BSR/PDA Standard 002-201x, 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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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, tissues and organs at very low temperatures. Although preservation standards are available for some biopharmaceuticals, these methods are not suitable for cell-based products. 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 potential 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 maybe used in research, development, and manufacturing of cell and gene therapy products. Generalized freezing, storage, and recovery flow charts are presented in this standard, and potential source of variability and important process considerations are identified. While best practice approaches to mitigate sources of variability and to meet current Good Manufacturing Practices (cGMP) are provided, it is important to note that specific protocol steps are likely to be cell, manufacturing process and product design dependent. This standard can serve as a general guide for industry in the development and assessment of cryopreservation processes for the manufacture of cell and gene therapy products. All cryopreservation process steps should be performed by appropriately trained and qualified operators. This philosophy is intrinsic to this standard at all levels of application. All facilities should follow policies, processes, and procedures within their quality system. Applicable regulatory requirements for integrity testing on the primary packaging may vary. Regulatory requirements should be consulted with regional regulatory authorities and guidance documents.

2 Scope

This document is a current Best Practices Guide on how to prepare, cryopreserve, and recover cells, cell lines, and cell-based products for use in cell and gene therapies and regenerative medicine manufacturing. This proposed American National Standard (ANS) 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 users in cell-based product manufacturing.

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.
- 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 that is stored under defined conditions.
- Cryopreservation – Maintenance of the viability and function of cells, tissues and organs by the process of cryoprotection, cooling and storing at very low temperatures. Cryoprotection refers to the

protection of cells, tissue and organs from damage that can occur during cooling and storing at very low temperatures ***(1)***.

- Cryopreservation reagents - Reagents, or a combination of reagents, that protect the cells against the stresses of cold and ice formation during the freezing and thawing procedure.
- Packaging component - Any single part of a container closure system ***(2)***. This includes primary packaging components and secondary packaging components.
- 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 ***(2)***.
- Potency - The specific ability or capacity of the product, as indicated by appropriate laboratory tests or by adequately controlled clinical data obtained through the administration of the product in the manner intended, to effect a given result ***(3)***.
- 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.
- Critical 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***(4)***
- 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 ***(5)***
- 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. The Master Cell Bank is used to derive all working cell banks. The qualification tests performed on a new Master Cell Bank (from a previous initial cell clone, Master Cell Bank or Working Cell Bank) should be the same as for the (original) Master Cell Bank unless justified ***(6)***.
- Working Cell Bank - The Working Cell Bank is prepared from aliquots of a homogeneous suspension of cells obtained from culturing the Master Cell Bank under defined culture conditions ***(6)***.
- Producer cells - Cells that are used to make gene therapy vector such as mammalian cells producing a recombinant viral vector.
- Container Closure Integrity -Per the FDA guidance, Integrity is a measure of the defined protection (from microbial and gas ingress) and therefore it can be considered one of its functions. ***(2, 7-10)*** .
- Suitability - Tests and studies used and accepted for the initial qualification of a component or a container closure system for its intended use ***(2)***.
- Quality Control – Checking or testing that specifications are met ***(11)***
- 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 ***(12, 13)***
- Leachables - Foreign organic and inorganic chemical entities that are present in a packaged drug product ***(12, 13)***. Leachables are typically a subset of extractables derived from the container.
- Validation - confirmation, through the provision of objective evidence, that the requirements for a specific intended use or application have been fulfilled***(14)***
- Qualification - Action of proving and documenting that equipment or ancillary systems are properly installed, work correctly, and actually lead to the expected results. Qualification is part of validation, but the individual qualification steps alone do not constitute process validation. ***(11)***
- Operational Qualification - A series of tests which ensure that equipment and its sub-systems will operate within their specified limits consistently and dependably.

- Glass Transition Temperature – The temperature below which the molecular motion slows down to a to form a glassy, state without ordered structures (e.g. ice crystals). Storage below Glass Transition is necessary to ensure long-term stability of the cells.

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
IATA	International Air Transport Association
ICH	International Council of Harmonization
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

The critical quality attributes (CQAs) of the cell-based product should be identified early in development and finalized as the manufacturing process is locked and product understanding, and knowledge are gained. The CQAs of the product should be considered through process development to determine critical process parameters and controls that define a product control strategy. The CQAs that are impacted by the cryopreservation process should be determined using a science- and risk-based approach where process parameters and controls linked to cryopreservation CQAs are defined.

While CQAs for cell -based products may vary, there are common attributes that could be affected by the cryopreservation process and therefore should be considered, e.g., potency and purity. Cell viability is considered a CQA as 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 and dead cells which could be detrimental to patient safety.

Biological activity or potency is a CQA of a cell-based product, and the cryopreservation process should 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 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 (both product- and process-related) that may be introduced into the cell-based product. The cryopreservation process should be developed using a science and risk-based approach in which any element that could adversely impact the CQA is considered, and controls are implemented to manage them in order to mitigate the associated risks. Details on this science 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 the product while maintaining product critical quality attributes. A science- and risk-based approach should be used to develop and validate the cryopreservation process ***(4, 5, 16-21)***. 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 product 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 product considerations (e.g. lot size, dose, container-closure system (CCS)).

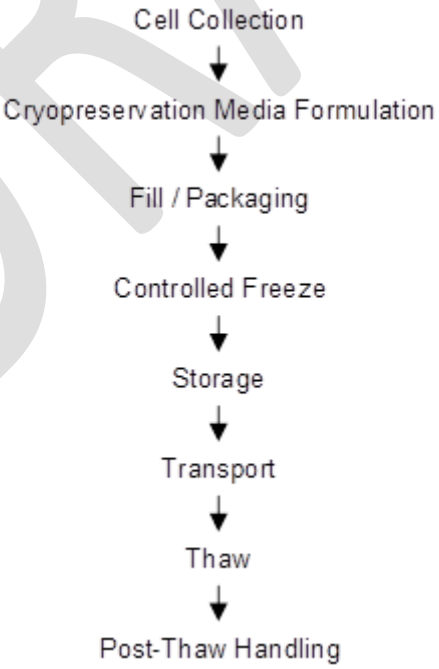

Figure 1. Standard process steps in cell cryopreservation process.

Unit operations involved in the cryopreservation process are listed in Figure 1. The process begins with the collection and isolation of the cells from either the source material or cell culture system. During collection, the target cells may be isolated, enumerated, and separated from the culture media (e.g. via

centrifugation and wash) to prepare the isolated cells for formulation in cryopreservation media. The isolated cells are then formulated to the desired cell concentration in cryopreservation media consisting of a cryoprotectant agent(s) (CPA) and other ingredients designed to protect the cell during the freezing process and storage at ultralow temperatures. The formulated cell suspension is then transferred (filled) and sealed (packaged) into an appropriate container closure system (e.g. cryobag or vial systems) engineered for ultralow temperature storage while maintaining container closure integrity. Established and validated cryopreservation protocols should be followed and supported by appropriate product stability studies. If shipping the cryopreserved product, specialized shipping systems capable of maintaining ultralow temperatures 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 media and/or dilution with an appropriate media/buffer. The thawed, reconstituted cell product would then be ready for intended use and administration to patients following the product manufacturer instructions.

6.1 Process parameters

Following a science - and risk-based approach to process development requires breaking the cryopreservation process down into its process steps and establishing the relationships between process inputs and outputs ***(4, 5, 17)***. Process inputs include materials, equipment, and process parameters, with process parameters being variables or conditions directly controlled in the process such as equipment settings, product volumes, cell and CPA concentrations, process and chronological times, temperatures, and other similar numerical variables. **Table 1** is a generalized list of important input variables involved in each process step. The list is not comprehensive and is intended to provide typical, important variables that should be considered in the development of a cryopreservation process for a cell-based product. A detailed discussion of important considerations for different steps, materials, equipment, parameters, and output measures are provided in the sections below.

Table 1. Generalized Inputs for Cell Cryopreservation Process

Process Step	**Process Input Variables**		
	Materials	**Equipment**	**Examples of Process Parameters and Points to Consider[1]**
Cell Preparation	(Starting) Cell culture Cryopreservation containers Cryopreservation media	Cell counter Dispensing equipment	Cryopreservation medium temperature 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) Sample volume Number of containers Container dimensions
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 system 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 profile (e.g. step rates, hold times and temperatures)

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

7 Cell Characterization and Testing

Master Cell Banks (MCB), Working Cell Banks (WCB) and producer cells used as 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 producer cells (cell banks) should be shown for the absence of contaminating adventitious agents, including bacteria, fungi, viruses and mycoplasma. Cell banks should have associated validated and/or verified assays to assess appropriate quality, including identity, purity and potency.

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 ***(22)***, and regional regulatory guidance documents such as FDA guidance "Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals"***(23)*** provide useful information on adventitious agent testing. Detailed information on tests for sterility, mycoplasma, endotoxin and viruses are provided in the relevant pharmacopoeias ***(24-27)***.

Use of compendial/pharmacopoeia and regional-specific (e.g. FDA, EMA) 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 ***(28)***. All the tests used for adventitious agents should be qualified/validated if these methods are not compendial methods.

Product qualification and release testing are performed prior to or following cryopreservation as appropriate. In addition, 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 ***(29)***. **Table 2** outlines the various recommended tests at process steps. Analytical methods should be validated. Also note that some of these tests could be used for release testing.

Table 2. Cell Characterization Testing

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	X	X	X	X	X	X	As applicable[3]
Formulation in Cryopreservation Media	X	X	X	X	X		
Fill/Packaging[2]	X	X	X	X	X	X	
Storage/Thaw		X	X	X			
Stability	X	X	X	X	X		

[2] Testing could be considered as release testing if applicable.
[3] Depending on the regulatory guidance for that region.

7.1 Material qualification

Cells as 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 human derived cells should be screened and tested as outlined in relevant international and/or regional regulations ***(6, 21, 30)***. For example, in the US, allogeneic cell sources require donor screening and testing ***(31)*** whereas autologous cell sources may require labelling requirements but not donor eligibility determination ***(32)***. Regardless of the use model (allogeneic or autologous), cells derived from donors should be characterized for safety, viability, identity, purity and function (potency).

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

In addition, appropriate controls should be in place to minimize the risk of introducing Bovine Spongiform Encephalopathy (BSE)/Transmissible Spongiform Encephalopathy (TSE) to humans from cell-based products in which animal-derived materials (e.g., derived from bovine, porcine, and ovine) are used during manufacture; for example, 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 of animal derived materials in manufacturing **(32).** Several USP **(33)** and WHO guidelines ***(34)*** provide useful information on risk reduction strategies for transmitting BSE/TSE.

7.2 Identity/Appearance

The cell source shall be identified using either phenotypic or genotypic characteristics. Appearance (visual examination for color, clarity, container defects, foreign visible particulates, etc.) might be performed in the cryopreservation containers after product 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 (PDA TR 79). Appearance (visual examination) should be performed in the cryopreservation containers after product fill and prior to storage for flaws or defects (e.g. container

defects, foreign visible particulates, discoloration). 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 (PDA TR 79).

7.3 Purity

Product purity is defined as relative freedom from extraneous material in the finished product, whether or not harmful to the recipient or deleterious to the product ***(3)***. Impurities can be product-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), and contaminants of microbial origin. Appropriate purity testing should include a measurement of contaminants that may impact patient safety. Considerations for selecting and designing purity assays are shown in ISO 23033 (in development).

7.4 Viability

Cell viability is often used as a rapid indication of cell performance both pre-freeze and post-thaw. Low cell viability correlates with poor cryopreservation survival. Common viability tests provide a rapid means of detecting cell death but are less effective at determining cell functionality and physical integrity post-thaw. The presence of non-viable cells in a cellular therapeutic product can be a concern, therefore the percentage of viable cells within the total number of cells in the cellular therapeutic product should be evaluated. Considerations for designing and selecting a viability assay are shown in ISO 23033 (in development).

7.4.1 Cell viability tests

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 essential structure within the cell, such as proteins, DNA, or RNA, and 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 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 coloured whereas those with a compromised cell membrane can be visualized. Dyes appropriate for the cryopreserved cell population should be qualified.

7.4.2 Cell viability assessment

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 variation. 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 validated for the assay. Samples thawed and assessed at 37°C may generate different results than those under ambient conditions and temperature should therefore be controlled and recorded. Viability of the cell population both pre-freeze and post-thaw should be determined.

7.4.3 Cell viability timepoint considerations

Post thaw viability is dependent on the time between thaw, 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.

7.5 Biological function (potency)

Potency is a cornerstone of an efficacious product. Per the US FDA's guidance document, potency is defined as "the specific ability or capacity of the product, as indicated by appropriate laboratory tests or by adequately controlled clinical data obtained through the administration of the product in the manner intended, to effect a given result." ***(3)***. A biological function or potency test is based on product attributes and must be specific to the product, and are performed to demonstrate that the manufactured lots meet defined specifications. Additionally, potency tests are also performed during stability testing and comparability studies. Potency tests must be performed to provide evidence that the product maintains biological activity and meets acceptance criteria. Potency assessments should be performed post-thaw to ensure that biological activity is not negatively impacted post-cryopreservation. The impact of different process parameters on potency should be assessed during process development to better understand their impact on the final product and may necessitate additional potency assessment points. Considerations for designing and selecting potency assays are shown in ISO 23033 (in development).

7.6 Sterility assurance and testing

Cellular products are aseptically manufactured because cells cannot be terminally sterilized or sterile-filtered. Additional manufacturing activities that can impact sterility of cellular products include, but are not limited to, cleaning/sanitization/decontamination and sterilization of freezing and storage equipment (when applicable) and other items used in the cryopreservation process, and pre-treatment procedures of container closure components (e.g., washing, sterilization and endotoxin reduction). Container closure systems used in aseptic processing should be included in validation/ qualification of aseptic processing. In addition, appropriate controls such as testing and acceptance criteria should be in place for raw materials (e.g., culture media, reagents, excipients, biologicals, chemicals) used in manufacturing to assure that cells are free of bacteria and fungus.

Final sterility testing is a required test for product release. ***(35)***. Sterility testing on cryopreserved in-process cells is also recommended upon thawing. In-process cells may also be tested for bacterial and fungal contaminants depending on the manufacturing procedures performed and quality of excipients/ reagents used. A compendial test method ***(25)*** is recommended for sterility testing when possible. If alternative or rapid microbiological test methods are used, these methods should be validated ***(36)***. In some cases, where product is administered to the patients before the results from a sterility test result are available, it is common to re-freeze a portion of the cryopreservation medium, which can be thawed later for re-examination if required. In the case of storage vessel contamination, appropriate steps should be taken to ensure sterility of all other products in the vessel before release for administation to the patient.

7.7 Mycoplasma

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

7.8 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 ***(6, 23, 37, 38)***. Cells are usually sampled for adventitious agent testing prior to long term cryo-storage.

7.9 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

assesses the impact of cryopreservation and storage on cell CQAs. An ongoing sampling program intended to assess adherence to cellular specifications throughout the expiration date should be adopted as appropriate. Stability studies should also be performed to evaluate stress conditions (e.g. excursion temperatures, temperature cycling, shipping conditions, etc.) as well as post-thaw hold-time (e.g. in-use) stability as applicable. 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)***.

For retention samples from nonclinical, clinical, and comparability studies, samples shall 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 Cryopreservation

8.1 Media

Media should be qualified before use and the extent of qualification depends on formula complexity and susceptibility to batch-to-batch variations. Cryopreservation media should be chemically defined and devoid of human or animal-derived components if possible, as outlined in 7.1 above. Media 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 or its component should be considered when determining the tests that are applicable for material qualification. Any container used or that comes into contact with the cryopreservation media should be sterile and pyrogen free.

8.2 Cryoprotectant agent

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

Cryoprotectant agents (CPA) are substances that protect cells during the cooling, freezing, and thawing process. Examples of commonly used CPAs are dimethyl sulfoxide (DMSO), glycerol, and propanediol along with various salts and sugars. Cryoprotectants should be used at the highest grade available. A risk assessment should be performed for the cryoprotectant agents used in a specific cell-based product to identify and evaluate the potential risks of use (especially for any novel excipients) and the safety (through nonclinical and/or clinical data) of the cryoprotectant agents must be demonstrated in product development.

8.2.2 Time and temperature of exposure and concentration of cryoprotective agent.

When possible, cryopreservation procedures should include a pilot study during process development that determines the optimal method of introducing and removing the CPA to and from cells. The study should include the concentration of CPA and time and temperature of exposure. Cell viability and functionality should be determined over an interval of time. Consideration for time intervals should include process parameters such as permissible time lapse prior to adding CPA, in-process storage or hold times, and in final fill-finish configuration. The CPA selected for best survival rate of cells and tissues are dependent on the type of cells, and may determine cooling rate, warming rate, and sample volume. Results of the study are documented and serve as the source for standard operating procedure development. Container closure systems (e.g., containers, closures, in-process materials) must be appropriate and meet defined, relevant quality standards ***(44)***. Materials that come into contact with cells

should be sterile, pyrogen free and of an appropriate grade for intended use and when possible, approved for human use by the applicable regulatory body.

8.3 Container closure systems

8.3.1 Selection, qualification, quality control and integrity testing

Selection of a primary container closure system is critical to preserve cell quality during cryo-storage and should be suitable for its intended use in freezing, long-term storage, shipping and thawing of cryopreserved cells. Contact surfaces of a primary container closure system should not be reactive, additive or absorptive ***(45)*** in a way that alters pre-determined cell quality attributes.

Suitability of a container closure system is determined with appropriate studies evaluating, but not limited to ability to perform in specified temperature range specifications. Appropriate quality controls such as testing and acceptance criteria for physical characteristics (e.g., appearance, shape and dimension), performance, sterility, pyrogen, particulate matter and materials of construction should be in place for all the components of container closure systems (primary and secondary) to ensure consistency for established and approved conditions and quality in accordance with relevant pharmacopoeia and regulatory requirements ***(44-46)***. Primary container closure system/packaging components should further be cleaned, sterilized and processed to remove pyrogenic properties and particulates ***(45, 47)***. Sterilization of primary packaging components by validated sterilization processes is required ***(44)*** and sterilized components should not be used after their expiry date.

All packaging components of container closure systems (e.g., bag/container, tubing sets, connectors, ports, caps etc.) should follow USP requirements and ISO standards ***(48-51)*** for biocompatibility. container closure systems should be evaluated for extractables and leachables as outlined in relevant pharmacopoeias, regulations and guidance documents ***(45, 47)***. Integrity test methods selected for intended use should be qualified and/or validated for evaluation of the final cell product container closure system ***(10)***. Container closure systems should be visually inspected ***(52-54)*** for leaks and other signs of damages, at least, following filling, upon thawing and at transport.

8.3.2. Container integrity/secondary container considerations

Hermetically sealed vessels are recommended for clinical products. Secondary containers such as 'overwrap' bags, 'over pouches,' or boxes can provide additional protection during cryostorage and transport. The secondary container should be composed of a similar low-temperature resistant material. Secondary packaging should be evaluated through appropriate studies to assess the impact of this packaging on cryopreservation, storage, and transport.

8.4 Cryopreservation procedures

Cellular products are sensitive to several key parameters during the freezing protocol. These cryopreservation parameters are cell-type dependent and should be optimized during development for the specific cellular product. Non-optimized cryopreservation protocols can reduce cell viability and function post-thaw. This section outlines typical, important steps that should be considered, but is not comprehensive and is intended to provide general guidance.

8.4.1. Cooling/Freezing rate

According to the USP 1046, 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 product. Best practices include contingencies for handling disruption of the freezing process and back up protocols for equipment failure. 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 heat into a system which could alter the product cooling protocol. Any opening of the controlled rate freezer during cooling should be automatically recorded. Excursions to freezing process (e.g., opening the freezer door, power failures) must be evaluated for cell quality attributes such as viability

Passive freezing methods refer to the use of an insulated container in a validated freezer (-80°C or -150°C) to maintain a controlled rate of temperature decline. The cooling rate should be evaluated, and temperature probes shall be used to monitor the temperature.

Validation of any freeze protocol used in the manufacture of the product is a CFR requirement and requires temperature mapping as part of the Operation Qualification process ***(55, 56)***. Upon completion of freezing, the actual temperature profile achieved should be recorded. Like other manufacturing procedures, planned 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 for manufacturing deviations. Deviations should be recorded, along with the action (or lack of action) that was taken.

8.4.2 Ice nucleation

The point of ice formation (nucleation) is a critical step during the freezing process that is dependent upon the cryopreservation media, 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 which is termed the latent heat of fusion. It is recommended that ice nucleation temperature be controlled where required and documented if possible, during the freezing profile.

8.4.3 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 keeps the samples at a constant temperature during the transfer process. It is recommended that samples be maintained below the glass transition temperature (~-125°C) to prevent cellular degradation.

8.4.4 Documentation

Documentation of the cryopreservation parameters, time, equipment, and operator is required. The date and time of cryopreservation must be recorded along with time samples are transferred into long-term storage. The start or end time can be recorded, along with the time between the start and end of the cooling process. This 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 administration to reduce the risk of mis-identification. The unique identifier shall be labelled on each sample as well as recorded in related documentation.

9 Transfer, Storage, and Transport (IATA and others)

9.1 Cryogenic storage

Cryogenic storage of products should be monitored with validated temperature monitoring devices. Temperature excursions should be documented and investigated to assess any potential product degradation. The procedures shall describe the operating instructions and requirements for the system used to monitor the storage temperature. This 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. Back-up systems should be in place for cryogenic storage. The storage conditions/criteria should be defined and validated as part of the validation of the

process. A defined inventory control system should be implemented to ensure samples and products can be tracked for storage and withdrawal

9.2 Alert limits and action

Control systems should be designed to reduce the likelihood of a temperature excursion. Alarms should be in place to react to any excursions and to alert in case of excursion outside of acceptable range (e.g., high temperature deviation). Excursions should be recorded and reported according to a facility's policies, procedures, and processes**.** Thorough investigation should be carried out to identify the root cause of the temperature excursion. The investigation should include a review of previous occurrence temperature excursion for any 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 taken 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 the Quality Unit. Any products stored within the affected area should not be released for use or supply prior to completion of the assessment and investigation.

9.3 In-process hold

The processes, workflows, and production processes should be documented and evaluated for any critical steps where product is temporarily held outside of its long-term storage condition ***(57)***, including the use of quarantine storage until release testing is complete and product is approved for transfer to long term storage.

9.4 Transfer

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.

9.5 Transport

The monitor and control of transportation and conditions includes managing traceability and maintaining chain of custody and establishing clear expectations and communications between cell product manufacturer and primary and secondary transportation service providers. These parameters all have significant impact on the quality of cells intended for therapeutic and can ultimately affect product safety and effectiveness. Validated processes for cell transportation should be appropriately and adequately planned, executed, traced, and documented to ensure container integrity and product quality. General requirements and points to consider for transportation service providers, clients, and senders to ensure cell quality, safety, and efficacy during the transportation process can be found in ISO 21973 (in development).

9.6 Label integrity

Types of labels and markings used should be designed to withstand extreme temperatures. Markings on labels should be legible and barcoded (if possible). Labels should not penetrate the container. If labels are too small to document all required information, additional information can be included in associated accompanying documentation. References for guidance in labelling can be found in ISBT 128. Different cell types should be appropriately segregated by physical, spatial or electronic separation from each other. Label qualification and control procedures shall be in place and implemented. Label qualification should include confirmation of adhesion to the container for long term storage ***(58, 59)***.

9.7 Equipment and qualification

Equipment used in freezing, cryogenic storage and thawing shall be installation, operational and performance qualified (IQ/OQ/PQ) for intended use ***(47, 56, 60)***. Qualified freezing equipment should be used to validate cell freezing process as outlined in available guidelines ***(5, 56)***. Freezing equipment should be temperature mapped for empty, minimum, and maximum (worst case) load configurations. Equipment used in cell freezing, storage and thawing should be in appropriate design, adequate size, and suitably located to facilitate operations for its intended use and for its cleaning and maintenance ***(60)***.

10 Thawing

Thawing of cryopreserved products is a critical step that may impact product quality. While thawing most products, it is important to maintain a consistent warming rate throughout the sample. During thaw, the temperature rises consistently and produces a temperature gradient across the vessel. As such, cells closer to the periphery of the vessel experience a higher temperature profile than those in the center and are exposed to cryoprotective agents in non-frozen conditions. Therefore, the thawing process needs to be adequately designed and qualified to achieve a uniform thawing temperature profile and rate across the vessel contents. Thawing and associated post-thaw processing should be evaluated during process development using a risk-based approach. Thawing can be performed using either automated devices or manual procedures using a water bath, bead bath or thermo block (when applicable). Manual thawing procedures should be qualified to minimize variability.

10.1 Duration of thaw

The time from removal from liquid nitrogen storage to full thaw (when no ice crystals are visible to the naked eye), is an important parameter that can directly affect product quality. An optimal thawing duration maximizes cell viability and recovery. Time limits for thawing and post thaw hold should be established to assure cell CQAs. Time limit for each phase of production is required ***(57)***. Additional measures of functionality may be conducted if the thawing process if found to impact product CQAs.

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

Cryopreservation media is commonly formulated with components that minimize temperature and freezing stress on cells. These components are not necessarily compatible with biological function of the cells and can be harmful during short- or long-term exposure at non-frozen temperatures. Hence, any holding of cells in cryopreservation media under non-frozen temperature should be minimized as 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 media to the cells during the holding period. Holding temperature and time should be validated during the process development to be applicable to the specific cellular product.

10.3 Washing

Depending on the component within the cryopreservation media, a washing step after thawing may or may not be necessary. In cases where the cryopreservation media contains components that are unapproved for in-human administration, the media should be considered an ancillary material. The cryopreservation media should be removed with washing after thawing process prior to next process step. This washing process should be validated during process development. In cases where the cryopreservation media 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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