

## Derivation of Induced Pluripotent Stem Cells from Human Fibroblasts Using a Non-integrative System in Feeder-free Conditions

Alvaro A. Beltran<sup>1</sup>, Sarahi G. Molina<sup>2</sup> and Adriana S. Beltran<sup>2, 3, \*</sup>

<sup>1</sup>Department of Neuroscience, University of North Carolina, Chapel Hill, NC 27599, USA; <sup>2</sup>Human Pluripotent Stem Cell Core, University of North Carolina, Chapel Hill, NC 27599, USA; <sup>3</sup>Department of Pharmacology, University of North Carolina, Chapel Hill, NC 27599, USA

\*For correspondence: [beltran@med.unc.edu](mailto:beltran@med.unc.edu)

**[Abstract]** Induced pluripotent stem cells (iPSCs) are genetically reprogrammed somatic cells that exhibit features identical to those of embryonic stem cells (ESCs). Multiple approaches are available to derive iPSCs, among which the Sendai virus is the most effective at reprogramming different cell types. Here we describe a rapid, efficient, safe, and reliable approach to reprogram human fibroblasts into iPSCs that are compatible with future iPSCs uses such as genome editing and differentiation to a transplantable cell type.

**Keywords:** Feeder-free, Fibroblasts, Sendai virus, Induced pluripotent stem cells, Transgene-free

**[Background]** Induced pluripotent stem cells (iPSCs) are genetically reprogrammed adult cells that exhibit morphological and functional qualities remarkably similar to those of embryonic stem cells (ESCs) (Takahashi and Yamanaka, 2006; Yu *et al.*, 2007). They offer a great opportunity not only for disease modeling, but for the development of therapeutic strategies for pathologies that involve tissue degeneration. Furthermore, the iPSCs promise relies on a safe replenishable cell source derived in chemically defined media and free of random DNA integration.

Reprogramming somatic cells into iPSCs requires the forced expression of transcription factors that support the pluripotent state, including OCT4, SOX2, KLF4, c-MYC, NANOG, and LIN-28 (Takahashi and Yamanaka, 2006; Takahashi *et al.*, 2007; Yu *et al.*, 2007). Multiple approaches are available to deliver the transcription factors into the cells, including those that require integration into the host chromosomes (Takahashi and Yamanaka, 2006; Kane *et al.*, 2010). Exogenous DNA integration can lead to unpredictable effects on the quality of the cells and safety after transplantation. Other approaches include DNA based vectors that exist episomally (Yu *et al.*, 2011; Weltner *et al.*, 2012) and thus, decrease the possibility of integration, and finally those that do not integrate into the host genome and are known as transgene-free. The transgene-free methods include mRNA (Warren and Wang, 2013), recombinant protein (Zhou *et al.*, 2009) and Sendai virus (Fusaki *et al.*, 2009). Delivering the pluripotency transcription factors as mRNA or recombinant protein is poorly effective and costly. In contrast, Sendai virus, is a highly effective RNA virus that efficiently reprogram different types of somatic cells.

Sendai is a cytoplasmic RNA replication incompetent virus (SeV) that safely and effectively delivers the reprogramming factors into somatic cells, and does not integrate into the genome or alter the genetic

information of the cell (Li *et al.*, 2000; Fusaki *et al.*, 2009). Furthermore, the virus is cleared out from the cells after a few passages assuring zero footprint of both the vectors and transgenes. The Sendai reprogramming system is commercially available as CytoTune iPS 2.0 for research purposes and as CTS CytoTune iPS 2.1 for clinical use. Both systems contain vectors encoding the four Yamanaka factors (OCT4, SOX2, KLF4, and c-MYC) optimized for generating iPSCs from human somatic cells, making Sendai virus the most rapid, efficient and cost-efficient method to generate transgene-free iPSCs.

Reliable and safe derivation of human iPSCs relies on the use of defined and qualified reagents that allow a smooth transition to downstream technologies and is compatible with GMP (Good Manufacture Practice) quality standards for large-scale cell production. Here we describe a reprogramming approach for human fibroblasts that uses a non-integrative system, chemically defined culture medium in feeder-free conditions. This approach enables rapid, efficient, safe and reliable derivation of iPSCs compatible with future uses including genome editing and differentiation to a transplantable cell type.

## **Materials and Reagents**

1. Falcon® 6-Well Flat-Bottom Plate, Tissue Culture-Treated (Falcon, catalog number: 38016)
2. Serological pipettes multiple sizes (Corning)
3. Falcon 15 ml conical centrifuge tubes (Falcon, catalog number: 352097)
4. Falcon 50 ml conical centrifuge tubes (Falcon, catalog number: 352070)
5. Cells: Human fibroblast expanded from skin biopsies preferentially at passage 1 to passage 5. Early passage fibroblast can be obtained from a commercial source (*i.e.*, ATCC), a repository (*i.e.*, Corriell Institute) or expanded in the lab from skin biopsies (Vangipuram *et al.*, 2013 describes the procedure in detail)
6. Corning® Matrigel® hESC-Qualified Matrix (Corning, catalog number: 354277)
7. CytoTune™-iPS 2.0 Sendai Reprogramming Kit (Thermo Scientific, catalog number: A16518)
8. DMEM/F-12 (Gibco, catalog number: 11320033)
9. Fetal Bovine Serum (Gibco, catalog number: 16000044)
10. MEM NEAA (Gibco, catalog number: 11140050)
11. Pen Strep (Gibco, catalog number: 15-140-122)
12. mTeSR™ 1 (STEMCELL Technologies, catalog number: 85850)
13. TrypLE™ Express Enzyme (1x) (Gibco, catalog number: 12605036)
14. Anti-Human TRA-1-60, Mouse monoclonal (Thermo Fisher Scientific, catalog number: 41-1000)
15. Anti-Human OCT4, Rabbit polyclonal, IgG1 (Abcam, catalog number: ab19857)
16. Anti-Human SOX2 Antibody, Rabbit polyclonal IgG1 (Abcam, catalog number: ab97959)
17. STEMdiff Trilineage Differentiation kit, STEMCELL Technologies, catalog number: 05230)
18. Universal Mycoplasma Detection Kit (ATCC, catalog number: 30-1012K)
19. Dulbecco's PBS (DPBS) without Calcium and Magnesium (Gibco, catalog number: 14190136)
20. Polybrene Hexadimethrine Bromide (Sigma-Aldrich, catalog number: H9268)
21. 0.5 M EDTA (Gibco, catalog number: 15575020)

22. Fibroblast culture medium (see Recipes)
23. 5 mM EDTA solution (see Recipes)
24. 10 mM Y27632 stock solution (see Recipes)

## **Equipment**

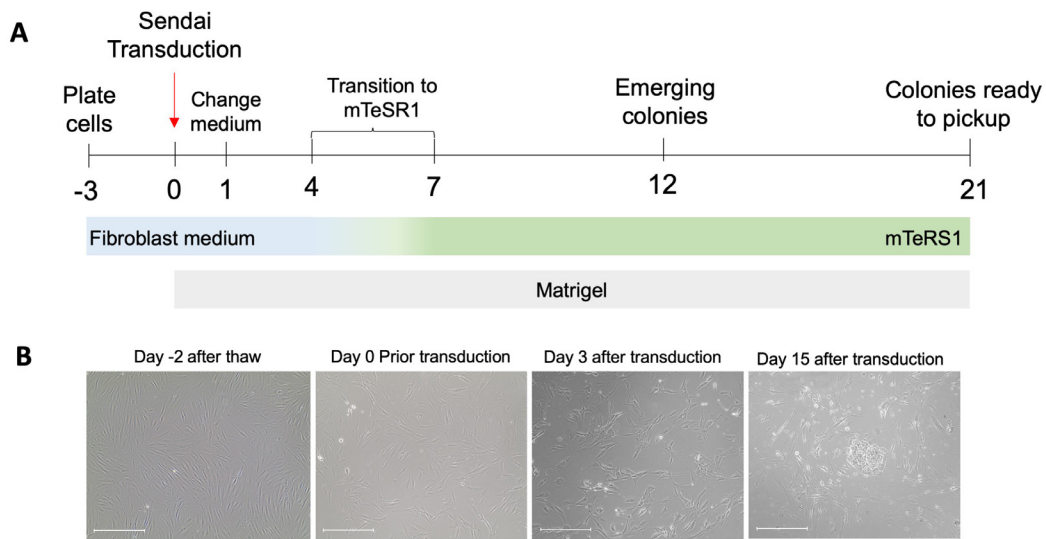
1. Tissue culture incubator Heracell™ 150i (Thermo Fisher Scientific, catalog number: 51026283)
2. Beckman Avanti J-30I Refrigerated with plate adapters (Beckman Coulter, catalog number: 17039)
3. Tissue culture laminar flow hood (NuAire Class II Type A2 NU-540)
4. Evos XL core microscope (Thermo Scientific, catalog number: AMEX1100)
5. Countess II FL Automated Cell Counter (Thermo Fisher Scientific, catalog number: AMQAF1000)
6. Water bath Isotemp (Fisher Scientific, catalog number: S35936)
7. Portable Pipet-aid (Drummond, model: DP-101, catalog number: 4-000-101)
8. Micro pipettes (Eppendorf)

## **Procedure**

### **Day -1: Plate dermal fibroblast for reprogramming**

Use low-passage (passage 1 to 5) human fibroblasts for reprogramming experiments. The reprogramming efficiency decreases with each passage. Hence, we don't recommend reprogramming fibroblast after passage 6. A schematic timeline and representative pictures can be seen in Figure 1.

1. Coat two wells of a 6 well plate with 2 ml of 5 mg/ml Matrigel and make sure it covers the entire well. Incubate at 37 °C for 30 min.
2. Inspect the fibroblast culture for the desired confluency (more than 70%), aspirate the culture medium and rinse twice with 2 ml DPBS.
3. Add 500 µl TrypLE™ Express enzyme and incubate at 37 °C and 5% CO<sub>2</sub> for 2 to 5 min or until fibroblasts have detached. TrypLE™ is used because it has lower cell toxicity than standard Trypsin and it is an animal-free product.
4. Add 1 ml fibroblast culture medium and transfer cell suspension to a 15-ml conical tube.
5. Centrifuge at 200 x g for 5 min. Remove and discard supernatant.
6. Resuspend cells in 1 ml of fresh fibroblast culture medium. Recipe available in the recipe section
7. Count fibroblasts using the desired method (*e.g.*, Countess Automated Cell Counter), and plate  $1 \times 10^5$  cells/well (10,000 cells/cm<sup>2</sup>) in the Matrigel-coated 6-well dish in 2 ml of fibroblast culture medium.
8. Incubate 24 h at 37 °C with a humidified atmosphere and 5% CO<sub>2</sub>.



**Figure 1. Derivation of induced pluripotent stem cells from fibroblast using Sendai virus and chemically defined medium.** A. Schematic view of general reprogramming procedure including culture medium used. B. Images of fibroblast morphology changes during the early stages of reprogramming. Scale bars: 650  $\mu\text{m}$ .

### Day 0: Transduction

1. Inspect the fibroblast culture, proceed with transduction if cell density is approximately 40-50% confluent, and cells have fully adhered and extended. If those parameters are not meet, wait another 24 h.
2. Warm 1 ml of fibroblast culture medium in a water bath for each well to be transduced calculate the volume of each premade virus needed to reach a multiplicity of infection of (MOI) of 5:5:3 (KOS MOI = 5, hc-Myc MOI = 5, hKlf4 MOI = 3) using the live cell count from the day of the seeding, and the titer information on the CoA (calculate based on the lot number, the CoA can be download from [thermofisher.com/cytotune](http://thermofisher.com/cytotune)). Calculate the volume of virus using the formula:

$$\text{Volume of virus } (\mu\text{l}) = \frac{\text{MOI} \left( \frac{\text{CIU}}{\text{cell}} \right) \times \text{cell number}}{\text{titer of virus} \left( \frac{\text{CIU}}{\text{ml}} \right) \times 10^{-3} \left( \frac{\text{ml}}{\mu\text{l}} \right)}$$

CIU = Cell infectious units

For example, to calculate the volume of hKOS virus with a titter of  $1.1 \times 10^8$  CIU;

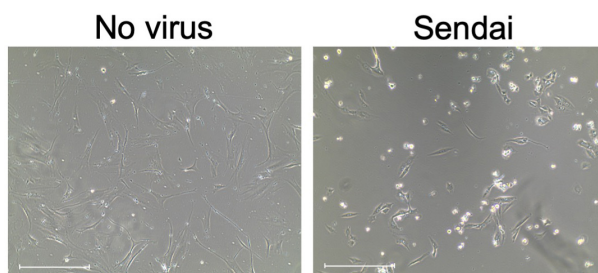
$$\text{Volume of virus } (\mu\text{l}) = \frac{5 \left( \frac{\text{CIU}}{\text{cell}} \right) \times 100,000}{1.1 \times 10^8 \left( \frac{\text{CIU}}{\text{cell}} \right) \times 10^{-3} \left( \frac{\text{ml}}{\mu\text{l}} \right)} = 4.5 \mu\text{l}$$

3. Thaw one set of CytoTune™ 2.0 Sendai aliquots from -80 °C and briefly centrifuge the tube. Place it immediately on ice until ready to use.

4. Add the calculated volumes of each of the three CytoTune™ 2.0 Sendai virus to 1 ml of pre-warmed fibroblast culture medium.
5. OPTIONAL: Add 4 µg/ml of polybrene to the medium. Ensure that the solution of virus and polybrene is thoroughly mixed by pipetting the mixture gently up and down. Complete the next step within 5 min. Polybrene increases the transduction efficiency of the virus, however, it can be toxic for some cell types. It neutralizes the charge repulsion between the virus and the cell surface increasing the overall transduction efficiency.
6. Aspirate the fibroblast culture medium from the cells, and add the reprogramming virus and polybrene mixture to the well containing the cells.
7. Close the 6-well dish, securely place on the plate adaptor, and centrifuge at 1,200 x g for 45 min at room temperature. Once the centrifugation is complete, add an additional 1 ml of prewarm fibroblast culture medium.
8. Incubate the plate overnight in a 37 °C incubator with a humidified atmosphere of 5% CO<sub>2</sub>.

#### Day 1: Remove virus

After 24 h, aspirate medium with viruses and add 2 ml fresh fibroblast culture medium. Expect to see cytotoxicity 24-48 h post-transduction, this is an indication of high uptake of the virus. See Figure 2.



**Figure 2. Images of morphological changes seventy-two hours after transduction with Sendai virus.** Scale bars: 650 µm.

#### Days 2 to 3

Remove 2 ml of used medium and add 2 ml of fibroblast culture medium. Expect to see changes in cell morphology.

#### Day 4: Transition to defined medium

There are many different chemically defined culture medium commercially available for deriving and maintaining iPSCs. We have used mTeSR1 medium (Stem Cell Technologies), STEMFLEX (Life technologies) and mTeSR plus (Stem Cell Technologies) agnostically.

1. Remove 500 µl of fibroblast culture medium and add 500 µl of fresh mTeSR1.

### Day 5

1. Remove 1 ml of culture medium and add 1 ml of fresh mTeSR1.

### Day 6

1. Remove 1.5 ml of culture medium and add 1.5 ml of fresh mTeSR1.

### Day 7

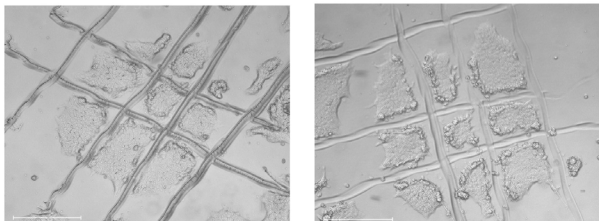
Remove 2 ml of medium culture medium and add the same volume of fresh mTeSR1.

### Days 8-20

Feed cells daily with 2 ml until colonies are ready to be passaged. Remove partially reprogrammed and differentiated colonies by scraping them before medium change. Fully reprogrammed colonies have round shape with well-defined borders, cells display identical morphology with a high ratio of nucleus to cytoplasm and prominent nucleoli. In contrast, partially reprogrammed colonies have undefined borders, an amorphous shape, and are composed of different types of cells.

### Day 21: First passage

1. When colonies are ready to pick, prepare one Matrigel coated dish per colony to be passage, and incubate at 37 °C for at least 30 min.
2. Aspirate Matrigel from plates and add 2 ml mTeSR1 + 10 µM ROCK inhibitor.
3. Use a 22 gauge needle or a pulled glass pipette to cut colonies in a grid-like pattern into small fragments. See Figure 3.
4. Use a 100 µl pipettor to scrape fragments and collect them. Immediately transfer the fragments to the Matrigel coated plates with mTeSR1 + 10 µM ROCK inhibitor.
5. Rock plate back-and-forth and side-to-side to evenly distribute the cell fragments and incubate overnight at 37 °C with a humidified atmosphere of 5% CO<sub>2</sub>.
6. Feed cells daily until ready to passage (usually every 6 to 7 days).
7. Repeat Steps 1 to 10 for 7 passages. We found that > 95% of the iPSCs clones at passage 7, are transgene free.



**Figure 3. Images of colonies cut in a grid-like pattern into small fragments. Scale bars: 650 µm.**



### IPSCs cloning and expansion

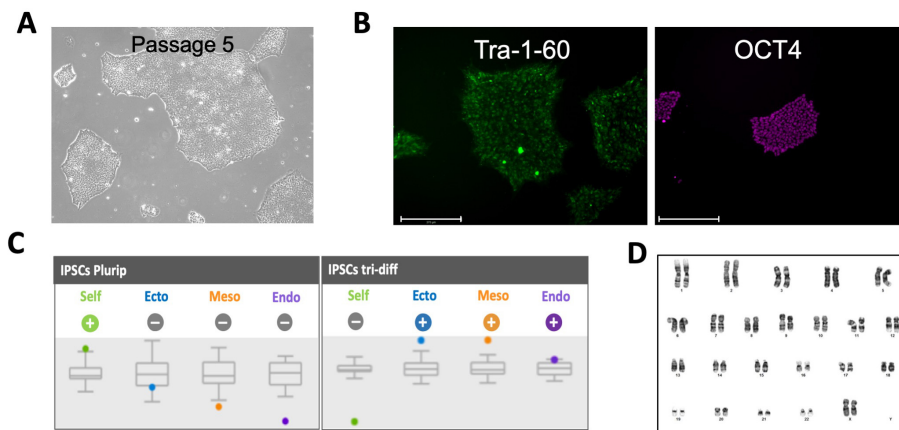
After passage 7, IPSCs are passage with EDTA buffer, as follow:

1. Coat 6 wells of a 6-well tissue culture plate with Matrigel and place in incubate at 37 °C for 30 min.
2. Prior to use, allow the Matrigel-coated plate to equilibrate to room temperature for at least 1 h.
3. Just before dissociating cells for passaging, aspirate the liquid Matrigel solution from the wells and replace with 1 ml of mTeSR1 + 10mM Y27632 cell culture media per well. Set aside.
4. Wash the cells with 2 ml 0.5 mM EDTA, aspirate.
5. Add 2 ml of room temperature 0.5 mM EDTA solution to the cells.
6. Incubate the culture at 37 °C for 3 to 5 min, or until cells begin to separate uniformly throughout the entire colony. Do not allow the cultures detach in the EDTA solution.
7. As soon as the cells appear rounded and uniform separation is seen throughout the colonies, carefully aspirate the EDTA solution from the well. Do not rinse.
8. Immediately add 1 ml of mTeSR1 + 10mM Y27632. With a 5 ml pipet, take up the 1 ml of media from the well, and very gently dispense it against the culture surface to dissociate the cells from the dish. Repeat 1 to 2 more times, if needed.
9. Be careful not to over-pipet the cell suspension.
10. Dispense the cells gently into the 15 ml conical tube containing an additional 3 ml of pre-warmed media.
11. Pipet the solution very gently 1 time to mix, and dispense 1 ml of the cell suspension drop-wise into each of the 6 new Matrigel-coated wells, and immediately rock plate back-and-forth and side-to-side to evenly distribute the colony pieces across the well.
12. Incubate undisturbed at 37 °C and 5% CO<sub>2</sub> overnight.
13. Replace cell culture media every day with 2.5 ml of fresh mTeSR1, warmed to room temperature.
14. Monitor cells daily and passage as needed.

### Characterization and cryopreservation

Cells are expanded for cryopreservation at passage 10. Then, iPSCs are characterized (Figure 4) and prepare for downstream usage in genome engineering experiments or differentiation into specific cell types. Characterization includes:

1. Immunofluorescence staining of cell surface markers such Anti-Human TRA-1-60, Mouse monoclonal and transcription factors such as anti-Human OCT4, Rabbit polyclonal, IgG1 and anti-Human SOX2 Antibody, Rabbit polyclonal IgG1.
2. Pluripotency and trilineage differentiation capabilities (using the STEMdiff Trilineage Differentiation kit, and the Taqman hPSC Score card Panel).
3. Test for mycoplasma contamination using commercially available kits such as The Universal Mycoplasma Detection Kit.
4. G-banding Karyotype.



**Figure 4. Characterization of iPSC derived from fibroblast.** A. Bright field images of iPSCs, B. Immunofluorescence staining for pluripotency markers Tra-1-60 and OCT4. C. Taqman ScoreCard assay for pluripotent iPSCs (left panel) and iPSCs differentiated into endoderm, mesoderm and ectoderm (right panel). D. G-banding karyotype.

## Recipes

### 1. Fibroblast culture medium

The following recipe is to prepare 500 ml of fibroblast culture medium. Aseptically mix the following:

445 ml high glucose DMEM

50 ml heat-inactivated fetal bovine serum (FBS)

5 ml non-essential amino acids

### 2. 5 mM EDTA solution

Dilute 0.5 ml of 0.5 M EDTA in 500 ml DPBS without Calcium and Magnesium. Store at room temperature

### 3. 10 mM Y27632 stock solution

Aseptically add 3.122 ml of sterile water to 10 mg of Y27632

Mix thoroughly by pipetting, aliquot in Eppendorf's and store at -80 °C

Avoid repeated freezing and thawing

## Acknowledgments

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## **Competing interests**

The authors do not have any financial or non-financial interest in the subject matter or materials discussed in this manuscript.

## **Ethics**

This research was carried out in according with the UNC-CH institutional guidelines.

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