## Week 4: Cryopreservation

**Date: February 8, 2024**

**​​General Notes**

DMSO is toxic to cells at room temperature, freeze cells soon after they are resuspended in freezing media.

**Materials**

* Freezing Media (90% Growth Medium, 10% DMSO)
* Cryovials
* 0.25% Trypsin-EDTA
* Sterile Filters
* PBS
* Cryobowl
* -80°C Freezer
* Liquid Nitrogen Storage

**Method**

1. Prepare 0.25% trypsin-EDTA (detachment enzyme) by warming it in the 37°C water bath.
2. Check your cells under the microscope. Take pictures and write down your observations. Introduce your flask to the biosafety cabinet.
3. Save some of the current culture media in the flask for trypsin inactivation/detachment enzyme dilution (later step) by pipetting out the current media into 15 or 50 ml conical tubes.
   * 8-10 ml per T175, 5 ml per T75, 1-2 ml per T25
     + Multiple flasks of the same cells can be pooled together in a big tube.
   * *Be sure to not disturb the adherent cell layer on the bottom of the flask (e.g do not allow the pipette to come in contact with the bottom of the flask).*
4. Aspirate the rest of the current culture media from each flask.
   * If there are many flasks (e.g more than 4-5) you may want to split this step and the next into batches to minimize the time the cells spend without liquid
   * *Be sure to not disturb the adherent cell layer on the bottom of the flask.*
5. Add sterile DPBS to the sidewall of each flask to rinse out residual culture media. Swish DPBS gently so that the bottom surface is completely covered.
   * Amounts are arbitrary, e.g 7 ml for T175, 5 ml for T75, 2-3 ml for T25
6. Aspirate the DPBS and add 0.25% trypsin-EDTA to the sidewall of the flasks. Swish the trypsin gently so that the bottom surface is completely covered.
   * Trypsin: 4 ml for T175, 2.5 ml for T75, 1 ml for T25
   * Ensure that the bottom surface is completely covered by the enzyme solution.
   * *Be sure to not disturb the adherent cell layer on the bottom of the flask when aspirating.*
7. Incubate the flasks at 37°C for 4-10 minutes.
   * Duration depends on cells and detachment enzyme
     + Trypsin usually 4-5 min, as short of a time as possible to minimize harm.
8. Check each flask for complete cell detachment with the light microscope, viewing several areas of the flask to be sure.
   * If all the cells are balled up but not all are detached, you should be able to gently smack the sidewall of the flask with your hand to dislodge them.
   * Detached cells appear to look like floating spheres.
9. Dilute/neutralize the detachment enzymes with the old media you saved (step 3).
   * Dispense old media/trypsin inhibitor onto the bottom surface of the flask to wash off any cells that might still be attached.
   * Collect in the 15 or 50 ml conical tubes that held the old culture media.
     + Multiple flasks of the same cells can be pooled together.
10. Make sure that the cell suspension is homogenous/well-mixed (triturate or pipet up and down 5-10x), then load a hemocytometer for cell counting
11. Centrifuge/Spin the cells down at 300 x g for 5 min, at RT. Count the cells while centrifuging.
    * Record the volume of the cell suspension for cell number calculations.
    * Remember to balance the centrifuge.
    * Viable Cell Number Calculations: [# cells/ml] \* [dilution factor if cell suspension is diluted] \* [total volume of the cell suspension in ml] \* [cell viability %]
    * To calculate the final resuspension volume (mL), divide [total # of cells] by the [desired cell concentration (# of cells/ml)].
12. After centrifugation, aspirate out the supernatant.
13. Resuspend your cell pellet in fresh, warmed BSC-GM such that the final concentration is roughly 1.1 million cells/ml.
14. Label cryovials with the cell type, passage number, your team name/initials, the date, and “1 million cells.”
    * NOTE: The passage number does not change until your cells touch new plastic flasks/plates.
    * You will need one cryovial for every 1 million cells.
15. Add DMSO such that the final concentration of DMSO is 10%. Mix well.
    * Freezing medium is 90% GM, 10% DMSO
16. Quickly transfer 1 ml of cell suspension in the freezing medium to each cryovial.
17. Transfer the vials to the isopropanol cooling container and place in -80°C.