

## **Title: Split Subculturing of C2C12 Cell Line to Setup for Differentiation**

### **Approvals:**

Preparer: W. H. Woodruff Date 20 June 2015

Reviewer: Maggie Bryans Date 12 July 2016

1. Purpose: This SOP will provide the necessary instructions to subculture the C2C12 mouse myoblast cell line, an adherent stem cell line, to prepare for differentiation into myotubes and osteoblasts.
2. Scope: These instructions will enable any qualified technicians to subculture any adherent cell line that can be dissociated with the trypsin enzyme.
3. Responsibilities:
  - 3.1. It is the responsibility of the supervisor to ensure that this SOP is performed as directed and to update the procedure when necessary.
  - 3.2. It is the responsibility of the technicians to follow the SOP as described and to inform the supervisor about any deviations or problems that may occur while performing the procedure.
4. References:
  - 4.1. SOP: ID# REA-006 Preparation of DMEM-HG Stock Reagent
  - 4.2. SOP ID# REA-002 Preparation of 1% and 0.25% Trypsin Stock Reagents
  - 4.3. SOP ID# REA-003 Preparation of DMEM-HG Growth Media
  - 4.4. SOP ID# REA-004 Preparation of Dulbecco's Phosphate Buffered Saline
  - 4.5. SOP ID# REA-005 Preparation of Penicillin and Streptomycin Reagent for Cell Culture
  - 4.6. SOP ID# BTC-010 Preparation of the Laminar Flow Safety Cabinet, Class II, for Aseptic Cell Culture Protocols
  - 4.7. SOP ID# BTC-011 Set Up and Operation of the Olympus Inverted Microscope for Cell Viewing
  - 4.8.
5. Definitions:
  - 5.1. Subculture: the process of expanding growing cells into new cultures vessels by dissociating and distributing the cells.
  - 5.2. Monolayer: single cell layer of growth of adherent cells in an appropriate culture vessel.
  - 5.3. DMEM-HG: Dulbecco's Modified Eagle's (aka, Essential) Media with high glucose. The original DMEM contained 1000 mg per liter glucose; the high glucose contains 4500 mg per liter.

6. Precautions:

- 6.1. All technicians must wear the appropriate PPE for aseptic cell culture work.
- 6.2. All equipment, reagents and cells must be handled aseptically.
- 6.3. Trypsin administered at a high concentration or for too long in contact with the cells will result in a lowered viability.

7. Materials:

- 7.1. Sterile 2 ml, 5 ml and 10 ml pipettes
- 7.2. Auto pipette aid
- 7.3. 12-well sterile culture vessel
- 7.4. 400 ml beaker for waste and discard liquids
- 7.5. 100 ml DMEM-HG growth media, 10% FBS (SOP #REA-003)
- 7.6. 100 ml Dulbecco's Phosphate Buffered Saline (D-PBS) (SOP #REA-004)
- 7.7. 1 ml 0.25% trypsin (SOP #REA-002)
- 7.8. Bone Morphogenic Protein - 2 (BMP-2)
- 7.8. 50 -60% confluent C2C12 healthy culture

8. Procedures:

8.1 General

- 8.1.1. Gather and inventory all the required materials.
- 8.1.2. Prepare the Laminar Flow Safety Cabinet for aseptic culture work (see SOP)
- 8.1.3. Disinfect with 75% ethanol (spray and wipe) and place all required materials into the Laminar Flow Safety Cabinet

8.2. Dissociating the cells from the flask surface

- 8.2.1. Obtain a C2C12 culture from the incubator and examine using the inverted microscope. Check for good morphology, no excess turbidity (sign of contamination), ~60 - 80% confluency and degree of floating, rounded cells. A small amount is an indication of cells dividing; large numbers are often an indicator of excessive cell death.
- 8.2.2. Aseptically remove the culture media in the flask and discard in the waste beaker.  
NOTE: gently "sloshing" the flask before removing the spent culture media will help increase the viability of your culturing by ensuring the dead cells are loose and floating for easier removal.
- 8.2.3. Rinse the cell monolayer with 5 ml of D-PBS.
  - 8.2.3.1. Add 5 ml of D-PBS to the flask

8.2.3.2. "Swirl" the flask to ensure rinsing in all corners and edges

8.2.3.3. Remove the D-PBS to the waste beaker

NOTE: the purpose of this step is to remove all residual Fetal Bovine Serum (FBS) which has an inhibitory effect on the trypsin

8.2.4. Repeat the rinse step (8.2.3.) once

8.2.5. Add 1 ml of 0.25% trypsin to the now empty T-25 flask with the C2C12 cells

8.2.5.1. Carefully swirl to ensure the entire cell layer is covered with trypsin

8.2.6. Place the flask into the 37°C incubator

8.2.6.1. Check the cells for dissociation after 4 – 5 minutes by looking at them under the inverted microscope. Dissociated cells will be rounded and many will be floating. If the majority of the cells are not yet rounded return the flask to the incubator and check again in 3 minutes. Continue this process until ~80% of the cells are rounded and/or floating.

8.2.7. When ready, gently rap the flask against the palm of your hand to dislodge the rounded cells from the surface of the flask into a single cell suspension.

8.2.8. Quickly add 4 ml of DMEM-HG Growth Media, 10% FBS to the 1 ml of trypsin/Cell suspension.

NOTE: the FBS in the growth media will inactivate the protease activity of the trypsin

8.2.9. Using a 5 ml pipette, triturate the cells by rapidly drawing up and expelling the cells while loosely holding the pipette in a corner of the flask. The goal is to achieve a single cell suspension without generating a lot of foam.

TIP: Do not draw up nor expel all of the liquid. Leaving ~ 0.5 ml in the flask or in the pipette will greatly reduce the amount of foam generated.

TIP: At this point, a check under the inverted microscope should reveal an ~95% single cell suspension. Repeat trituration if necessary.

8.2.10. Perform a cell count according to methods in your lab to determine the actual number of cells in the culture.

8.2.11. Dilute the C2C12 cells to 5 - 10 X 10<sup>3</sup> cells per ml

8.3. Distribution of cells to a 12-well plate

8.3.1. Prepare 3 wells of a 12-well plate

8.3.1.1. Using a Sharpie, label all vessels with the cell line, the growth media used, the date and your initials

8.3.1.2. Add a 1 ml volume of diluted cells to each of 3 wells in the plate

8.3.1.2.1. the 3 wells should be labeled 10% FBS, 2% HS (Horse Serum), and BMP-2.

NOTE: at this time all the wells contain cells in DMEM-HG + 10% FBS. The alternate media will be changed at a later step.

8.3.2. Place all plates in the incubator.

8.3.3 Check all cultures for contamination after 24 hours.

8.4. Addition of BMP-2 (day 1 of experiment)

NOTE: dilution of your sample of BMP-2 will be based on the instructions that are supplied with your product. the final concentration that I have found to be successful is 300 ng / ml. I set up my reagent in a 1 ml volume for easy addition.

8.4.1. Check that your culture is contamination free at 24 - 48 hours.

8.4.2. Remove the media from the well labeled BMP-2.

8.4.3. Add 1 ml of DMEM-HG + 10% FBS + 300 mg / ml to the well

8.4.4. return to the incubator.

8.5. Feeding 3 days post- BMP-2 addition.

NOTE: due to the expense of BMP-2, I usually do not feed this well. Having both fed and not fed at this point in different experiments, I have seen no difference in the outcome at day 6+.

8.5.1. Remove the media from the wells labeled 10% FBS and 2% HS

8.5.2. Add 1 ml of DMEM-HG + 10% FBS to the appropriately labeled well

8.5.3. Add 1 ml of DMEM-HG + 2% HS to the appropriately labeled well

8.5.4. Optional: Add 1 ml of DMEM-HG + 10% FBS + 300 ng / ml BMP-2 to the appropriately labeled well

8.5.5. Return to the incubator for analysis on day 6+.

9. History:

Name	Date	Amendment
W.H. Woodruff	20 June, 2015	Initial release
W.H. Woodruff	8 July, 2016	Modified to 12-well plates specifically
W.H. Woodruff	20 September, 2016	BMP-2 addition and feeding schedule