Generation of Human Induced Pluripotent Stem Cells Using a Defined, Feeder-Free Reprogramming System

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Human induced pluripotent stem cells (hiPSCs) offer great opportunities for the study of human development and disease modeling and have enormous potential for use in future clinical cell-based therapies. However, most current systems to create hiPSCs often expose the cells to animal feeder layers or xenogeneic reagents; this raises safety concerns about using hiPSC-derived cells for therapeutic purposes. Here, we describe protocols to generate hiPSCs without exposing the cells to xenogeneic materials that uses a defined, feeder-free reprogramming system. With this method, we were able to successfully reprogram not only patient-derived peripheral blood mononuclear cells but also amniocytes from the amniotic fluid of stillborn fetuses using two independent reprogramming platforms. Importantly, hiPSCs generated in this fashion expressed pluripotent markers and had normal karyotypes. The protocols allowed us to generate and culture hiPSCs under Good Manufacturing Practice–like conditions, a necessary step for the future clinical application of these cells.

Keywords: amniocytes • feeder-free reprogramming system • human induced pluripotent stem cells (hiPSCs) • peripheral blood mononuclear cells (PBMCs) • Sendai virus vector • STEMCCA lentiviral vector

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INTRODUCTION

The first human induced pluripotent stem cells (hiPSCs) were derived from dermal fibroblasts in 2007 by forced expression of the transcription factors Oct4, Sox2, Klf4, and c-Myc using individual retroviral vectors (Takahashi et al., 2007). Since then, several methods have been developed to generate hiPSCs using either viral delivery systems based on a retrovirus (Takahashi et al., 2007), lentivirus (Yu et al., 2007), or Sendai virus (Fusaki, Ban, Nishiyama, Saeki, & Hasegawa, 2009) or nonviral delivery systems based on a plasmid (Okita et al., 2011), transposons (Woltjen et al., 2009), or RNA (Warren et al., 2010). These hiPSCs have been derived from various types of somatic cells, such as skin fibroblasts (Takahashi et al., 2007), keratinocytes (Aasen et al., 2008), pancreatic beta cells (Stadtfeld, Brennand, & Hochedlinger, 2008a), and T lymphocytes (Seki et al., 2010; Staerk et al., 2010). hiPSCs hold enormous potential in studies of diseases, drug screening, and patient-specific cell therapies (Avior, Sagi, & Benvenisty, 2016). However, the full potential of hiPSC technology could be limited by the use of animal products for the cells' derivation and maintenance: most of the current reprogramming methods

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require exposure of the cells to animal feeder layers or xenogeneic reagents. This raises safety concerns for clinical translation (Ahrlund-Richter et al., 2009; Martin, Muotri, Gage, & Varki, 2005).

Here, we describe two protocols to generate hiPSCs under Good Manufacturing Practice)–like conditions. In these protocols, two different viral vector systems are employed to produce hiPSCs, namely, the STEMCCA vector and the Sendai virus vector. The STEMCCA lentiviral vector is a single floxed-excisable polycistronic lentivirus that we previously described as capable of very efficiently reprogramming murine and human cells (Sommer et al., 2009, 2010). The Sendai virus vector is a cytoplasmic RNA vector that does not integrate into the host genome. By using these two reprogramming systems, we were able to generate transgene-free hiPSCs.

Furthermore, two different somatic cell types can be used as sources for hiPSC generation, namely, peripheral blood mononuclear cells (PBMCs; Basic Protocol 1) and amniocytes (Basic Protocol 2). These cell types have advantages over other somatic cell types as a source for reprogramming. For example, PBMCs can be collected via a minimally invasive procedure that is relatively less traumatic and has lower risks to patients than skin biopsy. Regarding amniocytes, these cells can be easily obtained from amniotic fluid, even in cases where a stillborn fetus is affected by a disease impairing development.

The whole reprogramming protocol is performed under serum-free, feeder-free conditions, bringing us a step closer to the generation of hiPSCs that will be suitable for the clinical setting.

NOTE: Sterile techniques should be used when preparing all solutions and handling cells.

NOTE: All culture incubations are performed in a humidified 37°C, 5% CO2 incubator unless otherwise specified.

NOTE: All procedures should be performed in a Class II biological-hazard flow hood or a laminar flow hood.

BASIC PROTOCOL 1

GENERATION OF HUMAN INDUCED PLURIPOTENT STEM CELLS DERIVED FROM PERIPHERAL BLOOD MONONUCLEAR CELLS

This protocol involves reprogramming PBMCs using a feeder-free reprogramming system to generate hiPSCs. PBMCs are cultured and expanded in vitro based on a previously published protocol (Sommer et al., 2012) (Fig. 1) that tends to produce an enriched population of erythroblasts (Yang et al., 2012). In contrast to other protocols that use T lymphocytes (Seki et al., 2010; Staerk et al., 2010), the present protocol provides access to hiPSCs without a pre-rearranged T-cell receptor (TCR). The expanded PBMCs can then be transduced using either the STEMCCA lentiviral system or the Sendai viral reprogramming approach to derive hiPSCs. Upon reprogramming with STEMCCA, the integrated reprogramming cassette can be excised by utilizing Cre recombination

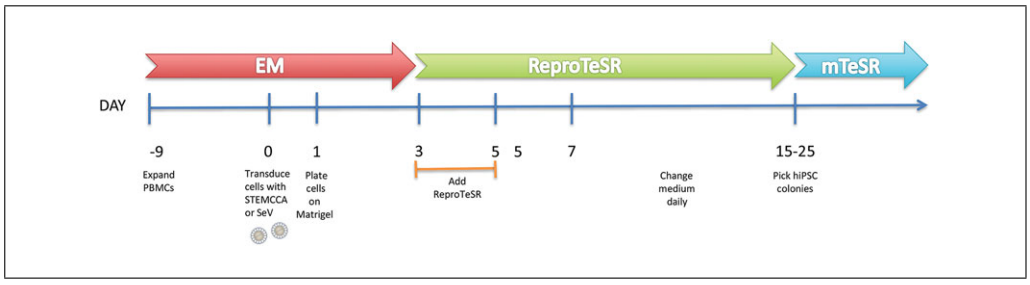

Figure 1 Timeline for reprogramming PBMCs. SeV, Sendai virus.

(Somers et al., 2010). Ultimately, this platform allows for the generation of integration-free hiPSCs in a serum-free, feeder-free manner.

Materials

Human peripheral blood
Phosphate-buffered saline without $CaCl_2$ and $MgCl_2$ (PBS; Invitrogen)
PBMC expansion medium (EM; see recipe), 37°C or room temperature
PBMC freezing medium (see recipe; optional), 4°C
Dry ice (optional)
70% (v/v) ethanol (optional)
QBSF-60 Stem Cell Medium (Quality Biological), 37°C
5 mg/ml polybrene (see recipe)
STEMCCA lentivirus *or* CytoTune-iPS 2.0 Sendai Reprogramming Kit (Invitrogen)
hESC-qualified Matrigel (Corning; store frozen in aliquots at –80°C)
DMEM/F12 (Invitrogen), 4°C
Complete ReproTeSR medium (see recipe), room temperature
Complete mTeSR medium (see recipe), room temperature
Y27632/Rho kinase (ROCK) inhibitor (STEMGENT)

BD Vacutainer CPT Cell Preparation Tube with Sodium Citrate (BD Biosciences)
Standard tabletop centrifuge with plate adaptor (e.g., Eppendorf Centrifuge 5810R with A-4-62 rotor)
P1000 and P20 pipet tips
15-ml conical tubes
12-well tissue culture plates
37°C, 5% CO_2 incubator
1-ml cryovials (optional)
Freezing container (e.g., Mr. Frosty or double-layered Styrofoam box; optional)
37°C water bath or thawing instrument (optional)
6-well tissue culture plate
Light microscope

Day –9: Isolate and expand PBMCs

1. Collect 4 ml human peripheral blood into a BD Vacutainer CPT Cell Preparation Tube with Sodium Citrate. After collection, keep tube upright at room temperature until centrifugation.
2. Mix blood sample right before centrifugation by gently inverting the tube 8 to 10 times. Then, centrifuge tube for 30 min at 1800 × *g* at room temperature.

   This step should be done within 2 hr of blood collection for best results.
3. Collect mononuclear cells within the buffy coat between the plasma and gel layers using a P1000 pipet tip. Transfer cells into a 15-ml conical tube.
4. Add PBS to bring volume to 10 ml and mix cells gently by inverting the tube five times.
5. Centrifuge tube for 15 min at 300 × *g*, room temperature.
6. Aspirate supernatant and resuspend cells in 10 ml PBS.
7. Perform cell count and transfer 1×10^6 to 2×10^6 cells (for fresh PBMC use) or $\geq 2 \times 10^6$ cells (for PBMC freezing) into a 15-ml conical tube, followed by centrifugation for 10 min at 300 × *g*, room temperature.

8a. *To expand fresh PBMCs:* Aspirate supernatant and resuspend cell pellet in 2 ml EM. Transfer cells into one well of a 12-well tissue culture plate and incubate in a 37°C, 5% CO_2 incubator for 3 days without media replenishment.

The number of wells can be scaled up according to the number of BD Vacutainer tubes used.

8b. *To expand frozen PBMCs:* Freeze ~2 × 10^6 cells in 1 ml cold PBMC freezing medium per 1-ml cryovial (see Support Protocol 1, step 11). For use, thaw cryovial of cells (see Support Protocol 2, steps 4 to 6), add cells to 10 ml QBSF-60 Stem Cell Medium in a 15-ml conical tube, and centrifuge 10 min at 300 × *g*, room temperature. Aspirate supernatant and resuspend cells using 2 ml EM. Transfer cells into one well of a 12-well tissue culture plate and incubate in a 37°C, 5% CO_2 incubator for 3 days without media replenishment.

Days –6 and –3: Keep expanding the PBMCs

9. At day –6, transfer cells into a 15-ml conical tube. Wash originating well using 1 ml QBSF-60 to collect adherent cells and add wash to the 15-ml conical tube.

10. Centrifuge cells for 10 min at 300 × *g*, room temperature. Aspirate supernatant and resuspend cells in 2 ml fresh EM.

11. Transfer cells into a new well of the 12-well plate from step 8a or 8b and incubate cells in a 37°C, 5% CO_2 incubator for 3 days without media replenishment. At day –3, repeat process from steps 9 to 11.

Day 0: Transduce the PBMCs

12. Transfer cells into a 15-ml conical tube and wash originating well with 1 ml QBSF-60 to collect adherent cells. Add wash to the 15-ml conical tube.

13. Centrifuge cells for 10 min at 300 × *g*.

Transduction using STEMCCA lentivirus

14a. Aspirate supernatant and resuspend cells in 1 ml fresh EM containing 5 μg/ml polybrene and STEMCCA lentivirus (MOI = 1 to 10).

The cationic polymer polybrene helps virions diffuse to the host cell surface by neutralizing charge repulsion. Polybrene thus has been proven to increase the efficiency of transduction.

15a. Mix cells with the virus by gently flicking the tube.

16a. Transfer cells into a new well of the 12-well plate from step 11 and centrifuge plate for 90 min at 1020 × *g*.

17a. After spinoculation, add an additional 1 ml fresh EM containing polybrene for a total of 2 ml medium and incubate cells in a 37°C, 5% CO_2 incubator.

Transduction using Sendai viruses

14b. Thaw CytoTune-iPS 2.0 Sendai Reprogramming Kit Sendai tubes according to the manufacturer's instructions and keep viruses on ice.

15b. Aspirate supernatant from step 13, resuspend cells in 1 ml fresh EM, and count cells.

16b. Add 2 × 10^5 cells and fresh EM containing 5 μg/ml polybrene to a final volume of 1 ml in a 15-ml conical tube.

17b. Based on the cell number, calculate volume of each virus needed to obtain the appropriate MOI (KOS/hc-Myc/hKlf4 = 5:5:3). Add each virus to the 1 ml of polybrene-containing EM containing the cells.

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

The titer of each virus is provided by the manufacturer.

18b. Mix cells with the viruses by gently flicking the tube.

19b. Transfer cells into a new well of the 12-well plate from step 11 and centrifuge plate for 90 min at 1020 × *g*.

20b. After spinoculation, add an additional 1 ml fresh EM containing polybrene for a total of 2 ml medium and incubate cells in a 37°C, 5% CO_2 incubator.

Day 1: Wash off the reprogramming virus(es) and re-plate the transduced cells

21. To prepare a 6-well tissue culture plate coated with hESC-qualified Matrigel, thaw an aliquot of Matrigel on ice and dilute it with 6.25 ml cold DMEM/F12. Distribute Matrigel mixture into plate at 1 ml/well and incubate plate for 1 hr at room temperature. Aspirate Matrigel mixture without scratching the coated surface right before plating the cells.

    hESC-qualified Matrigel should be aliquoted for one or two 6-well plates according to the lot-specific dilution factor provided by the manufacturer and stored at −80°C until use.

    The plate coated with hESC-qualified Matrigel should not be allowed to dry up.

22. Collect transduced cells and transfer them into a 15-ml conical tube. Wash well gently with 1 ml QBSF-60 to make sure that most cells are harvested.

23. Centrifuge cells for 10 min at 300 × *g*.

24. Remove supernatant and resuspend cells in 1 ml EM.

25. Perform cell count.

26. Plate 8.0×10^4 to 2.0×10^5 cells in 2 ml EM per well into the Matrigel-coated 6-well plate from step 21.

    Plating the cells at three different concentrations is recommended to make it easier to pick colonies.

    When the plate is spun down at low speed (e.g., 50 × g) to help the cells attach to the Matrigel-coated wells, relatively few cells attach and survive compared to cells without spinning. Therefore, the plate containing the infected cells should not be centrifuged, even at low speed.

27. Incubate cells overnight in a 37°C, 5% CO_2 incubator.

Day 2: Add more PBMC EM

28. Add 1 ml fresh EM to each well without aspirating original medium. Incubate cells overnight in a 37°C, 5% CO_2 incubator.

Days 3 and 5: Add reprogramming medium

29. On day 3, add 1 ml complete ReproTeSR medium to each well without aspirating original medium. Incubate cells in a 37°C, 5% CO_2 incubator. Repeat step on day 5.

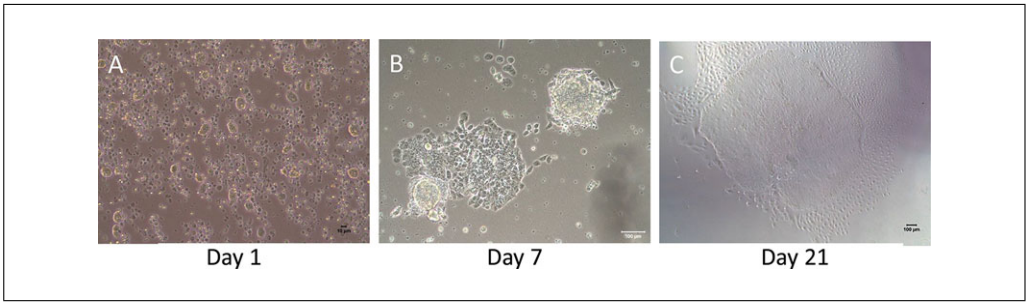

Figure 2 Representative images of morphological changes in the reprogrammed PBMCs. (**A**) Day 1: STEMCCA-infected or Sendai virus–infected PBMCs before plating onto hESC-qualified Matrigel (scale bar = 10 μm). (**B**) Day 7: There is a clear change in morphology, and the beginning of colony formation can be detected (scale bar = 100 μm). (**C**) Day 21: Typical hiPSC colonies appearing on the Matrigel are ready to be picked (scale bar = 100 μm).

Days 7 to 25: Feed the cells and pick putative hiPSC colonies

30. Aspirate old medium and add 2 ml complete ReproTeSR per well. Incubate cells in a 37°C, 5% CO_2 incubator.

31. Exchange medium daily with 2 ml fresh complete ReproTeSR per well.

32. Monitor cells every other day under a light microscope to check for the appearance of small colonies (Fig. 2).

    Once hiPSC colonies are observed, let them grow for 3 to 4 more days unless they start merging with each other. The bigger the colony, the easier it is to pick.

33. Once the hiPSC colonies become big enough (approximately 1.3 to 2.2 mm in diameter) to be picked, without having merged with each other, prepare a 12-well plate coated with hESC-qualified Matrigel (see step 21) by adding 500 μl per well of Matrigel mixture. Add 1 ml complete mTeSR medium with 10 μM Y27632/ROCK inhibitor per well and keep plate in a 37°C, 5% CO_2 incubator until use.

    If the hiPSC colonies start merging with each other, they are likely to start differentiating.

    If an hiPSC colony is still small but starts differentiating in the middle, it should be picked before the differentiated cells spread across the whole colony.

34. Manually pick putative hiPSC colonies using a P20 tip. Transfer each clone into a well of the 12-well plate coated with hESC-qualified Matrigel for further expansion or analysis.

    To pick only putative hiPSC colonies, remove differentiated, partially reprogrammed, or non-reprogrammed cells surrounding the colonies before picking. The identity of the generated hiPSCs can be confirmed by immunofluorescence staining using the ES Cell Characterization Kit from Millipore according to the manufacturer's protocol.

    Six clones should be picked from one sample, and only the best three clones should be expanded and frozen (Support Protocol 2) for future use.

    One colony should be divided into 10 to 30 cell clumps and plated in the same well of the 12-well plate.

FREEZING HUMAN INDUCED PLURIPOTENT STEM CELLS

SUPPORT PROTOCOL 1

Reprogrammed hiPSCs usually become stabilized within four passages. Stabilized and expanded hiPSCs can be frozen and stored at −150°C until thawing (Support Protocol 2) for future use.

Additional Materials *(also see Basic Protocol 1)*

mFreSR (STEMCELL Technologies; store at –20°C)
Stabilized and expanded hiPSCs (see Basic Protocol 1)
ReLeSR (STEMCELL Technologies), room temperature

1-ml cryovials
Cell scraper or lifter
Freezing container (e.g., Mr. Frosty or double-layered Styrofoam box)

1. Thaw mFreSR at room temperature or at 4°C overnight and keep it on ice after thawing.
2. Meanwhile, label 1-ml cryovials with information on the cells to be frozen (e.g., name of cell line, passage number, media type used for culture, date of freezing, and name of person freezing the cells).

   One cryovial should be used per well of a 6-well plate.

3. Wash stabilized and expanded hiPSCs with PBS.

   When the cells are around 70 to 90% confluent, they can be frozen.

4. Following aspiration of PBS, add 1 ml ReLeSR per well of a 6-well tissue culture plate and aspirate it within 1 min. Incubate cells in a 37°C, 5% CO_2 incubator for 2 to 5 min.

   The cells are covered with a thin layer of ReLeSR so that they do not dry up during incubation.

   ReLeSR can be replaced with other types of dissociation reagents (e.g., Dispase or Gentle Cell Dissociation Reagent, STEMCELL Technologies). In that case, the procedure should adhere to the reagent manufacturer's instructions.

5. Remove cells from the incubator and add 1 ml complete mTeSR medium per well. Induce cell detachment by gently tapping plate or scraping cells using a cell scraper or lifter.

   The cells should detach from the plate as cell aggregates. Be careful to avoid separating the aggregates into single cells.

   If most of the cells are not differentiated, they can be incubated for 2 min followed by addition of 1 ml complete mTeSR and harvesting by scraping. In this way, most of the cells can be harvested and transferred into a 15-ml conical tube for the next step. ReLeSR-treated cells often tend to float on the surface of the media and then stick to the plate again during transfer, so a fraction of the cells can be lost otherwise. If the cells include differentiated cells, incubate the cells for 5 min and then tap the plate gently 15 to 20 times, followed by addition of 1 ml complete mTeSR; only undifferentiated cells will detach from the plate.

6. Transfer cell aggregates into a 15-ml conical tube.

   If the cell aggregates are derived from the same cell line of the same batch, they can be combined into the same 15-ml conical tube.

7. Centrifuge cell aggregates for 5 min at 200 × *g* at room temperature.

   If the cell aggregates are still floating in the media, they can be centrifuged at 300 × g instead.

8. Gently aspirate supernatant without disturbing the cell pellet.

9. Gently resuspend cell pellet with 1 ml cold mFreSR by pipetting up and down and place on ice. Add additional mFreSR according to the number of harvested wells (1 ml/well).

   If the cells are being harvested from a whole 6-well plate, add 1 ml cold mFreSR to resuspend the cell pellet and add an additional 5 ml mFreSR, resulting in 6 ml mFreSR in total (1 ml/well).

   Be careful not to break up the cell aggregates.

10. Keeping the 15-ml conical tube containing the cells on ice, transfer 1 ml of cell aggregates into each labeled cryovial from step 2.

11. Freeze cryovials at −80°C overnight using a freezing container, which reduces the temperature at −1°C/min. Move frozen vials to −150°C on the following day.

SUPPORT PROTOCOL 2

THAWING HUMAN INDUCED PLURIPOTENT STEM CELLS

Previously frozen hiPSCs (Support Protocol 1) can be thawed when needed. Thawed hiPSCs can usually be recovered within 1 week, but recovery may vary depending on the cell line and freezing method used.

Additional Materials *(also see Basic Protocol 1 and Support Protocol 1)*

Frozen cryovial of hiPSCs (see Support Protocol 1)
Dry ice
70% (v/v) ethanol

37°C water bath or thawing instrument

1. Prepare a 6-well tissue culture plate coated with hESC-qualified Matrigel (see Basic Protocol 1, step 21).

   One cryovial of frozen cells can be thawed into 1 to 3 wells of a 6-well plate.

2. While coating the 6-well plate, warm complete mTeSR medium at room temperature.

3. Add 9 ml complete mTeSR to a 15-ml conical tube.

   A 15-ml conical tube containing 9 ml complete mTeSR should be prepared for each frozen cryovial to be thawed.

4. Remove frozen cryovial from −150°C and keep it on dry ice until thawing.

5. Quickly thaw frozen cells by gently shaking the cryovial in a 37°C water bath or using a thawing instrument until only a small ice pellet is left.

6. Remove cryovial from the water bath or thawing instrument. Spray 70% ethanol thoroughly over the entire cryovial and wipe it down for sterilization.

7. Transfer 1 ml of thawed cells from cryovial to the 15-ml conical tube from step 3.

8. Centrifuge tube for 5 min at 200 × *g* at room temperature.

   If the cell aggregates are still floating in the media, they can be centrifuged at 300 × g instead.

9. Meanwhile, prepare mTeSR with 10 μM ROCK inhibitor (assuming 2 ml/well of a 6-well plate). Aspirate hESC-qualified Matrigel from the 6-well plate from step 1 and add 1 to 1.5 ml mTeSR with ROCK inhibitor per well.

10. Gently aspirate supernatant from step 8 without disturbing the cell pellet.

11. Gently resuspend cell pellet with 1 ml mTeSR with ROCK inhibitor by pipetting up and down. Plate 0.5 to 1 ml cells into one or two wells so that the total volume is 2 ml cells in mTeSR with ROCK inhibitor.

    The cells should be plated at greater confluency than for routine passaging. Depending on the growth rate of the cells and the number of cell aggregates, the number of wells prepared can be adjusted.

12. Gently shake plate to evenly distribute the cell aggregates over the well(s).

13. Incubate cells in a 37°C, 5% CO_2 incubator and leave undisturbed until the following day.

14. Change media every day using mTeSR and passage cells when appropriate.

    Thawed cells are usually recovered and ready to be passaged within 1 week, but recovery may vary depending on the cell line and freezing method used and the confluency of the plated cells.

GENERATION OF HUMAN INDUCED PLURIPOTENT STEM CELLS DERIVED FROM AMNIOCYTES

BASIC PROTOCOL 2

In this protocol, we describe the generation of hiPSCs from amniocytes using an integration-free and feeder-free reprogramming system (Fig. 3). The integrated STEMCCA lentiviral vector can be excised by using Cre recombination, as previously described (Somers et al., 2010). Amniocytes are cultured and maintained based on a previously published method (Anchan et al., 2010), with modifications as described below. It is recommended to use low-passage amniocytes that are actively proliferating.

Additional Materials *(also see Basic Protocol 1)*

Amniocytes
Amniocyte medium (see recipe), 37°C
EmbryoMax 0.1% Gelatin Solution (Millipore)
0.05% trypsin (Invitrogen)
TrypLE Select (Invitrogen)

Day –2: Prepare amniocytes for transduction

1. To prepare a 6-well tissue culture plate with 0.1% gelatin, add 1 ml per well 0.1% gelatin solution and incubate it at room temperature for 15 to 30 min. Plate amniocytes in amniocyte medium into two wells of a 0.1% gelatin–coated 6-well tissue culture plate to obtain 2×10^5 to 5×10^5 cells on the day of transduction (which represents 50 to 80% confluency).

    The same number of cells should be plated into two wells. The cells in one well will be used for transduction and the ones in the other will be used for cell counting on Day 0.

    On day –2, 1×10^5 to 2.5×10^5 cells are usually plated to reach 50 to 80% confluency on the day of transduction. However, growth may vary depending on the cell line, so the cell number should be adjusted based on the cell line–specific growth rate.

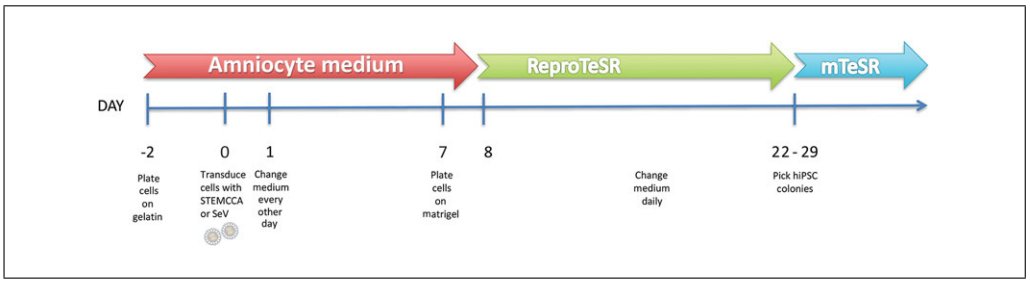

Figure 3 Timeline for generating hiPSCs derived from amniocytes. SeV, Sendai virus.

Day 0: Transduce the amniocytes

2. Aspirate old medium from one of the two wells of amniocytes and wash well using 1 ml PBS.
3. Add 1 ml of 0.05% trypsin and incubate cells in a 37°C, 5% CO_2 incubator for 3 to 5 min.
4. Inactivate trypsin by adding 2 ml amniocyte medium and transfer trypsinized cells into a 15-ml conical tube. Centrifuge for 5 min at 300 × *g* at room temperature.
5. Aspirate supernatant and resuspend cells using 1 ml amniocyte medium.
6. Count cells.

   These cells are only used for estimating the cell number in the other well.

   Transduction using STEMCCA lentivirus

7a. Thaw STEMCCA lentivirus on ice.

8a. Add STEMCCA lentivirus (MOI = 1 to 10) and 5 μg/ml polybrene to 1 ml amniocyte medium.

9a. Aspirate old medium in the remaining well from step 1 and add the 1 ml amniocyte medium containing STEMCCA and polybrene. Incubate cells in a 37°C, 5% CO_2 incubator.

   Transduction using Sendai viruses

7b. Thaw CytoTune-iPS 2.0 Sendai Reprogramming Kit Sendai tubes according to the manufacturer's instructions.

8b. Based on the cell number, calculate volume of each virus needed to obtain the appropriate MOI (KOS/hc-Myc/hKlf4 = 5:5:3). Add each virus and 5 μg/ml polybrene to 1 ml amniocyte medium.

9b. Aspirate old medium in the remaining well from step 1 and add the 1 ml amniocyte medium containing the Sendai viruses and polybrene. Incubate the cells in a 37°C, 5% CO_2 incubator.

Day 1: Wash off the reprogramming virus(es)

10. At 24 hr post-infection, aspirate medium containing the virus(es) and add 2 ml fresh amniocyte medium.
11. Incubate cells in a 37°C, 5% CO_2 incubator. Culture cells for 6 more days, exchanging the old medium for fresh amniocyte medium every other day.

Day 7: Plate the transduced cells onto a Matrigel-coated plate

12. Thaw hESC-qualified Matrigel according to the manufacturer's protocol and coat a sufficient number of 6-well plates for 1 hr at room temperature (see Basic Protocol 1, step 21).

    It is recommended to prepare small aliquots of hESC-qualified Matrigel ahead of time. Three wells of a 6-well plate are usually prepared per sample. However, this can be adjusted if more or fewer are needed.

13. After 1 hr, aspirate Matrigel from the 6-well plates and add 2 ml amniocyte medium per well. Keep plates in a 37°C, 5% CO_2 incubator until use.

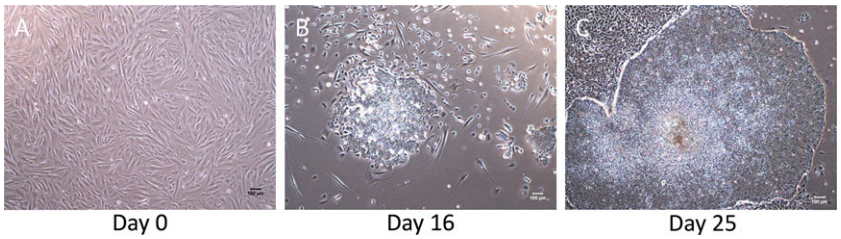

Figure 4 Representative images of morphological changes in the reprogrammed amniocytes. (**A**) Day 0: Amniocytes before infection should present around 50 to 80% confluency. (**B**) Day 16: Small colonies emerge between days 13 and 17. (**C**) Day 25: hiPSC colonies are ready to be picked (scale bar = 100 μm).

14. On day 7 post-infection, aspirate old medium in well from step 11 and wash cells with 1 ml PBS. Then, trypsinize transduced cells using 1 ml TrypLE Select for 2 to 3 min at room temperature.
15. Add 2 ml amniocyte medium to well and collect cells in a 15-ml conical tube.
16. Centrifuge 5 min at 200 × *g*. Aspirate supernatant and resuspend cells using 1 ml amniocyte medium.
17. Count cells.
18. Plate 1.0×10^4 to 8.5×10^4 cells/well into the 6-well plates coated with hESC-qualified Matrigel from step 12.

    Plating the cells at three different concentrations is recommended to make it easier to pick colonies. In particular, the cells can be plated at 1.5×10^4, 5.0×10^4, and 8.0×10^4 cells/well.

Days 8 to 29: Feed the cells and pick putative hiPSC colonies

19. Change medium daily at 2 ml complete ReproTeSR medium per well.
20. Monitor cells every other day under a light microscope to check for the appearance of small embryonic stem cell–like colonies (Fig. 4).
21. Once the hiPSC colonies become big enough (approximately 1.3 to 2.2 mm in diameter) to be picked, without having merged with each other, prepare a 12-well tissue culture plate coated with hESC-qualified Matrigel (see step 12). Add 1 ml complete mTeSR medium with 10 μM ROCK inhibitor per well and keep plate in a 37°C, 5% CO_2 incubator until use.

    Amniocyte-derived hiPSC colonies appear 2 to 3 days later than PBMC-derived hiPSC colonies.

22. Manually pick putative hiPSC colonies. Transfer each clone into a well of the 12-well plate coated with hESC-qualified Matrigel for further expansion or analysis.

    Amniocyte-derived hiPSC colonies are usually ready to be picked around days 21 to 25.

REAGENTS AND SOLUTIONS

For culture recipes and steps, use sterile tissue culture–grade water unless otherwise indicated.

Amniocyte medium

Alpha-MEM (Invitrogen)
10% (v/v) heat-inactivated fetal bovine serum, characterized (FBS; HyClone)

continued

1× non-essential amino acids (100×, Invitrogen)
1× GlutaMAX (100×, Invitrogen)
0.1 mg/ml Primocin (50 mg/ml, InvivoGen)
Store ≤2 weeks at 4°C

Ascorbic acid, 5 mg/ml

Dissolve L-ascorbic acid powder (MilliporeSigma) in deionized, distilled water at 5 mg/ml (100× stock solution). Store for 6 months or longer at −20°C.

Complete mTeSR medium

Basal mTeSR (STEMCELL Technologies)
1× mTeSR Supplement (50×, STEMCELL Technologies)
0.1 mg/ml Primocin (50 mg/ml, InvivoGen)
Store ≤2 weeks at 4°C

Complete ReproTeSR medium

Basal ReproTeSR (STEMCELL Technologies)
1× ReproTeSR Supplement (20×, STEMCELL Technologies)
1× ReproTeSR Supplement (500×, STEMCELL Technologies)
0.1 mg/ml Primocin (50 mg/ml, InvivoGen)
Store ≤2 weeks at 4°C or aliquot and store ≤6 months at −20°C

Dexamethasone, 50 μM

Dissolve dexamethasone powder in absolute ethanol at 1 mg/ml and add sterile DMEM/F12 (Invitrogen) to achieve a final concentration of 50 μM (50× stock solution). Store up to 12 months at −20°C. Keep protected from light.

Insulin-like growth factor 1 (IGF-1), 50 μg/ml

Reconstitute lyophilized recombinant human IGF-1 (R&D Systems) at 50 μg/ml in sterile PBS. Divide into appropriately sized aliquots and store up to 3 months at −20°C to −80°C under sterile conditions.

Interleukin 3 (IL-3), 10 μg/ml

Reconstitute lyophilized recombinant human IL-3 (R&D Systems) at 10 μg/ml in sterile PBS containing ≥0.1% bovine serum albumin. Divide into appropriately sized aliquots and store up to 3 months at −20°C to −80°C under sterile conditions.

PBMC expansion medium (EM)

QBSF-60 Stem Cell Medium (Quality Biological)
50 μg/ml ascorbic acid (see recipe)
50 ng/ml SCF (see recipe)
10 ng/ml IL-3 (see recipe)
2 U/ml EPO (R&D Systems)
40 ng/ml IGF-1 (see recipe)
1 μM dexamethasone (see recipe)
1× Pen/Step (100×, Invitrogen) or 0.1 mg/ml Primocin (50 mg/ml, InvivoGen)
Prepare fresh immediately before use

Use Pen/Strep while testing for mycoplasma contamination. After medium is confirmed as mycoplasma negative, switch to Primocin.

PBMC freezing medium

FBS (HyClone)
10% dimethyl sulfoxide (DMSO; MilliporeSigma)
Store ≤2 weeks at 4°C

Polybrene, 5 mg/ml

Dissolve polybrene powder (MilliporeSigma) in deionized, distilled water at 5 mg/ml (1000× stock solution). Store up to 3 years at −20°C.

Stem cell factor (SCF), 50 μg/ml

Reconstitute lyophilized recombinant human SCF (R&D Systems) at 50 μg/ml in sterile PBS containing ≥0.1% bovine serum albumin. Divide into appropriately sized aliquots and store up to 3 months at −20°C to −80°C under sterile conditions.

COMMENTARY

Background Information

In 2007, Shinya Yamanaka and his colleagues generated hiPSCs from somatic cells by inducing expression of several transcription factors, such as Oct4, Nanog, Sox2, c-Myc, *and* Klf4 (Takahashi et al., 2007). hiPSCs not only overcome many ethical concerns and potential immune rejection in future tissue transplantation but also have been shown to be equivalent to human embryonic stem cells (hESCs) in many other ways (Choi et al., 2015). hiPSCs provide great opportunities to study the pathophysiology of disease and to test drugs. Importantly, hiPSCs also hold great promise for personalized, patient-specific treatment (Shi, Inoue, Wu, & Yamanaka, 2016).

Initially, individual retroviruses and lentiviruses were used to derive hiPSCs. However, these viral reprogramming systems had a risk of introducing multiple random integrations into the host genome, potentially disrupting the host genome and inducing insertional mutagenesis. Eventually, this could potentially cause disruption or aberrant activation of neighboring genes and could also lead to the reactivation of reprogramming factors that can induce tumor formation (Ahrlund-Richter et al., 2009). To avoid these risks, several reprogramming systems capable of generating integration-free hiPSCs have been developed using excisable lentiviruses (Somers et al., 2010), adenoviruses (Stadtfeld, Nagaya, Utikal, Weir, & Hochedlinger, 2008b), plasmids (Okita et al., 2011), transposons (Woltjen et al., 2009), Sendai viruses (Fusaki et al., 2009), synthetic mRNAs (Warren et al., 2010), and recombinant proteins (Kim et al., 2009).

Conventionally, hiPSCs have been generated using animal feeder layers in the presence of xenogeneic reagents, which raises safety concerns for these cells' use in clinical applications. Therefore, in order to generate hiPSCs under GMP-like conditions, reprogramming should be done to obtain integration-free colonies obtained under feeder-free and xeno-free settings. To satisfy these criteria, we use a single excisable polycistronic lentiviral STEMCCA vector or Sendai virus vector for derivation of integration-free hiPSCs. In addition, defined serum-free culture conditions are used to generate and maintain hiPSCs. This reprogramming system brings us a step closer to the generation of clinical-grade hiPSCs.

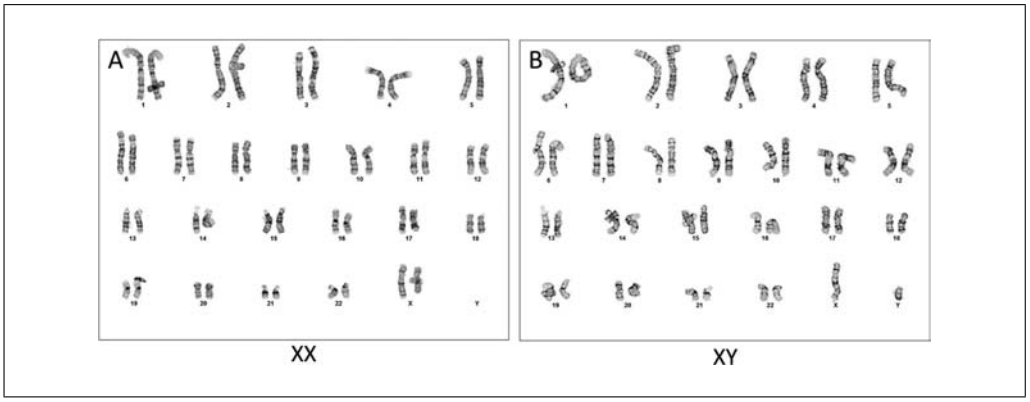

Figure 5 Karyotyping analysis of hiPSCs. The karyotypes of hiPSCs from both male and female samples are normal after reprogramming. (**A**) XX, female. (**B**) XY, male.

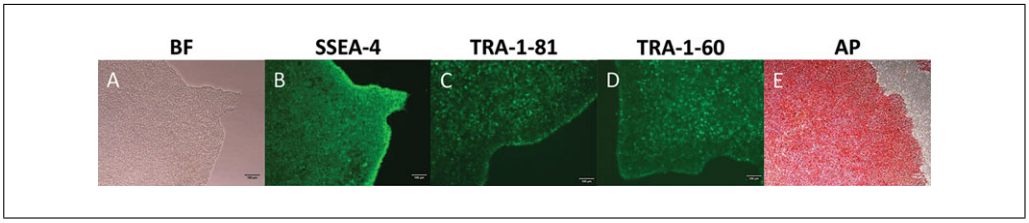

Figure 6 Characterization of hiPSCs generated from PBMCs or amniocytes. (**A**) Bright-field (BF) image (scale bar = 100 μm). Immunofluorescence analysis of hiPSCs derived from PBMCs or amniocytes shows expression of the pluripotency markers SSEA-4 (**B**), TRA-1-81 (**C**), and TRA-1-60 (**D**). (**E**) The hiPSCs generated from PBMCs or amniocytes display positive staining for alkaline phosphatase (AP).

Critical Parameters and Troubleshooting

Regarding PBMC expansion (Basic Protocol 1), the expansion medium is intended to expand erythroblasts among PBMCs; this ensures generation of hiPSCs devoid of pre-rearranged T/B-cell receptors.

When plating transduced PBMCs on hESC-qualified Matrigel, the plate containing the cells should not be spun down, which may otherwise prevent cell attachment and decrease cell viability.

To successfully reprogram amniocytes (Basic Protocol 2), we recommend using cells at a lower passage (<5).

It is important to keep the number of plated cells low to avoid high confluency, which can cause colonies to merge even before they become fully reprogrammed, making it very difficult to pick single-cell-derived hiPSC clones.

Anticipated Results

Using the protocols presented here, hiPSCs can be generated from PBMCs (Basic Protocol 1) or amniocytes (Basic Protocol 2). Both PBMC-derived and amniocyte-derived hiPSCs have normal karyotypes (Fig. 5). Moreover, they both positively express typical human pluripotent cell markers such as SSEA-4, TRA-1-81, and TRA-1-60 as well as alkaline phosphatase (Fig. 6). hiPSCs can be stably passaged and maintained under serum-free conditions or frozen down (Support Protocol 1) until thawing (Support Protocol 2) for later use.

Time Considerations

PBMCs transduced with either STEMCCA lentivirus or Sendai viruses start changing their morphology around day 7 post-infection. In our experience, PBMCs reprogrammed with the Sendai system tend to be ready for picking a few days earlier (around 17 to 19 days post-infection) than cells reprogrammed using the STEMCCA system. Regarding amniocytes, SeV-infected cells are usually ready to be picked around 22 to 25 days post-infection. STEMCCa-transduced cells are usually ready around 25 to 29 days post-transduction.

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Literature Cited

Aasen, T., Raya, A., Barrero, M. J., Garreta, E., Consiglio, A., Gonzalez, F., ... Belmonte, J. C. I. (2008). Efficient and rapid generation of induced pluripotent stem cells from human keratinocytes. *Nature Biotechnology*, *26*, 1276–1284. doi: 10.1038/nbt.1503.

Ahrlund-Richter, L., De Luca, M., Marshak, D. R., Munsie, M., Veiga, A., & Rao, M. (2009). Isolation and production of cells suitable for human therapy: Challenges ahead. *Cell Stem Cell*, *4*, 20–26. doi: 10.1016/j.stem.2008.11.012.

Anchan, R. M., Quaas, P., Gerami-Naini, B., Bartake, H., Griffin, A., Zhou, Y., ... Maas, R. L. (2010). Amniocytes can serve a dual function as a source of iPS cells and feeder layers. *Human Molecular Genetics*, *20*, 962–974. doi: 10.1093/hmg/ddq542.

Avior, Y., Sagi, I., & Benvenisty, N. (2016). Pluripotent stem cells in disease modelling and drug discovery. *Nature Reviews Molecular Cell Biology*, *17*, 1–13. doi: 10.1038/nrm.2015.27.

Choi, J., Lee, S., Mallard, W., Clement, K., Tagliazucchi, G. M., Lim, H., ... Hochedlinger, K. (2015). A comparison of genetically matched cell lines reveals the equivalence of human iPSCs and ESCs. *Nature Biotechnology*, *33*, 1–11. doi: 10.1038/nbt.3388.

Fusaki, N., Ban, H., Nishiyama, A., Saeki, K., & Hasegawa, M. (2009). 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. *Proceedings of the Japan Academy, Series B*, *85*, 348–362. doi: 10.2183/pjab.85.348.

Kim, D., Kim, C.-H., Moon, J.-I., Chung, Y.-G., Chang, M.-Y., Han, B.-S., ... Kim, K. S.

(2009). 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.

Martin, M. J., Muotri, A., Gage, F., & Varki, A. (2005). Human embryonic stem cells express an immunogenic nonhuman sialic acid. *Nature Medicine*, *11*, 228–232. doi: 10.1038/nm1181.

Okita, K., Matsumura, Y., Sato, Y., Okada, A., Morizane, A., Okamoto, S., ... Yamanaka, S. (2011). A more efficient method to generate integration-free human iPS cells. *Nature Methods*, *8*, 409–412. doi: 10.1038/nmeth.1591.

Seki, T., Yuasa, S., Oda, M., Egashira, T., Yae, K., Kusumoto, D., ... Fukuda, K. (2010). Generation of induced pluripotent stem cells from human terminally differentiated circulating T cells. *Cell Stem Cell*, *7*, 11–14. doi: 10.1016/j.stem.2010.06.003.

Shi, Y., Inoue, H., Wu, J. C., & Yamanaka, S. (2016). Induced pluripotent stem cell technology: A decade of progress. *Nature Reviews Drug Discovery*, *16*, 115–130. doi: 10.1038/nrd.2016.245.

Somers, A., Jean, J.-C., Sommer, C. A., Omari, A., Ford, C. C., Mills, J. A., ... Kotton, D. N. (2010). Generation of transgene-free lung disease-specific human induced pluripotent stem cells using a single excisable lentiviral stem cell cassette. *Stem Cells*, *28*, 1728–1740. doi: 10.1002/stem.495.

Sommer, A. G., Rozelle, S. S., Sullivan, S., Mills, J. A., Park, S.-M., Smith, B. W., ... Mostoslavsky, G. (2012). Generation of human induced pluripotent stem cells from peripheral blood using the STEMCCA lentiviral vector. *Journal of Visualized Experiments*, 1–5. doi: 10.3791/4327.

Sommer, C. A., Gianotti Sommer, A., Longmire, T. A., Christodoulou, C., Thomas, D. D., Gostissa, M., ... Mostoslavsky, G. (2010). Excision of reprogramming transgenes improves the differentiation potential of iPS cells generated with a single excisable vector. *Stem Cells*, *28*, 64–74. doi: 10.1002/stem.255.

Sommer, C. A., Stadtfeld, M., Murphy, G. J., Hochedlinger, K., Kotton, D. N., & Mostoslavsky, G. (2009). Induced pluripotent stem cell generation using a single lentiviral stem cell cassette. *Stem Cells*, *27*, 543–549. doi: 10.1634/stemcells.2008-1075.

Stadtfeld, M., Brennand, K., & Hochedlinger, K. (2008a). Reprogramming of pancreatic β cells into induced pluripotent stem cells. *Current Biology*, *18*, 890–894. doi: 10.1016/j.cub.2008.05.010.

Stadtfeld, M., Nagaya, M., Utikal, J., Weir, G., & Hochedlinger, K. (2008b). Induced pluripotent stem cells generated without viral integration. *Science*, *322*, 945–949. doi: 10.1126/science.1162494.

Staerk, J., Dawlaty, M. M., Gao, Q., Maetzel, D., Hanna, J., Sommer, C. A., ... Jaenisch, R. (2010). Reprogramming of human peripheral blood cells to induced pluripotent stem cells. *Cell Stem Cell*, *7*, 20–24. doi: 10.1016/j.stem.2010.06.002.

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., & Yamanaka, S. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell*, *131*, 861–872. doi: 10.1016/j.cell.2007.11.019.

Warren, L., Manos, P. D., Ahfeldt, T., Loh, Y.-H., Li, H., Lau, F., ... Rossi, D. J. (2010). Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. *Cell Stem Cell*, *7*, 618–630. doi: 10.1016/j.stem.2010.08.012.

Woltjen, K., Michael, I. P., Mohseni, P., Desai, R., Mileikovsky, M., Hämäläinen, R., ... Nagy, A. (2009). piggyBac transposition reprograms fibroblasts to induced pluripotent stem cells. *Nature*, *458*, 766–770. doi: 10.1038/nature07863.

Yang, W., Mills, J. A., Sullivan, S., Liu, Y., French, D. L., & Gadue, P. (2012). iPSC reprogramming from human peripheral blood using Sendai virus mediated gene transfer. In *StemBook*. Cambridge, MA: Harvard Stem Cell Institute. doi/10.3824/stembook.1.73.1.

Yu, J., Vodyanik, M. A., Smuga-Otto, K., Antosiewicz-Bourget, J., Frane, J. L., Tian, S., ... Thomson, J. A. (2007). Induced pluripotent stem cell lines derived from human somatic cells. *Science*, *318*, 1917–1920. doi: 10.1126/science.1151526.