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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)
- Dulbecco's PBS (DPBS) without Calcium and Magnesium (Cat. no. 14190-144)
- 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)
- Dimethyl sulfoxide (DMSO) (Sigma, Cat. no. D2650)
- 37°C water bath
- Appropriate tissue culture plates and supplies

Prepare media and materials

Essential 8™ Medium (500 mL of complete medium)

1. Thaw Essential 8™ Supplement (50X) at 2–8°C overnight. **Do not thaw 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
0.5 M 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) 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, below, 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 the **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

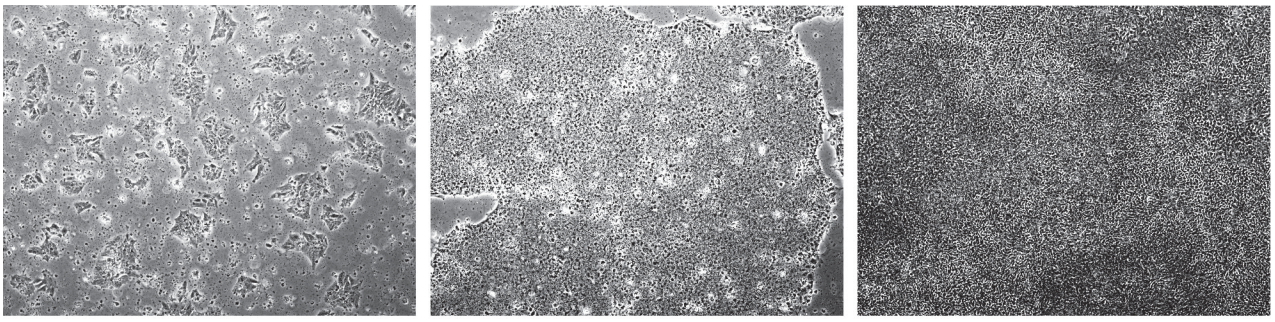
Passage PSCs

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.



A B C

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 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, below, for the recommended volumes.
3. Add 0.5 mM EDTA in DPBS to the vessel containing PSCs. Adjust the volume of EDTA for various dish sizes (refer to Table 2). Swirl the dish to coat the entire cell surface.
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 EDTA solution with a Pasteur pipette.
6. Add pre-warmed complete Essential 8™ Medium to the dish according to Table 2, below.

Table 2 Required volume of reagents

Culture vessel	Approximate surface area (cm^2)	DPBS (mL)	0.5 mM EDTA in DPBS (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 glass 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.

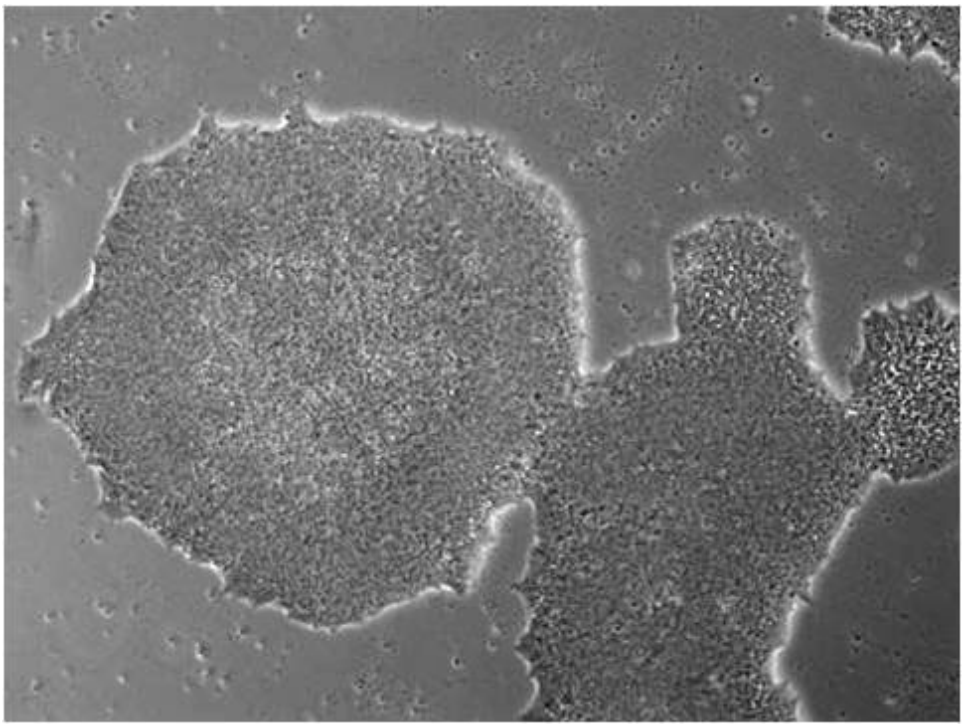
Note: Little or no extra pipetting is required to break up cell clumps after EDTA treatment.

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 EDTA 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. Move the vessel in several quick figure eight motions to disperse cells across the surface of the vessels.
11. Place dish gently into the 37°C, 5% CO_2 incubator and incubate the cells overnight.
12. Feed PSC cells beginning the second day after splitting. Replace spent medium daily.

Note: It is normal to see cell debris and small colonies after 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. Pre-warm the required volume of Essential 8™ Medium at room temperature until it is no longer cool to the touch. **Do not warm the medium in a 37°C water bath**.
2. Prepare Essential 8™ Freezing Medium. For every 1 mL of freezing medium needed, aseptically combine the components listed below in a sterile 15-mL tube:

Complete Essential 8™ Medium	0.9 mL
DMSO	0.1 mL

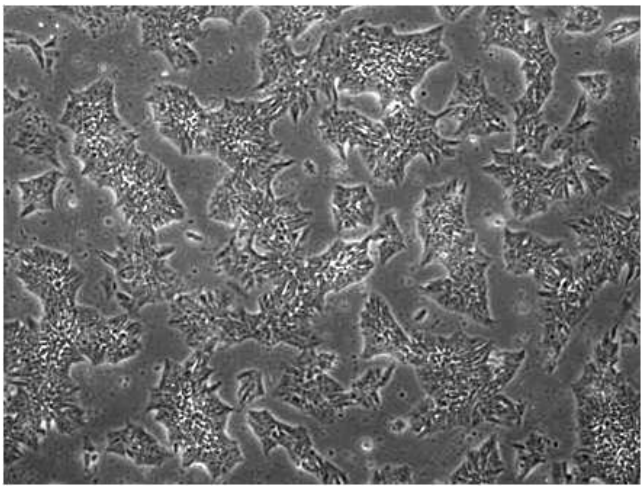
   Place the tube with Essential 8™ Freezing Medium on ice until use. Discard any remaining freezing medium after use.
3. Aspirate the spent medium from the dish using a Pasteur pipette, and rinse the cells twice with DPBS without Calcium and Magnesium (refer to Table 2, page 4).
4. Add 0.5 mM EDTA solution to the dish. Adjust the volume of EDTA for various dish sizes (refer to Table 2, page 4). Swirl the dish to coat the entire cell surface.
5. Incubate the dish at room temperature for 5–8 minutes or at 37°C for 4–5 minutes. When the cells start to separate and round up, and the colonies appear to have holes in them when viewed under a microscope, they are ready to be removed from the vessel.
6. Aspirate the EDTA solution with a Pasteur pipette.
7. Add 1 mL of ice-cold Essential 8™ Freezing Medium to each well of a 6-well plate.
8. Remove the cells by gently squirting the colonies from the well using a 5-mL glass pipette. Avoid creating bubbles. Collect the cells in a 15-mL conical tube and place on ice.
9. Resuspend the cells gently. Aliquot 1 mL of the cell suspension into each cryovial.
10. Quickly place the cryovials in a cryofreezing container (e.g., Nalgene® Mr. Frosty® Freezing Container) and freeze the cells by decreasing the temperature by 1°C per minute. Once frozen, transfer the cells to –80°C overnight.
11. After overnight storage at –80°C, transfer the cells to a liquid nitrogen tank vapor phase for long-term storage.

Thaw and recover PSCs

1. Place 10 mL of Essential 8™ Medium in a 50-mL tube and warm to room temperature.
2. Equilibrate an appropriate quantity of vitronectin-coated 6-well plates to room temperature.
3. Remove the vial of PSCs from liquid nitrogen storage using metal forceps. Transfer it on dry ice to the cell culture hood.
4. Immerse the vial in a 37°C water bath without submerging the cap. Swirl the vial gently.
5. When only an ice crystal remains, remove the vial from the water bath.
6. Spray the outside of the vial with 70% ethanol and place it in hood.
7. Pipet cells gently into a sterile 15-mL conical tube using a 5-mL sterile pipette.
8. Slowly add 10 mL of Essential 8™ Medium drop-wise to cells in the 15-mL conical tube. While adding the medium, gently move the tube back and forth to mix the PSCs. This reduces osmotic shock to the cells.
9. Rinse the vial with 1 mL of Essential 8™ Medium and add to the 15-mL tube with cells.
10. Centrifuge the cells at 200 × *g* for 5 minutes.
11. Aspirate and discard the supernatant.
12. Resuspend the cell pellet in 2 mL of Essential 8™ Medium by gently pipetting the cells up and down in the tube a few times.

13. Aspirate the spent medium from the pre-warmed vitronectin-coated plates. Slowly add the PSC suspension into the vitronectin-coated plate, plating one vial of thawed cells per well of the 6-well plate.
14. Move the dish in several quick figure eight motions to disperse cells across the surface of the wells.
15. Place the dish gently into the 37°C, 5% CO_2 incubator and incubate the cells overnight.
16. The next day, replace the spent medium with fresh complete Essential 8™ Medium.
17. Replace the medium daily thereafter until the cells are approximately 85% confluent.

Figure 3 PSCs cryopreserved in Essential 8™ Medium + 10% DMSO were thawed and plated onto vitronectin-coated dishes, and allowed to recover. The image below shows PSCs one day after the first passage.

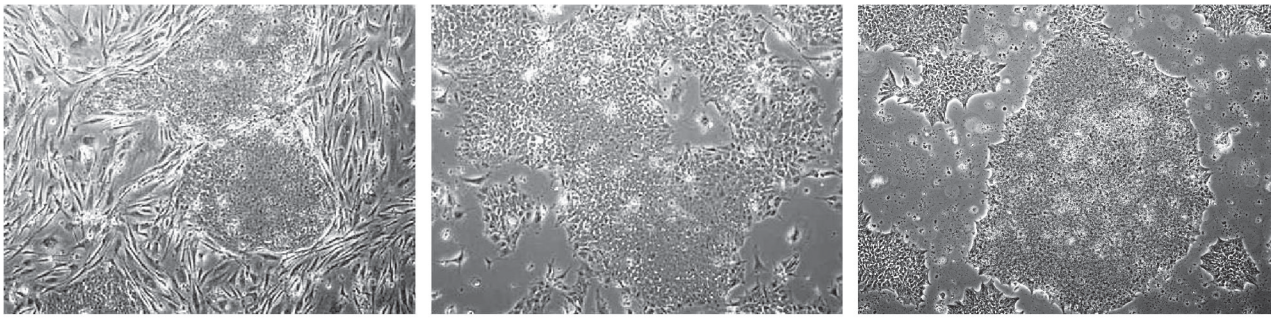


Adapt cells grown on feeders to Essential 8™ Medium

PSCs that were grown on feeders should not be thawed directly into Essential 8™ Medium. The cells should be thawed into the condition in which they were frozen. For example, if the cells were maintained in KnockOut™ Serum Replacement (KSR) Medium on Mouse Embryonic Fibroblasts (MEF) prior to freezing, then the cells should be thawed into KSR Medium on MEF feeder layer. Allow the cells to recover, and then passage into Essential 8™ Medium on vitronectin-coated culture vessels, using EDTA as the passaging reagent, as described in this protocol. The morphology of the PSCs may appear unusual in the first passage into Essential 8™ Medium on vitronectin-coated culture vessels, but should look normal by the next one or two passages.

Note: PSCs grown using mTeSR® Medium on Matrigel®-coated culture vessels can be thawed directly into Essential 8™ Medium on vitronectin-coated culture vessels, as described in this protocol.

Figure 4 A. PSCs in KSR Medium on MEF feeder layer, **B.** Two days after PSCs were passaged into Essential 8™ Medium on vitronectin-coated culture vessels using EDTA, **C.** PSCs after two more passages in Essential 8™ Medium on vitronectin-coated culture vessels.



Adaptation using Geltrex® LDEV-Free, hESC-Qualified Basement Membrane Matrix as an intermediary

Note: Some cell lines may be more difficult to adapt to Essential 8™ Medium and vitronectin-coated culture vessels. If the PSCs grown on feeders, passaged with EDTA and plated onto vitronectin-coated culture vessels in Essential 8™ Medium continue to look unusual after several passages, we recommend using Geltrex® Matrix in place of vitronectin substrate as an intermediary step.

1. Harvest PSCs using an enzymatic method, such as Collagenase Type IV (Cat. no. 17104-019) or Dispase (Cat. no. 17105-041).
2. Plate cells Geltrex® Matrix-coated plates in Essential 8™ Medium (see **Appendix** for coating plates with Geltrex® Matrix).
3. Incubate cells at 37°C, 5% CO_2 overnight.
4. Feed cells with Essential 8™ Medium beginning on the second day after splitting. Replace spent medium daily.
5. At the next passage, harvest the cells using EDTA and plate onto vitronectin-coated culture vessels in Essential 8™ Medium.
6. Cells should exhibit normal pluripotent stem cell morphology by the next one or two passages (see Figure 2, page 5).

APPENDIX

A. 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, below).
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 3 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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