⟨1044⟩ CRYOPRESERVATION OF CELLS

INTRODUCTION

Cryopreservation is the process of cooling and storing cells, tissues, or organs at very low temperatures to maintain their viability. The purpose of cryopreservation is to bank the cells and allow their future use in in vitro or in vivo applications for which post-thaw function is sufficiently representative of the cells' prefreeze function. Cryopreservation also minimizes the risk of genetic mutation or development of subpopulations due to cell replication. Depending on the application, sufficient postcryopreservation function may be assessed by the ability to divide, proliferate, differentiate, express genes, or to produce proteins, or by another specific functional property.

This chapter presents best practices for cryopreservation, maintenance, and use of a wide range of cells, cell therapy products, and cell banks derived from a variety of sources including human, animal, and microbial cultures (the chapter also contains an *Appendix* with additional guidance documents that are useful for particular cell types and applications). Cryopreserved cells provide a ready source of viable cells that can be used, either directly or indirectly, for the purposes of diagnostic tests, therapy, manufacture of drug products and vaccines, and for bioassays used to evaluate the potency of therapeutic drugs and vaccines. In some cases the cells themselves, after cryopreservation and thaw, constitute the patient therapy, and in other cases the cells are propagated or otherwise manipulated ex vivo in order to generate the product (e.g., a culture-expanded cellular therapy, a therapeutic protein, a monoclonal antibody, or a vaccine). In all cases, proper cryopreservation is essential for retention of required cellular properties and, ultimately, for application toward the advancement of patient therapies.

PRINCIPLES OF CRYOPRESERVATION

Overview

Understanding the role of water and the need to adequately remove it from cells or abrogate its ability to form ice crystals, which damage the cell membrane, is critical to successful cryopreservation. When cells are frozen in aqueous suspension, often they are destroyed. However, in the 1940s Polge and others discovered the cryoprotective properties of glycerol. Since then several chemicals, generically called *cryoprotectant agents* (CPAs), have been identified. The mechanism of action of CPAs is complex and is not fully understood. However, according to the commonly accepted theory of *colligative* action, CPAs increase solute concentration both within the cell and extracellularly, thereby suppressing ice formation. For this purpose, the so-called penetrating (or intracellular) CPAs [e.g., dimethylsulfoxide (DMSO), glycerol, propanediol, and methanol] must be able to cross the cell membrane readily and penetrate the cell without significant toxicity. There also is a group of nonpenetrating (or extracellular) CPAs (e.g., sucrose and trehalose) whose mechanism of action is thought to be related at least in part to their stabilizing interaction with cell membranes. This property also may explain the cryoprotective activities of certain large molecular weight compounds such as hydroxyethyl starch and polyvinylpropylene. Theoretical models of cryoprotection typically evoke the colligative theory, but full explanation of CPA action is yet to be established.

An alternative form of cell preservation, commonly called *vitrification,* whereby the cell suspension is loaded with high levels of penetrating CPAs (often several in combination), induces a glass-like state in which cellular and extracellular water cannot readily form ice crystals. When cell suspensions prepared in this way then are cooled very rapidly (cooling rates of 100°–1000°/min or more) the extreme viscosity prevents osmosis, and the water molecules are unable to form ice. This procedure has been widely used for complex structures including a variety of human, plant, and animal tissues and may help preserve those cell preparations that have variable degrees of cellular permeability or when standard cryoprotection cannot deliver the range of conditions required to optimally preserve viability in all the tissues' component cell types.

CPAs have biological activities beyond their cryoprotective properties. Some, like DMSO, can affect the cell membrane, cytoskeleton, and gene expression and may be toxic to cells following prolonged exposure. Therefore, during development of new cryopreservation protocols analysts should perform a toxicity assay in which the cells are exposed to the CPA over a range of time intervals to evaluate loss of viability or alteration of functionality.

Key Elements of Cryopreservation Practice

For any cellular sample or therapeutic product being cryopreserved, method development should address the following elements:

PREFREEZE PROCESSING AND CHARACTERIZATION

Optimizing the condition of the cells immediately before cryopreservation is critical to a successful outcome. The nature and extent of prefreeze processing depends on the state of the original cells harvested for preservation, the composition of the cell suspension, and the specific processing steps leading into cryopreservation. Prefreeze processing may include selection of subpopulations, ex vivo expansion, or incubation with activating or priming factors.

Precryopreservation characteristics and identity should be established during early process development. For cell banks in particular, the cell status and optimal growth conditions, as well as documented history (with traceability to a qualified cell bank or acceptable source), characteristics, and authenticity should be documented. Cell status and history typically are described in terms of the nature and number of manipulations and culture passages from the primary cells or original isolate. Finite or primary cells usually are cryopreserved at an early passage to maintain integrity of the original tissue, but continuous cell lines may be cloned and expanded, ensuring a homogeneous cell population. It is recommended to prepare cell banks from a single preparation or expanded population of cells since it is often necessary to pool cells for freezing from multiple culture vessels. Cells from cultures with different passage histories and certainly from different donors should not be pooled. In both cases, analysts should maintain detailed records of the procedures.

To prepare for cryopreservation of cultured cells, cells should be harvested during exponential or the most rapid phase of growth and before the culture enters stationary phase. Harvesting cells during this phase ensures that the cells are most viable and uniform. The optimal concentration of cells will depend on the cell type, purpose, and best recovery. Typically this lies between 10^6 and 10^7/mL for manufacturing cell banks but may be different for other purposes. Complete growth medium renewal a day before cell harvest also can be beneficial. Additionally, most cell suspensions benefit from washing by centrifugation and resuspension in an isotonic medium to a specific cell concentration. Prefreeze processing should not result in cells that are stressed before the start of the freezing process, or cell losses during freezing or after thaw will be higher than expected.

Optimizing the growth conditions of a cell line or primary cells is important to maintain high viability of the cells in culture. Typically, cells growing actively and in exponential phase have a low cytoplasm to nuclear volume ratio, which is conducive to successful cryopreservation with penetrating cryoprotectants. Suboptimal or improper culture conditions may result in lower viability and cell states that will be less robust for preservation and recovery. The culture medium should be optimized and the same medium should be used throughout all experiments, and each batch of animal-derived materials (e.g., serum) and other culture reagents

should be qualified (e.g., see the 2010 WHO guidance and the FDA 2010 guidance referenced in the *Appendix*). If possible, it is recommended to not use animal-derived components in the culture medium particularly for cells used for therapy or as manufacturing substrates.

Per the WHO 2010 guidance and based on a risk assessment, either the Master Cell Bank (MCB) or the Working Cell Bank (WCB) must be tested for adventitious agents. Ideally, samples of cells should be tested for adventitious agents before freezing. The specific testing regimen for potential microbial or viral contamination of cells depends on the donor source, the culture history, and the intended use. Detailed records of the cell history should be maintained as a basis for appropriate risk assessment to direct supplementary testing that may be required (e.g., exposure to bovine viruses in bovine serum albumin). Specific regulatory requirements for testing of cells, or donors of the cells, for products intended for a particular use (e.g., cell therapy or vaccine manufacture) are based on past experience regarding key agents that must be included or considered. *USP* general chapter *Cellular and Tissue-Based Products* ⟨1046⟩ contains guidance about sterility and safety testing requirements for cell therapy products.

REAGENTS AND CONTAINERS

All cryoprotectants, containers, etc., should be fit for purpose as indicated in relevant regulatory guidances. Sterile, single-use, disposable plastic bags, cryovials, or straws are customarily used for cryopreservation. Manufacturers' specifications should be carefully reviewed to ensure that the material used to manufacture the cryocontainer is appropriate for use at the storage temperature, is chemically compatible with the contents, minimizes the potential for leachables and extractables, and assures container closure integrity. If straws are used, then primary or secondary containment during storage is important to prevent direct contact of the preserved cells with liquid nitrogen. Cryovials should be selected based on their ability to provide adequate cell bank integrity.

Preservation of cells typically requires the use of specialized solutions that contain a base (typically an isotonic saline-based solution) with CPAs (most commonly DMSO but sometimes glycerol) and sometimes proteins (fetal bovine serum, human serum or plasma, conditioned medium, or human albumin). The optimum composition for different cell types may need to be determined.

The types of vials, labels, ink, or markers used should withstand extreme liquid nitrogen temperatures. The markings on the label should be legible and barcoded if possible. The minimum information on the label should include name or description of cell population, date of cryopreservation, lot number, and passage number if needed. Since most cryolabels are very small, additional information can be included on associated documentation. In certain applications it may also be necessary to sequentially number vials within a single lot as part of the minimum information on the label, to enable better control over movement of vials from a single bank, and to identify sectors of the bank which may have received different cryopreservation conditions.

ADDITION OF CRYOPROTECTANT SOLUTION

Cryoprotectant solutions typically are hypertonic and are not physiological. For example, a 10% DMSO solution used commonly in cell preservation has a concentration of approximately 1.4 osmolarity (Osm/L). Cells introduced into this type of solution rapidly dehydrate as water leaves the cell in order to reduce the difference in osmotic potential between the inside and outside of the cell. DMSO slowly permeates the cell to re-equilibrate. This may cause excursions in volume that can result in a loss of cell viability. Therefore, cryopreservation solutions commonly are added to a cell suspension in stepwise additions or gradually (e.g., using a syringe pump) or slowly dispensing down the side of the container to prevent cell losses resulting from osmotic stress. The method for introducing or removing a cryopreservation solution should be developed and evaluated for its impact on cell viability and functionality.

In the case of DMSO, a large latent enthalpy of mixing results in sample heating when the two solutions are mixed. This heating can be high enough to damage the cells, so solutions that contain DMSO commonly are precooled before mixing. Prechilling the solution reduces heating associated with mixing of the solution, reduces the osmotic volume changes that the cells experience, and reduces cell losses associated with exposure to DMSO. The time that cells are exposed to the cryoprotectant, prior to freezing, should be limited and the maximum time allowable, without deleterious effects, should be determined during development work for routine use.

COOLING

Two different types of freezing typically are used for cells: controlled-rate cooling (using programmable freezers) and passive cooling (including use of insulated containers). Controlled-rate freezers are attached to liquid nitrogen supplies. The temperature of the chamber should be controlled by increasing or decreasing the flow of cold nitrogen gas into the chamber according to a preprogrammed step. Controlled-rate freezing protocols typically involve several steps, each of which should be evaluated and qualified for a specific cell type.

The use of controlled-rate freezing provides more precise control of the freezing environment and therefore may provide more consistent (and higher) post-thaw recovery for cells that may have a narrow range of cooling rates associated with maximum survival or cells that are sensitive to the temperature at which ice forms in the extracellular solution. Temperature probes placed near the cells being frozen, or in a mock cell suspension that undergoes cryopreservation simultaneously, are used to monitor the freezing process and to provide process control. If release of the latent heat of fusion is delayed or poorly controlled, cells undergoing cryopreservation may be damaged and may have diminished viability after thaw.

Disruption of the controlled-rate freezing during the protocol may occur and typically is caused by failure of a valve in the controlled-rate freezer or cryogen. Protocols for handling disruption of the freezing process and backup plans should be prepared.

Passive freezing involves placing a product in a freezer (about −80° or −150°) and permitting the sample to cool in an uncontrolled fashion. Insulation or specially designed boxes are used to slow the cooling rate for the sample. The average cooling rate achieved for the majority of the process and the consistency of freezing curves should be evaluated and qualified for purpose. In general, control of the thermal environment during freezing results in improved post-thaw recovery, but certain cells exhibit comparable post-thaw recovery when they are passively cooled.

CRYOGENIC STORAGE, SAFETY, AND TRANSPORT

After the freezing process has been completed, products are transferred from controlled-rate or mechanical freezers to cryogenic storage units. Some microbial cell cultures can be suitably maintained in mechanical freezers but this should be demonstrated. Sample warming should be minimized during transfer of the cell product from the freezing device to storage. Cold tables or insulated transfer devices can be used to minimize warming during transfer. Newly cryopreserved cells commonly are placed in a quarantine cryogenic storage unit before completion of testing for adventitious agents. After testing, cells that test negative for adventitious agents can be released for transfer into long-term cryogenic storage units.

The inventory system (or repository) for the maintenance of the cryopreserved cells should be designed for easy access to minimize specimen handling, and the number of times per day that a repository is accessed should be limited because exposure to warmer temperatures may compromise cell viability and, consequently, longer-term stability. Cell banks (e.g., MCBs) or other cell cultures that are accessed infrequently should be stored separately from WCBs or other cell cultures that are accessed more often. Frequent retrieval from the cell bank/culture may cause shifts in temperature. This activity must not compromise the long-term stability and performance of the infrequently used cell bank/culture. It is also valuable to divide a bank and store it in multiple locations to decrease risks due to a catastrophic event at a particular site.

When storing cryopreserved cells, analysts should ensure that the storage temperature does not rise above a critical temperature called the glass transition temperature. For long-term storage of fastidious specimens such as cell lines and primary cell cultures, this critical temperature is not warmer than −130° for nonclinical specimens or not warmer than −150° for clinical material (to give an adequate margin of error) in the vapor phase of the liquid-nitrogen freezer. Liquid-nitrogen freezers are prone to temperature gradients in the vapor phase based on the shape and design of the freezer and the level of liquid nitrogen. Although storing cryopreserved cells in liquid nitrogen prolongs longevity, hazards associated with unsuitable containers or container use (e.g., exploding vials and rupturing

bags) have prompted greater use of nitrogen vapor phase storage. Liquid-nitrogen vapor phase provides a more convenient and safe environment for vial retrieval. If the liquid-nitrogen freezer is suitably configured, the working temperature in the vapor phase is commonly −150° or colder. The liquid-nitrogen freezer should be qualified, and the temperature of the vapor phase should be routinely checked to ensure that the temperature does not become warmer than −130° for cell lines or other frozen material or warmer than −150° for material used for clinical applications (e.g., cell therapies).

Temperature-monitoring systems should permit recording and storage of temperature history for quality control purposes. Storage units should be attached to alarms and facility monitoring systems. Critical storage units should be equipped with a multilevel alarm system to ensure backup in the monitoring and response. The storage units should be routinely monitored for temperature failure caused by power disruption and any other potential malfunctions. In the event of equipment or power failure, backup refrigeration should be available.

Proper operation of a repository requires monitoring of temperature and liquid-nitrogen levels and automatic filling. In addition, it is recommended to have a backup for emergency cooling (e.g., empty backup cryogenic storage) in case of freezer failure.

Only individuals who are trained for this purpose should access cryopreserved products or samples. In some cases, verification by a second person is required for source traceability. Personnel assigned to the implementation of the protocols should be trained in standard operating procedures (SOPs). Sample tracking systems that incorporate computer software and sometimes barcoding for identification, logging, and tracking of frozen samples are particularly useful for large sample repositories and may facilitate rapid retrieval of samples and minimize time that the entire repository is exposed to the risk of temperature excursions. In addition, it is recommended that all changes to cryostorage inventories be recorded in log books near the storage unit.

Products and samples such as primary cells, cell lines, and cell therapy products routinely are shipped among sites of collection, processing, storage, and use. Cryopreserved cells typically are shipped in liquid-nitrogen vapor shippers with temperature-monitoring systems to ensure that the unit does not become warmer than −130° for cell lines and −150° for clinical material during the shipping process. Shipping containers are subjected to significant vibrations and mechanical stresses during shipment and should be evaluated on a regular basis for proper function. Shipping validation studies should cover worst case scenarios and include temperature monitors for critical materials.

Cryopreserved cells, whether shipped nationally or internationally, should be transported using local postal, US Department of Transportation, and International Air Transport Association guidelines. Packages also should meet other regulatory requirements for quarantine, biosafety, and biosecurity. Cryopreserved cells should be retrieved, packed, and shipped in a manner that does not interfere with the integrity of the cells. For most cryopreserved cells, shipping in dry ice for short duration may be adequate, but the shipping process should be validated, shown to have no adverse impact on the cells, and temperature monitors should be included. However, some cells may require shipping in liquid-nitrogen vapor phase (Dewars). Prevalidation of the shipping methods may be required to determine the best option and prevalidation risk assessment should be performed even if only one option for transport is being considered. With the shipment, shippers should include instructions for proper storage upon cell receipt.

THAWING

Cells frozen using conventional methods (controlled rate or passive freezing) or by vitrification should be thawed as rapidly as possible, and the thawing process starts as soon as the frozen sample is removed from storage. Slow warming rates result in recrystallization damage or exposure of the cells to high extracellular concentrations of CPAs, either of which can result in cell death. For each cell therapy product and cell line the most appropriate thawing procedure (temperature, gradient, and time) needs to be developed. These products and cell lines typically are thawed in a warm-water bath or for therapeutic cell preparations in a bead bath or thermoblock. The water bath should be cleaned regularly and should contain sterile water or *Water for Injection*. The temperature of the bath also should be monitored. Many clinical labs use plastic overwrap bags to hold the primary container during rapid thaw to reduce the risk of product contamination in case the integrity of the inner container is impaired. Alternatively, warm bead baths (usually approximately 37°) can be used to reduce contamination risks. Thawing rates should be as rapid as possible (>1°/s for most mammalian cells). Increasing bath temperatures above 42° to increase the warming rate must be done with extreme caution because hyperthermic temperatures can damage cells, inducing necrosis or apoptosis.

POST-THAW PROCESSING AND EVALUATION

Because cryopreservation solutions are not physiological, it is not uncommon for some post-thaw processing to be performed. For cells preserved in DMSO, cells typically are washed or diluted immediately post-thaw because this CPA is harmful in particular to frozen and thawed cells. Cells are more sensitive to expansion than contraction, so CPA removal or dilution protocols must be carefully optimized to prevent cell losses from dilution or removal.

Quantifying the viability of cells post-thaw is important and may be performed by a variety of methods depending on the post-thaw requirements of the cells. Minimum viability limits should be set based on experience and thawed products with viabilities below the set limits should be discarded. The process of cryopreservation subjects the cells to significant stresses that can alter metabolic function, membrane structure, etc. Therefore, development and validation of suitable post-thaw assays are critical. Post-thaw function is most commonly assessed using physical integrity (e.g., membrane integrity), metabolic activity, mechanical activity (attachment or contraction), mitotic activity, or engraftment potential. The selection of assay depends strongly on the desired post-thaw function of the cell. Membrane integrity is used most often. Today, dyes like trypan blue are used less often to measure post-thaw physical integrity because the dye is difficult to validate on frozen and thawed cells. The method to test viability needs to be carefully selected and qualified for the particular cell type being measured with a protocol that specifies diluents and time. Fluorescent dyes are used with increasing frequency to determine cells' post-thaw physical integrity. Rigorous methods of post-thaw assessment typically involve multiple measures of cell viability and, in particular, at least two independent assays to measure post-thaw viability. For example, post-thaw attachment and proliferation commonly are used to evaluate viability. As the complexity of the desired cell function after thawing increases, so do the demands on post-thaw assessment. For example, post-thaw assessment of stem cells may require assay of membrane integrity as well as proliferation and the ability of the cells to differentiate into different lineages post-thaw. Post-thaw assays should be carefully developed and validated to avoid measurement bias. A certain fraction of cells will lyse during freezing, and methods of measuring cell recovery should include a complete assessment of cell losses (cells that have lysed as well as cells that are intact but not viable). Stability of cryopreserved cells can be assured by periodically thawing and testing a vial of the cells (also see ICH Q5D).

Testing for adventitious agents after the preparation of MCBs and WCBs should be routine, and *USP* general chapters *Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin* ⟨1050⟩ and *Virology Test Methods* ⟨1237⟩ provide additional testing guidance (see also the FDA 2010 guidance cited in the *Appendix*). Testing for mycobacterial contamination, which may not be isolated in standard sterility testing, also can be considered for some cell substrates. Representative vials should be retrieved and tested for contamination (bacteria, fungi, *Mycoplasma,* and viruses). Numerous well-established methods are available for detecting *Mycoplasma* in cell cultures (see *USP* general chapter *Mycoplasma Tests* ⟨63⟩).

The batch record should be detailed, including the history of the cells and all activities starting from their receipt to release of the cell banks or products for use. The record should include detailed information about the cryopreservation process, including the procedure, the equipment used (with unique identifier), and a printed record of the freeze profile. The viabilities of the cryopreserved cells should be monitored over time to ascertain the effectiveness of the freezing process and the storage conditions. The information captured in each batch file of a cell line must be traceable to the original source, and all documents should be maintained and updated according to the quality management system in place.

CRYOPRESERVATION OF HUMAN CELL THERAPY PRODUCTS

Cell preservation methods are used to ensure product stability during hold, storage, and transport steps for a wide range of human cell-based products (additional information is found in ⟨1046⟩). Cells can be preserved in liquid suspension for up to a few days, but quantitative and qualitative changes in the cellular product invariably occur over time. Although preservation in the frozen state also affects the cellular product, it allows more predictable preservation of specific product characteristics over much longer time intervals.

For any given cell product, the decision to use cryopreservation depends primarily on the timing of final product administration in relationship to cell source collection and product manufacturing steps. Many patient-specific autologous and allogeneic products are maintained in liquid suspension, without cryopreservation, from starting cell source through final formulation and are released as fresh products after a relatively short time. However, many clinical applications require cryopreservation of the cell source, intermediate products, or final product. In these cases, cryopreservation can permit optimization of workflow during manufacturing, completion of lot-release testing, maintenance and management of a product inventory, transport of the product to the clinical site, and coordination of product administration with the patient's medical or surgical regimen. For example, umbilical cord blood is cryopreserved and stored in public banks for subsequent transport to, and temporary storage at, clinical transplant centers, where it is thawed immediately before infusion into a patient.

Development of a cryopreservation process for a clinical cell therapy must consider the consequences for the product, the patient, and the overall feasibility of the therapy. For the cell product, the manufacturer must ensure that expected cell losses because of cryopreservation and thaw occur in a manner that is reasonably predictable and must ensure that the final product administered to the patient will meet specifications for cell number, viability, and functional characteristics.

For the patient, cryopreservation may affect the efficacy and safety of the final product. For example, the cryoprotectant DMSO is associated with risk of predictable dose-dependent gastrointestinal, cardiovascular, and neurologic toxicities that typically are ascribed to histamine release. Residual DMSO in the final product should be estimated or measured. DMSO is categorized by ICH as a Class 3 (relatively low risk) solvent or excipient in pharmaceutical products, and amounts of up to 50 mg/day, or less, are considered acceptable without justification (see ICH Q3C). Cryopreserved cellular products frequently contain 10–20 times this amount unless they are washed after thawing. Even higher amounts of DMSO may occur with administration of multiple cryopreserved products, and this occurs commonly with autologous peripheral blood stem cell transplantation. A DMSO limit of 1 g/kg recipient weight/day is commonly used in clinical cell therapy practice. Procedures to prevent DMSO toxicity also should be considered. It is common clinical practice to premedicate patients with diphenhydramine or other antihistamine agents to prevent DMSO toxicity. Product washing methods by either manual (centrifugation) or automated methods also can be considered but must be validated to ensure adequate postwash recovery and cell function.

Use of cryopreserved cell therapy products may require the clinical site to receive, store, thaw, and perform other final preparation steps on the cryopreserved product. Feasibility assessment requires consideration of the site's capabilities with regard to specialized personnel, training, equipment, and facilities to execute those tasks.

If cryopreservation is planned as part of the manufacturing process, development teams must consider the effect of cryopreservation on cell number and characteristics and should require reliable methods for cell enumeration and assessment of cell viability and function. During development runs, more assays often are performed than will be required eventually for in-process and final product testing. This is done in order to evaluate the effects of each manipulation of the product. Because some cells may be more susceptible than others to freeze–thaw damage, these studies should include assessment of selective losses of important cell subpopulations within the product.

As described in the *Introduction,* several critical processes influence the outcome of a cryopreservation protocol. Following is a brief discussion of issues unique to cell therapies.

Prefreeze Processing

If cells are harvested with plasma present, samples should be properly processed with an anticoagulant to prevent clotting. In addition, some cells are prone to clumping or aggregation when centrifuged, and some cell products may exhibit excessive damage or loss of one or more populations within the product. Cells harvested from adherent or nonadherent culture may include substantial numbers of dead or fragile cells. Therefore, centrifugation and wash steps should be optimized and specific for the product's cellular contents, suspension volume, suspension medium, and container, along with appropriate evaluation of the cellular product before and after these manipulations. Prefreeze processing should not result in cells that are stressed (e.g., cells that demonstrate elevated early apoptotic markers or temperature-shock responses) before the start of the freezing process, or cell losses will be higher than expected.

Reagents and Containers

Clinical-grade reagents and containers should be used whenever possible (see *Ancillary Materials for Cell, Gene, and Tissue-Engineered Products* ⟨1043⟩). It is customary to use sterile, single-use, disposable plastic bags or cryovials that have been qualified for the specific cryopreservation process and subsequent storage conditions. Cryopreservation media for cell therapy products usually consist of isotonic saline-based solutions with one or more CPA, typically the intracellular cryoprotectant DMSO at 5%–10% final concentration with or without an extracellular cryoprotectant such as hydroxyethyl starch. The use of human-derived protein additives such as human serum albumin, serum, or plasma is common, but they may need extensive qualification, so they should be avoided if possible and alternatives should be evaluated. Additives such as animal-derived heparin, citrate-based anticoagulants, and DNase sometimes are used. Many centers formulate their own cryopreservation media, but commercial cryopreservation media, which typically include 5%–10% DMSO and other proprietary components, increasingly are used by cell therapy manufacturers to eliminate variability and the need for additional qualification activities associated with local formulation.

Addition of Cryoprotectant Solution and Cooling

Procedures for introduction or removal of a cryopreservation solution should be assessed before freezing to ensure that cell losses resulting from this step are minimized. The cryopreservation medium usually is added to the cell suspension in steps or gradually (e.g., using a syringe pump) to prevent cell losses resulting from osmotic stress. It is common to prechill the cryopreservation medium and keep the cell suspension and the admixture chilled using cold packs, a frozen blanket, or a chilled work surface to prevent heat-related cell damage during addition of DMSO. Once the cryopreservation medium is added, the cell suspension typically is transferred to the precooled chamber of a controlled-rate freezer. During the freeze process, a record of chamber and product temperature over time, or freeze curve, is generated for inclusion in the production record. Product temperatures can be recorded from a probe placed on the product bag's outer surface or from the inside of a comparable product in a dummy bag or vial that undergoes concurrent freezing.

Storage and Transport

Cryopreserved cell therapy products typically are stored and transported at temperatures of −150° or colder. FDA requires screening and testing for evidence of transmissible disease only for allogeneic donors of cell therapy products and not for autologous donations. However, many centers test autologous donors as well and segregate products from autologous donors who are known to have transmissible diseases when their products must be stored. A report of hepatitis B cross-contamination of cellular products within a liquid-nitrogen storage tank led to the currently common practices of storage in the vapor phase of liquid nitrogen and the use of overwrap bags to reinforce product containment. Liquid-nitrogen vapor phase storage may be associated with vertical temperature gradients: products at the top of the storage tank may have a warmer temperature than those stored at the bottom of the tank. Temperature gradients should be monitored, and vertical temperature gradients should be minimized, e.g., by use of metal heat shunts. Overwrap bags may reduce the warming rate of the sample if used during the subsequent thaw process, and their use should be qualified as part of the overall cryopreservation process validation.

Transport of cryopreserved cell therapy products usually is accomplished by using dry shippers containing absorbent material that can be charged with liquid nitrogen to maintain vapor-phase temperatures for up to 2 weeks if properly charged. Data loggers are used to document the temperature history during transport. These shipping containers and procedures must be validated before cell therapy products for clinical use are shipped in the containers.

Warming (Thawing)

Although bedside thawing of cell therapy products before infusion has been a common clinical practice, the use of trained personnel in a controlled laboratory environment is now recognized as the preferred method for thawing because it allows a more standardized process and a higher degree of control when staff must respond to a container failure, which may require product salvage in a more sterile environment. Product thawing typically is done by immersion in 37° water baths and using overwrap bags to minimize product loss and contamination in case of primary container failure. Bags may be gently kneaded during thaw to reduce temperature gradients across the bag and to accelerate thawing. The product is removed from the water bath when some ice is still present in the product but the majority of the product is thawed.

Post-Thaw Processing

DMSO is toxic to cells in liquid suspension. Toxicity may be reduced by diluting or washing the cell suspension before infusion or further manipulation. Because cryopreserved, thawed cells are more sensitive to volumetric expansion when the cells transition from a hypertonic solution to an isotonic solution, the dilution and washing solutions and methods must be carefully designed and validated. Cell washing using either a conventional centrifuge or an automated device can result in additional mechanical stress to the cells, so cell losses must be assessed by an appropriate method before a specific method is implemented in clinical practice.

Quality Control Practices

Quality management of cryopreservation, storage, and thaw of clinical cell therapy products must incorporate quality system elements common to current Good Tissue Practices (cGTP) and current Good Manufacturing Practices (cGMP), including personnel qualification, facility controls, document control, control of equipment and materials, label control, and use of validated SOPs (21 CFR 1271, 210, and 211). Quality control practices specific to clinical cryopreservation typically include assessment and documentation of freeze curves for all products, retention of tubing segments and vials for subsequent testing, and regular monitoring of post-thaw product quality. Practices required by cGTPs and applicable to all human cell- and tissue-based products include measures to ensure accurate and complete labeling and records, to ensure that the correct product goes to the patient, and to allow tracking of the cell product from collection to infusion. The practical implications of these requirements are that labeling and records, including inventory systems, for cryopreserved products must be designed to prevent errors in identification of products. Identity verifications of products moving into and out of cryopreserved storage are performed routinely, e.g., with two people checking the product label against records. If necessary for additional verification of product identity, the contents of a tubing segment attached to the product bag can be thawed and tested before thaw of the entire product. ISBT 128, an internationally recognized system for labeling of blood and cell-therapy products, incorporates use of consistent product nomenclature and barcoding of the product from donor source through administration (see *Appendix*).

HEMATOPOIETIC STEM CELLS

Studies of the response of hematopoietic stem cells (HSCs) to freezing began in the 1950s, and cryopreserved HSCs have been widely used in clinical practice during the past 30 years. The most common method of cryopreserving HSCs for clinical applications involves the use of 10% DMSO and a controlled-rate freezer set at a cooling rate of 1°/min. Another method, less commonly used, involves the use of passive freezing of the HSC product in a −80° mechanical freezer and the use of 5% DMSO + 6% hydroxyethyl starch solution. Cells commonly are frozen at densities of 30–50 × 10^6 cells/mL. Exceeding cytocrits of 20% (v/v) has been shown to reduce cell recovery. Post-thaw assessment of the sample consists of enumeration of nucleated cells, viable $CD34^+$ cells, and hematopoietic colony-forming units, along with subsequent calculation of recoveries from corresponding prefreeze values.

HSCs cryopreserved for clinical use may be obtained from bone marrow, mobilized peripheral blood, or umbilical cord blood (UCB). Each source has unique requirements for preservation. For example, peripheral blood progenitor cell products contain larger numbers of cells and may be frozen in multiple bags with relatively high cell concentrations. Protocols for UCB preservation may include the use of syringe pumps to introduce cryopreservation solutions while minimizing osmotic stresses. For UCB, specialized solutions often are used after thaw to dilute or remove DMSO while minimizing osmotic stress for the cells.

MESENCHYMAL STEM CELLS

Research on the clinical use of mesenchymal stem cells (MSC) has grown rapidly since the mid-1990s. Reliable, safe, and efficient methods of cryopreservation and storage are critical, especially for allogeneic off-the-shelf MSC products manufactured in multiple product doses for treatment of a large number of patients with a range of clinical indications.

Because MSCs traditionally have been generated in cultures that contain fetal bovine serum (FBS), cryopreservation media for these cells often have incorporated FBS. More recently, alternatives to gamma-irradiated FBS are being explored for the culture expansion before cryopreservation, and cryopreservation has been successful in media that contain 5%–10% DMSO and other components without bovine sources of protein. Although there is no consistent method of post-thaw cell processing, some protocols include dilution or washing of the cells to mitigate the effects of DMSO. Emerging clinical applications for MSCs may require repeat dosing of the cellular product, a practice that requires attention to potential immunogenicity of components of the cryopreservation medium, e.g., FBS or other proteins.

Post-thaw assessment of MSCs typically has involved the use of membrane-integrity dyes such as trypan blue, surface antigen expression, and evidence that the cells are capable of multilineage differentiation. Because the mechanism of action of MSCs may involve the immunomodulatory or trophic properties of the cells, post-thaw assessment also should include relevant cell function.

LYMPHOID CELLS

Lymphocytes are used for a variety of clinical applications including immunotherapy to treat cancer, viral infection, and autoimmune disease. Therapy based on lymphocytes may consist of mixed lymphocyte populations or lymphocyte subpopulations that have been selected or activated ex vivo, e.g., regulatory T cells, natural killer cells, and activated T cells. As with hematopoietic cells, lymphocytes typically are cryopreserved using a 10% DMSO solution and a controlled cooling rate of 1°/min.

Lymphocytes may undergo extensive post-thaw apoptosis, which can influence the clinical efficacy of the cells. Highly purified populations of lymphocytes may exhibit higher levels of post-thaw apoptosis than mixed lymphocyte populations. Strategies such as caspase inhibition and cytokine rescue have been used to diminish post-thaw apoptosis of lymphocytes.

PRESERVATION OF HUMAN PLURIPOTENT STEM CELL LINES

Pluripotent stem cells (PSCs) are cells that appear to have the capacity to (1) undergo self-renewal and replicate indefinitely and (2) generate cells that are representative of the three germ-layer tissues required to create all cells of the human body as demonstrated by the capacity to generate teratomas in immune-deficient mice. The two predominant types of stem cell line used for in vitro laboratory research are human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs). hESCs are derived from donated surplus blastocysts by isolation and culture of the blastocyst inner cell mass (the tissue that would

have progressed to form the embryo). hiPSCs are created by artificial reprogramming of somatic cells (by the delivery of reprogramming factors using a variety of methods) to yield cells that express the critical properties of PSCs listed above. hiPSCs can be derived from a range of somatic cell types using an increasing range of methods to secure expression of certain reprogramming factors. A range of tissue-derived cultures are known to harbor stem cell populations in vitro (e.g., subcutaneous fat, bone marrow, cord and blood of the fetal umbilicus, primordial germ cells from the fetal neural crest, and neural stem cell spheroid cultures) that have been shown to have a limited capacity for in vitro replication and have not been established as stable diploid PSC lines. Such cultures will not be covered in this chapter, and the following sections will deal specifically with hESC and hiPSC lines.

PSC lines are complex multicomponent cell cultures and may contain a variety of different cell populations with greater or lesser degrees of differentiation or lineage commitment. However, the key property of a stem cell culture, self-renewal, must be sustained. In addition, the cells must retain the capacity to undergo asymmetric division to yield two different daughter cells: one that is a stem cell identical to the parent cell and one with a reduced degree of potency (i.e., ability to generate different cell lineages).

Development of Current Methodologies for PSC

Currently, individual labs have their preferred methodology, no single approach appears to dominate for routine use, and it seems possible to obtain acceptable levels of post-thaw viability and recovery by either vitrification or controlled slow rate cryopreservation; however, a common method that results in better cell recovery is described here. Briefly, this technique involves placing colonies of hESCs in a vitrification solution composed of 20% DMSO + 20% ethylene glycol (EG) + 0.5 mol/L sucrose after equilibration with lower-concentration DMSO + EG solutions. The colonies are loaded into straws and plunged into liquid nitrogen. Another common method involves placing colonies in a solution consisting of 10% (v/v) DMSO and using either a passive freezing device or a controlled-rate freezer designed to achieve an average cooling rate of approximately 1°/min.

Vitrified IPS or hES samples have special storage and shipping requirements. All cryopreserved or vitrified samples must be stored at temperatures below the glass transition temperature of the sample, and for vitrified samples this is much lower (e.g., −150°) than for traditionally cryopreserved cell samples. In addition, fluctuations in temperatures during storage or shipment can lead to crystallization and therefore degradation of the sample. Because vitrified samples are sensitive to these temperature fluctuations, they should be shipped at liquid nitrogen temperatures and should not be shipped on dry ice.

Challenges for the reliable and reproducible preservation of PSC lines lie not just in the preservation process itself but also in the preparation, cryoprotection, and recovery procedures. Specifically, methods of harvesting and handling before cryopreservation may result in significant cell losses. Processing procedures should be made as reproducible as possible and with the use of an SOP to enhance the reproducibility of preservation outcomes. Furthermore, the effective banking of PSC lines is challenged by the methods by which the cells typically are passaged and harvested for preservation (i.e., individual colonies dissected and transferred as small colony fragments to fresh culture flasks for expansion or to preservation medium for freezing). First, in order to avoid extensive loss of viability in the earlier harvested cells, cultures used to make a bank actually may require preservation in small batches over a working day. Second, preparation of PSC banks comprising smaller pools of cell colony fragments is not only time consuming and laborious, but it also makes it impossible to homogenize the preparation of cells before aliquoting into vials, as would happen with more traditional methods of preserving cell lines. Thus, consistency between vials of PSC lines is compromised. Third, freezing PSCs as colony fragments preserves the gap junctions known to form between cells, potentially leading to intercellular propagation of ice crystals and extensive loss of viability.

To avoid some of these issues, analysts can use enzymatic disaggregation of colonies to simplify and accelerate cell harvesting and to enable cryopreservation of more homogenous, single-cell suspensions before preservation. To be prudent, some laboratories choose to perform enzymatic disaggregation solely before cryopreservation but not for routine culture. Whenever possible, analysts should use non–animal-sourced enzymatic agents for cell dissociation. Another important characteristic of PSC lines is the common but variable incidence of undirected differentiation that occurs within colonies and may vary considerably across the many colonies in a culture. Colonies with a high proportion of differentiated cells should be discarded because the differentiated cells are an undesirable component of a PSC culture and may affect the properties of the undifferentiated cells within the colony.

Points to Consider in the Preservation of PSC Lines

Analysts should consider a number of important factors in the preservation of PSC lines that should be addressed at the levels of preparation, harvesting, banking, and testing of cryopreserved stocks.

EVALUATION AND HARVESTING

Analysts should observe cell cultures on a regular basis. Cultures that exhibit high levels of differentiated cells should be discarded. An individual investigator or banking facility should have a quality control program that evaluates differentiation of cultures on a regular basis and develops threshold levels of acceptable differentiation in a culture. The protocol for harvesting followed by equilibration of the sample with CPAs should be carried out rapidly in order to minimize cell losses because of harvesting.

THE BANKING PROCESS

Conventional cell banking procedures require the development of MCB and WCB. Cells in the MCB should be preserved at an early passage number (P10–P20) in order for experimental and cell line development work to be performed on cells at the lowest possible passage number. The first WCB should be established with the minimum passages to achieve the cell number required. To promote consistency, the WCBs subsequently can be replaced at the same passage level from the MCB as required.

VIABILITY

Determination of post-thaw viability and function for PSCs can be complicated and is often done incorrectly. The most common method of post-thaw assessment is use of a membrane integrity dye. As a measure of viability, the fraction of cells with intact membranes is compared to those without. Another fairly common method is to quantitate PSC colony formations. The total number of colonies seeded into a plate is counted and then is counted again after a certain incubation time. This method also evaluates other functional characteristics of the cells, such as their ability to attach and proliferate. Note that procedures such as harvesting and cryopreservation may induce apoptosis. Staining for early (phosphatidylserine on the cell surface) or later (Annexin VI) apoptotic markers may provide insights into the general health of frozen and thawed cultures.

HOMOGENEITY

As already described, preservation of colony fragments by vitrification can exacerbate the lack of homogeneity between vials or straws of cells. When larger banks of PSCs are established, analysts should test vials from early, middle, and late positions in the filling sequence of the cell bank for viability, growth rate, and key markers as indicated in *Viability*.

STEMNESS

In order to check that a PSC line has not lost any of its stem cell characteristics during preservation, analysts should check expression of a number of key stem cell-related markers. One extensive study analyzed 59 hESC lines and a panel of 94 genes and resolved five stem cell-related molecules for which mRNA was expressed consistently in hESCs. These genes now are included in commercially available microfluidics gene cards that are specifically designed for investigating

stem cell populations. If it is crucial to demonstrate that the PSCs have retained their pluripotency, then a number of characterization tests can be performed, including teratoma formation in immune-deficient mice, formation of trilaminar embryoid bodies, and directed differentiation to demonstrate that the culture can produce representatives of each of the three germ layer tissues that are required to form all of the cells of the human body.

GENETIC STABILITY

In both iPSC and hESC lines, it is not unusual for clones of abnormal karyotype to arise on extended passage and overgrow the culture. Thus analysts should monitor cultures for such abnormal cells. Traditionally, this has been performed by karyotypic studies of metaphase spreads of the cells using Giemsa staining. The occurrence of nondiploid cells, even at very low incidence, can be problematic. Guidance documents such as the 2009 International Stem Cell Banking Initiative are helpful to determine if such cultures should be discarded. However, newer procedures such as array comparative genome hybridization and single-nucleotide polymorphism arrays provide much more detailed analysis of genetic stability and can be used in parallel with Giemsa banding to give greater confidence in genetic stability.

BEST PRACTICES

In addition to following good cell culture practices, analysts should note the availability of a specific guidance that contains principles and best practices in the procurement, banking, testing, and storage of hESCs for research purposes (see the ISCB 2009 reference in the *Appendix*). This guidance is useful for both iPSC and hESC lines.

CELL SUBSTRATES USED IN PRODUCTION AND CHARACTERIZATION OF BIOTECHNOLOGY-DERIVED AND BIOLOGIC THERAPEUTIC PRODUCTS

A wide variety of both recombinant and nonrecombinant cells are cryopreserved and used in the production and characterization of human biologics and biotechnology-derived (B&B) products (more information can be found in ICH Q5D *Derivation and Characterisation of Cell Substrates Used for Biotechnological/Biological Products)*. The major groups include cell lines derived from mammals (including humans), insects (primarily moths), and selected strains of bacteria and yeast. The most common microbial substrates used for production of human biotechnology-derived products are recombinant strains of *Escherichia coli, Pichia pastoris,* or *Saccharomyces* spp. (yeasts). Despite the high degree of diversity among cell types used to manufacture B&B products, there is a surprising degree of uniformity across cryopreservation practices and the same principles apply to cells used for manufacture and those used in tests for adventitious agents and product potency.

Typically, a two- or three-tiered cell banking system is used for maintenance of manufacturing cell lines or microbial strains. For those that use a three-tier system, the first tier bank can be referred to as a research, seed, stock, accession, pre-MCB, or parent cell bank. The source of the pre-MCB can be a research or development laboratory, or cells can be purchased from a commercial repository. It is advisable to characterize the parental cell bank prior to its use in cloning. The second tier (or first tier in a two-bank system) MCB or Master Cell Stock (MCS) is prepared directly from this parent cell bank with minimal cell passages or generations. The MCB or MCS is extensively tested to confirm purity, phenotype, genotype, protein expression, or other important attributes. The WCB or Working Cell Stock (WCS) is derived from vials of the MCB after successive passages in culture. The WCB is the manufacturing cell substrate that is scaled up through repeated subcultures to seed the final production bioreactor, fermenter, or lot of culture vessels (e.g., roller bottles). At each tier in the cell banking system, proper cryopreservation is paramount to success in both product development and manufacturing. In some cases, the end of production cells also may be banked for testing purposes as part of cell bank qualification.

Mammalian and Insect Cell Lines

Mammalian cell lines are the cellular substrates of choice for the production of complex protein molecules. Mammalian cells possess the intrinsic biological machinery required for posttranslational glycosylation of proteins that often is critical for stability and bioactivity in humans. Both diploid and heteroploid cell lines (including hybridomas) are used for production of biotechnology-derived therapeutics. Diploid cell lines are common vaccine substrates [e.g., WI-38 and MRC-5 (human fibroblast cell lines), BHK-21 (baby hamster kidney cell line), and MDCK (Madin-Darby canine kidney)]. Additionally, the African green monkey Vero cell line (a heteroploid cell line) is used for several US-licensed vaccines. Today, commonly used heteroploid cell lines include various recombinant Chinese Hamster Ovary and human embryonic kidney cell lines. Cryopreservation methods are fairly well standardized for these cell lines. However, investigators may find it useful to investigate the toxicity of different cryoprotectants and concentrations when they use a new cell substrate.

Insect cell lines have proven their capability for production of various recombinant polypeptides. The most common cell lines are derived from the moths *Spodotera frugiperda* and *Trichoplusia ni,* and their established cell lines are called Sf9 and Tn5 (or High Five), respectively. Production of recombinant proteins employs recombinant baculovirus infection for transfer of heterologous genes. Although insect cell lines require different nutritional factors, lower incubation temperatures, and higher osmolarity than their mammalian counterparts, the same essential elements of cryopreservation apply to both groups.

In addition to the guidance given in the *Key Elements of Cryopreservation Practice* section, these additional points should be considered for animal cell line substrates:

PREFREEZE PROCESSING

Analysts should ensure that cells are not contaminated and are still viable. For diploid cells, analysts should grow to a passage level to maintain diploidy and below the intended level for use. Analysts pool cell cultures and perform a cell count to determine the number of viable cells available for banking. Harvested cells are centrifuged at a relatively low speed for a short duration, e.g., 100–200 × *g* for 5–10 min, preferably using a refrigerated centrifuge.

CPAS AND CRYOCONTAINERS

The membrane-permeable CPA of choice is 5%–10% (v/v) DMSO diluted in fresh growth medium. For certain sensitive cell lines, cell culture-conditioned medium can be added to supplement the cryopreservation medium. DMSO must be sterile and tissue-culture grade (>99% purity). The most appropriate cryocontainer is a presterilized polypropylene screw-cap vial designed for cryogenic storage in vapor phase liquid nitrogen.

INTRODUCTION OF CRYOPRESERVATION MEDIUM

Immediately following centrifugation of cells, growth medium is removed from cell pellets, and cells are gently resuspended by slow addition of cryopreservation medium that is often precooled for many cell types. The cell suspension is immediately diluted with an appropriate volume of cryopreservation medium based on viable cell count and targeted cell density (typically, cell banks are produced at a viable cell density of approximately 1×10^7 cells/mL). As mentioned in the previous section, cryopreservation medium is highly hypertonic, and exposure time should be limited. The final cell suspension is transferred to a vessel in which the cells can be gently mixed during vial filling to facilitate uniformity of the cell bank.

COOLING AND CONTROLLED FREEZING AND STORAGE

Vials can be filled manually by using a hand-held pipetting device or by using an automated vial-filling machine. In either case, vials are usually refrigerated as the filling progresses in order to minimize potential toxic effects of DMSO at higher temperatures. Time limits should be established for the entire filling process and recorded in the batch record. Immediately after the vials are filled, analysts should transfer them into a controlled-rate freezer or, alternatively, into an ultracold static freezer (e.g., −80°) using an insulated container designed for controlled cooling. Any controlled freezing system should be properly qualified to ensure expected cooling rates are delivered to all vials within the load. When using a controlled-rate freezer, analysts determine optimal cooling programs empirically, but a

rate in the range of 1°–5°/min after transition through heat of fusion should be acceptable for most cell lines (colder than about −40°, the rate can be increased, e.g., 10°/min). After vials have been frozen to approximately −80° or below in the freezing system, they are transferred as quickly as possible to liquid-nitrogen vapor phase storage units.

WARMING AND VIAL THAWING

In general, cells frozen at a slow cooling rate should be thawed as quickly as possible to maximize cell viability. In order to ensure uniformity of temperature, vials should be transferred directly from liquid-nitrogen vapor phase storage into a portable vapor phase (dry) shipper for transport to the laboratory. If a liquid-nitrogen Dewar is not available, then vials can sometimes be packed in dry ice, but this process should be demonstrated as suitable because it can result in detrimental pH changes. After transfer to the laboratory, vials are placed directly into a warm water bath (e.g., 37°, making sure caps are not immersed), a bead bath, or a thermoblock. Note that insect cell lines should be thawed at 27°–30°. Vials should be agitated to facilitate uniform thawing of cells. Immediately after thawing, vials should be transferred into the biological safety cabinet and sanitized before opening. Typically, growth medium is slowly added to thawed cells while agitating to dilute DMSO and to slowly reduce the osmolarity of the post-thaw milieu back to a physiological level.

POST-THAW PROCESSING

Different methods can accomplish this step, but analysts should achieve sufficient dilution of the DMSO and return the cells to their normal isotonic growth environment. Manipulation of cells immediately post-thaw should be minimized because of the stresses induced by the freeze–thaw process. For example, pipetting and centrifugation should be minimized.

Microbial Strains: *E. Coli,* Yeast, and Bacterial Vaccine Strains

Recombinant *E. coli* strains have a proven track record for production of a number of commercially viable biotechnology-derived products, including recombinant insulin analogs, human growth hormone, and parathyroid hormone. Compared to their mammalian and insect counterparts, recombinant *E. coli* strains are relatively simple to grow and scale up to large volumes, e.g., 40,000 L. However, bacteria lack the sophisticated cellular machinery for building more complex protein molecules that require post-translational modifications such as glycosylation. Yeasts are unicellular, eukaryotic cells that can be manipulated genetically to produce a wide range of recombinant proteins and peptides with limited complexity. Despite the evolutionary distances between the mammalian and microbial cell substrates, the same set of essential cryopreservation elements described above apply, with the following unique points:

PREFREEZE PROCESSING

Cultures should be propagated in shake flasks to late logarithmic or early stationary phase using strain-specific growth conditions.

CPAS AND CRYOCONTAINERS

The cell wall or cell membrane-permeable CPA of choice is glycerol (although DMSO is also sometimes used) at concentrations typically ranging from 5%–10% (v/v). Synthetic glycerol should be used for registration of commercial products along with other raw materials that are free of animal components.

INTRODUCTION OF CRYOPRESERVATION MEDIUM

Because microbial cells possess cell walls for protection and support of cytoplasmic contents, physical manipulation and osmotic shifts do not have the same negative impact seen in animal cell lines. Immediately after centrifugation, cell pellets are vigorously resuspended in the cryopreservation medium (to a dilution based on requirements of the cell bank for number of vials and viable colony-forming units/mL). This dilution step can be based on active measurement of culture optical density or other cell enumeration assays. The final cell suspension is transferred to a vessel in which the cells can be mixed during filling of final containers to facilitate uniformity of the cell bank. Because of potential toxicity, analysts should limit the time of exposure to glycerol, despite the relative robustness of microbial cells. Vials can be filled manually by using a hand-held pipetting device or by using an automated vial-filling machine.

COOLING AND CONTROLLED FREEZING AND STORAGE

Again, because of their inherent robustness, microbial cells do not require as strict control of cooling rate as do animal cell lines. Consequently, the choice of freezing system or method has a smaller effect on the viability of the cell bank. Filled cryocontainers simply can be transferred into an approximately −80° freezer overnight followed by transfer to vapor phase liquid nitrogen. Alternatively, microbial cell banks may not use liquid nitrogen storage but can be stored at approximately −80°. However, if liquid-nitrogen storage is available, it is preferred for long-term storage (e.g., years). If a controlled-rate freezer is employed, then it should be fully qualified to deliver a uniform cooling rate that is within an expected range, e.g., 1°–5°/min.

WARMING AND CONTAINER THAWING

Controlling the rates of cooling and warming is less critical for microbial cells, but the cells still should be thawed as quickly as possible using a warm water bath (e.g., 30°–35°). Temperatures can be adjusted to the incubation temperature of the strain.

POST-THAW PROCESSING

Growth medium is added to dilute the thawed cells to a desired level of colony-forming units/mL and to induce removal of intracellular glycerol.

All cells are not created equal and are divergent by nature. A broad span of evolutionary time separates the various cell substrates used for manufacture of human B&B products. Fortunately for the practitioner of industrial cell culture, cryopreservation strategies converge to a set of shared principles and methods that translate across evolutionary paths.

APPENDIX

Useful Guidances

- FDA. Guidance for industry: characterization and qualification of cell substrates and other biological materials used in the production of viral vaccines for infectious disease indications. 2010. *http://www.fda.gov/downloads/biologicsbloodvaccines/guidancecomplianceregulatoryinformation/guidances/vaccines/ucm202439.pdf*. Accessed 24 September 2012.
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