General Guidelines for Handling Human iPSC cells

- iPSC are cryopreserved in plastic cryovials and shipped on dry ice. If storing the iPSC before thawing, store in liquid nitrogen vapor. Storage directly in liquid nitrogen may result in cracking of o-rings.
- It is highly recommended that a small number of vials are cryopreserved as a master stock before beginning any experimentation.

Media and Reagents

Rho Kinase (ROCK) Inhibitor (Y-27632)

0.1% Gelatin solution

Table 1. Media

MEF medium	human iPSC growth medium	human iPSC cryopreservation medium
DMEM 10% FBS 1 mM Sodium Pyruvate $1x10^{-4}$M Non-essential amino acids 2mM L-glutamine	DMEM/F12 20% Knock-out Serum Replacement (KOSR) 2 mM L-glutamine $1x10^{-4}$M Non-essential amino acids 55 uM 2-mercaptoethanol bFGF (concentration is indicated on Certificate of Analysis)	90% ES grade FBS 10% DMSO

Table 2. Dissociation Enzymes – enzymes for dissociation should be chosen based on downstream assay or information supplied on the Certificate of Analysis supplied with each iPSC line.

Enzyme	Concentration	Incubation Period	Stop Medium
TrypLE™ Express	N/A	3 minutes or until edges of colonies begin to roll up.	growth medium
Collagenase	10 µg/ml	5 minutes or until edges of colonies begin to roll up.	growth medium

Plating Mouse Embryonic Fibroblasts (MEFs)

Irradiated MEFs may be obtained from a number of qualified vendors. MEF density ranges from 1 x 10^5 to 4 x 10^5 cells per well of 6-well plate. Optimal MEF density should be determined by individual labs each time a new lot of MEFs is obtained. When determining MEF density, thaw MEFs and plate at varying densities between 1 x 10^5 cells per well and 4 x 10^5 cells per well. Passage iPSCs onto MEFs and maintain for 5 days. Assess the ability of MEFs to maintain iPSCs in the undifferentiated state using morphology, growth rate and alkaline phosphatase expression.

1. Coat wells with 0.1% gelatin and incubate for 20 minutes at 37°C in incubator.
2. Thaw vial of irradiated MEFs by swirling in 37°C water bath and by adding the cell suspension dropwise to 10 ml of warm MEF medium.
3. Centrifuge at 1100 rpm for 5 minutes.
4. Aspirate medium and re-suspend pellet in 10 ml of MEF medium.
5. Remove gelatin-coated plate from incubator, aspirate gelatin and replace with 2 ml/well of MEF media.
6. Mix cell suspension and count viable cells by trypan blue exclusion.
7. Using viable cell number, calculate and aliquot appropriate number of cells to yield predetermined optimal cell density into each well of 6 well plate (be sure to mix cell suspension several times during plating to avoid settling of cells).
8. Place in incubator and rock plate back/forth, left/right and diagonally.

   NOTE: Failure to gently shake plate back and forth may result in uneven seeding.
9. Incubate overnight or up to 5 days before using as feeder layer for iPSC/ESC cells.

Thawing Human iPS cells

Optimally, MEFs should be plated on gelatin 1 day prior to use to allow adherence and flattening prior to

iPSC plating; MEFs should be plated no less than 8 hours before use. Perform the following steps in a time-efficient manner to obtain optimal cell viability.

1. Remove iPSC from liquid nitrogen vapor or dry ice and immerse the cryovial in in a 37°C water bath. Thaw quickly by gently swirling until only a small piece of frozen material remains. Spray the vial with 70% ethanol before transferring to a biological safety cabinet.

2. Gently add the thawed cell suspension dropwise to 10ml of warm culture medium in a sterile 15ml conical tube and gently mix cells by swirling.

3. Centrifuge conical tube containing cells at 1100 rpm for 3 min at room temperature.

4. Aspirate supernatant and gently re-suspend cells into 2 ml of warm culture medium supplemented with 10µM Y-27632 Rho kinase (ROCK) inhibitor (Y-compound).

5. Plate cells in 1 well of 6-well plate containing MEF feeder cells and gently rock plate to evenly distribute cells.

6. Replace with fresh medium without Y-compound. It is important to remove the Y-compound from the medium within 20 hours.

7. Maintain cells by daily medium exchange. **WARNING**: Failure to replace medium daily can result in spontaneous differentiation.

8. Colonies should be observed within 2-5 days.

Passaging of Human iPSC

Passage iPSCs when colonies approach borders of an adjacent colony. Ideally, iPSCs should be passaged before individual colonies begin differentiating in the center of colony (approximately 700 microns in diameter). To avoid spontaneous differentiation, do not allow colonies to overgrow.

Split ratios range from 1:3 to 1:6 and are indicated on the Certificate of Analysis for each iPSC line. We recommend that you record the passage information for each cell line to determine the exact growth kinetics of a particular line in your laboratory.

1. Remove spent medium from culture and rinse with PBS.

2. Add 1 ml/well enzyme solution for specified amount of time or until edges of colonies begin to roll up (see Table 2 and refer to the CofA for each cell line).

3. Aspirate enzyme solution.

4. Wash each well with 1ml of specified stop medium (see Table 2).

5. Add 1 ml of warm growth medium to cells and, using cell scraper, gently dislodge cells from plate.

6. Transfer the detached cell aggregates to a 15 ml conical tube. Rinse each well with 1 ml of growth medium to collect any remaining aggregates and add to conical tube containing cells.

7. Re-suspend cells in appropriate volume of growth medium for the appropriate cell density for cell culture vessel. We recommend passaging the cells without centrifugation ('no spin' method).

8. Seed cells onto prepared MEF-containing plates. Rock plates gently to achieve a uniform cell distribution.

Cyropreservation of Human iPSC

1. Remove spent medium from culture and rinse with PBS.

2. Add 1 ml/well enzyme solution for specified amount of time or until edges of colonies begin to roll up (see Table 2).

3. Aspirate enzyme solution.

4. Wash each well with 1ml of specified stop medium (see Table 2).

5. Add 1 ml of warm growth medium to cells and, using cell scraper, gently dislodge cells from plate.

6. Transfer the detached cell aggregates to a conical tube. Rinse each well with 1 ml of growth medium to collect any remaining aggregates and add to conical tube containing cells.

7. Centrifuge conical tube containing cells at 1100 rpm for 3 min at room temperature.

8. Remove supernatant and re-suspend cells in pre-cooled cryopreservation medium from Table 1 (1 ml for each well of a 6 well plate to be harvested).

9. Transfer 1 ml of cell suspension to each cryovial on ice.

10. Place cryovials in isopropanol freezing container and store at -80°C overnight.

11. Transfer vials to liquid nitrogen vapor.

Troubleshooting Tips

Problem	Observation of problem	Possible causes
Spontaneous differentiation	Morphology of differentiated cells can vary but is commonly characterized by 1. hypertrophic colonies (Image 2) 2. colonies without distinct borders (Image 3) 3. flattened cells (Image 4)	1. Low confluency or suboptimal passaging of cells (see "low viability after passage") 2. Poor or inappropriate feeder layer quality – this can be prevented by testing feeder layers prior to using in an experiment 3. Inadequate bFGF concentration; also cause 1. or 2. above
Non-uniform distribution of colonies within culture vessel	Areas within culture vessel with highly confluent iPSC colonies AND areas with few or no iPSC colonies	Failure to properly rock plates after plating cells. Rock plates back and forth gently immediately following plating - usually rocking plates back and forth and then side to side produces a fairly uniform distribution
Low Viability after Passage	Little to no cell colonies are visible 24-48 hours after passage	During passaging, clusters may have been disrupted into a single cell suspension – decrease pipetting of cells during passaging and decrease the incubation time with the passaging enzyme.
Low Viability after Recovery from Cryopreservation	Little to no cell colonies are visible within 5-7 days after recovery	Lack of or insufficient Rho kinase (ROCK) inhibitor in thawing medium

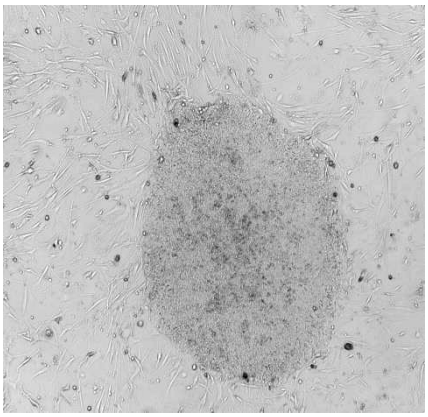

Image 1: Typical image of a human induced pluripotent stem cell (iPSC) colony. Note the distinct borders of the colony and morphology of the iPSCs.

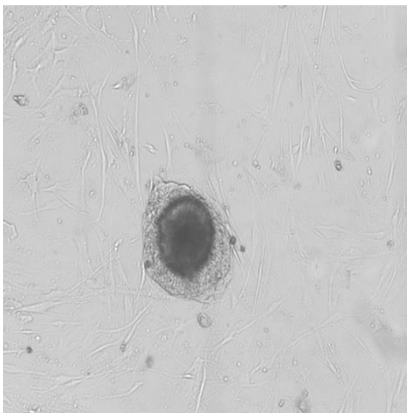

Image 2: Image of a colony that contains a hypertrophic center. This center can be a source of differentiation and should be removed from the culture.

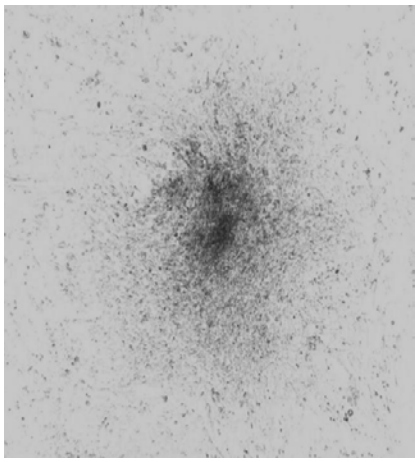

Image 3: Image of a differentiated colony. Note the lack of distinct borders and the large foci of dark cells within the center of the colony.

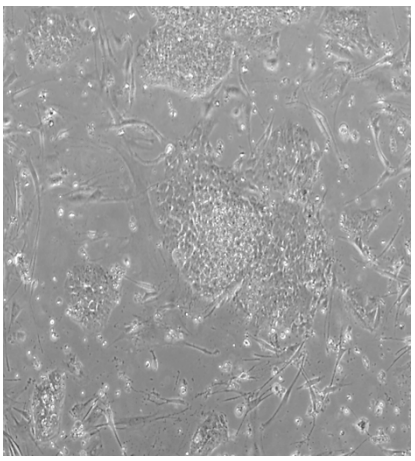

Image 4: Image of a differentiated colony. Note the flattened morphology of the cells on the outer portion of the colony.