

Ampoules and Vials

A variety of small containers, such as heat-sealable ampoules and screw-cap vials, can be used for storing cells at ultra-low temperatures. For ease of manipulation, 1.2- to 2.0-ml ampoules or vials are most frequently used. An aliquot of 0.5–1.0 ml of the cell suspension is usually dispensed into each storage container. Container selection depends on several factors including its cryotolerance, the storage temperature, type of cell to be stored, and safety considerations.

For storage below -100°C , choose ampoules and vials specifically designed to withstand cryogenic temperatures. A much broader variety of containers can be used for storage above -100°C where they are not subject to the stress of cryogenic temperatures. Keep in mind that thawing takes about one minute for a glass ampoule, 90 seconds for a plastic vial. The container's thawing time may be important for successful recovery of some cell lines.

Storage by immersion in liquid nitrogen is not advised. Storage in the vapor above the liquid nitrogen is preferred. It is possible over time for liquid nitrogen to enter a sealed glass ampoule through microfractures or channels resulting from improper sealing (1). When the ampoule is then transferred quickly from liquid nitrogen to ambient temperature, rapid conversion to the gaseous phase will result in explosion of the ampoule. Therefore, *it is not advisable to store hazardous material in sealed glass ampoules.*

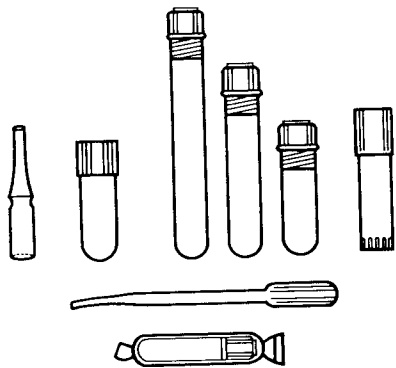


Fig. 4 Heat-sealable ampoules, screw-cap vials, and vial sleeves for use in cryogenic storage of cells

Glass ampoules can be torch-sealed, but exposure to the heat of sealing may damage sensitive cells. Use caution when torch-sealing. Even with specialized equipment, much practice is required to perfect the technique. After sealing, glass ampoules must be checked for improper seals. Place the ampoules in an aqueous bath with a dye, such as methylene blue, during the equilibration period. The dye will enter improperly sealed ampoules and be easily visible to the naked eye.

Plastic vials, even those with a screw cap, are not always leakproof in liquid nitrogen (5). Plastic vials do minimize the danger of explosion during retrieval. However, during warming, the liquid can spray from the cap/vial interface and expose personnel to the contents of the vial.

Rate of Cooling

Once the cells and cryoprotectant have been combined and dispensed, the next step is to cool the suspension. The rate of cooling controls the size of ice crystals and how fast they are formed, which may affect cell recovery. Different types of cells require different cooling rates. In most cases, a fast cooling rate is ineffective or may result in low cell recovery. A uniform cooling rate of -1°C per minute from ambient temperature is effective for a wide variety of cells. Generally, the larger the cells, the more critical slow cooling becomes.

A convenient method of slow cooling involves placing a preparation on the bottom of a mechanical freezer set at a low temperature (e.g., -55°C). Although this procedure does not result in a uniform cooling rate, it can be used to preserve a range of cell types. The initial rate of cooling is several degrees centigrade per minute, slowing to less than 1°C per minute as the preparation approaches the temperature of the freezer. Once the preparation reaches the temperature of the freezer (approximately 1.5 hours for volumes of 0.5–1.0 ml in a freezer set for -55°C) it can be moved to storage in a liquid-nitrogen freezer.

To achieve uniform, controlled cooling rates, use a programmable-rate freezing unit. Simple programmable units allow the selection of a single cooling rate for the entire cooling cycle. More complex units allow variable rates to be selected for different portions of the cooling cycle. With programmable-rate freezing units, it is possible to initiate rapid cooling to liquid-nitrogen temperatures after the slow cooling process is completed. The alternative is to remove the preparation from the freezing unit and quickly place it in a liquid-nitrogen freezer.

Whether controlled or uncontrolled cooling is used, most of the water present in the cells is transformed into ice crystals at -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 known as the latent heat of fusion. The temperature of the preparation can rise as much as 10°C during this phenomenon. If there is no compensation for this increase in heat, some types of cells will exhibit little or no recovery.

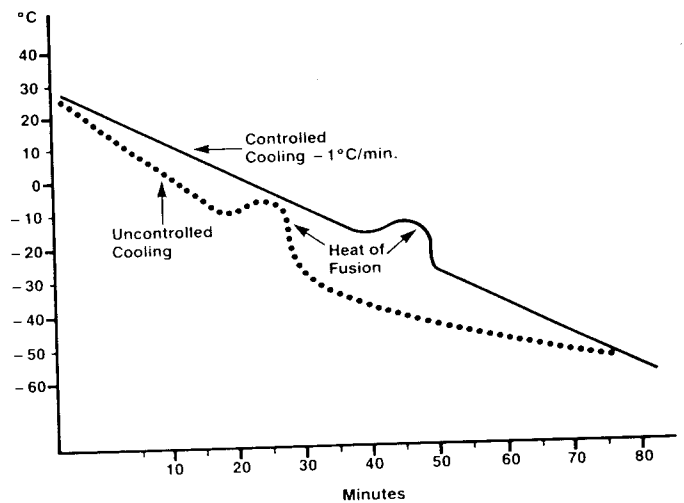


Fig. 5 Controlled and uncontrolled cooling rates. Note heat of fusion.

Storage

The temperature at which frozen preparations are stored affects the length of time after which cells can be recovered. The lower the storage temperature, the longer the viable storage period. Cells stored above -80°C may retain small amounts of unfrozen water. Over time the presence of liquid water, even at these temperatures, will permit slow chemical reactions that will eventually result in cell death.

By definition, cryogenic temperatures are those below -100°C . But final stability as a frozen preparation cannot be assured until a temperature of -130°C is reached. The viability of cells stored in freezers at temperatures above -130°C cannot be assured for long periods of time.

For ultimate security, living cells should be stored at liquid-nitrogen temperatures. However, there are risks in storing ampoules or vials directly in liquid nitrogen, as discussed above (e.g., leakage). Liquid-nitrogen units that provide all-vapor storage are ideal as long as the working temperature at the opening of the unit remains at -150°C or below. To assure that a liquid-nitrogen freezer maintains the proper working temperature, the volume of liquid nitrogen should be adjusted until the temperature just above the stored vials/ampoules is at -150°C when the lid of the

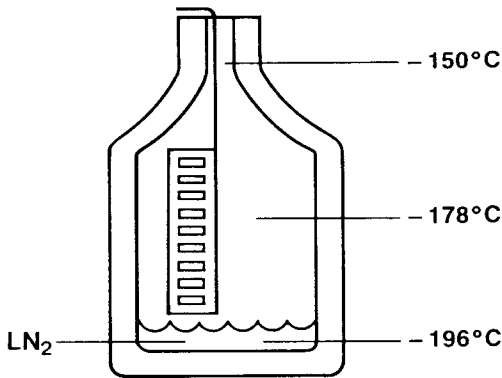


Fig. 6 Recommended temperatures in a liquid-nitrogen storage system

unit is open. A working temperature of $\approx -150^{\circ}\text{C}$ can be maintained in any type of liquid-nitrogen storage tank, but in certain models the amount of usable storage space will be limited by measures needed to maintain stable temperatures.

The level of the liquid nitrogen in the freezer must be continuously and carefully monitored. Any drop in the liquid level below a critical volume can result in a warming of the material stored at the top of the unit. The liquid level should be checked daily and liquid added to the unit as needed. Manually checking and recharging liquid-nitrogen units on a daily basis is the best assurance that the units are working properly. When the unit cannot be physically checked daily, such as over a weekend, the only assurance of proper performance is a liquid-level alarm system that is monitored 24 hours a day. Do not rely on automatic filling systems unless the unit is equipped with a continuously-monitored alarm system.

In addition to temperature of storage, handling during removal from storage will affect the viability of cells. Every time an ampoule/vial is exposed to a warmer environment, even briefly, it experiences a change in temperature. During slow warming, recrystallization of ice can occur (3) resulting in larger ice crystals with a potentially detrimental effect on viability. When removing an ampoule or vial from storage, avoid exposure of other containers to the warmer temperature. Repeated warming and cooling of stored preparations will reduce the viable storage time.

Liquid-nitrogen storage systems should be designed to minimize exposure of stored material to warmer temperatures and exposure of personnel to the extreme cold while retrieving a specific item. When using stacked boxes, maintain a small number of vials of each preparation in the top box of the stack, and store the remaining vials of each preparation in lower boxes. You can then retrieve one vial without exposing all vials of a particular culture or lot.



Fig. 7 Clear plastic sleeve allows identification of vials in an aluminum cane

To maximize the available space in the freezer and minimize exposure during retrieval, use aluminum canes or 5x5 boxes. Canes are designed to hold five or six standard 1.2- or 2.0-ml vials or ampoules; boxes hold 25 vials. Both provide a flat surface on top for coding their position in the freezer. Placing the canes into cardboard tubes for storage eliminates the possibility of vials falling from the canes during storage and retrieval. When properly coded, canes and boxes provide an inventory system that allows rapid retrieval of single vials without the risk of exposing other material.

Reconstitution (Thawing)

Although slow cooling is generally best to insure cell viability, the opposite is required when thawing from the frozen state. After removal from frozen storage, place the vial/ampoule in a 37°C water bath. Agitating the vial will accelerate the warming process but may be detrimental to fragile cells, such as hybridomas. Remove the vial from the water bath as soon as the contents have thawed. Allowing the preparation to reach the water bath temperature before proceeding is not advised.

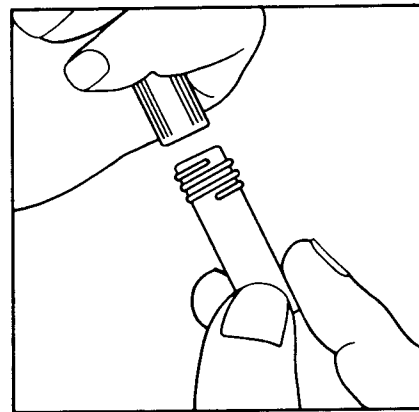


Fig. 8 Aseptic technique minimizes possibility of contamination

Thawing takes about one minute for a glass ampoule, 90 seconds for a plastic vial. Gently transfer the contents of the ampoule/vial to fresh growth medium immediately to dilute out the cryoprotectant. To minimize potential contamination when transferring culture material, dip the ampoule/vial in alcohol or wipe with alcohol-soaked gauze prior to opening.

Determination of Recovered Cells

There are several methods to accurately estimate the number of viable cells in a non-motile population. Visual inspection alone can be deceptive. You can determine the percentage of viable cells in the population using a dye exclusion test and counting the number of unstained (viable) cells relative to the total population. A dilution series determines the estimated number of viable cells capable of initiating a growing culture. There may be some vial-to-vial variation in a given lot, but with constant storage conditions, the number of recovered cells will generally be the same in all vials.

Inventory Control

Appropriate record-keeping is important in any laboratory. There are a number of possible methods for keeping records on cryopreserved materials. When establishing your own method, keep in mind that there are two major classes of information which will be important in the future: (a) the preservation methodology used, and (b) the location and identification of the stored material.

Identification starts with proper labelling of the vial itself. The label information should include an identification code for the frozen material, as well as a lot number. This label information should be kept with the records that include the location code for the vial. These records can be maintained on inventory cards (one for each item) or in computer files. Locator codes should be specific enough to

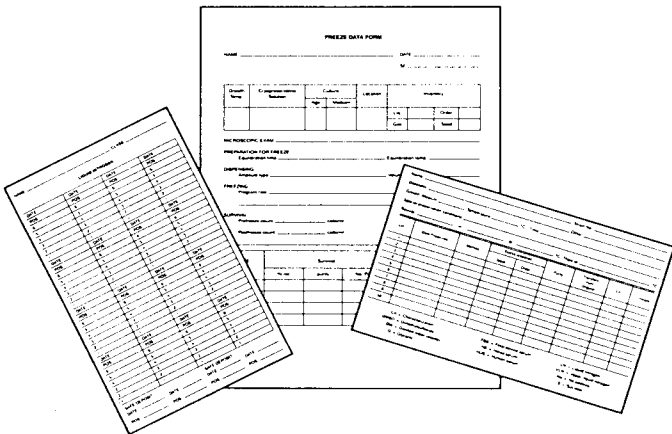


Fig. 9 Accurate record-keeping specifically for cryopreserved material. Full-size copies of these records are printed at the end of this manual and may be duplicated.

allow rapid and easy retrieval of a specific lot, and should include freezer unit number, a code for a freezer section or stainless steel rack, a box number and possibly even a grid spot within the box or a cane number, when appropriate. Detailed locator codes minimize hunting for material which risks warming the freezer unit, exposure of other materials and prolonged exposure of laboratory personnel to the cold.

Safety Considerations

Safety precautions should be observed when removing an ampoule from low temperature storage, especially when stored in liquid nitrogen. Wear a face shield, insulated gloves and laboratory coat. The face shield and coat serve to

protect the face, neck and arms from contact with liquid nitrogen and fragments of exploding ampoules/vials. Protect your hands and arms against exposure to ultra-low temperatures by wearing protective gloves at all times when working in a liquid-nitrogen freezer. Even when wearing gloves, lab personnel must use extreme care since the glove material will act as a wick when in contact with liquid nitrogen.

Exercise care when working with hazardous materials. Always thaw hazardous contents and open the vials in 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. Despite the harsh environment in liquid nitrogen, contaminants may survive and there is a risk of worker exposure. Extremely hazardous cultures should be stored separately from other materials. When a liquid-nitrogen freezer becomes contaminated, the entire freezer and its contents should be decontaminated as a unit after warming to room temperature. When closing down a liquid-nitrogen freezer for other reasons, remove all material to be retained, warm the unit to room temperature and decontaminate it prior to further handling.

Summary Outline

1. Harvest cells from late-log or early-stationary growth and concentrate to twice the desired final concentration in fresh growth medium.
2. Prepare presterilized DMSO or glycerol in twice the concentration desired (e.g., 20% starting for 10% final) in fresh growth medium.
3. Mix the cell suspension and the cryoprotective agent solution in equal quantities. Begin timing the equilibration period.
4. Gently dispense the cells into ampoules/vials.
5. After the minimum equilibration time has elapsed, begin cooling the cells:
 - a. Place the ampoules/vials on the bottom of a -55°C to -60°C mechanical freezer for 90 minutes, or
 - b. Cool the cells at approximately $1^{\circ}\text{C}/\text{min.}$ to -40°C in a programmable cooling unit.
6. Remove the cells from the freezer or cooling unit and place them into storage.
7. To reconstitute, remove an ampoule/vial from storage and place into a water bath at 37°C . When completely thawed, gently transfer the entire contents to fresh growth medium.

Selected References

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