SOP: AB100.6
Modified: 7/27/22 KE

Cell Line Maintenance (Suspension SP2/0 and Semi-adherent Hybridoma Cell Lines)

Materials and Reagents:

1. Complete RPMI, DMEM, or IMDM
   - Media is "complete" with the addition of FBS
2. Sterile 50 mL Falcon centrifuge tubes
3. Sterile 15 mL Falcon centrifuge tubes
4. Serological pipets (assorted volumes)
5. 0.65ml Eppendorf tubes
6. Tissue culture flasks (VWR Corning T25 (29185-302), T75 (BD353136))
7. P1000 pipet with 1000 µL ***filter*** tips
8. Automated pipet-aid
9. Ziploc baggie
10. Hemocytometer (SOP: SP067.2) or EVE™ Automatic Cell Counter (See SOP: SP078.1)
11. Inverted light microscope
12. 37°C water bath
13. 2.5 % Vesphene (or other disinfectant of choice)
14. 70% Ethanol
15. 5% CO_2 Humidified Tissue Culture Incubator 37°C
16. Biosafety cabinet
17. Tabletop centrifuge

Thaw and Growth Protocol:

1._____ Before thawing, check to see which complete media the cells were previously grown in (Note 1). When in doubt for older cell lines (incomplete history of media used), use complete RPMI media.

2._____ Prepare biosafety cabinet (BSC) for work under aseptic conditions (wipe down cabinet surface with 2.5% Vesphene (or disinfectant of choice) and then 70% ethanol. 70% isopropanol may be used if fungal contamination is of concern.

3._____ Warm up appropriate media to 37°C (~ 15 – 30 min) prior to thawing cells. Place in BSC immediately before thawing cells.

4._____ Remove cell line cryovial from liquid nitrogen tank (Note 2).

5._____ Immediately transfer cell line cryovial to 37°C water bath in sealed Ziploc baggie. It takes about 2-4 minutes to defrost until a little chunk of ice remains. When ready to transfer to BSC, wipe down outside of cryovial with 70% ethanol (make sure to write down label contents/save label to place in lab notebook) (Note 3).

6._____ Inside the BSC, gently pipet cells a couple of times with a P1000 pipetman and appropriate filter tips, then gently transfer to a sterile 15 mL conical.

7._____ Slowly add 9 mL of complete warmed media to cells. Add the first 1 mL of media dropwise and swirl cells gently a couple of times as to not shock the cells. Add the rest of the media, slowly. Media should be added by decanting down the side of the tube as to not blast the cells.

8._____ Centrifuge cells at 1,200 rpm, 5 min at room temperature.

9._____ Remove and discard the supernatant (biohazardous trash to be autoclaved) and gently re-suspend the cell pellet in 1-2 mL of complete warmed media.

10._____ IF needed, cell counts can be taken in this step to seed at desired concentration of cells per flask (Note 4).

11._____ Place the cell suspension into a T25 flask w/ 5-10 mL fresh complete warmed media. 10 mL of media is the largest volume the T25 flask can hold (laying down). If using vented cap flasks, screw the lid on firmly. If not using vented caps, make sure the cap is loose to allow for CO_2 exchange in the incubator.

12._____ Label flask appropriately (name, date, media, cell line and clone, "Dobos").

13._____ Transfer cells to a humidified 37°C incubator with 5% CO_2. Place T25 standing up for overnight incubation to encourage cell growth if cell count/density is low. If not, place T25 on the tissue culture-treated side (laying down).

14._____ Check cells daily under the microscope and for a change in media color (cells produce acidic byproducts when growing). It is imperative to check for contamination daily, as bacterial/fungal growth will also acidify the media and change its color (Note 5).

15._____ Once cells have reached appropriate confluency and/or there is a change in media color (yellow), feed or upscale/split the cells. SP2/0 cells and hybridoma cells usually need to be fed/split every day or every other day. *<u>One</u> T25 flask should be upscaled/split into <u>one</u> T75 and then subsequently upscaled/split into more T75 flasks as cells grow.* Lay T75 flasks on their tissue culture-treated side (Notes 6-9).

16._____ If cells are ready to be frozen down, see SOP: AB105.5.

Antibody Production Protocol (Hybridoma Cells Only):

1._____ For optimal *antibody production*, further upscale hybridoma cell line(s) to multiple T75 flasks (depending on how much supernatant product is needed).

2._____ Grow cells up to high confluency (~90-100 % of T75 surface area). Add 2-5 mL of warmed, fresh media every day whilst cells are growing to high confluency. Make sure not to add more media than the flask can hold (laying down).

3._____ Once cells have reach high confluency, leave cells sit in humidified 37°C CO_2 incubator for ~1 week ***without*** addition of any media (Note 10).

4._____ Harvest cell culture supernatant at complete cell death (Notes 11 & 12).

5._____ Collect culture supernatant and centrifuge at 3,000 rpm, 5 minutes at room temperature to remove cell debris.

6._____ 0.22 µm sterile vacuum filter cleared cell culture supernatant (Note 13).

7._____ *If desired/needed*, concentrate/crude purify cell supernatant via ammonium sulfate precipitation with a 50% cut (see reference #2 and SOP: PP057 – with an initial 50% ammonium sulfate cut from 0%).

8._____ Test concentrated antibody dilutions/titers via indirect ELISA (SOP: SP039.2) and Western blotting (SOP: SP011.3 or SP011a).

- **Sufficient reactivity is (generally) regarded as an OD405 of ≥ 1 at a supernatant dilution/titer of 1:1,000 for ELISA.**
- **Sufficient reactivity is (generally) regarded as a dark band (corresponding to appropriate molecular weight of antigen being tested) at a supernatant dilution/titer of 1:1,000 for Western blotting.**

9._____ Aliquot filtered culture supernatant accordingly.

Notes:

1. If the vial says 'HAT' the cells must be grown in HAT media. Otherwise, Complete RPMI, Complete DMEM (SOP: M027), or Complete IMDM (SOP: M028.2) should be used with 1X HT supplement added,

and 10-20% FBS. Other supplements are added and are noted in media-specific protocols. SP2/0 murine cancer cells should be grown in complete IMDM, 10%FBS without any HAT or HT supplements.

2. Make sure to update and save the LN2 Excel Inventory Sheet for any cells taken out.
3. It is important to note that the thawing process should be performed ***quickly*** as the dimethyl sulfoxide (DMSO) in the freezing media is toxic to the cells. A poor recovery of the cells could be due, in part, to a slow thawing process. Therefore, it is imperative that the BSC is set up and the appropriate culture media is warmed (37°C) prior to start of the thawing process. If the cells are not thawing quickly in the Ziploc baggie, remove the cryovial and submerse it into the 37°C water bath (making sure to ***not*** touch the cryovial cap/lid to the water bath water, as this can contaminate the contents of vial).
4. See **SOP: SP067.1** for counting cells using a hemocytometer. It may be necessary to dilute the cells prior to addition of trypan blue dye. Be sure to include any dilutions in the cell calculation. Viability is calculated by dividing the *number of live cells* by *the total number (live and dead) 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 is not included in the final count). However, if it is necessary to dilute the cells, the dilution factor must be included in the final count.
5. Cell debris may look like contamination; however, this is just Brownian motion and nothing to be concerned about in these cultures. Bacteria will move quickly and in a directional movement. Brownian motion will make the debris particles vibrate and not move in a particular motion (staying put, for the most part). Hybridomas grow rapidly and will change the media color quickly when growing well. Therefore, it is important to check for contamination under the microscope and not solely rely on media color change.
6. Hybridoma cells like to have at least 50% spent media while they are growing/expanding. This means that every time they are split, 50% of the volume should be old media from their previous flask and the other 50% should be warmed fresh media. If cell count is not important, and the goal is to keep expanding/upscaling cells, cell counting via hemocytometer, or an automatic cell counter is not necessary (until freezing). Confluency checks and upscaling will suffice (Notes 7 & 8).
7. Visually, if the cells need to be split, they are ~70-80% confluent and if the cells need to be fed, they are <50-60% confluent. Confluency is referred to as the percentage of the surface of a flask or plate that is covered by cells.
   a. **For hybridoma cells:**
      - **Feed** hybridoma cells by removing half of spent media (from opposite side of flask as to not disturb the semi-adherent hybridoma cells) and adding equal volume of fresh complete media (whether in a T25 or a T75 flask).
      - **Split** hybridoma cells by tapping flask gently on hand to detach the semi-adherent cells and rinsing the tissue culture treated side (where cells are attached) with cell suspension multiple times. Transfer half of the cell suspension to a new T75 flask. Add an equal volume of fresh complete media to each flask. Bubbles will happen, but hybridoma cells are very hardy and can handle some bubbles. However, bubbles can be sucked off top of media if there is an overabundance.

   b. **For SP2/0 cells:**
      - **Feed** SP2/0 cells by adding ~5 mL of fresh, warmed, complete IMDM media ***OR*** DMEM/F12 media with 10% FBS media.
      - **Split** SP2/0 cells by tipping flask up, re-suspending cells and washing treated side of flask a couple of times to make sure all cells are in suspension and splitting cell suspension in half into another flask.
        1. *Note that some cells may semi-attach and therefore the flask may be tapped gently and the tissue culture-treated side (where cells are attached) rinsed with cell suspension multiple times before transferring into another flask.*
        2. Add same volume of fresh complete IMDM, 10% FBS media to each split flask.
8. One may also check cell count via a hemocytometer or with an automatic cell counter (Note 2). ***Note that SP2/0 cells are usually not counted until fusion day (just visually checked for confluency).***
   a. Gently tap sides of flasks to release cells from tissue culture-treated side of flask and rinse the tissue culture-treated side with cell suspension multiple times.
   b. Re-suspend cell suspension well and take an aliquot of ~100 µL (place in a 0.65mL Eppendorf tube).
      - If the cell count is *lower* than 8 x 10^5 cells/mL, the ***culture needs to be fed***. Add approximately 5-10 mL of warmed fresh media to the existing culture volume. Make

sure the final cell density is no lower than $2x10^5$ cells/mL. The fewer the cells, the longer they will take to recover, but usually the density will be back up in 2-4 days.

1. **Flasks may be stood upright to encourage cell growth as well (less surface area).**
2. Make note on flask that it has been fed (+date)

- If the cell count is *greater* than $1x\ 10^6$ cells/mL, the ***culture should be split and expanded to new flasks***.
    1. Upscale from a T25 to 1 x T75, and so on with more T75 flasks. Do this by tapping the cells loose (gently) from flask and rinsing the tissue culture treated side (where cells are attached) with cell suspension multiple times, collecting cell suspension into a sterile 15mL conical, and spinning down the cells at 1,200 rpm, for 5 minutes at room temperature. Re-suspend in appropriate volume (T25 = 10mL, T75 = 15-20mL) of warmed fresh complete media and add to flasks. Lay the flask on its tissue culture-treated side.

9. A passage number should be noted when a cell line is thawed. One thaw/freeze equates to 1 passage. This is *relevant* if a cell line is being used to propagate then freeze, one can record a passage number for that thaw/freeze. However, some vials will be used for antibody production only. A passage number for these "terminal cells" (i.e. for antibody production purposes) is irrelevant.
10. Make sure to check for contamination every day whilst cells are dying. Adding 2-5 mL of warmed, fresh media every few days to replenish the antibiotic/antimycotic reagent. It may take cells longer than a week to die completely. However, 5-10% remaining confluency is adequate for "complete cell death" and cells can be collected (hybridoma cells like to survive). Media may also turn a very orange/yellow or yellow color.
11. Add 2.5% vesphene solution (or disinfectant of choice) to flasks after supernatant collection. Swirl vesphene (or disinfectant of choice) in flask and dispose of in biohazardous trash (to be autoclaved).
12. Hybridomas will persist for a long time and complete cell death is generally achieved for > 1 week after addition of media is suspended and cells are left to die. Make sure to add in some fresh media (2-3 mL) after 1 week to replenish antibiotic-antimycotic. ***However, if cells are ~5% confluent (≤ 1 week after left to die), the supernatant can be collected due to persistence of cell type. A sufficient amount of antibody has been made at this point and cells are just trying to hang on.***
13. Sterile filtered cell culture supernatant can be frozen at -20 °C until further use or purification.

Tips:

- Hybridomas are proliferative cells and should be checked daily. In general, they will need to be fed or split *every* day. However, media with 10% FBS will slow their growth (when compared to the media with 20% FBS). Media with 20% FBS can cause them to grow too quickly and kill them. However, if cells are not growing well or are growing slowly, media with 20% FBS can aid in recovery.
    - To step down the % FBS in culture media, slowly decrease % FBS in culture media over a couple of days to not shock the cells. This can be done by adding media without FBS (all other additional supplements) to the 20% FBS cultures until a 10% FBS culture is reached. Complete media with 10% FBS can then be used for the subsequent cultures.
    - % FBS should be considered for any assays downstream affected by FBS components. *IF needed*, these cells would need to be "weaned" off FBS gradually, following suggestions above.
    - Hybridoma cells can also be "weaned" off the 1X HT supplement if this supplement affects downstream assays, However, this is not recommended as this supplement aids in hybridoma cell growth.
- Hybridoma cells are generally incredibly hardy. Therefore, if a new base media is used (by accident or due to incomplete media history), the cells may initially be shocked but should recover. The additional supplements help support their growth. However, once a hybridoma cell line is established in a certain media, it is best not to switch back and forth.
- Hybridoma cells are a semi-adherent cell type. Therefore, *some* force is needed to detach them from the tissue culture-treated side of the flask. Gentle tapping and rinsing of the tissue culture-treated side of the flask should be enough to detach them (confirm by microscopy if not sure). Bubbling/some frothing of the cultures is OK, but nothing too vigorous. Again, hybridoma cells are incredibly hardy.
- When recovering cells for antibody collection (supernatant) or for generating more vials of a particular hybridoma cell line with an incomplete media history, a media consisting of base media, 1X HT

supplement, 10% FBS, 1X antibiotic/antimycotic, and 0.055 mM (1X) 2-Mercaptoethanol should be used (minimal ingredients).
    - 1X HT supplement helps stabilize and promote growth for these cells
    - 2-Mercaptoethanol helps with clumping of cells
- Additional supplements to consider (see desired media SOPs):
    - L-glutamine promotes cell growth and can be added in excess (compared to base media)
    - NCTC-109 media provides extra vitamins/factors
- SP2/0 cells are just as hardy as the hybridoma cells and can be cultured with the flask "standing up" or laying down (suspension cell line). They are just as proliferative as the hybridoma cells.
- Always look at cells under a microscope after manipulation (thawing, plating, upscaling, etc.).

References:

1. *Antibodies: A Laboratory Manual*. Ed Harlow and David Lane. Cold Spring Harbor Laboratory, New York. 1988. pp. 245-281.
2. Grodzki A.C., Berenstein E. (2010) Antibody Purification: Ammonium Sulfate Fractionation or Gel Filtration. In: Oliver C., Jamur M. (eds) Immunocytochemical Methods and Protocols. Methods in Molecular Biology (Methods and Protocols), vol 588. Humana Press