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Reprogramming Fibroblasts with the CytoTune™-iPS Reprogramming Kit

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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 (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 (ESCs); 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 transduction and chemical induction. While retroviral vectors require integration into host chromosomes to express reprogramming genes, DNA-based vectors, such as adenovirus, adeno-associated virus, and plasmid vectors exist episomally and do not require integration; however, they may still be integrated into host chromosomes at certain frequencies. Unlike these vectors, the CytoTune™ reprogramming vectors do not integrate into the host genome or alter the genetic information of the host cell (Fusaki *et al.*, 2009; Li *et al.*, 2000; Seki *et al.*, 2010).

CytoTune™-iPS Reprogramming System uses vectors based on replication in competent Sendai virus (SeV) to safely and effectively deliver and express key genetic factors necessary for reprogramming somatic cells into iPSCs. In contrastto many available protocols, which rely on viral vectors that integrate into the genome of the host cell, the CytoTune™ Reprogramming System uses vectors that are non-integrating and remain in the cytoplasm (i.e., they are zero-footprint). In addition, the host cell can be cleared of the vectors and reprogramming factor genes by exploiting the cytoplasmic nature of SeV and the functional temperature sensitivity mutations introduced into the key viral proteins.

The CytoTune™-iPS Reprogramming Kit contains four SeV-based reprogramming vectors, each capable of expressing one of the four Yamanaka factors (i.e., Oct4, Sox2, Klf4, and c-Myc) and are optimized for generating iPSCs from human somatic cells. The reprogramming vectors in this kit have been engineered to increase biological and environmental safety.

Materials Needed

- CytoTune™ Sendai Reprogramming Vectors

  Note: For successful reprogramming, you need all four reprogramming vectors.
- Human neonatal foreskin fibroblast cells (strain BJ; ATCC no. CRL2522) to reprogram
- Gibco® Mouse Embryonic Fibroblasts (Irradiated) (Cat. no. S1520-100)
- DMEM with GlutaMAX™-I (High Glucose) (Cat. no. 10569-010)
- KnockOut™ DMEM/F-12 (Cat. no. 12660-012)
- Fetal Bovine Serum (FBS), ES Cell-Qualified (Cat. no. 16141-079)
- KnockOut™ Serum Replacement (KSR) (Cat. no. 10828-028)
- MEM Non-Essential Amino Acids (NEAA) (Cat. no. 11140-050)
- GlutaMAX™-I Supplement (Cat. no. 35050-061)

- Basic FGF, Recombinant Human (Cat. no. PHG0264)
- β-Mercaptoethanol, 1000X (Cat. no. 21985-023)
- Penicillin-Streptomycin, Liquid (Cat. no. 15140-122)
- Attachment Factor (Cat. no. S-006-100)
- TrypLE™ Select Cell Dissociation Reagent (Cat. no. 12563) or 0.05% Trypsin/EDTA (Cat. no. 25300)
- DPBS Without Calcium or Magnesium (Cat. no. 14190-144)
- TRIzol® LS Reagent (Cat. no. 10296-010)
- SuperScript® VILO™ cDNA Synthesis Kit (Cat. no. 11754-050)
- AccuPrime™ SuperMix I (Cat. no. 12342-010)
- 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)
- Rabbit Anti-SeV Antibody (MBL International Corporation, Woburn, MA; Cat. no. PD029)
- 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)
- Sterile cell culture hood (i.e., biosafety cabinet) equipped with a stereo microscope
- 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 1½-inch needle

Guidelines for Reprogramming

- To maintain sterile culture conditions, carry out all of the procedures using sterile laboratory practices in a laminar flow hood.
- You can use the CytoTune™-iPS Reprogramming Kit to reprogram a wide range of cell types in proliferative and quiescent states. However, the reprogramming efficiency may vary among different cell types (~0.01%–1%).
- For successful reprogramming, transduce your cells using all four reprogramming vectors.

Note: For successful reprogramming, all four Yamanaka factors (i.e., Oct4, Sox2, Klf4, and c-Myc) need to be expressed in your host cell.

- Each CytoTune™-iPS Reprogramming Kit of four tubes supplies sufficient reagents to transduce cells in 2 wells of a 6-well plate (5×10^5 cells/well) at an MOI of 3.
- The titer of each CytoTune™ Sendai reprogramming vector is lot-dependent. For the specific titer of your vectors, refer to the Certificate of Analysis (CoA) available on our website. Go to **www.lifetechnologies.com/cytotune** and search for the CoA by product lot number, which is printed on the vial.
- Viral titers can decrease dramatically with each freeze/thaw cycle. Avoid repeated freezing and thawing of your reprogramming vectors. Viral titer is not guaranteed for kits that have been refrozen or thawed.
- Prior to starting, ensure that the media are equilibrated to 37°C and appropriately gassed.
- For positive control, we recommend performing a reprogramming experiment with human neonatal foreskin fibroblast cells (strain BJ; ATCC no. CRL2522). Note that experimental conditions may vary among target cells and need to be optimized for each cell type. The example given in the following protocol does not guarantee the generation of iPSCs for all cell types.

Preparing Media

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
10% KSR	10 µL

2. Aliquot and store at –20°C 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).

MEF/Fibroblast Medium (for 100 mL complete medium)

1. To prepare 100 mL of complete MEF/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. Complete MEF medium can be stored at 2–8°C for up to 1 week.

Human iPSC Medium (for 100 mL complete medium)

1. To prepare 100 mL of complete human iPSC medium, aseptically mix the following components:

KnockOut™ DMEM/F-12	78 mL
KnockOut™ Serum Replacement	20 mL
MEM Non-Essential Amino Acids Solution, 10 mM	1 mL
GlutaMAX™-I Supplement	1 mL
β-mercaptoethanol, 1000X	100 µL
Penicillin-Streptomycin (*optional*)	1 mL
bFGF (10 µg/mL)*	40 µL

   * Prepare the iPSC medium without bFGF, and then supplement with fresh bFGF when the medium is used.

2. Complete human iPSC medium can be stored at 2–8°C for up to 4 weeks.

Preparing MEF dishes

Gelatin coating culture vessels

1. Cover the whole surface of each new culture vessel with Attachment Factor (AF) solution and incubate the vessels for 30 minutes at 37°C or for 1 hour at room temperature.
2. Using sterile technique in a laminar flow culture hood, completely remove the AF solution from the culture vessel by aspiration just prior to use. Coated vessels may be used immediately or stored at room temperature wrapped in Parafilm® sealing film for up to 24 hours.

Note: It is not necessary to wash the culture surface before adding cells or medium.

Thawing Gibco® MEFs (Irradiated)

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. 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).

Plating MEFs

1. Centrifuge the remaining cell suspension (step 9, Thawing Gibco® MEFs) at 200 × *g* for 5 minutes at room temperature.
2. Aspirate the supernatant. Resuspend the cell pellet in MEF medium 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 Table 1, below).
5. Into each of these culture vessels, add the appropriate amount of MEF suspension (refer to Table 1, below).

Note: The recommended plating density for Gibco® Mouse Embryonic Fibroblasts (Irradiated) 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.

Table 1 Amount of Inactivated MEFs Needed

Vessel size	Growth area	Volume of media	Number of MEFs	Volume of MEF suspension
96-well plate	0.32 cm^2/well	0.1 mL	1.0×10^4/well	4 µL
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

Reprogramming Fibroblasts

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

Day –2: Prepare the cells for transduction

1. 2 days before transduction, plate human neonatal foreskin fibroblast cells into two wells of a 6-well plate at the appropriate density to achieve 5×10^5 cells per well on the day of transduction (Day 0).

Note: We recommend about 80–90% confluency on the day of transduction. Because overconfluency results in decreased transduction efficiency, we recommend replating your cells to achieve 80–90% confluency if your cells have become overconfluent during culturing.

2. Culture the cells for two more days, ensuring the cells have fully adhered and extended.

Day 0: Perform transduction

3. On the day of transduction, warm 2 mL of fibroblast medium in a water bath
4. Remove one set of CytoTune™ Sendai tubes from the –80°C storage. Thaw each tube one at a time by first immersing the bottom of the tube in a 37°C water bath for 5–10 seconds, and then removing the tube from the water bath and allowing it to thaw at room temperature. Once thawed, briefly centrifuge the tube and place it immediately on ice.
5. Add the indicated volumes of each of the four CytoTune™ Sendai tubes (3×10^6 CIU each; see the CoA for the appropriate volume) to 2 mL of fibroblast medium, pre-warmed to 37°C. Ensure that the solution is thoroughly mixed by pipetting the mixture gently up and down. Complete the next step within 5 minutes.
6. Aspirate the fibroblast medium from the cells, and add one half of the solution prepared in Step 5 to each of the two wells. Place the cells in a 37°C, 5% CO_2 incubator and incubate overnight.

Day 1: Replace medium and culture cells

7. 24 hours after transduction, replace the medium with fresh fibroblast medium.

Note: Depending on your cell type, you should expect to see some cytotoxicity 24–48 hours post-transduction, which can affect >50% of your cells. This is an indication of high uptake of the virus. We recommend that you continue culturing your cells and proceed with the protocol.

8. Culture the cells for 6 more days, changing the spent medium with fresh fibroblast medium every other day.

Note: Depending on your cell type, you may observe high cell density before Day 5. We do **not** recommend passaging your cells onto MEF culture dishes before 7 days post-transduction.

Day 5 or 6: Prepare MEF culture dishes

9. One to two days before passaging the transduced fibroblasts onto MEF feeder-cells, prepare 100-mm MEF culture dishes.

Day 7: Plate transduced cells on MEF culture dishes

10. Seven days after transduction (Step 6), fibroblast cells are ready to be harvested and plated on MEF culture dishes. Remove the medium from the fibroblasts, and wash cells once with DPBS.
11. To remove the cells from the 6-well plate, use 0.5 mL of TrypLE™ Select reagent or 0.05% trypsin/EDTA following the procedure recommended by the manufacturer and incubate at room temperature. When the cells have rounded up (1–3 minutes later), add 2 mL of fibroblast medium into each well, and collect the cells in a 15-mL conical centrifuge tube.

Note: Because the cells can be very sensitive to trypsin at this point, minimize trypsin exposure time and incubate the cells at room temperature.

12. Centrifuge the cells at 200 × g for 4 minutes, aspirate the medium, and re-suspend the cells in an appropriate amount of fibroblast medium.
13. Count the cells using the desired method (e.g., Countess® Automated Cell Counter), and seed the MEF culture dishes with 5×10^4–2×10^5 cells per 100-mm dish and incubate at 37°C, 5% CO_2 incubator overnight.

Note: We recommend plating 5×10^4, 1×10^5, and 2×10^5 cells per 100-mm dish. Depending on your cell type, you may need to plate most of your cells on the same plate to ensure sufficient numbers of colonies.

Note: Set aside any remaining cells for RNA extraction to be used as a positive control in the RT-PCR detection of the SeV genome.

Day 8 to 28: Feed and monitor the cells

14. 24 hours later, change the medium to iPSC medium, and replace the spent medium everyday thereafter.
15. Starting on Day 8, observe the plates every other day under a microscope for the emergence of cell clumps indicative of transformed cells (see Figure 1).

Note: For BJ fibroblasts, we normally observe colony formation on Day 12 post-transduction. However, depending on your cell type, you may need to culture for up to 4 weeks before seeing colonies.

16. Three to four weeks after transduction, colonies should have grown to an appropriate size for transfer. The day before transferring the colonies, prepare MEF culture plates using Attachment Factor-coated 12- or 24-well plates.

Note: We typically harvest colonies closer to three weeks to avoid differentiation.

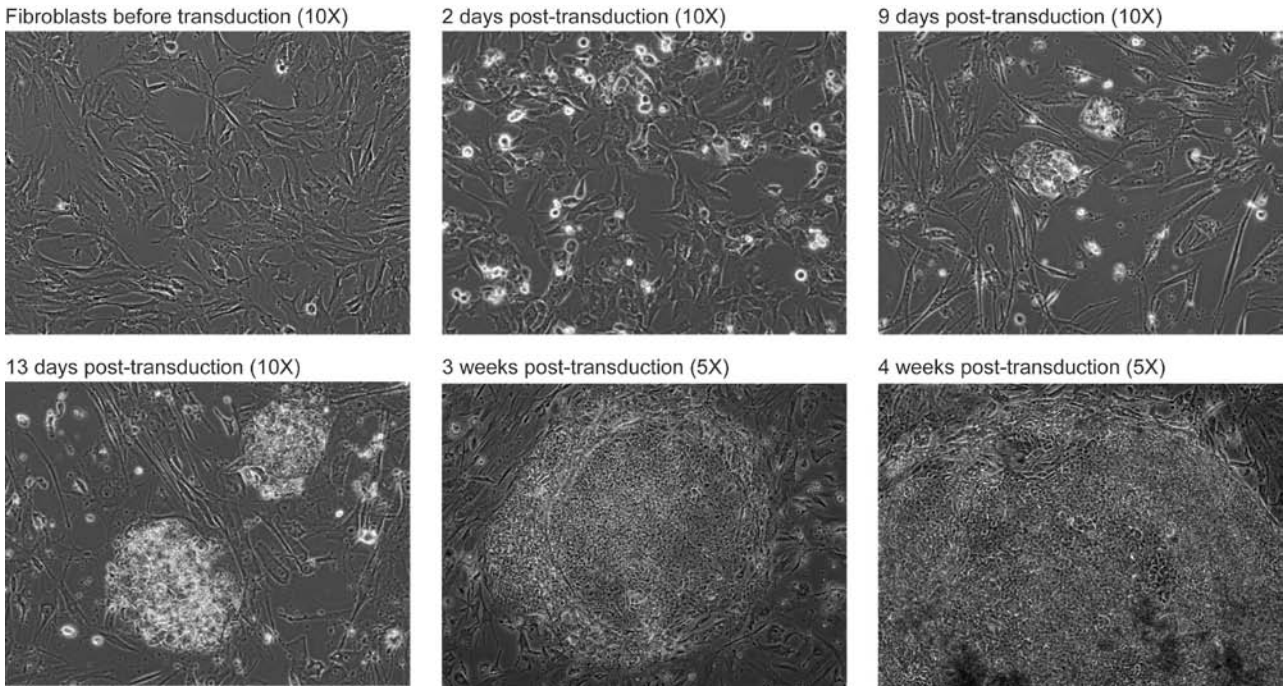
17. When colonies are ready for transfer, perform live staining using Tra1-60 or Tra1-81 for selecting reprogrammed colonies.
18. Manually pick colonies and transfer them onto prepared MEF plates.

Identifying iPSC Colonies

By Day 21 post-transduction, the cell colonies on the MEF culture dishes will have become large and compact, covering the majority of the surface area of the culture dish. 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 1, below). Therefore, we recommend that you perform live staining with Tra1-60 or Tra1-81 antibodies that recognize undifferentiated hESCs.

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

Figure 1 Human neonatal foreskin fibroblast cells (strain BJ) were transformed using the CytoTune™-iPS Reprogramming Kit and allowed to proliferate on MEF feeder layers in fibroblast medium. The images were obtained using a 5X or a 10X objective, as indicated.



Live Staining with Antibodies

One of the fastest and most reliable methods for selecting a reprogrammed colony is live staining with Tra1-60 or Tra1-81 antibodies that recognize undifferentiated iPSCs and enable the identification of reprogrammed cells from a variety of human cell types.

Note: Other methods of identifying iPSCs (such as alkaline phosphatase staining) are also acceptable.

1. Aspirate the medium from the reprogramming dish.
2. Wash the cells once with 1X KnockOut™ DMEM/F-12.
3. Add the diluted primary antibody 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.

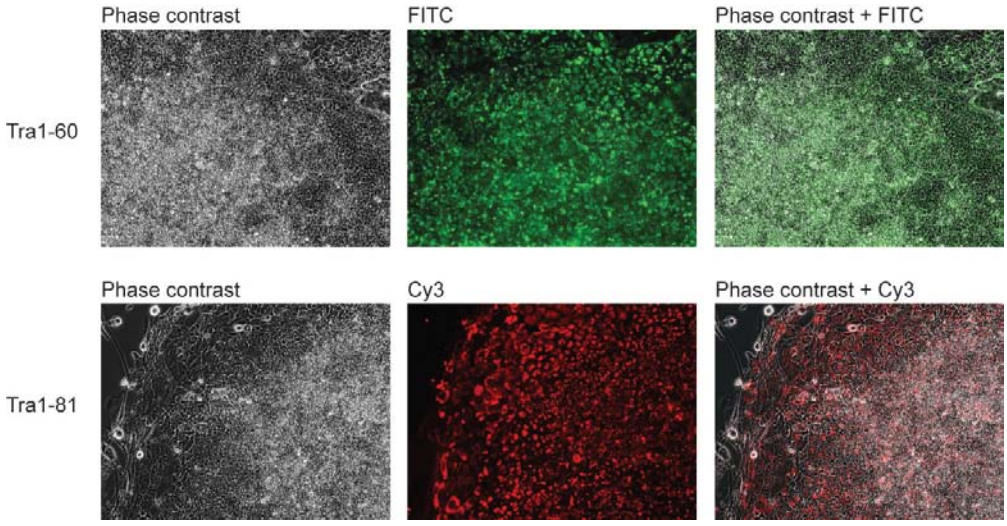
Note: The primary antibody solution can be stored at 4°C for 1 week and re-used up to 2 times.

6. Wash cells three times with KnockOut™ DMEM/F-12.
7. Add the diluted secondary antibody to the cells (6 mL per 100-mm dish).
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. Successful antibody staining can very specifically distinguish reprogrammed colonies from just plain transformed counterparts (see Figure 2), 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.

Figure 2 Human neonatal foreskin fibroblast cells (strain BJ) were transformed using the CytoTune™-iPS Reprogramming Kit and allowed to proliferate on MEF feeder layers in fibroblast medium. On Day 21, the cells were analyzed by live staining using the antibody against the cell surface markers Tra1-60 and Tra1-81. The images were obtained using a 10X objective.



Picking iPSC colonies

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 (see below).

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 containing human iPSC medium.
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.

Generating Vector-Free iPSCs

Guidelines for generating vector-free iPSCs

- The time needed to derive vector-free iPSCs may vary depending on culture and passage conditions. In the case of human neonatal foreskin fibroblast cells (strain BJ), it takes about 2 months after gene transduction to obtain iPSCs free of CytoTune™ Sendai reprogramming vectors.
- To obtain virus-free clones faster, we recommend that you perform single colony subcloning for the first few passages (minimum 5) instead of bulk or pooled-clone passaging.
- To perform single colony subcloning, pick from a single colony to transfer to another 6-well plate (Passage 1). From Passage 1, pick a single colony and transfer to another 6-well plate (Passage 2) and so forth. We recommend subcloning for 5 passages and then testing for virus free iPSCs.

Protocol for generating vector-free iPSCs

1. When passaging iPSC colonies, prepare duplicate plates; one for immunostaining and one for further passaging.
2. Perform immunostaining on one plate using anti-SeV antibodies (see below).
3. If any colonies stain positive, perform cell cloning on the other duplicate plate.
4. Repeat immunostaining with anti-SeV antibodies on the cloned colonies until all colonies in a plate are negative.
5. If all colonies are negative for anti-SeV antibodies, passage the cells and confirm the absence of the CytoTune™ Sendai reprogramming vectors by RT-PCR.

Immunocytochemistry with Anti-SeV Antibodies

1. Wash cells once with DPBS
2. Fix the cells in 4% paraformaldehyde for 5 minutes at room temperature.
3. Wash cells twice with DPBS.
4. Add the anti-SeV antibody (MBL, Cat. no PD029) diluted in 0.1% Triton® X-100 in DPBS to the cells and incubate for 1 hour at 37°C.

5. Remove the antibody solution. Wash the cells 3 times with DPBS.
6. Add the secondary antibody diluted in 0.1% Triton® X-100 in DPBS and incubate for 1 hour at 37°C.
7. Remove the secondary antibody solution from the dish. Wash the cells 3 times with DPBS.
8. Visualize the cells under a fluorescence microscope.

RT-PCR Protocol for Detecting the SeV Genome and Transgenes

1. Extract the total RNA from 5×10^6 iPSCs using the TRIzol® Reagent following the instructions provided with the reagent. As a positive control, use cells set aside at the last step of the reprogramming procedure.
2. Carry out a reverse transcription reaction using 1 µg of RNA (from step 1, above) and the SuperScript® VILO™ cDNA Synthesis Kit following the instructions provided with the kit.

Note: Because the CytoTune™ Sendai reprogramming vectors are based on SeV, which is an RNA virus, reverse transcription is required for detecting the presence of the SeV genome in your reprogrammed cells.

3. Carry out the PCR using 10 µL of cDNA from the reverse transcription reaction (step 2, above) and AccuPrime™ SuperMix I with the parameters below. For the RT-PCR primer sequences and the expected product size, refer to the Table 2.

Step	Temperature	Time	Cycles
Denaturation	95°C	30 seconds	30–35
Annealing	55°C	30 seconds	
Elongation	72°C	30 seconds	

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

Note: If you still detect CytoTune™ Sendai virus in your iPSC lines after more than 10 passages, and have performed RT-PCR to show that Oct4, Sox2, and Klf4 (these vectors do not have the temperature sensitive mutations) are absent from your cells, then you can perform temperature shift to remove the cMyc gene. CytoTune™ Sendai hc-Myc tends to persist in the cells longer than the other CytoTune™ Sendai reprogramming vectors. However, because this vector contains a temperature sensitivity mutation, you can enhance its removal and obtain complete absence of Sendai virus by incubating your cells at 38–39°C for 5 days.

Table 2 RT-PCR primer set used for detecting the SeV genome and transgenes in cells reprogrammed using the CytoTune™ Sendai reprogramming vectors

Target	Primer sets	Product size
SeV	Forward: GGA TCA CTA GGT GAT ATC GAG C* Reverse: ACC AGA CAA GAG TTT AAG AGA TAT GTA TC*	181 bp
Sox2	Forward: ATG CAC CGC TAC GAC GTG AGC GC Reverse: AAT GTA TCG AAG GTG CTC AA*	451 bp
Klf4	Forward: TTC CTG CAT GCC AGA GGA GCC C Reverse: AAT GTA TCG AAG GTG CTC AA*	410 bp
cMyc	Forward: TAA CTG ACT AGC AGG CTT GTC G* Reverse: TCC ACA TAC AGT CCT GGA TGA TGA TG	532 bp
Oct3/4	Forward: CCC GAA AGA GAA AGC GAA CCA G Reverse: AAT GTA TCG AAG GTG CTC AA*	483 bp

* Primer contains SeV genome sequences. Pairing of these primers with transgene-specific primers allows specific detection of transgenes carried by the CytoTune™ Sendai reprogramming vectors. Note that the same reverse primer is used for detecting Sox2, Klf2, and Oct3/4.

Troubleshooting

Problem	Possible cause	Solution
Cytotoxic effects observed after transduction	Viral load too high	Decrease the volume of CytoTune™ vector or increase the starting cell number.
Too many colonies on the plate	Too many cells plated	Decrease the number of cells plated after transduction.
No iPSC colony formation	Insufficient amount of virus used	• Check the volume of the CytoTune™ vector and the starting cell number. Changing the MOI may improve the results. • Not all cell types will be reprogrammed with the same efficiency. Check the levels of protein expression in your cell type using TaqMan® Protein Assays. • Do not re-freeze thaw or aliquot virus. Viral titer is not guaranteed for kits refrozen or thawed.
Too few iPSC colony compared to BJ fibroblasts	Cell type not efficiently reprogrammable	Not all cell types will have the same reprogramming efficiency. Increase the number of the cells plated.
iPSC colonies look differentiated	iPSC colonies transferred to MEF dishes too late	Perform staining earlier and transfer iPSC colony to fresh feeder cells.
Difficult to obtain vector-free iPSCs	Cell type cannot efficiently eliminate the CytoTune™ Sendai reprogramming vector	• Some cell strains may need longer time to eliminate the CytoTune™ Sendai vectors and become vector-free compared to other strains. Perform repeated cloning until you obtain negative cells as determined by immuno-cytochemistry with anti-SeV antibodies. • It may be easier to obtain SeV-negative colonies if cloning is performed by transferring a portion of a colony with a glass pipette. • The rate with which iPSC colonies eliminate the CytoTune™ Sendai vectors may increase if the cells are incubated for 5 days at 38–39°C **after** you have confirmed by RT PCR that Oct4, Sox2, and Klf4 genes are absent from your cells and only c-Myc remains.

APPENDIX

iPSC freezing medium

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 iPSCs

1. Prepare the required volume of fresh freezing medium and place it on ice (see page 12).
2. Aspirate the culture medium and rinse the dishes twice with DPBS without Ca^{2+} and Mg^{2+} (2 mL per 35-mm or 4 mL per 60-mm dish).
3. Gently add Collagenase IV solution to the culture dish (1 mL per 35-mm or 2 mL per 60-mm dish).
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 2 mL of iPSC culture medium or DMEM/F-12 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**. 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.

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.

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