Cryopreservation Guide

The Basics of Cellular Cryopreservation for Research and Clinical Use

Simplifying Progress

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1. Introduction

The primary purpose of cryopreservation is to preserve biological specimens. Individual cells and biological tissues can be cryopreserved in a living state of suspended cellular metabolism at the temperature of liquid nitrogen (-196 °C) (Bakhach, 2009). This practice is crucial for biomedical research, clinical medicine, zoology, botany, and biotechnology. When frozen and kept properly, specimens can remain in a state of suspended cellular metabolism indefinitely and can be thawed as needed.

Before the development of freezing media, cells were frozen in their own growth media or high serum media. Cryoprotective agent development has improved cell viability post-thaw as well as stem cell pluripotency. Increased knowledge of the physiochemical processes involved during cell cryopreservation has resulted in reproducible protocols (Mazur, 1984). Freezing techniques exist for tissues, single cells, embryos, and microorganisms. Challenges with cryopreservation include post-thaw cell growth, viability, maintenance of pluripotency, changes in gene expression, and the ability to differentiate post-thaw. By overcoming the challenges of cell freezing, researchers and clinicians will continue to bring valuable treatments to patients.

2. Cryopreservation Basics

The purpose of cryopreservation is to store cells indefinitely by halting the cell's metabolism with ultralow temperatures. The freeze-thaw process is stressful to all cells and tissues. Therefore, effective techniques were developed to prevent cell death and damage. One common cryopreservation technique involves changing cell maintenance media to culture media containing a cryopreservation agent, such as dimethyl sulfoxide (DMSO). Cells are then cooled at a rate of -1 °C/min (for mammalian cells) by transferring to -80 °C in a specialized cooling container. After cells have cooled to -80 °C, they are transferred to ultralow temperature storage of below -135 °C.

The most common ultralow temperature storage is liquid nitrogen in liquid or vapor form. After freezing, cells are stored as original seed and working stock assets.

Ultralow storage temperatures work by maintaining cells below the glass transition temperature (Tg) of pure water (Miller, 1969). This suspends all molecular processes and prevents free radical generation that negatively effects cryopreserved cultures (Baust J. , 2007; Baust, Corwin, Van Buskirk, & Baust, 2015). Cells survive by cooling the cultures to a "glassy" state slowly, so that less ice forms within the cell. Too much intracellular ice can cause mechanical damage to the cell when thawing. In addition, cells must not freeze too slowly because cells will shrink and dehydrate. Physical stress of shrinkage and dehydration results in high solute concentrations that increase toxicity (Mazur, Leibo, & Chu, 1969). Damage is also prompted by pH shifts and protein degradation that compromises the cell membrane or metabolic pathways.

Technologies for the cryopreservation of cells and tissues are constantly improving. The best cryopreservation results are obtained through research-based approaches that are adjusted for the specific purpose of cryopreservation. There are six applications of cryopreservation that include preservation of cells or organs, cryosurgery, biochemistry or molecular biology, food sciences, ecology or plant physiology, and medical applications (transfusion, bone marrow and cells transplantation, artificial insemination, and *in vitro* fertilization) (Jang, et al., 2017). This guide will cover the basics of cellular cryopreservation for research and clinical use.


hESC
Nutristem on matrigel
Endothel
HUVEC ATCC
HDMEC ATCC
DMSO
Vero, A549, Hep2
注意CAUTION
凍傷注意・手袋着用
USE PROTECTIVE GLOVES
霜を取除いて下さい
USE SCRAPER TO REMOVE ICE

3. Advantages of Cryopreservation

Cell Freezing Has Many Advantages

Proper cryopreservation of cells is the best way to save money, improve reproducibility of experiments, and protect important stocks of cells that might be difficult to replace. Cost savings occur because regular cell stock orders will not be necessary since cells can be split and multiplied. Expanding cells for cryopreservation will accommodate long experiments or industrial high-throughput screening efforts. Keeping cell passage numbers low increases the likelihood that populations will remain homogenous between experiments because changes occur when populations are expanding. Therefore, keeping a cryopreservation master cell bank and working stocks will improve reproducibility between similar experiments. Gene expression or other cellular changes can be documented if passages are frozen down, tested, and compared. In case of contamination, errors, or unintended variables occur then the original stocks can be revisited.

Why Should You Freeze Your Cells?

- Store your cells for future study.
- Cell storage acts as an insurance policy in case of contamination, failure, or cell shortage.
- Actively growing your cells over a long period of time can alter gene expression and differentiate cells. Continued growth and passage of cells can cause them to lose their original features, and this endangers reliable research results.
- Freezing and storage of cells is a crucial step to ensure long-term cell use and reproducible results.
- Save time and money—obtaining new cells is costly and time consuming.

4. Challenges of Cryopreservation

Government regulations of cells for medical use have increased the need for documented cell handling processes. To account for these regulations, protocols for cryopreservation must be established and adhered to by researchers and medical personnel. While optimized cryopreservation protocols exist for most areas of research and medicine, technical issues persist. These issues include low survival, altered gene expression and morphology, loss of cellular function, epigenetic changes, and differences in protein composition. (Baust J. , 2007; Van Buskirk, 2007; Baust, Corwin, Van Buskirk, & Baust, 2015; Allegrucci & Young, 2007; Stacey, 2007). If optimal conditions are not provided during cell freezing then damage and cellular apoptosis or necrosis can occur post-thaw.

Freezing cells includes intracellular and extracellular complex events that are not fully understood. During the freezing process ice forms within and outside of the cell and this is very much dependent on the cooling rate. If ice forms outside of the cell, water migrates out of the cell causing dehydration, shrinkage, and finally cell death. If too much water remains inside the cell during the freezing process, intracellular ice crystals form that damage cellular organelles and pierce the cell membranes during the thawing process. Rapid cooling minimizes the solute imbalance between the internal and external of the cell, but more intracellular ice is formed.

Slow cooling intracellular water migrates out of the cell resulting dehydration and shrinkage (Elliot, Wang, & Fuller, 2017). Cells can be successfully frozen when the cooling rate is slow enough to prevent intracellular ice formation but fast enough to prevent dehydration and damage. Osmotic pressure imbalance is the main concern during cryopreservation as it can cause dehydration, deformation, and injury by effecting the water movement in or out the cell. When freezing cells at too low cooling rate, water can leave the cell through the cell membrane to join the ice on the exterior (Meryman, 1971).

Since solutes are excluded from ice crystals, the solute concentration increases (Lovelock, 1954) and water moves from low solute concentration to high solute concentration to achieve osmotic balance. To mitigate osmotic imbalance, freezing solutions with specific cryoprotectants are used to prevent dehydration by replacing water and stabilizing the cell (Clegg, Seitz, Seitz, & Hazlewood, 1982). These cryoprotectants are added to cryopreservation media that can be individually formulated or bought commercially.

Rapid cooling can be used for cryopreservation by the vitrification technique. Vitrification is a fast cryopreservation method that avoid ice crystals formation within the cell. Vitrification method is usually successful when cryopreservation solutions contain high concentration of cryoprotective agents and it is used routinely for the cryopreservation of gametes and embryos.

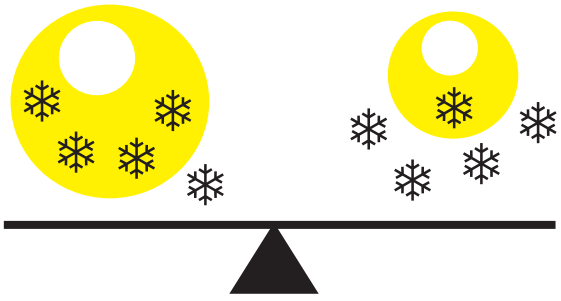

Figure 1: *A Delicate Balance Must Be Maintained While Freezing Cells.*

5. Freezing Methods and Storage

There are two main methods to cryopreserve cells for long-term: controlled rate freezers and step-down freezing. The more traditional approach of step-down freezing uses a specialized freezing container that is designed to cool cells at -1 °C/min in -80 °C freezers (e.g. Mr. Frosty). For this method cryovials are placed in specialized freezing containers in a -80 °C freezer, and the next day cells are transferred to liquid nitrogen storage. Controlled rate freezers can be programed for precise and active cooling protocols so that cells are frozen typically at -1 °C/min. The temperature is actively monitored while the freezer is cooled with liquid nitrogen. Some controlled rate freezers do not require any consumable cryogens. More recently, controlled rate freezers for the bench top have become available. Once cells are frozen, they can be kept in liquid nitrogen or mechanical storage as long as it is below -135 °C.

Several types of storage containers for stepdown cooling exist. Previous types of containers used alcohol to insulate and control cooling. Modern containers use insulation with a thermoconductive alloy core to control the rate of freezing and they are proven to freeze at -1 °C/minute. Cryovials are vessels designed for cell freezing and storage in cryopreservation solution. Most plastics and other materials might shatter or crack at low temperatures. Aliquots of cells are often made in 1 mL per tube. We recommend polypropylene plastic screw-capped 2 mL externally threaded vials with labels. These vials are designed to fit easily in freezing and storage containers and they withstand ultralow temperatures. Before committing to a vessel type, make sure the specifications meet your requirements.

Should You Store Your Cryovials in the Vaporous or Liquid Nitrogen Layer?
We recommend using the vaporous layer. The difference in temperature between layers is not great and the liquid phase is at greater risk for contamination.

Recommended:
After 24 hours we recommend checking one of the vials for viability and recovery.

Figure 2: *Freezing Mammalian Cells in Cryovials and Freezing | Storage Containers*

6. Creating a Master Cell Bank and Working Cell Bank

Biobanking is an important asset to both industrial and academic institutions. Successful cell banking will preserve primary cells and cell lines for research, cells for medical use, keep experiments reproducible, improve effectiveness of treatments, and keep operating costs low. Before generating a master or working stock, it is important to test incoming cells for malicious agents such as mycoplasma, bacteria, and fungi. Once cells have passed all testing during a quarantine period then they can be split for banking and further use. Cryopreservation of a large master stock is important in case the working stocks are lost or contaminated. The number of cells needed for experiments or treatments should be kept in mind to determine the number of master stock tubes.

Typically, master tubes can be any number from 10–100. Quality tests should be repeated at this level and if the master stocks pass then they can be used for preparing working stocks, which are typically 20–200 tubes. We recommend performing quality control tests with these stocks to determine cell viability, quality, and authentication. Working stocks should be kept at the same low passage number and experiments should be completed in a similar passage range. This way, if stocks run out then the smallest passage number can be split for more. When master stock tubes dwindle, more should be split and stored.

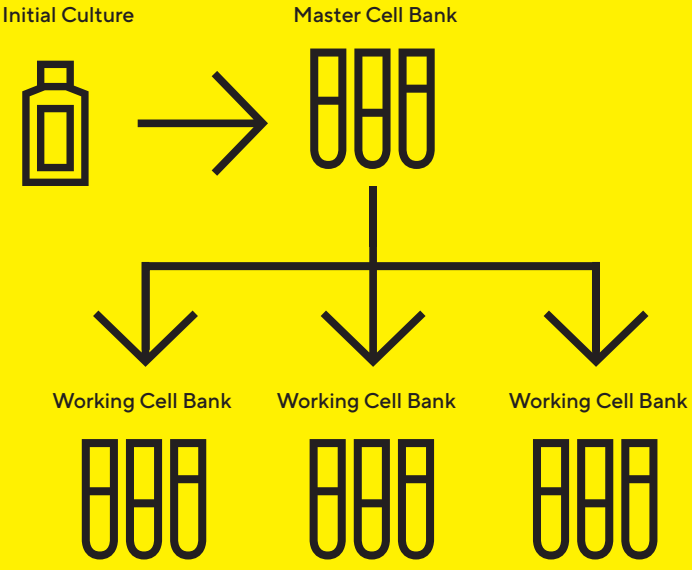

Figure 3: *Preparing a Master and Working Cell Bank*

Tips for Preparing Your Masterand Working Cell Bank

- One cryovial may contain 1–20 million cells in 1 mL. Start by freezing 1–5 million cells in 1 mL, depending on your cell type.
- When a healthy set of cells is ready for subculturing prepare your master cell bank (seed stock), consisting of 10 to 100 vials of cells. Use this seed stock to prepare two or more working cell banks (working stock), consisting of 20 to 200 vials of cells each.

7. Choosing a Cryopreservation Media

Cryoprotectants are necessary to lower solute concentration during the freezing process. Cryoprotectants are compounds that protect cells from intracellular ice formation. DMSO, glycerol, ethylene glycol, and propylene glycol are all permeating cryoprotectants. Their mechanism of action involves entering the cell freely and replacing water, lowering the amount of ice formed, and acting as a secondary solvent for salts (Lovelock, 1953; Pegg, 1984). Other cryoprotectants include high molecular weight compounds. Hydroxyethyl-starch and polyvinyl pyrollidone are non-permeating cryoprotectants that are thought to stabilize the cell membrane (Anchordoguy, Rudolph, Carpenter, & Crowe, 1987). The type of cryoprotectant desired depends on the cell type, tissue, or organism being frozen. DMSO and glycerol are the most commonly used cryoprotectants, with DMSO being more common for mammalian cells. DMSO can be used at a concentration of up to 10% w/v (1.28M) and can be filtered for cell culture use (Hunt, 2017). If single addition of DMSO media causes toxicity then multi-step addition can be used to gradually increase DMSO concentration and decrease osmotic damage. Another common cryoprotectant is glycerol. Glycerol may act as a nonelectrolyte to decrease electrolyte concentration in cell freezing solutions and is most commonly used for preservation of microorganisms, red blood cells, and spermatozoa (Jang, et al., 2017). While these agents protect cells during the slow freezing process they can also cause cell toxicity. The use of serum can help to protect the cells but this is not recommended for clinical or commercial uses that may require serum-free culture conditions. As an alternative to homebrew of cryopreservation media, commercially available media provide safe and reliable cryopreservation results. Defined freezing solutions, such as NutriFreez® Cryopreservation, can also be used (Figure 4).

When culturing cells in a serum-free environment, it is essential to also maintain serum-free conditions during cryopreservation.

Sartorius has developed a novel cell cryopreservation formulations which is proteinfree, serum-free and animal component-free. NutriFreez® Cryopreservation has been demonstrated to result in high rates of cell viability, proliferation, adherence (in relevant lines), and bioactivity and expression following freezing and thawing. Superior results were obtained both in comparison with serum-containing freezing media as well as competing serum-free products, making this an ideal product for both serum-containing and serum-free applications.

Figure 4: *NutriFreez® D10 Cryopreservation Medium*

8. Cryopreservation Protocols

8.1. General Instructions for Use for the Cryopreservation of Mammalian Cells

Notes:
- Keep NutriFreez® D10 Cryopreservation Medium on ice at all times during use.
- For freezing adherent cells, detach cells using a dissociation solution according to the manual instructions.

Freezing Procedure

1. To maintain aseptic work conditions, wipe the outer packaging with a cloth moistened in 70% ethanol | 70% isopropanol before opening the NutriFreez® D10 Cryopreservation Medium.
2. Centrifuge cells to obtain a cell pellet, 300 – 400 ×*g* for 4 – 5 minutes, then aseptically decant supernatant without affecting the cell pellet.
3. Suspend the pellet in cold (2 – 8°C) NutriFreez® D10 Cryopreservation Medium, mix thoroughly, and transfer the suspension to a cryovial (e.g., 1.0 mL of suspension in a 1.5 mL cryovial).

Note: If freezing multiple cryovials, keep the cells on ice at all times. Gently mix the resuspended cell solution frequently to ensure even distribution throughout the vials. Immediately transfer filled cryovials to ice before aliquotting the remaining cell solution.

4. Freeze the cells gradually (1 – 2 °C per minute) by using a controlled rate freezing system and store the vials in liquid nitrogen (vapor phase). Alternatively, place the vials in appropriate freezing container (e.g., Nalgene® Mr. Frosty™ freezing container) and transfer to -80°C for overnight. The following day transfer cryovials into liquid nitrogen (vapor phase recommended).

Note: Long-term storage at -80°C is not recommended.

5. We recommend determining the efficiency of cryopreservation by thawing one vial after 24 hours of storage in liquid nitrogen and following the thawing procedure outlined below.

Thawing of Cryopreserved Cells

1. Briefly warm culture medium of choice in a 37 °C water bath.
2. Rapidly thaw the cryovial of cells in a 37 °C water bath by gently shaking the vial and remove the vial when only a small frozen cell pellet remains. Do not vortex cells.
3. Disinfect the vial by wiping it down with a cloth moistened with 70% Ethanol or Isopropanol.
4. Suspend the cells in warmed growth culture medium at a ratio of at least 1:10 (cell suspension to culture medium).
5. Centrifuge cells to obtain a cell pellet, 300 – 400 ×*g* for 4 – 5 minutes, then aseptically decant supernatant without affecting the cell pellet and resuspend in growth medium as desired.
6. Culture the cells according to the recommended seeding density.

Tips for Cryopreservation

- Always use a reliable freezing media that is serum-free and animal-component free to insure that animal products will not influence the human cells.
- Serum is not necessary when freezing cells.
- Cryoprotectants, such as DMSO and glycerol, in freezing media help to protect cells from damage caused by ice crystal formation.

8.2. Instructions for Use for the Cryopreservation of Human Induced Pluripotent and Embryonic Stem Cells (hPSC)

Notes:

- hPSCs can be frozen as clumps or single cells with high viability and minimal differentiation post thaw.
- The single cells can be thawed onto recombinant laminin-coated culture ware without the addition of ROCK inhibitors. In case of using other matrices (e.g., Corning® Matrigel® matrix), ROCK inhibitor is required.
- Keep NutriFreez® D10 Cryopreservation Medium on ice at all times during use.

Freezing Procedure
(The Procedure Describes the Cryopreservation of Cells Cultured in a 6-Well Plate)

1. Using a vacuum aspirator and a sterile aspirator pipette, remove the hPSC culture medium from the culture vessel or well(s) to be harvested for cryopreservation.
2. Rinse wells with Dulbecco's PBS w/o Ca & Mg (Cat# 02-023-1), using approximately 2 mL of DPBS per 10 cm^2 culture surface area, then aspirate out the DPBS.
3. Determine the desired viable cell density and calculate the required volume of NutriFreez® D10 Cryopreservation Medium needed for a concentration of approximately 1×10^6 viable cells/mL.
4. Add dissociation solution as desired. Cells can be detached using the enzyme and method that the culture has been routinely passaged with. In case of using collagenase, dispase, or EDTA, incubate at 37 °C or at room temperature until the edges of the colonies begin to loosen from the plate.

Note: Incubation times vary between cell lines, colony size and the detachment solution used. Begin checking the culture after 3 minutes.

5. Cells cultured on laminin can be detached using Recombinant Trypsin-EDTA Solution (Cat# 03-079-1) to yield a single cell suspension.

Note: Once the cells are detached from the surface, neutralize the enzyme by adding 2–4 volumes of pre-warmed complete medium to the volume of the trypsin solution used. Alternatively, 1× Soybean Trypsin Inhibitor (SBTI) solution (Cat. No. 03-048-1) diluted in DPBS can be used to neutralize the trypsin.

6. Transfer the clumps or cell suspension to a centrifuge tube.
7. Centrifuge at 200 ×*g* for 5 minutes at room temperature then aseptically decant supernatant without affecting the cell pellet.
8. Resuspend the cell pellet in the predetermined volume of cold NutriFreez® D10 Cryopreservation Medium on ice (1 mL for every 1×10^6 viable cells). In case of aggregates; do not break up cell masses any more than necessary, two or three gentle pipetting motions are usually sufficient.
9. Dispense aliquots of this suspension into cryo vials (e.g., 1.0 mL of suspension in a 1.5 mL cryovial)

Note: If freezing multiple cryovials, keep the cells on ice at all times. Gently mix the resuspended cell solution frequently to ensure even distribution throughout the vials. Immediately transfer filled cryovials to ice before aliquotting the remaining cell solution.

10. Freeze the cells gradually (1–2 °C per minute) by using a controlled rate freezing system and store the vials in liquid nitrogen (vapor phase). Alternatively, place the vials in an appropriate freezing container (e.g., Nalgene® Mr. Frosty™ freezing container) and transfer to -80 °C overnight 11. The following day, transfer vials to liquid nitrogen storage (vapor phase).

Note: Long term storage at -80 °C is not recommended.

Thawing of Cryopreserved hPSC

1. Briefly warm NutriStem® hPSC XF Medium, or other growth culture media of choice, in a 37 °C water bath.
2. Add 9 mL of warmed NutriStem® hPSC XF Medium, or other growth culture media, into a centrifuge tube.
3. Rapidly thaw the cryovial of cells in a 37 °C water bath by gently shaking the vial and remove the vial when only a small frozen cell pellet remains. Do not vortex cells.
4. Disinfect the vial by wiping it down with a cloth moistened with 70% ethanol or isopropanol.
5. In a sterile biological safety cabinet, transfer the contents of the cryovial drop by drop into the 9 mL of culture medium in the previously prepared centrifuge tube. Gently rock to continually mix the cells as the new cell droplets are added to the tube.
6. Centrifuge the cells at 200 ×*g* for 5 minutes. Remove and discard supernatant.
7. Gently resuspend the cell pellet in NutriStem® hPSC XF Medium (Cat# 05-100-1) or other growth culture media, and plate as desired.
8. Refresh culture medium 48 hrs after plating.

Tips for Best Results While Freezing Human Pluripotent Stem Cells

- Follow institutional regulation and guidelines for all hPSC work.
- Cells can be frozen as aggregates or single cells.
- It is best to cryopreserve when cells are at their maximum growth rate (log phase). Cryopreservation at ~80% confluence should work best.

Figure 5: *Nutrifreez® D10 Cryopreservation Medium Is Proven Very Effective for Human Embryonic Stem Cell And Human Induced Pluripotent Stem Cell Cryopreservation. Cells Frozen With This Medium Maintain Pluripotency, Attachment Ability, and Viability (Nishishita, Muramatsu, & Kawamata, 2015).*

8.3. Instructions for Use for the Cryopreservation of Human Mesenchymal Stem Cells (MSC)

Notes:

- Keep NutriFreez® D5 Cryopreservation Medium on ice at all times during use

Freezing Procedure of hMSCs

1. Aseptically remove the hMSC culture medium from the culture vessel or well(s) to be harvested for cryopreservation.
2. Rinse wells with Dulbecco's PBS w/o Ca & Mg (Cat. No. 02-023-1), using approximately 2 mL of DPBS per 10 cm^2 culture surface area, then aspirate the DPBS.
3. Detach adherent hMSC using a sufficient volume of Recombinant Trypsin Solution (Cat. No. 03-078-1) to cover the entire cell culture surface and incubate the cells at room temperature or 37 °C for 3 to 5 minutes.

Note: Recombinant Trypsin-EDTA Solution (Cat. No. 03-079-1) can be used if the cells are over-confluent or are difficult to detach.

4. Observe the cells under a microscope. If less than 90% of the cells are detached from the culture surface, continue incubating and observe again at 1-minute intervals to check for complete detachment.

Note: Incubation times can vary between cells and confluency levels. Begin checking the cultures after 3 minutes. Do not over-incubate the culture, as MSC can be sensitive to enzymatic stress. Tap the vessel periodically to expedite cell detachment and monitor the progress of the enzyme solution.

5. Once the cells are detached from the surface, neutralize the action of the trypsin enzyme by adding a volume of pre-warmed complete medium that is 2 – 4 times the volume of the trypsin solution used.

Note: Alternatively, 1X Soybean Trypsin Inhibitor (SBTI) solution (Cat. No. 03-048-1) diluted in DPBS can be used to neutralize the trypsin.

6. Collect the cell suspension and transfer to a centrifuge tube. If needed, rinse the culture vessel with additional media to collect any remaining cells, and transfer to the same tube. Perform a viable cells count if neccessary.
7. Centrifuge at 300 ×*g* for 5 minutes at room temperature, and then aseptically remove supernatant without affecting the cell pellet.
8. Determine the required volume of NutriFreez® D5 Cryopreservation Solution needed according to the desired concentration.
9. Remove the supernatant from the centrifuge tube and quickly but gently resuspend the pellet in cold NutriFreez® D5 Cryopreservation Solution according to the freezing volume determined in the previous step.
10. Dispense aliquots of this suspension into cryovials (e.g., 1.0 mL of suspension in a 1.5 mL cryovial).

Note: If freezing multiple cryovials, keep the cells on ice at all times. Gently mix the resuspended cell solution frequently to ensure even distribution throughout the vials. Immediately transfer filled cryovials to ice before aliquotting the remaining cell solution.

11. Freeze the cells gradually (1 – 2 °C per minute) by using a controlled rate freezing system and store the vials in liquid nitrogen (vapor phase). Alternatively, place the vials in appropriate freezing container (e.g., Nalgene® Mr. Frosty™ freezing container) and transfer to -80 °C for at least 2 hours and up to 24 hours.
12. After 2 – 24 hours, transfer the cryovials into liquid nitrogen (vapor phase).

Note: Long-term storage at -80°C is not recommended.

Thawing of Cryopreserved hMSCs

1. Briefly warm 5–10mL of complete MSC NutriStem® XF Medium (Cat. No. 05-200-1 and 05-201-1) or other growth culture medium, in a 50 mL centrifuge tube.
2. Rapidly thaw a cryovial of hMSC in a 37 °C water bath, by gently shaking the vial and remove the vial when only a small frozen cell pellet remains. Do not vortex cells.
3. Disinfect the vial by wiping it down with a cloth moistened with 70% ethanol or isopropanol.
4. In a sterile biological safety cabinet, transfer the contents of the cryovial drop by drop into culture medium in the previously prepared centrifuge tube.
5. Gently rock to continually mix the cells as the new cell droplets are added to the tube, then resuspend the cells by carefully pipetting up and down.
6. Centrifuge cells at 300 ×*g* for 4–5 minutes at room temperature.

Note: It is possible to skip the centrifugation step after thawing by simply transferring the thawed cells directly onto a culture vessel with medium at a ratio of at least 1:10 (for the dilution of the DMSO).

Cryopreservation Tips

- We recommend mycoplasma testing before freezing.
- We recommend labelling your vials (cell, passage, lot #, date, your name) with a marker that will withstand alcohol and liquid N2. Printed labels also work well. We also recommend keeping the records online as well as having a hard copy.
- Make sure to follow each cryopreservation protocol carefully and quickly to prevent your freezing media from returning to room temperature. While you should work fast, make sure your speed isn't coming at the expense of sterility and your personal safety.

9. Safety Tips and Considerations

Close and prolonged contact with liquid nitrogen during cell storage can cause serious burns or asphyxiation if inhaled. Personal protective equipment (PPE) must be worn when storing cells. PPE includes a face shield, thermal gloves, a lab coat, and closed-toe shoes. Nitrogen levels should be checked regularly to make sure that complete evaporation doesn't occur. Vessels should be clear of frost build-up and any leaks. Alarm systems to monitor the performance of the liquid nitrogen vessels and the oxygen levels in the storage rooms can be installed as a precaution. Always use the correct storage vessel designed to withstand liquid nitrogen storage. Threaded cryovials should always be used to prevent exploding tubes.

DMSO is a combustible and toxic material that is easily absorbed through the skin (Cayman Chemical Company, 2017). It must be kept in a safety cabinet and should only be handled using appropriate PPE including gloves, eyewear, and a lab coat. If DMSO gets on your glove, remove the glove and get another. If there is skin or eye contact, wash your skin with soap or flush your eyes with water for 15 minutes. Treat DMSO and DMSO-soiled materials as chemical waste and dispose of them accordingly.

10. Troubleshooting

The practice of cryopreservation has become routine in research. Many troubleshooting methods are employed when issues arise. The most common concern with cell freezing and thawing is poor cell viability post-thaw. Viability issues are caused by cryoprotectant, improper cooling rate, post-freeze temperature flux, improper thaw, or incorrect cell density (Table 1). If contamination or other issues are detected in working stock cultures then return to master stock.

If master stock also exhibits the same issues then contact the company or source of the cultures. Cells that do not pass validation should be rejected once the source cells responsible for issues are identified. Proper maintenance of stored cells and tissues will help you ensure reproducible experiments and treatments.

Issue	Suggestions
Toxic Cryoprotectant	Use commercially available and defined media according to manufacturers' instructions. Remove cryoprotectant promptly after thaw. Do not allow cells to remain at room temperature in cryoprotectant media.
Improper Cooling Rate	Use a gradual cooling rate of -1 °C/min. To attain this rate, use a thermally insulated freezing container or a controlled rate freezer.
Post-Freeze Temperature Flux	Maintain the cryogenic temperature of cell vials after they reach <-130 °C. Keep cells on dry ice when transporting and make sure liquid nitrogen tanks are filled properly.
Improper Thawing Rate	Cells must be thawed quickly. Use a 37 °C water bath or dry thawer to thaw vials.
Incorrect Cell Density	Freeze and plate cells at the appropriate density for the cell type. Cell lines should have published density requirements. Typical density per frozen vial is 1×10^6 – 10×10^6 cells/mL. Testing for optimal freeze-thaw density might be necessary.

Table 1: *Troubleshooting: Viability Issues After Cryopreservation*

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