Cryogenic Preservation and Storage of Animal Cells

Protocol

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John A. Ryan, Ph.D.
Corning Incorporated,
Life Sciences
836 North St.
Building 300, Suite 3401
Tewksbury, MA 01876

Introduction

Cryogenic preservation (storage below -100°C) of cell cultures is widely used to maintain backups or reserves of cells without the associated effort and expense of feeding and caring for them. The success of the freezing process depends on four critical areas:

1. Proper handling and gentle harvesting of the cultures
2. Correct use of the cryoprotective agent
3. A controlled rate of freezing
4. Storage under proper cryogenic conditions

The last three points are discussed in more detail below.

A wide variety of chemicals provide adequate cryoprotection. However, dimethylsulfoxide (DMSO) and glycerol are the most convenient and widely used. DMSO is most often used at a final concentration of 5 to15% (v/v). 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. Glycerol is generally used at a final concentration of between 5 and 20% (v/v). Although less toxic to cells than DMSO, glycerol can cause 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 medium-cryoprotectant mixtures with 95% serum and 5% DMSO may be superior for some overly sensitive cell lines, especially hybridomas.

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 by 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. 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 systems 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 approach uses an alcohol-filled container designed to slowly freeze vials placed in the system. The filled container is placed in an ultracold mechanical freezer where the alcohol acts as a bath to achieve more uniform heat transfer and cooling. After freezing overnight, the vials are removed from the canister and transferred to their final storage locations.

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. 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.

Be Prepared for Emergencies

Learning that your liquid nitrogen freezer has failed without warning (destroying the cultures contained within) is a terrible but all too common event. Frequently check nitrogen levels in freezers; a schedule should be established and strictly adhered to. 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. Valuable or irreplaceable cultures should be stored in at least two separate facilities. ATCC provides a safe deposit service for this purpose; they can be contacted at 703.365.2700 for additional information on this service.

Using vapor phase storage greatly reduces the possibility of leaky vials or ampules exploding during removal.

The following procedure can be easily adapted to a wide variety of cell lines. For additional information and references refer to the *General Guide for Cryogenically Storing Animal Cell Cultures* on the Corning Life Sciences Technical Information web site at **www.corning.com/lifesciences**.

Supplies

Nonsterile

- Pipetting aids
- Disposal tray or bucket for discarding used pipets
- Bottle of 70% alcohol for wiping down work area
- Paper towels
- Marking pen
- Controlled rate freezer. There are a variety of mechanical freezing units that provide adequate control of the cooling rate and are relatively inexpensive: Stratagene StrataCooler (Cat. No. 400005 or 400006).
- Inverted phase contrast microscope
- Ice bucket
- Liquid waste container
- Hemacytometer or electronic cell counter
- Laminar flow hood

Sterile

- Cell culture medium appropriate for the cell line being frozen
- Healthy, near confluent cell culture in T-75 flask
- 1, 5, 10 and 25 mL pipets (Corning Cat. No. 4485, 4487, 4488 and 4489)
- 15 mL screw cap centrifuge tubes (Corning Cat. No 430055 or 430788)
- Phosphate Buffered Saline: Calcium- and Magnesium-free (CMF-PBS). Unlike Hanks' and Earle's buffered saline solutions, CMF-PBS is designed to maintain a physiological pH in an open system. The calcium and magnesium is removed because these cations play a role in cell-to-cell attachment.
- Trypsin solution or other dissociating agent in CMF-PBS. Trypsin concentration should be optimized so as to remove the cells as quickly as possible but with a minimum of stress or damage.
- 2 mL Cryogenic vials (Corning Cat. No. 430661, 2027 or 430489)
- Cryoprotective medium – complete culture medium containing 10% dimethylsulfoxide (DMSO). Always use reagent or other high purity grades that have been tested for cell culture suitability (American Type Culture Collection, 1.800.638.6597, ATCC Cat. No. 4-X). Sterilize DMSO by filtration through a 0.2 µm nylon membrane (Corning Cat. No. 431224) in a polypropylene or stainless steel housing and store in small quantities (5 mL).
- 0.04% Trypan blue solution for viability staining

Corning offers a variety of cryogenic vial sizes and cap styles.

Procedure

Examination

Prior to freezing, the cells should be maintained in an actively growing state to insure maximum health and a good recovery. Ideally, the culture medium should be changed the previous day. Using an inverted microscope, quickly check the general appearance of the culture. Look for signs of microbial contamination. It is also important to examine the culture with the unaided eye to look for small fungal colonies that may be floating at the medium-air interface and thus not visible through the microscope. It is best if the cultures are maintained antibiotic-free for at least one week prior to freezing to help uncover any cryptic (hidden) culture contaminants.

Cell Harvesting and Freezing

Treat the cells gently 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. You should be able to obtain up to 1.5 x 10^7 cells from a near confluent T-75 flask (depending on cell type and degree of confluency). This should be enough cells to set up at least several vials at 2 x 10^6 cells/vial.

1. Using a sterile pipette, remove and discard the old culture medium.
2. For a T-75 flask, rinse the cell monolayer with 5mL of calcium- and magnesium-free phosphate buffered saline (CMF-PBS) to remove all traces of fetal bovine serum.
3. Add 4 to 5 mL of the trypsin solution (in CMF-PBS) to the flask and allow cells to incubate for at least one minute. (Prewarming of the enzyme solution will decrease the exposure period.) Withdraw about 3 mL of the trypsin solution and allow the cells to round up and loosen.
4. Check the progress of the enzyme treatment every few minutes on an inverted phase contrast microscope. Once all of the cells have rounded up, gently tap the flask to detach them from the plastic surface. Then add 5 mL of growth medium to the cell suspension and, using the same pipette, vigorously wash any remaining cells from the bottom of the culture vessel.
5. Collect the suspended cells in a 15 mL centrifuge tube and place on ice. Take a sample for counting and then spin at 100 xg for 5 minutes to obtain a cell pellet. While the cells are spinning, do a viable cell count (with the trypan blue solution) and calculate the number of cells/mL and the total cell number.
6. Remove the supernatant from the centrifuged cells and resuspend the cell pellet in enough of the cryoprotective medium containing 10% DMSO (DMSO is most often used at a final concentration of 5 to 15%.) to give a final cell concentration of 1 to 2 x 10^6 cells/mL. Although not directly toxic, DMSO is a very powerful solvent and is able to rapidly penetrate intact skin (leaving a fishy or garlicky taste in your mouth). As a result, there is a POTENTIAL HAZARD associated with using this compound. It is very important to avoid contact with DMSO and dispose of any wastes containing DMSO properly.
7. Label the appropriate number of cryogenic vials with the cell line, and the date. Then, add 1.5 to 1.8 mL of the DMSO containing cell suspension to each of the vials and seal.
8. Place the vials in the controlled rate freezer overnight. After 24 hours, the cells should be transferred to a liquid nitrogen freezer for permanent storage.
9. Record the appropriate information about the cells in your cell repository records. Fully detail in these 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 its origin, history, growth parameters, special characteristics and applications, is also helpful and should be included whenever possible.

Caution

Take special care to avoid contact with solutions containing DMSO. It is a very powerful polar solvent capable of rapidly penetrating intact skin and carrying in with it harmful contaminants such as carcinogens or toxins.

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.

Caution

Corning strongly recommends that cryogenic vials always be stored in the vapor phase above the liquid nitrogen to reduce the possibility of the vials filling with liquid nitrogen during extended storage. Vials filled with liquid nitrogen may explode violently upon removal from the freezer.

Cell Thawing and Recovery

1. Using appropriate safety equipment, remove the vial 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.
2. 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:

a. 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 mL of culture medium and incubate normally. As soon as a majority of the cells have attached (usually 3 to 4 hours), remove the medium containing the now diluted cryoprotective agent and replace with fresh medium.

b. 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 xg. Discard the supernatant containing the cryoprotective agent and resuspend the cell pellet in fresh medium. Then transfer the cell suspension to a suitable culture vessel and incubate normally.

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References

1. Freshney, R.I., 1994. Culture of Animal Cells: A Manual of Basic Technique, p. 254-263. (3rd ed.). Wiley-Liss, New York.
2. Hay, R.J., 1978. Preservation of Cell Culture Stocks in Liquid Nitrogen, p. 787-790. TCA Manual 4.
3. Schroy, C.B. and P. Todd, 1976. A Simple Method for Freezing and Thawing Cultured Cells, p. 309-310. TCA Manual 2, Procedure Number 76035.
4. Shannon, J.E. and M.L. Macy, 1973. Freezing, Storage, and Recovery of Cell Stocks, p. 712-718. *In:* Tissue Culture: Methods and Applications. P.F. Kruse and M.K. Patterson Jr. (eds.). (Academic Press, New York).
5. Waymouth, C. and D.S. Varnum, 1976. Simple Freezing Procedure for Storage in Serum-free Media of Cultured and Tumor Cells of Mouse, p. 311-313. TCA Manual 2, Procedure Number 76165.

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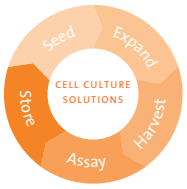

Corning Incorporated
Life Sciences
836 North St.
Building 300, Suite 3401
Tewksbury, MA 01876
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