



Thermo Scientific Nalgene and Nunc Cryopreservation Guide

NALGENE NUNC

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Written by Frank P. Simione, M.S. of the American Type Culture Collection (ATCC) in cooperation with Thermo Fisher Scientific

Introduction

To ensure reproducible results and continuity in research and biomedical processes, today's scientists are faced with the task of genetically stabilizing replicable materials such as living cells and organisms, and ensuring sub-cellular components such as nucleic acids and proteins are preserved unchanged. Serial subculturing of replicable materials is time consuming and can often result in contamination or genetic drift as smaller and smaller portions of a population are selected. Improper storage and handling of non-replicable materials can lead to divergent and irreproducible research results. However, a population of living cells or a suspension of subcellular components can be stabilized by subjecting them to cryogenic temperatures which, for all practical purposes, stops time.

The process of stabilizing biological materials at cryogenic temperatures is called cryopreservation, a practical application of cryobiology, or the study of life at low temperatures. Advances in cryopreservation technology have led to methods that allow low-temperature maintenance of a variety of tissues, cell types and subcellular materials. Techniques are available for the preservation of microorganisms, tissues, primary cells, established cell lines, small multicellular organisms, complex cellular structures such as embryos, as well as nucleic acid and proteins.

The freezing process involves complex phenomena that, even after decades of research, are not fully understood. Cryobiological studies have led to speculation on what occurs during the freezing of living cells and how adverse phenomena can be overcome. Since water is the major component of all living cells and must be available for the chemical processes of life to occur, cellular metabolism stops when all water in the system is converted to ice.

Ice forms at different rates during the cooling process. Slow cooling leads to freezing external to the cell before intracellular ice begins to form[1]. As ice forms external to the cell, water is removed from the extracellular environment and an osmotic imbalance occurs across the cell membrane leading to water migration out of the cell. The increase in solute concentration outside the cell, as well as intracellularly as water leaves the cell, can be detrimental to cell survival.[1] If too much water remains inside the cell, damage due to ice crystal formation and recrystallization during warming can occur and is usually lethal.

The rate of cooling has a dramatic effect on these phenomena (Fig. 1). Rapid cooling minimizes the solute concentration effects as ice forms uniformly, but leads to formation of more intracellular ice since water has not migrated out of the cell. Slow cooling on the other hand, results in a greater loss of water from the cell and less internal ice being formed, but results in an increase in the solution effects. Cell permeability affects the rate of water loss; more permeable cells are able to tolerate rapid cooling better than less permeable cells.[2] Mazur et al.[3] postulated that ice crystal formation and solution effects both play a role in cell damage, and that an optimum cooling rate minimizes the effect of each. With few exceptions, a cooling rate of 1°C per minute is preferred.

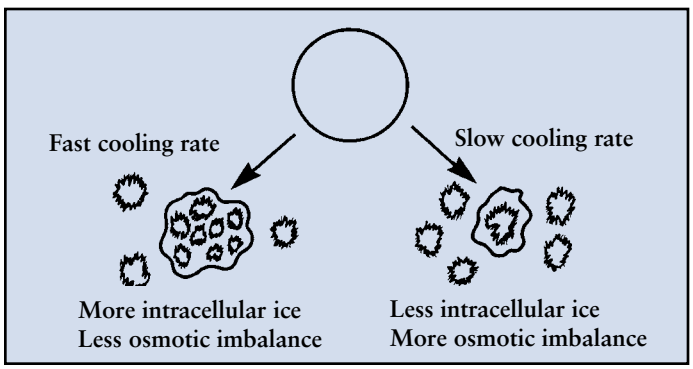


Fig. 1 Use a cooling rate of 1°C/minute and a cryoprotective agent to minimize damage due to osmotic imbalance and ice crystal formation.

Using cryoprotective additives, or chemicals that protect the cells during freezing also minimizes the detrimental effects of increased solute concentration and ice crystal formation. The most commonly used cryoprotective agents are dimethylsulfoxide (DMSO) and glycerol, although many other additives have been used for specific purposes. Additionally, maintaining frozen cells at the proper storage temperature and using an appropriate warming rate also contribute to minimizing damage to frozen cells and tissues.

A key element of a good cryopreservation program is standardization of the processes employed. Because of the complexity of the preservation process small variations in processing and storage can lead to subtle changes in the biological materials. By standardizing the methodologies there is greater assurance that research results will be consistent and comparable. Therefore, once a successful cryopreservation regimen is established efforts should be made to carefully document the methodology.

Seed Lot System

To aid in maintaining the genetic stability of cultured cells, the frequency of subculturing beyond the original established culture must be minimized. When freezing cells, use a system that ensures that early passage material is always available for producing new working stock. One method of preserving early passage material is to use a seed lot system.[4]

When preparing the first frozen lot of a culture, a portion of the lot is set aside as seed material. The vials designated as seed material are maintained separately from the working stocks to ensure that they remain unused and are not handled during retrieval operations (Fig 2). When the first lot of working stock is depleted, a vial is retrieved from the seed lot and used to prepare a second working stock. This continues until all seed vials, except one have been depleted. The last seed vial is then used to prepare a second seed lot. The second seed lot remains only one or two passages from the original material, but may be separated by many years if the lots are adequately sized.

The seed stock concept is similar to cell banking practices whereby a master cell bank that is fully qualified is developed and maintained for use in preparing working cell banks used in a manufacturing process.

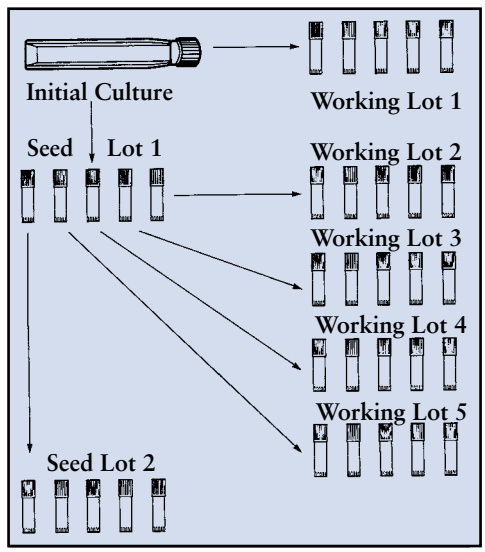


Fig. 2 Seed lot system.

A similar system could also be used replicable materials, such as DNA and proteins, except for a different purpose. In this case, since the material is not renewable, the seed material would be available solely for comparative purposes when changes in the working

material are suspected. Sufficient material should be retained as seed stock to allow for testing when necessary.

In addition to seed material, a small portion of the original lot, and even portions of working stocks, should be segregated and maintained in a location remote from all other material. This practice assures that the segregated materials are not handled during stocking and retrieval activities, and remain at a constant temperature. Backup materials should be stored in an off-site location if possible to ensure that preserved materials are not lost in the event of a physical disaster at the primary location. Using seed lots and maintaining off-site reserve material are of primary importance in ensuring continuity and longevity in any well-managed collection of biological materials.

Cryoprotective Agents

Many compounds have been tried as cryoprotective agents, either alone or in combination, including sugars, solvents and even serum. Although there are no absolute rules in cryopreservation, glycerol and DMSO have been widely used and traditionally have been demonstrated to be the most effective agents for preserving living cells and organisms. Other cryoprotectants that have been used occasionally, either alone or in combination, include: polyethylene glycol, propylene glycol, glycerine, polyvinylpyrolidone, sorbitol, dextran and trehalose.

The need to preserve tissues and whole organs has led to the development of novel preservation methodologies that are also applicable to enhancing the recovery of frozen cells and organisms.[4,5] These include variations on the concentration of cryoprotectants, and additives that protect cells against apoptosis, or programmed cell death. For many years the death of cells following freezing was assumed to be caused by events causing physical changes or damage to cells. More recently it has been discovered that more subtle events that contribute ultimately to cell death may be manageable with proper additives.[4,5]

Cryoprotective agents serve several functions during the freezing process. Freezing point depression is observed when DMSO is used which serves to encourage greater dehydration of the cells prior to intracellular freezing. Cryoprotective agents also seem to be most effective when they can penetrate the cell, delay intracellular freezing and minimize the solution effects.[1] The choice of a cryoprotective agent is dependent upon the type of cell to be preserved. For most cells, glycerol is the agent of choice because it is usually less toxic than DMSO. However, DMSO is more penetrating and is usually the agent of choice for larger, more complex cells such as protists. The cryoprotective agent should be diluted to the desired concentration in fresh growth medium prior to adding it to the cell suspension. This minimizes the potentially deleterious effects of chemical reactions, and assures a more uniform exposure to the cryoprotective agent when it is added to the cell suspension, reducing potential toxic effects. DMSO and glycerol are generally used in concentrations ranging from 5-10% (v/v), and are not usually used together in the same suspension with the exception of plant cells.

The optimum concentration of the cryoprotectant varies with the cell type and for optimal results the highest concentration the cells can tolerate should be used. For some materials it may be advantageous to examine the sensitivity of the cells to increasing concentrations of the cryoprotective agent to determine the optimum concentration. The Quick-Reference Chart (page 4) lists the recommended concentrations of cryoprotective agents for each group of cells and serves as a general guide to choosing the proper agent.

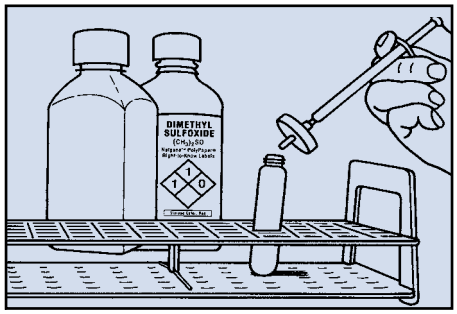


Fig. 3 Sterilization of DMSO by filtration.

Glycerol and DMSO should be of reagent grade or better, sterilized prior to use, and examined for undesirable properties. Each lot should be examined for toxic properties by exposing sensitive cells to concentrations previously used with success. Glycerol may be sterilized by autoclaving for 15 minutes at 121°C and 15 psig. Glycerol should be protected from light during storage. DMSO must be sterilized by filtration using a 0.2 micron nylon syringe filter or a Teflon® PTFE syringe filter which has been pre-washed with alcohol and rinsed with DMSO (Fig. 3). Cryoprotective agents should be prepared in single-use volumes to minimize the risk of contamination and moisture introduction with repeated use from one container.

> *Use caution when handling DMSO as it is quickly absorbed into the body through the skin and may transport harmful substances into the body with it.*

Non-replicable materials generally do not require additives when frozen except when certain characteristics need to be protected. For example, tissue specimens that are frozen to preserve morphology may benefit from suspension in materials such as optimal cutting temperature (OCT) compound to optimize the results. Normally tissues are frozen in blocks, however combining freezing in OCT with storage in cryovials improves the handling of small tissue specimens.[6]

Preparation of Biological Materials

How materials are processed in preparation for freezing can have an affect on the outcome of the preservation process. For non-replicable materials such as tissues, nucleic acids and proteins, the preparation process consists of ensuring that the materials are in the proper solution or freezing medium in order to maximize the intended use of the materials when recovered. However the stability and recoverability of living cells and organisms is affected by the growth conditions and pre-freezing processing. Several factors must be considered when preparing cells for cryopreservation. These include the type of cell, cell viability, growth conditions, physiological state of the cells, the number of cells, and how the cells are handled. When preparing the initial seed stock of a new isolate or cell line, the culture should be examined for identity and contaminating microorganisms at a minimum. This examination should be repeated after preservation and each time a new lot of the culture is prepared.

Microorganisms

Microbial cells, particularly bacteria and yeast, grown under aerated conditions demonstrate a greater resistance to the detrimental effects of cooling and freezing than non-aerated cells.[1] T. Nei et.al.[2] have demonstrated that cell permeability is greater in aerated cultures, and that the aerated cells dehydrate faster during cooling than non-aerated cells. Microbial cells harvested from late log or early stationary cultures also demonstrate greater resistance to the freezing process than younger or older cells.[1]

Generally, the greater the number of cells present initially, the greater the recovery. For most bacteria and yeast, approximately 10^7/mL cells are required to ensure adequate recovery.[7] These can be conveniently harvested from agar slants or plates, or

when greater quantities are required, grown in broth culture and harvested by centrifugation. In either case, cells are generally suspended in fresh growth medium containing the cryoprotective agent. Protists can also be concentrated by centrifugation, but are often suspended in the used medium and then diluted by adding an equal volume of fresh growth medium containing the cryoprotective agent.[7]

Spore-forming fungi require harvesting of spores and suspension of the spores in fresh growth medium containing the cryoprotective agent. When freezing fungal spores, care must be taken not to delay the freezing process too long to ensure that germination does not occur prior to freezing. For fungi that do not form spores, special procedures for harvesting mycelia prior to freezing must be utilized. For fungi with tough mycelia, the culture is harvested from agar growth by cutting and removing agar plugs containing the mycelia and placing the plugs into fresh growth medium containing the cryoprotective agent. Tough mycelia that do not adhere well to agar cultures are grown in broth culture and the mycelial mass is blended prior to freezing.[7]

The viability and an estimate of recovery should be determined both before and after freezing the culture. Viability is a measure of the culture's ability to grow and reproduce. For some material, such as protozoan cultures, this should include several passages to ensure stability. An estimate of the number of cells recovered can be made by several means including serial dilution, plate counts, or direct cell counting. A comparison of the counts prior to and after freezing gives an indication of the degree of recovery or the success of the preservation procedure.

Mammalian Cells

When preparing mammalian cells for cryopreservation, cell populations need to be adjusted to levels that ensure adequate recovery without unnecessarily growing large numbers of cells. For most mammalian cells, a starting population between 10^6 to 10^7 cells/mL is optimum.[7] The cell suspension should initially be prepared at a concentration twice that desired for preservation so that an equal volume of cryoprotectant (2 x cryoprotective agent + medium) can be added. Alternatively, the cell pellet can be resuspended in the cryoprotectant (1 x cryoprotective agent + medium) to the desired cell concentration. Gentle handling during cell harvesting and concentration procedures will ensure healthy cells prior to subjecting them to cold stress. Vigorous pipeting and high-speed centrifugation should be avoided if possible. Where appropriate, the pH should be maintained by gassing with 5% or 10% $C0_2$.

Factors which can affect the recovery of cryopreserved mammalian cells include: (a) type of cell, (b) the growth phase of the culture, (c) the stage of the cell in the cell cycle, and (d) the number and concentration of cells in the final suspension. Attempts to improve the viability of cryopreserved cells should consider these factors, as well as the nature of the cryoprotective agent and the freezing process.

Mammalian cell cultures are especially susceptible to contamination by other cells, such as HeLa[8], and contaminating microorganisms. The species of origin of cell lines can be verified by isoenzyme analysis, karyotyping, immunological assays, or genomic analysis. These should be performed prior to and following preservation. Contamination by viruses and Mycoplasma sp. is of particular concern.[9] A good characterization program for mammalian cell lines should include a check for contamination by bacteria, fungi, appropriate viruses, mycoplasma, and in some cases, protozoa.

Stem Cells

Stem cells are cryopreserved in a manner similar to other mammalian cells, with some exceptions to enhance recovery and clonogenic activity. Cryoprotection is normally afforded by using DMSO, sometimes in combination with serum, and freezing slowly is preferred. Trehalose can be used to reduce the potential toxicity associated with other cryoprotectants.[10] Rapid warming is also preferred, and viability may vary depending on the cell type.

Vitrification can also be used to preserve stem cells. The protocol involves suspending the cells in a concentrated mixture typically composed of more than one cryoprotectant. For vitrification, the freezing and re-warming processes are rapid to avoid ice crystal formation, and in some studies the vitrification process resulted in greater viability.[11] DMSO, glycerol and propylene glycol have all been used successfully to cryopreserve stem cells.

Plant Cells

Plant cells respond to cryopreservation in a manner similar to other cells.[12] The stage in the growth cycle from which they are harvested can affect their recovery, most optimum being late log phase. Also, cell density may play a role in recovery, the optimum cell density depending on the species being preserved.

Combinations of cryoprotective agents are sometimes more effective than agents used singly. The cooling rate is important, and in many cases a two-step cooling process where the cells are held at -30°C to -40°C for a period of time before cooling to liquid nitrogen temperatures, is beneficial. This process enhances the dehydration of the cytoplasm prior to freezing. Rapid thawing is preferred, but there is evidence that slow warming is just as effective in some cases. Vitrification can also be used to preserve plant cells by using concentrated cell suspensions and rapid rates of cooling.

Hardening of plants leads to greater tolerance of stressful conditions, such as experienced during the freezing process. Plants produce increased quantities of some compounds such as sugars and even glycerol which contribute to protecting the cells from osmotic stress during freezing. Undifferentiated callus tissue is often preserved in an effort to stabilize characteristics that can be affected by continued cultivation.

Preservation of seeds is also an acceptable method of stabilizing plant germplasm, and the most common method is storage at low humidity and cool temperatures. However some seeds are tolerant of the increased desiccation associated with freezing and cryogenic storage, and can be stored at liquid nitrogen temperatures.

Viruses

Most viruses can be frozen as cell-free preparations without difficulty and do not require controlled cooling.[7] The exceptions are those viruses cultured in viable infected cells which require controlled cooling. For cell-adapted viruses the preservation process should be applicable to survival of the host cell. When viruses are harvested from eggs, the high protein content of the allantoic fluid or yolk sac provides protection during the freezing process.

Plant viruses can be preserved either in infected plant tissue or as purified virus preparations. The virus preparations are suspended in DMSO or another cryoprotectant prior to freezing. Recovery is generally best when the cooling rate is controlled, although most plant viruses will tolerate a rapid freezing procedure. Recovery of plant viruses simply involves thawing in a warm bath, followed by inoculation into the appropriate plant host.

Embryos

Embryos have been preserved both by controlled cooling and vitrification. Recovery depends on the stage of embryonic development, and is measured by successful implantation leading to fetal development.

Genetically Modified Materials

Genetically modified cells and organisms can be cryopreserved in a manner similar to the unmodified host cell.[13,14]

Non-Replicable Materials

Non-replicable materials such as whole blood, serum, tissues, nucleic acids and proteins do not usually have any special requirements for successful preservation. The materials are generally frozen without a cryoprotectant, and the freezing process can be rapid. However, the process used depends on the end-use of the material. Successful recovery of the properties of whole blood requires cryoprotection and controlled cooling, and the quality of frozen tissues can be improved by using a suspension such as optimal cutting temperature (OCT) compound.

Equilibration

The period of time between mixing the cryoprotectant with the cell suspension and beginning the cooling process is called the equilibration period. For most cells, equilibration should occur for at least 15 minutes, but no longer than 45-60 minutes. The cryoprotective agent may be toxic to the cells if the equilibration time is too long. For tissues frozen in OCT, not technically a cryoprotectant, the period of equilibration is generally not significant since OCT does not usually penetrate the tissue but simply provides support during freezing and subsequent sectioning of the tissue.

Equilibration, which should take place at ambient temperature, allows time for the cryoprotective agent to penetrate the cells, with larger and less permeable cells as well as embryos requiring a longer equilibration period. During this period of equilibration the cell suspension may be dispensed into vials and otherwise manipulated in preparation for freezing. An optimal equilibration time should be determined empirically for the cells being cryopreserved to maximize later recovery.

Ampoules and Vials

A variety of small containers such as flame-sealed glass ampoules and screw-cap plastic vials can be used for storing cells at ultra-low temperatures. The most commonly used sizes are 1.2- to 2.0-mL cryovials, the size and configuration of which maximize storage capacity while retaining ease of handling during stocking and retrieval activities. Generally, 0.5-1.0 mL of the cell suspension is placed into each container. Several factors must be considered when selecting a container, including its cryotolerance, storage conditions, type of cells to be stored, and safety considerations.

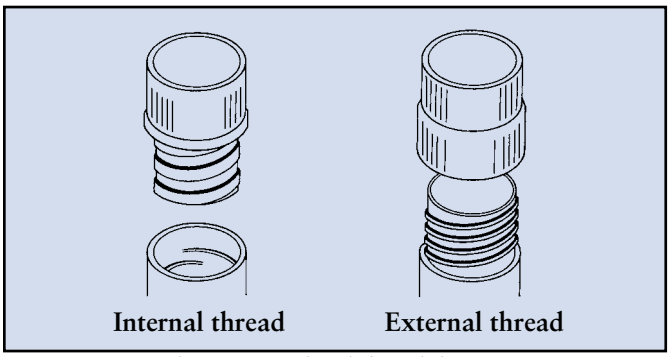


Fig. 4 Internal vs. External vial thread design

Quick-Reference Chart

(To be used as a general guide only)

Cell Type	No. of Cells	Cryoprotective Agent	Temperature
Bacteria	10^7/mL	Glycerol (10%)	-60°C*
Bacteriophage	10^8 pfu/mL	Glycerol (10%)	-80°C
Fungi			
Hyphae	†	Glycerol (10%)	-150°C
Spores	10^6/mL	Glycerol (10%)	-80°C
Yeast	10^7/mL	Glycerol (10%)	-150°C
Protozoa	10^5-10^7/mL	DMSO (5-10%) or Glycerol (10-20%)	-150°C
Algae	10^5-10^7/mL	Methanol (5-10%) or DMSO (5-10%)	-150°C
Plant Cells	**	DMSO (5 -10%) + Glycerol (5-10%)	-150°C
Animal Cells	10^6-10^7/mL	DMSO (5-10%) or Glycerol (5-10%)	-150°C
Hybridomas	10^7/mL	DMSO (5-10%) + Serum (20%)	-150°C
Stem Cells	10^5-10^6/mL	DMSO (5-10%) + Serum (20-90%)	-150°C
Sub/Non Cellular Materials			
Plant viruses	‡	None	-80°C
Animal viruses			
Cell Free	‡	None	-80°C
Infected Cells	10^6/mL	DMSO (7%) + Fetal Bovine Serum (10%)	-150°C
Plasmids	10^6/mL	Glycerol (10%)	-150°C
Phage Libraries	‡	Glycerol (10%)	-150°C
DNA	◊	None	- 80°C
RNA	◊	None	- 80°C
Protein	◊	None	- 80°C
Serum	◊	None	- 80°C
Multicellular			
Embryos	20	1,2-propanediol, glycerol or ethylene glycol	-150°C
Tissues	◊	OCT	- 80°C
Blood	◊	Glycerol	-150°C◊◊

Footnotes to Quick-Reference Chart:

* While -60°C is adequate for most organisms in the groups noted, some sensitive cells may not survive long periods of storage at this temperature.

† Mycelial masses are prepared for freezing of the hyphae of fungi without regard to numbers of cells.

** Plant cells are generally packed to 3-20% cell volume for freezing.

‡ The number of infectious particles has little effect on the recovery of viruses and bacteriophage.

◊ For non-replicable materials the concentration does not affect the ability to freeze the material, only the specific application for its intended use.

◊◊ Whole blood and most blood components can be stored at -80°C, however lymphocytes maintained for later development of established cell lines must be maintained at -150°C

For temperatures above -100°C where low-temperature mechanical stresses are less severe, a variety of containers may be used. However, when storing material at liquid nitrogen temperatures, containers specifically designed to withstand cryogenic temperatures must be used. A variety of containers specifically designed for cryogenic use are available. Plastic vials have screw-on closures with external or internal threads (Fig. 4). The rate of warming may be affected by the type of container used since plastic vials usually require a longer warming period for complete thawing than glass ampoules. This difference in warming rate may be significant for some fastidious cells, but for most cells does not contribute to a loss of viability.

Other containers can be used to store cryopreserved materials including straws traditionally used for embryos, and microtiter plates commonly used for freezing cell arrays or clones. The container of choice should be one that maximizes the ability to maintain viable material during storage, retrieval and handling. Glass ampoules may be flame sealed, however care must be taken that sealing is performed properly, since improperly sealed glass ampoules may have microchannels[15] that lead to liquid nitrogen penetration over time. When these are retrieved from liquid nitrogen to ambient temperature, rapid conversion of the liquid nitrogen to vapor inside the ampoule can result in explosion of the ampoule. Plastic vials with screwtop closures are also susceptible to liquid nitrogen penetration,[16] and while the explosion potential is minimized, liquid can spray from the cap/vial interface with potential dissemination of the vial contents during warming and handling.

Storage by immersion in liquid nitrogen is not advised.

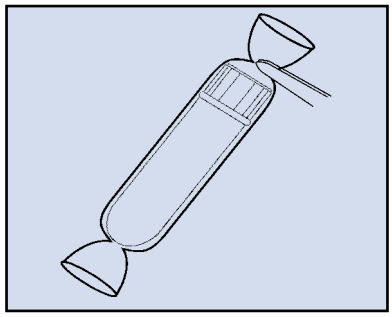


Fig. 5 If plastic vials are stored in the liquid phase of liquid nitrogen, the use of Thermo Scientific Nunc CryoFlex is strongly recommended (Nunc Cat. No. 343958). ***See warning on page 9.***

Rate of Cooling

Once the cells and the cryoprotectant have been combined and dispensed into vials, the next step is to cool the suspension. The rate of cooling is important since it affects the rate of formation and size of ice crystals, as well as the solution effects that occur during freezing. Different types of cells may require different cooling rates, however a uniform cooling rate of 1°C per minute from ambient temperature is effective for a wide variety of cells and organisms.

Generally, the larger the cells, the more critical slow cooling becomes. Most bacteria and spore-forming fungi will tolerate less-than-ideal cooling rates and can be frozen by placing the material at -80°C for a period of time. More fastidious bacteria and non-sporulating fungi require more uniform rates of cooling. Protists, mammalian cells and plant cells often require even greater control of the cooling rate including special manipulation to minimize the detrimental effects of undercooling and the heat liberated during the phase change from water to ice.

Despite the control applied to the cooling of cells, most of the water present will freeze at approximately -2°C to -5°C. The change in state from liquid to crystalline form results in the release of energy in the form of heat; this is known as the latent heat of fusion. Warming of the sample occurs until the equilibrium freezing point is reached, at which temperature ice continues to form. To minimize the detrimental effects of this phenomenon, undercooling must be minimized by artificially inducing the formation of ice. This can be accomplished by seeding the suspension with ice or some other nucleating agent, or by rapidly dropping the temperature of the external environment to encourage ice crystal formation.

To achieve uniform, controlled cooling rates, use a programmable-rate cell freezing apparatus. Simple units allow only the selection of a single cooling rate for the entire temperature range. More sophisticated units, however, allow a selection of variable rates for different portions of the cooling curve. Less costly and easier-to-use systems are available for simulating a controlled-rate cooling process by placing the vials in a mechanical freezer at -60°C to -80°C. In order to accomplish a uniform rate of cooling, the vials must be placed in specially designed containers.

Thermo Scientific Nalgene Mr. Frosty 1°C freezing container (Cat. No. 5100-0001) provides a simple-to-use system designed to achieve a rate of cooling very close to 1°C per minute. (Fig. 6) Typical cooling rates for homemade freezing systems lead to uncontrolled cooling that averages 1°C per minute but the cells actually experience more rapid rates of cooling during some parts of the cooling curve.[16] Homemade freezing systems are also non-repeatable. Nalgene® Mr. Frosty® eliminates the need for direct immersion in an alcohol bath. This feature eliminates the potential for contamination due to wicking of the alcohol, as well as the presence of residual alcohol on the exterior of the vials. During handling at colder temperatures, the presence of alcohol on the vials makes the vials colder to the touch and extremely slippery.

Cryopreservation of embryos requires even greater control of the process because of their multicellular structure. In addition to the controlled rate freezing commonly used for single cells, a vitrification process is also used for preserving embryos.[17] This requires suspending the embryos in a highly viscous solution, and rapidly cooling the suspension to eliminate the formation of ice crystals. The resulting frozen mass is a vitreous glass that requires storage at liquid nitrogen temperatures. If the storage temperature rises above -130°C ice crystals will form resulting in damage to the embryo.

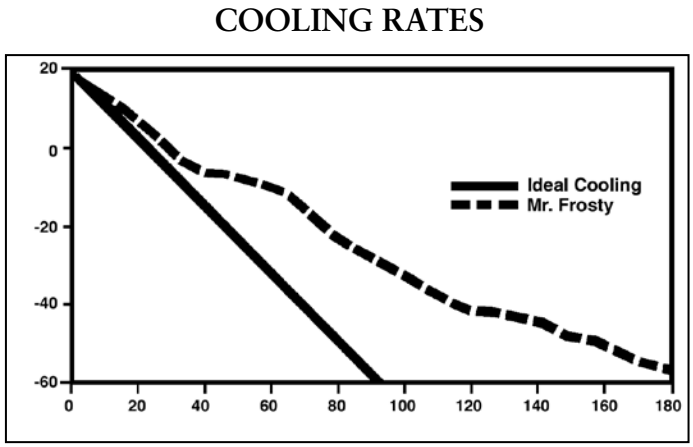


Fig. 6 The ideal cell cooling rate and the cooling rate provided by Nalgene Mr. Frosty 1°C freezing container.

Storage

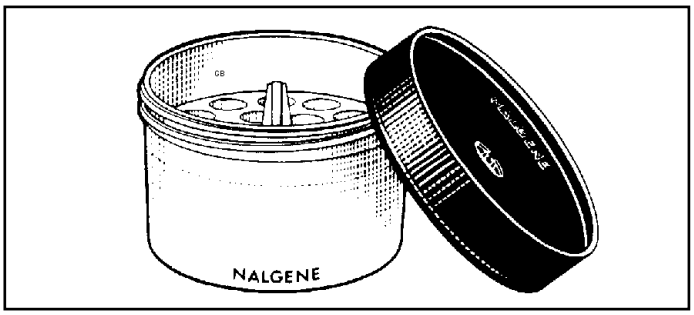
When the sample has been frozen for 48 hours, a vial should be thawed to determine whether the cells are viable and able to establish a cell population, (i.e. if they survived the freezing procedure).

See Determination of Recovered Cells, below.

The temperature at which frozen preparations are stored affects the length of time after which the material can be recovered. The lower the storage temperature, the longer the viable storage period. Ultimate stability of frozen cells cannot be assured unless the material is maintained below -130˚C.[18] Some bacteria and spore-forming fungi may tolerate storage temperatures of -60˚C to -80˚C for long periods of time. However, more fastidious cells, such as mammalian tissue cultures, hybridomas and stem cells must be maintained below -130˚C to assure long-term stability. It has been demonstrated that some cells survive for less than one year when stored at -80˚C.[1]

For ultimate security and maximum stability, living cells and embryos should be stored in liquid nitrogen freezers. However, there are risks associated with immersing vials directly into liquid nitrogen, as discussed previously. Liquid nitrogen units that provide all-vapor storage are ideal as long as the working temperature at the opening of the unit remains below -130˚C.

To assure that a liquid nitrogen freezer maintains the proper working temperature, the volume of liquid nitrogen in the unit should be adjusted to a level that results in a temperature of -150°C just above the stored material when the lid of the unit is removed[19] (Fig. 7). An adequate working temperature can be attained in most liquid nitrogen freezers; however, the design of some models requires that the amount of liquid nitrogen necessary to attain the proper working temperature will reduce the amount of usable storage space. If vials are to be immersed in the liquid phase of liquid nitrogen, they must be correctly sealed in Nunc™ CryoFlex™ tubing to prevent penetration by liquid nitrogen. Improper use may cause entrapment of liquid nitrogen inside the vial and lead to pressure build up, resulting in possible explosion or biohazard release. Liquid phase LN penetration can also be a source of contamination for submerged samples not properly protected by Nunc Cryoflex tubing. In most cases, vapor phase storage at –130˚C is adequate and avoids the hazards of liquid phase storage. Mechanical freezers that cool to -150˚C are also available.



Nalgene Mr. Frosty

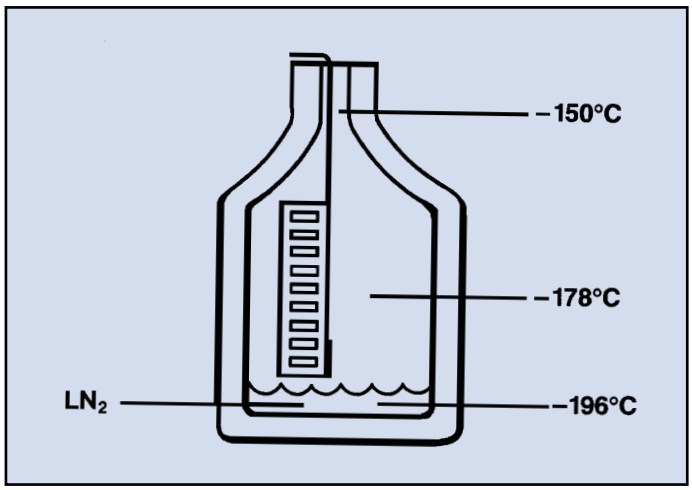


Fig. 7 Recommended temperatures in liquid-nitrogen storage system.

Improper handling of material maintained at cryogenic temperatures can have a detrimental effect on the viability of frozen cells. Each time a frozen vial is exposed to a warmer environment, even briefly, it experiences a dramatic change in temperature. Storage systems should be designed to avoid exposure of stored material to warmer temperatures, as well as minimizing prolonged exposure of personnel during specimen retrieval. Box stacking systems (i.e. stainless steel racks) necessitate exposure of boxes at the top to warmer temperatures when retrieving boxes at lower temperatures. When box stacking systems are used, maintain a small number of vials of each preparation in the top box of the rack and store the remaining vials of each preparation in lower boxes. By doing this, a vial of one preparation can be retrieved without exposing all vials of any particular culture or lot.[20]

To maximize the available space in liquid nitrogen freezers and minimize exposure of material during retrieval, use small storage boxes or aluminum canes. Press the vials onto the canes, putting no more than one lot of one culture on each cane. Canes provide a flat surface for coding their position and easy identification during retrieval. Place the canes into cardboard or plastic sleeves to eliminate the potential for vials to fall from the canes. (Fig. 8) When retrieving vials from canes, the cane should be lifted only to a level that exposes the first available vial, without removing the remaining vials from the working temperature of the freezer.[20]

Reconstitution (Thawing)

For most cells, warming from the frozen state should occur as rapidly as possible until complete thawing is achieved. To achieve rapid warming, place the frozen vial into a 37°C water bath. Remember, material frozen in plastic vials will take longer to thaw than that in glass ampoules, and sometimes gentle agitation of the vial during warming will accelerate the thawing process. Care must be taken, however, not to vigorously agitate vials containing fragile cells such as protists and mammalian cells. As soon as the contents of the vial have been thawed, remove the vial from the water bath. To minimize the risk of contamination during reconstitution, disinfect the external surface of the vial by wiping with alcohol-soaked gauze prior to opening.

Immediately transfer the contents of the vial to fresh growth



Fig. 8 Clear plastic sleeve allows identification of vials in an aluminum cane.

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medium following thawing to minimize exposure to the cryoprotective agent. For most cultures, the entire contents of the vial may be placed into fresh media, however further dilution may be necessary for cell lines. It is recommended that the cell suspension be centrifuged at 100 x g for 10 minutes after initial dilution, the supernatant removed, and the cells resuspended into fresh growth medium to remove residual cryoprotective chemicals.

Some materials that are not sensitive to the cryopreservation process may tolerate thawing and re-freezing. Most replicable cells will not tolerate refreezing unless they are in a resistant form such as a spore. However, for non-replicable materials such as serum, nucleic acids, and proteins, thawing and re-freezing may be acceptable. Keep in mind that each time an aliquot is thawed and re-frozen subtle changes may occur in the character of the material that could impact future use. An alternative to thawing and re-freezing is to store material in smaller aliquots for single use.

Determination of Recovered Cells

Methods used to estimate the number of viable cells recovered following freezing depend on the type of material preserved. Visual inspection alone can be deceptive, and although staining and dye exclusion are effective in determining the presence of viable cells for most mammalian cells, they do not indicate an ability to establish the cell population. For microbial cells, serial dilution and plate counts are effective in quantifying the population of cells recovered. Although there may be some vial-to-vial variation within a given lot, with constant storage conditions the number of recovered cells will generally be the same in all vials. Vial-to-vial variation may be an indication of problems occurring during storage and handling.

For stem cells the recovery is determined in the same manner as for other mammalian cells by estimating the number of viable cells. In addition measuring the differentiation capacity and clone forming capability are also important in assuring complete stem cell recovery[10]. Embryo recovery can be determined via morphological examination, and verified by implantation and fetal development. Recovery of non-replicable materials is determined by acceptable results following intended use.

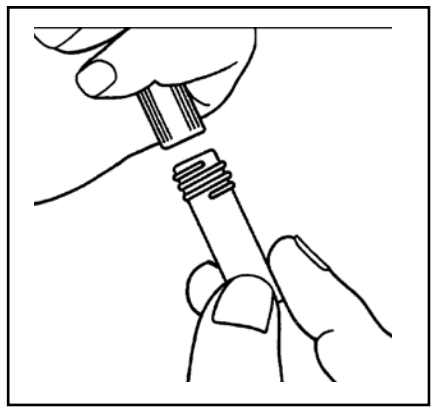
Inventory Control

Appropriate record keeping is important in any laboratory and there are a number of methods available for keeping records on cryopreserved materials.[20] When establishing your own method, keep in mind that there is key information which will be important for future use: (a) the preservation methodology used; (b) the location and identification of the stored material; (c) preservation date; and (d) number of passages for replicable material. The item number should be linked to associated data for that material, and for some purposes each container may require a unique identifying code linked to specific information for that particular aliquot.

Identification begins with proper labeling of the storage container. The label information should include a name or identification code for the frozen material, as well as a lot number. The information on the label should be kept with the inventory records that include the location code for each vial. These records can be maintained as paper documents, or preferably as electronic files. Duplicate inventory records should be maintained in a location separate from working records. Locator codes should be specific enough to allow rapid and easy retrieval for a specific lot and should include freezer unit number, a code for a freezer section or inventory rack, a box or canister number, and possibly even a grid spot within the box or a cane number when canes are used. Detailed locator codes minimize hunting for material which risks warming the freezer unit, exposure of other materials to warmer temperatures, and prolonged exposure of laboratory personnel to extremely cold temperatures.

Biological Materials Management

Despite its use in stabilizing living material, low-temperature preservation can stress cells. Care must be taken to ensure that preserved material remains unchanged following preservation. A good preservation program should include effective characterization and cataloging programs, both of which combined, provide optimum biological materials collection practices. There is little use in preserving material that is of little value, inadequately characterized, contaminated or misidentified. The first step in maintaining a collection of biological materials is to assess the material to be preserved to ensure that it is of use and is worth keeping. This practice should continue throughout the collection process to minimize the accumulation of preserved material to unmanageable levels. To avoid duplication of collection materials, a system of identification of each item should be established. This can be done by devising unique numbering systems or number/letter combinations. Each identification number should be cross-referenced to other information about the biological material. Preservation of living cells ensures stability but does not correct any problems already present in the material.[20] All material to be preserved should be examined thoroughly for contamination, proper identification, and other key characteristics unique to the cells, prior to preservation. Since freezing can stress cells and handling exposes cells to the risks of contamination, characterization must continue after successful preservation is accomplished. Each time a new lot of frozen material is prepared, complete characterization of the material should be carried out. Cataloging and data record keeping are important aspects of all biological material collection programs. Cataloging ensures that duplication of material does not occur and is especially useful when collection material is to be made available to others. Maintaining records on data generated during the characterization and preservation of collection materials ensures that any future problems can be adequately addressed. An important aspect of good biological materials management is constant assessment of the usefulness of the material, and removal of materials that are no longer needed.



Aseptic technique minimizes possibility of contamination.

Safety Considerations

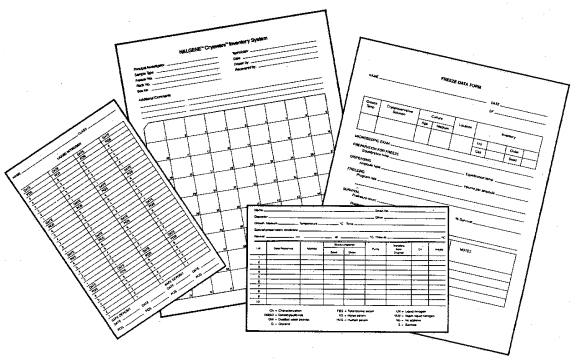
Safety precautions must be observed throughout the preservation and maintenance process. All work with hazardous cultures should be performed under proper containment, and U.S. Public Health Service Biosafety Level guidelines should be adhered to at all times.[21]

Human and other primate cells may contain adventitious viral agents that require special handling, and all primate cells that have not been thoroughly characterized should be handled at Biosafety Level II. At this level, laboratory staff must have training in handling pathogenic agents and work under the direction of a competent scientist. Access to the laboratory must be limited and biological safety cabinets must be used for large-volume work or when aerosols are generated.[21]

Low-temperature storage of cells presents unique hazards that necessitate safety precautions. Cryogenic temperatures can result in exposure of personnel to extremely cold conditions, and precautions must be taken to protect personnel during operations in liquid nitrogen freezers. Insulated gloves and long-sleeved laboratory coats or other garb protect the skin from exposure. It is extremely important to wear a full face and neck shield when working in the liquid portion of a liquid nitrogen freezer. As noted previously, improperly sealed glass ampoules may explode when retrieved from liquid nitrogen. To minimize the risk of potential explosions, leave vials retrieved from the liquid phase of the freezer in the vapor phase of the same freezer for a minimum of 24 hours. A face shield that provides neck protection should be mandatory when retrieving vials from liquid nitrogen. The use of Nunc CryoFlex is also strongly recommended. *See Warning on this page.*

Special precautions must be taken when working with hazardous biological materials at liquid nitrogen temperatures. Always thaw and open vials containing hazardous material inside a biological safety cabinet. Be prepared for exploding and leaking ampoules/vials. Broken ampoules in a liquid nitrogen freezer are a potential source of contamination and contaminants may survive, despite the extremely cold temperatures.[22]

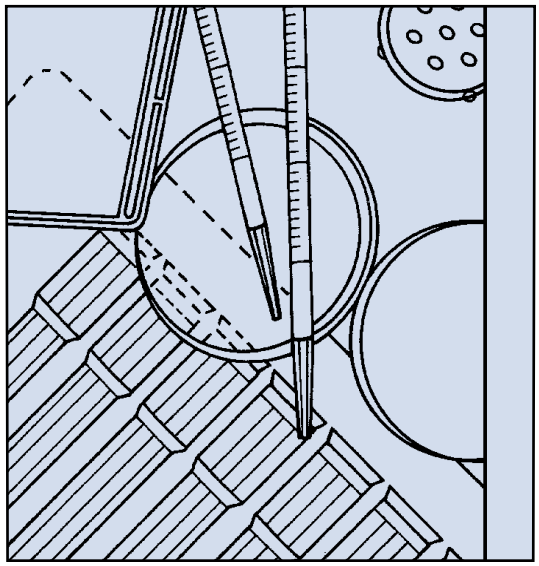
When a liquid nitrogen freezer becomes contaminated, the entire unit should be decontaminated after warming to room temperature. When closing down a liquid nitrogen freezer that is not obviously contaminated, remove all material to be retained, warm the unit to room temperature and disinfect it prior to further handling.



Accurate record keeping specifically for cryopreserved material. Full-size copies of these records are printed at the end of this manual and may be duplicated.

Step-by-Step for Cultured Cells
(Not Stem Cells)

1. Harvest cells from late log or early stationary growth. Scrape cells from the growth surface if they are anchorage dependent. Centrifuge broth or anchorage independent cultures to obtain a cell pellet, if desired.
2. Prepare presterilized DMSO or glycerol in the concentration desired in fresh growth medium. When mixing with a suspension of cells, prepare the cryoprotective agents in twice the desired final concentration.
3. Add the cryoprotectant solution to the cell pellet or mix the solution with the cell suspension. Begin timing the equilibration period.
4. Gently dispense the cell suspension into vials.
5. Begin cooling the cells after the appropriate equilibration time.
    a. Uncontrolled cooling—place the vials on the bottom of a -60°C freezer for 90 minutes.
    b. Semi-controlled cooling—use Mr. Frosty freezing container to freeze the vials in a -70°C freezer.
    c. Controlled cooling—use a programmable cooling unit to cool the cells at 1°C per minute to -40°C.
6. Remove the cells from the cooling unit and place them at the appropriate storage temperature.
7. To reconstitute, remove a vial from storage and place into a water bath at 37°C. When completely thawed, gently transfer the entire contents to fresh growth medium.



There are a number of tests you can perform to correctly identify cell lines. Correct identification is important to prevent cross-contamination.

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WARNING:

Do not use vials for storage in the liquid phase of liquid nitrogen unless correctly sealed in Nunc CryoFlex Tubing (Cat. No. 343958). Improper use may cause entrapment of liquefied nitrogen inside the vial and lead to pressure build-up, resulting in possible explosion or biohazard release. Use appropriate safety procedures as outlined in this manual when handling and disposing of vials.

For more information on Cryopreservation and Cryoware Products, visit us at www.thermoscientific.com/coldstorage

Cryoware Inventory System

Principle Investigator _____ Technician _____

Sample Type _____ Date _____

Freezer No. _____ Frozen by _____

Rack No. _____ Recovered by _____

Box No. _____

Additional Comments

_____ _____

_____ _____

_____ _____

1	2	3	4	5	6	7	8	9	10
11	12	13	15	15	16	17	18	19	20
21	22	23	24	25	26	27	28	29	30
31	32	33	34	35	36	37	38	39	40
41	42	43	44	45	46	47	48	49	50
51	52	53	54	55	56	57	58	59	60
61	62	63	64	65	66	67	68	69	70
71	72	73	74	75	76	77	78	79	80
81	82	83	84	85	86	87	88	89	90
91	92	93	94	95	96	97	98	99	100

Freeze Data Form

Name _____ Date _____

Growth Temp.	Cryopreserative Solution	Culture		Location	Inventory			
		Age	Medium					
					Storage Temp.		Seed	
					Gas		Working Lot	

Microscopic Exam _____

Preparation for Freeze

Equilibration Time _____ Equilibration Temp. _____

Dispensing

Vial Type _____ Volume per Vial _____

Freezing

Program Rate _____

Survival

Prefreeze count _____cells/ml Total vol. frozen _____ ml % Viable _____

Postfreeze count _____cells/ml Total vol. resuspended _____ ml

Date	Survival			Notes
	% Rec	Purity (free from contaminants)	No. pass	

Liquid Nitrogen Storage on Canes

Name _____

Date:	Date:	Date:	Date:
POS:	POS:	POS:	POS:
6			
5			
4			
3			
2			
1			
Date:	Date:	Date:	Date:
POS:	POS:	POS:	POS:
6			
5			
4			
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Date:	Date:	Date:	Date:
POS:	POS:	POS:	POS:
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Date:	Date:	Date:	Date:
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1			

Safe Deposit: Safe Deposit:

Date:_____ Date:_____ Date:_____ Date:_____

POS:_____ POS:_____ POS:_____ POS:_____

Liquid Nitrogen Storage on Canes

Name _____

Date:	Date:	Date:	Date:
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Safe Deposit: Safe Deposit:

Date:_____ Date:_____ Date:_____ Date:_____

POS:_____ POS:_____ POS:_____ POS:_____

Name _____ Strain _____

Growth Medium _____ Temperature _____ °C Time _____

Special preservation conditions _____

Thaw at _____ °C

Lot	Date Preserved	Method	Stocks prepared		Purity (free from contaminants	Transfers from original	Ch	Initials
			Seed	Order				
1								
2								
3								
4								
5								
6								
7								
8								
9								
10								

DMSO = Dimethylsulfoxide
DW = Distilled water (sterile)
G = Glycerol
No = No additive
FBS = Fetal bovine serum
HS = Horse serum
HuS = Human serum
LN_2 = Liquid nitrogen
VLN_2 = Vapor, liquid nitrogen
Ch = Characterization

Name _____ Strain _____

Growth Medium _____ Temperature _____ °C Time _____

Special preservation conditions _____

Thaw at _____ °C

Lot	Date Preserved	Method	Stocks prepared		Purity (free from contaminants	Transfers from original	Ch	Initials
			Seed	Order				
1								
2								
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6								
7								
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Ch = Characterization



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