

General Guide for Cryogenically Storing Animal Cell Cultures

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Introduction

Maintaining healthy, growing cell cultures is a demanding task made more difficult by the ever-present risk of their loss through accidents or contamination. In addition, actively growing cell cultures are not static but, like all populations of microorganisms, subject to age-related or environmentally-induced changes which can result in their ongoing evolution and potential loss.

These problems are reduced by using cryogenic preservation to stop biological time for cell cultures, effectively putting them into true suspended animation. This concept, long a favorite ploy of science fiction writers and movie producers, has been a reality since the important discovery by Polge, Smith and Parkes (11) in 1949 that glycerol prevents injury to cells caused by freezing. Many cook book-style protocols are now available for freezing cells and these procedures usually perform well (3,6,13-16). It is essential, however, when problems arise or protocol adaptations and improvements must be made, that the underlying concepts on which they are based are well understood. This guide examines both the basic theoretical concepts and practical aspects necessary for successfully freezing animal cells and managing a cell repository.

Advantages of Freezing Cell Cultures

Once successfully frozen and stored, cell cultures require little time and effort for their maintenance. The only real cost is the expense of maintaining an ultracold (-130°C or lower) mechanical freezer or liquid nitrogen supply. This limited expense compares very favorably with the time, effort and substantial cost of the media and supplies necessary for maintaining actively growing cultures, or for the cost of obtaining a new culture from a repository. Frozen cultures provide an important backup supply for replenishing occasional losses due to contamination or accidents and provide the assurance of a homogeneous culture supply. Cellular changes or alterations occur in all actively growing populations. These changes often result in the loss of important characteristics during evolution of the cultures thereby introducing unwanted variables into long-term experiments. Cryogenically preserved cultures apparently do not undergo any detectable changes once they are stored below -130°C (1,8). Therefore, the biological effects of *in vitro* cellular aging and evolution may be minimized by frequently returning to frozen stock cultures, allowing ongoing long-term culture experiments to be successfully completed without these unwanted variables. Frozen cultures also provide a valuable baseline against which future experimentally-induced changes may be compared or measured.

General Events During Cell Freezing

To understand why freezing protocols work, it is necessary to examine both the intracellular and extracellular events occurring in animal cell cultures during the freezing process (2,4,8). Initial cooling from room temperature to 0°C slows cellular metabolism, rapidly disrupting active transport and ionic pumping. Usually this disruption does not result in cellular damage if the culture medium is osmotically balanced. As cooling continues (0° to -20°C) ice crystals begin to form in the extracellular environment which

increases the solute concentration of the culture medium. As a result, water begins to move out of the cells and into the partially frozen extracellular medium, beginning the process of cellular dehydration and shrinkage.

When the cooling process is rapid, intracellular ice crystals form before complete cellular dehydration has occurred. These ice crystals disrupt cellular organelles and membranes and lead to cell death during the recovery (thawing) process (Figure 2).

When the cooling process is slow, free intracellular water is osmotically pulled from the cells resulting in complete cellular dehydration and shrinkage. This can also cause cellular death but there is little agreement on the mechanisms involved. The physical stresses of cellular shrinking may cause some damage resulting in irreparable membrane loss and cytoskeletal and organelle disruption. Damage may also be caused by the high concentrations of solutes in the remaining unfrozen extracellular medium (essentially a brine solution). These solutes attack cells both externally and internally, resulting in membrane damage, pH shifts and general protein denaturation.

However, when the cooling rate is slow enough to prevent intracellular ice formation, but fast enough to avoid serious dehydration effects, cells may be able to survive the freezing and thawing process. This survival zone or window is readily observed in many bacteria and other prokaryotes, but for most eukaryotic cells it is nonexistent or very difficult to find without using cryoprotective agents. These agents have little effect on the damage caused by fast freezing (intracellular ice crystal formation), but rather prevent or lessen the damage caused by slow freezing (dehydration and shrinkage) (8).

The final storage temperature is also critical for successful cryopreservation. To completely stop biological time, storage temperatures must be maintained below -130°C , the glass transition point below which liquid water does not exist and diffusion is insignificant. While many cell cultures are successfully stored at -70°C to -90°C for months or even years,

Advantages of Freezing Cultures

- ▶ less work — saves time and money
- ▶ serves as a backup supply for emergencies
- ▶ provides a more homogeneous population by minimizing culture aging and evolution

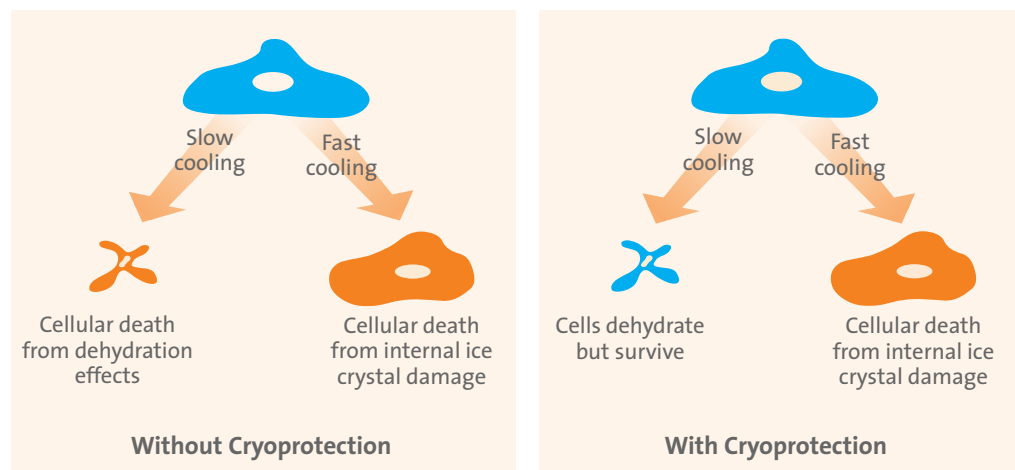


Figure 1. Effects of Freezing Rates on Cells

biological time is not stopped, only slowed, and cellular damage or changes will accumulate.

Storage in liquid nitrogen at -196°C effectively prevents all thermally driven chemical reactions. Only photo-physical effects caused by background ionizing radiation still operate at this temperature. Thousands of years are estimated to be necessary before background radiation will have a noticeable effect on cryopreserved cultures (2,8).

Practical Aspects of Cell Freezing

Under the best of circumstances the process of freezing remains stressful to all cell cultures. It is important that everything possible be done to minimize these stresses on the cultures in order to maximize their subsequent recovery and survival. The following suggestions and recommendations are designed to augment the protocols referred to earlier.

I. Cell Selection

First ensure that the cells are in their best possible condition. Select cultures near the end of log phase growth (approximately 90% confluent) and change their medium 24 hours prior to harvesting. Carefully examine the culture for signs of microbial contamination. Facilitate this by growing cultures in antibiotic-free medium for several passages prior to testing. This allows time for any hidden, resistant contaminants (present in very

low numbers) to reach a higher, more easily detected level. Samples of these cultures are then examined microscopically and tested by direct culture for the presence of bacteria, yeasts, fungi, and mycoplasmas.

Mycoplasmas present a special problem since they can be found in cultures at very high concentrations (up to 10^8 organisms per milliliter of medium) without any visible effects or turbidity. As a result, as many as 20% of all animal cell cultures are contaminated by these ubiquitous but unseen organisms. Although special efforts are required to detect mycoplasmas, the serious consequences of their presence makes testing frozen culture stocks absolutely essential (9,12).

Check for both the identity of the cultures and the presence of any expected special characteristics. Monitor cell identities by karyology and isoenzyme analysis, ensuring that they are, at the very least, the correct species (10).

II. Cell Harvesting

Start with the standard harvesting procedure generally recommended for the culture and be as gentle as possible. Remove all dissociating agents by washing or inactivation (especially important when using serum-free medium). Centrifugation, when absolutely necessary, should only be hard enough to obtain a soft pellet; $100 \times g$ for 5 to 6 minutes is usually sufficient. To ensure uniformity of the final

frozen stock, pool the contents of all harvested culture vessels. This also makes it much easier to perform essential quality control testing for microbial contamination and culture identity.

Count and then dilute or concentrate the harvested cell suspension to twice the desired final concentration, which is usually 4 to 10 million viable cells per milliliter. An equal volume of medium containing the cryoprotective agent at twice its final concentration will be added later to achieve the desired inoculum. Keep the cells chilled to slow their metabolism and prevent cell clumping. Avoid alkaline pH shifts by gassing with CO₂ when necessary.

III. Cryoprotection

As mentioned earlier, cryoprotective agents are necessary to minimize or prevent the damage associated with slow freezing. The mechanisms providing this protection, although not completely understood, appear to work primarily by altering the physical conditions of both the ice and solutions immediately surrounding (external to) the cells. Permeation of the cells by cryoprotectants does not appear to be necessary for their proper functions (4). Remember, protection against fast freezing damage (internal ice formation) is not provided by these agents, but rather by careful control of the freezing rate. A wide variety of chemicals provide adequate cryoprotection, including methyl acetamide, methyl alcohol, ethylene glycol and polyvinyl pyrrolidone (7). However, dimethylsulfoxide (DMSO) and glycerol are the most convenient and widely used. Many of these agents, although providing excellent cryoprotection, have toxic side effects on cultures making their use difficult.

DMSO is most often used at a final concentration of 5 to 15% (v/v). Always use reagent or other high purity grades that have been tested for suitability. Sterilize by filtration through a 0.2 micron nylon membrane in a polypropylene or stainless steel housing and store in small quantities (5mL). *CAUTION:* Take special care to avoid contact with solutions containing DMSO. It is a very powerful polar sol-

vent capable of rapidly penetrating intact skin and carrying in with it harmful contaminants such as carcinogens or toxins. Some cell lines are adversely affected by prolonged contact with DMSO. This can be reduced or eliminated by adding the DMSO to the cell suspension at 4°C and removing it immediately upon thawing. If this does not help, lower the concentration or try glycerol or another cryoprotectant.

Glycerol is generally used at a final concentration of between 5 and 20% (v/v). Sterilize by autoclaving for 15 minutes in small volumes (5mL) and refrigerate in the dark. Although less toxic to cells than DMSO, glycerol frequently causes osmotic problems, especially after thawing. Always add it at room temperature or above and remove slowly by dilution.

High serum concentrations may also help cells survive freezing. Replacing standard media-cryoprotectant mixtures with 95% serum and 5% DMSO may be superior for some overly sensitive cell lines, especially hybridomas.

Add cryoprotective agents to culture medium (without cells) immediately prior to use to obtain twice the desired final concentration (2X). Mix this 2X solution with an equal volume of the harvested cell suspension (also 2X) to obtain the inoculum for freezing. This method is less stressful for cells, especially when using DMSO as the cryoprotectant.

IV. Storage Vessels

After the cryoprotective solution is mixed with the cell suspension, the resulting inoculum is added in small aliquots (usually 1 to 2 milliliters) to each storage vessel. Due to the extremely low temperatures encountered during cryogenic storage, not all vessel materials or designs are suitable or safe. Many materials become very brittle at these temperatures; vessels made from them may shatter or crack during storage or thawing. Carefully check the vessel manufacturers' recommendations on proper selection and use.

Also important is selecting the sealing system or cap design used to maintain the integrity of the vessel, especially for

Preventing Freezing Damage

- ▶ Use slow freezing to remove all intracellular water.
- ▶ Use cryoprotective agents to minimize dehydration effects.
- ▶ Store below -130°C to completely stop biological time.



Figure 2. Corning Externally Threaded (left) and Internally Threaded (right) Cryogenic Vials

storage in liquid nitrogen. If these vessels leak during storage (as many do) they will slowly fill with liquid nitrogen. When they are eventually returned to room temperature, the liquid nitrogen quickly vaporizes causing a rapid pressure buildup. The vessels may then violently blow off their caps or explode to vent the pressure and release their contents into the atmosphere. This is a very dangerous situation, especially if the vessels contained pathogenic organisms or potentially toxic or harmful substances. Storage above liquid nitrogen to reduce these potential hazards is strongly recommended in such situations.

Two types of vessels are commonly used for cryogenic storage: heat-sealable glass ampules and plastic (usually polypropylene) screw-capped vials. Both are available in a variety of sizes (1 to 5 mL capacity) although the smaller sizes are preferred for cryogenic storage (See Figure 2).

Because of sealing and labeling problems, glass ampules are no longer widely used in cell culture laboratories. Invisible pinhole leaks may occur in vials during the sealing process; if these are later stored submerged in liquid nitrogen, they may explode when removed for thawing. Pinholes can usually be detected before freezing by immersing sealed ampules for 30 minutes in a chilled solution of 70% ethanol containing 1% methylene blue. This solution will rapidly penetrate and stain any leaky ampules; after rinsing with water, defective ampules are then easily detected and discarded.

Due to their greater safety and convenience, plastic vials have largely replaced glass ampules for cryogenic storage. The wide variety of styles and special features like printed marking areas and colored caps for easier identification also add to their popularity.

Several cap styles are available, some with an internally-threaded stopper, and others with externally-threaded designs which help minimize contamination (See Figure 3).

V. Labeling and Recordkeeping

Providing for long-term location and identification of frozen cultures is the most frequently overlooked area of cryogenic storage. A cryogenic cell repository is expected to outlast the laboratory workers who contribute to it, but poorly maintained or missing inventory records, and improperly or illegibly labeled vials and ampules may prevent this, especially after the people responsible have gone.

Labels must contain enough information to locate the appropriate records; usually the culture's identity, date frozen, and initials of the person responsible are sufficient. Most plastic vials have printed marking spots or areas for easy labeling. On vials and ampules without marking spots, use cloth labels with special adhesives formulated for cryogenic conditions.

Special ceramic-based inks are available for labeling glass ampules. These are applied prior to filling and then baked onto the glass, usually during dry heat sterilization. Permanent marking spots

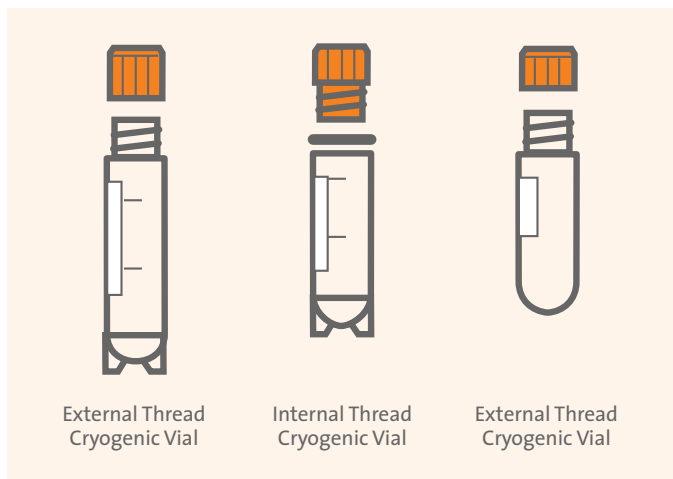


Figure 3. Corning Cryogenic Vial Cap Designs

can be applied on glass ampules with white nail polish. A laboratory marking pen is then used to write on the spot once it has dried.

No matter which labeling method is chosen, use special care to check its permanency under cryogenic conditions. Some marking spots, inks, and labels may flake off or fade during long-term storage; a trial run of at least several weeks is recommended.

Fully detail in the records the culture's storage conditions, including all of the following information: culture identity, passage or population doubling level, date frozen, freezing medium and method used, number of cells per vial, total number of vials initially frozen, and the number remaining, their locations, their expected viability and results of all quality control tests performed (sterility, mycoplasma, species, karyotype, etc.). Additional culture information, especially their origin, history, growth parameters, special characteristics, and applications, is also helpful and should be included whenever possible.

Make special efforts to keep all records up to date and to ensure everyone in the facility is properly using them. Use pre-printed forms to make the information recording process easier and more likely to be completed. Keep updated, duplicate copies of all critical records in a safe place removed from the laboratory area to guard against their accidental loss or destruction. This is especially important

if a computer-based recordkeeping system is used; a current back up copy should always be maintained in addition to the information stored in the computer.

VI. Cooling Rate

The cooling rate used to freeze cultures must be just slow enough to allow the cells time to dehydrate, but fast enough to prevent excessive dehydration damage. A cooling rate of -1°C to -3°C per minute is satisfactory for most animal cell cultures. Larger cells, or cells having less permeable membranes may require a slower freezing rate since their dehydration will take longer.

The best way to control cooling rates is using electronic programmable freezing units. Although expensive, they allow precise control of the freezing process, give very uniform and reproducible results, and can freeze large numbers of vials or ampules. Most units are available with chart recorders for a permanent record of the cooling process.

There are a variety of mechanical freezing units that provide adequate control of the cooling rate and are relatively inexpensive. Some units use racks designed to hold vials at predetermined depths in the neck of a liquid nitrogen freezer. The cooling rate is dependent on the total number of vials and the depth at which the rack is placed. Another design uses an alcohol filled metal or plastic canister containing a rack with a capacity of up to 24 vials. The filled canister is placed in an ultracold mechanical freezer where the alcohol acts as a bath to achieve more uniform heat transfer and cooling. After freezing 4 to 5 hours, the vials are removed from the canister and transferred to their final storage locations.

Insulated cardboard or polystyrene foam boxes are commonly used as freezing chambers in ultracold freezers. These homemade devices work well with many cell lines but do not always give controlled, reproducible, or uniform cooling. As a result, there may be serious differences in viability among the vials upon thawing. This homemade approach is not recommended for valuable or irreplaceable cultures.

No matter which cooling method is used, transfer from the cooling chamber or device to the final storage location must be done quickly to avoid warming of the vials. Use an insulated container filled with dry ice or liquid nitrogen as a transfer vessel to ensure that the cells remain below -70°C .

VII. Cryogenic Storage

Only freezers capable of continually maintaining temperature below -130°C should be considered for long-term cryogenic storage. Although most liquid nitrogen-cooled freezers and some specially designed mechanical freezers meet this requirement, most cell culture laboratories prefer liquid nitrogen freezers (See Figure 4). The final choice is often based on the availability of a reliable supply of liquid nitrogen, the storage capacity required, and the size of the budget. Liquid nitrogen freezers permit storage either in the vapor phase above the liquid at temperature between -140°C and -180°C , or submerged in the liquid at a temperature below -196°C . Using vapor phase storage greatly reduces the possibility of leaky vials or ampules exploding during removal. However, since the amount of liquid nitrogen in the freezer is reduced to provide space for vapor phase storage, the freezer's holding time (the period it can maintain its storage temperature without adding more liquid nitrogen) is also reduced. This lowers the freezer's margin of safety and requires more



Figure 4. Typical Cryogenic Freezers

frequent monitoring and filling. Give careful consideration to these safety issues when deciding upon a storage method.

Frequently check nitrogen levels in freezers; a schedule should be established and strictly adhered to. Nitrogen evaporation is dependent on both the degree of use and the static holding time of the freezer. Sudden, unexplained increases in the evaporation rate may signal damage to the insulation or other problems with the freezer and must be carefully investigated. Avoid frost or ice buildup around freezer openings; this increases the nitrogen evaporation rate and can cause elevated temperatures in the upper portion of vapor phase freezers. Audible alarm systems for detecting low liquid nitrogen levels are available to provide additional safeguards; however, they provide a false sense of security if not monitored 24 hours a day.

VIII. Thawing

CAUTION: Always use appropriate safety equipment when removing vials and ampules from liquid or vapor phase nitrogen freezers. A full face shield, heavy gloves and lab coat are strongly recommended for protection against exploding vials or ampules.

Remove the vial or ampule from its storage location and carefully check both the label and storage record to ensure that it is the correct culture. Place the vessel in warm water, agitating gently until completely thawed. Rapid thawing (60 to 90 seconds at 37°C) provides the best recovery for most cell cultures; it reduces or prevents the formation of damaging ice crystals within cells during rehydration.

IX. Recovery

Since some cryoprotective agents may damage cells upon prolonged exposure, remove the agents as quickly and gently as possible. Several approaches are used depending on both the cryoprotective agents and characteristics of the cells.

Most cells recover normally if they have the cryoprotective agent removed by a medium change within 6 to 8 hours of thawing. Transfer the contents of the

ampule or vial to a T-75 flask or other suitable vessel containing 15 to 20 milliliters of culture medium and incubate normally. As soon as a majority of the cells have attached, remove the medium containing the now diluted cryoprotective agent and replace with fresh medium.

For cells that are sensitive to cryoprotective agents, removing the old medium is easily accomplished by gentle centrifugation. Transfer the contents of the vial or ampule to a 15 mL centrifuge tube containing 10 mL of fresh medium and spin for 5 minutes at 100 x g. Discard the supernatant containing the cryoprotectant and resuspend the cell pellet in fresh medium. Then transfer the cell suspension to a suitable culture vessel and incubate normally.

When glycerol is used as the cryoprotectant, the sudden addition of a large volume of fresh medium to the thawed cell suspension can cause osmotic shock, damaging or destroying the cells. Use several stepwise dilutions with an equal volume of warm medium every 10 minutes before further processing to give the cells time to readjust their osmotic equilibrium.

X. Problem Solving Suggestions

Viability problems associated with cryogenic storage are usually noticed soon after cultures are thawed and plated. There are four major areas where problems occur:

1. During harvesting and processing of the cells. Problems may be caused by excessive exposure of the cells to dissociating agents; using a cryoprotective agent that is toxic; or allowing high density cell suspensions to remain too long at room temperature or at a pH that is too basic.
2. During the cooling (freezing) process. Excessive cell damage and reduced culture viability often result from using a cooling rate that is too fast or too slow, or when the cooling process is temporarily interrupted. Not using a suitable cryoprotective agent at an appropriate concentration will also result in viability problems.

3. During cryogenic storage. Culture viability is often reduced when vials are allowed to warm up during transfer to the freezer, or if the repository temperature is not consistently maintained at appropriate cryogenic temperatures.
4. During thawing and recovery. Problems arise when the thawing process is too slow or the cryoprotectants are improperly removed (see above).

These viability problems can often be corrected by using the following technique to identify the stage in the freezing process where the problem originates.

Harvest enough cells to prepare at least four vials. Then remove a sample of cell suspension, equivalent in cell number to that which will be placed into the vials, and immediately place it into a culture vessel with an appropriate amount of medium and incubate. This culture will be used as a control to compare with the cultures set up in the remaining steps.

Next, add the cryoprotective agent to the remaining cells and divide among three vials. Place one vial at 4°C for one hour. Then remove the cells from the vial, process as though they had just been thawed from the freezer, and plate in medium as above. This culture will be compared with the control culture to determine if there are any problems associated with cryoprotective agent.

Meanwhile, process the remaining vials through the slow cooling process as usual. One vial is then immediately thawed and processed as above. This culture will be compared with the control culture to determine if there are any problems associated with the slow cooling process.

The remaining vial is then transferred to the cryogenic freezer and stored overnight before being thawed and processed as above. This culture will be compared with the control culture to determine if there are any problems associated with the cryogenic storage conditions. If additional vials of cells are available, several different recovery techniques should be used to determine if the recovery technique is the source of the problem.

By comparing all of the cultures to the original culture, it should then be possible to determine at which stage of the freezing process the problem occurred. Once this is known, the information presented in this guide and its references should be enough to eliminate the problem.

Freezing Checklist

- ▶ Gently harvest cultures.
- ▶ Check cultures for contamination, especially mycoplasma.
- ▶ Check culture identity by karyotyping or isoenzyme analysis.
- ▶ Use tested cryoprotective agents.
- ▶ Only use vials tested for cryogenic conditions.
- ▶ Ensure labels are permanent and complete.
- ▶ Control the cooling rate.
- ▶ Store cultures below -130°C.
- ▶ Monitor liquid nitrogen levels frequently.
- ▶ Keep good records.

XI. Managing a Cell Repository

The effort and expenses of managing a repository should be kept in line with the value of the cultures stored within it. This value is determined by answering two questions: How much time, money and effort is already invested in these stored cell cultures? And, what are the consequences of losing them? Cultures that are easily replaced through other labs or commercial sources may not require special efforts, but unique cultures, such as hybridomas and other genetically engineered cells, are irreplaceable and require

that special efforts be made to ensure their safety. The answers to these questions will help determine just how extensive and thorough your efforts should be.

Next, identify the potential problem areas that can cause the loss of these cultures. Some of these areas, such as vessel selection, recordkeeping, labeling, freezer monitoring, storage conditions, and quality issues (contamination and species identity), have already been discussed in this guide. Decide what steps are necessary to eliminate or minimize these problems. Split irreplaceable or extremely valuable cultures among several freezers, with at least one freezer in a separate location to protect against fire or other natural disasters. Colleagues in other labs or buildings may be able to provide good backup storage, especially if a reciprocal arrangement is made for them.

One final step remains; plan ahead for emergencies! One of the most serious and unexpected emergencies is the failure of a cryogenic freezer. Careful monitoring of the liquid nitrogen level or charting the temperature may give an early warning that failure is occurring, but middle of the night failures can and do happen. Have plans prepared in advance to deal with freezer failure and other problems. If these involve a colleague's equipment, get permission and make all necessary arrangements in advance — late night phone calls are usually not appreciated.

This information has been compiled to provide a guide for better understanding of the cryogenic preservation process. For additional assistance in this area, please contact Corning Life Sciences Technical Services at 800.492.1110, 1.978.635.2200, or at clstechserv@corning.com.

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