

Video Article

Efficient Generation Human Induced Pluripotent Stem Cells from Human Somatic Cells with Sendai-virus

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Abstract

A few years ago, the establishment of human induced pluripotent stem cells (iPSCs) ushered in a new era in biomedicine. Potential uses of human iPSCs include modeling pathogenesis of human genetic diseases, autologous cell therapy after gene correction, and personalized drug screening by providing a source of patient-specific and symptom relevant cells. However, there are several hurdles to overcome, such as eliminating the remaining reprogramming factor transgene expression after human iPSCs production. More importantly, residual transgene expression in undifferentiated human iPSCs could hamper proper differentiations and misguide the interpretation of disease-relevant *in vitro* phenotypes. With this reason, integration-free and/or transgene-free human iPSCs have been developed using several methods, such as adenovirus, the piggyBac system, minicircle vector, episomal vectors, direct protein delivery and synthesized mRNA. However, efficiency of reprogramming using integration-free methods is quite low in most cases.

Here, we present a method to isolate human iPSCs by using Sendai-virus (RNA virus) based reprogramming system. This reprogramming method shows consistent results and high efficiency in cost-effective manner.

Video Link

The video component of this article can be found at <https://www.jove.com/video/51406/>

Introduction

Human embryonic stem cells (hESCs) have a capacity to self-renew *in vitro* and have pluripotency, which could be potentially useful for disease modeling, for drug screening, and to develop cell-based therapies to treat disease and tissue injuries. However, hESCs have a limitation for cell replacement therapy because of immunological, oncological and ethical barriers, and to study disease related genes, disease-specific hESCs could be isolated through pre-implantation genetic diagnosis (PGD) approaches, but it is still technically challenging and the embryo donations are pretty rare. These issues are related to the progress in stem cell biology, which has led to the development of induced pluripotent stem cells (hiPSCs).

Human iPSCs are genetically reprogrammed from human adult somatic cells and harbor pluripotent stem cell-like features similar to hESCs, which makes them a useful source for regenerative medicine such as drug discovery, disease modeling and cell therapy in patient-specific manner^{1,2}.

Till now, there are several methods to generate human iPSCs, including virus-mediated (retrovirus and adenovirus)³, non-virus mediated (BAC system and vectors transfection)⁴ gene transductions, and protein delivery system⁵⁻⁷.

Although a delivery of virus-mediated genes can ensure a certain level of efficiency, viral vectors could leave genetic footprint, because they integrate into host chromosomes to express reprogramming genes in an uncontrolled manner. Even when viral integration of transcription factors may activate or inactivate host genes⁸, it can cause an unexpected genetic aberration and the risk of tumorigenesis^{5,9}. On the other hand, the direct introduction of proteins or RNA into somatic cells were reported, but have some disadvantages such as labor-intensive, repeated transfection, and low level of reprogramming^{7,10}. Even episomal and non-integrating adenovirus, adeno-associated virus, and plasmid vectors are still relatively less efficient¹¹. For these reasons, it is plausible to choose non-integration reprogramming methods with high efficacy of iPSC generation and fewer genetic abnormalities. In this study, we use a Sendai-virus based reprogramming. This method is known to be non-integrated into the host genome and consistently produces human iPSCs without transgene integrations.

Protocol

1. Preparation of Cell and Media (Day 1)

1. Culture and expand human fibroblast with DMEM media containing 10% FBS.
2. Plate human fibroblasts (**Figure 1**) onto a 24-well plate at the appropriate density per well on the day before transduction.
NOTE: The following serial dilutions are recommended (200K, 100K, 50K, 25K, 12.5K and 6.25K) because different cell types have different attachment ability.
3. Incubate the cells for one more day in a 37 °C, 5% CO₂ incubator, ensuring the cells have fully adhered and extended.

2. Perform Transduction (Day 2)

1. On the day of transduction, check the cell density and chose the most efficient density wells (**Figure 2**). It is better to pick three different densities: high (80~90%), middle (50~70%) and low (20~40%).
2. At least 1 hr before transduction, aspirate the fibroblast media from the cells and change new 300 µl of fibroblast medium.
3. Remove one set of 4 different Sendai virus tubes from the -80 °C storage. Thaw each tube at the same time in a 37 °C water bath for few seconds, and then take the tubes out from the water bath. After that, thaw them to room temperature; centrifuge tubes at 6,000 x g for 10 sec and place them on ice till use. Do not re-freeze and thaw the virus since the titers will not maintain.
4. Add the indicated volumes of each of the four Sendai virus tubes (Oct-4, Klf-4, c- Myc and Sox-2) to micro-centrifuge tube. Make sure that the solution is mixed well by pipetting gently.
For example, if 50K cells/one well of 24-well plate lookwell for transduction:
(Titer based on the Certificate of Analysis from Life Technologies, can be different from batch to batch)
Tube hOct-4 = 6.0×10^7 CIU, 3 MOI = 5 µl
Tube hSox-2 = 6.5×10^7 CIU, 3 MOI = 4.6 µl
Tube hKlf-4 = 6.3×10^7 CIU, 3 MOI = 4.8 µl
Tube hc-Myc = 7.8×10^7 CIU, 3 MOI = 3.8 µl
Total = 18.2 µl of four virus factors mixture/one well (50K cells) of 24-well plate
5. Depending on cell densities; add 2X, 1X, and 0.5X volume of the virus mixture into the well. Gently shake the plate front to back, left and right (2X: 36.4 µl of virus mixture, 1X: 18.2 µl and 0.5X: 9.1 µl).
6. Place the cells in a 37 °C, 5% CO₂ incubator and incubate overnight.

3. Replacement of Culture Medium (Day 3 & Day 5)

1. 24 hr after transduction, replace the medium with 500 µl fresh fibroblast medium.
2. On Day 5, change Medium with fresh fibroblast media.

4. Prepare MEF Dishes for the Co-culture (Day 8)

1. Prepare MEF cells in 60 mm culture dishes with 10% FBS contained DMEM medium one day before passaging the transduced fibroblast onto MEF feeder-cells.
Note: We are using 5×10^5 of MEF in each 60 mm dish.

5. Start Co-culture Transduced Cells with MEF Feeder Cells (Day 9)

1. Before plating transduced cells, aspirate 10% FBS contained DMEM media from MEF feeder dishes and add fresh 10% FBS contained DMEM medium.
2. Remove the medium from the transduced fibroblasts that are in the 24-well plate and wash cells once with D-PBS. Add 200 µl of 0.25% Trypsin/EDTA into the fibroblast cells in the 24-well plate. After less than 5 min incubation with Trypsin/EDTA, when the cells start to detach from the plate, collect the cells with growth medium in 15 ml conical tube. Then centrifuge them in 6,000 x g for 4 min.
NOTE: Pooling 2X, 1X, and 0.5X virus transduced fibroblast is recommended.
3. Remove the supernatant medium and wash to re-suspend the cell pellet with 4-5 ml of fresh fibroblast medium for neutralization from trypsin. Then centrifuge them at 135 x g for 4 min.
4. Plate cells as serial dilution density and start to co-culture with fibroblast media onto MEF: such as 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, and 1/128 per 60 mm dish. Depending on cell death rate during the first week of transduction, one may not need to dilute to 1/2 or 1/128 density. Put aside remaining cells to extract total RNA as a positive control for Transgene RT-PCR.
5. Place 60 mm dishes in 37 °C, 5% CO₂ incubator overnight.

6. Feed Human Embryonic Stem Cell Medium and Monitor the Cells (Day 10)

1. The next day, change fibroblast media to human ES media with 10 µM Y-27632. After that, change medium daily with human ES medium: 800 ml DMEM/F12 + 200 ml Knockout Serum Replacement + 10 ml of L-Glutamine + 10 ml of 10 mM MEM minimum non-essential amino acids + 1 ml of 2-mercaptoethanol + 10 ng/ml of basic Fibroblast Growth Factor.

2. Do a daily media change with fresh human ES media. After a week, check the dishes once per 2~3 days under a microscope for the formation of ES colony-like cell clumps. Depending on the cell type, the culture will take more or less time before colonies are found.

7. Picking the Induced Pluripotent Stem Cell Colonies and Expand the Cells (Day 20~)

1. Three weeks after transduction, colonies should appear grown properly for picking.
2. The day before picking the colonies, prepare MEF feeder in 24-well plate.
NOTE: We are using 12.5×10^5 of MEF in one 24-well plate.
3. The next day, change medium of MEF dishes from fibroblast media to human ES media with 10 μ M Y-27632. Manually pick one colony at each time under the microscope in the picking-hood and make smaller clumps by pipetting and transfer them onto newly prepared MEF dishes. Try to pick several clones.
4. Passage and expand the each well from a 24-well plate to a 6 well plate initially. Then from a 6-well plate to a 60 mm dishes before further propagation.

8. Characterization of Human iPSCs (after 10 Passages)

1. **Immunofluorescence**
 1. Plate human iPSCs in 24-well plate and culture during 4-5 days with daily media change.
 2. For starting Immunofluorescence assay, wash the plate with D-PBS once. Then fix the cells immediately in 0.4% paraformaldehyde for 30 min. After that wash three times in D-PBS for 5 min.
 3. Treat fixed cells with 0.1% Triton X-100 solution in PBS for 15 min at room temperature.
 4. Aspirate the permeabilization solution (0.1% triton X-100 solution), then add 0.5% BSA blocking solution for 1 hr at room temperature.
 5. Perform a detection of Nanog, Oct-4, SSEA (stage-specific embryonic antigen) and TRA-1-81 (tumor rejection antigen) using an anti-human antibody in 0.5% BSA solution (check the material table for antibody dilution rate). Incubate Primary Antibody for 2 hr at room temperature. Then wash three times in D-PBS for 5 min each.
 6. Check using fluorescence microscopy after 1 hr of incubation with Alexa Fluor 488 Goat anti-mouse IgG antibodies as 2nd antibody, which are diluted 1:2,000 in 0.5% BSA solution at room temperature. Stain all the nuclei of each MEF feeder cells and hiPSCs with DAPI.
2. **RT-PCR of Transgene Confirmation**
 1. Extract total mRNA from cell pellet by using RNA extraction reagents.
 2. Do a reverse transcription on aliquots (1 μ g) of total RNA and the resultant cDNA for PCR amplification by Reverse Transcriptase.
 3. Prepare the PCR mixtures to contain 2 μ l of cDNA template, 10 μ l of 2X PCR master mix, 2 μ l of 2 pmol of primers (forward: 1 μ l and reverse: 1 μ l) and 6 μ l of DW. To detect the gene expression, do the RT-PCR with the primers listed in **Table 1**.
 4. Perform the RT-PCRs with GAPDH gene as a control for efficiency of the amplification reactions. Visualize and analyze PCR product by 1% agarose-gel electrophoresis.

Representative Results

Usually infected fibroblasts do not show any morphological changes in several days after Sendai virus transduction, but five days later, they start to have different shapes (**Figure 1**). As described in a right panel of **Figure 1**, cells do not have typical fibroblast morphology any more. They have a round shape and bigger nucleus than cytoplasm. Even when transduction is performed in 80% cellular confluency, it looks like they are less confluent in the well after they start to reprogram. If these kinds of morphological changes start to be seen in the most of the well, transduced fibroblasts are ready to be plated on MEF-feeder culture dishes.

In our protocol, we minimized cell culture scale and also gave various reprogramming possibilities, such as employing different fibroblast cell density and virus titer, and subsequent several re-plating ratio MEF, which will increase hiPSCs generation efficiency (**Figure 2**). Before transduction, the wells with different confluency (low, mid, and high) can be selected for further sendai-virus transduction; represented as red, green, and yellow color in the plate.

At the beginning of reprogramming on MEF feeders, it is hard to distinguish transduced cell types, but after over one week from co-culture, partially reprogrammed fibroblasts appear and look like loosened ES-like shape (**Figure 3**). Because of this reason when human iPSC colonies are ready for the passaging into new MEF feeder dish, manual picking is more efficient way for transfer instead of dissociation with enzyme.

In **Figure 3** and **Figure 4**, under the microscope only human iPSCs have clear edges, which make a clear distinction between human iPSCs (considered as 'fully reprogrammed cells') and 'incompletely reprogrammed cells' by morphology. Fully reprogrammed cells look like compact and tightly packed. Especially the colonies have clear boundary, whereas partially reprogrammed cells look like gathering together to make colony but very loose and fragile to break down. Even if we do a pipetting gently it is easily detached. Even after excluding partially reprogrammed human iPSCs colonies (during expanding them and passaging), fully reprogrammed human iPSCs need to be maintained by manual picking.

After picking colonies and expanding hiPSC clones in several weeks, expression of stemness markers needs to be determined. After expansion of hiPSC clones, we verify them with different sets of tests, including antibody staining (SSEA4, Oct-4, TRA-81 and Nanog) and RT-PCR of pluripotent marker (data is not shown) as a quality control.

Compared to H9, two different hiPSCs are positive to SSEA4, Oct-4, TRA-81, and Nanog. This result demonstrates that isolated human iPSCs acquired pluripotent quality (**Figure 5**).

Finally, **Figure 6** shows that we cannot detect any transgene expression in hiPSC clones by RT-PCR. At least over 10 passages hiPSCs, specific primers (**Table 1**) for exogenous Oct-4, Sox-2, Klf-4 and c-Myc can be used with positive control samples with strong transgene expression level.

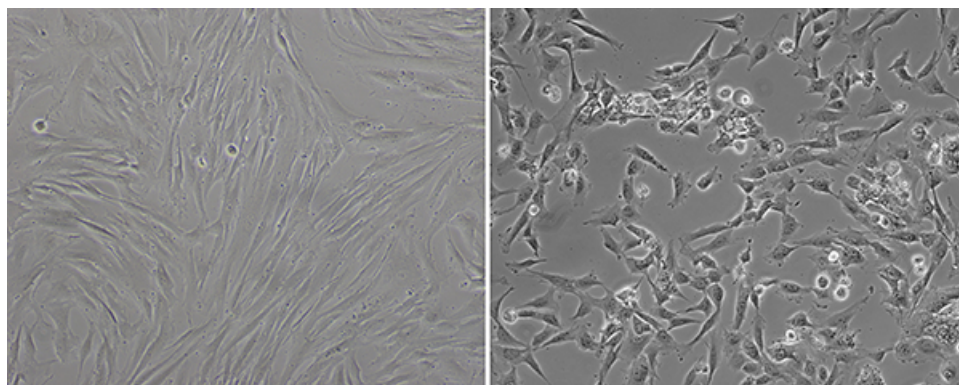


Figure 1. Morphological change after Sendai virus transduction. Before the transduction, fibroblasts show typical spindle-like shape (**left**). Approximately eight days after transduction, morphology of transduced cell change like much more round shape (**right**). [Please click here to view a larger version of this figure.](#)

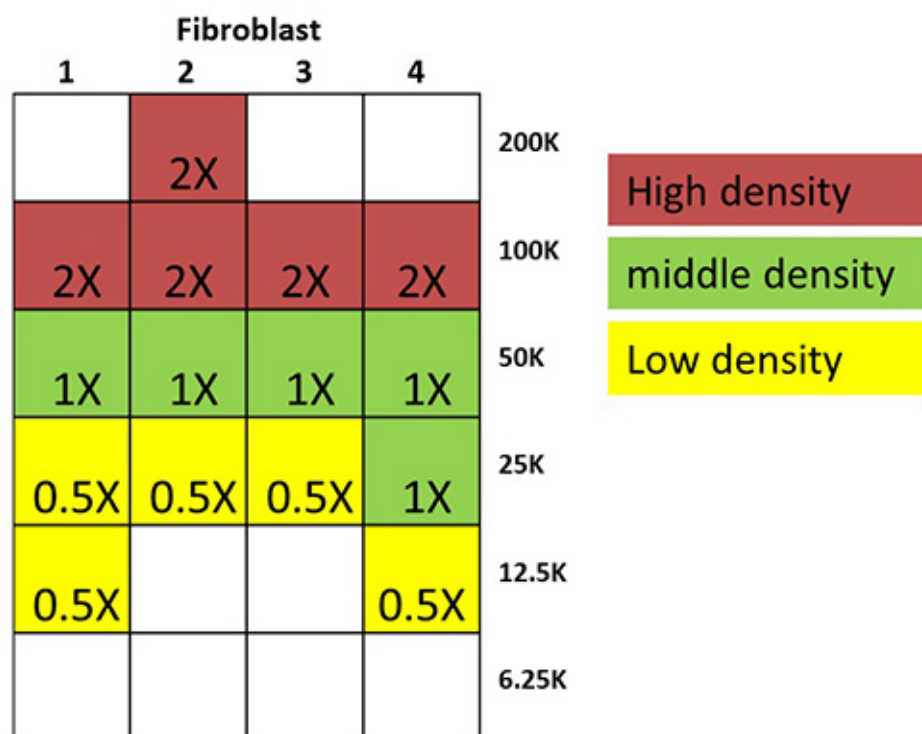


Figure 2. Diagram of induction plan to ensure maximum efficiency. Over four different cell types can be plated in a 24-well plate (from 1 to 4). Because their attachment ability and cell survival could be different depend on the cell types, it is suggested to plate different cell density (from 200K to 6.25K cells per well). In addition, virus concentration can be adjusted (0.5X, 1X and 2X).

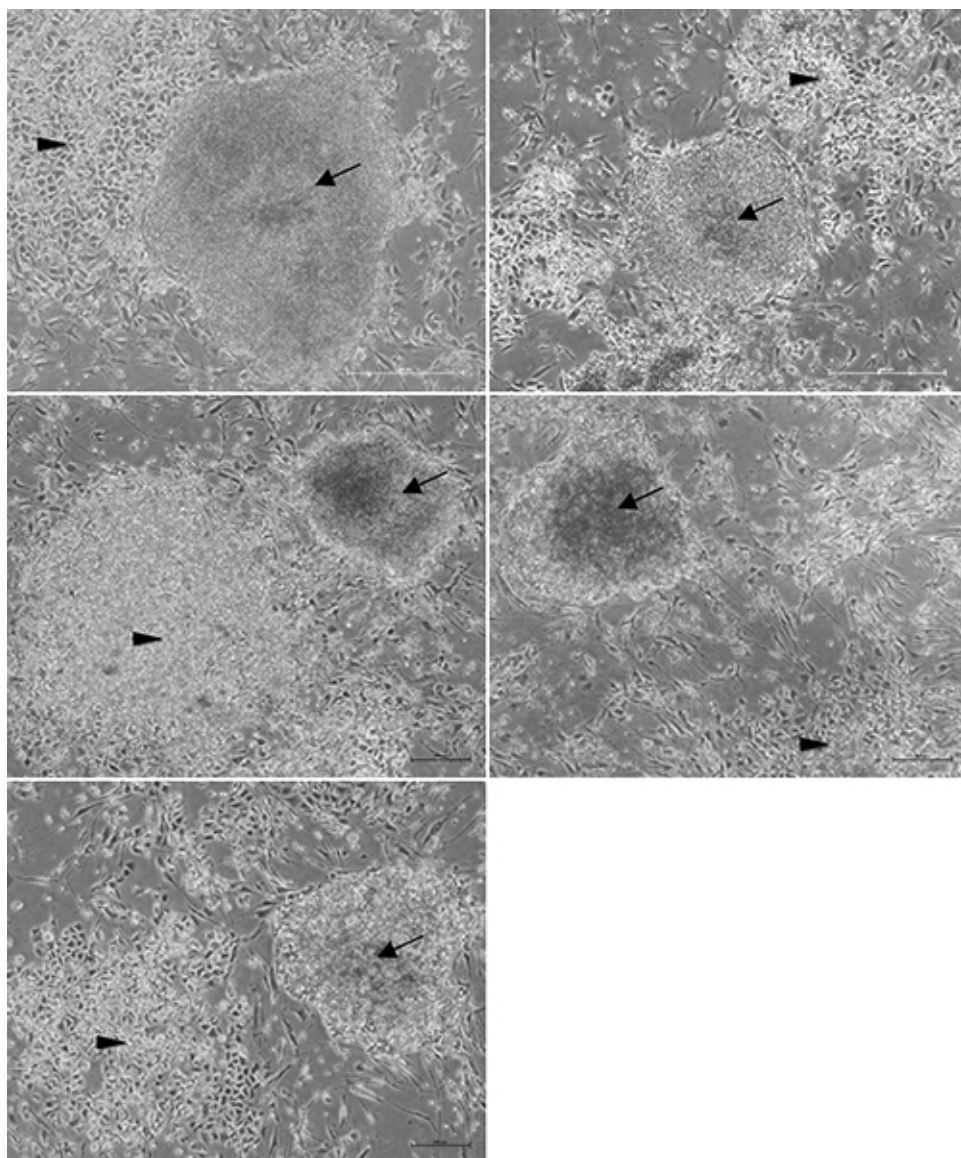


Figure 3. Formation of human induced pluripotent stem cell colony from transduced fibroblast. In about two weeks after re-plating them into MEFs, round colonies should appear and look like human embryonic stem cell colonies. Normally the fully reprogrammed human iPSC colonies have very clear boundaries. (Arrow: fully reprogrammed human iPSC colonies, arrowhead: partially reprogrammed human iPSCs). [Click here to view larger image.](#)

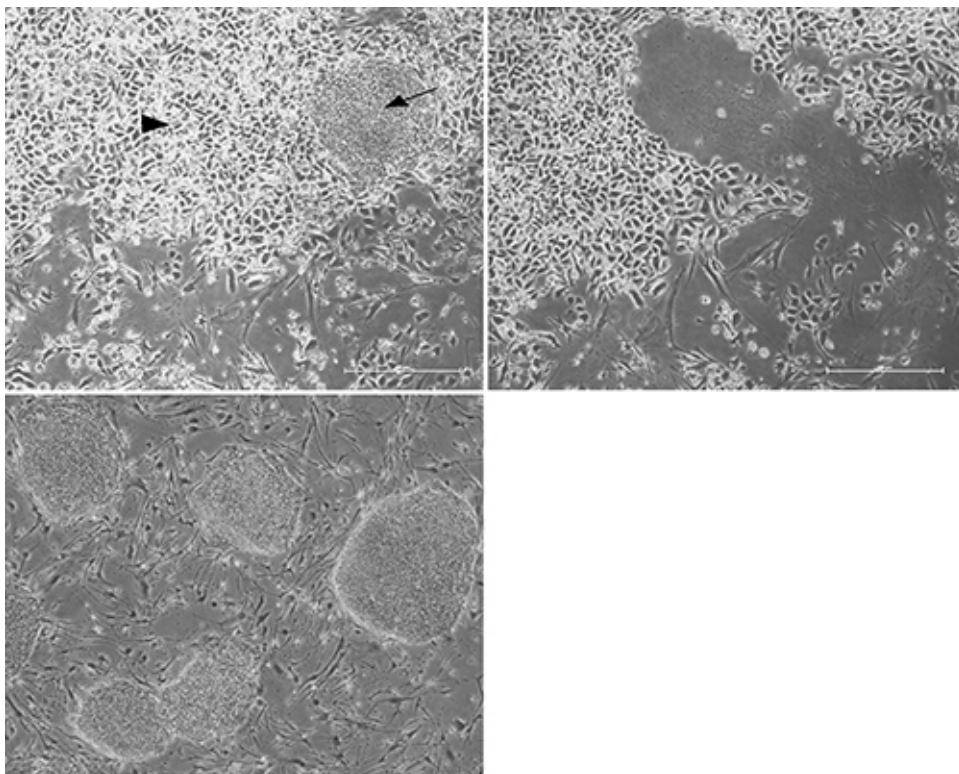


Figure 4. Picking up human induced pluripotent stem cell colonies. Human iPSC colonies are often surrounded by partially reprogrammed cells (**left**). Under the microscope, picking the ES-like colony only, break down into small clumps and then transfer (**right**). After 2~3 passages, there are undifferentiated hiPSC colonies (**bottom & left**). (Arrow: fully reprogrammed human iPSC colonies, arrowhead: partially reprogrammed human iPSCs) [Click here to view larger image.](#)

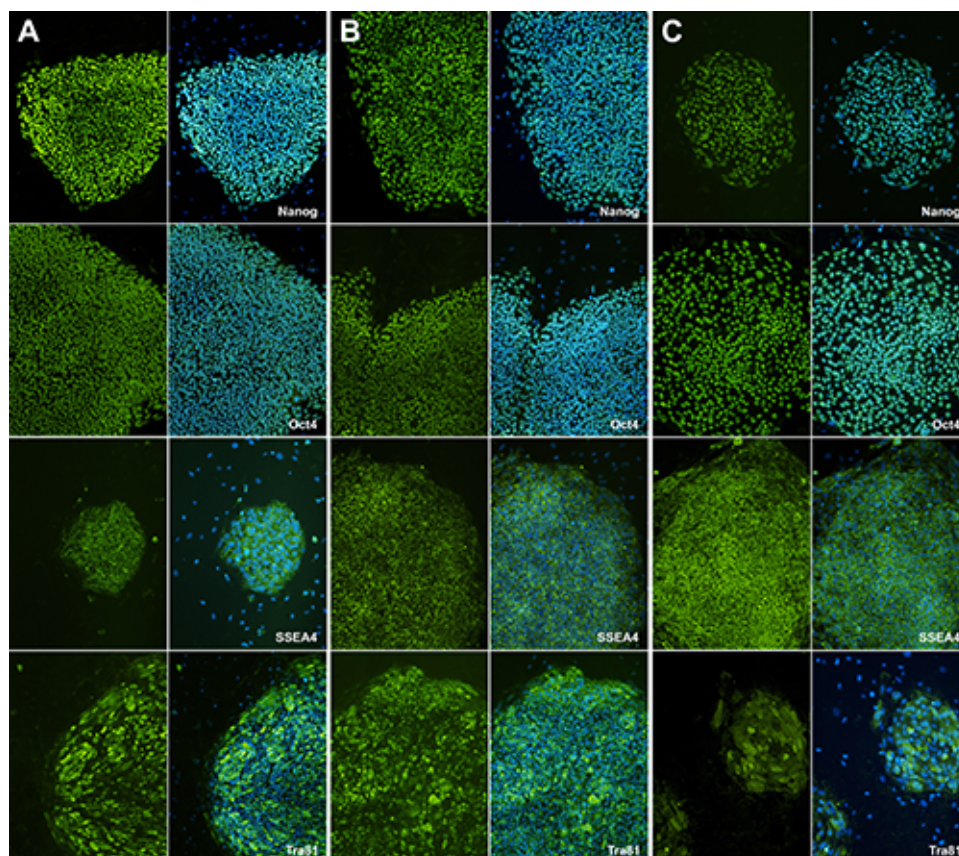


Figure 5. Immunofluorescence staining with stem cell pluripotent markers. Immunofluorescence assay shows human iPSCs have stemness features. SSEA-4, TRA-81, Nanog, Oct-4 as pluripotent markers and DAPI is a control for nucleus staining. (A) represents H9 staining as a control sample. (B) and (C) show human iPSC clones. [Click here to view larger image.](#)

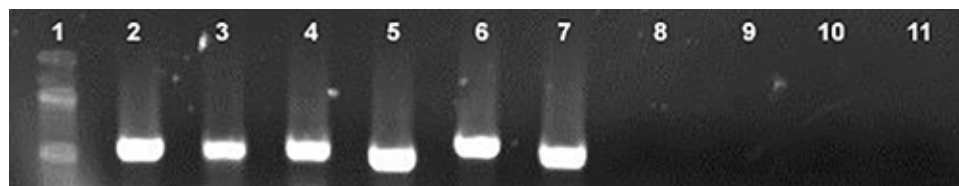


Figure 6. Confirmation of transgene expression level. At least after 10-passage culture of human iPSCs, silencing of exogenous Sox2, Klf-4, c-Myc, and Oct-4 gene expression levels is confirmed by reverse transcriptase polymerase chain reactions. hGAPDH is an internal control. For a positive control, RNA is extracted from leftover transduced fibroblasts (Day 7 after Sendai-virus infection). (Lane 1: Marker, lanes 2 - 6: GAPDH, Klf4, c-Myc, Oct-4, and Sox-2 in positive control, lanes 7 - 11: GAPDH, Klf4, c-Myc, Oct-4, and Sox-2 in hiPSCs).

Primer Name	Sequence
hOCT4 (F)	CCC GAA AGA GAA AGC GAA CCA G
hOCT4 (R)	AAT GTA TCG AAG GTG CTC AA
hMYC (F)	TAA CTG ACT AGC AGG CTT GTC G
hMYC (R)	TCC ACA TAC AGT CCT GGA TGA TGA TG
hSOX2 (F)	ACA AGA GAA AAA ACA TGT ATG G
hSOX2 (R)	ATG CGC TGG TTC ACG CCC GCG CCC AGG
hKLF4 (F)	ACA AGA GAA AAA ACA TGT ATG G
hKLF4 (R)	CGC GCT GGC AGG GCC GCT GCT CGA C
hGAPDH (F)	AGG TCG GAG TCA ACG GAT TTG
hGAPDH (R)	GTG ATG GCA TGG ACT GTG GT

Table 1. RT-PCR primers for transgene confirmation. These are the primer sequences that are used for transgene confirmation. T_m is 50.6 °C in all the primer sets.

Discussion

Reprogramming human somatic cells to hiPSCs holds unprecedented promises in basic biology, personal medicine, and transplantation¹². Previously, human iPSC generations required DNA virus that has integration risk into the host genome, which can create undesirable genetic mutations that limit further clinical applications such as drug development and transplantation therapies¹³. With this reason, many studies have been reported to generate vector- and transgene-free system human iPSCs by several alternative methods, but at the same time, the efficiency of isolating 'foot-print free' human iPSCs should be considered. For example, the RNA virus should have a minimal level of genetic aberration compare to other methods¹⁴, although there is no publication for whole genome sequencing to demonstrate the 'genomic safety' of sendai virus-mediated iPSC generation yet. Here, we present a method for generating hiPSCs with the Sendai-virus which has a high reprogramming efficiency in cost-effective way. The resulting human iPSCs are free of transgene with maintenance of cellular and molecular similarities to hESCs in proliferative and developmental potential.

We can reprogram different origins of fibroblast (>4) at the same time with only one set of the Sendai-virus. And in our method, we apply various cell confluences and virus dosage differences. This 'mix and match' combination technique can maximize the reprogramming efficacy. Fibroblasts (from a single patient) in each well have a different virus infection level, but by pooling together, the possibility of making hiPSCs colonies could increase based on our experience. Other issues of the clonal variation among hiPSC lines are proliferation rate, cell attachment/survival, status of X chromosome inactivation¹⁵, differentiation potentials¹⁶, etc. Indeed, we observed that each clone has a different neural propensity, which could be predicted by measuring the expression level of a mir-371 cluster. In addition we have succeeded in generating human iPSCs from human myoblasts with this method, which suggests that the Sendai-virus can be used for many different cell types in reprogramming process. Furthermore, using our method, we have generated hiPSCs in more than 10 different disease related fibroblasts. On average, from 5 to 10 hiPSC clones could be obtained from one fibroblast.

Although the derivation of transgene-free human iPS cells with the Sendai-virus is the most efficient reprogramming method which could be the most practical paths, there are a few limitations to be considered in this protocol;

- Depending on each sendai-virus batch, the virus titer needs to be calculated for consistent MOI (as described in 2.4).
- We observed the variation of infectivity among different somatic cells, which also needs additional titration for robust viral infection.
- The Sendai virus mediated reprogramming can be considered as one of the best choices for research purposes. But for the clinical trial, it might not be the first choice, since there is a licensing issue with the company that originally developed the Sendai virus system.
- It takes around two months until the reprogrammed somatic cells are free from transgenes, which is why we need to check the transgene expression after at least 10 passages.
- It seems like that the passage number of the primary cultured fibroblast may affect the efficiency of reprogramming with the Sendai-virus, although we don't have a direct comparison. The proliferation rate also needs to be determined. Further studies are needed to determine the effect of fibroblasts passage numbers and their proliferation on the reprogramming efficiency.
- In this study, we use a mouse feeder layer for hiPSC generation and maintenance, but a feeder free system may be an alternative way in a future study.

Our current protocol has been an important step toward studying patient-specific hiPSCs for disease modeling, regenerative medicine, and other applications.

Disclosures

The authors have nothing to disclose.

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References

1. Yu, J., *et al.* Induced pluripotent stem cell lines derived from human somatic cells. *Science*. **318**, 1917-1920, doi:10.1126/science.1151526 (2007).
2. Okita, K., Nakagawa, M., Hyenjong, H., Ichisaka, T., & Yamanaka, S. Generation of mouse induced pluripotent stem cells without viral vectors. *Science*. **322**, 949-953, doi:10.1126/science.1164270 (2008).
3. Zhou, W., & Freed, C. R. Adenoviral gene delivery can reprogram human fibroblasts to induced pluripotent stem cells. *Stem Cells*. **27**, 2667-2674, doi:10.1002/stem.201 (2009).
4. Woltjen, K., *et al.* piggyBac transposition reprograms fibroblasts to induced pluripotent stem cells. *Nature*. **458**, 766-770, doi:10.1038/nature07863 (2009).
5. Okita, K., *et al.* A more efficient method to generate integration-free human iPS cells. *Nature Methods*. **8**, 409-412, doi:10.1038/nmeth.1591 (2011).
6. Yusa, K., Rad, R., Takeda, J., & Bradley, A. Generation of transgene-free induced pluripotent mouse stem cells by the piggyBac transposon. *Nature Methods*. **6**, 363-369, doi:10.1038/nmeth.1323 (2009).
7. Kim, D., *et al.* Generation of human induced pluripotent stem cells by direct delivery of reprogramming proteins. *Cell Stem Cell*. **4**, 472-476, doi:10.1016/j.stem.2009.05.005 (2009).
8. Yu, J., *et al.* Human induced pluripotent stem cells free of vector and transgene sequences. *Science*. **324**, 797-801, doi:10.1126/science.1172482 (2009).

9. Okita, K., Ichisaka, T., & Yamanaka, S. Generation of germline-competent induced pluripotent stem cells. *Nature*. **448**, 313-317, doi:10.1038/nature05934 (2007).
10. Yoshioka, N., *et al.* Efficient Generation of Human iPSCs by a Synthetic Self-Replicative RNA. *Cell Stem Cell*. **13**, 246-254, doi:10.1016/j.stem.2013.06.001 (2013).
11. Dowey, S. N., Huang, X., Chou, B. K., Ye, Z., & Cheng, L. Generation of integration-free human induced pluripotent stem cells from postnatal blood mononuclear cells by plasmid vector expression. *Nature Protocols*. **7**, 2013-2021, doi:10.1038/nprot.2012.121 (2012).
12. Fluri, D. A., *et al.* Derivation, expansion and differentiation of induced pluripotent stem cells in continuous suspension cultures. *Nature Methods*. **9**, 509-516, doi:10.1038/nmeth.1939 (2012).
13. Nakagawa, M., *et al.* Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. *Nature Biotechnology*. **26**, 101-106, doi:10.1038/nbt1374 (2008).
14. Fusaki, N., Ban, H., Nishiyama, A., Saeki, K., & Hasegawa, M. Efficient induction of transgene-free human pluripotent stem cells using a vector based on Sendai virus, an RNA virus that does not integrate into the host genome. *P Jpn Acad B-Phys*. **85**, 348-362, doi:Doi 10.2183/Pjab.85.348 (2009).
15. Silva, S. S., Rowntree, R. K., Mekhoubad, S., & Lee, J. T. X-chromosome inactivation and epigenetic fluidity in human embryonic stem cells. *P Natl Acad Sci USA*. **105**, 4820-4825, doi:Doi 10.1073/Pnas.0712136105 (2008).
16. Kim, H., *et al.* miR-371-3 expression predicts neural differentiation propensity in human pluripotent stem cells. *Cell Stem Cell*. **8**, 695-706, doi:10.1016/j.stem.2011.04.002 (2011).