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Author manuscript
Methods Mol Biol. Author manuscript; available in PMC 2020 January 01.

Published in final edited form as:
Methods Mol Biol. 2019 ; 1576: 55–92. doi:10.1007/7651_2016_11.

Human Pluripotent Stem Cells (iPSC) Generation, Culture, and Differentiation to Lung Progenitor Cells

Mahboobe Ghaedi, **Laura E. Niklason**

Abstract

Induced pluripotent stem (iPS) cells are the product of adult somatic cell reprogramming to an embryonic-like state by inducing a "forced" expression of specific genes. They are similar to natural pluripotent stem cells, such as embryonic stem (ES) cells, in many aspects, such as the expression of certain stem cell genes and potency and differentiability. Human iPS cells are invaluable resource for basic research, cell therapy, drug discovery, and human organ tissue engineering. iPS cells can be derived from the patient to be treated and thus are genetically identical cells that may avoid immune rejection. The following protocols offer a general guideline for the induction of iPSCs from fibroblasts, and for culture and expansion to produce lung precursor cells.

Keywords

Human induced pluripotent stem cell; Reprogramming; Expansion; Cell culture; Differentiation; Progenitors

1 Introduction

Induced pluripotent stem cells (iPSCs) are typically derived by introducing a specific set of pluripotency-associated genes, or "reprogramming factors," into an adult cell type. These cells show qualities very similar to human embryonic stem cells. The original set of reprogramming factors (also called Yamanaka factors) are the genes Oct4 (Pou5f1), Sox2, cMyc, and Klf4. There are multiple methods to generate iPSCs, including retrovirus or lentivirus-mediated gene transduction and chemical induction. The retroviral vectors require integration into host chromosomes to express reprogramming genes, but DNA-based vectors and plasmid vectors do not generally integrate to the cell genome [6, 11]. To generate the iPSCs, each of the pluripotency factors can be also replaced by related transcription factors, miRNAs or small molecules [5, 14, 16]. iPSC generation is a slow and inefficient process. It takes 1–2 weeks for mouse cells and 3–4 weeks for human cells and the efficiencies are as low as 0.01–0.1 %. Recently, many advances have been made in improving the efficiency and the time it takes to derive iPSCs. After introduction of reprogramming factors, cells begin to form colonies very similar to human embryonic stem cells [1]. These iPSC colonies can be isolated based on their morphology, expression of pluripotent genes and surface markers and can be expanded in an appropriate culture system to keep pluripotency over

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several passages. The following protocols provide a general guideline for the induction of iPSCs from fibroblasts using an inducible lentiviral system.

Traditional human embryonic stem cells (hES cells) and induced pluripotent stem cells (hiPS cells) culture methods require the use of mouse or human fibroblast feeder layers [11, 16]. The feeder cell preparation requires significant time and effort, is labor-intensive and hard to scale. More recently, researchers have developed feeder free systems for both hES and hiPS cell culture. These systems include Matrigel, or other extracellular matrix (ECM) proteins such as vitronectin, to maintain hESCs and hiPSCs and the reduction or complete removal of serum from human stem cell culture [8]. To move toward feeder-free culture systems, researchers have also designed MTeSR™ and Essential 8™ medium. MTeSR™ 1 is the most widely published feeder-free cell culture medium for ES and iPS cells, with established protocols for applications ranging from derivation to differentiation. It has been used to successfully maintain hundreds of ES cell and iPS cell lines and has supported the main pluripotency genes expression in ES and iPS cells. Essential 8™ Medium is another xeno-free and feeder-free medium specially formulated for the growth and expansion of human pluripotent stem cells. Essential 8™ Medium has been extensively tested in multiple iPSC lines. In addition, both MTeSR™ and Essential 8™ Medium have been used to scale up production of iPSCs and have been shown to support iPSC growth for >50 passages without any signs of karyotypic abnormalities, along with maintaining the ability of iPSCs to differentiate into all three germ line lineages [9].

The lack of an abundant source of human lung epithelial cells is a major limitation for studying the lung disease phenotypes, drug screening and clinical application of these cells in respiratory disease. Recent advances in the stem cells field suggest that the use of induced pluripotent stem cells (iPSC) may be the most effective strategy to develop functional lung epithelial cells [3, 4, 7].

Embryonic lung arises from definitive endoderm (DE). Following developmental paradigms, directed differentiation of iPS cells to lung progenitors should proceed by generation of definitive endoderm, followed by patterning into anterior foregut endoderm (AFE). The AFE will then differentiate to lung progenitor cells and finally to lung alveolar and airway epithelial cells that cover the respiratory airways and alveoli. The following protocols offer a guideline for a stepwise differentiation of iPSC to human lung epithelial progenitors via definitive endoderm and anterior foregut endoderm (AFE) [4, 7, 12, 13, 15].

2 Materials

2.1 Feeder-Dependent iPSC Culture Protocol

Prepare all equipment, reagents, and solutions listed below:

2.1.1 Equipment and Supplies

1. Biosafety cabinet.
2. Centrifuge.
3. Microscope.

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4. 37 °C–5 % CO_2 incubator.
5. 37 °C water bath.
6. Cryogenic handling gloves and eye protection.
7. 6-well plates (Costar, 3516).
8. 5 mL sterile serological pipettes (Corning, 4487) and 10 mL sterile serological (Corning, 4488) pipettes or equivalent.
9. 2 mL aspirating pipette (Falcon, 357558).
10. 15 mL conical tube (Corning, 430791).
11. 50 mL conical tube (Corning, 430829).
12. 70 % ethanol (Dean lab Inc, 2701)

2.1.2 Reagents

1. DMEM/F-12 medium (Life Technologies, Gibco, 11330-032).
2. Knockout Serum Replacer (KOSR) (Invitrogen, 10828-028).
3. L-glutamine, non-animal, cell culture tested (Life Technologies, Gibco, 25030081).
4. MEM nonessential amino acid solution (Life Technologies, Gibco, 11140-050).
5. Basic fibroblast growth factor (b-FGF) (Life Technologies, Gibco, PHG0021) or equivalent.
6. β-mercaptoethanol (Life Technologies, Gibco, 21985-023).
7. High glucose DMEM Medium (Life Technologies, Gibco, 11965092).
8. Penicillin–streptomycin (Life Technologies, Gibco, 1510-122).
9. Fetal bovine serum (Hyclone, SH30084.03).
10. PBS without $CaCl_2$ or $MgCl_2$ (Invitrogen, 14190-144).
11. PBS with $CaCl_2$ and $MgCl_2$ (Invitrogen, 14040-141).
12. Bovine serum albumin (Sigma, A2153).
13. 2 % gelatin solution (Sigma G1393).
14. Mouse embryonic feeder (Global stem cells GSC-6201).
15. Rock Inhibitor (Y-27632 dihydrochloride; Ascent Scientist, Asc-129).
16. Sterile water (Sigma, W4502).
17. Collagenase Type IV (Invitrogen, 17104-019).
18. Defined FBS (Hyclone, SH30070.01).
19. Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, D2650).

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20. Dispase (StemCell Technologies, 07923).

2.1.3 Solution

Mouse Embryonic Feeder (MEF) Medium (250 mL): To make 250 mL feeder culture medium combine following components, filter-sterilize, store at 4 °C for up to 1 month.

- 217.5 mL high glucose DMEM medium.
- 25 mL fetal bovine serum.
- 2.5 mL 200 mM L-glutamine.
- 2.5 mL MEM nonessential amino acids.
- 2.5 mL penicillin–streptomycin.

Stem Cell Culture Medium (250 mL): To make 250 mL stem cell culture medium combine following components, filter-sterilize, store at 4 °C for up to 14 days.

- 195 mL DMEM/F-12 medium.
- 50 mL Knockout Serum Replacer.
- 2.5 mL 200 mM L-glutamine.
- 2.5 mL MEM nonessential amino acids.
- 0.5 mL 2 μg/mL Basic FGF solution (*See* below).
- 450 μL BME.

2 μg/mL Basic FGF Solution: To make 2 μg/mL Basic FGF Solution, for stem cell culture medium, dissolve 10 μg Basic FGF in 5 mL 0.1 % BSA in PBS with $CaCl_2$ and $MgCl_2$. Aliquot 0.5 mL/tube and store at −20 °C for up to 6 months. Each aliquot is enough to make 250 mL of stem cell culture medium. Thaw aliquot just prior to making Stem Cell Medium. Do not refreeze aliquots.

0.1 % Gelatin Solution: To prepare 0.1 % gelatin for coating the plate, dilute the 2 % gelatin solution with PBS with $CaCl_2$ and $MgCl_2$ to make 0.1 % gelatin solution. To make 200 mL 0.1 % gelatin solution, add 2 mL gelatin to 200 mL PBS with $CaCl_2$ and $MgCl_2$ and autoclave it and store it at 4 ° C up to 6 months.

Rock Inhibitor Solution: To make 10 mM Rock Inhibitor stock solution, dilute 1 mg Rock Inhibitor (FW 320.26) into 295 μL sterile water (*see* Note 1). Aliquot 20–50 μL/tube and store at −80 °C for up to 1 year. Aliquots can be stored up to 2 months at 4 °C. Rock Inhibitor working stock solution will be used at 1 μL to 1 mL final medium volume.

1 mg/mL Collagenase Solution: To make 1 mg/mL collagenase solution, dissolve the 25 mg collagenase Type IV powder in 25 mL DMEM/F-12, filter it and aliquot it to 5 mL and store at −20 °C. Collagenase solution can be stored at 4 °C for up to 14 days.

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[1]If FW of material is not 320.26, dilute appropriately to achieve a 10 mM solution.

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2× Cryopreservation Medium: To make 10 mL, 2× cryopreservation medium, add 4 mL defined FBS to 4 mL stem cell culture medium, filter-sterilize and then add 2 mL sterile DMSO. Keep on ice until use.

2.2 Feeder-Free Human iPSC Culture Protocol (Matrigel/ MTeSR Medium)

The mTeSR™1 medium from StemCell Technologies and Matri-gel™ Matrix from BD Bioscience, have been shown to be a successful combination for feeder-free maintenance of different human ES and iPS cell lines for up to 20 passages. BD Matrigel hESC-qualified Matrix is compatible with the MTeSR™1 medium from StemCell Technologies, in order to provide the reproducibility for hESC and hiPSC culture [2, 8, 9, 10].

Prepare all equipment, reagents, and solutions listed below:

2.2.1 Equipment and Supplies

1. Biosafety cabinet.
2. Centrifuge.
3. Microscope.
4. 37 °C / 5 % CO_2 incubator.
5. 37 °C water bath.
6. Cryogenic handling gloves and eye protection.
7. 6-well plates (Costar, 3516).
8. 5 mL sterile serological pipettes (Corning, 4487) and 10 mL sterile serological (Corning, 4488) pipettes or equivalent.
9. 2 mL aspirating pipette (Falcon, 357558).
10. 15 mL conical tube (Corning, 430791).
11. 50 mL conical tube (Corning, 430829).
12. 70 % ethanol (Dean lab Inc, 2701).

2.2.2 Reagents

1. hESC qualified Matrigel™ (BD Biosciences®, 354277).
2. DMEM/F-12 Medium (Life Technologies, Gibco, 11330-032).
3. MTeSR™1 medium (StemCell Technologies, 05850).
4. Dispase (StemCell Technologies 07923).
5. Defined FBS (Hyclone, SH30070.01).
6. Dimethyl sulfoxide (DMSO) 10 mL ampoules (Sigma-Aldrich, D2438).

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2.2.3 Solution

Preparing MTeSR™ Medium: Thaw the Supplement (50×) at 2–8 °C overnight. Do not thaw at 37 °C. To prepare 500 mL of complete MTeSR™ Medium, add the 100 mL supplement to the 400 mL basal medium. Aliquot it in 50 mL and store it in −20. Thaw each aliquot at 4 °C before use.

Aliquot Matrigel™

1. Keep the Matrigel™ frozen in −80 °C until you are ready to aliquot it. Matrigel™ is frozen at −20 °C to −80 °C, liquid at 4 °C, and gels rapidly at room temperature.
2. Calculate the volume of Matrigel™ needed per plate based on supplier instruction. Depending on volume of cell culture performed in the laboratory, different sized aliquots can be prepared. For provided catalog number above, 100 μL is enough for coating one 6-well plate. Label the concentration clearly on each tube. Each Matrigel™ aliquot is intended for one use.
3. Thaw the Matrigel™ overnight on ice in 4 °C refrigerator.
4. 2–3 h before aliquot the Matrigel™, place unopened box of the appropriate sized pipette tips, 1.5 mL tubes in the −20 °C or −80 °C freezer to keep them cold.
5. Aliquot the Matrigel™ while is on an ice using cold pipette tips in a cold 1.5 mL eppendorf tubes in sterile biosafety cabinet. Place each aliquot on ice and transfer them in −80 °C soon after finishing aliquot (*see* Note 2).

2× Cryopreservation Medium: To make 10 mL of 2× Cryopreservation Medium, add 4 mL defined FBS to 4 mL MTeSR™ Medium, filter-sterilize and then add 2 mL Sterile DMSO. Keep it on ice until use.

2.3 Feeder-Free Human iPSC Culture Protocol (Vitronectin/ Essential 8™ Medium)

Prepare all equipment, reagents and solutions listed below:

2.3.1 Equipment and Supplies

1. Biosafety cabinet.
2. Centrifuge.
3. Microscope.
4. 37 °C/5 % CO_2 incubator.
5. 37 °C water bath.
6. Cryogenic handling gloves and eye protection.
7. 6-well plates (Costar, 3516).

[2]Work quickly; if the Matrigel™ is allowed to warm at all, it will become gel and will not be appropriate for plating. Matrigel™ cannot be thawed and refrozen.

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8. 5 mL sterile serological pipettes (Corning, 4487) and 10 mL sterile serological (Corning, 4488) pipettes or equivalent.
9. 2 mL aspirating pipette (Falcon, 357558).
10. 15 mL conical tube (Corning, 430791).
11. 50 mL conical tube (Corning, 430829).
12. 70 % Ethanol (Dean lab Inc, 2701).

2.3.2 Reagents

1. Essential 8™ Medium, consisting of Essential 8™ Basal Medium and Essential 8™ Supplement (Life Technologies, A1517001).
2. Vitronectin, truncated recombinant human (Life Technologies, A14700).
3. PBS without calcium and magnesium (Invitrogen 14190-144).
4. UltraPure™0.5 M EDTA, pH 8.0 (Life Technologies 15575-020).
5. Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, D2650).

2.3.3 Solutions

Preparing Essential 8™Medium: Thaw the Essential 8™Supplement (50×) at 2–8 °C overnight. Do not thaw at 37 °C. To prepare 500 mL of complete Essential 8™ Medium, add the 10 mL Essential 8™ supplement to the 490 mL basal medium and store at 2–8 °C for up to 2 weeks (*see* Note 3).

0.5 mM EDTA: To prepare 50 mL of 0.5 mM EDTA in PBS, add 50 μL of 0.5 M EDTA to the 50 mL of sterile PBS. Filter-sterilize the solution and store it at room temperature for up to 6 months.

Aliquot Vitronectin: Thaw the vial of vitronectin on ice. Calculate the volume of vitronectin needed per plate based on supplier instructions. Depending on volume of cell culture performed in the laboratory, different sized aliquots may be preferred. For provided cat number above, 60 μL is enough for coating one 6-well plate. Make sure the concentration is clearly labeled on the tube. Freeze the aliquots at −80 °C or use immediately. Each aliquot is intended for one use.

Cryopreservation Medium: To make 10 mL of Cryopreservation Medium, combine 9 mL Complete Essential 8™ Medium with 1 mL, sterile DMSO in a sterile 15-mL tube. Keep it on ice until use.

2.4 Differentiation of Human iPSC to Lung Progenitors

Prepare all equipment, reagents and solutions listed below:

[3]Warm complete medium required for that day at room temperature until it is no longer cool to the touch. Do not warm the medium at 37 °C.

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2.4.1 Equipment and Supplies

1. Biosafety cabinet.
2. Centrifuge.
3. Microscope.
4. 37 °C/5 % CO_2 incubator.
5. 37 °C water bath.
6. Cryogenic handling gloves and eye protection.
7. 6-well plates (Costar, 3516).
8. 5 mL sterile serological pipettes (Corning, 4487) and 10 mL sterile serological (Corning, 4488) pipettes or equivalent.
9. 2 mL aspirating pipette (Falcon, 357558).
10. 15 mL conical tube (Corning, 430791).
11. 50 mL conical tube (Corning, 430829).
12. 70 % Ethanol (Dean lab Inc, 2701).

2.4.2 Reagents

1. RPMI 1640 (Life Technologies, Gibco 12633-012).
2. Recombinant human Activin A (Life Technologies, Gibco, PHG9014).
3. Penicillin–streptomycin (Life Technologies Gibco, 1510-122).
4. 100 m M L-glutamine, non-animal, cell culture tested (Life Technologies, Gibco, 25030081).
5. MEM nonessential amino acid solution (Life Technologies, Gibco, 11140-050).
6. 50× B27 supplement (Life Technologies Gibco, 17504-044).
7. Fetal bovine serum (Hyclone, SH30084.03).
8. Sodium pyruvate (Life Technologies Gibco 11360-070).
9. IMDM (Life Technologies, Gibco, 12440-053).
10. Recombinant human Noggin (Life Technologies, Gibco, PHC1506).
11. SB431542 (Tocris BioScience, 1614).
12. Basic fibroblast growth factor (Life Technologies, Gibco, PHC9394).
13. Bone morphogenetic protein-4 (BMP4) (Life Technologies, Gibco, PHC9531).
14. Wnt3a (R&D system, 5036 WN).
15. Retinoic acid (Sigma, R-2625).
16. Keratinocyte growth factor (KGF) (Life Technologies, Gibco, PHG0094).

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17. Epidermal growth factor (EGF) (Life Technologies, Gibco, PHG0311).

18. Fibroblast growth factor-10 (FGF10) (Life Technologies, Gibco, PHG0204).

2.4.3 Reagents

Differentiation of iPCs to Definitive Endoderm: Prepare all solutions listed below:

RPMI Basal Medium: To make 250 mL of RPMI as a basal medium for definitive endoderm differentiation combine the following components, filter-sterilize, store at 4 °C for up to 2 weeks.

- 245 mL RPMI 1640 Medium.
- 2.5 mL 100X penicillin–streptomycin.
- 2.5 mL 100 mM L-glutamine.

100 μg/mL Activin A Solution: To make 100 μg/mL Activin A Solution, for definitive endoderm medium, dissolve 5 μg Activin A in 5 mL 0.1 % BSA in PBS with $CaCl_2$ and $MgCl_2$. Aliquot 1 mL/tube and store at −20 °C for up to 6 months. Each aliquot is enough to make 10 mL of definitive endoderm medium. Thaw aliquot just prior to making the medium. Do not refreeze aliquots.

B27 Supplement: Thaw the B27 supplement at 4 °C overnight. Aliquot 0.5 mL/tube and store at −20 °C for up to 6 months. Each aliquot is enough to make 25 mL of definitive endoderm medium. Thaw aliquot just prior to making the medium.

Definitive Endoderm Medium (10 mL): To make 10 mL of definitive endoderm differentiation medium for the first 48 h of differentiation, combine the following components, filter-sterilize, store at 4 °C for up to 4 days.

- 10 mL RPMI 1640 basal medium.
- 1 mL of 100 μg/mL Activin A solution.

After 48 h, switch the medium to the following medium: to make 10 mL of the medium to be used from day3 to day 6, combine the following components:

- 10 mL RPMI 1640 basal medium.
- 1 mL of 100 μg/mL active solution.
- 0.2 mL of 50X B27 supplement.
- 50 μL 100 mM sodium butyrate.

Differentiation of Definitive Endoderm to Anterior Foregut Endoderm: To make 250 mL of IMDM/10 % FBS as a basal medium for anterior foregut endoderm differentiation, combine the following components, filter-sterilize, store at 4 °C for up to 4 days.

IMDM Basal Medium

- 217.5 mL IMDM Medium.

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- 25 mL FBS.
- 2.5 mL 100× penicillin–streptomycin.
- 2.5 mL 100 mM L-glutamine.
- 2.5 mL MEM nonessential amino acids.

200 μg/mL Noggin Solution: To make 200 μg/mL Noggin Solution, for anterior foregut endoderm differentiation, reconstitute the 20 μg Noggin in 10 mL 0.1 % BSA in PBS with $CaCl_2$ and $MgCl_2$. Aliquot 1 mL/tube and store at −20 °C for up to 6 months. Each aliquot is enough to make 10 mL of anterior foregut endoderm medium. Thaw aliquot just prior to making the medium. Do not refreeze aliquots.

10 mM SB431542 Solution: To make 10 mM SB431542 Solution, for anterior foregut endoderm medium, dissolve 1 mg of SB431542 (MW: 420.42) in 2.379 mL DMSO. Aliquot 100 mL/tube and store at −20 °C for up to 6 months.

Human Extracellular Matrix Solution: Human ECM protein comes in 1 mL volumes. Calculate the required amount of ECM protein solution for coating enough required wells. To make the human ECM solution, dilute it 1:100 in DMEM-F12 and use it freshly. Aliquot the human ECM in 100 μL/well and store it in −20 °C for up to 6 months. Thaw aliquot just prior to making the medium. Do not refreeze and thaw the aliquots.

Anterior Foregut Endoderm Medium (10 mL): To make 10 mL of anterior foregut endoderm differentiation medium, combine the following components, filter-sterilize, store at 4 °C for up to 4 days.

- 10 mL IMDM basal medium.
- 1 mL of 200 μg/mL NOGGIN solution.
- 10 μL of 10 mM SB431542.

Differentiation of Anterior Foregut Endoderm to Lung Progenitors

10 μg/mL BMP4 Solution: To make 10 μg/mL BMP4 Solution, reconstitute 10 μg BMP4 in 1 mL 0.1 % BSA in PBS with $CaCl_2$ and $MgCl_2$. Aliquot 50 μL/tube and store at −80 °C for up to 6 months. Each aliquot is enough to make 50 mL of differentiation medium. Thaw aliquot just prior to making the medium.

10 μg/mL KGF Solution: To make 10 μg/mL KGF (FGF7) Solution, reconstitute 10 μg KGF in 1 mL 0.1 % BSA in PBS with $CaCl_2$ and $MgCl_2$. Aliquot 50 μL/tube and store at −80 °C for up to 6 months. Each aliquot is enough to make 50 mL of differentiation medium. Thaw aliquot just prior to making the medium.

10 μg/mL bFGF Solution: To make 10 μg/mL bFGF Solution, dissolve 10 μg bFGF in 1 mL 0.1 % BSA in PBS with $CaCl_2$ and $MgCl_2$. Aliquot 50 μL/tube and store at −80 °C for up to 6 months. Each aliquot is enough to make 50 mL of differentiation medium. Thaw aliquot just prior to making the medium.

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100 μg/mL Wnt3a Solution: To make 100 μg/mL Wnt3a Solution, dissolve 100 μg Wnt3a in 1 mL 0.1 % BSA in PBS with $CaCl_2$ and $MgCl_2$. Aliquot 100 μL/tube and store at −80 °C for up to 6 months. Each aliquot is enough to make 10 mL of differentiation medium. Thaw aliquot just prior to making the medium.

0.5 mM Retinoic Acid Solution: To make 25 mM retinoic acid solution dissolve 100 mg (MW = 300.44) of retinoic acid powder in 12 mL DSMO. Aliquot 500 μL/tube and store at −80 °C for up to 6 months. To make 0.5 mM retinoic acid solution, add 100 μL of 25 mM retinoic acid solution to 500 μL DMSO.

Lung Progenitor Differentiation Medium (10 mL): To make 10 mL of lung progenitor differentiation medium, combine following components and store at 4 °C for up to 4 days.

- 10 mL IMDM basal medium.
- 10 μL of 10 μg/mL BMP4 solution.
- 10 μL of 10 μg/mL bFGF solution.
- 100 μL of 10 μg/mL Wnt3a solution.
- 10 μL of 10 μg/mL KGF solution.

10 μL of 0.5 mM retinoic acid solution.

2.5 iPSC Generation Using Lentiviral Vectors Protocol

Prepare all Equipment, Reagents and Solutions Listed Below:

2.5.1 Equipment and Supplies

1. Biosafety cabinet.
2. Centrifuge.
3. Microscope.
4. 37 °C/5 % CO_2 incubator.
5. 37 °C water bath.
6. Cryogenic handling gloves and eye protection.
7. 5 mL sterile serological pipettes (Corning, 4488).
8. 10 mL sterile serological pipettes (Corning, 4487).
9. 2 mL aspirating pipettes (Falcon, 357558).
10. 6-well tissue culture plates (Costar, 3516).
11. 24-well tissue culture plates (Costar, 3526).
12. 48-well tissue culture plates (Costar, 3548).
13. 96-well tissue culture plates (Costar, 3596).
14. 10 cm culture plate (Corning, 430167).

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15. 15 mL conical tubes (Corning, 430791).
16. 50 mL conical tubes (Corning, 430829).
17. 70 % Ethanol (Dean lan Inc, 2701).

2.5.2 Required Reagents

1. FUW-tetO-lox-hOCT4 plasmid (Add gene Plasmid 20728).
2. FUW-tetO-lox-SOX2 plasmid (Add gene Plasmid 20729).
3. FUW-tetO-lox-hKLF4 (Add gene Plasmid 20727).
4. FUW-tetO-lox-hNanog (Add gene Plasmid 20727).
5. FUW-tetO-lox-hcMyc (Add gene Plasmid 20324).
6. FUW-tetO-lox-m2rt TA (Add gene Plasmid 20342).
7. pMD2.G (Plasmid 12259).
8. psPAX2(Plasmid 12260).
9. DMEM-F12 Medium (Life Technologies, Gibco, 11330-032).
10. High glucose DMEM Medium (Life Technologies, Gibco, 11965092).
11. Penicillin–streptomycin (Life Technologies, Gibco, 1510-122).
12. Fetal bovine serum (Hyclone, SH30084.03).
13. PBS without $CaCl_2$ or $MgCl_2$ (Invitrogen, 14190-144).
14. Knockout Serum Replacer (KOSR) (Invitrogen, 10828-028).
15. L-glutamine, non-animal, cell culture tested (Life Technologies, Gibco, 25030081).
16. MEM nonessential amino acid solution (Life Technologies, Gibco, 11140-050).
17. Basic fibroblast growth factor (b-FGF) (Life Technologies, Gibco, PHG0021) or equivalent.
18. β-mercaptoethanol (Life Technologies, Gibco, 21985-023).
19. PBS with $CaCl_2$ and $MgCl_2$ (Invitrogen, 14040-141).
20. Bovine serum albumin (Sigma, A2153).
21. 2 % gelatin solution (Sigma G1393).
22. Mouse embryonic feeder (Global stem cells GSC-6201).
23. Rock Inhibitor (Y-27632 dihydrochloride; Ascent Scientist, Asc-129).
24. Sterile water (Sigma, W4502).
25. Collagenase Type IV (Invitrogen, 17104-019).
26. Defined FBS (Hyclone, SH30084.01).

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27. Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, D2650).
28. Dispase (StemCell Technologies 07923).
29. Polyberene (Sigma 107689, H9268).
30. Human HEK293 cell line (ATCC CRL1573™).
31. Doxycycline (Dox) (Sigma: D9891-1G).
32. Valproic acid (PVA) (Sigma, P4543).
33. Opti-MEM® Reduced Serum Medium (Life Technologies 31985070).
34. Lipofectamine® 2000 Transfection Reagent (Life Technologies 11668027).

2.5.3 Solution

DMEM Medium (To Culture HEK293T, Human Fibroblast and Mouse Embryonic Feeder (MEF) (250 mL): To make 250 mL feeder culture medium combine following components, filter-sterilize, store at 4 °C for up to 1 month.

- 217.5 mL high glucose DMEM Medium.
- 25 mL fetal bovine serum.
- 2.5 mL 200 mM L-glutamine.
- 2.5 mL MEM nonessential amino acids.
- 2.5 mL penicillin–streptomycin.

Cryopreservation Medium (HEK293T, Human Fibroblast): To make 10 mL of cryopreservation medium, combine 9 mL fibroblast culture medium with 1 mL, sterile DMSO in a sterile 15-mL tube. Keep it on ice until use.

Stem Cell Culture Medium (250 mL): To make 250 mL stem cell culture medium, combine following components, filter-sterilize, store at 4 °C for up to 14 days.

- 195 mL DMEM/F-12 medium.
- 50 mL Knockout Serum Replacer.
- 2.5 mL 100 mM L-glutamine.
- 2.5 mL MEM nonessential amino acids.
- 0.5 mL 2 μg/mL Basic FGF solution (*See* below).
- 450 μL BME.

2 μg/mL Basic FGF Solution: To make 2 μg/mL Basic FGF Solution, for stem cell culture medium, dissolve 10 μg Basic FGF in 5 mL 0.1 % BSA in PBS with $CaCl_2$ and $MgCl_2$. Aliquot 0.5 mL/tube and store at −20 °C for up to 6 months. Each aliquot is enough to make 250 mL of stem cell culture medium. Thaw aliquot just prior to making stem cell culture medium. Do not refreeze aliquots.

0.1 % Gelatin Solution: To prepare 0.1 % gelatin for coating the plates, dilute the 2 % gelatin solution with PBS with $CaCl_2$ and $MgCl_2$ to make 0.1 % gelatin solution. To make 200 mL 0.1 % gelatin solution, add 2 mL gelatin to 200 mL PBS with $CaCl_2$ and $MgCl_2$ and autoclave it and store it at 4 °C

1 μg/mL Doxycycline (Dox) Solution: Reconstitute 10 mg of powder in 10 mL PBS and filter with 0.2 μm filter, aliquot it and store at −20 °C.

1 M Valproic acid (VPA): Reconstitute 166 mg of VPA in 1 mL sterile H_2O to make 1 M solution. Add 1 μL to 1 mL medium to get 1000× dilution. Sometimes VPA is toxic, and sodium butyrate can be used instead of VPA.

Dox Induction Medium: To make 10 mL Dox induction medium combine the following components.

- 10 mL hESC Culture Medium.
- 10 ×L Dox Solution (2 mg/mL).

Thaw Dox Solution on ice and add to pre-warmed hESC medium.

Polyberene Solution: Polybrene is a polycation that increases binding between the pseudoviral capsid and the cellular membrane. Prepare a 6 mg/mL Polybrene stock solution in deionized, sterile water. Filter-sterilize it and aliquot the stock solution at 100 μL/tube and store at −20 °C for up to 1 year. The working stock can be stored at 4 °C for up to 2 weeks. Do not freeze/thaw the stock solution more than three times as this may result in loss of activity.

3 Methods

3.1 Feeder-Dependent iPSC Culture Protocol

3.1.1 Prepare Mouse Embryonic Feeder (MEF) Plates

1. Sterilize the biosafety cabinet for 20 min with UV light.
2. Turn on the blower and spray down the whole surface with ethanol and allow it to evaporate for 20 min prior to initiating cell culture.
3. Coat two 6-well plate with 0.1 % gelatin solution at least 2 h prior to thawing the MEF.
4. Remove a frozen vial of MEF (2×10^6 cells) from the liquid nitrogen tank and thaw by immersing the vial in a 37 °C water bath without submerging the cap. Swirl the vial gently (*see* Note 4).
5. Remove the MEF vial from the water bath when only a small ice crystal remains.

[4]Vials stored in liquid nitrogen may accidentally explode when warmed. Wear ultra-low temperature cryo gloves and also wear eye protection.

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6. Spray the vial with a 70 % ethanol to sterile the outside of the tube and transfer it into the sterile biosafety cabinet.
7. Transfer the MEF cells gently into a sterile 15 mL conical tube using a 1 mL sterile serological pipette.
8. Add 4 mL of warmed DMEM-10%FBS medium dropwise to the MEF cells in the 15 mL conical tube. To reduce osmotic shock to the cells, move the tube back and forth gently to mix the cells, while adding the medium.
9. Centrifuge the cells at 233 × *g* for 5 min.
10. Aspirate and discard the supernatant with a sterile aspirating pipette.
11. Resuspend the cell pellet in 24 mL of MEF medium; 2 mL for every well that will receive cells (2 M MEF cells are enough for 12 wells of a 6-well plate. It is based on the thaw recommendation from Global stem cells to get 2×10^5 cells per one well of 6-well plate).
12. Aspirate gelatin from each well and wash with PBS prior to transferring MEF into the gelatin coated plates.
13. Gently pipette cells up and down few times and add 2 mL of medium containing MEF cells to each well of 6-well plate.
14. Transfer the plates to 37 °C, 5 % CO_2 incubator (*see* Note 5).

3.1.2 Thawing the iPSC onto the Feeder (MEF)

1. 2–3 h prior to thawing the iPSC, aspirate MEF medium from each well, rinse it with 1 mL DMEM-F12 and add 1 mL of stem cell culture medium to each well of 6-well plates.
2. Transfer the MEF plate back to the 37 °C incubator.
3. Remove the frozen vial of iPSC from the liquid nitrogen storage tank.
4. Quickly remove the label or copy the information written on the tube in your notebook (*see* Note 6).
5. Immerse the vial in a 37 °C water bath and swirl the vial gently without submerging the cap. Remove the vial from the water bath when no ice crystals remain.
6. Spray the vial with a 70 % ethanol to sterilize the outside of the tube and transfer it into the sterile biosafety cabinet.
7. Transfer the cells gently into a sterile 15 mL conical tube using a 1 mL sterile pipette.

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[5]The feeder should be used within a week.

[6]Wear ultra-low temperature cryo gloves and eye protection when taking the cells from nitrogen tank.

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8. Slowly, add 4 mL of warmed stem cell medium dropwise to cells in the 15 mL conical tube. Gently move the tube back and forth to mix the cells while adding the medium.
9. Centrifuge the cells at 149 × *g* for 5 min.
10. Aspirate and discard the supernatant with a 2 mL sterile aspirating pipette.
11. Resuspend the cell pellet in 2.5 mL stem cell culture medium for every well that will receive cells (*see* Note 7).
12. To increase the iPS cell viability and attachment, add 1 mM Rock Inhibitor to stem cell medium only at first 24 h.
13. Gently pipette cells up and down in the tube a few times.
14. Transfer iPSC cells onto the feeder layer and place the iPSC plate into the incubator and gently shake the plate back and forth and side to side to evenly distribute the cells. Avoid circular motions to prevent pooling iPSCs colonies in the center of the well.
15. The next day, remove the spent medium using a sterile aspirating pipette and gently add 3 mL of stem cell culture medium to the well.
16. Place the plate into a 37 °C incubator.
17. Feed the iPS cells daily until ready to passage or freeze.

3.1.3 Feeding iPSC Cultured on MEF

1. Warm enough stem cell culture medium to feed the iPSC. Generally, 2–3 mL for each well that will be fed.
2. Aspirate the spent medium with a 2 mL sterile aspirating pipette.
3. Add 2 mL of warmed stem cell culture medium to each well for first 2 days. Starting from day 3, add 3 mL medium to each well (*see* Note 8).
4. Return the 6-well plate to the 37 °C incubator.
5. Feed the iPSCs daily until ready to passage or freeze (*see* Note 9).

3.1.4 Passaging iPSC Cultured on MEF—In general, iPS cells should be split when the MEF feeder layer is 10 days old or iPSC colonies are becoming too dense or too large or increased differentiation occurs. There are two methods for passaging the iPSC. An enzymatic method which is a standard passaging method, recommended when more than 10–20 colonies are in each well. And, a nonenzymatic or manual passaging method is recommended for sparse iPSC colonies or normally fewer than 10–20 colonies in each well.

[7]Number of wells receiving cells is based on the thaw recommendation found in the certificate of analysis if you bought it from the company. If you thaw the frozen vial prepared previously in laboratory follow the lab's instruction. For example: When the thaw recommendation is to thaw 1 vial into 1 well, resuspend the pellet in 3 mL of stem cell culture medium.

[8]To reduce the contamination potential, do not reinsert a used pipette into sterile medium for any reason. If feeding more than one plate, use a different pipette for each plate to reduce risk of contamination.

[9]Observe the pluripotent stem cells using a microscope. If they require passaging, follow the passaging protocol below.

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This method is also recommended when there is significant differentiation present and the culture must be maintained.

1. Prepare enough MEF plate as described in Section 3.1.1, the day prior to passaging the iPS cells.
2. Two hours prior to passaging the iPS cells, aspirate the MEF medium from the wells, and rinse with 1 mL/well warmed sterile DMEM/F-12.
3. Add 1 mL stem cell medium to each well and return the MEF plates to 37 °C incubator.
4. Remove differentiated colonies before passaging the iPS cells if necessary. Transfer the plate into a hood equipped with a stereomicroscope and remove all areas of differentiation with a sterile modified pastor pipette or sterile micropipette tip.
5. Return the plate in the biosafety cabinet. Aspirate the spent medium from the wells to be passaged with an aspirating pipette to remove floating picked differentiated colonies in medium.
6. Rinse it once with 1 mL warmed DMEM-F12.
7. Add 1 mL room temperature collagenase solution to each well to be passaged.
8. Incubate for 5–7 min at 37 °C incubator (*see* Note 10).
9. Check the iPSC colonies under a microscope to confirm appropriate incubation time. When the edges of the colonies just slightly folded back, aspirate the collagenase without disturbing the attached iPS cell colonies.
10. Gently add 1 mL of warmed DMEM/F-12 to each well with a 5 mL pipette and then aspirate off the medium.
11. Rinse each well with 1 mL of warmed DMEM/F-12 one more time (*see* Note 11).
12. Add 1 mL of stem cell culture medium to each well.
13. Gently scrape the surface of the plate using a sterile 2 mL glass pipette, while holding the pipette perpendicular to the plate and simultaneously dispensing medium.
14. Repeat it at different direction to cut colonies to small pieces as scrape the cells off the surface of the plate.
15. Pipette the medium slowly up and down to wash the cells off the surface.
16. Transfer iPSC pieces to 15 mL sterile conical tube after all wells are scraped and the cells are removed from the surface of the well.

[10]At least one well of cells should be left and used as a backup to protect against problems with the split.
[11]Make sure that the cells remain adhered to the plate. To avoid the iPSC colonies peeling off, do not dispense the medium in a continuous stream.

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17. Pipette cells up and down gently a few times in the conical tube to further break-up iPSCs colonies if needed.

18. Add 1–2 mL stem cell culture medium to the first well to wash and collect residual cells. Then take up the medium and transfer it into each subsequent well to collect all residual cells.

19. Determine how much additional medium is required based on the split ratio and the number of wells that will be used (*see* Note 12).

20. Add Rock Inhibitor at 1 μM concentration to the stem cell culture medium during the first 24 h after passaging the iPSC. This increases the viability and attachment of iPS cells.

21. Add 2 mL of cell suspension to each well of the new plate. In general, there should be a total of 3 mL of stem cell culture medium and cells in each of the new wells (2 mL of cell suspension + 1 mL of pre-plated stem cell culture medium on MEF cells).

22. Return the plate to the 37 °C incubator after plating the cells. Move the plate back-and-forth and side-to-side for a few times to further disperse cells across the surface of the wells (*see* Note 13).

23. Incubate cells overnight to allow colonies to attach.

24. Feed the iPSC as previously described until ready to passage or freeze (*see* Note 14).

3.1.5 Freezing iPSC Cultured on MEF

1. Label cryovials with the cell line, passage number, the freeze date, and your initials. Use an alcohol proof pen or labels that resist liquid nitrogen and ethanol.

2. Spray down the whole surface with ethanol and allow it to evaporate for 20 min prior to initiating cryopreservation.

3. Prepare the isopropanol freezing container "Mr. Frosty" and keep it in 4 °C, about 30 min before start (*see* Note 15).

4. Prepare the required amount of cryopreservation medium and keep it on ice until ready to use. 1 mL freezing media will be needed for every vial. Always make a little extra to account for pipet error.

5. Remove differentiated colonies before freezing the iPS cells if necessary.

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[12]iPS cells will grow at a different rate, and the split ratio will need to be adjusted every single time the iPSC cells are passaged. The split ratio is variable and generally is between 1:2 and 1:4. Always, as a general rule, observe the iPSC colonies from the last split ratio and adjust the ratio according to the appearance of the iPSC colonies. If the cells look healthy and colonies have enough space, split the iPSC using the same ratio, if they are dense and crowding, increase the ratio, and if the cells are sparse, decrease the ratio. iPS cells will need to be split every 4–7 days based on the morphology of the colonies.

[13]After splitting the iPSC, while cells are attaching, open and close the incubator carefully. This will prevent disturbing the even distribution of cells to the surface of the well.

[14]iPSC can be passaged with the same method using dispase enzyme solution (1 mg/mL). To use dispase at step 7, add 1 mL room temperature dispase instead of adding 1 mL room temperature collagenase solution to each well to be passaged. Incubate for 3–5 min at 37 °C and continue with the following steps after step 7.

[15]The isopropanol must be replaced every five uses.

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6. Aspirate stem cell medium from each well to remove floating picked differentiated colonies in medium.
7. Add 1 mL of room temperature collagenase solution to each well of each 6-well plate.
8. Incubate cells for 5–7 min at 37 °C incubator.
9. Check the plate under the microscope every 3 min and look for the edge of the colonies to be slightly folded back.
10. Aspirate collagenase solution from each well, rinse it with DMEM-F12 two times. Take care not to remove any floating colonies.
11. Add 1 mL of stem cell culture medium to each well. Take up 1 mL of medium from each well using 2 mL serological glass pipette and scrape the colonies of the plate while slowly expelling the medium to wash the cells off the surface.
12. Repeat several times and at different direction in each well to remove the cells from the well surface (*see* Note 16).
13. Collect the cells in a sterile 15 mL conical tube. Wash each plate with 2 mL of stem cell culture medium, transferring the medium from well to well and add the medium to the 15 mL conical tube.
14. Centrifuge at 149 × *g* for 5 min.
15. Aspirate the supernatant and resuspend each cell pellet in enough stem cell culture medium. Stem cells from each well go to 0.5 mL stem cell culture medium.
16. Freeze iPSC at 1 well/cryovial. Add 0.5 mL cryopreservation medium to each 0.5 mL of iPSC cells. For example, for three vials, add 1.5 mL of cryopreservation medium.
17. Add the cryopreservation medium very slowly and dropwise. Gently pipette up and down two times to mix. Do not break up the colonies.
18. Distribute 1 mL of cell suspension to each of the prepared cryovials, tighten caps and place cryovials into an isopropanol containing freezing container.
19. Place the freezing containers in the −80 °C freezer overnight and transfer cell vials to liquid nitrogen storage the following day (*see* Note 17).

3.2 Feeder-Free Human iPSC Culture Protocol (Matrigel/ MTeSR Medium)

3.2.1 Matrigel™ Coated Plates

1. Thaw as many as aliquots of Matrigel™ may be needed depending on volume of cell culture on ice.

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[16]Try not to break iPSC colonies up into small clumps. If iPSCs are frozen in large aggregates, they will recover from the thaw more efficiently.

[17]Once cells are in contact with DMSO, they should be aliquoted quickly and initiate freezing within 2–3 min.

2. For coating one 6-well plate, transfer 5 mL cold, sterile DMEM/F-12 medium to the 15 mL conical tube in sterile biosafety cabinet.
3. Add 1 mL of cold DMEM/F-12 medium to one of the 100 μL Matrigel™ aliquot and transfer it to 15 mL conical tube using 1 mL sterile pipette and pipette up and down to dissolve it.
4. Add 1 mL of Matrigel™solution to each well of a 6-well plate. This will be enough for one full 6-well plate.
5. Incubate the Matrigel™ coated plate 1 h at room temperature or 37 °C incubator before use or storage (*see* Note 18).

3.2.2 Thawing the iPSC on Matrigel

1. Remove the frozen vial of iPSC from the liquid nitrogen storage tank.
2. Quickly remove the label or copy the information written on the tube in your notebook (*see* Note 19).
3. Immerse the vial and swirl the vial gently in a 37 °C water bath, without submerging the cap. Remove the vial from the water bath when no more ice crystal remains.
4. Spray the vial with a 70 % ethanol to sterilize the outside of the tube and transfer the vial in the sterile biosafety cabinet.
5. Transfer the cells gently into a sterile 15 mL conical tube using a 1 mL sterile pipette.
6. Add 4 mL of warmed MTeSR™ medium dropwise to cells in the 15 mL conical tube. To reduce osmotic shock to the cells, gently move the tube back and forth to mix the cells while adding the medium.
7. Centrifuge the cells at 149 rpm for 5 min.
8. Aspirate the supernatant with a sterile aspirating pipette.
9. Resuspend the cell pellet in 2 mL of MTeSR™ medium for every well that will receive cells (*see* Note 20).
10. To increase the iPS cell attachment to the Matrigel™, add 1 mM Rock Inhibitor to MTeSR™ medium only for the first day.
11. Gently pipette cells up and down in the tube a few times.
12. Aspirate the Matrigel™ from each well gently without damaging the Matrigel™ layer.

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[18]Wrap the extra plates in Parafilm and store in refrigerator at 2–8 °C and use the plates within 7 days after preparation. If any portion of the well dries out, do not use the well.
[19]Vials stored in liquid nitrogen may accidentally burst when warmed due to influx of liquid nitrogen into the vial (rare). Wear ultra-low temperature cryo gloves and eye protection.
[20]number of wells receiving cells is based on the thaw recommendation found in the certificate of analysis if you bought the iPSC line from company. If you thaw the frozen vial of iPSC prepared previously in the laboratory, follow the lab instructions. For example: When the thaw recommendation is to thaw 1 vial into 1 well, resuspend the pellet in 2.5 mL of MTeSR™ medium.

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13. Transfer iPSC cells onto the Matrigel™ layer and place the iPSC plate into the incubator and gently move the plate back and forth and side to side to evenly distribute the cells.
14. The next day, remove the spent medium using a sterile aspirating pipette.
15. Gently add 2.5 mL of MTeSR™ Medium to the each well of a 6-well plate.
16. Place plate back into a 37 °C incubator.
17. Feed iPS cells daily until ready to passage or freeze.

3.2.3 Feeding iPSC Cultured on Matrigel

1. Warm enough MTeSR™ medium to feed 2–2.5 mL for each well that will be fed.
2. Aspirate the spent medium with a sterilized aspirating pipette.
3. Add 2 mL of warmed MTeSR™ medium to each well (*see* Note 21).
4. Return the 6-well plate to the 37 °C incubator.
5. Repeat procedure daily until ready to passage or freeze (*see* Note 22).

3.2.4 Passaging iPSC Cultured on Matrigel—Split iPS cells when iPSC colonies are becoming too dense or too large or increased differentiation occurs. iPS cells will grow at a different rates and the split ratio will need to be adjusted every single time you passage the cells.

1. Prepare Matrigel™plate as described in Section 3.2.1, 1 h or a day prior to passaging the iPS cells.
2. Remove all areas of differentiation in each well before passaging the iPS cells if necessary. Transfer the plate into the hood equipped with a stereomicroscope and remove differentiated colonies with a sterile modified pipette or sterile micropipette tip.
3. Transfer the plate in the biosafety cabinet and aspirate the spent medium from the wells to remove floating picked differentiated colonies in medium.
4. Rinse it once with 1 mL warmed DMEM-F12.
5. Add 1 mL room temperature dispase solution to each well to be passaged.
6. Incubate for 3–5 min at 37 °C incubator.
7. Check the colonies under a microscope to confirm appropriate incubation time. When the colonies appear to slightly fold back, aspirate the dispase solution without disturbing the attached iPS cell colonies.
8. Gently add 1 mL of warmed DMEM/F-12 to each well and then aspirate off the medium (*see* Note 23).

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[21]To reduce the contamination potential, do not reinsert a used pipette into sterile medium. If feeding more than one plate, use a different pipette for each plate.
[22]Observe the pluripotent stem cells using a microscope. Follow the passaging protocol below when they require passaging.

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9. Repeat rinsing each well with 1 mL of warmed DMEM/F-12 one more time.
10. Add 2 mL of MTeSR™ Medium to each well.
11. Using a sterile cell scraper, gently scrape the iPS cells colonies from the plate. Repeat in different directions if necessary.
12. Pipette the medium slowly up and down using 5 mL sterile pipette to wash the cells off the surface.
13. Transfer iPSC colonies to 15 mL sterile conical tube after all wells are scraped and the cells are removed from the surface of the well.
14. Pipette cells up and down gently a few times in the conical tube to further break-up iPS cell colonies if needed.
15. Take up 1–2 mL MTeSR™ in a 5 mL pipette and add it to the first well to wash and collect residual cells. Take up the medium and transfer it into each subsequent well to collect cells.
16. Determine how much additional medium is required. This is dependent on the split ratio and the number of wells used. There should be a total of 2 mL of MTeSR™medium and cells in each of the new wells (*see* Note 24).
17. Add 1 mM Rock Inhibitor to the MTeSR™ medium during first 24 h after passaging the iPSC to increase viability and attachment of cells to the Matrigel™.
18. Add 2 mL of cell suspension to each well of the new plate.
19. Return the plate to the incubator after plating the cells. Move the plate in several quick, short, back-and-forth and side-to-side motions to further disperse cells across the surface of the wells.
20. Incubate cells overnight to allow colonies to attach (*see* Note 25).

3.2.5 Freezing iPSC Cultured on Matrigel ™

1. Label enough cryovials with the cell line, passage number, the freeze date, and your initials with alcohol proof pen or labels that resist liquid nitrogen and ethanol.
2. Place the isopropanol freezing container and keep it in 4 °C, about 30 min before start.
3. Prepare the required amount of cryopreservation medium and keep it on ice until ready to use. Prepare 1 mL freezing medium for every vial plus a little extra for pipet error.

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[23]Make sure that the cells remain adhered to the plate. Do not dispense the medium in a continuous stream in one spot to avoid of detaching iPSC from the wells.
[24]The split ratio is variable, and is generally between 1:2 and 1:4. Always as a general rule, observe the last split ratio and adjust the ratio according to the appearance of the iPSC colonies in the wells. If the colonies on Matrigel™ have enough space, split using the same ratio, if they are dense and crowding, increase the ratio, and if the cells are sparse, decrease the ratio. Cells will need to be split every 5–7 days based on the iPSC cell colonies' appearance.
[25]To prevent disturbing the even distribution of cells to the surface of the well, try to limit opening and closing the incubator for few first hours after passaging the iPSC, while cells are attaching. Feed the iPSC daily until ready to passage or freeze.

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4. Remove differentiated colonies before passaging the iPS cells if necessary. Aspirate MTeSR™ medium from each well to remove floating picked differentiated colonies in medium.
5. Add 1 mL of room temperature dispase solution to each well of each 6-well plate.
6. Incubate cells for 3–5 min at 37 °C incubator.
7. Check the plate under the microscope every 2 min and look for the edge of the colonies to slightly be folded back.
8. Aspirate dispase solution from each well, rinse it with DMEM-F12 two times, taking care not to remove any floating colonies.
9. Add 1 mL of MTeSR™ medium to each well.
10. Using a sterile cell scraper, gently scrape the iPS cell from the plate. Repeat in different directions if necessary.
11. Pipette the medium slowly up and down to wash the cells off the surface.
12. Transfer iPSC pieces to 15 mL sterile conical tube after all wells are scraped and the cells are removed from the surface of the well.
13. Pipette cells up and down gently a few times in the conical tube to further break-up cell colonies if needed. Wash and collect residual cells from each well with 1–2 mL MTeSR™ medium and add them to 15 mL conical tube (*see* Note 26).
14. Centrifuge at 149 × *g* for 5 min.
15. Aspirate the supernatant and resuspend each cell pellet in enough MTeSR™ medium (stem cells from each well go to 0.5 mL MTeSR™ medium).
16. Freeze iPSC at 1 well/cryovial. Add 0.5 mL cryopreservation medium to each 0.5 mL of iPSC cells very slowly and dropwise. Pipette up and down two times to mix.
17. Distribute 1 mL of cell suspension to each of the prepared cryovials, tighten caps and place cryovials into an isopropanol containing freezing container.
18. Place the freezing containers in the −80 °C freezer overnight and transfer cell vials to liquid nitrogen storage the following day.

3.3 Feeder-Free Human iPSC Culture Protocol (Vitronectin/ Essential 8 ™ Medium)

3.3.1 Vitronectin Coated Plates

1. Thaw as many as aliquots of vitronectin may be needed depending on volume of cell culture on ice (each 60 μL is enough for one 6-well plate to get the concentration of 5 μg/mL).

[26] Try not to break the iPSC to small clumps. Cells will recover from the thaw more efficiently if frozen in large aggregates.

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2. For coating the wells of one 6-well plate, transfer 6 mL cold, sterile PBS to the 15 mL conical tube in sterile biosafety cabinet.
3. Add 60 μL of thawed vitronectin into a 15-mL conical tube and pipette up and down to resuspend it.
4. Add 1 mL of vitronectin solution to each well of a 6-well plate. Allow to set 1 h at room temperature or 37 °C incubator before use or storage (*see* Note 27).

3.3.2 Passaging iPSC Cultured in Essential 8 ™ Medium—Split cells when iPSC colonies are becoming too dense or too large or if increased differentiation occurs. iPS cells will grow at a different rates and the split ratio will need to be adjusted every single time when you passage the cells.

1. Prepare vitronectin coated plate as described in Section 3.3.1 1 h or a day prior to passaging the iPS cells.
2. One hour before starting, warm the vitronectin-coated plate to room temperature in the biosafety cabinet.
3. Aspirate residual vitronectin solution from each well. It is not necessary to rinse off the well after removal of vitronectin. Cells can be passaged directly onto the vitronectin-coated plate.
4. Add 1 mL pre-warmed Essential 8™Medium to each well of a coated 6-well plate and leave it in the biosafety cabinet.
5. Pre-warm the required volume of Essential 8™ Medium at room temperature until it is no longer cool to the touch (*see* Note 28).
6. Remove differentiated colonies before passaging the iPS cells if necessary in the hood equipped with a stereomicroscope. Remove all areas of differentiation with a sterile modified pipette or sterile micropipette tip.
7. Transfer the plate in the biosafety cabinet and aspirate the spent medium from the wells to remove floating picked differentiated colonies in medium.
8. Rinse it once with 1 mL warmed PBS without Ca^{+2} and Mg^{+2}.
9. Add 1 mL room temperature 0.5 mM EDTA solution to each well to be passaged.
10. Incubate the plate at room temperature for 5–8 min or 37 °C for 4–5 min (*see* Note 29).

[27]Wrap the extra plates in Parafilm and store in refrigerator at 2–8 °C. The plates should be used within a week after preparation. Do not allow the wells to dry. If any portion of the well dries out, do not use the well. Prior to use, pre-warm the plate to room temperature for at least 1 h.

[28]Warm complete medium required for that day at room temperature until it is no longer cool to the touch. Do not warm the medium at 37 °C.

[29]With certain cell lines, this may take longer than 5 min.

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11. Check the colonies under a microscope to confirm appropriate incubation time. When the cells start to separate and round up, and the colonies appear to have holes in them, they are ready to be removed from the plate (*see* Note 30).
12. Aspirate the EDTA solution gently from each well using 2 mL aspirating pipette.
13. Add 1 mL of warmed complete Essential 8™ Medium to each well. The initial effect of the EDTA will be neutralized quickly by adding the medium.
14. Wash the cells from the surface of the well by pipetting the colonies up using a 5 mL sterile pipette. Do not scrape the cells from the dish (*see* Note 31).
15. Collect cells in a 15 mL sterile conical tube after all the cells are removed from the surface of the well.
16. Pipette cells up and down gently a few times in the conical tube to further break-up iPS cell colonies if needed.
17. Determine how much additional medium is required. This is dependent on the split ratio and the number of wells used. There should be a total of 2 mL of Essential 8™ medium and cells in each of the new wells after the cell suspension has been added to each well (*see* Note 32).
18. Add 2 mL of cell suspension to each well of the new plate.
19. Return the plate to the incubator after plating the cells. Move the plate back-and-forth and side-to-side a few times to further disperse cells across the surface of the wells.
20. Incubate cells overnight to allow colonies to attach (*see* Note 33).
21. Feed iPSC cells beginning the second day after splitting. Then feed them daily until ready to passage or freeze.

3.3.3 Freezing iPSC Cultured in Essential 8™ Medium

1. Label cryovials with the cell line, passage number, the freeze date, and your initials. Use an alcohol proof pen or labels that resist liquid nitrogen and ethanol.
2. Place the isopropanol freezing container and keep it in 4 °C, about 30 min before start.
3. Prepare the required amount of cryopreservation medium and keep it on ice until ready to use. Prepare 1 mL cryopreservation medium for every vial plus a little extra for pipet error.
4. Pre-warm the required volume of Essential 8™ Medium at room temperature.

[30] At least one well of cells should be left and used as a backup to protect against problems with the split.

[31] Work quickly to remove cells after adding Essential 8™ Medium to the well. Do not passage more than one to three wells at a time.

[32] The split ratio is variable, though generally between 1:2 and 1:4. A general rule is to observe the last split ratio and adjust the ratio according to the appearance of the iPSC colonies. If the cells look healthy and colonies have enough space, split using the same ratio, if they are dense and crowding, increase the ratio, and if the cells are sparse, decrease the ratio.

[33] While cells are attaching, try to limit opening and closing the incubator doors, and if you need to access the incubator, open and close the doors carefully. This will prevent disturbing the even distribution of cells to the surface of the well.

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5. Remove differentiated colonies before passaging the iPS cells if necessary in the hood equipped with a stereomicroscope with a sterile modified pipette or sterile micropipette tip.
6. Transfer the plate in the biosafety cabinet and aspirate the spent medium from the wells to remove floating picked differentiated colonies in medium.
7. Rinse once with 1 mL warmed PBS without Ca^{+2} and Mg^{+2}.
8. Add 1 mL room temperature 0.5 mM EDTA solution to each well to be passaged.
9. Incubate the plate at room temperature for 5–8 min or 37 °C for 4–5 min.
10. Check the colonies under a microscope to confirm appropriate incubation time. When the cells start to separate and round up, and the colonies appear to have holes in them, they are ready to be removed from the plate.
11. Aspirate the EDTA solution gently from each well.
12. Add 1 mL of warmed complete Essential 8™ Medium to each well. Wash the cells from the surface of the wells by pipetting the colonies up using a 5 mL pipette. Do not scrape the cells from the dish.
13. Collect cells in a 15 mL sterile conical tube after all the cells are removed from the surface of the well.
14. Pipette cells up and down gently a few times in the conical tube to further break up iPS cell colonies if needed (*see* Note 34).
15. Centrifuge at 149 × *g* for 5 min.
16. Aspirate the supernatant and resuspend each cell pellet in enough cryopreservation medium.
17. Pipette up and down two times to mix. Freeze iPSC at 1 well/ cryovial (stem cells from each well go into 1 mL cryopreservation medium).
18. Distribute 1 mL of cell suspension to each of the prepared cryovials, tighten caps and place cryovials into an isopropanol containing freezing container.
19. Place the freezing containers in the −80 °C freezer overnight and transfer cell vials to liquid nitrogen storage the following day.

3.4 Differentiation of Human iPSC to Lung Progenitors

3.4.1 Differentiation of iPSCs to Definitive Endoderm

1. Aspirate the human stem cell medium or MTeSR medium from each well and add 1.5 mL of the endoderm differentiation medium directly to the each well of iPSC in a 6-well plate while iPSC colonies are still on MEF or Matrigel.
2. Change the medium the next day.

[34] Try not to break clumps into little collections of cells. Cells will recover from the thaw more efficiently if frozen in large aggregates.

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3. After 48 h, switch the medium to the same definitive endoderm medium, supplemented with 1× B27 supplement and 0.5 mM sodium butyrate.
4. Culture the cells in this medium for another 3–4 days with changing the medium daily.
5. Stain one well of differentiated cells to DE cells for CXCR4/ c-Kit or SOX17 to evaluate the efficiency of induction (*see* Note 35).

3.4.2 Differentiation of Definitive Endoderm to Anterior Foregut Endoderm

1. Thaw as many as aliquots of human ECM gel may be needed, depending on volume of differentiation, on ice.
2. For coating one 6-well plate, transfer 9 mL cold, sterile DMEM/F-12 medium to a 15 mL conical tube in sterile biosafety cabinet.
3. Add 1 mL of cold DMEM/F-12 medium to one of the 100 μL human ECM gel aliquot and transfer it to 15 mL conical tube using 1 mL sterile pipette and pipette up and down to dissolve it.
4. Add 1 mL of human ECM gel solution to each well of a 6-well plate.
5. Incubate it in 37 °C incubator 24 h before use (*see* Note 36).
6. Aspirate the DE medium from each well using 2 mL sterile aspirating pipette.
7. Wash each well with warmed DMEM-F12 to remove floating dead cells in medium.
8. Trypsinize the DE cells with diluted 0.25 % trypsin (1:3) for 1 min and check cells every 30 s to confirm appropriate incubation time.
9. When the cells start to be round and half are detached from the plate, add 1 mL warmed FBS to neutralize trypsin quickly.
10. Collect the cells and spin them down at 233 × *g* for 3 min.
11. Determine how much AFE medium is required based on the split ratio (The split ratio is 1:2) and the number of wells that will be used. In general, there should be a total of 2 mL of AFE medium in each of the new wells.
12. Aspirate excess ECM from each well gently without damaging the ECM on the plates.
13. Resuspend the cell pellets in the required amount of AFE medium and transfer to human ECM coated plate. Add 2 mL of cell suspension to each well of the ECM coated plate.

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[35]Proper maintenance of human iPSC in culture is critical for efficiency of endoderm induction. If the efficiency gradually decreases over several differentiations for a specific cell line, check the maintenance methods and reagents.

[36]Prepare a 6-well plate with human ECM protein 24–48 h before transferring DE cells onto the ECM coated plate. Wrap the extra plates in Parafilm and store in a refrigerator at 2–8 °C and use the plates within 7–10 days after preparation. If any portion of the well dries out, do not use the well.

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14. Add Rock Inhibitor at 1 μM concentration to the AFE medium during first 24 h after transferring DE cells onto ECM coated plate to increase the viability and attachment of DE cells.

15. Return the plate to the 37 °C incubator after plating the cells. Move the plate back-and-forth and side-to-side a few times to further disperse cells across the surface of the wells.

16. Change the AFE medium after 24 h.

17. Culture the cells in the AFE medium 48 h.

3.4.3 Differentiation of DE to AFE Cells While They Are Still on MEF or Matrigel

1. Aspirate the DE medium from each well using sterile aspirating pipette.

2. Wash the cells with 1 mL warmed RPMI to remove floating dead cells in the medium.

3. Add 2 mL of the anterior foregut endoderm differentiation medium directly to the each well of iPSC in a 6-well plate, while iPSC-derived DE are still on MEF or Matrigel.

4. Change the medium the next day.

5. Culture the cells in this medium for another 24 h (*see* Note 37).

3.4.4 Differentiation of Anterior Foregut Endoderm to Lung Progenitors

1. Beginning on day 9 of differentiation, switch the medium to the lung progenitor differentiation medium. Aspirate AFE medium from each well gently using 2 mL sterile aspiration pipette.

2. Add 2 mL of the lung progenitor differentiation medium to each well.

3. Return the plate to the incubator.

4. Change the medium every other day until day 15 of differentiation.

5. To check the differentiation efficiency to lung progenitor cells, stain cells for the co-expression of FOXA2 and NKX2.1 at day 15.

3.5 iPSC Generation Using Lentiviral Vectors Protocol (Reprogramming Fibroblasts by an Inducible Lentiviral System)

3.5.1 Skin Biopsy and Isolating Fibroblasts

1. To isolate the fibroblasts from the skin biopsy, cut the skin biopsy into several small pieces with razor blade.

[37]To assess the appropriate anterior foregut endoderm induction, stain a couple of wells without switching to lung progenitor differentiation medium for SOX2 and FOXA2.

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2. Put each small piece in one well of the 6-well plate and cover it with coverslip. The coverslip helps to keep skin from moving around.
3. Add 2 mL DMEM medium to each well of the 6-well plate.
4. After 3 days, the skin cut will attach and fibroblasts from skin start to sprout out. The coverslip can be taken out from each well and let the fibroblast cells grow.
5. Change the medium every 2 days.
6. When each well of a 6-well plate becomes confluent, expand the isolated fibroblasts to a 10 cm cell culture dish.
7. Aspirate the DMEM medium from the culture dish using sterile 2 mL aspirating pipette.
8. Rinse each well with 1 mL PBS without $CaCl_2$ or $MgCl_2$ using 5 mL serological pipette.
9. Add 1 mL pre-warmed 0.25 % trypsin solution to the each well of a 6-well plate.
10. Incubate the cells with trypsin for 3 min at 37 °C incubator.
11. Check the plate under the microscope every 2 min to find the appropriate incubation time.
12. When the majority of the cells look round and start detaching from the surface, add 2 mL of DMEM medium to each well to neutralize the trypsin enzyme.
13. Pipette the medium slowly up and down to wash the cells off the surface.
14. Transfer cells to 15 mL sterile conical tube after all the cells are removed from the surface of the flask.
15. Centrifuge at $149 \times g$ for 5 min.
16. Resuspend cells in 1 mL of DMEM medium.
17. Count the cells number using hemacytometer.
18. Plate 5×10^5 fibroblast cells in each 10 cm plate in 10 mL DMEM medium.
19. Return the plates to the 37 °C incubator and let the cells attach to the plate.
20. Feed the fibroblast cells every 2 days until ready to passage or freeze.

3.5.2 Freezing Human Fibroblasts

1. When each 10 cm plate becomes confluent, freeze the fibroblast at passage 2 or 3. Fibroblasts can be reprogrammed more efficiently at low passage numbers.
2. Label the cryovials with the cell line, passage number, the freeze date and your initials.
3. Aspirate the DMEM medium from the flask using sterile 2 mL aspirating pipette.
4. Rinse each well with 1 mL PBS without $CaCl_2$ or $MgCl_2$ using 5 mL serological pipette.

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5. Add 2 mL pre-warmed 0.25 % trypsin solution to the flask.
6. Incubate the cells with trypsin for 3 min at 37 °C incubator.
7. Check the plate under the microscope every 2 min. When the majority of the cells look round and start detaching from the surface, add 5 mL of DMEM medium to the flask to neutralize the trypsin enzyme.
8. Pipette the medium slowly up and down to wash the cells off the surface.
9. Transfer cells to 15 mL sterile conical tube after all the cells are removed from the surface of the flask.
10. Centrifuge at 149 × *g* for 5 min.
11. Resuspend cells in 1 mL of DMEM medium.
12. Count the cells number using hemacytometer.
13. Resuspend fibroblast cells in enough freezing medium. Freeze fibroblast cells at 5×10^4/cryovial.
14. Tighten caps and place cryovials into isopropanol containing freezing container.
15. Place the freezing containers in the −80 °C freezer overnight and transfer cell vials to liquid nitrogen storage the following day.

3.5.3 Thawing the HEK293T

1. Turn on the blower and spray down the whole surface of the hood with ethanol and allow it to evaporate for 20 min prior to initiating cell culture.
2. Remove a frozen vial of HEK293T (2×10^6 cells) from the liquid nitrogen tank and thaw by immersing the vial in a 37 °C water bath without submerging the cap. Swirl the vial gently.
3. Remove the HEK293T vial from the water bath when only a small ice crystal remains.
4. Spray the vial with a 70 % ethanol to sterilize the outside of the tube and transfer it into the sterile biosafety cabinet.
5. Transfer the HEK293 cells gently into a sterile 15 mL conical tube using a 1 mL serological pipette.
6. Slowly, add 4 mL of warmed DMEM medium dropwise to cells in the 15 mL conical tube. Gently move the tube back and forth to mix the cells, while adding the medium. This reduces osmotic shock to the cells.
7. Centrifuge the cells at 233 × *g* for 5 min.
8. Aspirate and discard the supernatant with a sterile aspirating pipette.
9. Resuspend the cell pellet in 10 mL DMEM medium; gently pipette cells up and down few times and add 2.5×10^6 cells/ 10 mL to the T-75 cell culture flask.
10. Transfer the flask to 37 °C incubator (*see* Note 38).

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3.5.4 Prepare HEK-293 Plates—To start generating the virus, split the HEK293T flask into a 10 cm dish when it is 70 % confluent.

1. Aspirate the DMEM medium from the flask using sterile 2 mL aspirating pipette.
2. Add 5 mL PBS without $CaCl_2$ or $MgCl_2$ to the flask using 5 mL serological pipette.
3. Aspirate the PBS using sterile 2 mL aspiration pipette.
4. Add 2 mL pre-warmed 0.25 % trypsin solution to the flask.
5. Incubate the cells with trypsin for 3 min in a 37 °C incubator.
6. Check the plate under the microscope every 2 min to find the appropriate incubation time.
7. When majority of the cells look round and start detaching from the surface, add 5 mL of DMEM medium to the flask to neutralize the trypsin enzyme.
8. Pipette the medium slowly up and down to wash the cells off the surface.
9. Transfer cells to 15 mL sterile conical tube after all the cells are removed from the surface of the flask.
10. Centrifuge at 149 × *g* for 5 min.
11. Resuspend cells in 1 mL of DMEM medium.
12. Count the cells number using hemacytometer.
13. Transfer the 5×10^6 HEK293T cells into 10 cm culture dish so at the day of transfection (next day) ideally you have around 6×10^6 cells/10 cm dish (around 70 % confluence) (*see* Note 39).
14. Return the plates to the 37 ° C incubator and let the cells attach to the plate.

3.5.5 Transfection of HEK-293

1. Two hours before transfection, replace the medium with 10 mL of fresh preheated at 37 °C DMEM medium without antibiotics.
2. For each 10 cm dish, prepare the following transfection mix in a 1.5 mL eppendorf tube and incubate at room temperature for 5 min:
   - 10 μg vector plasmid (with gene of interest, e.g., Oct4, Sox2)
   - 2.5 μg envelop plasmid (pMD2G codes for the broad range VSV-G envelope)
   - 7.5 μg packaging plasmid psPAX2 (It codes for packaging protein and is suitable for most studies)

[38]It is important to use low passage HEK293T cells for the production of viruses. To make sure the cells are always in the fastest growth phase, never let the cells grow to 100 % confluence.
[39]Prepare eight plates of HEK293T, each plate for making one type of virus.

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- 1.5 mL Opti MEM without FBS.

3. To prepare the Lipofectamine 2000 transfection solution, mix 36 μL of Lipofectamine 2000 with 1.5 mL Opti MEM without FBS in a 1.5 mL eppendorf tube and incubate for 5 min at room temperature.
4. Add the plasmid mix to the Lipofectamine solution dropwise, and then mix them gently by pipetting up and down.
5. Incubate the transfection mix for 20 min at room temperature.
6. Add dropwise 3 mL/dish of the transfection mix, and mix gently by rotating the 10 cm dish.
7. Transfer the plates to 37 °C incubator.
8. Remove medium around 14–16 h post-transfection and put 10 mL/dish of fresh warmed DMEM medium.
9. Collect supernatant for the first time 48 h after transfection.
10. Supernatant can be harvested two times, every 24 h. Collect supernatant for the second time 72 h after transfection. Keep supernatant at 4 °C during the collecting period.
11. Pool the collected supernatants for each individual virus and centrifuge 5 min at 524 × *g* to remove cell debris.
12. Filter the supernatant for each virus separately through 0.4 μm filter.
13. The cleared supernatants can be kept at 4 °C for 4–5 days. Supernatants can be used directly, stored at −80 °C in aliquots, or concentrated if needed.

3.5.6 Virus Concentration—To make more concentrated virus, each virus can be concentrated by ultracentrifuge.

1. Transfer the virus solution to the sterile ultracentrifuge.
2. Ultracentrifuge it at 47.000 × *g* for 2 h at 16 °C in a swinging rotor (or alternatively at 19.500 rpm in a Beckman SW32 Ti rotor).
3. After centrifuge is finished, transfer the tube to the biosafety cabinet.
4. Discard supernatant and resuspend pellet of each virus in 100–200 μL of sterile cold PBS (try to make a 100 or 1000-fold concentration).
5. The concentrated virus can be used directly or aliquot and store at −80 °C for future use.

3.5.7 Virus Titration—Titer the generated virus using a p24 ELISA kit, to confirm the success of virus packaging reaction and to avoid wasting time with your experiments. To perform consistent experiments, calculate the sufficient MOI for fibroblast transduction.

1. Allow all reagents to reach room temperature (18–25 °C).

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2. Select a sufficient number of 8-well strips to accommodate all standards, test samples, controls, and culture medium blanks in duplicate.

3. Label wells according to sample identity using the letter/number on the plastic frame.

4. Dispense 200 μL of each standard, sample, and blank into corresponding labeled duplicate wells and follow the manufacturer's instruction of Lenti-X™ p24 Rapid Titer.

5. After reading the absorbance values, calculate the virus particle from each plate based on P24 protein concentration. The following values and calculations can be used to determine approximate titers, and are based on the observation that each lentiviral particle (LP) contains approximately 2000 molecules of p24:

   - 1 LP contains 8×10^{-5} pg of p24 (derived from (2000) × (24 × 103 Da)/(6 × 1023).
   - ng p24 is equivalent to ~1.25×10^{7} LPs.
   - For a typical lentiviral vector, there is 1 IFU for every 100–1000 LPs.
   - Therefore, a supernatant titer of 10^{7} IFU/mL ≈ 109–1010 LP/mL or 80–800 ng p24/mL.

3.5.8 Prepare Fibroblast Cells for Reprogramming—The following protocol is based on human fibroblasts and will be applicable to most other cell types. If using skin fibroblast cells, follow the plating protocol listed below. If using another cell type, a different plating density may be required. Follow the instructions provided at the beginning of this section to prepare MEF medium, hESC medium, bFGF solution, etc. before starting the reprogramming process.

1. Plate 2×10^{5} fibroblast cells at passage 2 or 3 on one well of a gelatin coated 6-well plate (~70 % confluent) or 1×10^{5} cells in 3.5 cm dish in DMEM medium, one day before viral infection, including one well for GFP control. Incubate overnight at 37°C to reach up to 80 % confluency.

2. The following day, dilute enough amount of each lentivirus to make the recommended MOI below for each lentivirus to 1 mL culture medium without FBS and add polybrene at a concentration of 2 μg/mL.

   - FUW-tetOLoxP lentiviruses of hOct4: MOI = 15.
   - FUW-tetOLoxP lentiviruses of hSox4: MOI = 15.
   - FUW-tetOLoxP lentiviruses of hKlf4: MOI = 15.
   - FUW-tetOLoxP lentiviruses of hNanog: MOI = 15.
   - FUW-tetOLoxP lentiviruses of hc-Myc: MOI = 6.
   - M2rtTA: MOI = 30.

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3. Aspirate fibroblast medium from 6-well plate and add mix virus (1 mL) to each well of cells to be reprogrammed in the 6-well.
4. Move the cell culture dish gently side-to-side and back-and-forth to ensure that the medium is evenly distributed.
5. Incubate for 4 h at 37 °C.
6. Add 1 mL of fibroblast medium supplemented with 20 % FBS.
7. Return the plate to a CO_2 incubator and incubate overnight.
8. The following day, aspirate virus-containing medium and add 2 mL of fresh fibroblast medium to each reprogrammed well.
9. Change medium again after 48 h.

3.5.9 Determine Infection Efficiency—48 hours post infection; the efficiency can be assessed by immunostaining for 2–3 transcription factors.

1. Plate the extra human fibroblasts into a 24 well plate.
2. Transduce the cells with the same MOI and concentration of virus that was used to reprogrammed human fibroblasts, follow the instructions described in Section 3.5.8.
3. About 48 h after infection, perform immunocytochemistry staining for 1 transcription factor per well (*see* Note 40).

3.5.10 Re-plate Infected Cells—24 hours to 72 hours post viral treatment, re-plate the fibroblast cells onto a MEF layer.

1. Prepare the MEF in a 10 cm dish, the day prior to splitting the infected cells. Seed 1.2×10^7 MEF cells in MEF medium in each 10 cm dish.
2. Wash the well of infected fibroblasts with 2 mL of PBS using 5 mL sterile pipette.
3. Aspirate the PBS and add 1 mL of warmed 0.05 % trypsin enzyme.
4. Incubate the plate for 2 min at 37 °C.
5. When the majority of the cells look round and start detaching from the surface, add 2 mL of medium to each well to neutralize the trypsin enzyme.
6. Pipet the medium across the surface of the well until the cells appear completely detached.
7. Transfer cells to 15 mL sterile conical tube after all the cells are removed from the surface of the wells.

[40]For each transcription factor to be tested, incubate a Dox-induced (1 μL Dox for 48 h) and non-induced well with both the primary and secondary antibodies. The remaining Dox-induced well should be used as a negative control by incubating with the secondary antibody only.

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8. Centrifuge the cells for 5 min at 233 × *g*.
9. Remove the supernatant.
10. Resuspend cells 1 mL of fibroblast medium and count the total number of cells in solution using a hemacytometer.
11. Add the appropriate amount of fibroblast medium to the 15 mL conical tube to bring the cell suspension to 2.5×10^5 cells/mL.
12. Mix the cell solution gently in order to create a uniform suspension of single cells.
13. Seed the 2×10^5 infected skin fibroblasts onto MEF in DMEM medium.
14. Return the plates to the 37 °C incubator and let the cells attach to the plate.
15. About 24 h after plating of infected human fibroblast on MEF, aspirate the fibroblast medium from the plate.
16. Add 10 mL of Dox induction medium supplemented with 2 % FBS and 1 μL Dox and 1 mM VPA to induce the expression of pluripotency genes.
17. Feed the cells with 10 mL of Dox induction medium every 24 h for 6 days (Day 2 to day 7 after re-plating infected cells onto MEF) (*see* Note 41).
18. After 6 days, switch the medium to hESC medium supplemented with only 1 μg/mL of Dox for 14 days (Day 8 to day 25 after re-plating infected cells onto MEF).
19. Wait for 2–4 weeks for iPSC colonies to form (*see* Note 42).

3.5.11 Picking and Expansion of iPSC Colonies

1. Once iPSC colonies form, prepare the MEF cells in a 24-well plate at a concentration of 9×10^4 per well (80 % confluent).
2. Three hours before picking the colony, aspirate the MEF medium and rinse it with pre-warmed DMEM-F12 medium.
3. Add 200 μL of stem cell culture medium to each well and transfer the plate to the 37 °C incubator.
4. Pick colonies manually and transfer one colony into the one new MEF in 24-well plate (*see* Note 43).

[41] Add 10 mL of human iPSC culture medium to one plate, which will serve as a negative control.

[42] to ensure completion of the reprogramming process, it is necessary to remove Dox from the induction medium once colony with good morphology are observed. We recommend removing Dox from the medium at day 25. Removal of Dox ensures that the iPS cell colonies picked and passaged around day 30 are reliant on endogenous expression of pluripotency genes and are not the result of sustained induction of ectopic transcription factor expression by Dox.

[43] Some transduced fibroblasts in a 10 cm dish may reprogram later. To get the maximum reprogramming efficiency, trypsinize the rest of the cells into a 10 cm plate. Transfer the cells from each of the 10 cm plates into a new MEF cell plate and change medium every 48 h with hESC culture medium. Wait for another 1–2 weeks for iPSC colonies to develop.

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5. When MEF cells become too old (about 2 weeks) or a lot of iPSC colonies have developed in the 24-well plate, prepare new a MEF feeder layer in 6-well plates as described before to expand the iPSC colonies.
6. Using the same procedures described in the beginning of the chapter, iPSCs can be further expanded to meet your lab needs for iPSC analysis or down-stream applications.

References

1. Aasen T, Raya A, Barrero MJ, Garreta E, Consiglio A, Gonzalez F, Vassena R, Bili J, Pekarik V, Tiscornia G, et al. 2008; Efficient and rapid generation of induced pluripotent stem cells from human keratinocytes. Nat Biotechnol. 26(11):1276–1284. [PubMed: 18931654]
2. BD Biosciences. Assay methods protocol: human embryonic stem cell culture. 2013. https://www.daigger.com:1-7
3. Ghaedi M, Calle EA, Mendez JJ, Gard AL, Balestrini J, Booth A, Bove PF, Gui L, White ES, Niklason LE. 2013; Human iPS cell-derived alveolar epithelium repopulates lung extracellular matrix. J Clin Invest. 123(11):4950–4962. [PubMed: 24135142]
4. Huang SX, Islam MN, O'Neill J, Hu Z, Yang YG, Chen YW, Mumau M, Green MD, Vunjak-Novakovic G, Bhattacharya J, et al. 2014; Efficient generation of lung and airway epithelial cells from human pluripotent stem cells. Nat Biotechnol. 32(1):84–91. DOI: 10.1038/nbt.2754 [PubMed: 24291815]
5. Huangfu D, Maehr R, Guo W, Eijkelenboom A, Snitow M, Chen AE, Melton DA. 2008; Induction of pluripotent stem cells by defined factors is greatly improved by small-molecule compounds. Nat Biotechnol. 26(7):795–797. [PubMed: 18568017]
6. Kim D, Kim CH, Moon JI, Chung YG, Chang MY, Han BS, Ko S, Yang E, Cha KY, Lanza R, et al. 2009; Generation of human induced pluripotent stem cells by direct delivery of reprogramming proteins. Cell Stem Cell. 4(6):472–476. [PubMed: 19481515]
7. Longmire TA, Ikonomou L, Hawkins F, Christodoulou C, Cao Y, Jean JC, Kwok LW, Mou H, Rajagopal J, Shen SS, et al. 2012; Efficient derivation of purified lung and thyroid progenitors from embryonic stem cells. Cell Stem Cell. 10(4):398–411. [PubMed: 22482505]
8. Ludwig TE, Levenstein ME, Jones JM, Berggren WT, Mitchen ER, Frane JL, Crandall LJ, Daigh CA, Conard KR, Piekarczyk MS, et al. 2006; Derivation of human embryonic stem cells in defined conditions. Nat Biotechnol. 24(2):185–187. [PubMed: 16388305]
9. Stein GS, Borowski M, Luong MX, Shi M-J, Smith KP, Vazquez P. 2010; Human embryonic stem cell culture on BD Matrigel™ with mTeSR®1 medium. Human Stem Cell Technol Biol. doi: 10.1002/9780470889909.ch11
10. Stem Cell Technologies. Technical manual: maintenance of human pluripotent stem cells in mTeSR™1 and TeSR™2. 2012. http://www.stemcell.com:1-24
11. 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(5):861–872. [PubMed: 18035408]
12. Van Haute L, De Block G, Liebaers I, et al. 2009; Generation of lung epithelial-like tissue from human embryonic stem cells. Respir Res. 10:105. [PubMed: 19891764]
13. Wang D, Haviland DL, Burns AR, Zsigmond E, Wetsel RA. 2007; A pure population of lung alveolar epithelial type II cells derived from human embryonic stem cells. Proc Natl Acad Sci U S A. 104(11):4449–4454. [PubMed: 17360544]
14. Woltjen K, Michael IP, Mohseni P, Desai R, Mileikovsky M, Hämäläinen R, Cowling R, Wang W, Liu P, Gertsenstein M, et al. 2009; piggyBac transposition reprograms fibroblasts to induced pluripotent stem cells. Nature. 458(7239):766–770. [PubMed: 19252478]
15. Wong AP, Bear CE, Chin S, Pasceri P, Thompson TO, Huan LJ, Ratjen F, Ellis J, Rossant J. 2012; Directed differentiation of human pluripotent stem cells into mature airway epithelia expressing functional CFTRTR protein. Nat Biotechnol. 30(9):876–882. [PubMed: 22922672]

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16. Yu J, Hu K, Smuga-Otto K, Tian S, Stewart R, Slukvin II, Thomson JA. 2009; Human induced pluripotent stem cells free of vector and transgene sequences. Science. 324(5928):797–801. [PubMed: 19325077]