ProMab Biotechnologies, Inc.

Protocol

Generation of Human Induced Pluripotent Stem Cells (iPSCs) from Fibroblasts Using Episomal Vectors

OVERVIEW

Induced pluripotent stem cells (iPSCs) are genetically reprogrammed from adult cells, which are similar to natural pluripotent stem cells, such as embryonic stem cells (ESCs). iPSCs exhibit a pluripotent stem cell-like state, such as the expression of certain stem cell genes and proteins, chromatin methylation patterns, doubling time, teratoma formation, and potency and differentiability. While these artificially generated cells are not known to exist in the human body, they show qualities remarkably like those of embryonic stem cells. Therefore, iPSCs are an invaluable resource for drug discovery, cell therapy, and basic research.

Human iPSCs were first generated in 2007 through retrovirus- or lentivirus-mediated gene transduction. However, both viral vectors require integration into host chromosomes to express reprogramming genes. Integration-free human iPSCs have been generated using several methods, including adenovirus, Sendai virus, the piggyBac system, minicircle vector, episomal vectors, direct protein delivery and synthesized mRNA. The reprogramming efficiency of these integration-free methods is impractically low in most cases. Direct delivery of proteins or RNA is labor-intensive, requiring repeated delivery of the reprogramming factors. Modifying Sendai virus vectors or preparing synthesized mRNAs are technically demanding.

The Human iPS Cell Reprogramming Episomal Kit is an optimized mixture of multiple vectors that can reprogram somatic cells to iPSCs without integration. The episomal vectors have the oriP/EBNA-1 (Epstein-Barr nuclear antigen-1) backbone that delivers the reprogramming factors as well as puromycin resistance gene. This system has been successfully demonstrated in the reprogramming of fibroblasts, as well as other adult cells. High expression of transgenes due to oriP/EBNA-1 mediated nuclear import and retention of vector DNA allows iPSC derivation from a single transfection.

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The reprogramming efficiency is further enhanced by puromycin selection. In addition, silencing of the viral promoter which drives EBNA-1 expression and the loss of episomes at a rate of ~5% per cell cycle due to partitioning and defects in vector synthesis allows the removal of episomal vectors from the iPSCs without any additional manipulation. For optimal reprogramming with the Human iPS Cell Reprogramming Episomal Kit, culture the fibroblasts in Fibroblast Medium until the day of transfection. After transfection, allow the cells to recover in Fibroblast Medium for 24 hours, then add puromycin to remove the untransfected cells. Reseed the cells on feeders about 5 day of post transfection, and switch the culture medium to hESC culture medium the following day. This reprogramming protocol is very simple yet highly effective. Small molecules, such as PD0325901, CHIR99021, A-83-01, hLIF, and HA-100 are not required for reprogramming.

PROCEDURE FOR REPROGRAMMING

The following protocol has been optimized for human dermal fibroblast cells. We recommend that you optimize the protocol for your cell type.

1. Feeder-Free Reprogramming Protocol

Materials Needed

Human iPS Cell Reprogramming Episomal Vectors (cat. # RF202_1): 25 µl (1 µg/µl)
Episomal RFP control vector (cat. # RF202_2): 10µl (0.5µg/µl)
Neon Transfection Devices and kits (Invitrogen; 10 µl sets)
iPSGen Medium (StemRD)
PSGro Medium (StemRD)

Preparation of Fibroblast Medium with pen/strep
DMEM containing 10% FBS, 2mM GlutaMax, and 50 U and 50 µg/ml penicillin and streptomycin.

Preparation of Fibroblast Medium without pen/strep DMEM containing 10% FBS, 2mM GlutaMax.

Preparation of PSGro/mTeSR1 Medium
1:1 mix PSGro medium (StemRD) and mTeSR1 (Stemcell Technologies), and add penicillin and streptomycin to final concentration of 50 U and 50 µg/ ml, respectively.

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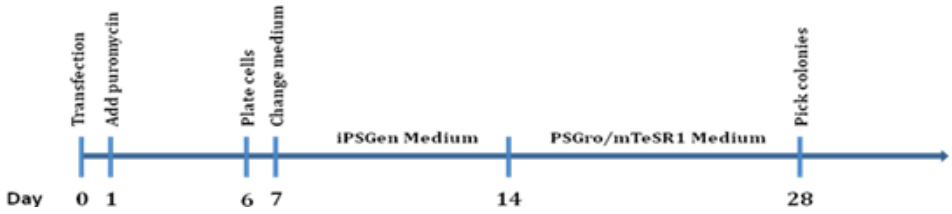
Coating plates with Matrigel

Matrigel (Cat.no. 354277, BD) should be aliquoted and stored at -80°C for long-term use.

1. Thaw matrigel on ice until liquid. Dilute Matrigel 1:50 with pre-chilled KO DMEM/F12.
2. Immediately use the diluted Matrigel solution to coat tissue culture-treated plates. For a 6-well plate, use 0.8 ml of diluted matrigel solution per well, and swirl the plate to spread the Matrigel solution evenly across the surface.
3. Let the coated plate stand for 1 - 2 hrs at 37°C or overnight at 4°C. If plate has been stored at 4°C, allow the plate to incubate at 37°C for at least 30 minutes before removing the Matrigel solution.

Procedure



Day-1: Seed Cells

1. Seed human fibroblasts at 1 x 10^6 cells in a gelatin coated T75 flask. Cells should reach approximately 75–90% confluent on the day of transfection (Day 0).

Day 0: Nucleofection of Episomal Plasmids to Human Fibroblasts

2. Prepare the Neon Transfection Devices and kits (using 10-µl tips in this protocol).
3. Prepare the gelatin-coated 6 well plate and warm up Fibroblast Medium (w/o P/S) in the plate.
4. Aspirate the medium from the fibroblasts in the T75 flask, and wash the cells in DPBS without calcium and magnesium. Add 2 ml of 0.05% Trypsin/EDTA to the flask, and incubate the flask at 37°C for 3 minutes.
5. Add 5 ml of Fibroblast Medium to the flask. Tap the flask to ensure the cells are dislodged from the flask, and carefully transfer the cells into a new 15-ml conical tube. Spin down the cells at 1,000 rpm for 5 minutes at room temperature.
6. Resuspend the cells in 2 ml of DPBS. Count the cells, and take 3 x 10^5 cells into each 1.5 ml tube for two tubes.

Note: You need 3 x 10^5 cells for one transfection, and the cell number can be modified to 1.5 – 6 x 10^5 cells per transfection.

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7. Spin down the cells at 2000 rpm for 5 min. In the meantime, add 3ml of solution E2 to the microporation tube. Mix 1.5 µg of reprogramming vectors in one 1.5 ml tube with 10 µl Solution R. In another tube, mix 0.5 µg for RFP control with 10 µl Solution R.

Note: If you use 100 µl tip, mix 3 µg of reprogramming vectors in 100 µl Solution R and 1 µg for RFP control in 100 µl Solution R, respectively.

8. Carefully aspirate most of the supernatant. Resuspend the cell pellet in Solution R with plasmids.

9. Turn on the Neon unit and enter the electroporation parameters in the Input window to 1,650 Volts of pulse voltage, 10 ms of pulse width, and 3 pulses.

Note: To increase the viability, you may use the electroporation parameters of 1,400 Volts of pulse voltage, 30 ms of pulse width, and 1 pulse.

10. Press the push-button on the Neon® Pipette to the first stop and immerse the Neon® Tip into the cell-DNA mixture. Slowly release the push-button on the pipette to aspirate the cell-DNA mixture into the Neon® Tip.

Note: Avoid air bubbles when pipetting to avoid arcing during electroporation. If you notice air bubbles in the tip, discard the sample and carefully aspirate fresh sample into the tip again without any air bubbles.

11. Insert the Neon® Pipette with the sample vertically into the Neon® Tube placed in the Neon® Pipette Station until you hear a click. Ensure that you have entered the appropriate electroporation parameters and press Start on the Neon® touchscreen to deliver the electric pulse.

12. Immediately after electroporation, the cell suspension solution is poured into warm Fibroblast Medium w/o P/S) in one well of a 6-well plate pre-coated with gelatin. Culture the electroporated cells in 37 °C, 5% CO_2 incubator.

Day 1, 3, 5: Change the Fibroblast Medium

13. Change medium to Fibroblast Medium with P/S, supplemented with 0.5g/ml of puromycin. **Note:** If RFP plasmid is used to monitor the transfection efficiency, the RFP expression can be detected during these days. By adding puromycin, non puro-resistant cells dislodge.

Note: Do NOT treat transfected cells with puromycin for more than three days.

Day 6: Replating the Transfected Cells

14. Aspirate the medium from the well with transfected fibroblasts, and wash the cells in DPBS without calcium and magnesium. Add 0.5 ml of 0.05% Trypsin/EDTA to the well, and incubate the plate at 37°C for 2 minutes.

15. Add 1 ml of Fibroblast Medium to the well. Tap the plate to ensure cells are dislodged from the plate, and carefully transfer cells to a new 15-ml conical tube. Spin down cells at 1,000 rpm for 5 minutes at room temperature.

16. Resuspend the cells in 2 of ml Fibroblast Medium. Count the cells. Seed 1 x 10^4, 2 x 10^4, and 4 x 10^4 transfected fibroblasts, respectively, in the wells of a 6-well plate pre-coated Matrigel.

Note: The cell numbers can be changed from 0.5 - 3 x 105 cells when using a 100-mm dish. The seeding day can vary according to the cells.

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Day 7: Switch to iPSGen Medium

17. Aspirate the medium from the reprogramming fibroblasts in the well, and add 2 ml of iPSGen Medium (StemRD).

Note: You may use N2B27 medium supplemented with 100 ng/ml of bFGF to replace iPSGen Medium. However, it may have some side effect.

18. Change the medium everyday up to day 14.

Day 14: Switch to PSGro/mTeSR1 Medium

19. Aspirate the medium from the reprogrammed fibroblasts, and add 2 ml of PSGro/mTeSR1 medium.
20. Change PSGro/mTeSR1 medium every day.

Day 24 – 30: Picking iPS-like Colonies

By Day 24 of post-transfection, the cell colonies in the Matrigel-coated plate consist of iPSCs, which exhibit a hESC-like morphology characterized by a flatter, cobblestone-like appearance with individual cells clearly demarcated from each other in the colonies.

21. Examine the culture plate containing the reprogrammed cells under 10X magnification of an inverted microscope, and mark the colony to be picked at the bottom of the culture dish.

Note: We recommend picking at least 10 distinct colonies by the end of each reprogramming experiment and expanding them in separate 24-well Matrigel-coated plate.

22. Transfer the culture dish to a biosafety cabinet equipped with a stereomicroscope.
23. Cut the colony to be picked into 5–6 pieces in a grid-like pattern using a 25-gauge 1½ inch needle.
24. Using a 200 µl pipette, transfer the cut pieces to a Matrigel-coated well of a 24-well plate containing mTeSR1 medium supplemented with 10 µM ROCK inhibitor (Y-27632, StemRD).
25. Culture the picked colonies in a 37°C, 5% CO2 incubator.
26. Allow the colonies to attach to the culture plate for 48 hours before replacing the spent medium with fresh mTeSR1 medium. Since then, change the medium every day.
27. Culture the reprogrammed colonies like normal human iPSC colonies; expand and maintain them using standard culture procedures.

Note: Newly derived iPSC lines may contain a fair amount of differentiation through passage 4. It is not necessary to remove differentiated material prior to passaging. By propagating the cells the overall culture health should improve throughout the early passages. Otherwise, pick up the iPS-like colonies and culture in Matrigel coated 24-well plate.

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II. **Reprogramming Protocol Using MEF Feeders**

Materials Needed

Human iPS Cell Reprogramming Episomal Vectors 25l (1 g/ l) Episomal RFP control vector 10 l (0.5g/l) Neon Transfection Devices and kits (Invitrogen; 10 µl sets) Feeder cells (mitomycin-c treated-SNL or MEF cells)

Preparation of Fibroblast Medium with pen/strep

DMEM containing 10% FBS, 2mM GlutaMax, and 50 U and 50 µg/ ml penicillin and streptomycin. Preparation of Fibroblast Medium without pen/strep DMEM containing 10% FBS, 2mM GlutaMax.

Preparation of human ES Medium

Knockout DMEM/F12 containing 20% knockout serum replacement, 2mM glutamine, 0.1 mM nonessential amino acids, 0.1 mM 2-mercaptoethanol, 10 ng/ml bFGF, and 50 U and 50 µg/ ml penicillin and streptomycin.

Preparation of hES/PSGro Medium

1:1 mix PSGro Medium (StemRD) and human ESC Medium, and add penicillin and streptomycin to final concentration of 50 U and 50 µg/ ml, respectively.

Preparation of hES/PSGro Medium

Preparation of mitomycin-C treated MEF or SNL feeders

MEF cells can be treated with mitomycin-C when they reach confluency, to halt the division of cells.

1. Add 6 ml of fresh MEF (or SNL) medium contain 50 µl of mitomycin-C solution (1 mg/ ml) to one T75 flask of confluent MEF (or SNL) cells, and swirl it briefly. The final concentration of mitomycin-C is 8 µg/ ml.
2. Incubate at 37°C for at least 3 hrs.
3. Aspirate the mitomycin-C containing medium off the cells and wash the cells twice with 10 ml PBS.
4. Aspirate PBS and add 2 ml of 0.25% trypsin-EDTA, swirl to cover the entire and incubate for 2 min at room temperature.
5. Add 5 ml Fibroblast Medium and break up the cells to single-cell suspension by pip and down. Count the number of cells.
6. Seed the cells on gelatin-coated dishes (3 x 10^6 cells per 100-mm dish, or 5 x 105 well of a 6-well plate).
7. Cells should be ready to use on the next day.

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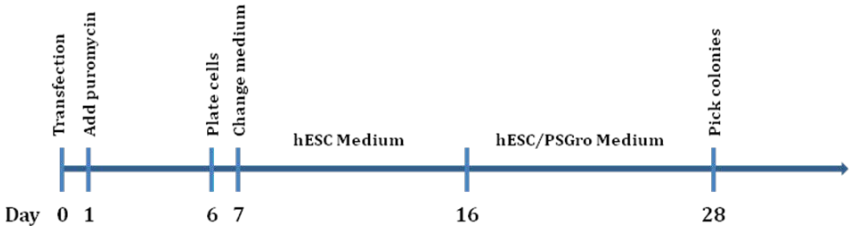
Procedure



Day -1: Seed Cells

1. Seed human fibroblasts at 1 x 106 cells in a gelatin coated T75 flask. Cells should reach approximately 75–90% confluence on the day of transfection (Day 0).

Day 0: Nucleofection of Episomal Plasmids to Human Fibroblasts

2. Prepare the Neon Transfection Devices and kits.
3. Prepare the gelatin-coated 6 well plates and warm up Fibroblast Medium (w/o P/S) in the plates.
4. Aspirate the medium from the fibroblasts in the T75 flask, and wash the cells with DPBS without calcium and magnesium. Add 2 ml of 0.05% Trypsin/EDTA to the flask, and incubate the flask at 37°C for 3 minutes.
5. Add 5 ml of Fibroblast Medium to the flask. Tap the flask to ensure the cells are dislodged from the flask, and carefully transfer the cells into a new 15-ml conical tube. Spin down the cells at 1,000 rpm for 5 minutes at room temperature.
6. Resuspend the cells in 2 ml of DPBS. Count the cells, and take 3 x 10^5 cells into each 1.5 ml tube for two tubes.

Note: You need 3 x 105 cells for one transfection, and the cell number can be modified to 1.5 – 6 x 10^5 cells per transfection.

7. Spin down the cells at 2000 rpm for 5 minutes. In the meantime, add 3ml of solution E2 to the microporation tube. Mix 1.5 µg of reprogramming vectors in one 1.5 ml tube with 10 µl Solution R. In another tube, mix 0.5 µg for RFP control with 10 µl Solution R.

Note: If you use 100 µl tip, mix 3 µg of reprogramming vectors in 100 µl Solution R and 1 µg for RFP control in 100 µl Solution R, respectively.

8. Carefully aspirate most of the supernatant, and resuspend the cell pellet in Solution R with plasmids.
9. Turn on the Neon unit and enter the electroporation parameters in the Input window to 1,650 Volts of pulse voltage, 10 ms of pulse width, and 3 pulses.

Note: To increase the viability, you may use the electroporation parameters of 1,400 Volts of pulse voltage, 30 ms of pulse width, and 1 pulse.

10. Press the push-button on the Neon® Pipette to the first stop and immerse the Neon® Tip into the

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cell-DNA mixture. Slowly release the push-button on the pipette to aspirate the cell-DNA mixture into the Neon® Tip.

Note: Avoid air bubbles when pipetting to avoid arcing during electroporation. If you notice air bubbles in the tip, discard the sample and carefully aspirate fresh sample into the tip again without any air bubbles.

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12. Immediately after electroporation, the cell suspension solution is poured into warm Fibroblast Medium (w/o P/S) in a 6-well plate pre-coated with gelatin. Culture the electroporated cells in 37 ° C, 5% CO2 incubator.

Day 1, 3, 5: Change the Fibroblast Medium

13. Change medium to Fibroblast Medium with P/S, supplemented with 0.5g/ml of puromycin.

Note: If RFP plasmid is used to monitor the transfection efficiency, the RFP expression can be detected during these days. By adding puromycin, non puro-resistant cells dislodge.

14. On day 5, seed the MEF/SNL cells in a gelatin-coated plate or dish (5 x 105 cells per well of a 6-well plate or 3 x 106 cells per 100-mm dish).

Day 6: Replating the Transfected Cells

15. Aspirate the medium from the transfected fibroblasts in the well, and wash the cells in DPBS without calcium and magnesium. Add 0.5 ml of 0.05% Trypsin/EDTA to the well, and incubate the plate at 37°C for 2 minutes.
16. Add 1 ml of Fibroblast Medium to the well. Tap the plate to ensure the cells have been dislodged from the plate, and carefully transfer the cells into an empty 15-ml conical tube. Spin down the cells at 1,000 rpm for 5 minutes at room temperature.
17. Resuspend the cells in 2 ml of Fibroblast Medium. Count the cells. Seed 1 x 10^4, 2 x 10^4, and 4 x 10^4 transfected fibroblasts, respectively, onto wells of a 6-well plate pre-seeded MEF/SNL feeder cells.

Note: The cell numbers can be changed from 5 x 10^4 to 3 x 10^5 cells when using a 100-mm dish. The seeding day can vary according to the cell types.

Day 7: Switch to hES Medium

18. Aspirate the medium from the fibroblasts in the well, and add 2 ml of hES Medium supplemented with 10 ng/ml of bFGF.
19. Change the medium everyday up to day 15.

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Day 16: Switch to hES/PSGro Medium

20. Aspirate the medium from the fibroblasts in the well, and add 2 ml of hES/PSGro Medium (StemRD).

Note: You may also use mTeSR1 (Stemcell Technologies) or Essential 8 Medium (Life Technologies) to replace PSGro medium.

21. Change hES/PSGro Medium every day.

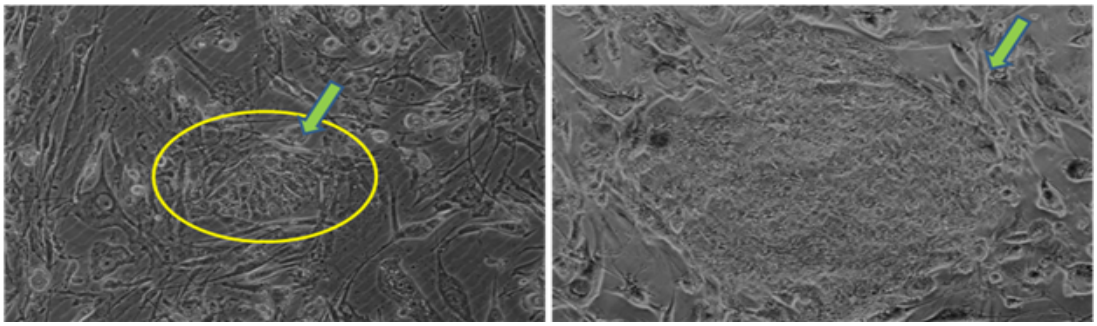

Figure 2. Expected morphology of emerging iPSCs during episomal reprogramming of human dermal fibroblasts as they undergo morphological changes and iPSC colonies begin to emerge.

Day 21 – 30: Picking iPS---like Colonies

By Day 21 of post-transfection, the cell colonies in the MEF plate consist of iPSCs, which exhibit a hESC-like morphology characterized by a flatter, cobblestone-like appearance with individual cells clearly demarcated from each other in the colonies (see Figure 2).

22. Examine the culture plate containing the reprogrammed cells under 10X magnification of an inverted microscope, and mark the colony to be picked at the bottom of the culture dish.

Note: We recommend picking at least 10 distinct colonies by the end of each reprogramming experiment and expanding them in separate 24-well Matrigel-coated plates.

23. Transfer the culture plate to a biosafety cabinet equipped with a stereomicroscope.
24. Cut the colony to be picked into 5–6 pieces in a grid-like pattern using a 25-gauge 1½ inch needle.
25. Using a 200-µL pipette, transfer the cut pieces to a well of a 24-well plate pre-seeded MEF/SNL feeder cells.

Note: If you want to culture the iPSCs in feeder-free conditions, transfer the cut pieces to a Matrigel-coated well of a 24-well plate containing mTeSR1 medium supplemented with 10 µM ROCK inhibitor (Y-27632, StemRD).

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23. Incubate the picked colonies in a 37°C, 5% CO2 incubator.
24. Allow the colonies to attach to the culture plate for 48 hours before replacing the spent medium with fresh hES medium. After that, change the medium every day.
25. Treat the reprogrammed colonies like normal human iPSC colonies; expand and maintain them using standard culture procedures.

Note: Newly derived iPSC lines may contain a fair amount of differentiation through passage 4. It is not necessary to remove differentiated material prior to passaging. By propagating the cells the overall culture health should improve throughout the early passages. Otherwise, pick the iPS-like colonies and culture in 24-well plate with feeder cells.