Human iPS Cell Culture

OVERVIEW

This protocol can be used for culturing human iPS cells. Human iPS cells were generated by transducing source cells with retroviruses individually encoding the four human transcription factors (Oct4, Sox2, Klf4, and c-Myc) that have been shown to induce the reprogramming of somatic cells to a pluripotent state. The cells were derived using morphological selection criteria and without the use of fluorescent markers or drug selection. When cultured under standard human ES cell culture conditions, the morphology of SBI human iPS cells is identical to that of human ES cells. The cells also express the pluripotency markers SSEA-4 and Nanog, and demonstrate a strong endogenous AP activity.

PROCEDURE

I. Feeder-free culture conditions

Preparation of feeder-free medium

1. Thaw mTeSR1 5X Supplement (Cat.no. 05850, STEMCELL Technologies) at room temperature or overnight at 4°C.
2. Add 100 mL of the thawed 5X Supplement to 400 mL Basal Medium (Cat.no. 05850, STEMCELL Technologies) for a total volume of 500 mL aseptically. Mix well. Filter through a 0.2 µm, low-protein binding filter, if desired.
3. Aliquot into appropriate amount according to usage and store the aliquots at 4°C.

Coating plates with Matrigel

Matrigel (Cat.no. 354277, BD) should be aliquoted and stored at -80°C for long-term use.
1. Thaw matrigel on ice completely. Dilute matrigel 1:30 to 1:50 with pre-chilled KO DMEM/F12 (Cat.no. 12660-012, Invitrogen).
2. Immediately coat tissue culture plates with the diluted matrigel solution. For a 6-well plate, use 0.8 mL of diluted matrigel solution per well, and swirl the plate to spread the matrigel solution evenly across the surface.
3. Let the coated plate stand for 1 h at 37°C or overnight at 4°C. If the plate has been stored at 4°C, incubate the plate at 37°C for at least 30 minutes before removing the matrigel solution.

Thawing cryopreserved human iPS cells

1. One hour before thawing human iPS cells, coat one well of a 6-well plate with 0.8 mL of diluted matrigel solution, swirl the plate to spread the matrigel solution evenly across the surface, and incubate it for an hour at 37°C.
2. Quickly thaw the human iPS cells in a 37°C water bath by gently shaking the cryovial continuously until half thawed. Remove the cryovial from the water bath and spray with 70% ethanol.
3. Transfer the contents of the cryovial to a 15 mL conical tube. Add 5 mL warm mTeSR1 dropwise to the tube, mixing gently.
4. Centrifuge cells at 200 *x g* for 5 minutes at room temperature.

5. After centrifugation, aspirate the medium from 15 mL tube. Gently resuspend the cell pellet in 2 mL of mTeSR1 containing 10 µM ROCK inhibitor (Y-27632, Cat.no. Y-05, StemRD). Take extra caution to maintain the cells as small cell clumps.
6. Remove the matrigel solution from the coated 6-well tissue culture plate. Transfer the medium containing the cell clumps into the well on the matrigel coated 6-well plate immediately.
7. Place the plate in a 37°C incubator and rock the plate to evenly distribute the clumps in the well. Culture the cells at 37°C, with 5% CO_2 and 95% humidity.
8. Change medium daily. Check for undifferentiated colonies that are ready to passage when colonies are big enough (approximately 7-10 days after thawing).

Passaging human iPS cells under feeder-free conditions

1. Use a microscope to identify regions of differentiation. Mark the differentiated colonies using a lens marker on the bottom of the plate.
2. Remove regions of differentiation by scraping with a pipette tip or by aspiration.
3. Aspirate medium from the human iPS cell culture and rinse with DPBS (2 mL/well, Cat.no. 14190, Invitrogen).
4. Add 0.5 mL per well of accutase (Cat.no. SCR005, Millipore, diluted 1:1 with DPBS before use). Let it stand at room temperature for 1 minute.
5. Remove accutase, and gently rinse each well with 2 mL of DMEM/F-12, 2 - 3 times to remove remaining enzymes.
6. Add 2 mL/well mTeSR1 and scrape colonies off with a cell scraper.
7. Transfer the detached cell aggregates to a 15 mL conical tube and rinse the well with an additional 2 mL mTeSR1 to collect any remaining aggregates. Add the rinse to the 15 mL tube.
8. Centrifuge the aggregates at 200 x *g* for 5 minutes at room temperature.
9. Aspirate the supernatant. Resuspend pellet in mTeSR1 containing 10 µM ROCK inhibitor by gently pipetting while ensuring that cells are maintained as aggregates.
10. Plate the human iPS cell aggregates with mTeSR1 onto a new matrigel-coated plate. (Remove matrigel solution before plating). *If the colonies are at an optimal density, the cells can be split every 5 - 7 days using 1:3 to 1:6 ratio.*
11. Rock the plate to distribute the clumps evenly in the wells. Place the plate into the 37°C incubator with 5% CO_2 and 95% humidity.
12. Change medium daily.

Cryopreserving human iPS cells

1. Prepare Human ESC freezing medium (Cat.no. ASM-5004, Applied StemCell, Inc.) on ice.
2. Perform steps 1-8 from **Passaging human iPS cells under feeder-free conditions**.
3. Gently aspirate the supernatant and loosen the cell pellet by tapping the bottom of the tube.
4. Gently resuspend the pellet in freezing medium, taking extra caution to leave the clumps larger than would normally be done for passaging.
5. Transfer 1 mL of clumps in freezing medium into each labeled cryogenic vial.
6. Place vials into an isopropanol freezing container and place the container at -80°C overnight.
7. Transfer to a liquid nitrogen tank on the following day.

II. Feeder-dependent culture conditions

Preparation of human ES medium

Knockout DMEM/F12 (Cat.no. 12660-012, Invitrogen) containing 20% knockout serum replacement (Cat.no. 10828-028, Invitrogen), 2mM glutaMAX (Cat.no. 35050-061, Invitrogen), 0.1 mM nonessential amino acids (Cat.no. 11140-050, Invitrogen), 0.1 mM 2-mercaptoethanol (Cat.no. 21985-023, Invitrogen), 10 ng/ml bFGF (Cat.no. 233-FB-025, R&D Systems), and 50 U/ml and 50 µg/ ml penicillin and streptomycin (Cat.no. 15140-122, Invitrogen), respectively.

Thawing cryopreserved human iPS cells

Warm medium to 37°C before use to ensure the highest level of cell viability. Due to the low survival rate of cryopreserved human iPS cells, the recovery is expected to take at least one week.

1. Quickly thaw the human iPS cells in a 37°C water bath by gently shaking the cryovial continuously until half thawed. Remove the cryovial from the water bath and spray with 70% ethanol.
2. Transfer the contents of the cryovial to a 15 mL conical tube. Add 5 mL warm human ES medium dropwise to the tube while gently mixing.
3. Centrifuge cells at 200 *x g* for 5 minutes at room temperature.
4. While centrifuging, remove MEF medium from the feeder cell plates, and wash the wells twice with Knockout DMEM/F12. Then add 1 ml of human ES Medium with 10 µM ROCK inhibitor (Y-27632, Cat.no. Y-05, StemRD) to the well on the 6-well plate.
5. After centrifugation, aspirate the medium from the 15 mL tube. Gently resuspend the cell pellet in 1 mL fresh human ES medium containing 10 µM ROCK inhibitor (Y-27632), taking care to maintain the cells as small cell clumps.
6. Transfer the medium containing the cell clumps to the well on the 6-well plate with MEF feeder cells.
7. Rock the plate gently to distribute the clumps evenly in the wells. Culture the cells at 37°C, with 5% CO_2 and 95% humidity.
8. Change medium daily. Check for undifferentiated colonies that are ready to passage when colonies are big enough (approximately 7-10 days after thawing).

Passaging human iPS cells under feeder-dependent conditions

1. Aspirate the medium and wash the cells twice with 1 ml of PBS.
2. Remove PBS completely and add 0.5 ml of Accutase (Cat.no. SCR005, Millipore, diluted 1:1 with DPBS before use) and incubate for 1-2 min at room temperature.
3. Tap the bottom of the plate to dislodge the cells from the bottom of the plate. Then aspirate the supernatant.
4. Add 1 ml of DMEM/F12 to the plate and gently wash off the feeder cells. Remove the supernatant. Repeat.
5. Add 1 ml of human ES medium containing 10 µM ROCK inhibitor to the plate and suspend the cell colonies by pipetting up and down, or by using a cell lifter (Cat.no. 3008, Corning) to harvest the human iPS cells. **It is important not to break up the colonies into single cells.**
6. Remove a plate of MEF feeder cells from the incubator. Aspirate the MEF medium. Wash once with KO DMEM/F12 medium.

7. Distribute 0.2 – 0.3 ml of the human iPS cell suspension into each well of a 6-well plate. Add human ES medium with ROCK inhibitor to a final volume of 2 ml per well. Right after plating the iPS cells, gently rock the plate and incubate at 37°C.
8. After 24 hours, remove the media and replace with human ES media (without ROCK inhibitor).
9. The human ES media must be changed every day and human iPS cells subcultured every 5-7 days. Track the passage number of the cells.

Cryopreserving human iPS cells

1. Prepare EZStem freezing medium (Cat.no. M050, ALSTEM) on ice.
2. Perform steps 1-6 from **Passaging human iPS cells under feeder-dependent conditions**.
3. Gently aspirate the supernatant and loosen the cell pellet by tapping the bottom of the tube.
4. Gently resuspend the pellet in freezing medium, taking extra caution to maintain the clumps larger than would normally be done for passaging.
5. Transfer 1 mL of clumps in freezing medium into each labeled cryogenic vial.
6. Place vials into an isopropanol freezing container and place the container at -80°C overnight.
7. Transfer to a liquid nitrogen tank the next day.