USER GUIDE

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CytoTune®-iPS Reprogramming Kit

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

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CYTOTUNE®-IPS REPROGRAMMING KIT

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Product information

Contents and storage

Kit contents

The CytoTune®-iPS Reprogramming Kit contains four CytoTune® reprogramming vectors, each capable of expressing one of the four Yamanaka factors (Oct3/4, Sox2, Klf4, c-Myc).

The kit is available in two sizes: 1 pack (1 × 4 vials) and 3 packs (3 × 4 vials), with each vial containing 100 µL of one of the CytoTune® reprogramming vector at a concentration of ≥3 × 10^7 cell infectious units/mL (CIU/mL).

Note: The titer of each CytoTune® 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.

Component	Cap color	Amount	
		A13780-01	A13780-02
CytoTune® Sendai hOct3/4	blue	100 µL	3 × 100 µL
CytoTune® Sendai hSox2	green	100 µL	3 × 100 µL
CytoTune® Sendai hKlf4	yellow	100 µL	3 × 100 µL
CytoTune® Sendai hc-Myc	purple	100 µL	3 × 100 µL

Shipping and storage

- CytoTune®-iPS Reprogramming Kit is shipped on dry ice.
- Immediately upon receipt, store each component at –85°C to –68°C.
- Use the kit by the expiration date specified on the Certificate of Analysis (CoA).

CAUTION!

This product must be used under Biosafety Level 2 (BL-2) containment with biological safety cabinet and laminar flow hood, and with appropriate personal safety equipment to prevent mucosal exposure/splash. For more information on BL-2 guidelines, see page 6.

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 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

CytoTune®-iPS Reprogramming System uses vectors based on replication-incompetent Sendai virus (SeV) to safely and effectively deliver and express key genetic factors necessary for reprogramming somatic cells into iPSCs. In contrast to 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 (see **Safety Features of the System**, page 6).

Sendai virus (SeV)

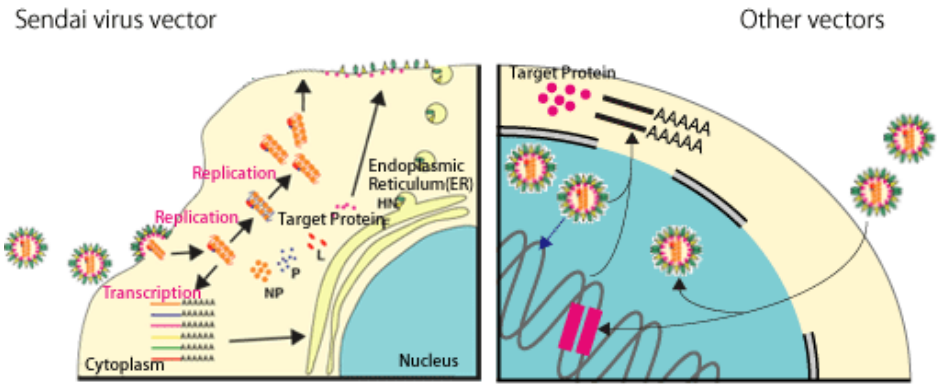
Sendai virus is a respiratory virus of mouse and rat, classified as mouse parainfluenza virus type I belonging to the *Paramyxoviridae* family. SeV was first isolated in Japan in the early 1950s (Kuroya *et al.*, 1953) and is also called Hemagglutinating Virus of Japan (HVJ). SeV is an enveloped virus of 150–250 nm in diameter whose genome is a single chain RNA (15,384 bases) in the minus sense. Six genes coding for viral proteins are situated sequentially on the genome of the wild-type SeV in the following order (starting from the 3′ end):

- Nucelocapsid protein (NP) forms the core nucleocapsid complex with the genome RNA.
- Phosphoprotein (P) is the small subunit of the RNA polymerase.
- Matrix protein (M) supports the envelope structure from the inside.
- Fusion protein (F) fuses the viral envelope with cell membrane when the virus enters the cell.

  Note: The gene encoding the F protein is deleted from the CytoTune® reprogramming vectors, rendering them incapable of producing infectious particles from infected cells (see page 6).
- Hemagglutinin-Neuraminidase (HN) recognizes the cell surface receptor, sialic acid.
- Large protein (L) is the large subunit of RNA polymerase.

Because SeV infects cells by attaching itself to the sialic acid receptor present on the surface of many different cells, it can infect a wide range of cell types of various animal species. Activation of F protein by a protease is required for the virus-cell fusion process to take place. After infection, the virus goes through genome replication and protein synthesis, and then daughter virus particles are assembled and released.

Figure 1 Comparison of the lifecycles of non-integrating SeV vectors and other, integrating vectors



Description of the system, continued

CytoTune® Sendai reprogramming vectors

The table below lists the CytoTune® Sendai reprogramming vectors included in the CytoTune®-iPS Reprogramming Kit. The reprogramming vectors include the four Yamanaka factors, Oct, Sox2, Klf4, and c-Myc, shown to be sufficient for efficient reprogramming (Takahashi *et al.*, 2007).

CytoTune® Sendai vector	Cap color	Factor	GenBank ID
CytoTune® Sendai hOct3/4	blue	Human Oct3/4	NM_002701.4
CytoTune® Sendai hSox2	green	Human Sox2	NM_003106.2
CytoTune® Sendai hKlf4	yellow	Human Klf4	BC029923.1
CytoTune® Sendai hc-Myc	purple	Human c-Myc	K02276.1

Advantages of CytoTune®-iPS Reprogramming Kit

- No genotoxicity: CytoTune® Sendai reprogramming vectors do not integrate into chromosomes of the target cells and potentially disrupt important genes.
- Wide range of targets: CytoTune® Sendai reprogramming vectors are capable of transducing a wide range of cell types in proliferative and quiescent states.
- High transduction efficiency with low multiplicity of infection (MOI).
- Short contact time of virus with target cells is sufficient to establish transduction.
- High level of expression of the transgenes.
- Fast expression of the transgenes: expression is detectable as early as 6–10 hours after transduction, with maximum expression detected more than 24 hours after transduction.
- Zero footprint: the vectors and transgenes can be eliminated from the cells.
- No production of infectious particles by the transduced cells.
- Derived from a virus that is non-pathogenic to humans.

Safety features of the system

Sendai virus (SeV) safety information

Host species: The host species for the Sendai virus (SeV) reported so far are mouse, rat, hamster, and guinea pigs, all of which have been described to be serologically positive.

Transmission: SeV is transmitted by aerosol and contact with respiratory secretions. The virus is highly contagious, but the infection does not persist in immunocompetent animals.

CytoTune® Sendai reprogramming vectors: CytoTune® Sendai reprogramming vectors in this kit are based on a modified, non-transmissible form of SeV, which has the Fusion protein (F) deleted, rendering the virus incapable of producing infectious particles from infected cells.

Inoculating animals with transduced cells: Although the CytoTune® Sendai reprogramming vectors are nontransmissible, cells that have been exposed to the virus should be tested with PCR or antibody staining to ensure the absence of the virus before being inoculated into animals. Animals that have already been infected with wild type SeV may be able to make infectious CytoTune®-Sendai virus.

Nontransmissible CytoTune® Sendai reprogramming vectors

SeV vectors used in this kit consist of viral proteins NP, P, M, F (activated), HN, and L, and the SeV genome RNA, from which the F gene is deleted. Because SeV infects cells by attaching itself to cell surface receptor sialic acid, present on the surface of many cell types of different species, the vectors maintain full infectivity to a wide range of cells; however, they are no longer capable of producing infectious particles from infected cells because the viral genome lacks the F-gene. In addition, the presence of functional mutations such as temperature sensitivity in the amino acid sequence of several SeV proteins (SeV/TSΔF and SeV/TS15ΔF) renders the vectors easily removable from transduced cells.

Note: SeV vectors used in this kit were developed by DNAVEC Corporation (**http://www.dnavec.co.jp**) and their rights for commercial use are the property of DNAVEC Corporation.

Biosafety Level 2

CAUTION! Although human is not the natural host for the SeV, and the virus is non-pathogenic to humans, appropriate care must be taken to prevent the potential mucosal exposure to the virus. This product must be used under Biosafety Level 2 (BL-2) containment with biological safety cabinet and laminar flow hood, and with appropriate personal safety equipment to prevent mucosal exposure/splash. In the event that the virus comes into contact with skin or eyes, decontaminate by flushing with plenty of water and consult a physician. For more information on BL-2 guidelines, refer to *Biosafety in Microbiological and Biomedical Laboratories*, 5th ed., published by the Centers for Disease Control, which is available for downloading at: **www.cdc.gov/od/ohs/biosfty/bmbl5/bmbl5toc.htm**.

Experimental outline

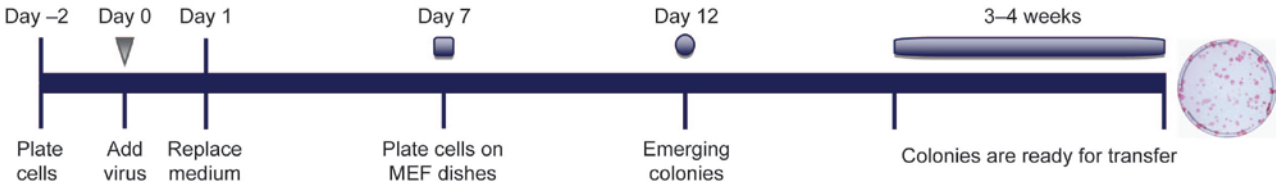
Workflow

The table below describes the major steps required for reprogramming somatic cells to generate iPSCs using the CytoTune®-iPS Reprogramming Kit.

Step	Action	Page
1	Plate your cells	11
2	Transduce cells with CytoTune® Sendai reprogramming vectors	11
3	Harvest your cells and plate them on MEF culture dishes (for preparing MEF culture dishes, see page 23)	12
4	Feed and culture the transduced cells on MEF culture dishes	12
5	Identify iPSC colonies	13
6	Perform live staining	14
7	Pick iPSC colonies	16
8	*Optional:* Adapt iPSC to feeder-free culture	25
9	Generate vector-free iPSCs	17

CytoTune®-iPS reprogramming timeline

Figure 2 Experiment timeline for the CytoTune®-iPS reprogramming experiment. These timeline are provided as a guideline for experimental planning; actual timelines can vary based on the cell type and experimental conditions.



Methods

Materials needed

Cells and vectors

- CytoTune® Sendai reprogramming vectors
  Note: For successful reprogramming, you need all four reprogramming vectors.
- Mammalian cells to reprogram
- Gibco® Mouse embryonic Fibroblasts (Irradiated) (Cat. no. S1520-100)
- *Optional*: Human neonatal foreskin fibroblast cells (strain BJ; ATCC no. CRL2522) as a positive reprogramming control

Media and reagents

- D-MEM with GlutaMAX™-I (high glucose) (Cat. no. 10569-010)
- KnockOut™ D-MEM/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 (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)
- D-PBS without Ca^{2+} or Mg^{2+} (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)

Antibodies for characterizing iPSCs

- 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)
- 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)

Materials needed, continued

Equipment

- Sterile cell culture hood (i.e., biosafety cabinet) equipped with a stereomicroscope
- Inverted microscope
- Incubator set at 37°C, 5% CO2
- 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

***Optional*: Products for growing iPSCs feeder-free**

- StemPro® hESC SFM (Cat. no. A1000701)
- Geltrex™ hESC-qualified Reduced Growth Factor Basement Membrane Matrix (Cat. no. A14133-02)
- StemProR EZPassage™ Disposable Stem Cell Passaging Tool (Cat. no. 23181-010)

Guidelines for generating iPSCs

Experimental 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 CytoTune®-iPS 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 (~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.
- One 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=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.

Positive control

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.

Reprogram somatic cells

Reprogramming protocol

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 onto 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 (see page 21 for recipe).
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 (see page 23).

Reprogramming protocol, continued

Day 7: Plate transduced cells on MEF culture dishes

10. Seven days after transduction (Step 6, previous page), fibroblast cells are ready to be harvested and plated on MEF culture dishes. Remove the medium from the fibroblasts, and wash cells once with D-PBS.
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 (see page 18).

Day 8 to 28: Feed and monitor the cells

14. 24 hours later, change the medium to iPSC medium (see page 22 for recipe), 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 **Visual identification** on page 13).

    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 (see page 14 for protocol).
18. Manually pick colonies and transfer them onto MEF plates prepared in step 15 (see page 16 for protocol).

Identify iPSC colonies

Visual identification

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 2, below). Therefore, we recommend that you perform live staining with Tra1-60 or Tra1-81 antibodies that recognize undifferentiated hESCs (see page 14).

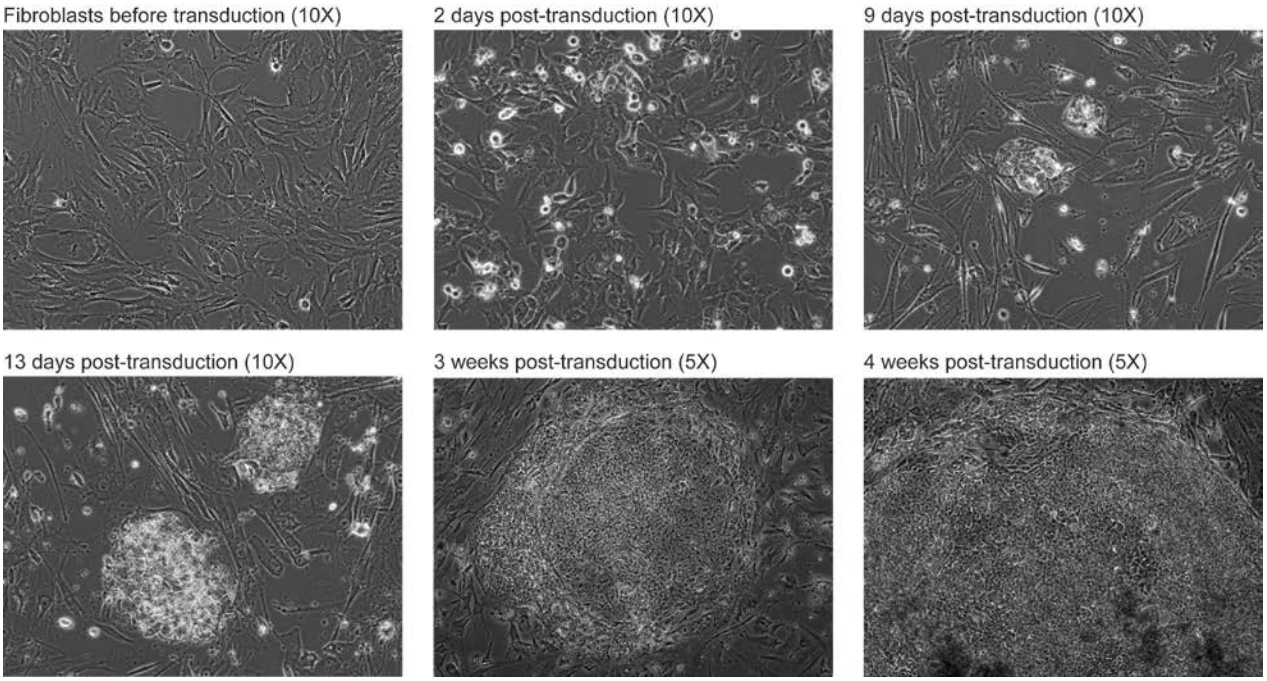
Note

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-transduction), resemble embryonic stem cells in morphology, and express the cell surface markers Tra1-60 and Tra1-81.

Morphology of reprogrammed cells

The images below show the morphology of human neonatal foreskin fibroblast cells (strain BJ) that were reprogrammed into iPSCs using the CytoTune®-iPS Reprogramming Kit.

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. The images were obtained using a 5X or a 10X objective, as indicated.



Identify iPSC colonies, continued

Live stain with antibodies

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

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

Required antibodies

Primary antibody:

- Mouse anti-Tra1-60 antibody (Cat. no. 41-1000) and/or mouse anti-Tra1-81 antibody (Cat. no. 41-1100), diluted 1:100 in KnockOut™ D-MEM/F-12

Secondary antibody:

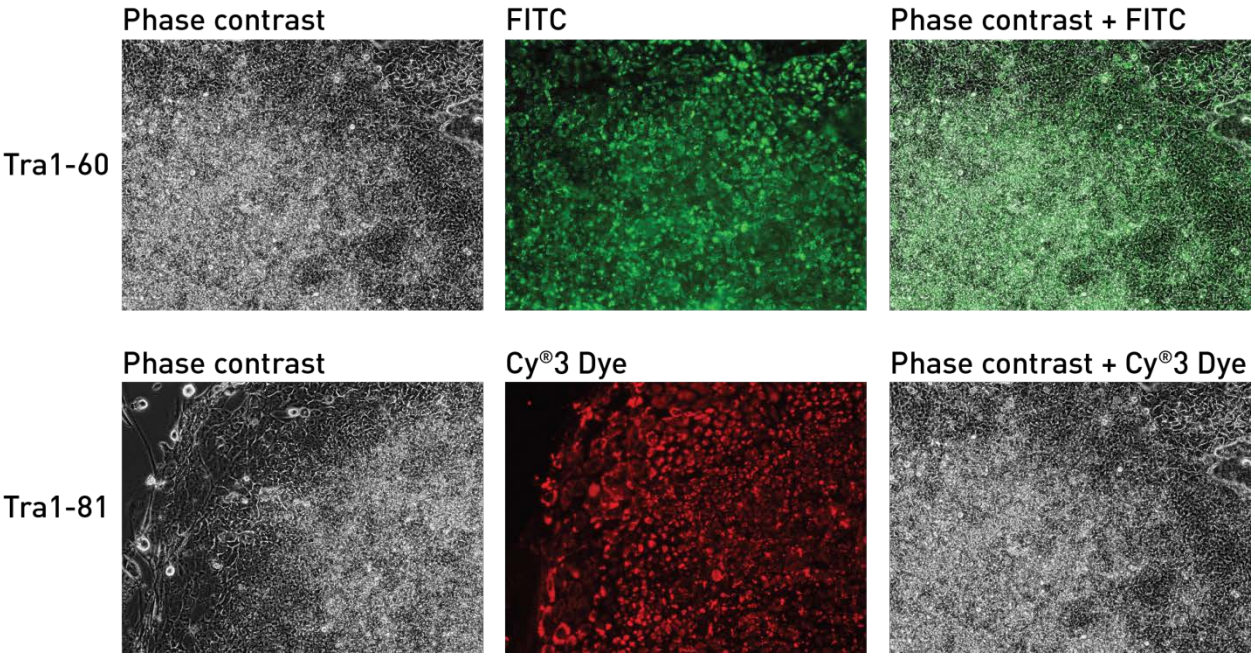
- Alexa Fluor® 488 goat anti-mouse IgG antibody (Cat. no. A11029) and/or Alexa Fluor® 594 goat anti-mouse IgG antibody (Cat. no. A11032), diluted 1:500 in KnockOut™ D-MEM/F-12

Live staining protocol

1. Aspirate the medium from the reprogramming dish.
2. Wash the cells once with 1X KnockOut™ D-MEM/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™ D-MEM/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™ D-MEM/F-12 and add fresh KnockOut™ D-MEM/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 (see page 16). Successful antibody staining can very specifically distinguish reprogrammed colonies from just plain transformed counterparts (see Figure 3, next page), 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.

Expected results

Figure 3 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.



Pick iPSC colonies

Protocol for 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 (see page 23) containing human iPSC medium (see page 22).
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 27).

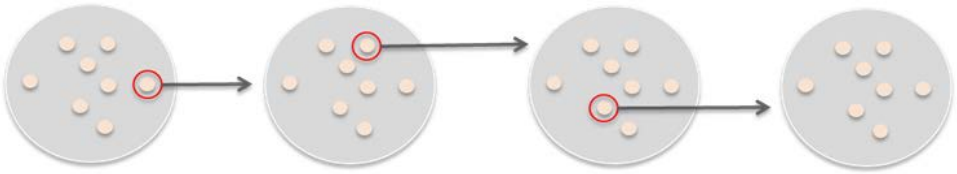
Adapt iPSCs to feeder-free culture

iPSCs cultured on MEF feeder layers can be adapted to feeder-free conditions in StemPro® hESC SFM. This can be done by directly thawing or splitting the iPSCs in MEF-conditioned (MEF-CM) medium and then replacing the culture medium daily with medium that contains increasing amounts of StemPro® hESC SFM (see page 25 for protocol).

Generate 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 (see page 18).

Immunocyto-chemistry with anti-SeV antibodies

1. Wash cells once with D-PBS
2. Fix the cells in 4% paraformaldehyde for 5 minutes at room temperature.
3. Wash cells twice with D-PBS.
4. Add the anti-SeV antibody (MBL, Cat. no PD029) diluted in 0.1% Triton® X-100 in D-PBS to the cells and incubate for 1 hour at 37°C.
5. Remove the antibody solution. Wash the cells 3 times with D-PBS.
6. Add the secondary antibody diluted in 0.1% Triton® X-100 in D-PBS to the cells and incubate for 1 hour at 37°C.
7. Remove the secondary antibody solution from the dish. Wash the cells 3 times with D-PBS.
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 (Cat. no. 15596-026) following the instructions provided with the reagent. As a positive control, use cells set aside during the reprogramming procedure (Step 13, page 12).
2. Carry out a reverse transcription reaction using 1 µg of RNA (from step 1) and the SuperScript® VILO™ cDNA Synthesis Kit (Cat. no. 11754-050) 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 (Cat. no. 12342-010) with the parameters below. For the RT-PCR primer sequences and the expected product size, refer to the table on the next page.

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°C –39°C for 5 days.

RT-PCR primer sets

The table below shows the 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.

Appendix A: Troubleshooting

Troubleshooting

The table below lists some potential problems and solutions that may help you troubleshoot your reprogramming experiments.

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 (see page 29 for ordering information). • 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 immunocytochemistry 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 (see **Note** on page 18).

Appendix B: Support protocols

Prepare media and reagents

Basic FGF stock solution

1. To prepare 1000 µL of 10-µg/mL Basic FGF solution, aseptically mix the following components:

Basic FGF	10 µg
D-PBS without Ca^{2+} and Mg^{2+}	980 µL
10% BSA	10 µL

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

Collagenase IV solution

1. To prepare 50 mL of a 1-mg/mL Collagenase IV solution, aseptically mix the following components:

Collagenase IV	50 mg
KnockOut™ D-MEM/F-12	50 mL

2. Sterilize the Collagenase IV solution through a 0.2 µm filter.
3. Aliquot and store the Collagenase IV solution at –20°C for up to 6 months.

Complete MEF/Fibroblast medium

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

Component	Concentration		Volume
	Stock	Final	
D-MEM with GlutaMAX™-I	—	1X	89 mL
FBS, ESC Qualified	—	10%	10 mL
MEM Non-Essential Amino Acids Solution	10 mM	100 µM	1 mL

Human iPSC medium

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

Component	Concentration		Volume
	Stock	Final	
KnockOut™ D-MEM/F-12	1X	1X	78 mL
KnockOut™ Serum Replacement	—	20%	20 mL
MEM Non-Essential Amino Acids Solution	10 mM	100 µM	1 mL
GlutaMAX™-I Supplement	100X	1X	1 mL
ß-mercaptoethanol	55 mM	100 µM	182 µL
Penicillin-Streptomycin (*optional*)	100X	1X	1 mL
Basic FGF*	10 µg/mL	4 ng/mL	40 µL

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

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.

Prepare 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 is a sterile 1X solution containing 0.1 % gelatin available from Life Technologies (see page 28 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.

Thaw 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. 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).

Prepare MEF culture dishes, continued

Plate 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 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) 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	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	3.8 cm^2/well	1 mL	1.0×10^5/well	40 µL
6-well plate	9.6 cm^2/well	2 mL	2.5×10^5/well	0.1 mL
60-mm dish	19.5 cm^2	5 mL	5.0×10^5	0.2 mL
100-mm dish	58.95 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

Adapt iPSCs to feeder-free culture

Feeder-free culture of iPSCs

iPSCs cultured on MEF feeder layers can be adapted to feeder-free conditions in StemPro® hESC SFM. This can be done by directly thawing or splitting the iPSCs in MEF-conditioned (MEF-CM) medium and then replacing the culture medium daily with medium that contains increasing amounts of StemPro® hESC SFM. Before thawing the iPSC, prepare MEF-CM and Geltrex™ matrix-coated culture dishes (see below for protocol).

Prepare MEF-conditioned medium (MEF-CM)

1. Prepare MEF culture dishes as described on page 23, but seed the MEFs at a density of 5.0×10^4 cells/cm^2 (i.e., 1×10^6 cells per 60-mm dish, 3×10^6 cells per 100-mm dish, 1.3×10^6 cells per T-25, 3.8×10^6 cells per T-75, or 8.8×10^6 cells per T-175).
2. Change the MEF medium to human iPSC culture medium after 24 hours of incubation.
3. Collect the human iPSC culture medium, now MEF-CM, from the MEF culture dishes every 24 hours for up to 7 days total.
4. Sterilize the MEF-CM through a 0.2 µm filter and supplement it with bFGF to a final concentration of 4 ng/mL before using it for iPSCs.

Prepare Geltrex™ matrix-coated culture dishes

1. Thaw one tube of Geltrex™ matrix (1 mL) slowly at 2–8°C and dilute it 1:100 in 99 mL of KnockOut™ D-MEM/F-12. Mix the solution gently.
2. Cover the whole surface of each culture dish with the Geltrex™ matrix solution (1 mL for a 35-mm dish, 1.5 mL for a 60-mm dish).
3. Seal each dish with Parafilm® sealing film to prevent drying and incubate the dishes for 1 hour at 37°C.

   Note: At this point you may store the Geltrex™ matrix-coated culture dishes at 2°C to 8°C for up to 1 month. Seal each dish with Parafilm® sealing film to prevent the Geltrex™ matrix from drying out.
4. Prior to using, transfer the Geltrex™ matrix-coated dishes to a laminar flow hood and allow them to equilibrate to room temperature (about 1 hour).

Adapt iPSCs to feeder-free culture, continued

Adaptation protocol

Adaptation to StemPro® hESC SFM can be performed on freshly passaged cells.

1. Pick the iPSCs as described on page 16, up to step 4.
2. Using a 200 µL pipette, transfer the cut pieces to a freshly prepared Geltrex™ matrix-coated plate.

   Caution: Do not make single-cell suspension by pipetting the cells up and down. iPSCs in single-cell suspension will **not** adhere to Geltrex™ matrix-coated culture dishes. The cells should be plated as small clumps. Not following the Geltrex™ matrix-coating procedure properly or plating the iPSCs in a single-cell suspension instead of small clumps may result in poor attachment.
3. For initial recovery, we recommend seeding cells at 6×10^4 to 1×10^5 cells/cm^2.
4. Incubate the Geltrex™ matrix-culture plate containing the picked colonies in a 37°C incubator with a humidified atmosphere of 5% CO_2. Allow the colonies to attach to the culture plate.
5. After 24 hours, replace the medium and adapt the cells to StemPro® hESC SFM medium in a sequential manner as described below.

   Day 1: 75% MEF-CM and 25% StemPro® hESC SFM

   Day 2: 50% MEF-CM and 50% StemPro® hESC SFM

   Day 3: 25% MEF-CM and 75% StemPro® hESC SFM

   Day 4: 100% StemPro® hESC SFM
6. Feed cells every day thereafter with 100% StemPro® hESC SFM until they are semi-confluent. When the cells are semi-confluent (each colony is touching their boundary), they are ready for passaging. It is preferable to split the cells at a 1:3 ratio.

   Note: Continued passaging may be performed using the StemPro® EZPassage™ Disposable Stem Cell Passaging Tool.

Freeze iPSCs

Freezing protocol

1. Prepare the required volume of fresh freezing medium and place it on ice (see page 22).
2. Aspirate the culture medium and rinse the dishes twice with D-PBS without Ca^{2+} and Mg^{2+} (2 mL per 35-mm or 4 mL per 60-mm dish).
3. Gently add Collagenase IV solution (page 21) 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 D-PBS without Ca^{2+} and Mg^{2+}.
7. Add 2 mL of iPSC culture medium or D-MEM/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 D-MEM/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.

Appendix C: Ordering information

Accessory products

Media, sera, and reagents

For more information about the following products, refer to our website at **www.lifetechnologies.com** or contact Technical support (page 30).

Product	Quantity	Catalog no.
Dulbecco's Modified Eagle Medium (D-MEM), high glucose	500 mL	10569-010
KnockOut™ D-MEM/F-12	500 mL	12660-012
StemPro® hESC SFM	1 kit	A1000701
Fetal Bovine Serum (FBS), ES-Cell Qualified	500 mL	16141-079
D-PBS without Ca^{2+} or Mg^{2+}	500 mL	14190-144
MEM Non-Essential Amino Acids Solution (10 mM)	100 mL	11140-050
KnockOut™ Serum Replacement	500 mL	10828-028
ß-Mercaptoethanol (1000X), liquid	50 mL	21985-023
FGF-basic, AA 1-155 Recombinant Human	10 µg	PHG0264
GlutaMAX™-I Supplement	100 mL	35050-061
Penicillin-Streptomycin, liquid	100 mL	15140-122
Trypsin/EDTA Solution	100 mL	25300-054
TrypLE™ Select Cell Dissociation Reagent	100 mL	12563-011
Attachment Factor	100 mL	S-006-100
Geltrex™ hESC-qualified Reduced Growth Factor Basement Membrane Matrix	5 mL	A14133-02
Collagenase Type IV	1 g	17104-01
TRIzol® reagent	100 mL	15596-026
SuperScript® VILO™ cDNA Synthesis Kit	50 reactions	11754-050
AccuPrime™ SuperMix I	200 reactions	12342-010

Cells

For more information about the following products, refer to our website at **www.lifetechnologies.com** or contact Technical support (page 30).

Product	Quantity	Catalog no.
Gibco® Mouse Embryonic Fibroblasts (Irradiated)	1 mL	S1520-100
Human Dermal Fibroblasts, neonatal (HDFn)	1 vial	C-004-5C
Human Dermal Fibroblasts, adult (HDFa)	1 vial	C-013-5C

Accessory products, continued

Antibodies

For more information about the following products, refer to our website at **www.lifetechnologies.com** or contact Technical support (page 30).

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

Equipment

For more information about the following products, refer to our website at **www.lifetechnologies.com** or contact Technical support (page 30).

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

TaqMan® Protein Assays

For more information about the following products, refer to our website at **www.lifetechnologies.com** or contact Technical support (page 30).

Product	Quantity	Catalog no.
TaqMan® Protein Assay Kit (hOct3/4)	100 reactions	4405489
TaqMan® Protein Assay Kit (hSox2)	100 reactions	4405495

Documentation and support

Obtaining support

Obtaining Certificates of Analysis

The Certificate of Analysis provides detailed quality control and product qualification information for each product. Certificates of Analysis are available on our website. Go to **www.lifetechnologies.com/support** and search for the Certificate of Analysis by product lot number, which is printed on the box.

Obtaining SDS

Safety Data Sheets (SDSs) are available at **www.lifetechnologies.com/support**.

Technical support

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- Obtain information about customer training
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Limited product warranty

Life Technologies and/or its affiliate(s) warrant their products as set forth in the Life Technologies General Terms and Conditions of Sale found on the Life Technologies web site at **www.lifetechnologies.com/termsandconditions**. If you have any questions, please contact Life Technologies at **www.lifetechnologies.com/support**.

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