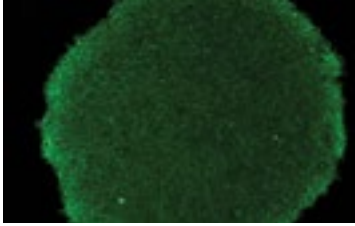
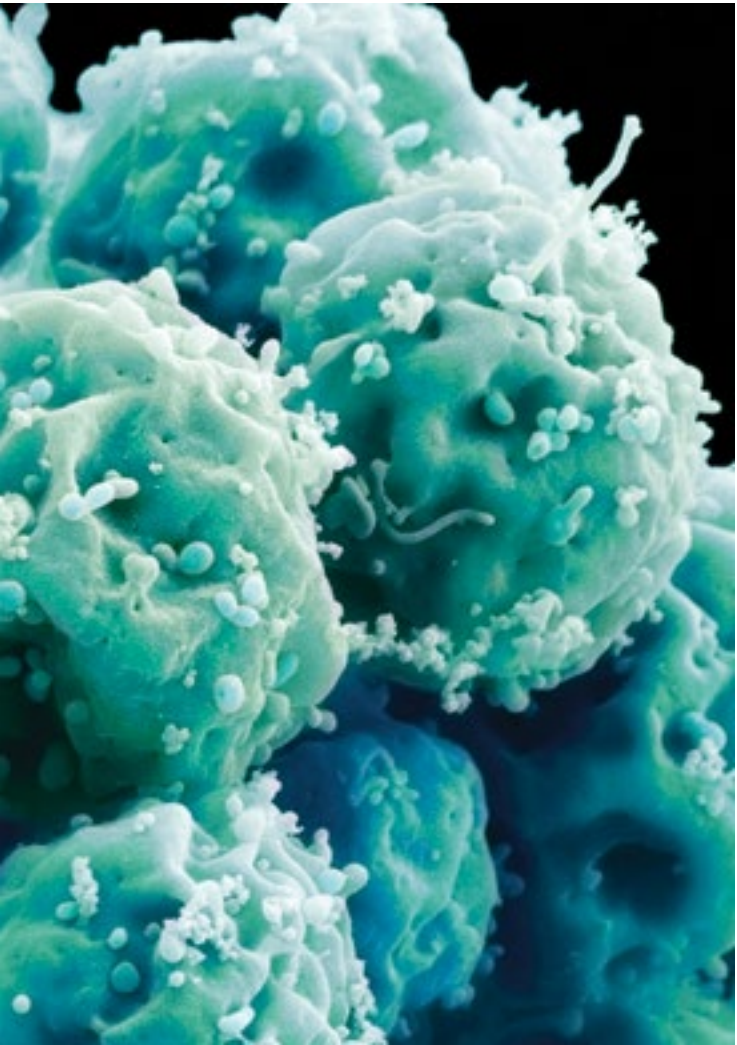


ATCC® STEM CELL CULTURE GUIDE

tips and techniques for culturing stem cells



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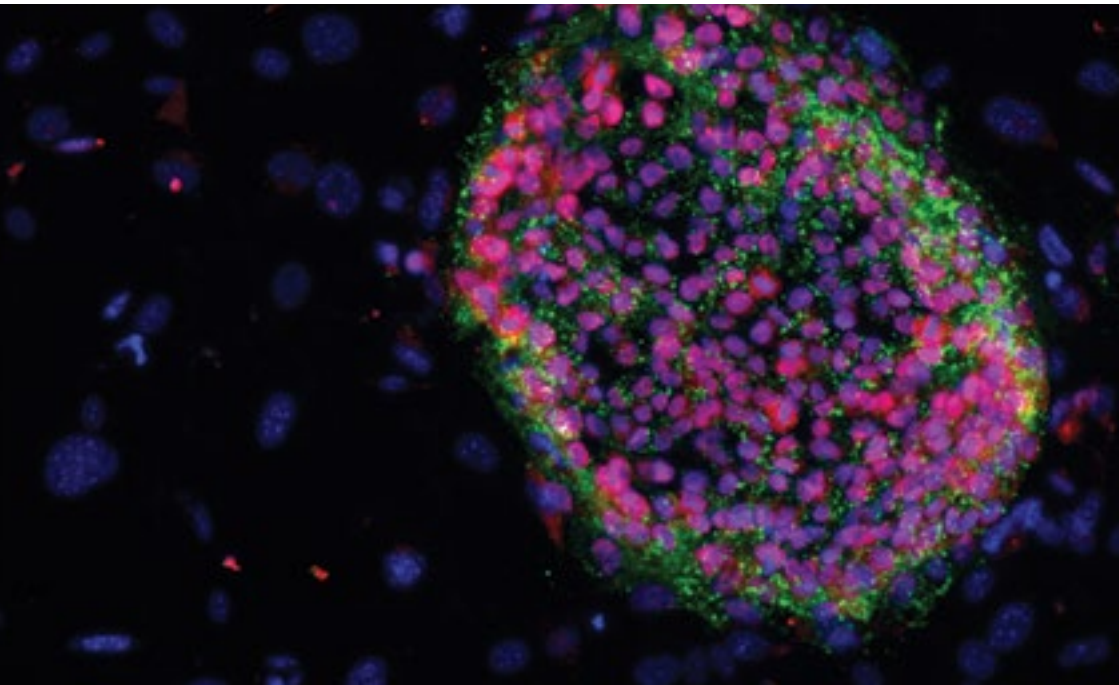

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1. Human induced Pluripotent Stem Cell (hiPSC) Guide

Human somatic cells can be "reprogrammed" to become stem-like by introducing combinations of genes typically expressed during early embryogenesis. These "reprogrammed" cells are known as human induced pluripotent stem cells (hiPSC)[1]. The ability to culture hiPSCs in an undifferentiated state and differentiate them to a specific lineage gives the cells great potential for developmental and tissue differentiation studies, disease-modelling, drug development, and regenerative medicine.

Undifferentiated hiPSCs can be maintained and proliferated on feeder-dependent or feeder-free (FF) culture systems. Traditionally, hiPSCs are co-cultured with mitotically inactivated mouse embryonic fibroblast (MEF) or human foreskin fibroblast (HFF) feeder cells. However, the use of feeders can be labour intensive and may pose a risk of transmitting animal pathogens to hiPSCs hindering clinical application of the cells. Recent advances in stem cell technology have made it possible to culture hiPSCs in a FF system by the utilisation of extracellular matrices.

hiPSC cultures have a natural tendency to differentiate. Thus, the *in vitro* maintenance of undifferentiated hiPSCs requires culture techniques that are different from conventional, continuous, or primary cells. The overall goal is to achieve a balance between promoting pluripotent cell growth and inhibiting spontaneous cellular differentiation. This manual describes the methods recommended by ATCC for the long-term maintenance of high quality hiPSC cultures.

1.1 hiPSC Lines

ATCC offers a wide variety of frozen hiPSCs, including cells reprogrammed using the Yamanaka factors (OCT4, SOX2, KLF4 and MYC)[2]. ATCC hiPSCs are well-characterised, robust, and are frozen at a low passage number. These hiPSC lines are optimised for use in feeder-free, serum-free culture, however, they can be easily adapted for use in feeder-dependent cell culture systems. Please see www.lgcstandards-atcc.org for a complete list of available hiPSCs.

Substantial variation in the differentiation potential has been reported between hiPSC lines. Many factors could contribute to the variation, such as the genetic background of the original material, derivation methods and culture conditions. ATCC offers hiPSC lines that have been fully characterised for the expression of pluripotent markers, differentiation potential for the three germ layers (ectoderm, endoderm, mesoderm), as well as karyotypic stability.

Human iPS Cells

High viability, low passage iPS cells are pre-adapted to serum-free, xeno-free and feeder-free culture conditions.

ATCC® No.	Designation	Reprogramming Method	Disease
ACS-1011™	ATCC-DYR0100 Human Induced Pluripotent Stem Cells	Retroviral expression of OCT4, SOX2, KLF4, and MYC genes	Normal
ACS-1007™	ATCC-HYR0103 Human Induced Pluripotent Stem Cells	Retroviral expression of OCT4, SOX2, KFL4, and MYC genes	Normal

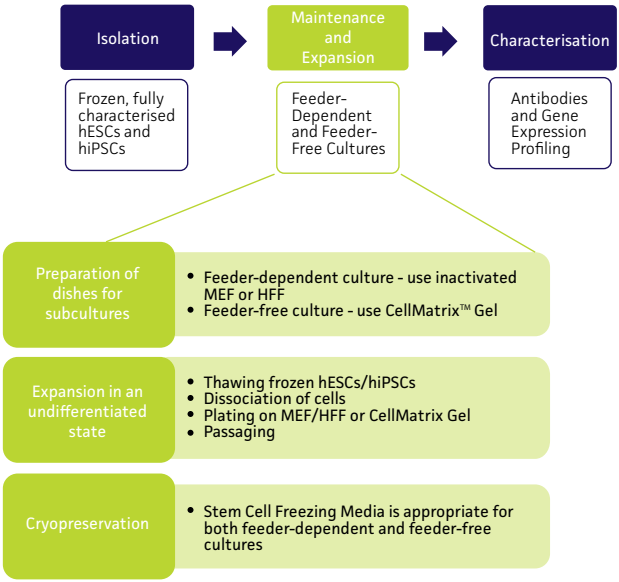
Human iPS Cells

High viability, low passage iPS cells are pre-adapted to serum-free, xeno-free and feeder-free culture conditions.

ATCC® No.	Designation	Reprogramming Method	Disease
ACS-1003™	ATCC-DYP0730 Human Induced Pluripotent Stem Cells	Episomal expression of OCT4, SOX2, KLF4, and MYC genes	Down syndrome
ACS-1004™	ATCC-DYP0250 Human Induced Pluripotent Stem Cells	Episomal expression of OCT4, SOX2, KLF4, and MYC genes	Cystic fibrosis; homozygous for the Delta 508 mutation in the CFTR gene
ACS-1012™	ATCC-DYR0530 Human Induced Pluripotent Stem Cells	Retroviral expression of OCT4, SOX2, KLF4, and MYC genes	Parkinson's disease, asthma, depression
ACS-1013™	ATCC-DYS0530 Human Induced Pluripotent Stem Cells	Sendai viral expression of OCT4, SOC2, KLF4 and MYC genes	Parkinson's disease, asthma, depression
ACS-1014™	ATCC-DYP0530 Human Induced Pluripotent Stem Cells	Episomal expression of OCT3/4, SOX2, KLF4, and MYC genes	Parkinson's disease, asthma, depression

1.2 Overview: Isolation, Maintenance, Expansion and Characterisation

The following sections focus on serum-free maintenance and expansion of hiPSC cultures, in both feeder-dependent and feeder-free systems.



1.3 Feeder-Free hiPSC Cultures

A significant advancement in stem cell culture is the development of feeder-free systems. Feeder-free cultures create culture conditions that are more reproducible and easy to use, while facilitating larger cultures. In addition, the concern of obtaining mixed cultures of stem cells and fibroblasts is eliminated. In a feeder-free system, a biological matrix is used in place of fibroblast feeders to provide a surface for the attachment of hiPSCs. Cell culture dishes are coated with CellMatrix Basement Membrane Gel, incubated for one hour and then are ready to use (Figure 1). The ATCC hiPSC lines are cultured in the feeder-free system and therefore, no adaptation is required when starting your cultures.

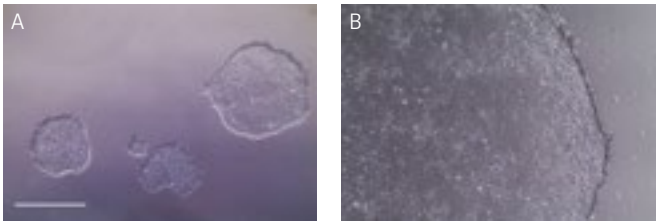


Figure 1. Characteristic morphology of hiPSCs grown on feeder-free cultures using Pluripotent Stem Cell SFM XF/FF on CellMatrix Basement Membrane Gel.

(A) Undifferentiated hiPSCs culture on CellMatrix gel in Pluripotent Stem Cell SFM XF/FF.

(B) Undifferentiated hiPSCs culture on CellMatrix gel in Pluripotent Stem Cell SFM XF/FF. Note the round colony with tightly packed hiPSCs with well-defined sharp edges. Individual cells within the colony exhibit prominent nucleoli with high nucleus-to-cytoplasm volume ratio.

1.3.1 Materials

ATCC® No.	Product Name	Size	Storage
ACS-3002	Pluripotent Stem Cell SFM XF/FF (consists of Basal Medium and Growth Supplement, combined prior to use)	500 mL	-20°C/2°C to 8°C
30-2006	DMEM: F-12 Medium	500 mL	2°C to 8°C
ACS-3010	Stem Cell Dissociation Reagent	250 mg	2°C to 8°C
ACS-3035	CellMatrix Basement Membrane Gel	5 mL	-80°C
ACS-3030	ROCK Inhibitor Y27632	10 mg	-20°C
30-2200	Dulbecco's Phosphate Buffered Saline (D-PBS)	500 mL	RT
---	70% ethanol	---	---

1.3.2 Preparation of Media Reagents

1. Complete Pluripotent Stem Cell SFM XF/FF
   a) Thaw Pluripotent Stem Cell Basal Medium at 2°C to 8°C over night. If 500 mL of medium will not be consumed within two weeks, aliquot the unused Pluripotent Stem Cell Basal Medium in desired volumes (e.g., 95 mL) and store at -20°C.

b) Allow medium to warm to room temperature.

c) Add 5 mL Pluripotent Stem Cell Growth Supplement to 95 mL Basal Medium. Adjust volumes accordingly to avoid storing complete medium for greater than 2 weeks.

d) Supplemented medium is stable for 2 weeks at 2°C to 8°C.

2. Aliquoting CellMatrix gel for long-term storage

a) Thaw CellMatrix gel on ice and swirl gently to mix. Keep CellMatrix gel, vials and pipette tips on ice at all times to prevent CellMatrix gel from solidifying. If air bubbles form, they may be eliminated by centrifuging CellMatrix gel at 300 x g for 10 minutes at 2°C to 8°C.

b) Determine the appropriate volume per aliquot based on concentration and usage. The concentration of CellMatrix gel is found on the product label.

NOTE:
Product stability is extended by aliquoting the product in working volumes to avoid repeated freezing and thawing. Note that CellMatrix gel is diluted just prior to use. A protein concentration of 150 µg/mL is recommended for the propagation of stem cells.

Example: 2 mL of Cell Matrix Gel at 150 µg/mL is required to coat one 6 cm dish. To coat four 6 cm dishes, prepare aliquots as follows:

Aliquot CellMatrix gel such that one vial when thawed and diluted in 8 mL DMEM:F-12 Medium is at a working concentration of 150 µg/mL:

Protein concentration of CellMatrix gel (on product label)=14 mg/mL.

$$\frac{(8\ \text{mL}) \times (0.15\ \text{mg/mL})}{14\ \text{mg/mL}} = 0.086\ \text{mL}$$

Aliquot 86 µL CellMatrix gel per tube.

c) Dispense appropriately-sized aliquots into pre-cooled tubes on ice and immediately place tubes at -20°C or -80°C for long-term storage.

3. Reconstitution of Stem Cell Dissociation Reagent

Lyophilised proteins tend to be hygroscopic. Bring the vial of Stem Cell Dissociation Reagent to room temperature before opening. The vial should not be cool to the touch. Once opened, the lyophilised material should be stored desiccated.

The specific activity of the reagent is found on the product label. Prepare a 0.5 U/mL working solution and aliquot into working volumes according to usage.

a) Dissolve the appropriate amount of Stem Cell Dissociation Reagent in DMEM: F-12 Medium to prepare a 0.5 U/mL working solution.

Example: To prepare 40 mL of a 0.5 U/mL working solution:
Specific activity of Stem Cell Dissociation Reagent (on product label)=1.46 U/mg

$$\frac{(40\ \text{mL}) \times (0.5\ \text{U/mL})}{1.46\ \text{U/mg}} = 13.7\ \text{mg}$$

Dissolve 13.7 mg Stem Cell Dissociation Reagent in 40 mL DMEM: F-12 Medium.

b) Filter sterilise through a 0.22 µm filter membrane.

c) Aliquot into working volumes according to routine usage.

d) Store aliquots at -20°C for up to three months. Avoid repeated freezing and thawing. Thawed aliquots may be kept at 2°C to 8°C for up to two weeks.

4. Reconstitution of ROCK Inhibitor Y27632

   ROCK Inhibitor Y27632 is soluble to 100 mM in water or Dulbecco's Phosphate Buffered Saline (D-PBS). We recommend preparation of a 10 mM stock solution as follows:

   a) Add 3 mL of sterile water or D-PBS to the 10 mg vial of ROCK Inhibitor Y27632. Mix thoroughly.

   b) Aliquot into working volumes according to routine usage. Note that ROCK Inhibitor Y27632 is used at a final concentration of 10 µM (1:1000 dilution) in the cell culture medium.

   c) Store aliquots at -20°C. Avoid repeated freezing and thawing. Thawed aliquots may be kept at 2°C to 8°C for two weeks.

1.3.3 CellMatrix Basement Membrane Gel

The protocol is designed for coating four 6 cm dishes. 2 mL of CellMatrix is required per 6 cm dish. Volumes can be directly scaled according to the size and number of tissue culture vessels used.

CellMatrix gel will solidify in 15-30 minutes if the temperature is above 15°C. Keep CellMatrix gel and labware (pipette tips, serological pipettes, conical tubes) on ice at all times to prevent the matrix from gelling prematurely. If necessary, CellMatrix gel may be returned to a liquid state by placing it at 2°C to 8°C, on ice, for 24-48 hours.

1. Remove one aliquot of CellMatrix gel from -20°C/-80°C storage and place on ice. Thaw CellMatrix gel in the refrigerator (2°C to 8°C), on ice, overnight.
2. Sterilise the vial by rinsing with 70% ethanol. All of the operations from this point on should be carried out under strict aseptic conditions.
3. Place 8 mL cold DMEM: F-12 Medium in a cold 15 mL conical tube on ice.
4. Add the thawed CellMatrix gel to the 8 mL of DMEM: F-12 Medium on ice. Rinse CellMatrix gel vial with cold DMEM: F-12 Medium and transfer to tube. Mix well. The final CellMatrix gel concentration should be 150 µg/mL (see section 4.2.2 on aliquoting concentrated CellMatrix gel).
5. Immediately coat the dishes with diluted CellMatrix gel. Use 2 mL for each 6 cm dish. Swirl dish gently to ensure that the entire dish is covered evenly.
6. Leave coated dishes at 37°C for one hour.
7. Aspirate coating solution and immediately plate the cells. It is critical that the coating does not dry out.

NOTE:
If air bubbles form when coating dishes, use a chilled pipette tip to break up the bubbles.

If the dishes will not be used the same day they are prepared, do not aspirate the coating solution. Seal the coated dishes with Parafilm and store at 2°C to 8°C for up to one week. Note that stored dishes should be warmed to room temperature in a biological safety cabinet for at least one hour before use.

1.3.4 Thawing of Cryopreserved hiPSCs

1. Pre-warm Complete Pluripotent Stem Cell SFM XF/FF in a 37°C water bath. If using a small volume of medium (50 mL or less), warm only the volume needed in a sterile conical tube. Avoid warming complete medium multiple times.

NOTE:
Before thawing cells, make certain culture dishes coated with CellMatrix gel are prepared; see section 4.3.

2. Remove cryovial of frozen cells from liquid nitrogen storage.
3. Thaw the cells by gently swirling in a 37°C water bath. To reduce the possibility of contamination, keep the O-ring and cap out of the water. Thawing should be rapid (approximately 1 to 2 minutes). Remove the cryovial from water bath when only a few ice crystals are remaining.
4. Sterilise the cryovial by rinsing with 70% ethanol.
5. Use a 1 mL or 5 mL pipette to gently transfer cell suspension to a 15 mL conical tube.
6. Slowly add 4 mL Complete Pluripotent Stem Cell SFM XF/FF drop-wise, to the conical tube. Use an additional 1 mL of media to rinse the cryovial and transfer the liquid to the 15 mL tube. Shake the conical tube gently to mix the cells while adding media.

NOTE:
Prepare Complete Pluripotent Stem Cell SFM XF/FF with ROCK Inhibitor by adding 20 µL of 10 mM ROCK Inhibitor Y27632 to 20 mL of medium.

7. Gently pipette the cells up and down several times to mix thoroughly. Avoid breaking apart the cell aggregates into a single-cell suspension.
8. Centrifuge cells at 200 x g for 5 minutes at room temperature.
9. Aspirate the supernatant and discard. Gently tap the bottom of the tube to loosen the cell pellet.
10. Add 1 mL of Complete Pluripotent Stem Cell SFM XF/FF in the presence of 10 µM ROCK Inhibitor Y27632 to the tube. Gently resuspend the pellet by pipetting up and down 5-6 times with a 1 mL tip, maintaining the cell aggregates.
11. Seed 0.25 mL of cell aggregates onto four 6 cm CellMatrix gel coated dishes, each containing 4 mL of Complete Pluripotent Stem Cell SFM XF/FF in the presence of 10 µM ROCK Inhibitor Y27632.
12. Change medium the next day and daily thereafter until the colonies reach 80% confluency. ROCK Inhibitor Y27632 is not required in subsequent cell culture medium changes.

1.3.5 Maintenance

1. Changing Media
   a) Pre-warm Complete Pluripotent Stem Cell SFM XF/FF in a 37°C water bath. If using a small volume of medium (50 mL or less), warm only the volume needed in a sterile conical tube. Avoid warming complete medium multiple times.

b) Remove the cells from the incubator and view each dish under the microscope to determine percent cellular confluence and morphology of undifferentiated cells (see section 4.5.2).

> *Characteristics of differentiation include colonies with less defined edges, dark areas, or non-uniform morphology.*

c) Carefully aspirate the medium without disturbing the monolayer.

d) Add 4 mL of fresh, pre-warmed Complete Pluripotent Stem Cell SFM XF/FF to the 6 cm dish and return the dish to the incubator.

e) Every 24 hours, view each dish under the microscope to determine percent cellular confluence. If not ready to passage, repeat steps c and d as described above. When cultures have reached approximately 80% confluence, with 90% undifferentiated cells, it is time to passage (see section 4.6).

> *It is essential that the cells are passaged before reaching confluence. Confluent colonies induce cell differentiation, along with exhibiting changes in morphology, slower proliferation and reduced differentiation capacity after passaging.*

2. Identification and Removal of Differentiated Cells

a) Undifferentiated hiPSCs grow as compact colonies and exhibit high nucleus-to-cytoplasm ratios and prominent nucleoli. The center of the cells will appear bright due to the multilayering of colonies. During the expansion and maintenance of hiPSCs, however, differentiated cells or other cell types may develop. Differentiating colonies have less-defined edges, dark areas, or exhibit a non-uniform morphology that is not typical of pluripotent stem cells such as fibroblast-like cells.

NOTE:
We recommend less than 10% differentiation to maintain high quality cultures. If differentiation is greater than 10%, differentiated cells must be removed from the cell culture.

b) Observe cultures under the microscope for the appearance of differentiated cells (Figure 2). Use a lens marker to mark areas of differentiation on the dish.

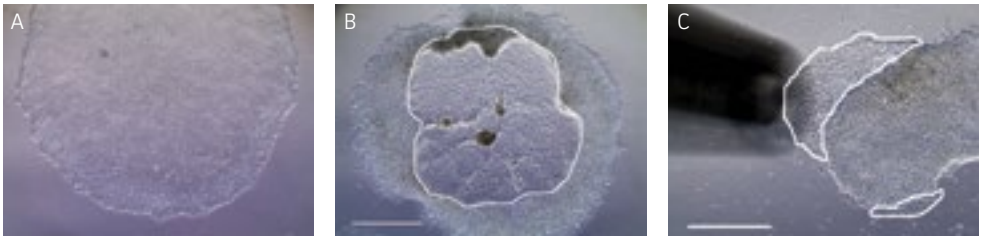


Figure 2. Characteristic morphology of hiPSCs grown on FF culture using Pluripotent Stem Cell SFM XF/FF on CellMatrix gel.

(A) Undifferentiated hiPSC colony cultured in Pluripotent Stem Cell SFM XF/FF.

(B) Partially differentiated hiPSC colony cultured on CellMatrix gel. The circled region in the center of the colony designates the area of differentiated hiPSCs that needs to be removed.

(C) Partially differentiated hiPSCs (circled) being removed/aspirated using a 200 µL pipette tip attached to the end of the aspirating pipette.

d) To remove clusters of differentiated cells, attach a fine-tipped aspirating pipette to a vacuum source. The tip can be made smaller by attaching a 200-µL pipette tip to the end of the aspirating pipette. Suction away portions of colonies which appear differentiated, as marked in step a). Take care not to allow the cultures to dry out.

3. When to Passage Cells

   Cells are typically split at a 1:4 ratio when cells are 80% confluent after approximately 4-5 days of growth (Figure 3). When colonies have grown such that adjacent colonies merge, or differentiation occurs in the center of each colony, the colonies are ready for passage.

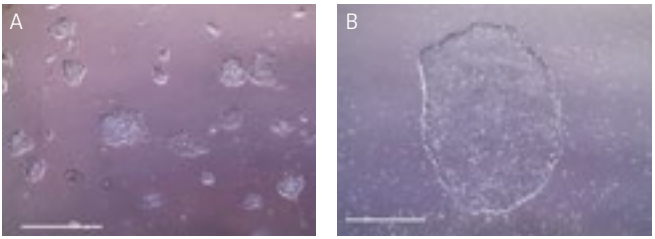


Figure 3. Morphology of hiPSCs cultured on CellMatrix gel.
(A) Undifferentiated hiPSCs 2 days post passage.
(B) Undifferentiated hiPSCs 5 days post passage, ready to split.

1.3.6 Passaging

The protocol is designed for the dissociation and expansion of cells on a 6 cm dish. Volumes should be adjusted according to the size of the tissue culture vessels used.

NOTE:
Make certain culture dishes coated with CellMatrix gel are prepared; see section 4.3

1. Remove an aliquot of 0.5 U/mL Stem Cell Dissociation Reagent working solution from the freezer and allow it to warm to room temperature (15°C to 25°C).
2. Pre-warm Complete Pluripotent Stem Cell SFM XF/FF in a 37°C water bath. If using a small volume of medium (50 mL or less), warm only the volume needed in a sterile conical tube. Avoid warming complete medium multiple times.
3. Aspirate the medium from the cells.
4. Rinse cells twice with 4 mL/dish of D-PBS.
5. Add 2 mL of Stem Cell Dissociation Reagent working solution to each dish.
6. Incubate at 37°C for 10-15 minutes. The reaction is complete when the edges of the individual colonies begin to loosen and fold back from the dish. View the plate under the microscope starting at 5 minutes as incubation time may vary depending on the cell line being used and colony size.

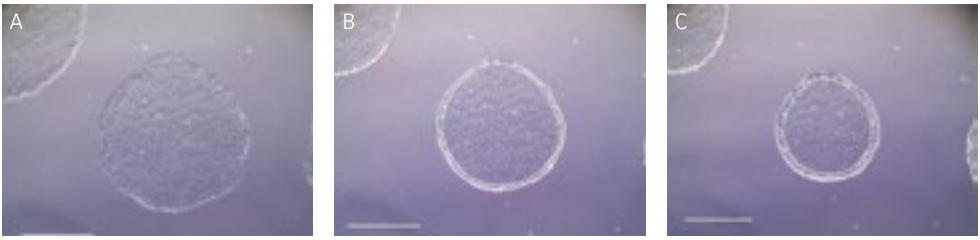


Figure 4. Time lapse images of passaging hiPSCs cultured on CellMatrix gel using Stem Cell Dissociation Reagent.
(A) Undifferentiated hiPSC colony ready for passaging.
(B) hiPSC colony after 5 minutes in Stem Cell Dissociation Reagent, edges of the hiPSC colony is starting to lift/curl.
(C) hiPSC colony after 10 minutes in Stem Cell Dissociation Reagent, ready to passage.

7. Carefully aspirate the Stem Cell Dissociation Reagent and gently rinse the colonies with 4 mL/dish of DMEM: F-12 Medium, taking care not to dislodge the cells during manipulation.
8. Add 2 mL of Complete Pluripotent Stem Cell SFM XF/FF to each dish. Detach cells by pipetting up and down several times with a 1 mL tip. Take care not to over-pipette the culture into a single-cell suspension as single cells will not establish colonies after seeding.

NOTE:
Stem Cell Dissociation Reagent must be completely removed from the cells since the reagent is not inactivated by medium or serum.

9. Transfer the cell aggregates to a 15 mL conical tube.
10. Add an additional 4 mL of Complete Pluripotent Stem Cell SFM XF/FF to collect any remaining cells on the surface of the dish. Transfer the rinse to the 15 mL conical tube containing the cell aggregates.
11. Centrifuge cells at 200 x g for 5 minutes at room temperature to pellet cells.
12. Aspirate the supernatant and discard. For each dish processed, add 2 mL of Complete Pluripotent Stem Cell SFM XF/FF in the presence of 10 µM ROCK Inhibitor Y27632 to the 15 mL tube.

NOTE:
Prepare Complete Pluripotent Stem Cell SFM XF/FF with ROCK Inhibitor by adding 20 µL of 10mm ROCK Inhibitor Y27632 to 20 mL of medium.

13. Gently resuspend the pellet by pipetting up and down 5-6 times with a 1 mL tip, maintaining the small cell aggregates.
14. Transfer 0.5 mL of the cell aggregates onto each CellMatrix gel-coated dish that contains 4 mL Complete Pluripotent Stem Cell SFM XF/FF in the presence of 10 µM ROCK Inhibitor Y27632 for a 1:4 split ratio.
15. Swiftly move the dishes in a forward to backward, then left to right pattern once to gently disperse the cells evenly across the surface of the dishes. Incubate plates overnight at 37°C and 5% CO_2.
16. Change medium daily until the colonies are big enough to passage (see section 4.5). ROCK Inhibitor Y27632 is not required in subsequent cell culture medium changes.

1.4 Feeder-Dependent hiPSC Cultures

A standard cell culture method utilised for maintaining hiPSCs is in co-culture with fibroblasts capable of conditioning the culture environment to support human pluripotent stem cells. In feeder-dependent cell culture systems, the fibroblast-seeded plates need to be prepared in advance, and the fibroblasts need to be mitotically inactivated, either by gamma irradiation or treatment with mitomycin C. Either mouse embryonic fibroblasts (MEF) or human foreskin fibroblasts (HFF) are recommended for hiPSC cultures. The hiPSCs are easily distinguishable from the fibroblasts as the colonies are well defined and compact. Individual cells within the colony are tightly packed and have a high nucleus-to-cytoplasm volume ratio (Figure 5).

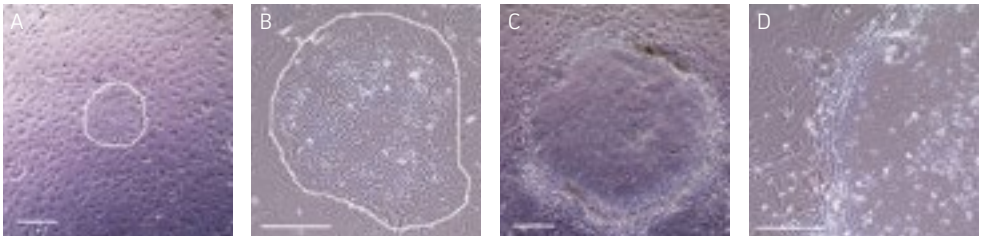


Figure 5. Characteristic morphology of hiPSCs grown on MEFs using Pluripotent Stem Cell SFM XF.

(A) A small hiPSC colony (circled) with loose colony morphology.

(B) A small hiPSC colony (circled) with loose colony morphology.

(C) Undifferentiated hiPSC colony ready to passage with tightly packed hiPSCs with well-defined sharp edges.

(D) Individual cells within the colony exhibit prominent nucleoli with high nucleus-to-cytoplasm volume ratio.

1.4.1 Materials

1. Media and Reagents

ATCC® No.	Product Name	Size	Storage
ACS-3001	Pluripotent Stem Cell SFM XF	500 mL	-20°C
30-2002	Dulbecco's Modified Eagle's Medium (DMEM)	500 mL	2°C to 8°C
30-2020	Fetal Bovine Serum (FBS)	500 mL	-20°C
30-2006	DMEM: F-12 Medium	500 mL	2°C to 8°C
ACS-3030	ROCK Inhibitor Y27632	10 mg	-20°C
30-2200	Dulbecco's Phosphate Buffered Saline (D-PBS)	500 mL	RT
30-2404	Erythrosin B Stain Solution	40 mL	RT
---	70% ethanol	---	---

2. Feeder Cells

Feeder cells condition the medium through metabolic leakage and provide a support matrix for cell attachment and proliferation. However, it is critical that the feeder cells do not overgrow the hiPSCs. The feeder cells listed in the appendix are either irradiated or treated with mitomycin C, which allows the feeder cells to continue to metabolise,

but not to proliferate. Untreated feeder cells are also available from ATCC, but they must be treated prior to use in hiPSC culture.

See Appendix for a list of ATCC feeder cell lines.

See www.lgcstandards-atcc.org for more details.

1.4.2 Preparation of Media Reagents

1. Pluripotent Stem Cell SFM XF

   Pluripotent Stem Cell SFM XF medium is ready to use; no supplements are required. However, if 500 mL of medium will not be consumed within two weeks, thaw and aliquot the medium into desired volumes (e.g., 100 mL) and store at -20°C.

2. DMEM + 15% FBS (Feeder medium)

   e) Thaw Fetal Bovine Serum (FBS) at room temperature.

   f) Add FBS to a final concentration of 15% in Dulbecco's Modified Eagle's Medium (DMEM).

   g) Complete medium can be stored at 2°C to 8°C for up to two weeks.

3. Reconstitution of Stem Cell Dissociation Reagent

   Prepare a 0.5 U/mL working solution in DMEM: F-12 Medium and aliquot into working volumes according to usage. See section 4.2.3 for details.

4. Reconstitution of ROCK Inhibitor Y27632

   Prepare a 10 mM stock solution in Dulbecco's Phosphate Buffered Saline (D-PBS). See section 4.2.4 for details.

1.4.3 Preparation of Dishes with Feeder Cells

> *Important: Cells should be plated 24 hours before use as a feeder layer and kept for no more than seven days.*

1. Pre-warm DMEM + 15% FBS (Feeder Medium) in a 37°C water bath. If using a small volume of medium (50 mL or less), warm only the volume needed in a sterile conical tube. Avoid warming complete medium multiple times.
2. Pipet 5 mL of DMEM + 15% FBS to a 15 mL conical tube.
3. Thaw one vial of MEF or HFF by gently swirling in a 37°C water. To reduce the possibility of contamination, keep the O-ring and cap out of the water. Thawing should be rapid (approximately 1 to 2 minutes). Remove the cryovial from water bath when a few ice crystals remain.
4. Sterilise the vial by rinsing with 70% ethanol. All of the operations from this point on should be carried out under strict aseptic conditions.
5. Use a 1 mL or 5 mL pipette to gently transfer cell suspension to a 15 mL conical tube containing DMEM + 15% FBS. Use an additional 1 mL of medium to rinse the vial and transfer the contents to the 15 mL tube.

6. Add 4 mL of DMEM + 15% FBS, bringing the total volume to 10 mL.
7. Gently mix and pellet the cells by centrifugation at 200 x g for 5 minutes.
8. Discard the supernatant and resuspend the cells with 10 mL of fresh, pre-warmed DMEM + 15% FBS.
9. Count viable cells (e.g., using erythrosin B exclusion). Cell viability should be > 85%.
10. Add the appropriate amount of fresh, pre-warmed DMEM + 15% FBS to plate cells at a seed density of 1.2 x 10^4 cells/cm^2. Refer to Table 1 for suggested plating volumes.
11. Incubate the feeder cells at 37°C and 5% CO_2. Cells should be plated 24 hours before use and stored at 37°C and 5% CO_2 for no more than 7 days.

NOTE:
Optimal feeder cell density is critical to maintain typical hiPSC morphology.

When storing plates, DMEM + 15% FBS should be changed twice during the week or when pH decreases. It is important to avoid excessive alkalinity of the medium during the recovery of the cells.

Table 1. MEFs/HFFs Seeding Densities for Different Culture Dishes (Calculated with 12,000 cells/cm^2)

Cell Culture Vessel	Growth Area (cm^2)	Total Number of Feeder Cells per Dish	Optimal Volume for Plating (mL)
24 well	2.0	2.4 x 10^4	0.5
12 well	4.0	4.8 x 10^4	1
6 well / 35 mm	9.5	1.2 x 10^5	2
6 cm	21	3.4 x 10^5	4
10 cm	56	9.4 x 10^5	10

1.4.4 Thawing of Cryopreserved hiPSCs

1. Pre-warm Pluripotent Stem Cell SFM XF in a 37°C water bath. If using a small volume of medium (50 mL or less), warm only the volume needed in a sterile conical tube. Avoid warming complete medium multiple times.
2. Replace the MEF or HFF feeder dish medium (DMEM + 15% FBS) with 4 mL of Pluripotent Stem Cell SFM XF in the presence of 10 µM ROCK Inhibitor Y27632. Place dish in the incubator for 15 minutes to allow the medium to reach its normal pH (7.0-7.6). Four 6 cm plates are needed for each vial of cells thawed.
3. Remove cryovial of frozen cells from liquid nitrogen storage.
4. Thaw the cells by gently swirling in a 37°C water bath. To reduce the possibility of contamination, keep the O-ring and cap out of the water. Thawing should be rapid (approximately 1 to 2 minutes). Remove the cryovial from water bath when only a few ice crystals are remaining.
5. Sterilise the cryovial with 70% ethanol. All of the operations from this point on should be carried out under strict aseptic conditions.

NOTE:
Before thawing cells, make certain culture dishes seeded with MEF or HFF are prepared; see section 5.3.

6. Use a 1 mL or 5 mL pipette to gently transfer cell suspension to a 15 mL conical tube.
7. Slowly add 4 mL Pluripotent Stem Cell SFM XF drop-wise, to the conical tube. Use an additional 1 mL of media to rinse the cryovial and transfer the liquid to the 15 mL tube. Shake the conical tube gently to mix the cells while adding media.
8. Gently pipette the cells up and down several times to mix thoroughly. Avoid breaking apart the cell aggregates into a single-cell suspension as single cells will not establish colonies after seeding.
9. Centrifuge cells at 200 x g for 5 minutes at room temperature.
10. Aspirate the supernatant and discard. Gently tap the bottom of the tube to loosen the cell pellet.
11. Add 1 mL of Pluripotent Stem Cell SFM XF in the presence of 10 µM ROCK Inhibitor Y27632 to the tube. Gently resuspend the pellet by pipetting up and down 5-6 times with a 1 mL tip, maintaining the cell aggregates.
12. Seed 0.25 mL of cell aggregates onto four 6 cm MEF or HFF feeder dishes, as prepared in step 2.
13. Change medium the next day and daily thereafter until the colonies reach 80% confluency. ROCK Inhibitor Y27632 is not required in subsequent cell culture medium changes.

1.4.5 Maintenance

1. Changing Media
   a) Pre-warm Pluripotent Stem Cell SFM XF in a 37°C water bath. If using a small volume of medium (50 mL or less), warm only the volume needed in a sterile conical tube. Avoid warming medium multiple times.

   NOTE:
   Characteristics of differentiation include colonies with less defined edges, dark areas, or non-uniform morphology. See action 5.5.2

   b) Remove the cells from the incubator and view each dish under the microscope to determine percent cellular confluence and morphology of undifferentiated cells (see section 5.5.2 on Identification and Removal of Differentiated Cells).
   c) Carefully aspirate the medium without disturbing the monolayer.
   d) Add 4 mL of fresh, pre-warmed Pluripotent Stem Cell SFM XF to the 6 cm dish and return the dish to the incubator.
   e) Every 24 hours, view each dish under the microscope to determine percent cellular confluence. If not ready to passage, repeat steps c and d as described above. When cultures have reached approximately 80% confluence, with 90% undifferentiated cells, it is time to passage (see section 4.6).

   Important: Cells should be plated 24 hours before use as a feeder layer and kept for no more than seven days.

2. Identification and Removal of Differentiated Cells

   We recommend less than 10% differentiation to maintain high quality cultures. If differentiation is greater than 10% differentiated cells must be removed from the cell culture.

a) Undifferentiated hiPSCs grow as compact colonies and exhibit high nucleus-to-cytoplasm ratios and prominent nucleoli. When cultured in Pluripotent Stem Cell SFM XF, small hiPSC colonies may initially exhibit loose colony morphology but will become more compact once the colony grows bigger. In addition, the colonies maintain a distinct border on feeder cells. During the expansion and maintenance of hiPSCs, however, differentiated cells or other cell types may develop. Differentiating colonies have less defined edges, dark areas, or exhibit a non-uniform morphology that is not typical of pluripotent stem cells such as fibroblast-like cells (Figure 6).

b) Observe cultures under the microscope for the appearance of differentiated cells. Use a lens marker to mark areas of differentiation on the dish.

c) To remove clusters of differentiated cells, attach a fine-tipped aspirating pipette to a vacuum source. The tip can be made smaller by attaching a 200-µL pipette tip to the end of the aspirating pipette. Suction away portions of colonies which appear differentiated, as marked in step a. Take care not to allow the cultures to dry out.

Figure 6. Characteristic morphology of hiPSCs grown on feeder-dependent culture on MEFs.

(A) Undifferentiated hiPSC colony cultured on MEFs.

(B) Partially differentiated hiPSC colony cultured on MEFs. The circled region designates the are of differentiated hiPSCs that needs to be removed.

(C) Partially differentiated hiPSCs (circled) being removed/aspirated using a 200 µL pipette tip attached to the end of the aspirating pipette.

3. Cells are typically split at a 1:4 ratio when cells are 80% confluent, approximately 4-5 days. When colonies have grown such that adjacent colonies merge, or differentiation occurs in the center of each colony, the colonies are ready for passage. (Figure 7)

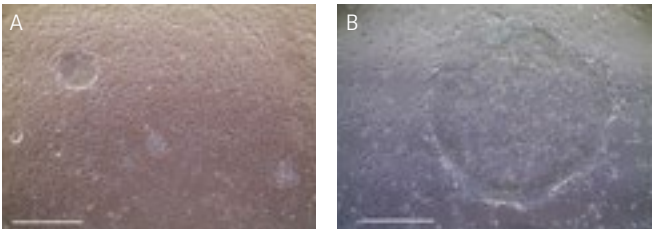


Figure 7. Morphology of hiPSCs cultured on MEF feeders.

(A) Undifferentiated hiPSCs 2 days post passage.

(B) Undifferentiated hiPSCs 5 days post passage, ready to split.

1.4.6 Passaging

The protocol is designed for the dissociation and expansion of cells on a 6 cm dish. Volumes should be adjusted according to the size and number of the tissue culture vessels used.

NOTE:
Make certain culture dishes coated with feeder cells are prepared; see section 5.3.

1. Remove an aliquot of 0.5 U/mL Stem Cell Dissociation Reagent working solution from the freezer and allow it to warm to room temperature.
2. Pre-warm Pluripotent Stem Cell SFM XF in a 37°C water bath. If using a small volume of medium (50 mL or less), warm only the volume needed in a sterile conical tube. Avoid warming complete medium multiple times.
3. Aspirate and discard the stem cell culture medium.
4. Rinse cells twice with 4 mL/dish of D-PBS.
5. Add 2 mL of Stem Cell Dissociation Reagent working solution to each dish.
6. Incubate at 37°C for 10-15 minutes. The reaction is complete when the edges of the individual colonies begin to loosen and fold back from the dish (Figure 8). View the plate under the microscope starting at 5 minutes as incubation time may vary depending on the cell line and colony size.

Figure 8. Time lapsed images of passaging hiPSCs cultured on MEFs using Stem Cell Dissociation Reagent.
(A) Undifferentiated hiPSC colony ready for passaging.
(B) hiPSC colony after 5 minutes in dissociation reagent, edges of the hiPSC colony is starting to lift/curl.
(C) hiPSC colony after 13 minutes in dissociation reagent, ready to passage.

7. Carefully aspirate the Stem Cell Dissociation Reagent and gently rinse the colonies with 4 mL/dish of DMEM: F-12 Medium, taking care not to dislodge the cells during manipulation.

Stem Cell Dissociation Reagent must be completely removed from the cells since the reagent is not inactivated by medium or serum.

8. Add 2 mL of Pluripotent Stem Cell SFM XF to each dish. Detach cells by pipetting up and down several times with a 1 mL tip. Take care not to over-pipette the culture into a single-cell suspension as single cells will not establish colonies after seeding.
9. Transfer the cell aggregates to a 15 mL conical tube.

10. Add an additional 4 mL of Pluripotent Stem Cell SFM XF to collect any remaining cells on the surface of the dish. Transfer rinse to the 15 mL conical tube containing the cell aggregates.
11. Centrifuge cells at 200 x g for 5 minutes at room temperature to pellet cells.

NOTE:
Prepare Pluripotent Stem Cell SFM XF with ROCK inhibitor by adding 20 µL of 10 mM ROCK Inhibitor Y27632 to 20 mL of medium.

12. Aspirate the supernatant. Gently tap the bottom of the tube to loosen the cell pellet.
13. For each dish processed, add 2 mL of Pluripotent Stem Cell SFM XF in the presence of 10 µM ROCK Inhibitor Y27632 to the 15 mL conical tube.
14. Gently resuspend the pellet by pipetting up and down 5-6 times with a 1 mL tip, maintaining the small cell aggregates.
15. If desired, fibroblasts can be depleted from cultures as follows:
    a) Add the entire suspension to an uncoated 6 cm tissue culture dish with 4 mL of Pluripotent Stem Cell SFM XF. Incubate for one hour at 37°C, 5% CO_2. During this time, the fibroblasts will adhere while the hiPSCs clusters remain in suspension.
    b) Collect the medium into a 15 mL conical tube and centrifuge at 200 x g for 5 minutes at room temperature to pellet cells.
    c) Aspirate the supernatant. Gently tap the bottom of the tube to loosen the cell pellet.
    d) For each dish processed, add 2 mL of Pluripotent Stem Cell SFM XF in the presence of 10 µM ROCK Inhibitor Y27632 to the 15 mL conical tube.
16. Transfer 0.5 mL of the cell aggregates onto MEF-or HFF-coated dishes that contain 4 mL Pluripotent Stem Cell SFM XF in the presence of 10 µM ROCK Inhibitor Y27632 for a 1:4 split ratio.
17. Swiftly move the dishes in a forward to backward, then left to right pattern, once, to gently disperse the cells evenly across the surface of the dishes. Incubate dishes overnight at 37°C and 5% CO_2.
18. Change medium daily until the colonies are big enough to passage. ROCK Inhibitor Y27632 is not required in subsequent cell culture medium changes.

1.5 Cryopreservation of hiPSCs

1.5.1 Materials

ATCC® No.	Product Name	Size	Storage
ACS-3002	Pluripotent Stem Cell SFM XF/FF for feeder-free cultures	500 mL	-20°C/2°C to 8°C
ACS-3001	Pluripotent Stem Cell SFM XF for feeder-dependent cultures	500 mL	-20°C
30-2006	DMEM: F-12 Medium	500 mL	2°C to 8°C
ACS-3010	Stem Cell Dissociation Reagent	250 mg	2°C to 8°C
ACS-3020	Stem Cell Freezing Media	20 mL	2°C to 8°C
30-2200	Dulbecco's Phosphate Buffered Saline (D-PBS)	500 mL	RT

1.5.2 Protocol for Cryopreservation

1. Remove an aliquot of 0.5 U/mL Stem Cell Dissociation Reagent working solution from the freezer and allow it to warm to room temperature.
2. Aspirate and discard the stem cell medium.
3. Rinse the cells twice with 4 mL/6 cm dish of D-PBS.
4. Add 2 mL of Stem Cell Dissociation Reagent working solution to each dish.
5. Incubate at 37°C for 10-15 minutes or until the edges of the individual colonies begin to loosen and fold back. View the dish under the microscope starting at 5 minutes as incubation time may vary depending on the cell line and colony size.
6. Aspirate the Stem Cell Dissociation Reagent and gently rinse the colonies with 4 mL/dish of DMEM: F-12 Medium, taking care not to dislodge the cells during manipulation.
7. Add 2 mL of stem cell culture medium to each dish and detach cells by pipetting up and down several times with a 1 mL tip. Take care not to over-pipette the culture into a single-cell suspension. It is best if the cells remain aggregated.
8. Transfer the cell aggregates to a 15 mL conical tube.
9. Add an additional 3 mL of stem cell culture medium to the dish to collect any remaining cells. Transfer this rinse to the 15 mL conical tube containing the cell aggregates.
10. Centrifuge cells at 200 x g for 5 minutes at room temperature to pellet cells. While cells are spinning, remove the Stem Cell Freezing Media from storage and swirl to mix. Keep cold. Decontaminate by dipping in or spraying with 70% alcohol.
11. Aspirate the supernatant and discard. Gently tap the bottom of the tube to loosen the cell pellet.

Note:
For optimal results, cells should be cultured to 80% confluency before freezing.

Note:
One 6 cm dish yields 2 mL of frozen cells. Cells are frozen in 1 mL aliquots.

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12. Add 2 mL of cold Stem Cell Freezing Media to the tube. Gently resuspend the pellet by pipetting up and down 5-6 times with a 1 mL tip, maintaining the small cell aggregates. Cells will recover from the freezing/thawing process more effectively if cells are frozen in clumps.
13. Immediately transfer 1 mL each of the cell suspension into two cryogenic storage vials.
14. Freeze the cells gradually at a rate of -1°C/min until the temperature reaches -70°C to -80°C. An isopropanol freezing container also may be used.
15. The cells should not be left at -80°C for more than 24 to 48 hours. Once at -80°C, frozen cryovials should be transferred to the vapour phase of liquid nitrogen for long-term storage.

1.6 Characterisation

1.6.1 Morphology

Undifferentiated hiPSCs grow as compact colonies and exhibit high nucleus-to-cytoplasm ratios and prominent nucleoli (Figures 9A and 9B). When cultured in Pluripotent Stem Cell SFM XF medium, small hiPSC colonies may initially exhibit loose colony morphology but will become more compact as the colony grows larger. In addition, the hiPSC colonies maintain a distinct border on feeder cells (Figure 9C). During the expansion and maintenance of hiPSCs, differentiated cells or obvious alternate cell types might emerge (Figure 9D, arrow). These cells should be removed before passaging the cultures by attaching a 200 µL pipette tip to the end of the aspirating Pasteur pipette (see section 1.4.5 on removal of differentiated cells).

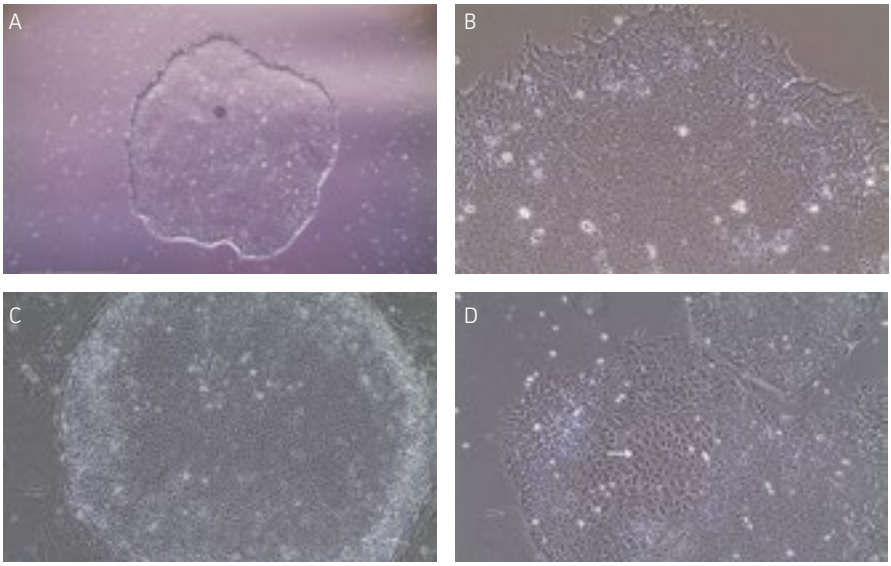


Figure 9. Characteristic morphology of hiPSCs grown on feeder-free or feeder-dependent culture.

(A) Undifferentiated hiPSC colony cultured on CellMatrix gel in Pluripotent Stem Cell SFM XF/FF grows as a compact colony with individual cells displaying high nucleus-to -cytoplasm ratios and prominent nucleoli.

(B) Undifferentiated hiPSC colony cultured on CellMatrix gel in Pluripotent Stem Cell SFM XF/FF at high magnification.

(C) Undifferentiated hiPSC colony cultured on MEF in Pluripotent Stem Cell SFM XF. Note the defined border on feeder cells.

(D) Arrow indicates partially differentiated hiPSCs grown on feeder-free culture. Colonies exhibit less defined edges and non-uniform morphology.

1.6.2 Characterisation Assays

1. Immunocytochemistry

   Immunocytochemistry is the most accessible and easiest method for most laboratories to assess the pluripotency of hiPSCs cultures. The cells are fixed and then incubated with primary antibodies to pluripotency markers. These antibodies are then detected with a secondary fluorophore conjugated antibody. Undifferentiated hiPCSs can be characterised by the expression of pluripotent markers such as SSEA4, Tra-1-60, Tra-1-81, and Nanog (Figure 10).

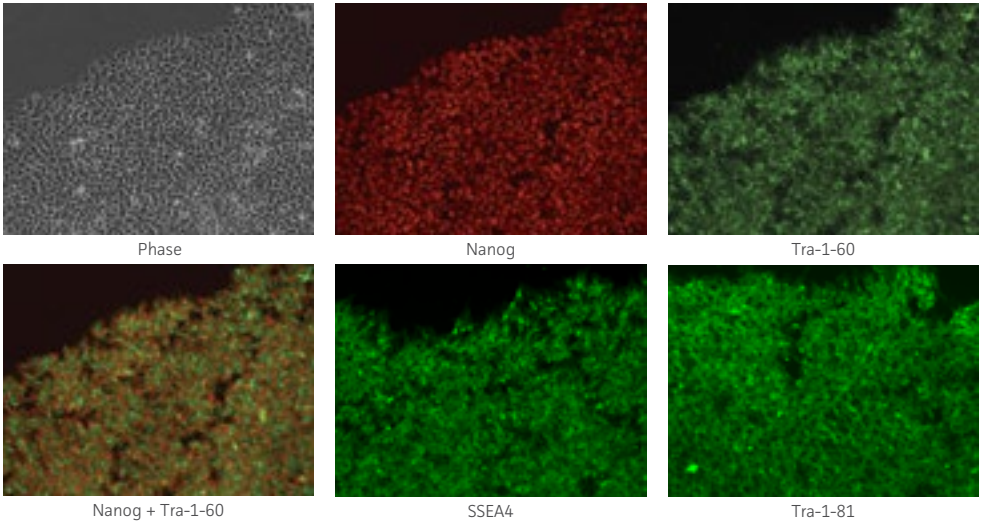


Figure 10. Immunocytochemistry of hiPSCs stained with Tra 1-60, Tra-I-81, SSEA4, and Nanog antibodies (10x).

2. Flow Cytometry

To quantitate the percentage of undifferentiated hiPSCs in culture, flow cytometric analysis of pluripotency markers and differentiation markers is performed. Samples of cells are stained with a fluorophore conjugated antibody to the pluripotency surface markers of interest. The cells are then counted by a flow cytometer. Upon differentiation of hiPSCs, Tra-1-60 and SSEA4 expression levels decrease while SSEA1 expression increases. For a pure, undifferentiated hiPSC line, the expression level of SSEA4 and Tra-1-60 pluripotency markers should be more than 90% of the total cells and the expression level of SSEA1 should be less than 15% of the total cells (Figure 11).

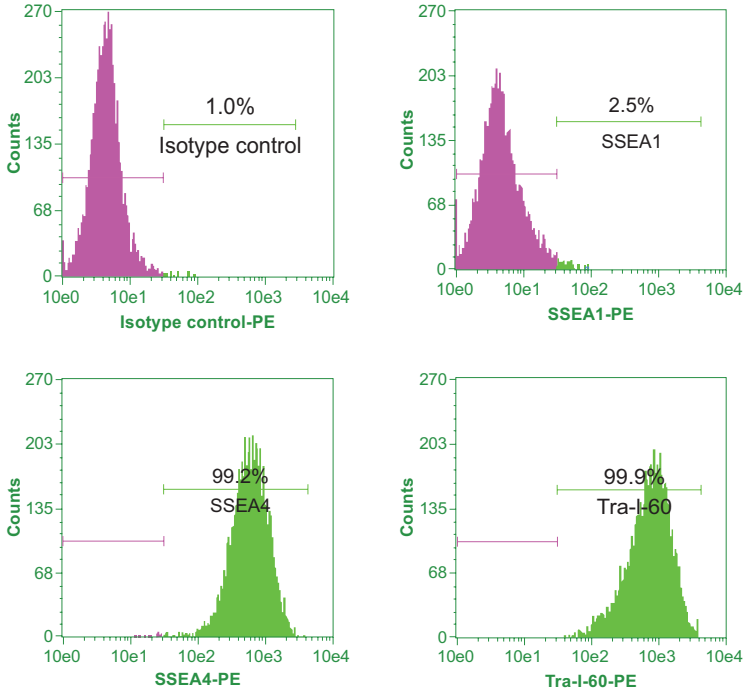


Figure 11. Flow cytometric analysis of expression of pluripotency markers in hiPSCs.

3. Karyotype

Stem cells can develop karyotypic mutations over time. It is good practice to verify that the hiPSCs are karyotypically normal after several passages. Various methods are available including comparative genomic hybridisation array, Giemsa banding, and fluorescent *in situ* hybridisation analysis.

4. STR Analysis

Short Tandem Repeat (STR) testing is a rapid, reproducible PCR-based technique used for the identification or authentication of hiPSC lines.

1.6.3 Characterisation Markers and Applications

Table 2. Markers Commonly Used for Characterising Pluripotent Stem Cells

Target	Subcellular Location	hiPSC expression
Alkaline Phosphatase	Intracellular	Upregulated in pluripotent cells
Tra-1-60	Surface Antigen	Upregulated in pluripotent cells
Tra-1-81	Surface Antigen	Upregulated in pluripotent cells
SSEA-1	Surface Antigen	Downregulated in pluripotent cells
SSEA-4	Surface Antigen	Upregulated in pluripotent cells
Nanog	Nuclear	Upregulated in pluripotent cells

Table 3. Comparison of Standard Methods Used for Characterising Pluripotent Stem Cells

Method	Application	Comments
Live stain	For selection of pluripotent colonies after reprogramming	Cells remain viable, allowing for continued expansion. Use when images from a limited number of markers is adequate.
Immunocytochemistry	Characterisation of one or multiple markers of pluripotency	Cells are fixed but can test for a broad range of markers. Excellent when vivid images are desired and loss of cell sample is not an issue.
Flow cytometry	Characterisation of one or multiple markers of pluripotency	Relative quantitation of percentage of pluripotent versus differentiated cells in a culture.

2. Mesenchymal Stem Cell Guide

Mesenchymal Stem Cells (MSCs) are self-renewing, multipotent adult stem cells. MSCs are traditionally found in the bone marrow, but can also be isolated from other tissues including adipose, human umbilical cord or cord blood, and peripheral blood. Adipose-derived MSCs are isolated from human adipose (fat) tissues by lipoaspiration or biopsy. MSCs derived from the umbilical cord are isolated from Wharton's Jelly, the gelatinous substance within the human umbilical cord. MSCs are isolated from connective tissue precursor cells and can differentiate into bone, fat, and cartilage upon treatment with ATCC® Osteocyte, Chondrocyte, or Adipocyte Differentiation Toolkits. Therefore, they are very useful in understanding cell differentiation as well as tissue engineering, orthopedic, and obesity research.

ATCC offers two human mesenchymal stem cell lines: Umbilical Cord-Derived Mesechymal Stem Cells (ATCC® No. PCS-500-010) and Adipose Derived Mesenchymal Stem Cells (ATCC® No. PCS-500-011). Both of these cell lines are isolated from single-donor tissue. Although MSCs from primary adipose tissue and Wharton's Jelly are considered to be relatively easy to obtain, consistent isolation of stem cell populations from such material is costly and time consuming. Lipoaspirates and umbilical cord tissue represent a heterogeneous mixture of cell types, including adipocytes, endothelial cells, smooth muscle cells, pericytes and progenitor cells. The heterogeneity of the source material increases the potential that the MSC culture will be contaminated and potentially over-run by another cell type. ATCC® MSCs are cryopreserved at second passage and tested for growth, morphology, marker expression[3] and differentiation potential.

MSCs are useful tools for stem cell differentiation research and for the creation of iPSC lines[4]. MSCs have also been useful in tissue engineering[5,6,7,8], cell therapy, and regenerative medicine applications[9,10,11,12].

2.1 Materials

Mesenchymal Stem Cells and complete growth medium

Mesenchymal Stem Cells	Growth Kit Options	Basal Medium
Adipose-Derived Mesenchymal Stem Cells, Normal, Human (ATCC® No. PCS-500-011)	Mesenchymal Stem Cell Growth Kit-Low Serum (ATCC® No. PCS-500-040)	Mesenchymal Stem Cell Basal Medium (ATCC® No. PCS -500-030)
Umbilical Cord-Derived Mesenchymal Stem Cells; Normal, Human (ATCC® No. PCS-500-010)		
Reagents for Subculture		
D-PBS (ATCC® 30-2200)		
Trypsin-EDTA for Primary Cells (ATCC® No. PCS-999-003)		
Trypsin Neutralizing Solution (ATCC® No. PCS-999-004)		

2.2 Preparation of Media Reagents

1. Obtain one Mesenchymal Stem Cell Growth Kit–Low Serum from the freezer; make sure that the caps of all components are secure.

2. Thaw the components of the growth kit just prior to adding them to the basal medium.
3. Obtain one bottle of Mesenchymal Stem Cell Basal Medium from cold storage.
4. Decontaminate the external surfaces of all growth kit component vials and the basal medium bottle by spraying them with 70% ethanol.
5. Using aseptic technique and working in a laminar flow hood or biosafety cabinet, transfer the indicated volume of each growth kit component, according to the following table, to the bottle of basal medium using a separate sterile pipette for each transfer.

Mesenchymal Stem Cell Growth Kit–Low Serum (ATCC® PCS-500-040)

Component	Volume	Final Concentration
MSC Supplement	10 mL	2% FBS 5 ng/mL rh FGF basic 5 ng/mL rh FGF acidic 5 ng/mL rh EGF
L-Alanyl-L-Glutamine	6 mL	2.4 mM

6. Tightly cap the bottle of complete growth medium and swirl the contents gently to assure a homogeneous solution. Do not shake forcefully to avoid foaming. Label and date the bottle.
7. Complete growth media should be stored in the dark at 2°C to 8°C (do not freeze). When stored under these conditions, complete growth media is stable for two weeks.

NOTE:
Refer to the batch specific information provided on the last page of the product information sheet for the total number of viable cells recovered from each lot of ATCC® Human Mesenchymal Stem Cells.

2.3 Thawing of Cryopreserved MSCs

1. Using the total number of viable cells, determine how much surface area can be inoculated to achieve an initial seeding density of 5,000 cells per cm^2.
2. Prepare the desired combination of flasks. Add 5 mL of complete growth medium per 25 cm^2 of surface area. Place the flasks in a 37°C, 5% CO_2, humidified incubator and allow the media to pre-equilibrate to temperature and pH for 30 minutes prior to adding cells.
3. While the culture flasks equilibrate, remove one vial of ATCC® Human Mesenchymal Stem Cells from storage and thaw the cells by gentle agitation in a 37°C water bath. To reduce the possibility of contamination, keep the O-ring and cap out of the water. Thawing should be rapid (approximately 1 to 2 minutes).
4. Remove the vial from the water bath as soon as the contents are thawed, and decontaminate by dipping in or spraying with 70% ethanol. All operations from this point onward should be carried out under strict aseptic conditions.
5. Add the appropriate volume of complete growth medium [volume = (1 mL x number of flasks to be seeded) – 1 mL] into a sterile conical tube. Using a sterile pipette, transfer the cells from the cryovial to the conical tube. Gently pipette the cells to homogenise the suspension. Do not centrifuge.

6. Transfer 1 mL of the cell suspension to each of the pre-equilibrated culture flasks prepared in steps 1 to 3 of Handling Procedure for Frozen Cells and Initiation of Culture. Pipette up and down several times, then cap and gently rock each flask to distribute the cells evenly.
7. Place the seeded culture flasks in a 37°C, 5% CO_2, incubator. Incubate for at least 24 hours before processing the cells further.

2.4 Maintenance

NOTE:
Adipose- and umbilical cord-derived stem cells are contact inhibited. It is essential that the cells be subcultured BEFORE reaching confluence as post-confluent cells exhibit changes in morphology, slower proliferation, and reduced differentiation capacity after passaging (Figure 12).

1. Pre-warm the complete growth media in a 37°C water bath. This will take between 10 and 30 minutes, depending on the volume. If using a small volume of medium (50 mL or less), warm only the volume needed in a sterile conical tube. Avoid warming complete growth media multiple times.
2. Remove the cells from the incubator, 24 hours after seeding, and view each flask under the microscope to determine percent cellular confluence.
3. Remove the spent media carefully without disturbing the monolayer.
4. Add 5 mL of fresh, pre-warmed complete growth medium per 25 cm^2 of surface area and return the flasks to the incubator.
5. View each flask under the microscope, 24 to 48 hours after seeding, to determine the percent cellular confluence. If not ready to passage, repeat steps 3 and 4 as described above. When cells have reached approximately 70% to 80% confluence, and are actively proliferating (many mitotic figures are visible), it is time to subculture.

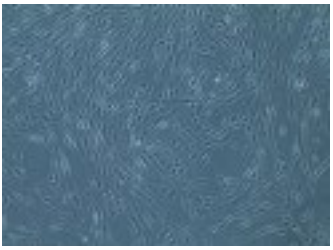


Figure 12. Phase contrast image of adipose tissue-derived MSCs at P2 (10x). Adipose tissue-derived MSCs (ATCC® PCS-500-011™) at passage 2 were seeded at 5,000 cells/cm^2 in T-75 flasks and were cultured in Mesenchymal Stem Cell Basal Medium (ATCC® PCS-500-030) supplemented with Mesenchymal Stem Cell Growth Kit - Low Serum (ATCC® PCS-500-040) in a 37°C, 5%CO_2 humidified incubator for 5 days. These cells are about 80% confluence ready for subculture.

2.5 Passaging

1. Warm both the Trypsin-EDTA for Primary Cells (ATCC® PCS-999-003) and the Trypsin Neutralizing Solution (ATCC® PCS-999-004™) to room temperature prior to dissociation. Warm the complete growth medium to 37°C prior to use with the cells.
2. Aspirate the spent media carefully without disturbing the monolayer.
3. Rinse the cell layer once with 3 to 5 mL D-PBS (ATCC® 30-2200) to remove residual medium.
4. Add pre-warmed trypsin-EDTA solution (1 to 2 mL for every 25 cm^2) to each flask.

5. Rock each flask gently to ensure complete coverage of the trypsin-EDTA solution over the cells.
6. Observe the cells under the microscope. When the cells pull away from each other and round up (typically within 1 to 3 minutes), remove the flask from the microscope and gently tap it from several sides to promote detachment of the cells from the flask surface.
7. When the majority of cells appear to have detached, quickly add an equal volume of the Trypsin Neutralizing Solution (ATCC® PCS-999-004™) to each flask. Gently pipette or swirl the culture to ensure all of the trypsin-EDTA solution has been neutralised.
8. Transfer the dissociated cells to a sterile centrifuge tube and set aside while processing any cells that remain in the culture flask.
9. Add 3 to 5 mL D-PBS (ATCC® 30-2200) to the tissue culture flask to collect any additional cells that might have been left behind.
10. Transfer the cell/D-PBS suspension to the centrifuge tube containing the trypsin-EDTA dissociated cells.
11. Repeat steps 10 and 11 as needed until all cells have been collected from the flask.
12. Centrifuge the cells at 150 x g for 3 to 5 minutes.
13. Aspirate neutralised dissociation solution from the cell pellet and re-suspend the cells in 2 to 8 mL fresh, pre-warmed, complete growth medium.
14. Count the cells and seed new culture flasks at a density of 5,000 viable cells per cm^2.
15. Place newly seeded flasks in a 37°C, 5% CO_2 incubator for at least 24 to 48 hours before processing the cells further. Refer to the section on Maintenance for guidelines on feeding.

2.6 Protocol for Cryopreservation

1. Harvest MSCs as described above.
2. Resuspend cells in complete MSC growth media into a single-cell suspension at cell concentration of 2 million cells/mL.
3. Add equal volume of 2x MSC freezing media (80% complete MSC growth media plus 20% DMSO, ATCC® 4-x).
4. Transfer 1 mL of the cell suspension (about 1 million cells/mL) into cryogenic storage vials.
5. Place cryovials into freezing containers and store overnight at a -70°C freezer.
6. Transfer frozen cryovials to the vapour phase of liquid nitrogen for long-term storage.

2.7 Adipose-derived Mesenchymal Stem Cell Differentiation Protocols

Adipocyte Differentiation

The Adipocyte Differentiation Toolkit (ATCC® PCS-500-050) contains medium and reagents designed to induce adipogenesis, with high efficiency, in actively proliferating Adipose-Derived Mesenchymal Stem Cells (ATCC® PCS-500-011), and support maturation of derived adipocytes during lipid accumulation.

Preparing Cells for Adipocyte Differentiation

1. Follow the instructions for the growth of Adipose-Derived Mesenchymal Stem Cells (ATCC® PCS-500-011). It is recommended that the cells not be passaged more than four times before initiating adipocyte differentiation.
2. When cells are 70-80% confluent, passage them into a tissue culture plate at a density of 18,000 cells/cm^2. Adjust the number of cells and volume of media according to the tissue culture plate used. Please see the example below.
3. Example: For a 6-well tissue culture plate with a surface area of 9.5 cm^2/well, add a total of 171,000 viable cells to each well containing 2 mL of Mesenchymal Stem Cell Basal Medium (ATCC® PCS-500-030) supplemented with Mesenchymal Stem Cell Growth Kit–Low Serum (ATCC® PCS-500-040) components.
4. Rock the plate gently back and forth and side to side to distribute the cells evenly before incubation. Do not swirl.
5. Incubate the cells in a 37°C, 5% CO_2, incubator for 48 hours before initiating adipocyte differentiation.

Adipocyte Differentiation Media Preparation

The adipocyte differentiation process requires two separate media preparations: one for initiation of differentiation and one for maintenance. Stock solutions of these media can be prepared in tandem, in advance as follows:

NOTE:
It may be necessary to shake the AD Supplement and the ADM Supplement upon warming to help re-dissolve any components that may have precipitated out of solution upon freezing.

1. Thaw all three components of the differentiation kit and warm to 37°C in a water bath.
2. Decontaminate the external surfaces of all three kit components by spraying them with 70% ethanol.
3. Using aseptic technique and working in a laminar flow hood or biosafety cabinet:
   a) Transfer 15 mL of Adipocyte Basal Medium and 1 mL of AD Supplement to a sterile 50 mL conical tube, using a separate sterile pipette for each transfer. This is your working stock of **Adipocyte Differentiation (AD) *Initiation* Medium** used during the first 48 hours of differentiation.
   b) Add 5 mL of ADM Supplement to the remaining 85 mL of Adipocyte Basal Medium. This is your working stock of **Adipocyte Differentiation *Maintenance* Medium**.
4. Tightly cap each container of medium and swirl the contents gently to assure a homogeneous solution. Do not shake forcefully to avoid foaming. Label and date the bottle.
5. Each container of differentiation medium should be stored in the dark at 2°C to 8°C (do not freeze). When stored under these conditions, the differentiation medium is stable for up to three weeks.

Adipocyte Differentiation Procedure

A. Initiation Phase

1. After incubating the prepared Adipose-Derived Mesenchymal Stem Cells as described above, carefully aspirate the media from the wells.
2. Immediately rinse the cells once by adding 2 mL of room-temperature D-PBS (ATCC® 30-2200) to each well, then carefully aspirate the PBS from the wells.

NOTE:
It is recommended that you transfer the required volume of media to a sterile tube for pre-warming prior to each feeding rather than repeatedly re-warming the entire working stock.

3. Add 2 mL of pre-warmed (37°C) **Adipocyte Differentiation *Initiation* Medium** to each well to begin the adipocyte differentiation process.
4. Incubate the cells in a 37°C, 5% CO_2 incubator for 48 hours.
5. Feed the cells by carefully removing half the volume of medium (1 mL) from each well and adding another 2 mL of pre-warmed (37°C) Adipocyte Differentiation Initiation Medium to each well.

   Important: DO NOT TILT the plate during aspiration. It is important that the cell monolayer is not exposed to air during this and subsequent steps to ensure that developing lipid vesicles do not burst.

B. Maintenance Phase

1. Incubate the cells in a 37°C, 5% CO_2 incubator for 48 hours.
2. Carefully remove 2 mL of medium from each well (leaving 1 mL) and replace with 2 mL of pre-warmed (37°C) Adipocyte Differentiation Maintenance Medium.

   Important: DO NOT TILT the plate during aspiration. It is important that the cell monolayer is not exposed to air during this and subsequent steps to ensure that developing lipid vesicles do not burst.

3. Repeat Steps 6 and 7 every 3-4 days for another 11 days until adipocytes reach full maturity. Full maturity will be reached 15 days after the beginning of initiation phase, or 17 days from initial plating of cells.
4. Cells can be used at any phase of adipocyte differentiation as predicated upon experimental design. To confirm lipid accumulation, cells can be fixed and stained with Oil Red O.

Additional differentiation protocols, including Chondrocyte and Osteocyte differentiation of Adipose-Derived Mesenchymal Stem Cells, are available online at www.lgcstandards-atcc.org.

3. Mouse Embryonic Stem Cell Guide

Mouse Embryonic Stem (ES) Cells were first isolated and propagated in culture in 1981. They have since proved to be an invaluable tool for analysing gene expression in development, cancer research, tissue engineering, and the production of transgenic mouse strains by homologous recombination. Mouse ES cells are typically isolated from blastocysts from the inner cell mass of 3.5 day-old embryos and can be maintained in an undifferentiated, pluripotent state by culture with Leukemia Inhibitory Factor (LIF) on a feeder layer of mitotically-arrested mouse embryonic fibroblasts or in a feeder-free environment using gelatin-coated flasks.

ATCC offers over 15 mouse ES cell lines, including lines that express Green or Yellow Fluorescent Protein (GFP/YFP) markers as well as Mouse ES Basal Medium (ATCC® SCRR-2011), ES cell qualified FBS (ATCC® SCRR-30-2020), and mouse embryonic fibroblast cell lines that may be used as feeder layers for the culture of Mouse ES cells.

To ensure the purity of ATCC Mouse ES Cells, each batch is isolated from a single mouse embryo of known genotype and tested for:

1. Mycoplasma, bacterial, and yeast contamination.
2. Authentication of growth and morphology, including adherence to plastic when cultured in optimised Mouse ES Basal Cell Media.

3.1 Materials

Mouse ES Cells

ATCC® No.	Cell Line Designation	Applications/Markers Expressed
CRL-1934 ™	ES-D3 (D3)	Producing transgenic and knock-out mouse lines
SCRC-1002™	ES-C57BL/6	
SCRC-1010™	J1	Gene expression studies, Disease models
SCRC-1011™	R1	
SCRC-1018™	RW.4	Producing transgenic and knock-out mouse lines
SCRC-1019™	B6/BLU	Contains a LacZ-β-globin reporter gene specifically expressed in red blood cells.
SCRC-1020™	SCC#10	Gene knock-out/knock-in
SCRC-1021™	EDJ#22	Target gene mutation/knock out.
SCRC-1023™	AB2.2	Target gene mutation/knock out. Tissue engineering
SCRC-1033™	7AC5/EYFP	Puromycin selection
SCRC-1036™	R1/E	
SCRC-1037™	G-Olig2	GFP-Olig2 for lineage-specific tracking
SCRC-1038™	CE-1	Lox targeting; Hygromycin resistance
SCRC-1039™	CE-3	Neural-differentiated lineages express GFP; Lox targeting; Hygromycin resistance

Complete Growth Medium

Medium and Reagents for Cell Culture and Subculture
ATCC® SCRR-2011 Mouse ES Cell Basal Medium
2-mercaptoethanol (Life Technologies Cat. No. 21985)
Mouse leukemia inhibitory factor (LIF) (EMD Millipore Cat. No. ESG1107)
ATCC® SCRR-30-2020 Fetal Bovine Serum, ES-Qualified
ATCC® 30-2300 Penicillin - Streptomycin
ATCC® SCRR-2201 PBS Ca^{+2}/Mg^{+2}-free
ATCC® 30-2101 Trypsin / 0.53 mM EDTA solution

3.2 Preparation of Media Reagents

1. Obtain one bottle of Mouse ES Cell Basal Medium from cold storage.
2. Decontaminate the external surfaces of all growth kit component vials and the basal medium bottle by spraying them with 70% ethanol.
3. Using aseptic technique and working in a laminar flow hood or biosafety cabinet, transfer the indicated volume of each component, as indicated in the following table, to the bottle of basal medium using a separate sterile pipette for each transfer.

Component	Final Concentration
2-mercaptoethanol	0.1 mM
Mouse leukemia inhibitory factor (LIF)	1,000 U/mL
Fetal Bovine Serum, ES-Qualified (ATCC® SCRR-30-2020)	15%

4. Cap the bottle of complete growth medium and swirl the contents gently to assure a homogeneous solution. Do not shake forcefully to avoid foaming. Label and date the bottle.
5. Complete growth media should be stored in the dark at 2°C to 8°C (do not freeze). When stored under these conditions complete growth media is stable for two weeks.

   This medium is formulated for use with a 5% CO_2 humid incubator.

3.3 Feeder Cells

Please see appendix for list of Feeder Cell Lines. Embryonic stem cells are best supported *in vitro* by growth on mitotically arrested feeder cells. Mitotic inactivation, by treatment of mouse embryonic fibroblasts (MEFs) and other feeder cell cultures with Mitomycin C or gamma-irradiation, prevents division of the fibroblasts while allowing the cells to survive and produce key proteins such as 4F that support the self-renewal cycle of the stem cells.

ATCC offers several different mouse embryonic fibroblast feeder cell lines that may be used to support the growth of mouse embryonic stem cells.

Mitotic inactivation can be achieved by irradiation, but most researchers choose to avoid the cost and safety issues involved with use of a gamma-irradiation source in the laboratory.

Alternatively, treatment with Mitomycin C (ATCC® SCRR-3020) is fast, convenient and does not require the use of sophisticated equipment. However, the use of Mitomycin C still requires the implementation of stringent safety measures during use, since the compound is toxic to both cultured cells and to humans.

3.4 Complete Medium for Feeder Cells

Feeder cells may be grown in Dulbecco's Modified Eagle's Medium (ATCC® 30-2002) and 10-15% Fetal Bovine Serum (ATCC® 30-20202). *Please note that SCRC-1007 also requires that 2-mercaptoethanol be added to the growth medium to a final concentration of 0.05 mM.* Please consult the product sheet provided for the feeder cell line you wish to use for specific medium requirements and subculturing procedures.

Feeder cells should be initiated 24-48 hours prior to inoculation with embryonic stem (ES) cells.

3.5 Thawing of Cryopreserved MSCs

1. At least one day before plating the ES cells, prepare the desired combination of flasks with feeder cells to accommodate an initial ES cell seeding density of 30,000 cells/cm^2 to 50,000 cells/cm^2.

   Refer to the batch specific information provided on the last page of the product information sheet for the total number of viable cells recovered from each lot of ATCC Mouse Embryonic Stem Cells.

2. Plate mitotically arrested mouse embryonic fibroblasts (MEFs) as a feeder layer at approximately 55,000 feeder cells/cm^2 in complete medium for feeder cells. *Refer to the product sheet for mitotically arrested MEF for detailed handling instructions.*

   Feeder cells should be used within one week of plating. It is best to use feeder cells within 24 or 48 hours of initiation.

3.6 Plating Embryonic Stem (ES) Cells

1. Pre-warm complete growth medium for ES cells at 37°C for at least 30 minutes before adding to cells.
2. Perform a 100% medium change for the MEF feeder cells using Complete Growth Medium for ES cells one hour prior to thawing the ES cells.
3. Thaw the vial of ES cells by gentle agitation in a 37°C water bath. To reduce the possibility of contamination, keep the O-ring and cap out of the water. Thawing should be rapid (approximately 90 seconds).
4. Remove the vial from the water bath before the contents are completely thawed, and decontaminate by dipping in or spraying with 70% ethanol.

   All of the operations from this point on should be carried out under strict aseptic conditions.

5. Transfer the contents of the vial plus 5 mL of complete growth medium for ES cells to a 15 mL centrifuge tube. Use an additional 1 mL of media to rinse the vial and transfer the liquid to the 15 mL tube. Add 4 mL of complete growth medium for ES cells to bring the total volume to 10 mL.

6. Spin the cells at 270 x g for 5 min. Aspirate the supernatant and resuspend the pellet in 2 mL of complete growth medium for ES cells.
7. Add the 2 mL of cell suspension to the appropriate size flask containing feeder cells and fresh complete growth medium for ES cells (see batch specific information). ES cells should be plated at a density of 30,000 – 50,000 cells/ cm^2.
8. Incubate the culture at 37°C, 5% CO_2, humid incubator.

Routine Handling

Perform a 100% medium change every day. Passage the cells every 1 to 2 days. If the colonies are close to, or touching each other the culture is overgrown. Overgrowth will result in differentiation.

Make sure that you have prepared a sufficient number of flasks pre-plated with MEF feeder layers to support frequent passage of the ES cells.

3.7 Passaging

Feeder Cell Preparation for Subcultures

1. *Daily* maintain a sufficient number of flasks that have been pre-plated with MEFs in complete medium for feeder cells.
2. *One hour before subculturing the ES cells,* perform a 100% medium change for the MEFs using complete growth medium for ES cells.

NOTE:
To ensure the highest level of viability, pre-warm media and Trypsin/EDTA to 37°C before adding to cells. Volumes used in this protocol are for T75 flasks. Proportionally adjust the volumes for culture vessels of other sizes. A split ratio of 1:4 to 1:7 is recommended.

Dissociation and Transfer of ES Cells

1. Aspirate the medium from the flask(s) containing ES cells.
2. Wash with PBS Ca^{+2}/Mg^{+2}-free (ATCC® SCRR-2201).
3. Add 3.0 mL of 0.25% (w/v) Trypsin / 0.53 mM EDTA solution (ATCC® 30-2101) and place in incubator. After about one minute the ES colonies will dissociate and all cells will detach from the flask.
4. Dislodge the cells by gently tapping the side of the flask then wash the cells off with 7-10 mL of fresh culture medium. Triturate cells several times with a 10 mL pipette in order to dissociate the cells into a single-cell suspension.
5. Spin the cells at 270 x g for 5 min. Aspirate the supernatant.
6. Resuspend in enough complete growth medium for ES cells to reseed new vessels at the desired split ratio (a split ratio of 1:4 to 1:7 is recommended). Perform a cell count to determine the total number of cells. ES cells should be plated at a density of 30,000 – 50,000 cells/ cm^2.
7. Add separate aliquots of the cell suspension to the appropriate size flask containing feeder cells and add an appropriate volume of fresh complete growth medium for ES cells to each vessel.
8. Incubate the culture at 37°C, 5% CO_2, humid incubator. Perform a 100% medium change every day, passage cells every 1-2 days.

3.8 Protocol for Cryopreservation

1. Harvest mouse ES cells including mouse feeder cells described above.
2. Resuspend cells in complete growth medium for ES cells into a single-cell suspension at cell concentration of 2 x 10^6 cells/mL.
3. Add equal volume of 2x ES freezing media (80% complete ES growth media plus 20% DMSO, ATCC® 4-x).
4. Transfer 1 mL of the cell suspension (about 1 x 10^6 cells/mL) into cryogenic storage vials.
5. Place cryovials into freezing containers and store overnight at a -70°C to -80°C freezer.
6. Transfer frozen cryovials to the vapour phase of liquid nitrogen for long-term storage.

For additional information about ATCC Mouse ES Cells, please visit www.lgcstandards-atcc.com.

Appendix

A.1 Safety Precautions

Biosafety Level

It is the responsibility of the investigator to determine appropriate safety procedures used with this material. Please refer to the latest edition of the publication "Biosafety in Microbiological and Biomedical Laboratories (BMBL)" at www.cdc.gov.

ATCC highly recommends that protective gloves and clothing always be used and a full face mask always be worn when handling frozen vials. It is important to note that some vials leak when submersed in liquid nitrogen and will slowly fill with liquid nitrogen. Upon thawing, the conversion of the liquid nitrogen back to its gas phase may result in the vessel exploding or blowing off its cap with dangerous force creating flying debris.

A.1 Equipment List

- Vertical laminar flow hood
- 37°C, 5% CO_2, humidified incubator.
- 37°C water bath
- Pipette-aid
- Inverted microscope with 2X, 4X, and 10X phase objectives
- Hemocytometer
- Isopropanol freezing container
- -80°C freezer
- -150°C freezer or liquid nitrogen tank
- Sterile conical tubes (15 mL, 50 mL)
- Sterile tissue culture-treated dishes
- Sterile serological pipettes

A.2 Recommended Volume of Media and Reagents for Different Cell Culture Dishes

Table 4. Recommended Volume of Media and Reagents for Different Cell Culture Dishes

Cell Culture Vessel	Growth Area (cm^2)	Pluripotent Stem Cell Culture Medium	CellMatrix Basement Membrane Gel	Feeder Medium (DMEM + 15% FBS)	Stem Cell Dissociation Reagent working solution	Stem Cell Freezing Media
6 well / 35 mm	9.5	2 mL	1 mL	2 mL	1 mL	1 mL
6 cm	21	4 mL	2 mL	4 mL	2 mL	2 mL
10 cm	56	10 mL	4 mL	10 mL	4 mL	4 mL

Feeder Cell Lines: Qualified for Stem Cell Research (Inactivated)

ATCC® No.	Product Name	Description
SCRC-1007.1™	AFT024 IRR	Irradiated mouse embryonic liver fibroblast
SCRC-1008.1™	MEF (C57BL/6) IRR	Irradiated mouse embryonic fibroblast
SCRC-1008.2™	MEF (C57BL/6) MITC	Mitomycin C-treated mouse embryonic fibroblast
SCRC-1040.1™	MEF (CF-1) IRR	Irradiated mouse embryonic fibroblast
SCRC-1040.2a™	MEF (CF-1) MITC	Mitomycin C-treated mouse embryonic fibroblast
SCRC-1041.1™	HFF-1 IRR	Irradiated human foreskin fibroblast
48-X™	IRR-3T3	Gamma-irradiation mouse embryonic fibroblast
56-X.2™	MITC-STO	Mitomycin C - mouse embryonic fibroblast

Feeder Cell Lines: Qualified for Stem Cell Research (Not Inactivated)

ATCC® No.	Product Name	Description
SCRC-1007	AFT024	Mouse embryonic liver fibroblasts
SCRC-1008	MEF (C57BL/6)	Mouse embryonic fibroblasts
SCRC-1040™	MEF (CF-1)	Mouse embryonic fibroblasts[1]
SCRC-1041™	HFF-1	Human foreskin fibroblasts
SCRC-1045™	MEF (DR4)	Multidrug resistant mouse fibroblasts
SCRC-1049™	SNL76/7	STO fibroblasts with G418 resistance and endogenous expression of LIF[2]
SCRC-1050™	SNLP 76/7-4	STO fibroblasts with resistance to G418 and puromycin plus endogenous expression of LIF[3]
SCRR-3020	Mitomycin C	Antineoplastic antibiotic to prepare inactivated feeder cells; detailed protocol provided for the preparation of high quality feeder cell layers[4]

[1] If the MEFs are being used as a feeder layer for ES cells, it is not recommended to use them past passage no.6 (P6).

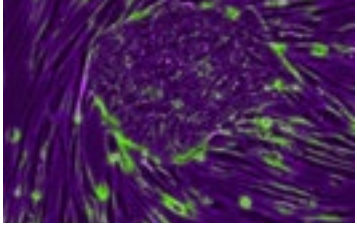
[2] Resistant to G 418 (neomycin): 350 µg/mL. Cells express high levels of LIF.

[3] SNLP 76/7-4 is a puromycin resistant derivative of SNL76/7.

[4] Mitomycin C is an antineoplastic antibiotic that inhibits DNA synthesis by cross-linking DNA at 5′–CpG–3′ sequences. It produces oxygen radicals and is preferentially toxic to hypoxic cells. Care must be taken when preparing the Mitomycin C solution. The fibroblasts must be extensively washed following treatment to prevent toxic effects to embryonic stem (ES) cells, or other cell types that require the use of a feeder cell layer for propagation.

References

1. Takahashi, K. and Yamanaka S. (2006) *Cell* 126:663-76.
2. Takahashi K., *et al.* (2007) *Cell* 131:861-72.
3. Dominici M, *et al. Cytotherapy* 8(4):315-317, 2006.
4. Safford KM, *et al. Exp. Neurol*. 187:319-328, 2004.
5. Zuk PA, *et al*. *Mol. Biol. Cell*. 13:4279-4295, 2002.
6. Freshney, RI. *Culture of Animal Cells: A Manual of Basic Technique*, 6th Ed. John Wiley & Sons, Hoboken, New Jersey, 2010.
7. Shaeffler A & Buechler C. *Stem Cells* 25:818-827, 2007.
8. Seo MJ, *et al*. B*iochem. Biophys. Res. Commun.* 328:258-264, 2005.
9. Kang SK, *et al.* J. *Cell Sci.* 117:4289-4299, 2004.
10. Safford KM, *et al. Current Drug Targets* 6:57-62, 2005.
11. Kingham PJ, *et al. Exp. Neurol.* 207:267-274, 2007.
12. Xu, *et al. Brain Res.* 1239:49-55, 2008.



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