SOP 19
In Vitro Cell Culture Expansion Procedure

Preparation

1) Wash hands, glove up; aseptically clean the bio-safety cabinet (hood) with 70% ethanol and sterile wipes
2) Warm medium to 37^{O} C in the water bath
3) Clean 1 bottle of PUCKS EDTA with 70% ethanol and place into hood
4) Gather flask(s) to be expanded and place into hood
5) Label new centrifuge tube(s) with cell line name
6) Using a pipette, remove all media+cells from the flask(s) and pipette into corresponding labeled centrifuge tubes
7) Grab a new pipette and add PUCKS into the flask(s) with a "sweeping motion" to evenly distribute the solution into the flask.
    a. Place flask(s) back into the correct incubator for 10-15minutes to allow time for the cells to detach
8) While waiting:
    a. Get the media from the hot bath; clean with 70% ethanol and place into hood
    b. Clean area
9) After 15 minutes, get the flask(s) from the incubator, tap the side of the flask with your hand to loosen cells and place into the hood
10) Pipette the PUCKS+cells into the same labeled centrifuge tubes used in step 6.
11) Centrifuge all but one tube (keep one set aside for combining and counting later in step 13) at 500 x g (1600rpm) for 6 minutes
12) After 6 minutes, pour off supernatant as waste without disrupting the new cell pellet
13) Using the set aside (unspun) centrifuge tube with media+cells, combine all cell pellets. Mix well
14) Proceed to count the cells

Counting Cells using Vi-Cell XR

1) Get a Vi-Cell™XR counting vial and add 500 µL of the mixed sample
2) Record the total volume of the centrifuge tube
3) Count the cells using the Vi-Cell™XR
    a. Click: Log in sample
    b. Enter: Sample ID, Cell Type, Dilution Factor (1)
    c. Click: Ok and start queue
4) Multiply total viable cells by the total volume and record the cell viability
5) Calculate how many cells are needed for each experiment moving forward (ie. those cells going back to culture, being pelleted, cryopreserved and/or for other purposes – each will need a separate calculation to determine cells/volume needed)
    a. Calculation: (Total cells needed ÷ Total viable cells) x total volume = volume needed to aliquot cell count needed
6) Once all calculations are complete, label new centrifuge tubes with cell line name and purpose of the tube.
7) Aliquot appropriate media+cell volumes to distribute the cells accordingly
8) Centrifuge all tubes at 500 x g (1600rpm) for 6 minutes, pour off supernatant as waste
9) Proceed to each appropriate next step

Cryopreservation

1) Cryopreservation materials:
    a. Outer-threaded vials
    b. 1 tube of pure FBS
    c. 1 tube of 15% DMSO in RPMI-1640 medium
    d. Isopropanol tub

e. Centrifuge tube containing cells for freezing

2) For every vial cryopreserved, add 0.8mL (800 µL) of FBS and 0.8 mL (800 µL) of 15% DMSO in RPMI-1640 medium directly to the centrifuge tube containing the cells to be frozen.
    a. Final concentrations of cryopreservation reagents are: 50% FBS and 7.5% DMSO
3) Mix with the serological pipette
4) Add 1.6 mL of the viable cells suspended in the cryopreservation medium + FBS to each 2 mL outer-threaded vial being frozen
    a. Label vials with: Cell line name, passage, cell count, viability, "Geno/Myco OK" todays date and your initials.
5) Place vials into the isopropanol control rate freezing tub and into a -80^{O}C freezer
    b. Samples are to be inventoried and transferred 12-24 hours later into a liquid nitrogen vapor freezer
6) Clean area with 70% ethanol

Pelleting

1) Re-suspend the cell pellet in PBS to wash the cells
2) Centrifuge at 500 x g (1600rpm) for 6 minutes, pour off PBS supernatant as waste
3) Re-suspend the cell pellet in 0.5mL PBS per vial
    a. If you are pelleting 5 vials at 5e^{6} cells each, re-suspend the 25e^{6} cell pellet in 2.5mL PBS total
4) Transfer 0.5mL of PBS+cells into each labeled 1.5mL microcentrifuge tube.
    a. Label vials with: Cell line name, passage, cell count, viability, "Geno/Myco OK" todays date and your initials.
5) Using a microcentrifuge, centrifuge the vials at roughly 3000-4000rpm for 1-2 minutes
6) Pipette off the PBS supernatant as waste
7) "Snap freeze" the dry pellets in liquid nitrogen for 10 seconds each and immediately store at -80^{O} C
8) Clean area with 70% ethanol

Re-Culturing Cells

1) Obtain desired number of flask(s)
2) Label flask(s): Cell line name, passage (increase by 1), today's date, and your initials
    a. Also add any additional notes necessary to your experiment to your flask(s)
3) Re-suspend (mix well) the cell pellet in the appropriate amount of media (see below) and place into the base of each newly labeled flask
    a. Amount of media will vary according to the number and size of the flasks you're expanding into
    b. Flask sizes and media amounts: T12.5 = 5mL, T25 = 7mL, T75 = 13mL, T150 = 25mL, 3-layer = 96mL, 5-layer = 160mL
4) Place the flask(s) back into their corresponding incubators
5) Clean area with 70% ethanol

Maintaining Cultures:

1) At the start and end of each experiment, submit a 2e6 dry cell pellet for STR analysis and 1mL of supernatant (no cells) for mycoplasma assay analysis.
2) Flasks need to be routinely checked via a microscope every 7-10 days.
    a. Incubators need to be monitored daily to verify that no flask is visibly contaminated or if the media needs replenishing earlier than 7-10 days.
    b. Watch for cell proliferation, contamination, and growth of non-cancerous cells via the microscope.
3) Medium needs to be changed every 7-10 days to maintain fresh nutrient concentrations
4) Flask(s) should be expanded when 80-90% confluent or when optimal number of cells needed for a specific experiment are apparent
5) Discard the cells immediately when microbial contamination is present.