# Human iPS Cell Derivation/Reprogramming

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## ABSTRACT

This unit describes a protocol for deriving induced pluripotent stem (iPS) cells from human fibroblast cells. Human fibroblast cells are infected with retroviral vectors expressing four transcription factors (Oct4, Sox2, Klf4, and Myc) and selected for 3 to 4 weeks under human embryonic stem (hES) cell culture conditions. iPS cell colonies are mechanically isolated using a dissection microscope and handled like hES cells thereafter. Human iPS cells share similarities with hES cells including the expression of pluripotency genes, and differentiation as embryoid bodies in vitro into three germ layers (EB) and in vivo as teratomas. *Curr. Protoc. Stem Cell Biol.* 8:4A.1.1-4A.1.8. © 2009 by John Wiley & Sons, Inc.

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## INTRODUCTION

This unit describes a protocol for deriving induced pluripotent stem (iPS) cells from human fibroblasts. hES cells have the property of self-renewal and pluripotency that provides an unlimited resource for research and medical applications. Recently, terminally differentiated murine and human somatic cells were induced to become pluripotent stem (iPS) cells by use of a four-transcription-factor cocktail (Oct-4, Sox-2, Klf4, and Myc; Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Yu et al., 2007; Park et al., 2008). In this unit, the production of retrovirus-expressing reprogramming factors, infection of human fibroblasts, and isolation of human iPS cells will be described.

*NOTE:* The following tissue culture procedures are performed in a Class II biological hazard flow hood or a laminar-flow hood.

*NOTE:* All procedures for producing the VSV-G pseudotyped retrovirus should be performed under BL2+ biosafety conditions (according to your Institute's Safety Department).

*NOTE:* All incubations are performed in a humidified 37°C, 5% $CO_2$ incubator.

## PRODUCTION OF VSV-G PSEUDOTYPED RETROVIRUS

***BASIC PROTOCOL 1***

This protocol is used for making retroviral vectors pseudotyped with the VSV-G (vesicular stomatitis virus G) envelope protein. The VSV-G pseudotyped retrovirus can be divided into aliquots and stored long-term at −80°C and then thawed and used to infect human fibroblasts.

### *Materials*

293T cells (ATCC, cat. no. CRL11268)
Fugene 6 (Roche Applied Science, cat. no. 1181509001)



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DMEM
DMEM/F12 (Invitrogen)
pMIG-OCT4 (Addgene, clone 17225), pMIG-SOX2 (Addgene, clone 17226), pMIG-KLF4 (clone 17227), and pMIG-MYC (requested from Dr. Cleveland from the Scripps Research Institute)
VSV-G (Addgene, clone 8454), and Gag-Pol (Addgene, clone 8455)
293T cell medium (see recipe)

10-cm dishes
0.45-μm filter
38.5-ml polyallomer centrifuge tube (Beckman, cat. no. 326823)
Cryovials

Additional reagents and equipment for determining the titer of the virus (Sastry et al., 2002; Tiscornia et al., 2006)

### *Transfect 293T cells with plasmids using Fugene 6*

1. One the day before transfection, split 293T cells ($0.5 \times 10^5$ cells/cm$^2$) into ten 10-cm dishes for each different virus at a confluency of 40%, aiming to have 70% to 80% confluency the next day.
2. For each 10-cm dish, add 20 μl of Fugene to 300 μl DMEM and incubate the mixture 15 min at room temperature. Add 2.5 μg of pMIG vector, 2.25 μg of Gag-Pol, and 0.25 μg of VSV-G vector and incubate for 15 min.

   *For ten 10-cm dishes, multiply the amounts by 10 to have 3 ml of DMEM with 200 μl Fugene, 25 μg pMIG vector, 22.5 μg Gap-Pol and 2.5 μg VSV-G. Use polystyrene tubes for maximum transfection.*
3. While the Fugene/plasmid mixture of step 2 is incubated, aspirate old medium from 293T cells and add 9 ml of new 293T medium.
4. Add 7 ml of 293T cell medium into 3 ml of Fugene/plasmid mixture (for ten 10-cm dishes from step 2) to make a total of 10 ml, mix well by pipetting and add 1 ml of each into each 10-cm dish of 293T cells in 9 ml medium.
5. Place the transfected 293T cells into BL2+ incubator.

   *After 293T cells are transfected with retroviral vectors, they need to be treated as BL2+ hazardous biomaterial.*

### *Concentrate VSV-G pseudotyped retrovirus*

6. Three days after transfection (do not change medium during 3-day incubation period), collect and filter the viral supernatant using 0.45-μm filter into a 38.5-ml polyallomer centrifuge tube.
7. Weigh the supernatant tube to make a balance tube for ultracentrifugation.
8. Centrifuge the supernatant 90 min at 70,000 × *g*, 4°C.
9. Remove the supernatant.

   *A white pellet should be visible when a polyallomer centrifuge tube is used.*
10. Add 1 ml DMEM/F12 and flick the tubes before storing overnight at 4°C to dissolve the pellet.
11. Next day, mix virus by pipetting up and down slowly, aliquot ~100 to 200 μl of virus into cryovials and store at −80°C for long-term storage.
12. Determine the titer of the virus following published protocols (Sastry et al., 2002; Tiscornia et al., 2006).

## INFECTION OF FIBROBLASTS AND ISOLATION OF iPS CELLS

*BASIC PROTOCOL 2*

This protocol is used to infect fibroblasts with the virus, and to isolate iPS cell colonies from them. During and after isolation, human iPS cells show similar colony morphology and require the same culture conditions as human ES cells. Therefore, it is highly recommended that scientists wishing to isolate and maintain human iPS cells first become skilled in handling hES cells, including the mechanical picking and passaging of colonies (*UNIT 1C.1*), or undergo training directly on iPS cell culture from experienced investigators.

### *Materials*

Human fibroblasts, acquired from skin biopsy (refer to *UNIT 1C.7*; split fibroblasts when they reach 70% confluency)
Human fibroblast medium (see recipe)
Protamine sulfate (see recipe)
Retroviral supernatants bearing the appropriate plasmids (Basic Protocol 1)
Phosphate-buffered saline, calcium- and magnesium-free (CMF-PBS; Mediatech, cat. no. 21-040-CV)
MEF (mouse embryonic fibroblasts), CF-1 strain, irradiated (Global Stem, cat. no. GSC-6001G)
MEF medium (see recipe)
0.1% (w/v) gelatin (see recipe)
0.05% trypsin/EDTA
hESC medium (see recipe)
Gelatin-coated 12-well plate preplated with MEFs at a density of $1 \times 10^4$ cells/cm$^2$
Collagenase IV (see recipe)
Gelatin-coated 6-well plate preplated with MEFs
Freezing medium (see recipe)
Liquid nitrogen

6-well plate
10-cm dish
Dissection microscope
20- and 1000-μl pipets
21-G needle or cell lifter (Corning, cat. no. CT-3008)
15-ml conical tube
Cryovials
−80°C freezer

### *Infect human fibroblasts with retrovirus*

1. At a time point 8 to 12 hr prior to infection, plate $1 \times 10^5$ human fibroblasts obtained from a skin biopsy in one well of a 6-well plate.
2. Aspirate medium to remove dead cells and add 2 ml of fresh human fibroblast medium. Add protamine sulfate at a final concentration of 5 μg/ml.
3. Into the fibroblast culture, add aliquots of the four retroviral supernatants (Basic Protocol 1) at a multiplicity of infection (MOI) of 5 for each virus.

   *This step and those following must be performed under BL2+ safety conditions.*

4. One day after infection, remove the viral supernatant, wash three times, each time with 3 ml PBS, and add 3 ml human fibroblast medium.
5. After 2 more days, replenish dish with 3 ml human fibroblast medium.
6. Four days after infection, plate $1 \times 10^4$/cm$^2$ MEFs in MEF medium on a 10-cm dish coated with 0.1% gelatin. Incubate until the next day.

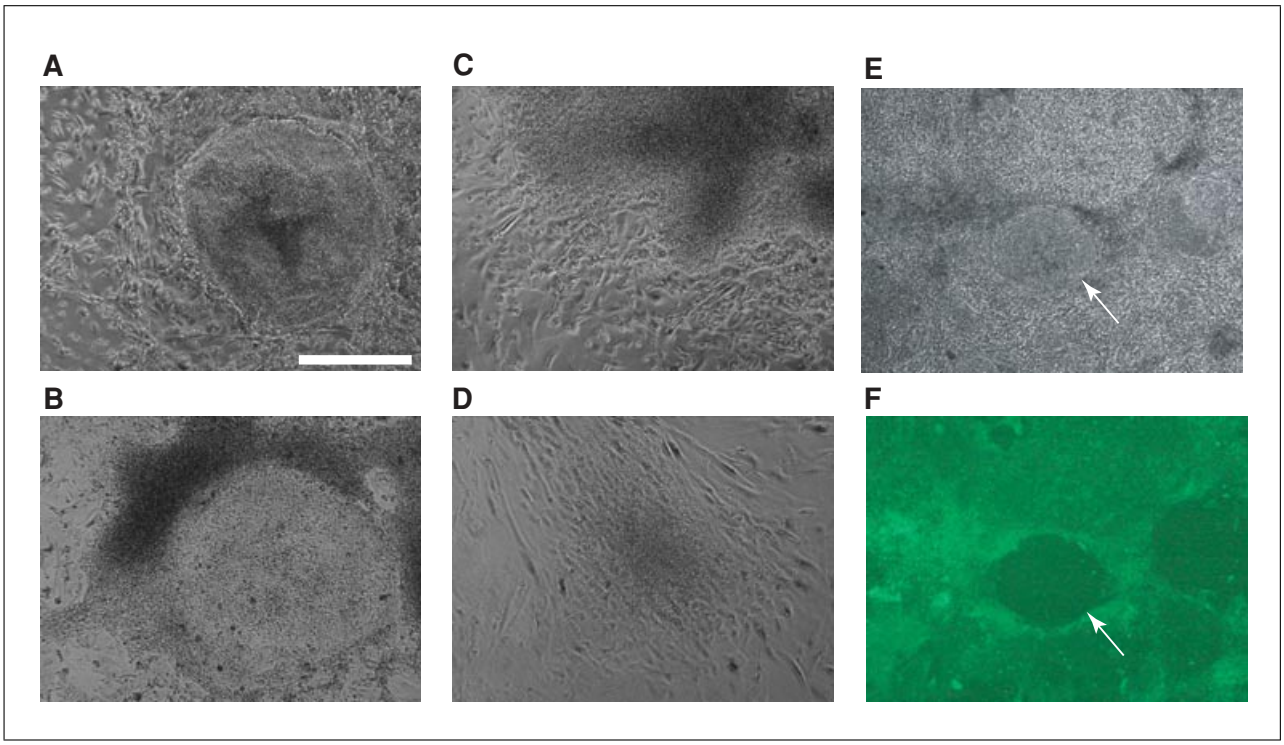


**Figure 4A.1.1** Identification of iPS colonies among heterogeneous types of colonies arising from infected human fibroblasts. Examples of colonies that should yield stable iPS colonies (A, B) and examples of colonies that do not (C, D). Silencing of retroviral gene expression in iPS colony (E, F). (**A**) iPS colony with a morphology comparable to hES cells. (**B**) iPS colony that is surrounded by outgrowth of infected fibroblasts. (**C**) Non-iPS colony from transformed cells. (**D**) Non-iPS colony from transformed cells. (**E**) iPS cell colony in the middle of field (arrow). (**F**) A reprogrammed iPS colony in E shows no fluorescence due to silencing of the infected retrovirus (arrow). Scale bar = 100 μm.

7. Five days after infection, incubate the infected fibroblasts with 1 ml 0.05% trypsin/EDTA for 3 min at 37°C. Stop the trypsinization with 11 ml human fibroblast medium and place all cells into one 10-cm dish preplated with MEFs (prepared in step 6).

8. Seven days after infection remove human fibroblast medium and add 10 ml hESC medium (containing KOSR and 10 ng/ml of bFGF).

9. Replenish the cells with 10 ml hESC medium every day and observe the cells for any sign of colony formation.

   *Typically, sometime around three weeks after infection, various types of colonies will appear, as exemplified in Figure 4A.1.1.*

10. Under a dissection microscope and using a 20-μl pipet, pick a colony that shows a morphology similar to hES cells and put the colony into one well of a 12-well plate that has been preplated with MEFs at a density of $1 \times 10^4$ cells/cm$^2$ on 0.1% gelatin-coated wells.

    *Fully reprogrammed iPS cells infected with GFP-containing viruses will lack GFP expression, as visualized under a fluorescence microscope (Fig. 4A.1.1F). Absence of GFP expression, a consequence of transcriptional silencing of retroviruses, is specific to embryonic or pluripotent cell types, and can be used as a surrogate marker to identify human iPS cells.*

### *Maintain and store human iPS cells*

Isolated clones of human iPS cells can be treated and maintained in a similar way as human ES cells. Please refer to the detailed protocol *UNIT 1C.1*.

11. When iPS cell colonies grow and start to touch other colonies, wash plate with 1 ml of DMEM/F12, add 0.5 ml collagenase IV, and incubate 10 min at 37°C.

12. Using appropriate tools (e.g., 21-G needle, or cell lifter), cut colonies into small pieces, detach pieces from the plate, collect with a 1000-µl pipet (similar to mechanical splitting of established human ES cells; *UNIT 1C.1*) into a 15-ml conical tube and centrifuge for 4 min at 200 × *g*, room temperature. Remove the supernatant and add 5 ml of fresh DMEM/F12 followed by centrifuging 4 min at 200 × *g*, room temperature.

13. Remove the supernatant.

14. For passaging of iPS cells, resuspend pieces of colonies from step 13 in 2 ml fresh hESC medium and transfer into one well of a gelatin-coated 6-well dish precoated with MEFs. Repeat steps 10 to 14 to expand iPS cells.

    *The split ratio used for the cells depends on cell density (ratio is usually 1:3 to 1:6)*

    *When you have more than three wells of iPS cells in a 6-well plate, freeze down cells before starting further analysis of iPS cells.*

***Freeze cells***

15. For freezing iPS cells, resuspend colonies from step 13 in 0.5 ml fresh hESC medium, add the same amount of 2× freezing medium dropwise and mix by gently pipetting up and down. Aliquot cells into cryovials and store overnight at –80°C. Transfer to liquid nitrogen next day.

## REAGENTS AND SOLUTIONS

*For culture recipes and steps, use sterile tissue culture–grade water. For other purposes, use deionized, distilled water or equivalent in recipes and protocol steps. For suppliers, see* ***SUPPLIERS APPENDIX****.*

### *293T cell medium*

*DMEM containing:*
10% (v/v) heat-inactivated fetal bovine serum (FBS)
2 mM L-glutamine
1× penicillin/streptomycin (add from 200× stock, e.g., Invitrogen)
Store up to 4 weeks at 4°C

### *Collagenase IV, 10×*

Dissolve collagenase IV (Invitrogen) at 10 mg/ml in DMEM/F12 (Invitrogen). Filter using 0.22-µm filter. Divide into 0.5- to 1.5-ml aliquots and store up to 1 year at −20°C.

Before splitting hES or iPS cells, dilute 10× stock solution in DMEM/F12 to make a 1× working stock.

### *Freezing medium, 2×*

Make a solution containing 20% dimethyl sulfoxide (DMSO), 60% FBS, and 20% human ES cell (hESC) medium (see recipe). Store up to 1 month at 4°C.

### *Gelatin, 0.1% (w/v)*

Dissolve 0.5 g of gelatin (from porcine skin) in 500 ml distilled water and autoclave. Store indefinitely at room temperature.

*To gelatinize plates:* Prior to addition of MEFs, coat all dishes or wells with enough 0.1% (w/v) gelatin solution to cover the surface. Remove gelatin after 5 min.

***Human embryonic cell (hESC) medium***

*DMEM/F12 (Invitrogen) containing:*
20% (v/v) Knockout Serum Replacement (KOSR; Invitrogen)
10 mM non-essential amino acids
2 mM L-glutamine
1× penicillin/streptomycin (add from 200× stock, e.g., Invitrogen)
50 mM 2-mercaptoethanol
10 ng/ml bFGF (see recipe)
Store up to 1 week at 4°C

***Human fibroblast medium***

*MEM-alpha containing:*
10% (v/v) heat-inactivated fetal bovine serum (FBS)
2 mM L-glutamine
1× penicillin/streptomycin (add from 200× stock, e.g., Invitrogen)
Store up to 4 weeks at 4°C

***MEF medium***

*DMEM containing:*
10% (v/v) heat-inactivated fetal bovine serum (FBS)
2 mM L-glutamine
1× penicillin/streptomycin (add from 200× stock, e.g., Invitrogen)
Store up to 4 weeks at 4°C

***Protamine sulfate, 1000×***

Dissolve protamine sulfate (Sigma-Aldrich) at 5 mg/ml in phosphate-buffered saline, calcium- and magnesium-free (CMF-PBS; Mediatech, cat. no. 21-040-CV). Filter using a 0.22-μm filter and store up to 1 year at 4°C for future use.

***Recombinant human basic fibroblast growth factor (bFGF)***

Resuspend lyophilized bFGF (PeproTech) to a final concentration of 10 μg/ml in CMF-PBS containing 0.1% (w/v) bovine serum albumin (BSA) and 1 mM DTT. Store at −80°C according to manufacturer's instructions.

## COMMENTARY

### Background Information

Human embryonic stem cells provide a valuable resource for research and regenerative medicine. However, human ES cells isolated to date are not matched to individual patients, and thus allow for generic studies but are limited in their relevance to specific diseases or treatments. Attempts to generate patient-specific stem cells include somatic cell nuclear transfer (SCNT), somatic cell fusion with pluripotent cells, direct cultural adaptation of germ cells, and direct reprogramming of somatic cells with defined factors (Jaenisch and Young, 2008). Each of these approaches has specific advantages and limitations. Since the report by Yamanaka's group that mouse embryonic and adult tail-tip fibroblasts can be reprogrammed to become ES cell–like pluripotent cells by expression of four transcription factors (Oct4, Sox2, Klf4, c-Myc), a similar strategy has been used to isolate human iPS cells. Our laboratory and Yamanaka's group successfully isolated human iPS cells from embryonic and adult human fibroblasts using the same four transcription factors (Takahashi et al., 2007; Park et al., 2008). A different combination of factors (Nanog and Lin28 in place of Klf4 and Myc) also produced human iPS cells (Yu et al., 2007). Yamanaka's reprogramming strategy also works even without Myc, although at lower efficiency, to produce iPS cells from both murine and human fibroblasts (Nakagawa et al., 2008).

The isolation of murine iPS cells has been facilitated by using fibroblasts that carry endogenous selectable markers. Fibroblasts from

mice that express neomycin- or puromycin-resistance genes under the control of the promoters for Fbx15, Oct4, or Nanog loci have been infected with four reprogramming factors, selected with neomycin or puromycin, and successfully reprogrammed to become iPS cells (Takahashi and Yamanaka, 2006; Maherali et al., 2007; Okita et al., 2007; Wernig et al., 2007). For most human fibroblasts, such endogenous selection systems do not exist, and thus selection has been based mostly upon colony morphology alone. By selecting colonies that show similar morphology to human ES cells, human iPS cells can be readily selected from a morphologically diverse background of colonies that arise when human fibroblasts are infected with several retroviruses.

Although reprogramming human fibroblasts into pluripotent cells will provide an alternative to human ES cells for certain research and clinical applications, current methods of generating iPS cells employ retroviral vectors that integrate into the fibroblast genome, and thus the resulting cells are potentially tumorigenic. Developing virus-free methods is desirable. Excision of ectopic Myc by Cre-mediated recombination has been shown to reduce the tumor formation potential of iPS cells (Hanna et al., 2007; Shi et al., 2008). The G9a inhibitor BIX01294 can replace Oct4, and only two factors are sufficient to make iPS cells from murine neuronal stem cells (Shi et al., 2008), suggesting that the right combination of excisable viruses and chemicals may provide a method to make iPS cells that lack persistent viral integration.

## Critical Parameters and Troubleshooting

Before starting to reprogram human fibroblasts, there are three important factors that determine success: the quality of the virus, MEFs, and target fibroblasts. When a new VSV-G pseudotyped virus is made and stored at –80°C, be sure to determine the titer of the virus, because it is essential to have a high-quality, high-titer virus. If the titer is $<0.5 \times 10^6$ IU/ml, make a new virus. The proper storage of the virus is important to maintain quality and titer, and we recommend freezing a large number of small aliquots of the virus, and limiting thawing and refreezing. If the virus has been stored for a long period of time, determine the titer again before starting another round of reprogramming. The quality of MEFs is also an important factor. Confirm the quality of the lot of MEFs by culturing hES cells on them before plating infected human fibroblasts. When low-quality MEFs are used to select iPS cells, the initial colonies of reprogrammed fibroblasts differentiate and do not form human ES cell-like colonies. The quality of human fibroblasts is another important factor to determine the efficiency of reprogramming. Although the mechanism is not yet clear, fibroblasts from younger donors appear to yield more iPS colonies than those from older donors. Beginners are recommended to start reprogramming with fetal or foreskin fibroblasts to experience the procedures of isolating iPS cells before attempting reprogramming of fibroblasts from older individuals.

## Anticipated Results

The efficiency of reprogramming human fibroblasts depends on parameters described in the troubleshooting section. By using a high-quality virus and MEFs, this protocol generates ~5 to 50 colonies from $1 \times 10^5$ cells, for a frequency of ~0.05%. When iPS cell lines are formed, they can be passaged over a year without karyotypic abnormality.

## Time Considerations

Two weeks are required to make a retrovirus carrying the reprogramming factors, including thawing of the 293T cells, transfecting of vectors, and determining of titer for the virus. While the retrovirus is being made, test the quality of the MEFs by culturing human ES cells on them. Three to four weeks are required to identify and isolate iPS cells, beginning from the day human fibroblasts are infected. Single iPS colonies need to be expanded for two to three weeks to generate stable cell lines that have an adequate number of cells for subsequent characterization.

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