SOP: AB105.5
Modified: 7/27/22 KE

Freezing Down Cell Lines

Materials and Reagents:

1. Freezing media (Note 1)
    - Fetal Bovine Serum (NOT heat inactivated), 500mL (Sigma, Cat# F2442-500ML).
        - **Heat inactivated FBS may be used if necessary for downstream assays/products.**
    - DMSO hybri-max, suitable **for hybridoma cell lines** (Sigma, Cat# D2650, 5x5mL ampules, sterile), *another DMSO reagent can be used for other cell lines*
        - ***Can get through PMF/ARC BIO stockroom – same vendor and Cat#***
2. 1.2 mL sterile, external thread Cryovials (Fisher Scientific, Cat# 09-761-74,)
3. Mr. Frosty container (ThermoFisher Scientific, Cat# 5100-0001)
    a. ***Use up to 5 times before changing the isopropyl alochol***
    b. ***TO CLEAN***: rinse all components with DI water, wipe down with 70% ethanol, add 250 mL of fresh isopropyl alcohol, **store at room temperature**
        i. ***MAKE SURE to thaw Mr. Frosty between uses, must be thawed to freeze cells down***
4. Isopropyl alcohol
5. Automated pipet-aid
6. P1000 pipetman with 1000 µL ***filter*** tips
7. 0.65mL Eppendorf tubes
8. 50 mL Falcon conical tubes
9. 15 mL Falcon conical tubes
10. Serological pipets (assorted volumes)
11. 0.22 µm Millipore Steriflip vacuum filter unit, PES membrane (Fisher Scientific, Cat# SCGP00525)
12. 5% CO_2 Humidified Tissue Culture Incubator, 37°C
13. Biosafety cabinet
14. -70°C Freezer
15. Liquid Nitrogen storage
16. Tabletop centrifuge (for large volumes)
17. Inverted light microscope
18. Hemocytometer (SOP: SP067.2) or an automatic cell counter (SOP: SP078.1) (both with 0.4% trypan blue dye solution)
19. 70% ethanol
20. 2.5% Vesphene or disinfectant of choice

Protocol:

1. _____ Prepare biosafety cabinet (BSC) for work under sterile conditions (wipe down BSC with 2.5% vesphene or choice of disinfectant and then 70% ethanol).

2. _____ Transfer culture flasks to BSC (wipe surface with Wypall sprayed with 70% ethanol).

3. _____ Remove cells from flasks by gently tapping the flasks on hand to detach the semi-adherent cells and rinsing the tissue culture treated side (where cells are attached) with cell suspension multiple times.

4. _____ Transfer cell suspension to a sterile 15 mL ***OR*** 50 mL conical tube.

5. _____ Make sure cell suspension is well mixed (re-suspend a few times) and take a small aliquot for counting. ***Record the cell suspension volume.***

6. _____ Centrifuge cells at 1,200 rpm, 5 min at 4°C (Note 3).

7. _____ While cells are spinning, ***count cells*** with a hemocytometer or an automatic cell counter (Note 2). Cells should be 80-90% viable (any lower and the cells may not be able to recover from the freezing process) and should have a density between $2x10^5$ – $8x10^5$ cells/mL ***live cells*** in original flask.

a. ***Label sterile cryovials with appropriate information***: *cell line and clone, date, initials, cell count (live cell count), growth media, freezing media, and passage # (see SOP:AB100.6, Note 9)*
b. The labels used on the label maker are best for freezing/liquid nitrogen storage. Extra labels should be made for lab notebooks.

8. _____ After centrifugation, wipe down conical tube containing cells with a Wypall sprayed with 70% ethanol and carefully transfer into BSC (careful not to disturb cell pellet).

9. _____ Remove supernatant carefully with a serological pipet and discard in biohazardous trash (to be autoclaved).

10. _____ Gently tap bottom of tube (on BSC grate) to break up cells and re-suspend cells in appropriate volume of cold freezing media to obtain necessary density of cells.

    a. Cells should be frozen at a density between $5x10^6$ – $10x10^6$ cells/mL (per vial) (Note 4). Freeze down according *to* ***live cell count only***.

11. _____ Immediately transfer cell suspension to appropriately labeled cryovial at 1 ml per vial via a P1000 pipetman with 1000 µL filter tips (***change tip for each vial***).

12. _____ Quickly transfer cell cryovials to a thawed Mr. Frosty container and transfer to a -70°C freezer (Notes 5 & 6).

13. _____ Let cells sit in Mr. Frosty overnight at -70°. The cells should ***never*** be stored at -20°C for any period of time. ***Do not transfer cells directly to liquid nitrogen tank***.

14. _____ After 24 hours, the cells may be transferred to liquid nitrogen storage.

    a. Make note of location in LN2 log Excel sheet, Antibody and Cell line list, and lab notebook. Cell viability decreases if the cells are kept at -70°C for an extended period of time.

15. _____ After at least a few days, ***if necessary***, thaw one vial from each lot of frozen cells to check viability/recoverability and for contamination during the freezing process (See **SOP: AB100.6** for hybridoma cell line & SP2/0 cell line thawing/cell maintenance).

Notes:

1. **Freezing Media (homemade)**:
   90% FBS
   10% DMSO

   Filter mixed freezing media solution through a 0.22µm Millipore Steriflip vacuum filter unit. Aliquot freezing media and store in -20°C until ready to freeze cells. For freezing, thaw freezing media in water at 4°C. Once thawed, keep cold at 4°C until ready to add to cells.

2. See **SOP: SP067.1** for counting cells using a hemocytometer. It may be necessary to dilute the cells prior to addition of trypan blue. Be sure to include any dilutions in your cell calculation. Viability is calculated by dividing the *number of live cells* by the *total (live and dead) number of cells*.

   See **SOP: SP078** for counting cells using EVE™ Automatic Cell Counter. The cell counter by default calculates for the 1:2 (10 µL cell suspension + 10 µL trypan blue dye) working *cell suspension: trypan blue dye* dilution (and therefore, not included in the final count). However, if it is necessary to dilute the cells, this dilution factor must be included in the final count.
3. Hybridoma cells grow quickly. Thus, their density may be higher than the upper limit of the suggested range. This is OK if cell viability is still high. A higher cell density aids in quick recovery of hybridoma cells. Cell densities of 1 x 10^6 cells/mL to 1 x 10^7 cells/mL ***(1 mL = 1 vial)*** can be used for these cell lines.
4. **Example**:
   a. Live cell count: $9.2x10^6$ cells/mL

b. Total volume: 10 mL
c. Total live cells: $9.2x10^6$cells/mL x 1 0mL = $9.2x10^7$ total live cells
d. Desired concentration: $5x10^6$ – $10x10^6$ cells/ 1 mL vial
    i. Re-suspend cell pellet after centrifugation in 10 mL of freezing media to get 10 x 1mL aliquots at a concentration of 9.2 $x10^6$ cells/1 mL.

5. Mr. Frosty containers allow for the cells to freeze at (approximately) -1°C/min in the -70°C freezer. This prevents the cells from freeze shock. Rinse Mr. Frosty with DI water and wipe down container with 70% ethanol (let dry). Fill Mr. Frosty with 250 mL of 100% isopropanol and assemble. After freezing, Mr. Frosty containers are then thawed and left at room temperature until next use. Mr. Frosty containers can be reused (freeze/thaw) up to 5 times before having to change the isopropanol.
6. Transferring the aliquoted cell cryovials in freezing media ***quickly*** to the -70°C is imperative so as to not let the cells sit in DMSO.

Tips:

- Good aseptic technique is *crucial* for the freezing process. Even though an antibiotic/antimycotic supplement is used for the hybridoma cell lines, it is imperative to not introduce any contaminants once cells are in freezing media. It is recommended to use sterile filter tips for freezing.
- Hybridoma cells like to be at high densities when frozen. This allows for quicker recovery when thawed. Cell counts will never be 100% accurate, with either counting method. Therefore, a higher density allows for some "wiggle room" for counting errors.
- **Always** freeze with the ***live cell count***. It is recommended to do an average of cell counts, for a more accurate live cell count.
- High cell viability is key for a better recovery after thawing.

References:

1. *Antibodies: A Laboratory Manual*. Ed Harlow and David Lane. Cold Spring Harbor Laboratory, New York. 1988. pp. 245-281.