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Generation of Human Induced Pluripotent Stem Cells (hiPSCs) from Fibroblasts using Episomal Vectors

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Introduction

Induced pluripotent stem cells (iPSCs) are genetically reprogrammed adult cells that exhibit a pluripotent stem cell-like state similar to embryonic stem cells.[1] 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.

There are multiple methods to generate iPSCs, including retrovirus-mediated gene transduction and chemical induction. While retroviral vectors require integration into host chromosomes to express reprogramming genes, DNA-based vectors and plasmid vectors exist episomally and do not require integration. The Episomal iPSC Reprogramming Vectors are an optimized mixture of three vectors that can reprogram somatic cells to iPSCs without integration. The three episomal vectors have the oriP/EBNA-1 (Epstein-Barr nuclear antigen-1) backbone that delivers the reprogramming genes, Oct4, Sox2, Nanog, Lin28, L-Myc, Klf4, and SV40LT. This system has successfully demonstrated reprogramming of fibroblasts, $CD34^+$ cells, and Peripheral Blood Mononuclear Cells (PBMCs). High transfection efficiency due to oriP/EBNA-1 mediated nuclear import and retention of vector DNA allows iPSC derivation in a single transfection.[2] In addition, silencing of the viral promoter driving EBNA-1 expression and the loss of the episomes at a rate of ~5% per cell cycle due to defects in vector synthesis and partitioning allows the removal of episomal vectors from the iPSCs without any additional manipulation.[3]

For optimal reprogramming efficiency with the Episomal iPSC Reprogramming Vectors, culture the cells in unsupplemented Fibroblast Medium until the day of transfection. After transfection, allow the cells to recover in Supplemented Fibroblast Medium overnight, and then switch to N2B27 Medium supplemented with bFGF and a cocktail of small molecules consisting of PD0325901 (MEK inhibitor), CHIR99021 (GSK3β inhibitor), A-83-01 (TGF-β/Activin/Nodal receptor inhibitor), HA-100 (ROCk inhibitor), and hLIF (human leukemia inhibitory factor). After 15 days of culture, transition the iPSCs into Essential 8™ Medium, a serum-free, xeno-free medium that minimizes variability while improving feeder-free culture conditions for iPSCs.

Materials needed

- Episomal iPSC Reprogramming Vectors (50 µL, 1 µg/µL) (Cat. no. A14703)
- Dulbecco's Modified Eagle Medium (DMEM) with GlutaMAX™-I (High Glucose) (Cat. no. 10569-010)
- KnockOut™ DMEM/F-12 (Cat. no. 12660-012)
- Fetal Bovine Serum (FBS), ESC-Qualified, US Origin (Cat. no. 16141-079)
- MEM Non-Essential Amino Acids Solution, 10 mM (Cat. no. 11140-050)
- Basic Fibroblast Growth Factor (bFGF) (Cat. no. PHG0264)
- HA-100 (ROCk inhibitor) (Santa Cruz, Cat. no. sc-203072)
- Bovine Albumin Fraction V Solution (BSA) (Cat. no. 15260-037)
- Essential 8™ Medium, consisting of Essential 8™ Basal Medium and Essential 8™ Supplement (50X) (Cat. no. A1517001)
- DMEM/F-12 with HEPES (Cat. no. 11330-057)

- N-2 Supplement (100X) (Cat. no. 17502-048)
- B-27® Supplement (50X) (Cat. no. 17504-044)
- GlutaMAX™-I (100X) (Cat. no. 35050-061)
- β-mercaptoethanol, 1000X (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) (Cat. no. PHC9461)
- Vitronectin, truncated recombinant human (VTN-N) (Cat. no. A14700) or Geltrex® LDEV-Free hESC-Qualified Reduced Growth Factor Basement Membrane Matrix (Cat. no. A1413301)
- 0.05% Trypsin-EDTA (1X), Phenol Red (Cat. no. 25300-054)
- UltraPure™ 0.5 M EDTA, pH 8.0 (Cat. no. 15575-020)
- Dulbecco's PBS (DPBS) without Calcium and Magnesium (Cat. no. 14190-144)
- Characterization reagents (surface marker staining):

  Mouse primary antibodies (one is required):

  - Mouse Anti-Tra1-60 Antibody (Cat. no. 41-1000)
  - Mouse Anti-Tra1-81 Antibody (Cat. no. 41-1100)
  - Mouse Anti-SSEA4 Antibody (Cat. no. 41-4000)

  Alexa Fluor® secondary antibodies (one is required):

  - Alexa Fluor® 488 Goat Anti-Mouse IgG (H+L) Antibody (Cat. no. A11029)
  - Alexa Fluor® 594 Goat Anti-Mouse IgG (H+L) Antibody (Cat. no. A11032)
  - Alexa Fluor® 488 Goat Anti-Rabbit IgG (H+L) Antibody (Cat. no. A11034)
  - Alexa Fluor® 594 Goat Anti-Rabbit IgG (H+L) Antibody (Cat. no. A11037)
- Detection reagents (for detection of episomal vectors using PCR)
  - CellsDirect™ Resuspension & Lysis Buffers (Cat. no. 11739-010)
  - AccuPrime™ Taq High Fidelity (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

Workflow

A typical reprogramming schedule using the Episomal iPSC Reprogramming Vectors is shown below:

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.

Prepare 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:

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

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

Fibroblast Medium (for 100 mL of complete medium)

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

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 2–8°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**:

HA-100 (ROCk inhibitor)	varies (final concentration = 10 µM)
bFGF (10 µg/mL)	40 µL (final concentration = 4 ng/mL)

2. 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 2–8°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:

Essential 8™ Basal Medium	490 mL
Essential 8™ Supplement (50X)	10 mL

3. Complete Essential 8™ Medium can be stored at 2–8°C for up to 2 weeks.

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:

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**.

PD0325901 (MEK inhibitor)	0.5 µM
CHIR99021 (GSK3β 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

Note: CHALP molecule cocktail is an optimized mixture of small molecules (**C**HIR99021, **H**A-100, **A**-83-01, h**L**IF, **P**D0325901) shown to greatly improve the episomal reprogramming efficiency.

3. N2B27 Medium (without CHALP molecules and bFGF) can be stored at 2–8°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:

DPBS without Calcium and Magnesium	50 mL
0.5 M EDTA	50 µL

2. Filter sterilize the solution. The solution can be stored at room temperature for up to 6 months.

Coat culture vessels with Vitronectin (VTN-N)

1. Remove a 1-mL vial of vitronectin from –80°C storage and thaw at 2–8°C overnight.
2. Prepare working aliquots by dispensing 60 µL of vitronectin into polypropylene tubes. The working aliquots can be frozen at –80°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. Refer to Table 1 for culture surface area and volume required.

   The optimal working concentration of vitronectin is cell line dependent. We recommend using a final coating concentration of 0.5 µg/cm^2 for human PSC culture.

$$\text{Working Conc.} = \text{Coating Conc.} \times \frac{\text{Culture Surface Area}}{\text{Volume Required for Surface Area}}$$

$$\text{Dilution Factor} = \frac{\text{Stock Concentration (0.5 mg/mL)}}{\text{Working Concentration}}$$

   Example: To coat a 6-well plate at a coating concentration of 0.5 µg/cm^2, you will need to prepare 6 mL of diluted vitronectin solution (10 cm^2/well surface area and 1 mL of diluted vitronectin/well; see Table 1) at the following working concentration:

$$\text{Working conc.} = 0.5\ \mu\text{g/cm}^2 \times \frac{10\ \text{cm}^2}{1\ \text{mL}} = 5\ \mu\text{g/mL}$$

$$\text{Dilution factor} = \frac{0.5\ \text{mg/mL}}{5\ \mu\text{g/mL}} = 100\text{X (i.e., 1:100 dilution)}$$

4. To coat the wells of a 6-well plate, remove a 60-µL aliquot of vitronectin from –80°C storage and thaw at room temperature. You will need one 60-µL aliquot per 6-well plate.
5. Add 60 µL 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 µg/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 cm^2/well) at 1 mL/well, the final concentration will be 0.5 µg/cm^2.

7. Incubate at room temperature for 1 hour.

   Note: Dishes can now be used or stored at 2–8°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 (see the **Appendix**, page 13).

Table 1 Volume of diluted Vitronectin required

Culture Vessel	Surface area (cm^2)	Diluted substrate volume (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

Reprogram 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

IMPORTANT! 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 1×10^6 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 (see Table 2).

Table 2 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.

IMPORTANT! 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 CO_2 incubator overnight.

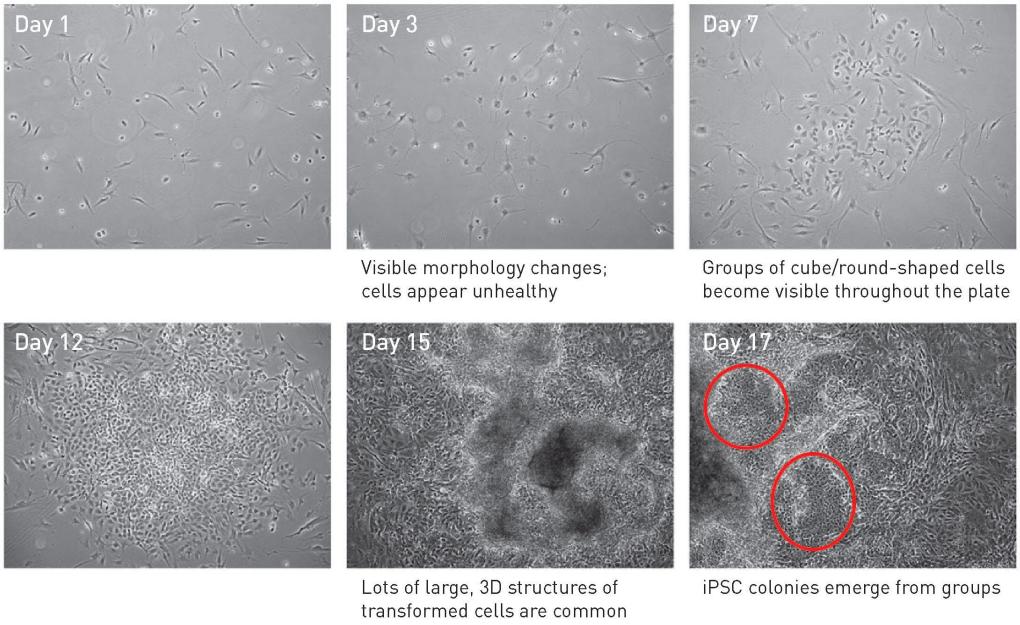
Day 1: Switch to Supplemented N2B27 Medium

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, page 8). Within 15 to 21 days of transfection, the iPSC colonies will grow to an appropriate size for transfer.

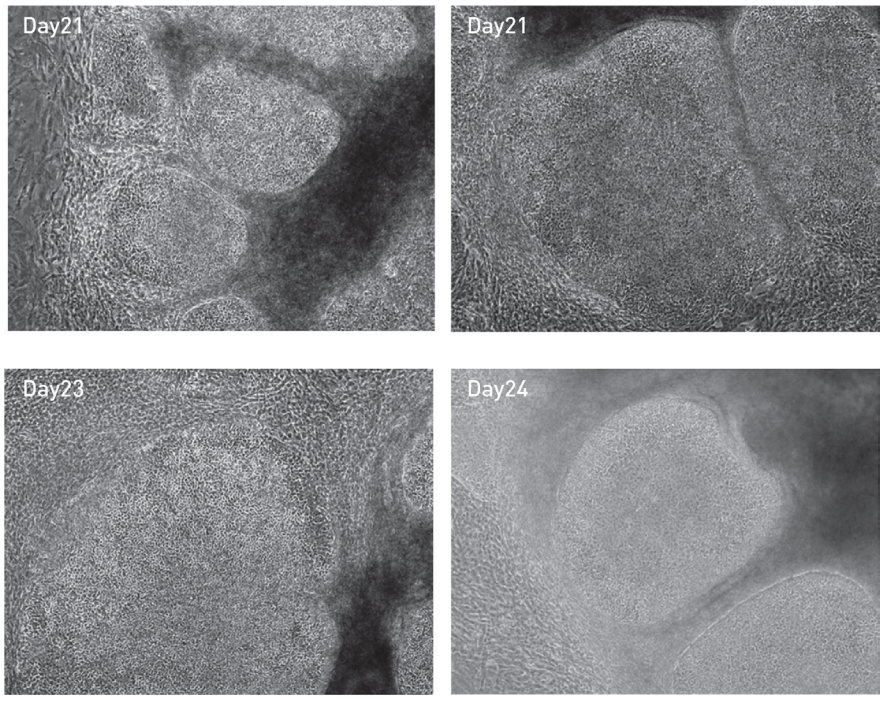
Figure 1 Expected morphology of cells during episomal reprogramming. The images show human neonatal foreskin fibroblast cells (strain BJ) as they undergo morphological changes and iPSC colonies begin to emerge.



Identify 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 stain 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).

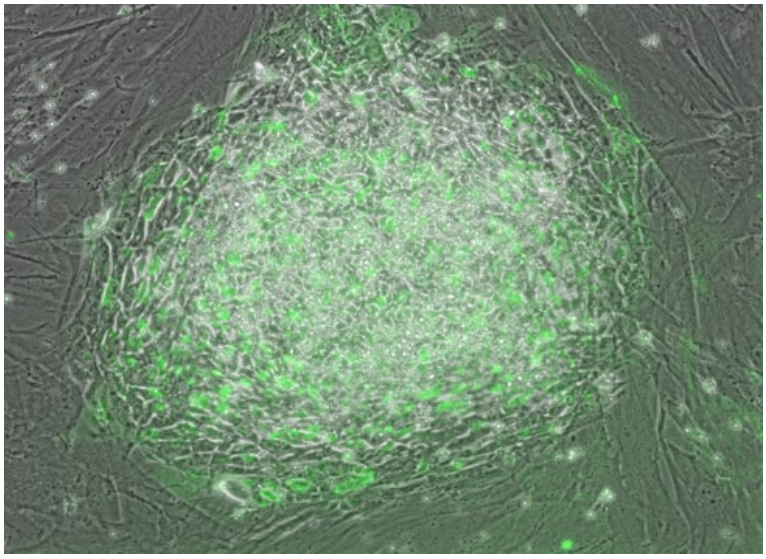
   Note: Any of the four Alexa Fluor® secondary antibodies listed in the **Materials Needed** section can be used.

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 2–8°C for one week and re-used up to two times.

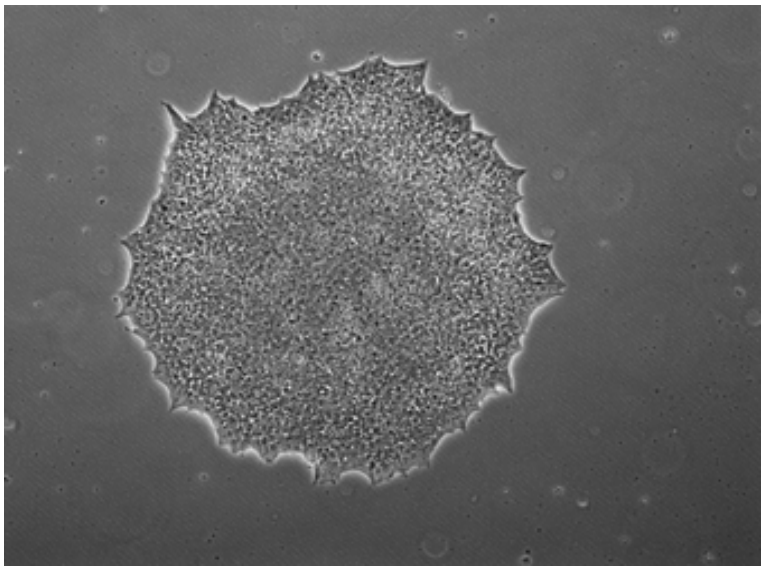
10. Wash cells three times with KnockOut™ DMEM/F-12. Add fresh KnockOut™ DMEM/F-12 to cover the surface of the cells (6 mL per 100-mm dish).
11. Visualize the cells under a standard fluorescent microscope and mark the successfully reprogrammed colonies for picking and expansion. Successful antibody staining can very specifically distinguish reprogrammed colonies from just plain transformed counterparts (see Figure 3, below), 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.

Figure 3 iPSC colony (10X) stained with Tra 1-81 antibody on Day 24 post-transfection.



Expected results

Figure 4 Human neonatal foreskin fibroblast cells were transformed using Episomal iPSC Reprogramming Vectors and allowed to proliferate on vitronectin-coated plates in fibroblast medium, shown here at passage 5.



Pick iPSC colonies

1. Examine the culture dish containing the reprogrammed cells under 10X magnification using an inverted microscope, and mark the colony to be picked on the bottom of the culture dish.

   Note: We recommend picking at least 10 distinct colonies by the end of each reprogramming experiment and expanding them in separate 24-well vitronectin-coated plates.

2. Transfer the culture dish to a sterile cell culture hood (i.e., biosafety cabinet) equipped with a stereomicroscope.
3. Cut the colony to be picked into 5–6 pieces in a grid-like pattern using a 25-gauge 1½ inch needle.
4. Using a 200-µL pipette, transfer the cut pieces to a freshly prepared 24-well vitronectin-coated plate containing Essential 8™ Medium.
5. Incubate the vitronectin-coated plate containing the picked colonies in a 37°C, 5% CO_2 incubator.
6. Allow the colonies to attach to the culture plate for 48 hours before replacing the spent medium with fresh Essential 8™ Medium. After that, change the medium every day.
7. Treat the reprogrammed colonies like normal human iPSC colonies; passage, expand, and maintain them using standard culture procedures until you have frozen cells from two 60-mm plates. Cells cultured in Essential 8™ Medium on vitronectin-coated culture vessels should be passaged using 0.5 mM EDTA in DPBS. Use of enzymes such as collagenase and dispase for passaging these cells results in compromised viability and attachment.

   Note: Newly derived iPSC lines may contain a fair amount of differentiation through passage 4**. It is not necessary to remove differentiated material prior to passaging.** By propagating/splitting the cells the overall culture health should improve throughout the early passages.

Detect episomal vectors by PCR

Prepare 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 3 for the recommended volumes.

Table 3 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.

IMPORTANT! **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 × *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 page 12).

PCR with AccuPrime™™ High Fidelity *Taq* DNA Polymerase

13. Add the following components to a DNase/RNase-free, thin-walled PCR tube as directed in Table 4. Forward and reverse primers are shown in Table 5. 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 4 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 5 Primers for standard PCR

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

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 6, below.

Table 6 PCR parameters

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

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

APPENDIX

A. Coat 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 (refer to Table 7, below).
5. Incubate the dishes in a 37°C, 5% CO_2 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 7 Required volume of Geltrex® hESC-qualified matrix

Culture vessel	Surface area (cm^2)	Diluted substrate volume (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. Cryopreserve 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:

Complete Essential 8™ Medium	0.9 mL
DMSO	0.1 mL

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 (refer to Table 3).
5. Add 0.5 mM EDTA solution to the dish. Adjust the volume of EDTA for various dish sizes (refer to Table 4). 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., Nalgene® Mr. Frosty® Freezing Container) 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.

References

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3. Nanbo, A., Sugden, A., and Sugden, B. (2007) The coupling of synthesis and partitioning of EBV's plasmid replicon is revealed in live cells. EMBO J *26*, 4252–4262.

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