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Reprogramming of Mouse, Rat, Pig, and Human Fibroblasts into iPS Cells

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Abstract

The induction of pluripotency in somatic cells by transcription factor overexpression has been widely regarded as one of the major breakthroughs in stem cell biology within this decade. The generation of these induced pluripotent stem cells (iPSCs) has enabled investigators to develop in vitro disease models for biological discovery and drug screening, and in the future, patient-specific therapy for tissue or organ regeneration. While new technologies for reprogramming are continually being discovered, the availability of iPSCs from different species is also increasing rapidly. Comparison of iPSCs across species may provide new insights into key aspects of pluripotency and early embryonic development. iPSCs from large animals may enable the generation of genetically-modified large animal models or potentially transplantable donor tissues or organs. In this unit, we describe the procedure for the generation of iPSCs from mouse, rat, pig and human fibroblasts. We focus on lenti- and retroviral infection as the main platform for pluripotent transcription factor overexpression since these reagents are widely-available and remain the most efficient way to generate iPSC colonies. We hope to illustrate the basic process for iPSC generation in these four species in such a way that would enable the lowering of the entry barrier into iPSC biology by new investigators.

Keywords

Induced Pluripotent Stem Cells; Reprogramming; Fibroblasts; Mouse; Rat; Human; Pig; Lentivirus; Retrovirus

Introduction

With the overexpression of just four transcription factors, Oct4, Sox2, Klf-4 and C-myc, Takahashi and Yamanaka (Takahashi & Yamanaka 2006) showed that terminally differentiated fibroblasts could be reprogrammed into becoming pluripotent, embryonic stem cell like cells, that they called induced pluripotent stem cells (iPSCs). These cells are

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able to proliferate indefinitely and were shown to differentiate into subtypes of all three germ-layers. iPSCs thus harbor potential for disease modeling as well as conducting patient-specific drug screens in vitro, without the ethical and technical challenges associated with embryonic stem cells (ESCs). In this unit, we describe the generation of iPSCs from four species: mouse, rat, pig, and human. To lower the barrier for new investigators to enter this exciting area of biology, we chose to focus on lenti- and retroviral infection-based strategies for somatic cell reprogramming since these approaches remain the easiest to generate iPSC lines. Although by no means exclusive, this protocol sets out to describe in detail the full procedure required for iPSC. Basic protocol 1 described the isolation and culture of fibroblasts, with a support protocol for lentiviral production. Basic protocol 2 describes the viral infection of fibroblasts, all of which can be applied to all four species. After this section, the procedure diverges from species to species; basic protocol 3 describes the establishment and maintenance of mouse iPSCs and there are support protocols for the same procedure in subsequently rat, pig and human cells.

Basic Protocol 1 : Isolation & Culturing of Fibroblasts

NOTE: This section describes the isolation and culturing of mouse embryonic fibroblasts. However, a slightly modified protocol can be followed for mouse dermal fibroblasts or tail-tip fibroblasts, as well as rat embryonic fibroblasts, rat dermal fibroblasts, rat tail-tip fibroblasts, pig dermal fibroblasts and human dermal fibroblasts. See notes below for details.

Materials—Pregnant female mice

Sterile PBS with CaCl and MgCl (Invitrogen 14040141)

Penicillin and Streptomycin (Invitrogen 15140-155)

Sterile forceps

100-mm petri dish (BD Falcon 351029)

100-mm cell culture dish (Corning 430167), pre-coated with 0.1% gelatin

Sterile scalpel blade

0.25% Trypsin-0.53mM EDTA (GIBCO 25200-056)

15 mL conical tube

MEF Medium

Centrifuge

37°C/5% CO₂ incubator

Hemocytometer

Laminar flow hood

Preparation of mouse embryonic fibroblasts

1. Euthanize pregnant female mice at 13.5 -15.5 day post coitum (dpc). Isolate the gravid uterus and briefly wash with PBS supplemented with antibiotics (Penicillin and Streptomycin at 5000 IU/ each/mL).
2. Dissect through the placenta and uteri until embryos are within view. Separate embryos from their placenta and surrounding fetal membranes with sterile forceps. Carefully remove the head, visceral organs and gonads from the embryos.
3. Wash body of the embryos thoroughly in a 100-mm petri dish containing fresh PBS supplemented with antibiotics. Mince well with sterile scalpel blade and digest the embryos in 1 mL of 0.25% Trypsin/EDTA in a 15 mL conical tube, and incubate at 37°C for 15 minutes. Using a P1000 pipette, gently triturate intermittently to allow the cells to disperse from the large tissue clumps.
4. Add 9 mL of MEF Medium to neutralize the trypsin. Again pipette up and down gently to dissociate as many cells as possible.
5. Incubate the samples for 5 min at room temperature (20–25°C) to allow the tissue debris to settle at the bottom and transfer the supernatant into a sterile 50 mL conical tube. Centrifuge at $200 \times g$ for 4 min at RT, discard the supernatant, and resuspend the pellet in 10 mL fresh MEF medium.
6. Count cells with a hemocytometer (see Appendix 3F) and adjust the concentration to 1×10^6 cells per mL with MEF medium. Generally, 1×10^7 cells can be obtained from a single embryo. Transfer the cell suspension to gelatinized 100-mm tissue culture dishes (1×10^7 cells per dish) with 10 ml of MEF media and incubate at 37°C with 5% CO₂. This is considered passage #0.
7. Remove non-adherent cells after 48 hours by washing with PBS or MEF media, then replenish with MEF media. Cells should be replenished with fresh MEF media every 48 hours until confluent.
8. When the cells have become confluent, remove MEF medium, wash once with PBS, and trypsinize with 2 mL of 0.25% trypsin EDTA for 4 min at 37°C. After detaching, add 9 mL of MEF medium and resuspend the cells by pipetting. Passage to new 100-ml dishes at 1:4 dilution (passage #1). Cells can also be frozen at this point (see below).

NOTE: For the generation of iPS cells it is advisable to use MEFs (mouse embryonic fibroblasts) up to and including passage #3 to avoid replicative senescence. As an alternative, other easily accessible tissues can be used for reprogramming, such as adult skin cells, tail tip fibroblasts, blood and cells from biopsy tissues (Park 2008a), but these cells show less reprogramming efficiency than embryonic fibroblasts within the first 5-7 passages.

NOTE: When using either skin fibroblasts or tail tip fibroblasts, the same procedure can be followed as above after mincing the sample finely using a sterile scalpel or razor blade.

NOTE: For rat samples the same procedure can be followed as described above while using pregnant female rats at 14.5 – 15.5 dpc for embryonic rat fibroblasts. Alternatively, skin samples can be used for dermal fibroblasts or tail tip fibroblasts.

NOTE: For pig and human samples a punch skin biopsy should be performed to obtain skin tissues. The same procedure can be followed as described above.

Support Protocol 1

NOTE: For retroviral production use PLAT-E cells with FuGENE HD Transfection Reagent.

Production of Lentivirus—This section describes the production of viral constructs in a lentiviral system. The protocols for infecting mouse, rat, pig, and human fibroblasts with lenti- and retroviruses is similar. Therefore the following applies to all four animal species.

Materials: 293FT cells (Invitrogen R700-07)

100-mm cell culture dish (Corning 430167) pre-coated with 0.1% gelatin [*Author: supplier or procedure for making these plates?]

293FT medium (see recipe)

Microcentrifuge tubes

DMEM/high glucose (GIBCO 11965092)

FuGENE HD Transfection Reagent (Roche 04 709 705 001)

rtTA plasmid (Addgene 19780)

STEMCCA (OKSM) Lentivirus Reprogramming Kit (Millipore SCR511)

D8.9/psPAX2 plasmid (Addgene 12260)

VSV-G plasmid (Addgene 8454)

Centrifuge

37°C and 5% CO₂ incubator

0.45µm disposable filters

Beckman Coulter Optima L-90K ultracentrifuge with SW-32 rotor

–80°C freezer

Lentiviral production

1. Culture 293FT cells in a gelatinized 10-cm dish in 293FT media until cells reach 80-90% confluency. Use 1 dish per viral construct or several if harvesting more virus.
2. Pre-warm DMEM/High Glucose and FuGENE HD Transfection Reagent to RT and mix together into a microcentrifuge tube. Use 770 μ L DMEM/High glucose and 50 μ L FuGENE Reagent per 10-cm dish. [*Author: do you typically prepare a single master mix or individual tubes of 770+50 μ l?]
3. Incubate at RT for 5 min.
4. For each infection: add 5.5 μ L VSV-G (5.5 μ g/infection) and 8.25 μ L D8.9 to each microcentrifuge tube and mix well.

Typical Infection Ratios of Lentiviral Helper Plasmids for Production of Lentiviral Vectors by FuGENE Transfection:

1 $\times$ VSV-G

1.5 $\times$ D8.9

2 $\times$ vector

5. Add a total of 11 μ g vector DNA (11 μ g/infection) to.
6. Mix by gently tapping tube several times (do not vortex).
7. Incubate 30 minutes at RT.
8. During incubation, add 10 mL of fresh 293FT medium to 293FT cells.
9. Add entire FUGENE/DNA complex to the 10-cm dish containing the 293FT cells in a drop-wise fashion around the dish. Gently rotate the plate to mix the contents. Incubate for 12-24 hours at 37°C/5% CO₂. [*Author: Mix in some way? Incubate 12-24 hr? at what temp?]
10. Change 293FT medium 12-24 hours later. Add in 10 mL of fresh medium at each medium replenishing step.
11. After 24 hours, collect all media into a 50-mL conical tube and store at 4°C until the collection procedure is completed. Add in 10 mL of fresh 293FT medium. Repeat every subsequent 24 hours for a total of 3 collections. This will allow a collection of 30 mL of virus medium per 10-cm dish. After completion, cells can be discarded using proper viral waste disposal procedures.
12. Filter collected viral medium through a 0.45 μ m filter. [*Author: Collect all media every 24 hr? Replace with new each time? Please give all necessary, detailed instructions.]
13. Transfer collected media into ultracentrifugation tubes (Beckman, #344058) (30 mL per tube) and spin down at 50,000 $\times$ g for 1.5h at 4°C (SW-32 rotor in Beckman Coulter Optima L-90K ultracentrifuge). Turn brakes off to avoid disturbance of pellet.

14. Decant supernatant into 10% bleach, place tube briefly “head-down” onto tissue paper to remove supernatant completely. This will take up to 1-2 minutes.
15. Add 200 μ L respective culture medium to the pellet, let stand overnight at 4 μ C.
16. Resuspend by carefully pipetting up and down and aliquot the virus in small volumes (e.g. 20 μ L per aliquot).
17. Store aliquots [*Author: up to how long?] at –80°C for up to 1 year.

Basic Protocol 2 : Infection of Fibroblasts using Pluripotency Factors

In this protocol we describe the infection of fibroblasts using viral constructs of the four pluripotency factors. This protocol can be applied to all four species described using the viral method described above.

Materials—embryonic fibroblasts or dermal fibroblasts (passage 3)

6-well plate pre-coated with 0.1% gelatin

PBS with CaCl and MgCl (Invitrogen 14040-141)

MEF medium (see recipe)

0.25% Trypsin-0.53mM EDTA (GIBCO 25200-056)

Polybrene (Millipore TR-1003-G)

Doxycycline (Sigma D9891)

Pre-aliquoted virus (see Support Protocol 1)

Laminar flow hood

1. Culture embryonic fibroblasts or dermal fibroblasts (passage 3) to approximately 90% confluency in a single well of a gelatin-coated 6-well plate (approximately 1×10^4 cells per dish).
2. Aspirate the culture medium from the MEF cells and wash with 2 mL of PBS.
3. Aspirate PBS, add 0.5 mL per well of 0.25% trypsin-EDTA, and incubate at 37°C for 4 min.
4. Add 2 mL of the MEF medium and triturate to obtain single cell suspension and transfer to a 15mL tube.
5. Centrifuge at $200 \times g$ for 4 min, discard the supernatant and resuspend the pellet in 1 mL of MEF media.
6. Count the number of cells using a haemocytometer and adjust the cell concentration to 1×10^4 cells per mL. (see Appendix 3F)

7. Add 1 μ L of Polybrene solution (8 mg/mL stock concentration) into each mL of cell containing media. Mix by gently pipetting up and down. The final concentration of Polybrene in the mixture should be 8 μ g/mL.
8. Thaw and freshly combine all four factor lenti- or retroviruses (Oct-3/4, Sox2, Klf4 and c-Myc) into a cocktail and add freshly into the cell mixture for mouse and rat iPS generation. For pig and human iPS a four-in-one mono-cassette virus is recommended. Mix by gently pipetting up and down. If using a doxycycline-inducible TetO viral system, a separate rtTa expressing virus should also be added at this point. Additionally, doxycycline should be added immediately and with each media change at a final concentration of 1 μ g/mL to induce gene expression. The amount of virus added should be calculated by making use of the equation mentioned below, using a Multiplicity of Infection (MOI) of at least 20.
9. Plate 1 mL of the cell/virus mixture onto a single well of a gelatin coated 6-well plate and incubate the cells for 24 h at 37°C and 5% CO₂.
10. After 24 hours, wash with warm MEF medium and replenish with 2 mL of fresh MEF medium and continue incubation at 37°C and 5% CO₂.
11. For continued protocol please see the respective section for each species you are reprogramming below.

NOTE: Using fresh virus gives dramatically better infection efficiency. Virus should not be stored for a longer period than 1 week at 4°C before use. Virus should never undergo more than 1 freeze/thaw cycle as multiple freeze/thaw cycles considerably reduce infection efficiency.

NOTE: For mouse and rat fibroblasts, mouse four factor plasmids are most widely used for making iPSCs. For pig and human fibroblasts it is preferable to use human four-in-one factor constructs, but individual human factors can also be used. A STEMCCA Cre-Excisable Constitutive Polycistronic (OKSM) Lentivirus Reprogramming Kit is commercially available (Millipore).

NOTE: For increased viral infection rate, multiple infections can be performed by aspirating and replenishing media with fresh virus containing media every 12 hours up until 36 hours after initial infection.

NOTE: When working with virus, use a designated laminar flow hood for virus-related work. Always wear protective disposable gowns and double gloves. Make sure to discard any viral waste into designated viral waste bins and rinse any disposables with bleach before disposing of them. The contents of a viral waste bin should be autoclaved in an autoclavable bag for 45 minutes in a standard autoclave using the sterilization program.

NOTE: As a feeder layer for iPS cells, MEFs can be used for all four species. The MEFs should be growth-inhibited by irradiation or mitomycin-C treatment. For best results, a single monolayer of feeder cells should be plated onto gelatinized culture plates (for mouse and rat) or Matrigel-coated culture plates (for pig and human).

NOTE: The following equation can be used to determine the volume of virus required to achieve a Multiplicity of Infection (MOI) of at least 20.

$$\text{Virus volume } (\mu\text{L}) \text{ required} = \frac{\text{Number of MEFs seeded for infection} \times \text{Desired MOI} \times 1000}{\text{Virus Titer (U/mL)}} \mu\text{L}$$

Example: If the number of cells in the well at the time of transduction is 1×10^5 , the viral titer is 3×10^8 IFU/mL, and a desired MOI is 20, then the volume of virus required is:

$$\frac{1 \times 10^5 \text{ cells}}{3 \times 10^8 \text{ U/mL}} \times \frac{20}{1} \times 1000 \mu\text{L} = 6.6 \mu\text{L} \text{ virus required for 1 well of a 6-well Plate}$$

The following sections describe the post-infection iPS cell colony selection and maintenance procedures for each species. Please refer to the specific section for information on iPSC generation procedure for your species of interest.

Basic Protocol 3 : Establishment and Maintenance of Mouse iPS Cells

This section describes the establishment and maintenance of mouse iPS Cells. Please revert back to the support protocols for the viral infection procedure.

Materials—15 mL conical tubes

6-wells plate, pre-coated with 0.1% gelatin

Irradiated CF1 MEFs (GlobalStem 6001G)

Mitomycin C

0.25% Trypsin-0.53mM EDTA (GIBCO 25200-056)

MEF medium (see recipe)

Mouse iPS cell medium (see recipe)

Doxycycline (Sigma D9891)

24-wells plate, pre-coated with 0.1% gelatin

37°C and 5% CO₂ incubator

37°C water bath

Centrifuge

Light Microscope with camera

Laminar Flow Hood

Freezing medium (see recipe)

PBS without CaCl and MgCl (Invitrogen 14190-250)

–80° C Freezer

Liquid Nitrogen Storage tank

Cryogenic handling gloves & eye protectors

Forceps

Refrigerator (4°C)

Biosafety cabinet

Micropipettes

Freezing chamber

2.0 mL Cryovials

Post-infection culturing of infected fibroblasts

1. After 48 hours, or when cells reach confluency, aspirate the medium from the infected cells and dissociate by adding 0.5 mL of 0.25% Trypsin-EDTA and incubating for 4 min at 37°C and 5% CO₂. Add 2 mL of warm MEF medium pipette up and down in order to obtain a single cell suspension. Transfer to a 15 mL tube containing 9 mL of warm MEF medium and centrifuge at $200 \times g$ for 4 minutes.
2. Discard the supernatant and dissolve the pellet in 8 mL of mouse iPS cell culture medium. For initial culture conditions use double the concentration of mouse LIF. After 2-3 passages, regular dose of mouse LIF can be used.
3. Aspirate the medium from 4 single wells of 6-wells plate that has been gelatin-coated and pre-seeded with growth-inhibited MEF cells. Add 2mL of the infected cell mixture per well onto the MEF feeder layer so that the infected cells are passaged in a 1:4 ratio. Incubate at 37°C and 5% CO₂.
4. Change iPS medium (supplemented with doxycycline if using a TetO lentiviral system) every 24 hours and check for colony formation. Colonies should start becoming microscopically visible approximately 7-10 days post-infection.
5. Let colonies grow into a reasonable size (roughly 50-100 cells/colony). This should take until approximately day 14 or 15 post-infection.

Picking and Establishing Mouse iPS clones

6. Pre-feed the cells 1 hour before picking iPSC colonies by replenishing with fresh mouse iPS media. This is especially important when the media has turned acidic (indicated by yellow color), since pre-feeding will increase cell survival after dissociation.

7. Before picking, select as many good colonies as necessary by circling the colony with a marker pen at the bottom of the plate to be able to retrieve the good colonies when the actual picking procedure is started. Suitable colonies should look translucent and circular. See Figure 2 for examples of suitable mouse iPS colonies that can be picked.
8. Picking should be done using a microscope inside the laminar hood to maintain sterile conditions. See Figure 1 for appropriate picking conditions.
9. Add 50 μ L of Trypsin-EDTA into several 15 mL tubes. Use a 20 μ L Pipette for the picking procedure.
10. Pick one individual colony by gently scratching with the pipette tip. Make sure not to touch any neighboring colonies. Pick up as many colonies as you can within 15 minutes to limit exposure of picked colonies to trypsin.
11. Transfer each picked colony into an individual 15 mL tube containing 50 μ L of trypsin. Dissociate the colony by gentle pipetting and incubate at 37°C in water bath for 4 minutes.
12. Add 5 mL of iPS medium to each 15 mL tube and pipette up and down to dissociate the colony further into a single cell suspension. Centrifuge at $200 \times g$ for 4 minutes.
13. Aspirate the supernatant and dissolve the pellet in 1 mL of mouse iPS medium. Transfer the cell suspension from each picked colony into a single well of a 24-well plate that has been gelatin-coated and pre-seeded with growth-inactivated MEF cells. Incubate at 37°C and 5% CO₂ and replenish media every 24 hours until nearly confluent. Cells are now ready to be passaged into a 6-well plate.

NOTE: When reprogramming cells using a doxycycline inducible system, wean your cells off doxycycline gradually after picking colonies. As a guideline, the following schedule can be applied: use 1/2 concentration of doxycycline after the first passage. Then gradually lower the concentration with each passage, so 1/4 concentration, then completely wean off doxycycline. If cell morphology begins to degrade, slow down the rate of weaning. Do not change media conditions at the time of passaging; rather wait until the cells have attached (usually after 12-24 hours) before making changes to the doxycycline concentration.

NOTE: Refer to Figure 3 for a schematic diagram of generating mouse iPSCs.

In Vitro Culture and Expansion of Mouse iPS cells

14. When iPS cell colonies reach 80-90% confluency in the dish or when individual iPS cell colonies become large (approximately >100 cells/colony), they should be passaged. For mouse iPS cells, a 1:6 passaging ratio is usually easily tolerated.
15. Aspirate the medium from the dish, and pre-feed the cells with mouse iPS media 1 hour before passaging.
16. After 1 hour aspirate the medium and wash the cells with 2 mL of PBS.

17. Aspirate the PBS, add 0.5 mL of 0.25% trypsin-EDTA and incubate at 37°C and 5% CO₂ for 4 min.
18. Add 1mL of the mouse iPS medium and dissociate the colonies by pipetting up and down into single cell suspension.
19. Transfer into a 15 mL tube containing 9 mL of warm mouse iPS medium and centrifuge at 200 × g for 4 minutes.
20. Discard the supernatant and dissolve the pellet in 12 mL of mouse iPS medium.
21. Aspirate MEF medium from 6 well plates containing mitomycin C growth-inactivated or irradiated MEFs and add 2 mL of cell suspension per well. Incubate the cells at 37°C and 5% CO₂ until cells reach 80–90% confluency. Replenish with fresh mouse iPS media every 24 hours. It is advisable to prepare frozen stocks of newly reprogrammed iPSCs at low passage for future use.

Freezing Mouse iPS Cells

22. Let cells grow until they reach 80-90% confluency.
23. Aspirate the cell culture medium from each well and wash cells with 2 mL of sterile PBS.
24. Remove PBS completely, add 0.5 mL of 0.25% trypsin/EDTA and incubate at 37°C and 5% CO₂ for 4 min (for 10cm plate use 2 ml of 0.25% trypsin/EDTA).
25. Add 2.5 ml of serum containing mouse iPS medium and suspend the cells by pipetting up and down to single cell suspension.
26. Transfer the cell suspension to a 15-ml tube and add in mouse iPS medium to a total volume of 10 mL. Count cells using a hemocytometer. Centrifuge at 200 × g for 4 min.
27. Aspirate the supernatant, resuspend the pellet in Freezing Medium (10% DMSO in FBS) to obtain a concentration of 2×10⁶ cells per mL.
28. Transfer the cell suspension to pre-labeled cryovials at 1mL per cryovial. Labeling should include cell line, passage number and date. Generally you can use two cryovials per well of a 6-well plate.
29. Quickly store the cryovials in a chilled cell-freezing container (4°C or on wet ice) and freeze at –80°C overnight. Cells should be transferred to –80°C rapidly, since DMSO is toxic to cells in liquid phase.
30. Next day: remove cryovials from freezing container and store in the gas phase of a liquid nitrogen tank for long-term storage.

NOTE: When working with DMSO-containing freezing media, it is key to work as quickly as possible to transfer the freezing stock rapidly into a –80°C freezer in order to initiate the freezing procedure. To minimize exposure at RT, you should pre-label the cryo-vials and have the freezing container ready.

Thawing Mouse iPS Cells

31. Use cryogenic hand gloves and eye protector as cryovials stored in the liquid nitrogen tank may explode unexpectedly when exposed to rapid thawing.
32. Remove the cryovial from the liquid nitrogen tank using forceps.
33. Immerse the vial in a 37°C water bath without submerging the cap.
34. Thaw vial rapidly until just a few ice crystals are left.
35. Note down the sample name and passage number and spray 70% ethanol on the outer surface of the vial then air dry quickly for few seconds in a sterile laminar flow tissue culture hood.
36. Transfer thawed cells to a 15-mL conical tube using a P1000 pipette. Rinse freezing vial once with 1mL of medium and add back into the tube.
37. Add 10 mL of cold culture medium drop-wise to cells in the 15 mL conical tube. While adding the medium, gently move the tube back and forth to mix the cells. This step reduces osmotic shock to the cells.
38. Centrifuge the cells at $200 \times g$ for 4 minutes at RT.
39. Aspirate the supernatant and resuspend the cell pellet in 2 ml of mouse iPS medium.
40. Slowly add the cell suspension in a drop-wise fashion into a well of a gelatin-coated 6-well plate, pre-seeded with growth-inhibited MEF cells.
41. Culture cells as described above in a 5% CO₂ incubator at 37°C.

Alternate Protocol 1; Establishment and Maintenance of Rat iPS Cells

This section describes the establishment and maintenance of rat iPS Cells. Please revert back to the support protocols for the viral infection procedure.

Materials—15 mL conical tubes

6-wells plate, pre-coated with 0.1% gelatin

Irradiated CF1 MEFs (GlobalStem 6001G)

Mitomycin C

0.25% Trypsin-0.53mM EDTA (GIBCO 25200-056)

MEF medium (see recipe)

Rat iPS cell medium (see recipe)

MEK Inhibitor (Stemgent 04-0006)

GSK3b inhibitor (Stemgent 04-0004)

Rat LIF (Millipore LIF3005)

Doxycycline (Sigma D9891)

24-wells plate, pre-coated with 0.1% gelatin

37°C and 5% CO₂ incubator

37°C water bath

Centrifuge

Laminar Flow Hood

Light Microscope with camera

Freezing medium (see recipe)

PBS without CaCl and MgCl (Invitrogen 14190-250)

–80° C Freezer

Liquid Nitrogen Storage tank

Cryogenic handling gloves & eye protectors

Forceps

Refrigerator (4°C)

Biosafety cabinet

Micropipettes

Freezing chamber

2.0 ml Cryovials

Post-infection culturing of infected fibroblasts

1. After 48 hours, or when cells reach confluency, aspirate the medium from the infected cells and dissociate by adding 0.5 mL of 0.25% Trypsin-EDTA and incubating for 4 min at 37°C and 5% CO₂. Add 2 mL of warm MEF medium pipette up and down in order to obtain a single cell suspension. Transfer to a 15 mL tube containing 9 mL of warm MEF medium and centrifuge at 200 × g for 4 minutes.
2. Discard the supernatant and dissolve the pellet in 8 mL of MEF media.
3. Aspirate the medium from 4 single wells of 6-wells plate that has been gelatin-coated and pre-seeded with growth-inhibited MEF cells. Add 2mL of the infected

cell mixture per well onto the MEF feeder layer so that the infected cells are passaged in a 1:4 ratio. Incubate at 37°C and 5% CO₂.

4. After 12-24 hours substitute the medium with rat iPS cell culture medium (supplemented with doxycycline if using a TetO lentiviral system). For initial culture conditions use double the concentration of rat LIF. After 2-3 passages, regular dose of LIF (1000u/mL) can be used.
5. Change rat iPS medium every 24 hours and check for colony formation. Colonies should start becoming microscopically visible approximately 7-10 days post-infection.
6. Let colonies grow into a reasonable size (> 50-100 cells/colony). This should take until approximately day 14 or 15 post-infection.

NOTE: Rat iPS medium should be supplemented with 0.5 μ M MEK inhibitor (e.g. PD0325901) and 3 μ M GSK3 β inhibitor (e.g. CHIR99021). These inhibitors will help maintain cells in an undifferentiated state.

NOTE: Cell death is a common problem when cells are transferred directly from serum-containing medium to serum-free medium or KOSR-containing medium, like rat iPS medium. This may appear quite dramatic initially as up to 50% of cells may fail to attach, but abundant viable and proliferative cells should be able to be apparent after medium change.

Picking and Establishing Rat iPS clones

7. Pre-feed the cells 1 hour before picking iPS cells colonies by replenishing with fresh rat iPS media. This is especially important in case the media has turned acidic (indicated by yellow color), since pre-feeding will increase cell survival after dissociation.
8. Before picking, select as many good colonies as necessary by circling with a marker pen at the bottom of the plate around the colony to be able to retrieve the good colonies when the actual picking procedure is started. Suitable colonies should look translucent and perfectly circular. See Figure 4 for examples of suitable rat iPS colonies that can be picked.
9. Picking should be done using a microscope inside the laminar hood to maintain sterile conditions. See Figure 1 for appropriate picking conditions.
10. Add 50 μ L of Trypsin-EDTA into several 15 mL tubes. Use a P20 (20 μ L) Pipette for the picking procedure.
11. Pick one individual colony by gently scratching with the pipette tip. Make sure not to touch any neighboring colonies. Pick up as many colonies as you can within 15 minutes to limit exposure of picked colonies to trypsin. See figure 4 for examples of good rat iPS colonies that can be picked.

12. Transfer each picked colony into an individual 15 ml tube containing 50 μ L of trypsin. Dissociate the colony by gentle pipetting and incubate at 37°C in water bath for 4 minutes.
13. Add 5 mL of rat iPS medium to each 15 mL tube and pipette up and down to dissociate the colony further into a single cell suspension. Centrifuge at $200 \times g$ for 4 minutes.
14. Aspirate the supernatant and dissolve the pellet in 1mL of rat iPS medium. Transfer the cell suspension from each picked colony into a single well of a 24-well plate that has been gelatin-coated and pre-seeded with growth-inactivated MEF cells. Incubate at 37°C and 5% CO₂ and replenish media every 24 hours until nearly confluent. Cells are now ready to be passaged into a 6-well plate.

NOTE: If you are reprogramming your cells using a doxycycline-inducible system, wean your cells off doxycycline gradually after picking colonies. As a guideline, you can use the following schedule: use $\frac{1}{2}$ concentration of doxycycline and then lower the concentration with each passage, so $\frac{1}{4}$ concentration, then no doxycycline. If cell morphology begins to degrade, slow down the rate of weaning. Do not change media conditions at the time of passaging; rather wait until the cells have attached (usually after 12-24 hours) before making changes to the doxycycline concentration.

NOTE: Refer to Figure 5 for a schematic diagram of generating rat iPSCs.

In Vitro Culture and Expansion of Rat iPS cells

15. When iPS cell colonies reach 80-90% confluency in the dish or when individual iPS cell colonies become large (approximately >100 cells/colony), they should be passaged. For rat iPS cells, a 1:6 passaging ratio is usually easily tolerated.
16. Aspirate the medium from the dish, and pre-feed the cells with rat iPS media 1 hour before passaging.
17. After 1 hour aspirate the medium and wash the cells with 2 mL of PBS.
18. Aspirate the PBS, add 0.5 mL of 0.25% trypsin-EDTA and incubate at 37°C and 5% CO₂ for 4 min.
19. Add 1mL of rat iPS medium and dissociate the colonies by pipetting up and down into single cell suspension.
20. Transfer into a 15 mL tube containing 9 mL of warm rat iPS medium and centrifuge at $200 \times g$ for 4 minutes.
21. Discard the supernatant and dissolve the pellet in 12 mL of rat iPS medium.
22. Aspirate the medium from 6 well plates containing mitomycin C inactivated MEF and add 2ml of cell suspension per well. Incubate the cells at 37°C and 5% CO₂ until cells reach 80–90% confluency. Replenish with fresh rat iPS media every 24 hours. It is advisable to prepare frozen stocks of newly reprogrammed iPSCs at low passage for future reference.

NOTE: There are two recipes for rat iPS medium; one for serum-free rat iPS medium and one for KOSR-containing rat iPS medium (see section “Reagents & Solutions”). Any of these media can be used for culturing rat iPSCs.

NOTE: Rat iPS medium should always be supplemented with 0.5 μ M MEK inhibitor (e.g. PD0325901), 3 μ M GSK3 β inhibitor (e.g. CHIR99021). These inhibitors will help maintain cells in an undifferentiated state.

Freezing Rat iPS Cells

23. Let cells grow until they reach 80-90% confluency.
24. Aspirate the cell culture medium from each well and wash cells with 2 mL of sterile PBS.
25. Remove PBS completely, add 0.5 mL of 0.25% trypsin/EDTA and incubate at 37°C and 5% CO₂ for 4 min (for 10cm plate use 2 ml of 0.25% trypsin/EDTA).
26. Add 2.5 ml of serum containing rat iPS medium and suspend the cells by pipetting up and down to single cell suspension.
27. Transfer the cell suspension to a 15-ml tube and add in rat iPS medium to a total volume of 10 mL. Count cells using a haemocytometer. Centrifuge at 200 $\times$ g for 4 min.
28. Aspirate the supernatant, resuspend the pellet in Freezing Medium (10% DMSO in FBS) to obtain a concentration of 2×10^6 cells per mL.
29. Transfer the cell suspension to pre-labeled cryovials at 1mL per cryovial. Labeling should include cell line, passage number and date. Generally you can use two cryovials per well of a 6-well plate.
30. Quickly store the cryovials in a chilled cell-freezing container (4°C or on wet ice) and freeze at –80°C overnight. Cells should be transferred to –80°C rapidly, since DMSO is toxic to cells in liquid phase.
31. Next day: remove cryovials from freezing container and store in the gas phase of a liquid nitrogen tank for long-term storage.

NOTE: When working with DMSO-containing freezing media, it is key to work as quickly as possible to transfer the freezing stock rapidly into a –80°C freezer in order to initiate the freezing procedure. To minimize exposure at RT, you should pre-label the cryo-vials and have the freezing container ready.

Thawing Rat iPS Cells

32. Use cryogenic hand gloves and eye protector as cryovials stored in the liquid nitrogen tank may explode when exposed to rapid thawing.
33. Remove the cryovial from the liquid nitrogen tank using forceps.
34. Immerse the vial in a 37°C water bath without submerging the cap.
35. Thaw vial rapidly until just a few ice crystals are left.

36. Note down the sample name and passage number and spray 70% ethanol on the outer surface of the vial then air dry quickly for few seconds in a sterile laminar flow tissue culture hood.
37. Transfer thawed cells to a 15-mL conical tube using a P1000 pipette. Rinse freezing vial with 1mL of medium.
38. Add 10 ml of cold culture medium drop-wise to cells in the 15 ml conical tube. While adding the medium, gently move the tube back and forth to mix the cells. This step reduces osmotic shock to the cells.
39. Centrifuge the cells at $200 \times g$ for 4 minutes at RT.
40. Aspirate the supernatant and resuspend the cell pellet in 2 ml of rat iPS medium.
41. Slowly add the cell suspension in a drop-wise fashion into a well of a gelatin-coated 6-well plate, pre-seeded with growth-inhibited MEF cells.
42. Culture cells as described above in a 5% CO₂ incubator at 37°C.

Alternate Protocol 2 : Establishment and Maintenance of Pig iPS Cells

This section describes the establishment and maintenance of pig iPS Cells. Please revert back to Basic Protocol 1 for the viral infection procedure.

Materials—15 mL conical tubes

0.1% gelatin solution (see recipe)

6-wells plate, pre-coated with 0.1% gelatin

24-wells plate, pre-coated with 0.1% gelatin

Irradiated CF1 MEFs (GlobalStem 6001G)

Mitomycin C

0.25% Trypsin-0.53mM EDTA (GIBCO 25200-056)

MEF medium (see recipe)

Pig iPS cell medium (see recipe)

Matrigel

Knock-Out DMEM medium (GIBCO 10829)

mTeSR1 medium

TeSR2 (STEMCELL Technologies 05860)

mFreSR (STEMCELL Technologies 05855)

Rock inhibitor Y27632 (Calbiochem 688000)

Dispase

Accutase

Collagenase IV

Doxycyclin (Sigma D9891)

24-wells plate, pre-coated with 0.1% gelatin

37°C and 5% CO₂ incubator

37°C water bath

Eppendorf Model 5810R benchtop centrifuge

Light Microscope with camera

Laminar Flow Hood

Freezing medium (see recipe)

PBS without CaCl and MgCl (Invitrogen 14190-250)

Cell lifter (Corning 3008)

–80° C Freezer

Liquid Nitrogen Storage tank

Cryogenic handling gloves & eye protectors

Forceps

Refrigerator (4°C)

Biosafety cabinet

Micropipettes

Freezing chamber

2.0 mL cryovials

70% ethanol

Post-infection culturing of infected fibroblasts

1. After 48 hours, or when cells reach confluency, aspirate the medium from the infected cells and dissociate by adding 0.5 mL of 0.25% Trypsin-EDTA and

incubating for 4 min at 37°C and 5% CO₂. Add 2 mL of warm MEF medium pipette up and down in order to obtain a single cell suspension. Transfer to a 15 mL tube containing 9 mL of warm MEF medium and centrifuge at 200 × g for 4 minutes.

2. Discard the supernatant and dissolve the pellet in 8 mL of MEF cell culture medium.
3. Aspirate the medium from 4 single wells of 6-wells plate that has been gelatin-coated and pre-seeded with growth-inhibited MEF cells. Add 2 mL of the infected cell mixture per well onto the MEF feeder layer so that the infected cells are passaged in a 1:4 ratio. Incubate at 37°C and 5% CO₂.
4. After 12-24 hours substitute the medium with pig iPS cell culture medium (supplemented with Doxycycline if using a TetO lentiviral system).
5. Change pig iPS medium every 24 hours and check for colony formation. Colonies should start becoming microscopically visible approximately 7-10 days post-infection.
6. Let colonies grow into a reasonable size (approximately >50 cells/colony). This should take until approximately day 21 post-infection.

NOTE: It is recommended to culture pig iPSCs on MEFs initially. Once stable colonies are established, the cells can be transferred to feeder-free conditions.

NOTE: Pig iPS medium should always be supplemented with human bFGF (10 ng/ml) when cultured on MEFs. This will help maintain the cells in an undifferentiated state.

Picking and Establishing Pig iPS clones

7. Pre-feed the cells 1 hour before picking iPS cells colonies by replenishing with fresh pig iPS media. This is especially important in case the media has turned acidic (indicated by yellow color), since pre-feeding will increase cell survival after dissociation.
8. Before picking, select as many good colonies as necessary by circling with a marker pen at the bottom of the plate around the colony to be able to retrieve the good colonies when the actual picking procedure is started. Suitable colonies should look translucent and perfectly circular. See Figure 6 for examples of suitable pig iPS colonies that can be picked.
9. Picking should be done using a microscope inside the laminar hood to maintain sterile conditions. See figure 1 for appropriate picking conditions.
10. Add 50 µL of pig iPS medium into several 15 mL tubes. Use a 20 µL Pipette for the picking procedure.
11. Pick one individual colony by gently scratching with the pipette tip. Make sure not to touch any neighboring colonies. See figure 6 for examples of good pig iPS colonies that can be picked.

12. Transfer each picked colony into an individual 15 ml tube containing 50 μ L of pig iPS medium. Dissociate the colony by gentle mechanical dissociation with the pipette tip and pipetting up and down. Ideally colonies should be dissociated into small cell clusters instead of single-cell dissociation.
13. Once the colony is properly dissociated, add in 1mL of pig iPS medium. Transfer the cell suspension from each picked colony into a single well of a 24-well plate that has been gelatin-coated and pre-seeded with growth-inactivated MEF cells. Alternatively, you can use feeder-free conditions using Matrigel-coated plates with mTeSR1 medium. Incubate at 37°C and 5% CO₂.
14. After 48 hours continuously replenish the medium every 24 hours until cells reach 80-90% confluency. This should take about 7-10 days. Cells are now ready to be passaged into a 6-well plate.

NOTE: If you are reprogramming your cells using a doxycycline inducible system, wean your cells off doxycycline gradually after picking colonies. As a guideline, you can use the following schedule: use $\frac{1}{2}$ concentration of doxycycline and then lower the concentration with each passage, so $\frac{1}{4}$ concentration, then no doxycycline. If cell morphology begins to degrade, slow down the rate of weaning. Do not change media conditions at the time of passaging; rather wait until the cells have attached (usually after 12-24 hours) before making changes to the doxycycline concentration.

NOTE: Refer to Figure 7 for a schematic diagram of generating pig iPSCs.

In Vitro Culturing and Expansion of Pig iPS Cells: **NOTE:** These instructions are for passaging cells grown on MEFs. For cells grown on Matrigel, one should use Dispase or Accutase instead of Collagenase IV. Trypsinization is not recommended, since more aggressive dissociation into single cells will not be well tolerated.

NOTE: If using MEFs plate 1 million MEFs per 10-cm plate or approximately 170,000 MEFs per well of a 6-well plate. Use gelatin-coated plates.

15. Before splitting, remove differentiated colonies under a microscope in sterile conditions (i.e. via slow-vacuum aspiration or pipet scraping). Be careful not to leave plate out too long and make sure cells do not dry out if using vacuum method. See Figure 6 for examples of good pig iPSC colonies as well as bad pig iPSC colonies that should be removed from the culture dish.
16. Wash cells with warm PBS. Aspirate PBS and add 1 mL of Collagenase IV and incubate 4 min at 37°C and 5% CO₂ (for pig iPSCs grown on MEFs). Alternatively, add 0.5 mL of Dispase or Accutase and incubate for 0.5-1 min at 37°C and 5% CO₂ (for pig iPSCs grown in feeder-free conditions). Expect to see visible curling or thickening of colonies around the edges.
17. Aspirate off the enzyme and wash with 2 mL of PBS. After aspirating off PBS, add 1 mL of pig iPS medium supplemented with ROCK inhibitor. Using a cell lifter, scrape the entire well to lift the colonies.

18. Transfer into a 15 mL conical tube; wash the well with 1 mL of pig iPS medium and transfer into tube.
19. Centrifuge $200 \times g$ for 4 min. Aspirate off the media, and resuspend pellet in pig iPS medium. Use 2 mL of medium per well of a 6-well plate that is going to be seeded. The ratio depends on cell density prior to passaging. Usually a 1:3 ratio (1 nearly confluent well can be passaged into 3 new wells) serves as a good guideline. For this example, resuspend the cell pellet in 6 mL of medium.
20. Triturate to get medium-small fragments (~50-200 cells per fragment). Avoid over-triturating since that will lead to cell death, especially when colonies are broken down to single cell suspensions.
21. Pre-wash gelatin-coated and MEF-seeded wells or Matrigel-coated wells with 1 mL of warm pig iPS medium, aspirate off and add in 2 mL of suspended cell solution into each well.
22. Replenish the cells with 2 mL of fresh pig iPS medium every 24 hours until cells reach confluency. This usually takes about 7-10 days. Cells are now ready for passaging. It is advisable to prepare frozen stocks of newly reprogrammed iPSCs at low passage for future reference. Please refer to the next paragraph for instructions.

NOTE: Cell death is a common problem when cells are transferred directly from serum-containing medium to serum-free medium, like mTeSR1 medium. This may appear quite dramatic initially as up to 50% of cells may fail to attach, but abundant viable and proliferative cells should be able to appear upon medium change.

NOTE: Always supplement pig iPS medium with human bFGF (10 ng/ml) when culturing on MEFs. This will help maintain the cells in an undifferentiated state.

Freezing Pig iPS cells: **NOTE:** For cells grown on Matrigel/mTeSR1, a similar procedure can be followed, except for using 500 μ L of mFreSR per 6-well. mFreSR is a defined, serum-free cryopreservation medium designed for the cryopreservation.

NOTE: It is very important to minimize the amount of pipetting to ensure cell survival later on.

23. Dissociate the cells of a nearly confluent well as described above. Use Collagenase IV for feeder/serum cultures and Dispase or Accutase for Matrigel/mTeSR1 cultures.
24. Generally you can freeze one cryovial per well of a 6-well plate or 5 cryo-vials per 10-cm dish. Gently resuspend the cell pellet in 250 μ L of pig iPS medium.
25. Add in 250 μ L of 2X freezing media, and carefully resuspend the pellet in the combined media (keeping cells in as large of chunks as possible; generally pipetting 2x should be enough).

26. Quickly transfer 500 μ L into a cryo-vial, and place inside isopropanol-containing freezing container. Store 24-48 hours at -80°C and then transfer to liquid nitrogen.

NOTE: When working with DMSO-containing freezing media, it is key to work as quickly as possible to transfer the freezing stock rapidly into a -80°C freezer in order to initiate the freezing procedure. To minimize exposure at RT, you should pre-label the cryo-vials and have the freezing container ready.

Thawing Pig iPS cells: NOTE: Each vial should be thawed into 1 well of a 6-well plate. The passage number and the name of the cell line should be noted. Ideally cells should be kept in large clumps in order to increase survival efficiency, so one must avoid vigorous pipetting.

27. Add 9 mL of cold pig iPS medium supplemented with 10 μM ROCK inhibitor into a 15 mL tube.
28. Use cryogenic hand gloves and eye protector as cryovials stored in the Liquid Nitrogen may accidentally explode when thawing.
29. Remove the iPS cells vial from the liquid nitrogen tank using forceps and immerse the vial in a 37°C water bath without submerging the cap. Thaw vial rapidly until just a few ice crystals are left.
30. Write down the sample name and spray 70% ethanol on the outer surface of the vial then air dry quickly a few seconds in the sterile laminar hood.
31. Transfer the thawed cell mixture in the tube containing 9 mL of cold pig iPS medium supplemented with ROCK inhibitor. Rinse the cryo-vial once with media and transfer to tube.
32. Centrifuge at $200 \times g$ for 4 min.
33. Meanwhile, wash one well of a 6-well gelatin-coated and MEF-seeded (growth inhibited) with PBS. Skip this step when using Matrigel plates.
34. Add in 2 mL of pig iPS media. It is highly recommended that you add 10 μM ROCK inhibitor Y-27632 for the first 24 hours to improve survival efficiency. Do not add ROCK inhibitor to medium replenishing steps that do not involve passaging or thawing.

NOTE: The ROCK Inhibitor Y-27632 enhances the survival rate of human embryonic stem cells following cryopreservation (Li 2008).

35. Aspirate the media from the cell pellet and gently resuspend in 1 mL of pig iPS media supplemented with ROCK inhibitor. Pipet slowly once or twice, avoiding disruption of the cell chunks. Transfer to one well of a 6-well plate.
36. Change the medium after 24 hours.
37. Feed cells daily with 2 mL medium. Colonies may not develop the next day, but may emerge anywhere from 3 to 10 days.

38. The first passage should be done mechanically, without the use of Collagenase IV or Dispase. Please refer to the section “Picking and Establishing Pig iPS Clones” for details. After this initial passage, cells can be cultured as described in the section “In Vitro Culture and Expansion of Pig iPS Cells”.

NOTE: It is highly recommended that you perform a mycoplasma test upon thawing.

Alternate Protocol 4 : Establishment and Maintenance of Human iPS Cells

This section describes the establishment and maintenance of human iPS Cells. Please revert back to the support protocols for the viral infection procedure.

Materials—15 mL conical tubes

0.1% gelatin solution (see recipe)

6-wells plate, pre-coated with 0.1% gelatin

24-wells plate, pre-coated with 0.1% gelatin

Irradiated CF1 MEFs (GlobalStem 6001G)

Mitomycin C

0.25% Trypsin-0.53mM EDTA (GIBCO 25200-056)

MEF medium (see recipe)

Human iPS cell medium (see recipe)

Matrigel (see recipe)

Knock-Out DMEM medium (GIBCO 10829)

mTeSR1 medium (see recipe)

TeSR2 (STEMCELL Technologies 05860)

mFreSR (STEMCELL Technologies 05855)

Rock inhibitor Y27632 (Calbiochem 688000)

Dispase (Invitrogen 17105-041)

Accutase (Innovative Cell Technologies AT-104)

Collagenase IV (Invitrogen 17104-019)

Doxycyclin (Sigma D9891)

24-wells plate, pre-coated with 0.1% gelatin

37°C and 5% CO₂ incubator

37°C water bath

Eppendorf Model 5810R benchtop centrifuge

Light Microscope with camera

Laminar Flow Hood

Freezing medium (see recipe)

PBS without CaCl and MgCl (Invitrogen 14190-250)

Cell lifter (Corning 3008)

–80° C Freezer

Liquid Nitrogen Storage tank

Cryogenic handling gloves & eye protectors

Forceps

Refrigerator (4°C)

Biosafety cabinet

Micropipettes

Freezing chamber

2.0 ml Cryovials

70% ethanol

Post-infection culturing of infected fibroblasts

1. After 48 hours, or when cells reach confluency, aspirate the medium from the infected cells and dissociate by adding 0.5 mL of 0.25% Trypsin-EDTA and incubating for 4 min at 37°C and 5% CO₂. Add 2 mL of warm MEF medium pipette up and down in order to obtain a single cell suspension. Transfer to a 15 mL tube containing 9 mL of warm MEF medium and centrifuge at 200 × g for 4 minutes.
2. Discard the supernatant and dissolve the pellet in 8 mL of MEF cell culture medium.
3. Aspirate the medium from 4 single wells of 6-wells plate that has been gelatin-coated and pre-seeded with growth-inhibited MEF cells. Add 2mL of the infected cell mixture per well onto the MEF feeder layer so that the infected cells are passaged in a 1:4 ratio. Incubate at 37°C and 5% CO₂.

4. After 12-24 hours substitute the medium with human iPS cell culture medium (supplemented with Doxycycline if using a TetO lentiviral system).
5. Change human iPS medium every 24 hours and check for colony formation. Colonies should start becoming microscopically visible approximately 7-10 days post-infection.
6. Let colonies grow into a reasonable size (approximately >50 cells/colony). This should take until approximately day 21 post-infection.

NOTE: It is recommended to culture human iPSCs on MEFs initially. Once stable colonies are established, the cells can be transferred to feeder-free conditions.

NOTE: Human iPS medium should be supplemented with human bFGF (10 ng/ml) when cultured on MEFs. This will help maintain the cells in an undifferentiated state.

Picking and Establishing Human iPS clones

7. Pre-feed the cells 1 hour before picking iPS cells colonies by replenishing with fresh human iPS medium. This is especially important in case the medium has turned acidic (indicated by yellow color), since pre-feeding will increase cell survival after dissociation.
8. Before picking, select as many good colonies as necessary by circling with a marker pen at the bottom of the plate around the colony to be able to retrieve the good colonies when the actual picking procedure is started. Suitable colonies should look translucent and perfectly circular. See Figure 8 for examples of suitable human iPS colonies that can be picked.
9. Picking should be done using a microscope inside the laminar hood to maintain sterile conditions. See figure 1 for appropriate picking conditions.
10. Add 50 μ L of human iPS medium into several 15 mL tubes. Use a P20 (20 μ L) Pipette for the picking procedure.
11. Pick one individual colony by gently scratching with the pipette tip. Make sure not to touch any neighboring colonies. See Figure 8 for examples of good human iPS colonies that can be picked.
12. Transfer each picked colony into an individual 15 ml tube containing 50 μ L of human iPS medium. Dissociate the colony by gentle mechanical dissociation with the pipette tip and pipetting up and down. Ideally colonies should be dissociated into small cell clusters instead of single-cell dissociation.
13. Once the colony is properly dissociated, add in 1 mL of human iPS medium. Transfer the cell suspension from each picked colony into a single well of a 24-well plate that has been gelatin-coated and pre-seeded with growth-inactivated MEF cells. Alternatively, you can use feeder-free conditions using matrigel-coated plates with mTeSR1 medium. Incubate at 37°C and 5% CO₂.

14. After 48 hours: continuously replenish human iPS medium every 24 hours until cells reach 80-90% confluency. This should take about 7-10 days. Cells are now ready to be passaged into a 6-well plate.

NOTE: If you are reprogramming your cells using a doxycycline-inducible system, wean your cells off doxycycline gradually after picking colonies. As a guideline, you can use the following schedule: use ½ concentration of doxycycline and then lower the concentration with each passage, so ¼ concentration, then no doxycycline. If cell morphology begins to degrade, slow down the rate of weaning. Do not change medium conditions at the time of passaging; rather wait until the cells have attached (usually after 12-24 hours) before making changes to the doxycycline concentration.

NOTE: Refer to Figure 9 for a schematic diagram of human iPSC generation.

In Vitro Culturing and Expansion of Human iPS Cells: **NOTE:** These instructions are for passaging cells grown on MEFs. For cells grown on Matrigel, one should use Dispase or Accutase in place of Collagenase IV. Trypsinization is not recommended.

NOTE: If using MEFs plate 1 million MEFs per 10-cm plate or approximately 170,000 MEFs per well of a 6-well plate. Use gelatin-coated plates.

15. Before splitting, remove differentiated colonies under a microscope in sterile conditions (i.e. via slow-vacuum aspiration or pipet scraping). Be careful not to leave plate out too long and make sure cells do not dry out if using vacuum method. See Figure 8 for examples of good human iPSC colonies as well as bad human iPSC colonies that should be removed from the culture dish.
16. Wash cells with warm PBS. Aspirate PBS and add 1 mL of Collagenase IV and incubate 4 min at 37°C and 5% CO₂ (for human iPSCs grown on MEFs). Alternatively, add 0.5 mL of Dispase or Accutase and incubate for 0.5-1 min at 37°C and 5% CO₂ (for human iPSCs grown in feeder-free conditions). Expect to see visible curling or thickening of colonies around the edges.
17. Aspirate off the enzyme and wash with 2 mL of PBS. After aspirating off PBS, add 1 mL of human iPS medium supplemented with ROCK inhibitor. Using a cell lifter, scrape the entire well to lift the colonies.
18. Transfer into a 15 mL conical tube; wash the well with 1 mL of human iPS medium and transfer into tube.
19. Centrifuge 200 × g for 4 min. Aspirate off the medium, and resuspend pellet with human iPS medium. Use 2 mL of medium per well of a 6-well plate that is going to be seeded. The ratio depends on cell density prior to passaging. Usually a 1:3 ratio (1 nearly confluent well can be passaged into 3 new wells) serves as a good guideline. For this example, resuspend the cell pellet in 6 mL of medium.
20. Triturate to get medium-small fragments (~50-200 cells per fragment). Avoid over-triturating since that will lead to cell death, especially when colonies are broken down to single cell suspensions.

21. Pre-wash gelatin-coated and MEF-seeded wells or Matrigel-coated wells with 1 mL of warm human iPS medium, aspirate off and add in 2 mL of suspended cell solution into each well.
22. Replenish the cells with 2 mL of fresh human iPS medium every 24 hours until cells reach confluency. This usually takes about 7-10 days. Cells are now ready for passaging. It is advisable to prepare frozen stocks of newly reprogrammed iPSCs at low passage for future reference. Please refer to the next paragraph for instructions.

NOTE: Cell death is a common problem when cells are transferred directly from serum-containing medium to serum-free medium, like mTeSR1 medium. This may appear quite dramatic initially as up to 50% of cells may fail to attach, but abundant viable and proliferative cells should be able to appear upon medium change.

NOTE: Supplement human iPS medium with bFGF (10 ng/ml) when culturing on MEFs. This will help maintain the cells in an undifferentiated state.

Freezing Human iPS cells: NOTE: For cells grown on Matrigel/mTeSR1, a similar procedure can be followed as described below, except for using 500 μ L of mFreSR per 6-well. mFreSR is a defined, serum-free cryopreservation medium designed for the cryopreservation of human embryonic and induced pluripotent stem cells (hESCs and hiPSCs). Together with mTeSR1 or TeSR2, mFreSR eliminates the use of feeders and serum. hESCs cryopreserved in mFreSR have thawing efficiencies 5-10 fold higher than reported conventional thawing methods using serum.

NOTE: It is very important to minimize the amount of pipetting to ensure cell survival later on.

23. Dissociate the cells of a nearly confluent well as described above. Use Collagenase IV for feeder/serum cultures and Dispase or Accutase for Matrigel/mTeSR1 cultures.
24. Generally you can freeze one cryovial per well of a 6-well plate or 5 cryo-vials per 10-cm dish. Gently resuspend the cell pellet in 250 μ L of human iPS medium.
25. Add in 250 μ L of 2X freezing medium, and carefully resuspend the pellet in the combined medium (keeping cells in as large of chunks as possible; generally pipetting 2x should be enough).
26. Quickly transfer 500 μ L into a cryo-vial, and place inside isopropanol-containing freezing container. Store 24-48 hours at -80°C and then transfer to liquid nitrogen.

NOTE: When working with DMSO-containing freezing medium, it is key to work as quickly as possible to transfer the freezing stock rapidly into a -80°C freezer in order to initiate the freezing procedure. To minimize exposure at RT, you should pre-label the cryo-vials and have the freezing container ready.

Thawing Human iPS cells: **NOTE:** Each vial should be thawed into 1 well of a 6-well plate. The passage number and the name of the cell line should be noted. Ideally cells should be kept in large clumps in order to increase survival efficiency, so one must avoid vigorous pipetting.

27. Add 9 mL of cold human iPS medium supplemented with 10 μ M ROCK inhibitor into one 15 mL tube.
28. Use cryogenic hand gloves and eye protector as cryovials stored in the Liquid Nitrogen may explode unexpectedly when thawing.
29. Remove the iPS cells vial from the liquid nitrogen tank using forceps and immerse the vial in a 37°C water bath without submerging the cap. Thaw vial rapidly until just a few ice crystals are left.
30. Write down the sample name and spray 70% ethanol on the outer surface of the vial then air dry quickly a few seconds in the sterile laminar hood.
31. Transfer the thawed cell mixture in the tube containing 9 mL of cold human iPS medium supplemented with ROCK inhibitor. Rinse the cryo-vial once with medium and transfer to tube.
32. Centrifuge at $200 \times g$ for 4 min.
33. Meanwhile, wash one well of a 6-well plate that has been pre-coated with gelatin and irradiated MEF with PBS. Skip this step when using Matrigel only plates.
34. Add 2 mL of human iPS medium. It is highly recommended that you add 10 μ M ROCK inhibitor Y-27632 for the first 24 hours to improve survival efficiency. ROCK inhibitor should not be added to any subsequent medium replenishing steps that do not involve passaging or thawing.

NOTE: The ROCK Inhibitor Y-27632 enhances the survival rate of human embryonic stem cells following cryopreservation. (Li 2008)

35. Aspirate the medium from the cell pellet and gently resuspend in 1 mL of human iPS medium supplemented with ROCK inhibitor. Pipet slowly once or twice, avoiding disruption of the cell chunks. Transfer to one well of a 6-well plate.
36. Change the medium after 24 hours.
37. Feed cells daily with 2 ml medium. Colonies may not develop the next day, but may emerge anywhere from 3 to 10 days.
38. The first passage should be done mechanically, without the use of Collagenase IV or Dispase. Please refer to the section “Picking and Establishing Human iPS Clones” for details. After this initial passage, cells can be cultured as described in the section “In Vitro Culture and Expansion of Human iPS Cells”.

NOTE: It is highly recommended that you perform a mycoplasma test upon thawing.

Reagents and Solutions—Unless otherwise noted, all solutions and media are 0.2µm-filter sterilized. All media must be stored at 4°C and should be used within 10 days of preparation, unless otherwise specified.

MEF Medium—

DMEM (high glucose) GIBCO 11965-092	81%	500.00 mL
FBS (heat inactivated) Benchmark 100-106	15%	94.00 mL
PEN/STREP (5,000 i.u./ea/ml)GIBCO 15070-063	2%	12.50 mL
L-GLUTAMINE (200mM) GIBCO 25030-081	1%	6.25 mL
NEAA (10mM or 100X) GIBCO 11140-050	1%	6.25 mL

Mouse iPS cell Medium(CJ7 Medium)—

DMEM (high glucose) GIBCO 11965-092	81%	500.00 mL
FBS (heat inactivated) Benchmark 100-106	15%	94.00 mL
PEN/STREP (5,000 i.u./ea/ml)GIBCO 15070-063	2%	12.50 mL
L-GLUTAMINE (200mM) GIBCO 25030-081	1%	6.25 mL
NEAA (10mM or 100X) GIBCO 11140-050	1%	6.25 mL
2 ME (or Beta ME) SIGMA M-6250	~10 ⁻⁴ M	4.4 µL
Mouse LIF (10 ⁷ u/ml stock) CHEMICON ESG1107	10 ³ u/mL	62.5 µL

NOTE: Complete medium is stable for ~2 week. Medium older than this should be resupplemented with fresh L-Glutamine and mouse LIF.

Rat iPS cell culture medium

Serum-free rat iPS cell medium: - To 100 mL DMEM-F12 Medium (Invitrogen 11330-057) add 1 mL N2 supplement (Invitrogen 17502048) 100X stock solution. Store at 4°C and use within 1 month.

- To 100 mL Neurobasal Medium (Invitrogen 21103) add 2 mL B27 (Invitrogen, 17504-044) and 0.5–1 mL of 200 mM L-glutamine (Invitrogen 25030-081). Store at 4°C and use within 1 month.

- Mix DMEM/F12-N2 medium with Neurobasal/B27 medium in a ratio of 1:1. Add β-mercaptoethanol (Sigma M7522) to a final concentration of 0.1 mM. Store at 4°C and use within 1 month.

NOTE: For establishing and culturing Rat iPS cells, rat iPS medium should always be supplemented with 0.5 µM MEK inhibitor (e.g. PD0325901, Stemgent 04-0006), 3 µM GSK3β inhibitor (e.g. CHIR99021, Stemgent 04-0004) and 1000 U/mL of Rat LIF (Millipore LIF3005). Add freshly before use. These supplements help maintain rat iPS in an undifferentiated state.

KOSR-containing rat iPS medium:

Knockout DMEM (GIBCO 10829)	400 mL
Knockout Serum Replacer (Invitrogen 10828-028)	100 mL
L-Glutamine (Invitrogen 25030-081)	5 mL
MEM-NEAA (Invitrogen 11140-050)	5 mL
Penicillin and Streptomycin (Invitrogen 15140-155) (optional)	5 mL
Beta-Mercapto Ethanol (Sigma M7522)	3.5 µL

Pig iPS cell medium (500 mL):

DMEM/F12 Medium (Invitrogen 11330-057)	400 mL
Knockout Serum Replacer (Invitrogen 10828-028)	100 mL
L-Glutamine (Invitrogen 25030-081)	5 mL
MEM-NEAA (Invitrogen 11140-050)	5 mL
Penicillin and Streptomycin (Invitrogen 15140-155)	5 mL
2-Mercaptoethanol (Sigma M7522)	3.5 µL
Human bFGF (Invitrogen-PHG0021)	5-10 µg

Note: Knock-Out DMEM medium (GIBCO 10829) can be used in place of DMEM/F12 medium. bFGF final concentration should be between 4-10 ng/mL. Addition of human bFGF will help maintain cells in an undifferentiated state.

Human iPS cell medium (500 mL):

DMEM/F12 Medium (Invitrogen 11330-057)	400 mL
Knockout Serum Replacer (Invitrogen 10828-028)	100 mL
L-Glutamine (Invitrogen 25030-081)	5 mL
MEM-NEAA (Invitrogen 11140-050)	5 mL
Penicillin and Streptomycin (Invitrogen 15140-155)	5 mL
2-Mercaptoethanol (Sigma M7522)	3.5 µL
Human bFGF (Invitrogen-PHG0021)	5-10 µg

Note: Knock-Out DMEM medium (GIBCO 10829) can be used in place of DMEM/F12 medium. bFGF final concentration should be between 4-10 ng/mL. Addition of human bFGF will help maintain cells in an undifferentiated state.

Preparing Matrigel-coated culture dishes: Matrigel (BD Biosciences 354277) should be thawed and aliquoted on ice. Aliquotes can be stored at –80°C. Mix the Matrigel and ice-cold Knock-Out DMEM medium (GIBCO 10829) in ratio of 1:100 on ice. Pipette to mix thoroughly and apply immediately to culture dishes, making sure to coat the entire bottom of

the plate. Use 2 mL/well of a 6-well or 1 mL/well of a 24-well plate. Incubate the plate at 37°C for 1 hour. Plates can be used immediately or wrapped with parafilm and stored at 4°C up to 15 days. Before use aspirate the Matrigel and wash once with cell culture medium.

Cryopreservation Medium:

FBS (Benchmark 100-106)	900 µL
DMSO (Sigma D-2650)	100 µL

NOTE: For pig iPSCs or human iPSCs grown on Matrigel (BD Biosciences, cat. no. 354277) and mTeSR1 medium (STEMCELL Technologies 05850), it is advisable to use mFreSR medium (STEMCELL Technologies 05855) for cryopreservation.

293-FT Cell Culture Medium (500mL):

DMEM (high glucose) GIBCO 11965-092	90%	400.00 mL
FBS (heat inactivated) Benchmark 100-106	10%	100.00 mL

Commentary

Background Information—Regenerative medicine has gained significant promise in recent years for treating chronic and debilitating diseases (Wu & Hochedlinger 2011). As the aging population increases in the U.S., it has become a medical and scientific imperative to find ways to alleviate the suffering of patients with some of the most challenging illnesses such as Alzheimer's disease, congestive heart failure, and emphysema. While the route to success in treating these diseases is far from clear at the moment, a number of scientific advances have enabled us to envision how cell replacement therapy may be accomplished. One of such approaches involves the use of pluripotent stem cells (PSCs) which exhibit both the ability to self-renew indefinitely and differentiate spontaneously into a wide variety of different cell types. Initial PSC studies in 1970s employed a germline tumor called teratocarcinoma, which exhibits features of pluripotent stem cells but with propensity for unchecked growth (Hogan 1976). Due to its cancer-like features, teratocarcinoma was relegated to nothing more than a developmental oddity. Then, in the early 1980s, with the isolation of mouse embryonic stem cells (ESCs) (Evans and Kaufman 1981), it became possible for investigators to engineer genome-modifications that could be studied by in vitro differentiation or, when introduced into the germline to even generate knockout mice for in vivo studies. Since mouse ESCs were karyotypically normal, they contributed efficiently to all adult tissues when injected into a developing blastocyst stage embryo. Furthermore, the creation of live-born animals exclusively from injected ESC in a tetraploid complementation assay validated the true developmental competency of these mouse ESCs (Nagy 1990, Eggan 2001). This has enabled the generation of countless numbers of genetic mouse models of human disease. However, despite these technical advances for deriving mouse

ESCs, it took yet another 18 years for the generation of ESCs from discarded human embryos (Thomson 1998).

Although mouse and human ESCs are now providing us with a platform for studies of early development, the use of human fetal-derived cells has been highly controversial. Furthermore, for a cell-based therapy to be successful, it would ideally involve immunohistocompatible donor cells with immunocompetent host individuals in order to avoid the need for immunosuppressant. In order to circumvent these issues, it would be desirable to have a source of pluripotent stem cell that can be derived easily from somatic cells. Early seminal work by Jon Gurdon and others (Gurdon 1962) demonstrated that the introduction of somatic cell nuclei from frogs into an enucleated oocyte can induce pluripotent gene expression in the nucleus of the injected somatic cell. This principle was subsequently used to generate “cloned” animals such as Dolly the sheep (Campbell 1996). Despite the success with nuclear cloning of multiple animal species including primates (Byrne 2007, Chan 2000), this approach has not lead to successful human nuclear cloning thus far. The reason for this is likely due to the limited availability of donor human eggs. The challenging social climate for human embryo work has also not been conducive to significant progress in this area.

To overcome these challenges in human somatic cell nuclear transfer, Takahashi and Yamanaka set out to prove that direct reprogramming by means of transcription factor overexpression could be feasible (Takahashi & Yamanaka 2006). In their seminal work, they systematically screened 24 transcription factors that are highly expressed in undifferentiated ESCs to find a combination of as few as four factors (Sox2, Oct4, Klf-4 and C-Myc) that could reprogram mouse embryonic fibroblasts into ESC-like cells. These induced pluripotent stem cells (iPSCs) express Nanog, Rex1, Oct4, Sox2 from its endogenous loci and are able to silence the expression of exogenously supplied transcription factors to enable their differentiation in vitro. Following this ground-breaking work, many investigators from around the world have been able to reproduce these findings (Okita 2007, Maherali 2007, Wernig 2007). Furthermore, the developmental competency of a subset of iPSCs lines were validated by their ability to generate “all-iPSC” mice in tetraploid complementation assays, (Boland 2009, Kang 2009, Zhao 2009, Stadtfeld 2010).

Remarkable advances have since been made to improve the efficiency of iPSC generation. While the use of viral vectors has established the feasibility of transcription factor-based reprogramming, concerns regarding the potential tumorigenicity from viral-mediated genome modification have spurred the development of reprogramming strategies that are virus-free. Such methods include nonintegrating adenoviral vectors (Stadtfeld 2008b), naked plasmids transfection (Okita 2008), mini-circle plasmids (Jia 2010), proteins (Zhou 2009), modified RNA (Warren 2010), and genome editing enzymes such as cre recombinase (Kaji 2009, Soldner 2009) and transposases (Woltjen 2009, Yusa 2009). While these non-integrating strategies induce minimal genome perturbation, their overall reprogramming efficiency is usually 100 to 1000 times lower than viral-based strategies. With the availability of lentiviruses carrying polycistronic cassettes encoding all four reprogramming factors, the efficiency for reprogramming have increased significantly (Carey 2009, Sommer 2009).

Given the high degree of conservation of pluripotency gene function, iPSCs have been derived from a number of different popular species, including mouse (Takahashi & Yamanaka 2006), rat (Li 2009, Kobayashi 2010), sheep (Li 2011), pig (Ezashi 2009), rhesus monkey (Liu 2008), and human (Takahashi 2007, Yu 2007, Park 2008b), as well as endangered species such as the drill and the nearly extinct northern white rhinoceros (Friedrich Ben-Nun 2011). This has been a remarkable feat since the derivation of true ESC lines from some of these species (e.g. rat, sheep, pig) has been technically challenging. The availability of iPSCs from these species now allows us to create genetic models in large animals that may exhibit more similar disease phenotype to human.

Critical Parameters—Efforts should be made in order to maximize reprogramming efficiency. Make sure to use fresh chemicals and unexpired media ingredients that have been handled and stored under recommended storage conditions. Fibroblasts should be used of low passage number (ideally below p3) to avoid replicative senescence. Cells should never be allowed to become overconfluent since this affects their reprogramming amenability. Similarly, do not let the feeder cells get overconfluent, or their ability as feeder cells may decrease. When cells have been successfully reprogrammed into iPSCs, it is also important not to let cells become overconfluent since this will affect their pluripotent state and cells will start differentiating or die. In order to achieve this, cells should be cultured in fresh feeder and iPS medium, that should be replenished regularly (ideally every 24 hours) and cells should be passaged when reaching approximately 80-90% confluency.

At the virus-producing step, it is advisable to transfect the virus-producing cells with a suitable control to monitor the transfection efficiency. For this purpose, generally a Lentiviral GFP or pMXs retroviral GFP vector can be used. This can be assessed by flow cytometry of the virus-producing cells. We routinely obtain 70-80% efficiency. High-efficiency transfection is crucial for iPS cell induction.

For viral overexpression of transcription factors, it is important to use a high titer virus with a high infection rate. Preferably use fresh virus and avoid multiple freeze/thaw cycle as this decreases the viral titer. When choosing a viral system, it is recommended to use a doxyxycyclin inducible lentiviral system, since this provides better control over transgene expression and silencing.

It is imperative for cell health and reprogramming efficiency to replenish with fresh media regularly. If the cell medium color changes to yellow (indicator of medium acidity), change the media immediately. It is always better to pre-feed 1 hour before passaging and/or freezing since this reduces excessive cell death. For long-term storage, keep frozen cells in the gas phase of a liquid nitrogen tank. The recovery of iPS cells after freezing is about 50%.

Troubleshooting—This protocol has proven to have an extremely high success rate in our hands, generating good iPSC colonies in almost 100% of times. In case no or inefficient reprogramming is achieved, there are a few issues that need to be considered before repeating again. First off, make sure to use fresh fibroblasts of low passage number (passage <3). High passage number cells show significantly lower reprogramming efficiency. Second, it is of eminent importance that the virus used for reprogramming is of good quality in order

for there to be a good viral infection rate. Only use fresh virus and limit freeze/thaw cycles to not more than one. It is worth considering to check whether the viral transfection rate in virus producing cells is efficient by using a GFP control vector (see Critical Parameters for details). In case of small number of colonies after viral infection, one can also increase the MOI empirically in order to increase reprogramming efficiency.

If iPSCs are showing decreased survival or are not attaching to the feeder after passaging or colony picking, it may be due to excessive exposure to trypsin or vigorous pipetting. Make sure to pipette the cells gently when tritulating and limit the exposure time to trypsin.

In case the colonies are differentiating or disintegrating, this may be due to partial reprogramming or improper culturing conditions. Pick only iPS clones of good morphology, good culture condition and pick only the good quality iPS clones. Make sure to use freshly prepared media at all times, with fresh reagents, to support the pluripotent conditions (especially LIF). Proof of pluripotency can be checked by analyzing the quantitative expression of pluripotent markers, teratoma assay in SCID mice and by chimeric mouse assays.

It is advisable to do regular periodic mycoplasma testing (once a month) to rule out an underlying mycoplasma infection as the reason for unsuccessful reprogramming or culturing.

Anticipated Results—The infection efficiency of fresh high titer virus in fibroblasts is more than 60%. Efficiency of iPS cell clone derivation from infected (integrated) is approximately 1% for MEFs and 0.1% for TTFs.

After 24 hours of viral infection, many fibroblasts may die due to viral cytotoxicity, but sufficiently large number of fibroblasts may proliferate abundantly and get confluent within two days.

High quality iPS cell clones mimic the ES cell like proliferation and morphology (e.g. round shape, large nucleoli and translucent cytoplasm). Poor quality iPS clones does not look like ES cell morphology and will not proliferate continuously when cultured in mouse iPS media.

Time Considerations—Isolation and culture of embryonic fibroblasts generally takes up to 10 days. Pause point: The mitomycin C-treated mouse feeder cells can be kept in the incubator for up to a week before use, but every three days fresh MEF media should be replenished.

Virus can be harvested within 2 days after transfecting virus-producing cells with viral plasmids. It is recommended to do this in advance, since reprogramming virus can be stored at -80°C up to 1 year until ready for use. After successful infection, colonies start forming after 10-21 days, depending on the species. Picking and establishing iPSC colonies can take an additional 3-4 weeks.

It is recommended to freeze down early passage iPS cells (immediately after complete doxycycline weaning in case of using inducible viral system). If necessary, cells can also be cultured and frozen at early passage for future reference. For long-term storage, keep frozen cells in the gas phase of a liquid nitrogen tank. The recovery of iPS cells after freezing is about 50%.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

D	Day(s)
ESCs	Embryonic Stem Cells
iPSCs	induced Pluripotent Stem Cells
KOSR	Knock Out Serum Replacer
MEFs	Mouse Embryonic Fibroblasts
Min	Minutes
mL	Milliliter
RT	Room Temperature
TTFs	Tail Tip Fibroblasts

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Figure 1.

Procedural set up for iPSC colony picking. a) Setup for picking iPSC colonies under a microscope in a laminar flow hood. b) Process of iPSC colony picking. Note the forearm is fixed against the microscope stage while looking through microscope c) Close-up of picking. Make sure not to touch any neighboring colonies.

Figure 2.

Typical morphology of good vs bad iPSC colony. a) A good mouse iPSC colony. Not the translucent appearance and sharp borders. b) Mouse iPSCs. Notice the one bad mouse iPSC colony (arrow) on the bottom right. This colony looks heterogeneous and differentiated.

Figure 3.
Schematic diagram of mouse iPSC generation.

Figure 4.

Examples of rat iPSC cell colonies. a) A good rat iPSC colony. Note the translucent appearance and sharp borders. b) a partially differentiated rat iPSC colony. This colony looks heterogeneous and differentiated. c) One good rat iPSC colony (center) amongst two differentiated iPSC colonies (top and bottom). Care should be taken when picking the center colony not to touch the differentiated colonies.

Figure 5.
Schematic diagram of Rat iPSCs generation.

Figure 6.

Examples of pig iPSC cell colonies. a) A developing pig iPSC colony. b) A good pig iPSC colony, cultured on MEFs. Note the translucent appearance and sharp borders. c) A good pig iPSC colony grown on Matrigel. d) A bad pig iPSC colony, grown on Matrigel. This colony looks heterogeneous and differentiated and should be removed from the culture dish.

Figure 7.
Schematic diagram of pig iPSC generation.

A.

B.

Figure 8.

Examples of human iPSC cells. a) A good human iPSC colony grown on Matrigel. Note the translucent appearance and sharp borders. b) A bad human iPSC colony grown on Matrigel. This colony looks heterogeneous and differentiated and should be removed from the culture dish. Images kindly provided by Dr. Emil M. Hansson at MGH Cardiovascular Research Center.

Figure 9.
Schematic diagram of human iPSC generation.