

# Generation of Human induced Pluripotent Stem Cells

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In the first protocol (Ver.1, released on July 4th, 2008), we have introduced how to establish human iPS cells using 4 reprogramming factors. In this revised protocol, which we designate Protocol Version 2, we explain our protocol of human iPS cell generation in a practical way focusing on problems we have encountered. A unique step is the introduction of mouse solute carrier family 7 (cationic amino acid transporter,  $\gamma^+$  system), member 1 (*Slc7a1*) gene encoding the ecotropic retrovirus receptor into human cells. This step enables ecotropic retroviruses to transduce human cells. In this manuscript, we particularly put in TROUBLESHOOTING, ANTICIPATED RESULTS and figures to make it easier to understand.

## 1. MATERIALS

### 1.1. Cells & Vectors

#### **pMXs retroviral vectors and Plat-E packaging cells<sup>1</sup>**

Available from Dr. Toshio Kitamura at the University of Tokyo

([kitamura@ims.u-tokyo.ac.jp](mailto:kitamura@ims.u-tokyo.ac.jp)). Also purchasable from Cell Biolabs, Inc.

(<http://www.cellbiolabs.com/>). pMXs consists of the 5' long terminal repeat (LTR) and 3' LTR of Moloney Murine Leukemia Virus (MMLV). Plat-E cells were derived from 293T cells and contain env-IRES-puro<sup>R</sup> and gag-pol-IRES-bs<sup>R</sup> cassettes driven by the EF1- $\alpha$  promoter.

**pMXs containing cDNA of *OCT3/4*, *SOX2*, *KLF4*, or *c-MYC***

Available from addgene ([http://www.addgene.org/Shinya\\_Yamanaka](http://www.addgene.org/Shinya_Yamanaka)). We use a pMXs retroviral vector encoding the green fluorescence protein (GFP) to monitor transfection efficiency. It also serves as a negative control for iPS cell induction. pMXs vector encoding GFP can be purchased from Cell Biolabs, Inc.

**pLenti6/UbC containing mouse *Slc7a1* gene**

Available from addgene ([http://www.addgene.org/Shinya\\_Yamanaka](http://www.addgene.org/Shinya_Yamanaka)). This vector was constructed by insertion of the mouse *Slc7a1* gene into pLenti6/UbC/V5-DEST (Invitrogen) with the gateway technology. Mouse *Slc7a1* gene is driven by the human Ubiquitin C promoter.

**Human cells**

Various primary human cells are available from the following resources:

Cell applications Inc. (<http://www.cellapplications.com/>)

Lonza (<http://www.lonza.com/group/en.html>)

American Type Culture Collection; ATCC (<http://www.atcc.org/>)

European Collection of Cell Cultures; ECACC (<http://www.ecacc.org.uk/>)

Riken Bioresource Center (<http://www.brc.riken.jp/>)

Japanese Collection of Research Bioresources (<http://cellbank.nibio.go.jp/>)

**SNL feeder cells<sup>2</sup>**

Available from Dr. Allan Bradley of the Sanger Institute

(<http://www.sanger.ac.uk/Teams/Team82/>) or European Collection of Cell

Cultures; ECACC (<http://www.ecacc.org.uk>). SNL cell is a subclone of STO cell and stably expresses the neomycin resistance and LIF genes.

### **293FT cells**

This cell line can be purchased from Invitrogen. 293FT cell is a subclone of HEK 293T cell line. These cells allow higher gene expression from vectors containing the simian virus 40 (SV40) replication origins because they constitutively express SV40 large T antigen.

### **1.2. Reagents**

- Dulbecco's modified eagle medium contains 4.5 g/l glucose (DMEM; 14247-15, Nacalai tesque, Japan)
- Phosphate-buffered saline without calcium and magnesium (PBS; 14249-95, Nacalai tesque, Japan)
- DMEM/F12 (10565-018, Invitrogen)
- Knockout serum replacement (KSR, 10828028, Invitrogen)
- L-glutamine (25030-081, Invitrogen)
- Non-essential amino acids solution (11140-050, Invitrogen)
- 2-mercaptoethanol (21985-023, Invitrogen)
- Sodium pyruvate (S8636, Sigma)
- Penicillin/streptomycin (15140-122, Invitrogen)
- Collagenase IV (17104-019, Invitrogen), see REAGENT SETUP
- Recombinant basic fibroblast growth factor, human (bFGF, 064-04541, WAKO, Japan), see REAGENT SETUP

- Y-27632 (253-00513, WAKO, Japan), see REAGENT SETUP
- Bovine serum albumin (810-661, ICN)
- 2.5% Trypsin (15090-046, Invitrogen)
- 0.25% Trypsin/1 mM EDTA solution (25200-056, Invitrogen)
- 0.5% Trypsin/5.3 mM EDTA solution (25300-054, Invitrogen), see REAGENT SETUP
- Gelatin (G1890, Sigma), see REAGENT SETUP
- G418 sulfite, 50 mg/ml solution (10131-035, Invitrogen)
- Puromycin (P7255, Sigma), see REAGENT SETUP
- Blastocidin S hydrochloride (KK-400, Funakoshi, Japan) , see REAGENT SETUP
- Virapower Lentiviral expression system (K4990-00, Invitrogen)
- Eugene 6 transfection reagent (1 814 443, Roche)
- Lipofectamine 2000 (11668-019, Invitrogen)
- OPTI-MEM I (31985-062, Invitrogen)
- Hexadimethrine Bromide (Polybrene; 17736-44, Nacalai tesque, Japan) , see REAGENT SETUP
- Acetamide (015-00115, WAKO, Japan), see REAGENT SETUP
- Propylene glycol (164-04996, WAKO, Japan)
- Dimethyl sulfoxide (D2650, Sigma)
- Human ES medium, see REAGENT SETUP
- SNL medium, see REAGENT SETUP
- 293FT medium, see REAGENT SETUP
- FP medium (for fibroblasts and Plat-E cells) , see REAGENT SETUP

- Povidone iodine (Isodine solution, Meiji Seika, Japan)
- Lidocaine (1% Xylocaine, Astrazeneka)

## **2. REAGENT SETUP**

### **Gelatin-coated culture dishes**

Gelatin stock is prepared as 10 x concentration (1% w/v) stocks. Dissolve 1 g of gelatin powder in 100 ml of distilled water, autoclave and store at 4°C. To prepare 0.1% (1 x) gelatin solution, thaw the 10 x gelatin stock by microwave, add 50 ml of this to 450 ml of distilled water. Filter the solution with a bottle-top filter (0.22 µm) and store at 4°C.

To coat a culture dish, add enough volume of 0.1% gelatin solution to cover the entire area of the dish bottom. For example, 1, 3 or 5 ml of gelatin solution is used for a 35, 60 or 100 mm dish, respectively. Incubate the dish for at least 30 min at 37°C. Before using, aspirate excess gelatin solution.

### **0.05% Trypsin / 0.53 mM EDTA solution**

To prepare 0.05% Trypsin / 0.53 mM EDTA, dilute 10 ml of 0.5% Trypsin/5.3 mM EDTA solution with 90 ml of PBS. To prepare 0.1% Trypsin/1 mM EDTA, add 20 ml of 0.5% Trypsin/5.3 mM EDTA to 80 ml of PBS. Aliquot and store at -20°C.

### **2 x Freezing medium**

Mix 2 ml of DMSO, 2ml of FBS and 6 ml of DMEM, and sterilize through a 0.22 µm filter.

**Puromycin**

Dissolve in distilled water at 10 mg/ml and sterilize through a 0.22  $\mu$ m filter. Aliquot and store at -20°C.

**Blasticidin S hydrochloride**

Dissolve in distilled water at 10 mg/ml and sterilize through a 0.22  $\mu$ m filter. Aliquot and store at -20°C.

**Hexadimethrine Bromide (Polybrene)**

Dissolve 0.8 g of polybrene in 10 ml of distilled water for a 10 x stock (80 mg/ml). Dilute 1 ml of 10 x stock solution with 9 ml of distilled water, and filter with a 0.22  $\mu$ m filter. Store at 4°C.

**Recombinant basic fibroblast growth factor, human**

To prepare PBS containing 0.1% bovine serum albumin (BSA), add 50  $\mu$ l of 10% BSA into 5 ml of PBS. Dissolve 50  $\mu$ g of bFGF in 5 ml of PBS containing 0.1% BSA (10  $\mu$ g/ml). Aliquot and store at -20°C.

**Y-27632**

Dissolve 5 mg of Y-27632 in 1.48 ml of distilled water (10 mM). Aliquot and store at -20°C.

**1 mg/ml collagenase IV**

Dissolve 10 mg of collagenase IV in 10 ml of distilled water, and through with a 0.22  $\mu$ m pore filter. Aliquot and store at -20°C.

**0.1 M CaCl<sub>2</sub>**

Dissolve 0.11 g of CaCl<sub>2</sub> in 10 ml of distilled water, and through with a 0.22  $\mu$ m pore filter. Store at 4°C.

**CTK solution**

Add 5 ml of 2.5% trypsin, 5 ml of 1 mg/ml collagenase IV, 0.5 ml of 0.1 M CaCl<sub>2</sub> and 10 ml of KSR into 30 ml of distilled water. Store at -20°C. Avoid repeated freezing and thawing.

**10 M acetamide**

Dissolve 5.9 g of acetamide in 10 ml of distilled water. Store at room temperature.

**DAP213 solution**

Add 1.43 ml of DMSO, 1 ml of 10 M of acetamide and 2.2 ml of propylene glycol into 5.37 ml of human ES medium. Store at -80°C.

**Human ES medium**

DMEM/F12 containing 20% KSR, 2 mM L-glutamine,  $1 \times 10^{-4}$  M non essential amino acids,  $1 \times 10^{-4}$  M 2-mercaptoethanol, and 50 units and 50 mg/ml penicillin and streptomycin. To prepare 500 ml of the medium, mix 100 ml of KSR, 5 ml of

L-glutamine, 5 ml of non-essential amino acids, 1 ml of 2-mercaptoethanol and 2.5 ml of penicillin/streptomycin, and then fill to 500 ml with DMEM/F12. Add 0.2 ml of 10 µg/ml basic fibroblast growth factor (bFGF) into 500 ml of the medium before use. Store at 4°C up to a week.

### **293FT medium**

DMEM containing 10% FBS, 2 mM L-glutamine,  $1 \times 10^{-4}$  M non essential amino acids, 1 mM sodium pyruvate, and 50 units and 50 mg/ml penicillin and streptomycin. To prepare 500 ml of the medium, mix 50 ml of FBS, 5 ml of L-glutamine, 5 ml of non-essential amino acids, 5 ml of sodium pyruvate and 2.5 ml of penicillin/streptomycin, and then fill to 500 ml with DMEM. Store at 4°C up to a week. Add 0.1 ml of 50 mg/ml G418 into 10 ml 293FT medium.

### **SNL medium**

DMEM containing 7% FBS, 2 mM L-glutamine, and 50 units and 50 mg/ml penicillin and streptomycin. To prepare 500 ml of the medium, mix 35 ml of FBS, 5 ml of L-glutamine and 2.5 ml of penicillin/streptomycin, and then fill up to 500 ml with DMEM. Store at 4°C up to a week.

### **FP medium (for fibroblasts and Plat-E cells)**

DMEM containing 10% FBS, and 50 units and 50 mg/ml penicillin and streptomycin. To prepare 500 ml of FP medium, mix 50 ml of FBS and 2.5 ml of penicillin/streptomycin, and then fill up to 500 ml with DMEM. Store at 4°C up to a



week. For Plat-E cells, add 1  $\mu$ l of 10 mg/ml puromycin stock and 10  $\mu$ l of 10 mg/ml blastcidin S into 10 ml of FP medium.

### **3. EQUIPMENT**

- Needle
- Thread (4-0 or 5-0 nylon)
- Scissors
- Tweezers
- Forceps
- 100 mm tissue culture dish (353003, Falcon)
- 6-well tissue culture plate (353046, Falcon)
- 24-well tissue culture plate (353047, Falcon)
- 96-well tissue culture plate (351172, Falcon)
- 15 ml conical tube (352196, Falcon)
- 50 ml conical tube (352070, Falcon)
- 1 ml plastic disposable pipette (357520, Falcon)
- 5 ml plastic disposable pipette (357543, Falcon)
- 10 ml plastic disposable pipette (357551, Falcon)
- 25 ml plastic disposable pipette (357525, Falcon)
- 0.22  $\mu$ m pore size filter (Millex GP, SLGP033RS, Millipore)
- 0.45  $\mu$ m pore size cellulose acetate filter (FP30/0.45 CA-S, Schleicher & Schuell)
- 10 ml disposable syringe (SS-10ESZ, Terumo, Japan)
- Coulter counter (Z2, Beckman Coulter)

- CO<sub>2</sub> incubator
- Cell scraper (9000-220, IWAKI)
- Pipette aid (Falcon)
- Pipetman and tips (GILSON)
- 2 ml cryovials (72. 694. 006, SARSTEDT)

## **4. PROCEDURE**

### **4.1. Preparation of fibroblasts (biopsy as an example in this protocol)**

Prepare primary human fibroblasts from skin biopsy specimens or purchase cells from vendors.

**CAUTION:** Prior to skin biopsy, proper informed consent should be obtained from donors.

#### **4.1.1. Biopsy**

1. Disinfect skin surface three times with povidone iodine.
2. Administer lidocaine as a local anesthesia.
3. Push and twist a disposable biopsy punch against the skin. Deep enough to reach the fatty layer of subcutaneous tissue.
4. Raise the biopsy punch and cut the tissue away from the donor.
5. Using tweezers, put the tissue into a 50 ml conical tube containing 10 ml of FP medium.
6. Suture the incision.

**4.1.2. Establishment of human skin fibroblasts;** TIMING 1 month

7. Transfer the skin biopsy sample to a 100 mm dish, and cut it into 1 mm pieces by using sterile scissors.
8. Transfer them to a 35 mm dish (or a well of 6-well plate) with sterile forceps.
9. Put the coverslip onto the tissues, and then add 2 ml of FP medium into the dish.
10. Incubate at 37°C, 5% CO<sub>2</sub>. Leave the dish untouched for a week, and later change the medium twice a week.

**NOTE:** Generally, you may be able to see the fibroblastic cells at the edge of tissues under the coverslip.

11. When cells cover 30~50% of the dish, it's time to transfer the cells to a larger dish (3~4 weeks from started).

**4.1.3. Passage of fibroblasts;** TIMING 0.5 hour

12. Aspirate the medium and wash once with PBS.
13. Add 0.3 ml of 0.05% Trypsin / 0.53 mM EDTA, and incubate the dish at 37°C for 10 minutes.
14. Neutralize by addition of 3 ml of FP medium, and transfer the cell suspension to a 15 ml conical tube.
15. Count the cells and plate them at  $3 \times 10^5$  cells to per 60 mm dish. When the cells become confluent, passage the cells to a 100 mm dish in the same manner.

**NOTE:** If the cell number is greater than  $5 \times 10^5$ , plate the cells onto a 100 mm dish. If it is lower, seed the cells onto a 60 mm dish.

**4.1.4. Making frozen stocks of fibroblasts;** TIMING 0.5 hour

When fibroblasts become confluent on a 100 mm dish, you should make frozen stocks.

16. Aspirate off the medium and wash with PBS.
17. Add 1 ml of 0.05% Trypsin/0.53 mM EDTA, and incubate the dish at 37°C for 10 min.
18. Add 9 ml of FP medium, suspend by pipetting up and down, and transfer the cell suspension to a 15 ml conical tube.
19. Count cell number, and spin the cells at 160 *g* for 5 minutes.
20. Aspirate off the supernatant, and add appropriate amount of FP medium to adjust concentration to  $1 \times 10^6$  cells/ml.
21. Add same amount of 2 x freezing medium to cell suspension, mix gently by pipetting up and down.
22. Aliquot 1 ml of cell suspension to per cryovial.
23. Transfer the tubes into a freezing container, and keep it in a -80°C freezer overnight.

**PAUSE POINT:** Fibroblasts can be stored at -80°C in short term. For long term storage, keep them in the gas phase of a liquid nitrogen tank.

**4.1.5. Thawing fibroblasts;** TIMING 0.5 hour

If you use fibroblasts obtained from cell banks or vendors, here is a starting point.

24. Prepare 9 ml of FP medium in a 15 ml conical tube.
25. Take a vial of frozen fibroblasts from the liquid nitrogen tank and put the vial into 37°C water bath until most (but not all) cells are thawed.

26. Wipe the vial with ethanol, open the cap, and transfer the cell suspension to the tube prepared in step 24.
27. Centrifuge at 160 *g* for 5 minutes, and then discard the supernatant.
28. Re-suspend the cells with 10 ml of FP medium, and transfer to a 100 mm dish (at least  $5 \times 10^5$  cells/dish). Incubate the cells in a 37°C, 5% CO<sub>2</sub> incubator, until the cells become 80~90% confluent. Change the medium every other day.

**4.1.6.Passaging fibroblasts;** TIMING 0.5 hour

29. Discard the medium and wash the cells once with PBS.
30. Aspirate PBS, add 1 ml per dish of 0.05% trypsin/0.53 mM EDTA, and incubate for 10 minutes at 37°C.
31. Add 9 ml of FP medium, and break up the cells into a single cell suspension by pipetting up and down several times.
32. Adjust the cell suspension to 40 ml by addition of FP medium, and transfer to dishes (10 ml per 100 mm dish). This splits the cells 1:4. Incubate the cells at 37°C, 5% CO<sub>2</sub> until the cells become 80~90% confluent (approximately  $2 \times 10^6$  cells per 100 mm dish, Fig. 2a). It commonly takes 4~5 days after passage.

**CRITICAL STEP:** We recommend 1:3~1:4 split ratio. Lower density would induce replicative senescence. Older fibroblasts may not be suitable for generation of iPS cells, because transduction efficiency of retrovirus is quite low (Fig. 2b).

## 4.2. Lentivirus production

CAUTION: The experiment using lentivirus should be performed in a safety cabinet. Wear a designated lab coat and gloves. Wastes should be treated with hypochlorous acid and following ethanol, and then autoclaved.

### 4.2.1. Passaging 293FT cells; TIMING 0.5 hour

33. Aspirate the medium and wash the cells with PBS.
34. Add 1 ml of 0.25% trypsin/1 mM EDTA and incubate the dish for 2 min at room temperature.
35. Add 10 ml of the medium and dissociate the cells by pipetting up and down (about 10 times)
36. Collect the cell suspension into a 15 ml conical tube, and count the cell number.
37. Adjust the concentration to  $4 \times 10^5$  cells per milliliter with 293FT medium without antibiotics. Seed cells at  $4 \times 10^6$  cells (10 ml) per 100 mm dish, and incubate overnight at 37°C, 5% CO<sub>2</sub>.

### 4.2.2. Transfection to 293FT cells; TIMING 1 hour

38. Dilute 9 µg of Virapower packaging mix (pLP1, pLP2 and pLP/VSVG mixture) and 3 µg of pLenti6/UbC encoding the mouse *Slc7a1* gene in 1.5 ml of OPTI-MEM I, and mix gently by finger tapping.
39. In a separate tube, dilute 36 µl of Lipofectamine 2000 in 1.5 ml of OPTI-MEM I. Mix gently by finger tapping and incubate for 5 min at room temperature.
40. After incubation, combine the diluted DNA with the diluted Lipofectamine 2000. Mix gently by finger tapping and incubate for 20 min at room temperature.

41. During incubation, remove the medium from 293FT dishes, and add 9 ml of fresh medium to each dish.
42. Then add 3 ml of the DNA-Lipofectamine 2000 complexes in each dish. Mix gently by rocking the dish back and forth. Incubate the dish overnight at 37°C, 5% CO<sub>2</sub>.
43. Twenty four hours after transfection, aspirate the medium containing the transfection cocktail, and add 10 ml of fresh FP medium. Incubate the dish overnight at 37°C, 5% CO<sub>2</sub>.

**4.2.3. Collection of virus-containing supernatant;** TIMING 0.5 hour

44. Forty eight hours after transfection, collect the supernatant of the 293FT culture with a 10 ml disposable syringe, and then filtrate it with a 0.45 µm pore size cellulose acetate filter.

**PAUSE POINT:** The lentivirus-containing medium can be stored at -80°C. Do not repeat freeze/thaw cycles to avoid the reduction of viral titer.

## **4.3. Lentiviral infection**

**4.3.1. Seeding fibroblasts;** TIMING 1 hour

45. Aspirate the medium and wash the cells with PBS.
46. Add 1 ml of 0.05% trypsin/0.53 mM EDTA and incubate the dish for 10 min at 37°C.
47. Add 9 ml of FP medium and dissociate the cells by pipetting up and down.
48. Collect the cell suspension into a conical tube. Count the cell number. Adjust the concentration to  $8 \times 10^4$  cells/ml by adding appropriate volume of FP

medium.

49. Seed the cells at  $8 \times 10^5$  cells (10 ml of cell suspension) per 100 mm dish.

Incubate overnight at 37°C, 5% CO<sub>2</sub>.

#### **4.3.2. Transduction of fibroblasts;** TIMING 0.5 hour

50. Replace medium with 10 ml/dish of the virus-containing supernatant, supplemented with 4 µg/ml polybrene. Incubate the dishes for 5 hours to overnight at 37°C, 5% CO<sub>2</sub> (See TROUBLE SHOOTING).

51. Twenty four hours after transduction, aspirate off the virus-containing medium, wash cells once with 10 ml of PBS (optional), and add 10 ml of fresh FP medium.

**CRITICAL POINT:** Three to 5 days after infection, transgene expression of lentivirus should reach the maximum level.

**PAUSE POINT:** You may use the cells immediately or freeze them at -80°C for storage.

### **4.4. Preparation of SNL feeder cells**

#### **4.4.1. Thawing SNL cells;** TIMING 0.5 hour

52. Prepare 9 ml of SNL medium in a 15 ml conical tube.

53. Remove a vial of frozen SNL cells from the liquid nitrogen tank and put the vial into 37°C water bath until most (but not all) cells thawed.

54. Wipe the vial with ethanol, open the cap, and transfer the cell suspension to the tube prepared in step 52. Pipet up and down several times to mix.

55. Centrifuge at 160 g for 5 minutes, and then discard the supernatant.

56. Re-suspend the cells with 10 ml of SNL medium, and transfer to a



gelatin-coated 100 mm dish. Incubate the cells in a 37°C, 5% CO<sub>2</sub> incubator, until the cells become 80~90% confluent. This usually take 3~4 days.

**CRITICAL STEP:** Do not let the cells get over-confluent, or their ability as feeder cells might deteriorate.

#### **4.4.2. Passage of SNL cells;** TIMING 0.5 hour

57. Discard medium and wash the cells once with PBS.
58. Aspirate PBS, and add 0.5 ml per 100 mm dish of 0.25% trypsin/1 mM EDTA, and incubate for 1 minute at room temperature.
59. Add 4.5 ml of SNL medium, and break up the clumps of cells into a single cell suspension by pipetting up and down several times.
60. Adjust the volume to 160 ml by adding SNL medium, and transfer to gelatin-coated dishes (10 ml per 100 mm dish). This splits the cells 1:16. Incubate the cells at 37°C, 5% CO<sub>2</sub> until the cells become 80-90% confluent. This should take 3~4 days after passage.

#### **4.4.3. Mitomycin C-inactivation of SNL cells;** TIMING 3 hours

61. Add 0.3 ml of 0.4 mg/ml mitomycin C solution directly to the culture medium of SNL dish, swirl it briefly, and incubate 2.25 hours at 37°C, 5% CO<sub>2</sub>. The final concentration of mitomycin C will be 12 µg/ml.
62. After incubation, aspirate the mitomycin C-containing medium off the cells, and wash the cells twice with 10 ml of PBS.
63. Aspirate off PBS, add 0.5 ml of 0.25% trypsin/1 mM EDTA, swirl to cover the entire surface, and let sit for 1 minute at room temperature.

64. Neutralize the trypsin by adding 5 ml of SNL medium, and break up the clumps of cells to a single cell suspension by pipetting up and down. Pour the cell suspension into a 50 ml conical tube and count the cell number. Seed the cells at  $1.5 \times 10^6$  cells per 100 mm dish, or at  $2.5 \times 10^5$  cells per well of 6-well plate.
65. Cells should be well spread with space between each other. They should become ready for usage of the next day.

**PAUSE POINT:** The mitomycin C-treated SNL dishes can be left for up to three days before use. In addition, you can make frozen stocks of mitomycin C-treated SNL cells with a standard technique at  $-80^{\circ}\text{C}$  or in vapor phase of liquid nitrogen tank. These stocks should be woken up in a gelatin-coated dish or plate within the 3 days before use.

**CRITICAL POINT:** Old SNL feeder (over three days after mitomycin C-treatment) may come off from the dish during over three weeks of iPS cell generation.

## **4.5. Preparation of Plat-E cells**

### **4.5.1. Thawing Plat-E cells; TIMING 0.5 hour**

66. Prepare 9 ml of FP medium in a 15 ml conical tube.
67. Take out a vial of frozen stocks from the liquid nitrogen tank and put the vial in a  $37^{\circ}\text{C}$  water bath until most (but not all) cells are thawed.
68. Wipe the vial with ethanol, open the cap, and transfer the cell suspension to a tube prepared in step 66.
69. Centrifuge at  $180\text{ g}$  for 5 minutes, and then discard the supernatant.
70. Resuspend the cells with 10 ml of FP medium, and transfer to a 100 mm dish. Incubate the cells in a  $37^{\circ}\text{C}$ , 5%  $\text{CO}_2$  incubator.

71. Next day, replace the medium with new media supplemented with 1 µg/ml of puromycin and 10 µg/ml of blasticidin S. Continue to incubate the cells in a 37°C, 5% CO<sub>2</sub> incubator until 80~90% confluent.

**4.5.2. Passage of Plat-E cells;** TIMING 0.5 hour

72. Wash the cells with PBS, add 4 ml per dish of 0.05% trypsin/0.53 mM EDTA, and incubate for 1 minute at room temperature. Detach cells from dishes by tapping, resuspend with 10 ml FP medium, and transfer to a 15 ml conical tube. Centrifuge it at 180 *g* for 5 minute, and aspirate the supernatant.
73. Add appropriate volume of FP medium, and break up the cells into a single cell suspension by pipetting up and down several times. Seed them on new 100 mm dishes at 1:4~1:6 dilution. Cells should become confluent within 2-3 days.

**4.6. Generation of iPS cells**

**4.6.1. Day 1 Plat-E preparation;** TIMING 1 hour

74. Wash the cells with PBS, add 4 ml of 0.05% trypsin/0.53 mM EDTA, and incubate for 1 minute at room temperature.
75. After incubation, add 10 ml FP medium into the Plat-E dish, suspend the cells by gently pipetting, and transfer the cell suspension to a 50 ml conical tube. FP culture medium used in this period contains neither puromycin nor blasticidin S.
76. Centrifuge the cells at 180 *g* for 5 minutes.
77. Discard the supernatant, break the pellet by finger tapping, and resuspend the cells in an appropriate amount of FP medium.
78. Count cell number and adjust the concentration to  $3.6 \times 10^5$  cells/ml with FP

medium.

79. Seed cells at  $3.6 \times 10^6$  cells (10 ml) per 100 mm dish, and incubate overnight at 37°C, 5% CO<sub>2</sub>.

**4.6.2. Day 2 Retrovirus production; Transfection into Plat-E cells;** TIMING 1 hour

80. Transfer 0.3 ml of OPTI-MEM I into a 1.5 ml tube.
81. Deliver 27 µl of Fugene 6 transfection reagent into the prepared tube in step 80, mix gently by finger tapping, and incubate for 5 minutes at room temperature.
82. Add 9 µg of pMXs plasmid DNA (respectively encoding Oct3/4, Sox2, Klf4, and c-Myc) drop-by-drop into the Fugene 6/OPTI-MEM I containing tube, mix gently by finger tapping, and incubate for 15 minutes.

**CRITICAL POINT:** Introduce one plasmid into one dish. Transfection of more than two plasmids into a dish causes reduction of efficiency of iPS cell generation.

83. Add the DNA/Fugene 6 complex drop-wise into the Plat-E dish, and incubate overnight at 37°C, 5% CO<sub>2</sub>.

**CRITICAL STEP:** Also transfect with a suitable control; we use pMXs retroviral vector GFP to monitor transfection efficiency. We routinely obtain efficiency more than 80%. High efficient transfection is crucial for iPS cell induction.

**4.6.3. Day 3 Retrovirus production (continued);** TIMING 0.5 hour

84. Aspirate the transfection reagent-containing medium, add 10 ml of fresh FP medium, and return the cells to the incubator.

**4.6.4.Preparation of fibroblasts;** TIMING 1 hour

85. When human fibroblasts expressing mouse *Slc7a1* gene from Step 51 reach to 80~90% confluence (Fig. 3), aspirate medium and wash once with 10 ml of PBS.
86. Discard PBS, add 1 ml per dish of 0.05% trypsin/0.53mM EDTA, and incubate at 37°C for 10 minutes.
87. Add 9 ml of the FP medium per plate, suspend the cells to a single cell by pipetting, and transfer to a 50 ml conical tube.
88. Count cell numbers, which should be  $\sim 2 \times 10^6$  cells per dish. Adjust the concentration to  $8 \times 10^4$  cells/ml with FP medium. Transfer 10 ml of cell suspension ( $8 \times 10^5$  cells) per 100 mm dish. Incubate the dish overnight at 37°C, 5% CO<sub>2</sub>.

**4.6.5.Day 4 Retroviral infection;** TIMING 0.5 hour

89. Forty eight hours post-transfection, collect the medium from each Plat-E dish by using a 10 ml sterile disposable syringe, filtering it through a 0.45 µm pore size cellulose acetate filter, and transferring into a 15 ml conical tube.
90. Add 5 µl of 8 mg/ml polybrene solution into the filtrated virus-containing medium, and mix gently by pipetting up and down. The final concentration of polybrene is 4 µg/ml.
91. Make a mixture of equal parts of the medium containing OCT3/4-, SOX2-, KLF4- and c-MYC-retroviruses.

**CRITICAL STEP: RETROVIRUSES SHOULD BE USED FRESHLY! DO NOT FREEZE, OR YOU WON'T OBTAIN iPS CELLS.** The titer of retrovirus is critical for iPS cell

generation. The titer of retrovirus used in our study is approximately  $5 \times 10^6$  TU/ml (determined in NIH3T3 cells). The freeze/thaw step decreases the titer of retrovirus.

92. Aspirate the medium from fibroblast dishes, and add 10 ml per dish of the polybrene/virus-containing medium. Incubate the cells from 4 hours to overnight at 37°C, 5% CO<sub>2</sub>.

**4.6.6.Day 5** TIMING 5 min.

93. After 4 hours ~ overnight culture, aspirate the medium from the transduced fibroblasts, and add 10 ml per dish of fresh FP medium.

**4.6.7.Day 6~10** TIMING 5 min each day.

94. Discard the medium, and add 10 ml per dish of FP medium.

CRITICAL POINT: At day 8~10, prepare mitomycin C-treated SNL feeder cells (see step 61-65)

**4.6.8.Day 11 *Replating transduced fibroblasts onto mitomycin C-treated SNL feeder.*** TIMING 1 hour

95. Aspirate the culture medium and wash with 10 ml per dish of PBS.

96. Discard PBS, add 1 ml per dish of 0.05% trypsin/0.53mM EDTA, and incubate at 37°C for 10 minutes.

97. Add 9 ml per dish of FP medium, suspend the cells to a single cell, and transfer to a 50 ml conical tube.

98. Count cell numbers, and adjust the concentration to  $5 \times 10^3$  or  $5 \times 10^4$  cells / ml.

Transfer 10 ml of cell suspension ( $5 \times 10^4$  or  $5 \times 10^5$  cells) per 100 mm dish with mitomycin C-treated SNL cells. Incubate the dishes overnight at 37°C, 5% CO<sub>2</sub> (See TROUBLE SHOOTING).

**4.6.9. Day 12~** TIMING 5 min per day

99. Replace the medium with 10 ml of human ES medium. Change the medium every other day until the colonies become large enough to be picked up. Colonies should become visible 2~3 weeks after the retroviral infection. They can be picked up at around day 30 when they become large enough (visible by the naked eyes, Fig. 4d) ..

**4.6.10.        *Picking up the iPS colonies*;** TIMING 1 hour

100. Aliquot 20 µl of human ES medium per well in 96-well plate.
101. Remove the medium from the dishes with iPS colonies, and add 10 ml per dish of PBS.
102. Aspirate PBS, and add 5 ml per dish of PBS.
103. Pick colonies from the dish under the stereomicroscope using a P2 or P10 Pipetman set at 2 µl, and transfer it into the 96-well plate prepared in Step 101. We usually pick up 6~12 colonies for a subject.
104. When colony picking is finished, add 180 µl of human ES medium to each well, and pipet up and down to break up the colony to small clumps (20~30 cells) carefully under the stereomicroscope.
105. Transfer cell suspension into 24-well plates with mitomycin C-treated SNL feeder cells, add 300 µl per well of human ES medium, and incubate in a

37°C, 5% CO<sub>2</sub> incubator until the cells reach 80~90% confluence. This usually takes ~7 days. At this point they should be passaged into 6-well plates.

**CRITICAL POINT:** Do not break up the cell clumps to single cells (See also TROUBLE SHOOTING).

**4.6.11.      *Passaging of iPS cells*; TIMING 0.5 hour**

106. Aspirate culture medium, and wash the cells with 0.5 ml per well of PBS.
107. Completely remove PBS, add 0.1 ml per well of CTK solution and incubate at 37°C for 2~5 minutes. When feeder cells are removed from the plate while iPS colonies still attach, go to Step 108.

**CAUTION:** Incubation time may depend on cell density. Please check the cells by your eyes every minute.

108. Aspirate off CTK solution, and add 0.5 ml per well of PBS.
109. Aspirate off PBS, and add 0.5 ml of PBS.
110. Remove PBS completely. Most of feeder cells can be removed by these PBS washings. Add 0.5 ml of human ES medium and suspend the cells to small clumps of 20~30 cells by pipetting up and down.
111. Transfer the cell suspension to a well of 6-well plates with mitomycin C-treated SNL feeder cells, add 1.5 ml of human ES medium, and incubate in a 37°C, 5% CO<sub>2</sub> incubator until cells become 80~90% confluent in 6-well plates. Change the medium every day.

**4.6.12.      *Expansion of iPS cells*; TIMING 0.5 hour**

112. Aspirate the medium, and wash the cells with 2 ml/well of PBS.



113. Remove PBS completely, add 0.5 ml per well of CTK solution and incubate at 37°C for 2~5 minutes.

**CAUTION:** Incubation time may depend on cell density. Please check the cells by your eyes every minute.

114. Aspirate off CTK solution, and add 2 ml per well of PBS.
115. Aspirate off PBS, and add 2 ml per well of PBS.
116. Remove PBS completely, add 2 ml of human ES medium.
117. Detach iPS cells by using cell scraper and break up the colonies to small clumps by pipetting up and down.
118. Transfer the cell suspension to a 60 mm dish with SNL feeder. Add 2 ml human ES medium, and incubate in a 37°C, 5% CO<sub>2</sub> incubator until cells reach 80~90% confluence. The cells should be passaged to a 100 mm dish with the same manner.

**4.6.13. Preparation of freeze stock;** TIMING 0.5 hour

When iPS cells reach 80~90% confluence in 100 mm dishes, frozen stocks should be prepared.

119. Before making frozen stocks, add 10 µl of 10 mM Y-27632, which is a specific inhibitor of p160-Rho-associated coiled-coil kinase (ROCK), to each dish (final concentration, 10 µM), and incubate at 37°C for 1 hour.
120. Aspirate the medium, and wash the cells with 10 ml of PBS.
121. Remove PBS thoroughly, add 1 ml of CTK solution and incubate at 37°C for 2~5 minutes.

**CAUTION:** Incubation time may depend on cell density. Please check the cells by your eyes every minute.

122. Aspirate off CTK solution, and wash twice with 10 ml per dish of PBS.
123. Remove PBS thoroughly. Add 12 ml of human ES medium. Detach colonies from dish by using cell scraper and transfer cell suspension to three 15 ml conical tubes (4 ml each).
124. Spin at 160 *g* for 5 minutes.
125. Remove the supernatant.
126. Suspend the pellet in 0.2 ml per tube of DAP213 solution by several times of pipetting.

**CAUTION:** Do not break up the colonies.

127. Transfer 0.2 ml of the cell suspension to freeze vials.
128. Put the vials quickly into liquid nitrogen.

**CRITICAL STEP:** Step 126~128 should be performed within 15 seconds for good cell viability.

**PAUSE POINT:** For long-term storage, store frozen cells in the gas phase of a liquid nitrogen tank.

#### **4.6.14.        *Waking up freeze stock*; TIMING 0.5 hour**

129. Prepare 10 ml of pre-warmed (37°C) human ES medium in 15 ml conical tubes
130. Take frozen stocks of iPS cells out from liquid nitrogen tank.
131. Add 0.8 ml of human ES medium prepared in Step 129 into each vial of frozen stocks, and thaw quickly by pipetting up and down for 2~3 times

with P1000 pippetman.

132. Transfer the cell suspension to the tube prepared in Step 129.
133. Spin at 160 g for 5 minutes.
134. Aspirate off the supernatant, and add 4 ml of human ES medium.
135. Transfer the cell suspension to 60 mm dishes seeded with mitomycin C-treated SNL feeder, and incubate in a 37°C, 5% CO<sub>2</sub> incubator until cells reach 80~90% confluence. Do not move the dish for 48 hours. Change the medium every day from day 2.

**CRITICAL POINT:** For the viability of the iPS cells, Step 130~132 should be as quick as possible. Do not break up cell clumps to single cells.

## **TROUBLESHOOTING**

### **PROBLEM**

Fibroblasts die or do not grow well after lentiviral transduction.

### **SOLUTION**

In some cases, lentivirus is toxic to fibroblasts. This problem should be overcome by doubling dilution of the supernatant with the fresh medium or shorting exposure time from overnight to 5 hours.

### **PROBLEM**

No ES-like colonies appear from fibroblast cultures after induction by the four factors.

### **SOLUTION**

Good preparation of retroviruses is ESSENTIAL. Viruses must be prepared freshly.

DO NOT FREEZE.

Passage numbers of fibroblast is also critical for iPS generation. Efficiency of retroviral transduction markedly decreases in older fibroblasts. We recommend using fibroblasts within passage 8 for iPS production.

When seeding retrovirally transduced fibroblasts onto feeder cells, cell density is critical for iPS cells generation. Overgrowth of fibroblasts inhibits generation of iPS cell colonies. On the other hand, if the cell number is too low, no colonies emerge. Optimal cell densities are different for each fibroblast culture. Two to three different densities should be tested.

#### PROBLEM

Isolated clone has an abnormal karyotype or is not pluripotent.

#### SOLUTION

We recommend to expand multiple clones.

#### PROBLEM

Characters and potentials of iPS cell change during culture.

#### SOLUTION

Frozen stocks should be prepared at early passages (within passage number 15).

#### PROBLEM

Cells detach from the bottoms of culture dishes during iPS cell generation.

#### SOLUTION

This can be caused by two reasons. First, feeder cells may be too old. We recommend to use SNL feeder cells within 3 days after mitomycin C-treatment.

Another possibility is that cell density may be too high when reseeding transduced cell onto feeder cells. Overgrowth of fibroblasts may result in peeling off from the edge of the dish.

#### PROBLEM

The viability of iPS cells is too low after passage or thawing. .

#### SOLUTION

Dissociation to single cells decreases the viability of iPS cells. Break colonies to small clumps of 20~30 cells.

#### PROBLEM

iPS cells spontaneously differentiate.

#### SOLUTION

In some iPS cells can be unstable during early passages. During the passaging procedures, you can remove the differentiated colonies by aspirating and transfer only undifferentiated colonies to a new dish. In addition, the qualities of feeder cells, such as density and freshness, are quite important

### **ANTICIPATED RESULTS**

When 293FT cells produced lentivirus, they become multinucleated (Fig. 3a, b). More than 90% of human fibroblasts can be infected with this lentivirus preparation (Fig. 3 c, d). And the transduction efficiency of subsequent retroviral infection can reach to more than 60% (Fig. 3e, f). The infection efficiency can be evaluated by analyzing GFP-infected cells with a flow cytometer. The morphology of

human dermal fibroblasts starts to change ~6 days after the introduction of *OCT3/4*, *SOX2*, *KLF4* and *c-MYC* (Fig. 4a).

From  $5 \times 10^4$  adult human fibroblasts that are commercially available (Cell applications Inc.), we usually observe more than 100 of non ES-like colonies around day 10 after retroviral transduction (Fig. 4b). In contrast, ~10 ES-like colonies emerge from  $5 \times 10^4$  fibroblasts by day 30 after retroviral infection (Fig. 4c, d). However, these numbers vary greatly among fibroblast cultures, since they are different in proliferation, retroviral transduction efficiency, and iPS cell generation efficiency. If the density of fibroblasts or the efficiency of iPS cell generation is too high, the dishes will be covered with numerous non-ES like cells and no iPS cell colony emerges (Fig. 4e).

Most, although not all, of clones can be established as iPS cell lines, which show similar proliferation, morphology, and gene expression to human ES cells (Fig. 4f). The average success rate is 60~100%. Most clones also express undifferentiated ES cell surface antigen, SSEAs and TRAs, and are positive for alkaline phosphatase activity. Differentiation potentials of human iPS cells can be evaluated by the methods established for human ES cells, such as embryonic body formation and teratoma experiments.

CTK solution developed by Dr. Suemori's group is suitable for passaging human iPS cells<sup>3</sup>. The treatment makes feeder cells detach from the culture plate faster than iPS cells (Fig. 4g). Simple washing with PBS can remove floating feeder cells (Fig. 4h). After washing, remaining human iPS cells can be collected by pipetting (Fig. 4i). We routinely passage human iPS cells once a week at a ratio of 1:3 or 1:4 (Fig. 4 j, k, l). We normally prepare three vials of frozen stocks per 100

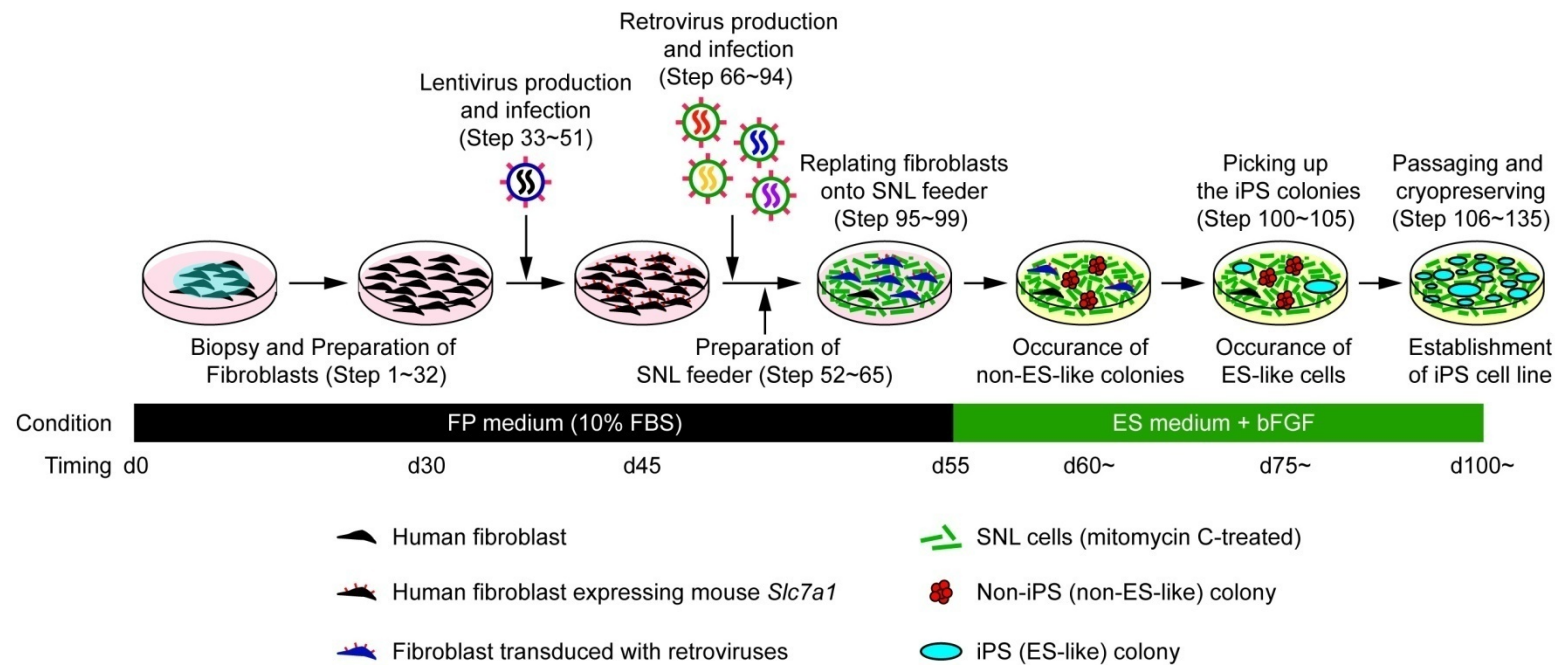
mm dish, with DAP213 solution<sup>3</sup>. Pretreatment with Y-27632 improves viability<sup>4</sup>. When one vial of stock is waken up on a 60 mm dish with mitomycin C-treated SNL feeder, cells become confluent in ~7 days. In addition to SNL feeder cells, human iPS cells can be maintained on mouse embryonic fibroblasts (MEF).

## **ACKNOWLEDGEMENT**

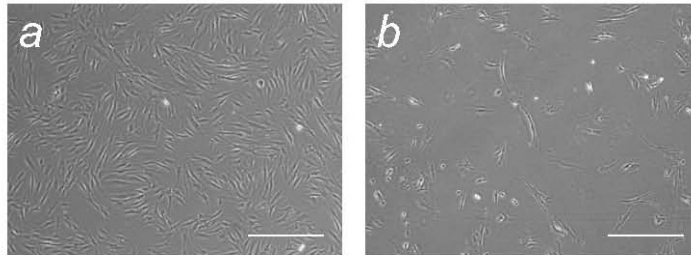
This protocol is originally prepared by Dr. Kazutoshi Takahashi at CiRA and PhD student Koji Tanabe at Stem Cell Biology, and modified by Research Management Office of CiRA, Kyoto University.

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**Figure 1****Figure 1. Schematic diagram of human iPS cell generation**

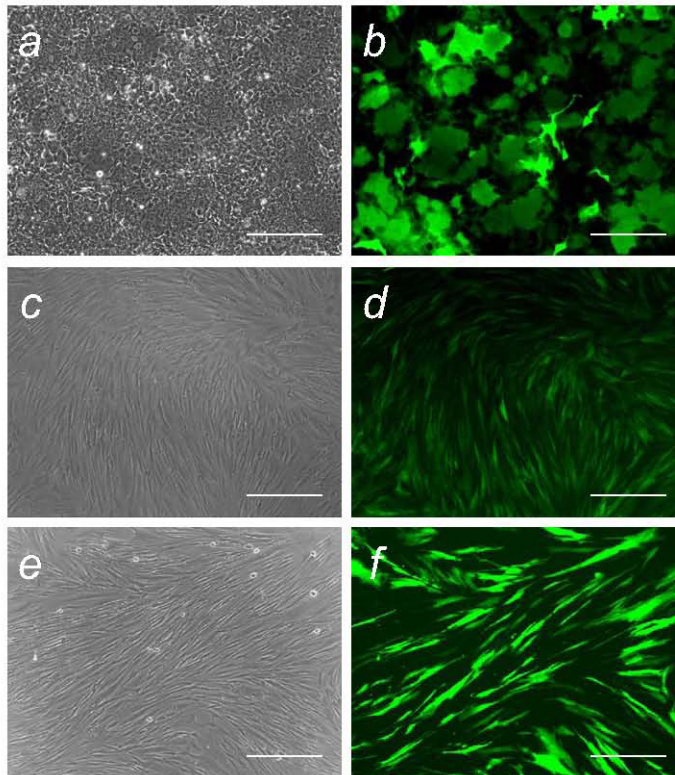


**Figure 2**

**Figure 2.**  
**Morphologies of human dermal fibroblasts**  
**used for iPS cell induction.**

**a.** Good fibroblast preparation suitable for iPS cell generation.

**b.** Cell undergoing senescence, with low transduction efficiency by retroviruses. Bar indicates 200  $\mu\text{m}$

**Figure 3****Figure 3.****Lentiviral and retroviral transduction system for iPS cell generation.**

**a.** Phase contrast image of 293FT cells transfected with plasmids for GFP lentiviruses.

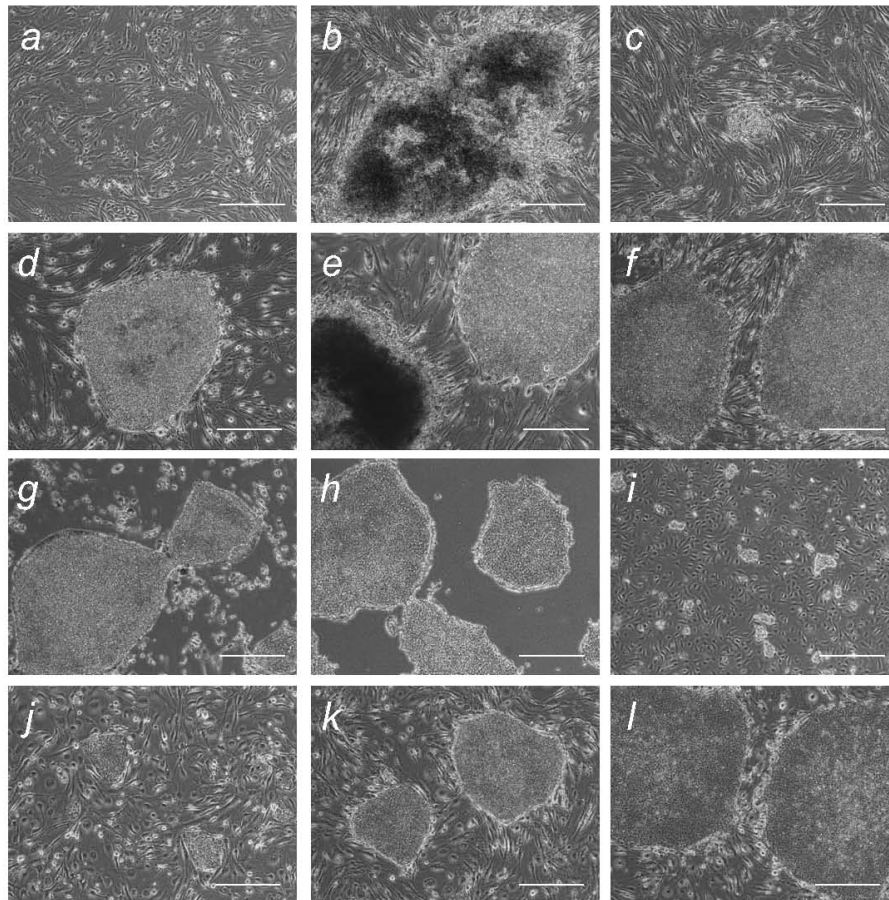
**b.** Fluorescent image of the cells shown in the panel (a).

**c.** Phase contrast image of human dermal fibroblasts infected with GFP lentiviruses.

**d.** Fluorescent image of the cells shown in the panel (c).

**e.** Phase contrast image of human dermal fibroblasts transduced with lentiviruses expressing mouse Slc7a1 gene and then transduced with GFP retroviruses.

**f.** Fluorescent image of the cells shown in the panel (e).  
Bars indicate 100  $\mu\text{m}$ .

**Figure 4****Figure 4.****Human iPS cells from dermal fibroblasts.**

- a.** Human dermal fibroblasts expressing mouse Slc7a1 introduced, day 6 after retroviral transduction of OCT3/4, SOX2, KLF4 and c-MYC.
- b.** Non-ES-like (non-iPS) colony.
- c.** Putative human iPS colony observed at day 20 after retroviral transduction.
- d.** Representative human iPS colony at day30.
- e.** Phase contrast images of iPS and non-iPS colonies in the same field.
- f.** Established human iPS cells at passage number 14.
- g.** iPS cells treated with CTK solution.
- h.** iPS cells after PBS washing.
- i.** iPS cells immediately after passage onto mitomycin C-treated SNL feeder cells.
- j.** iPS cells one day after passage.
- k.** iPS cells at day 3 after passage.
- l.** iPS cells at day 6 after passage. Bars indicate 200  $\mu$ m.