CHAPTER 1

QUALITY CONTROL PROCEDURES FOR STEM CELL LINES

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1.1. INTRODUCTION

The culture of human cells in vitro has provided important insights into cell biology, disease processes, and potential therapies. The advent of the culture of human embryonic stem cells has opened up an exciting new generation of possibilities including their potential for application to human regenerative medicine. However, in vitro cell culture brings a number of challenges; the cells and the cell culture environment are ideal for the growth of numerous microorganisms, and the cells themselves are prone to genetic changes. Furthermore, it is, unfortunately, common for cell cultures to be interchanged, cross-contaminated, or mislabeled during laboratory manipulations. This leads us to define three critical characteristics of cell cultures that are fundamental to the assurance of good-quality cell culture work. These are:

- Purity—The cells are free from microbiological contamination.
- Identity—The cells are what they are claimed to be.
- Stability—The genotype and phenotype remain stable during growth and passage in vitro.

Serial passage exposes cell cultures to the repeated risk of contamination by environmental microorganisms. Such contaminations are generally recognized by a significant change in the pH of the culture medium (as identified by a color shift in the medium) and the sudden appearance of turbidity or colonies of fungal organisms. In these situations the culture is generally not recoverable and should be discarded carefully to prevent contamination of other cultures. However, some microbial contaminants can establish subliminal persistent infections that are not obvious on visual inspection. These are commonly due to mycoplasma but may be caused by other organisms [*see*, e.g. Mowles et al., 1989; Buerhing et al., 1995].

Mycoplasma contamination is known to cause a broad range of permanent and deleterious effects on cells including chromosomal abnormalities [McGarrity et al., 1984] and cell transformation [Zhang et al., 2004, 2006; for a review *see* Rottem and Naot, 1998]. Mycoplasma contamination is not obvious by visual inspection, can spread rapidly to other cultures handled by the same or other operators, and is difficult to eradicate reliably. Accordingly, it is important to perform routine screening for this organism in cell cultures.

The first human cell line HeLa, established in 1952 [Gey et al., 1952], was widely distributed to laboratories also attempting to establish new cell lines. Within a few short years it became apparent that some of the "novel" cell lines being established were in fact cross-contaminated with HeLa cells [Gartler et al., 1967]. The problem was highlighted through the use of karyology and isoenzyme analysis [Nelson-Rees et al., 1981; O'Brien et al., 1977] but was only partially resolved and led to much scientific controversy [Gold 1983]. Since the identification of early cases of cross-contaminated cultures, cases have continued to be identified (Table 1.1). Despite periodic reminders from concerned cell culturists [e.g., Stacey et al., 2000] the problem appears to continue, and recent publications seem to indicate that part of this problem may be due to cross-contamination at the source of the cultures in the laboratories of the originators of the cell lines [MacLeod et al., 1999; Drexler et al., 2003]. It is vital that this situation does not develop with stem cell lines, as this could cause confusion in laboratory experimentation and major

TABLE 1.1 Publications Describing Cell Lines Not Matching Their Purported Origin

Reference	Cell Lines
Gartler [1967]	Breast cancer cell line cross-contamination
Nelson Rees et al. [1977]	Widespread cross-contamination of human breast tumor cell lines and others
Harris et al. [1991]	Putative human Hodgkin disease cell lines cross-contaminated with nonhuman cells
Masters et al. [1988]	Cross-contamination of bladder cancer cell lines
van Helden et al. [1988]	Cross-contamination among esophageal squamous carcinoma cell lines
Chen et al. [1990]	TE671 shown to be a derived from RD cells
Drexler et al. [1993]	Cross-contamination of a leukemia cell line
Reid et al. [1995]	Cross-contamination of U937 cells
MacLeod et al. [1997]	Dami megakaryocytes found to be HEL erythroleukemia cells
Dirks et al. [1999]	ECV304 endothelial cells found to be T24 bladder cancer cells
MacLeod et al. [1999]	18% leukemia cell lines submitted to DSMZ from originators cross-contaminated

problems in potential clinical application and hamper future development and acceptance of the technology.

One of the key issues in the development of hES cell lines has been the consistency and comparability among hES cells isolated at different centers under different conditions. Much work has been published on new culture and differentiation methods; however, each publication generally deals with a very limited number of cell lines. This leads hES cell researchers to ask whether the data produced can be applied more broadly to all hES cell lines. Approaches to technical standardization have been considered [Loring and Rao, 2006], and in addition one attempt to characterize hES cells on an international basis has been initiated [Andrews et al., 2005]. Generally, such attempts at standardization have been based on antibody markers developed for the study of early development in embryonal carcinoma models [Andrews et al., 2002]. Today a growing array of molecular and antibody markers for stem cells is developing that will be of use in the quality control of stem cell lines [Andrews et al., 2005; Loring and Rao, 2006; *see also* succeeding chapters]. In the following sections we explore the various techniques and methods that can be used to test stem cell lines to address issues of purity, identity, and stability and to qualify their use in stem cell research. Although much of this addresses hES cell lines, it is applicable equally to all cell lines whether derived from stem cells or not and whether embryonic, newborn, or adult.

1.2. THE CELL BANKING PRINCIPLE

To limit the chances of contamination and genetic change it is wise to keep the number of passages of cells to a minimum. An important approach to achieve this, called the master/working bank principle, has been adopted in industry for many years. This prescribes the establishment of a *master cell bank* that will provide the reference point for future work with a cell line. This bank should be well characterized and subjected to quality control tests. Ampoules from the master bank are then used to produce larger *working cell banks* that can be made available for experimental purposes or distribution

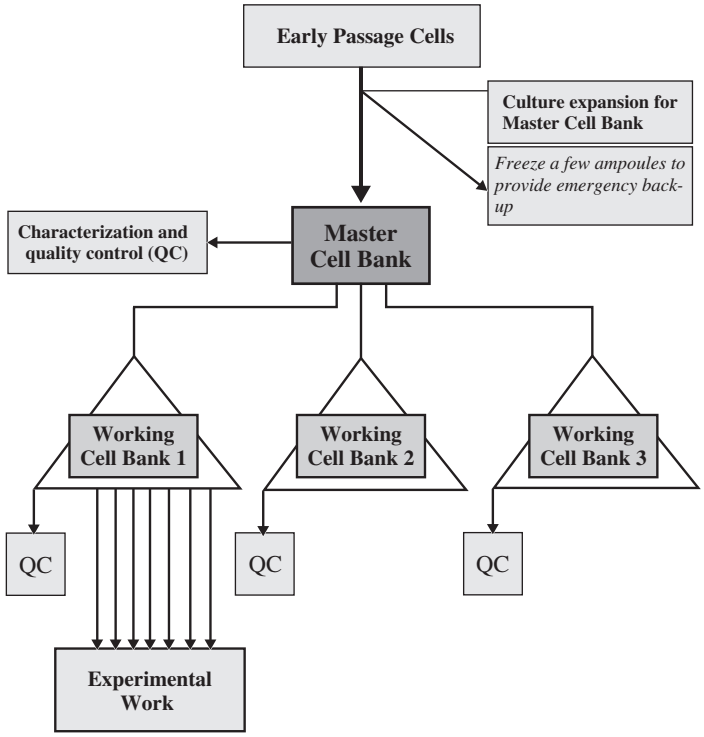

FIGURE 1.1. A scheme for the establishment of master and working cell banks.

to other workers [Hay et al., 2000]. The working cell bank should again be subjected to quality control, although this may be more limited and concerned mainly with identity and absence of contamination. If prepared correctly, this tiered master/working bank system (Fig. 1.1) can provide reproducible and reliable supplies of identical cultures for research work over many decades.

The quality control tests that should performed as a matter of routine for all cell banks include viability (typically Trypan Blue dye exclusion) and testing for absence of bacterial, fungal, and mycoplasmal contamination [Stacey and Stacey, 2000]. These tests should be performed on cultures after a period of at least 5 days, and preferably two passages of antibiotic-free culture, to ensure that any contaminants that may be suppressed by antibiotics do not go undetected. Other tests for authenticity (e.g., karyology, DNA fingerprinting, isoenzyme analysis, surface markers) and assays for the presence of viruses may be performed, but the exact profile of tests will depend on the type of cells involved and the intended use of the cells. For a general reference on cell banking and quality control *see* Stacey and Doyle [2000]. The specific approaches and methods are discussed in the following sections.

Effective cryopreservation protocols are clearly essential for cell banking (*see also* Chapters 2, 6, 8, 9). Standard methodologies for other cell lines and for preservation of mouse embryonic stem cell lines have not been reported as being very successful with hES cells compared to vitrification methods [Reubinoff et al., 2001; Zhou et al., 2004; *see also* Chapter 2]. Most vitrification methods used for hES cells have been adapted from methods established for bovine oocytes and embryos, and the most commonly referenced

modification used for hES cells is that of Reubinoff et al. [2001]. Successful methods reported generally utilize dimethyl sulfoxide and ethylene glycol as cryoprotectants, but details vary between publications. For a review of the methods currently used *see* Hunt and Timmons [2007]. Vitrification, while effective for preservation of hES cells, has a number of drawbacks including the need to carefully maintain storage temperatures close to liquid nitrogen temperatures to avoid devitrification, the costs of shipment under such storage conditions, the small volumes that can be frozen for each vial of cells required to be archived, and the rapid cooling rate required for successful vitrification.

1.3. CELL CHARACTERIZATION

1.3.1. Viability

Cell viability is obviously crucial but is also a characteristic that is all too often poorly addressed in cell culture. Numerous methods are available to determine viability. Each measures a different characteristic of cell biology (e.g., membrane integrity, membrane function, products released by cell damage or death, metabolic functions, enzyme activity, and clonogenic survival), and examples of techniques for measuring "viability" are given in Table 1.2. It is important to remember that the cell characteristics revealed in these tests can be affected differently by particular conditions in culture.

Trypan Blue dye exclusion [Patterson, 1979] is one of the most common methods used, although viability methods based on detection of apoptotic cells are also common [*see*, e.g., Sparrow and Tippet, 2005]. Whatever method is employed, it is important that it is relevant to the cell culture application and that it provides reproducible results. For stem cell cultures it is clear that a viability measurement cannot predict the proportion of stem cells present in a culture after a culture treatment or cryopreservation, but frozen stocks of cells still can and should be checked promptly for viability by recovering a vial of cells into culture immediately after cryopreservation.

1.3.2. Karyology

Visualization of the cell's chromosomes (karyotypic analysis) provides a valuable perspective on the physical structure of the genome. It has been used as a valuable tool for monitoring the genetic stability of a cell culture [*see*, e.g., Rutzky et al., 1980] and for recognizing the appearance of transformed cells, which are often aneuploid (having chromosome loss or duplication, or aberrant chromosomes with translocations, deletions, inversions, etc) and heteroploid (having a wide range of chromosome numbers per cell around or, more often, above the normal number).

The method of visualization of chromosomes most commonly used today employs colchicine or a similar compound to block cell division at metaphase when the individual chromosomes are separate and condensed and thus most readily visualized (*see* Chapter 5). The cells are then harvested, subjected to swelling with hypotonic saline, KCl, or saline citrate, and fixed in acetic methanol before applying them dropwise onto microscope slides to create the characteristic chromosome "spreads." These are then stained with Giemsa to visualize the condensed chromosomes. The ability to identify chromosome pairs and to resolve the nature of fine alterations in chromosome structure was realized through the use of trypsinization before Giemsa staining, which reveals banding patterns characteristic of each chromosome [Wang and Federoff, 1972]. Other

TABLE 1.2 Viability Testing for Animal Cell Cultures

Method	Principle and Comments
Dye exclusion (e.g., Trypan Blue, Naphthalene Black)	Dyes that penetrate cells are excluded by the action of the cell membrane in viable cells; thus cells containing no dye have functional membranes and are probably viable. **Advantages:** Rapid and usually easy to interpret **Disadvantage:** May overestimate viability since apoptotic cells continue to have active membranes and may appear viable.
Neutral red assay	Viable cells accumulate the red dye in lysosomes, and the dye incorporation is measured by spectrophotometric analysis. **Advantages:** Useful for certain toxicology assays **Disadvantages:** Time consuming and incubation conditions need to be optimized for each cell culture.
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay	MTT reduction is measured by the formation of a colored product, and this is indicative of biochemical activity. **Advantages:** Many tests can be performed rapidly in 96-well array in automatic plate readers. **Disadvantages:** Some inhibited cells show a low MTT reduction value that is not necessarily related to cell viability.
Fluorescein diacetate assay	Fluorescein diacetate enters the cell and is degraded by intracellular esterases, releasing fluorescein that cannot escape from cells with intact membranes, and thus the cells fluoresce when observed under UV light. **Advantages:** Rapid setup **Disadvantages:** Requirement for UV microscope or flow cytometer

techniques for studying karyology have been developed, such as Q and R banding and chromosome painting, but the Giemsa banding method described is the most widely applicable and generally useful method that has been used to characterize various stem cell culture systems. A typical method and review of other methods is given in Protocol 16.7 of Freshney [2005].

More recently, studies of hES cell cultures have revealed that they are prone to karyological changes, and a major challenge has emerged in maintaining the cells in the undifferentiated state while preserving a diploid karyotype. No culture system has been able to prevent completely the tendency of hESC lines to accumulate karyotypic abnormalities. It is felt that, owing to the nonideal culture systems for hESC lines, selection pressure is present in favor of chromosome duplications that confer an adaptive benefit [Draper et al., 2004; Andrews et al., 2005]. For hES cells there appear to be common patterns of chromosome alteration representing "adaptation" of these cells to in vitro culture conditions, notably changes involving chromosomes 12 and 17 [Draper et al., 2004]. An example of a karyotype of a normal hES cell line is shown in Figure 1.2.

It is important to verify that frozen stocks of cells retain the diploid karyotype and to check cells in use periodically to ensure that cells used to generate data for publication are

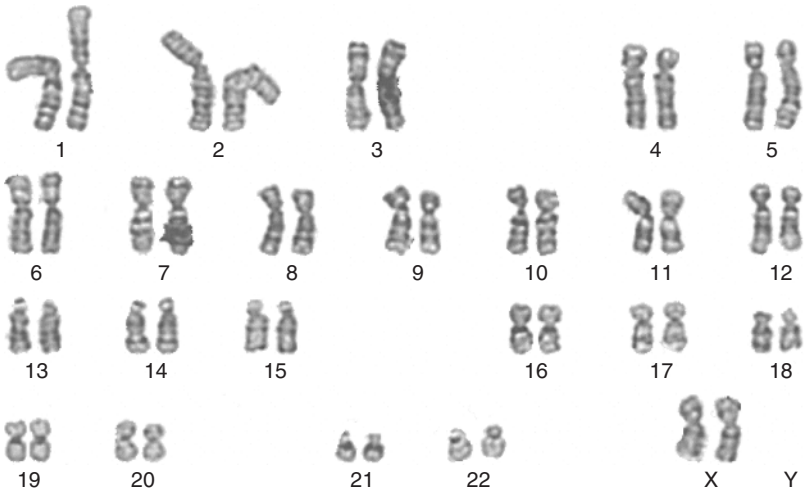

FIGURE 1.2. Diploid human ES cell karyotype.

diploid, unless deliberately studying transformed cultures. Many cytogenetics labs and some contract testing companies will provide a testing service for karyotype analysis. Routine diagnostic cytogenetic tests performed for clinical purposes often give analysis of 10–20 metaphase spreads. While such testing will identify the appearance of a transformed clone that is dominating the culture, it may not detect a low level of transformants occurring at the early stages of culture instability. It is often desirable to count at least 50 metaphase spreads in order to detect (or rule out) lower rates of mosaicism.

There are ongoing efforts to develop a chip-based or molecular assay for the karyotypic stability of hES cells in culture. One of these methods is based on single nucleotide polymorphism (SNP) genotyping. SNP arrays are very useful in mapping markers of genetic diseases and for detecting loss of heterozygosity (LOH) in cancer. Technological advances now enable the use of oligonucleotide SNP arrays to measure chromosomal copy number at high resolution [Zhao et al., 2004; Nannya et al., 2005]. This expands the utility of SNPs to detect nonreciprocal translocations, aneuploidy, and partial amplifications or deletions of chromosomes, and even amplifications or deletions of small chromosomal regions [Maitra et al., 2005]. The SNP array method has some advantages over conventional methods, mostly based on the resolution and size of genomic changes that can be detected. Based on a minimal detectable signal from 10 SNP sequences, currently available arrays of 550,000 SNPs have an effective resolution of about 28 kb, an array of 660,000 SNPs has an effective resolution of 25 kb, and, of course, increased density arrays that are sure to enter the field soon will improve this even further. One limitation that must be kept in mind, however, is the deficiency of molecular methods in analyzing heterogeneous or mosaic cell populations.

1.3.3. Identity Testing

1.3.3.1. Confirmation of Species of Origin. Isoenzyme analysis is based on measuring the charge-to-mass ratio of different isoenzyme activities using an agarose-based gel to separate the various polymorphic enzymes that can be identified for even just one enzyme reactivity. The cells are lysed, and the released enzymes are stabilized

in a buffer. Samples of this preparation are then subjected to agarose electrophoresis before the gel is treated with a specific enzyme substrate and the reaction is visualized by the formation of a purple formazan product. This method has been made more reliable with the advent of a commercial kit (AuthentiKit, Innovative Chemistry), and testing for specific enzymes can be performed within one working day. The enzymes usually used are selected for their ability to identify polymorphism between species while remaining monomorphic within species. A single enzyme test may not identify the species of origin, but certain enzyme substrates provide clear identification of the species of origin using just two or three enzyme substrates [Stacey et al., 1997; O'Brien et al., 1967; Doyle and Stacey, 2000] and may also identify embryonic isoforms. An example of an isoenzyme analysis is given in Figure 1.3.

Such typing enables rapid identification of the species of origin within one working day, and depending on the enzyme substrates used it may also allow the identification of the strain of origin for mouse cell lines. Such levels of differentiation will be valuable in a laboratory using cells from diverse species but may not be so useful in a laboratory that only cultures human cell lines.

Numerous molecular methods are now available for confirming the species of origin based on the amplification by the polymerase chain reaction (PCR) of conserved sequences [*see*, e.g., Stacey et al., 1997] and sequencing of specific genes such as cytochrome oxidase [Folmer et al., 1994; Herbert et al., 2003]. The latter method provides a sequence specific to each species that is supported by a growing database of sequence data maintained by the US National Center for Biotechnology Information [www.ncbi.nih.gov] and may well become a reference method for species identification.

Species identification is a useful part of cell authentication, and which method is used will be a decision based on the types of cell lines used in the laboratory, staff time available to carry out in-house testing, and access to appropriate facilities and equipment.

1.3.3.2. DNA Profiling for Cell-Specific Identification. Variable number tandem repeats (VNTRs) and short tandem repeats (STRs) are interesting sequences in the human genome that are comprised of repeated core units of sequences, some of which, when excised from the human genome with certain restriction enzymes, show polymorphism between individuals in the number of repeat units at a particular genomic locus. It was Alec Jeffreys who discovered that this variation might be used to identify and discriminate between human individuals by means of certain genomic probes and Southern blotting following electrophoresis [Jeffreys et al., 1985]. Other workers identified similar probes based on other VNTR sequences [Vassart et al., 1987].

Application of methods based on the hybridization of various probes to Southern blots of cell line DNA developed rapidly in the 1990s [Gilbert et al., 1990; Hampe et al., 1992; Stacey et al., 1992], and quickly these powerful methods, including PCR-based DNA profiling, revealed numerous cases of cross-contamination [e.g., MacLeod et al., 1999; van Helden et al., 1988].

STR loci consist of short, repetitive sequences, 3–7 base pairs in length. These repeats are well distributed throughout the human genome and are a rich source of highly polymorphic markers, which are routinely detected with PCR. Alleles of STR loci are differentiated by the number of copies of the repeat sequence contained within the amplified region and are distinguished from one another with radioactive, silver stain, or fluorescence detection after electrophoretic separation. Commercial kits that contain labeled primers to detect the number of repeats at 8 or 16 loci are available. These

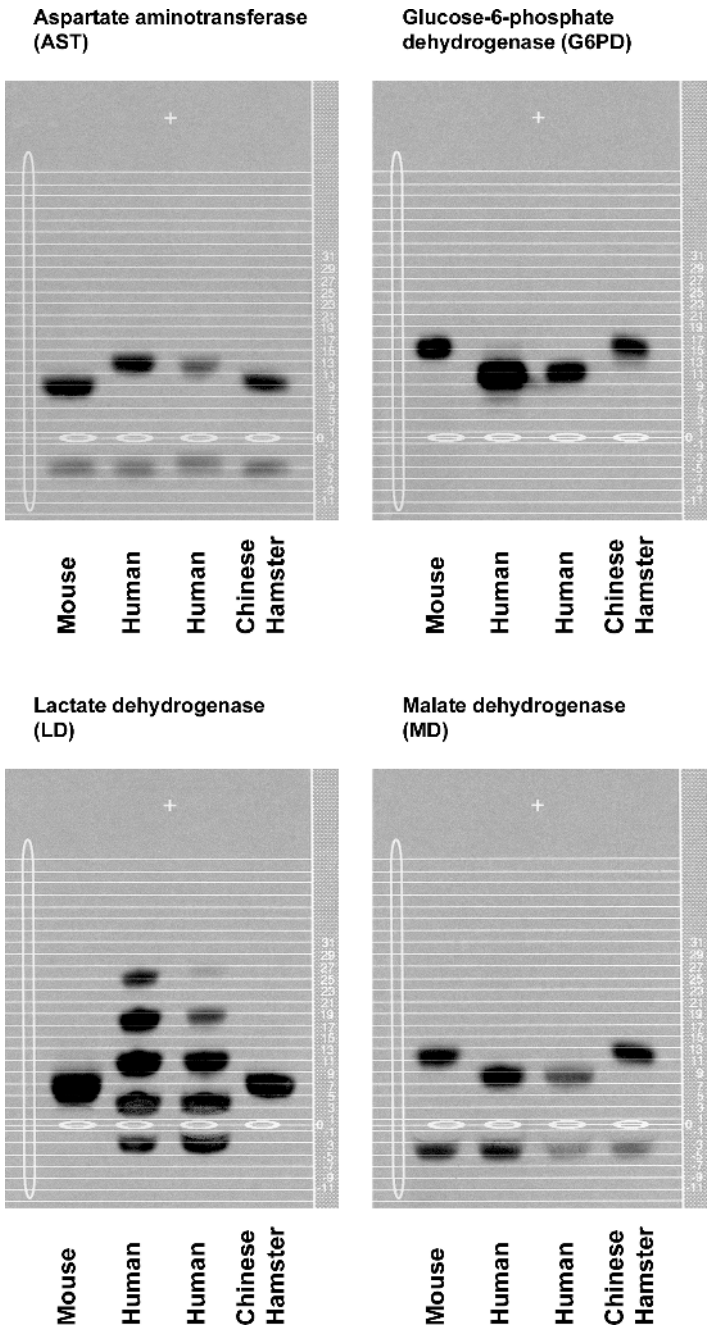

FIGURE 1.3. Isoenzyme profiles for cells from mouse, human, and Chinese hamster cell lines. (Photos and electropherograms courtesy of ATCC; modified from Freshney [2005].)

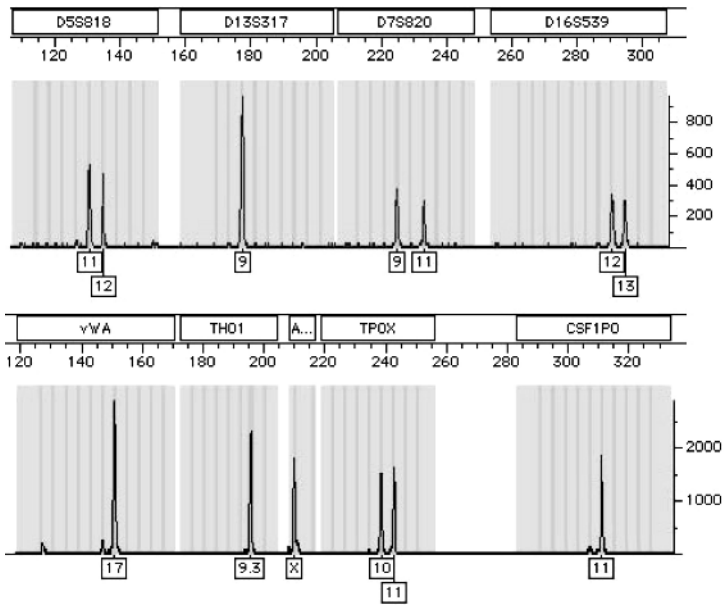

FIGURE 1.4. STR electropherograms.

loci satisfy the needs of several major standardization bodies throughout the world; for example, the US FBI has selected 13 STR core loci from the set of 16 to search or include samples in CODIS (COmbined DNA Index System), the US national database of profiles of convicted offenders. The matching probability of the 8 loci system ranges from 1 in 1.15×10^8 for Caucasian Americans to 1 in 2.77×10^8 for African-Americans, while the 16 loci system ranges from 1 in 1.83×10^{17} for Caucasian Americans to 1 in 1.41×10^{18} for African-Americans. A sample STR electropherogram is shown in Figure 1.4.

1.3.3.3. Antibody Markers. An important characteristic of any cell is its profile of antigen expression. A panel of antibodies has been commonly used to characterize hES cell antigens and show typical patterns of reactivity in such cultures (Table 1.3). Characterization of hES cells by immunophenotyping is best performed qualitatively by using immunohistochemistry together with quantitative analysis using flow cytometry. Several current markers are largely based on a single precursor (lactosylceramide) that undergoes biochemical modification including glycosylation to create the different epitopes representing the stage-specific early antigens (SSEAs) [Gooi et al., 1981; Kannagi et al., 1982, 1983]. Some of these markers have proven useful for tracking the differentiation of hES cells [Draper et al., 2002] and are key identifiers for hES cells, although they are not unique to this cell type. New markers are needed that have a direct functional relationship to stem cell biology.

It is important to take some care when sourcing antibodies for the characterization of stem cell lines. Each antibody used must be of the correct type, specificity, and titer, as described by the supplier. Fundamental approaches to this have been described elsewhere [Hybridoma Bank, USA www.uiowa.edu/∼dshbwww/], but at the very least new stocks of antibody should be compared in parallel with existing acceptable stocks and antibody isotype controls should be included in experimental work. It is important

TABLE 1.3 Typical Antigenic Marker Expression of hES Cells [Draper, 2002]

Antigen	Typical Reaction on Undifferentiated hES Cells	Typical Reaction on differentiated hES Cells
SSEA-1	–	+
SSEA-3	+	+*
SSEA-4	+	+*
Oct-4	+	+*
Alkaline phosphatase	+	+*
TRA-181	+	+*
TRA-160	+	+*

*May be downregulated during differentiation [Cai et al., 2006; Draper et al., 2002]

to differentiate between antibodies that might be involved in the detailed characterization of stem cell lines. Table 1.3 identifies a number of antibody markers that might be used. In characterization of master stocks and for publications, comprehensive antigenic characterization may be required. In the case of the UK Stem Cell Bank, the panel of markers such as those highlighted in Table 1.3 are applied to both master and distribution stocks (*see* Fig. 1.1) to ensure that the material released to researchers is of an acceptable quality. Some quality control on cultures used for experimental work is clearly desirable, but it is not practical to carry out detailed profiling in this situation. Observation of hES cell morphology may give an indication of the state of differentiation in routine observation of experimental and stock cultures, but it could be valuable to have flow cytometric data on a marker of differentiation, such as SSEA-1, at critical points in the use of cultures.

1.3.3.4. Gene Expression. The study of gene expression profiles is extremely valuable but is largely just developing at a research level. The application of such tests in routine work has yet to be validated, but there are a number of genes associated with stemness that should be checked (e.g., Nanog, Oct-4) as well as testing examples of genes that are associated with differentiation. Care should be taken to avoid primers that also detect pseudogenes and could cause confusion. At this point in time it is probably not wise to set a specific panel of genes for quality control until better culture methods and knowledge of the nature of stem cells are obtained. However, it will clearly be useful to gather information on gene expression profiles on candidate stem cell genes and markers of different differentiation lineages. Examples of genes being analyzed in parallel in a current international study of over 60 hES cell lines are given at www.stemcellforum.org.uk/.

Recent research comparing the transcriptomes of multiple hES cell lines has identified a set of approximately 100 genes that are highly expressed in undifferentiated hES cells [Bhattacharya et al., 2005]. In a study characterizing 17 different hES cell lines by whole genome gene expression array analysis, almost all of the cell lines show a similar expression pattern for these 100 genes [Josephson et al., 2006]. While global gene expression can be indicative of the state of differentiation of a particular hES cell line, it is most useful when compared side-by-side with a reference standard for the particular differentiation state under investigation. For example, human embryonal carcinoma cell lines and stable, karyotypically abnormal hES cell lines have been proposed as such standards [Plaia et al., 2006]. Microarray expression data are best verified by quantitative real-time reverse transcriptase PCR (RT-PCR), using a marker set for genes commonly associated

with the hES cell differentiation state of interest. For the undifferentiated state, a few accepted markers are listed in Table 1.3.

1.3.3.5. Pluripotency. This is clearly a key measure of stem cell line performance in which the expected outcomes may vary depending on the cell type (hES, mesenchymal stem cells, etc.). There are a number of ways of measuring pluripotency including the following:

- Germ line competence (only acceptable for nonhuman stem cells)
- Teratoma formation in immunocompromized mice (*see* Chapter 6)
- Generation of embryoid bodies with the three germ layers represented (*see* Chapter 3, 4, 6)
- Differentiation in vitro into cell types representing the three germ layers (*see* Chapters 2, 6).

In hES cell research, teratoma formation is generally accepted but is not yet well standardized—molecular assays of gene expression may help in the future. This is clearly a challenging area that requires considerable research effort before reliable QC methods can be selected with confidence. (*See* Chapter 6 for protocols and a discussion of teratoma formation.)

Briefly, undifferentiated hES cells are injected into immunodeficient mice. SCID/Beige is a commonly used strain. The cells are placed either intramuscularly into the hindlimb, in the testis, or under the kidney capsule. There have not been careful comparisons of injection locations, but all three mentioned above appear permissive for pluripotent hESC differentiation. Tumors form in all mice and are excised for analysis after approximately 8–12 weeks, based on tumor size. The first analysis performed is histological, preferably performed by an experienced pathologist. In this analysis, the pathologist attempts to identify tissues representative of the three germ layers (ectoderm, endoderm, and mesoderm). This may be followed by immunohistochemical and gene expression analyses to demonstrate that the teratomas contain specific terminally differentiated lineages. Typical teratomas are generally well demarcated from the host tissue and exhibit organized clusters of cells, which may include cartilage, mineralized bone, villi, smooth muscle, nerve bundles, neural rosettes, liverlike structures, ducts, cystic epithelium-lined spaces, and various types of epithelia.

1.4. STERILITY

Bacterial and fungal contamination generally prevents work with affected cultures as they become turbid with organisms that completely overwhelm and kill the cells. Contamination can arise from a variety of sources in the laboratory environment (e.g., water baths, fridges, sinks, cardboard boxes), and avoidance of contamination is most effectively achieved with good aseptic technique, correct use of class II safety cabinets (see Appendix 1), and maintenance of a clean and tidy cell culture laboratory [Freshney, 2005, Chapter 6]. The use of antibiotics may be helpful to avoid loss of cells in circumstances where the risk of contamination is high, for example, in primary mouse embryonic feeder cultures or in routine experimental work where environmental contamination is very high. However, routine use of antibiotics for cultures that should be "clean" is not

recommended and certainly not for the preparation of cell banks. Antibiotics can affect the function of cells, and routine use of specific antibiotics can encourage the development of resistance in microorganisms, leaving no fallback treatment for protection of critical cultures. They may also suppress but not eliminate the growth of mycoplasma, increasing the risk of false-negative results in mycoplasma testing (*see* Section 1.5).

Cultures can be tested by inoculation of the supernatant medium from a culture into bacteriological broth followed by incubation at both standard cell culture temperature (typically 35–37°C) and room temperature to reveal growth of contaminants with different optimal growth temperatures (see scheme in Fig. 1.5). Reliance on recognition of contamination by appearance of turbidity in inoculated broths can lead to difficulties because nonmotile organisms may not produce turbidity, while cell debris may cause turbidity. Accordingly, it is usual to subculture all broths onto solid nutrient agar media at the termination of their incubation period to detect any culturable organisms.

Detailed reference methods for this approach are published in national pharmacopoeia [European Pharmacopoeia, 2006a; US Food and Drug Administration, 2005a].

In addition to these standard culture methods, there are a number of kits available from tissue culture companies that may have useful applications in stem cell work. Such methods may also have valuable application alongside traditional culture methods because some are more rapid and may detect more fastidious organisms that can arise in cell cultures but would not be detected by the standard sterility tests described above. However, the range of organisms that could potentially be isolated in broth and agar cultures can be significantly expanded by supplementing these tests with additional growth media incubated in a CO_2-containing atmosphere [Cobo et al., 2005 and the references

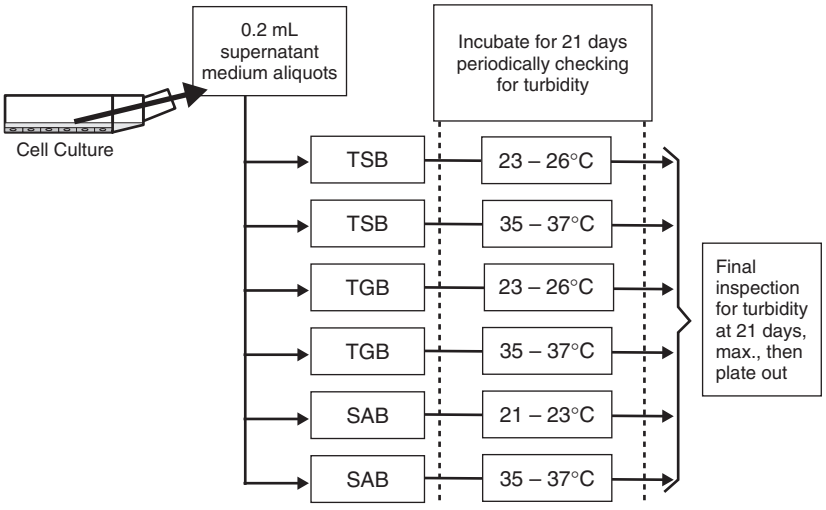

FIGURE 1.5. Example of a typical sterility test. TSB, trypticase soy broth used to isolate aerobic and facultative aerobic organisms. TGB, thioglycollate broth used to isolate anaerobic and microaerophilic organisms. SAB, Sabourard's broth used for isolation of fungi. Alternative media could include Todd–Hewitt broth (instead of TSB), brain-heart infusion broth (instead of TGB), and YM broth (instead of SAB). Additional media may be added that contain blood or serum (e.g., nutrient agar incorporating 5% defibrinated rabbit blood) [Cour, 2000].

therein]. By far the most common fastidious contaminants are mycoplasma species, and specific tests for these organisms are discussed in the next section.

1.5. MYCOPLASMA TESTING

Mycoplasmas are organisms of the order *Mollicutes*, which are much smaller than typical bacteria and, while similar to bacteria, have a number of distinct characteristics that give them special potential to cause problems in cell culture work. They have a degree of resistance to the antibiotics normally used in cell culture and can pass through standard bacteriological filters. Furthermore, they do not necessarily affect the growth rate of cells and do not usually produce visual turbidity in the supernatant medium of a contaminated culture, and thus may go unnoticed. Persistent contamination with mycoplasma can cause a diverse range of permanent deleterious effects on cell lines, and it is vital to carry out routine screening of new cell cultures coming into the laboratory to avoid potential spread to other cultures.

There are a number of techniques for mycoplasma detection that have been used widely, and examples are given in Table 1.4 [for reviews *see* Del Guidice and Gardella, 1984; Rottem and Naot, 1998]. The reference methods used in industry are Hoechst 33258 staining of Vero cells inoculated with culture supernate and culture using selective broth and agar media [European Pharmacopoeia, 2006b; US Food and Drug Administration, 2005b]. For routine screening, direct PCR or Hoechst 33258 staining (*see* Table 1.4) are useful, but these methods are generally not as sensitive in routine use as culture, which, in the absence of a more sensitive method, should be used for any important frozen stocks of cells that will be needed for future use. A scheme for a typical culture method and Hoechst 33258 staining is given in Table 1.5, which shows the added benefit of dual testing to give early screening results that will be confirmed some time later by the more sensitive culture method. It should be noted, however, that a considerable degree of skill is required to culture mycoplasma, and the need to include a positive control may not be acceptable in some laboratories without proper quarantine facilities. There are also a variety of proprietary methods available on the market, but, as indicated for sterility test kits (*see* Section 1.4), it is important to test these against a standard proven methodology before putting full confidence in them. This is important because the long-term consequences of missing positive cultures can be catastrophic in terms of wasted technical time and effort and invalidation of scientific data.

1.6. OTHER MICROBIAL CONTAMINANTS AND POTENTIAL BIOHAZARDS

A broad range of microorganisms could potentially contaminate human stem cell lines, and it would be impractical and too expensive to screen all cell lines for all of these organisms. While the future use of stem cell lines for therapy would require intensive investigation for microbial contamination, the use of these cells for research purposes should be based on sensible precautions that apply to any unscreened human cells. These precautions include a risk assessment based on any information available on the cells, use of aseptic technique, and good cell culture practice [Coecke et al., 2005]. Generally speaking, the risk associated with such cultures will be very low; however, there is no room for complacency because these cells could potentially carry a range of viruses due

TABLE 1.4 Comparison of Different Methods for Detection of Mycoplasma

Technique	Advantages	Disadvantages
Broth and agar subculture	Highly sensitive	Bacteria may grow on selective media
	Well-established method	Will not detect nonculturable strains
	Standard methods available in national pharmacopoeia	Long incubation periods (approx. 50 days total)
Vero cell culture inoculation and DNA stain	Results in 3 days	Vero test cells must be maintained and prepared
	Standard method available in national pharmacopoeia.	High-power (×100 objective) UV-fluorescence microscopy required.
		Nuclear fragments from cells and small bacteria may give false positives with inexperienced workers.
PCR	Results within 1 day	Sensitivity needs to be demonstrated and monitored carefully.
	Large numbers of samples readily screened	Nested PCR may give rise to false positives.
6-Methylpurine deoxyriboside (6-MPDR)	Simple end point (cell death)	Indicator cells must be maintained and prepared.
		Five days incubation required.
Added to sample and indicator culture (e.g., 3T3, Vero).		False negatives have been observed when compared with other methods [e.g., Uphoff et al., 1992].
Mycoplasma contamination detected due to mycoplasmal adenosine phosphorylase.		
Converts 6-MPDR to toxic metabolites that kill indicator cells.		
Mycoplasma RNA hybridization.	Sensitivity reported to be high but may vary.	Radioactive versions require scintillation counting equipment
		Difficult to discriminate between negative and low positive results.

to their origin in the human reproductive tract (e.g., herpes virus, HIV, hepatitis B), and a careful combination of risk assessment (to avoid use of cells with a significantly raised risk of contamination with serious human pathogens), containment (e.g., use of sealed culture vessels, use of a Class II safety cabinet [*see* Appendix 1]), treatment of cell culture waste as if infectious), and quarantine of cell cultures newly arrived in the

TABLE 1.5 Outlines of Typical Protocols for Reference Methods for Detection of Mycoplasma

Time (days)	Broth and Agar Culture	Hoechst stain
0	1. Inoculate 200-μL samples of supernatant medium into broth and onto an agar plate (contains thallous acetate and pig serum) and incubate in a anaerobic environment.	1. Replace medium on Vero cell monolayer (on glass coverslip) with supernatant medium to be tested and incubate in 5% CO_2:95% air.
Day 3		2. Remove medium and fix monolayer with fixative (1:3 acetic acid:methanol) for 3 min. Replace with fresh fixative for a further 3 min. 3. Drain fixative, air-dry slides and immerse in 2 mL of stain (0.1 μg/mL bisbenzimide Hoechst 33258 in Hanks' BSS without Phenol Red or PBSA) and incubate for 5 min at room temperature in the dark. 4. Remove stain, add nonfluorescent mountant, and apply a coverslip. 5. Scan the stained area (100× epifluorescence) for fluorescent cell nuclei (acts as a control for stain) and for small fluorescing particles over the cytoplasm.
Days 3–5	2. Inoculate 0.2 mL of sample from broth to a selective agar plate and observe original plate.	
Day 14	3. Inoculate selective agar plate from broth and observe plates inoculated at day 0 and days 3–5.	
Day 21	4. Check for pH change in broth and subculture if altered. Observe all plates and return to incubation conditions with final observation at 28 days for each plate.	
Day 42	5. Observe any remaining plates for colonies.	

Positive cultures should be discarded along with any media and reagents used for the affected cultures. It is possible to eliminate mycoplasma from cell lines with certain antibiotics [Uphoff and Drexler, 2004]. However, success rates for complete eradication are low and the toxic effects of the antibiotics used may alter the characteristics of the cell line.

laboratory until evaluations and basic quality control have been carried out should lower the risk.

1.7. QUALITY CONTROL OF CULTURE CONDITIONS, REAGENTS, AND MEDIA

In cultures of stem cells there is great potential for variability and instability. It is helpful in dealing with these issues to try to control the variation in the nutritional and environmental influences to which the cells are subjected. Calibration and monitoring of temperature and CO_2 levels are clearly important, especially in multi-user labs, where incubators may rarely reach standard culture conditions during the working day. Use of consistent sources and consistent composition of key media and supplements is also important. In addition, any critical reagents most likely to suffer from batch-to-batch variation, such as bovine serum and serum replacement formulations, should be tested before routine use, and a batch reserved, to ensure they provide acceptable growth of cultures.

It is also important to remember that feeder cells are also a potential cause of contamination and this is a particularly high risk with primary cell feeder cultures. To avoid this significant risk of contamination it is wise to establish large stocks of cryopreserved feeder cell preparations that can be quality controlled (i.e., viability, sterility, and mycoplasma as a minimum) before use (*see* Chapter 2).

1.8. CONCLUSIONS

The culture of hES cell lines is challenging, and they are prone to variation and instability. Culture methods and characterization of the stem cell nature of these cells are still developing, and standardization of some quality control methods can be difficult. Testing cell banks for the most common contaminants (bacteria, fungi, and mycoplasma) is essential to avoid spread of contamination in the laboratory, which can have a significant impact on efficiency and quality of laboratory work and scientific data. While characterization techniques in general will develop rapidly with time, there are current markers for confirming typical expression profiles of hES cells and some that give an indication of the level of cell differentiation. Genetic characterization is also important to confirm identity and also to check for chromosomal changes that indicate overgrowth of diploid cells by transformed cells that may no longer express all the characteristics of hES cells. As the basic science develops, it will be important to be responsive to review and update quality control methods and establish more quantitative methods for phenotypic analysis that may also become important factors in the future development of stem cell therapy.

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APPENDIX 1. SOME POINTS FOR CONSIDERATION IN THE USE OF CLASS II BIOSAFETY CABINETS

- Switch on the cabinet some time before use.
- Disinfect the work surface before you begin work.
- Disinfect bottles of media, etc. as they are passed into the cabinet.
- Do not clutter the cabinet or obstruct air grills (airflow).
- Separate waste and sterile reagents (e.g., on different sides of the work area) and manipulate cultures and reagents in the central zone.
- Handle only one cell line in the cabinet at one time.
- Remove reagents and disinfect surfaces after use.
- Leave cabinet switched on for a period after use (replace front cover).
- Periodically clean the cabinet thoroughly with an appropriate disinfectant.