BSR/PDA Standard 02-201x, Cryopreservation of Cells for Use in Cell Therapies, Gene Therapies, and Regenerative Medicine Manufacturing: An Introduction and Best Practices Approach on How to Prepare, Cryopreserve, and Recover Cells, Cell Lines, and Cell-Based Tissue Products

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Contents

1 Introduction

Cell-based products originate from biological starting material such as cells from tissue biopsies, blood, and bone marrow that can be developed and manufactured ex vivo into a clinical product. These cells require specialized processes to remain viable and functional throughout their lifecycle, including methods to enable short- and long-term storage and transport between manufacturing and clinical sites. Cryopreservation refers to the processes used to maintain the viability and function of cells at very low temperatures. Cryopreservation procedures for living cells have been studied extensively, but there are few guidance documents to ensure robustness of the cryopreservation, storage, and cell recovery process.

This standard is designed to provide guidance on best practice approaches to develop a robust protocol for the reliable freezing, storage, and recovery of cells intended for clinical applications. It focuses on primary cells or cell lines that are used in research, development, and manufacturing of cell and gene therapy products. A generalized freezing, storage, and recovery flow chart and table are presented in this standard, as well as potential sources of variability and important process considerations. While best practice approaches to mitigate sources of variability are provided, it is important to note that specific protocol steps are likely to be cell- and manufacturing-process dependent. This standard can provide general guidance during the assessment of regulatory requirements.

2 Scope

The purpose of this document is to provide guidance on how to establish suitable procedures for the cryopreservation and recovery of biological cells for use in cell and gene therapy products and regenerative medicine manufacturing. This document presents cryopreservation as a modular process and describes key details that should be considered when developing a cryopreservation and recovery process for a specific use-case. The guide emphasizes the effect cryopreservation and recovery may have on cell viability and cell function. Although best practices for users in cell-based product manufacturing are discussed, specific procedures will likely depend on the nature of the biological cells being cryopreserved.

This document is not intended to provide information on the terms and procedures directly associated with regulatory requirements governing cell-based products. The best practices and guidance details outlined in the document provide general procedural support for cryopreservation of cell-based products during both early and late phases of product development. References to scientific literature and other relevant documents are provided throughout this text for individuals interested in additional information.

This document is a current best practice standard and guide on how to establish suitable conditions for the cryopreservation and recovery of cells, cell lines, and cell-based products for use in cell and gene therapies and regenerative medicine manufacturing. This standard is intended to:

- Discuss considerations for cryopreservation;
- Address the challenges associated with maintaining the viable recovery and functionality of cell and gene therapy products; and
- Outline cryopreservation best practices for the manufacture of cell-based products.

3 Terms and definitions

- Allogeneic – Cells (such as stem cells, cord blood, bone marrow etc.), that are derived from a donor (related or unrelated) and are intended for use in another person that may or may not be genetically related to the recipient donor.
- Autologous – Cells that are derived from the patient being treated.
- Cell Bank – Collection of cells of uniform composition being representative of the original cell culture or cultures from which they are derived.
- Container Closure Integrity – Protection of the component from microbial and gas ingress. One function of the container closure system ***(1,2-5).***
- Critical Process Parameter – A process parameter whose variability has an impact on a critical quality attribute and therefore should be monitored or controlled to ensure the process produces the desired quality ***(6).***
- Quality Attribute – A physical, chemical, biological, microbiological property, or unique characteristic that should be within an appropriate limit, range, or distribution to ensure the desired product quality **(7).**
- Cryopreservation – Maintenance of the viability and function of cells, tissues and organs by the process of cryoprotection, cooling and storing at very low temperatures. This refers to the protection of cells, tissue and organs from damage that can occur during cooling and storing at very low temperatures ***(8).***
- Cryopreservation Formulation – Developing cocktail of compounds with properties found to be advantageous to overcome cryopreservation damages.
- Crypreservative – Reagents, or a combination of reagents, that protect the cells against the stresses of cold and ice formation during the freezing and thawing procedure.
- Excipients – Any inactive ingredients that are intentionally added to therapeutic and diagnostic products, but: (1) are not intended to exert therapeutic effects at the intended dosage, although they may act to improve product delivery (e.g., enhance absorption or control release of the drug substance); and (2) are not fully qualified by existing safety data with respect to the currently proposed level of exposure, duration of exposure, or route of administration.
- Extractables – Organic and inorganic chemical entities that can be released from a pharmaceutical packaging/delivery system, packaging component, or packaging material of construction and into an extraction solvent under laboratory conditions ***(9,10)***.
- Glass Transition Temperature – The temperature below which the molecular motion slows to form a glassy state without ordered structures (e.g., ice crystals).
- Leachables – Foreign organic and inorganic chemical entities that are present in a packaged drug product ***(9,10)***. Leachables are typically a subset of extractables derived from the container.
- Master Cell Bank – An aliquot of a single pool of cells which generally has been prepared from the selected cell clone under defined conditions, dispensed into multiple containers and stored under defined conditions. A Master Cell Bank is used to derive all working cell banks.
- Operational Qualification – A series of tests which ensure that equipment and its sub-systems will operate within their specified limits consistently and dependably.
- Packaging component – Any single part of a container closure system ***(1)***. This includes primary packaging components (those in direct contact with the component) and secondary packaging components (packaging components that are not in direct contact with the component).

- Potency – Potency is the specific ability or capacity of a product to achieve its intended effect. It is based on the measurement of some attribute of the product and is determined by a suitable quantitative method ***(11)***.
- Primary container closure system – The packaging whose material comes in direct contact with the product in a closed system. The assembled packaging components together contain and protect the cells during pre-freezing, freezing, storage, shipping/ transportation, thawing and post-thaw ***(1)***.
- Qualification – May apply to equipment, process and or personnel. Action of proving and documenting that systems are properly installed, work correctly, and lead to the expected results. Qualification is part of validation, but the individual qualification steps alone do not constitute process validation ***(12)***.
- Quality Control – Checking or testing that specifications are met ***(12).***
- Thawing – The process of changing the state of cells or tissues from frozen to liquid form during which ice crystals dissolve as a result of rise in the temperature of the cells or tissue.
- Validation – Confirmation, through the provision of objective evidence, that the requirements for a specific intended use or application have been fulfilled ***(13)***.
- Working Cell Bank – Cells prepared from aliquots of a homogeneous suspension of cells obtained from culturing the Master Cell Bank under defined culture conditions ***(14)***.

4 Acronyms

BSE	Bovine Spongiform Encephalopathy
CCS	Container Closure System
cGMP	(current) Good Manufacturing Practice
CPA	Cryoprotectant Agent
CQA	Critical Quality Attribute
DMSO	Dimethyl Sulfoxide
FDA	United States Food and Drug Administration
ICH	International Council of Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
IQ	Installation Qualification
ISO	International Standards Organization
MCB	Master Cell Bank
OQ	Operational Qualification
PDA	Parenteral Drug Association
PQ	Process Qualification
TR	Technical Report
TSE	Transmissible Spongiform Encephalopathies
USP	United States Pharmacopeia
WCB	Working Cell Bank
WHO	World Health Organization
7AAD	7-Aminoactinomycin D

5 Critical Quality Attributes of the Cell-based Product

Critical Quality Attributes (CQAs) for cell-based products evolve throughout the product development process. While CQAs vary depending on the product and its intended use, common attributes such as cell viability, cell number, and potency should be part of the risk-based approach used to develop cryopreservation protocols for cell therapy products. CQAs should be identified early in development and finalized as product understanding and knowledge are gained through the product lifecycle. While CQAs for cell-based products vary depending on the product and its intended use, there are common attributes, for example potency, purity, and cell viability, that could be affected by the cryopreservation process and therefore should be considered during development. Product CQAs that are impacted by the cryopreservation process should be determined using a science and risk-based approach, so that the process parameters and controls that may impact any CQA can be identified as part of the overall process control strategy. Some products involve the development and maintenance of Master Cell Banks (MCB) and Working Cell Banks (WCB), and producer cells (cell banks) that also rely on appropriate cryopreservation.

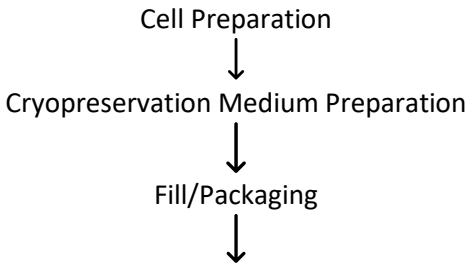
Viability is considered a CQA where the cells must be viable in vivo for biological activity. Cell viability should be maintained throughout cryopreservation to ensure a sufficient number of viable cells to meet dosing requirements and minimize cellular debris which could impact post-thaw biological activity. The cryopreservation process should also be designed to minimize the levels of residual impurities (i.e., reagents, serum, non-target cells).

Biological activity or potency should be identified as a CQA of a cell-based product, and the cryopreservation process should be developed to have minimal impact on potency. Due to the complexity of many cell-based products, multiple direct or surrogate assays to determine potency may be required. Additional information on potency can be found in the FDA guidance on Potency Tests for Cell and Gene Therapy Products ***(15)***.

CQAs related to safety that may be impacted by the cryopreservation process include, but are not limited to, sterility, endotoxin, adventitious viruses, and impurities introduced into the cell-based product (both product- and process-related). The cryopreservation process should be developed using a science and risk-based approach such that any process variable that could adversely impact a CQA is considered, and controls are implemented to manage them in order to mitigate the associated risks. Details on this science and risk-based approach to controlling and monitoring microbiological attributes throughout the cryopreservation process are discussed in subsequent sections of this document.

6 Cryopreservation Process Steps and Critical Process Parameters

Cryopreservation should protect cells while maintaining CQAs. A science and risk-based approach should be used to develop and validate the cryopreservation process as part of the process validation lifecycle approach ***(6,16-19)***. Cryopreservation process inputs and outputs should be defined, and risk assessments should be used to identify those inputs where variability could impact product CQAs. Utilizing a thorough understanding of the relationships (causality) and criticality between process inputs and outputs, a well-controlled cryopreservation process should be designed and developed that reliably maintains cell quality. The general process for cell cryopreservation will be similar across cell-based products but will differ depending upon individual cell types and according to manufacturing processes and units (e.g., lot size, dose, container-closure system (CCS)).



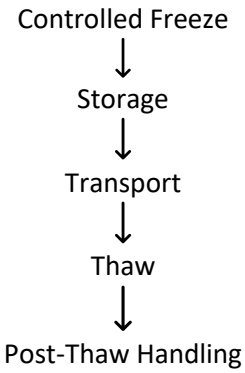


Figure 1. Generalized process steps in cell cryopreservation.

Typical steps involved in the cryopreservation process are listed in **Figure 1**. The process begins with the collection and isolation of cells, including those obtained from the source material or from the prior manufacturing stage. During collection, the target cells may be isolated, enumerated, and/or separated from the culture medium (e.g., via centrifugation and wash) to prepare the isolated cells for formulation in cryopreservation medium. The isolated cells are then formulated to the desired cell concentration in cryopreservation medium consisting of a cryoprotectant agent(s) (CPA) at an appropriate temperature. The formulated cell suspension is then transferred (filled) and sealed (packaged) into an appropriate CCS suitable for cryogenic temperature freezing and storage. Established and validated cryopreservation processes that include storage, thawing, and post-thaw activities should be followed and supported by appropriate product stability studies. If shipping the cryopreserved product, specialized shipping systems capable of maintaining cryogenic temperature storage should be adopted. Prior to use, the cells are removed from storage and thawed to safely recover them from cryopreservation. Equipment may be used to control the temperature profile and rate of thaw. Further process steps may be required post-thaw prior to use depending on manufacturer's requirements, an example of which may be the removal of cryopreservation medium and/or dilution with an appropriate medium/buffer.

6.1 Material qualification

Cells intended for use as starting material can be sourced from human donors (autologous and allogeneic). In some instances, animal-derived cells are used to support the growth of these cells during development and manufacturing. Donors/sources of these cells should be individually screened and tested as outlined in relevant international and/or regional requirements ***(14,19-20)***. For example, in the US, allogeneic cell sources require donor screening and testing ***(21)*** whereas autologous cell sources may require labelling requirements but not donor eligibility determination ***(22)***. Cells derived from donors should be assessed for safety, viability, identity, and purity.

In addition to cells, medium and ancillary materials should be examined for potential sources of contamination prior to formulation into cryopreservation medium. Appropriate controls for testing with acceptance criteria should be in place for raw materials (e.g., culture medium, reagents, excipients, biologicals, chemicals) used in manufacturing to assure that cells are free of contamination and adventitious agents. Assessment and guidelines for ancillary materials should be followed according to ISO/TS 20399-1-3:2018***(23)***.

Cells which were manufactured with animal-derived materials (e.g., derived from bovine, porcine, and ovine sources) should incorporate appropriate controls to minimize the risk of introducing Bovine Spongiform Encephalopathy (BSE)/Transmissible Spongiform Encephalopathy (TSE) to humans. A BSE/TSE compliance certificate (e.g., certificate of origin) and certificate of analysis should be requested from material suppliers/manufacturers and reviewed and evaluated prior to use during cryopreservation and/or manufacturing ***(22)***. Several USP ***(24)*** and WHO guidelines ***(25)*** provide useful information on risk reduction strategies for transmitting BSE/TSE.

6.2 Process parameters

Adherence to a science- and risk-based approach to process development requires breaking the cryopreservation process down into discrete steps and establishing the relationships between process inputs and outputs ***(6-7,17)***. Process inputs can include materials, equipment, and process parameters. Process parameters refer to variables or conditions directly controlled in the process such as equipment settings, product volumes, cell and cryoprotectant agent concentrations, process and chronological times, temperatures, and other similar numerical variables. **Table 1** is a generalized list of typical input variables that should be considered in the development of a cryopreservation process for a cell-based product. The list is not comprehensive and is merely intended to provide examples of important process variables. A detailed discussion of important considerations for different steps, materials, equipment, parameters, and output measures are provided in the sections below.

Table 1. Examples of Generalized Inputs for Cell Cryopreservation Process

Process Step	**Process Input Variables**		
	Materials	**Equipment**	**Examples of Process Parameters[1]**
Cell Preparation	(Starting) Cell Material Cryopreservation containers Cryopreservation medium	Cell counter Dispensing equipment	Cryopreservation medium temperature Concentration of cryopreservation medium components Total preparation process time Cell concentration
Controlled Freeze[1]	(Starting) Cells in cryopreservation medium in cryopreservation containers	Controlled temperature freezer (active and/or passive devices)	Temperature profile (e.g., step rates, hold times and temperatures, end temperature) Sample volume Batch Size (number of containers, fill volume) Type of container
Storage	(Starting) Cryopreserved cells in controlled rate or passive cooler	Ultralow mechanical freezer, dry ice, or LN2 storage dewar	Storage Temperature Liquid nitrogen level Closure container integrity Frequency of removal of stored articles Storage time
Transport	(Starting) Cryopreserved cells in long-term storage	Controlled temperature shipping system	Temperature throughout shipment Record of sample addition/removal/product orientation during shipment Shipping time closure system integrity
Thaw (Recovery)	(Starting) Cryopreserved cells	Controlled temperature warming equipment	Temperature

[1] Set points and ranges for operational process parameters should be determined.

7 Cell Characterization and Testing

All MCB, WCB, and cell stock should be cryopreserved. In-process and final product cells can also be cryopreserved when appropriate. Cryopreserved cells should be characterized and tested as described in this section.

MCB, WCB and cell stock should be shown to be absent of contaminating adventitious agents, including bacteria, fungi, virus and mycoplasma. Cell banks should have associated validated and/or verified assays to assess appropriate quality, including identify, purity, and biological activity.

International regulatory guidance documents such as ICH Q4 for Pharmacopoeias, ICH Q5A for Viral Safety Evaluation, ICH Q5D for Cell Bank Generation, Characterization and Testing ***(26)***, and regional regulatory guidance documents such as FDA guidance "Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals"***(27)*** provide useful information on adventitious agent testing. Detailed information on tests for sterility, mycoplasma, endotoxin and viruses are provided in the relevant pharmacopoeias ***(28-31)***.

Use of compendial/pharmacopoeia and regional-specific (e.g., FDA) approved/cleared test methods is recommended for adventitious agent testing when applicable. Compendial test methods do not need to be validated but should be verified for intended use under the actual conditions of use ***(32)***. All the tests used for adventitious agents should be qualified/validated if these methods are not compendial methods.

Cell testing is performed prior to or following cryopreservation as appropriate. Additionally, phase-appropriate cell qualification and release testing must also be performed prior to or following cryopreservation intended for clinical use as appropriate. Cell characterization and testing are performed throughout the entire cryopreservation process, from starting material through storage/thaw, and should include stability studies. All characterization methods should be qualified according to the facility's policies, procedures, and processes ***(33)*** and analytical methods should be qualified/ validated as appropriate. **Table 2** outlines the various recommended tests at process steps. Also note that some of these tests could be used for release testing.

Table 2. Cell Characterization Testing at Different Developmental Stages

Tests/Process Step	Identity/Appearance	Purity	Viability	Potency	Bacterial and Fungal Sterility	Mycoplasma	Viral and Other Adventitious Agents
Starting Materials	X	X	X	X	X	X	As applicable[3]
Cell Collection/Harvest	X	X	X	X	X	X	As applicable[3]
Formulation in Cryopreservation Medium	X	X	X	X	X		
Fill/Packaging[2]	X	X	X	X	X		
Storage/Thaw		X	X	X			
Stability	X	X	X	X	X		

[2] Release testing if applicable.

[3] Dependent upon regional regulatory guidance.

7.1 Identity/Appearance

The cell source shall be identified using either phenotypic or genotypic characteristics. Appearance (e.g., visual examination for color, clarity, container defects, cell aggregates, foreign visible particulates, etc.) might be performed in the cryopreservation containers after fill while the cell suspension is in non-frozen state if justified. Modifications or alternative approaches to visible particulate inspection methods may be necessary due to cells (inherent particulate) and container opacity making the cell suspensions difficult to inspect ***(34)***. Special consideration may be necessary for visible particulate inspection methods due to the cellular composition (inherent particulates) and opacity making the suspension difficult to inspect ***(34)***.

7.2 Purity

Cell purity is defined as relative freedom from extraneous material regardless the impact to the cell population ***(11)***. Impurities can be cell-related (e.g., unintended cellular phenotypes, dead cells, and debris), process-related (e.g., residual proteins or peptides used to stimulate, or pulse cells and reagents/components used during manufacture) or related to contaminants of microbial origin. Appropriate purity testing should include a measurement of contaminants that may impact patient safety if justified.

7.3 Viability

Cell viability is often used as a rapid indication of cryopreservation efficacy post-thaw, as low cell viability often correlates with poor cryopreservation efficacy. The presence of non-viable cells in a cellular therapeutic product may pose a safety risk, therefore the percentage of viable cells within the total number of cells should be determined.

7.3.1 Viability Testing

The most common viability tests for cell suspensions can be separated into two groups, cell membrane permeating and non-cell membrane permeating. Examples of cell permeating dyes include fluorescein diacetate and acridine orange. These compounds interact with an elemental structure within the cell, such as proteins, DNA, or RNA, or are metabolized into a new fluorescent state. Fluorescence indicates the number of viable cells within a population, and should be combined with a secondary agent, such as Hoechst 33342, which will generate fluorescence within all cells. Non-cell permeating dyes such as trypan blue and 7AAD are also common and can be added to a cell suspension. Cells with an intact cell membrane are not colored whereas those with a compromised cell membrane can be visualized. Additional viability indicators are available, and dyes chosen to evaluate cell populations post-thaw should be qualified.

Cell viability can be determined through manual counting such as with a hemocytometer, or through use of an automated cell counting system. Qualified automated systems are recommended to minimize operator error and inter-operator variability. The time between adding a dye and assessing viability should be constant, as should the time between thaw and addition of a dye. Sample temperature and storage conditions should also be standardized and qualified/validated for the assay. Samples thawed and assessed at 37°C may generate different results than those under ambient conditions and therefore, temperature should be controlled and recorded. Viability of the cell population both pre-freeze and post thaw should be determined.

7.3.2 Viability Timepoint Considerations

Cell post-thaw viability is dependent on the time between thaw, dilution or removal of cryoprotectant, and subsequent assessment. This is due to cryopreservation-induced delayed onset cell death and metabolic dysfunction during the cell recovery cycle. Performing viability tests at several timepoints post thaw is recommended to ensure the most accurate assessment of cell viability.

7.4 Biological function

A biological function or potency test is based on cell-specific attributes and demonstrate that the manufactured lots meet defined specifications. Functional assessments should be performed post-thaw to ensure that biological activity is not negatively impacted by the cryopreservation process. The impact of cryopreservation process parameters on biological function should be assessed during process development and may necessitate additional potency assessment points.

7.5 Sterility assurance and testing

Cells are aseptically processed because cells cannot be terminally sterilized and sterile filtered. Appropriate controls such as testing sterilization should be in place for raw materials, container closure systems, equipment, and other items used in the cell cryopreservation process to assure that cells are free of bacteria and fungus.

Sterility testing is required for cells intended for both product release ***(35)*** and banking ***(14)***. In-process cells may also be tested for bacterial and fungal contaminants. Because freezing may have negative impact on sterility testing, freezing of sterility test samples are not recommended and samples are taken for sterility testing prior to cryopreservation. A compendial test method ***(29)*** is recommended for sterility testing when possible. If alternative or rapid microbiological test methods are used, these methods should be validated ***(36)***.

7.6 Mycoplasma

Cells should be tested for the absence of mycoplasma prior to formulation in cryopreservation medium and filling using appropriate and validated methods.

7.7 Viral and other adventitious agent testing

Cells must be shown to be free of viral and other adventitious agents. The most appropriate tests will be determined by the starting material and history of the cells. Use of compendial/ pharmacopeial and FDA approved/cleared test methods are recommended for viral and other adventitious agent testing when applicable ***(14,27,37-38).*** Cells are usually sampled for adventitious agent testing prior to long term cryostorage.

7.8 Stability

Stability refers to the ability of a material, when stored under specified conditions, to maintain a value(s) for stated property(ies) within specified limits and for a specific period of time ***(39)***. A stability program includes the assessment of the impact of cryopreservation and storage on the quality of a cell preparation to determine shelf-life. A continuous sampling program intended to assess adherence to cellular specifications throughout the expiration date should be adopted as appropriate. Parameters assessed in a stability program may include all or any of the following: identity, viability, potency, sterility, viable cell number, and container closure integrity. Stability studies for the final product should be conducted with its actual container closure system whenever possible ***(40)*** or in a vessel of similar size and composition. Retention samples from nonclinical, clinical, and comparability studies should be maintained (preferably) in a short-term location that will be readily accessible without interfering with long–term storage. Storage conditions should be adopted and documented according to the facility's policies, procedures, and processes.

8 Formulation Development

8.1 Cryopreservation medium

Medium should be qualified before use and the extent of qualification depends on formula complexity and susceptibility to batch-to-batch variations. Cryopreservation medium should be evaluated for risks and confirm suitable testing and characterization if possible, as outlined in 7 above. Medium and material

used as part of a cryopreservation formulation must be sterile and free from adventitious agents including microorganisms and viruses. The origin of the material, components, and the final complex composition should be considered when determining applicable tests for material qualification.

8.2 Cryoprotectant agent

8.2.1 Qualification for cryoprotectant agents *(41-43)*

Cryoprotectant agents are comprised of substances intended to protect cells during the cooling, freezing, and thawing process. Examples of commonly used cryoprotectant agents include dimethyl sulfoxide (DMSO), glycerol, and propanediol, and human serum along with various salts and sugars. Components of the cryoprotectant agent should be of the highest quality available and pharmacopeial grade when possible. A risk assessment should be conducted on the components present in the cryoprotectant agents used for a specific cell-based product to identify and evaluate the potential risks of use (especially for any novel excipients) and safety (through nonclinical and/or clinical data where appropriate).

8.2.2 Cryoprotective agent (CPA) exposure

When possible, cryopreservation procedures should include a pilot study during process development that determines a suitable method of introducing and removing the cryoprotectant agent during freezing, storing, shipping (if part of process), and thawing. The study should include the concentration of cryoprotectant agent and time and conditions of exposure. Stability and functionality should be determined over an interval of time and can be determined through various assay types such as viability, potency, and biological activity. Consideration for time intervals should include process parameters such as permissible time lapse prior to adding cryoprotectant agent, in-process storage or hold times, and in the final fill/finish configuration. The selected CPA and its concentration within the cryopreservation formulation is also dependent on cell concentration. Results of the study are documented and serve as the source for standard operating procedure development.

9 Cryogenic Container Closure Systems

9.1 Cell Package selection, qualification, quality control and integrity testing

Selection of a primary container closure system is critical to preserve cell quality during cryostorage and should be suitable for its intended use in freezing, long-term storage, shipping and thawing of cryopreserved cells.

Suitability of a cryogenic container closure system is determined with appropriate studies evaluating, but not limited to, the ability to perform in specified temperature range specifications. Containers should allow for uniform thermal properties to prevent uneven heat transfer during the cryopreservation and thawing process. Appropriate quality controls such as testing and acceptance criteria for physical characteristics (e.g., appearance, shape, and dimension), performance, pyrogen, particulate matter, and materials of construction should be in place for all the components of CCSs (primary and secondary) to ensure consistency for established and approved conditions and quality in accordance with relevant pharmacopoeia and regional regulatory requirements.

Primary container closure system/packaging components should further be cleaned, sterilized, and processed to remove pyrogenic properties and particulates as suitable ***(44-45)***. Sterilization of primary packaging components by qualified and/or validated sterilization processes is required to prevent microbial contamination, and sterilized container components should not be used after their expiry date as recommended.

All packaging components of cryogenic container closure system (e.g., bag/container, tubing sets, connectors, ports, caps etc.) should follow applicable relevant pharmacopeial requirements and ISO standards ***(46-49)*** for biocompatibility. Contact surface of a primary container closure system, particularly for cell-based products, should not be reactive, additive, or absorptive ***(45)*** and container

closure systems should be evaluated for extractables and leachables as outlined in relevant pharmacopoeias, regulations, and guidance documents ***(50)***.

Integrity of the container closure system should be evaluated to ensure no microbial and virus contamination during cryogenic storage and shipping, and integrity test methods selected for intended use should be qualified and/or validated as suitable ***(5)***. Container closure systems should also be visually inspected ***(51-54)*** for leaks, breakages, and other signs of damages and non-conformities, following filling and upon thawing as applicable.

9.1.1 Container integrity/secondary container considerations

Hermetically sealed vessels are recommended for clinical products. Secondary containers such as 'overwrap' bags, 'over pouches,' or cassettes and boxes can provide additional protection for leaks, breakages, and other non-conformities during cryostorage and transport. The secondary container should be composed of a similar low-temperature resistant material ***(1)***. Secondary packaging should be evaluated through appropriate studies to assess the impact of this packaging on cryopreservation, storage, and transport. Manufactures should be aware that if thawing is being controlled, the secondary container can also impact the thawing profile.

10 Cryopreservation procedures

Cellular products are sensitive to several key parameters during the freezing protocol. This section outlines typical, important steps that should be considered, but is not comprehensive and is intended to provide general guidance. Processes should be designed to have physical separation so that only one (1) lot is processed at one time in one place and that proper cleaning between lots is performed.

10.1 Qualification of freezing device

Cryopreservation of cells can be conducted using controlled rate freezing (using programmable freezers) and passive cooling (including use of insulated containers). Freezing programs are generally stored within an electronic system. Temperature probes can be used in mock set-up or similar product containers to monitor the freezing process. The use of a controlled-rate freezer is the most precise choice but needs to be evaluated and qualified for each cell type or configuration. After cells are loaded into a controlled rate freezer, the system should remain closed until the cooling cycle is complete. Inappropriate opening of the freezing device during cooling can both expose a user to cold injury and introduce uncontrolled excursion into a system. Any opening of the controlled rate freezer during cooling should be automatically recorded. Excursions to the freezing process (e.g., opening the freezer door, power failures) must be evaluated for their influence on cell quality attributes such as viability. The acceptable temperature to end the cryopreservation program should be established.

Passive freezing methods refer to the use of an insulated container in a validated freezer (e.g. -80°C or -150°C) to maintain a controlled rate of temperature decline. The cooling rate should be evaluated, and temperature probes should be used to monitor the temperature. Passive freezing methods with more than few containers may have significant differences based on the geometry of the container placement within the unit. Passive freezing validation should show that all containers freeze nearly identically (typically, the samples on the edge freeze first).

While application of cGMP is required under section 501(a)(2)(B) of the FD&C Act ***(55)*** at all stages of clinical investigation, cGMP regulations ***(56)*** are not required for the manufacture of most investigational new drugs under Phase 1 INDs ***(57)***. Upon completion of freezing, the actual temperature profile achieved should be recorded to confirm the process parameters are within their acceptance criteria. Like other manufacturing procedures, deviations should be considered before any cooling process takes place. Any unplanned deviations between the programmed and actual cooling rate should be identified and addressed according to standard operating procedures.

10.2 Ice Nucleation

The point of ice formation (nucleation) is a critical step during the freezing process that is dependent upon the cryopreservation medium, cryopreservation volume, and sample form factor. Ice nucleation is stochastic and typically occurs a few degrees below the freezing point. It can be identified as a transient increase in temperature due to the latent heat of fusion. It is recommended that ice nucleation temperature be controlled where required and documented if possible, during the freezing profile.

10.3 Transfer

There is a risk of product warming during transfer to long-term storage. Any transfer should be carried out in an insulated pre-cooled container, and the process validated to ensure that the container maintains a constant sample temperature during the transfer process. Transfer to and from both long-term and in-process storage should be documented and evaluated for temperature excursions outside of a validated range. All transfers should be recorded to ensure chain of custody and chain of identity are maintained for traceability.

10.4 Documentation

Documentation of the cryopreservation parameters, time, equipment, and operator is required for batch manufacturing records. The date and time of cryopreservation must be recorded including the time that samples are transferred to long-term storage. The time reporting period is defined as starting when cells are loaded with cryoprotectant and ending when samples are transferred to long term storage. Each sample shall have its own unique identifier, which remains constant through all stages of manufacturing including cryopreservation, storage, thawing, and final use to reduce the risk of misidentification. The unique identifier shall be labelled on each sample as well as recorded in related documentation. Automated systems that allow for labelling and tracking such as bar codes and scanners, RFID, printed labels, and computer systems reduce the introduction of human errors and are recommended.

11 Cryogenic Storage and Transport

11.1 Cryogenic storage

The storage conditions/criteria should be defined and qualified to maintain cell quality attributes throughout the duration of storage. Cryogenic storage of products should be monitored with qualified temperature monitoring devices. The procedures should describe the operating instructions and requirements for the system used to monitor the storage temperature and includes maintaining the record of these temperature data within the retention period. Temperature monitoring records should be reviewed regularly and approved to ascertain whether an excursion occurred. Temperature excursions should be documented and investigated to assess any potential product degradation. Back-up systems should be in place for cryogenic storage. A defined inventory control system should be implemented to ensure samples and products can be tracked for storage throughout the product lifecycle ***(58)***.

11.2 Alert limits and action

Control systems should be designed to reduce the likelihood of a temperature excursion. Alarms should be in place to indicate any excursions and to alert in case of excursion outside of acceptable range. Any excursions should be recorded and reported according to a facility's policies, procedures, and processes**.** A thorough investigation should be carried out to identify the root cause of the temperature excursion. The investigation should include a review of any previous occurrence temperature excursion for indication of specific or recurring problems. Appropriate corrective actions and/or preventive actions should be identified and implemented following the investigation to prevent reoccurrence. The effectiveness of such actions should be monitored and assessed. The details of the investigation, corrective action and/or preventive actions should be recorded, and this record should be reviewed and approved by quality personnel. Any products stored within the affected area should not be released for use or supply prior to completion of the assessment and investigation.

627
628 ## 11.3 In-process hold

629 The processes, workflows, and production processes should be documented and evaluated for any critical
630 steps where product is temporarily held outside of its long-term storage condition ***(59)***, including the use
631 of quarantine storage until release testing is complete and product is approved for transfer to long term
632 storage. Product stability should also be evaluated under in-process hold conditions.

633 ## 11.4 Transport

634 Transportation control and monitoring includes managing traceability and maintaining chain of custody.
635 Clear expectations and communications must be established from the cell product manufacturer and the
636 primary and secondary transportation service providers to the receiver at the destination site (e.g.,
637 clinical site) where chain of custody ends. Validated processes for cell transportation should be
638 appropriately and adequately planned, executed, traced, and documented to ensure container integrity
639 and cell quality. General requirements and points to consider for transportation service providers,
640 clients, and senders to ensure cell quality, safety, and efficacy during the transportation process can be
641 found in ISO 21973:2020 ***(60).***

642 ## 11.5 Label integrity

643 Types of labels and markings used should be designed to withstand extreme temperatures. Markings on
644 labels should be legible. Labels should not penetrate the container. If labels are too small to contain all
645 required information, additional information can be included in associated accompanying
646 documentation. Different cell types should be appropriately segregated by physical, spatial, or electronic
647 separation from each other. Label qualification and control procedures shall be in place and implemented.
648 Label qualification should include confirmation of adhesion to the container for long term storage
649 ***(61,62).*** The International Council for Commonality in Blood Banking Automation provides information
650 on product coding and labelling for ISBT 128 ***(63).***
651

652 ## 11.6 Equipment and qualification

653 Equipment used in freezing, cryogenic storage, and thawing shall be installation, operational and
654 performance qualified (IQ/OQ/PQ) for intended use ***(64,65,66).*** Qualified freezing equipment should be
655 used to validate cell freezing process as outlined in available guidelines ***(6,65).*** Freezing equipment
656 should be temperature mapped for empty and maximum load configurations. Equipment used in cell
657 freezing, storage and thawing should be in appropriate design, adequate size, and suitably located to
658 facilitate operations for its intended use and for its cleaning and maintenance ***(66)***.

659 # 12 Thawing

660 Thawing of cryopreserved products is a critical step that may impact product quality. While thawing most
661 products, it is important to maintain a reproducible and consistent warming rate throughout the sample.
662 During thaw, the temperature rises consistently and produces a temperature gradient across the vessel.
663 As such, cells closer to the periphery of the vessel experience more rapid warming and are exposed to
664 cryoprotective agents in non-frozen conditions for longer duration. Therefore, the thawing process needs
665 to be adequately designed and qualified. Thawing and associated post-thaw processing should be
666 evaluated during process development using a risk-based approach. Thawing can be performed using
667 either automated devices or manual procedures using a water bath, bead bath or thermal block. Manual
668 thawing procedures should be qualified to minimize variability. This variability can be higher when cell
669 products are thawed at clinical sites; therefore, cell product manufacturers should provide established
670 thawing instructions (written thawing procedures) to clinical sites.
671

672 ## 12.1 Duration of thaw

673 The time from removal from liquid nitrogen storage to full thaw (when no ice crystals are visible to the
674 naked eye) is an important parameter that can directly affect cell quality. An optimal thawing rate

maximizes cell viability and recovery. Time limits for thawing and post thaw hold should be established to assure cell CQAs and are required for each phase of production ***(59)***. Additional measures of functionality such as stability studies may be conducted if the thawing process is found to impact product CQAs.

12.2 Holding before freezing or after thawing process (post-thaw hold)

Cryopreservation medium is commonly formulated with components that minimize temperature and freezing stress on cells. These components are not necessarily compatible with biological function and may be harmful during short- or long-term exposure at non-frozen temperatures. Hence, any holding of cells in cryopreservation medium under non-frozen temperature should be minimized when possible and further processing or administration of thawed drug product should be performed in a timely manner. If necessary, maintaining the temperature between 2-8 °C could help to minimize any adverse effects of the cryopreservation medium to the cells during the holding period. Holding temperature and time should be evaluated and determined to be applicable to the specific cellular product.

12.3 Washing

Depending on the components within the cryopreservation medium, a washing step after thawing may be necessary. In cases where the cryopreservation medium contains components that are unapproved for in-human administration, the medium should be considered an ancillary material. The cryopreservation medium should be removed with washing after the thawing process prior to the next process step. This washing process should be validated during process development. In cases where the cryopreservation medium contains components that are approved for in-human administration, product risk assessment should be performed during process development to determine whether a washing step after thaw is required.

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