

# Cryopreservation and banking of mammalian cell lines

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Published online 4 December 2008; doi:10.1038/nprot.2008.190

**This protocol describes the principles and methods used for the preparation of cryopreserved cell stocks. Following these procedures will ensure the availability of reproducible cultures for use within a single laboratory at different times and for different collaborating laboratories. Although the basic principle is simple, each cell line has characteristics that must be borne in mind when establishing and testing such stocks. The key requirements are reliable methods for culture, cryopreservation, characterization and quality control.**

## INTRODUCTION

### The need for cryopreserved cell banks and cell banking principles

Maintaining a constantly growing culture of cells over many months is bad practice. Any *in vitro* culture, whether prokaryotic or eukaryotic, is susceptible to genetic and phenotypic change during propagation. In addition, during constant culture, there is always the risk of an accident resulting in loss of the cells, cross-contamination with another cell line or contamination with microorganisms. The generation of a stable, cryopreserved stock of cells that can be regenerated at will has many advantages over constant culture, prime among which are the following:

- Phenotypic and genotypic drift and damage due to continuous passage are limited.
- Cell banks (i.e., characterized stocks) of aliquoted cells from a single passage can be stored ready to be used as required.
- These stocks can be extensively quality controlled and safety tested before they are used, enhancing reliability and reproducibility.

There are many situations where long-term storage of cell lines is required. Programmes of research can develop over decades and require standardized stocks of cells for research both within and between laboratories. Biomedical products derived from cells (e.g., monoclonal antibodies, recombinant therapeutic proteins, vaccines) take years to become established and licensed. In addition, patent applications involving cell culture may require storage of the cells in a stable state for a minimum of 30 years in the event of a challenge to the patent<sup>1</sup>.

To meet these requirements, a large number of cells are aliquoted and frozen as a single stock. This approach can provide replicate cultures for several years. However, if reproducible stocks of cells are needed over several decades, an archive or 'master' stock is essential to enable the working stock to be regenerated periodically. The tiered master and working bank enables the provision over decades of multiple working stocks of cells that have the same passage history and provenance.

### Principles of cryopreservation

For animal cell cultures, the preservation of cells is generally straightforward. For most cells, 10% (vol/vol) dimethylsulfoxide

is added as a cryoprotectant to the culture medium, followed by cooling at a rate of approximately 1 °C min<sup>-1</sup>. Understanding which elements of the cryopreservation process are critical to the successful preservation and recovery of viable cultures is essential to provide reliable frozen stocks of cells and deal with problematic cultures<sup>2</sup>. Key considerations for the successful preservation of cell lines include the following:

- Selection of cultures for cryopreservation in the exponential phase of growth.
- Minimization of exposure of cells to cryoprotectant during preparation (particularly at room temperature; 15–25 °C) to avoid toxicity
- Avoidance of temperatures close to 0 °C due to the risk of chilling injury.
- Establishment of procedures that deliver a reproducible smooth cooling rate.
- Maintenance of backup cultures until the cryopreserved cells have been checked for viability.
- Rapid thawing and dilution (or removal) of cryopreservative.
- Testing of one of the vials immediately after cryopreservation to test viability and to ensure lack of microbial contamination.

### Controlled freezing devices

In the research laboratory, relatively small numbers of vials may be frozen down at any one time and simple methods may be used. For example, the cells to be cryopreserved may be placed in vials in an insulated box or commercial freezing box (e.g., Mr Frosty, Invitrogen).

For industrial, agricultural and biomedical purposes, devices for freezing large numbers of vials of cells reproducibly have been developed, which will electronically document the cooling profile. Companies such as Taylor-Wharton, Grant and Planer Ltd supply instruments for routine and high volume preservation.

### Storage facilities

Failure to establish secure storage facilities can and has resulted in catastrophic loss of cryopreserved cells. Key elements to deal with this risk include the provision of high-temperature alarms on storage vessels, accurate documentation of storage inventories, reliable maintenance systems, periodic auditing of storage systems and procedures and off-site backup storage for critical material<sup>2</sup>.

A range of companies provide storage systems (e.g., Statebourne Ltd, MVE), usually based on large vacuum-lined vessels that contain a reservoir of liquid nitrogen in the base over which an inventory system contains the frozen stored materials in the vapor phase of the nitrogen (stored material under these conditions is typically around  $-150\text{ }^{\circ}\text{C}$  or below depending on the storage vessel). In general, storage of preserved material under liquid nitrogen is undesirable, as liquid nitrogen may penetrate screw cap vials, which, on rewarming, reseal and can subsequently explode as the nitrogen vaporizes inside the vial. Liquid-phase storage may also promote cross-contamination between stored materials, which can have tragic results in the case of cells stored for transplantation<sup>3</sup>. Electrical freezers can store material at  $-130\text{ }^{\circ}\text{C}$ , but the electrical supply needs to be robust. Liquid nitrogen emergency recovery systems can provide cover where electrical freezers are used.

**Factors that need to be considered when preparing a cell bank**

**Cell line acquisition.** Cell lines can be obtained from a variety of sources, including the originator, a colleague or another research laboratory or culture collection. Sourcing cell lines is a process that carries the risk of receiving cross-contaminated cells whatever the source<sup>4</sup>, as even the originator of a cell line may supply cross-contaminated cells<sup>5</sup>.

It is therefore important that suppliers of cells should address this risk, ideally using an approach described as a ‘cell line passport’<sup>6</sup>. Central elements in such a ‘passport’ include the passage history of the cells, a recent negative mycoplasma test, the passage level of cells supplied and evidence for the identity of the cells (currently a DNA profile for human cell lines).

The more laboratories a cell line has passed through, the greater the likelihood of contamination with microorganisms or cross-contamination with other cells. Most cell lines in culture collections were obtained on trust with no evidence of authentication. To date, very few cell lines have had their identity confirmed or authenti-

cated—in other words, proven to have come from the individual claimed. With the development of global expression profiling and proteomics, it has become apparent that a significant number of cell lines do not have the characteristics of the cell type from which they are claimed to have originated<sup>7</sup>.

Culture collections supplying cells should have quality assurance procedures in place to ensure not only that essential information is available, but also that it can be formally traced. Culture collections operate to different standards; a list of some cell culture collections that supply cells is provided in **Box 1**. It is therefore important to obtain as much of the information listed in **Table 1** as possible, and to select the source of cells on the basis of the completeness of the information available. It is also important to establish whether the information provided is on the basis of the supplier’s own characterization of the cells to be provided and is not simply published information or information provided by the originator of the cells.

Increasingly, cell lines are supplied only after a materials transfer agreement (MTA) has been signed, and it is important that researchers bear in mind the constraints that these may impose on their work or that could affect the ability of their sponsors to exploit any discoveries that are made with the cells. The issues an MTA typically addresses are as follows:

- Acknowledgement of source and original publication in publications using the cells.
- No third-party transfer without permission.
- Statement that all safety issues are addressed within receiving laboratory.
- Acknowledgement of any associated hazards (e.g., genetically modified organism (GMO), viral).
- Confirmation of compliance with local, national and international laws.
- No exploitation without previous negotiation.
- Disclaimer regarding applicability of patents or safety of cells.
- Statement on liability and ownership.

**BOX 1 | CELL LINE COLLECTIONS**

The following list indicates a number of culture collections that supply cell lines. The authors cannot confirm that cells supplied by these organizations will necessarily meet the user’s needs. Standards vary widely among these collections and these collections provide no guarantee of authenticity unless specifically stated. Therefore it is the buyer’s responsibility to check authenticity and other characteristics of the cells.

American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20108-1549, USA.

LGC Promochem, Queens Road, Teddington, Middlesex TW11 0LY, UK (European agency for ATCC).

Deutsche Sammlung von Mikroorganismen und Zellkulturen, Inhoffenstraße 7 B-38124 Braunschweig, Germany.

European Collection of Animal Cell Cultures, Health Protection Agency, Porton Down, Salisbury, Wiltshire SP4 0JG, UK.

Japanese Collection of Research Bioresources Cell Bank, Japanese Health Science Foundation Health Sciences Research Resources Bank ([http://www.jhsf.or.jp/English/index\\_e.html](http://www.jhsf.or.jp/English/index_e.html)).

RIKEN BioResource Centre, 3-1-1 Koyadai, Tsukuba Science City, Ibaraki 305-0074, Japan.

UK Stem Cell Bank, National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Herts, EN6 3QG, UK.

US Bank of Pluripotent Cells, WiCell, University of Wisconsin, Wisconsin, USA.



**TABLE 1** | Information that should be sought from potential suppliers of cell lines.

Information required	Rationale
Name of cell line	It is increasingly important to identify the correct name including subclone where relevant
Authentication/identity	DNA profile (services to provide these data are readily available for human cells, but it may be difficult to obtain such services for other species)
Cell type characterization	Any appropriate assays to confirm cell type and function
Morphology	Where a cell line has distinctive features, morphological evaluation can help to confirm that a cell culture is authentic and unchanged. Where the cell type is given, there should be some phenotypic or genotypic evidence, in addition to details of the identity of the original tissue used to derive the cell line
Passage level	Subtle genetic changes can occur on repeated passage and cells at very high passage levels may exhibit significant changes in their genotype or phenotype, which can affect their value for experimental purposes
Culture medium and supplements	In trying to replicate the work of other laboratories, it can be essential to know the specific sources and compositions of growth media and supplements. Names of reagent suppliers and appropriate grades or catalog numbers are helpful
Culture and passage methodology	The provider should recommend methods for culture of the cells and these protocols should be followed until the researcher has gained a good knowledge of the culture system. Some cell types, such as human embryonic stem cells, are technically demanding, and the provider's protocols will be especially important in these cases
Method of preservation	In general, methods of preservation vary little for different cell lines (generally on the basis of addition of 10% (vol/vol) dimethyl sulfoxide and cooling at $-1\text{ }^{\circ}\text{C}$ per min to at least $-100\text{ }^{\circ}\text{C}$ ), but for certain unusual cultures, such as human embryonic stem cell lines, the preservation and recovery method may be different and technically challenging. The supplier's recommendations on recovery of frozen cells may greatly assist in getting work off the ground quickly
Associated hazards and regulation	<p>Viral contamination: any evidence for the presence of human pathogens or other viral contaminants should be given to recipients as well as any results from microbiological testing</p> <p>Genetic manipulation: the supplier should confirm if the cells have been subject to genetic modification, as the use of such lines may present hazards and are often subject to certain legal obligations for the user</p> <p>Ethical issues: in many countries, regulations are now in place or are being established to ensure that appropriate donor consent is obtained for all human tissues used in research and for derivation of new cell lines. Although this may not be possible for most long-established cell lines where the identity of the donor is lost, the supplier should provide some statement relating to donor consent and the ethical status of the cell lines</p> <p>Commercial constraints: most cell lines will only be supplied once an MTA has been signed, and it is important to ensure that this does not apply unacceptable constraints on the work of the laboratory. Any commercial constraints should be referred to a legal representative who should sign any agreements before receiving the cells</p> <p><b>▲ CRITICAL</b> For any cultures representing an infectious hazard or that are subject to genetic or ethical regulation, it is important to consult the local laboratory rules and current national regulations and also to know that the supplier is operating in accordance with their own national laws</p>
Quality control applied to cells provided	It is helpful to know the results of any quality control tests performed by the supplier, including mycoplasma, sterility and identity. It is also helpful to know which test was used, as tests may vary in their sensitivity and specificity

Another issue that has become much more serious in recent years has been the demand for fully informed consent for the use of any tissue to derive cell lines. In many countries, the use of human cells and tissue without evidence of appropriate consent is prohibited. The use of cell lines must be carefully evaluated, considering the conditions under which cells have been donated for research, as donors of the tissue from which the cell line was derived may have applied constraints on its use.

**Controlling the process of cell banking.** Careful documentation of the cell banking process is vital to ensure that there is a detailed and accurate record of how and under what conditions the cells were grown, and how they were characterized and quality controlled. Guidance is given by Coecke *et al.*<sup>8</sup>.

Every culture flask should be clearly labeled with the name of the cells, the passage number, the date the container was seeded and dates of change of culture medium. Each frozen ampoule of cells should be labeled with sufficient information to unequivocally identify the frozen stock, including the cell name and date of cryopreservation or cell bank number. Inappropriate labeling of flasks or other tissue culture containers can result in switching of cell lines. Ideally, a bar coding system should be used to identify each ampoule.

Careful records of each passage, the media and reagents used and the characteristics of the cells including representative photographic records should be maintained. Ideally, the cell numbers seeded and harvested at each stage should be recorded to enable an estimation of population doublings for the final cell bank, as this is

## PROTOCOL

a better measure of replicative age of the cells than ‘passages’. These records will be important if a cell line becomes widely used for research and will be especially critical for the manufacture of commercial or biomedical products.

As a cell bank may be used for a wide range of applications over a long period of time, any culture conditions or events that could affect the quality of the cells for their intended purpose need to be carefully controlled. Therefore, additional factors to be considered include the following:

- Temperature and gas
- Batches of serum (which should be screened for growth-promoting capacity before use)
- Aseptic technique and culture in the absence of antibiotics, to ensure the absence of contamination in frozen stocks
- A quality control regime that addresses issues of generic importance (e.g., identity, contamination, viability) and also important characteristics specific to the cells in question.

**Quality control.** Each cell bank should be quality controlled for a number of generic issues that include viability, identity (typically a DNA profile for human cell lines) and the absence of microbial contamination. Detailed methods have been described<sup>9–12</sup>.

**Laboratory worker safety.** The culture of continuous cell lines carries two primary hazards: (i) development of tumors following accidental implantation of transformed cells into the operator and (ii) infection as a result of pathogens in the cells or culture reagents of animal origin. Although cell cultures are not considered to represent a serious risk to laboratory workers, there is always the possibility that the cells are infected with an organism that will present a hazard. Consequently, it is important to know the history of any cell line taken into the laboratory. Ideally, sources of human cell cultures should be tested for the most frequent serious human blood-borne pathogens. The potential hazards of working with cell culture are discussed in more detail in the *Nature Protocols* on changing medium and passaging cell lines<sup>13</sup>.

Extreme care should be taken in handling ampoules of cells taken directly from liquid nitrogen storage. Where vials are taken from low down in the tank, there is a risk that liquid nitrogen may have penetrated an ampoule. On warming, the gas generated (700× the volume of the original liquid) may be trapped and cause the ampoule to explode. Always wear appropriate safety garments including eye protection and take particular care with vials recently removed from storage.

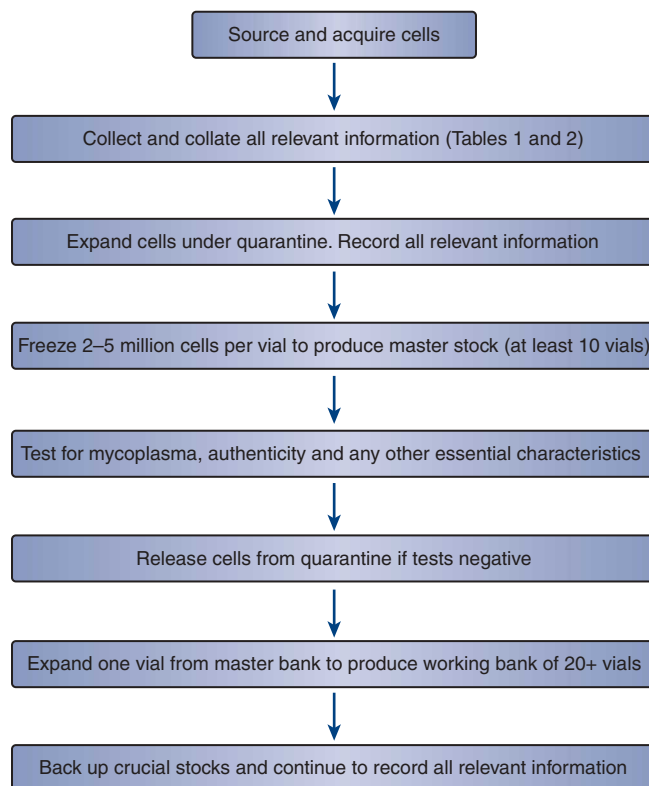
## MATERIALS

### REAGENTS

- Basic cell culture reagents<sup>13</sup>
- Dimethyl sulfoxide (tissue culture grade)
- Liquid nitrogen

### EQUIPMENT

- Certified Class II Biological Safety Cabinet (i.e., subjected to regular testing for filter performance and operator protection) **! CAUTION** Horizontal laminar flow hoods that force air over the work and toward the operator will expose the individual to any hazardous substances present in the cabinet.
- Inverted microscope (e.g., Olympus, Nikon, Zeiss, Leitz)
- Foot-operated pump and medium aspiration device
- 37 °C carbon dioxide incubator
- Indelible marker for labeling containers



**Figure 1** | An overview of the stages involved in cell banking.

Liquid nitrogen storage areas should be well ventilated, appropriate low oxygen alarms should be used and/or personal oxygen alarms should be worn by lab workers. There have been cases where a person working with liquid nitrogen has died of asphyxiation and these measures will be important in avoiding fatalities.

### Overview of procedure

In the procedure we describe the steps you will need to carry out to cryopreserve cells and to establish and test a cell bank; an overview of the stages involved is provided in **Figure 1**. Changing medium and passaging cells are described elsewhere<sup>13</sup>. The specific protocols will vary depending on the cell line used, but the principles are the same. When sourcing a cell line, it is important to ask any potential providers to answer a number of key questions regarding the cells they intend to supply, and these are outlined in **Table 1**.

- Controlled freezing device or passive freezing box (e.g., ‘Mr Frosty’, Invitrogen)
- Liquid nitrogen ‘Dewar’
- Cell reception logbook

### REAGENT SETUP

**Medium** Continuous cell cultures are usually grown in a standard cell culture medium (e.g., DMEM or RPMI-1640) supplemented with 5–20% (vol/vol) fetal bovine serum. For example, take a 500-ml bottle of medium (usually stored at 4 °C) and a 50-ml aliquot of serum (stored at –20 °C), thaw and mix (aseptically in the class II cabinet) and prewarm briefly in an incubator before use. **▲ CRITICAL** Media should not be left to prewarm for extended periods, as any free glutamine present will begin to break down to ammonia, which is toxic to cells.

**PROCEDURE**

**Reception and documentation**

1| On arrival, give the culture an accession number (or other specific reference) taken from a consecutive listing maintained in the cell culture laboratory to enable accurate traceability in experimental work. Check information from the supplier and complete a cell line master file (CLMF; see **Table 2**) created to provide a central source of information on the cell line. Any cell line that has been received previously should be given a new accession reference each time a new sample arrives at the laboratory, as different submissions, even from the same laboratory, may not be identical.

▲ **CRITICAL STEP** In some more highly regulated laboratory environments (e.g., good laboratory practice (GLP), current good manufacturing practice (cGMP)), elements of the CLMF may be located in dedicated parts of a quality assurance system, but it is still useful to have summaries in the CLMF with traceability to the stored data. Where cell lines are intended for clinical application, the CLMF may need to contain detailed and rigorously collated information on all aspects of the cells' history, including tissue donation (NB: should not include donor identity), cell line derivation in addition to records of banking, testing and characterization of seed stocks.

2| Document details of the culture received in the 'cell reception logbook' (see **Box 2**). This record should confirm the information provided previously by the supplier (see **Table 1**).

3| Immediately after receiving frozen cells, follow option A. Immediately after receiving fresh cells, follow option B.

**(A) Frozen cultures**

(i) Examine packaging and record in the cell reception logbook (see Step 2 and **Box 2**) any changes indicating that the ampoules have begun to thaw (e.g., no 'dry ice' remaining). Record all labeling on each ampoule and transfer the ampoules directly from dry ice to a quarantine location in liquid nitrogen (vapor phase) storage. Record the storage location in the appropriate inventory.

(ii) Set up a cell bank preparation record. When the bank has been completed, this should include name/reference number for cells, any reference for the frozen vial of cells used, viability data on original vial, number of ampoules prepared, number of 'viable' cells per ampoule, ampoule labeling, preparation of cells and cryopreservation media. This record could also include details of passaging and culture media used up to the point of cryopreserving cells as a master cell bank and the name of the person who prepared the cell bank.

▲ **CRITICAL STEP** The information recorded in this and the following step will be important to recall if any problems arise later that could have been associated with errors or contamination.

**TABLE 2** | Typical content of a 'Cell Line Master File'.

Information	Comments
Cell name and local 'accession number'	This should be checked to ensure that the correct scientific consensus on the nomenclature for the culture name is used
Authentication	DNA profile. Note that other methods can be used (e.g., isoenzyme analysis, cytogenetic analysis)
Supplementary information from the supplier	Information on testing, passage level, culture and preservation
Local cell line data sheet	This gives the key identifiers (such as name and accession number) along with specific culture media and conditions, seeding and harvest densities to be used locally. It should also include comments on hazards or constraints on the use of the cells (see regulatory information below), as people wishing to use the cell line for a new project should be directed to this sheet
Regulatory information	Records should include any risk assessments performed and local/national permissions including those relating to ethics and GMO regulation. Copies of any formal transfer agreements from the supplier should also be included. For cell lines established in recent years, there may be prescriptive and legal requirements to demonstrate traceability to fully informed consent relating to donation of the original tissue
Seminal and other external references	It is useful to ensure that copies of the seminal publications on the derivation and use of the cell line are included in the cell line master file. Also, other notable references that qualify the use of the cells or methodology for their use and characterization should be added as the bibliography on the cells develops over time
Cell banking records	Records of master and working cell bank preparation, preservation and testing
Key local data	It can be helpful to keep examples of data on the performance of cells in <i>in vitro</i> systems and assays. Copies of publications that incorporate local data on the cell line. Note that the source of the cells and the local reference should be given



## BOX 2 | INFORMATION THAT SHOULD BE DOCUMENTED IN THE CELL RECEPTION LOG BOOK

- Cell line name and any other reference used by the supplier (e.g., culture collection number)
- Name of provider
- Date of receipt and any comments on condition of cells when received (e.g., flask cracked or leaking, frozen vials partially thawed)
- Passage number of cells provided
- Immediate storage location
- ▲ **CRITICAL STEP** It is wise to store new cells away from tested cells until checked for mycoplasma and sterility.
- Note any supplementary cell and culture information supplied with the cells. This documentation should be transferred to a cell line master file (see **Table 2**).

- (iii) Prepare the growth medium and conditions as indicated by the supplier. Record batch numbers of growth media and supplements used on the cell bank preparation record and sign and date the record form.
- ▲ **CRITICAL STEP** Where only one frozen ampoule is available, a standard dilution of antibiotics (e.g., 100 U ml<sup>-1</sup> penicillin and 0.1 mg ml<sup>-1</sup> streptomycin) may be added for the initial passages until backup vials have been frozen down. Antibiotics should be avoided wherever possible during cell culture.
- (iv) Resuscitate one ampoule of the culture<sup>13</sup>. If the provider has supplied instructions they should be used, but if these are not provided, the following approach may be taken. In general, cells should be thawed rapidly at 37 °C in an incubator or heat block.
- (v) Transfer the ampoule rapidly to a class II safety cabinet (ideally one dedicated for quarantined cells), and after disinfecting the outside of the ampoule with 70% (vol/vol) isopropanol (preferred to ethanol as it is less flammable), gradually dilute the contents (over a few seconds) in an additional 0.5–1.0 ml of prewarmed growth medium (this will avoid unnecessary loss of cell viability due to osmotic shock). Dilute cells in a 15-ml sterile tube with 10 ml of prewarmed culture medium, mix gently, centrifuge (~80 g per 5 min), aspirate the supernatant and resuspend the cell pellet in 5 ml of medium. Perform a cell count (using a hemacytometer or other automated device), dilute the cells to the seeding density recommended by the supplier (see cell line data sheet above) and transfer to appropriate T-flasks in an appropriate incubator as described elsewhere<sup>13</sup>.
- (vi) Culture cells under quarantine conditions (in a separate room if possible, but if not in a dedicated incubator), until it is clear that there is no contamination (such as mycoplasma). New batches of cells entering a laboratory are a frequent cause of contamination, and therefore new cells should be maintained separately (quarantined) until all essential tests have been completed.
- (vii) Each day, examine the cultures macroscopically for evidence of microbial contamination and microscopically to confirm absence of gross bacterial or fungal contamination and to check density and appropriate morphology of the cultured cells.

### (B) Fresh, growing cultures

- (i) Immediately on receipt, check culture vessels for damage and examine cells under an inverted microscope for evidence of contamination, cell condition/density and appropriate morphology. Where instructions for initiation of culture are supplied by the provider, these should be followed. If this is not the case, then the following procedure can be used.
- (ii) Often on receipt, the culture vessel is filled with medium. Transfer cells to a class II safety cabinet (dedicated for quarantined cells, ideally in a separate room). Aspirate and replace with fresh medium; if cells do not appear healthy, contact the provider for advice, or if this is not possible, leave some of the original medium in the vessel overnight. Incubate some of the transport medium for 7 d to check for contamination. Cell cultures that grow in suspension will need to be centrifuged in a 15-ml centrifuge tube and resuspended in fresh prewarmed medium and transferred to a fresh flask. Then incubate cultures under the appropriate conditions of temperature and gas atmosphere under quarantine.
- (iii) Prepare a cell bank preparation record (see Step 3A(ii)).
- (iv) After overnight incubation (and at least within 24 h), check the density of cells, change medium and reincubate as required. If cultures are not ready for subculture, their general condition and density should be considered to decide if fresh medium should be added or if the cells need to be harvested and resuspended in a smaller volume of fresh medium to raise the cell density to promote cell growth and division. Such decisions are dependent on experience with the cells in question and advice should be sought from the supplier.

### Subculture

4| Once cells are ready, subculture them<sup>13</sup>, retaining one flask on antibiotics as a backup. Perform a cell count (using a hemacytometer or other automated device) on the harvested cell suspension such that the total cells harvested and the seeding density can be determined for calculation of population doublings (see ANTICIPATED RESULTS).

- 5| If only a single frozen ampoule or one growing culture was received, then prepare a reserve stock of frozen cells (up to three ampoules) within the first three passages or as soon as adequate numbers of cells have been received to permit a few ampoules of cells to be frozen. Follow the procedure described in **Box 3**.
- 6| As soon as cells are available from cultures passaged twice in the absence of antibiotics, carry out a preliminary test for mycoplasma. The test sample should comprise a mixture of cells and culture supernatant taken before subculture.
- 7| For cultures with characteristics that can be readily determined (e.g., surface markers, antibody secretion by hybridomas), appropriate samples may be taken at this stage and submitted for testing.
- 8| Repeated subcultures should be performed with cell counts to estimate the total number of cells harvested and the total number of cells seeded for the next passage level. Subculture should continue until adequate cells are available for the preparation of at least 14 ampoules at 2–5 million cells per ampoule (i.e., a minimum of 28 million viable cells). Allow up to 4

### BOX 3 | CRYOPRESERVATION PROTOCOL

The following method describes a basic protocol for cryopreservation of an adherent cell line. A similar method is used for suspension cultures after centrifugation of the cell suspension at  $\sim 100g$  for 5 min in a centrifuge tube, then continuing from Step 6 below.

Alternative methods may be recommended for preservation of certain cultures such as human embryonic stem cells, which may be preserved by a vitrification method. In such cases, it is recommended to use the supplier's method for recovery and preservation.

#### Materials

- Subconfluent monolayer cultures of cells, typically in T75 cm<sup>2</sup> flasks
- Growth medium
- Trypsin (0.25% (wt/vol) in EDTA)
- Phosphate-buffered saline (PBS)
- Cryoprotectant medium: fresh culture medium without antibiotics (70%, vol/vol), fetal calf serum (20%, vol/vol), dimethylsulfoxide (10%, vol/vol)
- **! CAUTION** Dimethylsulfoxide readily penetrates skin. Wear gloves and handle with care.
- Cryostorage ampoules (a wide range 'cryovials' are available for this purpose)

#### Selection and preservation of cell cultures

1. Examine all flasks by inverted-phase microscopy. Cultures used for preservation should be grown free of antibiotics, show no signs of microbial contamination and should be subconfluent (confluent cultures may be less amenable to freezing).
2. Remove the growth medium and wash twice in PBS ( $\sim 0.1$  ml cm<sup>-2</sup>).
3. Add prewarmed (37 °C) trypsin solution (0.5 ml per 25 cm<sup>2</sup>) to cover the cell monolayer, recap the flask and incubate at  $\sim 37$  °C for 5 min.
4. Examine the monolayer and tap the flask firmly on the bench to dislodge the cells. If the majority of the cells are not dislodged, continue the incubation for a further 5 min.
5. Harvest cells from each flask in 10 ml of medium containing serum or some other component that inactivates trypsin, bulk contents of all flasks and carry out a viable cell count by trypan blue dye exclusion or an alternative method, transfer to conical-based centrifuge tubes, mix by inversion and centrifuge at  $\sim 100g$  for 5 min.
6. Aspirate the supernatant and resuspend the cell pellet in cryoprotectant medium to give a final cell concentration of at least  $10^6$  cells per ml (ideally between  $2$  and  $5 \times 10^6$ ).
7. Mix the cell suspension by inversion in the cryoprotectant medium and divide into 1-ml aliquots in sterile labeled cryovials. Seal each vial immediately after it is filled.
8. Wrap the vials loosely in paper toweling (for insulation) inside a small polystyrene box and tape the box lid in place. Alternatively, proprietary devices such as the Mr Frosty (Invitrogen) can be used. Note that a variety of controlled rate freezing devices are marketed, which can help to minimize ice crystal damage and preserve greater cell viability. Nevertheless, the protocol described is adequate for most cell lines.
9. Transfer the box to a freezer overnight in a location where it will not be disturbed.
- ▲ **CRITICAL STEP** The freezer should be at  $-70$  °C or lower.
10. The next day, place the box directly into the vapor phase of liquid nitrogen and then transfer all vials to a storage location in the vapor phase of liquid nitrogen. Alternatively, if vapor phase is not an option and there is no biohazard, transfer the vials to a small quantity of liquid nitrogen and transfer to storage in liquid nitrogen. It is recommended to enclose the ampoules in case of explosion.
- **! CAUTION** Liquid nitrogen burns. Wear protective gloves and facial protection when using liquid nitrogen.
11. After overnight equilibration in nitrogen storage, recover one vial of cells and thaw rapidly at 37 °C in an incubator.
12. Gradually dilute the cell suspension by dropwise addition of prewarmed antibiotic-free growth medium.
13. Take a sample for a viable cell count and transfer the remaining cells to fresh growth medium in a T-25 culture flask and incubate and observe over several days to check for adequate adherence and growth.

▲ **CRITICAL STEP** Record the details.

## BOX 4 | PREPARATION OF SAMPLES FOR QUALITY CONTROL PROTOCOLS

After a minimum of 24-h storage of the master bank ampoules in the vapor phase of liquid nitrogen, the following samples should be tested as follows:

- (i) Submit one ampoule for DNA profiling (ideally, this should be performed with both a working bank sample and primary source material (e.g., paraffin-embedded tissue, Guthrie or FTA card blood spot) from the individual from whom the cell line was derived). A variety of companies can provide DNA profiling tests for human cell lines, such as GSL Ltd, UK; TDL, UK; or LGC, Twickenham, UK.
- (ii) One ampoule is resuscitated without antibiotics, and the freshly thawed cells are tested for total count and viability. These cells are maintained in a 25-cm<sup>2</sup> T-flask for 24–48 h before examination for typical cell morphology and gross bacterial or fungal contamination. This culture can then be subcultured to provide confluent monolayers in 25-cm<sup>2</sup> flasks: one for mycoplasma testing, one for confirmation of species of origin by isoenzyme analysis (if appropriate) and one for any in-house characterization, such as karyotyping or surface markers. Culture supernatant from one of the final 25-cm<sup>2</sup> flask cultures should be tested for sterility and mycoplasma. Note that for cell lines such as antibody-secreting hybridomas, culture supernatant may be retained for additional tests as required.
- (iii) Ampoules may also be allocated for other necessary tests identified for the Master Cell Bank, which may be performed by outside laboratories (e.g., viral testing, surface markers).

vials for quality control (see **Box 4**) and a minimum of 10 for the final master stock. Cultures showing poor growth may be cryopreserved at an earlier stage in a smaller number of ampoules.

▲ **CRITICAL STEP** Do not be tempted to dilute cells to achieve a larger number of ampoules, as recovery may be significantly slower.

**9|** Prepare a cell bank preservation record assigning each set of cultures to be frozen as a batch (e.g., Cell Bank number, which may be consecutive numbers assigned to each stock of cells preserved, cell line name and date).

**10|** For each culture, prepare sufficient sterile ampoules each labeled with the cell line name, date and the cell bank number. Prepare the cryopreservation medium (using sufficient dimethyl sulfoxide to give a final concentration of 10% (vol/vol) in antibiotic-free culture medium) and record batch numbers of all reagents and media used on the cell bank preservation record.

**11|** Harvest and cryopreserve cells, and prepare samples for quality control and authentication, from assigned frozen ampoules.

**12|** Transfer frozen ampoules to a temporary or 'in process' storage location until quality control procedures (i.e., viability, bacteria/fungi, mycoplasma) are complete and the culture shown to be satisfactory. Subsequent storage of the cell vials should be in a dedicated master bank storage location with at least one ampoule transferred for second-site safe storage. The location of second-site storage should be recorded in the cell bank preservation record.

**13|** Once the quality control is complete, collate a summary with all other relevant documentation and communications in a CLMF.

▲ **CRITICAL STEP** The stock of cells prepared as explained in Steps 1–12 represents the 'master' or archive stock that will be called upon for future work. As it holds a key role for future experimentation, it is vital that good records of its establishment are maintained and that it is carefully tested and characterized. Typically, 15–50 ampoules would be produced for a master stock, although a larger number may be established in certain circumstances such as a bank for commercial production. An ampoule of the master stock is taken to generate a working bank for routine use, and this is done in the same way as for the master stock. The number of ampoules required for a working cell bank should be calculated on the predicted midterm needs for that cell line, bearing in mind the recommendation to initiate fresh cultures every 3 months<sup>8</sup>.

### ANTICIPATED RESULTS

#### Acceptance criteria for cell banks

Master banks may be deemed appropriate for preparation of working stocks when they meet the following criteria:

*Viability and growth.* A freshly thawed ampoule should contain at least  $1 \times 10^6$  viable cells of which, for most cell types, at least 80% should be viable. Leukemia cell lines tend to have a lower viability after thawing, but still grow well.

Resuscitated cultures of typical mammalian cells should reach confluence within 2–5 d under the recovery conditions recommended for each cell line. Note that some cell lines, such as NIH 3T3 cells and Caco-2 cells, should not be allowed to reach 100% confluency, otherwise they may lose key characteristics.

*Characteristics.* Identity testing such as DNA profiling reveals that the cell line has the genotype of the species and individual of origin. If there is any doubt about the species, various tests are useful, such as isoenzyme analysis or PCR.

*Mycoplasma.* A negative mycoplasma test should also be obtained for cells recovered from that master stock.

#### Calculation of population doublings

In most cases, it may be adequate to record the total numbers for cells harvested and cells seeded at each passage as described above and to use the calculation: cells harvested/cells inoculated to give an approximate figure for the population doubling.

These calculations should be recorded on the cell bank preparation record and the total populations doublings (PDs) recorded in the cell bank preparation record. Population doublings can be calculated approximately using the following formula, which has been used for diploid fibroblasts<sup>14</sup>:

$$\text{Population doublings} = (\log_{10}(X) - \log_{10}(X_0)) \times 1/0.301$$

where  $X$  = final cell number harvested and  $X_0$  = original number of cells seeded.

For particular cell lines, it may be desirable to obtain a more accurate estimate of population doublings on the basis of published or in-house data for plating efficiency<sup>15</sup>.

### Action in the event of a test failure

Failure of tests for key characteristics does not necessarily lead to cell banks being discarded if a potential and valid use for the cells can be identified. However, the fact that the cells have failed critical tests for their original characteristics should be displayed prominently on any information where the cell line is quoted.

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