SOP SCB 020_v1	Title: **Embryoid Bodies (EBs) formation and three germ layer differentiation.**

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OBJECTIVE

Formation of EBs (Embryoid Bodies) from hPSC (Human Pluripotent Stem Cells) lines and their subsequent differentiation to the three germ layers: Ectoderm, Endoderm and Mesoderm.

MATERIALS

- Phosphate buffered saline (PBS) without magnesium and calcium (Biowest, Ref. L0615-500)
- EDTA 0.5M (Invitrogen, Ref. 15575-038)
- EB's differentiation media (For the preparation Media instructions see below):
  - Ectoderm (N2/B27).
  - Endoderm (EBm).
  - Mesoderm (EBm + AA).
- mTeSR1 Basal Medium Kit (StemCell Technologies, Ref. 05850)
- Matrigel (Corning, Ref. 356234)
- L-ascorbic acid powder (Sigma-Aldrich, Ref. A4544-25G)

PROCEDURE

Day 1:

1. Once colonies are compact and reach approximately 90% of the confluency of a 100 mm plate, aspirate the medium and wash the plate with 5 mL of PBS.
2. Eliminate PBS and add 3 mL of EDTA 0.05mM solution (Diluted in PBS and filter sterilized).
3. Incubate for 2 min at 37 °C and 5% CO_2.
4. Remove EDTA.
5. Lift colonies flashing with fresh mTSER complete medium **(**see **SOP SCB 003_v1)** and collect them in a 50 mL falcon tube, as if it was a split **(**see **SOP SCB 016_v1**).
6. Take 2/3 of the final volume (The other 1/3 could be used for other characterization procedures) and transfer it to a 50 mL falcon tube.
7. Complete with mTSER complete medium until getting a final volume of 15 mL.

8. Transfer the 15 mL to a multichannel pipette reagent reservoir and using a multichannel pipette seed 150 µl per well in a 96-well plate.
9. Centrifuge the 96-well plate at 800 g for 10 minutes.
10. Incubate for 24 hours at 37 °C and 5% CO_2.

Day 2:

1. Prepare two 60 mm ultra-low attachment (ULA) plates, one of them with 5 mL of mTSER and the other empty.
2. Using a microscope and a p1000 micropipette pick up the EBs formed in the 96-well plate one by one and place them at the empty ULA plate.
   Note: If the EBs break when they are being collected, let them incubate for another 24 hours.
3. After assuring that every EBs were collected from de 96-well plate transfer all of them to the ULA plate with the 5mL of fresh medium, trying not to transfer a large quantity of the previous media.
4. Incubate the EBs at 37 °C and 5% CO_2 for 24-48 hours.

Day 3:

1. Label 3 matrigel-coated Slide flasks (see **SOP SCB 004_v1**) with each one of the three-germ layer names (ECTO, ENDO, MESO).
2. Add 2 mL of the respective differentiation media and Matrigel solution at a final concentration of 0.092 mg/mL to each one of the slide flasks.
3. Pick up approximately 30 to 33 EBs and seed them in each slide flask.
4. Incubate at 37 °C and 5% CO_2.
5. Proceed to change the medium every 2 days. (Now without Matrigel)
   Note: For the Ectoderm differentiation media (N2/B27) changing, only eliminate 1 mL and add 1 mL of fresh medium.
6. Cultured conditions in the differentiation media would be held for 21 days for Endoderm and Mesoderm, and for 28 days in the case of ectoderm.
7. After the indicated days have passed, the slide flasks are fixed and analyzed by immunocytochemistry through IDIBELL's Histology Platform (See **SOP SCT-Hi-PNT-04002(ENG**)). Take confocal images using Leica TSC SPE/SP5 microscopes.

EBs DIFFERENTIATION MEDIA PREPARATION:

- **Ectoderm - N2/B27 Media:**
  50% Neurobasal medium (Gibco, Ref.21103-049)
  50% DMEM/F12 (Gibco, Ref. 21331020)
  0.5% N2 supplement (Gibco, Ref. 17502048)
  1% B27 supplement (Gibco, Ref. 17504-044)
  1% Glutamax (Gibco, Ref. 35050-038)
  1% Penicillin-Streptomycin (Gibco, Ref. 15140-122)

- **Endoderm – EBm:**

87% Knockout-DMEM (Gibco, Ref. 1082958)
10% Hyclone FBS (Cytiva, Ref. SV3016003)
1% NEAA (Gibco, Ref. 11140035)
0.1% β-mercaptoethanol (Gibco, Ref. 31350-010)
1% Glutamax (Gibco, Ref. 35050-038)
1% Penicillin-Streptomycin (Gibco, Ref. 15140-122)

- **Mesoderm – EBm + AA:**
  100% Endoderm medium
  0.5mM ascorbic acid solution*
  Note: Ascorbic acid must be added just before the media change.

  ***AA solution preparation:**
  1. Using a scale, weigh 88 mg of L-ascorbic acid powder in a microcentrifuge tube.
  2. Dissolve it in 1mL of KO DMEM media.
  3. Filter through a 0.22µm filter.
  4. Aliquot the final volume.
  5. Keep aliquots at -20°C until their use.
  NOTE: The aliquots ONLY must be kept for 1 month maximum.

NOTE: All the components of each media are mixed and then filtered through filtration unit MILLIPORE Express PLUS (0.22µm).