IPA SOP: Cryopreservation of Hybridomas
(Also applicable to all myeloma cell lines, unselected fused cells, or primed and pure lymphocytes)

PURPOSE:

To describe the method and materials necessary to successfully freeze hybridoma cell lines.

DESCRIPTION:

Hybridoma cell lines divide approximately every 18 - 24 hours. Cells are suspension and should be resuspended easily by pipetting them up off the bottom of the flask. No need to scrape or use trypsin. Split ½ or ¼ to feed as opposed to pelleting and seeding a new flask as hybridomas like to be in conditioned media.

Split cells ½ for at least 2 days before freezing while maintaining them at mid log. Freshly divided cells have stronger membranes that withstand the freezing process better.

Most hybridomas are frozen in either growth media with 20% fetal bovine serum (FBS) and 10% dimethylsulfoxide (DMSO) or 90% FBS plus 10% DMSO. Some replace DMSO with glycerol. IPA freezes hybridomas in 90% FBS plus 10% DMSO in 1mL (2 x 10^6 cells/vial) aliquots and stores them in liquid nitrogen.

Dimethylsulfoxide = DMSO (H₃C-S-CH₃)
DMSO is a cryoprotectant; its use avoids cellular damage caused by the formation of ice crystals during freezing. DMSO is used ice-cold at concentrations of 5-20% in media containing serum; DMSO is toxic to cells at higher temperatures. It is a solvent that dissolves ionic compounds and solvates cations well. It is an aprotic solvent (cannot form H-bonds) which acts as a positive center shielded from anions; therefore, it cannot solvate anions well (anions are poorly stabilized). The “naked” anions are thus highly reactive as bases or nucleophiles. DMSO should be kept sterile when used for freezing.

IPA uses Nalgene Cry 1 C Freezing Container with isopropanol which acts as an insulator. This allows the temperature of the cells to drop by 1°C/min (in a −80°C freezer) to prevent shock to the cells (snap freezing) caused by a rapid temperature decrease. Cells insulated in Styrofoam are also resistant to snap freezing. Snap freezing causes the formation of ice crystals in the cell, which destroys organelles and the cytoplasmic membrane.

NOTE: Ensure Freezing containers are filled with appropriate volume of fresh isopropanol. Keep a tally on the lid of the container each time you use it. Replace isopropanol after 5 uses. Alternatively, a styrofoam container can be used if freezing containers are unavailable.
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TIMEFRAME:

Time to freeze a cell line depends on how many vials are being frozen etc. Normally multiple vials of multiple cell lines are being frozen at any given time.

For 5 cell lines freezing 3x vials of each ➔ Including set-up; prepping freezing media, obtaining ice, labelling tubes, updating binder, resuspending, spinning, re-feeding and placing cell lines in -80°C should take about 1.5 hours.

SAFETY:

DMSO: Toxic combustible liquid, skin and eye irritant: Keep away from heat, sparks and flame. Avoid accumulation of static electricity. Avoid contact with eyes and skin. Wear eye protection and resistant gloves. Do not inhale vapours or mist, or take internally. Use only with ventilation. Keep container closed when not dispensing. Flash point is 94°C (OC).

Considerations for working with DMSO following this SOP:
DMSO is FLAMMABLE therefore do not flame the lid and be careful when working in the flow hood and when sterile flaming other objects.
- DMSO is TOXIC to cells therefore use gloves when handling. This is also an important consideration because DMSO toxicity will kill hybridomas and other cells as well if they are exposed to it at warmer temperatures for longer periods of time.
- Work quickly to minimize cell death, and keep solutions on ice as much as possible.

FIRST AID:

In case of contact with DMSO, immediately flush eyes and skin with plenty of water for at least 15 minutes. If swallowed do not induce vomiting. If affected by vapour, move to fresh air. If breathing has stopped, apply artificial respiration. Get medical attention immediately.

See Material Safety Data Sheet for more information
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REQUISITES/REAGENTS:

Fetal Bovine Serum (FBS)
Dimethylsulfoxide (DMSO)
Mid log healthy cell cultures
Sterile conical tubes
Freezing vials (cryo vials)
Sterile pipets
Nalgene Cry 1 C Freezing Container
Ice

Recipes and Set-up:

Freezing media

90% Fetal Bovine Serum
10% Dimethylsulfoxide

Calculate 1mL of freezing media for every vial of cells to be frozen.

Example: 0.5 mL DMSO added to 4.5 mL FBS.

- Add DMSO to FBS slowly and pipette up and down to mix thoroughly. If added too quickly proteins in the serum may precipitate.
- Hold on ice for entire procedure (put conical in small ice bucket at side of hood).

Cryovials

Label cryovials using label maker using liquid nitrogen resistant labels
Label with: freezing date, project name, clone name and isotype.
Example:

<table>
<thead>
<tr>
<th>IMMUNOPRECISE</th>
</tr>
</thead>
<tbody>
<tr>
<td>August 23/2012</td>
</tr>
<tr>
<td>Mouse anti-BSA mAb</td>
</tr>
<tr>
<td>Cell Line</td>
</tr>
<tr>
<td><strong>12D6</strong> IgG2b</td>
</tr>
<tr>
<td>For in vitro use only</td>
</tr>
</tbody>
</table>
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**PROCEDURE:**

Work in a Laminar Flow Hood or Biosafety Cabinet

Ensure that cells to be frozen are healthy, contamination-free and have been split ½ over the previous 2 days to ensure healthy membranes. Cell density should be at least $2 \times 10^5$ cells/mL. Inspect cells under the microscope before freezing.

Calculate for $2 \times 10^6$ cells per cryovial. This is roughly 10mL of a mid log phase culture ($2 \times 10^5$ cells/mL) per vial to be frozen down. Example: If you want to prepare 5 vials of one cell line. Use at least 50mL of mid log phase healthy tissue culture.

1. Label conical tubes with clone name in felt pen for spinning down cell cultures and label cryovials using label maker.

Hood set-up for re-suspending and freezing multiple clones.

2. Prepare freezing media in a sterile conical tube(s). Always make up 20% more than is needed. Hold on ice during entire procedure.
3. Transfer cell culture to be frozen to a labelled conical tube by pipeting cells thoroughly to resuspend them.

4. Spin down cells at 200 Xg for 6 minutes.

5. Pour off supernatant into waste beaker and “flick” tube once with your finger to loosen pellet.

6. Add appropriate volume of freezing media to the pellet. Resuspend the pellet well (pipette up and down several times, and avoid making bubbles) to resuspend the cells fully in the freezing media. The cell pellet can clump so ensure resuspension as cells need to be fully coated with freezing media.

7. Add 1 mL of cells/freezing media suspension per labelled freezing cryovial. Put cryovial on ice once lid has been tightened.
   - **NOTE:** If freezing down 5 vials of the same cell line, resuspend the pellet in 5mL of freezing media and aliquot into 5 cryovials. Resuspend well so that cells are equally distributed amongst the 5 vials.

8. Keep vials of cells in freezing media on ice until ready to place them in the −80°C freezer. Vials can be held on ice for up to 30 minutes.

9. Place vial(s) in iso-propyl alcohol Cryo 1° Freezing Container (C3H8O/IPA) in clock-wise order starting on inside holes at position 1. Each container holds 18 vials. Do not tighten lid of Cryo container too tight as it will tighten as it freezes. Place in −80°C freezer.
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10. Fill out a “freezing sheet” (project name, clone names, freezing container number etc.) and pin to the front of the -80°C freezer. This sheet is used for identifying cells when they are being sunk in liquid nitrogen and is added to the Project Binder after sinking.

11. Cells can be sunk into liquid nitrogen after a minimum of 4 hours. IPA normally sinks cells the following day. Cell lines location and tank number are recorded in the Liquid Nitrogen Binders and in the CryoManager software program.

QUALITY CONTROL:

One vial of each cell line frozen down is thawed from LN2 to perform quality control and ensure the freezing process has properly cryopreserved that lot of cells. Viability and antibody secretion are tested and noted. Cells that are <50% viable are re-frozen.

NOTES:

In the event of an emergency, it is crucial to have a secondary stock of your valuable biological materials stored at an alternative location. ImmunoPrecise provides you with a secure, off-site storage facility to safeguard your assets. We have the capability to store cell lines, tissues, RNA, DNA, plasmid constructs, antibodies, and many other biological materials. Should you require a quote for IPA’s cryostorage services, please reach us at 1-250-483-0308, via email, or try our online quote request form.

REFERENCES:

Nalgene Cry 1 C Freezing Container Product Info cite:
http://www.nalgenelabware.com/products/productDetail.asp?product_id=405&subcategory_id=139&category_id=139&brand_name=NALGENE+Labware&category_name=Cryoware&subcategory_name=
