

E14 ES Cell Embryoid Body Suspension Culture (Endothelial Cell 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
Fibronectin (1 mg/ml): 1:100 is 10 ug/ml on the plate, 50 ul into 5 ml PBS

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 – EC Medium: (made 50 ml at a time)

D10 Diff. Medium
VEGF Working Solution 50 ul

VEGF Solutions:

Mfg Stock, (20 ug/tube)
Working Stock (20 ug/ml): dissolve VEGF in 1 ml sterile water
(make 50 ul aliquots and freeze)
Culture Concentration (20 ng/ml): 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 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+EC 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 at a time as needed (approx. every 2 days); 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+EC medium.
 - 14) On Embryoid Body Day 5 (EB Day 5), prepare Fibronectin coated TC plates (10 ug/ml) using 50 ul Fibronectin in 5 ml sterile PBS to coat the plate for 30 min at 37 deg. C. Rinse the plate with sterile PBS before adding medium and cells.
 - 15) Harvest the EBs by suctioning off half of the medium, transferring the medium plus embryoid bodies to a 15 ml conical tube and allowing the EBs to settle to the bottom for 3-5 minutes.
 - 16) Rinse with 5 ml sterile PBS and allow EBs to re-settle before removing PBS. Then add 500 ul of Trypsin, allow 3-5 minutes of incubation at 37 deg. C, then pipette up and down to make a single cell suspension.

- 17) Wash the cells with 10 ml D10 Diff. Medium, 5 min. centrifugation at 200-250xg, then suction off the supernatant.
- 18) Resuspend the cells in 1 ml D10 – EC medium and transfer to the Fibronectin plate containing 10 ml D10 –EC medium.
- 19) Culture for 5 days for FLK1-GFP or 7 days for Tie1-GFP, with medium changes every 2-3 days.

** 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.