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Reference Transcriptome for Deriving Marmoset Induced Pluripotent Stem Cells

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Abstract

Limited access to primary tissue from various nonhuman primate (NHP) species represents a significant unmet need that hampers progress in understanding unique cellular diversity and gene regulation of specific tissues and organs in stem cell translational research. Most comparative biology studies have been limited to using postmortem tissue usually frozen specimens with limited utility for research. The generation of induced pluripotent stem cell (iPSC) lines from somatic cells, such as adult skin or blood cells, offers an alternative to invasive and ethically controversial interventions for acquiring tissue. Pluripotent iPSCs have virtually an unlimited capacity to proliferate and differentiate into all cell types of the body. We are generating high-quality validated NHP iPSC lines to offer to scientific community and facilitate their research programs. We use the non-integrative episomal vector system to generate iPSCs from NHP skin biopsies. In this chapter we describe the validation of NHP iPSC lines by confirming pluripotency and their propensity to differentiate into all three germ layers ectoderm, mesoderm, and endoderm according to established standards and measurable limits for a set of marker genes incorporated into a scorecard.

Keywords

Marmoset iPSCs; Pluripotency; Scorecard; iPSC characterization; Nonhuman primate iPSCs; Regenerative medicine

1 Introduction

Regenerative medicine focuses on creating vital functional cells to repair or replace tissue or organs damaged due to disease, injury, or congenital defects. This approach may provide treatments for currently intractable diseases. The burgeoning of this medical approach has been galvanized by the potential of pluripotent stem cells (iPSCs). The possibility to generate specialized organ-specific differentiated cells using human somatic cells induced to become iPSCs opens the door for individualized cell therapy. Individualized autologous or allogeneic cell therapy would be ideal for treating various diseases or injuries such as heart attacks, diabetes, stroke, neurodegenerative diseases, and others. Furthermore, current limited access to primary tissue from various parts of the body represents a significant unmet

⁵It is recommended to use marmoset ESCs as positive control.

Disclosures: Dr. Marcel M. Daadi is founder of the biotech company NeoNeuron.

need that hampers progress in understanding the unique cellular diversity and gene regulation of specific tissues and organs and in stem cell translational research. Most comparative studies have been limited to using postmortem tissue usually frozen, specimens with limited utility for research. Pluripotent iPSCs have virtually an unlimited capacity to proliferate and differentiate into all cell types of the body. Thus, the generation of iPSC lines will obviate the need for invasive interventions to acquire primary tissue and for the use of frozen tissues.

Animal models have historically made a significant contribution to our understanding of human diseases. However, disparities between results in animal studies and clinical trials have been identified, including failure to acknowledge the limitations of animal species and disease models [1]. Measuring the therapeutic potential of stem cell lines through testing in small and large animal models may predict the safety and efficacy of treatment strategies for clinical trials. NHP provide important disease models for translational regenerative medicine [2]. Given the similarities to humans, in anatomical structure, physiology, and pathology, NHP are also relevant in comparative biology and medicine. The creation of a resource of standardized, high-quality and validated nonhuman primates (NHP) iPSC lines will offer access to efficient and reliable model systems of all cell types of the body. This resource facilitates research and development in cell therapy, disease modeling, drug screening, and comparative functional genomics and medicine. Here, we describe methods and standards we use in our lab to derive defined pluripotent NHP iPSCs.

2 Materials

2.1 Equipment

1. Cell culture incubator (Nuaire, Plymouth, MN, USA).
2. Phase contrast microscope (Zeiss, Oberkochen, Germany).
3. StepOnePlus (Applied Biosystems, Waltham, MA, USA).
4. TaqMan hPSC Scorecard Kit, Fast 96-well (Applied Biosystems, Waltham, MA, USA).
5. MicroAmp Optical Adhesive Film (Applied Biosystems, Waltham, MA, USA).
6. Centrifuge (Eppendorf, Hamburg, Germany).
7. 6-, 24-, 48-, and 96-well cell culture plates (Corning, Oneonta, NY, USA).
8. Corning 6-well Clear Flat Bottom Ultra Low Attachment Multiple Well Plates (Corning, Oneonta, NY, USA).
9. 60 mm petri dish (BD biosciences, Franklin Lakes, NJ, USA).
10. Cell lifter (Fisher scientific, Pittsburgh, PA, USA).
11. Glass pipettes (Fisher scientific, Pittsburgh, PA, USA).
12. Centrifuge tubes (15 mL and 50 mL) (Corning, Oneonta, NY, USA).
13. Syringe filters (Corning, Oneonta, NY, USA).

14. Syringes (10, 20 and 60 mL) (BD, Franklin Lakes, NJ, USA).
15. Pipettes (2–25 mL) (Fisher scientific, Pittsburgh, PA, USA).
16. Pipette aids (10–1000 µL) (Eppendorf, Hamburg, Germany).
17. Pipette tips (10–1000 µL) (Accuflo, E&K Scientific, Santa Clara, CA, USA).
18. Water bath (Fisher scientific, Pittsburgh, PA, USA).
19. Hemocytometer (Hausser Scientific, Horsham, PA, USA) or automated cell counter (Countess, Invitrogen, Carlsbad, CA, USA).

2.2 Kits and Reagents

1. Episomal plasmids (pCXLE-hOCT^{3/4}-shp53-F, pCXLE-hSK, pCXLE-hUL, and pCXWB-EBNA1, Addgene, Cambridge, MA, USA).
2. NHDF Nucleofector Kit (Lonza, Walkersville, MD, USA).
3. Amaxa Nucleofector device 2b (Lonza, Walkersville, MD, USA).
4. Mycoplasma PCR detection kit (Sigma-Aldrich, St. Louis, MO, USA).
5. DMEM (Gibco, Life Technologies, NY, USA).
6. DMDM/F12 (Gibco, Life Technologies, NY, USA).
7. RNeasy Plus Mini kit (QIAGEN, Germantown, MD, USA).
8. Irradiated mouse embryonic fibroblasts.
9. KnockOut Serum Replacement (Gibco, Life Technologies, NY, USA).
10. L-Glutamine (Gibco, Life Technologies, NY, USA).
11. MEM Non-Essential Amino Acids Solution (Gibco, Life Technologies, NY, USA).
12. Penicillin-streptomycin (Gibco, Life Technologies, NY, USA).
13. β-Mercaptoethanol (Gibco, Life Technologies, NY, USA).
14. Basic fibroblast growth factor (Stemgent, Cambridge, MA, USA).
15. Fetal bovine serum (FBS, GE, Logan, Utah, USA).
16. Gelatin (Sigma-Aldrich, St. Louis, MO, USA).
17. Collagenase Type IV solution (Sigma-Aldrich, St. Louis, MO, USA).
18. Trypsin (Gibco, Life Technologies, NY, USA).
19. Trypsin neutralizer (Gibco, Life Technologies, NY, USA).
20. TaqMan hPSC Scorecard Kit, Fast 96-well (Applied Biosystems, Waltham, MA, USA).
21. SuperScript IV first-strand synthesis system (Invitrogen, Thermo Fisher, Waltham, MA, USA).

22. TaqMan Fast Advanced Master Mix (Applied Biosystems, Waltham, MA, USA).
23. Tris base (Fisher Bioreagents, Pittsburgh, PA, USA).
24. Phosphate buffered saline (PBS, Gibco, Life Technologies, NY, USA).
25. Double distilled water (Gibco, Life Technologies, NY, USA).
26. Ethanol (Fisher Bioreagents, Pittsburgh, PA, USA).

3 Methods

3.1 Preparation of Reagents and Media: Prepare All the Reagents Under Sterile Conditions in a Horizontal Laminar Flow Hood

1. *Preparation of 0.1% gelatin*: Dissolve 0.5 g gelatin in 500 mL ddH₂O. Autoclave (20 min at 120 °C) and store at room temperature under sterile conditions.
2. *Skin biopsy media (50 mL)*: Mix 40 mL of DMEM, 10 mL of fetal bovine serum (20%), and penicillin/streptomycin (1%). Filter sterilize and store at 4 °C.
3. *Fibroblast media (50 mL)*: Mix 45 mL of DMEM, 5 mL of fetal bovine serum (10%). Filter (2 µm) sterilize and store at 4 °C.
4. *Preparation of 10 mM Tris (25 mL)*: Dissolve 30.35 milligrams of Tris base (F.Wt, 121.4) in 15 mL of double-distilled water and adjust the pH to 7.6. Increase the volume to 25 mL and filter sterilize. Store at 4 °C.
5. *Preparation of basic fibroblast growth factor (bFGF) stock solution*: Briefly centrifuge the tube and reconstitute the bFGF (50 µg) in 2.5 mL of 10 mM Tris solution (pH 7.6) to prepare a 20 µg/mL stock solution. Aliquot and store at −20 °C.
6. *Preparation of human ESC (hESC) media (250 mL)*: Mix 196.25 mL of DMDM/F12, 50 mL of KnockOut Serum Replacement (20%), 2.5 mL of 10 mM MEM Non-Essential Amino Acids Solution, 1.25 mL of L-glutamine with 1.75 µL of β-mercaptoethanol, and 125 µL of 20 µg/mL bFGF (final 10 ng/mL). Filter sterilize and store at 4 °C.
7. *Preparation of Embryoid Body (EB) media (50 mL)*: Mix 40 mL of DMEM/F12, 10 mL KnockOut Serum Replacement (20%), 1 mM MEM Non-Essential Amino Acids Solution, and 55 µM β-mercaptoethanol. Filter sterilize and store at 4 °C.
8. *Preparation of Collagenase Type IV solution*: Dissolve 1 mg to 1 mL of ddH₂O and and filter sterilize. Aliquot and store at −20 °C.

3.2 Generation of Marmoset iPS Cells (CJ-iPSCs) from Skin Biopsy (See Note 1) (Fig. 1)

1. Coat the 6-well cell culture plate with 0.1% gelatin solution (1 mL per well) and incubate in 37 °C cell culture incubator for a minimum of 30 min.

2. Select 4- to 8-year-old healthy marmosets (*Callithrix Jacchus*) and take skin biopsies (4 to 6 mm) from a preferably hairless regions like abdomen or underarm (see Note 2).
3. Transport the skin biopsies in sterile PBS containing 1% penicillin and streptomycin to the cell culture facility.
The following steps are performed in the cell culture facility in a horizontal laminar flow hood under sterile conditions.
4. Using a sterile surgical blade, mince each skin piece into 1 mm or less pieces in a 60 mm petri dish.
5. Aspirate the gelatin solution from the culture plate using vacuum suction.
6. Using sterile forceps, carefully transfer the tissue pieces into the gelatin-coated wells. Typically 4–5 pieces of minced skin tissue can be placed into a single well of 6-well culture plate.
7. Carefully add 0.8 mL of skin biopsy media along the wall of the well without displacing the tissue pieces.
8. Incubate the plate at 37 °C incubator for 2 days. Do not move the culture plates until the biopsy pieces have attached to the bottom of wells.
9. After 2 days, add 0.2 mL of fresh media to the wells without removing the old media. Repeat the step every 2 days.
10. After 1 week, fibroblasts can be seen growing from the biopsy tissue.
11. Change media regularly until the fibroblasts become confluent.
12. When the fibroblasts become confluent, expand the cells to a T-25 cell culture flask.
13. Perform mycoplasma test using Hoechst 33258 DNA staining and mycoplasma PCR detection Kit.
14. Expand the fibroblasts to 2–5 passages for freezing stocks and for reprogramming.
15. The day before transfection, coat a 10 cm cell culture dish with gelatin and plate irradiated mouse embryonic fibroblasts (MEF) feeder with fibroblast media.
16. On the day of transfection, change the media of irradiated MEF feeder with fresh fibroblast media.

All the following steps should be performed on a bench that has been cleaned and sterilized with 70% ethanol.

¹-All nonhuman primate procedures should be authorized by local and regional governmental authorities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC international). All procedures involving animals should be performed in compliance with the guide for care and use of laboratory animals regulated by the Office of Laboratory Animal Welfare (OLAW) at the National Institutes of Health. The experimental procedures should be approved by an Institutional Animal Care and Use Committee (IACUC).

²-The skin biopsies should be taken by the veterinary staff according to standard operating procedures (SOP).

17. Set up NHDF Nucleofector kit on a clean bench (*see* Note 3).
18. Mix 82 μ L of NHDF Nucleofector solution with 18 μ L of supplement and add 3 μ g of human episomal plasmids (0.83 μ g of pCXLE-hOCT3/4-shp53-F, 0.83 μ g of pCXLE-hSK, 0.83 μ g of pCXLE-hUL, and 0.5 μ g of pCXWB-EBNA1).
19. Dissociate the marmoset fibroblasts with trypsin (passage number 3 to 6), and determine the cell density with cell counter or hemocytometer.
20. Prepare a cell suspension of 500,000 fibroblasts and centrifuge at 1000 rpm ($200 \times g$) for 5 min.
21. Remove the supernatant, and resuspend the cell pellet with Nucleofector solution-plasmids mixture.
22. Transfer cell-DNA mixture into a Nucleofector cuvette. Take care to prevent the formation of bubbles in the solution during transfer.
23. Load the cuvette into the Nucleofector device and start the program U-023.
24. After the completion of nucleofection, immediately transfer the cells onto irradiated MEF feeder and culture for 3 days.
25. On day 3 after transfection, replace the fibroblast media with fresh hESC media.
26. Replace old media with fresh hESC media every other day.
27. Culture the cells for 5 weeks or until hESC-like colonies emerge.
28. When the colonies emerge, prepare irradiated MEF feeder in a 24-well plate 1 day before colony picking.
29. On the day of colony picking, set up the microscope on a clean bench.
30. Change the media of irradiated MEF feeder with fresh hESC media.
31. Prepare a 96-well plate with 20 μ L of trypsin per well.
32. Using P10 pipettor, pick the colonies and transfer them to trypsin-containing wells (*see* Note 4).
33. Incubate the colony-loaded 96-well plate at 37 $^{\circ}$ C for 5 min.
34. Add 150 μ L of hESC media to each well and dissociate the colonies by pipetting 30 times.
35. Plate the dissociated cells on the irradiated MEF feeder in the 24-well plate.
36. Change the media daily with hESC media until the CJ-iPSCs become confluent.
37. Expand the CJ-iPSCs to 6-well plates to scale up and to prepare frozen stocks.

³.All reagents should be prepared 30 min in advance to allow them to reach ambient temperature for use.

⁴.The colonies should be picked within 20 min to prevent evaporation and drying of trypsin from the wells.

3.3 Embryoid Body (EB) Formation

1. Culture marmoset iPSCs (CJ-iPSCs) with irradiated MEF feeder layers in 6-well plate until they are confluent (Fig. 2).
2. Wash the cells with sterile DPBS and add 0.5 mL of collagenase type IV, and incubate at 37 °C for 5 min to remove the irradiated MEFs.
3. During incubation, check the cells every 2 min under the microscope to gauge the level of MEF detachment.
4. When the majority of the MEFs are detached (the marmoset iPSCs should not detach), proceed to the next step.
5. Aspirate collagenase-MEF supernatant and wash with sterile DPBS taking care not to detach marmoset iPSCs.
6. Add 1 mL of fresh hESC media, and detach all the marmoset iPSC colonies using a cell lifter.
7. Transfer the cell suspension into two 15 mL tubes and centrifuge at $200 \times g$ for 5 min.
8. Aspirate the supernatant carefully and store (short-term) the cells from one tube at -80°C for RNA isolation (undifferentiated iPSCs).
9. Add 2 mL of pre-warmed EB media to the other tube and gently resuspend the colonies while taking care not to dissociate them.
10. Transfer the cell suspension into a 6-well ultra-low attachment plate and culture for 7 days with media changed every other day with fresh EB media.
11. To change media, transfer the cell suspension into a 15 mL tube and allow the EBs to settle at the bottom of tube under gravity (for 5 min).
12. Carefully remove the supernatant and resuspend with fresh media.
13. On day 7, collect all the EBs by centrifugation ($200 \times g$ 5 min).

3.4 RNA Isolation Using RNeasy Plus Mini Kit (Fig. 3)

1. Thaw the frozen undifferentiated iPSCs stored at -80°C .
2. Prepare 1 mL of Buffer RLT and add 10 μL of β -mercaptoethanol.
3. Resuspend the CJ-iPSC pellet with 350 μL of RLT- β -mercaptoethanol mixture and triturate to lyse all the cells in the pellet.
4. Load the cell lysate into a gDNA eliminator spin columns and centrifuge at 13k rpm for 1 min and collect the flow-through.
5. Add 350 μL of 70% ethanol to the eluate and load the mixture into RNeasy spin column and centrifuge at 13k rpm for 1 min. Discard the flow-through.
6. Add 700 μL of RW1 and spin down 13k rpm for 1 min. Discard the flow-through.

7. Add 500 μL of RPE and spin down 13k rpm for 1 min. Discard the flow-through. Repeat this process.
8. Replace the column on the new 1.5 mL tube. Expel 30 μL of RNase-free water on the center of membrane in column. Hold for 1 min.
9. Centrifuge at 13k rpm for 1 min and elute the RNA.
10. Measure the concentration of RNA. Use 1 μg of RNA to determine RNA degradation by gel electrophoresis.

3.5 Complementary DNA Synthesis Using SuperScript IV First- Strand Synthesis System

1. In a PCR tube, dilute 1 μg of RNA in 11 μL of RNase-free water.
2. Combine 1 μL of 50 μM Oligo d(T)20 with 1 μL of 10 mM dNTP mix.
3. Place the mixture at 65 $^{\circ}\text{C}$ for 5 min followed by placement on ice for 1 min.
4. Add 4 μL of 5 $\times$ SSIV buffer, 1 μL of 100 mM DTT, 1 μL of ribonuclease inhibitor, and 1 μL of SuperScript IV Reverse Transcriptase.
5. Place the mixture at 50 $^{\circ}\text{C}$ for 10 min and then at 80 $^{\circ}\text{C}$ for 10 min for inactivation.

3.6 Scorecard Panel (Fig. 4)

1. Add ddH₂O to each cDNA sample to prepare a mixture with final volume 70 μL .
2. Add 70 μL of 2 $\times$ TaqMan Fast Advanced Master Mix.
3. Load 10 μL into each well of one row of TaqMan hPSC Scorecard Kit, Fast 96-well.
4. Seal the plate using MicroAmp Optical Adhesive Film and spin down at $600 \times g$ for 2 min.
5. Load in StepOnePlus and run in Fast Run mode.
6. Save the results as *eds* file.
7. Upload the files to the Thermo Fisher's TaqMan hPSC Score- card Panel and data analysis site. <https://www.thermofisher.com/us/en/home/life-science/stem-cell-research/taqman-hpsc-scorecard-panel/scorecard-software.html>.
8. Sign in with user ID and password.
9. Click "Create an analysis group" and label the folder.
10. Select "StepOnePlus System" under Instrument Type and "96 Wells Fast" under Block and then click "OK."
11. Click "Import Data" and load the eds files.
12. If done properly the message "Uploading to Thermo Fisher cloud successful" will appear.

13. Check the box on the left-hand side of each sample and then click “Plots” to compare expression, scores box plot, scores table, correlation, and assay QC.
14. The results can be downloaded as JPG by clicking “Download as JPG” located at the right upper corner of the screen.

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Fig. 1.

Process of generation, characterization, and validation of iPSC lines. Go/No-Go Workflow to generate, characterize and bank NHP iPSC lines. Adapted from [3]

Fig. 2. IPSC colonies derived by reprogramming skin fibroblast using the episomal approach. (a) Phase contrast photo showing example of two marmoset iPSC colonies. (b) Marmoset iPSC colonies express the pluripotent marker Nanog. Scale bar: 100 μ m

Fig. 3. Workflow of the scorecard: Representation of the steps leading to the pluripotency and differentiation standard tests—left the pluripotency and right the EB differentiation standard test. Adapted from [3]

Fig. 4. The scorecard test. (a) Scorecard assay pass for 7-day-old embryoid bodies (EBs) derived from marmoset iPSC line (CJO1). The EBs downregulated pluripotent self-renewal genes (self, green) as shown with the negative (−) sign and significantly upregulated ectoderm, mesoderm, and endoderm genes (Ecto, Meso, and Endo, respectively) as shown with the (+) sign. (b) Overview of Scorecard test of our marmoset iPSC line (CJO1) showing reference genes up- or downregulated. *CT* control, self-renewal = pluripotent, *ED* endoderm, *MS* mesoderm, *ME* mesoderm, *EC* ectoderm genes. The figure shows all the pluripotency genes downregulated (in blue) and the ME, EC, and ED genes upregulated. Adapted from [3]

Step	Temperature	Time (s)	Cycles
Hold	50 °C	20	1
Melt	95 °C	1	40
Anneal/extend	60 °C	20	