Induction of pluripotent stem cells from fibroblast cultures

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Clinical application of embryonic stem (ES) cells faces difficulties regarding use of embryos, as well as tissue rejection after implantation. One way to circumvent these issues is to generate pluripotent stem cells directly from somatic cells. Somatic cells can be reprogrammed to an embryonic-like state by the injection of a nucleus into an enucleated oocyte or by fusion with ES cells. However, little is known about the mechanisms underlying these processes. We have recently shown that the combination of four transcription factors can generate ES-like pluripotent stem cells directly from mouse fibroblast cultures. The cells, named induced pluripotent stem (iPS) cells, can be differentiated into three germ layers and committed to chimeric mice. Here we describe detailed methods and tips for the generation of iPS cells.

INTRODUCTION

Clinical application of human embryonic stem (ES) cells faces difficulties regarding use of human embryos, as well as tissue rejection after implantation. One way to circumvent these issues is to generate pluripotent cells directly from somatic cells[1]. Two strategies have been developed to this end: nuclear transfer to oocytes and fusion with ES cells. A recent study showed that fertilized eggs can be used for nuclear transfer[2]. Another study showed that fusion-mediated reprogramming is facilitated by overexpressing the transcription factor Nanog in ES cells[3]. However, these methods still require embryos or oocytes to generate pluripotent cells, thus are not free from ethical issues. In addition, fusion-mediated methods require elimination of ES-cell-derived chromosomes.

The fact that somatic cells can be reprogrammed by nuclear transfer into oocytes or by fusion with ES cells suggests that oocytes and ES cells contain factors that induce reprogramming. We hypothesized that factors that played important roles in the maintenance of pluripotency of ES cells also play pivotal roles in the induction of pluripotency in somatic cells. Long-term maintenance of pluripotency in mouse ES cells requires specific expression of transcription factors (e.g., Oct3/4, Sox2 and Nanog), and activation of widely expressed tumor-related genes (e.g., *Stat3*, *c-Myc*, *Klf4* and *β-catenin*)[4–7].

To test these candidates for their pluripotency-inducing activity, we have developed a system in which induction of pluripotency can be detected as marker gene expression (**Fig. 1**). In this system, we

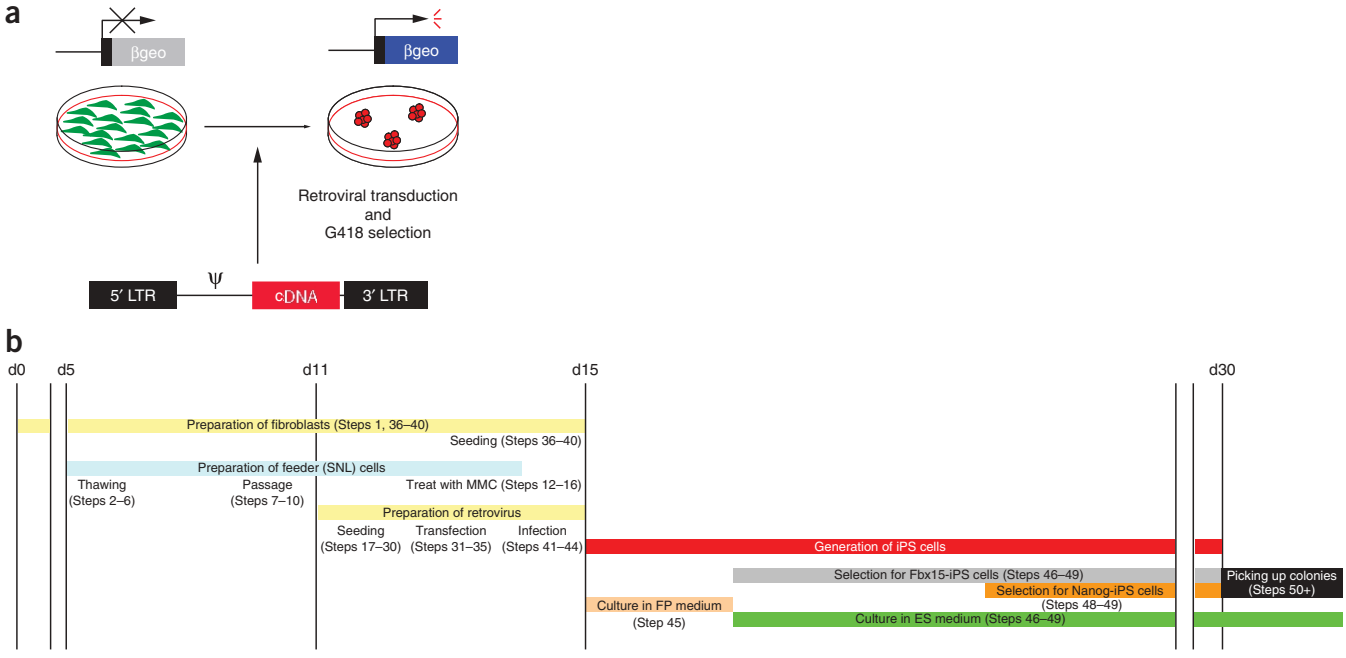

Figure 1 | Schematic diagram of induced pluripotent stem (iPS) cell generation. (**a**) A strategy for iPS cell generation by the four factors. (**b**) Approximate timetable of iPS cell generation. ES cells, embryonic stem cells; LTR, long terminal repeat; MMC, mitomycin C; SNL, Schlegel's nitrogen-limited.

utilized Fbx15 that is specifically expressed in mouse ES cells and early embryos, but is dispensable for self-renewal of ES cells and development[8]. We inserted the βgeo cassette (a fusion of β-galactosidase and the neomycin-resistant gene) into the mouse *Fbx15* genes by homologous recombination. ES cells homozygous for βgeo knock-in (*Fbx15*$^{\beta geo/\beta geo}$) were resistant to an extremely high concentration of G418 (up to 12 mg ml^{-1}), whereas somatic cells derived from *Fbx15*$^{\beta geo/\beta geo}$ mice were sensitive to the selection. We expected that even partial induction of pluripotency would make somatic cells resistant to G418 of normal concentration (0.3 mg ml^{-1}).

We introduced the candidate genes into *Fbx15*$^{\beta geo/\beta geo}$ mouse embryonic fibroblasts (MEFs) by retrovirus-mediated transfection and cultured them in ES cell medium containing G418. With any single factor, we did not obtain G418-resistant colonies. However, by combining four factors (Oct3/4, Sox2, c-Myc and Klf4), we obtained multiple G418-resistant colonies[9]. These cells showed morphology and proliferation similar to ES cells. When transplanted into nude mice, these ES-like cells produced teratomas containing various tissues of the three germ layers. More recently, by using Nanog as selection marker, we were able to induce germline competency by the four factors[10]. These data demonstrated that pluripotent cells can be generated from fibroblast culture with a few defined factors[1].

The generation of induced pluripotent stem (iPS) cells requires only the basic techniques in molecular and cellular biology. It does not require any special equipment or techniques. Here we describe the detailed methods and tips for the generation of iPS cells. **Figure 1b** gives an overview of the main stages of the protocol and illustrates how these stages need to be coordinated over time.

MATERIALS

REAGENTS

- pMXs retroviral vectors and Plat-E packaging cells[11] (see REAGENT SETUP)
- pMXs containing cDNAs of *Oct3/4*, *Sox2*, *Klf4* or *c-Myc* (see REAGENT SETUP)
- *Fbx15*$^{\beta geo/\beta geo}$ mice (see REAGENT SETUP)
- *Nanog*$^{GFP\text{-}IRES\text{-}Puro}$ mice (see REAGENT SETUP) ! CAUTION Experiments involving live rodents must conform to international and institutional regulations.
- SNL feeder cells[12] (see REAGENT SETUP)
- DMEM containing 4.5 g l^{-1} glucose (Nacalai tesque, cat. no. 14247-15)
- PBS without calcium and magnesium (Nacalai tesque, cat. no. 14249-95)
- L-GIn (Invitrogen, cat. no. 25030-081)
- Nonessential amino acid solution (Invitrogen, cat. no. 11140-050)
- 2-Mercaptoethanol (Invitrogen, cat. no. 21985-023)
- Penicillin/streptomycin (Invitrogen, cat. no. 15140-122)
- 0.25% (wt/vol) Trypsin/1 mM EDTA solution (Invitrogen, cat. no. 25200-056)
- 0.5% (wt/vol) Trypsin/5.3 mM EDTA solution (Invitrogen, cat. no. 25300-054) (see REAGENT SETUP)
- Gelatin (Sigma, cat. no. G1890) (see REAGENT SETUP)
- G418 sulfite, 50 mg ml^{-1} solution (Invitrogen, cat. no. 10131-035)
- Puromycin (Sigma, cat. no. P7255) (see REAGENT SETUP)
- Blasticidin S hydrochloride (Funakoshi, cat. no. KK-400) (see REAGENT SETUP)
- Fugene 6 transfection reagent (Roche, cat. no. 1 814 443)
- Hexadimethrine bromide (Polybrene; Nacalai tesque, cat. no. 17736-44) (see REAGENT SETUP)
- Crystal violet (Nacalai tesque, cat. no. 09804-52) (see REAGENT SETUP)
- ES medium (see REAGENT SETUP)
- SNL medium (see REAGENT SETUP)
- FP medium (for fibroblasts and Plat-E cells) (see REAGENT SETUP)
- MF-start medium (ready to use; Toyobo, cat. no. TMMFS-001)
- Methanol

EQUIPMENT

- 100-mm Tissue culture dish (Falcon, cat. no. 353003)
- 6-Well tissue culture plate (Falcon, cat. no. 353046)
- 24-Well tissue culture plate (Falcon, cat. no. 353047)
- 96-Well tissue culture plate (Falcon, cat. no. 351172)
- 1-ml Plastic disposable pipette (Falcon, cat. no. 357520)
- 5-ml Plastic disposable pipette (Falcon, cat. no. 357543)
- 10-ml Plastic disposable pipette (Falcon, cat. no. 357551)
- 25-ml Plastic disposable pipette (Falcon, cat. no. 357525)
- 0.22-μm Pore size filter (Millex GP; Millipore, cat. no. SLGP033RS)
- 0.45-μm Pore size cellulose acetate filter (FP30/0.45 CA-S, Schleicher & Schuell)
- 10-ml Disposable syringe (Terumo, cat. no. SS-10ESZ)
- Dissecting forceps ! CAUTION Sterilize by autoclave.
- Dissecting scissors ! CAUTION Sterilize by autoclave.
- Coulter counter (Z2, Beckman Coulter)
- Freezing container (Nalgene, cat. no. 5100-0001)

REAGENT SETUP

pMXs retroviral vectors and Plat-E packaging cells[11] Available from Dr. Toshio Kitamura at the University of Tokyo (kitamura@ims.u-tokyo.ac.jp). pMXs consists of the 5′-long terminal repeat (LTR) and 3′-LTR of Moloney murine leukemia virus (**Fig. 2a**). Plat-E cells were derived from 293T cells and contain env-IRES-puro^{R} and gag-pol-IRES-bs^{R} cassettes driven by the EF1-α promoter (**Fig. 2b**).

pMXs containing cDNAs of *Oct3/4*, *Sox2*, *Klf4* or *c-Myc* Available from Addgene. We use a pMXs retroviral vector encoding the GFP to monitor tranfection efficiency. It also serves as a negative control for iPS cell induction.

***Fbx15*$^{\beta geo/\beta geo}$ mice** Available from Riken Bioresource Center. The genomic region from exons 3 to 8 of the *Fbx15* gene was replaced with the reporter cassette IRES-βgeo-pA in RF8 ES cells by homologous recombination[8]. The expression of *βgeo* gene is driven by the endogenous *Fbx15* promoter. ES cell clones carrying *Fbx15*$^{\beta geo/+}$ were injected into blastocysts to generate *Fbx15*$^{\beta geo/+}$ and subsequently *Fbx15*$^{\beta geo/\beta geo}$ mice.

***Nanog*$^{GFP\text{-}IRES\text{-}Puro}$ mice** Available from Riken Bioresource Center. The reporter cassette GFP-IRES-Puro^{R} was introduced into 5′-untranslated region of the *Nanog* gene in a bacterial artificial chromosome (BAC)[10]. The modified BAC was linearized and introduced into RF8 ES cells by electroporation. ES cells containing the modified BAC were injected into blastocyst to generate *Nanog*–GFP reporter mice.

SNL feeder cells[12] Available from Dr. Allan Bradley of the Sanger Institute. SNL cells were clonally derived from a STO cell line and stably express a neomycin-resistant cassette and a leukaemia inhibitory factor expression

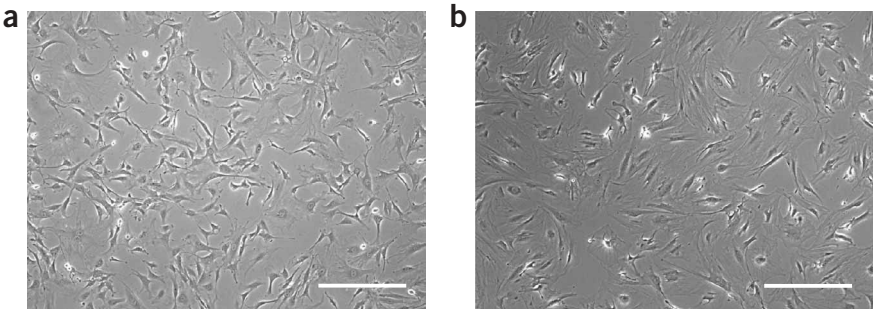

Figure 2 | Morphologies of mouse fibroblasts used for induced pluripotent stem (iPS) cell induction. (**a**) Mouse embryonic fibroblasts (MEFs) derived from E13.5 embryos. (**b**) Tail-tip fibroblasts (TTFs) from 15-week-old mouse. Bars indicate 200 μm. E, embryonic day.

construct[12]. *Note*: SNLP 76/7-4 feeder cell line, which is a puromycin-resistant derivative of SNL, is also available from Dr. Allan Bradley.

Gelatin coating the culture dishes Prepare gelatin stock at 10× concentration (1% wt/vol). Dissolve 1 g of gelatin powder in 100 ml of distilled water, autoclave and store at 4 °C. To prepare 0.1% (1×) gelatin solution, thaw the 10× gelatin stock with a 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.

0.5% Trypsin/5.3 mM EDTA solution To prepare 0.05% trypsin/0.53 mM EDTA, mix 10 ml of 0.5% trypsin/5.3 mM EDTA solution and 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.

Puromycin Dissolve in distilled water at 10 mg ml^{-1} and sterilize through a 0.22-μm filter. Aliquot and store at –20 °C.

Blasticidin S hydrochloride Dissolve in distilled water at 10 mg ml^{-1} and sterilize through a 0.22-μm filter. Aliquot and store at –20 °C.

Hexadimethrine bromide Dissolve 0.8 g of polybrene in 10 ml of distilled water for a 10× stock (80 mg ml^{-1}). Dilute 1 ml of 10× stock solution with 9 ml of distilled water, and filter with a 0.22-μm filter. Store at 4 °C.

Crystal violet Working solution is 0.1% (wt/vol). Dissolve 0.1 g of crystal violet in 100 ml of methanol.

ES medium DMEM containing 15% FBS (vol/vol), 2 mM L-GIn, 1×10^{-4} M nonessential amino acids, 1×10^{-4} M 2-mercaptoethanol, and 50 U and 50 mg ml^{-1} penicillin and streptomycin. To prepare 500 ml of the medium, mix 75 ml of FBS, 5 ml of L-GIn, 5 ml of nonessential amino acids, 1 ml of 2-mercaptoethanol and 2.5 ml of penicillin/streptomycin, and then fill to 500 ml with DMEM. Store at 4 °C for a week.

SNL medium DMEM containing 7% FBS, 2 mM L-GIn, and 50 U and 50 mg ml^{-1} penicillin and streptomycin. To prepare 500 ml of the medium, mix 35 ml of FBS, 5 ml of L-GIn and 2.5 ml of penicillin/streptomycin, and then fill to 500 ml with DMEM. Store at 4 °C for a week.

FP medium (for fibroblasts and Plat-E cells) DMEM containing 10% FBS, and 50 U and 50 mg ml^{-1} 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 to 500 ml with DMEM. Store at 4 °C for a week. For Plat-E cells, add 1 μl of 10 mg ml^{-1} puromycin stock and 10 μl of 10 mg ml^{-1} blastcidin S into 10 ml FP medium.

PROCEDURE

Preparation of fibroblasts

1| Obtain fibroblasts from mouse embryos (option A) or mouse tail-tips (option B). Generally, more iPS colonies are obtained after preparation from embryos.

(A) Preparation of fibroblasts from mouse embryos ● TIMING 15 d

(i) Kill 13.5-d pregnant female mice by cervical dislocation. Isolate uteri and wash with PBS briefly.

(ii) Separate embryos from their placenta and surrounding membranes with forceps. Remove the head, visceral tissues and gonads from isolated embryos.

(iii) Wash embryos by transferring it to a 100-mm dish containing fresh PBS. Hash out the remaining bodies by using a pair of scissors, transfer into a 50-ml conical tube containing 0.1% trypsin/0.1 mM EDTA solution (3 ml per embryo), and incubate at 37 °C for 20 min.

(iv) Add additional 0.1% trypsin/0.1 mM EDTA solution (3 ml per embryo), and incubate the mixture at 37 °C for 20 min.

(v) Add an equal amount of FP medium (6 ml per embryo), and pipette up and down a few times to help with tissue dissociation.

(vi) Keep the tissue/medium mixture still for 5 min at room temperature (20–25 °C) to remove debris, transfer the supernatant into a new 50-ml conical tube. Centrifuge at 200*g* for 5 min, discard the supernatant, and resuspend the pellet in fresh medium.

(vii) Count the number of cells and adjust the concentration to 1×10^6 cells per ml with FP medium. Generally, $\sim 1 \times 10^7$ cells can be obtained from a single embryo. Transfer the cell suspension to 100-mm tissue culture dishes (1×10^7 cells per dish) and incubate at 37 °C with 5% CO_2 for 24 h (passage 1).

(viii) The next day, remove floating cells by washing with PBS.

(ix) When the cells become confluent, remove FP medium, wash once with PBS, and trypsinize with 1 ml of 0.05% trypsin and 0.53 mM EDTA for a 5 min. After detaching, add 9 ml of FP medium and suspend by pipetting. Passage to new 100-ml dishes at 1:4 dilution (passage 2). For the generation of iPS cells, we used MEFs within three passages to avoid replicative senescence.

(B) Preparation of fibroblasts from mouse tail-tips ● TIMING 10 d

(i) Cut the tail from an adult mouse, and wash with PBS.

(ii) Make a lengthwise incision by an injection needle, peel superficial dermis by hands, and mince the remaining tail into 1-cm pieces with scissors. Place two pieces per well of gelatin-coated 6-well plates, add 2 ml of MF-start medium, and incubate at 37 °C for 5 d, during which time fibroblasts migrate out of the tails.

(iii) Remove the tissues of tails with sterile forceps and discard. Replace the medium with 2 ml of FP medium, and culture the cells when they reach confluent.

(iv) Aspirate the medium, wash twice with 2 ml of PBS and add 0.3 ml of 0.05% trypsin/0.53 mM EDTA, and incubate at 37 °C for 10 min.

(v) Add 2 ml of FP medium, suspend the cells, and transfer and pool to a 15-ml tube. Centrifuge the cells at 160*g* for 5 min.

(vi) Discard the supernatant, resuspend the cells with 10 ml of FP medium and plate to 100-mm tissue culture dish (passage 2).

(vii) When the cells become confluent, remove FP medium, wash once with PBS, and trypsinize with 1 ml of 0.05% trypsin and 0.53 mM EDTA for 5 min. After harvesting, resuspend with 9 ml of FP medium. Passage to new 100-ml dishes at 1:4 dilution (passage 3). These cells usually become confluent within 3–4 d. For the generation of iPS cells, we use tail-tip fibroblasts (TTFs) within three passages to avoid replicative senescence.

Thawing SNL cells ● TIMING 0.5 h

2| Prepare 9 ml of SNL medium in a 15-ml tube.

3| 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 are thawed.

4| Wipe the vial with ethanol, open the cap and transfer the cell suspension to the tube prepared in Step 2.

5| Centrifuge 160*g* for 5 min, and then discard the supernatant.

6| Resuspend 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_2 incubator, until the cells become 80–90% confluent.
▲ **CRITICAL STEP** Do not let the cells get overconfluent, or their ability as feeder cells may decrease.

Passage of SNL cells ● TIMING 0.5 h

7| Discard the medium and wash the cells once with PBS.

8| Aspirate PBS, and add 0.5 ml per dish of 0.25% trypsin/1 mM EDTA, and incubate for 1 min at room temperature.

9| Add 4.5 ml of SNL medium, and break up the cells into a single cell suspension by pipetting up and down several times.

10| Adjust the cell suspension to 160 ml by addition of SNL medium, and transfer to gelatin-coated dishes (10 ml per 10-cm dish). This splits the cells 1:16. Incubate the cells at 37 °C, 5% CO_2 until the cells become 80–90% confluent. This should happen 3–4 d after passage.

Mitomycin C-inactivation of SNL cells ● TIMING 3 h

11| Add 0.3 ml of 0.4 mg ml^{-1} mitomycin C solution directly to the culture medium of SNL dish, swirl it briefly, and incubate for 2.25 h at 37 °C, 5% CO_2. The final concentration of mitomycin C will be 12 μg ml^{-1}.

12| After incubation, aspirate all of the mitomycin C-containing medium off the cells, and wash the cells twice with 10 ml of PBS.

13| 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 min at room temperature.

14| Neutralize the trypsin by adding 5 ml of SNL medium, and break up the cells to a single cell suspension by pipetting up and down. Pool the cell suspension into a 50-ml tube and count the number of cells. Seed the cells on gelatin-coated dishes (1×10^6 cells per 100-mm tissue culture dish, or 1.5×10^5 cells per well of 6-well plate).

15| Cells should be nicely spread with little gaps in between. They should become ready for usage by the next day.
■ **PAUSE POINT** The mitomycin C-treated SNL dishes can be left for up to a week before use.

Thawing Plat-E cells ● TIMING 0.5 h

16| Prepare 9 ml of FP medium in a 15-ml tube.

17| Remove a vial of frozen Plat-E stocks from the liquid nitrogen tank and put the vial in a 37 °C water bath until most (but not all) cells are thawed.

18| Wipe the vial with ethanol, open the cap and transfer the cell suspension to a tube prepared in Step 17.

19| Centrifuge at 180*g* for 5 min, and then discard the supernatant.

20| Resuspend the cells with 10 ml of FP medium, and transfer to a gelatin-coated 100-mm dish. Incubate the cells in a 37 °C, 5% CO_2 incubator.

21| Next day, replace the medium with new media supplemented with 1 μg ml^{-1} of puromycin and 10 μg ml^{-1} of blastcidin S. Continue to incubate the cells in a 37 °C, 5% CO_2 incubator until they are 80–90% confluent.

Passage of Plat-E cells ● TIMING 0.5 h

22| Aspirate the PBS, and add 4 ml per dish of 0.05% trypsin/0.53 mM EDTA, and incubate for 1 min at room temperature. Detach cells from dishes by tapping, resuspend with 10 ml FP medium, and transfer to a 15-ml tube. Centrifuge at 180*g* for 5 min, and aspirate the supernatant.

23| 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 to new 100-ml dishes at 1:4–1:6 dilution. Cells should become confluent within 2–3 d.

Day 1: retrovirus production; Plat-E preparation ● TIMING 1 h

24| Wash the cells with PBS, add 4 ml of 0.05% trypsin/0.53 mM EDTA, and incubate for 1 min at room temperature.

25| 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 tube. FP culture medium used in this period contains neither puromycin nor blasticidin S.

26| Centrifuge the cells at 180*g* for 5 min.

27| Discard the supernatant, break the pellet by finger tapping, and resuspend the cells in an appropriate amount of FP medium.

28| Count the number of cells and adjust the concentration to 8×10^5 cells per ml with FP medium.

29| Seed cells at 8×10^6 cells (10 ml) per 100-mm culture dish, and incubate overnight at 37 °C, 5% CO_2.

Day 2: retrovirus production; transfection into Plat-E cells ● TIMING 1 h

30| Transfer 0.3 ml of DMEM into a 1.5-ml tube.

31| Deliver 27 µl of Fugene 6 transfection reagent into the prepared tube in Step 31, mix gently by finger tapping and incubate for 5 min at room temperature.

32| Add 9 µg of pMXs plasmid DNA (encoding Oct3/4, Sox2, Klf4 and c-Myc) drop-by-drop into the Fugene 6/DMEM-containing tube, mix gently by finger tapping and incubate for 15 min.

33| Add the DNA/Fugene 6 complex dropwise into the Plat-E dish, and incubate overnight at 37 °C, 5% CO_2.
▲ CRITICAL STEP Also transfect with a suitable control; we use pMXs retroviral vector GFP to monitor transfection efficiency. We routinely obtain efficiency >80%. High-efficient transfection is crucial for iPS cell induction.

Day 3: retrovirus production (continued) ● TIMING 0.5 h

34| Aspirate the transfection reagent–containing medium, add 10 ml of fresh FP medium, and return the cells to the incubator.

Preparation of fibroblasts ● TIMING 1 h

35| Culture MEF or TTF (passage <3) to ~90% confluency in 10-cm dishes (~2×10^6 cells per dish) (**Fig. 3**).

36| Aspirate the culture medium and wash with 10 ml of PBS.

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

38| Add 9 ml of the culture medium, suspend the cells to a single cell, and transfer to a 50-ml tube.

39| Count the number of cells, and adjust the concentration to 8×10^4 cells per ml. Transfer 10 ml of cell suspension (8×10^5 cells) to a 100-mm dish with mitomycin C-inactivated SNL cells (use puromycin-resistant feeder cells for *Nanog*$^{GFP\text{-}IRES\text{-}Puro}$). Incubate the dish overnight at 37 °C, 5% CO_2.

Day 4: retroviral infection ● TIMING 0.5 h

40| Collect the medium from the 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 tube.

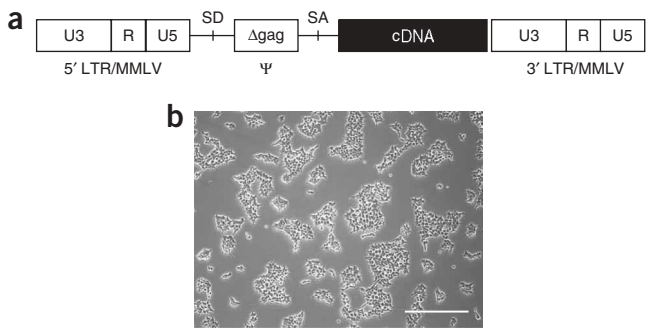

Figure 3 | Retroviral transfection system for induced pluripotent stem (iPS) cell generation. (**a**) Structure of the pMXs retroviral vector. Both 5′- and 3′-long terminal repeats (LTRs) consist of U3, R and U5. Ψ indicates packaging signal. Truncated *gag* sequence (Δgag) exists between splicing donor (SD) and splicing acceptor (SA). (**b**) Morphology of Plat-E packaging cells. Bar indicates 200 µm. MMLV, Moloney murine leukemia virus.

41| Add 5 µl of 8 mg ml^{-1} polybrene solution into the 10-ml filtrated virus-containing medium, and mix gently by pipetting up and down. The final concentration of polybrene will be 4 µg ml^{-1}.

42| Make a mixture of equal parts of the medium containing Oct-3/4-, Sox2-, Klf4- and c-Myc-retroviruses.
▲ CRITICAL STEP Retroviruses should be used freshly. Do not freeze, or you will not obtain iPS cells. The titer of retrovirus is absolutely important for iPS cell generation. The freeze/thaw step decreases the titer of retrovirus.

43| Aspirate the medium from a fibroblast dish, and add 10 ml of the polybrene/virus-containing medium. Incubate the cells from 4 h to overnight at 37 °C, 5% CO_2.

Day 5 and 6 ● TIMING 5 min each day
44| After 24 and 48 h aspirate the medium from a fibroblast dish, and add 10 ml of fresh FP medium.

Day 7 ● TIMING 5 min
45| Discard the medium, and add 10 ml of ES medium. For *Fbx15*$^{\beta geo/\beta geo}$ selection, the medium should be supplemented with 0.3 mg ml^{-1} of G418.

Day 8–10 ● TIMING 5 min each day
46| Change the medium every day (after 24, 48 and 72 h).

Day 11 ● TIMING 5 min
47| For *Nanog*$^{GFP\text{-}IRES\text{-}Puro}$ selection, add puromycin to the medium at the final concentration of 1.5 µg ml^{-1}.

Day 12 ● TIMING ~5 min each day
48| Change the medium every day until the colonies become big enough to be picked up. Colonies should first become visible approximately a week after the retroviral infection. They should become large enough to be picked up around day 20.
? TROUBLESHOOTING

Picking up the iPS colonies ● TIMING 1 h
49| Aliquot 20 µl of 0.25% trypsin/1 mM EDTA per well of 96-well plate.

50| Remove the medium from the dish, and add 10 ml of PBS.

51| Aspirate PBS, and add 5 ml of PBS.

52| Pick colonies from the dish using a Pipetman set at 2 µl, and transfer it into the 96-well trypsin plate. Pick up as many colonies as you can within 15 min. Incubate another 15 min in trypsin at 37 °C to dissociate cells in the colonies.

53| Add 180 µl of ES medium to each well, and pipette up and down to break up the colony to single cells.

54| Transfer cell suspension into the well of 24-well plates with SNL feeder cells, add 300 µl ES medium, and incubate in 37 °C, 5% CO_2 incubator until the cells reach 80–90% confluency. At this point they should be passaged into 6-well plates (see Step 60).

Counting the colonies: staining with crystal violet ● TIMING 1 d
55| After picking the colonies, aspirate PBS completely and then add 5 ml of methanol to fix the remaining cells. Incubate for 1 min at room temperature.

56| Wash the dishes twice with water.

57| Add 5 ml of 0.1% crystal violet solution into the dish and incubate for 5 min at room temperature.

58| Wash the dishes with water.

59| Photograph the dishes and count the number of colonies.

Expansion of iPS cells ● TIMING 1 h
60| Aspirate the medium, and wash the cells with 1 ml of PBS.

61| Remove PBS completely, add 0.1 ml of 0.25% trypsin/1 mM EDTA and incubate at 37 °C for 10 min.

62| Add 0.4 ml of the ES medium and suspend the cells by pipetting up and down to single cell suspension.

63| Transfer the cell suspension to a well of 6-well plate, add 1.5 ml ES cell medium, and incubate in a 37 °C, 5% CO_2 incubator until cells reach 80–90% confluency in 6-well plates. At this point, prepare frozen stock of the cells, as follows.
? TROUBLESHOOTING

Preparation of freeze stock ● TIMING 1 h
64| Aspirate the medium, and wash the cells with 2 ml of PBS.

65| Remove PBS completely, add 0.3 ml of 0.25% trypsin/1 mM EDTA and incubate at 37 °C for 10 min.

66| Add 2 ml of the ES medium and suspend the cells by pipetting up and down to single cell suspension.

67| Transfer the cell suspension to a 15-ml tube, count the number of cells and spin the cells at 160*g* for 5 min.

68| Discard the supernatant, resuspend the cells with ES medium to the concentration at 2×10^6 cells per ml.

69| Prepare 2× freezing medium (20% DMSO in ES medium) and aliquot it at 0.5 ml per vial.

70| Transfer 0.5 ml of the cell suspension to freeze vials and mix gently.

71| Put the vials in a cell-freezing container and keep it at −80 °C overnight.
■ **PAUSE POINT** For long-term storage, keep frozen cells in the gas phase of a liquid nitrogen tank.
? TROUBLESHOOTING

? TROUBLESHOOTING
Step 48
Problem: No drug-resistant colonies appear from fibroblast cultures after induction of the four factors.
Solution: The titer of retrovirus is important. The titer of retrovirus used in our study is $\sim 5 \times 10^6$ TU ml^{-1} (determined by using NIH3T3 cells). The retrovirus must be prepared freshly. **! CAUTION** Do not freeze retrovirus.
Age of fibroblast (passage number) is also critical for iPS generation. Efficiency of retroviral transduction markedly decreases in older fibroblasts. We recommend using MEFs or TTFs within passage 3 for iPS production.
Step 63
Problem: Isolated clone is not pluripotent.
Solution: Choose the superior clones by the morphologies and marker gene expression.
Step 71
Problem: The character and potential of iPS cell changes during culture.
Solution: iPS cells are less stable than ES cells. In some cases, granulated (not ES-like) cells emerge in both Fbx15- and Nanog-iPS cell cultures. These cells can be removed by antibiotic selection. Loosely attached GFP-negative cells can also emerge in Nanog-iPS culture. They can easily be washed out with PBS. Make large amount of freeze stocks of iPS clones at early passages. These stocks should be stored in the vapor phase of liquid nitrogen. The recovery of iPS cells after freezing is ~50%. Continuous selection with antibiotics facilitates stability of iPS cells.

ANTICIPATED RESULTS
When the procedure goes well, the retroviruses integrate into >70% of fibroblasts (both MEFs and TTFs). The infection efficiency can be evaluated by analyzing GFP-infected cells with a flow cytometer (**Fig. 4a**).

In the experiment using *Fbx15*$^{\beta geo/\beta geo}$ MEF, more than a hundred of G418-resistant colonies appear in a 100-mm dish transfected with *Oct3/4*, *Sox2*, *Klf4* and *c-Myc*, whereas no colonies emerge with the mock or enhanced GFP control (**Fig. 4b**). Generally, fewer colonies are obtained from TTF than from MEF. Estimated efficiency of iPS cells from MEFs that had incorporated all the four factors is ~1%. The frequency of iPS colony formation from TTFs is approximately one-tenth of that from MEFs. G418-resistant colonies are morphologically similar to those of ES cells (**Fig. 4c**). After picking up, most clones show ES-like proliferation and morphology, including a round shape, large nucleoli and scant cytoplasm in ES cell culture condition (**Fig. 4d**). However, some clones show non-ES-like morphology or fail to proliferate (**Fig. 4e**). Most clones express ES cell marker gene, such as *Nanog*, *ERas*, *Zfp42*, *Utf1* (primer sequences and PCR conditions are listed in **Table 1**). Most clones also express undifferentiated ES cell surface antigen, SSEA-1 (stage-specific embryonic antigen-1), and are positive for

TABLE 1 | PCR conditions.

Gene	Sense primer	Antisense primer	Initial denaturing	Denature	Annealing	Extension	Final extension	Hold	Cycle number
Ecat1	TGT GGG GCC CTG AAA GGC GAG CTG AGA T	ATG GGC CGC CAT ACG ACG ACG CTC AAC T	94°C, 1 min	94°C, 10 sec	60°C, 15 sec	72°C, 40 sec	72°C, 5 min	4°C	
Esg1	GAA GTC TGG TTC CTT GGC AGG ATG	ACT CGA TAC ACT GGC CTA GC	95°C, 1 min	95°C, 2 sec	68°C, 50 sec		68°C, 5 min	4°C	
ERas	ACT GCC CCT CAT CAG ACT GCT ACT	CAC TGC CTT GTA CTC GGG TAG CTG							
Fgf4	CGT GGT GAG CAT CTT CGG AGT GG	CCT TCT TGG TCC GCC CGT TCT TA							
Nanog	AGG GTC TGC TAC TGA GAT GCT CTG	CAA CCA CTG GTT TTT CTG CCA CCG	94°C, 1 min	94°C, 2 sec	70°C, 50 sec		70°C, 5 min	4°C	
NAT1	ATT CTT CGT TGT CAA GCC GCC AAA GTG GAG	AGT TGT TTG CTG CGG AGT TGT CAT CTC GTC							
Cripto	ATG GAC GCA ACT GTG AAC ATG ATG TTC GCA	CTT TGA GGT CCT GGT CCA TCA CGT GAC CAT	94°C, 1 min	94°C, 10 sec	60°C, 10 sec	72°C, 30 sec	72°C, 5 min	4°C	30
Dax1	TGC TGC GGT CCA GGC CAT CAA GAG	GGG CAC TGT TCA GTT CAG CGG ATC							
Gdf3	GTT CCA ACC TGT GCC TCG CGT CTT	AGC GAG GCA TGG AGA GAG CGG AGC AG							
Oct3/4 (end)	TCT TTC CAC CAG GCC CCC GGC TC	TGC GGG CGG ACA TGG GGA GAT CC							
Zfp42 (*Rex1*)	ACG AGT GGC AGT TTC TTC TTG GGA	TAT GAC TCA CTT CCA GGG GGC ACT							
Zfp296	CCA TTA GGG GCC ATC ATC GCT TTC	CAC TGC TCA CTG GAG GGG GCT TGC							
Utf1	GGA TGT CCC GGT GAC TAC GTC TG	GGC GGA TCT GGT TAT CGA AGG GT	95°C, 1 min	95°C, 5 sec	63°C, 10 sec	72°C, 30 sec	72°C, 5 min	4°C	
Sox2 (end)	TAG AGC TAG ACT CCG GGC GAT GA	TTG CCT TAA ACA AGA CCA CGA AA							

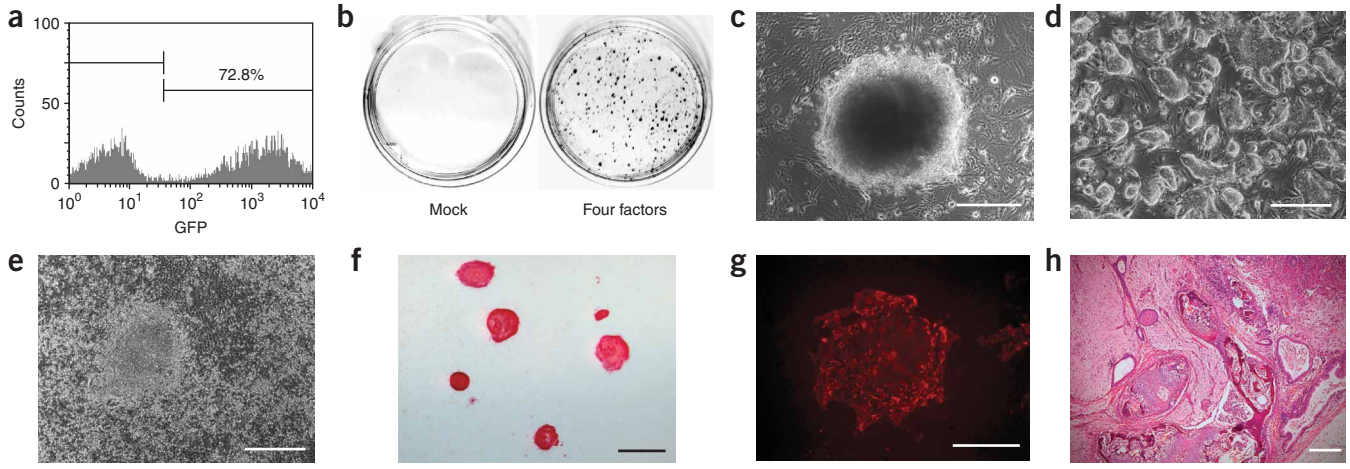

Figure 4 | Induced pluripotent stem (iPS) cells from mouse fibroblasts. (**a**) Transfection efficiency by pMXs retrovirus generated in Plat-E packaging cells. (**b**) G418-resistant colonies obtained after introduction of the four factors. (**c**) Morphology of a G418-resistant colony. Bar indicates 500 μm. (**d**) Morphology of *Fbx15*-selected iPS cells. Bar indicates 200 μm. (**e**) Morphology of non-embryonic stem (ES)-like cells. Bar indicates 200 μm. (**f**) Alkaline phosphatase staining of iPS cells. Bar indicates 1 mm. (**g**) SSEA-1 staining of an iPS cell colony. Bar indicates 500 μm. (**h**) H&E staining of teratoma derived from *Fbx15*-selected iPS cells. Bar indicates 200 μm.

alkaline phosphatase staining (**Fig. 4f** and **g**). Teratoma developed by s.c. injection into nude mice can be used to demonstrate pluripotency of these cells (**Fig. 4h**).

By using *Nanog*$^{\text{GFP-IRES-Puro}}$ MEF, GFP-positive cells became first apparent ~7 d after infection, and a few hundred colonies appeared within 3 weeks after infection. Among them, ~5% were positive for GFP. Most clones express ES cell marker genes, develop teratoma and contribute to adult chimeric mice[10].

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从成纤维细胞培养物中诱导多能干细胞

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胚胎干细胞 (ES)的临床应用面临着胚胎使用方面的困难，以及植入后的组织排斥。解决这些问题的一种方法是直接从体细胞中生成多能干细胞。通过将细胞核注射到去核的卵母细胞中或与 ES 细胞融合，体细胞可以重新编程为胚胎样状态。

然而，人们对这些过程背后的机制知之甚少。我们最近表明，四种转录因子的组合可以直接从小鼠成纤维细胞培养物中产生 ES 样多能干细胞。这些细胞被称为诱导多能干细胞 (iPS)，可以分化成三个胚层并形成嵌合小鼠。这里我们描述了生成 iPS 细胞的详细方法和技巧。

引言人类胚胎干细胞 (ES)的临床应用面临着使用人类胚胎的困难，以及植入后的组织排斥。避免这些问题的一种方法是直接从体细胞产生多能细胞 1。为此，已经开发了两种策略：核移植到卵母细胞和与 ES 细胞融合。最近的一项研究表明，受精卵可用于核移植 2。另一项研究表明，通过在 ES 细胞中过度表达转录因子 Nanog 可以促进融合介导的重编程 3。然而，这些方法仍然需要胚胎或卵母细胞来产生多能细胞，因此不能摆脱伦理问题。此外，融合介导的方法需要消除 ES 细胞衍生的染色体。

体细胞可以通过核移植到卵母细胞或与 ES 细胞融合而重新编程，这表明卵母细胞和 ES 细胞含有诱导重新编程的因子。我们假设在 ES 细胞多能性维持中起重要作用的因子在体细胞多能性的诱导中也起着关键作用。小鼠 ES 细胞多能性的长期维持需要转录因子（例如 Oct3/4、Sox2 和 Nanog）的特定表达，以及广泛表达的肿瘤相关基因（例如 Stat3、c-Myc、Klf4 和 b-catenin）的激活 47。为了测试这些候选基因的多能性诱导活性，

我们开发了一个系统，其中多能性的诱导可以作为标记基因表达来检测（图 1)。在这个系统中，我们

p u o r G g n i h s il b u P e r u t a N 7 0 0 2 natureprotocols /m o c .e r u t a n .w w w /:p t t h 5 LTR 3 LTR cDNA 逆转录病毒转导和 G418 选择 geo geo a b d0 d11 d5 d30 接种（步骤 3640）解冻（步骤 26）传代（步骤 710）用 MMC 处理（步骤 1216）接种（步骤 1730）选择 Nanog-iPS 细胞（步骤 4849）拾取菌落（步骤 50+)

成纤维细胞制备（步骤 1,3640）

ES 培养基培养（步骤 4649）

FP 培养基培养

Fbx15-iPS 细胞的选择（步骤 4649）

iPS 细胞的生成逆转录病毒的制备饲养细胞（SNL）的制备

d15（步骤 45）感染（步骤 4144）转染（步骤 3135）

图 1 |诱导多能干（iPS）细胞生成示意图。(a)通过四个因素生成 iPS 细胞的策略。(b)iPS 细胞生成的大致时间表。ES 细胞，胚胎干细胞；LTR，长末端重复序列；MMC，丝裂霉素 C；SNL，Schlegel 氮限制。

PROTOCOL

利用 Fbx15，该基因在小鼠 ES 细胞和早期胚胎中特异性表达，但对于 ES 细胞的自我更新和发育是可有可无的。我们通过同源重组将 bgeo 盒（b-半乳糖苷酶和新霉素抗性基因的融合）插入小鼠

Fbx15 基因中。bgeo 敲入纯合的 ES 细胞（Fbx15bgeo/bgeo）对极高浓度的 G418（高达 12 mg ml1）具有抗性，而来自 Fbx15bgeo/bgeo 小鼠的体细胞对选择很敏感。我们预计，即使部分诱导多能性也会使体细胞对正常 G418 具有抗性浓度（0.3 mg ml1）。我们通过逆转录病毒介导的转染将候选基因引入 Fbx15bgeo/bgeo 小鼠胚胎成纤维细胞（MEF），并在含有 G418 的 ES 细胞培养基中培养。使用任何单一因子，我们都没有获得 G418 抗性菌落。然而，通过结合四种因子（Oct3/4，Sox2，c-Myc 和 Klf4），我们获得了多个 G418 抗性菌落 9。这些细胞表现出与 ES 细胞相似的形态和增殖。当移植到裸鼠体内时，这些 ES 样细胞产生了含有三个胚层各种组织的畸胎瘤。最近，通过使用 Nanog 作为选择标记，我们能够通过四种因子诱导生殖系能力 10。这些数据表明，可以通过几种确定的因子从成纤维细胞培养物中产生多能细胞 1。诱导多能干细胞 (iPS)的生成仅需要分子和细胞生物学中的基本技术。它不需要任何特殊的设备或技术。这里我们描述了生成 iPS 细胞的详细方法和技巧。

图 1b 概述了协议的主要阶段，并说明了这些阶段需要如何随时间进行协调。

材料试剂

pMXs 逆转录病毒载体和 Plat-E 包装细胞 11（参见试剂设置）

含有 Oct3/4、Sox2、Klf4 或 c-Myc cDNA 的 pMXs（参见试剂设置）

Fbx15bgeo/bgeo 小鼠（参见试剂设置）

NanogGFP-IRES-Puro 小鼠（参见试剂设置）! 注意涉及活啮齿动物的实验必须符合国际和机构法规。

SNL 饲养细胞 12（参见试剂设置）

含有 4.5 g l1 葡萄糖的 DMEM（Nacalai tesque， 号 14247-15）

不含钙和镁的 PBS（Nacalai tesque， 号 14249-95）

L-GIn（Invitrogen， 号 25030-081）

非必需氨基酸溶液（Invitrogen， 号 11140-050）

2-巯基乙醇（Invitrogen， 号 21985-023）

青霉素/链霉素（Invitrogen， 号 15140-122）

0.25%（重量/体积）胰蛋白酶/1 mM EDTA 溶液(Invitrogen，
cat.no.25200-056)

0.5%(wt/vol)胰蛋白酶/5.3 mM EDTA 溶液 (Invitrogen，
cat.no.25300-054)（见试剂设置）

明胶 (Sigma，cat.no.G1890)（见试剂设置）

G418 亚硫酸盐，50 mg ml1 溶液 (Invitrogen，cat.no.10131-035)

嘌呤霉素 (Sigma，cat.no.P7255)（见试剂设置）

Blasticidin S 盐酸盐 (Funakoshi，cat.no.KK-400)
见试剂设置）
Fugene 6 转染试剂 (Roche，cat.no.1 814 443)
六亚甲基溴化铵 (Polybrene；Nacalai)tesque，　　号 17736-44)
参见试剂设置)
结晶紫 (Nacalai tesque，　　号 09804-52)（参见试剂设置）
ES 培养基（参见试剂设置）
SNL 培养基（参见试剂设置）
FP 培养基（用于成纤维细胞和 Plat-E 细胞）（参见试剂设置）
MF-start 培养基（即用型；Toyobo，　　号 TMMFS-001）
甲醇设备
100 毫米组织培养皿（Falcon，　　号
no.353003)
6 孔组织培养板 (Falcon，　　号 353046)
24 孔组织培养板 (Falcon，　　号 353047)
96 孔组织培养板 (Falcon，　　号 351172)
1 毫升塑料一次性移液器 (Falcon，　　号
357520)
5 毫升塑料一次性移液器 (Falcon，　　号
357543)
10 毫升塑料一次性移液器 (Falcon，　　号
357551)
25 毫升塑料一次性移液器 (Falcon，　　号
357525)
0.22 毫米孔径过滤器 (Millex GP；Millipore，　　号 SLGP033RS)
0.45 毫米孔径醋酸纤维素过滤器 (FP30/0.45 CA-S，Schleicher &Schuell)
10 毫升一次性注射器 (Terumo，　　号 SS-10ESZ)
解剖钳！注意用高压灭菌器灭菌。
解剖剪刀！注意用高压灭菌器灭菌。