

E14 ES Cell Embryoid Body Culture (Motor Neuron Directed Differentiation)

Materials:

E14 cell line plate 25 - 60% confluent cultured per HvR lab protocol;
"Non-MEF Stem Cell Culture Procedure"

Polystyrene (non-Tissue Culture plates)

Hemocytometer

1.5 ml microcentrifuge tube

Trypan Blue

0.25% Trypsin-EDTA

D10 Medium:

DMEM High Glucose (500 ml bottle)

FBS Standard (50 ml)

Pen./Strep. (5 ml)

D10 Diff. Medium: (made 250 ml at a time)

D10 Medium (250 ml)

2-Mercaptoethanol 250 ul

D10 - MN Medium (made 50 ml at a time)

D10 Diff. Medium

Shh Working Stock 50 ul

RA Working Stock 50 ul

Sonic Hedghog (Shh) Solutions:

Working Stock (100 ug/ml): add 250 ul of sterile PBS to each 25 ug vial
(make 50 ul aliquots in 1.5 ml tubes and store at -20 deg. C)

Culture Concentration (100 ng/ml): dilute working stock 1:1,000 in medium

Retinoic Acid (RA) Solutions (Very sensitive to light):

Working Stock (2mM): measure out 6 mg of RA into 10 mL of DMSO
in a foil wrapped 15 ml tube.

Filter sterilize through a PES 0.2 um filter and make 50 ul aliquots in amber 1.5 ml
tubes and store at -20 deg. C.

Culture Concentration (2 uM): dilute working stock 1:1,000 in medium.

2-Mercaptoethanol Solutions:

Mfg Stock: 14 Molar

Working Stock (50 millimolar): dilute 1:280 i.e. 7.14 ul into 2 ml
(filter sterilize, then make 50 ul aliquots and freeze)

Culture Concentration (50 nM/ml): dilute working stock 1:1,000 in medium

Protocol:

- 0) Warm medium and trypsin in the 37 deg. C water bath & label the polystyrene plates with cell line info, date & your initials.
- 1) Add 15 ml D10 Medium to the number of polystyrene plates desired for EB culture; and place in incubator.
- 2) Suction the growth medium off of the E14 culture plate, rinse with sterile PBS and suction off the PBS.

- 3) Add 1 ml of Trypsin solution to the plate and tilt/swirl to coat the surface, then tilt the plate suction off half of the Trypsin. Place plate in the incubator for 2-3 minutes.
 - 4) Prepare a 15 ml conical tube for washing the cells: add 12 ml of warm D10 medium to a 15 ml tube.
 - 5) Tap the side of the E14 cell line plate to loosen the cells, check under the microscope to see that the cells are floating, then pipette up and “wash” the plate surface with the Trypsin to dislodge the remaining cells.
 - 6) Transfer 1-3 ml of D10 medium from the 15 ml tube into the plate and “wash” the plate surface again.
 - 7) Transfer the Trypsin/Cell Slurry to the 15 ml conical tube and centrifuge for 5 minutes at 200g to pellet the cells.
 - 8) Re-suspend the cells in 500 ul D10 culture medium, pipetting up and down 2x.
 - 9) Use the Trypan Blue to count the cells according to the HvR Lab Method, “Adherent Culture Cell Counting”.
 - 10) Retrieve the plates from the incubator and transfer 100 thousand viable cells to each plate, and place the plates in in the incubator for culture.
 - 11) Culture the cells for 3 days adding 5 ml medium per day.
This culture step allows for the formation of tight spheroid embryoid bodies.
Differentiation occurs randomly as embryoid bodies initially develop.
 - 12) On Day 3 (which is referred to as Embryoid Body Day 0 (EB D0), tilt the plate and visualize the EBs descending toward the bottom edge of the plate. Carefully suction off as much of the medium as possible without losing EBs, then add 20 ml warm D10-MN medium.
- * One half of the medium must be changed every day after the 3rd day.
- 13) Culture the EBs for 5 days changing out half of the medium each day; tilt the plate and visualize the EBs descending toward the bottom edge of the plate. Carefully suction off half (~ 10 ml) medium as possible without losing EBs, then add 10 ml warm D10-MN medium.
 - 14) Replace the 10 ml of medium with fresh D10-MN medium and return the plate to the incubator.
 - 15) EBs can be harvested by transferring the medium plus embryoid bodies to a 15 ml conical tube and allowing the EBs to settle to the bottom for approximately 5 minutes before transferring for analysis or selection for further culture.

** If cells adhere to the bottom of the plate, immediately transfer the embryoid bodies and growth medium to a new plate so that the adherent cells do not exert phenotypic influence on the embryoid bodies.

Note: Purmorphamine can be substituted for Shh, ~ \$150.00 for 5 mg for a 50 mM stock and can be diluted 1:50,000 to 1 uM in the plates. Google & PubMed pull up ES differentiation articles for Motor Neurons.