Induced pluripotent stem cells (iPSCs)

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Abstract: All animal cells come from stem cells. Stem cell pluripotency means a stem cell having the potential to differentiate into any of the three germ layers: endoderm, mesoderm or ectoderm. Pluripotent stem cells can be differentiated to any fetal or adult cell type. However, the pluripotent stem cells cannot develop into a fetal or adult organism alone because they are lack of the potential to contribute to extraembryonic tissue, such as the placenta.
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1. Introduction

Stem cells describe all of the cells that can give rise to the different cells found in tissues. Under a right condition, a stem cell can become all type of the cells in the body. Cell pluripotency means a stem cell having the potential to differentiate into any of the three germ layers: ectoderm (epidermal tissues and nervous system, etc), endoderm (interior stomach lining, gastrointestinal tract and lungs, etc) or mesoderm (muscle, bone, blood and urogenital, etc). Pluripotent stem cells can be differentiated to any fetal or adult cell type. However, the pluripotent stem cells cannot develop into a fetal or adult organism alone because they are lack of the potential to contribute to extraembryonic tissue, such as the placenta. After the embryonic development stage is over, the stem cells no longer have this unlimited potential to develop into all cell types, the pluripotency is lost and they can only become certain types of cells.

Induced pluripotent stem cells (iPSCs) are genetically reprogrammed adult cells that exhibit a pluripotent stem cell-like state similar to embryonic stem cells. While these artificially generated cells are not known to exist in the human body, they show qualities remarkably similar to those of embryonic stem cells (ESCs); thus, iPSCs are an invaluable resource for drug discovery, cell therapy, and basic research.

iPSC technology was first started by Shinya Yamanaka's group in Kyoto of Japan in 2006 that they inserted 4 transcription factors genes [Oct4 (Pou5f1), Sox2, cMyc, and Klf4] to convert somatic cells into pluripotent stem cells through the reprogrammed.

Oct-4 (octamer-binding transcription factor 4) also known as **POU5F1** (POU domain, class 5, transcription factor 1) is a protein that in humans is encoded by the *POU5F1* gene. Oct-4 is a homeodomain transcription factor of the POU family. One of the problems is that: All cells in a body have exact same genes. This means that somatic cells have exact same genes as the embryonic stem cells. Why indroducing extra reprogramming factors genes [Oct4 (Pou5f1), Sox2, cMyc, and Klf4] can make the somatic cells reprogramed to pluripotent stem cells? What the original genes of these 4 transcript fators do?

There are many methods to deliver the transcription factors into target cells to generate iPSCs. The first method is retrovirus or lentivirus transduction. The problem of this technique is the genome integration of virus DNA which could possibly alter differentiation potential or other malignant transformation. The second method is adenoviral vectors to induce iPSC. The advantage of adenovirus vector based expression is that the transgenes will not integrate into the house genome, thus reduces the risk of tumorogenesis. The third one is a plasmid based transfection that can avoid the genome integration also. Recently, the Cre-recombinase excisable systems are used in iPSC induction and subsequent transgene removal making the iPSC technology closer to clinic applications.

After an egg is fertilized by a sperm, a single cell comes out. This fertilized egg is totipotent which has the potential ability to create an entire organism. However, the totipotent cells change to pluripotent cells that lost the ability of totipotent – the pluripotent cannot differentiate to an entire body.

There are several key types of pluripotent stem cells: (1) Embryonic stem cells are isolated from the inner cell mass of the blastocyst. (2) Embryonic germ cells are taken from aborted foetuses and these pluripotent cells are derived from very early cells. (3) Embryonic carcinoma or cancer cells are isolated from a type of tumour that sometimes occurs in a foetus.

Yamanaka and colleagues first demonstrated that retrovirus-mediated delivery and expression of

Oct4, Sox2, c-Myc and Klf4 is capable of inducing the pluripotent state in mouse fibroblasts, and they also reported the successful reprogramming of human somatic cells into induced pluripotent stem (iPS) cells using human versions of the same transcription factors delivered by retroviral vectors. The generation of patient-specific iPS cells circumvents an important roadblock to personalized regenerative medicine therapies by eliminating the potential for immune rejection of non-autologous transplanted cells. (Wu, Hamilton et al. 2009).

Mouse and human fibroblasts have been transformed into induced pluripotent stem (iPS) cells by retroviral transduction or plasmid transfection with four genes. Tumor formation has been found in offspring of mice generated from blastocysts made mosaic with iPS cells. The adenoviral vectors can reprogram human fibroblasts to pluripotent stem cells for use in individualized cell therapy without the risk for viral or oncogene incorporation (Zhou and Freed 2009).

Domesticated ungulate pluripotent embryonic stem (ES) cell lines would be useful for generating precise gene-modified animals. Many efforts have been made to establish domesticated ungulate pluripotent ES cells from early embryos without success. Wu, et al, reported that properties of porcine pluripotent stem cells that may facilitate the eventual establishment of porcine ES cells (Wu, Chen et al. 2009).

Pluripotent stem cells have the potential for treatment of many diseases. Pluripotent stem cells can evolve into specialized cells that ultimately can replace diseased cells and tissues. The positive uses of pluripotent stem cells are enormous but new research and ethical challenges must be taken into account before the public can reap the full benefits. For those who suffer from the many diseases that may be treated by pluripotent stem cells, additional knowledge and research will hopefully come sooner rather than later. The positive uses of pluripotent stem cells are enormous but new research and ethical challenges must be taken into account before the public can reap the full benefits.

Human induced pluripotent stem (iPS) cells hold great promise for cardiovascular research and therapeutic applications, but the ability of human iPS cells to differentiate into functional cardiomyocytes has not yet been demonstrated (Zhang, Wilson et al. 2009). Reprogramming differentiated human cells to induced pluripotent stem (iPS) cells has applications in basic biology, drug development, and transplantation. Human iPS cell derivation previously required vectors that integrate into the genome, which can create mutations and limit the utility of the cells in both research and clinical applications (Yu, Hu et al. 2009). Human induced pluripotent stem (iPS) cells derived from somatic cells hold promise to develop novel patient-specific cell therapies and research models for inherited and acquired diseases (Ye, Zhan et al. 2009).

Pluripotent stem cells have the potential to differentiate into almost any cell in the animal body. Under certain condition, the pluripotent stem cells from and embryo can produce all of the cells in the body. However, after the embryonic development stage, the stem cells will lost the ability and differentiate to adult cells. Pluripotent stem cells can evolve into specialized cells that ultimately can replace diseased cells and tissues.

After an egg is fertilized by a sperm, the zygote is created. The zygote is a single cell that has the totipotent ability. In the early hours and days (4 days for human) following fertilization, this single totipotent cell divides into more totipotent cells that are exact copies of the original zygote.

About 4 days after fertilization, the totipotent stem cells start to specialize and form a cluster of cells called the blastocyst. The blastocyst has a smaller group of cells called inner cell mass that are inner pluripotent stem cells. These pluripotent stem cells can differentiate to different somatic cells to form the animal body but lost totipotent ability.

Embryonic germ cells are taken from aborted foetuses and these pluripotent cells are derived from very early cells. These early cells can become sperm and eggs. Embryonic stem cells are isolated from the inner cell mass of the blastocyst. Embryonic carcinoma or cancer cells are isolated from a type of tumour that sometimes occurs in a foetus.

Invitrogen protocol examples (Invitrogen reagents are good as our experiences) (http://www.lifetechnologies.com) (Invitrogen, 2014):

Materials

- Episomal iPSC Reprogramming Vectors (50 µL, 1 µg/µL) (Invitrogen, Cat. no. A14703)
- Dulbecco's Modified Eagle Medium (DMEM) with GlutaMAX™-I (High Glucose) (Invitrogen, Cat. no. 10569-010).
- KnockOut™ DMEM/F-12 (Invitrogen, Cat. no. 12660-012).
- Fetal Bovine Serum (FBS), ESC-Qualified, US Origin (Invitrogen, Cat. no. 16141-079).
- MEM Non-Essential Amino Acids Solution, 10 mM (Invitrogen, Cat. no. 11140-050).
- Basic Fibroblast Growth Factor (bFGF) (Invitrogen, Cat. no. PHG0264).
- HA-100 (ROCk inhibitor) (Santa Cruz, Cat. no. sc-203072).

- Bovine Albumin Fraction V Solution (BSA) (Invitrogen, Cat. no. 15260-037).
- Essential 8™ Medium (Prototype), consisting of DMEM/F-12 (HAM) 1:1 and Essential 8™ Supplement (50X) (Invitrogen, Cat. no. A14666SA).
- DMEM/F-12 with HEPES (Invitrogen, Cat. no. 11330-057).
- N-2 Supplement (100X) (Invitrogen, Cat. no. 17502-048).
- B-27® Supplement (50X) (Invitrogen, Cat. no. 17504-044).
- GlutaMAX™-I (100X) (Invitrogen, Cat. no. 35050-061).
- β-mercaptoethanol, 1000X (Invitrogen, Cat. no. 21985-023).
- PD0325901(MEK Inhibitor) (Stemgent, Cat. no. 04-0006).
- CHIR99021 (GSK3β inhibitor) (Stemgent, Cat. no. 04-0004).
- A-83-01 (TGF-β/Activin/Nodal receptor inhibitor) (Stemgent, Cat. no. 04-0014).
- hLIF (Human Leukemia Inhibitory Factor) (Invitrogen, Cat. no. PHC9461).
- Vitronectin, truncated recombinant human (VTN-N) (Invitrogen, Cat. no. A14701SA) or Geltrex® LDEV-Free hESC Qualified Reduced Growth Factor Basement Membrane Matrix (Invitrogen, Cat. no. A1413301).
- 0.05% Trypsin-EDTA (1X), Phenol Red (Invitrogen, Cat. no. 25300-054).
- UltraPure™ 0.5 M EDTA, pH 8.0 (Invitrogen, Cat. no. 15575-020).
- Dulbecco's PBS (DPBS) without Calcium and Magnesium (Invitrogen, Cat. no. 14190-144).
- Characterization reagents (surface marker staining):
- *Mouse primary antibodies* (one is required):
    - Mouse Anti-Tra1-60 Antibody (Invitrogen, Cat. no. 41-1000).
    - Mouse Anti-Tra1-81 Antibody (Invitrogen, Cat. no. 41-1100).
    - Mouse Anti-SSEA4 Antibody (Invitrogen, Cat. no. 41-4000).
    - *Alexa Fluor® secondary antibodies* (one is required):
    - Alexa Fluor® 488 Goat Anti-Mouse IgG (H+L) Antibody (Invitrogen, Cat. no. A11029).
    - Alexa Fluor® 594 Goat Anti-Mouse IgG (H+L) Antibody (Invitrogen, Cat. no. A11032)
    - Alexa Fluor® 488 Goat Anti-Rabbit IgG (H+L) Antibody (Invitrogen, Cat. no. A11034)
    - Alexa Fluor® 594 Goat Anti-Rabbit IgG (H+L) Antibody (Invitrogen, Cat. no. A11037)
- Detection reagents (for detection of episomal vectors using PCR).
    - CellsDirect Resuspension & Lysis Buffers (Invitrogen, Cat. no. 11739-010).
    - AccuPrime Taq High Fidelity (Invitrogen, Cat. no. 12346-094).
    - Forward and Reverse primers for PCR (primer sequences are given in the PCR protocol).
- Electroporation instrument (e.g., Neon® Transfection System, Cat. no. MPK5000).
- 37°C water bath.
- Appropriate tissue culture plates and supplies.

Protocol Example

A typical reprogramming schedule using the Episomal iPSC Reprogramming Vectors is shown below (Table 1).

Table 1. A typical reprogramming schedule using the Episomal iPSC Reprogramming Vectors

Day –4 to –2:	Plate human fibroblasts into a T75 flask in Fibroblast Medium so that they are 75–90% confluent on the day of transfection (Day 0).
Day 0:	Transfect the cells using the Neon® Transfection System. Plate transfected cells onto vitronectin-coated culture dishes and incubate them overnight in Supplemented Fibroblast Medium.
Day 1 to 14:	Change the medium to N2B27 Medium supplemented with CHALP molecule cocktail and bFGF; replace the spent medium every other day.
Day 15:	Change the medium to Essential 8™ Medium and monitor the culture vessels for the emergence of iPSC colonies.
Day 21:	Pick and transfer undifferentiated iPSCs onto fresh vitronectin-coated culture dishes for expansion.

Preparing Media and Materials

10 µg/mL bFGF Solution (1000 µL)

1. To prepare 1 mL of 10 µg/mL bFGF solution, aseptically mix the following components (Table 2).

Table 2. Prepare 1 mL of 10 µg/mL bFGF solution

Component	Volume
bFGF	10 µg
DPBS without Calcium and Magnesium	980 µL
BSA	10 µL

2. Aliquot and store at –20°C (or for –20°C up to 6 months – 12 months.

Fibroblast Medium (for 100 mL of complete medium)

1. To prepare 100 mL of Fibroblast Medium, aseptically mix the following components (Table 3):

Table 3. Prepare 100 mL of Fibroblast Medium

Component	Volume
DMEM	89 mL
FBS, ESC-Qualified	10 mL
MEM Non-Essential Amino Acids Solution, 10 mM	1 mL

2. Fibroblast Medium can be stored at 4°C for up to 2 weeks.

Supplemented Fibroblast Medium (for 100 mL of complete medium)

Note: You will need 30 mL of Supplemented Fibroblast Medium per transfection.

1. To prepare 100 mL of Supplemented Fibroblast Medium, add the following components to Fibroblast Medium freshly, just prior to use:
2. HA-100 (ROCk inhibitor) varies (final concentration = 10 µgM).
3. bFGF (10 µg/mL) 40 iL (final concentration = 4 ng/mL)
4. Supplemented Fibroblast Medium must be used once HA-100 and bFGF are added to the medium.

Essential 8™ Medium (500 mL of complete medium)

1. Thaw Essential 8™ Supplement (50X) at 4°C overnight. Do not thaw the medium at 37°C.
2. To prepare 500 mL of complete Essential 8™ Medium, aseptically mix the following components:
3. DMEM/F-12 (HAM) 1:1 490 mL.
4. Essential 8™ Supplement (50X) 10 mL.
5. Complete Essential 8™ Medium can be stored at 4°C for up to 2 weeks.
6. *Note:* Before use, warm complete medium required for that day at room temperature until it is no longer cool to the touch. Do not warm the medium at 37°C.

N2B27 Medium (250 mL of complete medium)

1. To prepare 250 mL of N2B27 Medium, aseptically mix the following components (Table 4).

Table 4. Prepare 250 mL of N2B27 Medium

Component	Volume
DMEM/F-12 with HEPES	238.75 mL
N-2 Supplement (100X)	2.5 mL
B-27® Supplement (50X)	5.0 mL
MEM Non-Essential Amino Acids Solution, 10 mM	2.5 mL
GlutaMAX™-I (100X)	1.25 mL
β-mercaptoethanol, 1000X	454.5 µL

2. To supplement N2B27 Medium with CHALP molecule cocktail and bFGF, add the following components to the indicated concentration. These must be added freshly, just prior to use (Table 5).

Table 5. Supplement N2B27 Medium with CHALP molecule cocktail and bFGF

Component	Volume
PD0325901 (MEK inhibitor)	0.5 µM
CHIR99021 (GSK3â inhibitor)	3 µM
A-83-01 (TGF-β/Activin/Nodal receptor inhibitor)	0.5 µM
hLIF (Human Leukemia Inhibitory Factor)	10 ng/mL
HA-100 (ROCk inhibitor)	10 µM
bFGF (10 µg/mL)	100 ng/mL

3. N2B27 Medium (without CHALP molecules and bFGF) can be stored at 4°C for up to 1 week.

0.5 mM EDTA in DPBS (50 mL)

1. To prepare 50 mL of 0.5 mM EDTA in DPBS, aseptically mix the following components in a 50-mL conical tube in a biological safety cabinet.
2. DPBS without Calcium and Magnesium 50 mL0.5 M EDTA 50 ìL.
3. Filter sterilize the solution. The solution can be stored at room temperature for up to 6 months.

Coating Culture Vessels with Vitronectin (VTN-N)

1. Remove a 1-mL vial of vitronectin from –70°C storage and thaw at 4°C overnight.
2. Prepare working aliquots by dispensing 60 ìL of vitronectin into polypropylene tubes. The working aliquots can be frozen at –70°C or used immediately.
3. Prior to coating culture vessels, calculate the working concentration of vitronectin using the formula below and dilute the stock appropriately. The optimal working concentration of vitronectin is cell line dependent.
4. To coat the wells of a 6-well plate, remove a 60-ìL aliquot of vitronectin from –70°C storage and thaw at room temperature. You will need one 60-ìL aliquot per 6-well plate.
5. Add 60 uL of thawed vitronectin into a 15-mL conical tube containing 6 mL of sterile DPBS without Calcium and Magnesium at room temperature. Gently resuspend by pipetting the vitronectin dilution up and down. Note: This results in a working concentration of 5 ìg/mL (i.e., a 1:100 dilution).
6. Aliquot 1 mL of diluted vitronectin solution to each well of a 6-well plate (refer to Table 1 for recommended volumes for other culture vessels). Note: When used to coat a 6-well plate (10 cm2/well) at 1 mL/well, the final concentration will be 0.5 ug/cm2.
7. Incubate at room temperature for 1 hour. Note: Dishes can now be used or stored at 4°C wrapped in laboratory film for up to a week. Do not allow the vessel to dry. Prior to use, pre-warm the culture vessel to room temperature for at least 1 hour.
8. Aspirate the diluted vitronectin solution from the culture vessel and discard. It is not necessary to rinse off the culture vessel after removal of vitronectin. Cells can be passaged directly onto the vitronectin-coated culture dish. Note: Geltrex® LDEV-Free hESC-Qualified Reduced Growth Factor Basement Membrane Matrix may be substituted for vitronectin (Table 6).

Table 6. Volume of Diluted Vitronectin Required

Culture Vessel	Surface Area (cm^2)	Volume of Diluted Substrate (mL)
6-well plate	10 cm^2/well	1 mL/well
12-well plate	4 cm^2/well	0.4 mL/well
24-well plate	2 cm^2/well	0.2 mL/well
35-mm dish	10 cm^2	1 mL
60-mm dish	20 cm^2	2 mL
100-mm dish	60 cm^2	6 mL

Reprogramming Fibroblasts

The following protocol has been optimized for human neonatal foreskin fibroblast cells (strain BJ; ATCC no. CRL2522). We recommend that you optimize the protocol for your cell type.

Day –4 to –2: Seed Cells

1. Two to four days before transfection, plate human fibroblast cells in Fibroblast Medium into a T75 flask. Cells should be approximately 75–90% confluent on the day of transfection (Day 0). Note: Growth rate is dependent on the cell line and culture conditions. Depending on the seeding density and culture conditions, the cells may take up to 5 days to reach 75–90% confluency. *Note:* Since overconfluency results in decreased transfection efficiency, we recommend replating your cells to achieve 75–90% confluency if your cells have become overconfluent during culturing.

Day 0: Prepare the cells for transfection

Note: Gentle handling of the cells prior to transfection is essential for the success of the transfection procedure.

2. Add 6 mL of Supplemented Fibroblast Medium to a 15-mL conical tube for each transfection (1 tube per transfection). Incubate tube at 37°C until needed (see step 23).
3. Aspirate medium from vitronectin-coated plates and replace with 12 mL of fresh Supplemented Fibroblast Medium per plate. Place the coated plates at 37°C until ready for use. *Note:* You will need two 100-mm vitronectin-coated dishes for each transfection.
4. Aspirate the spent medium from the fibroblasts in T75 flasks.
5. Wash the cells in DPBS without Calcium and Magnesium.
6. Add 2 mL of 0.05% Trypsin/EDTA to each flask.
7. Incubate the flasks at 37°C for approximately 4 minutes.
8. Add 6 mL Supplemented Fibroblast Medium to each flask. Tap the plate against your hand to ensure cells have been dislodged from the plate, and carefully transfer cells into an empty 15-mL conical tube. *Note:* Each T75 flask provides plenty of cells for transfection, so any residual cells still clinging to the flask after Trypsin/EDTA treatment may be left behind.
9. Remove a 20-µL sample to perform a viable cell count and calculate the number of transfection to be performed. You will need 1 × 106 cells for one transfection. Number of transfections = Number of viable cells/(1×10^6).
10. Transfer enough cells for up to three transfections (i.e., 1×10^6 to 3×10^6 cells) into a new 15-mL conical tube.
11. Bring the volume to 10 mL in the new tube with Supplemented Fibroblast Medium and centrifuge cells at 1,000 rpm for 5 minutes at room temperature.
12. Carefully aspirate most of the supernatant, using a glass Pasteur pipette, leaving approximately 100–200 µL behind. Remove the remaining medium with a 200-µL pipette.

Day 0: Transfection

13. Resuspend the cell pellet in Resuspension Buffer R (included with Neon® Transfection kits) at a final concentration of 1.0×10^6 cells/0.1 mL.
14. Transfer the cells (100 µL per transfection reaction) to a sterile 1.5-mL microcentrifuge tube.
15. Turn on the Neon® unit and enter the electroporation parameters in the Input window (Table 7).

Table 7. Electroporation Parameters for Neon® Transfection System

Pulse Voltage	Pulse Width	Pulse Number	Cell Density	Tip Type
1650 V	10 ms	3	1×10^6 cells/0.1 mL	100 µL

16. Fill the Neon® Tube with 3 mL Electrolytic Buffer (use Buffer E2 for the 100 µL Neon® Tip).
17. Insert the Neon® Tube into the Neon® Pipette Station until you hear a click.
18. Transfer 8.5 µL Episomal Reprogramming Vectors to the tube containing cells and mix gently.
19. Insert a Neon® Tip into the Neon® Pipette.
20. Press the push-button on the Neon® Pipette to the first stop and immerse the Neon® Tip into the cell-DNA mixture. Slowly release the push-button on the pipette to aspirate the cell-DNA mixture into the Neon® Tip. Note: Avoid air bubbles during pipetting to avoid arcing during electroporation. If you notice air bubbles in the tip, discard the sample and carefully aspirate fresh sample into the tip again without any air bubbles.
21. Insert the Neon® Pipette with the sample vertically into the Neon® Tube placed in the Neon® Pipette Station until you hear a click.

22. Ensure that you have entered the appropriate electroporation parameters and press Start on the Neon® touchscreen to deliver the electric pulse. Note: After the electric pulse is delivered, the touchscreen displays "Complete" to indicate that electroporation is complete.
23. Remove the Neon® Pipette from the Neon® Pipette Station and immediately transfer the samples from the Neon® Tip into the 15-mL tube containing 6 mL of pre-warmed Supplemented Fibroblast Medium (prepared in step 2).
24. Mix the transfected cells by gentle inversion and pipette 3 mL into the 100-mm vitronectin-coated plate (two plates per transfection). Evenly distribute cells across plate. Discard the Neon® Tip into an appropriate biological hazardous waste container.
25. Repeat the process for any additional samples. Do not use Neon® tip more than twice.
26. Incubate the plates at 37°C in a humidified CO2 incubator overnight.

Day 1: Switch to Supplemented N2B27 Medium

27. Aspirate the spent Supplemented Fibroblast Medium from the plates using a Pasteur pipette.
28. Add 10 mL N2B27 Medium supplemented with CHALP molecule cocktail and bFGF (added freshly prior to use) to each 100-mm plate.
29. Replace the spent medium every other day, up to day 15 post-transfection.

Day 15: Switch to Essential 8™ Medium

30. Aspirate the spent medium and replace with Essential 8™ Medium. Resume medium changes every other day.
31. Observe the plates every other day under a microscope for the emergence of cell clumps indicative of transformed cells (see Figure 1). Within 15 to 21 days of transfection, the iPSC colonies will grow to an appropriate size for transfer.

Figure 1 Expected morphology of cells during episomal reprogramming. The images show human neonatal foreskin fibroblast cells (strain BJ) as they undergo morphological changes and iPSC colonies begin to emerge.

Identifying iPSC Colonies

By Day 21 post-transduction, the cell colonies on the vitronectin-coated plates are large and compact, covering the majority of the surface area of the culture vessel. However, only a fraction of these colonies will consist of iPSCs, which exhibit a hESC-like morphology characterized by a flatter cobblestone-like appearance with individual cells clearly demarcated from each other in the colonies (see Figure 2). Therefore, we recommend that you perform live staining with Tra1-60 or Tra1-81 antibodies that recognize undifferentiated iPSCs.

Figure 2 Expected morphology of emerging iPSCs generated by episomal reprogramming of human neonatal foreskin fibroblast cells (strain BJ). In these 5X images, lots of large, nested colonies are visible.

Live Staining with Antibodies

One of the fastest and most reliable methods for selecting a reprogrammed colony is live staining with Tra1-60 or Tra1-81 antibodies that recognize undifferentiated iPSCs and enable the identification of reprogrammed cells from a variety of human cell types. *Note:* Other methods of identifying iPSCs (such as alkaline phosphatase staining) are also acceptable.

1. Aspirate the medium from the reprogramming dish.
2. Wash the cells once with KnockOut™ DMEM/F-12.
3. Add the diluted primary antibody (Mouse Anti-Tra 1-60, Mouse Anti-Tra 1-81, or Mouse Anti-SSEA; see Materials Needed) to the cells (6 mL per 100-mm dish).
4. Incubate the primary antibody and the cells at 37°C for 60 minutes.
5. Remove the primary antibody solution from the dish. *Note:* The primary antibody solution can be stored at 4°C for one week and re-used up to two times.
6. Wash the cells three times with KnockOut™ DMEM/F-12.
7. Add the diluted secondary antibody to the cells (6 mL per 100-mm dish). Note: Any of the four Alexa Fluor® secondary antibodies listed in the Materials Needed section can be used.
8. Incubate the secondary antibody and the cells at 37°C for 60 minutes.
9. Remove the secondary antibody solution from the dish. *Note:* The secondary antibody solution can be stored at 4°C for one week and re-used up to two times.
10. Wash cells three times with KnockOut™ DMEM/F-12. Add fresh KnockOut™ DMEM/F-12 to cover the surface of the cells (6 mL per 100-mm dish).

11. Visualize the cells under a standard fluorescent microscope and mark the successfully reprogrammed colonies for picking and expansion. Successful antibody staining can very specifically distinguish reprogrammed colonies from just plain transformed counterparts (see Figure 3), and can be detected for up to 24–36 hours. This is particularly useful because it helps identifying and tracking of candidate iPSC colonies before picking and the day after they are transferred into a new culture dish for expansion.

Detecting Episomal Vectors by PCR

Preparing iPSCs for PCR

Note: Endpoint PCR is the suggested method for verifying the loss of the episomal vectors over time.

1. Aspirate the medium from the dish containing iPSCs with a Pasteur pipette, and rinse the dish twice with Dulbecco's PBS (DPBS) without Calcium and Magnesium. Refer to Table 8 for the recommended volumes.

Table 8. Volume of Reagents Required

Culture Vessel	Approximate Surface Area (cm^2)	DPBS (mL)	0.5 mM EDTA in DPBS (mL)	Complete Essential 8™ Medium (mL)
6-well plate	10 cm^2/well	2 mL/well	1 mL/well	2 mL/well
12-well plate	4 cm^2/well	1 mL/well	0.4 mL/well	1 mL/well
24-well plate	2 cm^2/well	0.5 mL/well	0.2 mL/well	0.5 mL/well
35-mm dish	10 cm^2	2 mL	1 mL	2 mL
60-mm dish	20 cm^2	4 mL	2 mL	4 mL
100-mm dish	60 cm^2	12 mL	6 mL	12 mL

2. Add 0.5 mM EDTA in DPBS to the dish containing iPSCs. Adjust the volume of EDTA for various dish sizes (refer to Table 3). Swirl the dish to coat the entire cell surface.
3. Incubate the vessel at room temperature for 5–8 minutes or 37°C for 4–5 minutes. When the cells start to separate and round up, and the colonies will appear to have holes in them when viewed under a microscope, they are ready to be removed from the vessel. *Note:* In larger vessels or with certain cell lines, this may take longer than 5 minutes.
4. Aspirate the EDTA solution with a Pasteur pipette.
5. Add pre-warmed complete Essential 8™ Medium to the dish according to Table 3.
6. Remove the cells by gently squirting the colonies from the well using a 5-mL glass pipette. Avoid creating bubbles. Collect cells in a 15-mL conical tube. *Note:* Do not scrape the cells from the dish. There may be obvious patches of cells that were not dislodged and left behind. Do not attempt to recover them through scraping. *Note:* Depending upon the cell line, work with no more than one to three wells at a time, and work quickly to remove cells after adding Essential 8™ Medium to the well(s). The initial effect of the EDTA will be neutralized quickly by the medium. Some lines re-adhere very rapidly after medium addition, and must be removed 1 well at a time. Others are slower to re-attach, and may be removed 3 wells at a time.
7. Centrifuge the cell suspension at 200 × g for 5 minutes to pellet cells.
8. Aspirate and discard the supernatant. Resuspend cell pellet in 500 µL DPBS and transfer resuspended cells to a thin-walled 0.5-mL PCR tube.
9. Centrifuge the cell suspension at 200 x~ g for 5 minutes to pellet cells.
10. Aspirate and discard the supernatant. Resuspend cell pellet in 20 µL of Resuspension Buffer with 2 µL of Lysis Solution added to the Resuspension Buffer.
11. Incubate the cells for 10 minutes in an incubator or thermal cycler that has been preheated to 75°C.
12. Spin the tube briefly to collect any condensation. Use 3 µL of the cell lysate in a 50-µL PCR reaction (see below).

PCR using AccuPrime™ High Fidelity Taq DNA Polymerase

13. Add the following components to a DNase/RNase-free, thin-walled PCR tube as directed in Table 9. Forward and reverse primers are shown in Table 10. For multiple reactions, prepare a master mix of common components to minimize reagent loss and enable accurate pipetting. Note: Assemble PCR reactions in a DNA-free environment. We recommend use of clean dedicated automatic pipettors and aerosol resistant barrier tips.

Table 9. Preparation of reactions for PCR

Component	Volume per reaction
10X PCR Buffer II	5 µL
Forward primer (10 µM stock)	1 µL
Reverse primer (10 µM stock)	1 µL
AccuPrime™ Taq Polymerase (5 units/µL)	1 µL
Cell Lysate	3 µL
Sterile distilled water	39 µL

Table 10. Primers for Standard PCR

Transgene	Primers	Sequence	Expected Size
oriP	pEP4-SF1-oriP pEP4-SR1-oriP	5'-TTC CAC GAG GGT AGT GAA CC-3' 5'-TCG GGG GTG TTA GAG ACA AC-3'	544 bp
EBNA-1	pEP4-SF2-oriP pEP4-SR2-oriP	5'-ATC GTC AAA GCT GCA CAC AG-3' 5'-CCC AGG AGT CCC AGT AGT CA-3'	666 bp

Note: These primers can detect all three episomal plasmids.

14. Cap the tube, tap gently to mix, and centrifuge briefly to collect the contents.
15. Place the tube in the thermal cycler and use the PCR parameters shown in Table 11:

Table 11. PCR Parameters

Step	Temperature	Time	Cycles
Initial Denaturation	94°C	2 minutes	—
Denaturation	94°C	30 seconds	
Annealing	55°C	30 seconds	35–40
Elongation	72°C	1 minute	
Final Elongation	72°C	7 minutes	—

16. Analyze the PCR products using 2% agarose gel electrophoresis.

Appendix

A. Coating Culture Vessels with Geltrex® LDEV-Free, hESC-Qualified Basement Membrane Matrix

1. Thaw a 5-mL bottle of Geltrex® LDEV-Free hESC-Qualified Reduced Growth Factor Basement Membrane Matrix at 2–8°C overnight.
2. Dilute the thawed Geltrex® solution 1:1 with cold sterile DMEM/F-12 to prepare 1-mL aliquots in tubes chilled on ice. These aliquots can be frozen at –20°C or used immediately. *Note:* Aliquot volumes of 1:1 diluted Geltrex® solution may be adjusted according to your needs.
3. To create working stocks, dilute a Geltrex® aliquot 1:50 with cold DMEM on ice, for a total dilution of 1:100. *Note:* An optimal dilution of the Geltrex® solution may need to be determined for each cell line. Try various dilutions from 1:30 to 1:100.
4. Quickly cover the whole surface of each culture dish with the Geltrex® solution (Table 12).
5. Incubate the dishes in a 37°C, 5% CO2 incubator for 1 hour. Note: Dishes can now be used or stored at 2–8°C for up to a week. Do not allow dishes to dry.
6. Aspirate the diluted Geltrex® solution from the culture dish and discard. You do not need to rinse off the Geltrex® solution from the culture dish after removal. Cells can now be passaged directly onto the Geltrex® matrix-coated culture dish.

Table 12. Volume of Geltrex® hESC-Qualified Matrix Required

Culture Vessel	Surface Area (cm^2)	Volume of Diluted Substrate (mL)
6-well plate	10 cm^2/well	1.5 mL/well
12-well plate	4 cm^2/well	750 µL/well
24-well plate	2 cm^2/well	350 µL/well
35-mm dish	10 cm^2	1.5 mL
60-mm dish	20 cm^2	3.0 mL
100-mm dish	60 cm^2	6.0 mL

B. Cryopreserving iPSCs

1. Pre-warm the required volume of Essential 8™ Medium at room temperature until it is no longer cool to the touch. Do not warm medium in a 37°C water bath.
2. Prepare Essential 8™ Freezing Medium. For every 1 mL of freezing medium needed, aseptically combine the components listed below in a sterile 15-mL tube (Table 13):

Table 13. Prepare Essential 8™ Freezing Medium

Component	Volume
Complete Essential 8™ Medium	0.9 mL
DMSO	

3. Place the tube with Essential 8™ Freezing Medium on ice until use. Discard any remaining freezing medium after use.
4. Aspirate the spent medium from the dish using a Pasteur pipette, and rinse the cells twice with DPBS without Calcium and Magnesium.
5. Add 0.5 mM EDTA solution to the dish. Adjust the volume of EDTA for various dish sizes. Swirl the dish to coat the entire cell surface.
6. Incubate the vessel at room temperature for 5–8 minutes or 37°C for 4–5 minutes. When the cells start to separate and round up, and the colonies will appear to have holes in them when viewed under a microscope, they are ready to be removed from the vessel.
7. Aspirate the EDTA solution with a Pasteur pipette.
8. Add 1 mL of ice-cold Essential 8™ Freezing Medium to each well of a 6-well plate.
9. Remove the cells by gently squirting the colonies from the well using a 5-mL glass pipette. Avoid creating bubbles. Collect cells in a 15-mL conical tube on ice.
10. Resuspend cells gently. Aliquot 1 mL of the cell suspension into each cryovial.
11. Quickly place the cryovials in a cryofreezing container (e.g., Mr. Frosty) to freeze the cells at 1°C per minute and transfer them to –80°C overnight.
12. After overnight storage at –80°C, transfer the cells to a liquid nitrogen tank vapor phase for long-term storage.

Reagents suggested:
Invitrogen, Catalog Number: A14666SA
Size: 500 ml
List Price: US$195.90

Description of some useful Reagents

Essential 8™ Medium (published as E8) is a xeno-free and feeder-free medium specially formulated for the growth and expansion of human pluripotent stem cells (PSCs). Originally developed by Guokai Chen et al. (1) in the laboratory of James Thomson (published as "E8") and validated by Cellular Dynamics International, Essential 8™ Medium has been extensively tested and is proven to maintain pluripotency in multiple iPSC lines. In addition, Essential 8™ Medium has been used to scale up production of iPSCs and has been shown to support iPSC growth for >50 passages without any signs of karyotypic abnormalities and maintains the ability of iPSCs to differentiate into all three germ line lineages.

- • **Consistent:** Reduced variability compared to existing feeder-free culture media.
- • **Robust** - Reliable and robust cultures with a xeno-free, cGMP, 8-component medium.
- • **Cost effective:** Economical and scalable PSC culture compared to other feeder-free media.

- Note: The "prototype" status of this product means that the product is currently undergoing real time stability studies in compliance with cGMP regulations.
- **Reduced Variability:** Essential 8™ Medium is xeno-free and contains only the eight essential components needed for stem cell culture. Unlike other media that contain over 20 highly variable ingredients, Essential 8™ Medium is produced under cGMP and has an optimized formulation and growth factor levels to help ensure maximum cell health, pluripotency, and growth, with minimal variability.
- **Reliable and Robust Cultures:** Essential 8™ Medium has been show to support pluripotent stem cell growth and provide cultures with superior morphology and growth kinetics compared to other feeder systems.
- **Cost Effective:** Essential 8™ Medium is provided in a convenient two component kit (500 ml basal & 10 ml supplement), and when used with vitronectin (VTN-N), provides a cost effective, defined system for feeder-free culture of human pluripotent stem cells (PSCs).
- (http://products.invitrogen.com/ivgn/product/A14666SA#).

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