Protocol for trilineage differentiation using STEMdiff™ Trilineage Differentiation Kit

A. PLATING HUMAN ES/iPS CELLS FOR TRILINEAGE DIFFERENTIATION

1. On day 0, warm to room temperature sufficient volumes of mTeSR™ Plus and DMEM/F-12 and Gentle Cell Dissociation Reagent for passaging.
2. Prepare Single-Cell Plating Medium by adding Y-27632 to mTeSR™ Plus to reach a final concentration of 10 µM.
3. Wash each well to be passaged with 2 mL of D-PBS (Without Ca++ and Mg++).
4. Aspirate the wash medium and add 1 mL/well of Gentle Cell Dissociation Reagent.
5. Incubate at 37°C for 3 - 5 minutes.
6. In each well, dislodge cells by pipetting up and down 1 - 3 times using a pipette with a 1 mL tip.
7. Immediately transfer cells to a tube containing 1 mL of DMEM/F-12 per well harvested. Wash each well once with 1 mL of DMEM/F-12 to collect any remaining cells and transfer to the tube. Centrifuge the tube at 300 x g for 5 minutes.
8. Remove supernatant. Resuspend cells in 1 mL Single-Cell Plating Medium (prepared in step 2).
9. Count viable cells using Trypan Blue and a hemocytometer.
10. Aspirate Geltrex from coated 6-well plates. Add 2 mL of Single-Cell Plating Medium per well.
11. Add the appropriate number of cells to the medium-containing wells. The required densities for each lineage are indicated in Table 1.

Table 1. Plating Densities for Ectoderm, Mesoderm, and Endoderm Lineages

Lineage	Cell density ($cell/cm^2$)	Total number of cells per well		
		24-well plate	12-well plate	6-well plate
Ectoderm	200000	400000	800000	2000000
Mesoderm	50000	100000	200000	500000
Endoderm	200000	400000	800000	2000000

12. Place the plate in a 37°C incubator. Move the plate in several quick, short, back-and-forth and side-to-side motions to evenly distribute the cells. Do not disturb the plate for 24 hours. Continue to section B for differentiation.

B. DIFFERENTIATING MONOLAYER CULTURES TO THREE GERM LINEAGES

1. On day 1, warm the three types of STEMdiff™ Trilineage media to room temperature (15 - 25°C).
2. Aspirate media from cell cultures.
3. Add the appropriate STEMdiff™ Trilineage medium to each well.

4. Incubate at 37°C for 24 hours.
5. Repeat steps 1 - 4 until day 5 (mesoderm and endoderm lineages) or day 7 (ectoderm lineage).