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Adaptation of hPSCs from Different Culture Systems to Essential 8™ Medium or Essential 8™ Flex Medium

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Introduction

This protocol describes the transition of human pluripotent stem cells (hPSCs) from different culture systems into Essential 8™ or Essential 8™ Flex Medium. It supplements the Essential 8™ and Essential 8™ Flex Medium user guides (Pub. nos. MAN0007569 and MAN0013988). Refer to the user guides for a description of Essential 8™ Medium (Cat. no. A1517001) or Essential 8™ Flex Medium (Cat. no. A2858501) and for detailed instructions on culturing hPSCs under feeder-free culture conditions in either medium.

Materials needed

- Essential 8™ Medium (Cat. no. A1517001) or Essential 8™ Flex Medium (Cat. no. A2858501)
- Dulbecco's Phosphate Buffered Saline (DPBS) without Calcium and Magnesium (Cat. no. 14190-250)
- Collagenase Type IV (Cat. no. 17104-019) or Dispase II, powder (Cat. no. 17105-041)
- Versene Solution (Cat. no. 15040-066) or UltraPure™ 0.5 M EDTA, 8.0 pH (Cat. no. 15575-020)
- 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
- 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
- Appropriate tissue culture plates and supplies

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 Calcium and Magnesium	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.

PSC medium (500 mL of complete medium)

1. To prepare 500 mL of complete PSC Medium, aseptically mix the following components:

DMEM/F-12 with GlutaMAX™-I	394.5 mL
KnockOut™ Serum Replacement	100 mL
MEM Non-Essential Amino Acids Solution	5 mL
2-Mercaptoethanol (55 mM)	500 µL

2. Sterilize through 0.22-µm filter and store at 2–8°C for up to 4 weeks.
3. Before use, add bFGF to a final concentration of 4 ng/mL (e.g., 0.4 µL of reconstituted bFGF at 10 µg/mL per mL of medium).

Collagenase IV solution (for 50 mL of 10 mg/mL solution)

1. To prepare 50 mL of Collagenase IV solution at a final concentration of 10 mg/mL, aseptically mix the following components:

Collagenase IV	500 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.

   Note: This solution can further be diluted to 1X concentration (1 mg/mL) with additional DMEM/F-12 as required.

Dispase II solution (for 50 mL of 10 mg/mL solution)

1. To prepare 50 mL of Dispase solution at a final concentration of 10 mg/mL, aseptically mix the following components:

Dispase II	500 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.

   Note: This solution can further be diluted to 2X concentration (2 mg/mL) with additional DMEM/F-12 as required.

0.5 mM EDTA in DPBS (50mL)

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. Sterilize through 0.22-µm filter and store at room temperature for up to six months.

Essential 8™ Medium or Essential 8™ Flex Medium (500 mL of complete medium)

1. Thaw Essential 8™ or Essential 8™ Flex Supplement (50X) at room temperature for approximately 1 hour. **Do not thaw at 37°C**.
2. To prepare 500 mL of complete Essential 8™ or Essential 8™ Flex Medium, aseptically mix the following components:

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

3. Complete Essential 8™ or Essential 8™ Flex 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**.

Adaptation of feeder-dependent cultures into Essential 8™ or Essential 8™ Flex Medium

Feeder-dependent cultures can be adapted into Essential 8™ or Essential 8™ Flex Medium using the modified Collagenase or Dispase passaging method shown below. Cells can be transferred directly into Essential 8™ or Essential 8™ Flex Medium with vitronectin or Geltrex™ matrix. Alternatively, for best results, cells can be transferred to rhLaminin-521 (Cat. no. A29248, A29249) or the complete Essential 8™ Adaptation Kit (Cat. no. A25935) as described in the product insert (MAN0014536) before transitioning to vitronectin.

Modified Collagenase or Dispase Passaging Method

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

1. Prepare 1:100 Geltrex™ matrix solution (for general applications) in DMEM/F-12 medium or 1:50 vitronectin solution (for xeno-free applications) in Dulbecco's Phosphate Buffered Saline (DPBS) without Calcium and Magnesium. Coat your culture dishes with your matrix of choice and incubate for one hour at 37°C (see APPENDIX, page 10, for the appropriate coating procedure).
2. Aspirate the spent PSC medium from the dish containing PSCs on feeder cells and wash once with DPBS without Calcium and Magnesium.
3. Aspirate the DPBS without Calcium or Magnesium, and add 2 mL of 1X Collagenase IV (1 mg/mL), pre-warmed to 37°C.

   Note: Alternatively, you can use Dispase II solution (2 mg/mL) in place of the Collagenase IV solution.
4. Incubate the cells for 30–45 minutes in the 37°C, 5% CO_2 incubator.

   Note: If using Dispase II solution (2 mg/mL), incubation time is 15–25 minutes in the 37°C, 5% CO_2 incubator.
5. Stop the incubation when the edges of the colonies begin to curl from the plate.

6. Add 2 mL of complete PSC medium and gently dislodge the colonies from the plate by washing off the colonies using a 5 mL serological pipette.

   Tip the plate at a 45 degree angle and rotate the plate as you begin to triturate the clusters of colonies into smaller fragments. Repeat until the desired fragment size is achieved.

   Note: Optimal fragment size is critical for successful adaptation. Colony fragments that are too large will form EB-like clusters when re-seeded, and fragments that are too small will differentiate.
7. Transfer the suspended colony clusters into a 15-mL conical tube.
8. Add an additional 2 mL of complete PSC medium to dislodge the remaining colonies and transfer them to the 15-mL tube.

   Note: If desired, you can triturate the PSC colonies by pipetting them up and down 5–7 times in the 15-mL conical tube rather in the tissue culture dish. This can reduce the chances of contamination.
9. Let the colony fragments sediment at the bottom of the 15-mL tube for 2–5 minutes by gravity.
10. Discard the supernatant, add 4 mL of complete PSC medium, and gently resuspend the sedimented colony fragments by pipetting up and down 2 times.
11. Gravity sediment the clusters again for 2–5 minutes.
12. While colony fragments are sedimenting, aspirate the matrix solution from the freshly prepared dish and add 4 mL of complete Essential 8™ or Essential 8™ Flex Medium.
13. Aspirate the supernatant from the sedimented clusters and add 4 mL of complete Essential 8™ or Essential 8™ Flex Medium.
14. Resuspend the PSC clusters by gently pipetting them up and down 2 times, taking care not to further break down the clusters; the goal is to just resuspend the PSC clusters for seeding (split ratio 1:4).
15. Distribute 1 mL of the suspended PSC clusters into each matrix-coated dish containing 4 mL of complete Essential 8™ or Essential 8™ Flex Medium. Move the dish in several quick figure eight motions to disperse cells across the surface of the dish.
16. Place the culture dishes containing the PSC clusters in the 37°C, 5% CO_2 incubator and incubate.

Figure 1. Adaptation procedure for PSCs: Performing the gravity sedimentation twice is essential for the attachment and adaptation of the colonies on vitronectin- or Geltrex™ matrix-coated plates.

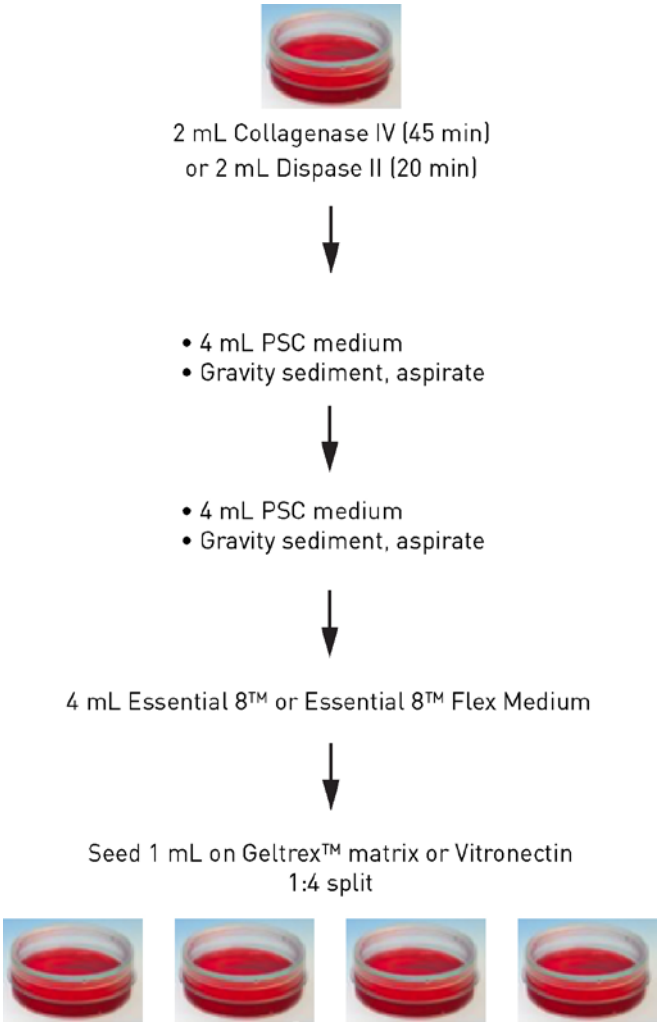
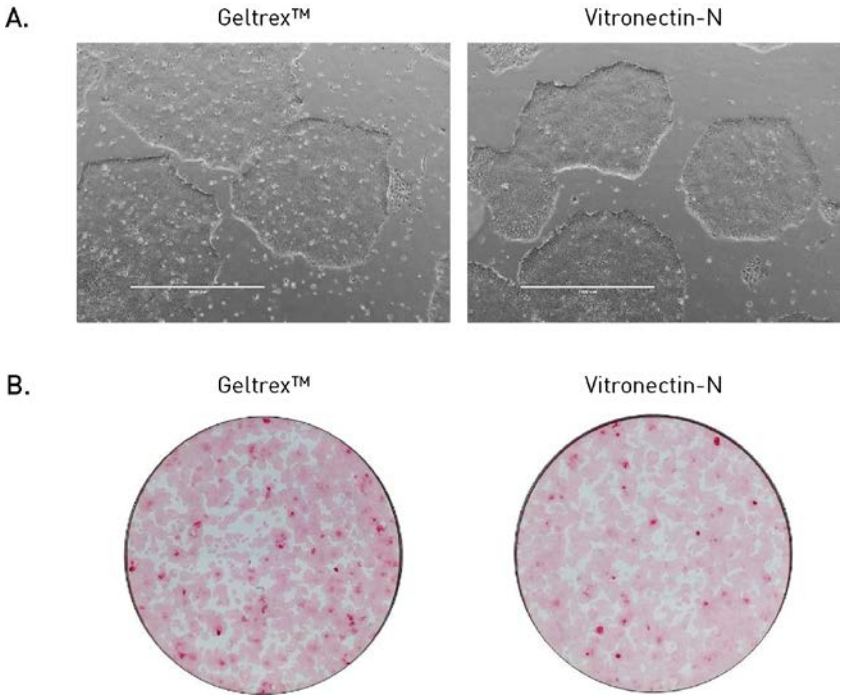


Figure 2. Examples of adapted colonies: (A) 50X phase contrast images and (B) whole-well Alkaline Phosphatase staining of PSCs adapted from feeder-dependent cultures into Essential 8™ Medium on Geltrex™ matrix- or vitronectin-coated dishes using Collagenase IV.

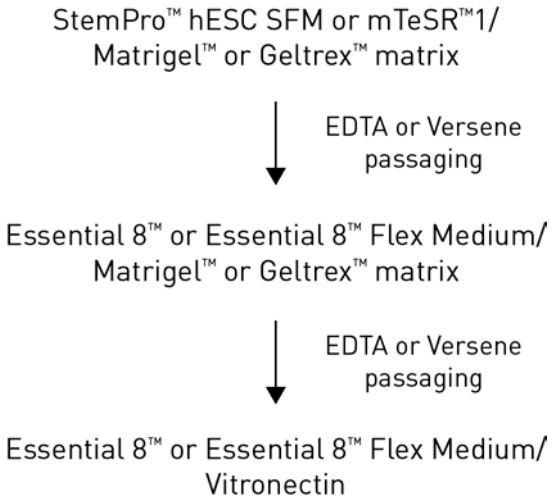


Adaptation of other feeder-free cultures into Essential 8™ or Essential 8™ Flex Medium

PSCs cultured in other feeder-free media like mTeSR1™ or StemPro™ hESC SFM can be adapted into Essential 8™ or Essential 8™ Flex Medium using the same EDTA or Versene passaging method that will later be used for routine passaging of the newly adapted cultures. When transitioning from a feeder-free culture grown on Matrigel® or Geltrex™ matrix, it is recommended to maintain the same matrix with Essential 8™ or Essential 8™ Flex Medium for at least one passage. The matrix can then be changed to vitronectin on the next passage. Use Figure 3 as a reference for the appropriate adaptation scheme.

Alternatively, for best results, cells can be transferred to rhLaminin-521 (Cat. no. A29248, A29249) or the complete Essential 8™ Adaptation Kit (Cat. no. A25935) as described in the product insert (MAN0014536) before transitioning to vitronectin.

Figure 3. Scheme for adapting PSCs from other feeder-free systems into Essential 8™ or Essential 8™ Flex Medium with vitronectin.



Versene or EDTA passaging protocol

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

1. Prior to starting, coat your dishes according to the coating protocols found in the appendix/product inserts. Equilibrate your pre-coated dishes to room temperature in the hood (this takes about one hour). Also pre-warm the required volume of Essential 8™ or Essential 8™ Flex 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, and rinse the vessel twice with 4 mL DPBS without Calcium and Magnesium.

3. Add 2 mL Versene or 0.5 mM EDTA in DPBS to the vessel containing PSCs. Swirl the dish to coat the entire cell surface.

4. Incubate the vessel 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.

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

5. Aspirate the Versene or EDTA solution.

6. Add 4 mL pre-warmed complete Essential 8™ or Essential 8™ Flex Medium to the dish.
7. Remove the cells from the dish by gently squirting medium and pipetting the colonies up and down using a 5 mL pipette. Avoid creating bubbles. Collect cells in a 15 mL conical tube.

   Note: Do not scrape the cells from the dish. There may be obvious patches of cells that were not dislodged. Do not attempt to recover them through scraping.

   Note: Little or no extra pipetting is required to break up cell clumps after Versene or 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™ or Essential 8™ Flex 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 dish at a time. Others are slower to re-attach, and may be removed 3 dishes at a time.
8. Aspirate residual matrix solution from the pre-coated dish.
9. Add the appropriate amount of Essential 8™ or Essential 8™ Flex Medium to each dish so that each dish contains 5 mL medium after the cell suspension has been added.
10. Mix the cell suspensions from step 7 by gently inverting the conical tube a few times. Keeping in mind the desired split ratio, transfer the appropriate volume of cell suspension into the dish containing pre-warmed complete Essential 8™ or Essential 8™ Flex Medium.
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. Whether using Essential 8™ or Essential 8™ Flex Medium, feed the cells the day after splitting. Replace spent medium daily when using Essential 8™ Medium. Essential 8™ Flex Medium cultures should be fed according to the protocol suggested in the Essential 8™ Flex Medium product insert.

    Note: It is normal to see cell debris and small colonies after passage.

Useful Tips:

1) In general, adaptation or regular passaging should be done 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 80-85% of the surface area of the culture vessel.

2) 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.

3) Once the cells are in Essential 8™ or Essential 8™ Flex Medium, passage using Versene or EDTA. Do not use Dispase or Collagenase.

4) During passaging, only incubate the cells with Versene or EDTA until small holes appear between colonies. Make sure not to over-incubate the cells in Versene or EDTA. The actual time may vary from line to line or from vessel to vessel so new users may incubate at room temperature on a microscope stage in order to determine the proper incubation time.

5) Do not overtriturate cells following Versene or EDTA passaging. Perform only 2–3 washes up and down the plate without directly scraping the cells off. If a lot of cells are left behind, then that is an indication that longer Versene or EDTA incubation times are required.

6) RevitaCell™ Supplement is not required for adaptation or for routine cell passaging with vitronectin and Essential 8™ or Essential 8™ Flex Medium. However, if the cells are too confluent or have been over-incubated or over-triturated, adding RevitaCell™ Supplement during the first 24 hours post-passaging may help maintain cell health.

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.

   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.

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

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

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.

   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).

   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.

   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.

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

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.

   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.

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

   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 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

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