CMBL – CRJ/CHK/JJR
8/10/07 (update 6/10/08)

Protocol: Subculturing

Materials:

- Cells in culture
- Trypsin* (cell specific, see below)
- Complete medium** (cell specific, see below)
- PBS (Invitrogen Cat# 10010)
- 37°C Water bath
- Tissue culture dishs
- Serological Pipets
- Pipet aid
- Pipette tips
- 200 µl, 1000 µl pipetter
- 15 ml, 50 ml Falcon tubes
- Tube rack
- Transfer pipet
- 0.4 % Trypan Blue Stain (Invitrogen Cat# 15250)
- Hemocytometer
- Microscope
- Microcentrifuge tubes
- Centrifuge
- Cell counter
- Timer
- Waste beaker
- Biohazard bag
- 70% ethanol
- Kimwipes
- Markers
- Nitrile or latex exam gloves

Procedure:

1. Heat complete medium, trypsin, and PBS to 37°C in a water bath
2. Observe the cells under microscope – check for contamination and note cell confluence
3. Prepare the hood:
   - place nitrile or latex gloves on hands
   - spray and wipe the hood surface with 70% ethanol
   - place the following materials into the hood: waste beaker, tube rack, pipet aid, serological pipets, and needed centrifuge tubes and tissue culture dishes
   - tape a biohazard bag to the front of the hood
4. Spray with 70% ethanol and place the pre-warmed PBS, trypsin, 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 (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[1], gently tap the dish and continue incubating @ 37°C
9. Once cells are detached, place 7 mL of complete medium[2] 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[3] (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

****Complete Media** (cell line specific)
MC3T3-E1: αMEM (Invitrogen cat# 12571) + 10 % Fetal Bovine Serum + 1% Pen/Strep (Invitrogen cat# 15140)
FAK - /- ; +/+: αMEM w/out nucleosides (Invitrogen cat# 12561) + 15 % Fetal Bovine Serum + 1% Pen/Strep
NIH 3T3: DMEM (Invitrogen cat# 11995-065) + 10% Calf Serum + 1% P.S.
See ATCC website for other cell lines (www.atcc.org)

***Trypsin** (cell line specific)
MC3T3-E1: 0.25 % Trypsin (Invitrogen cat# 15050)
FAK - /- ; +/+: 0.25 % Trypsin (Invitrogen cat# 15050)
NIH 3T3: 0.25% Trypsin/0.03% EDTA (Invitrogen cat# 25200-056)

1. If cell detachment does not occur within 10 minutes, the trypsin used may no longer be active (trypsin left at room temp. or in the 37^{o}C water bath too long will lose its activity).

2. The FBS in complete growth medium contains a trypsin inhibitor. Do not use medium without FBS at this step.

3. Trypan Blue Stain allows for both easier viewing/counting of live cells and identifying dead cells (will absorb the dye and appear a darker blue).