Embryoid body formation from cells cultured in Essential 8™ Medium

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WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from **thermofisher.com/support**.

Introduction

This protocol describes the generation of embryoid bodies (EBs) using KnockOut™ Serum Replacement (KSR) containing EB medium from pluripotent stem cells (PSCs) that were cultured in Essential 8™ Medium on Geltrex™ matrix- or vitronectin-coated culture vessels. EBs are floating spherical clusters of PSCs that allow you to test the *in vitro* differentiation potential of the cells. EBs can be initiated at a normally scheduled passage, but instead of replating the cells onto fresh Geltrex™ matrix- or vitronectin-coated dishes, the cells are plated onto non-tissue culture treated dishes to prevent attachment. Refer to the **Essential 8™ Medium user guide** (Pub. no. MAN0007569) for detailed instructions on culturing human pluripotent stem cells under feeder-free culture conditions in Essential 8™ Medium.

Materials needed

- Essential 8™ Medium (Cat. no. A1517001)
- Dulbecco's Phosphate Buffered Saline (DPBS) without calcium and magnesium (Cat. no. 14190-250)
- Collagenase Type IV (Cat. no. 17104-019)
- DMEM/F-12 (1X), Liquid (1:1), with GlutaMAX™-I (Cat. no. 10565-018)
- KnockOut™ Serum Replacement (Cat. no. 10828010)
- FGF-basic (AA 1-155) Recombinant Human (Cat. no. PHG0264)
- MEM™ Non-Essential™ Amino Acids Solution (100X) (Cat. no. 11140-050)
- 2-Mercaptoethanol (55 mM), Liquid (Cat. no. 21985-023)
- Geltrex™ LDEV-Free hESC-qualified Reduced Growth Factor Basement Membrane Matrix™ (Cat. no. A1413302) for general applications or Vitronectin (VTN-N) Recombinant Human Protein, Truncated (Cat. no. A14700) for xeno-free applications
- Non-tissue culture treated culture vessels or Nunclon™ Sphera™ 60-mm dishes (Cat. no. 12-566-437)
- Sterile cell culture hood (i.e., biosafety cabinet)
- Inverted microscope
- Incubator set at 37°C, 5% CO_2
- Water bath set at 37°C
- Sterile serological pipettes (5-mL, 10-mL)
- Centrifuge
- 15-mL centrifuge tubes
- Cell scrapers

Prepare media and reagents

Basic FGF solution (for 1 mL of 10 µg/mL solution)

1. To prepare 1 mL of basic FGF (bFGF) solution at a final concentration of 10 µg/mL, aseptically mix the following components:

bFGF	10 µg
DPBS without Ca and Mg	990 µL
KnockOut™ Serum Replacement	10 µL

2. Aliquot and store at –20°C for up to 3 months. Once the bFGF aliquot is thawed, store at 2–8°C and use within 7 days.

Collagenase IV solution (for 50 mL of 2 mg/mL solution)

1. To prepare 50 mL of 2X Collagenase IVsolution at a final concentration of 2 mg/mL, aseptically mix the following components:

Collagenase IV	100 mg
DMEM/F-12 with GlutaMAX™-I	50 mL

2. Sterilize through 0.22-µm filter and store at 2–8°C for up to 2 weeks.

EB medium (100 mL of complete medium)

1. To prepare 100 mL of complete EB Medium, aseptically mix the following components:

DMEM/F-12 with GlutaMAX™-I	79 mL
KnockOut™ Serum Replacement	20 mL
MEM™ Non-Essential™ Amino Acids Solution	1 mL
2-Mercaptoethanol (55 mM)	100 µL

2. Sterilize through 0.22-µm filter and store at 2–8℃ for up to 4 weeks.

3. When indicated (first 24 hours of the procedure), add bFGF to a final concentration of 4 ng/mL before use (e.g., 40 µL of reconstituted bFGF at 10 µg/mL per 100 mL of medium).

Essential 8™ medium (500 mL of complete medium)

1. Thaw frozen Essential 8™ Supplement (50X) at room temperature for ~1 hour. **Do not thaw the frozen supplement at 37°C.**

2. To prepare 500 mL of comp°Clete Essential 8™ Medium, aseptically mix the following components:

Essential 8™ Basal Medium	490 mL
Essential 8™ Supplement (50X)	10 mL

3. Complete Essential 8™ Medium can be stored at 2–8℃ for up to 2 weeks.

   Note: Before use, warm complete medium required for that day at room temperature until it is no longer cool to the touch. **Do not warm the medium at 37℃**.

Embryoid body (EB) formation

Note: The volumes given in the following procedure are for 60-mm culture dishes. For culture vessels with different sizes, adjust the volumes appropriately.

Prior to day 0

Culture cells under feeder-free conditions in Essential 8™ Medium on Geltrex™ matrix- or vitronectin-coated culture vessels.

Note: See the "Appendix" on page 4, for the appropriate coating procedures.

Day 0: EB formation

1. When the cultures are 80–85% confluent, the cells are ready to be harvested for EB formation.

   Note: It is important that the colonies are not small and overcrowded, but rather are allowed to grow robust in individual size for about 4 days (~1200 to 1500 µm in diameter).

2. Aspirate the spent medium from the culture vessel and briefly wash once with 5 mL of DPBS without Ca and Mg (for a 60-mm dish).

3. Aspirate the DPBS without Ca and Mg, and add 2 mL of 2X Collagenase IV solution (2 mg/mL), pre-warmed to 37℃. Ensure complete coverage of culture surface with the Collagenase IV solution.

4. Incubate the cultures grown on vitronectin-coated vessels for 5–10 minutes in a 37℃, 5% CO_2 incubator until the edges of the colonies begin to curl and detach from the plate. Do not overexpose the cultures to 2X Collagenase IV solution.

   Note: Cultures grown on Geltrex™ matrix may take longer to detach. Incubate cultures grown on Geltrex™ matrix for 15–20 minutes.

5. Aspirate off the 2X Collagenase solution and briefly wash the culture with 5 mL of pre-warmed DMEM/F- 12 medium.

6. Aspirate off the wash solution and add 3 mL of complete EB medium containing 4 ng/mL of bFGF to dilute any remaining Collagenase IV solution.

7. Gently dislodge the colonies from the plate using a cell scraper, and then wash by pipetting them up and down a few times in a 5-mL serological pipette.

   Note: Optimal fragment size for the colonies is critical for successful EB formation. Make sure not to triturate the colonies into very small fragments to ensure good fragment size.

8. Transfer the suspended colony clusters into a 15-mL conical tube.

9. Add an additional 2 mL of complete EB medium to the dish to dislodge the remaining colonies and transfer them to the 15-mL tube.

10. Let the colony fragments sediment at the bottom of the 15-mL tube for 5–7 minutes by gravity.

11. Gently aspirate off the supernatant, add 3 mL of complete EB medium with 4 ng/mL of bFGF, and gently resuspend the sedimented colony fragments by pipetting up and down 2 times.

12. 5 mL of complete EB medium is recommended for a 60-mm non-TC treated dish. Transfer the 3 mL cell suspension drop-wise to a non-TC treated culture dish that has been pre-aliquoted with 2 mL of complete EB Medium with 4 ng/mL of bFGF. This will give a final volume of 5 mL in the 60-mm culture dish.

13. Place the culture dish containing the cell clusters in a 37℃, 5% CO_2 incubator and incubate overnight.

Day 1:

1. Transfer the contents of the non-TC dish to a 15-mL conical tube. Use 2 mL of EB medium without bFGF to wash the dish to gather any remaining EBs and pool into the conical tube.
2. Gravity sediment the EBs for 5–10 minutes.
3. Aspirate off the supernatant; this step removes bFGF and single cells
4. Resuspend the sedimented EBs in 3 mL of EB Medium (without any bFGF from here on).
5. Transfer all 3 mL of EB suspension drop-wise to a new 60-mm non-TC treated dish or Nunclon™ Sphera™ dish that has been pre-aliquoted with 2 mL of EB Medium without bFGF.

Option A: Analysis on day 7 using the TaqMan® hPSC Scorecard™ panel

1. Continue incubation at 37°C, 5% CO_2 until Day 7, repeating the Day 1 harvest and feed procedure every other day.

   After Day 1, it is not necessary to use a new dish for each feed.

2. On Day 7, harvest the EBs for analysis using the TaqMan® hPSC Scorecard™ Panel (Cat. nos. A15870, A15871, A15872, and A15876).

   Note: For detailed instructions on sample preparation and qRT-PCR using the TaqMan® hPSC Scorecard™ Panel, refer to the **TaqMan® hPSC Scorecard™ Panel user guide** (Pub. no. MAN0008384).

Option B: Immunocytochemistry (ICC) on day 21

1. On Day 4, seed the EBs in EB medium without bFGF on Geltrex™ matrix-coated tissue culture multi-wells plates.
2. Change the spent medium every other day for three weeks.
3. On Day 21, perform ICC. Cells can be fixed with 4% paraformaldehyde for further staining.

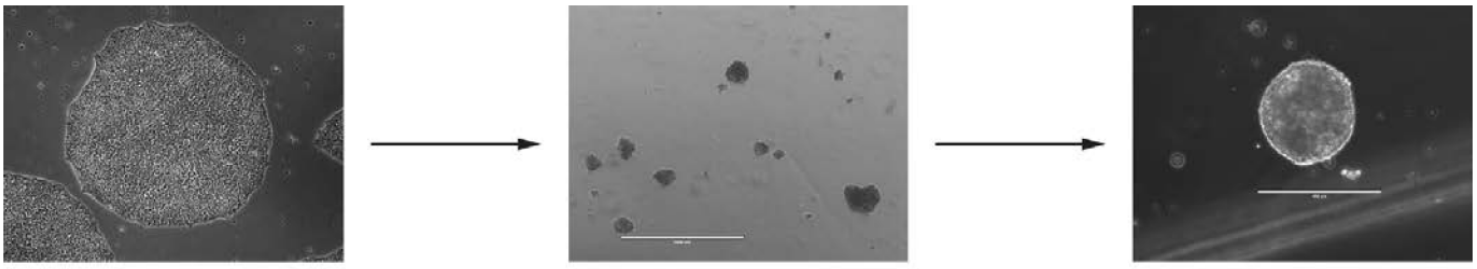

Figure 1 (A) iPSC derived using CytoTune™ iPSC reprogramming kits on feeders (BS3-C) and then cultured in Essential 8™ Medium on vitronectin-coated culture vessels were harvested using 2X Collagenase IV solution (2 mg/mL) and allowed to form EBs using the procedure described above. The EBs were harvested on Day 7 using TRIzol™ reagent and analyzed using the TaqMan® hPSC Scorecard™ Panel as described in the product user guide.

	Pluri	Endo	Meso	Ecto
BS3C-p47-E8-Undiff	−0.14	−0.57	−0.72	−1.76
BS3C-E8-Col-EB-sphera	−0.77	2.16	3.06	0.93
BS3C-E8-Col-EB-petri	−0.87	2.05	3.09	0.90

Gene expression relative to the reference standard	
x > 1.5	Upregulated
1.5 ⩾ x > 1.0	
1.0 ⩾ x > 0.5	
0.5 ⩾ x > −0.5	Comparable
−0.5 ⩾ x > −1.0	
−1.0 ⩾ x > −1.5	
x < −1.5	Downregulated

Figure 2 (B) The heat map analysis shows differentially expressed markers for pluripotent cells and the three expected differentiation lineages (endoderm, mesoderm, ectoderm) between undifferentiated and Day 7 EBs. Each input sample mean of t-statistic over gene categories of pluripotent and differentiation lineages is represented as a number with values –0.5 to 0.5 indicating comparable (white), <–0.5 lower expression (light blue to dark blue with increasing downregulation) and >0.5 higher expression (light red to dark red with increasing upregulation).

Appendix

Coat culture vessels with vitronectin (VTN-N)

1. Upon receipt, thaw the vial of vitronectin at room temperature and prepare 60-µL aliquots of vitronectin in polypropylene tubes. Freeze the aliquots at –80°C or use immediately.

2. Prior to coating culture vessels, calculate the working concentration of vitronectin using the formula below and dilute the stock appropriately. Refer to Table 1, for culture surface area and volume required.

   Note: The optimal working concentration of vitronectin is cell line dependent. We recommend using a final coating concentration of 0.5 µg/cm^2 for human PSC culture.

$$\text{Working Conc.} = \text{Coating Conc.} \times \frac{\text{Culture Surface Area}}{\text{Volume Required for Surface Area}}$$

$$\text{Dilution Factor} = \frac{\text{Stock Concentration (0.5 mg/mL)}}{\text{Working Concentration}}$$

Table 1 Required volume of diluted vitronectin substrate

Culture vessel	Approx. surface area	Diluted substrate volume
6-well plate	10 cm^2/well	1 mL/well
12-well plate	4 cm^2/well	0.4 mL/well
24-well plate	2 cm^2/well	0.2 mL/well
35-mm dish	10 cm^2	1 mL
60-mm dish	20 cm^2	2 mL
100-mm dish	60 cm^2	6 mL

3. To coat the wells of a 6-well plate, remove a 60-µL aliquot of vitronectin from –80°C storage and thaw at room temperature. You will need one 60-µL aliquot per 6-well plate.

4. Add 60 µL of thawed vitronectin into a 15-mL conical tube containing 6 mL of sterile DPBS without Calcium and Magnesium at room temperature. Gently resuspend by pipetting the vitronectin dilution up and down.

   Note: This results in a working concentration of 5 µg/mL (i.e., a 1:100 dilution).

5. Aliquot 1 mL of diluted vitronectin solution to each well of a 6-well plate (refer to Table 1, for recommended volumes for other culture vessels).

   Note: When used to coat a 6-well plate (10 cm^2/well) at 1 mL/well, the final concentration will be 0.5 µg/cm^2.

6. Incubate at room temperature for 1 hour.

   Note: Dishes can now be used or stored at 2–8°C wrapped in laboratory film for up to a week. Do not allow the vessel to dry. Prior to use, pre-warm the culture vessel to room temperature for at least 1 hour.

7. Aspirate the diluted vitronectin solution from the culture vessel and discard. It is not necessary to rinse off the culture vessel after removal of vitronectin. Cells can be passaged directly onto the vitronectin-coated culture dish.

Coat culture vessels with Geltrex™ LDEV-Free, hESC-Qualified basement membrane matrix

1. Thaw a 5-mL bottle of Geltrex™ LDEV-Free hESC-Qualified Reduced Growth Factor Basement Membrane Matrix™ at 2–8°C overnight.

2. Dilute the thawed Geltrex™ solution 1:1 with cold sterile DMEM/F-12 to prepare 1-mL aliquots in tubes chilled on ice. These aliquots can be frozen at –20°C or used immediately.

   Note: Aliquot volumes of 1:1 diluted Geltrex™ solution may be adjusted according to your needs

3. To create working stocks, dilute a Geltrex™ aliquot 1:50 with cold DMEM/F-12 on ice, for a total dilution of 1:100.

   Note: An optimal dilution of the Geltrex™ solution may need to be determined for each cell line. Try various dilutions from 1:30 to 1:100.

4. Quickly cover the whole surface of each culture dish with the Geltrex™ solution (refer to Table 2).

Table 2 Volume of Geltrex™ hESC-qualified matrix required

Culture vessel	Approx. surface area	Diluted substrate volume
6-well plate	10 cm^2/well	1.5 mL/well
12-well plate	4 cm^2/well	750 µL/well
24-well plate	2 cm^2/well	350 µL/well
35-mm dish	10 cm^2	1.5 mL
60-mm dish	20 cm^2	3 mL
100-mm dish	60 cm^2	6 mL

5. Incubate the dishes in a 37°C, 5% CO_2 incubator for 1 hour.

   Note: Dishes can now be used or stored at 2–8°C for up to a week. Do not allow dishes to dry.

6. Aspirate the diluted Geltrex™ solution from the culture dish and discard. You do not need to rinse off the Geltrex™ solution from the culture dish after removal. Cells can now be passaged directly onto the Geltrex™ matrix-coated culture dish.

Limited product warranty

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