Human PSC feeder-free culture protocol

Reagents and special tools:

1. StemPro® hESC SFM kit (Life Technologies#A1000701, which contains 500 ml DMEM/F12 with Glutamax, 10 ml STEMPRO® hESC Supplement, 50 ml 25% Bovine Serum Albumin)
2. Basic fibroblast growth factor (Life Technologies#PHG0026)
3. β-Mercaptoethanol (55 mM, Life Technologies#21985-023)
4. Geltrex™ (Life Technologies#12760-021)
5. Dispase (Life Technologies#17105-041)
6. DPBS without $CaCl_2$ and $MgCl_2$ (Life Technologies#14190-250)
7. Dimethyl sulfoxide (DMSO, Sigma-Aldrich#D2650)
8. StemPro EZPassge disposal stem cell passaging tool (Life Technologies#23181-010)
9. Cell scraper (BD Falcon#353085)
10. Knockout serum replacer (KSR, Life Technologies#10828-028)

Solution and medium preparation

Basic FGF Solution (10 µg/ml, For 1 ml)

Basic FGF	10 µg
PBS	996 µl
25% BSA	4 µl

Aliquot and store at -20°C for up to 6 months

Dispase Solution (1 mg/ml, For 50 ml)

Dispase	50 mg
DMEM/F12	50 ml

Sterilize through 0.22 µm filter and store at 4°C for up to 7 days.

StemPro hESC Medium (For 100ml)

DMEM-F12	90.8 ml
STEMPRO® hESC Supplement	2 ml
25% BSA	7.2 ml
β-Mercaptoethanol	182 µl
Basic FGF solution	80 µl

Add bFGF (final concentration 8 ng/ml) and β-Mercaptoethanol (final concentration 0.1 mM) at the time of medium change. Medium lasts for up to 7 days at 4°C.

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StemPro cryo-preservation medium A

StemPro hESC Medium (50%) + KSR (50%)

StemPro cryo-preservation medium B

StemPro hESC Medium (80%) + DMSO (20%)

Make fresh cryo-preservation medium A and B.

Coating with Geltrex™

1. Thaw Geltrex™ at 2 to 8°C overnight.
2. Remove DMEM/F-12 from 2 to 8°C storage. Dilute Geltrex ™ with DMEM/F-12 (1:30) and mix gently.
3. Cover the whole surface of each culture plate and dishes with the Geltrex™ solution (1 ml for each well of 6-well plate, 1.5 ml for a 60-mm dish, and 3-4 ml for a 100 mm dish).
4. Seal each dish with parafilm to prevent from drying up, and incubate 1 hour at 37°C or overnight at 4°C.
5. If the coated plates or dishes are not used right away, store the coated plates or dishes at 2 to 8°C for up to 1 week. Transfer plates or dishes to a laminar flow hood and allow them to equilibrate to room temperate (about 1 hour) before use.

Thawing and plating human PSCs

1. Coat culture dishes with Geltrex at least 1 hour before thawing human PSCs. Wear eye protection as cryo-vials stored in the liquid phase of liquid nitrogen may accidentally explode when warmed.

   Note: One 60 mm coated dish for one vial of frozen human PSCs (frozen vial contains cells from ½ of 60mm dish).
2. Wear ultra low temperature cryo-gloves. Remove a cryo-vial of PSCs from the liquid nitrogen storage tank using metal forceps.
3. Roll the vial between your gloved hands until the outside is free of frost. This should take between 10-15 seconds.
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. Quickly remove the sticker or copy the information written on the vial in your notebook. The writing may come off the vial after spraying outside of vial with 70% ethanol.
7. Spray outside of the vial with 70% ethanol and place it in hood
8. Pipette cells gently into a sterile 15 ml conical tube using a 1ml pipette.
9. Add 1 ml pre-warmed StemPro hESC medium into the vial to collect resident cells.

11. Using a pipette to remove StemPro hESC medium from the vial and add it to the 15 ml conical tube drop-wise. While adding the medium, gently move the tube back and forth to mix the human PSCs. This reduces osmotic shock to the human PSCs.
12. Centrifuge PSCs at 200 x g for 5 minutes and aspirate the supernatant.
13. Re-suspend the cell pellet in 5 ml StemPro hESC medium.
14. Centrifuge PSCs at 200 x g for 5 minutes and aspirate the supernatant.
15. Re-suspend the cell pellet in 5 ml StemPro hESC medium.
16. Aspirate Geltrex solution and label the Geltrex coated 60 mm dish with the passage number from the vial, the date and your initials.
17. Slowly add the human PSC suspension into the dish.
18. Place the dish gently into the incubator and move the dish in several quick back-and-forth and side-to-side motions to disperse cells across the surface of the dish.
19. Replace spent medium daily. If feeding more than one dish, use a different pipette for each dish to reduce risk of contamination. Examine cells under a microscope and colonies may be very small in the first 2 days.
20. Observe PSCs every day and passage cells whenever the colonies are too big or crowded. The ratio of splitting depends on the total number of PSC colonies in culture plate or dish (Approximately 1:3 for human PSCs at the first time of recovery).

Passaging human PSCs

Note: This protocol is for the culture on culture dishes and is not for human PSCs cultured in flasks because it is difficult to remove differentiated colonies in a flask. Also, StemPro EZPassge disposal stem cell passaging tool and cell scraper cannot reach cells in flask.

1. Coat culture dishes with Geltrex at least 1 hour before passage. Warm appropriate amount of dispase solution and StemPro hESC medium to 37°C in a water bath.
2. Remove PSC plates and dishes from incubator. Label differentiated colonies with a microscope marker.
3. Aspirate the spent medium with a Pasteur pipette. Remove differentiated colonies with a Pasteur pipette by aspirating.
4. Gently add 1 ml pre-warmed dispase solution to each well of 6-well plate, 3 ml to each 60 mm dish or 4 ml to each 100 mm dish.
5. Incubate for 3-5 minutes at 37°C until the edges of cell colonies begin to curl off.
6. Aspirate dispase solution and rinse with DPBS two times gently. Add 1 ml pre-warmed StemPro hESC SFM to each well of 6-well plate, 2 ml to each 60 mm dish or 3 ml to each 100 mm dish.
7. Roll StemPro EZPassge disposal stem cell passaging tool across the entire dish or plate in one direction (left to right). **When rolling, overlap the next area with the area rolled previously.** Rotate the culture

dish or plate 90 degree, and roll StemPro EZPassge disposal stem cell passaging tool across the entire dish or plate. **When rolling, overlap the next area with the area rolled previously.** This procedure produces relatively uniform size of cell clumps.

8. Use a cell scraper to gently detach the cells off the surface of the plates and dishes.
9. Gently transfer cell clumps using a 5-mL pipette and place into a 15 or 50 ml conical tube.
   Note: Do not break cell clumps into small pieces.
10. Add 1 ml pre-warmed StemPro hESC SFM to each well of 6-well plate, 2 ml to each 60 mm dish or 3 ml to each 100 mm dish to collect residual cells and add cell suspension to the tube.
11. Aspirate Geltrex solution from Geltrex coated vessels. Add 2.5 ml pre-warmed StemPro hESC SFM into each well of 6-well plate, 5 ml into each 60 mm dish or 10 ml into each 100 mm dish.
12. **Gently shake the tube to distribute cell clumps evenly.** Add **appropriate amount** of PSC suspension into each well of culture plate or dish according to **split ratio**.
    Note: The split ratio is variable, though generally between 1:4 and 1:6. 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, and if the cells are sparse, decrease the ratio. Cells will need to be split every 4-6 days based upon appearance.
12. Place vessels gently in an incubator and move culture vessels in several quick back-and-forth and side-to-side motions to disperse cells across the surface of vessels.
13. Gently change media the next day to remove non-attached cells, and change StemPro hESC SFM every day thereafter.
14. Observe cells every day and passage cells whenever the colonies are too big or crowded (approximately every 3 to 4 days).

Cryo-preserving human PSCs cultured in StemPro hESC SFM

1. Warm appropriate amount of dispase and StemPro hESC medium to 37°C in a water bath.
2. Aspirate the medium and gently add 1 ml of dispase solution into each well of 6-well plate, 2 ml into a 60 mm dish and 4 ml into a 100 mm dish.
3. Incubate for 3-5 minutes at 37°C until the edges of cell colonies begin to curl off.
4. Aspirate dispase solution and rinse with DPBS two times gently, and then add 1 ml StemPro hESC medium into each well of 6-well plate, 2 ml into a 60 mm dish and 4 ml into a 100 mm dish.
5. Roll StemPro EZPassge disposal stem cell passaging tool across the entire dish or plate in one direction (left to right). **When rolling, overlap the next area with the area rolled previously.** Rotate the culture dish or plate 90 degree, and roll StemPro EZPassge disposal stem cell passaging tool across the entire dish

or plate. **When rolling, overlap the next area with the area rolled previously.** This procedure produces relatively uniform size of cell clumps.

6. Use a cell scraper to gently detach the cells off the surface of the plates and dishes.
7. Gently transfer cell clumps using a 5-mL pipette and place into a 15 or 50 ml conical tube.
8. Wash vessels with 3 ml of StemPro hESC medium to collect resident cells and add cell suspension to the tube.
9. Centrifuge at 200 x g for 5 minutes.
10. Gently aspirate supernatant and re-suspend the cells with 1 ml StemPro cryo-preservation medium A for all cells from one 60 mm dish.
11. Add same volume of StemPro cryo-preservation medium B drop-wise.
12. Allocate 1 ml cell suspension into each cryotube and freeze cells at -80 °C overnight in isopropanol chamber.
13. Transfer cells into liquid nitrogen tank next day for long term storage.

Change of PSCs cultured on mouse embryonic fibroblasts (MEFs) to StemPro hESC SFM

If human PSCs are maintained on MEFs, use the following protocol to switch PSCs to feeder-free condition.

1. Coat culture dishes with Geltrex at least 1 hour before experiment.
2. When PSCs cultured on MEFs in dish or plate reach 80-90% confluence, aspirate spent medium and rinse cells with PBS two times.
3. Add 1 ml StemPro hESC medium into each well of 6-well plate, 2 ml into a 60 mm dish and 4 ml into a 100 mm dish.
4. Roll StemPro EZPassge disposal stem cell passaging tool across the entire dish or plate in one direction (left to right). **When rolling, overlap the next area with the area rolled previously.** Rotate the culture dish or plate 90 degree, and roll StemPro EZPassge disposal stem cell passaging tool across the entire dish or plate. **When rolling, overlap the next area with the area rolled previously.** This procedure produces relatively uniform size of cell clumps.
5. Use a cell scraper to gently detach the cells off the surface of the plates and dishes.
6. Gently transfer cell clumps using a 5-mL pipette and place into a 15 or 50 ml conical tube.
7. Wash vessels with 3 ml of StemPro hESC medium to collect resident cells and add cell suspension to the tube.
8. Aspirate Geltrex solution from Geltrex coated vessels. Add 2.5 ml pre-warmed StemPro hESC SFM into each well of 6-well plate, 5 ml into each 60 mm dish or 10 ml into each 100 mm dish.

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9. Gently shake the tube to distribute cell clumps evenly. Add appropriate amount of PSC suspension into each well of culture plate or dish according to split ratio **(**generally 1:3 to 1:4).
10. Place vessels gently in an incubator and move culture vessels in several quick back-and-forth and side-to-side motions to disperse cells across the surface of vessels.
11. Gently change media the next day to remove non-attached cells, and change StemPro hESC SFM every day thereafter.
12. Observe cells every day and passage cells whenever the colonies are too big or crowded (approximately every 3 to 4 days).

Note: The contaminated MEFs will die off after 2-3 passages in StemPro hESC SFM.