymphomas and myelomas. The following refers also to lymphoma- and myeloma-derived cell lines unless indicated otherwise.

Truly malignant cell lines must be discerned from Epstein-Barr virus (EBV)-immortalized normal cells, and so-called B-lymphoblastoid cell lines (B-LCL). Some types of malignant cell lines are indeed EBV-positive and some EBV-positive normal cell lines may also carry genetic aberrations ***(1)***.

Leukemia cell lines are now universally used and have become irreplacable tools in a multitude of research areas within the various scientific disciplines, e.g., immunology, cytogenetics, molecular biology, pharmacology, toxicology, and virology. Nevertheless, once cells are removed from their native environment and grown in cell culture, they are uncoupled from critical extracellular cues and may undergo phenotypic drift. During this process, transcriptional control mechanisms may change in ways that are difficult to predict. Therefore, in vitro-derived results must be interpreted with caution and confirmed in the context of the cell's native environment.

1.2. Basic Principles of Leukemia Cell Line Establishment

It is difficult to establish leukemia cell lines; the majority of attempts fail. The simple seeding of the malignant cells into suspension cultures is the most common approach. Although the success rate for establishing continuous cell lines is gener-

From: *Methods in Molecular Medicine, vol. 88: Cancer Cell Culture: Methods and Protocols*
Edited by: S. P. Langdon © Humana Press Inc., Totowa, NJ

Table 1
Advantages and Characteristics of Leukemia Cell Lines

- Unlimited supply of cell material.
- Worldwide availability of identical cell material.
- Indefinite storability in liquid nitrogen and recoverability.

- Monoclonal origin.
- Differentiation arrest at a discrete maturation stage
- Sustained proliferation in culture
- Stability of most features in long-term culture
- Specific genetic alterations

ally low, it depends on the subtype of leukemia, and varies according to the different reports in the literature.

It has been suggested that certain types of leukemia cells derived from patients at relapse or from cases with poor prognostic features have an enhanced growth potential in vitro when compared to samples obtained at presentation or from patients with good prognostic parameters. Indeed, a review of the literature showed that on aggregate the success rate for B-cell precursor (BCP)-cell lines established from patients at diagnosis was 6%, whereas the success rate was 29% for relapse samples ***(4)***. Furthermore, there seems to be a higher success rate in cases that have certain primary chromosomal aberrations or gene mutations, e.g., T-cell lines harboring an (8;14)(q24;q11) translocation and cell lines carrying alterations of the *P53, P15INK4B* or *P16INK4* genes ***(3,5)***.

It has been reported that a hypoxic environment (for example, 5% O_2) is more suitable to the culture and establishment of leukemic T- and BCP-cell lines than standard incubation in 5–10% CO_2 in air ***(6)***. According to this method, cells are cultured in wells with a feeder layer consisting of complete media, human serum, and agar supplemented with insulin-like growth factor. For the BCP-cell lines, the monocyte toxin L-leucine methyl ester and insulin were applied. Although the success rate of cell line establishment was reported to be high, some of these cell lines have long doubling times (10–14 d) limiting their usefulness. Currently, no other group has confirmed the reproducibility of this method.

The reasons for the frequent failure to establish cell lines remain unclear. The major causes appear to be culture deterioration with cessation of multiplication of the neoplastic cells and overgrowth by normal fibroblasts, macrophages, or lymphoblastoid cells. Although the lymphoblastoid cells may give rise to a continuous cell line (a nonmalignant EBV-positive B-LCL), human fibroblast and macrophage cultures are commonly not immortalized. Despite the fact that the proliferation of malignant hematopoietic cells in vivo seems to be independent of the normal regulatory mechanisms, these cells usually fail to proliferate autonomously in vitro even for short periods of time. In vivo, at least initially, these cells seem to require one or probably several hematopoietic growth factors for proliferation. The addition of regulatory proteins, e.g., recombinant hematopoietic growth factors or conditioned medium (CM) secreted by certain tumor cell lines (which often contains various factors), is a culturing technique

that appears to increase the frequency of success by overcoming the "crisis" period in which the neoplastic cells cease proliferating in vitro. These molecules enable the cells from the majority of patients to multiply for about 2–4 wk. Out of these short-term cultures containing surviving cells, continuous cell lines derived from the malignant cells can be established. For cell line immortalization, malignant cells must acquire a selective advantage in the cell culture environment. However, the mechanisms involved in the establishment of a leukemia cell line milieu are largely unknown.

Taken together, the efficiency of cell line establishment is still rather low and the deliberate establishment of leukemia cell lines remains by and large an unpredictable process. Difficulties in establishing continuous cell lines may be the result of inappropriate selection of nutrients and growth factors for these cells. Therefore, a suitable microenvironment for hematopoietic cells, either malignant or normal, cannot yet be created in vitro. Hence, this is the area with the greatest challenge in the culture of leukemia cells. A systematic investigation to define the optimal cell numbers, culture conditions, growth factor combinations, other supplements, matrix characteristics, and a multitude of different parameters has not been reported. Further work is required to achieve significant improvements in the success rate of leukemia cell line immortalization. A breakthrough will likely benefit leukemia research in a myriad of experimental questions. In the following subsection, some of the most commonly used techniques for establishing leukemia cell lines are described.

2. Materials

2.1. Establishment and Culture of Cell Lines

1. Ficoll-Hypaque solution: density 1.077 g/L; store at 4°C in the dark.
2. Cell culture medium: all are commercially available; the most commonly used are RPMI 1640, Iscove's Modified Dulbecco's Medium, α-MEM, or McCoy's 5A; store at 4°C in the dark; prepare aliquots of complete medium (maximally 100–200 mL) in a separate sterile glass bottle (to be used only for one specific cell line), which should be kept at room temperature in the culture laboratory in order to detect any microbiological contamination.
3. Fetal bovine serum (FBS): inactivate toxic components in the FBS prior to use in a 56 °C waterbath for 45 min; store aliquots of maximally 50 mL in plastic tubes at –20°C to 30°C.
4. Neubauer hematocytometer and trypan blue solution.
5. Recombinant growth factors: for example, erythropoietin (EPO), granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte-CSF (G-CSF), interleukin-2 (IL-2), IL-3, IL-6, stem cell factor (SCF), or thrombopoietin (TPO).
6. Conditioned medium (CM) from tumor cell line cultures: for example, the human bladder carcinoma cell line 5637 is known to produce and secrete large quantities of various growth factors *(7)*; aliquot the CM in 30- or 50-mL tubes and store at –20°C; test the 5637 CM in proliferation assays using an indicator cell line or determine the exact growth factor concentration with ELISA.

2.2. Freezing and Storage of Cell Lines

1. Dimethylsulfoxide (DMSO).
2. Freezing ampules (plastic cryo vials).
3. Controlled-rate freezer.
4. Cryofreezing container (Nalgene Cryo 1°C Freezing Container).

3. Methods

3.1. Establishment of Cell Lines

3.1.1. Acquisition of Cells (see **Notes 1** and **2**)

1. Collect heparin treated or otherwise anticoagulated specimens of peripheral blood, bone marrow, or other samples in sterile tubes (*see* **Notes 3** and **4**). Place lymph nodes and other solid tissues in sterile containers.
2. Specimens should be processed as soon as possible after receipt but may be stored overnight at room temperature. Peripheral blood and bone marrow should remain undiluted; solid tissues should be placed in culture medium.
3. Cryopreserved samples can also be used for attempts to establish cell lines. It appears to be of advantage to isolate the mononuclear cells prior to cryopreservation by Ficoll-Hypaque density gradient centrifugation (*see* **Subheading 3.1.2.).**

3.1.2. Isolation of Cells (see **Note 5**)

1. Cut solid tissue specimens (for example, lymph nodes) with scissors and force the particles through a fine metal mesh. Suspend the cells in 50–100 mL culture medium (possibly more, depending on the size of the tissue specimen). The most commonly used media are listed in **Note 6**.
2. Dilute the blood and bone marrow samples 1/2 with culture medium. Isolation of cells from a leukapheresis collection requires dilution of the sample with culture medium at 1/4.
3. Pipet the Ficoll-Hypaque density gradient solution (density 1.077 g/L) into a 15-mL or 30-mL conical centrifuge tube. Slowly layer the mixture of medium and sample over the Ficoll-Hypaque solution. Use equal volumes of sample mixture and Ficoll-Hypaque solution (*see* **Note 7**).
4. Centrifuge for 20–30 min at 450*g* at room temperature (with the centrifuge brakes turned off). A layer of mononuclear cells should be visible on top of the Ficoll-Hypaque phase as they have a lower density than the Ficoll-Hypaque solution. The anucleated erythrocytes and the polynucleated granulocytes are concentrated as a pellet below the Ficoll-Hypaque layer.
5. Using a sterile Pasteur pipet, transfer the interface layer containing the mononuclear cells to a centrifuge tube.
6. Wash the cells by adding culture medium plus 10% FBS (add about 5 times the volume of the mononuclear cell solution) and centrifuge for 5–10 min at 200*g* at room temperature (*see* **Note 8**).
7. Discard the supernatant, resuspend the cell pellet in culture medium plus 10% FBS and repeat the washing procedure.
8. Finally, resuspend the cells in 20 mL culture medium with 20% FBS. Count the cells and determine their viability (*see* **Notes 9** and**10**).

3.1.3. Culture Conditions

1. Adjust the cell suspension of the original patient's cells to a concentration of $2–5 \times 10^6$/mL in culture medium (*see* **Note 6**) with 20% FBS plus additional 10% CM of cell line 5637 (*see* **Note 11**) or with an appropriate concentration of purified or recombinant growth factors (*see* **Note 12**). For additional additives such as antibiotics and others *see* **Note 13**.
2. Place 5–10 mL of cell suspension in the complete culture medium in an 80-cm^2 plastic culture flask (*see* **Note 14**). If 24-well plates are used, add 1–2 mL cell suspension to each

well. Add 100–200 µL of cell suspension into wells of a 96-well flat-bottomed microplates (*see* **Note 15**).
3. Place the cells in a humidified incubator at 37°C and 5% CO_2 in air (*see* **Note 16**).
4. Expand the cells by exchanging half of the spent culture volume with culture medium plus 20% FBS plus 10% 5637 CM (or with appropriate concentrations of recombinant growth factors) once a week. After a few hours, some cells will become adherent (*see* **Note 17**).
5. During the first weeks, the neoplastic cells may appear to proliferate actively. If the medium becomes acidic quickly (yellow in the case of RPMI 1640 medium), change half of the volume of medium at 2–3 d (day) intervals. If the number of the cells increases rapidly, readjust the cells every week to a concentration of at least 1×10^6/mL in fresh complete medium by dilution or subdivision into new flasks or wells of the plate. The neoplastic cells from the majority of patients undergo as many as four doublings in 2 wk, but after 2–3 wk most malignant cells cease proliferating. Following a lag time of 2–4 wk (crisis period), a small percentage of cells from the total population may still proliferate actively and may continue to grow and form a cell line.
6. If the malignant cells continue to proliferate for more than 2 mo, there is a high possibility of generating a new cell line. Then, the task of characterizing the proliferating cells should begin as soon as possible ***(8)***. Prior to the characterization of the cells, freeze ampules of the proliferating cells containing a minimum of 3×10^6 cells/ampule in liquid nitrogen in order to avoid loss of the cells resulting from occasional contaminations or other accidents.
7. Limiting dilution of the cells in 96-well-plates leads to the generation of monoclonal cell lines (*see* **Note 18**).

3.1.4. Documentation of Cells

It is mandatory to freeze aliquots of the original cells (*see* **Subheading 3.2.**) and to store them in appropriate locations for later documentation, authentication, and comparisons. Once the cultured cells start to proliferate, ampules should be frozen at regular intervals during the initial period of the culture expansion. The morphology of the primary cell lines and also later of the cells from the resulting cell line should be documented on stained cytospin-slide preparation (*see* **Note 19**).

3.2. Freezing and Storage of Cell Lines

3.2.1. Freezing of Cells

It is generally assumed that cell lines can be cryopreserved at –196°C in liquid nitrogen for more than 10 yr, if not indefinitely, without any significant changes in their biological features. The viable cell lines can be recovered at any time when needed. Prior to freezing in liquid nitrogen, the cells are suspended in the appropriate medium containing 20% FBS and 10% DMSO, which can lower the freezing point in order to protect the frozen cells from damage caused by ice crystals (*see* **Note 20**). It appears that no single suspending medium and procedure will be perfect for processing and cryogenic storage of all cell cultures. However, the procedures described here are suitable for most cell lines and are compatible with prolonged preservation of viability and other characteristics of the cell lines.

1. Harvest the cells of the cell lines by transfering the flask contents to 30 mL or 50 mL centrifuge tubes. The cells should be harvested in their logarithmic growth phase and when they are healthiest (*see* **Note 21**). Sufficient numbers of primary cells should be

frozen for later documentation and authentication (*see* **Subheading 3.1.4.**); immediately upon isolation, primary cells can be frozen after thorough washing steps (*see* **Subheading 3.1.2.**).

2. Determine the total number of viable cells by counting the cell density as well as the viability of the cells using the trypan-blue dye exclusion method (*see* **Note 10**).
3. Prepare a sufficient volume of freezing solution consisting of 70% culture medium, 20% FBS, and 10% DMSO. Cool on wet ice (*see* **Note 22**).
4. Centrifuge the cell suspensions at 200*g* for 10 min; discard the supernatant.
5. Adjust the cells to a concentration of $5–10 \times 10^6$/mL for cell lines and $10–50 \times 10^6$/mL for primary material using freshly prepared freezing solution (*see* **Note 23**).
6. Distribute the cells into freezing ampules (plastic cryovials) with 1 mL/ampule, thus containing at least 5×10^6 cells (*see* **Note 24**).
7. Using a computer-controlled rate freezer (cryofreezing system) a cooling rate of 1°C per min can be achieved, from room temperature down to –25°C. When the temperature reaches –25°C, the cooling rate is increased to 5–10°C per min. Upon reaching –100°C, the ampules can be transferred quickly to a liquid nitrogen container (*see* **Note 25**).

3.2.2. Cryopreservation in Liquid Nitrogen

Permanent storage of ampules with primary cells or cells from cell lines should be in the liquid phase of the liquid nitrogen. However, long-term storage is also possible in the vapor phase of the liquid nitrogen tank. Long-term preservation of cell lines or primary cells at –80°C (beyond 1 wk) cannot be recommended as the cells will die under these conditions within months or even weeks.

3.3. Thawing, Expansion, and Maintenance of Cell Lines

3.3.1. Thawing of Cell Lines

Commonly, cell lines are stored frozen in liquid nitrogen. Frozen cells must be thawed carefully in order to minimize cell loss.

1. The thawing solution consists of 80% culture medium and 20% FBS at room temperature or 37°C.
2. Remove the frozen ampule from liquid nitrogen and thaw the cells rapidly in a 37°C water bath by gently shaking the ampule in the water (*see* **Note 26**).
3. Wipe the ampule with a tissue prewetted with 70% ethanol before opening the vial. As soon as the cell suspension is thawed, transfer the cell suspension into a centrifuge tube.
4. Dilute the cell suspension slowly by adding 10–20 mL culture medium plus 20% FBS to the tube; shake the tube gently (*see* **Note 27**).
5. Centrifuge the cells at 200*g* for 10 min. Discard the supernatant.
6. Wash the cells again using another 10–20 mL culture medium plus 20% FBS. During centrifugation determine the total cell number and the percentage of viable cells in the improved Neubauer hematocytometer with trypan blue vital staining (*see* **Note 10**).
7. Finally, resuspend the washed cells in the desired culture medium with the recommended FBS concentration at the optimal cell density.

3.3.2. Expansion of Cell Lines

After thawing, cells may be resuspended in complete medium at a general cell concentration of $0.2–1.0 \times 10^6$ cells/mL (*see* **Note 28**). Suspension cell lines may be cultured in flasks or 24-well plates (*see* **Note 29**).

1. Incubate the cells in a humidified 37°C incubator with 5% CO_2 in air. Loosen the top of the flask slightly to allow for free gaseous exchange into and out of the flask.
2. Feed the cells by exchanging half of the culture volume with culture medium plus FBS at 2–3-d intervals (*see* **Note 30**). Remove half of the spent medium from the flask (gently) and then add the same volume of new complete medium into the flask.
3. If the cells proliferate actively, the culture medium will soon change color as a result of the pH change caused by cellular metabolism. In this case, it is necessary to change the medium more frequently. Should the cells double, subdivide the cells from the original flask into a second flask by diluting the suspension 1/2 with new medium.
4. When changing the medium, it is important to calculate the total cell number by determining the density as well as the viability of the cells using trypan-blue dye exclusion (*see* **Note 10**).
5. A careful documentation of all manipulations, macroscopic, and microscopic observations, intentional and accidental changes in the cellular conditions, and data on cell density, viability, and total cell number at different time points is mandatory.

3.3.3. Maintenance of Cell Lines

The cell lines can be maintained as long as required (*see* **Note 31**). The cells can be harvested at any time for different uses. If the cells proliferate more quickly than needed, keep the cell growth at a slower pace by decreasing the FBS to a lower percentage in the medium, changing the medium at longer intervals, or discarding a certain amount of the cells (up to 75%) during the exchange of medium. There is, however, always the risk that a subclone with a growth advantage and somewhat different genotypic and/or phenotypic features than the parental cell line will overgrow the culture during long-term maintenance. For example, growth factor-dependent cell lines that during extended culture (1–2 mo) may become completely growth factor independent. Hence, establish a master and a working bank of any given cell line with a sufficient number of frozen samples so that cultures do not need to be kept for extended periods of time between experiments. A reasonable time frame for continuous uninterrupted cultivation of one cell line is in the range of 1–2 mo.

After extended usage, culture flasks or plates will often contain a certain amount of unused ingredients of the medium and metabolized molecules and cell detritus. It is recommended that the plastic culture vessel be changed once every 1–2 mo.

3.4. Further Considerations

3.4.1. General Considerations

Although in vivo malignant cells enjoy a selective growth advantage over normal hematopoietic cells, in vitro leukemia cells are difficult to grow and to maintain and attempts to establish cell lines meet more often with failure than with success. Although, currently there is no one single cell-culture system that assures consistent establishment of cell lines, several methods for immortalizing neoplastic cells have been developed. The technique of seeding cells in suspension cultures as described in this chapter is certainly the most used. Other methods recommended by several researchers have their advantages and might meet with success in some attempts.

Growth of leukemia cells in soft agar or methylcellulose offers the advantage that the colonies formed are well fixed and can be easily removed from the supporting

medium for further culture in other environments. Thus, a cell line might be established by passaging single colonies.

Some lymphocytic leukemia cells can be immortalized using transforming viruses. EBV can promote growth of malignant B-cell lines from some patients with mature B-cell malignancies, but the EBV can also transform normal B-lymphocytes. Human T-cell leukemia virus (HTLV)-I allows the growth of malignant T-cells by inducing the IL-2 receptor. Considering the fact that EBV and HTLV can also transform normal cells, it is necessary to ascertain the malignant origin of the established cell lines by means of karyotype and molecular genetic analysis ***(8)***.

The growth of malignant and normal hematopoietic cells in vitro and in vivo is the result of complex interactions between growth factors and their respective receptors. The addition of some growth factors into the culture medium can support the proliferation of the neoplastic cells and induce the formation of cell lines.

A number of completely synthetic media, including several media designed specifically for unique types of leukemia cells, have been used by researchers for establishment and maintenance of cell lines in suspension cultures. It appears that no single medium is well suited for the growth of all types of leukemia cells. Should one medium fail to support cell growth, it might become necessary to try another kind of medium.

FBS is the standard supplement in the suspension culture system of cell lines. It is commonly used in concentrations of 5–20%. Cell lines which do not require FBS in the culture medium have been described; however, they appear to be rather rare ***(3)***. Prior to usage, it is recommended that batches of serum be pretested for their ability to support vigorous cell growth and for viral (in particular bovine viral diarrhea virus), mycoplasmal and other bacterial contamination. If possible, a large supply of the FBS from a pretested batch that supports cell growth well and has no contamination should be purchased and stored at –20°C for future use. Alternatives are newborn-calf serum (usually at only 25% of the price of the expensive FBS) or serum-free media. However, not all cell lines will grow in newborn-calf serum as well as in FBS. Although serum-free media do not provide financial advantages, they do allow for certain experimental manipulations that are not possible with FBS. Because FBS is known to contain many unidentified ingredients at highly variable concentrations, one cannot control all the substances to which the cells are exposed.

3.4.2. Culture Environment

A minimal amount of oxygen is essential for the growth of most types of cells in suspension cultures. The majority of cell lines grow well when they are incubated in a humidified 37°C incubator with 5% CO_2 in air. However, the partial pressure of oxygen (pO_2) in the normal body fluids is significantly less than that of air. Studies have shown that the pO_2 in human bone marrow is 2–5%. This is considerably lower than the pO_2 (15–20%) existing in the typical cell culture incubator maintained at 5% CO_2 in air. It has been reported that growth of cultured cells could be improved by reducing the percentage of oxygen in the gaseous phase to between 1–10%. Growing leukemia cells under low oxygen conditions of 6% CO_2, 5% O_2, and 89% N_2 may be a useful method for the establishment of cell lines ***(6)***, but is logistically more difficult resulting from the necessity for specialized incubators and a rather large consumption of N_2.

3.4.3. Common Cell Culture Problems

In the attempts to establish cell lines, the overgrowth of fibroblasts and normal EBV -positive lymphoblastoid cells is the most common problem. Should the nutrients in the medium become exhausted too quickly, the adherent cells should be removed by passaging the suspension cells into new flasks containing fresh medium. The overgrowth of EBV-positive B-lymphocytes can become visible 2 wk after seeding of the new culture. The EBV-positive B-cells look small and have irregular contours with some short villi. They proliferate preferentially in big-floating clusters or colonies ***(9)***. The colonies can be picked out with a Pasteur pipet and the EBV genome should be detected as soon as possible.

It is always necessary to freeze aliquots of the fresh primary cells in liquid nitrogen before culture. If a cell line should subsequently become established, the original cells can be used as a control for characterization of the established cell line ***(8)***. In case of failure to immortalize a cell line, the frozen primary back-up cells can be used for further attempts.

Maintenance of cell lines requires careful attention. Every cell line appears to have its optimal growth environment; parameters such as culture medium, cell density, nutrition supplements, and pH all play a major role. If cell growth becomes suboptimal or cells inexplicably die during culture, some of the following problems should be considered: suitability of the culture medium or the growth supplements for this particular cell line; proper functioning of the incubator at the appropriate temperature, humidity, and CO_2 levels; and selection of the adequate cell density. Some cell lines clearly grow better in 24-well plates than in culture flasks.

Contamination with mycoplasma, other bacteria, fungus, and viruses and cross-contamination with other "foreign" cells are the most common problems encountered in the maintenance of cell lines ***(10,11)***. Therefore, analyses at regular intervals must be undertaken to ensure a contamination-free environment for cell growth. In particular, mycoplasma contamination should be examined regularly, for example, using the polymerase chain reaction (*see* **Chapter 31**). All solutions and utensils coming into contact with cells must be sterilized prior to use; sterile techniques and good laboratory practices must be followed strictly ***(12)***. Although antibiotics can be added to the culture medium to prevent bacterial infection, they do not inhibit virus, fungus, or mycoplasma infection. Therefore, antibiotics such as penicillin and streptomycin are not necessary if care is taken regarding cell culture techniques (*see also* **Note 13**). Because contamination can cause the loss of valuable cell lines, it is important to cryopreserve a sufficient amount of cell material from each cell line. In order to prevent cellular contamination and misidentification, it is mandatory to use a separate bottle of medium for each cell line. Furthermore, cell culturists should not handle more than one cell line at the same time.

3.4.4. Time Considerations

Generation of a cell line may require 2–6 mo. However, a cell culture should not be considered a continuous cell line until the cells have been passaged and expanded for at least 6 mo and ideally for 1 yr. Expansion of most cell lines after thawing takes 2–3

Table 2
Most Important Requirements for a New Leukemia Cell Line

- Immortality of cells
- Verification of neoplasticity
- Authentication of derivation
- Scientific significance
- Characterization of cells
- Availability to scientific community

wk. Depending on the parameters analyzed, 2–4 mo might be needed for a thorough characterization of the established cell line.

3.4.5. Availability of Cell Lines

The most important requirements for a new cell line are listed in **Table 2** ***(8)***.

The cell line must be immortalized, i.e., it should be able to grow indefinitely. To differentiate between normal and malignant cell lines, the neoplasticity of the cell line should be verified, for example, by the analysis of numerical and structural chromosomal alterations (*see* **Chapter 5**).

The derivation from the assumed patient must be proven by unequivocal authentication procedures, for example, forensic-type DNA fingerprinting (*see* **Chapter 4**). The scientific significance of a new cell line can be documented by its full characterization to uncover its most relevant features.

Finally, it is important that new cell lines are not only published but are also made available to outside investigators. On one hand, scientific authenticity requires reproducibility. On the other hand, a cell line is certainly much more useful if it is transferred to colleagues inside and outside the originator's own research field. The best option is the deposition of a cell line in a public cell line bank that is able to perform adequate quality and identity controls, further characterize the cells, store sufficiently large master and working stocks, and distribute the cells noncommercially to requesting scientists.

4. Notes

1. The most commonly used specimens are peripheral blood or bone marrow samples as these are relatively easily obtained from patients. Other liquid specimens (e.g., pleural effusion, ascites, cerebrospinal fluid) and solid tissue samples (e.g., lymph node, tonsil, spleen) are less often used but can be processed similarly to peripheral blood or bone marrow samples. Solid tissues require a prior step to dissociate mechanically. All solutions and utensils must be sterile. Working in a laminar flow cabinet (class 2 biological safety cabinet) under sterile conditions is recommended (for specifics on the biological safety, *see* **Note 2**).
2. During work with human blood or other tissues, including primary leukemia cells, established cell lines, and pathogenic and infectious agents, biosafety practices must be followed. Some cell lines may be virally infected. For example, EBV and human herpesvirus-8 (HHV-8) are assigned to biological safety risk category 2; human T-cell

leukemia virus (HTLV)-I/-II and human immunodeficiency virus (HIV) fall into risk category 3. Fresh primary material may contain hepatitis viruses B or C (both in risk category 3). A standard code of practice including safety and legal considerations has been published within the "UKCCCR (United Kingdom Co-Ordinating Committee on Cancer Research) Guidelines for the Use of Cell Lines in Cancer Research" ***(12)***.

3. A literature review of 439 new cell lines produced the following statistical findings with regard to the specimen site from which primary cells had been obtained: peripheral blood, 48%; bone marrow, 24%; pleural effusion, 14%; ascites, 5%; bulk tumor, 3%; lymph node, 3%; cerebrospinal fluid, 1%; pericardial effusion, 1%; spleen, 1%; others (liver, meninges, tonsils), <1%. Only data on well-characterized cell lines were counted, without sister cell lines or subclones (applies also for **Notes 4** and **6**).
4. According to a literature review on 413 new cell lines, it seems that cells obtained from patients at later stages of the malignancy are more likely to be immortalized than at earlier stages. Treatment status of patient when the sample was taken: 55% at relapse/refractory/terminal; 34% at diagnosis/presentation; 9% at blast crisis; 2% during therapy.
5. It appears to be advantageous to prepare single-cell suspensions from the patient-derived samples instead of placing the specimens directly into culture medium. In the case of peripheral blood or bone marrow, all erythrocytes, granulocytes, and thrombocytes that are end-stage cells without the potential to proliferate will die within days and the resulting metabolic byproducts may be toxic, hampering any potential proliferation of the blast cells.
6. A literature review of 454 cell lines showed that the following media were most commonly used for the establishment of cell lines: 74% RPMI 1640; 12% Iscove's Modified Dulbecco's Medium; 7% McCoy's 5A; 5% α-MEM, and 3% others (Cosmedium, Dulbecco's MEM, Eagle's MEM, Fischer's, Ham's F10, Ham's F12, L-15).
7. Do not disturb the Ficoll-Hypaque/sample interface. It is helpful to hold the centrifuge tube at a 45° angle.
8. The washing steps are performed to remove the acidic heparin or other anticoagulants that may harm the cells, to remove the patient's serum, which may inhibit the cell growth, and to remove the Ficoll-Hypaque, which is hypertonic for the cells. Two washes will generally suffice. FBS, inactivated prior to use in a 56°C waterbath for 30–45 min is included in order to prevent cells from adhering to one another.
9. In general, more than 90% of the mononuclear cells can be recovered by this procedure. Cell yields depend on the number of malignant cells in the specimen and are highly variable from patient to patient. On average, each milliliter of leukemia bone marrow yields $15–30 \times 10^6$ mononuclear cells and each milliliter of peripheral blood yields $1–2 \times 10^6$ mononuclear cells, the latter depending on the white blood cell count. Several hundred millions of mononuclear cells can usually be recovered from lymph nodes.
10. The cell count and cellular viability can be determined by trypan blue dye exclusion. Viable cells will not take up the trypan blue stain. Make a 1/10 dilution of the cell suspension in trypan blue by adding 10 µL of the cell suspension to 90 µL of the ready-for-use trypan blue solution in a test tube and mix well. Cells should be analyzed immediately as viable cells may also begin to take up the dye after about 5–10 min. Fill the counting chamber of an improved Neubauer hematocytometer. The chamber should not be under- or overfilled. Using a microscope, count the number of blue-stained (nonviable) and unstained (viable) cells in the 1-mm middle square and the four 1-mm corner squares. Cells touching the top and left lines of the square (but not those touching the bottom and right lines) are counted as well. Calculate the average number of viable and

nonviable cells per square (1 mm × 1 mm = 1 mm^2). The square is 0.1 mm deep, hence the volume is 0.1 mm^3 (0.1 µL). The number of cells per milliliter (cell density) and the total number of cells in the cell suspension and their percentage of viability can be calculated as follows:

a. Cell density: average number of cells per 1-mm square × dilution factor × 10,000 = cells per mL.
b. Total number of cells: cell count per mL × volume of cell suspension.
c. Percent cell viability: total number of viable cells × 100 divided by total number of viable plus nonviable cells. Ideally, there should be about 100 cells per square. Otherwise a lower dilution of the cell suspension (e.g., 1/2 or 1/5) or a higher dilution (for example, 1/20) should be used.

11. 5637 Conditioned medium (CM): the adherent bladder carcinoma cell line 5637 is known to produce and secrete large quantities of various growth factors, including G-CSF, GM-CSF, SCF and others (but not IL-3) ***(7)***. CM should be made in large volumes in order to guarantee continuity over a period of time; for example, 6 mo to 1 yr. At initiation of culture, seed out at approx 2×10^6 5637 cells/80 cm^2 in 10 mL of complete medium. Incubate at 37°C in 5% CO_2 in a humidified atmosphere, with the flask tops slightly loosened, until the cell monolayer becomes almost confluent. Replace the medium and grow for 4 d. Collect this medium and store it at 4°C. Add fresh complete medium to the 5637 culture. Repeat this process at 3–5-d intervals, pooling the culture supernatants. After 1–2 wk, split the confluent 5637 cultures 1/4 to 1/5 using trypsin/EDTA for 5 min and reseed the cells in fresh flasks. 5637 cells have a doubling time of about 24 h, leading to a cell harvest of approx $8–10 \times 10^6$ cells/80 cm^2 ***(13)***. Spin down the pooled conditioned medium at 200*g* for 5–10 min before filtering through sterile filters of 0.2 µm to exclude any contaminating nonadherent cells. Place aliquots into 30- or 50-mL tubes and store at –20°C. Test the 5637 CM in proliferation assays at 5%, 10%, and 20% (v/v), using specific growth factor-dependent cell lines, for example, cell line M-07e ***(13)***, to determine the optimum volume necessary for maximal stimulation. Alternatively, the exact growth factor concentrations in the 5637 CM can be determined with enzyme-linked immunoassays ***(7)***.
12. The most effective growth factors in terms of induction of proliferation appear to be GM-CSF, IL-3, and SCF, which may be used singly or in combination. A concentration of 10 ng/mL would provide a surplus of growth factors.
13. Most cell culturists add antibiotics (commonly penicillin-streptomycin) to their cell cultures, as ready-for-use commercially available 100X solutions. Although this does not appear to be harmful, it is not necessary if proper cell culture techniques ("good culturing practices") are used. L-glutamine (at 2 n*M*) and 2-mercaptoethanol (at 5×10^{-5} *M*) may also be added, but their effects are questionable. We strongly discourage the routine addition of antimycoplasmal reagents as this practice would lead to the development of resistant mycoplasma strains ***(14)***.
14. Most cell lines have been established using the "direct method" of placing cells into liquid culture in a humidified incubator at 37°C and 5% CO_2 in air. Alternative approaches are inoculation in semisolid media (methylcellulose, soft agar), initial heterotransplantation, and serial passage in immunodeficient mice with subsequent adaptation to in vitro culture, or culture on a temporary feeder layer (for example, on fibroblasts).
15. There are various types and sizes of plastic culture vessels: flasks (e.g., 25 cm^2, 80 cm^2, 175 cm^2) or plates with 12-, 24-, or 96-wells. The number of flasks or wells used depends

on the number of the primary leukemia cells available. As many of the malignant cells as possible should be used in attempts to establish a cell line. In theory, a cell line starts from one single cell. Thus, the more attempts, the higher the chances.

16. Alternatively (but seldom an option as this requires a special type of incubator and large quantities of N_2), incubate the cells in a humidified 37°C incubator with 6% CO_2, 5% O_2, and 89% N_2.
17. These adherent cells appear to be the source of colony-stimulating factors for both normal and malignant cells. During the first 2 wk, it is not necessary to remove the adherent cells from the culture unless there is a specific reason to do so, for example, because of the addition of a specific recombinant growth factor to the medium in order to obtain a unique cell line. After 2 wk, if the suspension cells grow rapidly, the adherent cells can be removed simply by transferring the suspension cells into new culture vessels to reduce the potential for overgrowth of fibroblasts and normal lymphoblastoid cells.
18. After prolonged culture in vitro, the cell line will become oligoclonal or monoclonal resulting from the outgrowth of selected cell clones. In most cases, it is not necessary to subclone the cell line by limiting dilution. In some types of cell lines, e.g., immature T- and BCP-cell lines, it might be difficult or virtually impossible to "clone" the cells.
19. Harvest the cells, count, and adjust the concentration to 0.5–1.0 × 10^6 cells/mL. Spin 50 µL, 70 µL, and 90 µL of cell suspension onto glass microscope slides in a cytocentrifuge (Shandon Scientific, Astmoor, England) at 500 rpm for 5 min and air dry at room temperature overnight (slides should not be left unstained for longer than 1 wk).

    Select the best preparations under the microscope: the cells should be neither too packed nor too isolated for an appropriate morphological examination. Stain with the May-Grünwald and Giemsa solutions as follows:

    a. Fix for 5 min in 100% methanol.
    b. Transfer for 5 min to May-Grünwald stain that has been diluted 1/2 with Weise buffer, and then rinse with Weise buffer.
    c. Transfer for 20 min to Giemsa stain which has been diluted 1/10 with Weise buffer
    d. Finally rinse thoroughly with Weise buffer. Let the staining dry overnight. Mount the stained cells with a cover slip using Entellan® (Merck) and examine the preparation under the microscope.

20. Glycerol has been substituted for DMSO but it is a bit cumbersome as a result of its high viscosity.
21. Freezing and storing the cells will not improve the status and quality of the cell culture prior to the freezing and may adversely affect the culture .
22. It is not necessary to sterilize the DMSO solution because pure DMSO is lethal to bacteria.
23. The freezing solution should be added quickly to the cells and then mixed thoroughly. Long-time exposure to DMSO at room temperature can trigger significant cellular changes such as activation and so-called "induction of differentiation." Keeping cells in DMSO-containing media on ice may minimize the effect of DMSO on the cells.
24. Seal the ampules that have been properly labeled with the name of the cell line and the date of freezing; keep a written record. More cells per ampule can be frozen if needed, depending on the cell type.
25. Alternatively if only a few ampules are frozen, the sealed ampules can be placed in a plastic box with an inset for ampules (Cryo 1°C Freezing Container; Nalgene); the box is half-filled with isopropanol and stored in a –80°C freezer for at least 4 h. With this method, a 1°C-per-minute cooling rate can be achieved as well.

26. It is important that the frozen cell solution be thawed in about 1 min. Rapid warming is necessary so that the frozen cells pass quickly through the temperature zone between -50°C and 0°C, where most cell damage is believed to occur. Slow thawing will harm the cells by formation of ice crystals in the cells causing hypertonicity and breakage of cellular organelles.
27. Cells frozen with DMSO are usually dehydrated. During the washing steps with medium, water will diffuse into the cells. Diluting the suspension slowly should reduce the loss of electrolytes, counteract extreme pH changes, and prevent denaturation of cellular proteins.
28. It appears that most cell lines grow better at higher cell concentrations than at lower concentrations. Some cell lines (for example, some BCP-cell lines) prefer a concentration higher than 1.0×10^6/mL. Usually, the optimal concentration of a cell line for expansion must be explored empirically. If after 2–3 d of culture, the cells do not grow well (perhaps because of the presence of many dead cells), it might be useful to concentrate the cells and to culture them at a higher cell density. First, resuspend the cells in medium containing 20% FBS. If the cells start to multiply and resume their expected growth activity, the percentage of FBS can be decreased stepwise.
29. General recommendations are 5–10 mL suspension into a 80-cm^2 flask or 1–2-mL suspension into each well of a 24-well plate. For "difficult" cell lines, it may be advantageous to suspend some cells in a flask and another aliquot in a 24-well plate (or even a 96-well microplate). There are distinct differences between flask and plate designations regarding exposure to CO_2, accessibility to microscopic observation, and possibilities of manipulation.
30. Before changing the medium, set the flasks upright in the incubator for at least 30 min in order to let the cells sink to the bottom of the flask. One of the advantages of plates is the fact that cells are always concentrated at the bottom of the wells.
31. There is a fundamental difference between "expansion" and "maintenance" of a cell line. Some cell lines will deteriorate over long-time culture under maintenance conditions. In such cases, it might then be better to freeze and rethaw the cells when needed.

References

1. Drexler, H. G., Matsuo, Y., and MacLeod, R. A. F. (2000) Continuous hematopoietic cell lines as model systems for leukemia-lymphoma research. *Leuk. Res.* **24,** 881–911.
2. Drexler, H. G. and Minowada, J. (2000) Human leukemia-lymphoma cell lines: Historical perspective, state of the art and future prospects, in *Human Cell Culture, Vol. III—Cancer Cell Lines Part 3: Leukemias and Lymphomas* (Masters, J. R. W. and Palsson, B. O., eds.), Kluwer Academic Publisher, Dordrecht, 1–18.
3. Drexler, H. G. (ed.) (2000) *The Leukemia-Lymphoma Cell Line FactsBook.* Academic Press, San Diego, CA.
4. Matsuo, Y. and Drexler, H. G. (1998) Establishment and characterization of human B cell precursor-leukemia cell lines. *Leuk. Res.* **22,** 567–579.
5. Drexler, H. G., Fombonne, S., Matsuo, Y., Hu, Z. B., Hamaguchi, H., and Uphoff, C.C. (2000) p53 alterations in human leukemia-lymphoma cell lines: In vitro artifact or prerequisite for cell immortalization? *Leukemia* **14,** 198–206.
6. Smith, S. D., McFall, P., Morgan, R., Link, M., Hecht, F., Cleary, M., and Sklar, J. (1989) Long-term growth of malignant thymocytes in vitro. *Blood* **73,** 2182–2187.
7. Quentmeier, H., Zaborski, M., and Drexler, H. G. (1997) The human bladder carcinoma cell line 5637 constitutively secretes functional cytokines. *Leuk. Res.* **21,** 343–350.

8. Drexler, H. G. and Matsuo, Y. (1999) Guidelines for the characterization and publication of human malignant hematopoietic cell lines. *Leukemia* **13,** 835–842.
9. Drexler, H. G. and Matsuo, Y. (2000) Malignant hematopoietic cell lines: In vitro models for the study of multiple myeloma and plasma cell leukemia. *Leuk. Res.* **24,** 681–703.
10. Drexler, H. G., Dirks, W. G., and MacLeod, R. A. F. (1999) False human hematopoietic cell lines: Cross-contaminations and misinterpretations. *Leukemia* **13,** 1601–1607.
11. Drexler, H. G., Uphoff, C. C., Dirks, W. G., and MacLeod, R. A. F. (2002) Mix-ups and mycoplasma: The enemies within. *Leuk. Res.*. **4**,329–333.
12. UKCCCR (2000) UKCCCR guidelines for the use of cell lines in cancer research. *Br. J. Cancer* **82,** 1495–1509.
13. Drexler, H. G., Dirks, W., MacLeod, R. A. F., Quentmeier, H., Steube, K. G., and Uphoff C.C. (eds.) (2001) DSMZ Catalogue of Human and Animal Cell Lines, 8th Edition, Braunschweig, Germany. http://www.dsmz.de
14. Drexler, H. G. and Uphoff, C. C. (2000) Contamination of cell cultures, mycoplasma, in *The Encyclopedia of Cell Technology* (Spier, E., Griffiths, B., and Scragg, A. H., eds.), Wiley, New York, 609–627.

13

Cell Sensitivity Assays: *Clonogenic Assay*

Jane A. Plumb

1. Introduction

The use of cell culture systems to assess the toxicity of anticancer agents began over 50 years ago following the observation of the antineoplastic effects of nitrogen mustard ***(1)***. There are a wide variety of assays designed to evaluate cellular drug sensitivity and they have been previously described in the literature. These assays essentially fall into two groups: those that measure cell survival and those that measure cytotoxicity. Cytotoxicity assays include methods such as trypan blue dye exclusion, ^{51}Cr release, and ^{3}H-thymidine incorporation ***(2–4)***. These assays assess the structural integrity and metabolic function of the cells after drug exposure. In contrast, cell-survival assays measure the end result of these effects on the cell, which can be either cell death or recovery. A cell-survival assay requires a measure of the ability of cells to proliferate and this is usually an estimate of the ability of individual cells to form colonies. However, cytotoxicity assays can also measure the ability of cells to proliferate if the cells are allowed a period of growth following drug exposure. In a clonogenic assay, this recovery time is comparable to the time for formation of colonies.

Clonogenic assays are regarded as the "gold standard" cellular-sensitivity assay. This idea originates from the early 1960s when radiobiologists were comparing the radiosensitivities of tumor cell lines in vitro. This involved estimation of multiple logs of cell kill and it was thought that only a clonogenic assay would have sufficient sensitivity to be able to assess cell kill at low percentage survivals (<1%). However, the results obtained with a cell growth assay were similar to those obtained with a clonogenic assay ***(5)***. Nevertheless, the clonogenic assay has retained its superior status ***(6)***. Many factors influence cellular drug sensitivity and no single assay can take account of all these variables.

The human tumor stem-cell assay has been widely used in attempts to predict the response of tumors to chemotherapeutic agents ***(7)***. In order to distinguish between normal and tumor cells present within a biopsy, the assay measures the ability of cells to undergo substrate independent proliferation in agar. Overall, the ability of the assay

From: *Methods in Molecular Medicine, vol. 88: Cancer Cell Culture: Methods and Protocols*
Edited by: S. P. Langdon © Humana Press Inc., Totowa, NJ

to predict treatment response is good but only about 30% of biopsies processed result in sufficient colony numbers to allow evaluation ***(8)***. This is a major difficulty with the assay and many human tumor cell lines show a poor cloning efficiency in agar (<1%), whereas the cloning efficiency on tissue culture treated plastic can be >50%. The clonogenic assay described is based on monolayer cloning. which is widely applicable to continuous cell lines.

In the standard clonogenic assay, cells in the exponential phase of growth are exposed to a cytotoxic drug. The drug exposure time depends on a number of factors. If the drug is cell cycle specific (i.e., specific for cycling as opposed to noncycling cells) a short exposure may be sufficient and this can be related to the estimated duration of exposure in the clinic. In contrast, if the drug is phase specific it may be necessary to extend the exposure period to take account of the cell doubling time. Cells are normally exposed to drug in the exponential phase of growth since the majority of cytotoxic drugs are active against cycling cells. However, the assay can be used equally well with confluent noncycling cells provided that they will reenter the cell cycle on subculture. Following drug exposure, the cells are disaggregated to form a single-cell suspension and are plated out at low density to allow colony formation. The colonies are fixed, stained, and counted. Each colony is assumed to be derived from a single cell and thus the colony count is an estimate of the number of cells that survived the drug treatment.

The number of cells within each colony depends on the number of cell doublings and can be used as an estimate of the effects, if any, of the drug on the cell doubling time. A clonogenic assay can thus discriminate between cytotoxic (cell kill) and cytostatic (decreased growth rate) effects. Since a cytostatic effect may be lost upon removal of the drug, a cytotoxicity assay based on colony formation is also described since this allows continuous drug exposure.

2. Materials

1. 6 cm Petri dishes (tissue culture grade).
2. 25 cm^2 Tissue culture flasks.
3. 30 mL Universal containers.
4. Plastic box.
5. Wash bottles.
5. Growth medium.
6. Phosphate buffered saline (PBS, Dulbecco's A).
7. 0.25% Trypsin + 1 m*M* EDTA (in PBS).
8. Methanol.
9. 1% Crystal violet (Merck).

3. Methods

3.1. A Standard Clonogenic Assay

1. Trypsinize a subconfluent monolayer culture and collect cells in growth medium containing serum. Centrifuge the suspension (200*g*, 5 min) to pellet cells and resuspend in fresh growth medium. Use a hemocytometer to count the cells and ensure that a single cell suspension is obtained (*see* **Note 1**). Dilute cells to a density of 8×10^4 cells/ mL (*see*

Note 2) in a total volume of 10 mL. Add 4 mL of culture medium to each of 9 tissue culture flasks (25 cm^2) and transfer 1 mL of the cell suspension to each flask (*see* **Note 3**). Equilibrate with CO_2 and incubate cells at 37°C for 2–3 d to ensure that cells are in the exponential phase of growth for drug addition.

2. Prepare a serial 5-fold dilution of the cytotoxic drug in growth medium to give 8 concentrations (*see* **Note 4**). Pipet 6 mL of growth medium into each of 7 Universal containers (30 mL). Prepare 10 mL of the highest concentration of the drug and transfer 1.5 mL of this solution to the first Universal container. Mix and then transfer 1.5 mL to the next Universal. Continue until the seventh Universal is reached. The concentrations should be chosen so that the highest concentration kills most of the cells and the lowest kills none of the cells (*see* **Note 5**).
3. Label the 9 flasks with a label for each of the 8 drug concentrations and a control. Remove the medium from the flasks of cells. Add 5 mL of growth medium to the control flask and 5 mL of the appropriate drug solution to the other eight. Equilibrate the flasks with CO_2 and incubate at 37°C for 24 h (*see* **Note 6**).
4. Remove the medium from the 9 flasks, add 1 mL of trypsin solution and incubate at 37°C. While waiting for the cells to detach, label the Petri dishes on the side of the base (*see* **Note 7**). Use 3 dishes for the control and for each drug concentration. When the cells have detached, add 4 mL of growth medium to each flask. Disperse the cells by repeat pipeting to give a single-cell suspension and transfer the flask contents to a 30 mL Universal container. Count the cells from the control flask only and dilute to give a density of 10^3 cells/ mL and a total volume of 4 mL (*see* **Note 8**). Follow the same dilution steps for the cell suspensions from each of the drug treated flasks (*see* **Note 9**). Transfer 1 mL of the control cell suspension to each of the 3 labeled Petri dishes. Repeat for each of the drug treatments. Finally, add 4 mL of growth medium to each Petri dish (*see* **Note 10**). Place the Petri dishes in a plastic box and incubate for 10 d in a humidified atmosphere at 37°C (*see* **Note 11**).
4. Fill a wash bottle with PBS and a second with methanol. Remove and discard the lids from the Petri dishes. Pour the medium from the Petri dish into a container for disposal and carefully add about 5 mL of PBS to wash off the remaining medium. Pour off the PBS and add about 5 mL of methanol and leave for 5 min. Repeat for all dishes. After 5 min pour off the methanol and add another 5 mL of methanol to each dish and leave for 5 min. Pour off the methanol and allow dishes to dry.
5. Add 5 mL of crystal violet to each Petri dish and leave for 5 min. Pour off the stain and rinse the dish under running tap water to remove excess dye. Invert dishes and leave to dry.
6. Count the colonies in each Petri dish (*see* **Note 12**). If the drug has a cytostatic effect this will be seen as a reduction in the size of the colonies and should be apparent to the naked eye. In this case, count the total number of colonies per dish and the number of cells per colony (*see* **Note 13**).
7. Calculate the mean colony count for each of the treatments. Divide the number of colonies in the drug treated dishes by the number of colonies in the control dishes and express as a percentage. Plot a graph of percent survival (*y*-axis) against drug concentration (*x*-axis). Results are usually expressed as the IC_{50} value which is the drug concentration required to kill 50% of the cells or, as in this assay, to reduce the number of colonies to 50% of that in the control untreated dishes (**Fig. 1**). Values for the IC_{10} and IC_{90} can be determined in the same manner. The shape of the survival curve depends on a number of factors. For a cycle specific drug and a homogeneous cell population the curve can be very steep such that only a small increment of drug is required to go from 0–100% cell

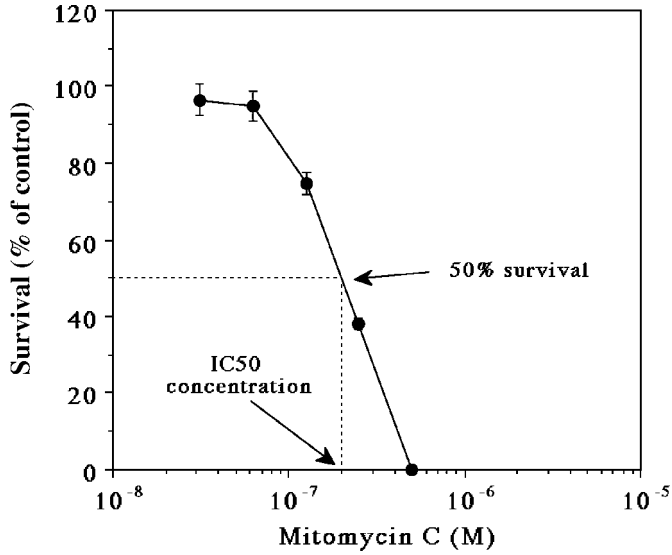

Fig. 1. A typical dose–response curve obtained by clonogenic assay. The human colon tumor cell line HT29 in exponential growth was exposed to mitomycin C for 3 h and then plated out at a density of 500 cells per 6 cm Petri dish. The mean colony count in the control dishes was 281, which is a cloning efficiency of 56%. Three flasks of cells were used at each dose level and each point is the mean ± standard error of the mean of the three estimates. Estimation of the IC_{50} value (the drug concentration required to kill 50% of the cells) is shown by the lines.

kill. Occasionally, a tail is seen on the curve such that cell kill does not reach 100% even at high drug concentrations. This can result from the presence of a resistant subpopulation. It can also occur when a phase specific drug, such as camptothecin, is used and the duration of drug exposure is less than the cell doubling time. In this case the tail should not be apparent if the drug exposure time is increased.

3.2. A Cytotoxicity Assay Based on Colony Formation

1. Trypsinize a subconfluent monolayer culture and collect cells in growth medium containing serum. Centrifuge the suspension (200*g*, 5min) to pellet cells, resuspend in growth medium and count cells. Dilute cells to a density of 10^3 cells/ mL (*see* **Note 7**). Label Petri dishes (6 cm) allowing three per treatment and add 1 mL of cell suspension to each dish. Add 3 mL of medium to the dishes and place in a plastic box. Incubate in a humidified atmosphere at 37°C for 4 h to allow cells to adhere (*see* **Note 14**).
2. Prepare a range of concentrations of the cytotoxic drug in growth medium (*see* **Notes 3** and **4**). The drug is diluted fivefold when added to the Petri dishes so these solutions should be prepared at five times the required final concentration.
3. Add 1 mL of the drug solution to the 4 mL of medium in each of the three Petri dishes. Incubate for 10 d in an humidified atmosphere at 37°C (*see* **Notes 11** and **15**).
4. Fix and stain the colonies and evaluate as for the standard clonogenic assay.

4. Notes

1. It is essential that a single-cell suspension is plated out, and it may be necessary to adjust the trypsin concentration or duration of exposure to achieve this.
2. A density of 8×10^4 cells per 25 cm^2 culture flask is a suggested density for cells with a doubling time of about 24 h and a plating efficiency of around 60%. Clearly the density may need to be increased or decreased depending on the cell line used. The aim is to obtain a subconfluent culture of cells in the exponential phase of growth for drug treatment.
3. An experimental design based on one control and eight drug treatments is a suggested starting point and it should be noted that this does not include replicates. The number of flasks that can be set up in one experiment is limited by the time required to carry out **step 4**.
4. The drug solution should be prepared just before use and should be sterile. Many cytotoxic drugs are insoluble in water. Any diluents used to solubilize the drug should be included as a separate control, usually at the highest concentration to be used. DMSO can be used and since this is self sterile it avoids the possible loss of drug as a result of binding to the filter. Most cells will tolerate up to 1% DMSO in culture medium.
5. If the cytotoxicity of the drug is not known, a serial dilution with a starting concentration of 10^{-5} *M* can be used. Once the cytotoxicity is known the drug concentration range can be reduced to cover the area of interest.
6. The drug exposure period can be varied. As a rule, cytotoxicity increases with increasing drug exposure. The most marked effects are seen during the first 24 h and sensitivity usually shows a plateau by 72 h. Factors to take into account are the mechanism of action of the drug. If it is S-phase specific, the exposure period should allow for all cells to have passed through S-phase. The stability of the drug in culture medium should also be taken into account. For drug exposure periods of greater than 24 h it is recommended that the drug is replaced at 24-h intervals.
7. Do not label the lids because these are removed when the colonies are fixed. Make sure that the marker pen used is resistant to methanol.
8. Accuracy of the dilution is important and it is recommended that individual dilution steps are no greater than 1 in 10 and that the volume of cell suspension used is greater than 200 µL. A density of 10^3 cells/ mL is a suggested density assuming a plating efficiency for the cell line of about 50%. This would yield 500 colonies in the control dishes. The aim is to retain separated colonies in the control dishes at the end of the experiment but to still have a sufficient number of colonies in the drug treated dishes to allow accurate estimation of survival at the higher drug concentrations. It is possible to compensate for the low survival at higher drug concentrations by increasing the number of cells plated out for these concentrations. To do this, either increase the volume of cell suspension used or reduce the dilution factor.
9. It is not necessary to count the cells in the drug-treated flasks since all flasks contained the same number of cells at the start of the experiment. Any difference in cell counts between the flasks at this stage is a result of the effects of the drug and is thus part of the experiment. Remember to resuspend the cells well before diluting.
10. Care must be taken to ensure an even distribution of cells in the Petri dish. This is achieved if the cells are added first and then the bulk of the medium added. Do not swirl the dishes to mix the cells because this results in the cells accumulating in the center of the dish and forming one large colony.
11. The incubation time will vary depending on the doubling time of the cell line used but is usually between 8–12 d. This allows for about 10 doubling times. It is advisable to check the dishes after about 8 d and colonies should be clearly visible to the naked eye.

12. Following drug treatment some cells will plate and undergo a few cell divisions before the damage is expressed. This leads to the formation of small colonies that fail to develop further. These cells are not viable and the colonies should not be counted. Limit the counts to those colonies that have undergone more that 5 cell doublings, i.e., those containing more than 50 cells. Colonies can be counted by sight and this is easiest if colonies are marked as they are counted. A colony-counting pen is available from Camlab (Cambridge, UK) that marks and counts colonies. Alternatively, an imaging system such as the Protos from Synoptics (Don Whitley Scientific, Shipley, UK) can be calibrated to count colonies.
13. A cytostatic effect will result in a reduction in the number of cell doublings in a given time and thus a reduction in the number of cells within a colony. There are several ways of quantifying a cytostatic effect. The most direct method is to count the number of cells in 50 representative colonies per dish. Alternatively, it can be estimated by measuring the diameter of the colony and thus calculating the area of the colony. This method assumes that there is no change in cell size.
14. For most continuous cell lines, 4 h is sufficient for adherence to plastic. The time can be increased but it should not exceed the doubling time for the cell line since the assay relies on colonies originating from single cells. In some protocols, the cells and drug are added together. The disadvantage of this approach is that the drug may have an effect on the plating of the cells independent from effects on cell survival.
15. This assay protocol is best suited to continuous drug exposure but it is possible to limit the drug exposure time by replacing the medium. However, as explained in **Note 14** the total time for plating and drug exposure should not exceed the doubling time for the cell line.

References

1. Dendy, P. P. and Hill, B. T. (eds.) (1983) *Human Tumour Drug Sensitivity Testing in Vitro: Techniques and Clinical Applications.* Academic Press, London.
2. Wilson, A. P., Ford, C. H. J., Newman, C. E., and Howell, A. (1984) A comparison of three assays used for the in vitro chemosensitivity testing of human tumours. *Br. J. Cancer*, **49,** 57–63.
3. Weisenthal, L. M. (1981) In vitro assays in preclinical antineoplastic drug screening. *Semin. Oncol.* **8,** 362–376.
4. Weisenthal, L. M., Dill, P. L., Kurnick, N. B., and Lippman, M. E. (1983) Comparison of dye exclusion assays with a clonogenic assay in the determination of drug-induced cytotoxicity. *Cancer Res.* **43,** 258–264.
5. Nias, A. H. W. and Fox, M. (1968) Minimum clone size for estimating normal reproductive activity of cultured cells. *Br. J. Radiol.* **41,** 468–474.
6. Weisenthal, L. M. and Lippman, M. E. (1985) Clonogenic and nonclonogenic in vitro chemosensitivity assays. *Cancer Treat. Rep.* **69,** 615–632.
7. Salmon, S. E. (1980) *Cloning of Human Tumour Stem Cells.* Liss, New York.
8. Von Hoff, D. D. (1990) He's not going to talk about in vitro predictive assays again, is he? *J. Natl.Cancer Inst.* **82,** 96–101.

14

Cell Sensitivity Assays: *The MTT Assay*

Jane A. Plumb

1. Introduction

The clonogenic assay described in the Chapter 13 is not suitable for all cell lines. Many adherent cell lines do not form colonies, and clearly it is not applicable to nonadherent cell lines. Furthermore, it is slow and time consuming. This chapter describes an alternative cytotoxicity assay that has a number of advantages when compared with a clonogenic assay. It is quick and easy and allows a large number of assays to be carried out in one batch. This is an important consideration when making comparisons between cell lines, between cytotoxic agents, or when evaluating combinations of drugs. No one cytotoxicity assay is ideal, and it is always advisable to support results with those obtained from alternative assays where possible.

Cytotoxicity assays are widely used especially in the field of new drug development. Clonogenic assays are not amenable to automation and centers such as the American National Cancer Institute (NCI) as well as the pharmaceutical industry have developed rapid-throughput microtitration assays to screen new cytotoxic agents. These assays measure the effect of a drug on the growth of a population of cells and the endpoint is an estimate of cell number. Use of a tetrazolium dye (MTT) as an indirect measure of cell number was first reported in the early 1980s ***(1)***. The NCI evaluated MTT dye reduction as a possible endpoint in a rapid screening assay ***(2)***, and this stimulated interest in the wider scientific community. At that time a major limitation of microtiter assays was that the endpoint tended to involve the use of a radioisotope.

The basis of the assay is as follows. Cells in the exponential phase of growth are exposed to a cytotoxic drug. The duration of exposure is usually determined as the time required for maximal damage to occur but is also influenced by the stability of the drug. After removal of drug, the cells are allowed to proliferate for two to three doubling times in order to distinguish between cells that remain viable and are capable of proliferation and those that remain viable but cannot proliferate. Surviving cell numbers are then determined indirectly by MTT dye reduction. MTT is a yellow water-soluble tetrazolium dye that is reduced by live cells ***(3)*** to a purple formazan product that is insoluble in aqueous solutions. The amount of MTT-formazan pro-

From: *Methods in Molecular Medicine, vol. 88: Cancer Cell Culture: Methods and Protocols*
Edited by: S. P. Langdon © Humana Press Inc., Totowa, NJ

duced can be determined spectrophotometrically once solubilized in a suitable solvent. A drawback of the assay is that it does not distinguish between a cytotoxic (cell kill) and a cytostatic (reduced growth rate) effect.

There is no such thing as "The MTT Assay." Many laboratories use cytotoxicity assays that are based on MTT dye reduction but the assay protocols differ markedly. Many factors affect the reduction of MTT. The cells require an adequate energy supply from the culture medium, and reduction is inhibited by some cytotoxic drugs. For these reasons the NCI chose not to use MTT reduction in their screening assay ***(4)***. The assay described here has been optimized for a number of cytotoxic drugs to give the same results as are obtained with a standard clonogenic assay ***(5)***. It is not suitable for the estimation of survival following X-irradiation since the assay will not exclude cells that show late radiation damage. These cells can undergo two or three divisions before damage is expressed. They would not be regarded as surviving cells in a clonogenic assay because the colonies would contain less than 50 cells. An important feature of the assay is the inclusion of a growth period after removal of the drug. This allows cells to recover from the effects of the drug, or to die, and also avoids possible interference of drug in the reduction of MTT.

2. Materials

1. Microtiter plates (Iwaki from Becton Dickinson).
2. Multichannel pipet (Costar).
3. Tip box, autoclavable (ICN Flow).
4. Pipet tips (ICN-Flow).
5. 18-G, 24-G Hypodermic needles.
6. 5-cm and 10-cm Petri dishes (Sterilin).
7. 30-mL and 100-mL Universal containers (Sterilin).
8. Plastic box.
9. Growth medium.
10. Phosphate buffered saline (PBS, Dulbecco's A).
11. 0.25% Trypsin +1 m*M* EDTA in PBS.
12. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) prepared at a concentration of 5 mg/mL in PBS. This is filter sterilized, which also removes any reduced MTT, and can be stored at 4°C in the dark for 1 mo.
13 Sorensen's glycine buffer: 0.1 *M* glycine, 0.1 *M* NaCl adjusted to, pH 10.5, with 1 *M* NaOH.
14. Dimethylsulfoxide (DMSO).
15. ELIZA plate reader (Molecular Devices, Wokingham, UK).
16. Plate carriers for centrifuge (for nonadherent cell lines).

3. Methods

3.1. Adherent Cell Lines

1. Trypsinize a subconfluent monolayer culture and collect cells in growth medium containing serum. Centrifuge the suspension (200*g*, 5 min) to pellet cells, resuspend in growth medium, and count cells. Dilute cells to a density of 5×10^3 cells/mL (*see* **Note 1**) allowing 60 mL of cell suspension for 3 microtiter plates. Transfer cell suspension to a 10-cm Petri dish and with a multichannel pipet add 200 µL to each well of the central 10 columns

of a flat-bottomed 96-well plate (80 wells per plate) starting with column 2 and ending with column 11 (*see* **Note 2**). Add 200 µL of growth medium to the 8 wells in columns 1 and 12. Put plates in a plastic box and incubate in a humidified atmosphere at 37°C for 2–3 d to ensure that cells are in the exponential phase of growth for drug addition.

2. Prepare a serial fivefold dilution of the cytotoxic drug in growth medium to give 8 concentrations (*see* **Note 3**). Pipet 6 mL of growth medium in to each of 7 Universal containers (30 mL). Prepare 10 mL of the highest concentration of the drug and transfer 1.5 mL of this solution to the first Universal container. Mix and then transfer 1.5 mL to the next Universal. Continue until the seventh Universal is reached. The concentrations should be chosen so that the highest concentration kills most of the cells and the lowest kills none of the cells (*see* **Note 4**). Normally, three plates are used for each drug to give triplicate determinations within one experiment.
3. Remove the medium from all the wells in columns 2 to 11. This can be achieved with a hypodermic needle attached to a suction line. Cells in the 8 wells in columns 2 and 11 are fed with 200 µL of fresh growth medium. Transfer the drug solutions to 10-cm Petri dishes and add 200 µL to all 8 wells of a column. For ease of analysis, arrange the drug solutions in order so that the highest is in column 3 down to the lowest in column 10. Plates are returned to the plastic box and incubated for 24 h (*see* **Note 5**).
4. At the end of the drug exposure period, remove the medium from all wells containing cells and feed the cells with 200 µL of fresh medium.
5. Feed plates daily for two more days.
6. Feed plates with 200 µL of fresh medium and add 50 µL of the MTT solution (5 mg/mL in PBS) to all wells in columns 1 to 11. Wrap the plates in aluminium foil and incubate for 4 h in a humidified atmosphere at 37°C (*see* **Note 6**).
7. Remove the medium and MTT from the wells and dissolve the purple MTT-formazan crystals by adding 200 µL of DMSO to all wells in columns 1 to 11 (*see* **Note 7**). Add 25 µL glycine buffer (per well) to all wells containing DMSO (*see* **Note 8**).
8. Record absorbance at 570 nm with the wells in column 1 that contain medium, MTT, but no cells used as a blank.
9. A graph is plotted of absorbance (*y*-axis) against drug concentration (*x*-axis). The mean absorbance reading from the wells in columns 2 and 11 is used as the control absorbance and the IC_{50} concentration is determined as the drug concentration required to reduce the absorbance to half that of the control. IC_{10} or IC_{90} values can be determined in the same manner (*see* **Note 9**).

3.2. Nonadherent Cells

1. Prepare a cell suspension in fresh growth medium from a flask of cells that are in the exponential growth phase and count the cells. Dilute cells to a density of 10^4 cells/mL (*see* **Note 1**) allowing 30 mL of cell suspension for 3 plates. Transfer the cell suspension to a 10-cm Petri dish and with a multichannel pipet add 100 µL to the central 10 columns of a round-bottomed microtiter plate. Add 200 µL of growth medium to the wells in columns 1 and 12. Put plates in a plastic box and incubate in a humidified atmosphere at 37°C while the drug solutions are prepared.
2. Prepare the eight drug dilutions essentially the same as for adherent cells except prepare the solutions at twice the desired final concentration.
3. Add 100 µL growth medium to the wells in columns 2 and 11. Add the drug solutions as for the adherent cells but only 100 µL is added to the 100 µL of cells already in the wells. Place the plates in the plastic box and incubate for 24 h.

4. Centrifuge (200*g*, 5 min) the plates to pellet cells before removal of the medium. A fine-gage needle (24G) is recommended to prevent removal of the cell pellet. Feed the cells with 200 µL of growth medium.
5. Plates are processed as for the adherent cells except that they are centrifuged each time the medium is removed.

4. Notes

1. A cell density of 10^3 cells per well is suggested as a starting point and is usually suitable for cell lines with a doubling time of around 24 h. The aim is for the cells to remain in exponential growth throughout the assay. If cells in the control wells (i.e., those not exposed to drug) become confluent they will stop dividing, while the drug-treated cells will continue to grow. As a result, drug sensitivity will be underestimated since the control absorbance will be lower than it should be. The initial cell density can range between 5×10^2 to 10^4 cells per well depending on the cell line. An easy way to determine the optimum density is to plate out cells at a range of densities in a microtiter plate and feed them daily for the duration of the assay. Incubate the plates with MTT and select the density that gives an absorbance value of approx 1. Remember to prepare a cell suspension at 5 times the density required per well since only 200 µL is added to the well (10 times for nonadherent cells).
2. Microtiter plates vary in their overall plating efficiency and in the variability of plating between wells. The assay requires that all wells are replicates, and the choice of plates is therefore a compromise between the optimum overall plating efficiency and the reproducibility between wells. A detailed statistical evaluation of microtiter plates showed the Iwaki range (obtained from Beckton Dickinson) to be suitable. Furthermore, these plates do not demonstrate the so called "edge effect," in which plating is noticeably reduced in the outer wells. Cells are not plated in columns 1 and 12 because these are used as a blank for the plate reader to allow for the absorbance of the residual medium and MTT in the wells.
3. The drug solution should be prepared just before use and should be sterile. Many cytotoxic drug are insoluble in water. A diluent used to solubilize the drug should be included as a separate control, usually at the highest concentration to be used, and added in medium to the cells in column 11. DMSO can be used and since this is self sterile it avoids possible loss of drug as a result of binding to the filter. Most cells will tolerate up to 1% DMSO in culture medium.
4. If the cytotoxicity of the drug is not known, a serial dilution with a starting concentration of 10^{-5} *M* can be used. Once the cytotoxicity is known, the drug concentration range can be reduced to cover the area of interest.
5. The drug-exposure period can be varied. As a rule, cytotoxicity increases with increasing drug exposure. The most marked effects are seen during the first 24 h and sensitivity usually shows a plateau by 72 h. The mechanism of action of the drug, for example, if it is S-phase specific, the exposure period should allow for all cells to have passed through S-phase. The stability of the drug in culture medium should also be taken into account. For drug exposure periods of greater than 24 h it is recommended that the drug be replaced at 24 h intervals. Regardless of the drug exposure period, the cells must be allowed to undergo two to three cell doubling times after removal of the drug. It is not necessary to use eight wells for each drug concentration. Reproducible results can be obtained from four wells per concentration and it is practical to divide the plate so that one drug is used in rows A–D and a second in rows E–H.

6. MTT supplied by Sigma has a variable amount of the reduced formazan present. Formazan is insoluble in PBS and can be filtered out when the solution is prepared. The solution can be stored, but if it is not sterile or is exposed to light, the MTT will undergo reduction. Check that the solution is bright yellow and if not refilter. Plates are wrapped in foil to minimize the reduction of MTT by light. The 4-h incubation period is the time required for maximal MTT reduction. For convenience, plates can be incubated for up to 6 h without loss of accuracy.
7. DMSO can damage some multichannel pipets. Costar pipets are essentially resistant but DMSO should still be dispensed with caution. Alternatively the Labsystems Microplate Dispenser (ThermoQuest; cat. no. 5840 127) can be used to dispense both DMSO and glycine buffer.
8. MTT reduction is used to estimate cell numbers at the end of the assay. The validity of the endpoint depends on a linear relationship between MTT reduction and cell number. The absorption spectrum of MTT-formazan is pH-dependent and the pH of the MTT-formazan solution in DMSO depends on the cell density in the well. Glycine buffer is added to shift the pH of all wells to, pH 10.5. At this pH, the spectrum shows a single peak with an absorption maximum at about 570 nm. In contrast, at pH 7, the spectrum shows two absorption maxima, at 500 nm and 570 nm, and measurement at a single wavelength underestimated the amount of MTT-formazan present.
9. Microtiter plate readers were developed for ELISA techniques and this is reflected in the computer software available. The E_{max} reader (Molecular Devices, Wokingham, UK) is recommended since the software (Softmax PRO) was developed for diverse applications and contains a curve-fitting facility that is ideal for analysis of survival curves.

References

1. Mosman, T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J.Immunol. Methods,* **65,** 55–63.
2. Alley, M. C., Scudiero, D. A., Monks, A., et al. (1988) Feasibility of drug screening with panels of human tumour cell lines using a microculture tetrazolium assay. *Cancer Res.*, **48,** 589–601.
3. Liu, Y., Peterson, D. A., Kimura, H., and Schubert, D. (1997) Mechanism of cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction. *J. Neurochem***. 69,** 581–593.
4. Vistica, D. T., Skehan, P., Scudiero, D., Monks, A., Pittman, A., and Boyd, M. R. (1991) Tetrazolium-based assays for cellular viability: A critical examination of selected parameters affecting formazan production. *Cancer Res.*, **51,** 2515–2520.
5. Plumb, J. A., Milroy, R., and Kaye, S. B. (1989) Effects of the pH dependence of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-formazan absorption on chemosensitivity determined by a novel tetrazolium-based assay. *Cancer Res.*, **49,** 4435–4440.

15

PARP Cleavage as a Means of Assessing Apoptosis

Peter Mullen

1. Introduction

Although cell death is often seen as a pathological process, there are classically two types of cell death in biological systems, namely necrosis (accidental cell death) and apoptosis (programmed cell death). Although it was recognized from the early fifties that cell death was a natural process in normal tissue development and embryogenesis ***(1,2)***, the term apoptosis was first used by Wyllie et al. to describe a number of molecular and morphological processes in which individual cells actively undergo a programmed, genetically-regulated cell death ***(3–5)***. The term, derived from a Greek word meaning "the dropping off of leaves from trees," was thus introduced to distinguish this mode of death from the more familiar process of necrosis and to emphasize its physiological role (programmed cell death and apoptosis are often used interchangeably).

Apoptosis is a normal physiological process involving many genes and requires the active consumption of energy in the form of ATP to safely dispose of cells once they have fulfilled their intended biological role. It is of widespread biological significance and is critical for normal development where cell proliferation and cell death must be balanced to maintain tissue homeostasis. As well as being involved in both normal cell turnover and the removal of abnormal cells ***(4)***, it is crucial in determining tissue size and shape during embryogenesis, metamorphosis, and differentiation. It is also particularly important for the regulation and function of the immune system. Abnormal functioning or inappropriate regulation of the apoptotic processes can contribute to a variety of pathological conditions with reduced apoptosis being associated with cancer, autoimmunity, immunodeficiency, and viral infections. Enhanced apoptosis can be seen in ischemic heart disease and stroke, neurodegenerative disease, sepsis, and multiple organ dysfunction syndrome ***(6,7)***.

Apoptotic cell death can be induced by a variety of stimuli, including ligation of cell surface receptors, serum deprivation, growth factor deprivation, heat shock, hypoxia, exposure to ultraviolet radiation, DNA damage, viral infection, and exposure to dexamethasone and cytotoxic or chemotherapeutic agents. The process is characterized by a number of stereotypical morphological features ***(8)*** whereby the cell first shrinks and

From: *Methods in Molecular Medicine, vol. 88: Cancer Cell Culture: Methods and Protocols*
Edited by: S. P. Langdon © Humana Press Inc., Totowa, NJ

deforms. After becoming detached from its neighbors it then undergoes chromatin condensation and internucleosomal cleavage of the DNA before fragmenting into compact membrane-enclosed structures termed "apoptotic bodies." These apoptotic bodies are then engulfed by macrophages before elimination from the tissue with no significant inflammatory damage to surrounding cells. This is in contrast to necrosis, where cells suffer a major insult resulting in the loss of membrane integrity, swelling, and then rupture, after which the contents of the cell are released in an uncontrolled manner into the cell's environment, resulting in further damage to surrounding cells and a strong inflammatory response in the tissue. Although apoptosis and necrosis are considered conceptually distinct forms of cell death, cells exposed to physical, biochemical, or biological injury activate a common series of stress-response genes regardless of how they are elicited. Cells suffering only minimal insults will often recover while those suffering greater damage will succumb to individual cell death or apoptosis with the cell dying before being recycled. If the insult overwhelms a large number of cells then necrosis will occur. Because both apoptosis and necrosis can occur simultaneously in tissues or cell cultures exposed to the same stimulus, the intensity of the same initial insult will often decide the prevalence of either apoptosis or necrosis. There is also increasing evidence that the processes of apoptosis and necrosis represent only the extreme ends of a wide range of possible morphological and biochemical deaths.

Signaling for apoptosis occurs through a limited number of independent pathways via extracellular signals and intracellular mediators that are initiated from triggering events from either within or outside the cell ***(9)***. Both intrinsic and extrinsic pathways for the activation of apoptosis are known to occur with cross-talk between the two pathways taking place. Although specific death signals can act through death ligands/receptors and their respective intracellular signaling pathways, all apoptosis signaling pathways converge on a common machinery of cell destruction that is activated by a family of cystein proteases known as caspases ***(10)***. Although the initiation of cell death involves a number of signaling systems, the activation of caspases has a key role in the progression towards the final morphological changes that take place ***(11)***. Fourteen such mammalian caspases have been identified, three of which (caspase-3, -6, and -7) are thought to coordinate the late execution phase of apoptosis by cleaving the aspartate residues of various structural and repair proteins ***(12,13)***. Cleavage of chromosomal DNA into nucleosomal units is accomplished in a number of ways following activation of such caspases. Control of this process requires the functioning of subcellular organelles (particularly the mitochondria) and involves members of the BH3-containing proapoptotic (for example, *Bax*) or antiapoptotic (for example, *Bclxl*) subfamilies of the *Bcl2* gene family.

The enzyme poly(ADP-ribose) polymerase, or PARP, whose expression is triggered by DNA-strand breaks, was the first such protein identified as a substrate for caspases ***(14)***. PARP is involved in DNA damage repair and functions both by catalyzing the synthesis of poly(ADP-ribose) and by binding to DNA-strand breaks and modifying nuclear proteins. In cells undergoing apoptosis, it is cleaved from a full-length 116 kD peptide into 89 kD and 24 kD polypeptides by caspase-3 during the degradation of cellular DNA, thus preventing DNA damage repair (*see* **Note 1; Fig. 1**). DNA topoi-

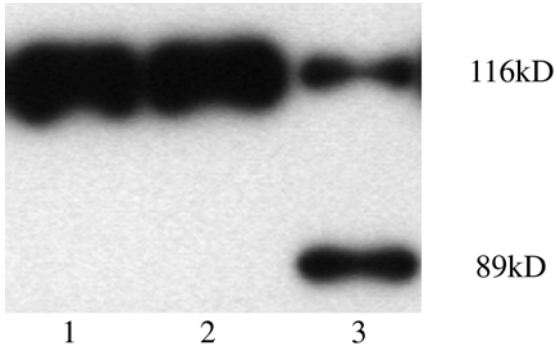

Fig. 1. Western blot illustrating SKOV-3 ovarian cancer cells treated with c-Raf antisense oligonucleotide (lane 3) and c-Raf mismatch oligonucleotide (lane 2). Untreated control cells are shown in lane 1. The three upper bands represent the full length 116 kD product while the lower band (lane 3) represents the 89 kD cleaved PARP product associated with apoptosis. No such band was seen in control cells (lane 1) or cells treated with mismatch oligonucleotide (lane 2).

somerase II, which is a nuclear enzyme essential for DNA replication and repair, is then also inactivated, resulting in further DNA damage. The first stage of the apoptotic process thus involves inactivating PARP on extensively fragmented DNA and consequently preventing DNA repair processes. The breakdown of structural nuclear proteins, such as lamins, by caspase-6 then gives rise to the chromatin condensation commonly observed in apoptotic cells. Subsequent fragmentation of DNA into the typical nucleosomal DNA ladder is then brought about by an enzyme known as CAD, or caspase activated DNase. CAD usually exists as an inactive complex along with the inhibitor of CAD (ICAD), which is itself cleaved by caspases, including caspase-3, to release active CAD during apoptosis. Such PARP cleavage prevents induction of necrosis during apoptosis and ensures appropriate execution of caspase-mediated cell death ***(15)***. The activation of CAD prior to the cleavage of PARP after DNA fragmentation results in a catastrophic consumption of nicotinamide adenine dinucleotide (NAD)+ energy ***(16,17)***. This consumption of energy can disrupt the ordered completion of the apoptotic process, alternatively diverting the cell to a necrotic fate with resulting tissue damage.

Apoptotic cell death can be measured in a number of ways although no single assay is suited to all cell models. Determination of apoptosis levels should therefore ideally be made using more than one method. Assessment of apoptosis can be carried out by a variety of methods, including the analysis of cells stained for DNA content, the TUNEL assay, annexin labeling, or the PARP cleavage assay. It should, however, be pointed out that morphological changes visualized by electron microscopy would be regarded as the definitive method of confirming the presence of apoptosis. The first and simplest method of assessing apoptosis involves staining cells for total DNA content using propidium iodide to give typical G_0/G_1, S, and G_2/M cell cycle distribution. A sub-G_0/G_1 peak is taken to be indicative of enhanced apoptosis, although

necrotic cells or cell debris with mitochondrial DNA can also stain positively and present as an undefined sub-G_1 region. As an assay of apoptosis, the technique therefore has limited value. The TUNEL assay relies on strand breaks within the DNA brought about by the characteristic DNA cleavage taking place during apoptosis. The enzyme terminal deoxynucleotidyl transferase (TdT) is used to add biotinylated, BrdU, or digoxygenin-labeled nucleotides to these strand breaks, and they can in turn be detected using a fluorochrome-labeled streptavidin conjugate, an anti-BrdU antibody, or a fluorochrome-labeled antidigoxygenin antibody, respectively. The method is applicable to all cell types, is quantitative, and has the advantage that apoptosis can be directly correlated with cell-cycle kinetics. It is, however, technically more demanding as well as being relatively more expensive. Annexin-V labeling measures translocation, or "flipping," of phosphatidylserine (PS) from the inside to the outside of the cell membrane during the early stages of apoptosis and requires Ca^{++} for binding. Detection is made possible using fluorescein isothiocyanate (FITC)-labeled annexin-V, a Ca^{++} dependent phospholipid-binding protein. The assay utilizes propidium iodide exclusion as a membrane integrity dye because nonviable, necrotic cells will be leaky and hence take up the DNA dye, whereas viable cells will exclude low concentrations of propidium iodide. Dual-staining with FITC-conjugated annexin-V thus allows discrimination of healthy viable cells, early apoptotic cells, and late apoptotic/necrotic cells. This useful and informative method is particularly suited to nonadherent cells; it can be equally useful when studying adherent cell systems, although the mechanical or enzymatic detachment of cells can give artificially high values, particularly if the membranes are compromised during the harvesting procedure. Finally, cleaved PARP in the form of 89 kD and 24 kD polypeptides derived from the 116 kD full length peptide during the degradation of cellular DNA can be measured by Western blotting combined with enhanced chemiluminescence (ECL) detection. Essentially, floating and adherent cells are pooled, washed in PBS, sonicated on ice in a volume of loading buffer proportional to the cell count, and then probed with one of the many commercially available PARP antibodies. The specific rate of change in PARP activity depends on a number of factors, including cell type, method of induction of DNA damage or apoptosis, as well as culture conditions. Specific proteolytic cleavage of PARP has nevertheless been shown to be a reliable marker of apoptosis in a wide variety of cell lines. The anti-PARP antibody C2-10 used in this study recognizes both full length PARP and the larger of the two cleavage products (89 kD). In contrast to the annexin-V assay (which measures early-stage events in apoptosis), this methodology specifically looks at late-stage apoptosis after the activation of caspase-3. Although the induction of apoptosis can be achieved through a variety of methods such as the use of glucocorticoids ***(18)***, we will illustrate the use of the PARP cleavage assay by inducing apoptosis using an antisense oligonucleotide to c-Raf1 since we have an interest in antisense therapy ***(19)***.

2. Materials

2.1. Cell Culture and Induction of Apoptosis

1. SKOV-3 ovarian cancer cells (ECACC, Porton Down, Wiltshire, UK).
2. RPMI 1640 culture media (Gibco-BRL, Life Technologies, Paisley, Scotland). Add penicillin/streptomycin (Gibco-BRL, Life Technologies) to final concentrations of 100 U/mL

and 100 µg/mL, respectively. Add 10% fetal calf serum (FCS; Advanced Protein Products, Brierley Hill, England) (*see* **Note 2**).
3. Optimem culture media (Gibco-BRL, Life Technologies).
4. Lipofectin (Gibco-BRL, Life Technologies).
5. Phosphate buffered saline (PBS) (Oxoid, Basingstoke, Hampshire, UK).
6. 75-cm^2 Culture flasks (Life Technologies).
7. Apoptosis induction agent, for example, cRaf antisense oligonucleotide sequence TCCCGCCTGTGACATGCATT (ISIS Pharmaceuticals, Carlsbad, CA).

2.2. Preparation of Cell Lysate

1. 1X Trypsin-EDTA (Gibco-BRL).
2. Gel loading buffer: 62.5 m*M* Tris-HCl, pH 6.8, 6 *M* urea, 10% glycerol, 2% SDS and 0.00125% bromophenol blue in 20 mL distilled water. Dispense into 2-mL aliquots and freeze at –20°C. Fresh β-mercaptoethanol (50 µL/mL) should be added just before use.
3. Sonicator (Decon Ultrasonics, Hove Sussex, UK).

2.3. SDS-Polyacrylamide Gel Electrophoresis and Western Blot Analysis

1. Protean II Electrophoresis Tank Apparatus and power pack (Bio-Rad Laboratories, Hercules, CA).
2. 30% Acrylamide/Bisacrylamide (37.5/1) (Severn Biotech, Kidderminster, UK).
3. Ammonium persulfate, TEMED (*N,N,N′,N′*-tetramethylethylenediamine), isopropylalcohol, ethanol, Whatman No. 1 filter paper.
4. 1 *M* Tris-HCl, pH 8.85; 0.375 *M* Tris-HCl, pH 6.8.
5. Molecular weight markers.
6. Vaseline.
7. 10% Sodium dodecylsulfate (SDS): 100 g in 1 L distilled water. Store at room temperature (*see* **Note 3**).
8. Electrode buffer: 9.09 g Tris-HCl base, 43.26 g glycine, 30 mL 10% SDS in 3 L distilled water.

2.4. Transfer Onto Nitrocellulose Membranes

1. Transfer Tank Apparatus and power pack (Bio-Rad Laboratories).
2. Transfer buffer: 9.09 g Tris-HCl base, 43.26 g glycine in 3 L distilled water.
3. Immobilon P transfer membranes (Millipore, Bedford, MA; no. IPVH304FO).
4. Methanol (BDH, Poole, Dorset, UK).
5. Whatman No. 1 filter paper (Whatman International, Maidstone, Kent, UK).

2.5. Antibody Detection and ECL Visualization

1. PBS/Tween: 3 mL Tween-20 in 3 L PBS (Bio-Rad Laboratories).
2. Marvel dried skimmed milk powder (Premier Brands UK, Spalding, Lincs, UK).
3. Anti-PARP primary antibody C2-10 (R&D Systems Europe, Abingdon, UK; no. 4338-MC) (*see* **Note 4**).
4. Goat antimouse IgG-HRP 2 antibody (Autogen Bioclear UK, Calne, Wilts, UK; sc-2031).
5. ECL detection kit (Roche Diagnostics, Mannheim, Germany; 1520709).
6. Developer/Fixer (Agfa-Gevaert NV, Mortsel, Belguim).
7. Hyperfilm ECL photographic film (Amersham Pharmacia, Little Chalfont, Bucks, UK).
8. Darkroom cassette, shaking platform, plastic lunchboxes with lids, acetate sheets.

3. Methods

3.1. Cell Culture and Induction of Apoptosis

1. Suspend 2.5 $\times 10^5$ SKOV-3 ovarian cancer cells in 25 mL RPMI/PS/10%FCS and transfer to a 75-cm^2tissue culture flask (one flask for each experimental condition). Place in a 5% CO_2 humidified incubator at 37°C and allow cells to grow for 48–72 h.
2. Remove Optimem culture media from the fridge and place in a water bath at 37°C.
3. Add 60 µL lipofectin to a sterile beaker containing 10 mL of Optimem (final concentration, 6 µL/mL) and incubate at 37°C for 15 min (*see* **Note 5**).
4. In the meantime, mix a working concentration of 5 n*M* antisense oligonucleotide by diluting 5 µL of stock antisense oligonucleotides (0.5 µ*M*) into 500 µL of lipofectin/Optimem. Place on ice.
5. Remove culture media from tissue culture flasks and wash the cells in 10 mL PBS, removing any excess with a pastette.
6. Add the pre-incubated 10 mL Optimem/lipofectin to each flask, followed by antisense oligunucleotide (400 µL/10 mL; final concentration 200 n*M*). Place back in the incubator for 3 h.
7. Remove the antisense oligonucleotides, wash the flasks with PBS, and then replenish with fresh RPMI/PS/10% FCS (25 mL). Leave for 48 h.

3.2. Preparation of Cell Lysates

1. Remove spent media from the flasks and transfer to labeled 50-mL conical tubes. *Do not discard*.
2. Wash cells with 10 mL PBS and discard. Add 3 mL trypsin/EDTA to each flask and replace in the incubator until the cells have become detached *see* **Note 6**).
3. Transfer the trypsinized cell suspensions (3 mL) to the tubes containing their respective media (*see* **Subheading 3.2.1.**). Wash the flask with 10 mL of fresh media and combine with both the spent media and the cell suspension (total volume 38 mL).
4. Centrifuge at 1800*g* for 5 min and then pour off the media. Resuspend the pellet in RPMI/PS/10% FCS (2 mL). Syringe with a pastette to break up the pellet and then count a 200-µL aliquot.
5. Transfer 0.8 $\times 10^6$ to fresh Universal containers and then spin at 500*g* for 5 min at 4°C.
6. Wash the pellet in ice cold PBS and then again centrifuge at 500*g* for 5 min at 4°C. Discard supernatant.
7. Remove an aliquot of gel loading buffer from the freezer and add β-mercaptoethanol (50 µL/mL) immediately prior to use. Resuspend cells in gel loading buffer (200 µL per sample) using a 1-mL pipet. Gently draw the sample up and down inside the pipet until it becomes viscous as a result of the release of DNA during lysis. Transfer to 1.5-mL microcentrifuge tubes.
8. Sonicate the samples on ice for 10 seconds (s), rest on ice for 10 s, and then resonicate for a further 10 s. Store at –20°C prior to Western blot analysis.

3.3. SDS-Polyacrylamide Gel Electrophoresis and Western Blot Analysis

1. Clean the glass plates of the gel apparatus using ethanol in order to remove any dirt or grease. Assemble the plates in the casting stand as per the manufacturer's instructions.
2. Make up sufficient resolving gel (10% SDS-PAGE) for the volume between the plates

(1.5 × 14 × 16 cm) by adding 16 mL of 30% acrylamide/bisacrylamide, 15 mL 1 *M* Tris-HCl, pH 8.85, 0.4 mL 10% SDS, 8.6 mL distilled water (*see* **Note 7**).

3. Add 100 µL TEMED and 100 µL AMPS (2-acrylamido-2-methyl-1-propanesulfonic acid) in order to initiate polymerization of the gel (*see* **Note 8**).
4. Pour the resolving gel between the two glass plates up to the level indicated on the apparatus, taking care not to spill it. Immediately afterwards, drop isopropyl alcohol between the plates to form a layer of about 1–2 mm on top of the gel. This will not only ensure that the surface of the gel is smooth and even, but prevent it drying out. Leave at room temperature to polymerize.
5. When the gel has set, tip the casting stand over the sink, and pour away the isopropyl alcohol. Gently wash with distilled water between the top of the two plates and again remove excess moisture from the top of the gel by tipping the casting stand on its side and blotting dry with filter paper, being careful not to touch the gel (*see* **Note 9**).
6. Make stacking gel by adding 3.6 mL 30% acrylamide/bisacrylamide, 10 mL 0.375 *M* Tris-HCl, pH 6.8, 0.3 mL 10% SDS, 16 mL distilled water, 0.1 mL AMPS, and 0.1 mL TEMED, adding TEMED and AMPS last. Pour the stacking gel on top of the resolving gel but stop approx 1 cm from the top of the plates. Starting at one end, insert the comb of choice between the plates and into the gel, and then firmly push it down until it rests horizontally on the outer glass plate. Continue to fill the gel to within approx 2 mm from the top of the plates (otherwise the wells will become fused together when the comb is removed). Gently tap the top of the comb and use a fine-tipped pastette to remove any air bubbles or excess acrylamide. Allow the gel to set. It may be helpful at this stage to draw a line at the bottom of each well with a marker pen to facilitate loading of the gel.
7. Remove the gel from the casting stand and assemble the electrophoresis tank as per the manufacturer's instructions. Fill the upper chamber and then the bottom of the tank with 3 L electrode buffer and let sit for a few minutes to ensure that the glass plates have formed a watertight seal (if not, the buffer will leak out of the top chamber into the tank below and the electrode will overheat, causing the plastic to melt). The rubber gasket which forms the seal along the bottom edge of the upper chamber can be lightly smeared with Vaseline to ensure a better seal if necessary. Remove any air bubbles which may be trapped on the underside of the glass plates within the tank.
8. Flush out each individual well with a fine-tipped pastette to ensure there is no residual acrylamide at the bottom of the well that may impair movement of proteins through the gel.
9. Remove samples from the freezer and denature for 10 min at 65°C. If molecular weight markers are to be used for reference they should be denatured at the same time (95°C for 5 min). Allow all tubes to cool prior to loading (*see* **Note 10**).
10. Carefully load an equal number of cells (75 µL) to each lane of the gel using extra long gel loading tips—a duck-billed tip can be used if preferred (*see* **Note 11**).
11. Connect a rubber hose to the tank and turn on the water supply to keep the gel cool throughout the run (*see* **Note 12**).
12. Place the lid onto the tank, ensuring that the positive and negative contacts on the lid match those of the tank. Connect the leads to the power pack, again ensuring correct polarity. Run each gel for 35 min at 60 mA (120 mA for two gels) through the stacking gel, followed by 3–4 h at 35 mA (70 mA for two gels) through the resolving gel (or until the dye front runs off the bottom of the gel into the tank). Turn off the power before dismantling the apparatus.

3.4. Transfer Onto Nitrocellulose Membranes

1. Cut one piece of Immobilon-P (Millipore) membrane (15 cm × 17 cm) and four pieces of filter paper (16 cm × 18 cm) so that they are approx 1–2 cm larger than the area of the resolving gel.
2. Using flat-nosed forceps (do not touch with hands) soak the membrane in methanol for 12 s, followed by distilled water for 2 min and then transfer buffer for 5 min (*see* **Note 13**).
3. Lay the black side of the sandwich apparatus on the bench with the hinge away from yourself, the clear side being on top. Open the sandwich completely so that the clear side is then also on the bench but furthest away from you
4. Soak one of the sponge pads in transfer buffer and then lay it on top of the black surface.
5. Hold two of the filter papers together and soak in transfer buffer. Lay the filter papers on top of the sponge pad. Gently, but firmly, roll a clean pipet over the surface of the filter paper to push away any air bubbles.
6. Disassemble the electrophoresis tank and carefully separate the two glass plates using one of the plastic spacers to lever the two sides apart. Cut off the uppermost stacking gel and discard.
7. Flood the resolving gel in transfer buffer and then carefully lift it by the two top corners. Carry it over to the filter paper and lower the bottom edge of the gel onto the lower surface of the filter paper. Carefully lay the gel down (from bottom to top) onto the filter paper trying to prevent it from tearing. Roll the pipet over the gel to again remove any air bubbles (*see* **Notes 14** and **15**).
8. Place the membrane over the center of the gel. Roll the pipet over the gel to again remove any air bubbles.
9. Soak two more filter papers in transfer buffer and lay on top of the gel. Roll the pipet over the top to again remove any air bubbles.
10. Finally, soak the second sponge pad in transfer buffer and then lay on top of the filter papers. Close the sandwich plate and secure with the clip.
11. Fill the transfer tank with buffer and then immerse the sandwich into the tank with the black plate towards the negative electrode (black) and the clear/red plate towards the positive electrode (red). Place a magnetic stirring bar in the tank and check the polarity before putting on the lid. Connect the leads to the power pack and again check the polarity. Place the tank on a magnetic stirrer in a cold room and run at 30 V overnight.

3.5. Antibody Detection and ECL Visualization

1. Make up 100 mL blocking agent containing 5 g/100 mL (5%) "Marvel" (Premier Brands) nonfat dried milk in PBS-Tween.
2. Disassemble the transfer apparatus and lay the black sandwich plate on the bench with the hinges away from you. Open the top of the sandwich plate away from you and remove the upper sponge pad. Remove and discard the two upper filter papers, exposing the membrane below. Trim the membrane with a scalpel if necessary and then carefully lift it up with flat-nosed forceps and place into a sandwich box containing 50 mL of the blocking agent. Flipping the membrane over from left to right will ensure that the proteins are on the upper surface and the samples are in the correct left to right orientation. Place on a shaking platform for 1 h at room temperature with gentle agitation.
3. Discard the blocking agent and incubate the membrane with a 1/10,000 dilution of (10 µL/20 mL) of anti-PARP antibody (in blocking agent) overnight at 4°C on a rocking platform (*see* **Notes 16** and **17**).

4. Wash the membrane in PBS-Tween for 10 min (three times) by gentle agitation on a rocking platform.
5. Incubate the membrane with a 1/2000 dilution (10 µL/20 mL) of goat antimouse IgG-HRP secondary antibody (blocking agent) for 1 h at room temperature on a rocking platform.
6. Before continuing with the final three washes, prepare the ECL detection solution by combining starting solution B with substrate 150 µL/15 mL solution A in a ratio of 1/100. Mix and leave at room temperature for 30 min prior to use.
7. Wash the membrane in PBS-Tween for 10 min (three times) by gentle agitation on a rocking platform.
8. Pour off the final PBS-Tween wash and add the ECL detection solution. Gently swirl the solution over the membrane for 60 s and discard. Carefully lift the membrane up and lay it down on top of an overhead–projector acetate sheet placed on a flat surface. Place another similarly sized sheet on top of the membrane and bring together to make a sandwich. Smooth out any air bubbles with the back of your finger working from the center outwards—if bubbles remain lift the top sheet up and start again. When all the bubbles have disappeared, place the sandwich inside a darkroom–cassette and fasten down the sides with tape to prevent movement (you later want to trace the positions of any molecular weight marker bands onto your exposed photographic film, hence it must be firmly located).
9. Take the cassette into the dark room, close the door, and turn on the safe light. Open the cassette and place one sheet of Hyperfilm-ECL over the membrane, securing against one corner of the cassette to hold it in place. Expose for 10 s, remove the film, and then develop/fix using a commercial developer or conventional trays of developer, fixer, and wash. Repeat using different exposure times to get an optimal image (*see* **Note 18**).
10. Place the developed image back into the cassette, again locating it against the same corner, and mark the position of the molecular weight markers onto the film. Label the lanes according to original loading arrangement and identify position of bands using relative positions of the molecular weight markers.

4. Notes

1. Some reports refer to a cleavage product of 85 kD in size while others speak of a 121-kD full-length peptide.
2. Fetal calf serum must be heat-inactivated prior to use by heating in a water bath for 30 min at 56°C. It can then be placed in aliquots and stored at –20°C.
3. Gloves and a mask should be worn when weighing out SDS to prevent inhalation of the fine powder.
4. Although anti-PARP antibodies are available from various commercial sources, some detect both the full-length peptide and the cleaved product while others simply pick up the cleaved product. Check that you have the correct antibody for the specific application in mind.
5. Although the manufacturers state that Lipofectin has an indefinite shelf life if stored correctly, its efficiency will be quickly compromised if not kept at 4°C.
6. The time interval for cells to become detached from the plastic is dependent on the cell line being used but is usually in the order of 5–10 min. Suspension cultures do not require the trypsinization stage (*see* **Subheading 3.2.2.**).
7. Acrylamide/bisacrylamide is extremely hazardous and should not be purchased separately in powder form unless absolutely necessary. It can, however, be obtained premixed in a

variety of concentrations from various suppliers. The protocol described uses a 30% solution. If a different concentration is used the volume required will need to be adjusted.

8. TEMED and AMPS must be added last and immediately prior to pouring the gel because the polymerization will commence as soon as these two agents are added.
9. The resolving gel can be poured the day before it is needed if precautions are taken to prevent it drying out. This is best achieved by dropping distilled water onto the gel between the plates and then covering completely in cling film to prevent evaporation. The gel can then be stored vertically in the casting stand at 4°C overnight.
10. Although the samples should be kept on ice to prevent degradation by protease enzymes, chilling immediately prior to loading onto the gel can make the glycerol in the loading buffer become viscous which can in turn make it difficult to accurately dispense equal volumes into each well.
11. When loading the gels, it is recommended to try and leave the first and last lanes free as the gel may sometimes smile if it has been run to fast—this phenomenon becomes more of a problem towards the edges of the gel.
12. Cooling of the tank during operation will enhance resolution by preventing distortion of the gel if high voltages are used (which may cause it to melt). This can be achieved by placing a rubber hose from the tap to one side of the tank and a return hose down the drain creating a simple heat exchanger.
13. In order to prevent scratching the delicate membranes, use flat-nosed forceps when handling.
14. When transferring the gel onto the membrane, keep it wet and try to be quick. Higher density gels will be more robust than those of lower density. Clumsiness will inevitably result in the gel tearing and subsequently splitting in two.
15. For optimal transfer while keeping orientation of the gel from left to right during the transfer process, it is suggested that the gel is in fact flipped over so that the left side of the gel lies down the right side of the membrane, i.e., placed on the membrane up-side-down with the molecular weight markers running down the right side of the membrane. When the membrane is then later taken out of the tank, the inverted samples will again be in the correct order from left to right.
16. The primary antibody should not be repeatedly freeze/thawed but rather reconstituted and placed in aliquots of smaller volumes for storage at –20°C or –70°C degrees.
17. The primary antibody incubation can alternatively be carried out for one hour at room temperature, although this may be prone to higher background activity depending on the antibody used.
18. When placing the photographic film into the cassette, it may be useful to fold over or snip the bottom right hand corner in order to orient the image after exposure.

References

1. Glucksmann, A. (1951) Cell deaths in normal vertebrate ontogeny. *Biol. Rev.* **26,** 59–86.
2. Saunders, J. W. (1966) Death in embryonic systems. *Science*. **154,** 604–612.
3. Kerr, J. F. R., Wyllie, A. H., and Currie, A. R. (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer*. **26,** 239–257.
4. Wyllie, A. H., Kerr, J. F. R., and Currie, A. R. (1980) Cell death: the significance of apoptosis. *Int. Rev. Cytol.* **68,** 251–306.
5. Wyllie, A. H. (1981) Cell death: a new classification separating apoptosis from necrosis, in: *Cell Death in Biology and Pathology* (Bowen, I. D. and Lockshin, R. A., eds.) Chapman and Hall, New York, pp. 9–34.

6. Afford, S., and Randhawa, S. (2000) Apoptosis. *Mol. Pathol.* **53,** 55–63.
7. Kam, P. C., and Ferch, N. I. (2000) Apoptosis: mechanisms and clinical implications. *Anaesthesia* **55,** 1081–1093.
8. Wyllie, A. H., Duvall, E., and Blow, J. J. (1984) Intracellular mechanisms in cell death in normal and pathological tissues, in *Cell Aging and Cell Death*, (Davies, I. and Sigee, D. C., eds.) Cambridge University Press, Cambridge, UK, pp. 269–294.
9. Nicotera, P., Leist, M., and Ferrando-May, E. (1999) Apoptosis and necrosis: different execution of the same death. *Biochem. Soc. Symp.* **66,** 69–73.
10. Strasser, A., O'Connor, L., and Dixit, V. M. (2000) Apoptosis signalling. *Annu. Rev. Biochem.* **69,** 217–245.
11. Gallaher, B. W., Hille, R., Raile, K., and Kiess, W. (2001) Apoptosis: live or die - hard work either way. *Horm. Metab Res.* **33,** 511–519.
12. Bredesen, D.,E. (2000) Apoptosis: overview and signal transduction pathways. *J. Neurotrauma* **17,** 801–810.
13. Slee, E. A., Adrain, C., and Martin, S. J. (2001) Executioner caspase-3, -6, and -7 perform distinct, nonredundant roles during the demolition phase of apoptosis. *J. Biol. Chem.* **276,** 7320–7326.
14. Lindahl, T., Satoh, M. S., Poirier, G. G., and Klungland, A. (1995) Post-translational modification of poly(ADP-ribose) polymerase induced by DNA-strand breaks. *Trends Biochem. Sci.* **20,** 405–411.
15. Herceg, Z. and Wang, Z. Q. (1999) Failure of poly(ADP-ribose) polymerase cleavage by caspases leads to induction of necrosis and enhanced apoptosis. *Mol. Cell.Biol.* **19,** 5124–5133.
16. Berger, N. A. (1985) Poly (ADP-ribose) in the cellular response to DNA damage. *Radiat. Res.* **101,** 4–15.
17. Schraufstatter, I. U., Hyslop, P. A., Hinshaw, D. B., Spragg, R. G., Sklar, L. A., and Cochrane, C. G. (1986) Hydrogen peroxide-induced injury of cells and its prevention by inhibitors of poly(ADP-ribose) polymerase. *Proc. Natl. Acad. Sci. USA.* **83,** 4908–4912.
18. Wyllie, A. H., and Morris, R. G. (1982) Hormone induced cell death. Purification and properties of thymocytes undergoing apoptosis after glucocorticoid treatment. *Am. J. Pathol.* **109,** 78–87.
19. McPhillips, F. Mullen, P., Monia, B. P., et al. (2001) Association of c-Raf expression with survival and its targeting with antisense oligonucleotides in ovarian cancer. *Br. J. Cancer.* **85,** 1753–1758.

16

Detection of Apoptosis by the TUNEL Assay

John Lawry

1. Introduction

Apoptosis is the name given to a normal cell process—that of programmed cell death. As the term implies, this is normally a well controlled and highly regulated series of events leading to the elimination of an "unwanted" cell. Without this process, many stages of embryogenesis would not be complete and immune responses would not be terminated ***(1)***. Failure to initiate apoptosis can induce many disease states, the most well documented being arthritis and cancer. In these conditions, the normal constraints on the life span of a differentiated cell are not present, so the cell is not eliminated ***(2)***.

The process of apoptosis results in nuclear and cytoplasmic fragmentation,and the formation of membrane-bound apoptotic bodies. This is one means of differentiating apoptosis from necrosis. Necrosis is a process of cellular lysis with a corresponding release of cytoplasmic components into the surrounding tissue. This invokes an inflammatory response. There is no such response with apoptosis, as the apoptotic bodies are phagocytized by adjacent cells leaving no trace of the original cell, except perhaps a "hole" if visualized in formaldehyde-fixed, paraffin-embedded tissue sections.

The apoptotic process includes a nuclease activation stage. Nucleases degrade the higher order chromatin structures of DNA to produce fragments initially between 50 and 300 kilobases (kb) in size that are further cleaved to fragments around 200 base pairs in length ***(3)***. These fragments enable "DNA ladders" to be visualized by agarose-gel electrophoresis for some cell types ***(4)***.

The formation of large numbers of DNA fragments, by definition, leads to the presence of large numbers of exposed DNA 3'-hydroxyl (OH) ends. It is this feature that has enabled the development of "DNA end-labeling" assays, or "*in situ* end labeling" (ISEL) which can be adapted for measurement by fluorescence microscopy, or more frequently, by flow cytometry ***(5,6)***. One form of the end labeling assay utilizes the enzyme terminal deoxynucleotidyl transferase (TdT) to catalyze the template-independent addition of deoxyribonucleoside triphosphates to the 3'-hydroxyl (OH)

From: *Methods in Molecular Medicine, vol. 88: Cancer Cell Culture: Methods and Protocols*
Edited by: S. P. Langdon © Humana Press Inc., Totowa, NJ

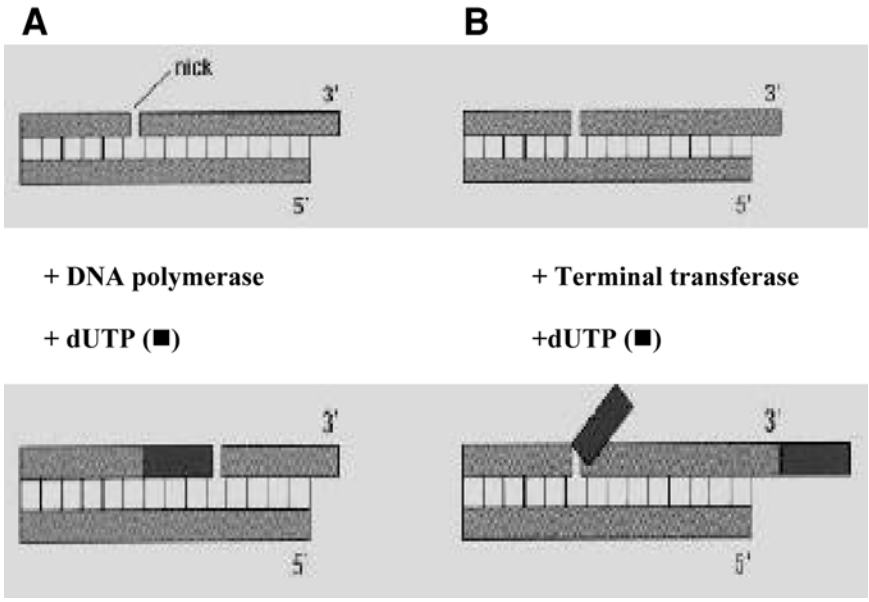

Fig. 1. *In situ* nick translation and *in situ* end labeling (TUNEL).

ends of double-stranded, or single-stranded DNA, irrespective of whether the ends are recessed, overhanging, or blunt. This is the TUNEL assay (**T**dT-mediated d**U**TP **N**ick **E**nd **L**abeling) *(7)*.

1.1. Principles of the TUNEL Assay

Two parallel staining techniques have been developed: the *in situ* nick translation method and the *in situ* TUNEL method:

- *Nick translation* involves labeling DNA strand breaks with modified nucleotides using exogenous DNA polymerase. This method primarily results in the labeling of *mid-strand breaks.*
- The *TUNEL* method utilizes exogenous terminal transferase to label strand breaks by incorporating modified nucleotides. This method primarily produces *end labeling.*

Both methods are then developed to visualize the incorporated nucleotides with a secondary detection system which incorporates a reporter molecule (e.g., fluoroscein isothiocyanate [FITC]). This is illustrated in **Fig. 1.** DNA polymerase I catalyzes the template addition of 2'-deoxyuridine 5'-triphosphate (dUTP) at free 3'-OH termini when one strand is broken ("nicked"), so random DNA fragmentation will be detected in addition to apoptotis-induced strand breaks. This would include endonuclease action during processes such as necrosis, so the *in situ* labeling procedure can result in more nonapoptotis-specific labeling. In contrast, TdT only labels blunt ends

of double-stranded DNA and is template independent. Labeling that is detected by this procedure should closely parallel true apoptotic counts.

The deoxyribonucleoside triphosphate used as the end label in the TUNEL assay can be bromolated to form Br-dUTP. This has been found to be more readily incorporated into the genome of apoptotic cells than deoxynucleotide triphosphates complexed to larger molecules, such as digoxigenin, biotin, or fluorescein. The greater the level of incorporation of dUTP, the greater will be the detection signal, and the more sensitive the assay. All assays suffer from background binding in the system. Although this is normally low in nonapoptosing cells, false positives may be associated with elevated levels of actively dividing cells, or as a result of harsh fixation procedures. Therefore, the greater the resolution between nonlabeled cells, low-intensity background staining, and high-intensity true positive (apoptotic) cells, the easier it will be to take qualitative measurements ***(8,9)***.

After Br-dUTP incorporation, labeled sites on the DNA can be tagged using an anti-BrdU monoclonal antibody. To facilitate dual-staining protocols, and to simplify the assay, the anti-BrdU antibody is usually conjugated directly to a suitable fluorochrome, usually FITC. Other indirect procedures include biotin-dUTP and FITC streptavidin, or digoxigenin-dUTP with FITC antidigoxygenin.

The TUNEL assay can be further simplified by using the enzyme TdT to catalyze the template-independent addition of directly labeled dUTP (FITC-dUTP) to the 3'-hydroxyl (OH) terminus of the DNA fragment. This is therefore a single-step labeling procedure. However, this system may be a compromise between increased simplicity and the lack of a signal amplification stage, which may reduce sensitivity in some cell types.

The advantage of the flow cytometric TUNEL assay is that the detection fluorochrome (usually FITC) is compatible with dual staining with a DNA fluorochrome such as propidium iodide (PI) or 7-aminoactinomycin D (7-AAD), or the UV excited dye, DAPI (4',6-diamidino-2-phenylindole), to visualize ploidy and cell-cycle phase subpopulations ***(10)***. It is therefore possible to trace any dominant subpopulation (ploidy or cell-cycle phase) responsible for the generation of most of the apoptotic cells. Conversely, this approach can also be used to identify any populations that seem to be resistant to apoptosis induction.

For the detection of DNA strand breaks, there is a requirement for pre-fixing cells with a crosslinking agent such as formaldehyde. Unlike ethanol, which is used as a fixative for many other methods, formaldehyde or paraformaldehyde prevent the extraction of degraded DNA, thereby retaining DNA levels at, or close to, that of normal unfixed cells.

2. Materials

1. Phosphate buffered saline (PBS), pH 7.2.
2. 1% Paraformaldehyde in PBS, pH 7.0. Warm to 56°C to dissolve the powder form of this reagent in the PBS.
3. 70% (v/v) Methanol.
4. 5X TdT reaction buffer: 125 m*M* Tris-HCl, pH 6.6, 1.25 mg/mL bovine serum albumin (BSA), 1*M* sodium cacodylate. Weigh out 1.96 g Tris-HCl, 16 g Na-cacodylate, and 0.125 g BSA into 100 mL distilled water.

5. TdT enzyme mixture: 20 U TdT, 2 µL 2 m*M* Br-dUTP, 5 µL 25 n*M* $CoCl_2$, 10 µL TdT reaction buffer, 35 µL distilled water. Use 50 µL per sample, so multiply for the test size adding an extra 50 µL to allow for volume loss. Make up immediately prior to use. Do not store.
6. Wash buffer: PBS, 0.2% Triton X-100, 1% BSA. Add 100 µL Triton X-100, 100 mL PBS, 1% BSA and warm to dissolve. Store at 4°C.
7. Antibody labeling mix: 5 µL FITC anti-BrdU antibody (1 mg/mL) and 95 µL wash buffer. Use 100 µL per test to multiply up for the whole assay. Add one extra measure (100 µL) to allow for volume loss.
8. DNA fluorochromes: 10 µg/mL propidium iodide in PBS, 10 µg/mL 7-AAD in PBS, or 10 µg/mL DAPI in PBS.
9. DNAse-free RNAse A for use with propidium iodide (200 µg/mL PBS plus 0.1% azide).

3. Methods

TUNEL assays are easy to perform with only three processing stages:

1. Sample preparation (in vitro apoptosis induction, harvest and fix cells).
2. The staining assay.
3. Analysis on the flow cytometer.

3.1. Fixation

Cells may be prepared using a variety of apoptosis-inducing treatments (cytotoxic drugs, ionizing radiation, FAS ligand/receptor activation, serum depletion, hypoxia, and, for some cells, substrate detachment) ***(11)***. Some cell lines spontaneously undergo apoptosis in culture (e.g., T47D breast cancer cell line, Y79 retinoblastoma cell line).

All assays benefit from some form of positive control. Assay kits usually include positive and negative control cells (often HL60 cells) that have been incubated with 10 µ*M* camptothecin for 4 h, then harvested and fixed in alcohol. These cell preparations are, therefore, stable for considerable periods of time. It is not difficult to identify a suitable cell type and store aliquots in alcohol for each experiment. Both control and test cells are then harvested and fixed using the same procedure, namely:

1. Suspend 1–2 × 10^6 cells in 0.5 mL of PBS.
2. To the cell suspension, add 5 mL of 1% (v/v) paraformaldehyde in PBS and place on ice for 10 min after vortexing.
3. Centrifuge cells for 5 min at 300*g* and discard the supernatant.
4. Wash the cells in 5 mL of PBS then pellet the cells by centrifugation.
5. Resuspend the cells in 0.5 mL of PBS.
6. Add 5 mL of ice-cold 70% (v/v) methanol and vortex. Allow cells to be fixed and permeabilized for at least 15 min prior to commencing staining.

Alternatively cells can be stored after stage 6 for several days at 4°C, or for longer periods at –20°C (*see* **Note 1**).

3.2. The TUNEL Assay

1. Resuspend all cell suspensions (positive and negative controls and test cell samples) by gently vortexing the vials. Remove aliquots of the cell suspensions so as to have approx 1

$\times 10^6$ cells per test, and place in 12×75-mm flow cytometry round bottom tubes. Centrifuge all the cell suspensions for 5 min (300*g*) and remove the 70% (v/v) ethanol by aspiration being careful to not disturb the cell pellet.
2. Resuspend each cell sample with a short vortex mix, and add 1 mL of the wash buffer to each tube. Centrifuge as before and remove the supernatant by aspiration.
3. Repeat the wash stage.
4. Resuspend each cell sample with a short vortex mix, and add 50 µL of the TdT Enzyme Mixture and incubate for 60 min at 37°C in a water bath. Shake the tubes every 15 min to resuspend cells that may settle.
5. At the end of the incubation time add 1.0 mL of the wash buffer to each tube and centrifuge (300*g*) for 5 min. Remove the supernatant by aspiration and gently vortex to resuspend the cell pellet.
6. Repeat the wash stage. Resuspend the cells pellet in 0.1 mL of the antibody labeling mix (prepared as described above). Incubate in the dark for 30 min at room temperature. Wash all samples twice using the wash buffer as before.
7. Resuspend the cell pellets and add 0.9 mL PBS. Add 100 µL propidium iodide/RNase A solution to the tube, or an alternative DNA fluorochrome if required. If the cell density is low, decrease the amount of PI/RNase A solution to 0.5mL.
8. Incubate the cells in the dark for 30 min at room temperature.
9. Analyze the cells by flow cytometry by leaving them in the propidium iodide/RNase solution.
10. Analyze the cells within 3 h of staining.

3.3. Analysis on the Flow Cytometer

Samples may need to be filtered (50 µm mesh) before analysis. If samples need to be diluted, use further aliquots of the propidium iodide/RNase solution, not PBS alone as this will alter DNA staining by propidium iodide.

The combination of FITC antibody and propidium iodide DNA fluorochrome can be analyzed on any cytometer with a blue argon ion laser (488 nm emission). 7-AAD is also excited by this laser, but DAPI requires UV excitation.

1. Run the negative control sample and obtain a dot cluster on a dual-forward scatter versus side-scatter display. Threshold some of the debris on the forward scatter parameter, but do not threshold too close to the main population or apoptotic cells will be excluded (*see* **Note 2**).
2. Using linear red detection (usually FL3, but FL2 can be used) as the *x*-axis, and log green detection (FL1) as the *y*-axis, plot a second dual-parameter dot display. Adjust the amplification until the DNA G1-phase cell cluster is about one-third of the distance along the *x*-axis, and the FL1 (FITC) cluster is within the first log decade (*see* **Note 3**).
3. If possible, establish doublet discrimination gates on a plot of DNA area versus DNA width or peak, and use this gate for all other displays.
4. Acquire and save the list mode data for the negative control sample.
5. Run the positive control sample, and a clear positive cluster (FL1-FITC) should be seen one to two log decades above the negative cells. Controls usually contain between 10 and 20% TUNEL-positive cells.
6. Acquire and save the list mode data for the positive control sample and proceed with all other samples in the assay.
7. Examples are illustrated in **Figs. 2** and **3**.

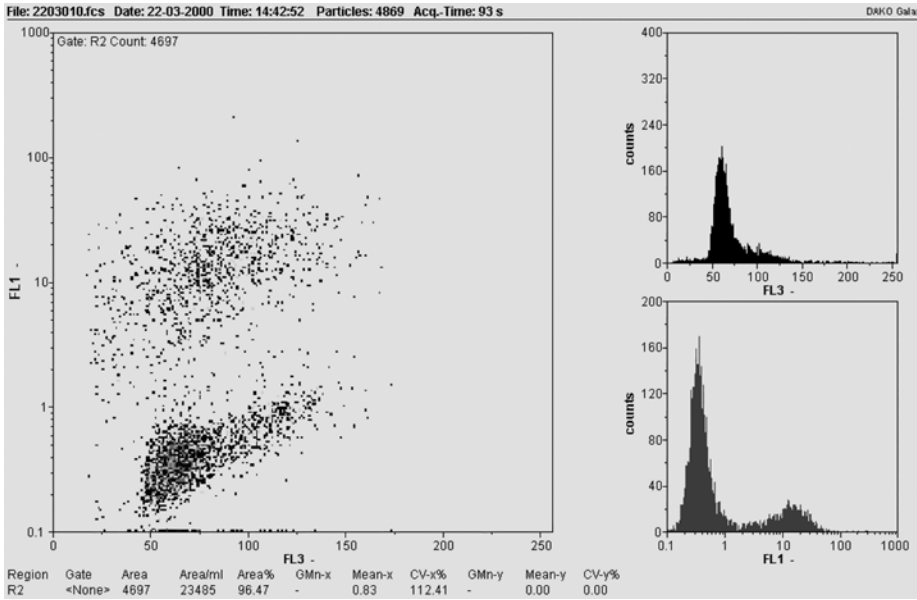

Fig. 2. Y79 cells labeled with FITC TUNEL with propidium iodide DNA fluorochrome.Y79 (retinoblastoma) cells were labeled using the TUNEL assay and propidium iodide as the DNA fluorochrome. TUNEL-positive cells can be clearly seen above the nonapoptotic TUNEL-negative cells. Most of the positive cells seem to be centered between G1 and G2 phases suggesting that cells were apoptosing from S-phase.

3.4. Summary

The TUNEL method is now considered to be sensitive, fast, and capable of identifying early stage DNA fragmentation. It is able to identify early stage apoptotic cells better than the nick translation technique, which also identifies necrotic cells. The assay can be developed "in house" or purchased as a commercial kit. Samples produced by this technique can be analyzed on any cytometer so this procedure can be exploited in all laboratories interested in gaining a greater insight into the pathogenesis and treatment of disease ***(12)***.

4. Notes

1. DNA fragmentation occurs in numerous biological and artificial situations, so background or baseline values may vary from cell type to cell type. For example, fragmentation is seen in:
   - Necrosis.
   - Sample preparation (fixation).
   - Tissue sectioning or dissociation.
   - Detergent and proteinase K treatment.
   - Normal cell division, DNA repair and recombination.

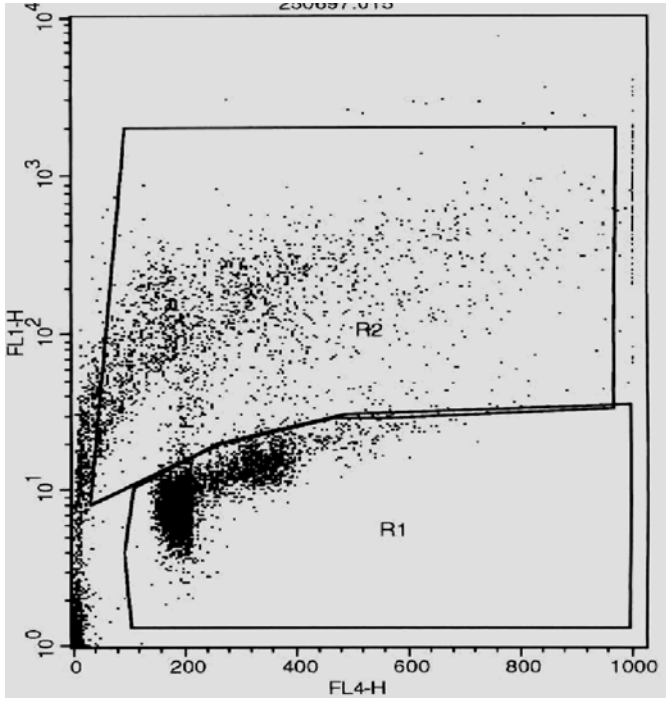

Fig. 3. Y79 cells labeled with FITC TUNEL with DAPI DNA fluorochrome. Y79 cell samples were stained with TUNEL and the UV-excited DNA fluorochrome DAPI. In this example, low thresholding was used so a lot of signals appear to the far left and bottom corner of the display (outside regions 1 and 2). Clearly these represent small fragments of a whole cell, as they have fractional amounts of DNA relative to the G1 peak. These must be apoptotic bodies, not intact cells (*see* **Note 4**).

2. For many cell types, improved results can be obtained after fixation, by storing the cells at –20°C in 70% (v/v) ethanol overnight, prior to staining for apoptosis detection.
3. Control cells from kits may have a different total DNA content to the test cells, so the DNA signal (FL3 or FL2) may need to be increased or decreased, so as to bring the G1-phase population back to a position approximately one-third of the way along the axis.
4. Different cells may follow different forms of the apoptotic pathway. Not all apoptosis assays therefore identify apoptotic cells as clearly. Classic morphology is still the gold standard for the confirmation of the apoptotic phenotype, with apoptotic body formation readily seen using fluorescence microscopy with a cytospin of the cell sample labeled with the DNA fluorochrome. DAPI is the fluorochrome of choice but good results can also be seen with propidium iodide ***(13)***.

Acknowledgments

The Flow Cytometry Facility in the Institute for Cancer Studies is funded by Yorkshire Cancer Research.

References

1. Wyllie, A. H., Kerr, J. F. R., and Currie, A. R. (1980) Cell death: the significance of apoptosis. *Int. Rev. Cytolog.* **68,** 251–306.
2. Kerr, J. F. R., Wyllie, A. H., and Currie, A. R. (1972) Apoptosis: a basic biological phenomenon with wide ranging implications in tissue kinetics. *Br. J. Cancer* **26,** 239–257.
3. Arends, M. J., Morris, R. G., and Wyllie, A. H. (1990) Apoptosis: the role of endonuclease. *Am. J. Pathol.* **136,** 593–608.
4. Gorczyca, W., Bigman, K., Mittelman, A., et al. (1993) Induction of DNA strand breaks associated with apoptosis during treatment of leukaemias. *Leukaemia* **7,** 659–670.
5. Gorczyca, W., Bruno, S., Darzynkiewicz, R. J., Gong, J., and Darzynkiewicz, Z. (1992) DNA strand breaks occurring during apoptosis: their early in-situ detection by the terminal deoxynucleotidyl transferase and nick translation assays and prevention by serine protease inhibitors. *Int. J. Oncol.* **1,** 639–648.
6. Gorczyca, W., Gong, J., and Darzynkiewicz, Z. (1993) Detection of DNA strand breaks in individual apoptotic cells, by the in-situ terminal deoxynucleotidyl transferase and nicktranslation assay. *Cancer Res.* **52,** 1945–1951.
7. Negoescu, A., Lorimier, P., Labat-Moleur, F., et al. (1997) TUNEL: improvement and evaluation of the method for in-situ apoptotic cell identification. *Biochemica* **2,** 12–17.
8. Li, X. and Darzynkiewicz, Z. (1995) Labelling DNA strand breaks with BrdUTP. Detection of apoptosis and cell proliferation. *Cell Prolif.* **28,** 571–579.
9. Li, X., Traganos, F., Melamed, M. R., and Darzynkiewicz, Z. (1995) Single-step procedure for labelling DNA strand breaks with fluorescein or BODIPY conjugated deoxynucleotides: detection of apoptosis and bromodeoxyuridine incorporation. *Cytometry* **20,** 172–180.
10. Li, X., Melamed, M. R., and Darzynkiewicz, Z. (1996) Detection of apoptosis and DNA replication by different labeling of DNA strand breaks with fluorochromes of different colour. *Exp. Cell Res.* **222,** 28–37.
11. Steller, H. (1995) Mechanisms and genetics of cellular suicide. *Science*, **267,** 1445–1449.
12. Thompson, C. B. (1995) Apoptosis in the pathogenesis and treatment of disease. *Science* **267,** 1456–1462.
13. Darzynkiewicz, Z., Bruno, S., Del Bino, G., et al. (1992) Features of apoptotic cells measured by flow cytometry. *Cytometry* **13,** 795–808.

17

Apoptosis Measurement by Annexin V Staining

Eric Miller

1. Introduction

Several methods exist for the detection of apoptosis using features of the cell as it undergoes the various stages leading to the death of the cell ***(1,2)***. One of the earliest uses of flow cytometry was the detecting of a sub-G_0 peak in the DNA histogram that showed fragmentation of the nucleus ***(3)***. The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay also detects fragmentation of DNA by labeling the broken ends of DNA sequences with fluorescein isothiocynate conjugate (FITC)-labeled nucleotides ***(4,5)***. Unfortunately, DNA fragmentation occurs at a late stage in the process of apoptosis ***(6)*** and is not specific to apoptotic cells. Necrotic cells can also produce these appearances in a flow-cytometric assay. Recently, PARP cleavage ***(7)*** and annexin V ***(8–11)*** have claimed more specificity. PARP cleavage occurs early in the response as a result of the activity of caspase-3 and correlates well with chromatin condensation ***(12)*** . PARP cleavage is associated with the condensed chromatin in apoptotic cells as a measure of apoptosis. It may appear as early as 3 h post the apoptosis inducing event and precedes the ability to detect actual DNA fragmentation. The annexin V assay has been widely accepted as a marker of apoptosis ***(13–15)*** and the remainder of this chapter will be devoted to its measurement by flow cytometry.

Annexin V is a Ca^{++}-dependent phospholipid-binding protein that binds strongly to phosphatidylserine residues on the cell membrane. In a normal cell these residues are on the inner surface of the membrane and therefore inaccessible to annexin V unless the cell membrane becomes permeable. At an early stage of apoptosis the phosphatidylserine residues are translocated to the outside of the cell ***(16)***. This is considered irreversible at this point; the cell is now committed to death by apoptosis ***(17)***.

By allowing annexin V to react with these cells, any cell that has been committed to apoptosis will have exposed phosphatidylserine residues and thus will bind annexin V. If the annexin V is conjugated to FITC, the proportion of cells giving a positive FITC signal will provide a measure of the proportion of the population undergoing apoptosis. An important consideration is the fact that if the cell membrane is compro-

From: *Methods in Molecular Medicine, vol. 88: Cancer Cell Culture: Methods and Protocols*
Edited by: S. P. Langdon © Humana Press Inc., Totowa, NJ

mised, the phosphatidylserine residues within the cell will also stain and thus affect the result of the assay. One way around this problem is to incorporate a propidium iodide (PI) staining step in the protocol. If the cell membrane is compromised, the propidium iodide will be able to stain the DNA in the nucleus and thus can be used to discriminate the apoptotic cell from the damaged or necrotic cell.

Reference will be made to the use of a kit from R & D Systems ***(18)*** to perform the assay, and the results will be analyzed on a Becton Dickinson ***(19)*** FacsCalibur, but the principles of the assay remain the same and apply to all flow cytometers.

2. Materials

1. Cell line of interest.
2. Phosphate buffered saline (PBS).
3. Microcentrifuge tubes.
4. Microcentrifuge.
5. Pipets.
6. Flow cytometer.
7. Trypsin/EDTA.
8. Annexin V-FITC (R & D Systems).
9. 10X Binding buffer: 100 m*M* HEPES-NaOH, pH 7.4, 1.5 *M* NaCl, 50 m*M* KCl, 10 m*M* $MgCl_2$, 18 m*M* $CaCl_2$ in distilled water.
10. Propidium iodide (PI) (R & D Systems).

3. Methods

3.1. Cell Preparation and Treatment

3.1.1. Harvest

The annexin V assay is ideal for a single-cell suspension but can be equally successful using adherent cells, as long as care is taken to preserve the floating cell population. Floating cell populations represent late stage apoptotic and necrotic cells and excluding these may compromise the results. Cells may be harvested by the following method:

1. Decant supernatant from flask and set aside.
2. Wash flask twice in warm PBS to remove serum.
3. Detach cells in trypsin/EDTA mixture.
4. Add 10 % fetal calf serum (FCS) in media to inactivate trypsin and centrifuge for 5 min at 600*g*.
5. Resuspend in media.
6. Leave in incubator at 37°C for 30–45 min.
7. Treat as for suspension cultures.

3.1.2. Controls

Generally, 10^5 to 10^6 cells are recommended for each assay, but as three controls are required to run a sample, we find that 2×10^6 cells are needed. The following groups are prepared:

1. Unstained cells to measure background fluorescence.
2. Cells stained with PI alone.
3. Cells stained with annexin V/FITC alone.
4. Cells stained with PI and annexin V/FITC.

3.1.3. Compensation Settings

Samples 2 and 3 will be used to assess the compensation settings on the flow cytometer and need not be used for every sample.

1. Prepare the following solutions:
   a. Binding buffer, 1/10 dilution of 10X concentrate. You will need approx 1 mL per tube.
   b. Staining buffer minus PI: 0.25 µg/mL annexin V/ FITC in 1X binding buffer, 100 µL per tube.
   c. Staining buffer minus annexin V/FITC: 5 µg/mL PI in 1X binding buffer, 100 µL per tube.
   d. Full staining buffer: 0.25 µg/mL annexin V/FITC, 5 µg/mL PI in 1X binding buffer, 100 µL per tube (*see* **Notes 1** and **2**).
2. Keep all solutions on ice until needed.
3. Divide cells into four aliquots of 10^5 to 10^6 cells and wash in cold PBS.
4. Add 100 µL of solutions A–D to four aliquots of cells prepared at **step 1**. Incubate for 15 min in the dark at room temperature.
5. Add 400 µL binding buffer to each tube and analyze within 1 h on a flow cytometer.

3.2. Analysis

1. Two dot plot displays will be needed on the flow cytometer. The first dot plot display should show FSC vs SSC. Adjust the flow cytometer until all cells are between the upper and lower display boundaries. The second display should show log green vs log red fluorescence.
2. Using unstained cells, adjust red and green fluorescence until all cells are contained within lower left quadrant and approximately equal in intensity. It is important not to adjust the detector voltages after the unstained sample is removed (*see* **Fig. 1**).
3. Remove this tube and substitute the green-only stained tube (annexin - FITC) (FL1). The display should now show two populations on the lower half of the display. Adjust the degree of compensation FL2-FL1 until both populations have an equal (background) red fluorescence (*see* **Fig. 2**).
4. Replace the tube with the red-only tube (PI) (FL2) and repeat as for second tube. Populations should be visible along the left-hand side of the display. Adjust FL1-FL2 until both populations have an equal green fluorescence (*see* **Fig. 3**).

The cytometer is now set up and the test sample can be run. If the procedure is successful up to four populations should be visible, one in each quadrant of the display. The percentage in each quadrant will define the amount of apoptosis present.

3.3. Interpretation

A typical result is shown in **Fig. 4**. Cells in each quadrant can be interpreted in the following manner:

1. Lower left quadrant. These cells are negative for both annexin V and PI and can be regarded as viable nonapoptotic cells
2. Upper left quadrant. Cells are negative for annexin V but positive for PI. These are cells that have lost their cell membrane and are dead, either end-stage apoptotic or necrotic.
3. Upper right quadrant. These are positive for both annexin V and PI. Although the cells show evidence of phosphatidyl residues these are not necessarily on the cell surface,

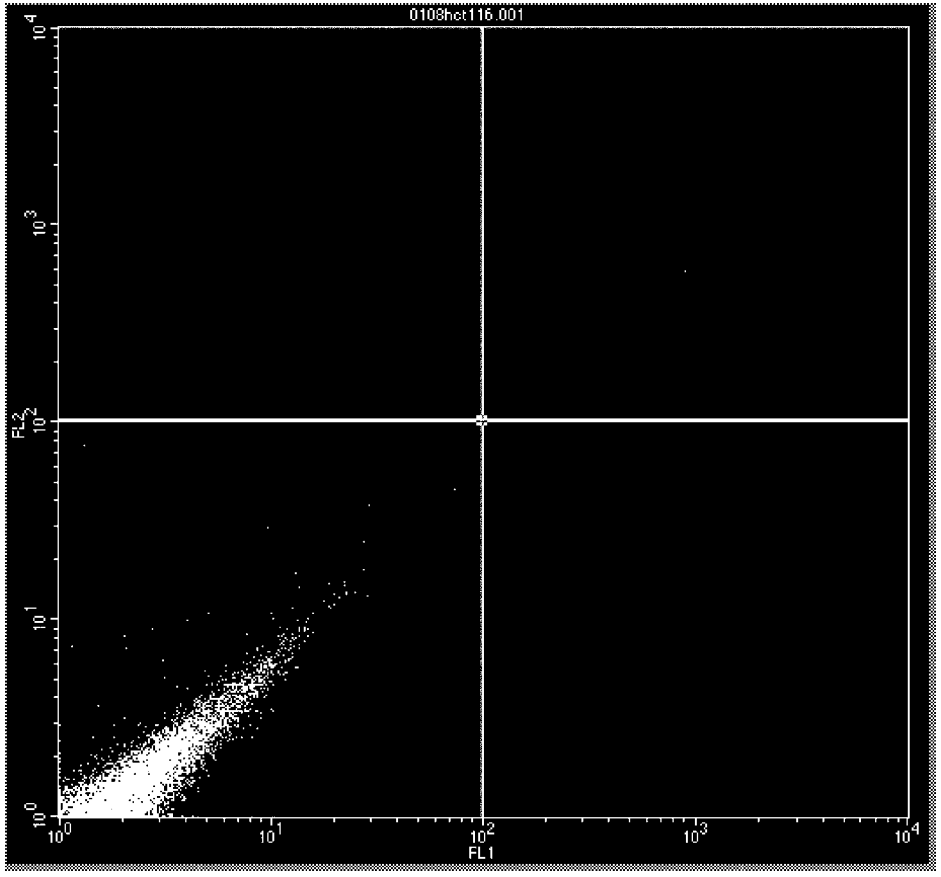

Fig. 1. Fluorescence of unstained cells: first control. Red and green fluorescence values are adjusted until all cells are within the lower left hand quadrant and approximately equal in intensity.

because the cell membrane is permeable, shown by PI positivity. Cells here are damaged cells, perhaps late stage apoptotic.

4. Lower right quadrant. Annexin V positive but PI negative. Annexin V positivity here must be the result of external phosphatidyl residues, because the cell membrane remains intact, shown by lack of PI staining. Cells here are in early stage apoptosis and this percentage should be reported as the percentage of apoptotic cells.

3.4. Troubleshooting

The next section will be devoted to common problems, how they appear on the cytometer, and how to correct them.

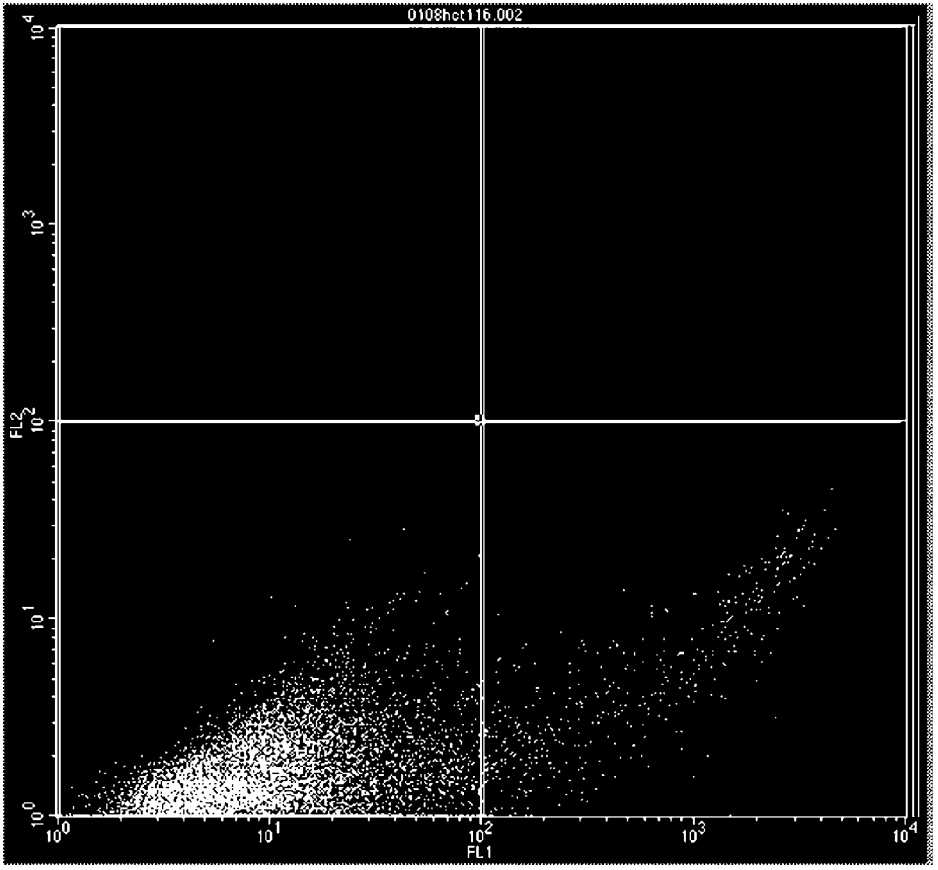

Fig. 2. Fluorescence of annexin V-FITC cells only: second control (green channel, FL1). The degree of compensation (FL1-FL2) is adjusted until both populations have an equal (background) red fluorescence.

3.4.1. Reagent Missing or Not Working

In this example (*see* **Fig. 5**) the researcher neglected to add the annexin V/FITC reagent. The appearance is identical to the PI only control.

3.4.2. Compensation Not Set Up Correctly

Insufficient compensation on FL2-FL1 (*see* **Fig. 6**).

Instead of the apoptotic cells appearing in the bottom right quadrant they are in the top right. The cells in the top left quadrant are actually dual positive late apoptotic/necrotic cells. This situation is easiest to salvage by returning to the control samples and starting from scratch.

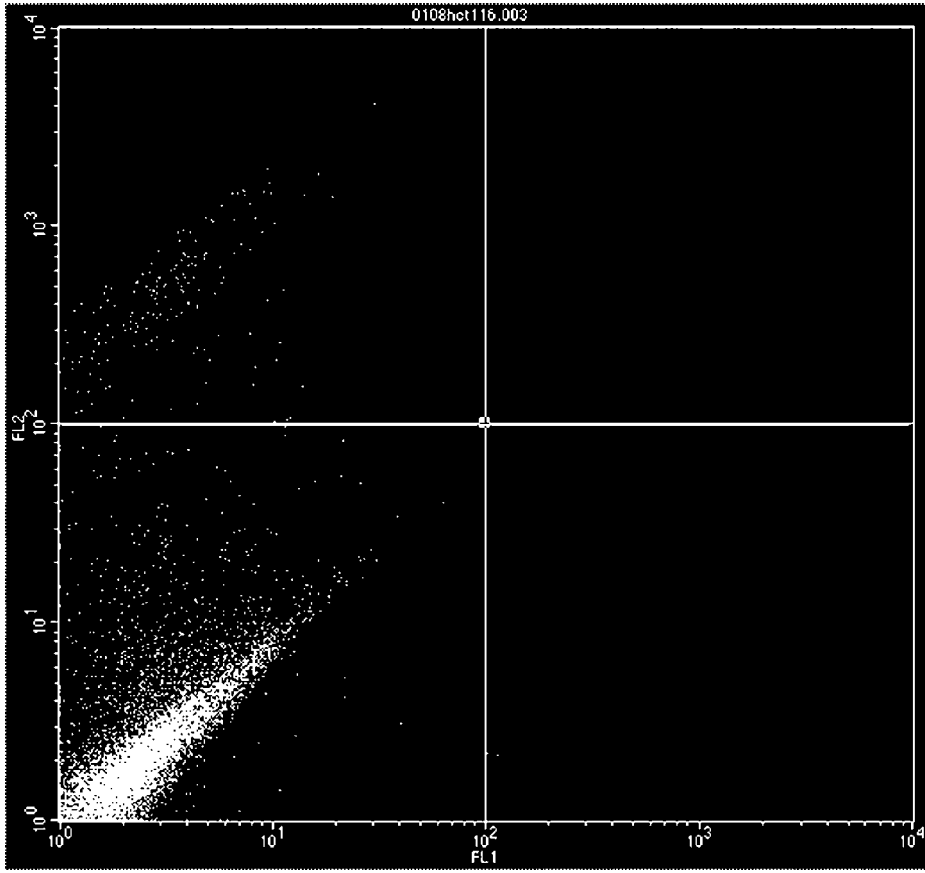

Fig. 3. Fluorescence of PI cells only: third control (red channel, FL2). The degree of compensation (FL1-FL2) is adjusted until both populations have an equal (background) green fluorescence.

3.4.3. Damaged Cells (e.g., Rough Handling, Vortexing)

If cells have been damaged in processing, the result may be as shown in **Fig.7**. Note the appearance of two parallel bands of dots along the top left quadrant. Rough handling has damaged the cell membrane of all viable cells and allowed saturation of the DNA with PI. The cells in the top right have damaged cytoplasmic membranes, while those in the top right have lost their cytoplasm altogether. Essentially this is now a cell-cycle preparation viewed through a log amplifier. The FL2 signal alone appears as shown in **Fig. 8**.

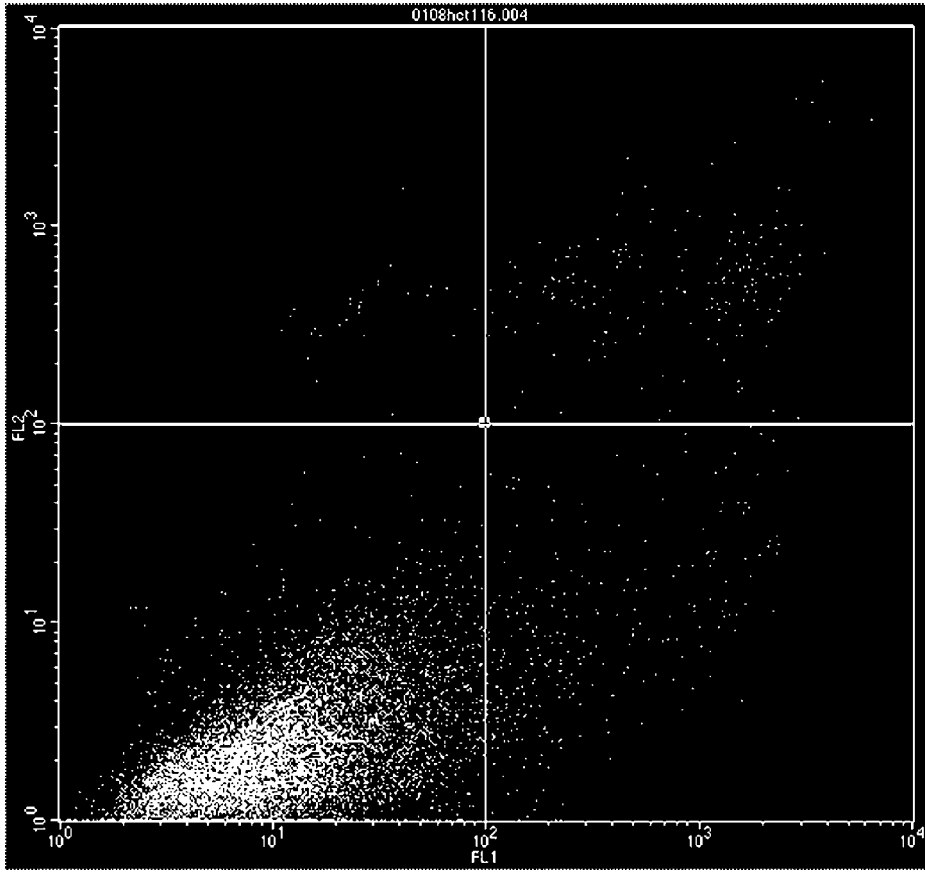

Fig. 4. Test sample, dual stained for both annexin-V and PI. Cells in the lower left quadrant are negative for both annexin V and PI and are regarded as viable and nonapoptotic. Cells in the lower right quadrant are annexin V-positive but PI-negative and are regarded as apoptotic. Cells in the upper quadrants are PI-positive and although the left-hand quadrant cells are likely to be necrotic, cells in the upper right may be either necrotic or late-stage apoptotic.

Note the two peaks in the third decade corresponding to the G_0 and G_2 peaks of the DNA histogram. The best cure for this situation is to handle the cells more gently. Anything that affects the integrity of the cell membrane will affect the results of the annexin V assay, either resulting in false positives or uninterpretable dot plots. The assay relies on the integrity of the cell membrane, which in turn relies on the care of the operator preparing the samples.

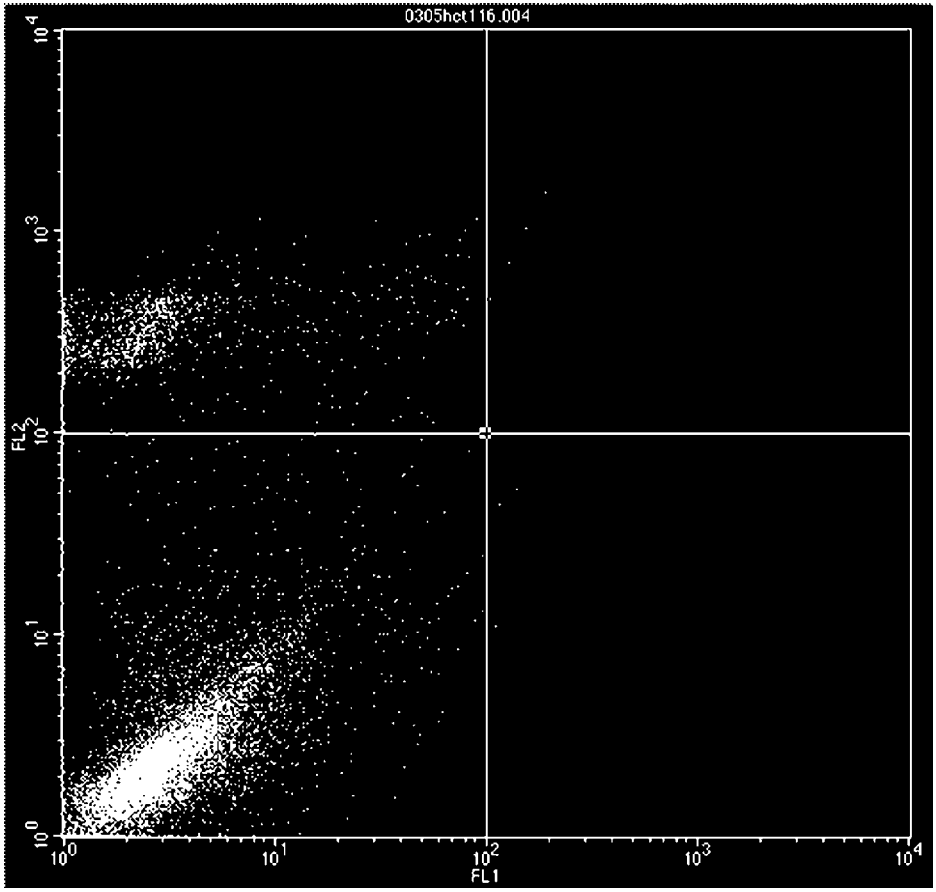

Fig. 5. Example of a profile in which a reagent is missing. In this example annexin-FITC has been omitted. Thethe resulting appearance is equivalent to the PI-only control.

3.5. Summary

Annexin V/PI staining has a wide variety of applications ***(20–24)***. It is a rapid and sensitive method for distinguishing apoptotic cells from necrotic cells in a flow cytometry based assay. Its major drawbacks are that cells cannot be stored for a batch analysis and it requires careful handling of the cells to produce good results.

4. Notes

1. Propidium iodide is toxic and should be treated with caution.
2. The concentration of annexin V-FITC may be modified if the cells have less or more phosphatidylserine residues.

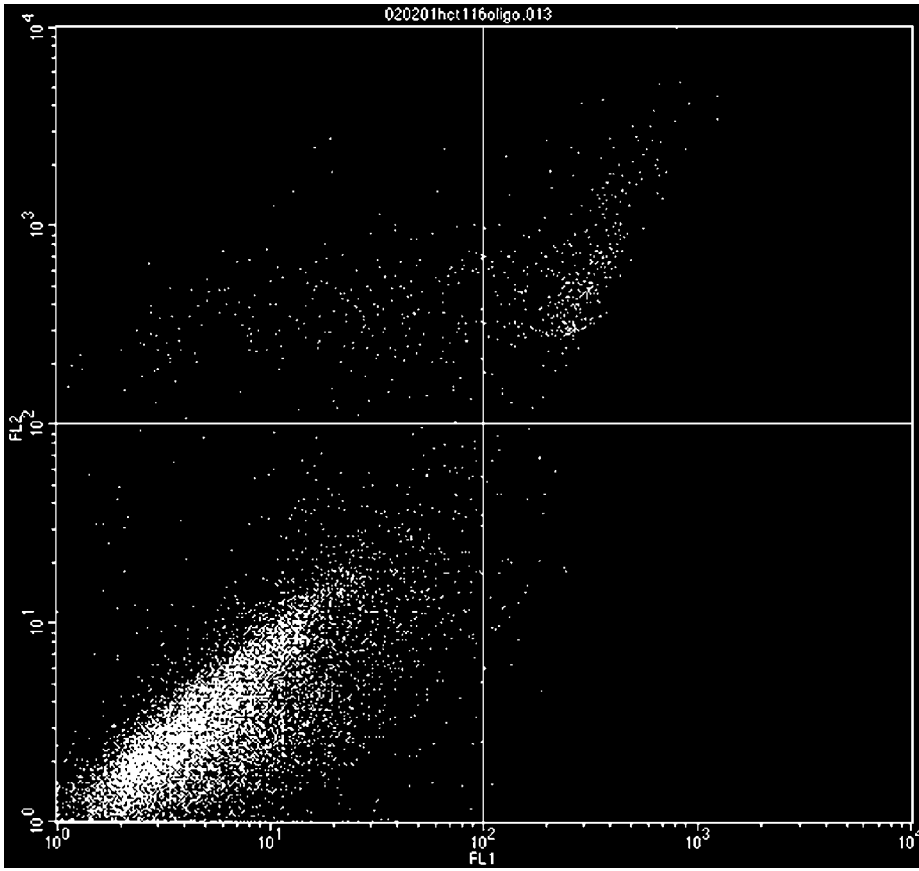

Fig. 6. Example of a profile in which there has been insufficient compensation (FL1-FL2). Cells in the upper right quadrant should be level with cells in the bottom left quadrant.

References

1. Darzynkiewicz, Z., Bedner, E., and Smolenski, P. (2001) Flow cytometry in analysis of cell cycle and apoptosis. *Semin. Hematol.* **38,** 179–193.
2. Kerr, J. F. R., Wyllie, A. H., and Currie, A. R. (1972) Apoptosis: a basic biological phenomenon with wide ranging implications in tissue kinetics. *Br. J. Cancer* **26,** 239–257.
3. Sailer, B. L., Valdez, J. G., Steinkamp, J. A., and Crissman, H. A. (1998) Apoptosis induced with different cycle-perturbing agents produces differential changes in the fluorescence lifetime of DNA-bound ethidium bromide. *Cytometry* **31,** 208–216.
4. Gorczyca, W., Bruno, S., Darzynkiewicz, R. J., Gong, J., and Darzynkiewicz, Z. (1992) DNA strand breaks occur during apoptosis: their early in situ detection by the terminal

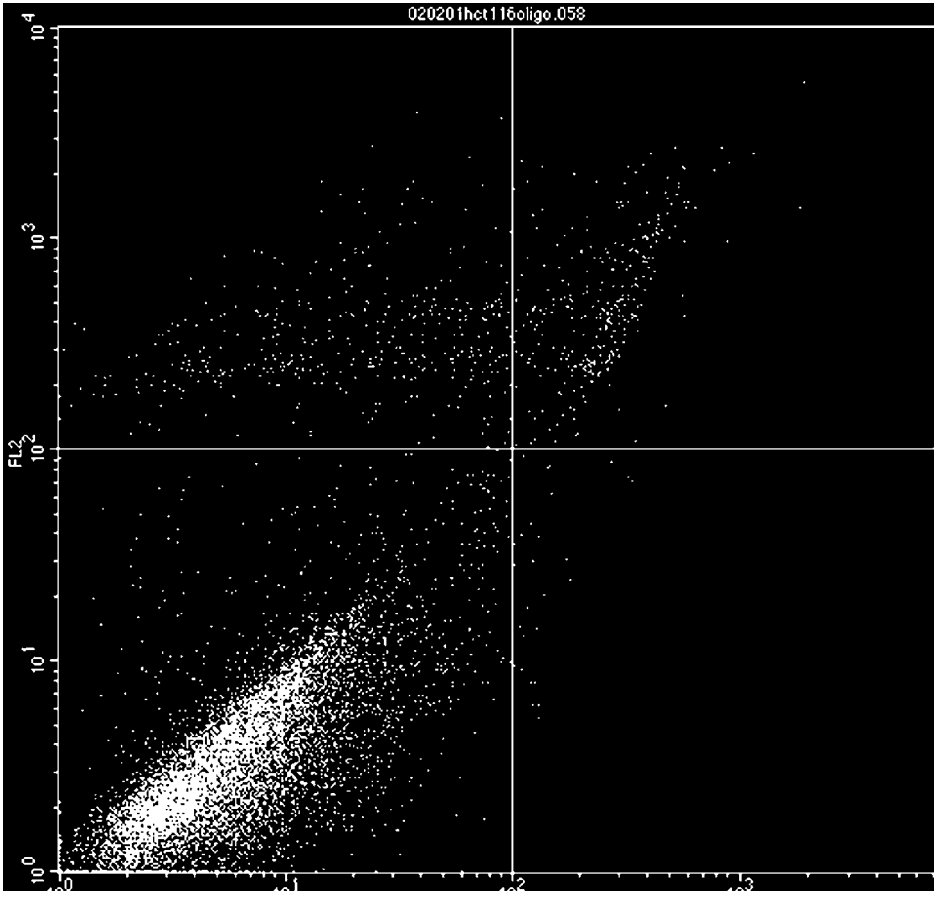

Fig. 7. Example of a profile in which there has been cellular damage during processing. Cells have stained to saturation with PI. Note the appearance of two parallel bands of dots along the top left quadrant.

deoxynucleotidyl transferase and nick translation assays and prevention by serine protease inhibitors. *Int. J. Oncol.* **1,** 639–648.

5. Gorczyca, W., Gong, J., and Darzynkiewicz, Z. (1993) Detection of DNA strand breaks in individual apoptotoic cells, by the in situ terminal deoxynucleotidyl transferase and nick translation assay. *Cancer Res.* **52,** 1945–1951.
6. Glucksmann, A. (1951) Cell deaths in normal vertebrate ontogeny. *Biol. Rev.* **26,** 59–86.
7. Slee, E. A., Adrain, C., and Martin, S. J. (2001) Executioner caspase-3, -6, and -7 perform distinct, non-redundant roles during the demolition phase of apoptosis. *J. Biol. Chem.* **276,** 7320–7326.

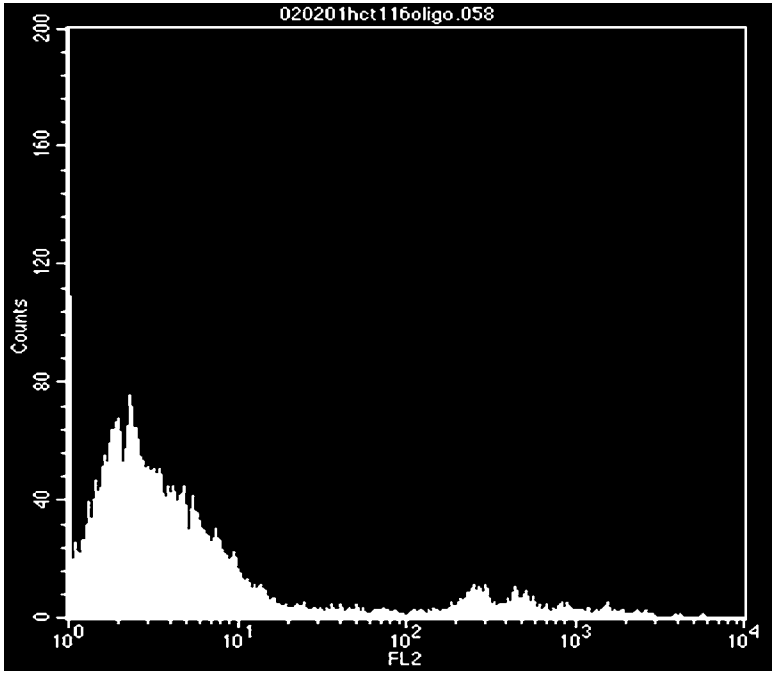

Fig. 8. Histogram of the red fluorescence from **Fig. 7**. Note the two peaks in the third decade corresponding to G_0 and G_2 peaks of the DNA histogram.

8. van Engeland, M., Nieland, L. J., Ramaekers, F. C., Schutte, B., and Reutelingsperger, C. P. (1998) Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure. *Cytometry* **31,** 1–9.
9. Zhang, G., Gurtu, V., Kain, S. R., and Yan, G. (1997) Early detection of apoptosis using a fluorescent conjugate of Annexin V. *Biotechniques***23,** 525–531.
10. Stuart, M. C., Damoiseaux, J. G., Frederik , P. M., Arends, J. W., and Reutelingsperger, C. P. (1998) Surface exposure of phosphatidylserine during apoptosis of rat thymocytes precedes nuclear changes. *Eur. J. Cell Biol.* **76,** 77–83.
11. van Engeland, M., Ramaekers, F. C., Schutte, B., and Reutelingsperger, C. P. (1996) A novel assay to measure loss of plasma membrane asymmetry during apoptosis of adherent cells in culture. *Cytometry* **24,** 131–139.
12. Lindhal, T., Satoh, M. S., Poirier, G. G., and Klungland, A. (1995) Post translational modification of poly(ADP-ribose) polymerase induced by DNA strand breaks. *Trends Biochem. Sci.* **20,** 405–411.
13. Darzynkiewicz, Z., Bruno, S., Del Bino, G., et al. (1992) Features of apoptotic cells measured by flow cytometry. *Cytometry* **13,** 795–808.

14. Gorczyca, W., Gong, J., Ardelt, B., Traganos, F. and Darzynkiewicz, Z. (1993) The cell cycle related differences in susceptibility of HL-60 cells to apoptosis induced by various antitumor agents. *Cancer Res.* **53,** 3186–3192.
15. Schmid, I., Uittenbogaart, C. H., and Giorgi J. V. (1994) Sensitive method for measuring apoptosis and cell surface phenotype in human thymocytes by flow cytometry. *Cytometry* **15,** 12–20.
16. Kroemer, G., Petit, P., Zamzami, N., Vayssiere, J. L., and Mignotte, B. (1995) The biochemistry of programmed cell death. *FASEB J.* **13,** 1277–1287.
17. Wyllie, A. H., Duvall, E., and Blow, J. J. (1984) Intracellullar mechanisms in cell death in normal and pathological tissues. In: *Cell Aging and Cell Death* (Davies, I. and Sigee, D. C., eds.) Cambridge University Press, Cambridge, UK, pp. 269–294.
18. R&D Systems Europe Ltd. 4–10 The Quadrant, Barton Ln. Abingdon, Oxon OX14 3YS, United Kingdom.
19. BD Biosciences, European Office, Denderstraat 24, 9320 Erembodegem, Belgium.
20. Cichorek, M., Kozlowska, K., Witkowski, J. M., and Zarzeczna, M. (2000) Flow cytometric estimation of the plasma membrane diversity of transplantable melanomas, using Annexin V. *Folia Histochem. Cytobiol.* **38,** 41–43.
21. Geldwerth, D., Helley, D., de Jong, K., et al. (1999) Detection of phosphatidylserine surface exposure on human erythrocytes using Annexin V-ferrofluid. *Biochem. Biophys. Res. Commun.* **258,** 199–203.
22. Hammill, A. K., Uhr, J. W., and Scheuermann, R. H. (1999) Annexin V staining due to loss of membrane asymmetry can be reversible and precede commitment to apoptotic death. *Exp. Cell Res.* **251,** 16–21.
23. Glander, H. J., and Schaller, J. (1999) Binding of Annexin V to plasma membranes of human spermatozoa: a rapid assay for detection of membrane changes after cryostorage. *Mol. Hum. Reprod.* **5,** 109–115.
24. van Heerde, W. L., Robert-Offerman, S., Dumont, E. et al. (2000) Markers of apoptosis in cardiovascular tissues: focus on Annexin V. *Cardiovasc Res.* **45,** 549–559.

18

Cell Adhesion Assays

William N. Scott

1. Introduction

Interactions with cell adhesion molecules determine the organization of tissues and mediate precise cell migrations during embryonic development, inflammation, the immune response, and wound repair ***(1–6)***. They are involved in the regulation of gene expression, growth, and differentiation, and are central to invasion and metastasis in cancer ***(7–13)***. Viral and bacterial pathogens may also use these molecules to selectively gain entrance to specific cells or tissues ***(14–16)***.

Cell adhesion molecules function by forming specific protein–protein or protein–carbohydrate bonds at the cell surface to mediate cell interactions. In addition, cell adhesion molecules or their receptors often form direct associations with cytoplasmic-based proteins, which allow these complexes to interact with the cytoskeleton and signal transduction pathways. In this respect, cell adhesion not only connects cells with other cells and the extracellular matrix, but also assists in communication between the external environs and signal transduction pathways within cells ***(17–19)***.

Molecules that mediate cell–cell or cell–matrix adhesion fall into two broad structural classes. One class is anchored to the plasma membrane, often as a transmembrane protein. These membrane-anchored molecules are generally receptors, homophilic adhesion molecules, or counter-receptors. They consist of an extracellular domain containing one or more specific cell interaction domains or sites, as well as a stalk region, a hydrophobic transmembrane domain, and a cytoplasmic domain or tail. This type of adhesion molecule is often involved in the transmembrane transmission of signals after binding to their target molecule and includes the cadherins, selectins, integrins, and the immunoglobulin superfamily.

The second broad class of adhesion molecules consists of cell-surface or extracellular matrix proteins that contain domains involved in cellular adhesion. Nearly all matrix proteins, including fibronectin, laminins, vitronectin, collagens, and many other extracellular proteins contain such sites. These proteins contain one or more cell-binding domains that are composed of a primary recognition motif consisting of a short

From: *Methods in Molecular Medicine, vol. 88: Cancer Cell Culture: Methods and Protocols*
Edited by: S. P. Langdon © Humana Press Inc., Totowa, NJ

peptide sequence (e.g., Arg-Gly-Asp or Leu-Asp-Val) and often a synergy site or other structural feature that substantially enhances receptor-binding specificity and affinity.

One feature common to both classes of adhesion molecule is their relatively moderate ligand affinity (K_d 10^{-6} to 10^{-4} *M*). This modest affinity is an important process that allows the dynamic changes that occur in cell attachment and detachment during cell migration.

Cell adhesion is often categorized into cell-to-cell adhesion and cell-to-substrate adhesion. In each case, the physical structures that mediate cell adhesion can be either specialized adhesive structures or extended regions of plasma membrane. For example, cells can initially adhere to other cells along large areas of plasma membrane using general-purpose adhesive molecules such as cadherins. They can also form attachments to each other by using specialized adhesive structures, such as desmosomes, adherens junctions, and tight junctions. Each type of junctional complex involves specific adhesion-molecule components, such as cadherins and associated cytoplasmic components involved in linkage to the actin-containing cytoskeleton. Fibroblasts can also form adhesions to other fibroblasts, but they characteristically form adhesive interactions with extracellular matrix molecules. Such cell-to-matrix adhesion sites can also be either broad zones or specialized structures. For example, rapidly migrating cells typically interact with tissue culture substrates through broad areas of contact. The entire basal surface of epithelial cells adheres via integrins to their underlying basement membranes. Specialized adhesive structures include the hemidesmosomes of certain epithelial cells and the focal adhesions of fibroblastic and endothelial cells. The protein composition of the complexes involved in cell-to-matrix adhesions can sometimes involve a few of the same proteins as in cell-to-cell adhesions, such as vinculin and actin, but they mainly consist of distinct types of adhesive, cytoskeletal, and signal transduction molecules.

1.1. Major Families of Cell Adhesion Molecules

1.1.1. Cadherins

Cadherins comprise a large family of proteins centrally involved in cell-to-cell adhesive interactions (*see* **Fig. 1**). These molecules mediate adhesion over broad expanses of cell–cell contact or through more organized foci of adhesion such as adherens junctions. Cadherins on one cell generally bind in homophilic fashion to the same type of cadherin on other cells by means of specific cell-interaction domains that can include the short peptide recognition sequence His-Ala-Val ***(20)***. In this way they act as both receptor and ligand.

Sharing a considerable degree of homology at the amino acid level (40–60%), cadherins are a family of calcium dependent molecules of which there are at least 11 different human forms ***(21–23)***. They are responsible for selective cell–cell adhesion during embryogenesis and assist in the distribution and positioning of cells during development ***(21,24)***. Despite their importance, little is known about the regulatory mechanisms controlling the differential expression of these molecules.

The principle forms of cadherins are neuronal (N)-cadherin, placental (P)-cadherin and epithelial (E)-cadherin. The N-cadherin is predominant in neural tissues and has

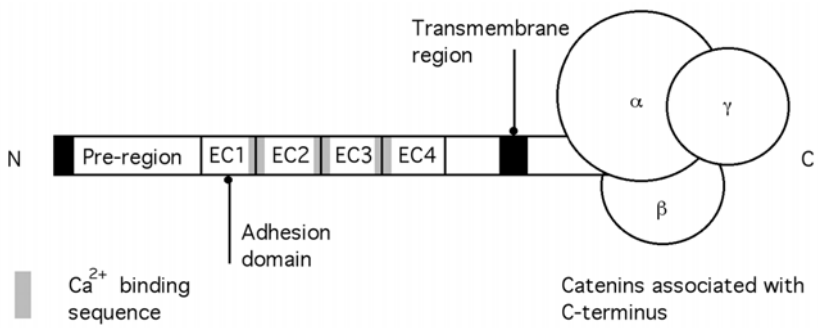

Fig. 1. Human E-cadherin structure. Mature cadherins are derived from a precursor by cleavage of the Pre-region. Each cadherin molecule has a number of cadherin repeats (EC1–EC4) with integrated calcium binding regions. The cytoplasmic sequence that interacts with catenins is highly conserved between cadherins.

been identified in a soluble form, fragments of which have been shown to retain positive biological activity ***(25)***. Permanent expression of P-cadherin is limited to epidermis, mesothelium, and corneal epithelium ***(2)***. E-cadherin expression is restricted to nonneuronal epithelial tissue ***(26)***. Loss of E-cadherin has been linked with the invasive behavior of tumor cells ***(13,27,28)***, and elevated levels of soluble E-cadherin fragments have been found in the circulation of patients with advanced stages of cancer ***(8)***.

Cadherins are synthesized as a precursor polypeptide which undergoes posttranslational modifications to form a mature protein between 723 and 748 residues long containing 3–5 internal repeats of approx 110 amino acids in the external domain ***(23, 29)***. Repeats 1–3 each contain Ca^{2+} binding sites with the motif DxNDN or DxN, in addition to the highly conserved cluster, LDRExxxYxL ***(21)***.

The cytoplasmic domain of cadherin molecules characteristically binds to catenins, which provide direct linkages to the actin cytoskeleton (e.g., via α- , β-, and γ catenins). Catenins are essential for cadherin function and assist in the formation of the cell junctions in epithelial cells that maintain cell polarity ***(3,28,30)***. A number of gene products of the *src* proto-oncogene family have been reported to be expressed at adherens junctions, and it has been suggested that these kinases may be associated with cadherin phosphorylation and signal transduction ***(19,21,22)***.

1.1.2. Integrins

Integrins are nearly ubiquitous cell surface receptors for a wide variety of extracellular matrix proteins, as well as for counter-receptor ligands on other cells ***(31,32)***. They consist of a head domain containing a ligand-binding site, two spacer runs, and usually short cytoplasmic domains. There are more than 20 distinct integrin subunits, which are combined to form heterodimers that always consist of one α and one β subunit (**Fig. 2**).

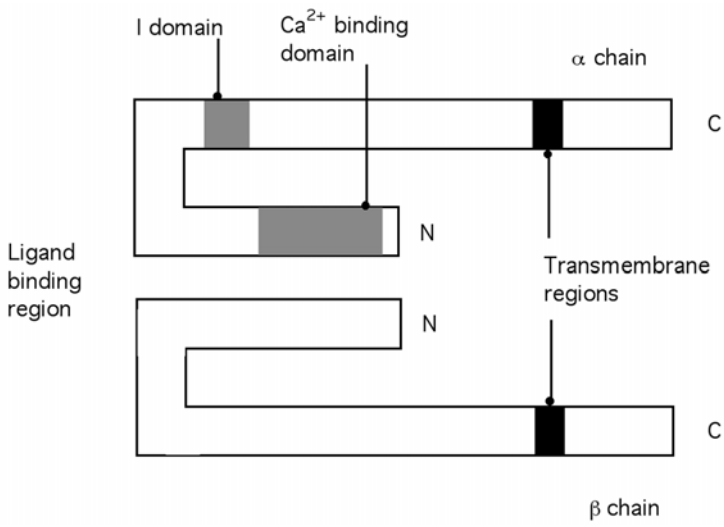

Fig. 2. Human basic integrin structure showing the constitutive α and β subunits.

Genetic loss of almost any integrin subunit leads to disease or death, often during embryonic development or near the time of birth ***(32–34)***. In addition, there is considerable data implicating integrins in signaling pathways, transmitting signals both into and out of cells ***(35–37)***.

The integrins bind to a wide variety of extracellular matrix proteins, including fibronectin, fibrinogen, laminin, collagen, thrombospondin, and vitronectin. They may also bind to members of the immunoglobulin superfamily. Most integrins bind to more than one ligand and exhibit different specificities depending on the cell type in which they are expressed. The existence of alternatively spliced variants differing in the extracellular domain may be important in altering ligand binding affinity.

The binding site for some integrins is an Arg-Gly-Asp sequence on extracellular matrix proteins ***(38)***. Integrin binding is often inhibited by depletion of divalent cations. The cytoplasmic domains of both subunits also appear to be required for binding to cytoskeletal components.

Integrin mediated adhesion generally requires activation of the receptor by specific signals resulting in a conformational change that enables ligand binding. Ligand occupation may then trigger a series of intracellular events.

Studies have also shown that the regulation of integrin function may involve phosphorylation of the β-subunit cytoplasmic domain ***(39).*** Serine and tyrosine phosphorylation on the β4 subunit and a number of other β subunits all possess potential tyrosine phosphorylation sites. The α subunit cytoplasmic domain does not appear to be involved in the role of ligand binding although it may be important in the translation of signaling into subsequent cellular events ***(37,40)***.

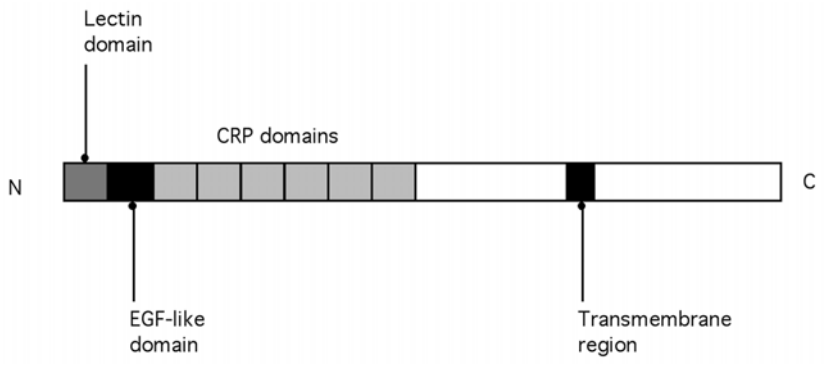

Fig. 3. Human E-selectin structure showing the location of the three types of protein domain.

It is of interest that multiple integrins that bind to the same ligand may be co-expressed on the same cell type. The apparent redundancy may be explained by the fact that differing cellular responses may be affected by different integrin cytoplasmic domains in response to common ligands. As such, the presence of variant cytoplasmic domains on several integrin subunits may add even more versatility to intracellular signaling processes.

1.1.3. Selectins

The selectins **(Fig. 3)** are represented by a group of three Ca^{2+}-dependent carbohydrate binding proteins (E-, P-, and L-selectin), that are involved in the initial attachment of flowing leukocytes to the blood vessel wall during the capture and rolling step of the inflammatory adhesion mechanism. They are constructed from three types of protein domain. At the C-terminal lies the lectin domain, similar to the Ca^{2+}-dependent configurations found in a range of proteins, including serum glycoproteins and extracellular matrix proteoglycans. This is followed by a repeat, initially identified in epidermal growth factor (EGF), with varying numbers of short consensus repeats similar to those found in complement-regulatory proteins (CRP domains) and a short cytoplasmic tail ***(41)***. Considerable data indicate that both the lectin and EGF domains are directly involved in cell adhesion and may determine binding specificity ***(42)***. Monoclonal antibodies, which block selectin-mediated adhesion, map to the lectin domain confirming the importance of this region. The role for the EGF-domain is unclear, as it has been shown for E-selectin that deletion of this domain abolishes cell adhesion ***(43)***. However, it may be required to maintain the structural conformation of the lectin domain, although it may have a more direct role in the cell through recognition of a ligand protein component ***(44)***. The role of the CRP repeats also remains unresolved, although it has been suggested that they assist with counter-receptor interactions and act by extending the lectin-EGF domain away from the cell surface and by increasing molecular flexibility.

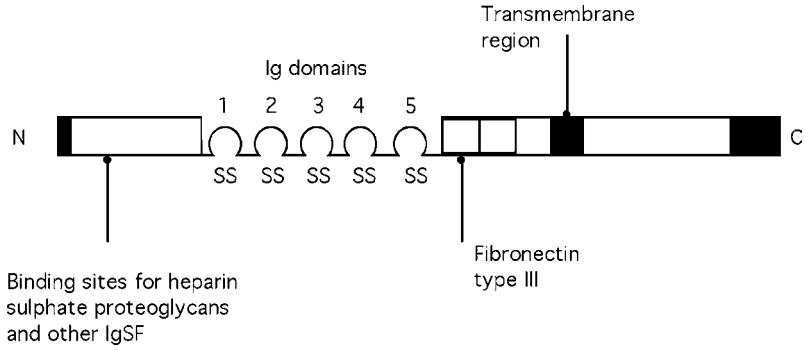

Fig. 4. Generalized structure of immunoglobulin superfamily.

Recent studies suggest that the selectins are involved in some of the earliest events leading to leukocyte extravasion. In regions of inflammation, leukocytes move towards the capillary edge and begin to role along the endothelium. This has been demonstrated in vitro and in vivo for both L- and P-selectin. A function with E-selectin in leucocyte rolling has yet to be identified.

The rolling process requires an interaction between a receptor on the endothelial cell and a counter-receptor on a transient leucocyte. This interaction is then broken and the cycle repeated. Under these conditions, when cells are moving rapidly in relation to one another, it is the rate of reaction rather than the absolute affinity that may be more important.

1.1.4. Immunoglobulin Superfamily

This group of adhesion molecules is the most abundant of cell surface molecules, accounting for approx 50% of leukocyte surface glycoproteins. They function during development and in the regulation of the immune system; activities that involve complex interactions among a diverse array of cell types ***(45,46)***. They are multifunctional, with the majority of molecules serving both as adhesion molecules and signal transducers ***(47,48)***. Their structure is composed of repeated domains considered to have evolved from a single ancestral unit. The basic motif is similar to that found in immunoglobulins and contains between 70 and 110 amino acid residues organized into two parallel β sheets **(Fig. 4)**. The immunoglobulin domain has evolved by mutation and selection to serve many different functions, although adhesion appears to be its prime purpose ***(49)*.**

With the exception of a few highly conserved residues, there is an enormous variation in the primary structure of these adhesion molecules, although the tertiary structure remains relatively constant. It has been proposed that this basic structure serves as a template on which unique determinants can be displayed ***(49)***. This creates diversity in domain interaction between individual members of this group; binding sites can be localized to single domains or involve extended faces and multiple domains.

The sites can be located in *N*-terminal membrane distal domains or positioned internally. Studies have also located these sites in membrane proximal domains ***(50)***. As such, molecules can interact using the *N*-terminal distal domains in a heterotypic manner or by homotypic binding mediated by internal domains and requiring antiparallel alignment of interdigitated molecules ***(49,50)***. Quantitative studies have shown that binding between individual immunoglobulin superfamily members generally of low affinity although considerable adhesive strength can be effected through multivalent presentation ***(51)***.

1.1.5. Extracellular Matrix Proteins

As previously mentioned, the second broad class of adhesion molecules consists of cell surface or extracellular matrix proteins that contain domains directly involved in cellular adhesion. Nearly all matrix proteins including fibronectin, laminins, vitronectin, collagens, and many other extracellular proteins contain such sites. These proteins contain one or more cell-binding domains composed of a primary recognition motif consisting of a short peptide sequence (e.g., Arg-Gly-Asp or Leu-Asp-Val) and often a synergy site or other structural feature that substantially enhances receptor-binding specificity and affinity.

One interesting aspect of their function involves their effects on adsorption or attachment to a substrate. Molecules such as fibronectin and vitronectin circulate in blood at relatively high concentrations (e.g., 100–300 μg/mL), yet binding to cell surface receptors is relatively limited. On the other hand, even very low concentrations of the same proteins (1–10 μg/mL) bind to substrates and then mediate strong cell adhesion. This functional enhancement of binding of cells to molecules already attached to a substrate has been ascribed to the activation of molecules such as fibronectin and vitronectin by conformational changes. These changes may be the result of binding to a substrate, to enhanced ability to interact with cells as a result of multivalency, or to a physical chemical enhancement in free energy (overall avidity) resulting from immobilization of a ligand. Whatever the mechanisms, it is clear that the binding of extracellular adhesion molecules to substrates can enhance functional activity, which appears to be important biologically to generate strong adhesion only when a circulating ligand is immobilized. For example, in wounds, immobilized fibronectin forms a crucially important provisional matrix that permits adhesion and migration of cells to close the wound.

1.2. Adhesion Assays

As the name suggests, adhesion assays measure cell adhesion. However, because adhesion is a complex process involving receptor-ligand binding, modulation of intracellular signaling, and cytoskeletal modulation, these assays can also be a source of information about other cellular events and a means of probing the contribution of these processes to adhesion. In general, adhesion assays are used to test whether a certain cell type can adhere to a specific adhesive substrate and to test the sensitivity of a particular cell-substrate interaction to inhibitors.

There are two main types of cell adhesion assay and these may be used to quantitate either cell attachment or spreading. Each assay measures different parameters of the

adhesion process. Cell attachment assays generally determine the numbers of cells that can attach to a substrate after a specific period of time, and cell spreading systems gauge the percentage of cells showing spread morphologies on a specific substrate.

Both the spreading and attachment assays measure percent adhesion, and the level of adhesion obtained depends on the cell type and adhesive substrate under study. In spreading assays, a level of 80% should be anticipated, although higher levels can be obtained; background level of spreading should be as low as possible. The level of adhesion in attachment assays is usually not as high as for spreading, but 60–70% should be attainable. The background level of attachment should also be as low as possible in both types of assay.

The effective analysis of cell adhesion depends on the characterization of both the cells and substrate in vitro. Cell–substrate adhesion assays are frequently used to examine the ability of cells to attach to matrix molecules, such as collagen, fibronectin, or laminin, and to determine which specific adhesion receptors are involved by using antibodies or synthetic peptide inhibitors. The functions of cytoplasmic elements such as cytoskeletal proteins and signaling molecules can also be evaluated with these assays by using inhibitors of intracellular function.

A number of considerations will affect the decision to use a cell spreading assay or a cell attachment assay. Spreading assays take longer to perform, but are less prone to nonspecificity. For example, many molecules can mediate attachment of cells that are not physiologically relevant, but very few of these molecules can mediate morphological spreading. Importantly, by observing cells in a spreading assay, a lot of information can be gained about the biological response of the cells to the substrate. The morphology of cells can differ even if the percent spreading is the same. Additionally, now that signaling mechanisms that control cell morphology are better understood, spreading assays can give indirect indications of the intracellular events that are triggered by certain substrates. Spreading assays are more sensitive when used to measure the inhibitory activity of an exogenous agent. The readout from the spreading assay is more reliant on multiple adhesive interactions, and partial disruption by an inhibitor is sufficient to see a biological effect. A greater degree of receptor blockade is probably needed to observe an inhibitory effect in an attachment assay. Finally, as spreading assays do not need replicate wells, it is easier to construct detailed dose-response curves.

Nonetheless, use of attachment assays is sometimes obligatory, because not all cells are able to spread and because some cells only spread on certain substrates. It is misleading to think that attachment assays measure single adhesive contacts; multiple contacts are needed for a cell to withstand the washing steps in an attachment assay. Nevertheless, fewer contacts are needed for attachment than for a cell to spread.

In this chapter, two semiquantitative assays for measuring cell adhesion to a substrate are detailed. The first is a cell spreading assay in which adhesion is evaluated by determining the percentage of cells that spread on a substrate, using microscopy to count spread cells. The second assay quantitates cell attachment after washing out nonattached cells, using a colorimetric assay to determine cell number. The relative advantages of each of these widely used assays are compared.

2. Materials

2.1. Spreading Assay

1. Cells of interest.
2. Adhesion molecule of interest, e.g., fibronectin, laminin, or collagen IV.
3. Phosphate buffered saline (PBS).
4. 96-well tissue culture plates.
5. Bovine serum albumin (BSA) or casein.
6. Ethylene glycol tetraacetic acid (EGTA) or trypsin/ethylenediaminetetraacetic acid (EDTA).
7. Dulbecco's modified Eagle medium (DMEM).
8. 3% Glutaraldehyde in 0.1 *M* cacodylate buffer, pH 7.4.
9. 0.1% Sodium azide in PBS.
10. Microscope.

2.2. Attachment Assay

1. Cells of interest.
2. Adhesion molecule of interest e.g., fibronectin, laminin, or collagen IV.
3. PBS.
4. 96-Well tissue culture plates.
5. BSA or casein.
6. EGTA or trypsin/EDTA.
7. DMEM medium.
8. 10% Formaldehyde in PBS.
9. 0.1% Crystal violet.

3. Methods

3.1. Spreading Assay

In this protocol, cells are added to a microtiter plate coated with an adhesive molecule and are incubated to allow spreading. After the incubation period, all cells are fixed with glutaraldehyde and the wells examined for the percent of cell spread using morphological criteria and phase-contrast microscopy (*see* **Note 1**).

1. Dissolve the adhesion molecule in a suitable solvent and dilute in PBS prior to coating microtiter plates (*see* **Note 2**). For adhesion to extracellular matrices or purified extracellular matrix molecules a concentration of 1–20 µg/mL is usually adequate. If a nonmatrix molecule or a complex mixture is to be tested, a higher concentration may be required (*see* **Note 3**).
2. Add the diluted adhesion molecule (100 µL) to the appropriate wells of a 96-well, bacteriological grade microtiter plate (*see* **Note 4**). Let blank wells remain empty to allow the measurement of background spreading on blocked plastic (*see* **Note 5**).
3. Incubate plates for 60 min at room temperature or overnight at 4°C. Time-course studies have shown that there is substantial coating of proteins on plastic within an hour at room temperature. This procedure allows the assay to be performed quickly. However, if the adhesion molecule binds weakly to plastic, the wells can usually be dried onto the plastic without detrimental effect.
4. After coating with adhesion molecule, block the wells for nonspecific attachment. Rinse each well with PBS (3X, 100 µL) and 200 µL 0.2% BSA or casein solution added to each

well. The plates should then be incubated for 45 min at 37°C and then washed extensively with PBS (3X, 100 µL) (*see* **Note 6**).

5. While the plates are being blocked, a suspension of the cells to be examined can be prepared by detachment from monolayer by means of treatment with 0.02% EGTA (a milder chelating agent than EDTA). Freshly isolated cells from tissue can also be used, but results are much more variable (*see* **Notes 7** and **8**).
6. Count cells using a hemocytometer, and resuspend at a population density of 10^5 cells/mL in warm DMEM preequilibrated with 5% CO_2 (*see* **Note 9**).
7. Aspirate the blocking solution and wash the wells with 100 µL PBS.
8. To determine spreading of specific cell types on given adhesion molecules, add 50 µL PBS followed by 50 µL cell suspension to the appropriate wells. To examine the effects of exogenous agents (e.g., antibodies, peptides) on spreading, add 50 µL of exogenous agent followed by 50 µL cells. In this case, also add 50 µL PBS followed by 50 µL cells to control wells (*see* **Note 10**).
9. Incubate at 37°C in a 5% CO_2 incubator for 60–90 min. To ensure good spacing of cells, lay the plate on a level surface and avoid excessive disturbance (*see* **Note 11**).
10. Aspirate medium and fix cells by directly adding 100µL of 3% glutaraldehyde in 0.1 *M* cacodylate buffer, pH 7.4, to each well and incubate for 30 min at room temperature.
11. Remove fixative and store cells in 200 µL PBS containing 0.1% NaN_3.
12. Fixed assay plates can be stored at 4°C for several weeks protected from evaporation.
13. Using an inverted phase-contrast microscope, quantify percent spreading in each well by counting three separate, randomly selected fields of 100 cells each. Use specific morphological criteria, such as a phase-dark cell body and cytoplasm that is visible around the entire circumference of the nucleus, to identify cells with spread morphology. Alternatively, image analysis software can be used to measure average cell area.

3.2. Attachment Assay

This procedure measures the attachment of cells to microtiter plates coated with an adhesion molecule. After incubation, nonattached cells are removed and the remaining cells fixed with formaldehyde, washed, and stained with crystal violet. The assay is similar to the spreading assay just described.

1. Dissolve substrates in a suitable solvent and dilute in PBS. Prepare sufficient wells to perform assays in triplicate or quadruplicate. Leave sufficient blank wells to determine 100% attachment at three cell concentrations and to determine background binding of crystal violet to plastic (*see* **Notes 1–3**).
2. Incubate 60 min at room temperature or overnight at 4°C. Alternatively, the substrate can be dried onto the plastic (*see* **Note 4**).
3. Rinse with PBS (3X, 100 µL) and block with 200 µL 0.2% BSA or casein solution added to each well.
4. The plates should then be incubated for 45 min at 37°C and then washed extensively with PBS (3X, 100µL) (*see* **Note 6**). Block wells for background crystal violet binding, but not those for 100% attachment.
5. While the plates are being blocked, prepare a suspension of the cells by means of treatment with 0.02% EGTA (*see* **Note 8**). Prepare a sufficient number of cells to perform assays at least in triplicate. Determine cell density and prepare working cell suspensions at 5×10^5 cells/mL in warm DMEM preequilibrated with 5% CO_2 (*see* **Notes 9** and **12**).
6. Incubate for 10 min at 37°C with the lid off in a CO_2 incubator.

7. Aspirate the BSA solution from the plates and wash the wells with 100 µL PBS. To estimate 100% attachment, dilute cells to 20, 50, and 100% of the working cell suspension using warm DMEM equilibrated with 5% CO_2.
8. Add 50 µL PBS followed by 50 µL cells to uncoated wells (*see* **Note 13**).
9. Using cell dilutions and extrapolating the resulting graph is the most accurate way of determining 100% attachment, as the absorbance value for 100% attachment with undiluted cells may be off the linear range of the plate reader.
10. To ensure good spacing of cells, guard against swirling, tapping, or shaking the wells once cells have been added. In the author's experience, a single pipeting of cells down the side of the well and into the DPBS solution produces good dispersion (*see* **Note 14**).
11. To test attachment of specific cell types to given adhesion molecules, add 50 µL PBS followed by 50 µL cells to the appropriate wells. To examine the effects of exogenous agents on attachment, add 50 µL of exogenous agent followed by 50 µL cells. In this case, also add 50 µL PBS followed by 50 µL cells to control wells (*see* **Note 10**).
12. Incubate 15–20 min at 37°C in a 5% CO_2 incubator with the microtiter plate lid off (*see* **Note 15**).
13. Fix cells only in the wells to be used for determining 100% attachment by adding 100 µL of 10% formaldehyde in PBS. Remove nonadherent and loosely attached cells from remaining wells by gently washing the wells with 100 µL PBS and cut-off pipet tips (*see* **Note 16**).
14. Aspirate the final wash and fix attached cells by adding 100 µL of 4% formaldehyde and incubating for 30 min at room temperature.
15. Wash wells three times with 100 µL water and allow to air dry.
16. Add 100 µL of 0.1% crystal violet solution to each well and incubate for 30 min at room temperature.
17. Wash wells three times with 200 µL water and again air dry. Solubilize dye in 100 µL acidified methanol and incubate with gentle agitation for 5 min at room temperature. Measure absorbance at 570 nm using a microtiter plate reader. Subtract background crystal violet staining from all experimental and 100% attachment results. Plot data from 20, 50, and 100% inocula and determine the value for 100% attachment by extrapolation. As an alternative, cell number may be determined by MTT assay (*see* Chapter 14).

Notes

1. The major problem to be encountered in both assays is that cells may fail to adhere. Many factors can lead to this, including coating plates with insufficient amounts of adhesive substrate, using bad batches of adhesive substrate, using poor protein-binding plates, using cultures that are growing poorly or that have a mycoplasma infection, having variations in pH during adhesion, and (for attachment assays) overwashing.
2. It is important to remember that the handling of adhesion molecules prior to dilution varies; some molecules (for example, fibronectin) are best thawed quickly at 37°C, while others (for example, laminin) are best thawed over night at 4°C. Occasionally, it may be necessary to measure adhesion of cells to peptides rather than to proteins. In this case, peptides may be conjugated to inert protein carriers in order to permit more efficient immobilization
3. The concentration of adhesion molecule required for coating depends on a number of factors, including the size of the molecule, the efficiency with which it coats plastic, and the apparent affinity with which it is bound by cellular receptors. In most cases, spreading assays are used to measure the adhesion of cells to extracellular matrices or

purified extracellular matrix molecules. The key components of such matrices are usually large macromolecules that coat plastic relatively well; they are also bound with at least moderate affinity by cells. For these reasons, a concentration range between 1 and 20 μg/mL is usually adequate, although it is advisable to carry out a range-finding dose-response experiment before focusing on a narrow range. If a nonmatrix molecule or a complex mixture is to be tested, a higher concentration should be used.

4. Tissue culture plates are not ideal for spreading and attachment experiments as these plates may enhance the nonspecific attachment of cells. Bacteriological grade plates are preferable although these plates may be more difficult to coat with protein substrate.
5. There is generally no need to carry out spreading assays with replicate wells, as quantification is normally performed by counting multiple fields from within the same well.
6. The time can be varied at this step; 45 min is probably the minimum time for efficient blocking of plastic, but longer times are not detrimental. Even overnight blocking at 4°C may also be used although this increases the possibility of substrate leaching.
7. For cell spreading assays, an important parameter is the health of the cells. Cultures should be actively growing, but should have been passaged >24 hr previously. Relatively poor spreading responses have been observed in cells that were passaged the day before a spreading assay.
8. Trypsin, EDTA, or trypsin/EDTA solutions may also be used to detach adherent cells although the cells may require a period of recovery prior to assay. It is important to guard against clumping or aggregation of cells during centrifugation and resuspension.
9. For most cells, this density prevents cells added to the microtiter plate from aggregating. If cells contact each other, substrate-dependent adhesive effects can be altered; on the other hand, if cells are too sparse, quantification becomes difficult.
10. When cells and PBS are combined in the coated well, it is important that the DMEM/PBS mixture has the opportunity to equilibrate as rapidly as possible with CO_2 in order to reestablish the buffer system.
11. A number of adhesion studies have focused on divalent cation-dependence, particularly when integrins are involved. Calcium, magnesium, and manganese ions are the most frequently studied. It is worth noting that manganese ions will precipitate phosphate from PBS. Therefore, in the presence of manganese, HEPES-buffered saline should be used in place of PBS.
12. Use pipet tips that have their ends cut off for attachment assays. This helps to prevent coated proteins and/or cells from being washed off directly by a fine stream of liquid.
13. Cells can also be plated on poly-L-lysine-coated plastic to determine 100% attachment.
14. For attachment assays, the key parameter is the washing protocol. When washing unattached cells, different cells respond differently to tapping and washing, and it is recommended to vary the number of tapping or washing cycles to obtain the best signal-to-noise ratio (i.e., attachment to an adhesive substrate compared to attachment to BSA-blocked plastic). Cut-off pipet tips are particularly important at this stage to avoid removal of attached cells. More specific problems include clumping of cells either in the center of a well or around the perimeter (which may be caused by swirling the plate). In addition, large errors in attachment assays can result from inaccurate pipeting, which can come from the use of multichannel pipets, or suboptimal washing of wells.
15. The incubation time for attachment assays may need to be adjusted depending on the cell type, as some cells adhere more quickly than others.
16. This is the most critical stage in an attachment assay, and needs to be optimized for each cell type used.

References

1. Yoshida-Noro, C., Suzuki, N. and Takeichi,M. (1984) Molecular nature of the calcium-dependent cell–cell adhesion system in mouse teratocarcinoma and embryonic cells studied with a monoclonal antibody. *Dev. Biol.* **101,** 19–27.
2. Nose, A. and Takeichi, M. (1986) A novel cadherin cell adhesion molecule: its expression patterns associated with implantation and organogenesis of mouse embryos. *J. Cell Biol.* **103,** 2649–2658.
3. Ranscht, B. (1994) Cadherins and catenins: interactions and functions in embryonic development. *Curr. Opin. Cell Biol.* **6,** 740–746.
4. Cartwright, J. E., Whitley, G. S., and Johnstone, A. P. (1995) The expression and release of adhesion molecules by human endothelial cell lines and their consequent binding of lymphocytes. *Exp. Cell Res.* **217,** 329–335.
5. Borradori, L. and Sonnenberg, A. (1999) Structure and function of hemidesmosomes: more than simple adhesion complexes. *J. Invest. Dermatol.* **112,** 411–418.
6. Bloom, B. J., Nelson, S. M., Alario, A. J., Miller, L. C., and Schaller, J. G. (2002) Synovial fluid levels of E-selectin and intercellular adhesion molecule-1: relationship to joint inflammation in children with chronic arthritis. *Rheumatol. Int.* **22,** 175–177.
7. Navarro, P., Gomez, M., Pizarro, A., Gamallo, C., Quintanilla, M., and Cano, A. (1991) A role for the E-cadherin cell–cell adhesion molecule during tumor progression of mouse epidermal carcinogenesis. *J. Cell Biol.* **115,** 517–533.
8. Katayama, M., Handa, M., Araki, Y., et al. (1994) Soluble E-cadherin fragments increased in circulation of cancer patients. *Br. J. Cancer* **69,** 580–585.
9. Behrens, J. (1995) Cell contact, differentiation, and invasiveness of epithelial cells. *Invasion Metastasis* **14,** 61–70.
10. Figarella-Branger, D., Lepidi, H., Poncet, C., et al. (1995) Differential expression of CAM, neural CAM and E-cadherin in ependymomas and choroid plexus tumors. *Acta Neuropathol.* **89,** 248–257.
11. Gardener, M. J., Jones, L. M., Catterall, J. B., and Turner, G. A. (1995) Expression of cell adhesion molecules on ovarian tumour cell lines and mesothelial cells, in relation to ovarian cancer metastasis. *Cancer Lett.* **91,** 229–234.
12. Giannelli, G. and Antonaci, S. (2000) Biological and clinical relevance of laminin-5 in cancer. *Clin. Exp. Metastasis* **18,** 439–443.
13. Joo, Y. E., Rew, J. S., Choi, S. K., et al. (2002) Expression of E-cadherin and catenins in early gastric cancer. *J. Clin. Gastroenterol.* **35,** 35–42.
14. Shirayoshi, Y., Nose, A., Iwasaki, K., and Takeichi, M. (1986) N-linked oligosaccharides are not involved in the function of a cell–cell binding glycoprotein E-cadherin. *Cell Struct. Funct.* **11,** 245–252.
15. Sansonetti, P. J., Mounier, J., Prevost, M. C., and Mege, R. M. (1994) Cadherin expression is required for the spread of Shigella flexneri between epithelial cells. *Cell* **76,** 829–839.
16. Mengaud, J., Ohayon, H., Gounon, P., Mege, R. M. and Cossart, P. (1996) E-cadherin is the receptor for internalin, a surface protein required for entry of *L. monocytogenes* into epithelial cells. *Cell* **84,** 923–932.
17. Gutwein, P., Oleszewski, M., Mechtersheimer, S., Agmon-Levin, N., Krauss, K., and Altevogt, P. (2000) Role of Src kinases in the ADAM-mediated release of L1 adhesion molecule from human tumor cells. *J. Biol. Chem.* **275,** 15,490–15,497.
18. Kim, L. T., Wu, J., Bier-Laning, C., Dollar, B. T. and Turnage, R. H. (2000) Focal adhesion kinase up-regulation and signaling in activated keratinocytes. *J. Surg. Res.* **91,** 65–69.

19. Alahari, S. K., Reddig, P. J. and Juliano, R. L. (2002) Biological aspects of signal transduction by cell adhesion receptors. *Int. Rev. Cytol.* **220,** 145–184.
20. Nose, A., Tsuji, K., and Takeichi, M. (1990) Localization of specificity determining sites in cadherin cell adhesion molecules. *Cell* **61,** 147–155.
21. Takeichi, M. (1990) Cadherins: A molecular family important in cell–cell adhesion. *Ann. Rev. Biochem.* **59,** 237–252.
22. Koch, P. J. and Franke, W. W. (1994) Desmosomal cadherins: Another growing multigene family of adhesion molecules. *Curr. Opin. Cell Biol.* **6,** 682–687.
23. Chothia, C. and Jones, E. Y. (1997) The molecular structure of cell adhesion molecules. *Ann. Rev. Biochem.* **66,** 823–862.
24. Edelman, G. M. and Crossin, K. L. (1991) Cell adhesion molecules: Implications for a molecular histology. *Ann. Rev. Biochem.* **60,** 155–190.
25. Paradies, N. E. and Grunwald, G. B. (1993) Purification and characterisation of NCAD90, a soluble endogenous form of N-cadherin, which is generated by proteolysis during retinal development and retains adhesive and neurite-promoting function. *J. Neurosci. Res.* **36,** 33–45.
26. Shimoyama, Y., Hirohashi, S., Hirano, S., et al. (1989) Cadherin cell-adhesion molecules in human epithelial tissues and carcinomas. *Cancer Res.* **49,** 2128–2133.
27. Birchmeier, W. and Behrens, J. (1994) Cadherin expression in carcinomas: role in the formation of cell junctions and the prevention of invasiveness. *Biochim. Biophys. Acta.* **1198,** 11–26.
28. Mialhe, A, Louis, J., Montlevier, S., et al. (1997) Expression of E-cadherin and alpha-, beta- and gamma-catenins in human bladder carcinomas: are they good prognostic factors? *Invasion Metastasis* **17,** 124–137.
29. Yap, A. S., Brieher, W. M., and Gumbiner, B. M. (1997) Molecular and functional analysis of cadherin-based adherens junctions. *Ann. Rev. Cell Dev. Biol.* **13,** 119–146.
30. Hajra, K. M. and Fearon, E. R. (2002) Cadherin and catenin alterations in human cancer. *Genes Chromosomes Cancer*. **34,** 255–268.
31. Albelda, S. M. and Buck, C. A. (1990) Integrins and other cell adhesion molecules. *FASEB J.* **4,** 2868–2880.
32. Haas, T. A. and Plow, E. F.(1994) Integrin-ligand interactions: a year in review. *Curr. Opin. Cell Biol.* **6,** 656–662.
33. Larson, R. S. and Springer, T. A. (1990) Structure and function of leukocyte integrins. *Immunol. Rev.* **114,** 181–217.
34. Tang, D. G. and Honn, K. V. (1995) Adhesion molecules and tumor metastasis: an update. *Invasion Metastasis* **14,** 109–122.
35. Hemler, M. E., Kassner, P. D., and Chan, B. M. (1992) Functional roles for integrin alpha subunit cytoplasmic domains. *Cold Spring Harb Symp Quant Biol.* **57,** 213–220.
36. Ruoslahti E. (1991) Integrins *J. Clin. Invest.* **87,** 1–5.
37. Hynes R. O. (1992) Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* **69,** 11–25.
38. D'Souza, S. E., Ginsberg, M. H., and Plow, E. F. (1991) Arginyl-glycyl-aspartic acid (RGD): a cell adhesion motif. *Trends Biochem. Sci.* **16,** 246–250.
39. Hibbs, M. L., Xu, H., Stacker, S. A., and Springer, T. A. (1991) Regulation of adhesion of ICAM-1 by the cytoplasmic domain of LFA-1 integrin beta subunit. *Science* **251,** 1611–1613.
40. Chan, B. M. C., Elices, M. J., Murphy, E and Hemler M. E. 1992. Adhesion to vascular cell adhesion molecule 1 and fibronectin. Comparison of $\alpha 4\beta 1$ (VLA-4) and $\alpha 4\beta 7$ on the human B cell line JY. *J. Biol. Chem.* **267,** 8366–8370.

41. Rosen, S. D. and Bertozzi, C. R. (1994) The selectins and their ligands. *Curr. Opin. Cell Biol.* **6,** 663–673.
42. Tedder, T. F., Steeber, D. A., and Pizcueta, P. (1995) The selectins: vascular adhesion molecules. *FASEB J.* **9,** 866–873.
43. Pigott. R., Needham, L. A., Edwards, R. M., Walker, C., and Power, C. (1991) Structural and functional studies of the endothelial activation antigen endothelial leucocyte adhesion molecule-1 using a panel of monoclonal antibodies. *J. Immunol.* **147,** 130–135.
44. Seigelman, M. H., Cheng, I. C., Weissman, I. L. and Wakeland, E. K. (1990) The mouse lymph node homing receptor is identical with the lymphocyte cell surface marker Ly-22: role of the EGF domain in endothelial binding. *Cell* **61,** 611–622.
45. Yoshihara, Y., Oka, S., Ikeda, J., and Mori, K. (1991) Immunoglobulin superfamily molecules in the nervous system. *Neurosci. Res.* **10,** 83–105.
46. Nelson, R. M., Venot, A., Bevilacqua, M. P., Linhardt, R. J., and Stamenkovic, I. (1995) Carbohydrate-protein interactions in vascular biology. *Ann. Rev. Cell Dev. Biol.* **11,** 601–631.
47. Crossin, K. L. and Krushel, L. A. (2000) Cellular signaling by neural cell adhesion molecules of the immunoglobulin superfamily. *Dev. Dyn.* **218,** 260–279.
48. Juliano, R. L. (2002) Signal transduction by cell adhesion receptors and the cytoskeleton: functions of integrins, cadherins, selectins, and immunoglobulin-superfamily members. *Ann. Rev. Pharmacol. Toxicol.* **42,** 283–323.
49. Holness, C. L., and Simmons, D. L. (1994) Structural motifs for recognition and adhesion in members of the immunoglobulin superfamily. *Cell* **107,** 2065–2070.
50. Williams, A. F., and Barcaly, A. N. (1988) The immunoglobulin superfamily-domains for cell surface recognition. *Ann. Rev. Immunol.* **6,** 381–412.
51. Van der Merwe, P. A., Brown, M.H., Davis, S. J., and Barclay, A. N. (1993) Affinity and kinetic analysis of the interaction of the cell adhesion molecules rat CD2 and CD48. *EMBO J.* **12,** 4945–4954.

19

Radial Monolayer Cell Migration Assay

Michael E. Berens and Christian Beaudry

1. Introduction

Cell migration underlies fundamental features of embryonic development, wound healing, immune cell trafficking, and pathological processes such as cancer metastasis ***(1–3)***. Techniques that assess the biochemical and molecular mechanisms of cell migration afford insight into normal biological processes and the underpinnings of pathology.

The following technique describes a method by which cell migration can be simply, reproducibly, conveniently, and affordably investigated ***(4)***. The method involves guided deposition of cells to a predetermined circular location on a defined substrate of the investigator's choosing or construction. Once deposited, analysis of the rate of radial dispersion of the cells enables assessment of cell locomotion response to a solid substrate (haptokinesis), directional movement along a gradient of substrate (haptotaxis), movement towards or away from a soluble factor (chemotaxis), and locomotion changes coincident to different concentrations of soluble factors (chemokinesis) ***(5–7)***.

The platform of the assay is a modified microscope slide that enables subsequent immunostaining of the migrating cells for epitopes of interest, including adhesion molecules ***(8)***, signaling cascade mediators ***(9)***, cytoskeletal components ***(10)***, and cell-derived matrix proteins ***(11)***. Modulation of the migrating cells by treatment with pharmacological agents ***(12)***, antisense oligonucleotides ***(13)*** and antibodies ***(14,15)*** is readily achieved in this small-volume assay system. Washout studies can be performed to determine recovery after treatment removal or to follow pulse-chase changes. Multiple phenotypic endpoints can be measured in the same system, including migration, proliferation ***(16)***, gene expression ***(17,18)***, biochemical colocalization, and morphological correlates ***(19)***. Conventional upright epifluorescent microscopy and confocal microscopy can be employed following immunofluorescent staining for detailed assessment of colocalization of biochemical determinants of cell movement in the context of provoked cell response to taxis or kinesis treatments. Guided cell sedimentation of target cells onto anatomical structures from cryostat sections ***(20)***, onto monolayers of other cell types ***(21)***, or onto cell-derived complex extracellular matrices are readily accomplished ***(22)***.

From: *Methods in Molecular Medicine, vol. 88: Cancer Cell Culture: Methods and Protocols*
Edited by: S. P. Langdon © Humana Press Inc., Totowa, NJ

2. Materials

All the materials used in this protocol can be obtained from commercial sources.

1. Cell Sedimentation Manifold (CSM), sterilized and stored at 4°C (Creative Scientific Methods, Phoenix, AZ, www.cre8ive-sci.com), or custom glass cell sedimentation cylinders (*see* **Note 1**).
2. Manufactured silicone suspension pads (Creative Scientific Methods).
3. Teflon-printed microscope slides, sterilized by autoclave prior to use (Creative Scientific Methods).
4. Glass microscope slides, sterilized by autoclave prior to use.
5. Media appropriate to cell line.
6. 1X Phosphate-buffered saline (PBS), pH 7.4.
7. Bovine serum albumin (BSA).
8. Sterile water.
9. Surface treatment or protein coating (extracellular matrix) (*see* **Notes 2** and **3**).
10. 150-mm diameter Petri dish.
11. 35-mm diameter Petri dish.
12. 0. 2–2.0 µL and 40–200 µL Pipets

3. Methods

All manipulations must be executed in a sterile environment.

3.1. Sterilization of the Materials

1. Package the Cell Sedimentation Manifold in autoclave bags. Steam autoclave the manifold for 1 h to sterilize. Store at 4°C until ready to use.
2. Package the Teflon-masked and plain slides in autoclave bags. Steam autoclave the slides for 1 h to sterilize. Store at room temperature until ready to use.

3.2. Assembly of the Migration Assay

1. Place the slide in a 150-mm diameter Petri dish (capillary force created by adding a drop of water under the ends of the slide will prevent the slide from moving). Using the surface coating of choice, add 20 µL onto each well then incubate 1 h at 37°C.
2. After coating, rinse the wells with three changes of PBS. Block nonspecific binding sites with 0.1% BSA in PBS for 30 min at 37°C. Rinse twice with PBS and fill the wells with 40–50 µL of PBS and store at 4°C until ready to use.
3. Harvest the cells from culture according to standard protocols. Adjust cell concentration to approx 2000/µL (2×10^6/mL). Optimal cell concentration may vary depending on the cell type. Keep cell suspensions on ice.
4. Aspirate PBS from each well, pipet 40–50µL of media of choice onto each well. Carefully set the prechilled (4°C) CSM onto the Teflon-printed slide. Verify that the side ridges of the CSM rest over each side of the slide. Keep the slide with CSM in a 150-mm diameter Petri dish.
5. It is important to ensure that no air bubbles reside in the channels, which would prevent cells from falling through the channels onto the slide. Using a 0. 2–2.0-µL pipet with a sterile tip, place the empty tip vertically into the top of each channel, then carefully and slowly aspirate 2 µL of media up into each channel of the CSM, checking that media has filled each channel.
6. Slowly, place 1 µL of the cell suspension into each channel using a 0.2–2.0-µL pipet with a sterile tip held vertically. Be sure to avoid injecting an air bubble into the chan-

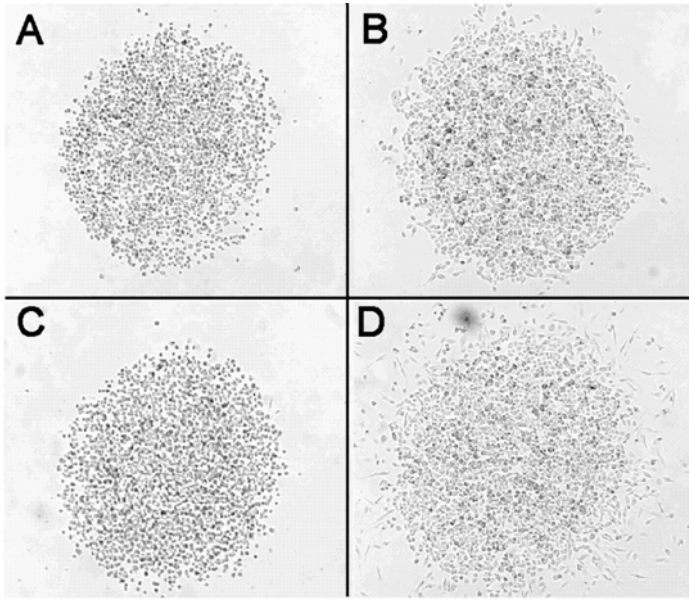

Fig. 1. Photomicrographs of SF767 human glioma cells on 0. 1% BSA **(A,B)** and on 10-µg/mL laminin **(C,D)**. Images shown in A and C were captured immediately after the CSM manifold had been removed. Images shown in B and D are images of the cells shown in A and C captured 24 h later.

nels (*see* **Note 4**). The cells must be carefully deposited into the channel opening. If the cells are injected too quickly into the channel, cells will disperse out of the bottom and become unusable for migration studies. Be sure to repeatedly resuspend the cell suspension before loading into channels and several times during use (*see* **Notes 5** and **6**).

7. Place the slides with CSM into a 37°C, 5% CO_2 tissue culture incubator for several hours to overnight to allow cell attachment. Place a 35-mm Petri dish filled with sterile water without a cover into the Petri dish to ensure 100% humidity.
8. After verifying cell attachment (*see* **Note 7**), gently remove the manifold and refeed each well very gently with appropriate solution using a 40–200-µL pipet with a sterile tip.

3.3. Assessment of Cell Migration

1. To prevent wells from drying and to facilitate optical imaging, a glass microscope slide can be used as a suspended cover slide. The cover slide can be mounted onto the Teflon-coated microscope slide using manufactured silicone suspension pads, which fit over the ends of the Teflon-coated slide and serve to suspend the cover slide 1 mm above the cell surface. This cover slide can be removed to allow treatment of the migrating cells or to enable media changes or pulse chases, for example.
2. Cell migration can be measured or monitored by inverted microscopy (*see* **Fig. 1**). Serial measurements of the diameter/radius of the seeded population allow assessment of the specific lateral movement (migration) of the cell population (*see* **Fig. 2**). Recommended compatible equipment can be used to assess serial measurements (*see* **Note 8**).

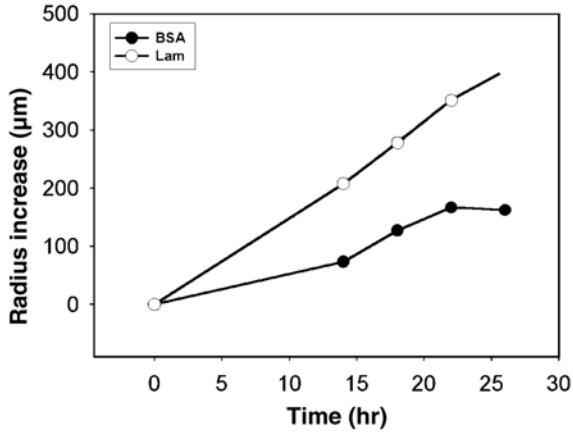

Fig. 2. Graph showing the radius increase of SF767 human glioma cells shown in **Fig. 1**. Cells seeded on 10-µg/mL laminin show a rapid radius increase over time compared to cells seeded on 0.1% BSA. Values represent a single data point. Typically, replicate measures are within 10% errors.

3. After the migration has been followed for the desired time period, the cells can be fixed and processed for microscopic evaluation by numerous techniques (immunofluorescence, *in situ* hybridization, or conventional staining, for example).

4. Notes

1. Custom glass cell sedimentation cylinders can also be used for depositing the cells onto the slides. The cylinders can be created by sectioning a 1-mL glass pipet into 0.5-cm segments. The cylinders are placed in the center of each well and filled with appropriate media. Cells are carefully deposited into the cylinders. Chicoine and Silbergeld adopted conventional 5-mm cloning rings to sediment cells at precise locations ***(23)***, and Akiyama et al. produced metal devices to accomplish the same outcome ***(24)***. From a logistical view, the smaller the circle of cells, the greater the fraction of perimeter, migration-unimpeded cells.
2. The extracellular matrix (ECM) proteins used for coating the slides can be reused (≤2×). Simply aspirate the protein solution that is left on each well using a 40–200-µL pipet and dispense in an appropriate tube.
3. Cell-derived ECM can also be used for coating the Teflon-masked slides. The cells use to generate the ECM are grown on the slides in appropriate media to postconfluence. Cells are removed by treatment with 0.5% Triton X-100 for 30 min at room temperature (RT), followed by 0.25 *M* NH_4OH 3–5 min at RT and thoroughly rinsed with PBS ***(25)***. The slides are stored at 4°C until ready to use.
4. Injecting air bubbles into the channels can be overcome by simply overfilling the pipet with cells (1.3 µL) and expressing only to the first stop on the pipet.
5. This assay required a small number of cells, which is amenable to primary cultures.
6. Adherent cells or nonadherent cells that have been activated to adhere can be used in this system (**Fig. 3**).

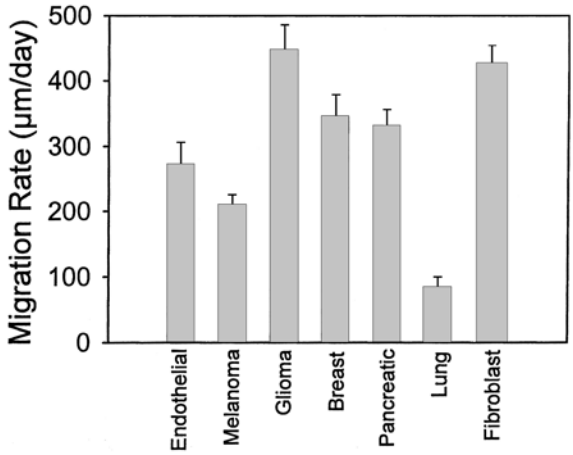

Fig. 3. Migration rate of different human cell lines on 10-µg/mL laminin. Values are means ±SE of 5 replicates. Cell lines of any given histopathological subtype may show highly variable migration rates and/or ECM preference for migration. Primary cultures or early passage tumor and endothelial cells perform well in this system.

7. Cell attachment interval varies with the ECM proteins used for coating the slides and with the cell type. Some cells (i.e., endothelial) perform very poorly if the system is cold (below RT).
8. A macro used to assess serial migration measurements can be downloaded from the Creative Scientific Methods website at www.cre8ive-sci.com. Recommended compatible equipment can also be found on the Website (i.e., Scion Image software, LG-3 frame grabber).

References

1. Fong, C. J., Sutkowski, D. M., Kozlowski, J. M., and Lee, C. (1992) Utilization of the Boyden chamber to further characterize in vitro migration and invasion of benign and malignant human prostatic epithelial cells. *Invasion Metastasis* **12,** 264–274.
2. Wells, A. (2000) Tumor invasion: role of growth factor-induced cell motility. *Adv. Cancer Res.* **78,** 31–101.
3. Bittner, M., Meltzer, P., Chen, Y., Jiang, Y., Seftor, E., Hendrix, M., et al. (2000) Molecular classification of cutaneous malignant melanoma by gene expression profiling. *Nature* **406,** 536–540.
4. Berens, M. E., Rief, M. D., Loo, M. A., and Giese, A. (1994) The role of extracellular matrix in human astrocytoma migration and proliferation studied in a microliter scale assay. *Clin. Exp. Metastasis* **12,** 405–415.
5. Alt, W. (1980) Biased random walk models for chemotaxis and related diffusion approximations. *J. Math. Biol.* **9,** 147–177.
6. Stokes, C. L., Rupnick M. A., Williams, S. K., and Lauffenburger, D. A. (1990) Chemotaxis of human microvessel endothelial cells in response to acidic fibroblast growth factor. *Lab. Invest.* **63,** 657–668.

7. Maheshwari, G., and Lauffenburger, D. A. (1998) Deconstructing (and reconstructing) cell migration. *Microsc. Res. Tech.* **43,** 358–368.
8. Mercurio, A. M., Rabinovitz, I., and Shaw, L. M. (2001) The alpha 6 beta 4 integrin and epithelial cell migration. *Curr. Opin. Cell Biol.* **13,** 541–545.
9. Wu, C., and Dedhar, S. (2001) Integrin-linked kinase (ILK) and its interactors: a new paradigm for the coupling of extracellular matrix to actin cytoskeleton and signaling complexes. *J. Cell Biol.* **155,** 505–510.
10. Kassis, J., Lauffenburger, D. A., Turner, T., and Wells, A. (2001) Tumor invasion as dysregulated cell motility. *Semin. Cancer Biol.* **11,** 105–117.
11. Geiger, B., Bershadsky, A., Pankov, R., and Yamada, K. M. (2001) Transmembrane crosstalk between the extracellular matrix—cytoskeleton crosstalk. *Nat. Rev. Mol. Cell Biol.* **2,** 793–805.
12. Gillespie, G. Y., Soroceanu, L., Manning, T. J., Jr., Gladson, C. L., and Rosenfeld, S. S. (1999) Glioma migration can be blocked by nontoxic inhibitors of myosin II. *Cancer Res.* **59,** 2076–2082.
13. Mariani, L., McDonough, W. S., Hoelzinger, D. B., et al. (2001) Identification and validation of P311 as a glioblastoma invasion gene using laser capture microdissection. *Cancer Res.* **61,** 4190–4196.
14. Treasurywala, S., and Berens, M. E. (1998) Migration arrest in glioma cells is dependent on the alphav integrin subunit. *Glia* **24,** 236–243.
15. Mariani, L., Beaudry, C., McDonough, W. S., et al. (2001) Death-associated protein 3 (Dap-3) is overexpressed in invasive glioblastoma cells in vivo and in glioma cell lines with induced motility phenotype in vitro. *Clin. Cancer Res.* **7,** 2480–2489.
16. Giese, A., Loo, M. A., Tran, N., et al. (1996) Dichotomy of astrocytoma migration and proliferation. *Int. J. Cancer* **67,** 275–282.
17. McDonough, W., Tran, N., Giese, A., Norman, S. A., and Berens, M. E. (1998) Altered gene expression in human astrocytoma cells selected for migration: I. Thromboxane synthase. *J. Neuropathol. Exp. Neurol.* **57,** 449–455.
18. Mariani, L., Beaudry, C., McDonough, W. S., et al. (2001) Glioma cell motility is associated with reduced transcription of proapoptotic and proliferation genes: a cDNA microarray analysis. *J. Neurooncol.* **53,** 161–176.
19. Miyamoto, S., Akiyama, S. K., and Yamada, K. M. (1995) Synergistic roles for receptor occupancy and aggregation in integrin transmembrane function. *Science* **267,** 883–885.
20. Giese, A., Laube, B., Zapf, S., Mangold, U., and Westphal, M. (1998) Glioma cell adhesion and migration on human brain sections. *Anticancer Res.* **18,** 2435–2447.
21. Owens, G. C., Orr, E. A., DeMasters, B. K., et al. (1998) Overexpression of a transmembrane isoform of neural cell adhesion molecule alters the invasiveness of rat CNS-1 glioma. *Cancer Res.* **58,** 2020–2028.
22. Cukierman, E., Pankov, R., Stevens, D. R., and Yamada, K. M. (2001) Taking cell-matrix adhesions to the third dimension. *Science* **294,** 1708–1712.
23. Chicoine, M. R., and Silbergeld, D. L. (1995) Assessment of brain tumor cell motility in vivo and in vitro. *J. Neurosurg.* **82,** 615–622.
24. Akiyama, Y., Jung, S., Salhia, B., et al. (2001) Hyaluronate receptors mediating glioma cell migration and proliferation. *J. Neurooncol.* **53,** 115–127.
25. Vlodavsky, I., Levi, A., Lax, I., Fuks, Z., and Schlessinger, J. (1982) Induction of cell attachment and morphological differentiation in a pheochromocytoma cell line and embryonal sensory cells by the extracellular matrix. *Dev. Biol.* **93,** 285–300.

20

Invasion and Motility Assays

William N. Scott

1. Introduction

A key feature of malignant cells is their capacity to invade surrounding tissue and metastasize to distant sites ***(1,2)***. The process of metastasis involves a complex series of events that include cell transformation and proliferation, vascular invasion at primary growth site with associated basement membrane degradation, transport through capillary or lymphatic vessels, attachment of tumor cells to endothelial or subendothelial structures at the secondary site, and the subsequent growth of the secondary tumor mass ***(3)***.

In order to metastasize to different organs, tumor cells must acquire a number of highly differentiated characteristics that define the metastatic phenotype. None of the functions of metastasizing cells are unique to cancer since they can be demonstrated in trophoblast implantation, mammary gland involution, embryonic development, and tissue remodelling ***(4)***. The difference between the normal processes and the nature of cancer invasion is basically one of dysregulation and can be attributed to high mutation rates and genetic instability ***(3)***.

This high mutation rate assists in the development of clonal populations with metastatic capabilities, and for most tumors the neoplastic element must expand considerably before invasion and metastasis can occur ***(5,6)***.

Several studies have compared the similarities between metastasis and the responses of inflammatory cells, an essential component of both being the recruitment and migration of cells through basement membranes and connective tissues. In inflammation, the migration is initiated by bacterial or complement-mediated signalling; in metastasis the response is less well defined.

1.1. Models of Invasion and Motility

Major emphases on cancer invasion motility have focused on in vivo models in which tumor cells are injected intravenously or subcutaneously into mice and the number of metastases determined ***(7–9)***. However, the procedure is relatively expen-

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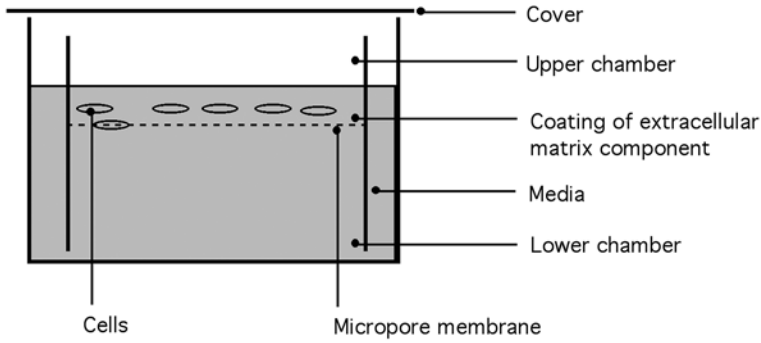

Fig. 1. Modified Boyden chamber system for the in vitro determination of cell invasion.

sive and is not well suited to rapid screening of potential antiinvasive or motility-inhibiting agents.

Alternatives to this invasion model have been developed using modifications of the Boyden chamber (*see* **Fig. 1**), where a porous membrane is interposed between cells and potential attractants; the membrane can be precoated with extracellular matrix components to give a more accurate reflection of the in vivo process.

Similarly, a number of assays exist for the determination of cell motility and these have evolved from models primarily designed to measure the chemotactic response of leukocytes and other inflammatory cells. Procedures are detailed for the relative quantitation of these key metastatic events.

2. Materials

2.1. Invasion Assay

1. Cells of interest.
2. Extracellular matrix component, e.g., collagen IV, laminin, or fibronectin.
3. Cell culture medium: Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS).
4. Transwell systems (8.0-µm pore size; Costar).

2.2. Cell Motility Assay

1. Cover slip (22 × 22 mm).
2. 5% Detergent (7X ; ICN Biomedicals, High Wycombe, UK).
3. Ethanol.
4. Gelatin.
5. Na_2CO_3.
6. $AuHCl_4$.
7. 0.1% Formaldehyde solution.
8. Petri dishes.
9. Cells of interest.
10. Cell culture medium: DMEM containing 10% FCS.

11. 3% Glutaraldehyde in cacodylate, pH 7.2.
12. Microscope with phase-contrast objectives and imaging software.

3. Methods

3.1. Invasion Assay

1. Coat the upper surface of a 24-well transwell system (8-µm pore size) with 250 µL/well of cell attachment matrix (30 µg/mL) (e.g., collagen IV, fibronectin, or laminin) and allow to incubate overnight in a laminar air flow cabinet.
2. Aspirate off excess solution and allow to air dry. UV sterilize, then add chemo-attractant to the lower well if required.
3. Inoculate the upper well with approx 2.5×10^3–10^4 cells and incubate for 12 h. Count cells in upper and lower chambers (*see* **Note 1**).

3.2 Cell Motility Assay

This assay is based on the principal that migrating cells ingest, push aside, or collect small particles in their path on their dorsal surface. Quantification of cell migration may be achieved using computer-assisted image analysis. Basically, glass surfaces are coated with particles of colloidal gold embedded in serum proteins. Cells are then seeded onto the gold monolayers and as the cells migrate, a clear, particle-free phagokinetic track is formed leaving a permanent record of their movement. Variations of the assay have been used in studies of the movement of a wide spectrum of cell types including fibroblasts ***(10)***, neutrophils ***(11)***, keratinocytes ***(12)***, and endothelial cells ***(13)***.

As such, the method provides a relatively inexpensive and reproducible screen for studies requiring an index of cell migration as an end-point.

1. Remove grease from cover slip (22×22 mm) by treating in 5% phosphate free detergent (at 100°C) and washing in alcohol.
2. After drying, immerse in gelatin solution at 90°C for 10 min (300 Bloom; 0.5 g in 300 mL distilled H_2O) and place in a 70°C oven for 45 min (*see* **Note 2**).
3. Prepare colloidal gold suspension by adding 11 mL distilled H_2O and 6 mL Na_2CO_3 (36.5 m*M*) to 1.8 mL $AuHCl_4$ (14.5 m*M*) in a 50-mL glass beaker.
4. With constant stirring, heat the mixture to 95°C at which point add 1.8 mL of freshly prepared 0.1% formaldehyde solution; maintain temperature at 95°C. A suspension of colloidal gold will form which is brown to absorbed light and blue to transmitted light.
5. Allow the gold suspension to cool to 75°C and add 3 mL to Petri dishes containing individual gelatin coated cover slip. Incubate the plates at 37°C for 45 min.
6. After washing with calcium-free saline (3×4 mL) to remove unattached gold particles, transfer the cover slip to 6-well cluster dishes and UV sterilized in 0.3 mL culture media.
7. The cover slip should remain in this media until required for assay in order to prevent drying of the prepared surface (*see* **Note 4**). Using these prepared gold monolayers, the migration of various cell types in response to external influences can be readily monitored.
8. Seed the cells in culture medium (2 mL DMEM containing 10% FCS) onto the prepared cover slip at an approximate density of 5×10^3 cells per well in media.
9. Allow the cells to plate down for 2 h at 37°C after which the treatments may be added in a further 2 mL culture media.
10. Assay systems should then be maintained for approx 18 h, after which the cells can be fixed using 3% gluteraldehyde in cacodylate buffer, pH 7.2 (*see* **Note 5**).

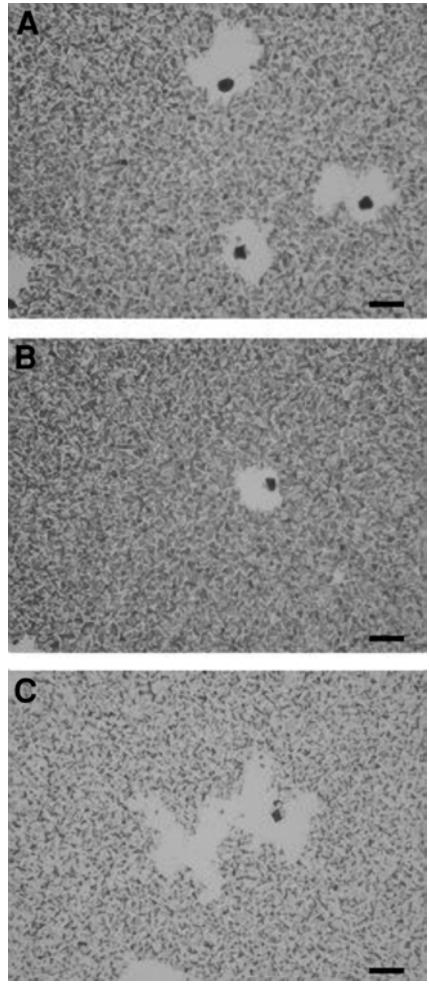

Fig. 2. Motility tracks of SK Hep-1 endothelial cells ***(14)*** showing: **(A)** basal rate motility; **(B)** inhibition of motility in response to mEGF(33–42), a peptide derived from the C-loop of murine epithelial growth factor (EGF); and **(C)** stimulation of motility by Lam.β1 (925–933) from the β1 chain of murine laminin-1. Both peptides were added to give a final concentration of 500 n*M*. Bars represent 10 μ*M*.

11. After mounting, cover slips are best examined using a phase contrast microscope and imaging software. We routinely use a Leica DM1RB phase contrast microscope and Q500MC image analysis system incorporating a JVC TK-1280E color camera (Leica, Milton Keynes, UK). The track images of at least 30 cells are video-captured and the area (representing migration response) determined for each. Representative tracks are detailed in **Fig. 2**.

4. Notes

1. The chemoattractant should normally be added at tenfold its optimal concentration. This assay may also be used to determine the effects of competing inhibitor, which can be incorporated into the attachment matrix or as a media additive. A longer time interval may be needed
2. Individual drying times may vary depending on the oven used; it is essential that the gelatin coating is not allowed to totally dehydrate but forms a gel rather than a thin film.
3. It is important that triple glass-distilled H_2O be used in the preparation of all reagents used in this assay.
4. It is recommended that the protocol be adhered to with regard to all volumes and temperature settings in order to ensure an even coating of gold colloid. The method does not allow for compromise. Prepared cover slips should be used immediately and not stored.
5. Individual seeding densities and incubation times should be optimized for each cell type under investigation.

References

1. Hirohashi, S. (1998) Inactivation of the E-cadherin-mediated cell adhesion system in human cancers. *Am. J. Pathol.* **153,** 333–339.
2. Liotta, L. A., Steeg, P. S., and Stetler-Stevenson, W. G. (1991) Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. *Cell* **64,** 327–336.
3. Dickson, R. B. (1992) Regulation of tumor-host interactions in breast cancer. *J. Steroid Biochem. Molec. Biol.* **41,** 3–8.
4. Weiss, L. (1985) *Principles of Metastasis.* Academic Press, New York.
5. Fidler, I. J. and Hart, R. I.(1982) Biological diversity in metastatic neoplasms: origins and implications. *Science* **217,** 998–1003.
6. Clark, R., Dickson, R. B., and Brunner, N. (1990) The process of malignant progression in human breast cancer. *Ann. Oncol.* **1,** 401–407.
7. Ozzello, L. and Sordat, M. (1980) Behaviour of tumors produced by transplantation of human mammary cell lines in athymic nude mice. *Eur. J. Cancer* **16,** 553–559.
8. Shafie, S. M. and Liotta, L. A. (1980) Formation of metastasis by human carcinoma cells (MCF-7) in nude mice. *Cancer Lett.* **16,** 553–559.
9. Sommers, C. L, Papageorge, A., Wilding, G., and Gelmann, E. P. (1990) Growth properties and tumourigenesis of MCF-7 cells transfected with isogenic mutants of rasH. *Cancer Res.* **50,** 67–71.
10. Albrecht-Buehler, G. (1977) The phagokinetic tracks of 3T3 cells. *Cell* **11,** 395–404.
11. Kawa, S., Kimura, S., Hakomori, S., and Igarashi, Y. (1997) Inhibition of chemotactic motility and trans-endothelial migration of human neutrophils by sphingosine 1-phosphate. *F.E.B.S. Letters* **420,** 196–200.
12. Ando, Y. and Jensen, P. J. (1993). Epidermal growth factor and insulin-like growth factor I enhance keratinocyte migration. *J. Invest. Dermatol.* **100,** 633 –639.
13. Scott, W. N., McCool, K., and Nelson, J. (2000) Improved method for the production of gold colloid monolayers for use in the phagokinetic track assay for cell motility. *Anal. Biochem.* **287,** 343–344.
14. Heffelfinger, S. C., Hawkins, H. H., Barrish, J., Taylor, L. and Darlington, G. J. (1992) SK HEP-1: a human cell line of endothelial origin. *In Vitro Cell Dev. Biol.* **28A,** 136–142.

21

Identification of Senescence in Cancer Cells

Lesley J. Jardine

1. Introduction

1.1. Senescence as a Tumor Suppressive Mechanism

Senescence is thought to be a programmed response to protect cells from DNA damage by preventing cell cycle progression under stress conditions ***(1)***. The prolonged arrest associated with senescence limits the number of mutational hits required for tumorigenesis, supporting a role for senescence as a tumor suppressive mechanism.

Replicative senescence was initially identified in human diploid fibroblasts and shown to have a limited number of genetically determined cell divisions in culture ***(2)***. When the critical number of cell divisions is reached the cell undergoes changes in morphology and gene expression and permanently stops growing with a characteristic senescence-like phenotype ***(3)***. Replicative senescence is associated with a decrease in the length of the protective caps (or telomeres) at the ends of chromosomes. Additional repeats are added to the telomeres by an enzyme called telomerase; however, in normal somatic cells telomerase is inactive and the telomeres shorten with each cell division, as a result of under replication. Upon reaching a critical telomere length the cells enter senescence ***(4)***. Tumor cells can overcome this mitotic clock and escape senescence by reactivating telomerase ***(5)***.

Primary cells in culture can be induced into a senescence-like arrest after extensive passaging or under conditions of cellular stress induced by ionizing radiation ***(6)***, DNA damaging drugs ***(7)***, and activated oncogenes such as *ras* and *raf* ***(8,9)***. This stress-induced senescence occurs more rapidly than replicative senescence and appears to be independent of telomere shortening; moreover, reintroduction of telomerase activity, by overexpression of a catalytically active subunit of telomerase (hTERT), allows cells to escape from replicative senescence but not from *ras*-induced senescence ***(10,11)***.

Of particular interest is that some tumor cells have retained a number of the elements required for senescence growth arrest and when treated with anticancer drugs can be induced into a senescence-like growth arrest ***(12–14)***.

From: *Methods in Molecular Medicine, vol. 88: Cancer Cell Culture: Methods and Protocols*
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1.2. The Senescence-Like Phenotype (SLP)

The downstream responses of replicative and stress-induced senescence are phenotypically similar. Cells entering senescence become large, flat, and more granular in appearance. A number of senescent cells are also positive for senescence-associated β-galactosidase activity detected at pH 6.0 ***(15)***.

Senescent cells are permanently growth arrested at G_1 and remain metabolically active; however, they fail to enter S phase in response to mitogenic stimuli. Senescent cells, therefore, can be identified in culture by a lack of DNA synthesis under optimal growth conditions, distinguishing senescent cells from quiescent or terminally differentiated cells. A number of senescent cell lines, including human fibroblasts, also show resistance to apoptosis.

Another characteristic feature of senescent cells is the change in gene expressison of a number of cell cycle inhibitory proteins seen in gene expression. Both replicative and stress-induced senescence have been shown to require p53 activation ***(16)*** and the cyclin-dependent kinase inhibitors p21 ***(17)*** and p16, ***(18)*** as well as other members of the p16/pRB and the p53/ARF pathways. Moreover, tumor derived cell lines have been shown to enter a senescence-like growth arrest after overexpression of the cell cycle inhibitory proteins p53, p21, and p16 ***(19–21)***.

1.3. Induction of Senescence in HCT116 Colon Cancer Cells

As an example of cells showing the characteristic SLP this chapter will focus on the induction of senescence of HCT116 colon cancer cells that express wild type p53. Upon treatment with the senescence-inducing agent SN38, a DNA topoisomerase I inhibitor, the cells enter a senescence-like growth arrest ***(14)***.

2. Materials

2.1. Cell Line

Cell line of interest, for example, HCT116 colon cancer parental cell line that expresses wild type p53 (from B. Vogelstein) is maintained in RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum (FBS) and 100 U/mL penicillin/streptomycin (all from GIBCO Life Technologies). The cells are incubated at 37°C with 5% carbon dioxide in a humidified incubator.

2.2. Cell Staining

1. Paraformaldehyde (4%): 4% (w/v) Paraformaldehyde in phosphate-buffered saline (PBS) heated to 60–80°C in a covered container; stir until it dissolves to give a clear solution. Adjust to pH 7.2–7.4; when cool, filter and store at –20°C for up to 1 mo in aliquots. Paraformaldehyde is harmful by inhalation and contact with the skin.
2. PBS: 137m*M* NaCl, 10 m*M* Na_2HPO_4, 1.8 m*M* KH_2PO_4, pH 7.2.
3. X-Gal: 20 mg/mL stock 5-bromo-4-chloro-3-indolyl βD-galactoside (X-Gal) (Sigma) made up in dimethylformamide (DMF); store at –20°C protected from light.
4. β-Galactosidase stain: 1 mg/mL X-Gal, 40 m*M* citric acid/sodium phosphate, pH 6.0, 5 m*M* potassium ferricyanide, 150 m*M* NaCl, 2 m*M* $MgCl_2$. Make up the stain fresh and protect from light.

2.3. BrdU Cell Proliferation Assay

All the reagents used for this BrdU cell proliferation assay were obtained from Oncogene Research Products.

2.4. Western Analysis

1. Lysis buffer: 50 m*M* Tris-base, pH 7.5, 5 m*M* ethylenediaminetetraacetic acid (EDTA), 150 m*M* NaCl, 0.5% (v/v) NP-40, nonylphenol ethoxylate (IGEPAL); can be stored at room temperature. Add fresh 1 m*M* phenylmethylsulfonyl fluoride (PMSF), 2 µg/mL aprotinin and 5 µg/mL leupeptin prior to use.
2. SDS sample buffer: 62.5 m*M* Tris-base, pH 6.8, 2% (v/v) SDS, 10% (w/v) sucrose, 0.002% (w/v) bromophenol blue, 25% (v/v) β-mercaptoethanol. Store at –20°C in 1-mL aliquots.
3. Electrophoresis running buffer: 25 m*M* Tris-base, 190 m*M* glycine, 3.5 m*M* sodium dodecyl sulfate.
4. Transfer buffers: 25 m*M* Tris-base, 190 m*M* glycine.
5. TNET: 10 m*M* Tris-base, pH 7.5, 2.5 m*M* EDTA, 50 m*M* NaCl, 0.1% Tween-20.
6. Blocking solution: 5% powdered milk in TNET.
7. Antibodies: p53 monoclonal antibody (Ab-6) at 1/1000 dilution and p21 monoclonal antibody (Ab-1) at 1/1000 dilution from Oncogene Research Products. Actin monoclonal antibody (Calbiochem) at 1/80,000 dilution. Secondary antibodies, horseradish peroxidase (HRP)-conjugated specific for mouse and rabbit antibodies (Calbiochem).

3. Methods

3.1. Morphology of the Senescent Cells

Induction of senescence shows a number of characteristic morphologies that can be observed with a light microscope.

1. Seed cells into six-well plates at a low density and treat with the senescence-inducing agent (*see* **Note 1**). In the experiment shown in **Fig. 1** the HCT116 cells are treated with SN38 (Aventis) at IC_{80} concentration of 6 n*M* for 24 h.
2. Replace with fresh media and incubate cells at 37°C.
3. The morphology of the cells starts to change at day (d) 5 post-drug treatment (*see* **Note 2**); as the cell size increases they become flattened and a more granular appearance can be seen.

3.2. Acidic β-Galactosidase Staining

A biomarker for cells in senescence is acidic β-galactosidase activity, which can be detected in senescent cells by X-Gal and forms a local blue precipitate when cleaved ***(15)***. This activity is cell type specific and not all cells that enter senescence will show positive staining.

1. Seed cells at a low density into six-well plates and treat with senescence-inducing agent (*see* **Subheading 3.1.1.**).
2. Post-drug treatment: wash the cells twice with PBS and fix with 4% paraformaldehyde in PBS for 5 min at room temperature.
3. Wash the cells twice with PBS and incubate with β-galactosidase stain at pH 6.0, overnight at 37°C without CO_2 (*see* **Notes 3** and **4; Fig. 1**).

3.3. Bromodeoxyuridine (BrdU) Cell Proliferation Assay

To confirm lack of proliferation in senescent cells an assay can be used that measures newly synthesized DNA in dividing cells. There are a number of assays avail-

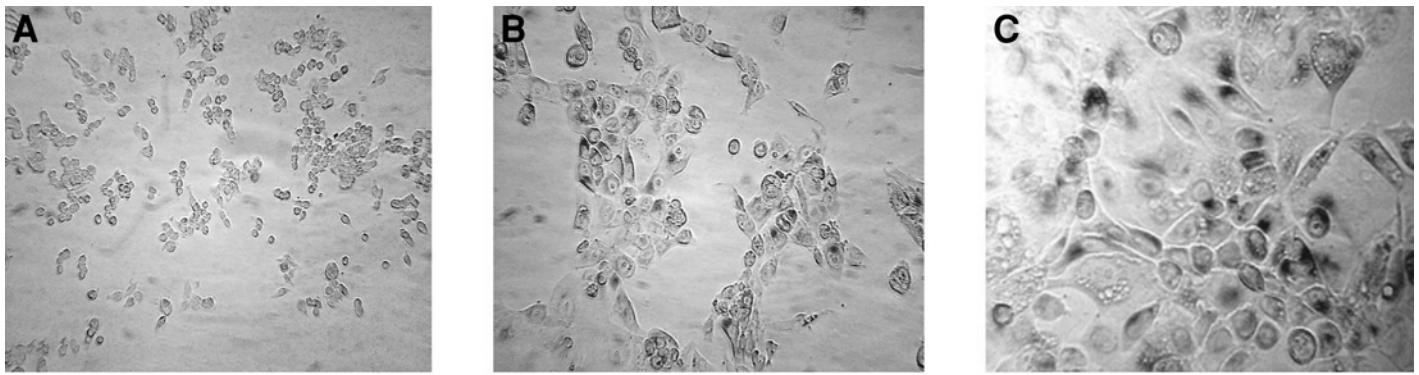

Fig. 1. Induction of the senescence-like phenotype in HCT116 cells after exposure to SN38. HCT116 cells were treated with either DMSO used as the drug carrier **(A)** or SN38 6nM **(B**,**C)** for 24 h before incubation in fresh media. Samples were taken at various times post-drug treatment and the cells fixed and stained for senescence-associated β-galactosidase activity. Note the enlarged granular phenotype and positive β-galactosidase staining which can be seen initially at d 5 post drug treatment (B) and more evident at d 11 shown in (C).

able and the method described here is a nonisotopic immuno cell proliferation assay from Oncogene Research Products. Add BrdU to the cells and any cells that are dividing will incorporate the BrdU into their newly synthesized DNA. This in turn is detected by an antiBrdU antibody. A secondary antibody, which is conjugated to HRP, is added and binds to the primary antibody. The HRP-conjugated antibody catalyzes a colorimetric reaction that is proportional to the amount of incorporated BrdU in the cells and therefore a measure of DNA synthesis.

1. Seed cells at 1.5×10^5 cells per milliliter in a final volume of 100 µL in a 96-well culture dish and incubate overnight to allow the cells to adhere to the dish. Each sample is assayed in triplicate and control samples include a blank (no cells) and background (cells without BrdU).
2. Treat cells with senescence-inducing agent for the appropriate time (**Fig. 1**).
3. Take samples post-drug treatment at various times and assay for BrdU activity.
4. Dilute the BrdU label 1/2000 in media, add 20 µL to each well and incubate for 24 h at 37°C.
5. Replace the BrdU label with 200 µL of Fix/denaturing solution for 30 min at room temperature and remove by inverting the plate.
6. Dilute the BrdU antibody 1/100 in dilution buffer and add 100 µL to each well for 1 h at room temperature.
7. Wash each well 3 times with wash buffer, inverting the plate after each wash to remove buffer.
8. Dilute the secondary antibody (anti-mouse IgG HRP conjugated) in conjugate diluent and add 100 µL to each well for 30 min.
9. Wash the wells three times with wash buffer and one time with dH_2O.
10. Remove the dH_2O and add 100 µL of substrate solution to each well and incubate for 15 min at room temperature in the dark.
11. Add 100 µL of stop solution to the wells and measure absorbance at 450–540 nm.

3.4. Western Blot Analysis to Identify Senescence-Regulated Proteins

In order to identify senescence-regulated proteins, lyse the cells post drug treatment and separate by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transfer to a PDVF membrane, and probe with antibodies specific to the protein of interest.

1. Seed cells in a 60-mm cell culture dish and grow until 50% confluent.
2. Treat cells with senescence-inducing agent for the appropriate time.
3. Post drug treatment, wash the cells with ice cold PBS.
4. Remove the PBS and add 500 µL of ice cold lysis buffer, remove the cells into a 1.5-mL Eppendorf using a cell scraper.
5. Place the lysis solution on ice for 30 min with occasional vortexing to ensure cell lysis is complete.
6. Centrifuge the lysed cells at 8000*g* for 15 min at 4°C to pellet the cell debris.
7. Transfer the supernatant to a clean 1.5-mL Eppendorf tube and keep on ice.
8. Measure the concentration of cellular protein using the Bio-Rad Protein Assay solution.
9. Resolve the cellular proteins by SDS-PAGE using a 10% polyacrylamide gel and transfer onto a polyvinylidene fluoride (PVDF) membrane.
10. Soak the PVDF membrane in blocking solution for a minimum of 1 h and rinse with TNET solution.

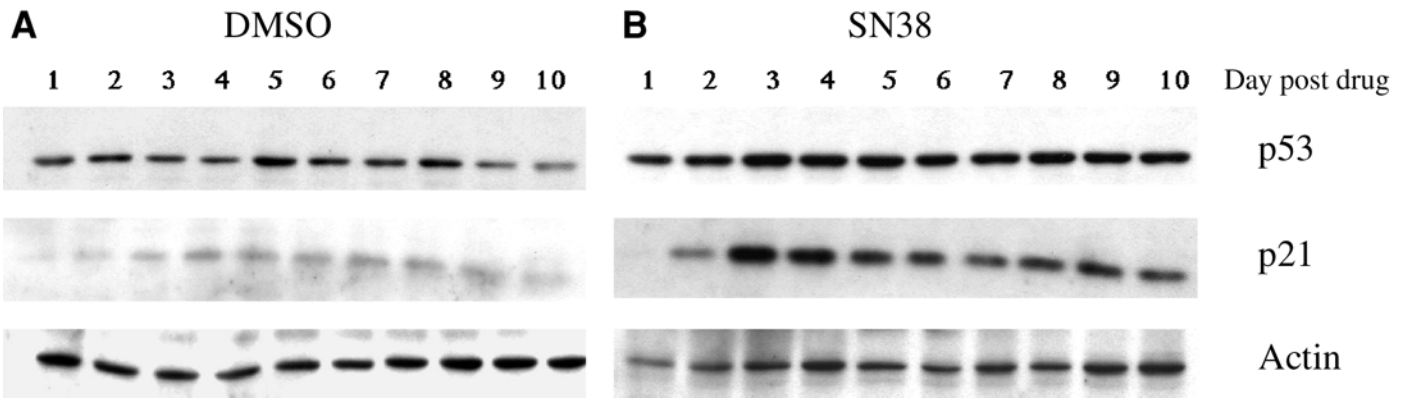

Fig. 2. Detection of senescence induced proteins p53 and p21 after treatment with SN38 in HCT116 cells. HCT116 cells were treated with either DMSO, the drug diluent, shown in **(A)** or SN38 6 n*M* **(B)** for 24 h before incubation in fresh media. Samples were taken for Western blot analysis at various times post drug treatment (indicated) and probed with antibodies specific for p53 and p21. Actin was used as a loading control.

11. Dilute the primary antibody in TNET solution and add to the membrane for 1 h with shaking or at 4°C overnight (*see* **Note 5**). The primary antibodies used in **Fig 2.** are specific for p53 and p21 and actin.
12. Wash the membrane three times with TNET shaking for 10 min each time.
13. Dilute the secondary antibody (HRP-conjugated) in TNET solution and add to the membrane for 1 h with shaking.
14. Wash the membrane quickly with TNET and then three times with TNET shaking for 10 min each time.
15. Add enhanced chemiluminescence (ECL) reagents for 1 min to the membrane and expose to film for an appropriate time (between 5 sec and 10 min).
16. To demonstrate this, HCT116 cells were treated with SN38 at 6 n*M* for 24 h. Samples were taken post drug treatment at the times shown and probed with antibodies specific for p53 and p21 **(Fig. 2)**.

4. Notes

1. In order to see the change in morphology the cells should be cultured at a low confluency. The effects of passage number must be taken into consideration when assaying the affect of premature senescence in primary cells. Examples of senescence-inducing agents would include ionizing radiation, DNA damaging drugs, or overexpressed oncogenes.
2. The senescent phenotype is evident after approx 5 d post drug treatment when a small percentage of the cells show a characteristic SLP. By d 11 post drug treatment approximately all of the cells are positive for SLP (*see* **Fig. 1**).
3. The endogenous β-galactosidase activity can only be detected when the β-galactosidase staining solution is at pH 6.0. The blue staining is evident after 4 h but maximal at 12–16 h. Lysosomal β-galactosidase activity can be detected at pH 4.0. Presenescent and senescent cells stain equally well for this.
4. Tissue samples can also be used to identify cells staining positive for β-galactosidase activity. Sections of the tissue samples should be frozen, mounted with Tissue-Tek optimal cutting temperature (OCT) medium, and thin sections cut and mounted onto glass slides. Fix in 1% Formalin in PBS for 1 min at room temperature before washing with PBS. Add the β-galactosidase stain overnight and counterstain with eosin.
5. Antibodies used for Western blotting can be stored at 4°C and reused for multiple Western blots. If the antibody of interest has high levels of background staining 5% powdered milk can be added to the primary antibody dilution in TNET. In addition to this 5% powdered milk can be added to the TNET washes after primary and secondary antibody binding.

References

1. Weinberg, R. A. (1997) The cat and mouse games that genes, viruses, and cells play. *Cell* **88,** 573–575.
2. Hayflick, L., and Moorhead, P. S. (1961) The serial cultivation of human diploid strains. *Exp. Cell Res.* **25,** 585–621.
3. Stein, G. H., and Dulic, V. (1995) Origins of G1 arrest in senescent human fibroblasts. *Bioessays* **17,** 537–543.
4. Martens, U. M., Chavez, E. A., Poon, S. S., Schmoor, C., and Lansdorp, P. M. (2000) Accumulation of short telomeres in human fibroblasts prior to replicative senescence. *Exp. Cell Res.* **256,** 291–299.
5. Stewart, S. A., and Weinberg, R. A. (2000) Telomerase and human tumorigenesis. *Cancer Biol.* **10,** 399–406.

6. Di Leonardo, A., Linke, S. P., Clarkin, K., and Wahl, G. M. (1994) DNA damage triggers a prolonged p53-dependent G1 arrest and long-term induction of Cip1 in normal human fibroblasts. *Genes Dev.* **8,** 2540–2551.
7. Robles, S. J., and Adami, G. R. (1998) Agents that cause DNA double strand breaks lead to $p16^{INK4a}$ enrichment and the premature senescence of normal fibroblasts. *Oncogene* **16,** 1113–1123.
8. Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D., and Lowe, S. W. (1997) Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and $p16^{INK4a}$. *Cell* **88,** 593–602.
9. Zhu, J., Woods, D., McMahon, M., and Bishop, J. M. (1998) Senescence of human fibroblasts induced by oncogenic raf. *Genes Dev.* **12,** 2997–3007.
10. Bodnar, A. G., Ouellette, M., Frolkis, M., et al. (1998) Extension of life-span by introduction of telomerase into normal human cells. *Science* **279,** 349–352.
11. Wei, S., Wei, W., and Sedivy, J. M. (1999) Expression of catalytically active telomerase does not prevent premature senescence caused by overexpression of oncogenic Ha-Ras in normal human fibroblasts. *Cancer Res.* **59,** 1539–1543.
12. Chang, B. D., Xuan, Y., Broude, E. V., et al. (1999) Role of p53 and $p21^{waf1/cip1}$ in senescence-like terminal proliferation arrest induced in human tumor cells by chemotherapeutic drugs. *Oncogene* **18,** 4808–4818.
13. Chang, B. D., Broude, E. V., Dokmanovic, M., et al. (1999) A senescence-like phenotype distinguishes tumor cells that undergo terminal proliferation arrest after exposure to anticancer agents. *Cancer Res.* **59,** 3761–3767.
14. te-Poele, R. H., Okorokov, A. L., Jardine, L. J., Cummings, J., and Joel, S. P. (2002) DNA damage is able to induce senescence in tumour cells in vitro and in vivo. *Cancer Res.* **62,** 1876–1883.
15. Dimri, G. P., Lee, X., Basile, G., et al. (1995) A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc. Natl. Acad. Sci. USA* **92,** 9363–9367.
16. Atadja, P., Wong, H., Garkavtsev, I., Veillette, C., and Riabowol, K. (1995) Increased activity of p53 in senescing fibroblasts. *Proc. Natl. Acad. Sci. USA* **92,** 8348–8352.
17. Noda, A., Ning, Y., Venable, S. F., Pereira-Smith, O. M., and Smith, J. R. (1994) Cloning of senescent cell-derived inhibitors of DNA synthesis using an expression screen. *Exp. Cell Res.* **211,** 90–98.
18. Alcorta, D. A., Xiong, Y., Phelps, D., Hannon, G., Beach, D., and Barrett, J. C. (1996) Involvement of the cyclin-dependent kinase inhibitor p16 (INK4a) in replicative senescence of normal human fibroblasts. *Proc. Natl. Acad. Sci. USA* **93,** 13,742–13,747.
19. Sugrue, M. M., Shin, D. Y., Lee, S. W., and Aaronson, S. A. (1997) Wild-type p53 triggers a rapid senescence program in human tumor cells lacking functional p53. *Proc. Natl. Acad. Sci. USA* **94,** 9648–9653.
20. Vogt, M., Haggblom, C., Yeargin, J., Christiansen-Weber, T., and Haas, M. (1998) Independent induction of senescence by $p16^{INK4a}$ and $p21^{CIP1}$ in spontaneously immortalised human fibroblasts. *Cell Growth Differ.* **9,** 139–146.
21. Uhrbom, L., Nister, M., and Westermark, B. (1997) Induction of senescence in human malignant glioma cells by $p16^{INK4a}$. *Oncogene* **15,** 505–514.

22

Angiogenesis Assays

William N. Scott

1. Introduction

Considerable data on the mechanisms of angiogenesis and neovascularization form the basis for clinical interventions aimed at both inhibiting and promoting vessel growth. Antiangiogenic therapy is desirable in proliferative diabetic retinopathy and in some forms of macular degeneration, as well as being an alternative mode of antitumor therapy ***(1)***. In contrast, proangiogenesis is directed towards the formation of new vessels or collaterals. This outcome is desirable in disorders characterized by tissue ischemia, such as myocardial infarction or peripheral vascular disease ***(2)***.

1.1. The Angiogenic Process

Angiogenesis is the process by which new capillaries evolve from existing microvessels, most commonly capillaries and small venules ***(1,3–5)*** but in some cases from arterioles ***(6)***. Neovascularization occurs when the endothelial cells of a microvessel receive an angiogenic stimulus, resulting in the development of sprouts from the parent vessel in the general direction of the signal. These sprouts eventually anastomose to form distinct loops (**Fig. 1**).

At the cellular level the initial response to an angiogenic signal is the localized degradation of the basement membrane of the parent vessel by the responding endothelial cells ***(7,8)***. These cells then migrate directionally from the vessel ***(4,9)***.

During migration the cells align linearly to form a capillary sprout ***(9)***. Proliferation of endothelial cells occurs in the parent vessel as the sprout elongates to facilitate lumen formation ***(10)***. This is followed by anastomosis of adjacent sprouts and resultant capillary loop formation ***(11)***. After the initiation of blood flow, the production of a new basement membrane and incorporation of pericytes leads to the maturation of the new vessel ***(12,13)***.

1.2. Pathological Angiogenesis

Angiogenesis is key to the development of a range of conditions characterized by pathological growth of new capillaries, the most notable of which involves the growth

From: *Methods in Molecular Medicine, vol. 88: Cancer Cell Culture: Methods and Protocols*
Edited by: S. P. Langdon © Humana Press Inc., Totowa, NJ

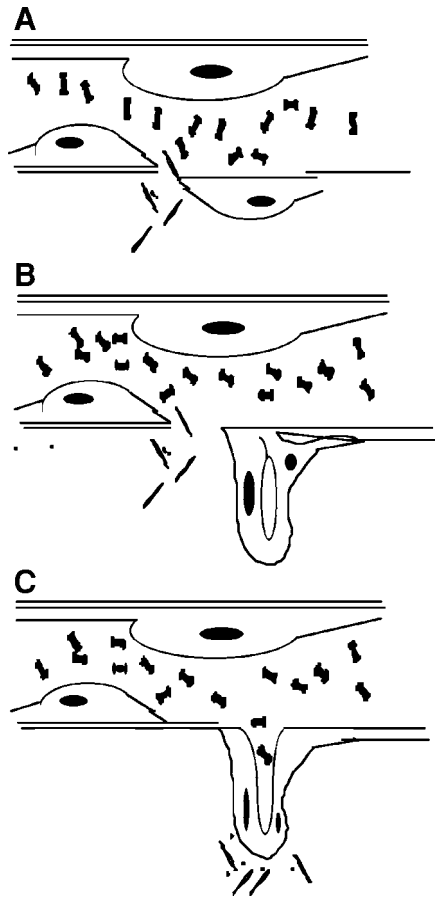

Fig. 1. Key events in the angiogenic process: **(A)** proteolytic degradation of the extracellular matrix, and associated migration of endothelial cells; **(B)** endothelial cells form a sprout and proliferate; **(C)** deposition of new extracellular matrix, formation of tubules, and anastomosis of the newly formed vessel.

of solid tumors ***(14)***. Indeed, much of the current level of understanding of angiogenesis is derived from studies of tumor neovascularization. The concept that solid tumors are angiogenically dependent originally derived from the observation that without circulatory support, tumors fail to develop beyond 1–2 mm in diameter ***(15)***. Subsequent studies involving the transplantation of tumors into the anterior chamber of the eye have confirmed this theory ***(16–18)***. Furthermore, such experiments have shown that avascular tumors have the ability to induce neovascularization ***(19)***. In addition, the capillaries induced by tumors have been reported to grow rapidly and are structurally distinct from nontumor-associated microvasculature; the former are frequently devoid of pericytes and have little or no basement membrane ***(18,20,21)***.

Endothelial cells from tumor induced capillaries are similarly distinct from those of normal vasculature. The cells are relatively undifferentiated, containing numerous mitochondria and a large nucleus, but lacking typical endothelial features, such as micropinocytotic vesicles ***(20)***. It has also been noted that the doubling time of tumor-derived capillary endothelium is markedly less than that of normal capillaries ***(18,20)***.

Recently, it has been shown that tumor and embryonic vessels can be distinguished immunologically suggesting that the endothelial cells of rapidly growing vessels differ not only in their ultrastructure, but also in their ability to express distinct epitopes ***(18)***.

1.3. Assay Systems

Despite the intense interest currently focused on angiogenesis research, the relative inaccessibility of the process and lack of suitable model systems for study have limited experimental progress.

A number of in vivo systems have been developed, including the rabbit cornea model and the chick chorioallantoic membrane (CAM) and vitelline membrane (VIM) assays. However, these systems are impractical for the study or screening of large numbers of samples and are far removed from angiogenesis in a human system. The in vitro methods currently in use have generally isolated the different components of the angiogenic process and have either studied endothelial cell proliferation ***(22)*** or endothelial cell migration (*see* Chapter 20).

Although none of these assay systems accurately reflects the angiogenic process in its entirety, they are generally considered to be responsive to known inhibitors and stimulators of angiogenesis.

A number of alternatives to these model systems have recently been developed which closely resemble the capillary network found in the CAM assay and also allow for rapid throughput screening ***(23–26)***. The general procedure exploits the ability of endothelial cells to associate into tubules in contact with various matrix proteins.

2. Materials

2.1. Isolation of Endothelial Cells

2.1.1. Avian Embryonic Endothelial Cells

1. Fertilized chicken eggs.
2. Petri dishes.
3. Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS).

2.1.2. Bovine Retinal Capillary Endothelial Cells

1. Bovine eyes.
2. MEM medium containing 30 m*M* HEPES (MEM-HEPES).
3. 53-µm Nylon mesh.
4. Pronase.
5. Deoxyribonuclease.
6. Collagenase.
7. Insulin.

8. Heparin.
9. Streptomycin
10. Penicillin.
11. Fungizone.
12. Human serum.
13. Gelatin.

2.1.3. Human Umbilical Vein Endothelial Cells

1. Umbilical cords of neonates delivered by Caesarean section.
2. MEM-HEPES containing 10% FCS.
3. Phosphate-buffered saline (PBS).
4. Collagenase.
5. MEM containing 10% FCS.

2.1.4. Tubule Assay

1. Trypsin-EDTA (ethylenediaminetetraacetic acid).
2. Matrigel.
3. Culture medium: DMEM or MEM containing 10% FCS.
4. 24-Well tissue culture plates.
5. Eppendorf tubes.
6. Pipets.
7. Microscope (phase contrast).

3. Methods

The tubule-forming assay can be used in the determination of both positive and negative angiogenic effects. The system is reproducible and permits the rapid comparison of different treatment regimes. Procedures are detailed for use with primary cultures, although the assay can also utilize established endothelial cell lines.

3.1. Isolation of Endothelial Cells

3.1.1. Avian Embryonic Endothelial Cells

1. Each isolation uses three dozen fertilized chicken eggs, available commercially from various sources. Spray the eggs with 70% ethanol and crack into individual sterile Petri dishes.
2. Dissect out the embryos, remove the area vasculosa from each and bulk the membranes in 10 mL ice cold DMEM supplemented with 10% FCS.
3. Carefully homogenize and then centrifuge the resultant suspension at 800*g* for 10 min.
4. Remove the supernatant and resuspend the pellet in 10 mL DMEM containing 10%FCS.
5. Divide the suspension between two 25 cm^2 tissue culture flasks and incubate at 37.5°C in a humidified atmosphere containing 5% CO_2.

3.1.2. Bovine Retinal Capillary Endothelial Cells

1. Collect freshly removed bovine eyes and store at 4°C overnight in DMEM.
2. Rinse in fresh DMEM and dissect out the retinal vasculature from twenty eye specimens.
3. Homogenize the vasculature in 10 mL ice-cold MEM containing 30 m*M* HEPES buffer until fragments of approx 4 mm^2 are obtained (*see* **Note 1**).
4. Centrifuge the homogenate at 800*g* for 10 min. Discard the supernatant and resuspend the pellet in fresh MEM-HEPES (10 mL).

5. Filter through an 85-µm nylon mesh to retain microvessels.
6. Collect the microvessels and suspend in 5 mL MEM-HEPES containing 0.5 mg/mL pronase, 0.5mg/mL deoxyribonuclease, and 50 µg/mL collagenase.
7. Incubate at 37°C on a rotation wheel for 30 min or until individual microvessels are observed. Stop the reaction by the addition of 10 mL ice cold MEM-HEPES and filter through a 53-µm nylon mesh.
8. Resuspend the retained microvessels in MEM-HEPES (10 mL) and centrifuge at 1000*g* for 10 min.
9. Discard the supernatant and resuspend the pellet in DMEM supplemented with 7.5% platelet poor human serum, 4 µg/mL insulin, 10 µg/mL heparin, 100 U/mL penicillin, 100 µg/mL streptomycin and 1% (v/v) fungizone.
10. Plate the suspension in 25 cm^2 tissue culture flasks precoated with 0.5% gelatin solution and maintain at 37°C in a humidified atmosphere containing 5% CO_2 (*see* **Note 2**).

3.1.3. Human Umbilical Vein Endothelial Cells

HUVEC cells can be isolated from umbilical cords of neonates delivered by caesarean section.

1. The specimens should be transferred to the laboratory from the delivery suite as quickly as possible in ice-cold MEM-HEPES containing 10% FCS (*see* **Note 3**).
2. Remove the cords from the transport medium and cut into 10-cm lengths.
3. Cannulate and wash several times with PBS to rinse out blood. Close one end of the vein with a surgical artery clamp and introduce 0.1% collagenase in MEM-HEPES.
4. Incubate overnight at 4°C.
5. Rinse out the vein with PBS and collect the effluent containing liberated endothelial cells.
6. Spin at 800*g* for 10 min and resuspend the pellet in MEM supplemented with 10% FCS.
7. Divide the suspension between two 25-cm^2 tissue culture flasks and incubate at 37°C in a humidified atmosphere containing 5% CO_2 (*see* **Note 2**).

3.2. Tubule Assay Procedure

1. Prepare a single cell suspension of endothelial cells by trypsinization of the monolayer and centrifuge at 800*g* for 5 min.
2. Resuspend the cell pellet in the ice-cold media containing 10% FCS to give a population density of approx 10^5 cells/mL (*see* **Note 4**).
3. Add equal volumes of the cell suspension and Matrigel to an Eppendorf tube.
4. Maintain on ice and mix immediately.
5. Dispense 100-µL aliquots into the individual wells of a 24-well tissue culture plate.
6. Gently rock the plate to ensure an even coating of the cell suspension.
7. Incubate for 30 min at 37°C in a humidified atmosphere with 5% CO_2. This initial incubation allows the formation of the cell support system prior to the addition of test substances in 0.5 mL medium (*see* **Note 5**).
8. Subsequent to the addition of media and supplements, the plates are incubated further and the media containing test substances refreshed every 72 h (*see* **Note 6**).
9. The cell layer should be examined for tubule formation every 24 h until the end of the experimental period (typically 15 d), using phase contrast microscopy.
10. The extent of tubule formation is not easily determined in unstained culture systems. However, the cells may be fixed with 70% ethanol at the end of the incubation and stained for any one of a number of endothelial cell markers (*see* **Note 7**).

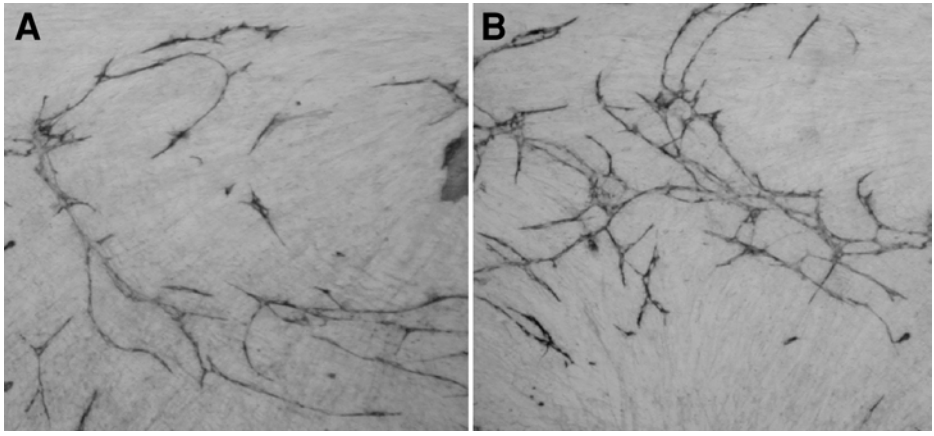

Fig. 2. Digital micrograph of tubule formation in **(A)** untreated SK Hep-1 endothelial cells and **(B)** in cells treated with 0.1 ng/mL vascular endothelial growth factor, showing an increase in the vascular network (reduced from original magnification ×40).

11. Cultures can then be scored manually or by means of image analysis software. Typical results are shown in **Fig. 2**.

4. Notes

1. It is important that the temperature of the homogenization buffer is not allowed to rise as the cells may be adversely affected by resulting proteolytic activity.
2. It is important to examine the cultures frequently during the initial incubation period for any signs of contamination.
3. Patient consent should be obtained and use of the cells fully documented as requested by current legislation.
4. This population density serves only as a guideline. Optimal cell numbers will depend on the individual characteristics of the cells used in the assay system. It is important that the cell suspension and Matrigel both be maintained on ice prior to mixing or the support will gel.
5. Modifications of this technique allow for the treatment of Matrigel to remove potential mitogens and angiogenic contaminants by means of low molecular weight cut-off filters.
6. Care should be taken to ensure that the wells are not allowed to dry out during media changes. Similarly, care must be taken to avoid damaging the cell layer with pipets.
7. Antibodies for specific endothelial cell markers and individual staining procedures are available from a number of commercial sources.

References

1. Folkman, J. (1997) Angiogenesis and angiogenesis inhibition: an overview. *EXS* **79,** 1–8.
2. Isner, J. M. and Asahara, T. (1999) Angiogenesis and vasculogenesis as therapeutic strategies for postnatal neovascularization. *J. Clin. Invest.* **103,** 1231–1236.
3. Cogan, D. G. (1949) Vascularisation of the cornea, its experimental induction by small lesions and a new theory of its pathogenesis. *Arch. Ophthalmol.* **41,** 406–416.

4. Auspruck, D. H. and Folkman, J. (1977) Migration and proliferation of endothelial cells in preformed and newly formed blood vessels during tumour angiogenesis. *Microvasc. Res.* **14,** 53–66.
5. Myrhage, R. and Hudlicka, O. (1978) Capillary growth in skeletal muscle. *Microvasc. Res.* **16,** 73–90.
6. Zarem, H. A., Zweifach, B. W., and McGehee, J. M. (1967) Development of microcirculation in full thickness autogenous skin grafts in mice. *Am. J. Physiol.* **212,** 1081–1085.
7. Nicosia, R. F., Tchao, R., and Leighton, J. (1983) Angiogenesis dependent tumor spread in reinforced fibrin clot culture. *Cancer Res.* **43,** 2159–2166.
8. Gross, J. L., Moscatelli, D., and Rafkin, D. B. (1983). Increased capillary endothelial cell protease activity in response to angiogenic stimuli in vitro. *Proc. Natl. Acad. Sci. USA* **80,** 2623–2627.
9. Folkman, J. (1985). Regulation of angiogenesis: a new function of heparin. *Biochem. Pharmacol.* **34,** 905–909
10. Ausprunk, D. H., Knighton, D. R., and Folkman, J. (1974) Differentiation of vascular endothelium in the chick chorioallantois: a structural and autoradiographic study. *Dev. Biol.* **38,** 237–248.
11. Harris-Hooker, S. A., Gadjusek, C. M., Wight, T. N. and Schwartz, S. M. (1983) Neovascular responses induced by cultured aortic endothelial cells. *J. Cell Physiol.* **114,** 302–310.
12. Zweifach, B. W. (1980) Integrity of vascular epithelium. *Adv. Microcirc.* **9,** 206–225.
13. D'Amore, P. A. and Thomson, R. W. (1987) Mechanisms of angiogenesis. *Ann. Rev. Physiol.* **49,** 453–464.
14. Moses, M. A. and Langer, R. (1991) Inhibitors of angiogenesis. *Biotech.* **9,** 630–634.
15. Folkman, J. (1972) Anti-angiogenesis: new concept of therapy of solid tumours. *Ann. Surg.* **175,** 409–416.
16. Gimbrone, M. A., Leapman, S. B., Cotran, R. S. and Folkman, J. (1972) Tumour dormancy in vivo by prevention of neovascularization. *J. Exp. Med.* **136,** 261–276.
17. Carlsson, J., Stalnacke, C. G., and Acker, H. (1979) The influence of oxygen on viability and proliferation in cellular spheroids. *Int. J. Radiat. Oncol. Biol. Phys.* **5,** 2011–2020.
18. Blood, C. N. and Zetter, B. R. (1990) Tumor interactions with the vasculature: angiogenesis and tumour metastasis. *Biochem. Biophys. Acta* **1032,** 89–118.
19. Gimbrone, M. A., Cotran, R. S., Leapman, S. B., and Folkman, J. (1974) Tumor growth and neovascularization: an experimental model using rat cornea. *J. Natl. Cancer Inst.* **52,** 413–427.
20. Schor. A. M. and Schor, S. L. (1983) Tumour angiogenesis. *J. Pathol.* **141,** 385–413.
21. Paku, S. and Paweletz, N. (1991) First steps of tumor-related angiogenesis. *Lab. Invest.* **65,** 334–346.
22. Connolly, D. T., Heuvelman, D. M., Nelson., R., Olanter, J. V. and Eppley, B. L. (1989) Tumour vascular permiability factor stimulates endothelial cell growth and angiogenesis. *J. Clin. Invest.* **84,** 1470–1478.
23. Donovan, D., Brown, N. J., Bishop, E. T., and Lewis, C. E. (2001) Comparison of three in vitro human 'angiogenesis' assays with capillaries formed in vivo. *Angiogenesis* **4,** 113–121.
24. Bussolati, B., Dunk, C., Grohman, M., Kontos, C. D., Mason. J., and Ahmed, A. (2001) Vascular endothelial growth factor receptor-1 modulates vascular endothelial growth factor-mediated angiogenesis via nitric oxide. *Am. J. Pathol.* **159,** 993–1008.

25. Houston, P., Campbell, C. J., Svaren, J., Milbrandt, J. and Braddock, M. (2001) The transcriptional corepressor NAB2 blocks Egr-1-mediated growth factor activation and angiogenesis. *Biochem. Biophys. Res. Commun.* **283,** 480–486.
26. Drinkwater. S. L., Smith, A., Sawyer, B. M., and Burnand, K.G. (2002) Effect of venous ulcer exudates on angiogenesis in vitro. *Br. J. Surg.* **89,** 709–713.

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Flow Cytometric DNA Analysis of Human Cancer Cell Lines

Peter Mullen

1. Introduction

Flow cytometry is a rapid technique allowing simultaneous analysis of multiple cellular parameters, including DNA content. The technique relies on a single-cell suspension being passed within a stream of sheath fluid through an optically focused excitation light source, either a laser or an arc lamp. The most common excitation wavelength used in flow cytometers is a 488 nm wavelength light from an argon laser. Lasers provide a single wavelength of coherent light while arc lamps produce a mixture of incoherent wavelengths that must be filtered. When a laser light source is used, the amount of light scattered in the forward direction is detected in the forward angle light scatter (FALS) channel and its intensity is roughly proportional to the size of the cells or particles. Light scattered perpendicular to the path of the laser is detected in the side scatter channel (SSC) and its intensity is more closely related to granularity. If the cells have been stained with fluorochromes they will also emit fluorescence intensities at levels that directly correspond to the density of fluorochrome on or within the cell. The fluorescence signal emitted by any specific fluorochrome is collected through separate channel detectors (photomultipliers) by means of a series of optical filters and mirrors that guide the beam of light. In order to simultaneously measure more than one fluorescence signal from any given cell, multiple channels/detectors are used. A more comprehensive resume can be found elsewhere ***(1)***.

DNA dyes (such as propidium iodide) are useful in that they can quantify how much DNA is present within a given cell. Tumor cells are often found to be "aneuploid," that is, having too many or too few chromosomes compared to the normal diploid DNA number for the species (*see* **Note 1**). In addition, such dyes can quantify the proportion of cells in the different phases of the cell cycle, namely:

1. G_0, resting, nondividing cells, or any cycling cells in G_1,
2. S phase (when DNA is synthesized), and
3. G_2 or in the process of mitosis.

From: *Methods in Molecular Medicine, vol. 88: Cancer Cell Culture: Methods and Protocols*
Edited by: S. P. Langdon © Humana Press Inc., Totowa, NJ

Abnormal nuclear DNA content has been shown to be a useful marker of malignancy ***(2)*** while a number of reports have shown an improved survival rate associated with normal or near normal DNA content in breast ***(3–7)***, ovarian ***(8–10)***, and prostate cancer ***(11)***. Although some studies have found DNA content to be an independent prognostic indicator of survival in colorectal carcinoma ***(12–15)***, other studies suggest that conventional histological parameters remain the best predictors of prognosis, with no flow cytometric DNA variable being able to significantly improve on the conventional prognostic variables of Duke's stage, patient age, and tumor differentiation ***(16)***. Similarly, others have reported that DNA content and ploidy status do not appear to be major prognostic indicators in ovarian cancer ***(17,18)***. There are also reports showing that the proportion of proliferating cells in S phase of the cell cycle may be of prognostic significance in breast ***(19)*** and lung cancer ***(20)***—indeed , it has also been reported in meningioma patients that the proportion of S-phase tumor cells may represent the most important parameter for predicting disease-free survival ***(21)***. Because flow cytometry is such a rapid and precise method of measuring tumor DNA content, it may therefore be of both diagnostic and prognostic value as well as being useful when studying the mechanism of the action of drugs by means of alterations in the cell cycle distribution ***(22)***.

Although the methodology described in this chapter is specifically aimed at preparing and staining nuclei from established cell lines for the purpose of DNA, other protocols can be found for studying clinical material derived from a number of pathological sources, including fresh, frozen, paraffin-embedded, or fine-needle aspirated tissue ***(23–26)***.

2. Materials

2.1. Preparation of Solutions

1. Citrate storage buffer: Add 85.50 g sucrose (250 m*M*) and 11.76 g trisodium citrate, 2 H_2O (40 m*M*) to a beaker of distilled water with 50 mL of dimethyl sulfoxide (DMSO). After adjusting to pH 7.6, fill to 1 L and store at 4°C. This buffer is used to store frozen samples prior to analysis ***(27)***.
2. Stock solution: dissolve 2 g trisodium citrate · 2 H_2O (3.4 m*M*), 2 mL NP40 (0.1% v/v), 1044 mg spermine tetrahydrochloride (Sigma Aldrich, Poole, Dorset, UK, cat. no. S2876) (1.5 m*M*) and 121 mg Tris [(hydroxymethyl)-aminomethane] (Sigma Aldrich, cat. no. T1378) (0.5 m*M*) in 2 L of distilled water after adjusting to pH 7.6. This "stock solution" is used to make up the digestion/staining buffers ***(28)***.
3. Solution A: 15 mg trypsin (Sigma Aldrich, cat. no. T0303) in 500 mL of stock solution (adjusted to pH 7.6).
4. Solution B: 250 mg trypsin inhibitor (Fluka Chemicals, Poole, Dorset, UK, cat. no. 93621) and 50 mg RNase A (Sigma Aldrich, cat. no. R4875) in 500 mL of stock solution (adjusted to pH 7.6).
5. Solution C: 208 mg propidium iodide (Fluka Chemicals) and 580 mg spermine tetrahyrdrochloride made up in 500 mL of stock solution, adjusted to pH 7.6. Protect solution from the light by using tinfoil throughout the preparation, storage, and the staining procedures (*see* **Note 2**).
6. All solutions were stored in 20-mL aliquots at –70°C.

2.2. Cell Culture

1. Select cell line of interest, for example, MCF-7 breast cancer ***(29)*** or PE01 ovarian cancer ***(30)*** cells.
2. RPMI/PS/10% FCS: combine 500 mL RPMI 1640 culture media (Gibco-BRL, Life Technologies Ltd., Paisley, Scotland) with penicillin/streptomycin (P/S) (Gibco-BRL, Life Technologies Ltd.) at final concentrations of 100 U/mL and 100 µg/mL, respectively. Add 55 mL (10%) fetal calf serum (FCS; Advanced Protein Products, Brierley Hill, England) (*see* **Note 3**).
3. Dulbecco's phosphate buffered saline (PBS) (Oxoid, Unipath, Basingstoke, England).
4. 1X Trypsin-EDTA (Gibco-BRL, Life Technologies, Paisley, Scotland).
5. 75-cm^2, and 150-cm^2 Tissue culture flasks (Life Technologies).
6. 10-mL Syringes and 21G needles (Becton Dickinson, Franklin Lakes, NJ).
7. FACS tubes (Becton Dickinson).

2.3. Preparation of Samples for DNA analysis

1. Water bath and Wirlimixer.
2. RNase (R4875, Sigma Aldrich, Poole, Dorset, UK).

2.4. Acquisition of Flow Cytometric DNA Histograms

1. FACSCalibur™ flow cytometer (Becton Dickinson) (*see* **Note 4**).

2.5. Analysis of Flow Cytometric DNA Histograms

1. Cell cycle analysis software, for example, ModFit LT™ (Verity Software House).

3. Methods

3.1. Cell Culture

1. Suspend 0.5×10^6 MCF-7 breast cancer or PE01 ovarian cancer cells in 25 mL RPMI/PS/10% FCS and transfer to a 75-cm^2 tissue culture flask. Place in a 5% CO_2 humidified incubator at 37°C.
2. Allow cells to grow to confluence, feeding twice per week by aspirating the spent media, washing with 20 mL PBS, and then adding 25 mL fresh tissue culture media.
3. When cells become confluent, remove spent tissue culture media and wash cells in 10 mL PBS. Remove excess PBS.
4. Add 5 mL trypsin/EDTA to each flask and place back in the incubator until the cells have become detached (*see* **Note 5**).
5. Transfer cell suspension to a sterile Universal container using a sterile pipet; pour 20 mL RPMI/PS/10% FCS (to deactivate the trypsin) into the flask in order to wash it out and then pool with the cell suspension in the Universal container (total volume of 25 mL).
6. Centrifuge at 600*g* for 5 min.
7. Pour off the media and resuspend the pellet in 5 mL RPMI/PS/10% FCS. Syringe with a 21G needle (3×) to break up the pellet, and then make up to 25 mL with RPMI/PS/10% FCS.
8. Count total number of cells using a hemocytometer.
9. Transfer aliquots of 1×10^6 cells to FACS tubes (regardless of volume) and then centrifuge again at 600*g* for 5 min.
10. Resuspend pellet in 100 µL citrate buffer, cover tubes, and store at –40°C prior to analysis.

3.2. Preparation of Samples for Analysis

1. Thaw solutions A and B, along with the frozen whole cell suspensions (in 100 µL) (but not heated) in a water bath at 37°C before preparing and staining nuclei for DNA analysis. Allow solution C to thaw at room temperature before placing on ice.
2. Digest cell suspensions down to nuclei by adding 450 µL of 0.003% trypsin solution (Solution A), mix and leave at room temperature for 10 min (*see* **Notes 6** and **7**).
3. Prevent further degradation by adding 0.05% (w/v) trypsin inhibitor solution and 0.01% (w/v) RNase A (Solution B) in a final volume of 375 µL, mix, and leave for 10 min.
4. Finally, stain cells by adding 416 µg/mL ice-cold propidium iodide/1.16 mg/mL spermine tetrahydrochloride solution (Solution C) in a final volume of 250 µL and leave the samples on ice in the dark for an additional 10 min prior to analysis.

3.3. Acquisition of Flow Cytometric DNA Histograms

1. This protocol assumes that the user is familiar with the principles and practices of flow cytometry and is able to run samples according to the operators manual pertaining to the instrument being used.
2. For the purposes of collecting data, all plots must be formatted for "Acquisition".
3. Plot a two-parameter dot-plot of Forward Light Scatter (FLS) vs Side Scatter (SS).
4. Plot a single-parameter FL2 (area) histogram with linear *x*-axis to illustrate relative DNA content (propidium iodide fluorescence is usually assigned to the FL2 channel; *see* **Note 8**).
5. Plot a two-parameter dot-plot of FL2 (area) vs FL2 (width) to monitor doublets (*see* **Subheading 3.4.**).
6. Select the signal threshold (the point at which a signal will be accepted as a positive event) to FL2 and then set an appropriate value to gate out debris (a value of 20 should suffice in the first instance).
7. No compensation is required since only one fluorochrome (propidium iodide) is present.
8. Introduce the sample and set the machine to "Run". Using the appropriate settings panel, adjust both FLS and SS photo multiplier tube (PMT) voltages so that the majority of dots in the first two-parameter dot-plot (FLS vs SS) are contained roughly within the center of the box (*see* **Fig. 1A**).
9. Adjust the FL2 PMT voltage up or down until the peak appears in the graph. The voltage can then be fine-tuned so that the main peak is approximately one quarter of the way along the *x*-axis within the linear graph (*see* **Fig. 1B**). This voltage will allow sufficient space along the *x*-axis for the G_2/M peak (which will have twice as much DNA per cell) to be held in the graph and not over-spill the end. The histogram can also be monitored for any tetraploid cells that may be present.
10. After setting up the machine, 10,000 ungated events are collected. Data files are stored in an appropriate folder for subsequent retrieval/analysis using cell cycle software provided with the machine being used.

3.4. Analysis of Flow Cytometric DNA Histograms

1. Provisional analysis of data can be conducted in a manner similar to data acquisition with all histograms being formatted for "Analysis" rather than "Acquisition."
2. Plot a two-parameter dot-plot of FL2 (area) vs FL2 (width), open the first data file, and set the "gates" around the majority of cells contained within the FL2 (area) vs FL2 (width) dot-plot. Define as "gate 1" (**Fig. 2A**).

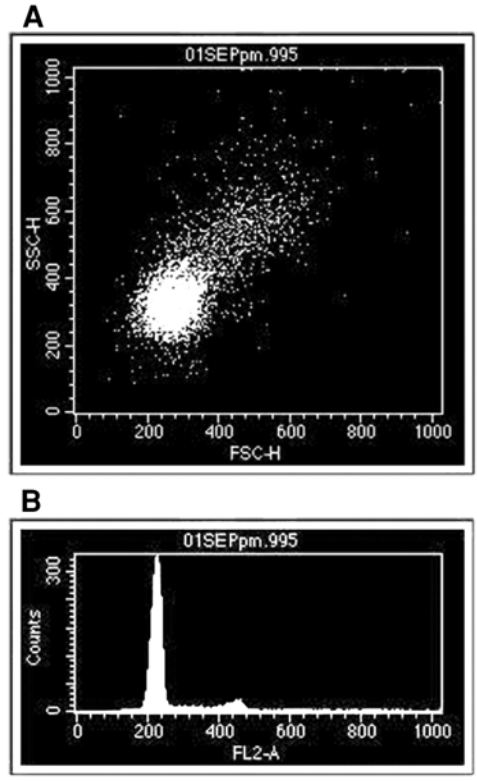

Fig. 1. **(A)** Data acquisition is optimized by separately adjusting forward angle light scatter (FALS, FLS, FSC) and side scatter (SS, SSC) voltages to fall on a diagonal line from lower left to upper right with the majority of cells lying roughly within the center of a two-parameter FLS vs SS dot-plot. Increasing the FLS voltage will stretch the signals along the *x*-axis, whilst increasing SS voltages will pull the signals up the *y*-axis. **(B)** DNA content can then be visualized by increasing FL2 voltage until the main G_0/G_1 peak can be seen to appear in the linear graph approximately one quarter of the way along the *x*-axis. By keeping the main peak to the left of the histogram channel 200, for example, sufficient space to the right will be left for G_2/M cells with twice as much DNA per cell (channel 400) as well as any tetraploid G_0/G_1 cells that may be present (channel 400) and their respective G_2/M component (channel 800). Data acquisition can then be activated and the required number of events accrued.

3. Plot a single-parameter FL2 (area) histogram with linear *x*-axis to represent relative DNA content. Format the histogram by deselecting the default "ungated" events and choose "gate 1".
4. Place cursors around the first (G_0/G_1), intermediate (S), and second (G_2/M) populations of cells in a manner appropriate to your machine and choose the appropriate statistics of interest **(Fig. 2B)**. Successful analysis should yield the appropriate proportion of cells in the G_0/G_1-phase, S phase, and G_2/M phases of the cell cycle.

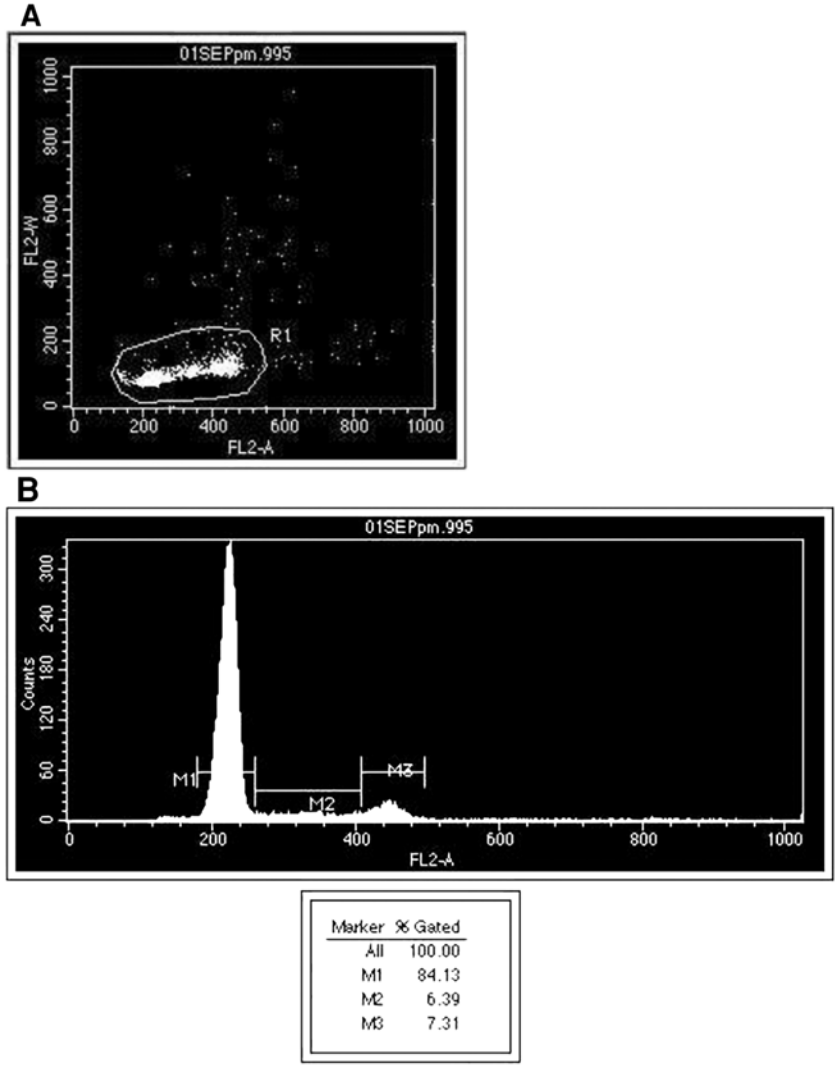

Fig. 2. **(A)** Data analysis is performed by retrieving the saved data file into a histogram template within a suitable analysis package (i.e., CellQuest™ [Becton Dickinson]). A two-parameter dot-plot of FL2A (area) vs FL2W (width) is created and electronic gates drawn around the main cluster of cells. Activation of the gate during subsequent analysis will then consider and process data contained only within the defined region (for example, Region 1). **(B)** A single-parameter FL2A (area) histogram, with linear *x*-axis to represent relative DNA content, is plotted and formatted by selecting the predefined gate 1 (rather than the default setting of ungated events). Cursors can then be placed around the first (G_0/G_1), intermediate (S), and second (G_2/M) populations of cells and the appropriate statistics of interest selected. Comparative cell cycle distribution data (i.e., the appropriate proportion of cells in the G_0/G_1 phase, S phase and G_2/M phases of the cell cycle) can then be made on an appropriate sample series.

5. Accurate cell cycle analysis must be performed using dedicated software supplied with the instrument being used. Users are expected to be familiar with such software and the appropriate statistical models to use for such analysis. For the purposes of this protocol, analysis was carried out using the ModFit software provided with the FACSCalibur flow cytometer.
6. Open the ModFit program and select the appropriate **FILE**.
7. Choose the parameter for analysis; in this case select FL2A for relative DNA content.
8. Define "gate 1" by selecting FL2A (x) and FL2W (y). Drag each of the points of the gate (R1) to include the entire cell population of interest.
9. Choose a specific **MODEL** to analyze the data or use the suggested model according to specified parameters, such as whether samples were fresh or frozen or paraffin embedded; of diploid, aneuploid or tetraploid DNA content; whether aggregates were present; or if there is a visible G_2/M fraction. The model can also account for the presence of internal standards should they be included.
10. Check the position and **RANGE** of the markers that are automatically placed on the histogram and adjust their position if necessary (this may be the case, particularly if the S-phase fraction becomes relatively high).
11. Calculate the relative cell cycle distributions using the **FIT** option (**Fig. 3**). Repeat the process for all other samples, making sure that the cell population of interest is within the defined "gate." Adjust the gate if necessary.
12. Data can then be tabulated and exported to a suitable presentation package, for example, Excel.

4. Notes

1. Many cancer cell lines are not true diploid but rather aneuploid, where aneuploidy is defined as having a greater than 10% deviation from the DNA index of 1.0. Many primary tumors can produce one or more aneuploid cell lines in addition to the normal diploid state. Established cancer cell lines usually exhibit a single diploid or aneuploid population.
3. Propidium iodide is a DNA intercalating agent and must therefore be treated appropriately and discarded in a suitable manner.
3. FCS must be heat-inactivated prior to use by heating in a water bath for 30 min at 56°C. It can then be placed in 60-mL aliquots and stored at –20°C.
4. Although much of this protocol pertains to a specific instrument, namely the Becton Dickinson FACSCalibur, the methodology is applicable to other machines. The manner in which histograms are set up and the cell cycle analysis software may be different.
5. The time interval for cells to become detached from the plastic is dependent upon the cell line being used, but is usually in the order of 5–10 min.
6. Chicken erythrocyte nuclei (CEN) and trout erythrocyte nuclei (TEN) can be added to samples prior to trypsinization and used as internal controls in order to calculate the ploidy (diploid DNA content) of the cell lines in question ***(31)***. Because CEN and TEN have less total DNA per cell than human cells (35% and 80%, respectively), they have by definition DNA indexes of 0.35 and 0.8 relative to a human diploid index of 1.0. Relative positions (channel numbers) of the CEN or TEN peaks can therefore be used to determine the relative DNA index of unknown samples. Although both internal standards can be included when determining the ploidy value of a given cell line, cell cycle distribution is made difficult since the G_2/M peak of TEN will fall within the S-phase fraction of normal diploid cells. CEN alone can therefore be used if a ploidy check is deemed necessary while at the same time cell cycle data is required.

7. There is considerable debate as to whether CEN and TEN controls are the most appropriate internal standards. A human blood preparation (with a true ploidy value of 1.0) may be more relevant, providing that the cell lines in question are not true diploid in nature (in which case the two peaks will overlap owing to their identical relative DNA contents).
8. All single-parameter histograms should be of 1024 channels resolution (rather than 256) to obtain maximum resolution.

References

1. Shapiro, H. M. *Practical Flow Cytometry, 4th Ed.*, Alan R Liss, ISBN 0-471-41125-6.
2. Barlogie, B., Raber, M. N., Schuman. J., et al. (1983) Flow cytometry in clinical cancer research. *Cancer Res.* **43,** 3982–3997.
3. Auer, G. U., Caspersson, T. O., and Wallgren, A. S. (1980) DNA content and survival in mammary cancer. *Anal. Quant. Cytol. Histol.* **2,** 161–165.
4. Baildam, A. D., Zaloudik, J., Howell, A., et al. (1987) DNA analysis by flow cytometry, response to endocrine treatment and prognosis in advanced carcinoma of the breast. *Br. J. Cancer* **55,** 553–559.
5. Kallioniomi, O. P., Hietanen, T., Mattila, J., Lentinen, M., Lauslahti, K., and Koivula, T. (1987) Aneuploid DNA cancer and high S-phase fraction of tumour cells are related to poor prognosis in patients with primary breast cancer. *Eur. J. Cancer Clin. Oncol.* **23,** 277–282.
6. Pinto, A. E., Andre, S., and Soares, J. (1999) Short term significance of DNA ploidy and cell proliferation in breast carcinoma: a multivariate analysis of prognostic markers in a series of 308 patients. *J. Clin. Pathol.* **52,** 604–611.
7. Bracko, M., Us-Krasovec, M., Cufer, T., Lamovec, J., Zidar, A., and Goehde, W. (2001) Prognostic significance of DNA ploidy determined by high resolution flow cytometry in breast carcinoma. *Anal. Quant. Cytol. Histol.* **23,** 56–66.
8. Evans, M. P., and Podratz, K. C. (1996) Endometrial neoplasia: prognostic significance of ploidy status. *Clin. Obstet. Gynecol.* **39,** 696–706.
9. Ozalp, S., Yalcin, O. T., Gulbas, Z., Tanir, H. M., and Minsin, T. (2001) Effect of cellular DNA content on the prognosis of epithelial ovarian cancers. *Gynecol. Obstet. Invest.* **52,** 93–97.
10. Kimmig, R., Wimberger, P., Hillemanns, P., Kapsner, T., Caspari, C., and Hepp, H. (2002) Multivariate analysis of the prognostic significance of DNA-ploidy and S-phase fraction in ovarian cancer determined by flow cytometry following detection of cytokeratin-labelled tumor cells. *Gynecol. Oncol.* **84,** 21–31.
11. Fordham, M. V., Burdge, A. H., Matthews, J., Williams, G., and Cooke, T. (1986) Prostatic carcinoma cell DNA content measured by flow cytometry and its relation to clinical outcome. *Br. J. Surg.* **73,** 400–403.
12. Wolley, R. C., Schreiber, K., Koss, L. G., Karas, M., and Sherman, A. (1982) DNA distribution in human colon carcinomas and its relationship to clinical behaviour. *J. Natl. Cancer Inst.* **69,** 15–22.
13. Emdin, S. O., Stenling, R., and Roos, G. (1987) Prognostic value of DNA content in colorectal carcinoma. A flow cytometric study with some methodologic aspects. *Cancer* **60,** 1282–1287.
14. Lanza, G., Gafa, R., Santini, A., et al. (1998) Prognostic significance of DNA ploidy in patients with stage II and stage III colon carcinoma: a prospective flow cytometric study. *Cancer* **82,** 49–59.

15. Salud, A., Porcel, J. M., Raikundalia, B., Camplejohn, R. S., and Taub, N. A. (1999) Prognostic significance of DNA ploidy, S-phase fraction, and P-glycoprotein expression in colorectal cancer. *J. Surg. Oncol.* **72,** 167–174.
16. Deans, G. T., Williamson, K., Heatley, M., et al. (1993) The role of flow cytometry in carcinoma of the colon and rectum. *Surg. Gynecol. Obstet.* **177,** 377–382.
17. Resnik, E., Trujillo, Y. P., and Taxy, J. B. (1997) Long term survival and DNA ploidy in advanced epithelial ovarian cancer. *J. Surg. Oncol.* **64,** 299–303.
18. Coley, H. M., Sargent, J. M., Titley, J., and Taylor, C. G. (1999) Lack of prognostic significance of ploidy and S-phase measurements in advanced ovarian cancer. *Anticancer Res.* **19,** 2111–2116.
19. Duigou, F., Herlin, P., Marnay, J., and Michels, J. J. (2000) Variation of flow cytometric DNA measurement in 1,485 primary breast carcinomas according to guidelines for DNA histogram interpretation. *Cytometry* **15,** 35–42.
20. Emri, S., Akbulut, H., Zorlu, F., et al. (2001) Prognostic significance of flow cytometric DNA analysis in patients with malignant pleural mesothelioma. *Lung Cancer* **33,** 109–114.
21. Maillo, A., Diaz, P., Blanco, A., et al. (1999) Proportion of S-phase tumour cells measured by flow cytometry is an independent prognostic factor in meningioma tumors. *Cytometry* **38,** 118–123.
22. Koester, S. K., Maenpaa, J. U., Wiebe, V. J., et al. (1994) Flow cytometry: potential utility in monitoring drug effects in breast cancer. *Breast Cancer Res. Treat.* **32,** 57–65.
23. Hedley, D. W., Friedlander, M. L., Taylor, I. W., Rugg, C. A., and Musgrove, E. A. (1984) Method for analysis of cellular DNA content of paraffin-embedded pathological material using flow cytometry. *J. Histochem. Cytochem.* **31,** 1333–1335.
24. Schultz, D. S. and Zarbo, R. J. (1992) Comparison of eight modifications of Hedleys method for flow cytometric DNA ploidy analysis of paraffin-embedded tissue. *Am. J. Clin. Pathol.* **98,** 291–295.
25. Alanen, K. A., Klemi, P. J., Joensuu, H., Kujari, H., and Pekkala, E. (1989) Comparison of fresh, ethanol-preserved, and paraffin-embedded samples in DNA flow cytometry. *Cytometry* **10,** 81–85.
26. Levack, P. A., Mullen, P., Anderson, T. J., Miller, W. R., and Forrest, A. P. M. (1987) DNA analysis of breast tumour fine needle aspirates using flow cytometry. *Br. J. Cancer* **56,** 643–646.
27. Vindeløv, L. L., Christensen, I. J., Keiding, N., Spang-Thomsen, M., and Nissen, N. I. (1983) Long term storage of samples for flow cytometric DNA analysis. *Cytometry* **3,** 317–322
28. Vindeløv, L. L., Christensen, I. J., Nissen, N. I. (1983) A detergent-trypsin method for the preparation of nuclei for flow cytometric DNA analysis. *Cytometry* **3,** 323–327.
29. Soule, H. D., Vazquez, J., Long, A., Albert, S., and Brennan, M. T. (1973) A human cell line from a pleural effusion derived from a breast carcinoma. *J. Nat. Cancer Inst.* **51,** 1409–1413.
30. Langdon, S. P., Lawrie, S. S., Hay, F. G., et al. (1988) Characterisation and properties of nine human ovarian cancer cell lines. *Cancer Res.* **48,** 6166–6172.
31. Vindeløv, L. L., Christensen, I. J., and Nissen, N. I. (1983) Standardisation of high-resolution flow cytometric DNA analysis by the simultaneous use of chicken and trout red blood cells as internal reference standards. *Cytometry* **3,** 328–331.

24

DNA-Mediated Gene Transfer

Amanda J. M. O'Donnell

1. Introduction

Transfection is the process by which exogenous DNA is transferred into eukaryotic cells. This allows the functional study of a specific gene product within a cellular context. To facilitate expression of the gene of interest, it is first cloned into a vector plasmid DNA that supplies necessary transcriptional and translational control elements (*see* **Fig. 1**). There are two types of transfection; transient transfection and stable transfection. Both have advantages and limitations.

1.1. Transient Transfection

Transfected plasmid DNA is frequently unstable since it exists as an extrachromosomal unit that is not inherited by progeny cells. Expression of the transferred gene is eventually lost after a few days, as the plasmid is either degraded by nucleases or diluted by cell division, but this time period is usually sufficient for functional analysis. Higher gene expression can be obtained in this system by using a replicating vector. This increases intracellular copy number, which in turn increases the level of gene product. A commonly used replicating vector is the SV40 vector, which contains the SV40 origin of replication (ori). This plasmid will replicate in COS cells, a line of monkey kidney cells that expresses SV40 large-T ***(1)***.

1.2. Stable Transfection

Stable transfectants are cells in which the transfected exogenous DNA has been spontaneously integrated into the host genome by nonhomologous recombination ***(2)***. This is a rare event; usually less than 0.02% of a population of transfected cells are stably transformed. To allow isolation of stable transfectants, the transfected vector DNA must contain a selectable marker, such as the gene for neomycin phosphotransferase, which confers resistance to the G-418 antibiotic. It should be noted that creation of stable cell lines involves integration of foreign DNA into the host genome. The selection of a site for integration is a random event and may cause gross chromosomal rearrangement. Because no two cells will have the same site of integra-

From: *Methods in Molecular Medicine, vol. 88: Cancer Cell Culture: Methods and Protocols*
Edited by: S. P. Langdon © Humana Press Inc., Totowa, NJ

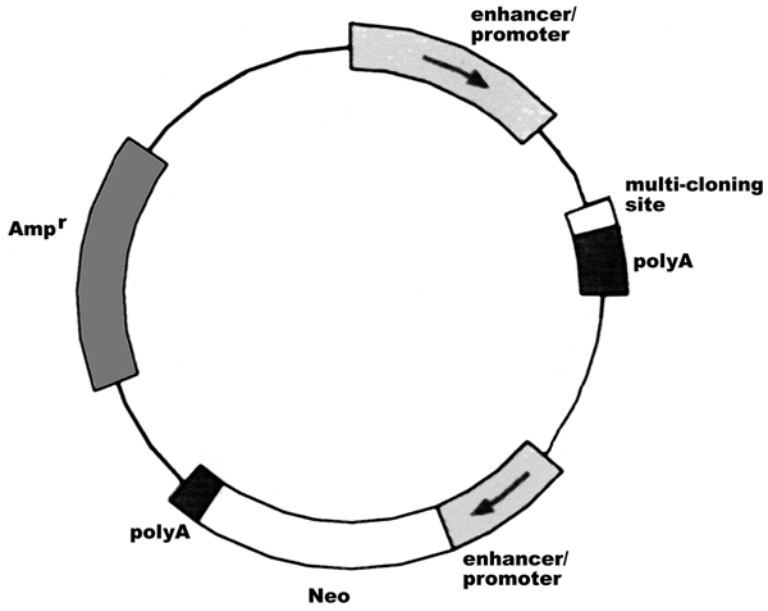

Fig. 1. Typical mammalian expression vector. The gene of interest is cloned into a multicloning site flanked by an RNA polymerase binding site, to drive transcription, and a polyadenylation site, to terminate transcription and enhance translation. An upstream enhancer/promoter aids expression. The cloned gene insert must provide the translational start site ATG. Also in this plasmid are genes encoding neomycin phosphotransferase, which gives protection to transfected cells from the G-418 antibiotic when creating stable transfectants, and β-lactamase, which gives transformed bacteria protection from ampicillin antibiotic when replicating the vector.

tion, and the location of the integration site may affect expression of both exogenous and endogenous genes, it is essential to clone several cell lines, each derived from a single transfectant. Stable transfection is commonly used with inducible gene expression vector plasmids, allowing expression of the integrated gene to be switched on and off. This is particularly important if prolonged expression of the transfected gene is detrimental to the cell.

1.3. Methods of DNA-Mediated Gene Transfer

Several methods can be used to transfer DNA into cells. No single method works best for all applications. Deciding factors in the choice of method include cost, reproducibility, toxicity, mechanism of delivery, ease of use, and efficiency.

1.3.1. DEAE-Dextran

Diethylaminoethyl (DEAE)-Dextran is a polymeric cation that associates tightly with the negatively charged DNA and transports it into the cell ***(3)***. This method is

successful for transient but not stable transfection of cells ***(1)***. DEAE-Dextran is, however, very toxic and is only suitable for transfecting certain cell lines. It cannot be used to transfect cells that cannot tolerate serum-free media. Concentration and exposure times must be optimized for each cell line.

1.3.2. Calcium Phosphate

Transfection mediated by calcium phosphate involves mixing DNA with $CaCl_2$ and a phosphate buffer to form a fine coprecipitate of calcium phosphate and DNA ***(4)***. These complexes adhere to the cell membrane and enter the cell by phagocytosis. This method can be used for both transient and stable transfection. Transfection efficiency (i.e., the proportion of transfected cells within an exposed population) is relatively low but this method is very inexpensive.

1.3.3. Cationic Lipids and Polymers

The cationic head of the lipid compound associates with the negatively charged DNA. The lipid-DNA fuses, or associates, with the negatively charged cell membrane, which results in internalization of the nucleic acid into the cell through endosomes and lysosomes ***(5)***. Lipids can deliver DNA to transfection-resistant cell types more efficiently than chemical methods. This method can be costly but transfection efficiency is very high and more reproducible than traditional chemical methods.

1.3.4. Electroporation

A specific electrical field transiently alters cell membranes so that macromolecules such as DNA can enter the cell ***(6,7)***. The success of gene transfer is no longer dependent on cell division. High gene transfer efficiencies are achieved even in resting cells, and the time required to detect gene expression is reduced. Extensive optimization of conditions is required however, as high levels of cell death occur. This method is inexpensive, after the initial cost of the electroporation machine. It has been shown, however, that electrically induced changes to the physiology of the cell membrane may affect cell-signaling pathways.

1.4. Optimizing Transfection Efficiency

The key to successful transfection is the careful optimization of transfection conditions for each cell type. Several transfection protocols should be tried in order to determine the most efficient method. The following factors, which may influence transfection efficiency, should also be considered.

1. Condition of cells: cell lines may change as they are maintained in culture, accumulating mutations, gross chromosomal rearrangements, or changes in gene expression; therefore, greater reproducibility will be achieved by using low passage number cells (< 50 splitting cycles; *see* **Note 1**).
2. Cell density: transfection efficiency is very sensitive to culture confluence. Confluent or stationary phase cells do not transfect well. Adherence to a standard seeding protocol is mandatory to eliminate density variations between experiments.
3. Amount of DNA: this should remain constant for each transfected plate. Use empty vector plasmid DNA or salmon sperm DNA if necessary.
4. Incubation time can be anywhere between 2–24 h. Check toxicity on cells.

5. Growth media: use only fresh media and supplements of highest quality available. Serum is subject to significant biological variation; if possible keep to one batch.

To monitor transfection efficiency, cells are cotransfected with a vector plasmid encoding a reporter gene. The protocol shown here uses the β-galactosidase reporter gene. *In situ* staining with X-Gal substrate can easily identify transfected cells expressing this gene ***(8)***.

2. Materials

Conditions used in the protocols below serve as a good starting point when using 60-mm culture dishes. If a different size of culture plate is used, the amounts of reagents can be scaled up/down in proportion to the surface area.

2.1. DNA

The plasmid DNA in the transfection should be free of protein, RNA, and chemical contamination (*see* **Note 2**). Ethanol-precipitated DNA should be resuspended in sterile Tris-EDTA (TE) buffer (10 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.0) at a concentration of 0.2–1.0 mg/mL and stored at 4°C (*see* **Note 3**).

2.2. Transfection Reagents

2.2.1. DEAE-Dextran Method

1. 10 mg/mL DEAE-Dextran in phosphate-buffered saline (PBS), warmed to 37°C.
2. 8 m*M* Chloroquine (*see* **Note 4**).
3. PBS: 137 m*M* $NaCl_2$, 2.68 m*M* KCl, 4.3 m*M* NaH_2PO_4, 1.47 m*M* KH_2PO_4, final pH 7.4, warmed to 37°C.
4. 10% Dimethyl sulfoxide (DMSO) in PBS, warmed to 37°C.

2.2.2. Calcium Phosphate Method

1. 2 *M* $CaCl_2$, cell culture grade (*see* **Note 5**).
2. 2X HBS: 50 m*M* HEPES, 280 m*M* NaCl, 1.5 m*M* Na_2HPO_4, pH 7.1 (*see* **Note 6**).
3. Sterile, nuclease-free water.
4. PBS (as in **Subheading 2.2.1., item 3**).

2.2.3. Liposome-Mediated Method

1. Serum-free medium.
2. Cationic lipid reagent (*see* **Note 7**).

2.2.4. Electroporation Method

PBS (as in **Subheading 2.2.1., item 3**).

2.3. In Situ β*-Galactosidase Assay to Measure Transfection Efficiency*

1. PBS (as in **Subheading 2.2.1., item 3**).
2. Fixative: 0.25% (v/v) glutaraldehyde in PBS (*see* **Note 8**).
3. X-Gal stain: 0.2% X-Gal, from 2% stock in dimethylformamide (*see* **Note 9**), 2 m*M* $MgCl_2$, 5 m*M* $K_4Fe(CN)_6$, 5 m*M* $K_3Fe(CN)_6$ (*see* **Note 10**). Prepare fresh in PBS, and filter immediately prior to use.

2.4. Stable Transfection

1. 50 mg/mL G-418 (Geneticin).
2. Cloning rings.

3. Methods

3.1. Preparation of Cells

1. Plate the cells the day before the transfection experiment. The appropriate density depends on the cell line used. Aim for approx 80% confluency on the day of transfection (*see* **Note 11**). As a general guideline, plate approx 5×10^5 cells per 60-mm culture dish.
2. Three hours prior to the transfection remove the medium from the cells and replace it with fresh growth medium.

3.2. Transfection

3.2.1. DEAE-Dextran Method

1. For each transfection, prepare 6 µg DNA in PBS to 475 µL in an Eppendorf tube.
2. Add 25 µL of 10 mg/mL DEAE-Dextran to each tube.
3. Aspirate media from cells, wash with PBS, and replace with DEAE-Dextran-DNA mix. Incubate at 37°C for 1 h (*see* **Note 12**).
4. Add 3.5 mL media and 35 µL chloroquine to each plate (*see* **Note 13**). Incubate at 37°C for 2.5 h. Monitor cells closely for cytotoxicity.
5. Aspirate media and add 10% DMSO for 2 min (*see* **Note 14**).
6. Aspirate and wash with media, replace with fresh media, and incubate in a 37°C, 5% CO_2 incubator.

3.2.2. Calcium Phosphate Method

1. For each transfection, prepare DNA in sterile water to 225 µL in an Eppendorf tube (use empty vector DNA to make up to 10 µg DNA total for each transfection).
2. Add 25 µL of 2.5 *M* $CaCl_2$ to each Eppendorf tube.
3. Dispense 250 µL 2X HBS into a 7-mL bijou bottle (72-mm × 75-mm sterile tube) for each transfection.
4. Gently vortex the HBS solution while slowly adding the DNA/calcium phosphate solution dropwise. When the DNA addition is complete, the solution should appear cloudy resulting from the formation of the calcium phosphate-DNA coprecipitate. Incubate the solution for 30 min at room temperature.
5. Vortex the solution again just prior to adding it to the cells. Add the solution dropwise to the plates. Swirl the plates to distribute evenly over the cells (*see* **Note 15**). Return the plates to a 37°C, 5% CO_2 incubator.
6. Change the culture medium 4–16 h after transfection. Optimize the length of incubation for individual cell lines. Primary cell lines are particularly sensitive and should not be exposed to calcium phosphate for more than 4 h. Washing cells 2–3 times with warm PBS may be necessary to remove the calcium phosphate DNA from the cells.

3.2.3. Liposome-Mediated Method

1. For each transfection, dilute 6 µg DNA in 300 µL serum free medium in an Eppendorf tube.
2. Dilute 2–25 µL liposome reagent in 300 µL serum-free medium in a 7-mL bijou bottle for each transfection. Leave at room temperature for 15 min (*see* **Note 16**).

3. Combine the two solutions in the aliquot and incubate at room temperature for 15–30 min.
4. Wash cells with serum-free medium.
5. Add 2.4 mL serum-free media to each bijou then gently add to cells. Return the plates to a 37°C, 5% CO_2 incubator (*see* **Note 17**).
6. The culture medium should be changed 2–24 h after transfection and replaced with 10% serum-containing media

3.2.4. Electroporation Method

1. Harvest cells using trypsin-EDTA, then resuspend cells in 5 mL growth medium to inactivate trypsin. Centrifuge 125*g* for 5 min. Aspirate medium, wash cells with PBS, and repeat centrifugation.
2. Resuspend cells in 400 µL cation-free PBS and place into a 0.4-cm electroporation cuvet (*see* **Note 18**).
3. Add 25 µg of plasmid DNA to be transfected. Mix gently and let stand at room temperature for 10 min.
4. Electroporate cells using recommended settings for your electroporation device. Optimization required for maximum transfection efficiency with minimal cell death.
5. Allow cells to sit in cuvet for 10 min following electroporation before placing in 60-mm culture dish with 5mL growth media. Return the plates to a 37°C, 5% CO_2 incubator.

3.3. Harvesting Transfected Cells

3.3.1. Transient Expression

Cells can be harvested 24–72 h after transfecting. Expression levels usually peak by 48 h; however, if a replicating plasmid is used in an appropriate host (for example, SV40-ori plasmid in COS cells), higher expression will be observed at 72 h.

3.3.2. Stable Expression

1. Twenty-four hours after transfection, trypsinize cells and plate 1/10 to allow space for cell division (*see* **Note 19**).
2. Forty-eight hours after transfection, add media containing selective antibiotic (*see* **Note 20**). Refeed cells twice a week for first 3 wk, then once a week as the nontransfected cells die off (*see* **Note 21**).
3. At around 2–5 wk, resistant cells may be picked by placing a cloning ring over the top and pipeting the cells off into a 24-well plate (*see* **Note 22**). Maintain selection in selective antibiotic media.

3.4. Measuring Transfection Efficiency Using β-Galactosidase

1. Cotransfect cells with expression vector for β-galactosidase along with gene of interest plasmid. Allow 1–2 d for expression.
2. Wash cells twice with PBS.
3. Fix cells in 2 mL fixative for 5 min at room temperature.
4. Remove fixative and wash 3 times with PBS (*see* **Note 23**).
5. Stain for 2–16 h at 37°C with 1 mL of X-Gal stain.
6. Remove stain and cover cells with PBS, observe on light microscope, score dark blue cells positive.

4. Notes

1. Some cell lines, over time, will become more resistant to transfection. Thaw out a fresh ampule of cells if transfection efficiency begins to drop.

2. Transfection-grade plasmid DNA should be purified using CsCl-gradient centrifugation or a commercially available kit. UV spectrophotometry of the prepared DNA should give an A_{260}/A_{280} ratio of 1.8–1.9. Check that the DNA is predominantly in a supercoiled form by running on an agarose gel.
3. Degradation by nucleases and stress during handling or improper storage may influence transfection efficiency. DNA resuspended in TE will remain stable at 4°C for months. Stocks stored at –20°C should not be repeatedly frozen and thawed. DNA is not stable at 4°C when resuspended in water.
4. 8 m*M* Chloroquine should be filter sterilized and stored in the dark at –20°C.
5. 2 m*M* $CaCl_2$ should be filtered and stored at –20°C in small aliquots. Once thawed, do not refreeze.
6. Final pH of 2X HBS is critical to transfection success. It must be 7.1 ± 0.05 and should be filtered and stored at –20ºC in small aliquots. Once thawed, do not refreeze.
7. A number of reagents are commercially available. Most manufacturers supply a range of different formulations to suit different cell types.
8. Glutaraldehyde is a carcinogen and should be diluted in a fume hood. Avoid contact with skin and avoid inhalation.
9. Store X-Gal in dimethylformamide (DMF) in dark polypropylene tubes at –20°C. DMF will dissolve polystyrene; use glass or polypropylene pipets, or tips.
10. 50 m*M* Stocks of potassium ferricyanide and potassium ferrocyanide can be stored at 4°C.
11. Best results are obtained when cells are transfected at a high density and in the log phase of growth.
12. Shaking plate gently every 15 min may improve transfection efficiency.
13. This step is optional. Chloroquine may enhance transfection efficiency by binding to DNA and inhibiting its degradation by lysosomes. It may be too toxic for some cell lines.
14. DMSO shock is optional. The exact mechanism of action is unknown but it may modify cell membrane structure to enhance uptake of DNA.
15. Check that the precipitate is fine by using a light microscope. Course precipitate will reduce the efficiency of uptake.
16. Some serum proteins can interfere with lipid/DNA complex formation. For sensitive cells, serum can be re-added to the medium just before adding the complexes to the cells.
17. Antibiotics should not be used as lipid reagents increase the permeability of the cell membrane allowing antibiotics into the cell.
18. PBS must be cation free to prevent arcing during electroporation.
19. Do not use the antibiotics penicillin and streptomycin when creating stable cell lines, as they may inhibit the action of the transfectant selective antibiotic.
20. The concentration of selective antibiotic required for selection of resistant transfectants should be determined from a kill curve for each cell type. Prepare 6-well culture plates with 2 mL medium containing antibiotic at a range of concentrations from 0–1200 µg/mL. Add 400 trypsinized cells to each well and incubate at 37°C, 5%CO_2. Replace medium with antibiotic twice a week. After 10–14 d, aspirate the supernatant and wash cells with PBS. Stain the cells with 0.5% methylene blue/50% methanol for 20 min. Determine the lowest concentration of antibiotic that kills all the cells and use the next higher concentration for transfectant selection.
21. If no colonies appear, it is possible that your gene of interest is toxic to cells when overexpressed. Include a control transfection of empty vector. If colonies appear with vector alone then your gene product may be toxic. In this case, consider creating an inducible stable cell line.

22. An alternative method, if cells adhere strongly to plate, is to use a p1000 tip dipped in vaseline to make a ring around the selected clone. Trypsin can then be applied within the ring to release the clone.
23. Wash carefully as residual glutaraldehyde may inhibit the activity of β-galactosidase.

References

1. Gluzman, Y. (1981) SV40-transformed simian cells support the replication of early SV40 mutants. *Cell* **23,** 175–182.
2. Botchan, M., Stringer J., Mitchison T., and Sambrook J. (1980) Integration and excision of SV40 DNA from the chromosome of a transformed cell. *Cell* **20,** 143–152.
3. McCutchan, J. H. and Pagano, J. S. (1968) Enhancement of the infectivity of simian virus 40 deoxyribonucleic acid with diethylaminoethyl-dextran. *J. Natl. Cancer. Inst.* **41,** 351–357.
4. Graham, F. L. and Van der Eb, A. J. (1973) A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* **52,** 456–467.
5. Felgner, P. L., Gadek, T. R., Holm, M., et al. (1987) Lipofectin: A highly efficient, lipid-mediated DNA-transfection procedure. *Proc.Natl. Acad. Sci. USA* **84,** 7413–7417.
6. Wong, T. K. and Neumann, E. (1982) Electric field mediated gene transfer. *Biochem. Biophys. Res. Commun.* **107,** 584–587
7. Neumann, E., Schaefer-Ridder, M., Wang, Y., and Hofschneider, P. H. (1982) Gene transfer into mouse lyoma cells by electroporation in high electric fields. *EMBO J.* **1,** 841–845.
8. Lim, K., and Chae, C. B. (1989) A simple assay for DNA transfection by incubation of the cells in culture dishes with substrates for β-galactosidase. *Biotechniques* **7,** 576–579.

25

Development of Drug-Resistant Models

Helen M. Coley

1. Introduction

1.1. Background

The observation that tumors are capable of developing resistance to anticancer agents is a well-established fact in the clinic. In order to explain this phenomenon in the laboratory, fluctuation analysis has been used in a number of studies involving tumor cell lines ***(1–3)***, although it was first used by Luria and Delbruck ***(4)*** in the study of bacterial cultures. Subsequently, work by Goldie and Coldman ***(5)*** using fluctuation analysis focused on the genetic instability of tumor cells as pivotal to the emergence of drug-resistant cells. The hypothesis suggested that a drug-resistant cell emerged from the clonal expansion of spontaneously mutated cells and not from changes in cellular function induced by drugs. The methodology used in fluctuation analysis is described elsewhere ***(3)*** and can be used to assess the mutation rate of drug treated tumor cells.

The description of a P388 leukemia cell line with acquired resistance to actinomycin D was published by Biedler and Riehm in 1970 ***(6)***. This landmark paper was one of the first reports describing the use of cell line models with in vitro-derived anticancer drug resistance. A salient feature of the cell lines described was the cross-resistance seen to other natural product anticancer drugs such as vinblastine and daunorubicin. This paper was one of the first reports to describe the multidrug-resistant (MDR) phenotype, as we know it today.

Drug-resistant cell line models, for example, those demonstrating MDR and others, serve as useful paradigms for the clinical scenario of anticancer drug resistance. Cell-line models with acquired resistance to a broad range of types of anticancer drugs have served us well in our quest to clarify mechanisms underlying clinical anticancer drug resistance. An additional and very important role for the drug-resistant cell line model is to elucidate the mechanism of action of new, developmental anticancer agents. Cellular and/or molecular changes seen in a drug-resistant cell line, when compared to its drug-sensitive counterpart, can suggest a locus of drug action inferred by the presence of those particular alterations. A specific example of this is seen in VP-16 (etoposide) resistant cell lines, which often show a mutation or deletion of topoisomerase-IIα.

From: *Methods in Molecular Medicine, vol. 88: Cancer Cell Culture: Methods and Protocols*
Edited by: S. P. Langdon © Humana Press Inc., Totowa, NJ

1.2. Developing Drug-Resistant Cell Line Models

The first consideration when developing a drug-resistant cell line is the choice of parental cell line. This will depend on a number of factors, such as tumor type and its relevance to the selecting agent in question. Additionally, specific molecular features of the cell line may be related to the mechanism (putative or confirmed) of action of the selecting agent.

A method that may be suitable for the development of anticancer drug-resistant cell lines is the limiting dilution technique following treatment of cells with a sublethal dose of drug. This technique develops a drug-resistant cell line that is derived from a clone (obtained from initial treatment of the parental cell line) and may display a more homogeneous drug-resistant phenotype/genotype than other methods.

The most common method for development of drug-resistant tumor cell line models is incubation over a period of time in the presence of the drug in question. Although this technique has relied on a good deal of empiricism, there are several considerations (discussed in detail later) that are crucial to ensure success.

2. Materials

1. Standard cell culture laboratory equipment.
2. Mycoplasma-free parental cell lines at lowest passage number possible.
3. Inducing agent, i.e., cytotoxic drug at appropriate concentration. The drug should be stored as frozen aliquots and regularly made up (avoid repeated freeze thawing).

3. Methods

3.1. Development of Drug-Resistant Cell Lines by Growing in Step-Wise Increases in Cytotoxic Drug

It is important to establish the drug sensitivity of the parental cell line. This information is crucial to establishing the starting dose for treatment of the cell line. In our experience the best policy is to commence treatment at around 10–20% of the chronic IC_{50} dose.

1. Seed the cells initially into a flask to be around 20% confluent when drug treatment is carried out 24 h later.
2. Add the drug to the medium in a Universal container and then add it to the cells in the tissue culture flask under standard aseptic conditions.
3. As the cells become confluent, the cells can be subcultured in the usual manner.

It is important to document the development of a drug-resistant cell line. Duplicate flasks should be set up. One flask will be drug-treated and the other flask allowed to grow for that passage in drug-free medium. Thus, at each passage of the developing drug-resistant cells the nondrug-treated flask (from the previous passage) will be discarded. The drug-treated flask of cells is then used to subculture each of the flasks to give approx 20–30% confluence after 24 h.

The increase in drug doses generally follows the pattern of doubling the concentration, sometimes at every passage. It is vital that the cells are monitored following drug treatment. In general, cultures will tolerate the lower doses of drug well, but a slowing of cell growth will be evident at various stages in the cell line development. At this point the empirical nature of drug-resistant cell line development becomes apparent.

When the cell line appears not to have tolerated a drug treatment it is unwise to repeat another round of drug treatment. The cells should be grown in drug-free medium for at least one passage to recover. With cytotoxic drugs, such as doxorubicin, a doubling of drug doses generates resistance. However, for some agents this may not be possible and smaller increments in dosing will be a successful approach. It is not possible to predict the types of agents likely to behave in this manner. However, the author has had trouble increasing the dose of the selecting agent when developing cell lines with resistance to new developmental dual I and II topoisomerase inhibitors and to certain alkylating agents.

It is very easy to lose a drug-resistant cell line that has taken several weeks to generate either owing to contamination of the culture or because a high proportion of the cells die and the cell line cannot recover. Aliquots of cells should be frozen in liquid nitrogen at regular intervals, safeguarding the loss of the culture. In addition, provided there is always a duplicate flask of cells set up growing up in drug-free medium at every passage, there should always be a supply of "resistant" cells. The length of time to develop a resistant cell line can vary enormously from a few weeks to many months (*see* **Note 1**).

3.2. Development of Drug-Resistant Cell Lines Using the Limiting Dilution Technique

This technique is used after the plating efficiency of the parental cell line exposed to a particular drug concentration has been established using soft agar cloning.

1. After making a single cell suspension of the cell line (usually by mechanical disaggregation), the cells are diluted in tissue culture medium containing 10% fetal calf serum (FCS) and drug or inducing agent. The seeding density should range from 10–200 cells/mL. The optimal cell number for this procedure is based on the plating efficiency of the cells. The drug dose should be 10–20% of the IC_{50}.

   Two hundred µL of the diluted cell suspension is then distributed to each well of a 96-well flat bottomed microtiter plate. When a well contains a large colony following 1–3 wk of incubation under standard tissue culture conditions, the colony is isolated and propagated as a resistant subline. Colony isolation is carried out by application of trypsin-EDTA to the well following aspiration of the culture medium. Alternatively, use a sterile loop.
2. Following propagation by growing up the colony as a single culture in a maintenance dose of drug, the tumor line is then subjected to the limiting dilution technique with increasing drug concentration.
3. This procedure can be repeated with an increase in the maintenance dose each time. A description of this method is given in Hong et al. *(7)*.

3.3. Titration of Drug Sensitivity of Developing Drug-Resistant Cell Lines

As a cell line develops a drug-resistant phenotype, it is useful to monitor its progress. Take a sample of the resistant cells in sufficient number to set them up in a 96-well plate for standard chemosensitivity testing. The end point for readout may be MTT, SRB, or methylene blue whatever the method of choice is in your laboratory. It is essential to set up the parental, drug sensitive cells at the same time for use alongside the drug-resistant line. It may become clear early on that the drug-resistant cell

line grows more slowly than the parental line, i.e., possesses a longer doubling time. This will affect the conduct of cytotoxicity testing. Hence, more cells that are drug-resistant will have to be seeded to allow for similar cell densities for the untreated (control) wells when the assay is terminated.

It may be important to monitor the progress of the development of the drug-resistant line, especially if a certain level of resistance to an agent is an issue, i.e., 10-fold rather than 1000-fold resistance. It may be relevant for a study to use a low level of drug resistance, for example, as this mirrors the clinical situation of anticancer drug resistance. On the other hand, cell lines with high levels of resistance to a particular agent may be useful as analytical tools in certain circumstances.

3.4. Confirmation of Level of Drug Resistance

Once the desired level of drug resistance is achieved (or the drug dose cannot be increased any further) then the culture should be expanded and frozen down in liquid nitrogen. The drug dose that the cell line has been growing in at the most recent passages should then be designated the maintenance dose (*see* **Note 2**). It is important that the cells are grown up in the presence of the cytotoxic agent in order to maintain their drug-resistant profile. In many instances drug-resistant cell lines are grown up in the presence of the selecting agent at every passage. However, this may not always be suitable for some cell lines as drug treatment at every single passage may alter the stability of the drug resistance and the overall viability of the culture. This aspect of developing cell lines with acquired resistance to anticancer drugs can be variable and may depend on the type of cell line being used and the nature of the anticancer agent. In essence, this is an empirical aspect of anticancer drug development lacking hard and fast rules. If a cell line is drug-resistant then it should be able to tolerate continual drug treatment. However, some cytotoxic drugs may prove difficult to use in this way and alternate passage with drug treatment may be the most practical way for maintaining a particular drug-resistant variant.

It is important to check the drug-resistant phenotype of cells maintained in culture. This applies to their relative level of drug sensitivity and to any drug-resistant features such as ABC transporters, for example, P-glycoprotein. It is preferable to return to original stocks of cells after a certain number of passages (between 10 and 20) in continuous culture (*see* **Note 3**).

Drug-resistant variant cell lines often differ from their parental counterparts. **Figures 1** and **2** show a selection of cell lines with acquired resistance to various anticancer drugs.

3.5. An Example of the Development of a Drug-Resistant Cell Line

In order to develop a human breast-carcinoma cell line with acquired resistance to VP-16 (etoposide) the mycoplasma-free status of the parental cell line was confirmed. A flask of cells was set up so that they would be in early exponential phase 24 h later (approx 10% confluence). For this particular parental cell line, the (chronic) IC_{50} dose for VP-16 was previously estimated to be 5–10 μ*M*. The first dose used was 0.5 μ*M*: passage one. All drug treatments were begun 24 h following the passage of the culture.

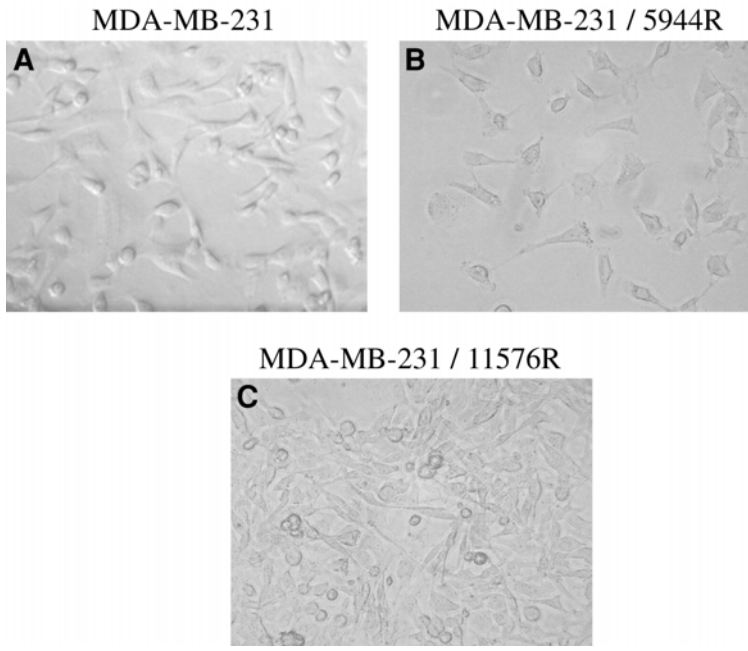

Fig. 1. **(A)** MDA-MB-231 human breast adenocarcinoma cell line; **(B)** MDA-MB-231/5944R cell line with acquired resistance to a developmental topoisomerase inhibitor; **(C)** MDA-MB-231/11576R cell line with acquired resistance to a developmental topoisomerase inhibitor.

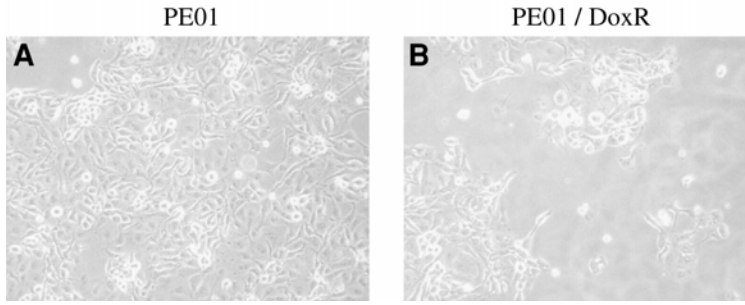

Fig. 2. **(A)** PEO1 human epithelial ovarian cancer cell line; **(B)** PEO1DoxR cell line with developing resistance to doxorubicin.

The cells appeared to slow their growth, but became confluent 8 d following treatment. For passage two, a dose of 0.5 μ*M* was repeated and rapidly came to confluence within a week. Using a dose of 1 μ*M* at passage three the culture reduced its growth considerably and confluence was not reached for 2 wk. The cells started to assume a vacuolated appearance and a large number of cells were found floating in the medium,

having become detached. Five weeks into the development of the VP-16 resistant cell line, a drug-free passage was conducted because the culture was showing reduced viability. A dose of 1 μ*M* VP-16 was repeated and the cells did not recover for another 2 wk. Passage 8 was carried out using an additional treatment with 1 μ*M* VP-16. The cells appeared to tolerate this and became confluent after a week. Over the next 6 wk, a dose of 2 μ*M* was used to treat the cells. The culture slowed its growth and subsequently died out at passage 13. The accompanying nontreated flask of cells was then used and this showed poor growth. After 3 wk in drug-free medium the cells were retreated at the dose of 1 μ*M* until growth appeared healthy, up to passage 18. Over the next two passages a dose of 1.5 μ*M* was tried, which was well tolerated, followed by 2 μ*M*. This latter dose was applied for the next three passages, followed by one drug-free passage. At passage 26, the drug sensitivity of the VP-16 resistant line was titrated alongside the parental MDA-MB-231 cells. The IC_{50} data obtained (using the MTT assay) indicated that the drug-resistant cells had a level of drug resistance approximating sevenfold. Over the following 2 mo passages using 2 μ*M* VP-16 were applied, which appeared to be well tolerated. Three successive MTT assays were then set up within a 2-wk period and the level of drug resistance in the VP-16 resistant line was 10-fold. Two drug-free passages confirmed that the resistance was stable in the absence of the selecting agent for a short time period. The cell line culture was expanded to allow freezing down of aliquots of the cells (approx 20 cryovials), after confirmation that the culture was mycoplasma-free.

4. Notes

1. The time to develop resistance can vary and depends on the type of selecting agent being used. Doxorubicin resistance, for example, can evolve rapidly (within a few weeks), but resistance to cisplatin may take months. The example shown above in **Subheading 3.5.** of this section is typical for the development of many drug-resistant variants. There are a number of points that this exercise has highlighted:
   a. Be prepared to wait for your cell line to develop the desired drug-resistant phenotype.
   b. Good record keeping is essential during the development of the drug-resistant cell line (e.g., dates of drug treatments, morphological changes, time to grow to confluence).
   c. Regularly freeze down the evolving drug-resistant variant (taking care to record the passage number and drug treatment history).
   d. Decide what you require from the drug-resistant cell line before you start to develop it. Which tumor cell line should be used? Choice of drug to be used? How the drug-resistant cell line can be used to help answer specific questions, relating to such aspects as the mechanism of action of a developmental anticancer drugs.
2. Can the cell line be maintained in the absence of the selecting agent? If the cell line has achieved a stable level of drug resistance then withdrawal of the selecting agent from the growth medium should not result in loss of the resistant phenotype for a number of passages. However, the titration of the level of resistance will often reveal a reduction with successive drug-free passaging.
3. A report by Scudiero et al. ***(8)*** describing the MCF-7 line MCF-7ADR with acquired resistance to doxorubicin serves as an important warning. Although a number of studies had been carried out using this cell line pair, including a description of a difference in p53 status ***(9)***, it was not until restriction fragment length polymorphism (RFLP) analysis of

the cell lines was carried out that it was realized that MCF-7ADR could not have originated from the parental MCF-7 line. The need to be scrupulous with regard to labeling of cultures, for example, is emphasized in this report.

References

1. Chen, G., Jaffrezou, J. P., Fleming, W. H., Duran, G. E., and Sikic, B. I. (1994) Prevalence of multidrug resistance related to activation of the mdr1 gene in human sarcoma mutants derived by single step doxorubicin selection. *Cancer Res.* **54,** 4980–4987.
2. Jaffrezou, J. P., Chen, G., Duran, G. E., Kuhl, J. S., Sikic, B. I. (1994) Mutation rates and mechanisms of resistance to etoposide determined from fluctuation analysis. *J. Natl. Cancer Inst.* **86,** 1152–1158.
3. Beketic-Oreskovic, L., Duran, G. E., Chen, G., Dumontet, C., Sikic, B. I. (1995) Decreased mutation rate for cellular resistance to doxorubicin and suppression of mdr1 gene activation by the cyclosporin PSC833. *J. Natl. Cancer Inst.* **87,** 1593–1602.
4. Luria, S. E., and Delbruck, M. (1943) Mutation of bacteria from virus sensitivity to virus resistance. *Genetics* **28,** 491–511.
5. Goldie, J. H., and Coldman, A. J. (1979) A mathematical model for relating the drug sensitivity of tumours to the spontaneous mutation rate. *Cancer Treat Rep.* **63,** 1727–1733.
6. Biedler, J. L., and Riehm, H. (1970) Cellular resistance to actinomycin D in Chinese hamster cells in vitro: cross-resistance, radioautographic and cytogenetic studies. *Cancer Res.* **30,** 1174–1184.
7 Hong, W. S., Saijo, N., Sasaki, Y., et al. (1988) Establishment and characterisation of cisplatin-resistant sublines of human lung cancer cell lines. *Int. J. Cancer* **41,** 462–467.
8. Scudiero, D. A., Monks, A., Sausville, B. A. (1998) Cell line designation change: multidrug-resistant cell line in the NCI anticancer screen. *J. Natl. Cancer Inst.* **90,** 862.
9. O'Connor, P. M., Jackman, J., Bae, I., et al. (1997) Characterisation of the p53 tumor suppressor pathway in cell lines of the National Cancer Institute anticancer drug screen and correlations with the growth inhibitory potency of 123 anticancer agents. *Cancer Res.* **57,** 4285–4300.

26

Immortalization of Human Prostate Cells With the Human Papillomavirus Type 16 E6 Gene

Norman J. Maitland, Catherine A. Macintosh, Christian Schmitz, and Shona H. Lang

1. Introduction

A survey of the Medline titles for the years 2000–2001 revealed 125 papers whose titles included the name of the androgen-sensitive prostate cancer cell line LNCaP, and 36 papers also included PC3, the most common androgen independent line. With the addition of DU145 the list of human experimental prostate cell systems is almost exhausted, although recently new sets of cell lines, immortalized by viral and nonviral genes, have begun to appear to augment the list ***(1–5)***.

Why do we require cell lines when the study of clinical material and the increasing availability of animal models should provide sufficient material for genetic and functional studies? Monotypic cell lines (epithelial and fibroblastic) offer both convenience and the ability to produce cellular reconstitutions (*see* Lang and Maitland, Chapter 28). In these experiments, primary cells, which retain the phenotype of the original tissue components, are vastly superior. However, such primary cells have a very limited life span in culture, and are rapidly eliminated within several passages. Clearly these cells derive from elderly patients with shortened telomeres, and senescence is the likely mechanism of loss.

However, in the case of prostate adenocarcinoma cells, in which telomerase is activated ***(6)***, the induction of senescence is clearly more complex ***(7)***. A combination of these mechanisms is likely the cause of the lack of prostate carcinoma cell lines; in contrast to the multitude of breast adenocarcinoma lines.

One solution to the problem of senescence and the reluctance of prostate epithelial cells to establish in monoculture is to exploit the ability of a number of viral oncogenes to extend the lifespan of their host cells, to prolong the lifespan of the host in the production of progeny virus.

The key to the immortalization function is to inactivate the ability of the tumor virus to replicate, either by using individual cloned genes or by inactivation of the viral

From: *Methods in Molecular Medicine, vol. 88: Cancer Cell Culture: Methods and Protocols*
Edited by: S. P. Langdon © Humana Press Inc., Totowa, NJ

origin of replication. The original immortalizing gene was SV40 T antigen. A construct with the SV40 genome inserted into a plasmid vector, but with the SV40 ori deleted (SV40 ori-) ***(8)*** was initially used to establish extended life span cultures from Wilms' tumor ***(9)***. In this case, the tumor origin of the cells was established by measuring loss of heterozygosity, relative to normal tissue (or peripheral blood lymphocyte) DNA ***(9)***. Recent advances to the SV40 system include the use of temperature-sensitive mutations in the T antigen gene, which are nonfunctional at 37°C. Therefore the initial immortalization is carried out at reduced temperature (32°C), and when immortalized cultures are observed the T antigen function is switched off ***(10)***.

Our approach with prostate epithelium is to employ the immortalizing genes E6 and E7 of human papillomavirus (HPV). A number of researchers have successfully used this approach in both breast and prostate (for example ***(11,12)***. After initial experimentation with plasmid transfer systems that had an initially low frequency of transfection, we decided to employ retroviral infection as the method of choice for HPV gene transfer. The preliminary experiments also indicated that the dual expression system (E6/E7), while giving a high frequency of immortalization, also produced cells with a higher than normal proliferative index. We therefore chose to employ just the E6 gene, which has the dual properties of inactivating p53 by targeting it for degradation ***(13)*** and activating telomerase ***(14)***. For all of the initial experiments the pLXSNE6 vector, originally reported by Halbert et al. ***(15)*** was used, although second generation vectors are now available with improved safety characteristics.

The protocols describe techniques to generate and propagate the recombinant retroviruses, infection of primary prostate epithelial cultures, and a number of techniques for preliminary genotyping of the extended lifespan cells before they have undergone too many cell doublings. Maitland et al. describes a complete account of the characterization of cell lines in human prostatic cancer ***(16)***.

2. Materials

1. The PA317 murine packaging cell line (ATCC CRL-9078) was obtained from the American Tissue Culture Collection.
2. Retroviral transfer vectors pLNCX and pLXSN can be obtained as part of the Retro-X kit marketed by Clontech.
3. Transport medium: RPMI 1640, 3% (v/v) horse serum, 50 µg/mL gentamycin (Sigma), 2.5 µg/mL Fungizone. All tissue culture supplies were purchased from Life Technologies
4. DF10 complete medium: 1/1 DMEM/Nutrient Mix F12, 10% FCS, 2 m*M* L-glutamine, 100 U/mL penicillin, 100 µg/L streptomycin, and 20 m*M* HEPES, sterilized by 0.2 µm filtration.
5. D10 complete medium: Dulbecco's modified eagle medium (DMEM) with the supplements listed for DF10 complete medium.
6. Trypsin solution: 0.25% (v/v) trypsin/EDTA in phosphate buffered saline (PBS).
7. DNA extraction buffer containing proteinase K: Extraction buffer contains 0.01*M* Tris-HCl, pH 7.9, 0.5 m*M* EDTA, pH 8.0, 1% (v/v) Tween-20, and 0.4 mg /mL proteinase K.
8. Dosper liposomal transfection reagent.
9. Supor® disc filters (0.45 µm; Gelman Sciences).
10. BDH Giemsa staining solution.

3. Methods

3.1. Setting Up Primary Prostate Epithelial Cultures for Retroviral Transduction

This method has been optimized for prostatic epithelium, but any method of tissue disaggregation can be employed. A critical step for the amphotrophic retroviral procedure is to obtain dividing cell cultures, as the viral life cycle is not completed in G_0 cells (in this case a lentiviral vector could be substituted).

3.1.1. Tissue Collection

After obtaining patient consent, obtain the prostate tissue by transurethral resection (TURP) and place the "tissue chips" into transport medium. Remove a 5-mm^2 slice of tissue, blot dry, and store at –80°C for later histological analysis. The remainder can be stored overnight at 4°C in transport medium.

3.1.2. Preparation of Biopsy for Culture

Mechanically disaggregate tissue (chop) in a sterile Petri dish producing pieces 1 mm^2 in diameter in 1 mL of transport medium (for alternatives *see* **Note 1**).

3.1.3. Seeding of Biopsy Material in Explant Culture

Using a disposable transfer pipet, aspirate the disaggregated biopsy and transfer to 25-cm^2 tissue culture flasks with 0.2-µm vented lids (Corning). The medium of choice for the primary culture is described in Lang and Maitland, Chapter 28 (primary culture medium). Seed the majority of the disaggregated tissue specimens directly onto tissue culture plastic, but other substrata such as polylysine and collagen can be used to aid adhesion of the explants.

3.2. Maintenance and Growth of Recombinant Amphotrophic Retroviruses

To generate producer cell lines for retrovirus, the recombinant DNA transfer vector must be transfected into the packaging cell line (PA317). This requires the insertion of the immortalizing gene into a transfer plasmid vector manipulated in bacteria that mimics the proviral form of the retrovirus in its most primitive form, i.e., a transgene coding region flanked by the viral long terminal repeat (LTR) sequences. Many such transfer vectors exist, and the immortalizing gene used in this example is inserted in pLXSN (Genbank accession number M28248) ***(17)***. This vector also contains an SV40 promoter-driven neomycin/G418 resistance gene to allow selection of the producer cells. The immortalizing gene (the E6 gene from HPV ***(15)***, is under the control of the retroviral promoter in the LTR. A more elegant (and ultimately safer alternative) is to use the related pLNCX transfer vector (Genbank accession number M28247), in which the retroviral promoter in the LTR is inactive and the immortalizing gene is under the control of a separate but stronger cytomegalovirus (CMV) immediate early promoter. Complete kits for the generation and manipulation of amphotrophic retroviruses are now available commercially (Retro-X from Clontech).

Table 1
Transfection Mixtures for Various Tissue Culture Vessel Types

Tissue culture dish	A (cm^2)	Liposomal transfection reagent (µL)	Total DNA per dish (µg)	Serum-free medium (µL)
T150	150.0	78.0	26.0	1,195
T75	75.0	39.0	13.0	598
T25	25.0	13.0	4.3	199
10-cm	58.1	30.2	10.1	463
6-well	9.6	5.0	1.7	77
12-well	4.5	2.4	0.8	36
24-well	2.3	1.2	0.4	18
8-well chamber	0.9	0.5	0.2	7

3.2.1. Maintenance of Retroviral Producer Cell Line

The murine fibroblast cell line PA317 is one of several effective hosts for recombinant retroviruses (*see* **Note 2**). It contains the *gag*, *pol* and *env* open reading frames from the transfer vector pMAM3 co-transfected into 3T3 cells with an HSV1TK gene. The *gag*, *pol* and *env* genes are constitutively expressed and provide the "packaging" function for any small RNA (<9kb) with appropriate packaging signals derived from the retroviral terminal LTR sequences, such as pLXSN.

1. The cell line is maintained in D10 medium and is subcultured 1:10 to 1:40 every 4–5 days (d) using standard techniques (*see* **Note 3**).
2. Replicate frozen stocks of PA317 are prepared in 40% DMEM, 50% fetal calf serum (FCS), and 10% dimethyl sulfoxide (DMSO), and stored in liquid nitrogen.

3.2.2. Generation and Storage of Recombinant Amphotropic Retroviruses

All transfections into PA317 are performed utilizing Dosper™ transfection reagent (Roche).

1. Passage adherent cells 1:2, 48 h before transfection, and again 24 h before transfection at 50–80% confluency.
2. Mix 1 µg of transfer plasmid DNA with 3.125 µL of Dosper reagent and add serum-free medium to about 0.1 mL. Incubate the reaction at 20°C for 15 min. **Table 1** gives optimal volumes for various vessels.
3. Immediately before transfection, add 10 volumes (vol) of the appropriate complete medium to the transfection mixture and gently pipet the complete mixture onto the cells (*see* **Note 4**).
4. Conduct transfections at 37°C for 5–12 h, afterwards replace the transfection mixture with fresh complete medium.
5. Incubate transfected cells for 6–16 h, after which the cells are washed once with PBS, and then add 1 mL D10 medium per 25 cm^2 for a further 24–48 h.
6. Collect transiently produced retroviruses by removing the growth medium and remove the floating cells by 0.45 µm disc filtration.
7. The retrovirus-containing supernatant can be stored at 4°C for up to several months or frozen at –80°C without further modifications.

3.2.3. Generation of Stable Retroviral Producer Cell Lines

According to the resistance encoded by the retroviral vector used, the stably infected producer cells are selected with 1–2 mg/mL G418® (pLNCX and pLXSN vector series). Stable retroviral producer cell lines are always maintained under selection conditions. However, when retroviruses are to be used for transduction experiments, drug-free medium is applied for a period of 24–48 h.

3.2.4. Titration of the Virus Stock

1. Plate prepared human keratinocyte cell line (HaCaT) cells ***(18)*** into a 6-well tissue culture plate at 25–30% confluency, and leave for 12–24 h to completely adhere.
2. Replace the medium with 0.7–1 mL fresh DF10 containing 8 µg/mL hexadimethrine bromide (polybrene). Add 10 µL of serial diluted retroviral supernatant (1:1, 1:2, 1:10, 1:100, 1:1000, 1:10000) to the HaCaT cells and incubated for 4–12 h at 37°C.
3. After the transduction is complete, remove the retroviral supernatant, wash the cells several times with PBS, and maintain for a further 48 h in complete DF10 medium.
4. Incubate HaCaT cells with selection medium containing 500 µg/mL G418® (pLNCX or pLXSN series). Carry out the selection for 10–20 d, changing medium every 3–4 d.
5. Stain the cells with Giemsa staining solution for visualization of generated colonies. Remove the medium, wash the cells once with PBS, and add 500 µL Giemsa staining solution to each well. Incubate cells for 10–20 min and remove excess staining solution with several washes of tap water. Air dry the fixed cells and count the colonies.
6. The titer is calculated as follows and expressed in colony-forming units per milliliter (cfu/mL) of virus ***(19)***.

$$\text{G418–resistant cfu/mL} = \frac{\text{number of colonies}}{\text{retroviral dilution volume (mL)}}$$

3.3. Immortalization of Primary Prostatic Epithelium

The actual immortalization procedure is an extension of the preceding protocol, as used to assay the recombinant viral stock. Prostatic epithelial cells are difficult to transfect by conventional precipitation or liposome-mediated techniques, but our experience with retroviruses indicates that the cells are readily infectable with an efficiency as high as most mammalian cell lines.

3.3.1. Infection of the Primary Prostatic Epithelial Cells With Retrovirus Stock

For infection of primary prostatic epithelial cells undiluted viral stock is used.

1. Draw off the medium from the prostatic cell outgrowths.
2. Wash the cells twice for 5 min in PBS prior to addition of 1.5 mL of virus with polybrene at 8 µg/mL per 25-cm^2 flask. Incubate cells in the presence of the virus for 2 h at 37°C, 5% CO_2.
3. Remove the polybrene-containing medium and wash the cells twice for 5 min with PBS. Replace with fresh D10 medium and incubate the cells for a further 48 h prior to selection with G418 at 25 µg/mL.

3.3.2. Ring Cloning of Transfected Cells

After 10–14 d of drug selection discrete colonies are observed that can be individually ring cloned into 12.5-cm^2 flasks.

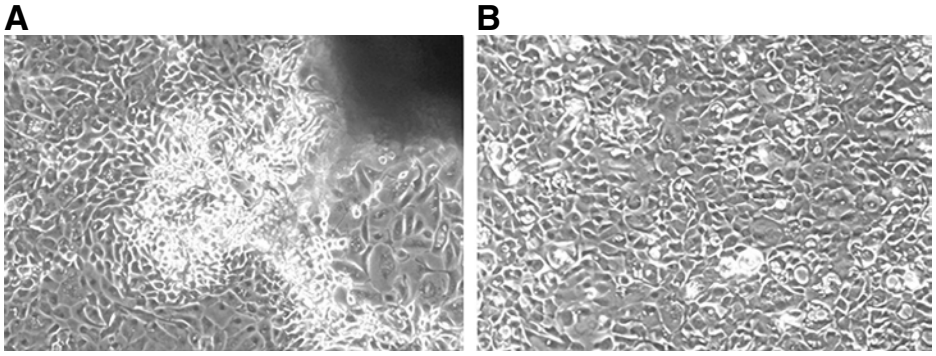

Fig. 1. Comparative morphology of primary epithelial outgrowth and the E6 immortalized culture. **(A)** Epithelial outgrowth from a fragment of prostate tissue. The tissue is the large black object at the top right of the panel. **(B)** Epithelial component from this outgrowth has been infected with a recombinant E6-expressing retrovirus and a cloned epithelial culture produced. Note the similar morphology.

1. Wash the cells twice for 3 min in PBS-Ca^{2+}/Mg^{2+} and cut off the lid of the flask under sterile conditions.
2. Dip a sterile glass ring (10 mm in diameter) in autoclaved petroleum jelly and place over the individual colonies, creating a seal.
3. Place 500 µL of 0.25% (v/v) trypsin/EDTA in the ring and immediately aspirate off with the PBS.
4. Apply trypsin again and monitor the cells. As they begin to round up, gently pipet the cells up and down and place in flasks containing D10 culture medium.
5. The isolated colonies are incubated at 37°C, 5% CO_2, until they can be subcultured into larger tissue culture flasks (*see* **Note 5**).

3.4. Analysis of Transformed Cells

3.4.1. Immortalization or Extended Life Span?

Introduction of the E6 gene into a primary prostatic cell culture extends the lifespan of the cells. For prostatic epithelium, any extension beyond passage 3 or 4 represents an extension of life-span. In the initial stages, the cells are genetically stable and resemble the original culture in morphology (*see* **Fig. 1**). The cells are NOT however immortal at this stage, and require passing through a crisis for full immortalization to occur. After the crisis period, the cells will still be epithelial in morphology, but certain chromosomal rearrangements will have occurred.

The extended lifespan cells, produced after the first retroviral infection, are genetically stable, and behave in a similar way to the original primary cells in most of the biological assays for up to 12 population doublings. If extremely large cell numbers are not required, then the extended life span cells are preferable. To maintain these cells, a proportion of the culture should be preserved by standard cryopreservation at every passage, particularly while the cells are proliferating.

3.4.2. Passage Numbers

For most E6 extended-life-span cultures, at least 25 passages are possible after introduction of the E6 gene and selection of cell clones. An exact number is unpredictable and may depend on the age of the patient. However, we have seen no relationship to the tumorigenic phenotype. Almost inevitably, after up to 30 passages the epithelial cells enter a crisis, and a prolonged G_0 phase. The requirement now is the patience to maintain the cultures for up to 6 wk by feeding essentially static and apparently dead cells. Spontaneously immortalized cells emerge from this crisis infrequently and often resemble the original culture but with a less epithelial phenotype (expression of vimentin—a stromal marker—is sometimes upregulated compared to the original culture).

3.4.3. Cell Lifts for DNA Purification

To monitor the immortalization process, and to provide an indication of the origin of the cells immortalized, a microassay from the growing immortalized colonies of epithelial cells can be carried out. Using the procedure described in this subheading, sufficient cells are obtained to carry out a PCR amplification of known genes (to compare mutation status between the original tumor and the cellular outgrowths) or a microsatellite/single nucleotide polymorphism analysis ***(16)***. Full protocols for the latter analysis are available elsewhere.

1. Aspirate the medium from growing cells, then wash twice for 5 min in PBS.
2. Cut squares of 3MM paper measuring 3 mm^2 and sterilize by autoclaving in a glass Petri dish.
3. Using sterile forceps, place the 3MM squares onto the cells, leave for 20 seconds (s), and then remove into an Eppendorf tube containing 200 µL of DNA extraction buffer.
4. Restore fresh medium to the cultured cells, which can then continue growing.
5. Process the 3MM squares as follows. Add 50 µL (or more) of DNA extraction buffer to an Eppendorf tube containing the 3MM square and incubate overnight at 42°C.
6. Next day, inactivate the proteinase K by incubation at 95°C for 8–10 min, and centrifuge the paper either to the bottom of the tube or carefully remove with a sterile tip.
7. The resulting solution is ready for PCR amplification and further analysis, when used to make up no more than 10% of a PCR reaction final volume.

3.4.4. Detection of E6 DNA in Infected Cells

1. Pellet cells by centrifugation and extract the DNA by standard methods. Use 20 ng of the DNA as a substrate for PCR.
2. Reaction mixtures contain 2 m*M* dNTPs, 0.05% W-1, 1.5 m*M* $MgC1_2$, 0.3 µ*M* forward and reverse primers (*see* **Note 6**), and 0.5 U of *Taq* DNA polymerase. Thirty-five cycles of amplification, with annealing at 55°C are sufficient to detect the low copy numbers of E6 retained in the immortalized cells.
3. Detect PCR product (455 bp) by electrophoresis in a 1% (w/v) agarose gel.

3.4.5. RT-PCR to Detect E6 mRNA

1. Pipet 5 µg total cell RNA into a diethylpyrocarbonate (DEPC)-treated Eppendorf tube with 0.5 µL of RNA guard solution (Boerhinger Mannheim), 500 ng of oligo-dT primer, and sterile double-distilled (dd) H_2O to a volume of 10.5 µL.

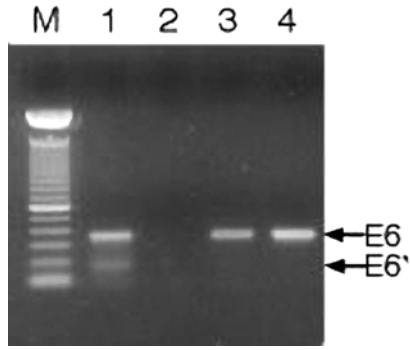

Fig. 2. Detection of E6 DNA and mRNA in the immortalized cultures by RT-PCR. Agarose gel electrophoresis of PCR products from an E6-transformed prostatic epithelial cell. Marker lane (M) is a 100-bp ladder from Life Technologies. Lane 1 is the amplification of cDNA from the cell line showing both E6 and E6*-specific products. Lane 2 is a negative control; lane 3 contains DNA from the same cell line (455-bp product only), and lane 4 is the CaSki cell DNA positive control.

2. Heat the tube to 70°C for 10 min and snap-cool on ice. Pulse the Eppendorf tube in a microfuge to collect the contents.
3. Set up the cDNA synthesis reaction by adding 0.5 µL RNAguard, 1X Superscript buffer (Boehringer Mannheim), 10 m*M* dithiothreitol (DTT), 1 m*M* dNTPs, and 200 U of Superscript enzyme.
4. Mix the contents of the tube gently and incubate at 42°C for 1 h. After this period, precipitate the cDNA at –80°C for 1 h by adding 0.05 vol glycogen solution, 0.5 vol 3 *M* NaCl, and 3 vol of absolute ethanol.
5. Pellet the cDNA by centrifugation at 12,000*g* at 4°C for 5 min and following a final wash with ice-cold 70% ethanol, air dry the pellet and resuspend in 20 µL sterile ddH2O.
6. Use 2 µL of cDNA as a substrate for PCR to detect E6 expression. The reaction mixture also consists of 2 m*M* dNTPs, 0.05% W-1 detergent, 1.5 m*M* $MgCl_2$, 3 pmoles each of the forward and reverse primers (*see* **Note 6**), 1X PCR buffer, and 1 U of *Taq* DNA polymerase (Gibco-BRL).
7. Resolve products (*see* **Note 7**) by electrophoresis in a 1% (w/v) agarose gel as shown in **Fig. 2**.

3.4.6. Immunofluorescent Detection of E6 Protein

1. Remove the medium from cells grown to 60–100% confluency and wash the cells twice for 5 min in PBS to remove residual sera and debris.
2. Fix cells in 4% paraformaldehyde (v/v) in PBS (0.45 µm filter-sterilized) for 10 min at room temperature.
3. Wash cells once for 5 min prior to permeabilization with 100% methanol for 2 min.
4. Wash the fixed cells with 4 changes of PBS over 5 min and air-dry for storage at –80°C until use, or drain and use immediately.

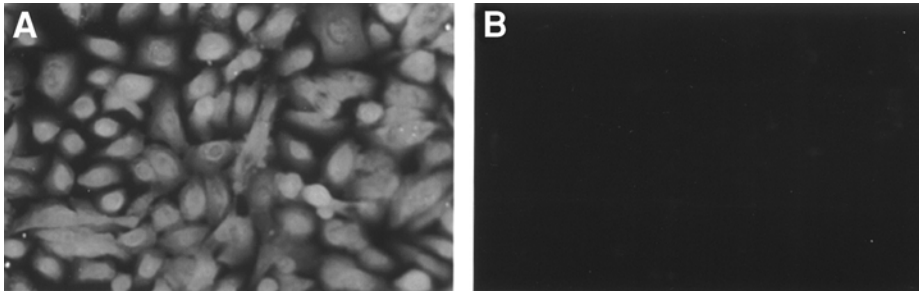

Fig. 3. Immunodetection of E6 protein in E6 transformed prostatic epithelial cells. **(A)** Positive immunostaining (mainly pancellular) with an anti-E6 antisera ***(20)*** of the same cell line as analyzed for DNA and RNA (shown in **Fig. 2**. **(B)** Corresponding negative control in which the primary antibody has been replaced with PBS in the full staining procedure.

5. Demarcate areas for staining with either a PAP pen (The Binding Site, Birmingham, UK) or a diamond pen, and rehydrate with 2% (w/v) bovine serum albumin (BSA) (Sigma) in PBS (0.45 µm filter sterilized) for 10 min at room temperature.
6. Block cells with 1% (v/v) horse serum in PBS for 20 min at room temperature in a humidified chamber.
7. After two 10-min washes with 2% (w/v) BSA/PBS, incubate cells for 1 h at room temperature with the primary antibody: an anti-E6 polyclonal antibody at a dilution of 1:50 in 2% (w/v) in PBS ***(20)***.
8. Detect the rabbit primary antibody by incubation for 30 min at room temperature in a humidified chamber, using an FITC-linked antirabbit secondary (Sigma) at a 1:20 dilution in 2% (w/v) BSA/PBS.
9. After a final set of three washes for 5 min each in 2% (w/v) BSA/PBS, cover slips (BDH) are mounted using Citifluor (Agar Scientific) and sealed with clear nail varnish (*see* **Note 8**).
10. Observe fluorescent staining using a Nikon FXA fluorescent microscope (*see* **Fig. 3**).

4. Notes

1. Alternative methods of disaggregation including enzymatic digestion overnight at 37°C with collagenase and hyaluronidase (both at 30 U/mL), are possible and can produce a single cell suspension (*see* Lang and Maitland, Chapter 28).
2. A common and "safer "host for amphotrophic retroviruses, and an alternative to PA317, is PT67 (ATCC number CRL-12284). PT67 also has variant *env* protein (10A1), which broadens the host range to most human cell types ***(21)***. The techniques involved do not change when this is substituted for PA317.
3. Retrovirus producer cells grow quickly and must be maintained in a healthy state to optimize transduction and yield of recombinant retrovirus.
4. Transfection mixtures are prepared in plastic tissue culture vials obtained from various suppliers but NOT Eppendorf type tubes.
5. When growing up E6-transfected epithelial cells it is vital not to "push" them by subculturing too soon. This will limit the time to crisis in the cultures.

6. The E6 primers for PCR are: 5'-GCAAGCAACAGTTACT-3 and 5'-CTGGGTTT CTCTACGTGTTC-3'; CaSki (an HPV-16 infected cervical carcinoma cell line) can be used (ATCC number CRL1550) as a positive control.
7. In most cells two forms of E6, full length (455 bp) and the spliced E6* form (180 bp) are detected by RT-PCR.
8. Fluorescently labeled cells can be stored in the dark (sealed in aluminium foil) at 4°C until viewing.

Caution should be exercised in the production and handling of recombinant retrovirus. In accordance with biological safety regulations, retroviral supernatants should only be handled in a class II laboratory environment. Appropriate national, NIH, and institutional guidelines and permission should be obtained.

References

1. Bello, D., Webber, M. M., Kleinman, H. K., Wartinger, D. D., and Rhim, J. S. (1997) Androgen responsive adult human prostatic epithelial cell lines immortalized by human papillomavirus 18. *Carcinogenesis* **18,** 1215–1223.
2. Sramkoski, R. M., Pretlow, D. G. , Giaconia, J. M., et al. (1999) A new human prostate carcinoma cell line, 22Rv1. *In Vitro Cell. Dev. Biol. Anim.* **35,** 403–409.
3. Macoska, J. Beheshti, A., Rhim, B., et al. (2000) Genetic characterization of immortalized human prostate epithelial cell cultures: Evidence for structural rearrangements of chromosome 8 and i(8q) chromosome formation in primary tumor-derived cells. *Cancer Genet. Cytogenet.* **120,** 50–57.
4. Yasunaga, Y., Nakamura, K., Ewing, C. M., Isaacs, W. B., Hukku, B., and Rhim, J. S. (2001) A novel human cell culture model for the study of familial prostate cancer. Cancer Res. 61, 5969–5973.
5. Yasunaga,Y., Nakamura, K., Ko, D., et al. (2001) A novel human cancer culture model for the study of prostate cancer. *Oncogene* **20,** 8036–8041.
6. Zhang, W., Kapusta, L. R., Slingerland, J. M., and Klotz, L. H. (1998) Telomerase activity in prostate cancer, prostatic intraepithelial neoplasia, and benign prostatic epithelium. *Cancer Res.* **58,** 619–621.
7. Urquidi, V., Tarin, D., and Goodison, S. (2000) Role of telomerase in cell senescence and oncogenesis. *Annu. Rev. Med.* **51,** 65–79.
8. Gluzman, Y., Frisque, R. J., and Sambrook, J. (1980) Origin- defective mutants of SV40. *Cold Spring Harb. Symp. Quant. Biol.* **44,** 293–300.
9. Poirer, V., Tyler, S. J., Brown, K. W., Shaw, A. P., and Maitland, N. J. (1988) SV40 transfection of human kidney epithelial cells and stability of chromosome 11. *Int. J. Cancer* **42,** 887–894.
10. Smalley, M., Leiper, K., Tootle, R., McCloskey, P., O'Hare, M. J., and Hodgson, H. (2001) Immortalization of human hepatocytes by temperature-sensitive SV40 large-T antigen. *In Vitro Cell. Dev. Biol. Anim.* **37,** 166–168.
11. Band, V., Zajchowski, D., Kulesa,V., and Sager, R. (1990) Human papilloma virus DNAs immortalize normal human mammary epithelial cells and reduce their growth factor requirements. *Proc. Natl. Acad. Sci. USA* **87,** 463–467.
12. Bright, R. K., Vocke, C. D., Emmert-Buck, M. R., et al. (1997) Generation and genetic characterization of immortal human prostate epithelial cell lines derived from primary cancer specimens. *Cancer Res.* **57,** 995–1002.

13. Scheffner, M., Werness, B. A., Huibregtse, J. M., Levine, A. J., and Howley, P. M. (1990) The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation or p53. *Cell* **63,** 1129–1136.
14. Klingelhutz, A. J., Foster, S. A., and McDougall, J. K. (1996) Telomerase activation by the E6 gene product of human papillomavirus type 16. *Nature* **380,** 79–85.
15. Halbert, C. L., Demers, G. W., and Galloway, D. A. (1991) The E7 gene of human papillomavirus type 16 is sufficient for immortalization of human epithelial cells. *J. Virol.* **65,** 473–478.
16. Maitland, N. J., Macintosh, C. A., Hall J., Sharrard, M., Quinn, G., and Lang, S. (2001) In vitro models to study cellular differentiation and function in human prostate cancers. *Radiat. Res.* **155,** 133–142.
17. Miller, A. D., and Rosman, G. J. (1989) Improved retroviral vectors for gene transfer and expression. *Biotechniques* **7,** 980–990.
18. Boukamp, P., Petrussevska, R. T., Breitkreutz, D., Hornung, J., Markham, A., and Fusenig, N.E. (1988) Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *J. Cell Biol.* **106,** 761–771.
19. Brown, A. M. C. and Dougherty, J. P. (1995) DNA cloning 4. A Practical Approach., IRL Press, Oxford, 113–140
20. Daniels, P. R., Sanders, C. M., and Maitland, N. (1998) Characterisation of the interactions with human papillomavirus type 16 E6 with p53 and E6-associated protein in insect and human cells. *J. Gen. Virol.* **79,** 489–499.
21. Miller, A. D. (1996) Cell-surface receptors for retroviruses and implications for gene transfer. *Proc. Natl. Acad. Sci. USA* **93,** 11,407–11,413.

27

The Use of Matrigel to Facilitate the Establishment of Human Cancer Cell Lines as Xenografts

Peter Mullen

1. Introduction

Matrigel is a solubilized tissue basement membrane matrix rich in extracellular matrix proteins that was originally isolated from the Engelbreth-Holm-Swarm (EHS) mouse tumor. Although composed mainly of laminin, collagen IV, heparan sulphate, proteoglycans, and entactin (nidogen), various growth factors such as transforming growth factor-β (TGF-β), fibroblast growth factor (FGF), and tissue plasminogen activator are also present. Under normal physiological conditions Matrigel polymerizes to produce a reconstituted, biologically active stable matrix, that is effective for the attachment and differentiation of normal and transformed anchorage-dependent cell types ***(1–2)***.

The establishment of xenografts using material originating from primary human tumors (of any type) is arguably one of the most informative in vivo models available and one that has been well documented. Despite such importance, the use of cell lines for the establishment of human tumor xenografts in immunodeficient mice has historically proven to be difficult for many tissue types. These problems are further exacerbated when attempting to establish xenografts from primary clinical material where "take rates" are often low ***(3–5)***. However, the inclusion of Matrigel has been shown to significantly enhance the tumorigenicity of a wide range of cancer cell lines in vivo, including breast ***(6–8)***, ovarian ***(8,9)***, endometrioid ***(10)***, lung ***(11,12)***, prostate ***(13–16)***, glioblastoma ***(17,18)***, and retinoblastoma ***(19,20)***. There are also reports of Matrigel enhancing the tumorigenic potential of primary breast material that has traditionally been difficult to transplant ***(21–23)***. Interestingly, xenografts originally established in the presence of Matrigel can be excised, transferred into fresh recipient animals, and subsequently grown without the need for Matrigel, suggesting that it is only required during the initial establishment of tumors ***(8)***. Matrigel is therefore useful for the development of routine xenografts from a wide variety of cell lines that would otherwise prove difficult to "take" (*see* **Fig. 1**).

From: *Methods in Molecular Medicine, vol. 88: Cancer Cell Culture: Methods and Protocols*
Edited by: S. P. Langdon © Humana Press Inc., Totowa, NJ

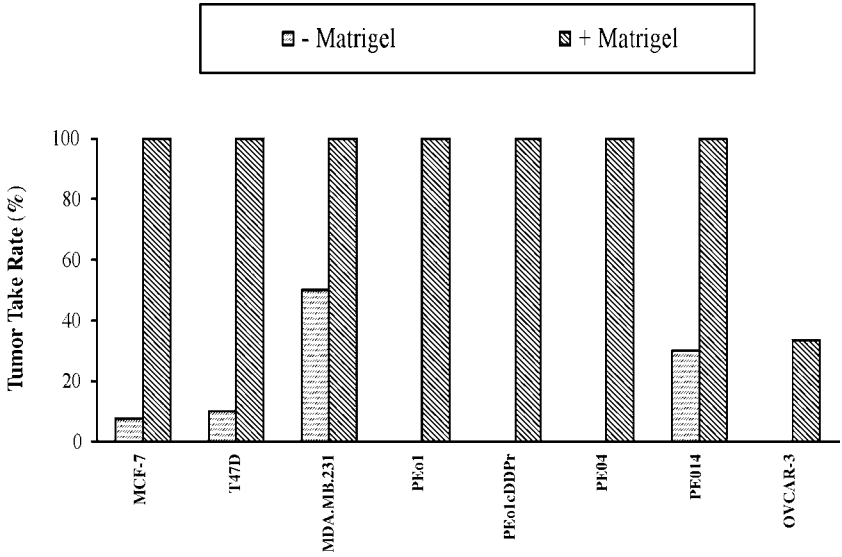

Fig. 1. Comparative take rates for three breast- and five ovarian-cancer cell lines inoculated in the presence/absence of Matrigel. The three breast cancer cell lines (MCF-7, T47D, and MDA.MB.231) and five ovarian cancer cell lines (PE01, PE01cDDPr, PE04, PE014, and OVCAR-3) were all injected at 4×10^6 cells/implant.

2. Materials

2.1. Preparation of Matrigel

Matrigel (Collaborative Biological Research, Bedford, MA).

2.2. Animals and Animal Husbandry

1. Female nude (nu/nu) mice (Harlan Olac, Oxford, England).
2. Negative-pressure isolator (Moredun Isolators Ltd., Edinburgh, Scotland).
3. Open filter-box cages (North Kent Plastics, Erith, Kent, England).
4. RM3(E) animal feed irradiated to 2.5 mrad (SDS Diet Services, Witham, England).
5. Sterile water (Baxter Travenol, Thetford, England).
6. Bedding (BS&S (Scotland) Ltd., Edinburgh, Scotland).
7. Ear identification tags (Brookwick Ward, Glenrothes, Fife, Scotland).

2.3. Cell Culture

1. Cell line of interest, e.g., MCF-7 breast cancer ***(24)*** or PE01 ovarian cancer ***(25)*** cells.
2. Dulbecco's phosphate buffered saline (PBS) (Oxoid, Unipath Ltd., Basingstoke, England).
3. 1X Trypsin-EDTA (Gibco-BRL, Life Technologies Ltd., Paisley, Scotland).
4. RPMI/PS: RPMI-1640 (Gibco-BRL, Life Technologies Ltd., Paisley, Scotland), 100 U/mL penicillin/100 µg/mL streptomycin (Gibco-BRL, Life Technologies Ltd., Paisley, Scotland).
5. RPMI/PS/10% fetal calf serum (FCS): RPMI-1640, 100 U/mL penicillin/100µg/mL streptomycin, 10% FCS (Advanced Protein Products, Brierley Hill, England) (*see* **Note 1**).
6. 150-cm^2 Tissue culture flask (Life Technologies Ltd., Paisley, Scotland).

3. Methods

3.1 Preparation of Matrigel

1. Place Matrigel in the refrigerator overnight at 4°C (*see* **Notes 2** and **3**).
2. Transfer the Matrigel on ice into a tissue culture hood, along with sterile microcentrifuge tubes (1.5 mL) and Gilson 1-mL tips (*see* **Notes 4** and **5**).
3. Let chill for 5–10 min.
4. Wipe top of vial with 70% ethanol and leave to air-dry.
5. Open vial carefully with gloved hands to maintain sterility.
6. Gently swirl vial to disperse Matrigel evenly, taking care not to let it warm up.
7. Using a prechilled 1-mL tip, aliquot 250 µL from one 10-mL bottle of Matrigel into each of 40 prechilled sterile microcentrifuge tubes.
8. Immediately refreeze Matrigel aliquots at –20°C prior to use, thus minimizing the number of freeze/thaw cycles.
9. Each ampule can be removed from the freezer and thawed on ice just prior to use.

3.2. Animals and Animal Husbandry

1. At the point of delivery, female nude (nu/nu) mice are placed in negative-pressure isolators until ready for use. RM3(E) animal feed (irradiated to 2.5 mrad) and sterile water are made available *ad libitum*. All bedding is changed twice per week, prior to experimentation.
2. Remove mice from the isolator and transfer to open filter-box cages. Affix ear identification tags to aid identification of individual mice if required.

3.3. Cell Culture

1. Suspend 2.5×10^6 cells of the cell line of interest in 50 mL RPMI/PS/10%FCS and transfer to a 150-cm^2 tissue culture flask. Place in a 5% CO_2 humidified incubator at 37°C.
2. Allow cells to grow to confluence, feeding twice per week by aspirating the spent media, washing with 20 mL PBS, and then adding 50 mL fresh tissue culture media .
3. When cells become approx 80% confluent, remove spent tissue-culture media, and wash cells in 10 mL PBS. Remove excess PBS.
4. Add 5 mL trypsin/EDTA to each flask and place in the incubator until the cells have become detached (*see* **Note 6**).
5. Transfer cell suspension to a sterile universal container using a sterile pipet; pour 20 mL RPMI/PS/10%FCS (to deactivate the trypsin) into the flask in order to wash and then pool the media with the cell suspension in the Universal container (total volume 25 mL).
6. Centrifuge at 600*g* for 5 min.
7. Pour off the media and resuspend the pellet in 5 mL RPMI/PS/10% FCS. Syringe with a 21-gage needle (X3) to break up the pellet and then make up to 25 mL with RPMI/PS/10% FCS. Aliquot 5 mL into each of five 150-cm^2 flasks (1 in 5 split) and add a further 50 mL of media. Replace in incubator.
8. Repeat splitting process (**Subheading 3.3., steps 3–7**) until sufficient cells are available for inoculating into the desired number of mice.

3.4. Preparation and Delivery of Inoculations

1. Harvest cells as described in **Subheading 3.3., steps 3–6**.
2. Resuspend pellet in 5 mL RPMI/PS (no FCS from this point on).
3. Syringe the cell suspension with a 10-mL syringe plus a 21-gage needle (X3) to remove clumps—make up to 25 mL with RPMI/PS.
4. Count total number of cells using a hemocytometer.

5. Prepare a suspension of cells at a concentration of 4×10^6 cells per 250 µL.
6. Allow cells to sit on ice for 5 min to chill.
7. Remove desired number of aliquots of Matrigel from freezer and allow to thaw out on ice.
8. Transfer 250 µL of cell suspension to the sterile microcentrifuge vials containing 250 µL of Matrigel (test) or 250 µL RPMI/PS (control), giving a final volume of 500 µL.
9. Close all microcentrifuge lids and transfer the cell suspension (on ice) to the inoculation site.
10. Place all syringes and needles on ice to chill for 5 min prior to inoculation (*see* **Notes 4** and **5**).
11. Gently mix tubes before drawing Matrigel/cell suspension up into the syringe.
12. Inoculate all mice in the flank (*see* **Note 7**), being careful to prevent back-spill when the needle is removed. Apply a preparatory aerosol sealer if back-spill becomes a problem (*see* **Note 8**). Make one inoculation on each side of the mouse, allowing each animal to have its own negative control if required.
13. Mice are maintained in open filter-box cages and measurements made twice per week using callipers.

4. Notes

1. FCS must be heat-inactivated prior to use by heating in a water bath for 30 min at 56°C. It can then be placed in 55-mL aliquots and stored at –20°C.
2. Matrigel is shipped frozen and should be stored at –20°C until ready for use. However, it naturally polymerizes as the temperature rises above 4°C. Since this polymerization is nonreversible, once removed from the freezer Matrigel must be kept on ice at all times prior to inoculation.
3. Although Matrigel is commercially available from more than one supplier, some sources are better than others—some are completely ineffective. We would therefore recommend that Matrigel is initially obtained from the source cited.
4. Since tissue culture requires aseptic technique, and Matrigel must be kept on ice at all times, the most practical way of achieving both requirements is to obtain a large shallow polystyrene box that can be filled with ice and then placed into the tissue culture hood. All preparatory manipulations involving Matrigel can then be conducted on ice within a sterile environment.
5. To prevent Matrigel polymerizing within the barrel of the syringe during inoculation, chill all tubes, tips, syringes, and needles prior to use.
6. The time interval for cells to become detached from the plastic is dependent on the cell line being used but is usually in the order of 5–10 min.
7. There is evidence that better take-rates are often obtained if the inoculations are made into the mammary fat pad of the mice ***(26)***, but this was not evident to us, probably because the mouse fat pad is small in relation to the large volumes being introduced. Therefore, mice were simply inoculated subcutaneously.
8. It is possible that the cell suspension can leak out of the inoculation site when the needle is removed, probably as a result of the relatively large volumes (500 µL) being introduced. A preparatory aerosol sealer can be applied in order to seal the wound and retain the cell suspension if this becomes a problem.

References

1. Kleinman, H. K., McGarvey, M. L., Liotta, L. A., Robey, P. G., Tryggvason, K. and Martin, G. R. (1982) Isolation and characterisation of type IV procollagen, laminin and heparan sulfate proteoglycans from the EHS sarcoma. *Biochemistry* **21,** 6188–6193.
2. Baatout, S. (1997) Endothelial differentiation using Matrigel. *Anticancer Res.* **17,** 451–455.
3. Bailey, M. J., Gazet, J. C., and Peckham, M. J. (1980) Human breast cancer xenografts in immune-suppressed mice. *Brit. J. Cancer* **42,** 524–529.
4. Rae-Venter, B., and Reid, L. M. (1980) Growth of human breast carcinomas in nude mice and subsequent establishment in tissue culture. *Cancer Res.* **40,** 95–100.
5. Giovanella, B. C., Vardeman, D. M., Williams, L. J., et al. (1991) Heterotransplantation of human breast carcinomas in nude mice. Correlation between successful heterotransplants, poor prognosis, and amplification of the *HER-2/neu* oncogene. *Int. J. Cancer* **47,** 66–71.
6. Noel, A., Simon, N., Raus, J., and Foidart, J. M. (1992) Basement-membrane components (Matrigel) promote the tumorigenicity of human breast adenocarcinoma MCF-7 cells and provide an in-vivo model to assess the responsiveness of cells to estrogen. *Biochem. Pharmacol.* **43,** 1263–1267.
7. Noel, A., De Pauw-Gillet, M. C., Purnell, G., Nusgens, B., Lapiere, C. M., and Foidart, J. M. (1993) Enhancement of tumorigenicity of human breast adenocarcinoma cells in nude-mice by Matrigel and fibroblasts. *Brit. J. Cancer* **68,** 909–915.
8. Mullen, P., Ritchie, A., Langdon, S. P,. and Miller, W. R. (1996) Effect of matrigel on the tumorigenicity of human breast and ovarian carcinoma cell lines. *Int. J. Cancer* **67,** 816–820.
9. Yoshida, Y., Kamitani, N., Sasaki, H., Kusumi, K., Tominaga, T., and Kotsuji, F. (1998) Establishment of a liver metastatic model of human ovarian cancer. *Anticancer Res.* **18,** 327–331.
10. Yoshida, Y., Kamitani, N., Sasaki, H., and Kotsuji, F (1998) Establishment of a cell line from a neuroendocrine carcinoma of the endometrium and an invasion model. *Anticancer Res.* **18,** 1569–1577.
11. Fridman, R., Giaccone, G., Kanemoto, T., Martin, G. R., Gazdar, A. F., and Mulshine, J. L. (1990) Reconstituted basement membrane (Matrigel) and laminin can enhance the tumorigenicity and the drug resistance of small cell lung cancer cell lines. *Proc. Natl. Acad. Sci. USA* **87,** 6698–6702.
12. Yamaura, T., Murakami, K., Doki, Y., et al. (2000) Solitary lung tumors and their spontaneous metastasis in athymic nude mice orthotopically implanted with human non-small cell lung cancer. *Neoplasia* **2,** 315–324.
13. Fridman, R., Kibbey, M. C., Royce, L.S., et al. (1991) Enhanced tumour growth of both primary and established human and murine tumor cells in athymic mice after coinjection with matrigel. *J. Nat. Cancer Inst.* **83,** 769–774.
14. Pretlow, T. G., Delmoro, C. M., Dilley, G. G., Spadofora, C. G., and Pretlow, T. P. (1991) Transplantation of human prostatic carcinoma into nude mice in Matrigel. *Cancer Res.* **51,** 3814–3817.
15. Krupski, T., Harding, M. A., Herce, M. E., Gulding, K. M., Stoler, M. H., and Theodorescu, D. (2001) The role of vascular endothelial growth factor in the tissue specific in vivo growth of prostate cancer cells. *Growth Factors* **18,** 287–302.
16. Culp, L. A., Holleran, J. L., and Miller, C. J. (2001) Tracking prostate carcinoma micrometastasis to multiple organs using histochemical marker genes and novel cell systems. *Histol. Histopathol.* **16,** 945–953.
17. Rubenstein, M., Shaw, M., Mirochnik, Y., et al. (1999) In vivo establishment of T98G human glioblastoma. *Methods Find. Exp. Clin. Pharmacol.* **21,** 391–393.

18. Wyrick, B. J., Ozawa, T., Lamborn, K. R., Bollen, A. W., and Deen, D. F. (1997) Effects of Matrigel on the SF-767 malignant glioma athymic mouse tumor model. *Anticancer Res.* **17,** 2419–1425.
19. Albini, A., Melchiori, A., Garofalo, A., et al. (1992) Matrigel promotes retinoblastoma cell-growth in vitro and in-vivo. *Int. J. Cancer* **52,** 234–240.
20. Cowell, J. K., Ramani, P., Song, Y., Evans, M., and Morgan, G. (1997) The use of SCID mice for the growth of retinoblastoma cell lines and for the establishment of xenografts from primary tumours. *Eur. J. Cancer* **33,** 1070–1074.
21. Mehta, R. R., Graves, J. M., Hart, G. D., Shilkaitis, A., and Dasgupta, T.K. (1993) Growth and metastasis of human breast carcinomas with matrigel in athymic mice. *Breast Cancer Res. Treat.* **25,** 65–71.
22. Mehta, R. R., Graves, J. M., Shilkaitis, A., and das Gupta, T. K. (1998) Development of a new metastatic human breast carcinoma xenograft line. *Br. J. Cancer* **77,** 595–604.
23. Elenbaas, B., Spirio, L., Koerner, F., et al. (2001) Human breast cancer cells generated by oncogenic transformation of primary mammary epithelial cells. *Genes Dev.* **15,** 50–65.
24. Soule, H. D., Vazquez, J., Long, A., Albert, S., and Brennan, M. T. (1973) A human cell line from a pleural effusion derived from a breast carcinoma. *J. Nat. Cancer Inst.* **51,** 1409–1413.
25. Langdon, S. P., Lawrie, S. S., Hay, F. G., et al. (1988) Characterisation and properties of nine human ovarian cancer cell lines. *Cancer Res.* **48,** 6166–6172.
26. Price, J. E., Polyzos, A., Zhang, R. D., and Daniels, L. M. (1990) Tumorigenicity and metastasis of human breast carcinoma cell lines in nude mice. *Cancer Res.* **50,** 717–772.

28

Coculture of Prostate Cancer Cells

Shona H. Lang and Norman J. Maitland

1. Introduction

1.1. In Vitro Culture of Human Primary Prostatic Cells

The progression of prostate cancer is accompanied by the breakdown of normal epithelial-ductal architecture. This breakdown is attributable to the loss of normal homeostatic interactions between the epithelial and stromal compartments of prostatic tissue. Complex in vitro models, unlike basic monolayer cultures, are capable of representing several cellular compartments and 3D glandular architecture to study these processes. Such models complement existing animal models because they are less complex than a whole animal and therefore more easily manipulated. Moreover, employing human primary cell cultures for in vitro models is likely to be more informative than studying animal cells or systems, particularly in the prostate where organogenesis may differ between mouse and human.

This chapter describes the isolation of epithelial and stromal cells from human primary tissue and its subsequent use in producing acinus-like structures (or spheroids) in basement membrane gels, illustrated in **Fig. 1**. Basement membrane preparations can maintain both epithelial morphology and the expression of tissue specific genes in vitro ***(1,2)***. The ability to coculture epithelia with stromal cell compartments further enhances epithelial differentiation ***(3,4)*** and moves closer to a physiologically relevant model. In particular, stromal coculture increases cellular polarization and enhances tissue specific gene expression (*see* **Fig. 1**; **ref. *4***). The basic model described in this chapter can be manipulated to study the effects of coculturing cells from normal or cancer-derived tissues, with or without any number of growth factors or hormones. The method assumes the reader has a basic knowledge of cell culture techniques.

2. Materials

All media listed should be stored at 4°C, and warmed to 37°C before use unless otherwise stated.

From: *Methods in Molecular Medicine, vol. 88: Cancer Cell Culture: Methods and Protocols*
Edited by: S. P. Langdon © Humana Press Inc., Totowa, NJ

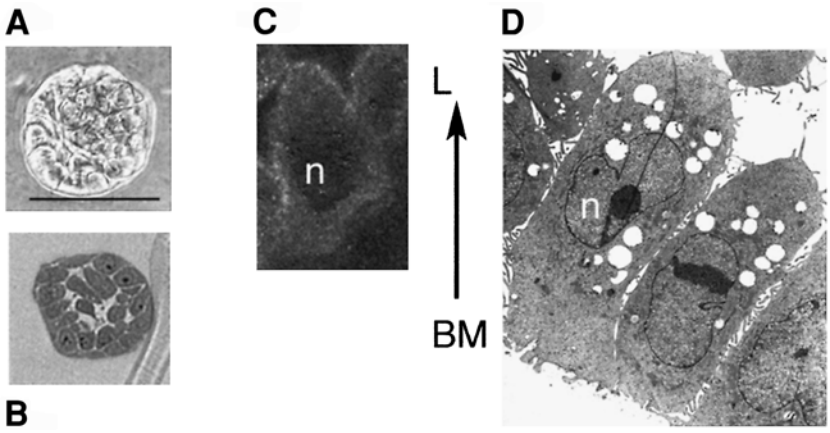

Fig. 1. Acinus-like spheroids produced by coculture of prostatic epithelia and stromal cells. **(A)** Phase contrast of a epithelial spheroid. Bar measures 80 µm. **(B)** 1-µm sections of a spheroid stained with toluidene blue. The acinus-like nature of the spheroids is evident; they will develop one or two epithelial layers of cuboidal/columnar cells. **(C)** Detection of prostate specific antigen (PSA) by confocal microscopy of a columnar epithelium. Intense luminal membrane staining indicates PSA secretion becomes polarized towards the spheroid lumen (L) away from the basement membrane (BM). n, nucleus. **(D)** Transmission electron microscopy of columnar epithelia from the spheroid depicted in B. In the presence of stromal coculture the epithelia show increased luminal polarization of secretory vesicles and Golgi apparatus. In addition, tight and desmosomal-like junctions form between adjacent cells.

2.1. Preparation of Human Prostatic Cell Cultures

1. Transport medium: RPMI 1640 culture medium, 5% fetal calf serum (FCS) (v/v), 1% antibiotic/antimycotic solution (v/v).
2. Collagenase solution: RPMI 1640 culture medium, 200 U/mL collagenase type I, 1% FCS (v/v).
3. Washing medium: phosphate buffered saline (PBS), 1 m*M* EDTA, 1% bovine serum albumin (BSA) (w/v).
4. Trypsin solution: washing medium, 0.1% trypsin (w/v).
5. Epithelial cell culture medium: keratinocyte serum free medium, 5 ng/mL epidermal growth factor, 50 µg/mL bovine pituitary extract, 2 m*M* glutamine, 1% antibiotic/antimycotic solution (v/v).
6. Stromal cell culture medium: RPMI 1640 culture medium, 10% FCS (v/v), 2 m*M* glutamine, 1% antibiotic/antimycotic solution (v/v).

2.2. Coculture in Matrigel

1. 0.4-µm Cell culture inserts (Millipore PIHP, cat. no. 01250).
2. BD Matrigel™ (Becton Dickinson, Oxford, UK, cat. no. 354234).
3. 5-mL Round bottom tubes (Falcon, cat. no. 352058).
4. Coculture growth medium: epithelial cell culture medium, 2% FCS.

2.3. Preparation of Spheroids Grown in Matrigel for Further Analysis

1. OCT Compound (BDH, Poole, UK, cat. no. 36160 3E)
2. Super frost microscope slides (BDH, cat. no. 406 0179 00)
3. BD Matrisperse™ (Becton Dickinson, Oxford, UK, cat. no. 354253).
4. 40-µm Cell sieve (Falcon, cat. no. 2340)
5. Electron microscopy fixative: 100 m*M* phosphate buffer, pH 7.6, 4% paraformaldehyde (w/v), and 2.5% Ultrapure glutaraldehyde (v/v).

3. Methods

3.1. Preparation of Human Prostatic Cell Cultures

3.1.1. Precautions for Handling Human Tissues

1. It is recommended that workers are vaccinated against hepatitis B infection. Tissue samples from any patients known to have or suspected of hepatitis B or HIV infections should not be used.
2. Keep detailed records of all tissue samples used. For example, note the patient number or tissue number allocation, date of operation, hospital of origin, name of clinician supplying the tissue, name(s) of people handling tissue, how the tissue was used.
3. Use class II flow cabinets and disposable tissue culture equipment. All discarded biological samples or contaminated equipment should be treated with a suitable disinfectant (for example, virkon) or autoclaved, followed by incineration.

3.1.2. Prostate Tissue Collection

1. Obtain full patient consent and ethical permission before using human tissue for experimentation.
2. Obtain nonmalignant tissues from patients undergoing transurethral resection for benign prostatic hyperplasia or cystoprostatectomy. Obtain malignant samples from patients undergoing transurethral resections or radical prostatectomy for prostate cancer.
3. Store 30-mL tubes containing 15 mL of transport medium at 4°C in proximity to the operating room. Collect tissue samples and immediately place them into the medium (*see* **Note 1**). Collect tissue and process within 24 h (*see* **Note 2**).
4. Divide all samples into two parts, send half for histopathological analysis to confirm tissue and disease status, and half is used for culture (*see* **Note 3**).

3.1.3. Digestion of Prostate Tissues

1. Weigh tissue.
2. Empty tissue onto a sterile Petri dish. Remove any collagenous material or burnt areas using a disposable scalpel and sterile forceps (if the tissue has suffered heat damage during resection). If the sample is from a tumor, take care dissecting any apparent tumor nodules from the surrounding normal tissue. Slice the remaining tissue into 1–2-mm^3 cubes (*see* **Note 4**).
3. Place tissue into a sealed and sterile conical flask containing 8 mL collagenase solution per gram of tissue. Place the flask in a shaking 37°C incubator overnight (*see* **Note 5**).
4. Pipet the digested solution until all visible lumps have disappeared, then centrifuge the solution for 5 min at 800*g*.
5. Add 20 mL washing medium to the pellet and pipet until lumps disappear again. Centrifuge as before.

6. Add 20 mL trypsin solution to the pellet, break up the lumps with a pipet, and place into a fresh conical flask. Place in shaking 37°C incubator for 30 min. This step helps to break down the protein debris and is essential if you aim to use the final preparation of a single epithelial cell suspension rather than an explant culture.
7. Pipet the digested solution until all visible lumps have disappeared, then centrifuge the solution for 5 min at 800*g*. Repeat this step two times or more or until the supernatant becomes clear.
8. Resuspend the pellet into 20 mL epithelial cell medium, pipet until all lumps disappear, and then centrifuge at 360*g* for 30 seconds (s).
9. Carefully remove the supernatant into a separate tube; this represents a fraction enriched for stromal cells, while the pellet is enriched for epithelia.

3.1.4. Stromal Cell Culture

1. Centrifuge the stromal supernatant prepared in **Subheading 3.1.3., step 9** for 5 min at 800*g*. Resuspend the pellet in 20 mL of stromal cell media and again centrifuge for 5 min at 800*g*.
2. Resuspend the pellet in 20 mL of stromal cell medium and seed one 75-mL tissue culture flask per 2 g of original tissue (using 10 mL stromal cell medium per flask).
3. Feed stromal cell cultures weekly by the removal of 5 mL spent medium and the addition of 5 mL fresh medium. Cultures take 2–4 wk to become confluent (*see* **Note 6**).
4. After one or two passages these cultures will contain 95–100% fibroblasts and 5–40% smooth muscle cells. Stroma derived from nonmalignant tissue will grow for 6–15 passages, while stroma from malignant tissues will only grow for 2–6 passages.

3.1.5. Epithelial Cell Culture

1. Take the epithelial pellet prepared in **Subheading 3.1.3., step 9** and resuspend in 20 mL epithelial cell growth medium. Pipet until all lumps have disappeared, then pass the cell solution through a 19-gage needle to disrupt any small lumps.
2. Place a 40-µm cell sieve onto a 50-mL tube and pass the cell solution through. The cellular debris left on the sieve is used to grow explant cultures, while the cells that have passed through the sieve provide a single epithelial cell solution.
3. Explant cultures are grown by seeding the cellular debris into one 25-mL flasks per 2 g original tissue (use 6 mL epithelial growth medium per flask). Confluent epithelial cultures generally form between 5–14 days (d) and are then used for further experimentation.
4. The number of viable epithelial cells (large and phase bright) in the single cell solution is counted using a hemocytometer (*see* **Note 7**). A cell count of $1–2 \times 10^6$ cells/4 g tissue is usually obtained. At this stage, the epithelia are stored by freezing in liquid nitrogen for use as required (*see* **Note 8**).

3.2. Coculture in Matrigel

The setup of these cultures is illustrated in **Fig. 2** (*see* **Note 9**).

1. Place the required number of 0.4-µm cell culture inserts into 24-well tissue culture plates.
2. A subconfluent stromal culture (between passages 2 and 5) is trypsinized and counted.
3. A single cell solution is prepared in stromal cell medium to a concentration of 10^5 cells/mL.
4. Plate 0.5 mL of the cell suspension into the inserts and 0.5 mL of stromal cell medium into the surrounding well. Allow the cultures to adhere and grow for 2–3 d.
5. Thaw sufficient aliquots of Matrigel on ice from frozen stocks (–20°C).

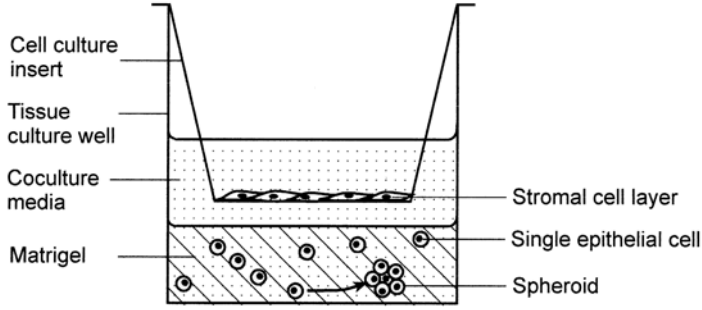

Fig. 2. The arrangement of components for a coculture within one well of a 24-well plate. The growth of a spheroid from a single cell (time course is usually 1 wk). Redrawn from Lang et al. ***(4)***.

6. Place 5-mL round bottom tubes, 1-mL syringes, and 24-well culture plates on ice or into a fridge to cool (*see* **Note 10**).
7. A 5–14-d explant culture is trypsinized and counted.
8. Prepare a single cell solution in coculture medium to a concentration of 6×10^4 cells/mL (*see* **Note 11**) and place on ice.
9. Place a 5-mL round bottom tube on ice and add an equal volume of epithelial cell solution to Matrigel. Slowly mix the two solutions using a 1-mL syringe to avoid producing air bubbles.
10. Carefully place the cell/Matrigel mix into 0.25–0.5-mL aliquots and place into the required number of wells of a 24-well plate.
11. Gently tap the plate to ensure the solution in the wells is evenly coated (*see* **Note 12**). Avoid plating more than ten aliquots at once to ensure that even coating and setting of the Matrigel occurs.
12. Incubate the 24-well plate for 30 min at 37°C to set the Matrigel.
13. Wash the inserts containing the stromal cultures twice using 0.5 mL of epithelial growth medium inside the well and 0.5 mL to the well.
14. Once the Matrigel has set, add 0.5 mL coculture medium to each well and then add an insert with stromal cells to the top of this. Add an additional 0.5 mL coculture medium to the inside of the insert.
15. The cultures medium is changed every 3 d by the removal of 0.5 mL of spent medium and the addition of 0.5 mL of fresh medium.
16. Grow cultures for 1–2 wk before further analysis (*see* **Note 13**).

3.3 Preparation of Spheroids Grown in Matrigel for Further Analysis

3.3.1. Preparation for Fluorescent Immunostaining

1. Remove spheroids in Matrigel from 24-well plates by loosening the gel with a scalpel blade and lifting the gel using a spatula.
2. Cups formed from aluminium foil (diameter 1–2 cm and height 3–4 cm) are filled with OCT Compound to a depth of 0.5 cm. Place this in liquid nitrogen until almost frozen, at which point remove and add the gel sample to the unfrozen OCT compound. Add addi-

tional OCT compound to the gel sample in the cup until it is covered and return to the liquid nitrogen until all is snap frozen.
3. Mount embedded gels in a cryostat and cut and mount 7-µm sections on Superfrost microscope slides. Leave these overnight at room temperature to allow the sections to adhere properly.
4. Store embedded gels or slides airtight at –20°C until use.
5. For immunofluorescence, observe the best results using the unfixed samples.

3.3.2. Release of Spheroids and Cells from Matrigel

Release spheroids or single cells from culture in Matrigel for reculture or molecular analysis. Carry out the following method on ice using medium and equipment pre-cooled to 4°C.

1. Remove culture medium from spheroids grown in Matrigel and wash three times within the 24-well plates using 0.5 mL PBS.
2. Add 0.5 mL of MatriSperse (Collaborative Biomedical/Becton Dickinson) per well.
3. Scrape the cell and matrix into a 15-mL tube sitting on ice.
4. Wash the empty well twice with 1 mL MatriSperse and add the washes to the tube.
5. Invert the tube several times to mix and leave on ice for 1 h. Apply further mixing at intermittent periods.
6. Centrifuge the cell matrix solution at 360*g* for 5 min at 4°C.
7. If gel is still apparent in the pellet, repeat **steps 2–6**. Gently wash the cell pellet twice more using PBS once the gel has dissolved.
8. At this stage, the majority of epithelia remain as spheroids, which can be used as required. If single cells are required, the pellet is spun down and resupended in 5 mL of 1% trypsin (w/v).
9. The trypsin and cell solution is placed in a 37°C shaking incubator for 5–10 min.
10. The cells are centrifuged at 800*g* for 5 min.
11. The cell pellet is resuspended in 10 mL coculture medium and passed through a 40-µm cell sieve placed on a 50-mL tube.
12. Place the collected cells on ice and use as required.
13. A cell count is required to check that a single cell solution has been produced and to measure cell viability.

3.3.3. Fixation of Cultures for Transmission Electron Microscopy

1. Remove culture medium from spheroids grown in Matrigel and wash three times within the 24-well plates using 0.5 mL PBS, pH 7.6.
2. Fix for 1 h at room temperature in prepared fixative.
3. Using standard techniques, dehydrate fixed cultures and embed within the 24-well plates before removal for sectioning. For additional methods see Allen and de Wynter *(7)*.

4. Notes

1. The use of tissue from patients treated with either catheters or antibiotics should be avoided since these tissues often yield cultures that carry microbial contamination.
2. Tissues can be stored for several days and still yield good cellular growth in culture. However, the best cell growth is achieved with fresh tissue (less than 24 h storage). In addition, consistent results are likely to be achieved between cultures from different patients if a set time from operation to experimentation is adhered to.

3. Samples that are large in size and from younger patients (generally under 65 yr old for prostate samples) provide the best in vitro cell growth.
4. At this stage, it is worth checking the sterility of the tissue. This can be done by adding a sample of tissue to equal volumes of used transport medium and bacteriological nutrient broth. Place this mixture in a 37°C incubator overnight. Any contamination should cause the medium to go cloudy.
5. A minimum of 6–8 h is usually required.
6. A precise count can be made by labeling the cells with cytokeratin and doing fluorescence-assisted cell sorter (FACS) analysis. This requires a large sample if you intend to use the same sample for additional experimentation. A cell suspension prepared as described contains 30–80% epithelia. In our experience, the majority of other cells are red and white blood cells. Epithelia can be further isolated if required using a variety of techniques, such as, magnetic cell sorting.
7. Although subconfluent explant cultures are routinely used to establish cocultures, it is useful to prepare frozen stocks of epithelia, so that several samples can be compared at once in the presence of a particular stromal culture or growth factor.
8. It is advantageous to produce a large frozen stock of stromal cultures that grow well and at a low passage number. This allows analysis of many epithelial samples against one standard stromal culture. It is also worth characterizing the stromal cultures for the proportion of fibroblast and smooth muscle cells ***(6)***.
9. Variation in spheroid size, morphology, and growth will occur between different primary epithelial cell cultures. Cell lines can be used to replace the primary cell cultures described here; however, the method described has been optimized for primary cells. Fibroblast cell lines can be substituted for the stromal cultures without altering the method. Epithelial cell lines often require different coculture medium, in most cases the cell lines' normal growth medium is the best supplement. Not all epithelial cell lines will form hollow-acinus-like spheroids; some form solid cell masses ***(2,5)***.
10. It is useful to prepare 0.5-mL and 1-mL aliquots of Matrigel to prevent multiple freeze-thawing. In addition, it is useful to purchase large batches of Matrigel at one time to ensure sufficient quantities of the same batch for many experiments. A note of the Matrigel batch number should be kept along with laboratory notes in case of discrepancies occurring between different batches.
11. Explant cultures consistently grow better than freshly isolated single epithelia cell preparations. If using frozen stocks of epithelia, these should be pregrown like the explant cultures in a tissue culture flask for 5–14 d, until a healthy growth is observed in coculture.
12. It is best to use small volumes of Matrigel solutions (less than 5 mL) as this prevents the gel setting prematurely within either the plates, syringe, or tubes.
13. Analysis of spheroids is most informative between weeks one and two. The morphology of the epithelia is most accurate during this period, as over time, the spheroids age and the epithelia lose their columnar appearance becoming less polarized and adopt a more cuboidal/flattened appearance. Spheroids have been grown for 2–3 mo, after which time the cellular structures start to breakdown the Matrigel, and thus would not be useful beyond such time points.

References

1. Barcellos-Hoff, M. H., Aggeler, J., Ram, T. G., and Bissell, M. J (1989) Functional differentiation and alveolar morphogenesis of primary mammary cultures on reconstituted basement membrane. *Development* **105,** 223–235.

2. Webber, M. M., Bello, D., Kleinman, H. K., and Hoffman, M. P. (1997) Acinar differentiation by non-malignant immortalized human prostatic epithelial cells and its loss by malignant cells. *Carcinogenesis* **18,** 1225–1231.
3. Darcy, K. M., Zangani, D., Shea-Eaton, W., et al. (2000) Mammary fibroblasts stimulate growth, alveolar morphogenesis, and functional differentiation of normal rat mammary epithelial cells. *In Vitro Cell. Dev. Biol. Anim.***36,** 578–592.
4. Lang, S. H., Stark, M., Collins, A., Paul, A. B., Stower, M. J., and Maitland, N. J. (2001) Experimental prostate epithelial morphogenesis in response to stroma and three-dimensional Matrigel culture. *Cell Growth Differ.* **12,** 631–640.
5. Lang, S. H., Sharrard, R. M., Stark, M., Villette, J. M., and Maitland, N. J. (2001) Prostate epithelial cell lines form spheroids with evidence of glandular differentiation in three-dimensional Matrigel cultures. *Br. J. Cancer* **85,** 590–599.
6. Lang, S. H., Stower, M. J., and Maitland, N. J. (2000). In vitro modelling of epithelial and stromal interactions in non-malignant and malignant prostates. *Br. J. Cancer.* **82,** 990–997.
7. Allen, T. D and de Wynter, E. A. (1993) Visualization of haemopoietic and stromal cell types and their interactions in bone marrow culture. In: *Haemopoiesis. A Practical Approach* (Testa, N.G. and Molineux, G., eds.) IRL Press, Oxford, UK, pp. 201–218.

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Coculture of Ovarian Cells Using Porous Tissue Culture Inserts

Katherine E. Snow and Simon P. Langdon

1. Introduction

Interactions between epithelial and stromal cells are critical to the development and maintenance of tissue development. Some of these interactions can be modeled using coculture systems. Published studies have described experiments in which ovarian carcinoma cells were cultured separately from fibroblasts but could interact ***(1,2)*** and also where the two populations have been mixed and cultured together ***(3,4)***. These studies have investigated the paracrine regulation that carcinoma cells can exert on tenascin secretion from fibroblast cells ***(1)*** and the effects that endothelins secreted by carcinoma cells can have on both the carcinoma and fibroblast cells ***(2)***. Ovarian cancer cells have been shown to regulate the production of the matrix metalloprotease MMP-9 in monocytes ***(3)*** and enhance release of MMP-2 from fibroblasts ***(4)***. The cells can be cocultured as a mixture of two (or more) populations or kept physically separate by the use of special porous tissue culture inserts. These inserts provide a means of investigating contact-independent modulation of cell behavior. The inserts have a high density of pores to allow diffusion of soluble factors.

The method described here involves the coculture of ovarian cancer cell lines with primary ovarian fibroblasts cultured from ascitic fluid.

2. Materials

1. Cell culture medium: Dulbecco's modified Eagle's medium (DMEM) supplemented with L-glutamine (2×10^{-3} *M*), 10% fetal calf serum (FCS), 100 U/mL penicillin and streptomycin (100 µg/mL).
2. Phosphate buffered saline (PBS).
3. Trypsin/EDTA.
4. For serum-free conditions: DMEM plus HITS (10 n*M* hydrocortisone, 5 µg/mL insulin, 10 µg/mL transferrin, 30 n*M* sodium selenite).
5. Tissue culture plastics: porous tissue culture inserts, 24-well trays, tissue culture flasks (*see* **Note 1**).

From: *Methods in Molecular Medicine, vol. 88: Cancer Cell Culture: Methods and Protocols*
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6. Monoclonal antibodies: 5B5 (Dako) targets the β subunit of prolyl-4-hydroxylase and the disulphide isomerase and reacts positively with fibroblasts. E29 (Dako) stains epithelial cells while 2B11 (Dako) targets leukocyte common antigen (CD45).

3. Methods

Carry out all culture experiments under sterile conditions in an appropriate tissue culture facility. Described following is a method for combining established ovarian cancer cell lines with primary ovarian fibroblast cells.

3.1. Establishment of Primary Fibroblast Cells

The primary fibroblast cell lines are initiated from ascitic cells obtained from patients with primary ovarian cancer.

1. Transfer freshly obtained ascitic fluid drained from the peritoneum of an ovarian cancer patient at the time of paracentesis or staging laparotomy to a sterile environment, for example, a class II hood (*see* **Note 2**).
2. Centrifuge the fluid for 20 min at 3000*g* and 4°C to produce a cell pellet. Remove the fluid and wash the cells in PBS. Repeat the centrifugation step to pellet the cells.
3. Use the cells immediately or store them in liquid nitrogen (*see* Chapter 2) until required.
4. Rapidly thaw cryopreserved cells at 37°C and wash twice with cell culture media (centrifuging the cell pellet by using a 5-min spin at 600*g*).
5. Resuspend the cell pellet in 10 mL of cell culture medium supplemented with 10% FCS and seed into 25-cm^2 flasks.
6. Allow cells to adhere for 2–4 h; in this time period the fibroblasts will rapidly adhere to the plastic substrate while few if any of the carcinoma cells will attach. Remove media containing unattached cells, leaving a predominantly fibroblast population.
7. If primary cell lines are used it is essential that they are well characterized. This can be done by examining the morphology of the cells and staining with appropriate antibodies to check for specific contamination by other cell types. The 5B5 antibody will stain fibroblasts positively, while contamination with epithelial cells can be detected by the E29 antibody and leukocytes by the 2B11 antibody.

3.2. Maintenance of Cells and Cell Lines

Both the established ovarian epithelial cancer cells and the primary fibroblast lines are cultured at 37°C in an atmosphere of 5% CO_2/95% air. Carry out regular passaging of cells during routine culture to ensure cells remain at optimal viable density. It is essential that both populations of cells are growing in the same media. Grow cells to 70% confluence before beginning a coculture experiment.

3.3. Coculture Using Porous Well Inserts

For coculture experiments, the fibroblast and epithelial populations are initially cultured separately (*see* **Fig. 1**).

1. Trypsinize and count fibroblasts. Suspend in DMEM containing 10% (FCS).
2. Place fibroblasts into the wells of 24-well plates at an appropriate cell density for the length of time to be cultured. For growth experiments over a 5 day (d) duration use 5×10^4 cells/well and for more rapid assays, such as measurement of protein secretion over 48 h, use 2×10^5 cells/well.

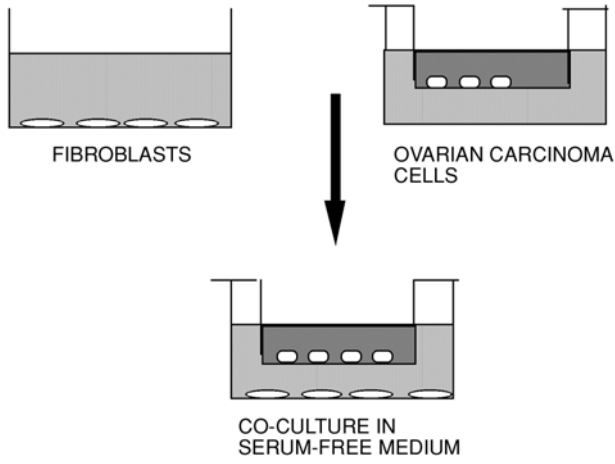

Fig. 1. Coculture system used. Fibroblast cells are initially cultured in the wells and ovarian cancer cells within inserts in serum-containing medium. Inserts are then placed into the wells containing cells for coculture and either serum-free or serum-containing conditions can be used.

3. Remove the insert from its sterile packaging using forceps, taking care not to contaminate the insert or pierce its membrane. Inserts have a variety of pore sizes. Sizes of 0.4 and 3.0 µm are sufficiently small to prevent cell transfer.
4. Plate ovarian epithelial cancer cell lines into the inserts (2.5×10^4 cells/insert for 5-d culture and 2×10^5 cells/insert for 48-h culture), and place into separate wells containing tissue culture media.
5. Culture the two populations separately for 24 h in media containing FCS to allow attachment.
6. After 24 h, wash the cells to remove any trace of serum if serum-free conditions are required. For example, in experiments exploring the production and secretion of proteins or the effects of paracrine regulation of growth, it is desirable to work in serum-free conditions. Medium supplemented with HITS replaces the serum-containing medium for 12–16 h.
7. Carefully transfer the inserts into the wells containing the fibroblast cells and add fresh media to the well and the insert. The wells should contain a volume of 500 µL and the inserts contain 300 µL (*see* **Note 3**).
8. To measure growth, coculture the cells for periods of time up to 5 d before trypsinizing and counting in a Coulter counter or hemocytometer. To measure protein secretion, collect media from the wells after 48 h and assay by specific ELISA (*see* **Note 4**).

4. Notes

1. Several suppliers including Nunc and Costar make Transwell inserts.
2. Treat all ascitic samples with care and carry out all sterile procedures within class II containment facilities.
3. Add specific inhibitors to the culture system in order to block possible interactions, thereby identifying the paracrine factors causing the effects seen.

4. This system of culture is suitable for investigating the effects of soluble factors on numerous aspects of cell behavior and function. RNA can be prepared from either population of cells to investigate the levels of mRNA for specific genes of interest by PCR. Protein lysates can be prepared for use in Western blotting experiments or ELISA.

References

1. Wilson, K. E., Bartlett, J. M., Miller, E. P., Mullen, P., Miller, W. R. and Langdon, S. P. (1999) Regulation and function of the extracellular matrix protein tenascin-C in ovarian cancer cell lines. *Br. J. Cancer* **80,** 685–692.
2. Moraitis, S., Miller, W. R., Smyth, J. F., and Langdon, S. P. (1999) Paracrine regulation of ovarian cancer by endothelin. *Eur. J. Cancer* **35,** 1381–1387.
3. Leber, T. M. and Balkwill, F. R. (1998) Regulation of monocyte MMP-9 production by TNF-alpha and a tumour-derived soluble factor (MMPSF). *Br. J. Cancer* **78,** 724–732.
4. Boyd, R. S. and Balkwill, F. R. (1999) MMP-2 release and activation in ovarian carcinoma: the role of fibroblasts. *Br. J. Cancer* **80,** 315–321.

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Cell Culture Contamination

An Overview

Simon P. Langdon

1. Introduction

For the cell culturist, two types of contamination require careful monitoring and constant vigilance: the contamination of cell cultures with microbiological organisms and the contamination of one cell line with another. Both forms of contamination are extremely prevalent and cannot be underestimated. Neither type can be eliminated, only controlled and managed to minimize the possibility of occurrence. Contamination consequences can range from minor inconvenience (a flask of cells becoming contaminated with bacteria) to a major disaster (published results that may be invalid owing to cross-contamination of one cell line with another). Other types of contaminants, such as chemical contamination, may also cause problems (e.g., deposits of disinfectants or detergents on glassware; residues, impurities, and toxins in water, media or sera), but the common recurring problems are likely to be biological in origin.

2. Microbial Contamination

The major microbial contaminants in cell culture are mycoplasma, bacteria, fungi, yeasts, and viruses (*see* **Table 1**). Sources of contamination include the cells themselves, (e.g., for viruses and mycoplasma), the media or serum, poor aseptic technique, and airborne contamination. Even when excellent aseptic technique is in place, it is essential to monitor and test for contamination. In general, bacteria, yeasts, and fungi are easy to detect while mycoplasma and viruses are more difficult (**Table 2**). In the absence of antibiotics, they will grow rapidly; however, if antibiotics are routinely used, low level infections may develop that may be more difficult to observe.

2.1. Mycoplasma

Mycoplasmas are the smallest prokaryotes (approx 0.3–0.8 μm diameter) and their presence is generally not obvious in cultures either macroscopically or microscopi-

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Table 1
Tissue Culture Contaminants

Contaminant	General indications	Microscopic appearance	Common source
Bacteria	pH change Turbidity/cloudiness Precipitation	Fine granules	Cell culturist Water bath
Yeast	Cloudiness PH change	Oval organisms Budding/chains	Airborne
Fungus	Spores Furry growths PH change	Thin spores	Airborne
Mycoplasma	Often covert Poor cell adherence Reduced growth	—	Cell culturist Other cell lines
Virus	Sometimes cytopathic	—	Serum Cell lines

cally. Screening in the United States by the Food and Drug Administration for over 30 yr indicated that of 20,000 cell cultures examined, over 3000 (15%) were contaminated with mycoplasma ***(1)***. In studies conducted in Japan and Argentina, incidence rates of mycoplasma contamination of 80% and 65% respectively have been reported ***(1)***. It is likely that laboratories that do not routinely screen and eliminate sources of mycoplasma will be rife with the organism and may have all cell lines contaminated. Contaminating organisms include members of both the Mycoplasmataceae (Mycoplasma) and Acholeplasmataceae (Acholeplasma) families, and although at least 20 distinct species have been isolated from continuous cell lines, the most common species include *M. fermentans* (human), *M. orale* (human), *M. arginini* (bovine), and *A. laidlawii* ***(1)***. The major sources of mycoplasma contamination are incoming cell cultures or cell lines, sera, or human contact (poor aseptic technique).The level of contamination can reach densities of 10^6 to 10^8 organisms/mL. The consequences of mycoplasma contamination on any individual cell line or culture are often difficult to predict but can include effects on growth rates ***(2)***, chromosome aberrations ***(1,3)***, nucleic acid and amino acid synthesis, and metabolism ***(4–6)***, and membrane alterations ***(7)***. Their presence can also modulate the effects of viruses ***(1)*** and influence results of techniques such as the MTT assay ***(8)***.

Many methods of detecting mycoplasma contamination have been described, and each has advantages and disadvantages with respect to cost, time, reliability, specificity, and sensitivity. These include culture methods, DNA staining techniques, immunological methods, transmission electron microscopy, nucleic acid hybridization, and polymerase chain reaction (PCR) identification. Traditionally, the two most widely used methods of detection involve staining with Hoescht 33258 ***(6)*** and microbiological culture. The Hoeschst 33258 dye binds to DNA producing fluorescence that can

Table 2
Methods to Identify Contaminants

Mycoplasma	Bacteria/fungi	Viruses
PCR	Microbiological culture	PCR
DNA fluorescence		Transmission electron microscopy
Microbiological culture		Immunodetection
Transmission electron microscopy		Hemadsorption
Nucleic acid hybridization		
^{3}H-Thymidine		

be observed by fluorescence microscopy. Mycoplasma-infected cultures will demonstrate extranuclear staining. An indicator cell line is often used, which allows for a level of standardization. Culture techniques are more sensitive but can take several weeks and require more expertise. Furthermore, certain mycoplasmas do not grow readily and may be overlooked. With the widespread use of PCR technology in most cell and molecular biology laboratories, a very sensitive, specific, and rapid option has now become available and an example of this method is described in Chapter 31. The target choice for primer design is the 16S rRNA. Use of size determination of PCR fragments, together with restriction enzyme analysis, can allow detection of species-specific sequences.

The elimination of mycoplasma is not straightforward and several approaches using antibiotics are described in detail in Chapter 32. This may be the only option for rescuing unique samples that have become infected. For cultures that cannot be returned to previous or frozen stocks, this is the normal course of action. In addition to applying appropriate aseptic technique, a number of simple precautions will help minimize mycoplasma contamination. Cells should be tested for mycoplasma at frequent intervals if they are to be maintained in long-term culture. Ideally cell lines should only be cultured for a limited number of passages before returning to frozen stocks. Cell lines imported into a laboratory should be quarantined until verified mycoplasma-negative.

2.2. Bacteria

Bacteria are another widespread cell culture contaminant. These are usually introduced through poor aseptic technique and are often first recognized in culture medium. A downward shift in pH (yellow with phenol red as indicator) with aerobic bacteria and an increased turbidity or cloudiness will be evident. Microscopic inspection indicates many fine granules if there is gross contamination (**Fig. 1A**). This should be distinguished from cell debris, media, or sera precipitates. Under high magnification, several principal forms of bacteria can be distinguished including cocci (round-shaped), bacilli (rod-shaped), and spirilla (spiral-shaped). Movement may also be observed. To detect lesser levels of contamination, media from the suspect cells is cultured and observed for the growth of colonies in antibiotic-free media for 2–3 wk.

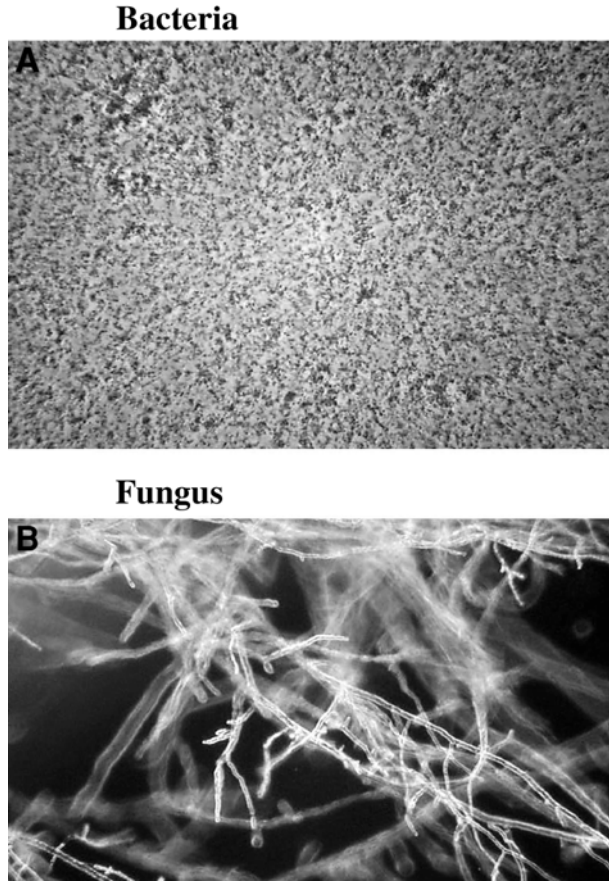

Fig. 1. Microscopic appearance of bacteria and fungus, **(A)** bacteria, **(B)** fungus.

Good aseptic technique should avoid contamination with bacteria. One of the major sources of bacterial contamination is the water bath used to warm media and sera before use.

Once recognized, contaminated flasks should be discarded immediately and new stocks used to regenerate the cell culture. If the culture is unique and irreplaceable, then antibiotics may be able to eliminate the bacteria. It may be necessary to try several antibiotics to find an effective one, and when one has been identified, the culture should be maintained in the presence of antibiotic for a number of subcultures. Eradication of the contaminant should be confirmed by testing cell-conditioned media antibiotic-free media. There is divergence of opinion as to whether antibiotics should be used routinely in cell culture. If they are added, they will reduce the incidence of bacterial infection but resistant organisms may appear. Penicillin and streptomycin are widely used in combination.

2.3. Fungi

Fungi (molds) routinely appear as a contaminant either as thin filamentous mycelia or as clumps of spores (**Fig. 1B**). In their advanced stages, they can take over a culture as furry growths that can vary in color from white to black. Fungi are ubiquitous in the environment and generally enter cultures via the air. Seasonal changes, air-conditioning, and heating can all have a major effect on levels in the atmosphere. Antimycotics such as amphotericin B (Fungizone) and mycostatin (Nystatin) can be used to treat essential cultures if the fungus is not too advanced.

2.4. Yeast

Yeasts characteristically appear as small oval shaped organisms that are substantially smaller than mammalian cells. They can be seen "budding" from other yeast particles in short chains. Eventually these chains will form multibranches. Yeasts generally infect cell culture materials by airborne routes and are transferred easily by contact. Mycostatin (Nystatin) can be used to treat essential cultures in early stages of infection.

2.5. Viruses

Viruses are the most difficult contaminants to detect, but unless they are cytopathic may have little effects on their host cells. Viral contaminants have been detected in bovine serum intended for use in tissue culture and in cell lines including bovine viral diarrhea virus (BVDV), bovine herpes virus (BHV), bovine parainfluenza-3, and epizootic hemorrhagic disease ***(9)***. Because BVDV was widely present in serum, it may be present in many cell lines unless the cultures have been grown in rigidly tested sera or sera of nonbovine origin. A variety of methods have been used to identify viruses, including identification of cytopathic effects in susceptible cells, hemadsorption, electron microscopy, and immunofluorescence, but more recently PCR-based techniques have been developed to detect these contaminants ***(9)***. Some ATCC human cell lines are known to contain Epstein-Barr virus, human T cell leukemia virus, and hepatitis virus, and this information is available in their catalogue and data sheets. Their published recommendation is to use caution when handling any human cell line and to regard it with the same level of biosafety caution as a line known to carry human immunodeficiency virus.

The possible hazard from bovine spongiform encephalopathy (BSE) has become a concern over the last few years and although the risk for tissue culture workers from fetal calf serum (FCS) contamination may be trivial, some regulatory authorities now insist that FCS used for culture should be obtained from countries where BSE has not been diagnosed. Therefore, countries such as the USA will only allow import of cells cultured in serum from BSE-free areas.

2.6. Sources of Contamination

There are many different entry points for contaminants into the culture system. The first is via tissue culture materials. Sterilize all plastics and glassware prior to use and although many plastics are sterilized commercially, care should be taken when packages are unsealed. Similarly, tissue culture liquids, such as media, sera, and washing

solutions should be filter sterilized. When bottles are opened, frequent observation and monitoring is required to check that the liquid is not infected. All equipment entering the tissue culture cabinet should be sprayed with 70% ethanol or an alternative disinfectant (e.g., dilute hypochlorite or 1% benzalconium chloride). One major source of contamination are the still damp external surfaces of bottles taken from water. These should be sprayed well with ethanol, as should tissue culture flasks that have been used for some period of time, because they are effective at attracting fungi to their external surfaces. A dirty lab coat is another excellent source of contamination. Working within the sterile environment of the cabinet, care should be taken to avoid constant or unnecessary moving in and out of the hood. Aerosols should be minimized as far as possible. Airborne contamination may also increase seasonally, for example in spring and summer when pollen counts can be high and during construction work when there may be an increased amount of dirt and dust. When spillages occur, they should be cleaned immediately and incubators and cabinets should be cleaned thoroughly at regular intervals. Minimizing personnel traffic in cell culture rooms and reducing the opening and closing of incubators reduces the probability of spreading microorganisms. Finally, one of the common means of transferring infections is via the transfer of cell lines into a laboratory from another location. Cell lines new to a laboratory should be quarantined and treated with caution until proven free of microorganisms, unless they have been confirmed free prior to transfer (for example, if obtained from a cell bank).

2.6. Prevention of Microbial Contamination and the Use of Antibiotics

The cornerstone to preventing microbial contamination is the practice of aseptic technique. The use of antibiotics in routine culture varies but is generally discouraged for a number of reasons. First, good practice should make their addition unnecessary. Although antibiotics may suppress infection, they may not eliminate the infection and may permit resistant organisms to develop. Poor technique should not rely on assistance from antibiotic use, and it is important to detect infection as early as possible. Evidence to support this view was obtained in one analysis where 72% of cultures grown continuously in antibiotics were shown to be mycoplasma-positive while only 7% grown in the absence of antibiotics were infected ***(1)***. Secondly, the antibiotics may influence the biochemistry of the cultured cells, which may influence the experimental endpoint. However, the short-term use of antibiotics may have useful strategic value in a number of different situations. These include initial culture of primary samples and also curing unique and essential contaminated samples. The latter will be dependent on the extent of contamination and is only feasible if still at a relatively early stage. In most instances, contaminated culture should be autoclaved.

2.7. General Procedure for Decontaminating Infected Cultures

For cultures that are considered unique and become infected, it may be feasible to disinfect and eliminate the infection. Having established the type of infection (bacterial, fungal, yeast, or mycoplasmal), a range of antibiotics are available to treat the problem (*see* **Table 3**). As a higher concentration of antibiotic is likely to be more effective, it can be worthwhile evaluating the antibiotic over a range of concentrations

Table 3
Commonly Used Antibiotic and Antimycotic Treatments

		Responsive microorganisms			
Reagent	Working Conc	Bacteria	Yeast	Fungus	Mycoplasma
Amphotericin B	2.5 mg/L	+	–	+	–
Ampicillin	100 mg/L	+	–	–	–
Dihydrostreptomycin	100 mg/L	+	–	–	–
Erythromycin	100 mg/L	+	–	–	–
Gentamycin sulfate	50 mg/L	+	–	–	+
Kanamycin sulfate	100 mg/L	+	–	–	+
Neomycin sulfate	50 mg/L	+	–	–	–
Nystatin	100,000 U/L	–	+	+	–
Penicillin G	100,000 U/L	+	–	–	–
Polymyxin B sulfate	50 mg/L	+	–	–	–
Streptomycin sulfate	100 mg/L	+	–	–	–
Tetracycline hydrochloride	10 mg/L	+	–	–	–
Tylosin tartrate	10 mg/L	+	–	–	+

to establish a maximum tolerated concentration, i.e., a concentration that is just subtoxic. Having established this concentration, culture infected cells for several (3–4) passages in antibiotic to eliminate infection.

Cells should be cultured in antibiotic-free conditions for several passages and then tested for the presence of infection. Repeat this process if the infection is not fully eliminated.

3. Cell Line Cross-Contamination

The use of multiple cell lines in any laboratory leads to the possibility of contamination of one cell line with another. The cross-contamination of cultures has plagued many researchers, often leading to mistaken results, retractions of results, cover-ups, and some out-and-out falsification of data and results following inadvertent use of the wrong cells ***(10)***. The realization that this was not a trivial problem came initially in the 1960s and 1970s with the observation that many cell lines had become cross-contaminated with HeLa cells, the first established human cell line. This had occurred to such a degree that a number of cell lines, such as KB, Hep-2, and INT407 were so cross-contaminated that it was unclear if the original cell line existed any longer or had been replaced completely by HeLa. In 1967, Gartler, using isoenzyme analysis reported that 20 human cell lines had been contaminated by HeLa cells ***(11)***. Nelson-Rees and colleagues systematically investigated large numbers of cell lines and demonstrated that widespread contamination of cell lines with HeLa had

occurred ***(12–14)***. In 1977, this group demonstrated that of a series of 253 cultures examined, 21 were of the wrong species and 15 were contaminated with HeLa ***(15)***. Another contemporary study investigating 246 cultures showed that 14% of cultures were contaminated with another species while 25% were of HeLa origin ***(16)***. In 1984, a study by Hukku showed that the situation had not improved and testing of 275 cultures received by that laboratory for analysis indicated that 36% of cultures were cross-contaminated: 25% by cells of another species and 11% by another human line ***(17)***. In a study published in 1999 by investigators from the German Collection of Microorganisms and Cell Cultures (DSMZ), analysis of 252 human cell lines indicated cross-contamination in 45 (18%) of cell lines ***(18)***. This recent report indicates that complacency about this issue still exists despite the ongoing warnings of the dangers ***(19,20)***.

Most cross-contamination of this nature is the result of poor tissue culture technique and the culture of multiple cell lines at one time. In addition to accidental mixing of cell lines, the use of pipets or media bottles for multiple cell lines and the creation of aerosols can allow contamination to occur. As with microbial contamination, microscopic monitoring of cultures is likely to be the first indication of cross-contamination, and the observation of mixed morphologies or changed growth rates should alert the culturist to potential problems. A number of recommendations to minimize the possibility of cross-contamination ***(19,21)*** include:

1. When new cell lines are derived, archive representative samples of the original tissue, cells, or DNA for later authentication of stocks.
2. Disseminate cell lines from authenticated sources, such as cell collections if possible, rather than transfer them frequently through multiple laboratories.
3. Cell lines transferred between laboratories should be confirmed free of mycoplasma, and full information should accompany the cell line attesting to its authenticity.
4. Rapidly expand new cell lines to produce frozen stocks.
5. Treat changes in cell morphology and characteristics with suspicion.
6. Only one cell line should be used at one time when working in a microbiological safety cabinet. After removal of the line, disinfect the cabinet and run for several minutes before the introduction of another line.
7. Dedicate bottles of media for use with individual cell lines.
8. The formation of aerosols should be kept to a minimum.
9. After 3 months or 10 passages, discontinue the working cell line and obtain a new stock of cells from liquid nitrogen storage.
10. All culture vessels should be carefully and correctly labelled.

Following these recommendations should minimize the risk of contamination.

References

1. Rottern, S. and Barile, M. F. (1993) Beware of mycoplasmas. *Trends Biotechnol.* **11,** 143–151.
2. McGarrity, G. J., Phillips, D., and Vaidya, A. (1980) Mycoplasma infection of lymphocyte cultures: infection with *M. salivarium*. *In vitro* **16,** 346–356.
3. Aula, P. and Nichols, W. W. (1967) The cytogenetic effects of mycoplasma in human leucocyte cultures. *J. Cell Physiol.* **70,** 281–290.

4. Perez A. G., Kim, J. H., Gelbard, A. S. , and Djordjevic, B. (1972) Altered incorporation of nucleic acid precursors by mycoplasma-infected mammalian cells in culture. *Exp. Cell. Res.* **70,** 301–310.
5. Levine, E. M., Thomas, L., McGregor, D., Hayflick, L., and Eagle, M. (1968) Altered nucleic acid metabolism in human cell cultures infected with mycoplasma. *Proc. Natl. Acad. Sci. USA* **60,** 583–589.
6. Stanbridge, E. J., Hayflick, L., and Perkins, F. T. (1971) Modification of amino acid concentrations induced by mycoplasmas in cell culture medium. *Nature* **232,** 242–244.
7. Wise, K. S., Cassell, G. H., and Action, R. T. (1978). Selective association of murine T lymphoblastoid cell surface alloantigens with mycoplasma hyorhinis. *Proc. Natl. Acad. Sci. USA* **75,** 4479–4483.
8. Denecke, J., Becker, K., Jurgens, H., Gross, R., and Wolff, J. E. (1999) Falsification of tetrazolium dye (MTT) based cytotoxicity assay results due to mycoplasma contamination of cell cultures. *Anticancer Res.* **19,** 1245–1248.
9. Kappeler, A., Lutz-Wallace, C., Sapp, T., and Sidhu, M. (1996) Detection of bovine contamination in fetal bovine sera and modified live viral vaccine using polymerase chain reaction. *Biologicals* **24,** 131–135.
10. Nelson-Rees, W. A. (2001) Responsibility for truth in research. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **356,** 849–851 .
11. Gartler, S. M. (1968) Apparent HeLa contamination of human heterodiploid cell lines. *Nature* **217,** 750–751.
12. Nelson-Rees, W. A., Flandermeyer, R. R., and Hawthorne P. K. (1974) Banded marker chromosomes as indicators of intraspecies cellular contamination. *Science* **184,** 1093–1096.
13. Nelson-Rees, W. A. and Flandermeyer, R. R. (1976) HeLa cultures defined. *Science* **191,** 96–98.
14. Nelson-Rees, W. A., Daniels, D. W., and Flandermeyer, R. R. (1981) Cross-contamination of cells in culture. *Science* **212,** 446–452.
15. Nelson-Rees, W. A., and Flandermeyer, R. R. (1977) Inter- and intraspecies contamination of human breast tumor cell lines HBC and BrCa5 and other cell cultures HeLa cultures defined. *Science* **195,** 1343–1344.
16. Stulberg, C. S., Peterson, W. D., and Simpson, W. F. (1976) Identification of cells in culture. *Am. J. Hematol.* **1,** 237–242.
17. Hukku, B., Halton, D. M., Mally, M., and Peterson, W. D. (1984) Cell characterization by the use of multiple genetic markers. *Adv. Exp. Med. Biol.* **172,** 13–31.
18. MacLeod, R. A. F., Dirks, W. G., Matsuo, Y., Kaufmann, M., Milch, H., and Drexler, H. G. (1999) Widespread intraspecies cross-contamination of human tumor cell lines arising at source. *Int. J. Cancer* **83,** 555–563.
19. Stacey, G. N., Masters, J. R. W., Hay, R. J., Drexler, H. G., MacLeod, R. A. F., and Freshney, R. I. (2000) Cell contamination leads to inaccurate data: we must take action now. *Nature* **403,** 356.
20. MacLeod, R. A. F., Dirks, W. G., and Drexler, H. G. (2002) Persistent use of misidentified cell lines and its prevention. *Genes Chromosomes Cancer* **33,** 103–105.
21. UKCCCR (2000). UKCCCR Guidelines for the use of cell lines in cancer research. *Brit. J. Cancer* **82,** 1495–1509.

31

Detecting Mycoplasma Contamination in Cell Cultures by Polymerase Chain Reaction

Cord C. Uphoff and Hans G. Drexler

1. Introduction

1.1. Mycoplasma Contaminations of Cell Lines

Contamination of cell cultures with bacteria, fungi, and yeasts represents a major problem in cell culture. Whereas these microorganisms are easily detected during routine cell culture by the turbidity of the culture and observation under the inverted microscope, one class of bacteria regularly evades detection. These bacteria belong to the class of Mollicutes, commonly known as mycoplasma. Mycoplasma may persist undetected in cell cultures for a long time without visibly affecting the culture. Nevertheless, mycoplasma can cause extensive alterations in the cell cultures ***(1)***.

The frequency of contamination is about 10–30% with pronounced variations in series regarding the laboratory, type of cell culture examined, and the origin of the culture, for example. The infecting mycoplasmas are limited to a few species of the genera *Mycoplasma* and *Acholeplasma* with human, swine, and bovine as predominant natural hosts. The ubiquitous use of cell cultures and their transfer from one laboratory to another has lead to widespread dissemination of such infections. Hence, adequate detection methods need to be established and frequently employed in every laboratory applying cell cultures. Every incoming cell culture should be kept in quarantine until mycoplasma detection assays are completed and the infection status is determined. Positive cultures should either be discarded and replaced by clean cultures or cured with specific antibiotics (*see* Chapter 32). Only clean cultures should be used for research experiments and for the production of biologically active pharmaceuticals. Additionally, stringent rules for the prevention of further mycoplasma contamination of cell cultures should be followed ***(2)***.

1.2. Mycoplasma Detection

Polymerase chain reaction (PCR) provides a sensitive and specific option for the direct detection of mycoplasma in cell cultures. PCR is useful for the routine screen-

From: *Methods in Molecular Medicine, vol. 88: Cancer Cell Culture: Methods and Protocols*
Edited by: S. P. Langdon © Humana Press Inc., Totowa, NJ

ing of cell lines newly introduced into the laboratory, for initial analysis of primary cell cultures, and for the periodical monitoring of growing cell cultures. The advantages of the PCR assay are sensitivity, specificity, speed, cost efficiency, and the potential to screen a large number of samples. Furthermore, an objective result is obtained that is much easier to interpret than most other conventional assays as long as the appropriate control reactions are included in the PCR assay.

Some investigators have described the use of primer sequences that are complementary to highly conserved regions of the eubacterial 16S rRNA in order to detect any bacterial contamination, either by PCR or reverse transcription PCR (RT-PCR). Here, we describe the application of a mixture of oligonucleotides as primers for the visualization of mycoplasma-specific sequences ***(3)***. The advantage of this specific approach is that common airborne bacterial contaminations or contaminations that may be present in solutions—those used for washing steps, DNA extraction, and the PCR reaction—or may be introduced by other materials will not be detected and therefore not lead to false-positive results. Nevertheless, major emphasis should be placed on the preparation of the template DNA, the amplification of positive and negative control reactions, and the observance of general rules for the preparation of PCR reactions.

To exclude inhibitors of the Taq polymerase that may be present in crude cell culture solutions (which sometimes cannot be eliminated by serial dilutions), it is important that the DNA of the samples is extracted and purified. This can be achieved by conventional phenol-chloroform extraction, column extraction, or by DNA binding to matrix. To control the integrity of the PCR reactions and the preceding template preparation steps, it is also essential to perform the appropriate control reactions; including internal, positive, and negative control reactions. The internal control represents DNA containing the same primer sequences but with an additional stretch of interspersed nucleotides, resulting in a gel band of different size than the expected amplicon of the contaminant. This internal control should be added to the PCR reaction in a limiting dilution to recognize any inhibiting components. The following protocols detail an established PCR method for monitoring potential mycoplasma contaminations in any laboratory ***(4)***.

2. Materials

2.1. Sample Collection and DNA Extraction Buffers

1. Phosphate-buffered saline (PBS): 140 m*M* NaCl, 27 m*M* KCl, 7.2 m*M* Na_2HPO_4, 14.7 m*M* KH_2PO_4, pH 7.2; autoclave.
2. 50X TAE buffer: 2 *M* Tris-base, 5.71% glacial acetic acetate (v/v), 100 m*M* EDTA, pH 8.0.
3. Wizard DNA Clean-Up System (Promega, Madison, WI, USA).
4. 80% Isopropanol (v/v).
5. Double-distilled water prewarmed to 80°C.
6. Disposable 2-mL syringes.

2.2. PCR Reaction

1. GeneAmp 9600 thermal cycler (Applied Biosystems, Weiterstadt, Germany).
2. Taq DNA polymerase (Qiagen, Hilden, Germany).

3. 6X Loading buffer: 0.09% bromophenol blue (w/v), 0.09% xylene cyanol FF (w/v), 60% glycerol (v/v), 60 m*M* EDTA.
4. Primers (any supplier) (*see* **Note 1**):

5' primers:	3' primers:
cgc ctg agt agt acg ttc gc	gcg gtg tgt aca aga ccc ga
w	r
cgc ctg agt agt acg tac gc	gcg gtg tgt aca aaa ccc ga
tgc ctg ggt agt aca ttc gc	gcg gtg tgt aca aac ccc ga
r	
tgc ctg agt agt aca ttc gc	
cgc ctg agt agt atg ctc gc	
cgc ctg ggt agt aca ttc gc	

   Stock solution: 100 µM in dH_2O, stored frozen at –20°C.
   Working solutions: mix of forward primers at 5 µM each and mix of reverse primers at 5 µM each, placed in 25–50 µL aliquots, and stored frozen at –20°C.
5. Internal control DNA: may be obtained from the DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). A limiting dilution should be determined experimentally by performing a PCR with a dilution series of the internal control DNA.
6. Positive control DNA: any mycoplasma-positive sample prepared as described below, or may be obtained from the DSMZ.

3. Methods

3.1. Sample Collection and Preparation of DNA

1. Take the samples to be tested from at least 2–3-day-old cell cultures to ensure that the titer of the mycoplasmas is above the detection level of the assay. Take one milliliter of the supernatant of adherently growing cells or of cultures with settled suspension cells for the analysis. The samples do not need to be free of eukaryotic cells. On the contrary, some viable or dead cells in the sample are of advantage, because some mycoplasma species tend to adhere firmly to the cell membranes of the eukaryotic cells. Several mycoplasma species are also described to live intracellularly. Thus, it is also not necessary to centrifuge the sample to eliminate the eukaryotic cells. For collection of a series of samples over a period of time, the crude cell culture supernatants can be stored at 4°C for several days or frozen at –20°C for several weeks.
2. Centrifuge the cell culture suspension in an Eppendorf tube at 13,000*g* for 5 min. Remove the supernatant with a disposable pipet and discard. Resuspend the remaining pellet in 1 mL PBS by vortexing.
3. Centifuge the suspension again and wash one more time with PBS as described in **step 2**.
4. After the washing step, resuspend the pellet in 100 µL PBS by vortexing, and heat to 95°C for 15 min.
5. To isolate the DNA present in the solution, the commercially available Wizard DNA extraction kit from Promega is applied (*see* **Notes 2** and **3**). Add one milliliter of mixed DNA clean-up resin to the sample solution and mix by pipeting. Keep the solution at room temperature for a few minutes.
6. Fit the minicolumn to a syringe barrel after removing the plunger, transfer the solution into the syringe barrel, and insert into a 1.5-mL Eppendorf tube. Using the plunger, press the resin onto the minicolumn.

7. Detach the syringe from the minicolumn and remove the plunger. Discard the collected supernatant. Reattach the syringe and the minicolumn, insert into the empty tube, and fill the syringe with 2 mL 80% isopropanol for washing the bound DNA. Press the isopropanol through the minicolumn directly into a waste container.
8. Remove the syringe and centrifuge the minicolumn with the tube for 2 min at 13,000*g* to remove the isopropanol.
9. Transfer the minicolumn to a new 1.5-mL tube and add 50 µL of preheated water onto the minicolumn. Centrifuge for 20 seconds (s) at 13,000*g* to collect the eluate quantitatively. The DNA is stored at –20°C until the PCR reaction is performed.

3.2. PCR

The amplification procedure and the parameters described here are optimized for the application in 0.2-mL reaction tubes in an Applied Biosystems GeneAmp 9600 thermal cycler. An adjustment to any other equipment might be necessary (*see* **Note 4**). Amplified positive samples contain large amounts of target DNA for the next PCR run. Thus, established rules to avoid DNA carry over should be strictly followed:

1. Separate the DNA extraction area from the PCR set-up area and the gel run locations.
2. Store all reagents in small aliquots to provide a constant source of uncontaminated reagents.
3. Avoid reamplifications.
4. Reserve pipets, tips, and tubes for their use in the PCR only and irradiate the pipets frequently by UV-light.
5. Follow strictly the succession of the PCR set up in the protocol.
6. Wear gloves during the sample preparation and PCR set up.

It is also fundamental to integrate the appropriate control reactions, such as internal, positive, and negative control, and the water control reaction.

1. Prepare a premaster mix (calculated for 15 µL per reaction, plus 1 additional reaction) containing 1X PCR buffer (use the Taq DNA polymerase buffers recommended by the manufacturer) with 1.5 m*M* $MgCl_2$, 0.165 m*M* of each dNTP, 0.33 µ*M* of each oligonucleotide (*see* **Note 1**), fill up with dH_2O to 13 µL per reaction.
2. Transfer 13 µL of the premaster mix for the water control reaction to a 0.2-mL PCR reaction tube and add 2 µL dH_2O.
3. Prepare the Taq DNA polymerase mix (10 µL per reaction, plus 1 additional reaction) containing 1X PCR buffer and 1 U Taq polymerase per reaction.
4. Add 1 µL per PCR reaction (plus one additional reaction) of a limiting dilution of the internal control DNA (*see* **Note 5**) to the premaster mix and place 14 µL of the resulting master mix into the appropriate number of 0.2-mL PCR reaction tubes (*see* **Note 6**).
5. Store all reagents used for the preparation of the master mix and take out the samples of DNA to be tested and the positive control DNA. Do not handle the reagents and samples simultaneously. Add 1 µL of the DNA preparation to the reaction solutions.
6. Transfer the reaction mixtures without Taq polymerase to the thermal cycler and start one thermocycle with the following parameters:

   Cycle step 1: 7 min at 95°C
   Cycle step 2: 3 min at 72°C
   Cycle step 3: 2 min at 65°C
   Cycle step 4: 5 min at 72°C

   During cycle step 2, open the thermal lid and add 10 µL of the Taq polymerase mix to each tube to perform a hot start PCR. For many samples, the duration of this step can be

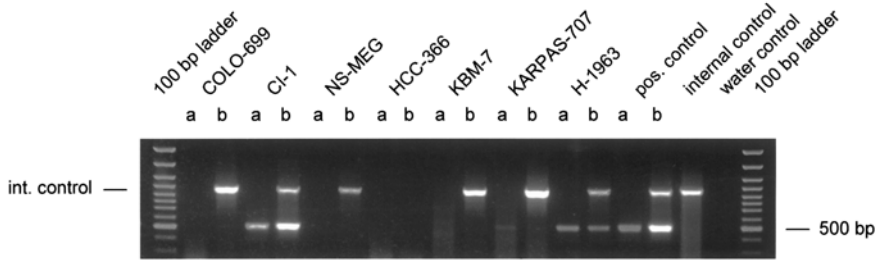

Fig. 1. PCR analysis of mycoplasma status in cell lines: a typical ethidium bromide-stained gel containing the reaction products following PCR amplification with the primer mix listed in **Subheading 2.2.** Products of about 510 bp were obtained; the differences in length reflect the sequence variation between different mycoplasma species. Shown are various examples of mycoplasma-negative and -positive cell lines. Two paired PCR reactions were performed: one PCR reaction contained an aliquot of the sample only *(a)* and the second reaction contained the sample under study plus the control DNA as internal standard *(b)*.

Cell lines COLO-699, NS-MEG, and KBM-7 are mycoplasma-negative; cell lines CI-1 and H-1963 are mycoplasma-positive. The analysis of cell line HCC-366 cannot be evaluated as the internal control was not amplified and is not visible. Additionally, the one single positive band did not appear. In this case the analysis needs to be repeated. KARPAS-707 post-sparfloxacin treatment shows a weak but distinctive band in the reaction without internal control and also needs to be repeated after a longer antibiotic-free period posttreatment, because the apparently low concentration might result from residual DNA of dead mycoplasmas.

prolonged. Open and close each reaction tube separately to prevent evaporation of the samples. Allow at least 30 s after closing the lid of the thermal cycler for equilibration of the temperature and removal of condensate from the lid before continuing to the next cycle step.

7. After this initial cycle, perform 32 thermal cycles with the following parameters:
   Cycle step 1: 4 s at 95°C
   Cycle step 2: 8 s at 65°C
   Cycle step 3: 16 s at 72°C plus 1 s of extension time during each cycle.
8. The reaction is finished by a final amplification step at 72°C for 10 min and the samples are then cooled down to room temperature.
9. Prepare a 1.3% agarose-TAE gel containing 0.3 µg of ethidium bromide per mL. Submerge the gel in 1X TAE and add 12 µL of the amplification product (10 µL reaction mix plus 2 µL of 6X loading buffer to each well) and run the gel at 10 V/cm.
10. Visualize the specific products on a UV screen and document the result.

3.3. Interpretation of the Results

Figure 1 shows a representative ethidium bromide-stained gel with samples that produced the results as described following. Ideally, all samples containing the internal control DNA show a band at 986 bp. This band might be more or less bright, but the band has to be visible if no other bands are amplified (*see* **Note 7**). The absence of this band indicates that the reaction was contaminated with inhibitors from the sample preparation. In this case, it is usually sufficient to repeat the PCR run with the same DNA solution as before. It is not necessary to collect a new sample.

If the second run shows no band for the internal control, the whole procedure has to be repeated.

Mycoplasma-positive samples show an additional band or only a single band at 502 to 520 bp, depending on the mycoplasma species. In the case of *Acholeplasma laidlawii* contamination and applying the DSMZ internal control DNA, a third band might be visible between the internal control band and the mycoplasma-specific band. This is formed by hybridization of the complementary sequences of the single-stranded long internal control DNA and the single-stranded short mycoplasma DNA form.

Contaminations of reagents with mycoplasma-specific DNA or PCR product are indicated by a band in the water control and/or in the negative control sample. Very weak mycoplasma-specific bands can sometimes occur after treatment of infected cell cultures with antimycoplasma reagents for the elimination of mycoplasma or when other antibiotics such as penicillin/streptomycin are applied routinely. In these cases the positive reaction might either be a result of residual DNA in the culture medium derived from dead mycoplasma cells, or from viable mycoplasma cells present at a very low titer. Therefore, special caution should be taken when cell cultures are tested that were treated with antibiotics. Prior to PCR testing, cell cultures should be cultured for at least 2–3 wk without antibiotics, or retested at frequent intervals.

3.4. Further Considerations

Although the method described is sufficient to detect mycoplasma contaminations, it might be of advantage to know the infecting mycoplasma species to determine the source of contamination. This PCR allows the identification of the mycoplasma species most commonly infecting cell cultures. In case of a contamination detected by PCR, the PCR has to be repeated in a 50 µL volume and without internal control to amplify the mycoplasma-specific PCR fragment. This DNA fragment is then digested in parallel reactions with the restriction endonucleases *Asp*I, *Hpa*II, and *Xba*I (8-µL PCR reaction mix plus 1 µL of the appropriate 10X restriction enzyme buffer plus 1 µL of the restriction enzyme). Depending on the restriction pattern (*see* **Table 1**), the species can be determined directly. Otherwise, two more digestions with the restriction enzymes *Hae*III and *Sfu*I have to follow for the exact identification of the species. Of course, this analysis allows only the determination of those mycoplasma species that most often (>97%) occur in cell cultures and is not suitable for the global identification of all types of mycoplasma species. Cell culture infections are commonly restricted to five or six mycoplasma species.

4. Notes

1. The primers can be designed using the degenerated code to incorporate two different nucleotides to form a mixture of two primers (**Subheading 2.2.**). When all oligonucleotides are mixed and prepared in aliquots for use in the PCR reaction, remember that the molarities are reduced by 50%. The primer solutions should be placed into small portions (25 µL aliquots) and stored frozen at –20°C to avoid multiple freeze-thawing cycles and to minimize contamination risks.
2. Any other DNA extraction and purification method that eliminates PCR inhibitors, should work as well. We tested normal phenol/chloroform extraction and ethanol precipitation,

Table 1
Restriction Fragment Patterns of the PCR Amplicons Digested With Various Restriction Enzymes

	*Asp*I	*Hpa*II	*Xba*I	*Hae*III	*Sfu*I
A. laidlawii	–	–	–	–	436/81
M. arginini	–	–	266/253	–	–
M. bovis	303/213	–	–	–	–
M. fermentans	–	357/111/48	–	356/160	–
M. hominis	–	–	263/253	336/180	–
M. hyorhinis	303/213	–	263/253	–	–
M. orale	–	288/230	266/252	–	–

The numbers represent the sizes of the restriction fragments of the PCR product which was digested with the respective restriction enzyme; –, not digestable.

High Pure PCR Template Preparation Kit from Roche (Mannheim, Germany), and Invisorb Spin DNA MicroKit III from Invitek (Berlin, Germany). Following these methods, the amplification of the mycoplasma sequences was similar to the one after the Wizard preparation when the DNA was dissolved or eluted in 50 µL of water.

3. We do not recommend using the crude samples directly in the PCR reaction after heating to 95°C as described in some publications. Even different dilutions of the unextracted samples should not be used, because endogenous substances might act as inhibitors of the Taq polymerase at very low concentrations and cause false-negative results. Even after DNA extraction, the PCR reaction can be inhibited by the unidentified substances. For this reason, internal control reactions need to be incorporated in the test series.
4. The use of thermal cyclers other than the GeneAmp 9600 might require some modifications in the amplification parameters, for example, duration of the cycling steps, which are short in comparison to other applications. In addition, Mg^{2+}, primer, or dNTP concentrations might need to be altered. The same is true if another Taq polymerase is used, either polymerases from different suppliers or different kinds of Taq polymerase; for example, we found that the parameters described were not transferable to HotStarTaq with a prolonged denaturation step (Qiagen).
5. The limiting dilution of the internal control DNA can be used maximally for 2 or 3 mo when stored at 4°C. After this time, the amplification of the internal control DNA might fail even when no inhibitors are present in the reaction, because the DNA concentration might be reduced owing to degradation or attachment to the plastic tube.
6. Applying the internal control DNA, the described PCR method is competitive only for the group of mycoplasma species that carries primer sequences identical to the one from which the internal control DNA was prepared. The other primer sequences are not used up in the PCR reaction as a result of mismatches. Usually one reaction per sample is sufficient to detect mycoplasma in "naturally" infected cell cultures. To avoid the possibility of performing a competitive reaction and of decreasing the sensitivity of the PCR reaction, for example, after antimycoplasma treatment or for the testing of cell culture reagents, two separate reactions can be performed. First, run a reaction without internal control DNA to make all reagents available for the amplification of the specific product. Second, an additional reaction including the internal control DNA to demonstrate the integrity of the PCR reaction **(Fig. 1)**.

7. During the analysis of heavily infected cultures, sometimes the internal control is not visible in the gel, owing to overwhelming amounts of target DNA in the sample compared to the trace amounts of the internal control DNA. In these cases, the correct run of the PCR reaction is demonstrated by the appearance of the band that is indicative of mycoplasma positivity.

References

1. Drexler H. G. and Uphoff CC. (2000) Contamination of cell cultures, mycoplasma. In: *The Encyclopedia of Cell Technology* (Spier E., Griffiths B., Scragg A. H., eds), Wiley, New York, pp.609–627.
2. Uphoff, C. C. and Drexler H. G. (2001) Prevention of mycoplasma contamination in leukemia-lymphoma cell lines. *Human Cell* **14,** 244–247.
3. Hopert, A., Uphoff, C. C., Wirth, M., Hauser, H., and Drexler, H. G. (1993) Specificity and sensitivity of polymerase chain reaction (PCR) in comparison with other methods for the detection of mycoplasma contamination in cell lines. *J. Immunol. Methods* **164,** 91–100.
4. Uphoff, C. C. and Drexler, H. G. (2002) Detection of mycoplasma in leukemia-lymphoma cell lines using polymerase chain reaction. *Leukemia* **16,** 289–293.

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Elimination of Mycoplasma from Infected Cell Lines Using Antibiotics

Cord C. Uphoff and Hans G. Drexler

1. Introduction

1.1. Mycoplasma Elimination: A Necessity

Although many mycoplasma contaminations of cell cultures remain undetected over many years and thus seem to have no apparent influence on the growth or other characteristics of the cells, this is by far a misinterpretation of the symbiotic relationship of eukaryotic cells and mycoplasmas. Mycoplasmas indeed have a multitude of different effects on the various eukaryotic cells. However, the effects are not constantly seen in all infected cultures, but depend on the mycoplasma species, the type of cell culture, and the cell culture conditions. Nearly every parameter measured in cell culture or experimental investigations may be affected by mycoplasmas ***(1)***.

The spectrum of effects range from more or less marked growth inhibition, altered levels of protein, RNA, and DNA synthesis, induction of chromosomal aberrations, alteration of the membrane composition, including surface antigen and receptor expression combined with alterations of signal transduction. The specificity of the infections is indicated by the adverse effects of induction or inhibition of activation, proliferation, and differentiation of hematopoietic cells, the induction or suppression of cytokine and growth factor expression, enhanced immunoglobulin secretion by B-cells, the increase or decrease of virus propagation, and a variety of other parameters. Many of the effects mentioned above are attributable to the deprivation of essential culture medium components and the degradation of specific nucleic acids and their precursors, amino acids, lipids, and so forth. Moreover, the metabolites can be harmful for the eukaryotic cells because of the production of acids from the fermentation (by fermentative mycoplasmas) or ammonia (by the arginine-hydrolyzing mycoplasmas) that is highly toxic to the eukaryotic cells ***(2)***.

Thus, mycoplasma contaminations cannot be regarded as harmless but may have detrimental consequences. Moreover, contaminated cell cultures represent a source of infection for other cell cultures. Consequently, cell cultures harboring mycoplasmas

From: *Methods in Molecular Medicine, vol. 88: Cancer Cell Culture: Methods and Protocols*
Edited by: S. P. Langdon © Humana Press Inc., Totowa, NJ

should either be autoclaved and discarded immediately or taken into quarantine and cured from the infection ***(3)***.

1.2. Antibiotic Treatment of Mycoplasma-Infected Cell Cultures

Taking into account that 10–30% of all cell lines maintained in laboratories are infected with mycoplasmas, and many of these cannot be replaced by uncontaminated cell cultures, there is a clear need for effective and feasible methods for the elimination of mycoplasma. A number of methods have been described to cure cell lines from the contaminants. These include physical, chemical, immunological, and chemotherapeutic procedures ***(2)***. Many of the elimination methods are of limited value for use in cell culture, because they are not always effective, and the mycoplasmas reappear after a while, are too laborious or require special equipment or facilities, or show detrimental effects on the eukaryotic cells ***(4)***. The application of chemotherapeutic reagents, such as antibiotics, currently seems to be the method of choice, because it is practicable for almost any cell culture laboratory.

Concerning morphology, biochemistry, and genetics, mycoplasmas exhibit special features which make them unique among the eubacteria. The antibiotic agents commonly used in cell culture, mainly penicillin and streptomycin, have no effect on the viability of mycoplasmas, although sometimes may suppress their growth (*see* **Note 1**). Thus specific antimicrobial agents have to be applied.

Over recent years, three unique groups of antibiotics have been shown to be highly effective against mycoplasmas, both in human/veterinary medicine and in cell culture: tetracyclines, macrolides, and quinolones. Tetracyclines and macrolides inhibit protein synthesis by binding to the 30S and 50S ribosomal subunits, respectively. Quinolones inhibit the bacterial DNA gyrase, essential for the replication of the DNA. It is important to have a number of antibiotics available which act differently against mycoplasmas, because the development of resistant clones and the possible cross-reactivity with weakened eukaryotic cells can be overcome by the application of an antibiotic from an unrelated group of reagents.

It should be mentioned that these antibiotics are not intended to be used as general supplement in cell culture to avoid mycoplasma infections, but only for the short time-period during antimycoplasma treatment.

Here, we describe the use of several antibiotics for the treatment of mycoplasma-contaminated cells, the rescue of heavily infected cultures, salvage treatment of resistant cultures, and some pitfalls during and after the treatment.

2. Materials

As the antibiotics are light sensitive, protect the stock and working solutions from the light as well as the cell cultures containing the antibiotics as far as possible.

1. BM-Cyclin (Roche, Mannheim, Germany, cat. no. 799050) contains the macrolide tiamulin (BM-Cyclin 1) and the tetracycline minocycline (BM-Cyclin 2), both in lyophilized states. Dissolve the antibiotics in 10 mL sterile dH_2O, aliquot in 1-mL fractions and store at –20 °C. Repeated freezing and thawing of the solutions is not critical for the activity of the antibiotics. The dissolved solutions can be used as 250-fold dilutions in cell culture (at 10 µg/mL and 5 µg/mL, respectively).

2. Ciprobay 100 (Bayer, Leverkusen, Germany) contains 2 mg/mL ciprofloxacin and can be used 1/200 in cell culture (at 10 µg/mL). One-mL aliquots should be taken in a sterile manner from the bottle and stored at 4°C.
3. Baytril (Bayer) contains 100 mg/mL of enrofloxacin and is diluted 1/100 with RPMI 1640 medium immediately prior to the treatment. The dilution should be prepared freshly for every antimycoplasma treatment. This solution is used as 1/40 final dilution in cell culture (at 25 µg/mL).
4. Zagam (Rhône-Poulenc, France) contains the antibiotic sparfloxacin as powder, and the stock solution is prepared by dissolving the antibiotic in freshly prepared 0.1 *N* NaOH to a concentration of 20 mg/mL. This solution can be stored at 4 °C. Before treatment, the stock solution is diluted 1/1 with RPMI 1640 medium and used in cell culture at a 1/1000 final dilution (at 10 µg/mL).
5. MRA (Mycoplasma Removal Agent, ICN, Eschwege, Germany) contains 50 µg/mL of a 4-oxo-quinolone-3-carboxylic acid derivative and is used in the treatment of cell cultures in 1/100 dilutions (at 0.5 µg/mL).
6. Phosphate-buffered saline (PBS): 140 m*M* NaCl, 27 m*M* KCl, 7.2 m*M* $Na_2HPO_4 \cdot 12\ H_2O$, 14.7 m*M* KH_2PO_4, pH 7.2; autoclave.

3. Methods

3.1. General Considerations

1. Prior to the treatment, aliquots of the contaminated cell line should be stored frozen, but isolated from non-infected cultures, either at –80 °C for short time (maximally 2–3 mo) or preferably in liquid nitrogen in separate tanks (*see* **Note 2**). The ampoules have to be marked properly as mycoplasma-positive to prevent a mix-up of ampoules containing cured or infected cells. After successful cure, these mycoplasma-positive ampoules will be removed and the cells destroyed by autoclaving.
2. The mycoplasma infection often impedes growth and negatively affects the general appearance of the eukaryotic cells; in other words the cells are objectively and subjectively not in the best of health. These impaired conditions are further aggravated by the exposure of the cells to the antibiotics, as the agents appear to affect those cells more than cells in good condition. Sometimes the desired anti-mycoplasma effect is cancelled by the negative effect of the antibiotics on the eukaryotic cells. Therefore, a few general rules should be followed to improve the culture conditions and to reduce the stress of infection and treatment on the eukaryotic cells:
   a. Increase the FBS concentration to 20%, even if the cells grow well at lower concentration.
   b. Culture the cells at a medium cell density and keep this density almost constant over the period of treatment and a few weeks posttreatment.
   c. Observe the culture daily under the inverted microscope to recognize quickly any alteration in general appearance, growth, morphology, decrease in cell viability, detachment of cells, formation of granules, vacuoles, and so forth.
   d. In the case of deterioration of the cell culture, interrupt the treatment for a few days and let the cells recover (but this should only be the last resort).
   e. Detach frequently even slowly growing adherent cells in order to facilitate the exposure of all mycoplasmas to the antibiotic; the contaminants should not have the opportunity to survive in sanctuaries such as cell membrane pockets. It is similarly helpful to break up clumps of suspension cells by vigorous pipeting or using other reagents, for example, trypsin.

f. Store the antibiotics at the recommended concentrations, temperatures, and usually in the dark, and do not use them after the expiration date.
g. Prepare the working solutions freshly for every treatment and add the solution directly to the cell culture and not to the stored medium.
h. Keep the concentration of the antibiotic constant during the treatment period; degradation of the antibiotic can be avoided by frequent complete exchange of the medium.

3. Generally, two different methods are applied for the treatment of cell cultures: the use of quinolones, which is basically the same procedure for each antibiotic of that group, and the use of BM-Cyclin, which is a combination therapy applying the two antibiotics minocycline (tetracycline) and tiamulin (macrolide) in alternating cycles ***(5)***. The latter method is more time consuming, but also more effective. We recommend applying both types of treatment in parallel.

A schematic overview of the procedure is given in **Fig. 1**; an exemplary representation of the treatment with BM-Cyclin is shown in **Fig. 2**.

3.2. Antibiotic Treatment

3.2.1. Treatment With BM-Cyclin

1. Bring the cells into solution (detach adherent cells, break up clumps by pipeting or using other methods) (*see* **Note 3**); determine the cell density and viability by trypan blue exclusion staining. Seed out the cells at a medium density (*see* **Note 4**) in a 25-cm^2 flask or one well of a 6- or 24-well-culture plate with the appropriate fresh and rich culture medium (10 mL for the flask, and 2 mL and 1 mL for the wells, respectively). Add 4 µL of a 2.5-mg/mL solution BM-Cyclin 1 (tiamulin) per milliliter medium. Incubate the cell culture for 2 days (d).
2. Remove all cell culture medium in flasks or wells containing adherent cells or after centrifugation of suspension cells. If applicable, dilute the cell cultures to the medium cell density. Add fresh medium and the same concentration of BM-Cyclin 1 as used in **step 1**. Incubate for another day. This procedure will keep the concentration of the antibiotic approximately constant over the 3-d cycle of applying tiamulin.
3. Remove the medium and wash the cells once with PBS to remove the residual antibiotic agent completely from the cells and loosely attached mycoplasmas (*see* **Note 5**). Seed out the cells at the appropriate density and add 4 µL of the 1.25 mg/mL solution BM-Cyclin 2 per milliliter medium. Incubate the culture for 2 d.
4. Remove the culture medium and substitute with fresh medium. Add the same concentration of BM-Cyclin 2 as used in **step 3**. Incubate the cell culture for 2 d to complete the 4-d cycle of minocycline-treatment.
5. Repeat **steps 1–4** twice (three cycles of BM-Cyclin 1 and BM-Cyclin 2 altogether). Proceed with protocol in **Subheading 3.3.**

3.2.2. Treatment With Quinolones

1. Bring the cells into solution (detach adherent cells, break up clumps by pipeting or using other methods) (*see* **Note 3**); determine the cell density and viability by trypan blue exclusion staining. Seed out the cells at a medium density (*see* **Note 4**) in a 25-cm^2 flask or one well of a 6- or 24-well-culture plate with the appropriate fresh and rich culture medium (10 mL for the flask, and 2 mL and 1 mL for the wells, respectively). Add one of the following antibiotics to the cell culture and incubate for 2 d:
   a. 25 µL of a 1 mg/mL solution enrofloxacin (Baytril) per mL medium.
   b. 10 µL of a 50 µg/mL solution MRA per mL medium.

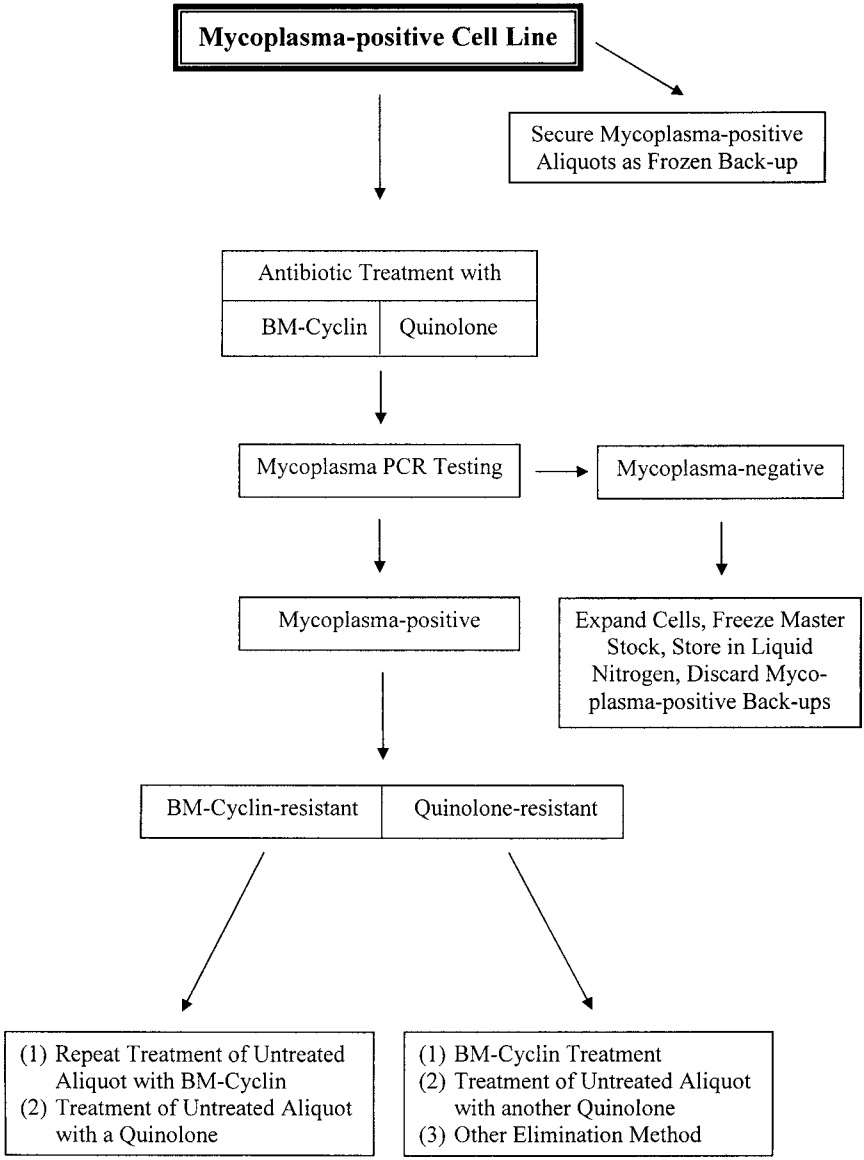

Fig. 1. Recommended scheme for mycoplasma eradication. An arsenal of different antibiotics can be used to treat mycoplasma-contaminated cell lines with a high rate of expected success. We recommend: (1) cryopreservation of original mycoplasma-positive cells as back-ups; and (2) splitting of the growing cells into different aliquots. These aliquots should be exposed singly to the various antibiotics. It is important to point out that quinolone-resistant cultures could still be cleansed by BM-Cyclin. Posttreatment mycoplasma analysis and routine monitoring with a sensitive and reliable method (for example by PCR) are of utmost importance.

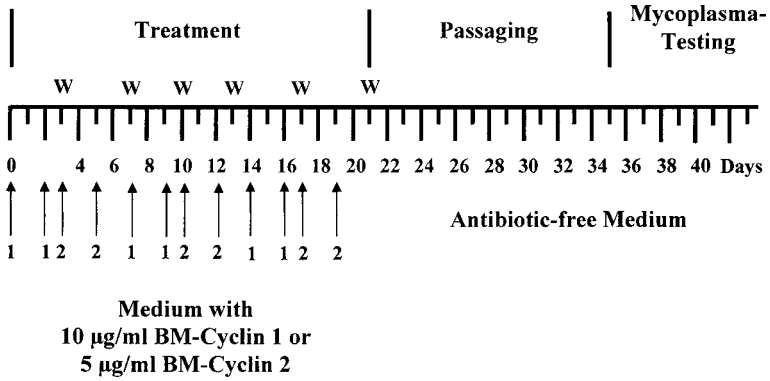

Fig. 2. Treatment protocol for BM-Cyclin. Antibiotics are given on the days indicated by arrows. Cells are washed (indicated by W) with PBS prior to the cyclical change of antibiotics to avoid formation of resistant mycoplasmas that occur when concentrations of the antibiotics are low. At the end of the decontamination period, cells are washed with PBS and suspended in antibiotic-free medium. After a minimum of 2 wk posttreatment, the mycoplasma status of the cells is examined with sensitive and robust methods (for example by PCR).

c. 1 µL of a 10 mg/mL solution sparfloxacin (Zagam) per mL medium.
d. 5 µL of a 2 mg/mL solution ciprofloxacin (Ciprobay) per mL medium.

2. Remove all cell culture medium in flasks or wells containing adherent cells or after centrifugation of suspension cells. If applicable, dilute the cell cultures to the medium cell density. Add fresh medium and the same concentration of the respective antibiotic as used in **step 1**. Incubate for another 2 d.
3. Applying enrofloxacin, MRA, or sparfloxacin, repeat **step 2** another two times (altogether 8-d treatment). Employing ciprofloxacin, repeat **step 2** five times (altogether 14-d treatment). Proceed with with protocol in **Subheading 3.3.**

3.3. Culture and Testing Post-Treatment

1. When the treatment is completed, remove the supernatant, wash the cells with PBS, and seed out the cells as described in **Subheading 3.2.** Keep the cells at the higher cell density and use the enriched medium, but do not add any antibiotic compounds, also no penicillin/streptomycin or similar. Thus, the cells should be cultured for at least another 2 wk. Even if initially the cells appear to be in good health after the treatment, we found that the cells might go into a crisis after the treatment, especially following treatment with BM-Cyclin. Thus, the cell status should be frequently examined under the inverted microscope.
2. After passaging, test the cultures for mycoplasma contamination. If the cells are clean, freeze and store aliquots in liquid nitrogen. The cells in active culture have to be retested periodically to ensure continued freedom from mycoplasma contamination.
3. After complete decontamination, expand the cells and freeze master stocks of the mycoplasma-free cell line and store them in liquid nitrogen to provide a continuous supply of clean cells. Discard the ampoules with mycoplasma-infected cells.

3.4. Further Considerations

In our experience, it is advantageous to employ both types of treatments (BM-Cyclin and one of the quinolones) in parallel, as usually at least one of the treatments is successful. In the rare event of resistance, cells of the untreated frozen back-up aliquots can be thawed and treated again with another antibiotic. As MRA, ciprofloxacin, enrofloxacin, and sparfloxacin all belong to the group of quinolones, it is likely that the use of an alternative compound from the same group will produce the same end result (cure, resistance, or culture death). In the case of loss of the culture during or after the treatment, aliquots can be treated with quinolones, as these are usually better tolerated by the eukaryotic cells. We recommend using MRA which shows almost no effect on the growth parameters during the treatment of 1 wk. The use of 5 µg/mL sparfloxacin might be an alternative to the treatment procedure described, as this concentration was also shown to be effective against mycoplasma in most cases. One of the latter two treatments is also recommended when the cells are already in a very bad condition prior to treatment and the number of available cells would suffice only for one single treatment. Sometimes, the cells recover rapidly after starting the treatment because of the immediate reduction of the mycoplasmas and the ensuing release of the cells from the mycoplasmal stranglehold.

If the cytotoxic effects of the antibiotics appear to be too strong, so that one would risk the loss of the cell culture (occasionally observed when BM-Cyclin is employed), the procedure might be interrupted for a short time, i.e., one or two days, to allow the cells sufficient time for recovery. This should be done when the first symptoms of serious cell culture deterioration begin to appear. If the cells are already beyond a certain degree of damage, it is difficult to reverse the progression of apoptosis.

4. Notes

1. The general use of antibiotics in cell culture is not recommended except under special circumstances and then only for short durations. Use of antibiotics may lead to lapses in aseptic technique, to selection of drug-resistant organisms, and to delayed detection of low-level infection with either mycoplasmas or other bacteria.
2. The storage in liquid nitrogen might be one of the potential contamination sources of cell cultures with mycoplasmas. Mycoplasmas were shown to survive in liquid nitrogen even without cryopreservation. Once introduced into the nitrogen, mycoplasmas may persist in the tank for an indefinite time, not proliferating, but being able to contaminate cell cultures stored in the liquid phase of the nitrogen. The infection might happen, when the ampoules are inserted into the tank, cooled down to –196°C and the unfilled part of the ampoule is filled with liquid nitrogen because the pressure is low. Thus, we strongly recommend storing the ampoules in the gaseous phase of the nitrogen to prevent contamination. Additionally, contaminated cell cultures and those of unknown status should be stored separately from noninfected cells, preferably in separate tanks. If this is not possible, be sure to store the ampoules at different locations of one tank and in the gaseous phase (high positions in the tank). Do not fill with liquid nitrogen above a certain level.
3. It is important to break up the clumps and clusters and to detach the cells from the surface of the culture vessels. Although the antibiotics are in solution and should be accessible to all parts of the cells, the membranes might be barriers which cannot be passed by the

antibiotics. Mycoplasmas trapped within clumps of eukaryotic cells or even in cavities formed by the cell membrane of a single cell might be protected from the antibiotic. This is also the reason for the advice to keep the concentration of the antibiotic constantly high by frequently exchanging the medium.

4. Depending on the growth rate of the cell line, which might be severely altered by the antibiotic, the cell density should be diluted, kept constant, or even concentrated. If no data are available at all for a given cell culture, or if the cell culture is in a very bad condition, the cell density, growth rate, and viability should be recorded frequently to improve the condition of the culture.
5. The concentration of the antibiotics should be kept at a constant level throughout the treatment period. Low antibiotic concentrations attributable to degradation in culture or dilution by passaging the cell culture may lead to the development of resistant mycoplasma strains. Thus, exchange of the complete medium and washing the cells with PBS is recommended.

References

1. Barile, M. F. and Rottem, S. (1993) Mycoplasmas in cell culture. In: *Rapid Diagnosis of Mycoplasmas* (Kahane, I. and Adoni, A., eds.), Plenum Press, New York, pp. 155–193.
2. Drexler, H. G. and Uphoff, C. C. (2000) Contamination of cell cultures, mycoplasma. In: *The Encyclopedia of Cell Technology* (Spier, E., Griffiths, B., Scragg, A. H., eds.), Wiley, New York, pp. 609–627.
3. Uphoff, C. C. and Drexler, H. G. (2001) Prevention of mycoplasma contamination in leukemia-lymphoma cell lines. *Human Cell* **14,** 244–247.
4. Uphoff, C. C., Meyer, C., and Drexler, H. G. (2002) Elimination of mycoplasma from leukemia-lymphoma cell lines using antibiotics. *Leukemia* **16,** 284–288.
5. Schmidt, J. and Erfle, V. (1984) Elimination of mycoplasmas from cell cultures and establishment of mycoplasma-free cell lines. *Exp. Cell Res.* **152,** 565–570.

Appendix 1

Formulations of Commonly Used Cell Culture Media

Formulations of Commonly Used Cell Culture Media

Component	BME mg/L	Eagle's MEM mg/L	DMEM mg/L	Iscove's DMEM mg/L	Ham's F12 mg/L	RPMI 1640 mg/L	CMRL 1066 mg/L
Amino Acids							
L-Alanine	-	-	-	25	8.9	-	25
L-Arginine	-	-	-	-	-	240	-
L-Arginine.HCl	21	126	84	84	211	-	70
L-Asparagine	-	-	-	-	-	50	-
L-Asparagine.H_20	-	-	-	28.4	15.01	-	-
L-Aspartic acid	-	-	-	30	13.3	20	30
L-cysteine	-	-	-	-	-	-	-
L-cystine	12	31	48	-	-	50	200
L-cystine.2HCl	-	-	-	70	-	-	20
L-cystine.HCl.H_20	-	-	-	-	35.12	-	-
L-glutamic acid	-	-	75	14.7	20	75	-
L-glutamine	-	292	584	584	146	300	100
Glycine	-	-	30	30	7.5	10	50
L-histidine	8	-	-	-	-	15	-
L-histidine.HCl.H_20	-	42	42	42	20.96	-	20
L-hydroxyproline	-	-	-	-	-	20	10
L-isoleucine	26	52	105	105	3.94	50	20
L-leucine	26	52	105	105	13.1	50	60
L-lysine.HCl	29	73	146	146	36.5	40	70
L-methionine	7.5	15	30	30	4.48	15	15
L-phenylalanine	16.5	33	66	66	4.96	15	25
L-proline	-	-	-	40	34.5	20	40
L-serine	-	-	42	42	10.5	30	25
L-threonine	24	48	95	95	11.9	20	30
L-tryptophan	4	10	16	16	2.04	5	10
L-tyrosine	18	36	72	104	5.4	20	40
L-valine	23.5	47	94	94	11.7	20	25

Formulations of Commonly Used Cell Culture Media *(Cont.)*

Component	BME mg/L	Eagle's MEM mg/L	DMEM mg/L	Iscove's DMEM mg/L	Ham's F12 mg/L	RPMI 1640 mg/L	CMRL 1066 mg/L
Inorganic salts							
$CaCl_2$	-	-	200	165	-	-	200
$CaCl_2.2H_2O$	264	264	-	-	44	-	-
$Fe(NO_3)_3.9H_2O$	-	-	0.10	-	-	-	-
KCl	400	400	400	330	224	400	400
KH_2PO_4	60	-	-	-	-	-	
$MgCl_2.6H_2O$	100	-	-	-	122	-	-
$MgSO_4$	-	-	-	97.67	-	-	-
$MgSO_4.7H_2O$	200	200	200	-	-	100	200
NaCl	6800	6800	6400	4505	7599	6000	6799
$NaHCO_3$	2200	2200	3700	3024	1176	2000	2200
$NaH_2PO_4.H_2O$	140	140	125	125	-	-	140
$Na_2HPO_3.7H_2O$	-	-	-	-	268	1512	-
KNO_3	-	-	-	0.076	-	-	-
$Na_2SeO_3.5H_2O$	-	-	-	0.0173	-	-	-
$CuSO_4.5H_2O$	-	-	-	-	0.00249	-	-
$FeSO_4.7H_2O$	-	-	-	-	0.834	-	-
$ZnSO_4.7H_2O$	-	-	-	-	0.863	-	-
$CaNO_3.4H_2O$	-	-	-	-	-	100	-

Formulations of Commonly Used Cell Culture Media *(Cont.)*

Component	BME mg/L	Eagle's MEM mg/L	DMEM mg/L	Iscove's DMEM mg/L	Ham's F12 mg/L	RPMI 1640 mg/L	CMRL 1066 mg/L
Vitamins							
Biotin	1	-	-	0.013	0.0073	0.2	0.01
D-Ca pantothenate	1	1	4	4	0.48	0.25	0.01
Choline chloride	1	1	4	4	13.96	3	0.5
Folic acid	1	1	4	4	1.3	1	0.01
i-inositol	1.8	2	7.2	7.2	18	35	0.05
Nicotinamide	1	1	4	4	0.04	1	0.025
Pyridoxal HCl	1	1	4	4	-	-	0.025
Riboflavin	0.1	0.1	0.4	0.4	0.038	0.2	0.01
Thiamine HCl	1	1	4	4	0.34	1	0.01
Vitamin B_{12}	-	-	-	0.013	1.36	0.005	-
Pyridoxine HCl	-	-	-	-	0.062	1	0.025
Cholesterol	-	-	-	0.02	-	-	0.2
p-aminobenzoic acid	-	-	-	-	-	1	0.05
L-ascorbic acid	-	-	-	-	-	-	50

Formulations of Commonly Used Cell Culture Media *(Cont.)*

Component	BME mg/L	Eagle's MEM mg/L	DMEM mg/L	Iscove's DMEM mg/L	Ham's F12 mg/L	RPMI 1640 mg/L	CMRL* 1066 mg/L
Other components							
D-Glucose	1000	1000	4500	4500	1802	2000	1000
Phenol red	10	10	15	15	12	5	20
Sodium pyruvate	-	-	110	110	110	-	-
HEPES	-	-	-	5958	-	-	-
Hypoxanthine	-	-	-	-	4	-	-
Linoleic acid	-	-	-	-	0.084	-	-
Putrescine.2HCl	-	-	-	-	0.161	-	-
Thymidine	-	-	-	-	0.73	-	10
Glutathione	-	-	-	-	-	1	10

- In addition to the above, CMRL 1066 also contains carboxylase (1 mg/L), coenzyme A (2.5 mg/L), deoxyadenosine (10 mg/L), deoxycytidine HCl (10 mg/L), deoxyguanosine (10 mg/L), dihydrophosphopyridine nucleotide. $4H_2O$ (7 mg/L), ethanol (16 mg/L), flavin adenine dinucleotide (1 mg/L), 5-methyl-deoxycytidine (0.1 mg/L), sodium acetate (83 mg/L), sodium glucuronate (4 mg/L), triphosphopyridine nucleotide (1 mg/L), Tween 80 (5 mg/L) and uridine triphosphate (1 mg/L).

Appendix 2

Human Cancer Cell Lines Available from the ATCC and DSMZ Cell Banks (Combined List)

Human Cancer Cell Lines Available from the ATCC and DSMZ

BLADDER CANCER

5637	647-V	BC-3C	BFTC-905	CAL-29
Hs 172.T	Hs 195.T	Hs 228.T	Hs853.T	HT 1197.T
HT 1376.T	HT-1197	HT-1376	J82	JMSV-1
KU-19-19	RT-112	RT4	SCaBER	SW 780
SW-1710	T24	TCCSUP	UM-UC-3	VM-CUB1

BONE CANCERS

143.98.2	143B	CAL-72	G-292	H39.T
HOS	Hs 14.T	Hs 184.T	Hs 188.T	Hs 3.T
Hs 387.T	Hs 704(A).T	Hs 704.T	Hs 706.T	Hs 735.T
Hs 737.T	Hs 755(B).T	Hs 781.T	Hs 792(B).T	Hs 805.T
Hs 811.T	Hs 814.T	Hs 821.T	Hs 822.T	Hs 845.T
Hs 846.T	Hs 860.T	Hs 863.T	Hs 864.T	Hs 866.T
Hs 870.T	Hs 871.T	Hs 883.T	Hs 888.T	Hs 889.T
Hs 890.T	Hs 894.T	Hs 899.T	Hs 900.T	Hs454.T
Hs454.T	Hs819.T	Hs88.T	Hs903.T	Hs919.T
HT 1080.T	HT 728.T	KHOS	MG-63	MNNG/HOS
RD-ES	SaOS	SaOS-2	SJSA-1	SK-ES-1
SW 1353	T1-73	TE 130.T	Te 417.T	Te 418.T
TE 76.T	TO 203.T	U-2OS	VA-ES-BJ	

BRAIN CANCERS

8-MG-BA	42-MG-BA	A172	BE(2)-C	BE(2)-M17
CCF-STTG1	CHP-126	CHP-212	D283 Med	D341 Med
Daoy	DBTRG-05MG	DK-MG	GAMG	GOS-3
G-MS-10	H4	Hs 683	IMR-32	KELLY
LN-405	M059J	M059K	MC-IXC	MHH-NB-11
NCI-H1915	SH-SY5Y	SIMA	SK-N-AS	SK-N-BE(2)
SK-N-DZ	SK-N-FI	SK-N-MC	SK-N-SH	SNB-19
SW 1088	SW1783	T98G	TE 615.T	TE671
U-118MG	U-138MG	U-87MG		

BREAST CANCER

AU565	BT-474	BT-483	CAL-51	CAL-85-1
CAL-120	CAL-148	CAMA-1	COLO-824	DU-4475
EFM-19	EFM-192A	EFM-192B	EFM-192C	EVSA-T
GL-101A	HCC 1008	HCC 1569	HCC 1954	HCC1143
HCC1187	HCC1395	HCC1419	HCC1428	HCC1500
HCC1569	HCC1599	HCC1806	HCC1937	HCC202
HCC2157	HCC2218	HCC38	HCC70	HDQ-P1
Hs 274.T	Hs 280.T	Hs 281.T	Hs 343.T	Hs 574.T
Hs 739.T	Hs 741.T	Hs 742.T	Hs 902.T	Hs362.T
Hs540.T	Hs566(B).T	Hs605.T	HS606	KPL-1
MB157	MCF-7	MDA-MB-175-VII	MDA-MB-231	MDA-MB-361
MDA-MB-415	MDA-MB-435S	MDA-MB-436	MDA-MB-453	MDA-MB-468
MFM-223	MT-3	SK-BR-3	T-47D	UACC-812
UACC-893	ZR-75-1	ZR-75-30		

Human Cancer Cell Lines Available from the ATCC and DSMZ *(Cont.)*

BURKITT'S LYMPHOMA

2F7	BL-41	BL-70	CA46	Daudi
DG-75	EB1	EB2	EB-3	GA-10
HS-Sultan	Jiyoye	NAMALWA	NC-37	P3HR-1
P-3J	Raji	Ramos (RA)	Ramos.2G6.4C10	ST486
TE 161.T				

CERVICAL CANCER

C-33A	C-41	C-41 II	C4II	CaSki
DoTc2 4510	Hela	Hs 588.T	Hs 636.T	HT-3
KB-3-1	ME 180	MS751	SiHa	SISO
SW756				

CHORIOCARCINOMA

AC-1M32	AC-1M46	AC-1M59	AC-1M81	AC-1M88
ACH1P	BEWO	JAR	JEG-3	

COLORECTAL CANCER

(HRT-18)	C2BBE1	Caco-2	CL-11	CL-14
CL-34	COLO 201	COLO 205	COLO-206F	COLO 320
COLO 320DM	COLO320HSR	COLO 678	CX-1	DLD-1
HCT 116	HCT-15	Hs 200.T	Hs 207.T	Hs 219.T
Hs 241.T	Hs 255.T	HS 257.T	HS 586.T	HS 587.Int
Hs 674.T/cc	Hs 698.T	Hs 722.T	HT-29	LoVo
LS 123	LS 174T	LS 180	LS1034	LS411N
LS513	NCI-H498	NCI-H498	NCI-H508	NCI-H508
NCI-H548	NCI-H630	NCI-H716	NCI-H716	NCI-H747
NCI-H747	NCT-8	NCT-8	SK-CO-1	SNU-C1
SNU-C2A	SNU-C2B	SW1116	SW1417	SW1463
SW 403	SW430	SW48	SW480	SW620
SW948	T84	WiDr		

EMBRYONAL CANCERS

NCCIT	Tera-1	Tera-2

ENDOMETRIAL CANCER

AN3 3CA	EFE-184	ESS-1	HEC-1-A	HEC-1-B
KLE	MES-SA	MFE-280	MFE-296	MFE-319
RL95-2	SK-UT-1	SK-UT-1B		

ESOPHAGEAL CANCER

COLO-680N	KYSE-30	KYSE-70	KYSE-140	KYSE-150
KYSE-180	KYSE-270	KYSE-410	KYSE-450	KYSE-510
KYSE-520				

Human Cancer Cell Lines Available from the ATCC and DSMZ *(Cont.)*

EWING'S SARCOMA

C-433	CADO-ES1	MHH-ES-1	RD-ES	SK-ES-1
TC-71				

FIBROSARCOMA

Hs 15.T	Hs 414.T	Hs 778(A).T	Hs 778(B).T	Hs 868.T
Hs 913(B).T	Hs 913(C).T	Hs 913(D).T	Hs 913(F).T	Hs 93.T
HT-1080	SW 684	TE115.T		

HODGKIN'S LYMPHOMA

HD-MY-Z	HDLM-2	Hs 388.T	Hs 445	Hs 604.T
Hs 611.T	Hs 616.T	Hs 751.T	L-428	L-540
RPMI 6666	TO 175.T			

LEUKEMIA / LYMPHOMA (see Hodgkin's and Burkitt's lymphomas also)

1069	1A2	295R	380	697
ALL-SI	ARH-77	BC-1	BC-2	BC-3
BCP-1	BE-13	BONNA-12	BV-173	CCRF-CEM
CMK	CML-T1	CRO-AP2	CRO-AP3	CRO-AP5
CTV-1	DB	DOHH-2	EHEB	EM-2
EM-3	EOL-1	GDM-1	GRANTA-519	H9
HC-1	HEL	HH	HL60	HPB-ALL
Hs 313.T	Hs 491.T	Hs 505.T	Hs 518.T	Hs 602
Hs 697.Ln	Hs 777.T	HSB-2	HT	HT 1417
HuT 102	HuT78	IM-9	JK-1	JM1
JOSK-I	JOSK-M	JURKAT	JVM-2	JVM-3
K562	KARPAS-299	KARPAS-422	KARPAS-45	KARPAS-620
KASUMI-1	KASUMI-2	KCL-22	KE-37	KG-1
KG-1A	KMOE-2	KU-812	L-363	LAMA-84
LAMA-87	LOUCY	LP-1	M-07e	MC116
MCC-116	MEC-1	MEC-2	MEG-01	MHH-CALL-2
MHH-CALL-3	MHH-CALL-4	MHH-PREB-1	ML-2	MN-60
MOLT-13	MOLT-14	MOLT-16	MOLT-17	MOLT-3
MOLT-4	MONO-MAC-1	MONO-MAC-6	MU	MUTZ-2
MUTZ-3	MUTZ-5	NALM-1	NALM-19	NB-4
NCI-H929	NK-92	NK-92MI	NV4-11	OCI-AML2
OCI-AML5	OPM-2	PC12-ICHIKAWA		PEER
PF-382	PLB-985	REH	RH9	RH9/CB
RH9/HTLV	RH9/MSC	RL	RO	RPMI-8226
RPMI-8402	RS4:11	SIG-M5	SK-MM-2	SK-W-3
SPI801	SPI-801	SPI-802	SR-786	SU-DHL-1
SUDHL-4	SUP-B15	SUP-M2	SUP-T1	TALL-1
TANQUE	TF-1	THP-1	TMM	TUR
U-266	U-698M	U-937	UT-7	WSU-NHL
YT				

Human Cancer Cell Lines Available from the ATCC and DSMZ *(Cont.)*

LIVER CANCER

C3A	Hep.3B	Hep 3B2.1-7	Hep G2	HEP G2/2.2.1
LCLC-97TMI	NCI-H1755	PLC/PRF/5	SHU-398	SK-HEP-1
SNU-182	SNU-387	SNU-423	SNU-449	SNU-475

LUNG CANCER

A-427	A549	BEN	CAL-12T	Calu-1
Calu-3	COLO-677	COLO-699	CPC-N	DMS 114
DMS 153	DMS 79	DMS53	DV90	EPLC-272H
H69AR	HCC-15	HCC-33	HCC-366	HLF-a
Hs 284.Pe	Hs 57.T	Hs 57.T	Hs 618.T	Hs229.T ChaGo-K-1
LCLC-103H	LOU-NH91	LXF289	MSTO-211H	NCI-H1048
NCI-H1059	NCI-H1092	NCI-H1105	NCI-H1155	NCI-H1184
NCI-H1238	NCI-H128	NCI-H1284	NCI-H1299	NCI-H1304
NCI-H1339	NCI-H1341	NCI-H1355	NCI-H1373	NCI-H1395
NCI-H1404	NCI-H1417	NCI-H1435	NCI-H1436	NCI-H1437
NCI-H146	NCI-H152	NCI-H1563	NCI-H1568	NCI-H1573
NCI-H1581	NCI-H1618	NCI-H1623	NCI-H1648	NCI-H1650
NCI-H1651	NCI-H1666	NCI-H1672	NCI-H1688	NCI-H1693
NCI-H1694	NCI-H1703	NCI-H1734	NCI-H1781	NCI-H1792
NCI-H1793	NCI-H1819	NCI-H1836	NCI-H1838	NCI-H187
NCI-H1870	NCI-H1876	NCI-H1882	NCI-H1926	NCI-H1930
NCI-H1944	NCI-H196	NCI-H1963	NCI-H1975	NCI-H1993
NCI-H1994	NCI-H2009	NCI-H2023	NCI-H2029	NCI-H2030
NCI-H2059	NCI-H2066	NCI-H2073	NCI-H2081	NCI-H2085
NCI-H2087	NCI-H209	NCI-H2106	NCI-H2107	NCI-H2108
NCI-H211	NCI-H2122	NCI-H2126	NCI-H2141	NCI-H2170
NCI-H2171	NCI-H2195	NCI-H2196	NCI-H2198	NCI-H220
NCI-H2227	NCI-H2228	NCI-H2250	NCI-H226	NCI-H2286
NCI-H2291	NCI-H23	NCI-H2330	NCI-H2342	NCI-H2347
NCI-H2405	NCI-H250	NCI-H292	NCI-H295	NCI-H345
NCI-H358	NCI-H378	NCI-H446	NCI-H460	NCI-H510A
NCI-H520	NCI-H522	NCI-H524	NCI-H526	NCI-H596
NCI-H60	NCI-H647	NCI-H650	NCI-H660	NCI-H661
NCI-H676B	NCI-H69	NCI-H711	NCI-H719	NCI-H735
NCI-H740	NCI-H748	NCI-H774	NCI-H810	NCI-H82
NCI-H838	NCI-H841	NCI-H847	NCI-H865	NCI-H889
NCI-H920	NCI-H969	NCI-HH64	NCI-N417	NCI-N592
SHP-77	SK-LU-1	SK-MES-1	SW 1271	SW 900
SW1573				

Human Cancer Cell Lines Available from the ATCC and DSMZ *(Cont.)*

MELANOMA

A 101D	A2058	A375	A-375	A375.S2
A7	C32	C32TG	CHL-1	CHL-2
COLO679	COLO800	COLO818	COLO783	COLO829
COLO849	G-361	HMCBHs 432.T	Hs 600.T	Hs 688(A).T
Hs 688(B).T	Hs 695T	Hs 834.T	Hs 839.T	Hs 852.T
Hs 895.T	Hs 906(A).T	Hs 906(B).T	Hs 908.Sk	Hs 934.T
Hs 935.T	Hs 936.T	Hs 936.T(C1)	Hs 939.T	Hs940.T
HT-144	IGR-1	IGR-37	IGR-39	IPC-298
Malme-3M	MEL-HO	MEL-JUSO	MeWo	RPMI-7951
RVH-421	SH-4	SK-MEL-1	SK-MEL-2	SK-MEL-24
SK-MEL-28	SK-MEL-3	SK-MEL-30	SK-MEL-31	SK-MEL-5
WM-115	WM-266-4	Hs 294T		

OVARIAN CANCER

Caov-3	Caov-4	COLO-704	EFO-21	EFO-27
ES2	FU-OV-1	MDAH 2774	NIH:OVCAR-3	OV-90
PA-1	SK-OV-3	(SW626)	TOV-112D	TOV-21G

PANCREATIC CANCER

AsPC-1	BxPC-3	Capan-1	Capan-2	CFPAC-1
DAN-G	HPAC	HPAF-II	Hs 766T	HUP-T3
HUP-T4	KCI-MOH1	MIA PaCa-2	MPanc-96	PANC-1
PA-TU-8902	PA-TU-8988S	PA-TU-8988T	PL45	Su-86.86
SW 1990	YAPC			

CANCERS OF THE PHARYNX

Detroit 562	FaDu	Hs 840.T

PLASMACYTOMA

MC/CAR	MC/CAR-Z2	NCI-H929	RPMI 8226	SKO-007
U266B1				

PROSTATIC CANCER

22Rv1	BM-1604	CA-HPV-10	DU145	LNCaP
LNCaP clone FGC		LNCaP-FGC	MDA Pca 2b	PC-3

RENAL CANCER

769-P	786-O	A498	A704	ACHN
BFTC-909	Caki-1	Caki-2	CAL-54	G-402
Hs 891.T	Hs 926.T	SV7tert	SW 156	SW 839

RETINOBLASTOMA

WERI-Rb-1	Y79

Human Cancer Cell Lines Available from the ATCC and DSMZ *(Cont.)*

RHABDOMYOSARCOMA

130T	A204	A673	A-673	Hs 729
Hs 729.T	RD	RH-18	RH-30	SJRH30
T 174	TE 159.T	TE 381TTE 441.T		TE 617.T
TE.671				

SKIN CANCERS

166-ME SK	182-PF SK	A253	A431	Hs 156.T
Hs 295.T	Hs 416.T	Hs 892.T	Hs 898.T	Hs 925.T
Hs 941.T	Hs357.T	Hs398.T	Hs63.T	Sar Nis
TE354.T				

STOMACH CANCER

AGS	Hs 692(A).T	Hs740.T	Hs746T	KATO III
NCI-87	NCI-SNU-1	NCI-SNU-5	NCI-SNU-6	RF-1
RF-48				

CANCERS OF THE TESTIS

Cates-1B	Hs 444(B).T	NTERA-2cl.D1

THYROID CANCER

8305C	8505-C	B-CPAP BHT-101		CAL-62
CGTH-W-1	ML-1	ONCO-DG-1	S-117	TT2609-CO2

TONGUE CANCER

CAL3	CAL 27	SCC-15	SCC-25	SCC-4
SCC-9				

VULVAL

CAL-39	SK-LMS-1	SW692	SW 954

OTHERS

639-V	23132/87	A-253	BHY	Calu-6
CAL-78	DEL	EGI-1	HN	Hs 132.T
Hs 51.T	Hs 701.T	Hs 769.T	Hs 789.T	Hs696
Hs700T	HuTu 80MKN-45	NCI-H295	RPMI 2650	
SW 872	SW 982	SW579	Te 206.T	Te 206.T
TFK-1	TT	U-2197	VA-ES-BJ	W5-6