Prepared by: Steve Brown	**SOP – SKIN BIOPSY PROCESSING**
Revised by: SB	**October 10, 2006**

PURPOSE:

To establish a standard operating procedure for processing a skin biopsy for tissue culture.

PROCEDURE:

1. Prior to taking biopsy, collection tubes should be prepared, filled with collection medium, and kept on ice.
   1.1. COLLECTION MEDIUM: Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 4500mg/l glucose, 50%Fetal Calf Serum, 1%pennicillin/streptomycin/glutamine solution.
   1.2. Tubes should be almost filled to facilitate immersion of biopsy, which adheres easily to dry surfaces.
2. Biopsies are standard 2mm round dermal punches, taken from upper arm or buttocks.
   2.1. Samples (generally 2 per patient) are immediately immersed in cold medium.
   2.2. Biopsies in this solution, on ice, can be kept for several days. (4 days tested)
   2.3. **Biopsies can be shipped on wet ice at this stage, and are still processable after 4 days.**
3. Place each punch in a separate 4cm culture dish. Incubate in 2ml **warm** DMEM medium supplemented with 20%FCS, 1/100 amphotericin B (Sigma A2942 or equivalent) and 0.2ml Liberase Blendzyme 3 (Roche 1988468) for desired time; see below.
   3.1. Remove samples from tube with tweezers, but be careful not to completely close the tweezers and crush the sample.
   3.2. Amphotericin is supplied as a ready-to-use stock. Liberase should be dissolved in DMEM, 9mg or 28WU in 8ml, and frozen at -20C in 0.2ml aliquots.
   3.3. Concentration and time of digestion will vary according to batch of enzyme. Hence, it is advantageous to titrate a large quantity of enzyme at once.
      3.3.1. Goal: At end of digestion and adherence, multiple clumps containing tens to hundreds of cells. If all cells are dispersed, reduce enzyme up to 4x or time up to 4x or both. If all tissue fragments are whole, increase accordingly.
      3.3.2. A good starting point is 1/100 amphotericin B, 4 hours of digestion.
   3.4. If in doubt, use different conditions on the two digested biopsies.
4. Digest samples 4-14h at 37 degrees / 5-7% CO2. Do not digest longer than 20h.
5. At end of digestion period, remove digestion medium with pipet tip whose end has been cut to allow passage of larger fragments. Place into pointed Falcon tube containing 10ml warm PBS. Rinse plate with a bit more PBS to obtain all fragments.
6. Spin samples 5 minutes at 1000 rpm. Remove supernatant.
7. Resuspend supernatant in 0.2 ml DMEM/20%FCS + amphotericin B, and place in center of fresh 4cm. dish. Overlay fragments with Millicell CM membrane disc (Millipore PICMORG50 or equivalent).

7.1. Prior to use, “feet” should be cut from Millipore filter disc so that it presses tissue fragments against the bottom of the plate.

8. Add 1.5ml growth medium + amphotericin B to interior of disc, 0.5ml to exterior.
   8.1. Spread medium by gently inclining plate in each direction.
9. Incubate plate 1 day without disturbance at 37 degrees / 5-7%CO2.
10. Carefully remove overlay disc from biopsy. Replace with 2ml fresh growth medium + amphotericin B.
    10.1. Tissue fragments should adhere to plate when disc is very gently inclined and then removed.
    10.2. Remove medium by inclining plate and then aspirating from corner.
    10.3. Replace medium with pipet tip against wall of dish to avoid creating waves that might dislodge fragments.
    10.4. If there is lots of blood, wash VERY gently with PBS once prior to replacing medium.
11. Incubate plates for a few weeks at 37 degrees / 5% CO2.
    11.1. Change medium every 3-4 days. One week after biopsy, amphotericin B can be omitted from growth medium.
    11.2. Length of culture will vary with the number of viable fibroblast foci.
      11.2.1. This varies with age of subject, optimization of digestion.
    11.3. Cells are ready to harvest when the combined volume of their foci is about ½ the volume of the plate to which they will be amplified.
12. Trypsinize and replate cells after foci are of reasonable size.
    12.1. I usually trypsinize when cells cover 1/6-1/4 the surface of the plate, and I plate to a single well of a 1-well plate.
    12.2. Trypsinization as for normal cells:
      12.2.1. Aspirate medium and rinse plates with warm PBS.
      12.2.2. Cover with a few drops warm trypsin solution (0.4 ml).
      12.2.3. Incubate 3’ at 37 degrees, or until cells detach.
      12.2.4. Resuspend in 2 ml growth medium.
      12.2.5. Dilute into 10ml PBS in pointed Falcon tube, and spin 5’ at 1000 rpm.
      12.2.6. Remove supernatant. Resuspend cells in 1ml growth medium.
      12.2.7. Place cells into well of 12-well plate.
    12.3. Fragments of tissue are now irrelevant: they can come or stay as it happens.
    12.4. Incubate cells in incubator until they reach confluence (1-4 days).
13. Amplify cells each time they reach confluence, splitting 1:2 or 1:3, until they reach the equivalent of 3x4cm dishes.
    13.1. Cells can be left at confluence up to 2 days without ill effects.
    13.2. It is not necessary to spin and rinse cells after first passage described above.
    13.3. Date and passage number should be noted. Cells will become senescent and useless around passage 20.
14. Freeze cells in growth medium + 10%DMSO, as normally. I use 1x4cm dish per vial.

At this stage the samples can be transported on dry ice, if required (see above for direct transport post-biopsy).