PRESERVATION OF CELL-CULTURE STOCKS IN LIQUID NITROGEN

Submitted by

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I. INTRODUCTION

The routine method used at the American Type Culture Collection (ATCC) for the freezing and recovery of cultured cell lines is outlined below. In addition, reference is made to modifications in the procedure or equipment for cell preservation when using comparatively lower numbers of ampuls. These modifications may be of use in laboratories engaged in less extensive cell-culture banking. Rationale and other details have been presented elsewhere (1-3).

Special CAUTION should be exercised in removing sealed ampuls or vials from liquid nitrogen. A protective full face mask and gloves must be worn during retrieval and thawing.

Key words: cell culture; preservation; freezing; storage.

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II. MATERIALS

Ampuls: Unscored, No. 651463; and scored, Gold Band, No. 651483 Wheaton[1]
Ampul covers, No. TC200 Lurex[2]
Ampul canes, No. A545 NASCO[3]
Labeling machine, No. 135A Markem[4]
Specialty ink, No. 575625[4]
Ceramic ink, No. 36085-005 VWR[5]
Anodized aluminum ampul rack, No. 064 of desired size, Dixie[6]
Glycerol, Baker reagent, No. 1-2136[5]
Dimethylsulfoxide, Baker reagent, No. 1-9224[5]
Selas filter, 03 porosity, No. FPS 168-03[7]
Celstir units, 50- to 500-ml, No. 356676-82[2]
Cornwall automatic syringe, No. BD3052[5]
Syringe, disposable, 5-ml, No. BD5603[5]
Cannulas, No. PD1789[5]
Semiautomatic sealing device: No. K-6 161[8]; No. AS200 VirTis[9]
Dual arm torch, No. 18360-006[5]
Programmed freezing unit with controller, BF4-2 BF-6A Landsel[10]
Biological freezer, Linde No. BF-5[10]
Liquid-nitrogen refrigerators: No. MVE-LZ100 MVE[11]; and No. LR-30[10]
Liquid-nitrogen refrigerator control unit, Cryo-Med. Model 400ABCD[12]
Liquid nitrogen storage reservoirs: Nos. LR-31, VST-150, and LS-160B[10]
Alarm unit for liquid nitrogen refrigerator (LR30), No. CY30[10]
Full face mask, No. 33007-107[5]
Insulated gloves, No. 32918[5]
Ethanol, 70%
Methylene blue, 0.05%

[1] Wheaton Scientific, Millville NJ.
[2] Lurex Manufacturing Co., Vineland NJ.
[3] Nasco, Fort Atkinson WI.
[4] Markem Co., Keene NH.
[5] VWR Scientific, Inc., Baltimore MD.
[6] Dixie Sheet Metal, Falls Church VA.
[7] Selas Flotronics, Spring House PA.
[8] Kahlenberg-Globe Equipment Co., Sarasota FL.
[9] VirTis Co., Inc., Gardiner NY.
[10] Landsel Cryogenics, Hyattsville MD.
[11] Minnesota Valley Engineering, New Prague MN.
[12] CRYO Associates, Washington DC.

III. PROCEDURE

A. Ampul preparation

1. Mark ampuls using the Markem labeling machine or by hand with a straight marking pen and ceramic ink.
2. Place the ampuls on a suitable rack and heat in a hot-air oven at 120° C for 60 min or more to anneal the ink.
3. Place a cap on each ampul, wrap the rack, ampuls and covers, and sterilize by dry heat (270° C for 2 hr).

B. Preparation of the freeze medium

1. Sterilize reagent grade dimethylsulfoxide (DMSO) by filtration using a Selas filter (03 porosity). Store the sterile cryoprotective agent in 10- to 15-ml aliquots in the refrigerator.

2. Sterilize reagent grade glycerol by autoclaving and store an appropriate volume for each freeze protected from light in the refrigerator.
3. Just before use prepare the freeze medium by simple admixture of fresh growth medium and the required cryoprotective agent (dimethylsulfoxide and/or glycerol). The concentrations of choice vary slightly with the cell line and cryoprotective agent used (3) ranging from 5% to 10% (v/v). Discard residual portions of any used aliquot of cryoprotectant.

C. Dispensing procedure

1. Obtain a cell suspension by using the same procedures employed for routine subcultivation.
2. Collect the cells in pellet form by centrifugation at about $100 \times g$ for 10 min and resuspend in an appropriate volume of freeze medium at room temperature. Concentrations of 10^6 to 10^7 cells per ml are generally satisfactory and practical. For some applications it may be desirable to increase or decrease this by as much as 1 log.
3. If the total volume is large, as for a production-level freeze, maintain the cell suspension with gentle agitation in an apparatus such as the Wheaton Celstir unit. Set this up in advance complete with a Cornwall automatic syringe.

NOTE: If the cell suspension is in a small volume (10 to 20 ml), dispense by means of a syringe fitted with an 18-ga needle or cannula. Mix repeatedly during this process.

4. Maintain the pH when necessary by gassing with an appropriate mixture of water-saturated air/CO_2.
5. Dispense the cell suspension in 1-ml aliquots to the ampuls taking care to proceed rapidly and with uniformity. Cells sediment quickly under unit gravity in the Cornwall tubing and inaccuracies can result if the process is interrupted or arythmic.

D. Ampul sealing and testing

1. Seal each ampul by means of a dual arm gas-oxygen torch. Accomplish this by pulling the neck of the ampul as it is rotated in the highest heat zone of the flame. This pull-seal technique is preferred since it reduces the risk of permitting pinhole leaks in the sealed tip.

NOTE: For routine large-scale freezes use a semiautomatic ampul sealing device. For less extensive freezing requirements (eg. 5 to 30 ampuls per freeze), and in absence of a semiautomatic sealing device, seal ampuls by means of a manual unit.

2. Place sealed ampuls on an ampul cane and totally immerse upright in a suitable cylindrical vessel containing aqueous methylene blue (0.05%) at 4° C.

3. After 30 to 45 min remove each cane, wash briefly with cold running tap water and discard any ampuls containing the blue dye.

4. Dry the cane and ampul thoroughly in preparation for freezing.

E. Ampul freezing and records

1. Place ampuls on canes into a programmed freezing unit (eg. Linde BF4-2 freezer with BF-6A controller) and cool at 1° to 3° per min to −30° C.
   a. Monitor the drop in temperature using thermocouple probes in dummy ampuls within the freezing container and a temperature chart recorder (see manufacturers' directions). The optimum rate of cooling varies somewhat among different cell lines and can best be determined empirically in advance.
   b. At any point below −30° C cool the ampul rapidly to −150° C.

   NOTE: For a small-scale freeze (eg. 1 to 9 ampuls) and when a mechanical programmed freezer is not available, cool the ampuls in a cone-type unit (Linde BF-5) that is compatible with small liquid-nitrogen refrigerators (Linde LR-30). Adjust the rate of cooling by using the O-ring setting as directed by the manufacturer.
2. Remove the ampuls and transfer immediately and rapidly to the liquid-nitrogen refrigerator (MVE LZ100 for large repositories; LR30 for smaller scale cell banking).
3. Record the location and specifics of the cell line in question. Two separate cross-index files providing both subject and location cards (Fig. 1) are used for this purpose.

F. Reconstitution

1. Protect face and neck by wearing a full mask and use insulated gloves to retrieve ampuls. These PRECAUTIONS are essential in any case in which ampuls or vials have been stored in the liquid-nitrogen phase.

Approved by Senior Author
Signature [signature] Date 9/18/78

SUBJECT CARD

TL, CRL or CCL No. (Identifying No.)	CELL LINE	FREEZE NO.
Location (List Refrigerators by No.) ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ 6		
Section _____ Box(es) _____ Cane(s) _____		
Date Frozen _____ No. Ampuls _____ No. Cells/Ampul _____		
Culture Medium _____ Passage _____ Population Doubling _____		
Type of Freeze (Seed, Distribution, etc.) _____		
Final Disposition of Freeze _____ Date _____		
Comments:		

OVERSIDE

No. Ampuls Thawed	Date	Initials	No. Ampuls Remaining

LOCATION CARD

SPECIFIC REFRIGERATOR NO.	SECTION	BOX(ES)	CANE(S)
Cell Line _____ TL,CRL or CCL No. _____			
Freeze No. _____ No. Ampuls Frozen _____			

Ampuls Thawed	Date	Initials	Ampuls Thawed	Date	Initials

FIG. 1. Example index cards for inventory of ampuls stored in liquid-nitrogen refrigerators.

Procedure No. 18601

2. Remove the selected ampul rapidly and plunge directly into a warm water bath (37° C).
   a. Agitate the ampul contents until the suspension has thawed completely (20 to 60 sec).
   b. Immerse the ampul in 70% ethanol at room temperature.
3. Score standard ampuls using a small file that has been dipped previously in ethanol. Gold Band Cryules or prescored ampuls require no such additional treatment.
4. Use a sterile towel to pick up the ampul; break sharply at the neck at the prescored point.
5. Transfer the contents, by means of a sterile pasteur pipette or syringe, to a minimum of 10 ml of complete growth medium. Centrifuge at 100 × *g* for 10 min.
6. Remove the supernate, add a fresh aliquot of medium, and mix the suspension.
7. Initiate the culture by standard procedure.

IV. DISCUSSION

Considerations of safety deserve additional emphasis. Danger to the operator derives mainly from the possibility that liquid nitrogen can penetrate ampuls via hairline leaks during storage. On warming, rapid evaporation of the nitrogen within the confines of such an ampul can cause a sharp explosion with shattered glass flying at high force almost instantaneously in all directions. Fortunately the frequency of such traumatic accidents declines dramatically as the operator gains experience in ampul sealing and testing. However, even with highly accomplished laboratory workers, the remote possibility of explosion still exists. The PRECAUTIONS indicated in the Introduction and Procedure sections should be mandatory.

Aspects relating to safe storage for the ampuls produced also are of obvious concern. National repository liquid-nitrogen refrigerators, such as those of this institution (eg. MVE LZ100), require constant monitoring. Each refrigerator is equipped with controls including an alarm (Cryo-Med Model 400 ABCD) which is activated if the liquid nitrogen falls below expected levels. The alarm systems are checked regularly on a 24-hr schedule by the American Type Culture Collection (ATCC) personnel. In addition, during off-hours the systems are monitored externally (as are other mechanical freezers, refrigerators and incubators), and maintenance personnel are notified by telephone of any irregularity. Reserve replacement units are available in case of failure. Furthermore, secondary storage banks have been established at a site distant geographically from the central repository in case of catastrophic damage to this physical plant. All liquid-nitrogen freezers are serviced semiautomatically via a central large reservoir tank (Linde VST 150) which is replenished twice weekly.

Of course these PRECAUTIONS would not be practical or necessary for smaller cell-culture repositories established in research laboratories. An alarm system (Linde CY30) is available for smaller liquid-nitrogen refrigerators (eg. Linde LR-30) requiring manual replenishment. In addition, these units can be monitored by routine inspection using the frost line on a dipstick to indicate liquid levels and/or by direct weight measurement. Reservoir units (Linde LS-160B) or reserve storage tanks (Linde LR-31) are available for regular refilling of lower capacity refrigerators. Laboratory managers must maintain constant vigilance over this type of facility to avoid loss of frozen stocks due to human negligence or error.

V. REFERENCES

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