CMBL – CRJ/CHK/JJR
8/10/07

Protocol: Subculturing 3T3 Fibroblasts

Materials:

- 3T3 Fibroblasts (ATCC)
- 0.25% Trypsin/0.03% EDTA (Invitrogen #25200-056)
- Complete medium
  450 ml DMEM (Invitrogen #11995-065) + 50 ml Calf Serum (Invitrogen #16010-159)
- PBS (Invitrogen #10010)
- Water bath
- Tissue culture dish
- Serological Pipets
- Pipet aid
- Pipette tips
- 200 µl, 1000 µl pipetter
- 15 ml, 50 ml Falcon tubes
- Transfer pipet
- Trypan blue
- Hemocytometer
- Microscope
- Microcentrifuge tubes
- Centrifuge
- Cell counter
- Timer
- Waste beaker
- Biohazard bag
- 70% ethanol
- Kimwipes
- Markers
- Gloves

Procedure:

1. Heat complete medium, trypsin/EDTA, and PBS to 37^{o}C in a water bath
2. Observe the cells under microscope – check for contamination and note cell confluence
3. Prepare the hood:
   - spray and wipe the hood surface with 70% ethanol
   - place the following materials into the hood: waste beaker, tube rack, centrifuge tubes, dishes (tissue culture or petri), pipetter, and pipettes
   - tape a biohazard bag to the front of the hood
4. Place the pre-warmed PBS, trypsin/EDTA, and complete medium in the hood
5. Place the cells in the hood, remove the cell culture media, and wash the cells 2X with PBS
6. Mix the trypsin/EDTA (pipet up and down), and place 3 mL on the cells (for a 100 mm dish)
7. Gently swirl the dish and return the dish to the incubator for 3 to 4 min.
8. Observe the cells on a microscope
   - if the cells are not detached, gently tap the dish and continue incubating @ 37^{o}C
9. Once cells are detached, place 7 mL of complete medium in the dish to neutralize the trypsin
10. Transfer the cell suspension from the dish into a sterile centrifuge tube.
11. Pellet cells by centrifuging for 10 min @ ~300 x g (remember to counterbalance)
12. Place the cells back in the hood and carefully remove the supernatant without disturbing the cell pellet
13. Resuspend the cells in 5 mL of complete medium
14. Count cells:
    - add 50 uL of cell suspension to a microfuge tube
    - add 50 uL of trypan blue vital stain (making a 1:1 solution of cell suspension and trypan blue); pipet up-and-down to mix
    - load both sides of a hemocytometer with the trypan blue cell solution
    - count the live cells in 5 large squares and compute the average per large square
    - calculate the total # of cells

    Total # of cells = [5 (mL of cell suspension)] x [2 (tyrpan blue dilution factor)] x [10,000 (0.1 uL per large square on hemocytometer)] x [average count] = [100,000] x [average count]
15. Dilute the cell suspension such that each new tissue culture dish receives 1-2 mL of the suspension.
16. Pipette the cell suspension into dishes, and add complete medium (10 mL total for a 100 mm dish)
17. Label the dishes (cell type, passage #, date, your name, and seeding density)
18. Place the cells back in the incubator (you can check for cell attachment in ~40 min)
19. Clean the hood and place all material that contacted cells/medium in a biohazard waste bag; clean the waste beaker by adding bleach for ~15 min. before washing