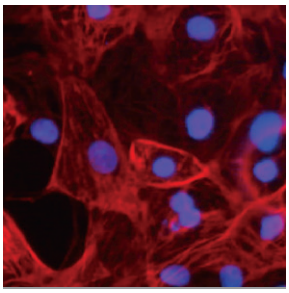

Stem Cell Application Protocol

Spin Embryoid Body Formation and Confirmation of Pluripotency

With the Cellartis® DEF-CS™ 500 Culture System

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I. Introduction

Embryoid bodies (EBs) are three-dimensional aggregates comprised of human pluripotent stem cells (hPSCs). hPSCs within EBs undergo differentiation and cell specification along the three germ layers, which are commonly used as an assessment of the initial hPSCs' pluripotency. The protocol for formation of spin EBs can also be used as a basis for the development of protocols for directed differentiation.

II. Materials Required

- 24-well plates, cell culture treated, flat bottom
- 96-well plates, untreated, V bottom
- Advanced RPMI 1640 (with glucose, sodium pyruvate, and non-essential amino acids; without L-glutamine and HEPES)
- B-27 Supplement (50X), serum free
- DEF-CS COAT-1
- GlutaMAX-I (100X; 200 mM)
- PBS Dulbecco's with Ca^{2+} & Mg^{2+} (D-PBS +/+)
- PBS Dulbecco's w/o Ca^{2+} & Mg^{2+} (D-PBS –/–)
- Penicillin-streptomycin (PEST; 10,000 units/ml of penicillin and 10,000 µg/ml of streptomycin)
- TrypLE Select Enzyme (1X), w/o phenol red
- Y27632

III. Protocol

A. Medium Preparation

Preparing the *In Vitro* Differentiation (IVD) Medium

Prepare the IVD medium by adding 10 ml B-27 Supplement (50X), 5 ml GlutaMAX-I (100X), and 5 ml PEST to 500 ml of Advanced RPMI 1640. Mix the solution properly and carefully. The medium expires one month after the date of preparation.

Preparing the Seeding Medium

Prepare the seeding medium by adding Y27632 (to a final concentration of 5 µM) to the IVD medium. Prepare fresh medium on the day of intended use.

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B. Formation of Spin EBs

1. Warm the seeding medium to 37°C ± 1°C and all other reagents to room temperature (RT, 15–25°C) before use.
2. Wash **one** T25 flask containing confluent hPSCs with 5 ml D-PBS –/–.

   NOTE: The entirety of this flask will be used for spin EB formation. Other flasks or banked cells of equivalent passage number from the original line should be set aside if spin EB formation is being used to assess the pluripotency of that line.

3. Add 500 µl of TrypLE Select (20 µl/cm^2) and place the cells in an incubator at 37°C ± 1°C, 5% CO_2, and >90% humidity for 5 min, or until cells start to detach.
4. Add 5 ml of seeding medium and dissociate the cells, pipetting up and down until a single-cell suspension is achieved.
5. Count the cells to determine the initial cell concentration.
6. Make a cell suspension of 20 ml seeding medium containing 2.5 x 10^5 cells/ml.
7. Seed 200 µl of the final cell suspension into each well of a 96-well plate (untreated, V bottom), generating a seeding density of 5 x 10^4 cells/well.
8. Centrifuge at 400*g* at RT for 5 min.
9. Place the 96-well plate in the incubator at 37°C ± 1°C, 5% CO_2, and >90% humidity and let it sit undisturbed for 7 days to form EBs.

   NOTE: Throughout this process, the cells are undergoing spontaneous differentiation. During the incubation period, directed differentiation protocols can be optimized and applied. Alternatively, if pluripotency is to be assessed, continue with Section III.C.

C. Spontaneous Differentiation of Spin EBs

Coating of the Cell Culture Plate

1. Dilute the required volume of DEF-CS COAT-1 in D-PBS +/+ before use. Make a 1:20 dilution.
2. Mix the diluted DEF-CS COAT-1 solution gently and thoroughly by pipetting up and down.
3. Add the appropriate volume of diluted DEF-CS COAT-1 solution to a 24-well plate (use 0.2 ml/cm^2), making sure the entire culture surface of each well is covered.
4. Place the plate for a minimum of 20 min in an incubator at 37°C ± 1°C, 5% CO_2, and >90% humidity for 0.5–3 hr at RT.
5. Aspirate DEF-CS COAT-1 solution from the plate immediately before use.

Transferring of Spin EBs

1. Warm IVD medium to 37°C ± 1°C.
2. Add 1.5 ml of fresh, warm IVD medium to each well of the coated 24-well plate.
3. Carefully detach and transfer the EBs using a pipette. Transfer 5–7 EBs to each well of the 24-well plate.
4. Place the 24-well plate in the incubator at 37°C ± 1°C, 5% CO_2, and >90% humidity and let it sit undisturbed for 4 days.

Exchanging the Media

NOTE: Complete a 100% media change every 2–3 days.

1. Warm IVD medium to 37°C ± 1°C.
2. Completely exchange the media in each well of the 24-well plate (1.5 ml/well).

   NOTE: 18–21 days after the start of spin EB formation, the cells are ready to be analyzed for the presence of specialized cells along the three germ layers. The number of days needed depends on the cell line.