



CRYOGENIC STORAGE OF ANIMAL CELLS

Cryogenic preservation (storage below -130°C) of cell cultures is widely used to maintain reserves of cell cultures. Besides providing a valuable back-up supply, properly stored cultures also reduce alterations in or loss of culture characteristics. This “culture drift” results from the phenotypic and genotypic changes that accumulate over time in most cell lines as they age and evolve in culture. The success of the freezing process depends on four critical areas:

1. Proper handling and gentle harvesting of the cultures
2. Use of appropriate cryoprotective agents
3. A controlled rate of freezing
4. Storage at cryogenic temperatures

The procedure described below is for a typical mammalian monolayer cell culture grown in medium containing fetal bovine serum. However, this procedure can be easily adapted to a wide variety of cell cultures; see references for additional information.

SUPPLIES

Nonsterile

- Marking pen suitable for labeling plastic cryogenic vials
- Controlled-rate freezing unit
- Hemocytometer

Sterile

- Complete cell culture medium containing serum or any other supplements necessary for the culture being frozen
- Pipettes
- 15-mL screw-capped centrifuge tubes
- Calcium- and magnesium-free PBS (CMF-PBS) for rinsing the cultures
- Trypsin or other appropriate dissociating solution
- 2-mL screw-capped plastic vials specially designed for cryogenic use
- Cryoprotective medium: complete culture medium containing 5% (v/v) dimethyl sulfoxide (DMSO) or 10% (v/v) glycerol
- Trypan blue solution or other vital staining solution for determining the viability of the cells during counting

PROCEDURE

1. Culture selection and examination:

Prior to freezing, the culture should be maintained in an actively growing state (log phase or exponential growth) to ensure optimum health and good recovery. Ideally, the culture medium should be changed 24 hours prior to harvesting. It is also recommended that the culture be tested for the presence of microbial contaminants, especially mycoplasma, and have its identity (species and cell type) confirmed by appropriate methods.

Using an inverted-phase contrast microscope, check the general appearance of your culture. Look for signs of microbial contamination. It is also important to examine the culture with the unaided eye to look for small, isolated fungal colonies that may be floating at the culture medium-air interface and thus are not easily detected with the microscope. If antibiotics have been used for growing your cultures, then maintaining the cultures antibiotic-free for at least one to two weeks prior to freezing will make it easier to uncover any cryptic culture contaminants.

2. **Cell harvesting:** Your standard protocol routinely used for subculturing your cell cultures can generally be used. Be as gentle as possible during harvesting since it is very difficult for cells damaged during harvesting to survive the additional damage that occurs during the freezing and thawing processes. The amounts of reagents recommended in this procedure are for a 75-cm² (T-75) flask; volumes should be adjusted accordingly for other culture vessel sizes.

- 2a. Using a sterile pipette, remove and discard the culture medium. Any materials and solutions coming into contact with cells should always be disposed of properly.
- 2b. Rinse the cell monolayer with 5 to 10 mL of CMF-PBS to remove all traces of fetal bovine serum.
- 2c. Add 3 to 5 mL of trypsin (in CMF-PBS) or other appropriate dissociating agent to the flask and incubate at 37°C. Prewarming the enzyme solution will usually shorten the exposure period.
- 2d. Check the progress of the enzyme treatment every few minutes on an inverted phase-contrast microscope. Once



the cells have rounded up, gentle tapping of the flask should detach them from the plastic surface. Add 5 mL of serum-containing growth medium to inactivate the trypsin solution. Vigorous pipetting may be required to wash off any remaining cells from the bottom of the culture vessel or to break up cell clumps into a single cell suspension. The removal or inactivation of the dissociating agent is critical for optimum recovery of frozen cells. If the dissociating agent cannot be directly inactivated, then it will be removed when the cells are centrifuged in the next step (see below).

2e. Collect the suspended cells in a 15-mL centrifuge tube. Remove and set aside a small sample for cell counting and then spin the remaining cell suspension at approximately 100 x *g* for 5 minutes to obtain a soft cell pellet. Count the cells using a hemocytometer while the tube is spinning. Use the trypan blue solution to check their viability.

3. **Cryoprotective agents:** Cryoprotective agents are necessary to minimize the damage that occurs to cells during the freezing process. A wide variety of chemicals have been used to provide cryoprotection, including polyvinyl-pyrrolidone, ethylene glycol, methanol, and methyl acetamide. However, the most common cryoprotectants are dimethylsulfoxide (DMSO) and glycerol. DMSO (ATCC® cat. no. 4-X, 5 x 5 mL) is most often used at a concentration of 5 to 10% (v/v) in freezing media; the optimum concentration varies with the cell line.

WARNING: *Although not directly toxic, DMSO is a very powerful solvent and is able to rapidly penetrate intact skin. As a result there is potential hazard associated with using this compound. It is very important to avoid contact with DMSO and safely dispose of any wastes containing DMSO. In addition, because it is a powerful solvent, some batches of commercially available DMSO may contain toxic substances and should not be used for freezing cells. ATCC sells DMSO that has been thoroughly tested to assure it is nontoxic (ATCC cat. no. 4-X, 5 x 5 mL).*

Glycerol is most often used in freezing media at a final concentration of 5 to 15%; again, the optimum concentration depends on the cell line. Increasing the serum concentration in the cryoprotective medium is often used to increase the survival rate of cells that are difficult to preserve. Serum concentrations as high as 90% to 95% (no medium, just serum plus the cryoprotective agent) are sometimes used, especially with sensitive hybridoma cell lines. For cells normally grown in serum-free medium, adding 50% conditioned medium (serum-free medium in which the cells were grown for 24 hours) to both the cell freezing and the recovery media may improve their post-freezing recovery and survival. The addition of 10% to 20% cell culture-grade albumin to serum-free freezing medium may also increase post-freezing survival.

3a. Remove and discard the supernatant from the centrifuged cells and resuspend the cell pellet in enough of the cell freezing medium to give a final cell concentration of 2 to 5 million viable cells per mL.

3b. Label the appropriate number of plastic cryogenic vials with at least the name of the cell line and the date. Then add 1.0 to 1.8 mL of the cell suspension to each of the vials and seal.

4. **Cell freezing:** A slow and reproducible cooling rate is very

important to ensure good recovery of cultures. A decrease of –1 to –3°C per minute will usually work for most animal cell cultures. The best way to control the cooling process is to use a programmable electronic freezer unit. However, there are several commercially available mechanical freezer units that give a satisfactory and reproducible cooling rate when placed overnight in a –70°C to –90°C freezer (Mr. Frosty from Nalg Nunc International, catalog no. 5100-0001; or StrataCooler from Stratagene®, catalog no. 400005, for example).

4a. Freeze the labeled vials in an appropriate controlled rate freezer unit according to the manufacturer's directions. If no commercial freezing unit is available, a homemade substitute can be constructed by placing the vials in a small box of polystyrene foam or insulated cardboard, which is then placed in a –70°C to –90°C freezer overnight. While this approach works with many cell lines, it does not give controlled, uniform, or reproducible cooling and is not recommended for valuable or irreplaceable cultures.

4b. No matter which cooling method is used, it is important that the transfer to the final storage location be done quickly and efficiently. If the transfer can not be done immediately, the vials can be placed on dry ice for a short time. This will avoid damage to cultures by inadvertent temporary warming during the transfer process. Warming during this transfer process is a major cause of variation in culture viability upon thawing.

5. **Cryogenic storage:** Always keep the storage temperature below –130°C for optimum survival. Cells may survive storage at higher temperatures but viability will usually decrease over time. The ideal storage container is a liquid nitrogen freezer where the cultures are either stored submerged in the liquid nitrogen or suspended in the vapor phase above the liquid nitrogen. For safety reasons (#6 below) storage in vapor phase is preferred. There are several important points to remember for maintaining a liquid nitrogen-based cell repository:

5a. Make sure that the labeling system is suitable for (permanent) cryogenic storage.

5b. Keep good records of the locations of the frozen cultures and a full history of their characteristics and growth and handling requirements. Remember that the frozen cultures may be around longer than the person who froze them.

5c. Be prepared for emergencies! Liquid nitrogen freezers can fail. There should be a person responsible for frequent checks (preferably daily or at least weekly) on the status of a liquid nitrogen freezer. There are a variety of commercially available alarm systems that can be used to continually monitor their status. Cultures that are very valuable or irreplaceable should be stored in at least two separate locations. ATCC provides a safe deposit service for this purpose; contact ATCC Professional Services at (703) 365-2700 for additional information on this service.

6. **Culture recovery:** Special care should be taken when thawing cultures that have been stored submerged in liquid nitrogen instead of in the vapor phase above liquid nitrogen. If a vial leaks during submerged storage it will slowly fill with liquid nitrogen; upon thawing, the conversion of the liquid nitrogen back to its gas phase may result in the vessel exploding or blowing off its cap with dangerous force creating flying debris.

SAFETY PRECAUTION: Always wear protective clothing, gloves and a face mask or safety goggles when the vial is removed from the liquid nitrogen storage vessel and thawed.

- 6a. Thaw the vial by gently agitating it in a water bath at 37°C. To reduce the possibility of contamination, the O-ring and cap should be kept out of the water. Thawing should be rapid (approximately 2 minutes). Remove the vial from the water bath as soon as the contents are thawed, and then decontaminate it by dipping in or spraying with 70% ethanol. All of the operations from this point on should be carried out under strict aseptic conditions.
- 6b. Transfer the vial contents to a 25-cm² tissue culture flask or 100 mm tissue culture dish and dilute it with complete culture medium (approximately 4 to 5 mL for the flask, 10 mL for the dish). For most cell lines it is not necessary to immediately remove the cryoprotective agent. Normally, the culture medium is changed to remove the cryoprotective agent 8 to 24 hours after thawing. However, for those cell lines that may be sensitive to the presence of cryoprotective agents, the cell suspension may be centrifuged to remove the cryoprotective agent. Then incubate the culture at 37°C in a suitable incubator. It is important to avoid excessive alkalinity of the medium during recovery of the cells. It is suggested that, prior to the addition of the vial contents, the culture vessel containing the growth medium be placed into the incubator for at least 15 minutes to allow the medium to reach its normal pH. Some cell lines may undergo osmotic shock if a large volume of fresh medium is added too quickly to the cell suspension upon thawing. Several smaller volume stepwise additions (1 to 2 mL each time) of fresh medium to the cell suspension at 20°C to 37°C over a period of 10 to 20 minutes may improve their recovery and survival.

REFERENCES

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