Title: Transfer of Embryoid Bodies into Gelatin-coated Plate for Differentiation With and Without Retinoic Acid

Approvals:
Preparer: W. H. Woodruff Date 15 July 2016
Reviewer: Maggie Bryans Date 17 July 2016

1. Purpose: This SOP describes the techniques and materials used to transfer the embryoid bodies from the non-binding petri plate into gelatin-coated wells for the purpose of initiating the final steps to differentiation into cardiomyocytes, neurons, and other cell types.

2. Scope: This SOP applies to any mouse embyonic stem cell line that is able to differentiate under these conditions.

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# REA-021 Preparation of 0.1% Gelatin
   4.2. SOP: ID# REA-008 Preparation of mESC Differentiation Media
   4.3. SOP: ID# REA-010 Preparation of 5 mM Retinoic Acid
   4.4. SOP: ID# BTC-010 Preparation of the Laminar Flow Safety Cabinet, Class II, for Aseptic Cell Culture Protocols
   4.5. SOP: ID# BTC-011 Set Up and Operation of the Olympus Inverted Microscope for Cell Viewing
   4.6. SOP: ID# BTC-012 Set Up and Operation of the EVOS Inverted Microscope for Cell Viewing

5. Definitions:
   5.1. mESC Embryoid Bodies (EBs): spherical structures formed by mESCs grown in suspension. The EBs are the pre-stage to differentiation into a variety of cell types.
   5.2. Cardiomyocytes: In culture, these differentiated cells are identified as "beating" cell clusters.
   5.3. Other cell types: May include fibroblasts, epithelial cells, astrocytes, among others.

6. Precautions:

6.1. All activities must be carried out using aseptic techniques and wearing PPE.
6.2. No other precautions are noted for this protocol.

7. Materials:
7.1. Sterile 2 ml, 5 ml and 10 ml pipettes
7.2. Auto pipette aid
7.3. Sterile, 12-well tissue culture plate
7.4. 400 ml beaker for waste and discard liquids
7.5. 1X sterile 15 ml conical centrifuge tube
7.6. 0.5 - 10 ul micropipette + sterile tips
7.5. 10 ml 0.1% gelatin solution, sterile
7.6. 100 ml mESC Differentiation Media
7.7. 25 ul 5mM Retinoic Acid
7.8. Culture of embryoid bodies at ~day 7 in a petri plate

8. Procedure:
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 70% ethanol (spray and wipe) and place all required materials into the Laminar Flow Safety Cabinet

8.2. Gelatin coat the plating wells
8.2.1. Using a sterile serological pipette, put 0.5 ml of 0.1% gelatin into all wells
8.2.2. Incubate for 5 minutes at room temperature
8.2.3. Aspirate off the gelatin solution into a waste beaker
8.2.3.1. The wells are now ready for use
8.2.4. Label the 12-well plate for EBs without RA (spontaneous differentiation) OR with RA (directed differentiation)

8.3. Plating EBs with fresh media without Retinoic Acid (spontaneous differentiation)
8.3.1. Label one (1) sterile 15 ml conical centrifuge tube for each EB plate
8.3.2. Obtain the EB plate from the incubator labeled "w/o RA" and check
8.3.3. Transfer the entire 10 ml of EB suspension into the centrifuge tube
8.3.4. Rinse the petri plate with 5 ml of mESC Differentiation Media and add to the same centrifuge tube
8.3.5. Place the centrifuge tube with the EBs into the incubator for 10 minutes to allow the EBs to settle to the bottom of the tube

8.3.6. Retrieve the EBs / centrifuge tube from the incubator and **carefully** aspirate the supernatant into the waste beaker

8.3.7. Gently resuspend the EBs in ~10 ml of mESC Differentiation Media

8.3.8. Distribute 0.8 to 1.0 ml of the suspended EBs into each gelatin-coasted well

NOTE: DO NOT use a micropipettor, since the opening of the tips is narrow and will crush the EBs, unless you have wide-bore pipette tips.

NOTE: Since the EBs are heavy, they tend to settle to the tip of the pipette fast. It may be helpful to mix the EBs by pipetting gently in between dispensing to each well to get a more homogeneous mix.

8.4. Plating EBs with fresh media with Retinoic Acid (directed neuron differentiation)

8.3.1. Label one (1) sterile 15 ml conical centrifuge tube for each EB plate

8.3.2. Obtain the EB plate from the incubator labeled "w/RA" and check

8.3.3. Transfer the entire 10 ml of EB suspension into the centrifuge tube

8.3.4. Rinse the petri plate with 5 ml of mESC Differentiation Media and add to the same centrifuge tube

8.3.5. Place the centrifuge tube with the EBs into the incubator for 10 minutes to allow the EBs to settle to the bottom of the tube

8.3.6. Retrieve the EBs / centrifuge tube from the incubator and **carefully** aspirate the supernatant into the waste beaker

8.3.7. Gently resuspend the EBs in ~10 ml of mESC Differentiation Media

8.3.8. Spray the barrel of the micropipette with 75% EtOH and attach a sterile tip

8.3.9. Transfer 10 ul of the 5 mM Retinoic Acid to the tube and mix gently but well

8.3.10. Distribute 0.8 to 1.0 ml of the suspended EBs into each gelatin-coasted well

NOTE: DO NOT use a micropipettor, since the opening of the tips is narrow and will crush the EBs, unless you have wide-bore pipette tips.

NOTE: Since the EBs are heavy, they tend to settle to the tip of the pipette fast. It may be helpful to mix the EBs by pipetting gently in between dispensing to each well to get a more homogeneous mix.

8.5. Place the 12-well plate into the incubator and leave for evaluation of differentiation for 2+ days

9. History:

Name	Date	Amendment
W.H. Woodruff	15 July, 2016	Initial release