

## **EMBRYONIC RAT PRIMARY SKELETAL MUSCLE CELL CULTURE**

1. The **day before** establishing the primary cultures prepare the following items:
  - Coat forty 60 mm culture plates with a diluted solution of Vitrogen 100 (dilute 1:4 in sterile water). Add 1 mL. Per plate and swirl to cover the entire surface of the plate; leave uncovered overnight in the Cell culture hood under the UV light.
  - Clean the surgical tools with water and place under the UV light in the cell culture hood overnight.
  - Cut a filter (~20-mesh nylon screen). Do not mark the screen with lead or pen (lead or ink may be cytotoxic) prior to cutting; just cut it while holding the screen against the filter holder. Leave under the UV light in the cell culture hood overnight.
  - Check the volumes of PBS and Primary Growth medium to ensure there will be enough for the procedure. Also, check for contamination. Note: the DMEM used for the Primary cultures are Penicillin- and Streptomycin-free. Apparently, Streptomycin inhibits myoblast fusion (see Culture of Animal Cells: A Manual of Basic Technique- R. Ian Freshley, Chapter 20, p270 of the 2<sup>nd</sup> edition).
  
2. Prior to beginning the procedure make sure the following is prepared:
  - PBS
  - Two 150 mm petri plate (not a culture plate)
  - One Biohazard bag
  - Two coffee cans
  - Sterile surgical tools (leave two forceps in the cell culture hood)

- Sterile surgical tools (take the scissors and two forceps to the fume hood).
- A container of 70% ethanol
- A squirt bottle of 70% ethanol
- Put the Primary Growth medium into the 37°C water bath.
- Sterile 50 mL. Conical centrifuge tubes
- Sterile 60 cc syringe
- Assemble the sterile filtration unit (leave it in the cell culture hood)
- Sterile Pasteur Pipets
- Hemacytometer

3. Place the rats in the coffee cans. This is most easily accomplished by tipping the can on its side; picking the rat up by its tail and allowing the rat to seek protection within the can. Return the can to its upright position and cover.
4. Expose the animals to CO<sub>2</sub> until death occurs (-3-4 Minutes depending on the level of gas flow)
5. Squirt ethanol on the belly of the animal and wipe off any debris. Make an incision through the skin but do not puncture the body wall. Incise the skin from stern to stern being careful to keep the surface of the body wall clean; pull the skin back and away from the “sterile” area. Taking a sterile pair of forceps and scissors cut through the body wall without puncturing the internal organs (pull the tissue up away from the organs and then make the incision). Remove the uterus, which contains the fetuses, and place them in a sterile 150mm plate (remove the uteruses from both animals before proceeding). Remove the fetuses from the uteruses and place in the 150mm plate containing PBS (cool from the PBS stored at 4°C).
6. Remove the limbs from the fetuses. Using the scissors cut medio-laterally (from the inside to the arm out towards the shoulder girdle; or from the inside of the leg towards the pelvic girdle) and put the excised limb into a 50mL conical centrifuge tube containing PBS (cool from the PBS stored at 4°C). Note: If it is necessary to hold the

tissue for a few hours put a 50mL. Conical centrifuge tube containing ~ 35 mLs of PBS on **ice** to collect the limbs. The tissue should remain viable for ~2-3 hours).

7. After removing, all the limbs cover the tube and take it into the cell culture hood (after wiling the exterior of the tube with ethanol). Rinse the tissue in PBS ~ 3 times to remove blood. Remove the skin from the limbs and take off the “foot”. Put the skimmed muscle into a 60 mm plate containing 1 mL of PBS. It is easier to dissociate the tissue by “tweezing” if it is slightly moist. Remove any bone that is visible because it will ease filtration later on in the procedure.
8. Pipet 4.5 mLs of PBS into a sterile, uncoated 60mm plate. Taking two opposing tweezers, dissociate the tissue by picking up a small pile and gently tease the muscle apart. The consistency will change as the tissue dissociates; it will become more apt to “hang” together: become more cohesive. Once it becomes more “cohesive”, place the tissue into the 60 mm plate containing 4.5 mLs Of PBS and disperse the tissue in the plate to check the size of the “pieces” (~1 mm x 1 mm seems sufficient).
9. When all the tissue has been “teased”, apart pipet 0.5mLs of a2.5% Trypsin stock solution into the plate and triturate (~5 times up and down) with a 5mL pipet to mix and further dissociate the tissue. Incubate at 37° C, 8% CO<sup>2</sup> for 5 minutes.
10. Following the incubation, add 5.0mLs of Primary Growth medium (the growth medium will interrupt the enzymatic action of the trypsin) to the plate and triturate (this tissue has a great capacity to aggregate and “glom” together). Remove the suspension to a sterile 50mL, conical centrifuge tube containing 2.5 mLs of Primary Growth medium and triturate further (~10 times up and down). Bring the total volume to 25-30 mLs with Primary Growth medium.
11. filter the cell/tissue suspension through the 20-mesh nylon screen using the 60 cc syringe and catch the filtrate in a clean, sterile 50mL conical centrifuge tube. Allow the large pieces of tissue to settle to the bottom and start by filtering the top fluid;

filter the large pieces of tissue last (to prevent excessive clogging of the filter). Allow the majority of the suspension to filter by gravitational pressure but express the last 10mLs of suspension with the plunger of the syringe; the muscle cells seem to aggregate and the yield appears greater when increased pressure is used when filtering.

12. Count the filtered suspension using the hemacytometer. See the procedure for Trypan Blue exclusion (description for using the hemacytometer). Briefly, count 5 squares; the middle and the four corners, take the average of the five counts and multiply by  $10^4$  (there's no dilution factor) and this will give # of cells/mL. Multiply the # of cells/mL by the volume in the conical centrifuge tube to get total # of cells. Dilute the suspension to a concentration between  $1.5 \times 10^6$  and  $2.0 \times 10^6$  cells per mL in Primary Growth medium and dispense 3 mLs per 60 mm Vitrogencoated plate ( $4.5 \times 10^6$  cells to  $6.0 \times 10^6$  cells per plate). Incubate the cells at  $37^\circ\text{C}$ , 8%  $\text{CO}_2$ .