

# 15 Fundamental Issues for Cell-Line Banks in Biotechnology and Regulatory Affairs

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## 15.1 INTRODUCTION

For many years, animals and primary cell cultures from animal tissues have been used for research and for the manufacture and testing of vaccines. In the 1960s, human diploid fibroblast cell lines were developed for the manufacture of vaccines, and more recently, continuous cell lines from other species have been used in biotechnology. Notable examples include BHK cells for foot-and-mouth disease vaccine, CHO cells for production of recombinant proteins, and hybridoma cell lines for the production of monoclonal antibodies (Griffiths and Doyle, 1999; Stacey, 2000). The increased use of cell lines for the manufacture of a range of biological medicines has been driven

by the need for more reproducible and safe cell substrates. In the 1950s, batches of polio vaccine were found to be contaminated with SV40 virus originating from the primary monkey kidney cells used in the manufacturing process. Although studies of vaccinees failed to show any direct adverse effects at the time, this incident in particular highlighted the need for safe and standardized cell substrates. Cell lines appeared to offer a solution but were susceptible to genetic variation and contamination if maintained by serial passage. Fortunately, mammalian cell lines are generally amenable to cryopreservation, and this made it possible to prepare reliable cell banks of homogenous aliquots that could be stored in a stable state at ultra-low temperature. Sample ampoules from the banks could then be thoroughly investigated for infectious agents before use, thus, enhancing the safety of vaccines produced from the banked cells. The benefits of standardized cell banks can be applied to all applications of cell lines, and cell-banking procedures are a vital preliminary step for any application of cell lines in which reproducibility and reliability are key issues.

Animal cell lines play an increasingly important role in the establishment of *in vitro* methods for diagnosis, prophylaxis, and treatment of human and animal diseases. They provide important substrates for biological assays of vaccine potency, vaccine efficacy, product toxicology, and detection of adventitious agents in products. In these applications, a primary driving force for use of cell lines is the international pressure to refine, reduce, and replace the use of the use of animals (the “3Rs” principle).

The principles of cell banking and associated safety testing and quality control are now enshrined in regulatory guidelines for the manufacture of products from animal cell substrates. They are also implicit in the development of cell lines used in processes subject to patent applications and in the provision of cell cultures from public collections for research and development. This chapter deals with key issues in the establishment, maintenance, and use of cryopreserved cell line banks.

Author: This phrase (the “3 Rs”) was used for something else in a different chapter – rephrase?

## 15.2 FUNDAMENTAL ISSUES IN CELL CULTURE

Once a cell line has been established that has useful characteristics, its value is only sustained if the culture remains free of contaminating microorganisms (pure) and other cells (authentic) and shows stability of its characteristics on passage *in vitro*.

Cell culture growth media will readily support the growth of many bacteria and fungi, and thus, contamination with these organisms from the laboratory environment is often the cause of overwhelming infection and cell death. A more subtle form of contamination may occur because of mycoplasma, which are often closely associated with the cell membrane and do not produce the turbidity or colonies typical of bacterial and fungal infections, respectively. Mycoplasma are also smaller in size than most bacteria. The presence of these organisms may thus not be suspected, even when the cells are viewed by microscopy. Care must be taken to exclude environmental sources of microorganisms (e.g., via routine disinfection of waterbaths and sinks, regular laboratory cleaning regimes, exclusion of cardboard and other carriers of fungal spores) and to screen cultures for contaminants including mycoplasma (see following). The cell-doubling times of microorganisms are much shorter than for animal cells (i.e., 1 to 2 h vs. 20 to 30 h), and thus, trace contamination of cryopreserved stocks may result in overwhelming infection when thawed and recovered into culture. Therefore, wherever practicable, the use of antibiotics should be avoided, and especially when establishing cell banks for future use.

Cells may also be contaminated with viruses that can affect the characteristics of the culture or that may present a hazard to laboratory workers or patients treated with animal cell products. Viral contaminants may be introduced via growth media components of animal origin (notably bovine serum and porcine trypsin) or from the tissue of origin of the cell culture (Doblhoff-Dier et al., 1991; Erickson et al., 1991; Hallauer et al., 1978). Although viable virus titer may be reduced by freezing, the use of cryoprotectants to preserve cell cultures may also enhance the survival of cell-free virus and intracellular virus. In the case of cell-associated virus, snap-freezing of cells

Author: Year in Refs. is 1971 – which is correct?

Author: No Doblhoff-Dier et al. 1991 in the Refs. – please add.

**TABLE 15.1**  
**Publications Describing Cell Lines Not Matching Their Purported Origin**

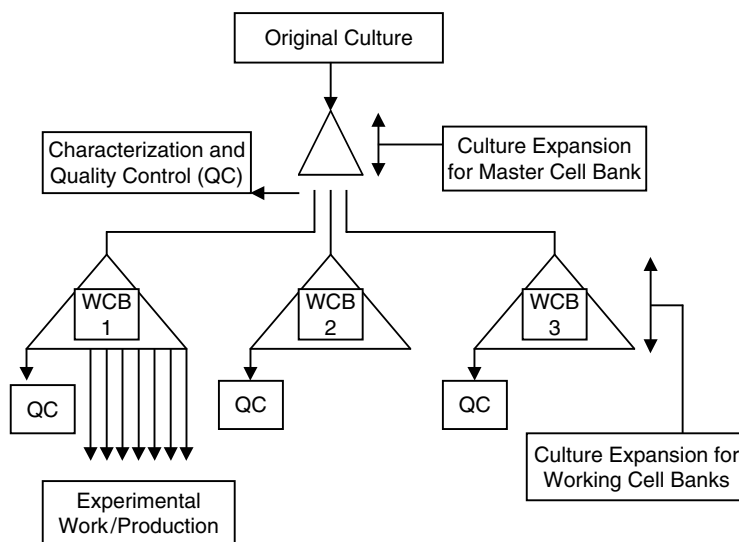
	Reference	Cell Lines
Author: Year is 1981 in Refs. – which is cor- rect?	Gartler (1967)	Breast cancer cell line cross-contamination
	Culliton (1974)	HeLa cell contamination of cells worldwide
	Nelson Rees et al. (1977)	Widespread cross-contamination of human breast tumor cell lines and others
	Harris et al. (1991)	Putative human Hodgkins Disease cell lines cross-contaminated with nonhuman cells
	De Benedetti et al. (1987)	Retraction of paper because of discovery of contaminated cells
	Masters et al.(1988)	Cross-contamination of bladder cancer cell lines
Author: Year is 1989 in Refs. – which is cor- rect?	van Helden et al. (1988)	Cross-contamination amongst 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 leukaemia cell line
	Reid et al. (1995)	Cross-contamination of U937 cells
	MacLeod et al. (1997)	Dami megakaryocytes found to be HEL erythroleukaemia cells
	Dirks et al. (1999)	ECV304 endothelial cells found to be T24 bladder cancer cells
	MacLeod et al. (1999)	18% original human tumor cell lines cross-contaminated
Drexler et al. (1999)	16% human haematopoietic cell lines cross-contaminated	

and other treatments that cause cell lysis can significantly increase the level of free viable virus in suspension on thawing. Freeze–thaw cycles are sometimes used to promote release of intracellular virus, and thus enhance virus detection.

Maintenance of cell cultures over extended periods by serial passage is a high-risk approach to provision of cells for both research and product manufacture. Even continuous cell lines that appear to be stable may show genotypic and phenotypic variation over extended periods of serial passage. Long-term passage also raises the risk of laboratory accidents, contamination with microorganisms, or cross-contamination with other cells. The latter occurrence has been well documented since the early days of cell culture, and Table 15.1 gives selected examples of reported cases of cross-contamination. Despite such frequent reports, the occurrence of cross-contaminated or mislabeled cell lines continues to be a serious scientific issue that receives insufficient attention (MacLeod et al., 1999; Stacey et al., 2000). The preparation of cryopreserved cell banks that are well characterized is central to resolving such concerns.

### 15.3 STANDARDIZATION

When a new cell line is established *de novo* or received in the laboratory, a viable stock of cryopreserved cells should be established at an early stage, and an initial stock of 2 to 3 ampoules will provide vital backup material in the event of accidental loss. Whether or not a cell line is likely to be used over an extended period of time, it is wise to expand cells from the initial stock of frozen cells to establish a master cell bank. It is also good practice to keep a careful note in laboratory records of early culture passages, including details of the culture medium and growth conditions. As the master cell bank will provide the reference point for all future work with a cell line, it should be well characterized and subjected to appropriate quality control tests. For future reference, it is useful to compile a record of all characterization performed on the master cell bank and other passage material. Such characterization may include cytogenetics, molecular investigations, morphology, biochemical functions, secreted products, and surface markers. Ampoules from the master bank are used to produce larger working cell banks that can be used for experimental or manufacturing purposes. The working cell bank should again be subjected to quality control. If prepared correctly, this tiered master/working bank system (Figure 15.1) can provide reproducible and reliable supplies of identical cultures over many decades.



**FIGURE 15.1** Preparation of master and working cell banks.

The quality control tests that should be performed as a matter of routine for all cell banks include viability (typically trypan blue dye exclusion), sterility (i.e., absence of bacteria and fungi), and testing for mycoplasma (Cord et al., 1992; Stacey and Stacey, 2000). These tests should be performed following a period of antibiotic-free culture to ensure that any contaminants that may be suppressed by antibiotics do not go undetected. Other investigations for authenticity (e.g., karyology, DNA fingerprinting, isoenzyme analysis, surface markers) and 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). Where cells are intended for use in the manufacture of medicines or as part of medical therapies to which patient tissues are directly exposed, a range of additional requirements are invoked that will be necessary for acceptance and licensing of any respective pharmaceutical or biological product (e.g., World Health Organization, 1998; International Conference on Harmonization, 1997). In particular, these requirements include testing for potential viral contaminants of the cells, which may have established persistent infection of a culture without any cytopathic effect. Such infections include human retroviruses (such as the case of the MT4 human leukaemic cell line) and Epstein-Barr virus expressed by human B-lymphoblastoid cell lines, but a range of murine viruses (including some pathogenic for humans) have also been found in nonhuman cell lines (Nicklas et al., 1993). In addition, cell lines of mouse and hamster origin (e.g., mouse myeloma cell lines, L929, CHO) are known to express endogenous retroviruses. However, these are not considered to represent a serious health hazard, and their elimination can be demonstrated during downstream processing of cell culture products.

## 15.4 ESTABLISHMENT AND VALIDATION OF PROCEDURES

### 15.4.1 OPTIMIZATION

The preservation of mammalian cell cultures is generally assumed to be straightforward, and the most commonly used protocols involve dimethyl-sulphoxide at 5 to 10% v/v as cryoprotectant with a linear cooling rate of approximately  $-1^{\circ}\text{C}/\text{min}$  down to a terminal temperature of at least  $-70^{\circ}\text{C}$  before transfer to ultra-low-temperature storage. However, there are a number of issues in the

**TABLE 15.2**  
**Viability Testing for Animal Cell Cultures**

Method	Principle and Comments
Dye exclusion (e.g., trypan blue, Evans 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 Rapid and usually easy to interpret Such methods overestimate viability, and apoptotic cells continue to have active membranes; thus, cells committed to this form of cell death may appear viable
Neutral red assay	The red dye is accumulated in the lysosomes of active cells and is measured by spectrophotometric analysis Commonly used in toxicology assays Relatively time-consuming, and incubation conditions should be optimized for each cell culture
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylterazolium (MTT) assay	Reduction of MTT is measured, and this is indicative of degree of biochemical activity Reduced metabolism in a short-term assay may be reversible and is therefore not necessarily related to cell viability
Fluorescein diacetate assay	The diacetate is split by active membrane esterases releasing fluorescein, which cannot pass through the membrane, and being trapped in the cell produces fluorescence in viable cells observed under ultraviolet light

cryopreservation of cell cultures in general that may need to be considered to ensure reliable recovery of thawed cells. When preserving an animal cell culture for the first time, it may be valuable to assess and validate the preservation method and its potential effects on the culture. A first step is to perform cryoprotection toxicity tests that will establish the optimum conditions (temperature, concentration, duration, etc.) for cryoprotection. The cooling profile may also be investigated using a cryomicroscope stage that enables direct visualization of cells as they cool and freeze. This may be particularly useful if the culture contains cells of variable morphology that may show differential survival on cooling. The optimized profiles can then be applied practically using the versatility of rate-controlled freezing equipment (see following). Subsequently, the viability and recovery of thawed cells can be investigated to establish the optimum recovery procedures (rate of warming, recovery medium, removal of cryoprotectants, etc.). The choice of a technique for viability testing can be critical, and there are a number of methods used widely in animal cell culture (Table 15.2). Techniques such as trypan blue dye exclusion may significantly overestimate cell viability and may well assess cells committed to programmed cell death (apoptosis) as viable, as their membrane function is retained (Solis-Recendez et al., 1994). Viability assays are obviously only one indicator of the success of the preservation process, and functional assays should be employed to validate appropriate performance of recovered cells.

#### 15.4.2 VALIDATION OF PRESERVATION PROCEDURES AND QUALIFICATION OF CELL BANKS

Homogeneity and reproducibility is a fundamental requirement of cell banks. The numbers of cells preserved in individual ampoules should be demonstrated as sufficient to regenerate cultures that faithfully reproduce the characteristics of the parental culture. Diluting out cells simply to achieve a larger number of ampoules for the bank is often a false economy, as cultures from such banks usually take much longer to achieve adequate cell density for further passage, thus delaying progress. A change of culture procedure such as adaptation to serum-free medium (see following) or scale-up of the preservation process for larger cell banks will also require careful validation.

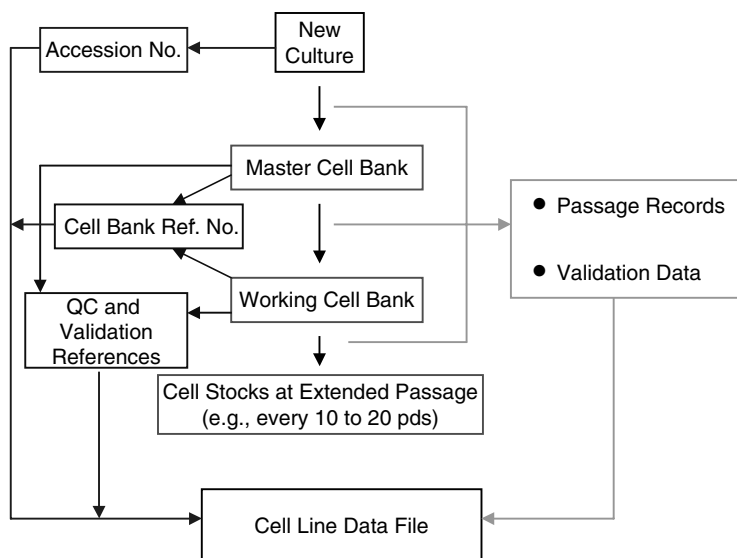


FIGURE 15.2 Documentation of cell banks and validation.

Documentation of procedures, cultures, and reagents used are important to promote good-quality scientific work but will also be crucial to provide traceability where the cultures are used for the manufacture or testing of medicinal products. Not only will the qualification of cell banks need to be documented, but it is also important to establish the stability of critical characteristics of the culture with passage to ensure reliable performance of cells derived from cell banks. The types of documentation and how they are used to qualify the testing and validation of cell lines are illustrated in Figure 15.2.

### 15.4.3 PRESERVATION OF SERUM-FREE CULTURES

Where cell cultures are used in a manufacturing process (e.g., recombinant therapeutic proteins from CHO cells, viral vaccine from cell substrates, monoclonal antibodies from hybridomas), manufacturers try to avoid the use of bovine serum in the cell growth medium to simplify the downstream processing and purification and to avoid the risks associated with raw materials of animal origin. However, the transfer of cultures to serum-free growth medium generally subjects the cells to stress and may lead to permanent changes in the culture. Thus, it is important to recognize that following adaptation to serum-free growth medium a cell culture should be evaluated and characterized again to establish whether any changes have occurred that may affect performance, quality, and safety of the cell substrate. As part of this program, cell banks should be established under serum-free conditions. The removal of serum from the cryopreservation medium may significantly influence cell survival, as serum is a complex protective agent that may help to prevent cell damage during freezing and thawing. Cryoprotectants have been developed for serum-free preservation of cell lines, and some are available commercially.

For cell lines used in the manufacture of biological medicines, there are generally restrictions placed on the maximum number of population doublings permitted between master cell bank and the cells used for the production process. This is particularly important where human diploid fibroblast cultures are used that have a finite lifespan *in vitro* (Wood and Minor, 1991). Thus, in the development of new serum-free cryoprotective solutions, it may be necessary to confirm that cells recovered after thawing and on serum-free passage have not been subject to high levels of cell death, which would increase the number of population doublings at each passage and may also lead to alteration of the characteristics of the culture.

#### 15.4.4 COOLING DEVICES

To ensure the reliability of an established cryopreservation method, it is important to have a device that will ensure a reproducible rate of cooling. This may be achieved by two-stage “passive freezing,” whereby the cells are placed in a static system exposed an environment of ultra-low temperature. Some means of insulation between cells and the ultra-low-temperature environment then permits a progressive cooling profile in the cells until they reach the ambient low-temperature environment, at which point they are transferred directly to a liquid nitrogen storage vessel. Many laboratories achieve this by packing ampoules of cells insulated with paper toweling or wadding inside a small polystyrene box that is then placed in a  $-80^{\circ}\text{C}$  freezer overnight. Alternative methods are also used whereby the ampoules of cells are suspended in the vapor phase of liquid nitrogen, where they gradually cool and freeze. Careful adjustment of the position of ampoules and monitoring with thermocouples can enable establishment of an effective and reliable preservation procedure. Devices for reproducible passive cooling have been developed commercially (e.g., Handi-Freeze, Taylor Wharton, UK; Mr Frosty, Invitrogen, UK), but these should be validated to ensure correct performance under local laboratory conditions before using with valuable cell stocks.

The passive freezing devices described above appear to be generally effective and have been used widely in research and routine cell culture laboratories. However, the performance of these devices may be affected by variation in the levels of liquid nitrogen in “Dewar” vessels, or by interference with cells undergoing the critical process of freezing in a  $-80^{\circ}\text{C}$  freezer. Please note that although longer-term storage of cells at  $-80^{\circ}\text{C}$  has been demonstrated to be successful (Sugiura et al., 1968), this should not be considered as a long-term solution in general, as in many laboratories such storage systems are open to interference when other material is retrieved, and there is a high risk of significant loss of viability even within a few months. Enhanced control and recording of the cooling process may be required, especially in development and production programs for biological products where large cell banks comprising hundreds of ampoules are required. For such critical cryopreservation procedures, mechanical regulation of cooling enables exquisite a control of the cooling rate that can be used to optimize the cryopreservation process. In addition, the machines available commercially (e.g., Biotronics, UK; Kryo-10, Planer Products, UK) provide data outputs to enable each cryopreservation run to be carefully documented, which may be important for cell banking performed under certain quality standards (see following).

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### 15.5 STORAGE FACILITIES

#### 15.5.1 GENERAL CONSIDERATIONS

Having invested time and resources to prepare a cryopreserved a cell bank, it is wise to ensure that the facility used to maintain the bank will provide a secure, clean, and stable environment for long-term storage. Security for stored material is assured through adoption of appropriate management systems to restrict access to authorized personnel, appropriate alarms for nitrogen storage vessels, and documented procedures for filling and maintenance of nitrogen storage. Storage management systems should include an inventory of all stored material (this is a legal requirement for infectious and recombinant materials under health and safety legislation in some countries) and routine documentation of withdrawals and entries. However, the best-designed systems will fail if they not monitored properly, and for any important stored material there should be an auditing process to ensure that maintenance and documentation are kept up to date and that any procedural changes are appropriate and recorded.

#### 15.5.2 FACILITY SPECIFICATION AND DESIGN

Storage areas should be selected and established with a number of key criteria in mind: They should provide adequate space to maintain and hold storage vessels in a controlled area, preferably where

access to vessels is restricted by use of a dedicated room or locks on vessels; to avoid infection and contamination risks (Fountain et al., 1997) areas prone to heavy environmental microbial contamination such as corridors and storage rooms with access from outdoors are undesirable and should not be used for storage of cell cultures, particularly where intended for aseptic and antibiotic-free cell culture; the storage area should be well ventilated to prevent oxygen depletion during periods of high nitrogen gas release resulting from filling procedures or accidental spills.

Liquid nitrogen storage involves significant safety issues for laboratory workers. Documented emergency procedures and use of oxygen-depletion alarms (both visible and audible) will be important features. A fixed oxygen sensor should be located below head height to detect oxygen depletion. In such systems, dual-level alarms are useful, as they can be used to trigger ventilation fans when oxygen levels are slightly depressed (e.g., 18%) thus minimizing the incidence with which a lower danger-level alarm (17%) is triggered to activate alarms and initiation of emergency procedures. It is important that if ventilation extract fans are required they should be located close to ground level, as nitrogen vapor is more dense than air.

It is important to establish whether storage will be in the liquid or vapor phase of nitrogen or whether electrical freezers ( $-100^{\circ}\text{C}$  or below) are to be considered. In theory, the liquid phase of nitrogen provides the lowest and most stable storage temperature and is the method of choice for long-term storage. However, the risks of transmission of pathogenic virus should be considered, as highlighted in the past for stored bone marrow (Tedder et al., 1995). Vapor-phase storage may increase the risk of temperature cycling in stored materials, but it is generally more convenient and safer for regular access to stored material than liquid-phase storage. Furthermore, some manufacturers (e.g., CBS) are now producing vapor-phase storage systems in which liquid nitrogen is retained in the vessel walls, which improves safety and appears to provide a temperature profile superior to standard vapor-phase systems. Electrical storage systems provide a very practical and maintenance-free low-temperature storage solution. However, such systems in a multiuser environment may be more prone to the effects of temperature cycling than liquid nitrogen vapor-phase storage (see long-term storage below), and this may have more serious consequences for materials stored at  $-135^{\circ}\text{C}$  compared with  $-150^{\circ}\text{C}$  freezers. It should also be noted that electrical freezer storage is a high-risk form of storage in which power supplies may not be reliable, and even where this is not the case, liquid nitrogen or carbon dioxide back-up systems will be required to cope with emergencies.

Where liquid nitrogen storage is used, the means of supply is also an important consideration in terms of safety and cost. Commercial deliveries of nitrogen are tested for gas composition at source and are generally considered to be free of microbial contamination. However, delivery trucks visit many sites, including centers storing infectious organisms, patient material, and a variety of other reagents. It is therefore possible that a supply pipe recently used, for example, to fill an open storage tank for infectious agents may go on to be used at the next location for filling tanks of patient material for transplantation. It will therefore be helpful to know what procedures are used by the nitrogen suppliers to exclude the possibility of contamination being transferred via filling devices. For critical storage systems, it may be appropriate to have on-site generation of liquid nitrogen (e.g., Cryomech; Sterling Cryogenics). Although there will be significant capital cost to set up such systems, the quality and source of nitrogen can be assured and it may provide economical benefits in the long run.

Transport of nitrogen around an organization can be a hazardous process, especially where large, unwieldy, pressure vessels must be moved along public or staff corridors. Piping from the main delivery point is expensive and not efficient over long distances. Wheeled vessels with low centers of gravity or motorized "trucks" to carry or tow vessels will reduce risk significantly, but where such vessel movements are frequent, it is sensible to consider a centralization of stored material. Obviously the materials stored in a common location must be scrutinized carefully to ensure that infectious materials are physically separated in the interests of the safety of laboratory workers and subsequent cell culture products. However, centralized storage can yield economic

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benefits where the burden of storage maintenance can be shared and security, including out-of-hours surveillance and emergency responses, can be easier and more effective.

When setting up a new storage facility, the types of material to be stored and their physical size are obvious considerations, as is the need to separately isolate hazardous materials and those in quarantine. It is also useful to differentiate between material intended for archive storage and other material that will be accessed regularly. Material for archiving is obviously most secure when stored in vessels giving low nitrogen loss. These normally have narrow access apertures that have longer standing-times; that is, maintain acceptable storage temperatures without refilling for longer periods. For routine access, vessels with wide "bin"-type lids are most convenient and can provide a useful low-temperature working area in the top of the vessel. However, these vessels obviously lose nitrogen vapor at a much higher rate and will tend to have shorter standing-times than low-loss, narrow-necked vessels. In addition, in the upper levels of the inventory systems, "bin"-type vessels are likely to be exposed to significant temperature cycles (see Section 15.6). A decision also has to be made between use of electrical ultra-low-temperature freezers and liquid nitrogen storage vessels. Where electrical freezers are considered, the reliability of electrical supplies, provision of emergency backup (often liquid nitrogen), and need for air conditioning should be considered.

Careful consideration of the location of the storage site is also important to ensure it can be made secure (with access restricted to authorized staff) and well ventilated at all times with an appropriate alarm system (see above). Layout of the storage area and positioning of services must also be considered to ensure staff safety. For example, liquid nitrogen delivery points should not be positioned in thoroughfares, and condensation on cold delivery systems should not cause electrical hazards; that is, electrical supplies and connections should be above any transfer pipes even if insulated.

### 15.5.3 SAFETY OF PERSONNEL

A risk assessment should be performed in collaboration with the local safety representative (Sheeley, 1998) to establish that the proposed facility and associated procedures are appropriate and would cope with catastrophic failure and total release of liquid nitrogen. Such precautions may seem excessive, but recent cases of fatal accidents involving liquid nitrogen storage in the United Kingdom indicate the importance of careful design and risk assessment of such storage facilities. Emergency procedures should also include contingency plans for transfer of critical materials to back-up storage vessels.

Liquid nitrogen presents a serious frostbite hazard, and protective gloves, masks, and aprons designed for cryogenic work should be readily available and used whenever working with storage and supply vessels. In some facilities, it may also be wise for staff to carry personal oxygen monitors. Mechanized vessel filling is usually achieved via solenoid valves. These can freeze in the open position, thus presenting a serious hazard of overfilling storage vessels. Dual solenoid valves in series reduce this risk, but a more robust system would also incorporate a compressed air-activated shut-off valve (e.g., Thames Cryogenic, UK) and an in-line ice filter that can be drained periodically.

On occasion, ampoules stored in the liquid phase can explode as a result of rapid vaporization of liquid nitrogen trapped in the ampoule on warming. Serious penetration injuries can be sustained when this occurs, and this is especially grave when working with infectious materials. To reduce this risk, newly recovered ampoules destined for use in the laboratory may be held for a period of time in the vapor phase, and when ampoules are subsequently transferred to the laboratory, staff should continue to wear protective gloves and masks and to keep the ampoules covered until thawed. Solid carbon dioxide or "cardice," commonly used for shipment of cryopreserved cultures, carries similar risks of frostbite and asphyxiation to those of liquid nitrogen, and its use should be assessed in the same way. Emergency procedures for exposure to liquid nitrogen or cardice and low-oxygen conditions should be documented and included in staff training programs.

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**TABLE 15.3**  
**Traceability through Reference Numbers on Laboratory Records**

Reference No.	Accession Day Book	Cell Bank Ampoules	Cell Banking Records	Quality Control Records	Media Batch Preparation Records	Sterilisation Records
Accession no.	+	+	+			
Cell bank no.		+	+	+		
QC reference no.			+	+		
Media batch reference no.				+	+	+
Sterilization reference					+	+

#### 15.5.4 DOCUMENTATION OF STORED MATERIALS

Accurate records of stored materials are not only helpful to retrieve ampoules of cells efficiently but may also be a legal requirement for storing genetically modified, infectious, or other hazardous materials. Numerous commercial database systems are available that are specially designed for this purpose, but it is important to select a system that is flexible to the full range of user requirements. It is wise to have up-to-date, hard-copy printouts of these records and to ensure that amendments to storage records for additions or withdrawals can be made at the storage site to avoid transcriptional errors.

For critical applications, such as manufacture of biological medicines, it is important to establish reference numbering for important stages in the process of cell banking to enable accurate two-way traceability of laboratory procedures (see Figure 15.2), which enable independent audits and tracking of reagents and cells when adverse events arise in the use of cell cultures. Key references include a cell line accession number (assigned on each receipt of a cell culture), cell bank reference number (specific to each homogenous batch of preserved cells), and quality control and media batch references that are linked to ensure two-way traceability as illustrated in Table 15.3.

#### 15.6 ISSUES FOR LONG-TERM STORAGE

Where cells are stored at low temperature over long periods, they may be prone to a number of potential hazards such as variation in storage temperature and contamination. Stored cell lines may be subjected to temperature cycles during routine access and maintenance of storage vessels. Intermittent warming of ampoules may be particularly marked in inventory systems in which vessels are opened frequently, and particularly where the individual storage racking must be completely withdrawn into ambient room temperature to gain access to stored vials and ampoules. The storage racking material may also be a significant factor, as good conductors of heat will promote warming cycles and temperature gradients within the storage vessel. One procedure that is guaranteed to have significant and potentially disastrous effects on viability of stored cells if not performed correctly is filling and maintenance of nitrogen storage vessels. Anecdotal cases of massive loss of important material as a result of vessel failure or breakdown in filling procedures are all too frequent given that the solutions to many of these incidents are often relatively simple.

Build-up of microbial contamination from environmental sources is known to occur (Fountain et al. 1997). Thus, long-term storage vessels will benefit from periodic cleaning at least to remove the ice-sludge that accumulates at the bottom of such vessels. Careful disinfection of recovered ampoules is also important to prevent contamination of cultures, and the use of sealed ampoules or storage boxes will also help to provide protection against microbial contamination.

Natural radiation has also been considered a potential cause of loss of viability or mutation in stored cells and tissues. However, there does not appear to be any evidence for the adverse effects of long-term storage in well-maintained nitrogen vessels, even for biological systems that might be expected to be more sensitive to such effects, such as embryos (Glenister et al., 1984).

A number of straightforward procedures can be used to enhance the security of important archived material. Manual or automatic monitoring of temperature or liquid nitrogen levels can provide useful monitoring data to identify trends in the quality of maintenance over time. A system to document any filling (especially manual filling) and other maintenance procedures (e.g., visual inspection, electrical testing, vessel vacuum checks) is important and should be checked regularly and periodically audited (e.g., during safety inspections).

It is wise to store important archive material in the bottom of storage vessels or, ideally, in separate archive vessels of with long standing-times (i.e., low-nitrogen loss systems). Vessels suited to regular access with large access lids have a high nitrogen loss and, as described above, are more likely to suffer significant temperature cycling. In cases in which temperature cycling is a significant risk, it may be helpful to consider establishing "sentinel" banks of cells that are recovered periodically to determine any trends in level of viability at points in the storage vessel that may be most prone to this effect (Stacey, 1998). However, the approaches described above do not protect against catastrophic failure of vessels (usually resulting loss of the insulating vacuum). Accordingly, contingency planning is also an important aspect of the management of cryopreserved cell line banks. A primary contingency measure is to have back-up vessels available that can be brought into use rapidly should vessel failure occur (it should be born in mind that large vessels at room temperature may take several hours to cool down sufficiently for transfers to take place). In addition, risk of damage to the storage site must be considered (e.g., failure of nitrogen filling, failure of power supply for electrical freezers, fire, sabotage), and to counter against such risk cell banks may be split to storage vessels in different locations on site and possibly also distributed to a second site. In the latter case, it is important to be sure that the storage maintenance procedures and general quality of storage are at least equivalent to those in the originating center, and periodic audits will help to assure this.

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## 15.7 REGULATION AND QUALITY ASSURANCE OF CELL BANKS OF ANIMAL CELL SUBSTRATES

### 15.7.1 GUIDELINES ON CELL SUBSTRATES

A range of guidelines on best practice in cell and tissue culture have been published (Doblhof-Dier and Stacey, 2000; Freshney, 1994; Stacey et al, 1998; UK Coordinating Committee for Cancer Research, 1999). Cell culture processes involved in the preparation of biological medicines are subject to more stringent guidelines from official national and international regulatory bodies including the U.S. Food and Drug Administration, the World Health Organization, and the International Conference on Harmonization (Center for Biologics Evaluation and Research 1993a; International Conference on Harmonization, 1998; World Health Organization, 1998; EMEA at <http://www.emea.int>). The referenced guidelines were established to apply to cell substrates used in the manufacture of biological medicines. They are based on key issues relating to

- Sterility (i.e., absence of bacteria, fungi, and mycoplasma)
- Viruses and TSE contamination of cells and media
- Genetic and phenotypic stability on passage
- Tumorigenesis of cells and oncogenicity of cell DNA

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Although these references deal with the substrates used in the manufacture of biological products, there is a diverse range of new cell-based therapies that will need guidelines and regulations.

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Regulations for aspects of gene therapy have been developed (e.g., Center for Biologics Evaluation and Research, 1993b; EMEA, 2002), and guidelines for other forms of cell therapy are under development in a number of countries. These new regulations on cell therapy and tissue engineering introduce new issues for safety and quality of medicines that include:

- The effects of combining more than one cell type in a product
- Residual tumor cells (including tumorigenesis of stem cells)
- Influence of the scaffold materials (toxicity, adsorption, and leaching of compounds)
- Specification and validation of raw materials for clinical use

In the future, the use of embryonic stem cell lines for therapy will deliver a new set of challenges. Issues that will need to be considered include genetic imprinting, activation of endogenous viruses, and standardization of growth and differentiation. In addition, tumorigenicity studies in animal models may be difficult to assess, as teratoma formation is considered to be a positive functional indicator.

### 15.7.2 QUALITY STANDARDS

Quality is the fitness for purpose of a particular product or process, and this is demonstrated in the context of a documented quality system. There are a number of formal quality standards within which the process of cell banking may be operated. The ISO9000 quality systems provide for consistent provision of a service or product that may include provision of cells from a cell bank (BS EN ISO9000, 1994; <http://www.iso-9000-2000.com/>). ISO9001 specifically addresses design, processing, and final inspection, whereas ISO9002 may be applied to research and development. However, the ISO9000 quality systems will only monitor for consistency of cell banking procedures against in-house standards, without reference to external measures of quality.

Where the cells are used in the manufacture of biological medicines (e.g., recombinant proteins, antibodies, viral vaccine) or critical testing purposes such as vaccine batch control, more stringent national and international quality systems are required, including current Good Manufacturing Practices (cGMP), for which there are formal regulations published for Europe, by the World Health Organization (<http://www.who.int/vaccines-documents/DocsPDF/www9651.pdf>), and for some individual countries. Traditionally, cGMP has applied to the final formulation of a product, although during auditing of GMP facilities, the downstream processing will normally be expected to meet the same requirements as the final steps. Raw materials, including cell banks, used in GMP processes may also be expected to meet the requirements of cGMP.

Testing of cell banks in relation to their safety for use in manufacturing is usually performed under **GLP accreditation** based on the guidelines established by the **OECD in 1982** (OECD, 1982). Various organizations operate worldwide to accredit such testing procedures, and these organizations are coordinated through the International Laboratory Accreditation Cooperation, established in 1996, which mediates international cooperation in this field (<http://www.ilac.org/>).

### 15.8 BIOLOGICAL RESOURCE CENTERS

Collections of microbial cultures have existed for more than a hundred years, and the first acknowledged service collection providing cultures for industrial use was established by Kraal in 1889. Since then numerous large collections of bacteria, yeast, and fungi have been established as public service collections, and these now include a number of collections of animal and human cell lines. These public collections are coordinated internationally through organizations such as the European Culture Collection Organization (<http://eccosite.org>) and the World Federation of Culture Collections (<http://wdcm.nig.ac.jp/>). Culture collections and other institutions with specialist expertise in provision of cell lines are important sources of quality-controlled cell cultures that

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enable researchers to obtain reliable supplies of cultures. These organizations provide cell lines for various purposes, including

- Cells representative of particular species or tissue
- Controls and reference strains for biological assays
- Seed stocks for product development (see above)
- Sources of genomic DNA carrying specific genetic lesions as controls
- Supplies of cells/DNA for genomics/proteomics research

Biological resource centers should be the first port-of-call for researchers seeking new cell lines. They provide the kind of quality-controlled and authenticated cells that will give confidence in the authenticity of the cells supplied. In addition, many resource centers also provide advice and training in culture, preservation, and quality-control techniques that will be invaluable to anyone starting out in cell culture.

### 15.9 OWNERSHIP OF CELL LINES AND PATENTS

Many cell lines in use for research and development have been passed between laboratories for many years and are considered to be in the public domain. Public collections, described above, maintain quality-controlled stocks of such cells for many years and have historically not claimed ownership over the cells themselves. However, in recent years, as the potential use of cell lines in the manufacture of medical products and laboratory reagents has increased, much greater attention has been directed at the ownership of cell lines in which there may be a number of interested parties.

Where the cell line has been derived from clinical procedures, the patient of origin may claim an interest in exploitation of their cells. A small number of such cases have been pursued by patients and their families, notably in relation to HeLa cells. Whereas the HeLa cell story (Gold, 1986) may be an exceptional case, it highlights the problems that may be encountered where proper informed consent has not been obtained before using patient tissues. Today it is standard practice, usually as part of local ethics approval, that each candidate patient is asked to sign a patient consent form that may specifically identify the uses to which their cells can be put or waive any rights to the ownership of the cells or any subsequent discoveries or developments relating to them. Such an agreement may pass the ownership of the cells to a particular sponsor, often a research or commercial organization, and in some cases, such as embryonic stem cell lines, this has led to a significant delay in the open availability of important new cell lines for research.

Public service collections (see above) have played a valuable role in establishing quality-controlled stocks of cell lines, making them available for research and development and securing their availability for later generations of scientists. This role as custodian of cell lines means that some of these resource centers now claim, with some justification, that they have rights to a share of any intellectual property arising from cell lines supplied by them. Most collections require that a materials transfer agreement is signed by the organization receiving cell lines and that restricts third party distribution and requires that any commercial exploitation of the material is notified to the collection. As discussed above the originators of the cells may also have supplementary conditions on use of cells received from resource centers. Thus, when embarking on a line of research that may ultimately lead to, or otherwise assist the development of, a commercial product or process, the researchers should carefully consider the potential effect of any materials transfer agreements they sign to obtain the cells. At an early stage of project development, alternative sources of the cell line or different candidate cells can be assessed and appropriate agreements can be put in place to ensure that the development of a process or product will ultimately be commercially exploitable.

Patent applications based on a novel cell line or involving the use of a cell line as a critical part of the patent may require that samples of the cell line or a representative cell bank must be

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submitted to a recognized patent depository (Fritze and [Weih](#), 2001; for general references see Cook 1999; Crespi, 1998). Such depositories act as independent laboratories to test and hold those cultures and act as a reference point for any procedure to verify or challenge the veracity of the patent. Patents may be filed on a national basis or through the U.S. or European Patent Office. The World International Property Office is an organization that aims to provide for coordination, mutual international acceptance, and harmonization of patent procedures. Under the Budapest Treaty (1979), certain laboratories are registered to act as International Depository Authorities (IDAs). IDAs verify the viability and purity of the deposited materials and hold them in a stable preserved state for at least 30 years. The latter commitment for these centers is a significant challenge for some cultures such as plant cell cultures, where the preservation methods now in use may not have been available to researchers until quite recently. The depository centers will have a specific list of requirements that must be met by the depositor before the patent culture accession number is formally approved, and it is wise to prepare a master cell bank and perform key tests such as sterility testing and mycoplasma tests before submitting samples of cells to the patent depository. Typical requirements of the IDA include:

- A minimum number of ampoules (typically 10 to 15) for storage and quality control
- Freedom from microbial contamination (primarily bacteria, fungi, and mycoplasma)
- Payment for the deposit application

Alternatively, the culture may be submitted to the IDA for its own in-house testing before making the patent application. Although this may prove expensive, it carries the advantage that a culture deposited with an IDA for safekeeping may be subsequently translated to a patent deposit while retaining the original deposit date. This may be a critical advantage when the patent must be accepted urgently.

## 15.10 CONCLUSIONS

When a cell line is first established, a cryopreserved archive stock, at low passage, is essential to protect against accidental loss of the culture. For cell lines likely to be used over an extended period of time, reliable supply of cultures is achieved through the establishment of a two-tiered master and working bank system. Confirmation of viability, key characteristics, and quality control of cultures recovered from cryopreserved cells should then be performed at the earliest opportunity. To establish reliable larger scale cell banks such as those used in manufacture of recombinant proteins and vaccines, it may be necessary to adapt and validate scale-up of the cryopreservation process to avoid loss of viability or variability between ampoules. Such banks will also be subjected to far more stringent characterization under appropriate regulatory guidelines. Cell banks stored for long-term use, including patent deposits, will require consideration of the additional important challenges, and particular attention should be paid to the maintenance and monitoring of storage conditions.

The establishment of well-characterized and quality-controlled cell banks enables enhanced reproducibility of research work and enhanced standardization of diagnostic and manufacturing processes using cell line substrates. The ability to standardize cell culture procedures in different locations and at different times is a major contribution to high-quality research and development that is dependant on the availability of cryopreserved cell banks.

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