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Culturing Pluripotent Stem Cells (PSCs) in Essential 8™ Medium

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Introduction

Essential 8™ Medium is a fully defined, feeder-free medium formulated for the growth and expansion of human pluripotent stem cells (PSCs). Originally developed by Chen *et al.*[1] in the laboratory of James Thomson, and validated by Cellular Dynamics International, Essential 8™ Medium has been extensively tested and is proven to be able to maintain pluripotency in multiple PSC lines. Unlike most feeder-free media, Essential 8™ Medium does not require the presence of BSA (bovine serum albumin) or HSA (human serum albumin) that contributes to lot-to-lot variability. In addition, most serum-free media consist of more than 20 components, adding complexity, time, and cost, while Essential 8™ Medium is comprised of only eight components. Complete Essential 8™ Medium is prepared by adding Essential 8™ Supplement (50X) to Essential 8™ Basal Medium, which are provided with the product.

Standard physical growth conditions for human PSCs in complete Essential 8™ Medium are 37°C in a humidified atmosphere of 5% CO_2. Cultures are grown in complete Essential 8™ Medium on vitronectin-coated tissue culture-treated vessels and must be passaged with EDTA. Cells are typically passaged approximately 24 hours sooner than they would be in other feeder-free media, with passaging occurring when the cells are 85% confluent. This uncomplicated, xeno-free medium minimizes batch variability and improves feeder-free culture conditions for pluripotent stem cells.

Materials needed

- Essential 8™ Medium, consisting of Essential 8™ Basal Medium and Essential 8™ Supplement (50X) (Cat. no. A1517001)
- Vitronectin, truncated recombinant human (VTN-N) (Cat. no. A14700) or Geltrex™ LDEV-Free hESC-Qualified Reduced Growth Factor Basement Membrane Matrix (Cat. no. A1413301)
- Collagenase Type IV (Cat. no. 17104-019)
- Dulbecco's PBS (DPBS) without Calcium and Magnesium (Cat. no. 14190-144)
- Versene Solution (Cat. no. 15040-066) or UltraPure™ 0.5 M EDTA, pH 8.0 (Cat. no. 15575-020)
- Dulbecco's Modified Eagle Medium (DMEM) with GlutaMAX™-I (Cat. no. 10569-010)
- PSC Cryopreservation Kit (Cat. No. A2644601)
- 37°C water bath
- Appropriate tissue culture plates and supplies

Prepare media and reagents

Essential 8™ Medium (500 mL of complete medium)

1. Thaw Essential 8™ Supplement (50X) at room temperature for ~1 hour. **Do not thaw frozen supplement at 37°C**.
2. To prepare 500 mL of complete 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°C 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°C**.

0.5 mM EDTA in DPBS (50 mL)

1. To prepare 50 mL of 0.5 mM EDTA in DPBS, aseptically mix the following components in a 50-mL conical tube in a biological safety cabinet:

DPBS without Calcium and Magnesium	50 mL
0.5M EDTA	50 µL

2. Filter sterilize the solution. The solution can be stored at room temperature for up to six months.

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, page 3, for culture surface area and volume required.

   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.

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

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

   Example: To coat a 6-well plate at a coating concentration of 0.5 µg/cm^2, you will need to prepare 6 mL of diluted vitronectin solution (10 cm^2/well surface area and 1 mL of diluted vitronectin/well; see Table 1, page 3) at the following working concentration:

$$\text{Working conc.} = 0.5\ \mu\text{g/cm}^2 \times \frac{10\ \text{cm}^2}{1\ \text{mL}} = 5\ \mu\text{g/mL}$$

$$\text{Dilution factor} = \frac{0.5\ \text{mg/mL}}{5\ \mu\text{g/mL}} = 100\text{X (i.e., 1:100 dilution)}$$

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.

   Note: Geltrex™ LDEV-Free hESC-Qualified Reduced Growth Factor Basement Membrane Matrix may be substituted for vitronectin (see Appendix, page 8).

Table 1 Required volume of diluted Vitronectin substrate

Culture vessel	Approximate surface area (cm^2)	Diluted substrate volume (mL)
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

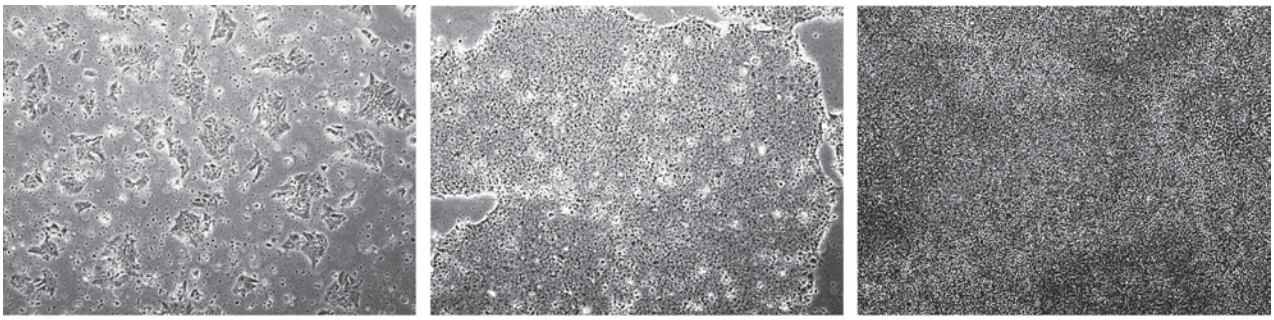
Passage PSCs

When to split cells

In general, split cells when one of the following occurs:

- PSC colonies are becoming too dense or too large.
- PSC colonies are showing increased differentiation.
- The colonies cover approximately 85% of the surface area of the culture vessel, usually every 4 days. **Even if the colonies are sparse and small, it is important to split the culture every 4 to 5 days.**

Figure 1 A. PSCs growing in Essential 8™ Medium on vitronectin 24 hours after a passage, prior to changing the medium. **B.** PSCs growing in Essential 8™ Medium on vitronectin that are ready for passage. **C.** PSCs growing in Essential 8™ Medium on vitronectin that are over-confluent.



Split ratio

- The split ratio can vary, though it is generally between 1:2 and 1:4 for early passages and between 1:3 and 1:12 for established cultures. Occasionally, cells will grow at a different rate and the split ratio will need to be adjusted.
- A general rule is to observe the last split ratio and adjust the ratio according to the appearance of the PSC colonies. If the cells look healthy and colonies have enough space, split using the same ratio. If they are overly dense and crowding, increase the ratio. If the cells are sparse, decrease the ratio.

Passage PSC colonies with Versene or EDTA

Note: Newly derived PSC lines may contain a fair amount of differentiation through passage 4**. It is not necessary to remove differentiated material prior to passaging.** By propagating/splitting the cells the overall culture health should improve throughout the early passages.

IMPORTANT! **Enzymes such as collagenase and dispase do not work well with cells cultured in Essential 8™ Medium and on vitronectin**. Use of these enzymes for passaging cells results in compromised viability and attachment.

1. Prior to starting, equilibrate your vitronectin-coated dishes to room temperature in the hood (this takes about one hour). Pre-warm the required volume of Essential 8™ Medium at room temperature until it is no longer cool to the touch.

   Note: Do not warm medium in a 37°C water bath.

2. Aspirate the spent medium from the vessel containing PSCs with a Pasteur pipette, and rinse the vessel twice with Dulbecco's PBS (DPBS) without Calcium and Magnesium. Refer to Table 2, for the recommended volumes.
3. Add 1X Versene solution to the vessel containing PSCs. Adjust the volume of Versene for various dish sizes (refer to Table 2). Swirl the dish to coat the entire cell surface.

   Note: 0.5 mM EDTA in DPBS may be substituted for Versene solution.

4. Incubate the vessel at room temperature for 5–8 minutes or 37°C for 4–5 minutes. When the cells start to separate and round up, and the colonies will appear to have holes in them when viewed under a microscope, they are ready to be removed from the vessel.

   Note: In larger vessels or with certain cell lines, this may take longer than 5 minutes.

5. Aspirate the Versene solution with a Pasteur pipette.
6. Add pre-warmed complete Essential 8™ Medium to the dish according to Table 2.

Table 2 Required volume of reagents

Culture vessel	Approximate surface area (cm^2)	DPBS (mL)	1X Versene solution (mL)	Complete Essential 8™ Medium (mL)
6-well plate	10 cm^2/well	2 mL/well	1 mL/well	2 mL/well
12-well plate	4 cm^2/well	1 mL/well	0.4 mL/well	1 mL/well
24-well plate	2 cm^2/well	0.5 mL/well	0.2 mL/well	0.5 mL/well
35-mm dish	10 cm^2	2 mL	1 mL	2 mL
60-mm dish	20 cm^2	4 mL	2 mL	4 mL
100-mm dish	60 cm^2	12 mL	6 mL	12 mL

7. Remove the cells from the well(s) by gently squirting medium and pipetting the colonies up using a 5-mL serological pipette. Avoid creating bubbles. Collect cells in a 15-mL conical tube.

IMPORTANT! **Do not scrape the cells from the dish**. There may be obvious patches of cells that were not dislodged and left behind. Do not attempt to recover them through scraping.

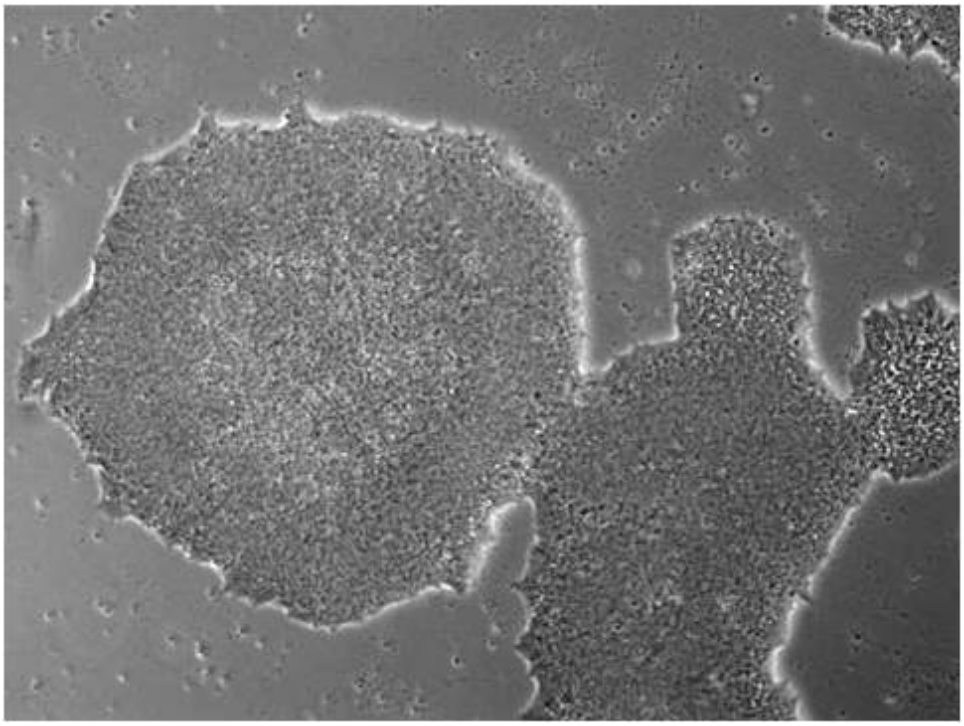
Note: Little or no extra pipetting is required to break up cell clumps after Versene treatment. Two to three triturations should be sufficient. Do not pipet vigorously or the colonies will break apart.

Note: Depending upon the cell line, work with no more than one to three wells at a time, and work quickly to remove cells after adding Essential 8™ Medium to the well(s). The initial effect of the Versene will be neutralized quickly by the medium. Some lines re-adhere very rapidly after medium addition, and must be removed 1 well at a time. Others are slower to re-attach, and may be removed 3 wells at a time.

8. Aspirate residual vitronectin solution from the pre-coated dish.
9. Add an appropriate volume of pre-warmed Essential 8™ Medium to each well of a coated 6-well plate so that each well contains 2 mL medium after the cell suspension has been added. Refer to Table 2, page 4, for volumes for other culture vessels.
10. Mix the cell suspensions from step 7 by gently inverting a few times, then transfer the appropriate volume of cell suspension into each well containing pre-warmed complete Essential 8™ Medium according to the desired split ratio.
11. Move the vessel in several quick figure eight motions to disperse cells across the surface of the vessels.
12. Place dish gently into the 37°C, 5% CO_2 incubator and incubate the cells overnight.
13. Feed PSCs the day after splitting. Replace spent medium daily.

    Note: It is normal to see cell debris and small colonies after passage.
14. *(Optional)*: To improve cell survival, you can add RevitaCell™ Supplement (Cat. no. A26445) to 1X final concentration (i.e., 20 µL per 2 mL of cell suspension) for the first 24 hours post-passage.

Figure 2 Normal pluripotent stem cell morphology. The expected morphology of PSCs is demonstrated specifically by tightly packed colonies with defined borders and a high nucleus-to-cytoplasm ratio. The image below shows PSCs at passage 6.



Cryopreserve PSCs

Freeze PSCs

1. Thaw and pre-chill the PSC Cryomedium (Cat. No. A2644601) at 2°C to 8°C.

   Note: Use of Essential 8™ Medium + 10% DMSO for cryopreservation instead of PSC Cryomedium is possible, but will result in lower relative performance.

2. Harvest PSCs according to standard cell passaging protocols.
3. Centrifuge the cell suspension at 200 × *g* for 4 minutes.
4. Aspirate the medium, being careful not to disturb the cell pellet.
5. Add PSC Cryomedium (chilled to 2°C to 8 °C) dropwise to the cells while gently rocking the tube back and forth followed by gentle resuspension of cell pellet.

   Note: In general, from a 100-mm dish, 8–12 vials containing $1x10^6$ viable cells/mL can be generated.

6. Dispense aliquots of the suspension into cryogenic vials according to manufacturer's specifications (i.e., 1.5 mL in a 2-mL cryovial).

   Note: Mix the cell suspension in PSC Cryomedium frequently to maintain a homogenous suspension. If utilizing clumped passaging methods at cell harvest, then mix cell suspension by gentle inversion to prevent breaking cells into smaller clumps.

7. Cryopreserve cells in an automated or manual controlled rate freezing apparatus following standard procedures (approximately 1°C decrease per minute).
8. Transfer frozen cell vials to liquid nitrogen (vapor phase); we recommend storage at –200°C to –125°C.

Thaw and recover PSCs

1. Quick-thaw cryopreserved PSCs in a 37 °C waterbath until only a small ice crystal remains.
2. Gently pipet the thawed cells up and down to create a cell suspension and transfer to a 50-mL conical tube.
3. Dilute the cell suspension with 3 mL of growth medium per mL of cryopreserved cells, adding it dropwise while gently rocking the tube back and forth to avoid osmotic shock to the cells.
4. Centrifuge cell suspension at 200 × *g* for 4 minutes.
5. Aspirate the medium, being careful not to disturb the cell pellet.
6. Gently resuspend the cells in growth medium supplemented with RevitaCell™ Supplement at a 1X final concentration (i.e., 100 µL of RevitaCell™ Supplement in 10 mL of growth medium).

   Note: Do not add any additional ROCK inhibitors to the growth medium.

7. Transfer the cell suspension to an appropriate growth vessel and incubate for 18–24 hours in the recommended culture environment.

   Note: Refer to Table 3 (page 7) for recommended cell seeding densities.

8. Following 18–24 hour incubation, aspirate the growth medium supplemented with RevitaCell™ Supplement and replace it with unsupplemented growth medium (i.e., without the addition of RevitaCell™ Supplement) for the remainder of the culture.

Table 3 Recommended cell seeding densities and volumes of medium for plating (per dish or per well)

Culture vessel (surface area)	Number of viable cells added[1]		Essential 8™ Medium + 1X RevitaCell™ Supplement[2]
	20,000 cell/cm^2	40,000 cells/ cm^2	
6-well (10 cm^2)	200,000	400,000	2 mL
12-well (4 cm^2)	80,000	160,000	1 mL
24-well (2 cm^2)	40,000	80,000	0.5 mL
35-mm (10 cm^2)	200,000	400,000	2 mL
60-mm (20 cm^2)	400,000	800,000	4 mL
100-mm (60 cm^2)	1,200,000	2,400,000	12 mL

[1] Time to confluency is 4–5 days for a seeding density of 20,000 cells/cm^2 and 3–4 days for seeding density of 40,000 cells/cm^2

[2] For resuspension

APPENDIX

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 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 3).
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.

Table 4 Volume of Geltrex™ hESC-qualified matrix required

Culture vessel	Approximate surface area (cm^2)	Diluted substrate volume (mL)
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	3.0 mL
100-mm dish	60 cm^2	6.0 mL

Reference

1. Chen G., Gulbranson D.R., Hou Z., Bolin J.M., Ruotti V., Probasco M.D., Smuga-Otto K., Howden S.E., Diol N.R., Propson N.E., Wagner R., Lee G.O., Antosiewicz-Bourget J., Teng J.M., Thomson J.A. (2011) Chemically defined conditions for human iPSC derivation and culture. *Nat Methods* 8(5):424–429.

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