Derivation of mouse induced pluripotent stem cells (iPSCs) from mouse embryonic fibroblasts (MEFs) using CytoTune™-iPS 2.0 Sendai Reprogramming Kit

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WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from **thermofisher.com/support**.

Introduction

Induced pluripotent stem cells (iPSCs) are genetically reprogrammed cells that exhibit a pluripotent stem cell-like state similar to embryonic stem cells (ESCs), making them valuable tools in scientific research. There are multiple methods to generate iPSCs: Retroviral vectors require integration into host chromosomes to express reprogramming genes. On the other hand, 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. CytoTune™-iPS Reprogramming System uses vectors based on replication-incompetent Sendai virus (SeV) to effectively deliver and express key genetic factors necessary for reprogramming somatic cells into iPSCs. Unlike the abovementioned vectors, the CytoTune™ reprogramming vectors do not integrate into the host genome or alter the genetic information of the host cell. In this protocol, we describe how to reprogram mouse embryonic fibroblasts (MEFs) using the CytoTune™ system in order to derive iPSCs.

Required materials

Item	Source
DMEM, high glucose, GlutaMAX™ Supplement, pyruvate	1056010
DMEM/F-12, GlutaMAX™ Supplement	10565018
MEM Non-Essential Amino Acids Solution (100X)	11140050
2-Mercaptoethanol	21985023
LIF Recombinant Mouse Protein	PMC9484
KnockOut™ Serum Replacement–Multi-Species	A3181502
Attachment Factor Protein (1X)	S006100
C57BL/6 Mouse Embryonic Fibroblasts, Irradiated	MTI-GlobalStem, Cat. No. GSC-6202G
CF-1 Mouse Embryonic Fibroblasts, Untreated	MTI-GlobalStem, Cat No. GSC-6001
TrypLE™ Express Enzyme (1X), no phenol red	12604013
Fetal Bovine Serum, embryonic stem cell-qualified	16141061
Penicillin-Streptomycin	15140122
DPBS, no calcium, no magnesium	14190144
CytoTune™-iPS 2.0 Sendai Reprogramming Kit	A16517
Nunc™ Cell-Culture Treated Multidishes	140675
Alkaline Phosphatase Live Stain	A14353
37°C water bath	—

Safety features of CytoTune™-iPS 2.0 Sendai Reprogramming Kit

- **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™-iPS 2.0 Sendai reprogramming vectors:** CytoTune™-iPS 2.0 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™-iPS 2.0 Sendai reprogramming vectors are non-transmissible, 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™-iPS 2.0 Sendai virus.

Reprogramming guidelines

- To maintain sterile culture conditions, carry out all of the procedures using sterile laboratory practices in a laminar flow hood.
- For successful reprogramming, transduce your cells using all three reprogramming vectors supplied with the kit.

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

- The titer of each CytoTune™-iPS 2.0 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 **thermofisher.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.

Note: 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.

Prepare media and materials

Prepare MEF Medium (500 mL of complete medium)

Aseptically mix the following components:

Reagent	Volume
DMEM, high glucose, GlutaMAX™ Supplement, pyruvate	444.5 mL
Fetal Bovine Serum, embryonic stem cell-qualified	50 mL
MEM Non-Essential Amino Acids Solution	5 mL
2-Mercaptoethanol	500 µL
Penicillin-Streptomycin (optional)	5 mL

Complete MEF Medium can be stored at 2–8°C for up to 1 week.

Prepare mPSC Culture Medium (500 mL of complete medium)

Aseptically mix the following components:

Reagent	Volume
DMEM/F-12, GlutaMAX™ Supplement	395 mL
KnockOut™ Serum Replacement–Multi-Species	100 mL
MEM Non-Essential Amino Acids Solution	5 mL
2-Mercaptoethanol	500 µL
LIF Recombinant Mouse Protein[1], 10 µg/mL	500 µL

[1] Alternatively, for best results, add LIF fresh to an aliquot of medium before use. Final concentration should be 10 ng/mL.

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

Reprogram MEFs

Day –2: Seed untreated MEFs for reprogramming

Two wells of untreated MEFs are required to enable induction and cell count. Use MEFs of as early passage as possible.

1. Two days before induction, remove the vial of untreated MEFs from liquid nitrogen storage using metal forceps.
2. Roll the vial between your gloved hands until the outside is free of frost.

   This should take ~10–15 seconds.
3. Immerse the vial in a 37°C water bath without submerging the cap, and swirl the vial gently.
4. When only an ice crystal remains, remove the vial from the water bath.
5. Spray the outside of the vial with 70% ethanol and place it in hood.
6. Pipet the thawed cells gently into a sterile 50-mL conical tube.

7. Slowly add 10 mL of pre-warmed MEF Medium drop-wise to cells in the 50-mL conical tube.

   While adding the medium, gently move the tube back and forth to mix the MEFs. This reduces osmotic shock to the cells. Aseptically, transfer the contents to 15 mL conical tube.

8. Centrifuge the cells at 200 × g for 4 minutes.

9. Aspirate the supernatant, then resuspend the cell pellet in MEF Medium to a density of approximately 5×10^6 cells/mL.

10. Add the appropriate volume of MEF Medium into each culture vessel, and the appropriate amount of MEF suspension. See Table 1.

    Note: Depending on the mouse line of origin, the required density can vary. For CF-1 untreated MEFs, the recommended plating density is 5×10^4 cells/cm^2.

11. Move the culture vessels in several quick figure eight motions to disperse the cells across the surface of the vessels.

12. Place MEF culture vessels into a 37°C, 5% CO_2 incubator.

Table 1 Amount of MEFs needed

Culture vessel	Surface area (cm2)	Number of MEFs	Optimal volume
6-well plate	10 cm^2/well	5.0 × 10^5/well	2 mL/well
12-well plate	4 cm^2/well	2.0 × 10^5/well	1 mL/well
24-well plate	2 cm^2/well	1.0 × 10^5/well	0.5 mL/well
35-mm dish	10 cm^2	5.0 × 10^5	2 mL
60-mm dish	20 cm^2	1.0 × 10^6	5 mL
100-mm dish	60 cm^2	3 × 10^6	10 mL

Day 0: Count cells and perform transduction

1. Harvest the cells from one well to perform a cell count.

   These cells will not be transduced, but will be used to estimate the cell number in the other well(s) plated at Day –2. We generally recommend transducing 250,000 to 300,000 cells per sample.

   Note: Cells may have decreased in number. Proceed with transduction using the live cell count.

2. Remove one set of CytoTune™-iPS 2.0 Sendai tubes from the –80°C storage.

3. 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.

4. Transduce the cells by adding the Klf4, Oct4, and Sox2 (KOS) virus, the c-Myc virus, and the Klf4 virus at an MOI of 5 (KOS MOI=5, c-Myc MOI=5, Klf4 MOI=5) in a total volume of 1 mL pre-warmed MEF media.

   Complete the next step within 5 minutes.

5. Aspirate the fibroblast medium from the unharvested well, and add the reprogramming virus mixture prepared in Step 4.

   Incubate the cells overnight in a 37°C incubator with a humidified atmosphere of 5% CO_2

   Note: We recommend initially performing the transductions with MOIs of 5, 5, and 5 as described above. This MOI can be optimized for your specific application and experimental conditions.

Day 1: Remove CytoTune™-iPS 2.0 Sendai virus and culture cells

1. The next day, aspirate the spent media from the culture plate and add 2 mL of pre-warmed MEF medium to each well.

2. Incubate the cells at 37°C , 5% CO_2

Day 2 to 6: Replace spent medium

1. The next day, remove the spent medium from the cells and replace it with fresh complete MEF Medium.

2. Replace the spent medium everyday thereafter.

3. On Day 6, plate inactivated MEF feeder cells on a gelatin- or Attachment Factor-coated 6-well plate in preparation for passaging the transduced cells on the next day.

   See "Appendix: Prepare inactivated MEF dishes" on page 4.

Day 7: Transition to feeders

1. Seven days after transduction, wash each well once with DPBS and add 1 mL TrypLE™ Express enzyme per well of a 6-well plate and incubate at 37°C for 2–3 minutes.

2. Add 3 ml of MEF media. Harvest the cells in a conical tube and spin 200 × g for 4 minutes.

3. Resuspend the pellet in 1–2 mL of MEF media and perform a cell count.

4. Plate 15,000 to 75,000 cells per well of a 6-well plate coated with feeders (from Day 6).

   Culture the cells with MEF Medium.

Day 8: Transition to mPSC Culture Medium

1. Prepare 500 mL of mPSC Culture Medium as described.

2. Aspirate the spent medium and add 2 mL of pre-warmed mPSC Culture Medium.

Day 9 to 16: Feed and monitor cells

1. From Day 8 onwards, feed cells with mPSC Culture Medium every day.

2. Observe the plates every other day under a microscope for the emergence of cell clumps indicative of transformed cells.

   At day 16 to 21 after transduction, colonies should have grown to an appropriate size for analysis/cloning. See Figure 1. Emerging colonies can be more easily identified by staining for mPSC markers using tools like the Alkaline Phosphatase Live Stain (Cat. No. A14353).

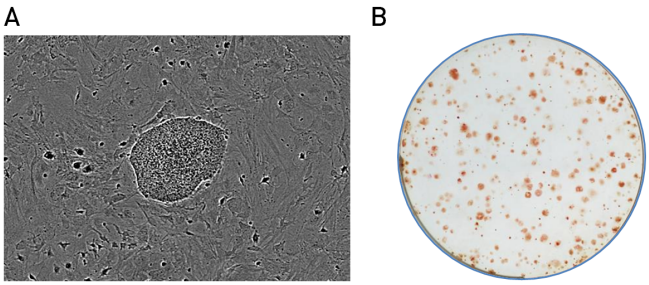

Figure 1 Emerging iPSC colonies

At Day 6 post-transduction, 50,000 cells were seeded per well of a 6-well plate already containing inactivated MEFs. At Day 16 post-transduction, emerging MEF-derived miPSC colonies were viewed by (A) phase contrast microscopy or (B) stained for alkaline phosphatase (red) and subjected to whole well imaging.

Appendix: Prepare inactivated MEF dishes

Coat culture vessels with Attachment Factor (optional)

Attachment Factor (AF) is a sterile solution (1X) containing 0.1% gelatin.

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 2 hours at room temperature.

2. Use sterile technique in a laminar flow culture hood, and completely remove the AF solution from the culture vessel by aspiration just prior to use. It is not necessary to wash the culture surface before adding cells or medium. Coated vessels may be used immediately or stored at room temperature for up to 24 hours.

   Note: Coating the vessel with AF improves attachment and spreading of MEFs but this step is dispensable.

Thaw and plate mitotically inactivated MEFs

Mitotically inactivated MEF feeder layers are ideally used one day after seeding.

1. Remove the vial of inactivated MEFs from liquid nitrogen storage using metal forceps.

2. Roll the vial between your gloved hands until the outside is free of frost.

   This should take ~10–15 seconds.

3. Immerse the vial in a 37°C water bath without submerging the cap, and swirl the vial gently.

4. When only an ice crystal remains, remove the vial from the water bath.

5. Spray the outside of the vial with 70% ethanol and place it in hood.

6. Pipet the thawed cells gently into a sterile 50-mL conical tube.

7. Slowly add 10 mL of pre-warmed MEF Medium drop-wise to cells in the 50-mL conical tube.

   While adding the medium, gently move the tube back and forth to mix the MEFs. This reduces osmotic shock to the cells.

8. Aseptically transfer the contents to a 15-ml conical tube, then centrifuge the cells at 200 × g for 5 minutes.

9. Aspirate the supernatant, then resuspend the cell pellet in MEF Medium to a density of approximately 5×10^6 cells/mL.

10. Aspirate the AF solution from the pre-coated culture vessels.

11. Add the appropriate volume of MEF Medium into each pre-coated culture vessel and add the appropriate amount of MEF suspension as shown in Table 2:

Table 2 MEF plating conditions

Vessel size	AF coating volume	Media volume	Number of MEFs
6-well plate	1 mL	2 mL	3.0×10^5/well
12-well plate	0.5 mL	1 mL	1.5×10^5/well
24-well plate	0.3 mL	0.5 mL	6.0×10^4/well
35-mm dish	1 mL	2 mL	3.0×10^5
60-mm dish	3 mL	3 mL	6.0×10^5
100-mm dish	9 mL	10 mL	1.8×10^6

   Depending on the mouse line of origin, the required feeder density can vary. For C57BL/6 strain cells, the recommended MEF plating density is 3×10^4 cells/cm^2.

12. Move the culture vessels in several quick figure eight motions to disperse the cells across the surface of the vessels.

13. Place inactivated MEF culture vessels into a 37°C, 5% CO_2 incubator.

14. Maintain inactivated MEF culture by changing medium every 2 days.

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