

Title: Myoblast to Osteoblast: Analysis of C2C12 Differentiation Using BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate / nitro blue tetrazolium)

Approvals:

Preparer: W. H. Woodruff Date 20 June 2015
Reviewer: Maggie Bryans Date 12 July 2016

1. Purpose: This SOP will describe the techniques and materials necessary to detect the level of alkaline phosphatase activity in C2C12 cells treated and untreated with bone morphogenic protein-2 (BMP-2).
2. Scope: This protocol is used to determine the success of C2C12 myoblast differentiation under the influence of BMP-2 into osteoblast by comparing the level of alkaline phosphatase (AP) activity. Myoblast have little to no AP activity while osteoblast exhibit very high levels of AP activity. BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate / nitro blue tetrazolium) will produce a dark bluish-to-blackish precipitate in the presence of AP at a pH of 9.5.
3. Responsibilities:
 - 3.1. It is the responsibility of the course instructor /lab assistant to ensure that this SOP is performed as directed and to update the procedure when necessary.
 - 3.2. It is the responsibility of the students/technicians to follow the SOP as described and to inform the instructor about any deviations or problems that may occur while performing the procedure.
4. References:
 - 4.1. SOP: ID# BTC-011 Set Up and Operation of the Olympus Inverted Microscope for Cell Viewing
 - 4.2. SOP: ID# BTC-012 Set Up and Operation of the EVOS Inverted Microscope for Cell Viewing
5. Definitions:
 - 5.1. Alkaline phosphatase: A hydrolase enzyme found in your bloodstream, it helps break down proteins and is mostly produced in the liver, but also in bones.
 - 5.2. BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate / nitro blue tetrazolium): a compound purchased from Sigma (Catalog #B5655) in tablet form that when dissolved in lab grade water acts as a substrate and chromagen to the AP while also creating an isotonic, 9.5 pH environment in which the reaction will occur.
 - 5.3. C2C12: a multipotent mouse myoblast stem cell line that will spontaneously differentiate when over crowded into myotubes, an early stage of muscle development.

- 5.4. Osteoblast: a pre-chondrocyte type cell in the bone lineage
 - 5.5. Bone Morphogenic Protein-2 (BMP-2): belongs to the TGF- β superfamily of structurally related signaling proteins. BMP-2 is a potent osteoinductive cytokine, capable of inducing bone and cartilage formation
6. Precautions:
 - 6.1. All technicians must wear the appropriate PPE for this lab work
 - 6.2. No other precautions are noted.
7. Materials:
 - 7.1. 2, 5 ml serological pipettes
 - 7.2. waste beaker for discard liquid and reagents
 - 7.3. 1X tablet of Sigma*Fast* BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate / nitro blue tetrazolium)
 - 7.4. 10 ml lab grade water
 - 7.5. 12-well plate of 6 day incubated C2C12 with one well untreated (D-MEM-HG + 10% FBS), one well treated with D-MEM_HG + 2% Horse Serum (HS), and one well treated with D-MEM-HG + 10% FBS + 300 nG/ml BMP-2
8. Procedure:
 - 8.1 General
 - 8.1.1. Gather and inventory all the required materials.

NOTE: This protocol does not require aseptic techniques, you may work at an open bench to complete this activity.
 - 8.2. Dissolve 1 tablet of the BCIP/NBT in 10 mL of lab grade water
 - 8.3. Obtain the 12-well plate of 6 day incubated C2C12 with one well untreated (D-MEM-HG + 10% FBS), one well treated with D-MEM_HG + 2% Horse Serum (HS), and one well treated with D-MEM-HG + 10% FBS + 300 ng/ml BMP-2
 - 8.3.1. Compare the differing growth patterns displayed by the BMP-2 treated vs. the untreated. By now you should be able to discern the focal-type pattern of the osteoblast cells. The myoblast should be dense enough to have differentiated and fused into multinucleated myotubes. If the myotubes are established and anchored at both ends, it is possible to see muscle twitch movement within the culture.
 - 8.4. Remove the media from the wells of the untreated controls and treated with BMP-2
 - 8.4.1. It is not necessary to perform this assay on the 2% HS-treated well

8.5. Replace the discarded media with 1 mL per well of the dissolved BCIP/NBT reagent

8.6. Incubate at room temperature for 5 - 20 minutes, until the precipitate is noticeable

8.6.1. Note the difference in the intensity of the precipitate between the control (10% FBS) and the treated (BMP-2) wells. The greater reaction in the BMP-2 treated wells is an indicator of directed differentiation into osteoblast.

9. History:

Name	Date	Amendment
W.H. Woodruff	16 July, 2016	Initial release

NOTE: The usual growth media for C2C12 is D-MEM-HG + 10% FBS. Multipotency is maintained by keeping confluency under ~70 - 80% as fusion occurs when the cells are in high density and close (crowding) to each other. FBS contains growth signals that encourage a high rate of proliferation in the C2C12 cell line and, therefore, it must be monitored regularly to avoid the high cell density that leads to spontaneous differentiation into multinucleated myotubes. The switch to 2% HS is to aid in the fusion of the cells into myotubes. This happens because HS has very little constituent growth signals and the myoblast cells will tend to apply their energy to fusion.

In the interest of full disclosure, I have seen a video of a twitching myotube but have not witnessed this directly in any of my own (or my students') cultures. Perhaps I have not maintained the differentiated cultures long enough for this event to happen.