USER GUIDE

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Epi5™ Episomal iPSC Reprogramming Kit

For efficient, integration-free reprogramming of somatic cells into induced pluripotent stem cells (iPSC)

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Product Information

Kit Contents and Storage

Kit contents

The Epi5™ Episomal iPSC Reprogramming Kit (Cat. no. A15960) contains **five** reprogramming vectors in two separate tubes. Tube A contains an optimized mixture of three episomal vectors that deliver the five reprogramming factors Oct4, Sox2, Klf4, L-Myc, and Lin28. Tube B contains an optimized mixture of two vectors expressing mp53DD (a dominant negative mutation of p53) and EBNA1, which together improve the reprogramming efficiency of the system.

The Epi5™ Episomal iPSC Reprogramming Kit contains sufficient material for 20 reprogramming experiments.

Component*	Part no.	Amount	Vectors	Genes
Epi5™ Reprogramming Vectors	A15958	20 µL	pCE-hOCT3/4	Oct4
			pCE-hSK	Sox2, Kfl4
			pCE-hUL	L-Myc, Lin28
Epi5™ p53 & EBNA Vectors	A15959	20 µL	pCE-mP53DD	mp53DD
			pCXB-EBNA1	EBNA1

Shipping and storage

- Epi5™ Episomal iPSC Reprogramming Kit is shipped on dry ice.
- Immediately upon receipt, store each component at –20°C.
- Before use, briefly centrifuge the thawed vectors to collect them at the bottom of the tube.
- Use the kit by the expiration date specified on the Certificate of Analysis (CoA).

Description of the System

Induced pluripotent stem cells (iPSC)

Induced pluripotent stem cells (iPSCs) are genetically reprogrammed adult cells which exhibit a pluripotent stem cell-like state similar to embryonic stem cells (Meissner *et al.*, 2007; Park *et al.*, 2008; Takahashi *et al.*, 2007; Takahashi & Yamanaka, 2006; Wernig *et al.*, 2007; Yu *et al.*, 2007). 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 (ESC); thus, they are an invaluable new source of pluripotent cells for drug discovery, cell therapy, and basic research.

There are multiple methods to generate iPSCs, including retrovirus-mediated gene transfection 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 Epi5™ Episomal iPSC Reprogramming Kit contains an optimized mixture of **five** vectors that can reprogram somatic cells to iPSCs without integration.

Epi5™ Reprogramming Vectors

The Epi5™ Reprogramming Vectors contain an optimized mixture of three episomal vectors with an oriP/EBNA-1 (Epstein-Barr nuclear antigen-1) backbone for delivering the reprogramming genes, Oct4, Sox2, Lin28, L-Myc, and Klf4. High transfection efficiency due to oriP/EBNA-1 mediated nuclear import and retention of vector DNA allows iPSC derivation in a single transfection (Yu *et al.*, 2011). 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 (Nanbo *et al.*, 2007).

Epi5™ p53 & EBNA Vectors

Epi5™ p53 & EBNA Vectors provide additional improvements to the reprogramming system.

The p53 protein is highly involved in cell cycle regulation and tumor suppression. With the activation of cytotoxic responses, p53 expression results in cell cycle arrest or cell death to prevent further complications within the system. Knockdown of p53 has been shown to improve reprogramming efficiencies as well as to prevent differentiation via the introduction of a variety of knockdown agents (Hong *et al.*, 2009; Spike & Wahl, 2011). The mp53DD is a dominant negative mutation of the p53 protein providing higher efficiency knockdown than traditional shRNA systems (Kawamura *et al.*, 2009). The presence of this gene in an episomal system allows for transient expression of the dominant negative mutant over an extended period of time, diluting out with normal passaging of iPSC.

Supplemental expression of the EBNA1 gene from a vector solely dedicated to this purpose allows for high expression of plasmids containing the origin of replication present on the reprogramming plasmids. This plasmid, however, lacks the OriP gene, thus providing only transient expression for the early stages of reprogramming.

Continued on next page

Comparison of episomal reprogramming vectors

The table below provides a comparison of the Episomal iPSC Reprogramming Vectors (Cat. no. A14703) and the Epi5™ Episomal iPSC Reprogramming Kit (Cat. no. A15960).

	Episomal iPSC Reprogramming Vectors	Epi5™ Episomal iPSC Reprogramming Kit
Genes	Oct4, Sox2, Nanog, Lin28, Klf4, and L-Myc	Oct4, Sox2, Lin28, L-Myc, Klf4, mp53DD, additional EBNA
Vector size	12–17.5 kb	6–11 kb
Polycistronic elements	IRES element	2A sequence
Promoter	EF1α	CAG
Small molecules	required	not required
Number of vectors	3	5

Advantages of Epi5™ Episomal iPSC Reprogramming Kit

- As a transgene-free and viral-free reprogramming system, it is a safe alternative to other reprogramming methods such as lentiviral delivery for all stages of iPSC research.
- Allows the reprogramming of a variety of somatic cell types and provides flexibility in somatic cell selection.
- Optimized for feeder-free reprogramming, enabling defined and feeder-free reprogramming when used with the Essential 8™ Medium.
- High transfection efficiency due to oriP/EBNA-1 mediated nuclear import and retention of vector DNA allows iPSC derivation in a single transfection.
- The episomal vectors are removed from the iPSCs without any further manipulation due to the silencing of the viral promoter driving EBNA-1 expression and the loss of the episomes due to defects in vector synthesis and partitioning.
- Do not require the use of small molecules for reprogramming.

Materials and Equipment Needed

Cells and vectors

- Epi5™ Episomal iPSC Reprogramming Kit (Cat. no. A15960)
- Human neonatal foreskin fibroblast cells (strain BJ; ATCC no. CRL2522)

Media and reagents

- Dulbecco's Modified Eagle Medium (DMEM) with GlutaMAX™-I (high glucose) (Cat. no. 10569)
- KnockOut™ DMEM/F-12 (Cat. no. 12660-012)
- Fetal Bovine Serum (FBS), ESC-Qualified (Cat. no. 16141-079)
- MEM Non-essential Amino Acids (NEAA) (Cat. no. 11140-050)
- Basic Fibroblast Growth Factor (bFGF), recombinant human (Cat. no. PHG0264)
- Bovine Albumin Fraction V Solution (BSA) (Cat. no. 15260-037)
- Essential 8™ Medium (Prototype), consisting of DMEM/F-12 (HAM) 1:1 and Essential 8™ Supplement (50X) (Cat. no. A14666SA)
- DMEM/F-12 with HEPES (Cat. no. 11330-057)
- N-2 Supplement (100X) (Cat. no. 17502-048)
- B-27® Serum-Free Supplement (50X) (Cat. no. 17504-044)
- GlutaMAX™-I Supplement (Cat. no. 35050-061)
- β-mercaptoethanol (Cat. no. 21985-023)
- 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)

Characterization reagents

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 (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)

Continued on next page

Materials and Equipment Needed, continued

Reagents for PCR detection of episomal vectors

- CellsDirect Resuspension & Lysis Buffers (Cat. no. 11739-010)
- AccuPrime™ *Taq* DNA Polymerase High Fidelity (Cat. no. 12346-094)
- Forward and Reverse primers for PCR (see page 27)

Equipment

- Electroporation instrument (see **Note**, below).
- Sterile cell culture hood (i.e., biosafety cabinet) equipped with a stereomicroscope
- Inverted microscope
- Incubator set at 37°C, 5% CO_2
- Water bath set at 37°C
- Sterile serological pipettes (5-mL, 10-mL)
- Centrifuge
- 15-mL centrifuge tubes
- 60-mm and 100-mm tissue culture-treated dishes
- 6-well tissue culture-treated plates
- 25 gauge 1 inch needle

The protocols in this user guide describe electroporation using the Neon® Transfection System (Cat. no. MPK5000) and the Neon® Transfection System 10 µL Tip Kit (Cat. no. MPK1025). Electroporation parameters must be optimized for other electroporation devices.

Methods

Guidelines for Generating iPSCs

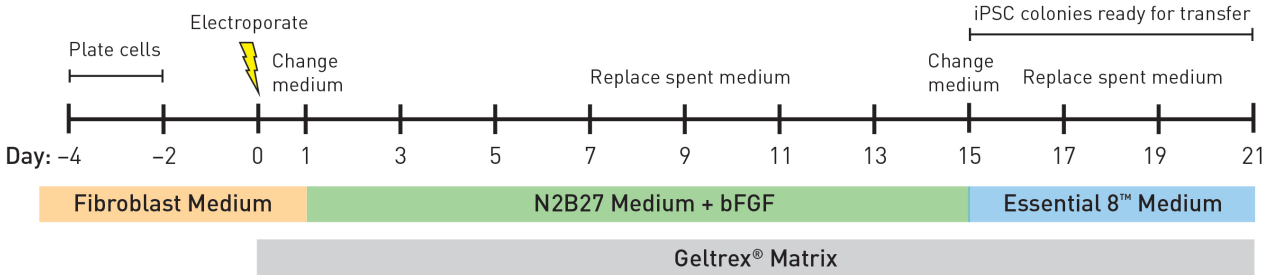
Experiment guidelines

- To maintain sterile culture conditions, carry out all of the procedures in this manual using sterile laboratory practices in a laminar flow hood.
- You can use the Epi5™ Episomal iPSC Reprogramming Kit to reprogram a wide range of cell types in proliferative and quiescent states. However, the reprogramming efficiency may vary between different cell types.
- Each reprogramming experiment requires 1×10^5 cells and 1 µL each of the Epi5™ Reprogramming Vectors and the Epi5™ p53 & EBNA Vectors.
- The Epi5™ Episomal iPSC Reprogramming Kit contains sufficient material for 20 reprogramming experiments. Before use, briefly centrifuge the thawed vectors to collect them at the bottom of the tube.
- Cells should be approximately 75–90% confluent on the day of transfection (Day 0). Since overconfluency results in decreased transfection efficiency, we recommend replating the cells to achieve 75–90% confluency, if they have become overconfluent during culturing.
- We recommend reprogramming patient cells at the earliest passage possible. However, it is important to have the cells growing and healthy, which can take between 1–4 weeks. The cells are usually ready to reprogram once they have gone through a total of 3–4 passages.
- Prior to starting your experiments, ensure that the media are equilibrated to 37°C and appropriately gassed.
- You do not need to manipulate the culture conditions to remove the reprogramming vectors. In the absence of drug selection, approximately 5% of the episomal vectors are lost each cell cycle due to silencing of the viral promoter driving EBNA-1 expression and defects in vector synthesis and partitioning.
- 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.

Reprogramming Fibroblasts

Workflow

The major steps required for reprogramming human neonatal foreskin fibroblast cells to generate iPSCs using the Epi5™ Episomal iPSC Reprogramming Kit are shown below. Note that the timeline is provided as a guideline for experimental planning; actual timeline can vary based on the cell type and experimental conditions.



Fibroblast reprogramming timeline

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 Geltrex® matrix-coated culture dishes, and incubate them overnight in complete Fibroblast Medium.

Day 1 to 14: Change the medium to N2B27 Medium supplemented with 100 ng/mL 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 Geltrex® matrix-coated culture dishes for expansion.

Media for reprogramming fibroblasts

For optimal reprogramming efficiency with the Epi5™ Episomal iPSC Reprogramming Kit, culture the cells in Fibroblast Medium on Geltrex® matrix-coated culture vessels until the day of transfection.

After transfection, allow the cells to recover in Fibroblast Medium overnight, and then switch to N2B27 Medium supplemented with 100 ng/mL bFGF.

After 15 days of culture in Fibroblast Medium and on Geltrex® matrix, transition the reprogrammed cells into Essential 8™ Medium, a serum-free, xeno-free medium, and continue culturing them on Geltrex® matrix-coated culture vessels until iPSCs emerge and are ready to be picked.

See page 28 for instructions on preparing media and reagents.

Reprogramming Fibroblasts, continued

Fibroblast reprogramming protocol

The following reprogramming 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

2–4 days before transfection, plate human fibroblast cells in Fibroblast Medium (see page 28 for recipe) into a T75 flask. Cells should be approximately 75–90% confluent on the day of transfection (Day 0).

- 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.
- Because 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

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

1. Aspirate the medium from Geltrex® matrix-coated 6-well plates (see page 31) and replace with 2 mL of fresh Fibroblast Medium per well. Place the coated plates at 37°C until ready for use.

   Note: One Geltrex® matrix-coated 6-well plate can be used for six transfections.
2. Aspirate the spent medium from the fibroblasts in the T75 flask (from Step 1).
3. Wash the cells in DPBS without calcium and magnesium.
4. Add 2 mL of 0.05% Trypsin/EDTA to the culture flask containing the cells.
5. Incubate the flask at 37°C for approximately 4 minutes.
6. Add 6 mL of Fibroblast Medium to each flask. Tap the plate against your hand to ensure cells have been dislodged from the flask, and carefully transfer cells into an empty, sterile 15-mL conical tube.
7. Wash the culture flask with an additional 4 mL of Fibroblast Medium and add to the wash to the same 15-mL conical tube to ensure that all of the cells have been transferred.
8. Remove a 20-µL sample to perform a viable cell count and calculate the number of transfections to be performed. You will need 1×10^5 cells per transfection.
9. Centrifuge the cells at 1,000 rpm for 5 minutes at room temperature.
10. 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.

Continued on next page

Day 0: Transfect cells using the Neon® Transfection System

1. Thaw the Epi5™ vectors at 37°C and place them on wet ice. Before use, briefly centrifuge the thawed vectors to collect them at the bottom of the tube.
2. Resuspend the cell pellet in Resuspension Buffer R (included with Neon® Transfection kits) at a final concentration of 1.0×10^6–1.4×10^6 cells/0.1 mL.
3. Transfer the cells (10 µL per transfection reaction) to a sterile 1.5-mL microcentrifuge tube.
4. Turn on the Neon® unit and enter the following electroporation parameters in the Input window.

Pulse Voltage	Pulse Width	Pulse Number	Cell Density	Tip Type
1650 V	10 ms	3	1×10^5 cells/0.1 mL	10-µL

5. Fill the Neon® Tube with 3 mL Electrolytic Buffer (use Buffer E for the 10-µL Neon® Tip).
6. Insert the Neon® Tube into the Neon® Pipette Station until you hear a click.
7. Transfer 1 µL of Epi5™ Reprogramming Vectors to the tube containing cells.
8. Transfer 1 µL of Epi5™ p53 & EBNA Vectors to the tube containing cells and mix gently.
9. Insert a 10-µL Neon® Tip into the Neon® Pipette.
10. 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.
11. Insert the Neon® Pipette with the sample vertically into the Neon® Tube placed in the Neon® Pipette Station until you hear a click.
12. 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.
13. Remove the Neon® Pipette from the Neon® Pipette Station and immediately transfer the samples from the Neon® Tip into one well of the pre-warmed Geltrex® matrix-coated 6-well plate containing Fibroblast Medium.

    Note: Evenly distribute cells over the well in a dropwise manner.
14. Discard the Neon® Tip into an appropriate biological hazardous waste container.
15. Repeat the process for any additional samples. Do not use Neon® tip more than twice.
16. Incubate the plates at 37°C in a humidified CO_2 incubator overnight.

Continued on next page

Day 1: Switch to N2B27 Medium

1. Aspirate the spent Fibroblast Medium from the plates using a Pasteur pipette.
2. Add 10 mL of N2B27 Medium supplemented with bFGF (added freshly prior to use) to each well (see page 29 for recipe).
3. Replace the spent medium every other day, up to day 15 post-transfection.

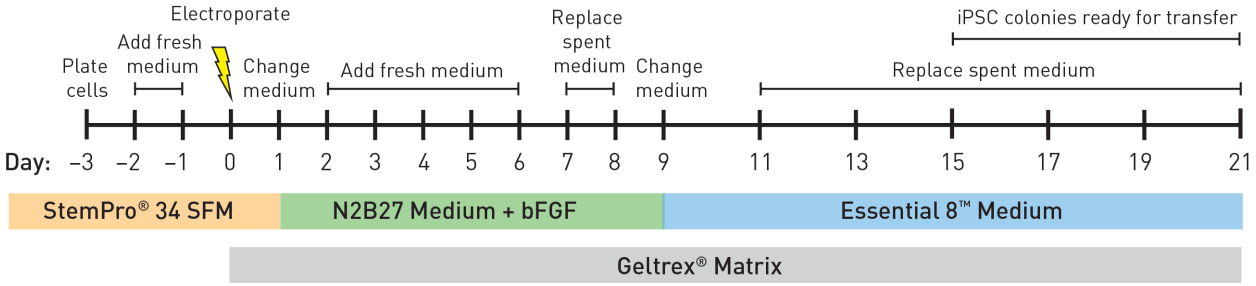
Day 15: Switch to Essential 8™ Medium

1. Aspirate the spent medium and replace with Essential 8™ Medium (see page 29 for recipe). Resume medium changes every other day.
2. Observe the plates every other day under a microscope for the emergence of cell clumps indicative of transformed cells (see "Identifying iPSC Colonies", page 20). Within 15 to 21 days of transfection, the iPSC colonies will grow to an appropriate size for transfer.

Reprogramming StemPro® CD34⁺ Cells (Feeder-Free Culture)

Workflow

The major steps required for reprogramming StemPro® CD34⁺ cells to generate iPSCs using the Epi5™ Episomal iPSC Reprogramming Kit and the subsequent culture of the reprogrammed cells under feeder-free conditions are shown below. Note that the timeline is provided as a guideline for experimental planning; actual timeline can vary based on the cell type and experimental conditions.



StemPro® CD34⁺ (feeder-free) reprogramming timeline

Day –3: Plate StemPro® CD34⁺ cells into a 24-well plate in complete StemPro®-34 medium containing cytokines (i.e., SCF, IL-3, and GM-CSF).

Day 0: Transfect the cells using the Neon® Transfection System. Plate transfected cells onto Geltrex® matrix-coated culture dishes, and incubate them overnight in complete StemPro®-34 medium containing cytokines.

Day 1: Change the medium to N2B27 Medium supplemented with 100 ng/mL bFGF; replace the spent medium every other day.

Day 2 to 6: Replenish the culture every day by adding fresh medium.

Day 7 to 8: Replace the spent medium with fresh medium every day.

Day 9: Change the medium to Essential 8™ Medium and monitor the culture vessels for the emergence of iPSC colonies. Replace the spent medium every other day.

Day 15 to 21: Pick and transfer undifferentiated iPSCs onto fresh Geltrex® matrix-coated culture dishes for expansion.

Media for reprogramming StemPro® CD34⁺ cells (feeder-free)

For optimal reprogramming efficiency with the Epi5™ Episomal iPSC Reprogramming Kit, culture the cells in complete StemPro®-34 medium containing cytokines (i.e., SCF, IL-3, and GM-CSF) until the day of transfection.

After transfection, allow the cells to recover in complete StemPro®-34 medium with cytokines on Geltrex® matrix-coated culture vessels overnight, and then switch to N2B27 Medium supplemented with 100 ng/mL bFGF.

After 9 days of culture in supplemented N2B27 Medium on Geltrex® matrix-coated culture vessels, transition the reprogrammed cells into Essential 8™ Medium, and continue culturing them on Geltrex® matrix-coated culture vessels until iPSCs emerge and are ready to be picked.

See page 28 for instructions on preparing media and reagents.

Reprogramming StemPro® CD34+ Cells (Feeder-Free Culture), continued

StemPro® CD34+ reprogramming protocol (feeder-free culture)

The following reprogramming protocol has been optimized for StemPro® CD34+ cells (Cat. no. A14059), which are human hemopoietic progenitor cells (HPCs) derived from the human umbilical cord blood of mixed donors. We recommend that you optimize the protocol for your cell type.

Day –3 to –1: Seed and expand cells

1. 3 days before transduction (day –3), thaw 1 vial of StemPro® CD34+ cells (0.5×10^6 cells) and gently transfer into a 15-mL sterile, conical tube. Drop-wise add 1 mL of StemPro®-34 SFM to the cells while gently agitating to mix.
2. Centrifuge the cell suspension at 200 × *g* for 10 minutes, discard the supernatant, and resuspend the cells in 1 mL of complete StemPro®-34 medium containing cytokines (i.e., SCF, IL-3, and GM-CSF) into one well of a 24-well plate (see page 30).

   Note: The recommended final concentrations for the cytokines are 100 ng/mL for SCF, 50 ng/mL for IL-3, and 25 ng/mL for GM-CSF.

   Note: We recommend using the wells in the middle section of the 24-well plate to prevent excessive evaporation of the medium during incubation.
3. 2 days before transduction (day –2), count the cells to ensure that they are expanding, and add 0.5 mL of fresh complete StemPro®-34 medium containing cytokines without disturbing the cells.
4. One day before transduction (day –1), count the cells to ensure that they are continuing to expand, gently remove 0.5 mL of the medium, and add 1.0 mL of fresh complete StemPro®-34 medium containing cytokines without disturbing the cells.

Day 0: Transfect cells using the Neon® Transfection System

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

1. Aspirate the medium from Geltrex® matrix-coated 6-well plates (see page 31) and replace with 2 mL of complete StemPro®-34 medium containing cytokines (see page 30) per well. Place the coated plates at 37°C until ready for use.

   Note: One Geltrex® matrix-coated 6-well plate can be used for six transfections.
2. Thaw the Epi5™ vectors at 37°C and place them on wet ice until ready for use. Before use, briefly centrifuge the thawed vectors to collect them at the bottom of the tube.
3. Count the number of cells using a hemacytometer or the Countess® Automated Cell Counter to determine the viability and total number of cells; the cells should have more than doubled in number.
4. 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.
5. Resuspend the cell pellet in Resuspension Buffer T (included with Neon® Transfection kits) at a final concentration of 1.0×10^6 –1.4×10^6 cells/0.1 mL.

   Note: Buffer T is required for blood cell transfections.

Continued on next page

Day 0: Transfect cells using the Neon® Transfection System, continued

6. Transfer the cells (10 µL per transfection reaction) to a sterile 1.5-mL microcentrifuge tube.
7. Turn on the Neon® unit and enter the following electroporation parameters in the Input window.

Pulse Voltage	Pulse Width	Pulse Number	Cell Density	Tip Type
1650 V	10 ms	3	1×10^5 cells/0.1 mL	10-µL

8. Fill the Neon® Tube with 3 mL Electrolytic Buffer (use Buffer E for the 10-µL Neon® Tip).
9. Insert the Neon® Tube into the Neon® Pipette Station until you hear a click.
10. Transfer 1 µL of Epi5™ Reprogramming Vectors to the tube containing cells.
11. Transfer 1 µL of Epi5™ p53 & EBNA Vectors to the tube containing cells and mix gently.
12. Insert a 10-µL Neon® Tip into the Neon® Pipette.
13. 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.
14. Insert the Neon® Pipette with the sample vertically into the Neon® Tube placed in the Neon® Pipette Station until you hear a click.
15. 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.
16. Remove the Neon® Pipette from the Neon® Pipette Station and immediately transfer the samples from the Neon® Tip into one well of the pre-warmed Geltrex® matrix-coated 6-well plate containing 2 mL of complete StemPro®-34 medium with cytokines.

    Note: Evenly distribute cells over the well in a dropwise manner.
17. Discard the Neon® Tip into an appropriate biological hazardous waste container.
18. Repeat the process for any additional samples. Do not use Neon® tip more than twice.
19. Incubate the plates at 37°C in a humidified CO_2 incubator overnight.

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Reprogramming StemPro® CD34⁺ Cells (Feeder-Free Culture), continued

Day 1: Switch to N2B27 Medium

1. Do not aspirate the spent medium from the plates. Add 1 mL of N2B27 Medium supplemented with 100 ng/mL bFGF (added freshly prior to use; see page 29 for recipe) to each well.
2. Replace the spent medium every other day, up to day 15 post-transfection.

Day 2 to 6: Add fresh N2B27 Medium

1. Carefully aspirate 0.5–1 mL of the supernatant from the top of the well, trying to not remove any of the suspension StemPro® CD34⁺ cells while they are still attaching.
2. Add 0.5–1 mL of N2B27 medium supplemented with 100 ng/mL bFGF every day, up to day 6 post-transfection.

Day 7 to 9: Replace spent N2B27 Medium

1. Carefully aspirate the spent medium and replace with 2 mL of N2B27 medium supplemented with 100 ng/mL bFGF.
2. Replace spent medium with 2 mL fresh medium every day, up to day 9 post-transfection.

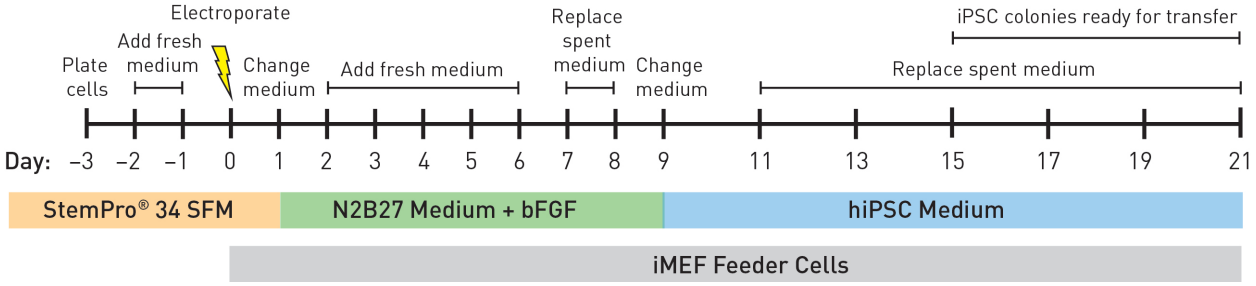
Day 9: Switch to Essential 8™ Medium

1. Aspirate the spent N2B27 medium and replace it with complete Essential 8™ Medium (see page 29 for recipe). Resume medium changes every other day.
2. Observe the plates every other day under a microscope for the emergence of cell clumps indicative of transformed cells (see "Identifying iPSC Colonies", page 20). Within 15 to 21 days of transfection, the iPSC colonies will grow to an appropriate size for transfer.

Reprogramming StemPro® CD34+ Cells (Feeder-Dependent Culture)

Workflow

The major steps required for reprogramming StemPro® CD34+ cells to generate iPSCs using the Epi5™ Episomal iPSC Reprogramming Kit and the subsequent culture of the reprogrammed cells on MEF feeder layers are shown below. Note that the timeline is provided as a guideline for experimental planning; actual timeline can vary based on the cell type and experimental conditions.



StemPro® CD34+ (feeder-dependent) reprogramming timeline

Day –3: Plate the StemPro® CD34+ cells into a 24-well plate in complete StemPro®-34 medium containing cytokines (i.e., SCF, IL-3, and GM-CSF).

Day 0: Transfect the cells using the Neon® Transfection System. Plate transfected cells onto MEF feeders, and incubate them overnight in complete StemPro®-34 medium containing cytokines.

Day 1: Change the medium to N2B27 Medium supplemented with 100 ng/mL bFGF.

Day 2 to 6: Replenish the culture every day by adding fresh medium.

Day 7 to 8: Replace the spent medium with fresh medium every day.

Day 9: Change the medium to human iPSC Medium and continue culturing the reprogrammed cells on MEF feeders, monitoring the culture for the emergence of iPSC colonies and replacing the spent medium every other day.

Day 15 to 21: Pick and transfer undifferentiated iPSCs onto fresh culture dishes with MEF feeder layers for expansion.

Media for reprogramming StemPro® CD34+ cells (feeder-dependent)

For optimal reprogramming efficiency using the Epi5™ Episomal iPSC Reprogramming Kit, culture the cells in complete StemPro®-34 medium containing cytokines (i.e., SCF, IL-3, and GM-CSF) until the day of transfection.

After transfection, allow the cells to recover in complete StemPro®-34 medium with cytokines on MEF feeders overnight, and then switch to N2B27 Medium supplemented with 100 ng/mL bFGF.

After 9 days of culture in supplemented N2B27 Medium on MEF feeder layers, transition the reprogrammed cells into human iPSC Medium, and continue culturing them on MEF feeders until iPSCs emerge and are ready to be picked.

See page 28 for instructions on preparing media and reagents.

Reprogramming StemPro® CD34+ Cells (Feeder-Dependent Culture), continued

StemPro® CD34+ Reprogramming Protocol (Feeder-Dependent Culture)

The following reprogramming protocol has been optimized for StemPro® CD34+ cells (Cat. no. A14059), which are human hemopoietic progenitor cells (HPCs) derived from the human umbilical cord blood of mixed donors. We recommend that you optimize the protocol for your cell type.

Day –3 to –1: Seed and expand cells

1. 3 days before transduction (day –3), thaw 1 vial of StemPro® CD34+ cells (0.5×10^6 cells) and gently transfer into a 15-mL sterile, conical tube. Drop-wise add 1 mL of StemPro®-34 SFM to the cells while gently agitating to mix.
2. Centrifuge the cell suspension at 200 × *g* for 10 minutes, discard the supernatant, and resuspend the cells in 1 mL of complete StemPro®-34 medium containing cytokines (i.e., SCF, IL-3, and GM-CSF) into one well of a 24-well plate (see page 30).

   Note: The recommended final concentrations for the cytokines are 100 ng/mL for SCF, 50 ng/mL for IL-3, and 25 ng/mL for GM-CSF.

   Note: We recommend using the wells in the middle section of the 24-well plate to prevent excessive evaporation of the medium during incubation.
3. 2 days before transduction (day –2), count the cells to ensure that they are expanding, and add 0.5 mL of fresh complete StemPro®-34 medium containing cytokines without disturbing the cells.
4. One day before transduction (day –1), count the cells to ensure that they are continuing to expand, gently remove 0.5 mL of the medium, and add 1.0 mL of fresh complete StemPro®-34 medium containing cytokines without disturbing the cells.

Day 0: Transfect cells using the Neon® Transfection System

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

1. Aspirate the medium from the 6-well plates containing MEF feeder cells (see page 32) and replace it with 2 mL per well of complete StemPro®-34 medium containing cytokines (see page 30). Place the coated plates at 37°C until ready for use.

   Note: One 6-well plate with MEF feeder layers can be used for six transfections.
2. Thaw the Epi5™ vectors at 37°C and place them on wet ice until ready for use. Before use, briefly centrifuge the thawed vectors to collect them at the bottom of the tube.
3. Count the number of cells using a hemacytometer or the Countess® Automated Cell Counter to determine the viability and total number of cells; the cells should have more than doubled in number.
4. 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.

Continued on next page

Day 0: Transfect cells using the Neon® Transfection System, continued

5. Resuspend the cell pellet in Resuspension Buffer T (included with Neon® Transfection kits) at a final concentration of 1.0×10^6 –1.4×10^6 cells/0.1 mL.

   Note: Buffer T is required for blood cell transfections.

6. Transfer the cells (10 µL per transfection reaction) to a sterile 1.5-mL microcentrifuge tube.
7. Turn on the Neon® unit and enter the following electroporation parameters in the Input window.

Pulse Voltage	Pulse Width	Pulse Number	Cell Density	Tip Type
1650 V	10 ms	3	1×10^5 cells/0.1 mL	10-µL

8. Fill the Neon® Tube with 3 mL Electrolytic Buffer (use Buffer E for the 10-µL Neon® Tip).
9. Insert the Neon® Tube into the Neon® Pipette Station until you hear a click.
10. Transfer 1 µL of Epi5™ Reprogramming Vectors to the tube containing cells.
11. Transfer 1 µL of Epi5™ p53 & EBNA Vectors to the tube containing cells and mix gently.
12. Insert a 10-µL Neon® Tip into the Neon® Pipette.
13. 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.

14. Insert the Neon® Pipette with the sample vertically into the Neon® Tube placed in the Neon® Pipette Station until you hear a click.
15. 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.

16. Remove the Neon® Pipette from the Neon® Pipette Station and immediately transfer the samples from the Neon® Tip into one well of the pre-warmed 6-well plate with MEF feeders and containing 2 mL of complete StemPro®-34 medium with cytokines.

    Note: Evenly distribute cells over the well in a dropwise manner.

17. Discard the Neon® Tip into an appropriate biological hazardous waste container.
18. Repeat the process for any additional samples. Do not use Neon® tip more than twice.
19. Incubate the plates at 37°C in a humidified CO_2 incubator overnight.

Continued on next page

Reprogramming StemPro® CD34⁺ Cells (Feeder-Dependent Culture), continued

Day 1: Switch to N2B27 Medium

1. Do not aspirate the spent medium from the plates. Add 1 mL of N2B27 Medium supplemented with 100 ng/mL bFGF (added freshly prior to use; see page 29 for recipe) to each well.
2. Replace the spent medium every other day, up to day 15 post-transfection.

Day 2 to 6: Add fresh N2B27 Medium

1. Carefully aspirate 0.5–1 mL of the supernatant from the top of the well, trying to not remove any of the suspension StemPro® CD34⁺ cells while they are still attaching.
2. Add 0.5–1 mL of N2B27 medium supplemented with 100 ng/mL bFGF every day, up to day 6 post-transfection.

Day 7 to 9: Replace spent N2B27 Medium

1. Carefully aspirate the spent medium and replace with 2 mL of N2B27 medium supplemented with 100 ng/mL bFGF.
2. Replace spent medium with 2 mL fresh medium every day, up to day 9 post-transfection.

Day 9: Switch to human iPSC Medium

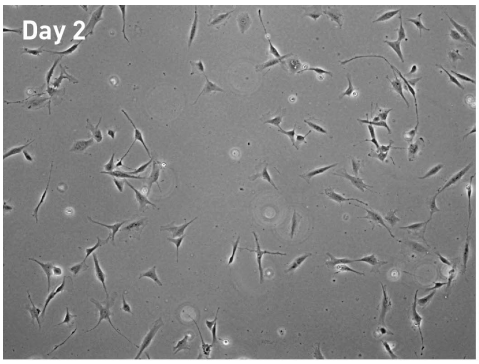
1. Aspirate the spent N2B27 medium and replace it with complete human iPSC Medium (see page 30 for recipe). Resume medium changes every other day.
2. Observe the plates every other day under a microscope for the emergence of cell clumps indicative of transformed cells (see "Identifying iPSC Colonies", page 20). Within 15 to 21 days of transfection, the iPSC colonies will grow to an appropriate size for transfer.

Identifying iPSC Colonies

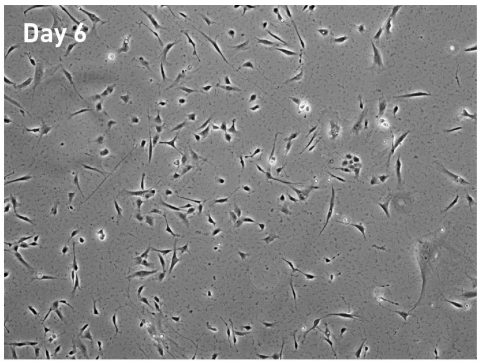
Visual identification

By day 21, the colonies on the plates will appear large and compact and cover 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 "Morphology of reprogrammed cells", page 21). Therefore, we recommend that you perform live surface marker staining with Tra1-60 or Tra1-81 antibodies that recognize undifferentiated iPSCs (see "Live Staining with Antibodies", page 22). Note that other methods of identifying iPSCs, such as alkaline phosphatase staining, are also acceptable.

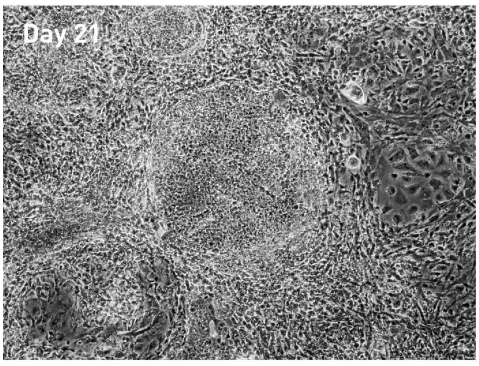
The images below illustrate the expected morphological changes as iPSC colonies emerge from human neonatal foreskin fibroblast cells (strain BJ) that were reprogrammed using the Epi5™ Episomal iPSC Reprogramming Kit. The phase contrast images were obtained at 5× magnification on Day 2, Day 6, and Day 21 post-transfection.



Visible morphology changes; cells appear unhealthy.



Groups of cube/round-shaped cells become visible throughout the plate



iPSC colonies emerge.

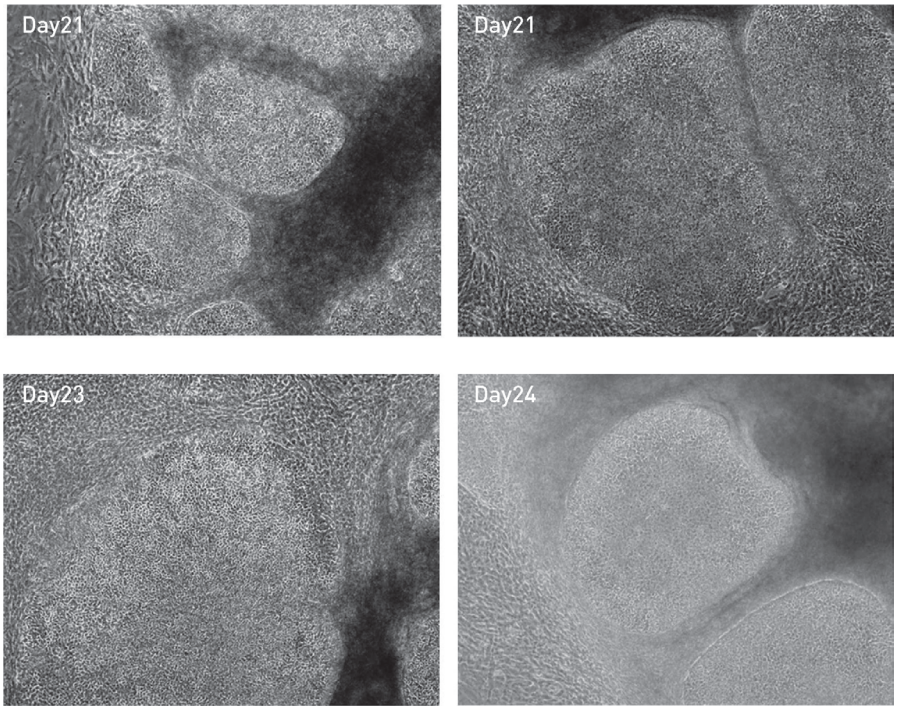
Although colonies of "transformed" cells may emerge as early as 7 days after transfection, most of these colonies will not be correctly "reprogrammed" cells. iPSCs usually emerge a little later (around day 14 post-transfection), resemble embryonic stem cells in morphology, and express the cell surface markers Tra1-60 and Tra1-81.

Continued on next page

Identifying iPSC Colonies, continued

Morphology of reprogrammed cells

The images below show the 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 that other methods of identifying iPSCs (such as alkaline phosphatase staining) are also acceptable.

Required antibodies

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)

Live staining protocol

1. Aspirate the medium from the reprogramming dish.
2. Wash the cells once with 1X KnockOut™ DMEM/F-12.
3. Add the primary antibody, diluted 1:100 in KnockOut™ D-MEM/F-12, to the cells (1 mL per well of a 6-well plate).
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 1 week and re-used up to 2 times.
6. Wash cells three times with KnockOut™ DMEM/F-12.
7. Add the secondary antibody, diluted 1:500 in KnockOut™ D-MEM/F-12, to the cells (1 mL per well of a 6-well plate).
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 1 week and re-used up to 2 times.
10. Wash cells three times with KnockOut™ DMEM/F-12 and 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.

Expected results

Successful antibody staining can very specifically distinguish reprogrammed colonies from just plain transformed counterparts, and can be detected for up to 24–36 hours. This is particularly useful because it helps identifying and tracking of candidate iPS colonies before picking and the day after they are transferred into a new culture dish for expansion.

Picking iPSC Colonies

Protocol for picking iPSC colonies (feeder-free culture)

1. Place the culture dish containing the reprogrammed cells under an inverted microscope and examine the colonies under 10X magnification.
2. 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 Geltrex® matrix-coated 24-well culture plates.
3. Transfer the culture dish to a sterile cell culture hood (i.e., biosafety cabinet) equipped with a stereomicroscope.
4. Using a 25 gauge 1½ inch needle, cut the colony to be picked into 5–6 pieces in a grid-like pattern.
5. Using a 200-µL pipette, transfer the cut pieces to a freshly prepared Geltrex® matrix-coated 24-well plate containing Essential 8™ Medium.
6. Incubate the Geltrex® matrix-coated-coated plate containing the picked colonies in a 37°C, 5% CO_2 incubator.
7. 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.
8. 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 (see page 34).

- Cells cultured in Essential 8™ Medium on Geltrex® matrix-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.
- 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.

Continued on next page

Protocol for picking iPSC colonies (feeder-dependent)

1. Place the culture dish containing the reprogrammed cells under an inverted microscope and examine the colonies under 10X magnification.
2. 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 MEF culture plates.
3. Transfer the culture dish to a sterile cell culture hood (i.e., biosafety cabinet) equipped with a stereomicroscope.
4. Using a 25 gauge 1½ inch needle, cut the colony to be picked into 5–6 pieces in a grid-like pattern.
5. Using a 200 µL pipette, transfer the cut pieces to a freshly prepared 24-well MEF culture plate (see page 32) containing human iPSC medium (see page 30).
6. Incubate the MEF culture plate containing the picked colonies in a 37°C incubator with a humidified atmosphere of 5% CO_2.
7. Allow the colonies to attach to the culture plate for 48 hours before replacing the spent medium with fresh human iPSC medium. After that, change the medium every day.
8. Treat the reprogrammed colonies like normal human ESC colonies and passage, expand, and maintain them using standard culture procedures until you have frozen cells from two 60-mm plates (see page 35).

Adapting iPSCs to feeder-free culture

iPSCs cultured on MEF feeder layers can be adapted to feeder-free conditions in Essential 8™ Medium.

1. Harvest PSCs using an enzymatic method, such as Collagenase Type IV (Cat. no. 17104-019) or Dispase (Cat. no. 17105-041).
2. Plate cells on Geltrex® Matrix-coated plates in Essential 8™ Medium (see page 31 for coating plates with Geltrex® Matrix).
3. Incubate cells at 37°C, 5% CO_2 overnight.
4. Feed cells with Essential 8™ Medium beginning on the second day after splitting. Replace spent medium daily.

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.

Detecting Episomal Vectors by PCR

Harvesting iPSCs for PCR (feeder-free culture)

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 below for the recommended volumes.
2. Add 0.5 mM EDTA in DPBS to the dish containing iPSCs. Adjust the volume of EDTA for various dish sizes. 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 and add pre-warmed complete Essential 8™ Medium to the dish according to the table below.
5. 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.
6. Centrifuge the cell suspension at 200 × *g* for 5 minutes to pellet cells.
7. Aspirate and discard the supernatant. Resuspend the cell pellet in 500 µL DPBS and transfer resuspended cells to a thin-walled 0.5-mL PCR tube.
8. Centrifuge the cell suspension at 200 × *g* for 5 minutes to pellet cells.
9. Aspirate and discard the supernatant. Resuspend the cell pellet in 20 µL of Resuspension Buffer with 2 µL of Lysis Solution added to the Resuspension Buffer.
10. Incubate the cells for 10 minutes in an incubator or thermal cycler that has been preheated to 75°C.
11. Centrifuge the tube briefly to collect any condensation. Use 3 µL of the cell lysate in a 50-µL PCR reaction (see page 27).

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

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Harvesting iPSCs for PCR (feeder-dependent culture)

1. Aspirate the MEF medium from a dish containing inactivated MEFs and add pre-warmed human iPSC Medium to the dish, 3–4 hours before harvesting hESCs.
2. Aspirate the spent medium from the dish with a Pasteur pipette.
3. Add Collagenase Type IV (1 mg/mL) solution to the dish containing hESCs. Adjust the volume of Collagenase Type IV for various dish sizes (e.g., 35-mm dishes require 1 mL of Collagenase IV).
4. Incubate the dish(es) for 30–60 minutes in a 37°C, 5% CO_2 incubator. Note that the incubation times may vary among different batches of collagenase; therefore, examination of the colonies is needed to determine the appropriate incubation time.

   Note: As an alternative to Collagenase Type IV, you may use 2 mg/mL of Dispase and incubate the dish for 2–3 minutes in a 37°C, 5% CO_2 incubator.
5. Stop the incubation when the edges of the colonies are starting to pull away from the plate.
6. Aspirate the Collagenase Type IV Solution with a Pasteur pipette. Remove the collagenase carefully without disturbing the attached cell layer.
7. Add human iPSC Medium to the dish. Use a 5-mL pipette to gently blow the cells off the surface of the dish while pipetting up and down. Make sure to pipet gently to minimize the formation of bubbles.
8. After the cells have been removed from the surface of the well, pool the contents of the wells into a 15-mL conical tube.
9. Using a 5-mL pipette, add human iPSC Medium to the dish to wash and collect any residual cells. Pipet up the medium and cells, and then add the collected cells to the 15-mL tube.
10. Pipet cells up and down gently a few times in the 15-mL tube to further break up cell colonies. Pipet carefully to reduce foaming.

    Note: Avoid making a single cell suspension.
11. Centrifuge the cell suspension at 200 × *g* for 5 minutes to pellet cells.
12. Aspirate and discard the supernatant. Resuspend the cell pellet in 500 µL DPBS and transfer resuspended cells to a thin-walled 0.5-mL PCR tube.
13. Centrifuge the cell suspension at 200 × *g* for 5 minutes to pellet cells.
14. Aspirate and discard the supernatant. Resuspend the cell pellet in 20 µL of Resuspension Buffer with 2 µL of Lysis Solution added to the Resuspension Buffer.
15. Incubate the cells for 10 minutes in an incubator or thermal cycler that has been preheated to 75°C.
16. Centrifuge the tube briefly to collect any condensation. Use 3 µL of the cell lysate in a 50-µL PCR reaction (see page 27).

Continued on next page

Detecting Episomal Vectors by PCR, continued

PCR primers for detecting Epi5™ Episomal Vectors

You can detect the presence of Episomal iPSC Reprogramming Vectors in reprogrammed iPSC colonies by endpoint PCR using the PCR primers listed below. The EBNA-1 primer set can detect all five episomal plasmids in the kit. The oriP primer set can detect all episomal plasmids in the kit except pCXB-EBNA1, which lacks the OriP gene.

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'	

PCR using AccuPrime™ High Fidelity *Taq* DNA Polymerase

1. Add the components listed below to a DNase/RNase-free, thin-walled PCR tube. 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.

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

2. Cap the tube, tap gently to mix, and centrifuge briefly to collect the contents.
3. Place the tube in the thermal cycler and use the PCR parameters shown below.

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	—

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

Appendix A: Recipes

Media and Reagents

Basic FGF stock solution

1. To prepare 10 mL of 10-µg/mL Basic FGF solution, aseptically mix the following components:

Basic FGF	100 µg
DPBS without Ca^{2+} and Mg^{2+}	9.8 mL
10% BSA	100 µL

2. Aliquot and store the Basic FGF solution at –20°C for up to 6 months.

Note: If desired, you may use 100 µL of KnockOut™ Serum Replacement (KSR) instead of 10% BSA.

0.5 mM EDTA in DPBS

1. To prepare 50 mL of 0.5 mM EDTA in DPBS, aseptically mix the following components in a 50-mL conical tube:

DPBS without Ca^{2+} and Mg^{2+}	50 mL
0.5 M EDTA	50 µL

2. Filter sterilize the solution and store at room temperature for up to 6 months.

1 mg/mL Collagenase Type IV Solution

1. Add DMEM/F-12 to Collagenase Type IV to make a 10 mg/mL stock solution. Gently vortex to suspend, and filter sterilize the solution. This solution can be aliquoted and frozen at –20°C until use.
2. Make a working solution of 1 mg/mL Collagenase Type IV in DMEM/F-12. The working solution can be used for 2 weeks if properly stored at 2–8°C (store in aliquots to avoid repeated warming).

Fibroblast Medium

To prepare 100 mL of complete Fibroblast Medium, aseptically mix the following components. Complete Fibroblast Medium can be stored at 2–8°C for up to 1 week.

Component	Volume
DMEM with GlutaMAX™-I	89 mL
FBS, ESC Qualified	10 mL
MEM Non-Essential Amino Acids Solution (10 mM)	1 mL

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N2B27 Medium

To prepare 250 mL of complete N2B27 Medium, aseptically mix the following components. N2B27 Medium (without bFGF) can be stored at 2–8°C for up to 1 week.

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 µL
Basic FGF* (10 µg/mL)	2.5 mL

* Prepare the N2B27 Medium without bFGF, and then supplement with fresh bFGF to a final concentration of 100 ng/mL when the medium is used.

Essential 8™ Medium

1. Thaw the frozen Essential 8™ Supplement at 2–8°C overnight. **Do not thaw the frozen supplement at 37°C.**
2. Mix the thawed supplement by gently inverting the vial a couple of times, remove 10 mL from the bottle of DMEM/F-12 (HAM) 1:1, and then aseptically transfer the entire contents of the Essential 8™ Supplement to the bottle of DMEM/F-12 (HAM) 1:1.
3. Swirl the bottle to mix and to obtain 500 mL of homogenous complete medium.
4. Complete Essential 8™ Medium can be stored at 2–8°C for up to 2 weeks. 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.**

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StemPro®-34 Medium

1. Thaw the frozen StemPro®-34 Nutrient Supplement at 2–8°C overnight.
2. After thawing, mix the supplement well by gently inverting the vial a couple of times, and then aseptically transfer the entire contents of the vial to the bottle of StemPro®-34 SFM. Swirl the bottle to mix and to obtain a homogenous medium.
3. Aseptically add GlutaMAX™-I (100X) supplement (Cat. no. 25030) to a final concentration of 1X (5 mL of 100X GlutaMAX™-I supplement to 500 mL of medium).
4. Before use, add the following cytokines to the indicated final concentration into the medium. **These must be added freshly, just prior to use**.

Recombinant human SCF	100 ng/mL
Recombinant human IL-3	50 ng/mL
Recombinant human GM-CSF	25 ng/mL

5. Complete StemPro®-34 Medium (without the cytokines) can be stored at 2–8°C **in the dark** for up to 4 weeks. The thawed nutrient supplement has a shelf life of 2 weeks when stored at 2–8°C, in the dark.

Human iPSC Medium

To prepare 100 mL of Human iPSC Medium, aseptically mix the components listed below. Human iPSC Medium can be stored at 2–8°C for up to 1 week.

Component	Volume
DMEM/F-12 (1X) with GlutaMAX™-I	78 mL
MEM Non-essential Amino Acids Solution (10 mM)	1 mL
β-Mercaptoethanol (1000X)	100 µL
KnockOut™ Serum Replacement (KSR)	20 mL
Basic FGF* (10 µg/mL)	1 mL

* Prepare the iPSC Medium without bFGF, and then supplement with fresh bFGF to a final concentration of 100 ng/mL when the medium is used.

MEF Medium

To prepare 100 mL of complete MEF medium, aseptically mix the components listed below. Complete MEF Medium can be stored at 2–8°C for up to 1 week.

Component	Volume
DMEM/F-12 (1X) with GlutaMAX™-I	89 mL
FBS, ESC-Qualified	10 mL
MEM Non-essential Amino Acids Solution (10 mM)	1 mL
β-Mercaptoethanol (1000X)	100 µL

Appendix B: Preparing Culture Vessels

Coating Culture Vessels with Geltrex® Matrix

Coating protocol

1. Thaw a 5-mL bottle of Geltrex® LDEV-Free hESC-Qualified Reduced Growth Factor Basement Membrane Matrix at 2–8°C overnight.
2. For reprogramming experiments using fibroblasts, dilute the thawed Geltrex® matrix solution 1:1 with cold sterile DMEM/F-12 to prepare 1-mL aliquots (or another volume suitable for your needs) in tubes chilled on ice. These aliquots can be frozen at –20°C or used immediately.

   For reprogramming experiments using hemopoietic progenitor cells (HPCs), such as StemPro® $CD34^+$ cells, do **not** dilute the Geltrex® matrix solution 1:1 to create the intermediate dilution. The Geltrex® matrix solution needs to be twice as strong for reprogramming experiments with HPCs.
3. To create working stocks, dilute an aliquot of Geltrex® matrix solution 1:50 with cold DMEM on ice. This creates a final dilution of 1:100 for fibroblasts or a final dilution of 1:50 for StemPro® $CD34^+$ cells.

   Note: An optimal dilution of the Geltrex® matrix 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® matrix solution (see table below).
5. Incubate the dishes in a 37°C, 5% CO_2 incubator for 1 hour.
6. Geltrex® matrix-coated culture dishes can now be used or stored at 2–8°C for up to a week. Do not allow dishes to dry.
7. Aspirate the diluted Geltrex® matrix solution from the culture dish and discard. You do not need to rinse off the Geltrex® matrix solution from the culture dish after removal. Cells can now be passaged directly onto the Geltrex® matrix-coated culture dish.

Culture vessel	Surface area	Volume of Geltrex® matrix dilution
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

MEF Culture Dishes

Gelatin coating culture vessels

1. Cover the whole surface of each culture vessel with Attachment Factor (AF) solution and incubate the vessels for 30 minutes at 37°C or for 2 hours at room temperature.

   Note: AF (Cat. no. S-006-100) is a sterile 1X solution containing 0.1 % gelatin available from Life Technologies (see page 37 for ordering information).

2. Using sterile technique in a laminar flow culture hood, completely remove the AF solution from the culture vessel by aspiration.

   Note: It is not necessary to wash the culture surface before adding cells or medium. Coated vessels may be used immediately or wrapped in Parafilm® sealing film and stored at room temperature for up to 24 hours.

Thawing MEFs

1. Remove the cryovial containing inactivated MEFs from the liquid nitrogen storage tank.
2. Briefly roll the vial between hands to remove frost, and swirl it gently in a 37°C water bath.
3. When only a small ice crystal remains in the vial, remove it from water bath. Spray the outside of the vial with 70% ethanol before placing it in the cell culture hood.
4. Pipet the thawed cells gently into a 15-mL conical tube.
5. Rinse the cryovial with 1 mL of pre-warmed MEF medium (see page 30). Transfer the medium to the same 15-mL tube containing the cells.
6. Add 4 mL of pre-warmed MEF medium **dropwise** to the cells. Gently mix by pipetting up and down.

   Note: Adding the medium slowly helps the cells to avoid osmotic shock.

7. Centrifuge the cells at 200 × *g* for 5 minutes.
8. Aspirate the supernatant and resuspend the cell pellet in 5 mL of pre-warmed MEF medium.
9. Remove 20 µL of the cell suspension and determine the viable cell count using your method of choice (e.g., Countess® Automated Cell Counter).

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MEF Culture Dishes, continued

Plating MEFs

1. Centrifuge the remaining cell suspension (step 9, previous page) at 200 × *g* for 5 minutes at room temperature.
2. Aspirate the supernatant. Resuspend the cell pellet in MEF medium (see page 30) to a density of 2.5×10^6 cells/mL.
3. Aspirate the gelatin solution from the gelatin coated culture vessel.
4. Add the appropriate amount of MEF medium into each culture vessel (refer to the table below).
5. Into each of these culture vessels, add the appropriate amount of MEF suspension (refer to the table below).

   Note: The recommended plating density for Gibco® Mouse Embryonic Fibroblasts (Irradiated) (Cat. no. S1520-100) is 2.5×10^4 cells/cm^2.
6. Move the culture vessels in several quick back-and-forth and side-to-side motions to disperse the cells across the surface of the vessels.
7. Incubate the cells in a 37°C incubator with a humidified atmosphere of 5% CO_2.
8. Use the MEF culture vessels within 3–4 days after plating.

Vessel size	Approximate growth area	Volume of MEF medium	Number of MEFs	Volume of MEF suspension
24-well plate	2 cm^2/well	0.5 mL	5.0×10^4/well	20 µL
12-well plate	4 cm^2/well	1 mL	1.0×10^5/well	40 µL
6-well plate	10 cm^2/well	2 mL	2.5×10^5/well	0.1 mL
60-mm dish	20 cm^2	5 mL	5.0×10^5	0.2 mL
100-mm dish	60 cm^2	10 mL	1.5×10^6	0.6 mL
25-cm^2 flask	25 cm^2	5 mL	6.3×10^5	0.25 mL
75-cm^2 flask	75 cm^2	15 mL	1.9×10^6	0.75 mL

Appendix C: Cryopreserving iPSCs

Freezing iPSCs (Feeder-Free Culture)

Freezing feeder-free iPSC cultures

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 the medium in a 37°C water bath**.
2. Prepare Essential 8™ Freezing Medium. For every 1 mL of freezing medium needed, aseptically combine the following components 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 Ca^{2+} and Mg^{2+} (see table below).
5. Add 0.5 mM EDTA solution (see page 28) to the dish. Adjust the volume of EDTA for various dish sizes (see table below). Swirl the dish to coat the entire cell surface.
6. Incubate the dish at room temperature for 5–8 minutes or at 37°C for 4–5 minutes. When the cells start to separate and round up, and the colonies 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 ice-cold Essential 8™ Freezing Medium to the dish (see table below).
9. Remove the cells by gently squirting the colonies from the well using a 5-mL glass pipette. Avoid creating bubbles. Collect the cells in a 15-mL conical tube and place on ice.
10. Resuspend the 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) and freeze the cells by decreasing the temperature by 1°C per minute. Once frozen, transfer the cells 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.

Culture Vessel	Approximate surface area	DPBS (mL)	0.5 mM EDTA in DPBS (mL)	Essential 8™ Freezing Medium (mL)
6-well plate	10 cm^2/well	2 mL/well	1 mL/well	1 mL/well
35-mm dish	10 cm^2	2 mL	1 mL	1 mL
60-mm dish	20 cm^2	4 mL	2 mL	2 mL
100-mm dish	60 cm^2	12 mL	6 mL	6 mL

Freezing iPSCs (Feeder-Dependent Culture)

iPSC freezing medium for feeder-dependent culture

1. Prepare the Freezing Media A and B immediately before use.
2. In a sterile 15-mL tube, mix together the following reagents for every 1 mL of **freezing medium A** needed:

Human iPSC medium	0.5 mL
KnockOut™ Serum Replacement	0.5 mL

3. In another sterile 15-mL tube, mix together the following reagents for every 1 mL of **freezing medium B** needed:

Human iPSC medium	0.8 mL
DMSO	0.2 mL

4. Place the tube with freezing medium B on ice until use (you can keep freezing medium A at room temperature). Discard any remaining freezing medium after use.

Freezing feeder-dependent iPSC cultures

1. Prepare the required volume of fresh freezing medium and place it on ice.
2. Aspirate the culture medium and rinse the dishes twice with DPBS without Ca^{2+} and Mg^{2+} (see table on page 36).
3. Gently add Collagenase IV solution (see page 28) to the culture dish. Adjust the volume of Collagenase IV solution for various dish sizes (see table on page 36).
4. Incubate the dish with cells for 5–20 minutes in a 37°C incubator with a humidified atmosphere of 5% CO_2.

   Note: Incubation times may vary among different batches of collagenase. Therefore, the appropriate incubation time should be optimized by examining the colonies periodically under microscope during incubation.
5. Stop the incubation when the edges of the colonies are starting to pull away from the plate.
6. Remove the culture dish from the incubator, aspirate the Collagenase IV solution, and gently rinse the dish with DPBS without Ca^{2+} and Mg^{2+}.
7. Add human iPSC Medium or DMEM/F-12 (see table on page 36) and gently dislodge the cells off the surface of the culture dish using a sterile pipette or a cell scraper. Transfer the cells to a sterile 15-mL centrifuge tube. Rinse the dish with additional iPSC Medium or DMEM/F-12 to collect any leftover colonies.
8. Centrifuge the cells at 200 × *g* for 2–4 minutes at room temperature.
9. Discard the supernatant, gently tap the tube to dislodge the cell pellet from the tube bottom, and resuspend the cells in **freezing medium A** (see table on page 36). After the cell clumps have been uniformly suspended, add an equal volume of **freezing medium B** to the cell suspension in a drop-wise manner while gently swirling the cell suspension to mix.

   Note: At this point, the cells are in contact with DMSO, and work must be performed efficiently with no or minimum delays. After the cells come into contact with DMSO, they should be aliquoted and frozen within 2–3 minutes.

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Freezing feeder-dependent iPSC cultures, continued

10. Aliquot 1 mL of the cell suspension into each cryovial.
11. Quickly place the cryovials containing the cells in a cryo freezing 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.

Culture Vessel	Approximate surface area	DPBS (mL)	Collagenase IV solution (mL)	Human iPSC medium (mL)	Freezing Medium A or B (mL)
6-well plate	10 cm^2/well	2 mL/well	1 mL/well	1 mL/well	0.5 mL/well
35-mm dish	10 cm^2	2 mL	1 mL	1 mL	0.5 mL
60-mm dish	20 cm^2	4 mL	2 mL	2 mL	1 mL
100-mm dish	60 cm^2	12 mL	6 mL	6 mL	3 mL

Appendix D: Ordering Information

Accessory Products

Epi5™ episomal reprogramming products

For more information about the following products, refer to our website at **www.lifetechnologies.com** or contact Technical Support (page 40).

Product	Quantity	Catalog no.
Epi5™ Episomal iPSC Reprogramming Kit	1 kit	A15960

Media, sera, and supplements

For more information about the following products, refer to our website at **www.lifetechnologies.com** or contact Technical Support (page 40).

Product	Quantity	Catalog no.
Dulbecco's Modified Eagle Medium (DMEM) with GlutaMAX™-I (high glucose)	500 mL	10569-010
DMEM/F-12 with HEPES	500 mL	11330-032
KnockOut™ DMEM/F-12	500 mL	12660-012
KnockOut™ Serum Replacement	500 mL	10828-028
Essential 8™ Medium (Prototype) (50X)	500 mL	A14666SA
StemPro®-34 SFM (1X), Liquid	500 mL	10639-011
MEM Non-Essential Amino Acids Solution (10 mM)	100 mL	11140-050
Basic Fibroblast Growth Factor (bFGF), recombinant human	10 µg	PHG0264
Bovine Albumin Fraction V Solution (BSA), 7.5%	100 mL	15260-037
Fetal Bovine Serum (FBS), ES-Cell Qualified	500 mL	16141-079
β-Mercaptoethanol (1000X), liquid	50 mL	21985-023
GlutaMAX™-I Supplement	100 mL	35050-061
N-2 Supplement (100X)	5 mL	17502-048
B-27® Serum-Free Supplement (50X)	10 mL	17504-044
DPBS without Ca^{2+} or Mg^{2+}	500 mL	14190-144
DMSO, anhydrous	10 × 3 mL	D12345

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Matrices and dissociation reagents

For more information about the following products, refer to our website at **www.lifetechnologies.com** or contact Technical Support (page 40).

Product	Quantity	Catalog no.
Geltrex™ hESC-qualified Reduced Growth Factor Basement Membrane Matrix	5 mL	A14133-02
Attachment Factor	100 mL	S-006-100
UltraPure™ 0.5 M EDTA, pH 8.0	4 × 100 mL	15575-020
0.05% Trypsin-EDTA (1X), Phenol Red	100 mL	25300-054
Collagenase, Type IV, powder	1 g	17104-019
Dispase II, powder	5 g	17105-041

Cells

For more information about the following products, refer to our website at **www.lifetechnologies.com** or contact Technical Support (page 40).

Product	Quantity	Catalog no.
Gibco® Mouse Embryonic Fibroblasts (Irradiated)	1 mL	S1520-100
StemPro® CD34+ Cell Kit (contains 0.5×10^6 cells)	1 kit	A14059
Human Dermal Fibroblasts, neonatal (HDFn)	1 vial	C-004-5C
Human Dermal Fibroblasts, adult (HDFa)	1 vial	C-013-5C

Antibodies

For more information about the following products, refer to our website at **www.lifetechnologies.com** or contact Technical Support (page 40).

Product	Quantity	Catalog no.
Mouse anti-Tra1-60 antibody	100 µg	41-1000
Mouse anti-Tra1-81 antibody	100 µg	41-1100
Mouse anti-SSEA4	100 µg	41-4000
Alexa Fluor® 488 goat anti-mouse IgG (H+L) antibody	0.5 mL	A11029
Alexa Fluor® 594 goat anti-mouse IgG (H+L) antibody	0.5 mL	A11032
Alexa Fluor® 488 goat anti-rabbit IgG (H+L) antibody	0.5 mL	A11034
Alexa Fluor® 594 goat anti-rabbit IgG (H+L) antibody	0.5 mL	A11037

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Reagents for PCR Detection of Episomal Vectors

For more information about the following products, refer to our website at **www.lifetechnologies.com** or contact Technical Support (page 40).

Product	Quantity	Catalog no.
CellsDirect Resuspension & Lysis Buffers	1 kit	11739-010
AccuPrime™ *Taq* DNA Polymerase High Fidelity	200 reactions	12346-086

Equipment

For more information about the following products, refer to our website at **www.lifetechnologies.com** or contact Technical Support (page 40).

Product	Quantity	Catalog no.
Countess® Automated Cell Counter (includes 50 Countess® cell counting chamber slides and 2 mL of Trypan Blue Stain)	1 unit	C10227
StemPro® EZPassage™ Disposable Stem Cell Passaging Tool	10 units	23181-010
Neon® Transfection System	1 unit	MPK5000
Neon® Transfection System 10 µL Tip Kit	50 reactions	MPK1025
Neon® Transfection System 100 µL Tip Kit	50 reactions	MPK10025

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- Download software updates and patches

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