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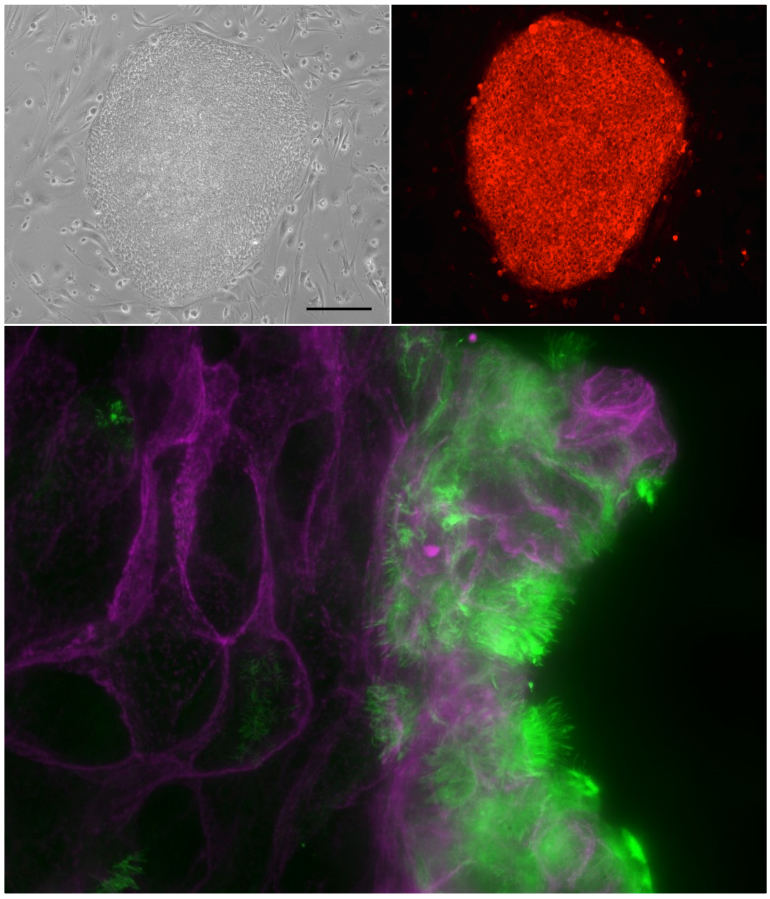
IPSC CORE

The Fourth Annual

Training Course in Human Induced Pluripotent Stem Cells and their Differentiation into Endoderm and Lung Progeny



April 1st-5th, 2019
Boston, MA

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iPSC CORE

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Cover: Human iPS cells stained with SSEA-4 (top, iPSC Core) and W1282X Day 19 ALI stained with EPCAM (violet) and Ac α-Tubulin (green): passaged on Differentiation Day 55 from 3D to Matrigel Coated Transwells; exposed to Air after 4 Days (bottom, M.L.B and FH, Hawkin's Lab)

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Training Course Description

This five-day course will focus on deriving, maintaining and characterizing pluripotent stem cells (hiPSCs) and their differentiation to endoderm and lung progeny. The course is designed for research scientists working with or planning to work with human iPSC culture who have prior experience in general cell culture techniques. It will include hands-on training, lectures, and demonstrations from leading experts and educators in the field of stem cell biology from the Center for Regenerative Medicine (CReM) at Boston University and Boston Medical Center. Small class size will enable researchers to learn the process of reprogramming from somatic cell preparations, including iPS cell identification, isolation and characterization, and current approaches in directed differentiation to endodermal and lung lineages. Additional topics will include lectures and training in novel gene editing techniques, including the design and use of CRISPR/Cas9 in iPSCs, and development of 3D culture systems.

LECTURE TOPICS

- Isolation and expansion of somatic cells (blood and fibroblast cells) prior to reprogramming
- Overview of different reprogramming methods for the generation of iPSC lines
- iPSC culture methods using feeder-dependent and feeder-free systems
- Characterization (IF staining; qPCR) and validation (fingerprinting, karyotyping) of iPSC colonies
- Directed differentiation to definitive endoderm/NKX2.1+ lung progeny
- Gene editing and CRISPR/Cas9 design and construction
- Understand principles of flow cytometry (FACS) in the context of iPSC research
- Discussion of immunofluorescence-stained iPSC colonies using antibodies against stem cell surface markers
- Discussion and troubleshooting with course instructors
- Assay for mycoplasma contamination

PRACTICAL LABORATORY SESSIONS

- Expansion of PBMCs for reprogramming
- Reprogramming of PBMCs: observe OKSM viral transduced PBMCs (using both feeder-free (Matrigel™) and MEF feeder systems) at early- and late-stages of reprogramming process and observe morphological changes
- iPS cell culture propagation and maintenance on feeder (MEFs) and feeder-free matrices
- Colony passaging by manual picking and cell dissociation reagents; removing (“cleaning”) differentiated cells
- Cryopreservation and thawing of hiPSC onto MEF feeders and feeder-free dishes
- Preparation of definitive endoderm
- Identify NXK2-1+ lung progenitors by FACS analysis; hands-on introduction to flow cytometry (FACS)
- NKX2-1 immunostaining
- Proximal vs distal lung protocol
  - 3D CX plating onto matrigel
  - observe distal spheres and passage alveolospheres
- Observation of immunofluorescence-stained iPSC colonies using pluripotentcy markers

iPSC Training Course Schedule

	MONDAY	TUESDAY	WEDNESDAY	THURSDAY	Friday
9:00am	Welcome (9:05) (DK) Reprogramming (GM)	ESCs and the embryo (DK) Pluripotency Endoderm	Breakfast with Instructors and Students	Gene Editing (GM)	Lung (DK)
10:00am	General iPSC culture methods (MJ). Detailed description of culture, passaging, etc. Intro to week	Mycoplasma (Greg)	Observe Monday and Tuesday iPS cells	CRISPR design (George)	Intro characterization (MJ)
		Detailed description freeze/thaw methods (MJ)	Thaw hiPSC from previous day onto Matrigel™		Observe all cells done during the week Observe proximal and distal spheres Dispase alveolospheres (KDA/LS/DR)
11:00am	- View expanded PBMCs - View reprogrammed cells	Freeze hiPSCs		Observe plated endoderm cells from Wed LUNG PROGENITORS: NKX2-1+ Observe Day 15 Cells and see analysis of these cells by FACS (RW, MLB, NL)	
	Plate Matrigel™		(ARR/NL/MJ)		
noon	Lunch	Lunch	Lunch	Lunch	Lunch.
1:00pm	Passage hiPSCs onto Matrigel™ with Gentle Cell™	Directed Differentiation/ Definitive Endoderm (Finn)	Observe cells from the start of lung differentiation on Tues	Prepare Day 15 cells for FACS/replating (RW, MLB, DR)	Passage alveolospheres (KDA/LS/DR)
		Start lung directed differentiation: Passing cells onto Matrigel™ (AB/NL/KA)	ENDODERM: CKIT/CXCR4 See analysis of cells by FACS Plate definitive endoderm		
2:00pm	Plate inactive MEFs (demo)			3-D matrigel culture of lung progenitors	Q+A
		Practice Picking hiPSCs onto MEFs/Matrigel™			
3:00pm	Passage hiPSCs onto MEFs (1:3 and 1:10) with collagenase type IV		(KDA/NL/KA)	Proximal v distal lung protocol Nkx2-1 Immunostaining (3:45)	Beer hour, all CReM invited! 
4:00pm		Feed hIPSCs (ARR/NL/MJ)	Feed hIPSCs (ARR/MJ)	Feed hIPSCs (ARR/MJ)	
	(ARR/NL/MJ/)				

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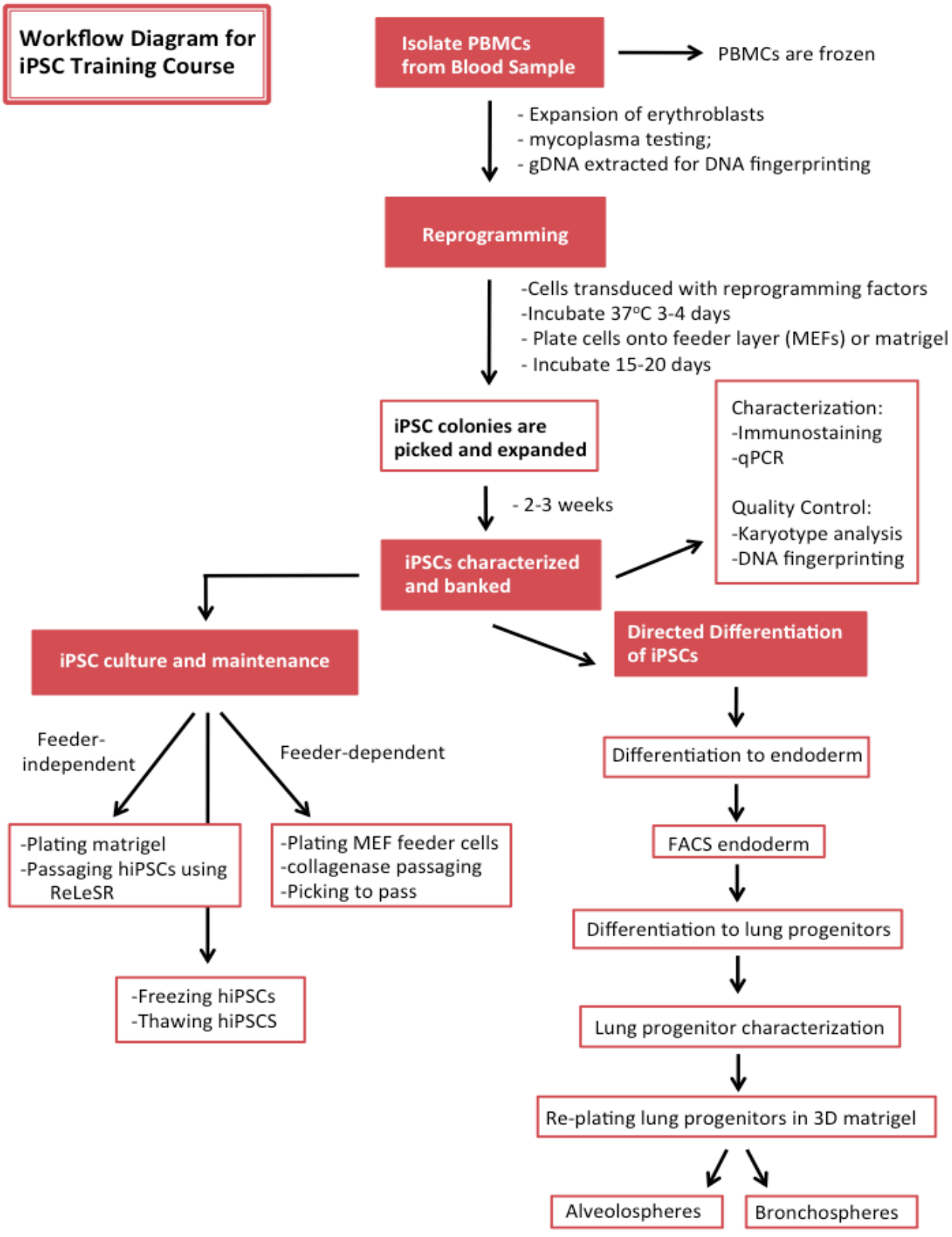

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Isolation of Peripheral Blood Mononuclear Cells (PBMCs) for Reprogramming

Materials and reagents:

- Chlorhexidine chloraprep applicators (Fisher Cat# 14-910-43)
- Sterile Alcohol Prep Pads (Dynarex 1103)
- BD vacutainer CPT cell preparation tubes with sodium citrate (BD Cat# 362760): http://www.bd.com/vacutainer/pdfs/bd_cpt_VDP40104.pdf
- Dulbecco's PBS (Invitrogen Cat# 14190-144)
- Fetal Bovine Serum (FBS) Hyclone Defined (Cat# SH30070.03), 0.22 micron filter before use
- DMSO (Sigma Cat #D2650)
- 1.2 mL cryovials (Corning Cat# 430487)

1. Clean venipuncture site and top of blood draw tube thoroughly with chlorhexidine prep or 70% isopropyl alcohol pads and allow to dry.

2. Draw 4 mLs of peripheral blood into each of two BD Vacutainer Cell Preparation Tubes (CPTs) with sodium citrate. Invert the tubes 8 to 10 times and keep upright at room temperature (RT)

3. Centrifuge at 1,800 x g for 30 min at RT. Ideally, this step should be done within 2 hrs of collection.

4. Using a 1 mL pipette tip, collect the mononuclear cells (MCs) by pipetting the buffy coat (cell layer between gel barrier and plasma) into a sterile 15 mL conical centrifuge tube.

5. Bring total volume to 10 mLs with sterile Dulbecco's phosphate-buffered saline (DPBS), pipette to mix or invert several times.

6. Centrifuge at 300 x g for 15 min at RT and aspirate supernatant

7. Resuspend cell pellet in 10 mL of sterile DPBS and perform cell count

8. Centrifuge at 300 x g for 10 min at RT and aspirate supernatant

9. Resuspend cell pellet in fetal bovine serum (FBS) containing 10% DMSO to freeze. Aliquot ~$2x10^6$ cells/cryovial in 1 mL volume. Place cryovials in a pre-cooled freezing container and freeze at -80°C overnight. Transfer cryovial to liquid nitrogen storage the following day for long term storage. Typical yield is ~$1x10^6$ cells/mL blood drawn; for 8 mLs blood drawn, should obtain 6-$8x10^6$ cells for 3-4 frozen cryovials.

Reference:

Sommer AG, Rozelle SS, Sullivan S, Mills JA, Park SM, Smith BW, Iyer AM, French DL, Kotton DN, Gadue P, Murphy GJ, Mostoslavsky G. Generation of human induced pluripotent stem cells from peripheral blood using the STEMCCA lentiviral vector. J Vis Exp. 2012 Oct 31;(68). doi:pii: 4327. 10.3791/4327. PMID: 23149977 (see online video demo.).

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Generation of iPS Cells on MEFS from Human Peripheral Blood

Induced pluripotent stem cells (iPSCs) can be generated from freshly collected or previously frozen peripheral blood (PB) or bone marrow (BM) mononuclear cells (MNCs). This method describes the steps used to reprogram human PBMCs to iPS cells on mouse embryonic fibroblasts (MEF) feeder layers.

Materials and reagents:

- QBSF-60 serum free medium (Fisher Cat# NC0823508)
- Primocin (InvivoGen Cat# ant-pm-2)
- Ascorbic Acid (SIGMA Cat# A4544)
- SCF/c-kit Ligand (R&D Systems Cat# 255-SC)
- IL-3 (R&D Systems Cat# 203-IL)
- Epo (R&D Systems Cat# 287-TC)
- IGF-1 (R&D Systems Cat# 291-G1-01M)
- Dexamethasone (SIGMA Cat# D4902)
- FGF basic protein (R&D Systems Cat# 233-FB-500)
- CytoTune-iPS 2.0 Sendai Reprogramming Kit (Invitrogen, ThermoFisher, Cat# A16517)
- Polybrene (SIGMA Cat# H9268)
- DMEM/F12 (Life Tech. Cat# 11330-057)
- Fetal Bovine Serum (FBS) Hyclone Defined (Cat# SH30070.03), 0.22 micron filter before use
- IMDM medium (Life Tech. Cat# 12440-061)
- GlutaMAX supplement (100X, 200 mM; Fisher Cat# 35050061)
- NEAA (MEM non-essential amino acids, ThermoFisher Cat# 11140050)
- hESC medium
- Inactive MEF feeder cells
- 2-mercaptoethanol (BME) 55mM 1000x (Gibco ThermoFisher Cat# 21985023)
- Rock Inhibitor (Reprocell Cat# 04001202; Fisher Cat# NC0454602)
- 12-well plates (Fisher Cat# 07-200-84)
- 6-well plates (Fisher Cat# 07-200-83)
- 15 mL conical centrifuge tubes (Fisher Cat# 14-959-70C)

Day (-9): Erythroblast expansion

1. Transfer freshly isolated PBMCs (~ 2x10^6 cells) or, alternatively, thaw 1 vial of previously frozen PBMCs into 10 mL of QBSF medium and centrifuge at 300 RCF for 10 min at room temperature (RT).

2. Re-suspend cell pellet in 2 mL of EM (below) and transfer to 1 well (12 well plate). Incubate at 37^{o}C.

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Generation of iPS Cells on MEFs from Human Peripheral Blood (continued)

Expansion Medium (EM)* *Prepare fresh*	**Working Conc**	**Final Conc**	**Volume (5 mLs)**
QBSF-60 (serum free medium)			up to 5 mLs
Primocin	500X	100 µg/mL	10 µL
Ascorbic Acid (AA)	5 mg/mL	50 µg/mL	50 µL
Growth Factors:			
SCF	100 µg/mL	50 ng/mL	2.5 µL
IL-3	50 µg/mL	10 ng/mL	1.0 µL
EPO	2 U/µL	2 U/mL	5.0 µL
IGF-1	25 µg/mL	40 ng/mL	8.0 µL
Dexamethasone**	$5x10^{-5}$ M	1 µM	0.1 mL

*EM = QBSF-60 + Primocin + AA + growth factors; **Keep dexamethasone protected from light (discard every 2 weeks)

Days (-3 and -6): Erythroblast expansion and washing cells

3. Transfer cells to sterile 15 mL conical tube. Wash well with 1 mL QBSF-60 to collect adherent cells and combine in 15 mL conical tube.

4. Centrifuge cells at 300 RCF for 10 min at RT.

5. Aspirate supernatant and resuspend cell pellet in 2 mLs of fresh EM

6. Transfer cells to new well 12 well dish and continue culturing for an additional 3 days

Day (-9)/Day 0: Transduction

Transduce cells with reprogramming vectors that include the four Yamanaka factors, Oct3/4, Sox2, Klf4 and c-Myc using the Sendai virus delivery and expression system (Cytotune-iPS 2.0 Sendai Reprogramming Kit).

7. Transfer cells to sterile 15 mL conical tube. Wash well with 1 mL QBSF-60 to collect adherent cells and combine in 15 mL conical tube.

8. Centrifuge cells at 300 RCF for 10 min and resuspend in 1 mL of fresh EM plus polybrene (5 *µ*g/mL)

9. Count cells and plate 2 x 10^5 cells into one well of a 12 well dish.

10. Thaw each viral vector and calculate the volume of each virus needed to reach the target MOI using the live cell count and the titer information on the Certificate of Analysis (CoA). Refer to lot-specific CoA to obtain viral titer.

$$\text{Volume of virus (µL)} = \frac{\text{MOI (CIU/cell) x number of cells}}{\text{titer of virus (CIU/mL) x } 10^{-3} \text{ (mL/µL)}}$$

11. Add virus to cells and gently rock to distribute virus.

12. Spinnoculation: Spin plate at 2250 RPM at 25°C for 90 min. After spin, incubate plate in 37°C incubator.

13. At end of day, add additional 1 mL of EM to cells for a 2 mL total volume and return plate to incubator.

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Generation of iPS Cells on MEFs from Human Peripheral Blood (continued)

Day 1: Wash cells to remove virus

14. Transfer cells to 15 mL conical tube. Rinse well with 1 mL of QBSF to collect adherent cells and add to 15 mL conical tube. Centrifuge the cells at 300 RCF for 10 min at RT.

15. Aspirate supernatant and resuspend cell pellet in 2 mL fresh EM and continue to culture in 1 well of a 12 well plate.

Day 3: Plate MEF feeder layer and prepare MEF media

16. Plate inactive MEFs in MEF media onto 4-6well, gelatin(0.1%)-coated plates and incubate overnight.

MEF media	STOCK	Final Conc	Volume (50 mLs)
IMDM			up to 50 mLs
FBS	100%	10%	5 mL
GlutaMAX	100X	1X	0.5 mL
NEAA	100X	1X	0.5 mL
BME	55 mM	0.05 mM	45 µL
Primocin	500X	100 µg/mL	0.1 mL

Day 4: Plate cells onto MEFs

17. Prepare fresh 10 mLs MEF media supplemented with bFGF (10ng/mL) and Ascorbic Acid (AA) and growth factors/cytokines at concentrations described for EM media (above).

18. Remove MEF plate from incubator, aspirate MEF media and add 1.5 mL/well of MEF media supplemented with bFGF, AA and growth factors/cytokines. Return plate to incubator.

19. Collect cells into 15 mL conical tube. Wash well with 1 mL QBSF and combine with cells in 15 mL conical tube. Centrifuge at 300 RCF for 10 min.

20. Aspirate supernatant and resuspend cell pellet in 4 mLs MEF media supplemented with bFGF, AA and growth factors/cytokines.

21. Add 1 mL of cells to each of 4 MEF wells for a total volume of 2.5 mLs media per well. *(plate cells between 1 x 10^4- 5 x 10^4 cells per well)*

22. Centrifuge plate at 500 RPM at 25^{o}C for 30 min and return to incubator.

Day 6: Feed cells

23. Feed cells every other day with 2.5 mL of MEF media with *only* bFGF and AA supplements (no growth factors/cytokines).

24. Aspirate and discard floating cells with each feeding.

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Generation of iPS Cells on MEFs from Human Peripheral Blood (continued)

Days ~ 8 - 28: View small colonies and feed daily

25. Once small colonies appear, feed cells daily with 2 mLs of human embryonic stem cell (hESC) media.

Days ~ 16 - 21: Pick colonies

26. Each colony is manually picked into one well of a 12-well feeder plate with 1 mL hESC media and Rock Inhibitor (10 μM final concentration). (*Typically 6 clones per sample are picked and the three of the best clones are further expanded and banked. One of the 3 clones is fully characterized and validated by karyotyping and immunofluorecent staining with stemness markers).*

27. Feed hiPS cells daily thereafter with hESC medium (without Rock Inhibitor).

References:

1. *Chou, B-K., et al. 2011. Efficient human iPS cell derivation by a non-integrating plasmid from blood cells with unique epigenetic and gene expression signatures. Cell Res. 21(3):518-29*
2. *Van den Akker, E., et al. 2010. The majority of the in vitro erythroid expansion potential resides in CD34(-) cells, outweighing the contribution of CD34(+) cells and significantly increasing the erythroblast yield from peripheral blood samples. Haematologica 95(9): 1594-98*
3. *Leberbauer, C., et al. 2005. Different steroids co-regulate long-term expansion versus terminal differentiation in primary human erythroid progenitors. Blood 105(1):85-94*
4. *Yang W. et al., 2008-. June 2012. iPSC Reprogramming from Human Peripheral Blood Using Sendai Virus Mediated Gene Transfer StemBook. Cambridge, MA: HSCI*
5. *CytoTune-iPS 2.0 Sendai Reprogramming Kit User Guide*

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Generation of iPS Cells from Human Peripheral Blood under feeder-free conditions

Induced pluripotent stem cells (iPSCs) can be generated from freshly collected or previously frozen peripheral blood (PB) or bone marrow (BM) mononuclear cells (MNCs). This method describes the steps used to reprogram human PBMCs to iPS cells using a feeder-free system.

Materials and reagents:

- QBSF-60 serum free medium (Fisher Cat# NC0823508)
- Primocin (InvivoGen Cat# ant-pm-2)
- Ascorbic Acid (SIGMA Cat# A4544)
- SCF/c-kit Ligand (R&D Systems Cat# 255-SC)
- IL-3 (R&D Systems Cat# 203-IL)
- Epo (R&D Systems Cat# 287-TC)
- IGF-1 (R&D Systems Cat# 291-G1-01M)
- Dexamethasone (SIGMA Cat# D4902)
- FGF basic protein (R&D Systems Cat# 233-FB-500)
- CytoTune-iPS 2.0 Sendai Reprogramming Kit (Invitrogen, ThermoFisher, Cat# A16517)
- Polybrene (SIGMA Cat# H9268)
- Matrigel Corning™ Matrigel™ hESC-qualified Matrix (Corning Cat# 354277)
- ReproTeSR (STEMCELL Tech. Cat# 05920)
- mTeSR (STEMCELL Tech. Cat# 05850)
- Rock Inhibitor (Reprocell Cat# 04001202; Fisher Cat# NC0454602)
- 12-well plates (Fisher Cat# 07-200-84)
- 6-well plates (Fisher Cat# 07-200-83)
- 15 mL conical centrifuge tubes (Fisher Cat# 14-959-70C)

Day (-9): Erythroblast expansion

1. Transfer freshly isolated PBMCs (~ 2x10^6 cells) or, alternatively, thaw 1 vial of previously frozen PBMCs into 10 mL of QBSF medium and centrifuge at 300 RCF for 10 min at room temperature (RT).

2. Re-suspend cell pellet in 2 mL of EM and transfer to 1 well (12 well plate). Incubate at 37°C, 5% CO_2.

Expansion Medium (EM)* *Prepare fresh*	**Working Conc**	**Final Conc**	**Volume (5 mLs)**
QBSF-60 (serum free medium)			up to 5 mLs
Primocin	500X	100 µg/mL	10 µL
Ascorbic Acid (AA)	5 mg/mL	50 µg/mL	50 µL
Growth Factors:			
SCF	100 µg/mL	50 ng/mL	2.5 µL
IL-3	50 µg/mL	10 ng/mL	1.0 µL
EPO	2 U/µL	2 U/mL	5.0 µL
IGF-1	25 µg/mL	40 ng/mL	8.0 µL
Dexamethasone**	$5x10^{-5}$ M	1 µM	0.1 mL

*EM = QBSF-60 + Primocin + AA + growth factors
**Keep dexamethasone protected from light (discard every 2 weeks)

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Generation of iPS Cells from Human Peripheral Blood under feeder-free conditions (Continued)

Days (-3 and -6): Erythroblast expansion and washing cells

3. Transfer cells to sterile 15 mL conical tube. Wash well with 1 mL QBSF-60 to collect adherent cells and combine with cells in 15 mL conical tube.

4. Centrifuge cells at 300 RCF for 10 min at RT.

5. Aspirate supernatant and resuspend cell pellet in 2 mLs of fresh EM.

6. Transfer cells to new well of a 12 well dish and continue culturing for an additional 3 days.

Day 0: Transduction

Transduce cells with reprogramming vectors that include the four Yamanaka factors, Oct3/4, Sox2, Klf4 and c-Myc using the Sendai virus delivery and expression system (Cytotune-iPS 2.0 Sendai Reprogramming Kit).

7. Transfer cells to sterile 15 mL conical tube. Wash well with 1 mL QBSF-60 to collect adherent cells and combine with cells in 15 mL conical tube.

8. Centrifuge cells at 300 RCF for 10 min and resuspend in 1 mL of fresh EM plus polybrene (5 μg/mL)

9. Count cells and plate 2 x 10^5 cells into one well of a 12 well dish.

10. Thaw each viral vector and calculate the volume of each virus needed to reach the target MOI using the live cell count and the titer information on the Certificate of Analysis (CoA). Refer to lot-specific CoA to obtain viral titer.

$$\text{Volume of virus (µL)} = \frac{\text{MOI (CIU/cell) x number of cells}}{\text{titer of virus (CIU/mL) x } 10^{-3} \text{ (mL/µL)}}$$

11. Add virus to cells and gently rock to distribute virus.

12. Spinnoculation: Spin plate at 2250 RPM at 25°C for 90 min. After spin, incubate plate in 37°C incubator.

13. At end of day, add additional 1 mL of EM to cells for a 2 mL total volume and return plate to incubator.

Day 1: Wash cells to remove virus and plate cells onto Matrigel-coated wells

14. Prepare multiple Matrigel-coated wells of a 6-well plate according to manufacturer's instructions.

15. Collect transduced cells and transfer cells to 15 mL conical tube. Rinse well with 1 mL of QBSF-60 to collect adherent cells and combine with cells in 15 mL conical tube.

16. Centrifuge the cells at 300 RCF for 10 min at RT.

17. Resuspend cell pellet in 1 mL fresh EM and perform cell count.

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Generation of iPS Cells from Human Peripheral Blood under feeder-free conditions (Continued)

18. Plate 20,000 – 60,000 of cells/well of a 6-well Matrigel-coated plate. Plating serial dilutions of cells into multiple Matrigel-coated wells is recommended. Bring total volume of each well to 2 mLs EM.

19. Incubate cells overnight at 37°C, 5% CO_2.

Day 2: Add additional EM

20. Add 1 mL fresh EM to each well without aspirating original medium. Incubate cells overnight at 37°C.

Days 3 and 5: Add reprogramming medium

21. On day 3, add 1 mL complete ReproTeSR™ medium each well without aspirating original medium. Incubate cells overnight at 37°C. Repeat step on day 5.

Days 7 – 28: Feed cells and pick putative hiPSC colonies

22. Aspirate the old medium entirely and feed with 2 mLs complete ReproTeSR™ medium per well. Incubate cells overnight at 37°C.

23. Change the medium daily with 2 mL complete ReproTeSR™ medium per well. Monitor the cells until hiPS cell colonies appear. Once hiPSC colonies are observed, let them grow for 3 to 4 more days unless they start to merge with each other.

24. Prepare multiple Matrigel-coated wells of a 12-well plate according to manufacturer's instructions. Add 1 mL complete mTeSR medium supplemented with 10 μM ROCK inhibitor per well and keep plate at 37°C, 5% CO2 incubator until use.

25. Manually pick putative individual large iPS cell colonies and transfer to new individual wells of a 12-well dish. Carefully break up the colony into small fragments by gently and slowly pipetting with a P1000 pipet tip. Distribute colony fragments by rocking plate and return to incubator.
If necessary, prior to picking, carefully scrape away any partially reprogrammed and/or differentiated cells surrounding the putative iPS cell colony. Human iPS cell colonies typically are picked between days 16 and 24 but may vary depending on cell type, vector system used and transduction efficiency.

26. *Typically 6 clones per sample are picked and the three best clones are further expanded and banked. One of the 3 clones is further characterized and validated by karyotyping and immuofluorescent staining with stemness markers.*

References:

1. *Park, S. and G. Mostoslavsky. 2018. Generation of human induced pluripotent stem cells using a defined, feeder-free reprogramming system. Curr Protoc Stem Cell Biology. 45(1): 1-15.*
2. *ReproTeSR™ Blood reprogramming medium for human iPS cell induction. STEMCELL Technologies. Catalog #05920.*
3. *CytoTune-IPS 2.0 Sendai Reprogramming Kit User Guide. Invitrogen. Catalog # A16517.*

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Human Dermal Fibroblast Isolation

6mm Punch Biopsy from Arm
Placed immediately into 15 mL conical centrifuge tube containing DMEM with 1% pen-strep.

1. In sterile hood, transfer the skin sample to a 15 mL conical tube with waiting 1 mL digestion media*. Cap the tube tightly and place in 37°C tissue culture incubator overnight.
2. The next day vortex for 20 seconds to disrupt the skin—observe separation of the epidermis and disintegration of the dermis.
3. Wipe off the outside of the conical tube with 70% alcohol to sterilize it before entering hood.
4. In a sterile tissue culture hood, add 7 mLs of fibroblast culture media** to the conical tube, pipette up and down to mix 2-3 times and then plate entire contents into a T75 tissue culture flask.
5. Incubate for 3 days without touching the flask to allow cells to settle/adhere—check at 72 hours to assess whether fibroblasts are starting to grow.
6. On day 6-7 the culture will have started to turn yellow or will be at risk of drying out— in this case, add 7 mls more of fibroblast culture media (but with 10% FBS DMEM, rather than 20% FBS).
7. When 80% confluent, passage 1:3 using 0.25% trypsin/EDTA. Upon passaging, FBS can be reduced from 20% to 10%.
8. A small aliquot should be taken for mycoplasma testing by PCR
9. Begin reprogramming at passage 3 and freeze down backup vials for liquid nitrogen storage.

Digestion media* (per Andrea Bujor, MD):
8 mL FBS (final conc=20% FBS)
32 mL DMEM (high glucose)
100 mg Collagenase type I, 0.25% final. (Worthington-biochem.com; CLS-1; 235U/mg, lot specific)
20 mg DNAse I, 0.05% final (Sigma Cat# DN25-100MG)
1% (final conc.) pen-strep
mix, filter, and divide into 1 mL aliquots in 15cc conical tubes; keep at -20C.

Human fibroblast media**:
20% FBS
DMEM-high glucose with sodium pyruvate and L-glutamine added (Gibco #10569-010)
1% pen-strep

Reference: *D. Kotton 8/17/2010 As learned from Robert Lafyatis, MD.*

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Human Fibroblast Reprogramming to make human iPS Cells using a Lentiviral Stem Cell Cassette

1. Plate 1x10^5 human dermal fibroblasts in fibroblast media (DMEM with 10% FBS) on a gelatin-coated 35mm plastic tissue culture dish. For fresh adult cells, passage 3-4 works best and reprogramming efficiency declines with each passage.

2. The next day, change to 1 mL of fibroblast media adding polybrene to the media (5μg/ml).

3. Add EF1α-hSTEMCCA-loxP lentivirus (4 factors, excisable vector) at a multiplicity of infection (MOI) = 0.1-1 (usually from 0.2 to 5 μl of concentrated lentiviral supernatant, depending on the viral titer). Infect overnight (around 16 hours).

4. The next day (day 1) change to fresh fibroblast media without polybrene.

5. On day 2, change media to serum-free hESC media

6. On day 6, trypsinize the well (0.25% trypsin/EDTA) and pass at a 1:16 split by plating the cells onto one 10 cm gelatin-coated culture dish (pre-seeded the day before with mitomycin C-inactivated mouse embryonic fibroblast (MEF) feeder cells). Discard the other cells (or keep to passage if more plates/ colonies are needed). Re-feed the cells every 2 days or so and observe occasionally to watch the reprogrammed cells changing morphology as early colonies form. Resist the urge to pick colonies early prior to day 30. If dense overgrowth and too many colonies are observed then repeat the reprogramming from step 1, but passage 1:40 onto MEFs on day 6 (by discarding 80% of the fibroblasts on day 6 and passing the remainder onto a 10 cm plate of feeders).

7. On day 30, mechanically pick colonies of good morphology with a P200 pipette. An inverted microscope cleaned and placed in a sterile culture hood is recommended to visualize colonies for picking. (For 3 factor reprogramming, where indicated, GSK3 inhibitor (Bio) (EMD Biosciences, 361550; 10 µM) can be added to the culture media on days 7-30 of reprogramming. Colonies take longer to emerge and are far fewer when reprogramming with the 3 factor hSTEMCCA-RedLight-loxP vector).

8. The picked colonies are placed in 1 well of a 24 or 96 well plate, pre-plated with inactivated MEF feeders on gelatin. The picked colony during picking should be gently mechanically broken up by pipetting but kept in chunks. Do not trypsinize.

9. After outgrowth of the picked colony, passage the well to a new 24 well plate and then expand as usual with passaging (see below).

Reference:
Somers A. et al., 2010. Generation of transgene-free lung disease-specific human iPS cells using a single excisable lentiviral stem cell cassette. Stem Cells. 28: 1728-1740.

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Preparing Feeder-Dependent Tissue Culture Plates

Inactive MEF feeder plates can be prepared several days prior to passaging hiPS cells. It is best to use MEF feeder plates within the first two days of seeding but may be used up to four days after seeding. Inactive MEF cells are plated on gelatin-coated dishes at a concentration of ~ 2-4x10^5 cells/well (6-well dish). It is critical to obtain optimal cell density of MEFs for expansion and maintenance of healthy hiPS cells.

1. Coat 6-well tissue culture dishes (35 mm) with 1 mL sterile 0.1% gelatin solution (EMD Millipore Cat# ES-006-B) and incubate at 37°C for 30-60 min.
2. Aspirate gelatin solution and wash plate with 1 mL of serum-free DMEM.
3. Add 2 mLs DMEM Complete Medium (DMEM, 10% FBS, 1x L-glutamine and 100 *m*g/mL primocin) to each well and return to incubator.
4. Thaw frozen vial of inactive MEFs (~ 3.75x10^6 cells/mL) briefly in 37°C water bath, wipe vial with 70% alcohol and transfer cells to 15 mL conical centrifuge tube with 10 mLs DMEM Complete Medium.
5. Spin cells 300 *x g* for 5 min.
6. Aspirate medium and resuspend cell pellet in DMEM Complete Medium in 3 mLs and perform cell count.
7. Add approximately 2.25-2.4x10^5 cells/well of a 6-well gelatin-coated dish with 2 mLs DMEM Complete Medium (from steps 1-3).
7. Rock gently to evenly distribute cells and establish cell monolayer.
8. Incubate cells at 37°C overnight.
9. Prior to passaging hiPS cells onto MEFs, aspirate DMEM Complete Medium from each MEF-seeded well and wash with 2 mLs of DMEM/F12.
10. Add 2 mL hESC medium to each MEF-seeded well and return to 37°C incubator until use.

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Passaging hiPS Cells on Feeders using Collagenase IV

In general, the cells should be split when the colonies become large and before they start touching each other. iPSC lines are typically passaged approximately every five days at a 1:3 to 1:20 split ratio. hiPSCs are routinely passaged as clumps, rather than single cells. Collagenase Type IV (1 mg/mL) is an enzyme used for passaging the cells as clumps. It is used to loosen the cells from the dish before mechanically scraping to passage. The clumps should have a small-medium size (around 50 to 200 cells). For most clones, pipetting up and down three times is enough to break the colonies into optimal clump size.

In order to maintain hiPSC cultures in an undifferentiated state, differentiated colonies may be removed or 'cleaned' by mechanical scraping with the use of a pipette tip prior to collagenase passaging.

Alternatively, hiPSCs may also be maintained in the undifferentiated state by mechanical passaging of individual colonies. Individual undifferentiated colonies may be lifted off the culture dish with the use of a pipette tip. The free-floating colony is transferred to a secondary dish with a small volume of hESC. As described above, the colonies are broken into smaller clumps by pipetting up and down. These clumps are then transferred to a fresh MEF feeder plate and distributed evenly by gentle rocking.

Materials and reagents:

- Collagenase Type IV (1 mg/mL; StemCell Technologies Cat# 07909)
- DMEM/F12 (Life Technologies Cat# 11330-057)
- DPBS (Life Technologies Cat# 14190-144)
- Cell scrapers (Corning Cat# 3010)
- hESC medium
- MEF feeder plate (6 well dish)

1. To prepare a fresh MEF feeder plate for hiPSCs, aspirate the 10% DMEM complete culture medium and wash well twice with 1-2 mL DMEM/F12. Add 2 mL of hESC medium per well of a 6-well plate (1 mL per well of a 12-well plate) and return plate to the incubator.

2. Under the microscope, pinpoint any differentiated hiPSC colonies with a marker on the outside of the well and remove them with a pipette tip *('cleaning').*

3. Aspirate the medium from the hiPSC culture dish, removing the previously marked differentiated colonies.

4. Wash well twice with 1 mL of DPBS.

5. Add 1 mL of collagenase (warm) per well of a 6-well plate (500 μL per well of a 12-well plate) and incubate the cells for 3 – 5 minutes at RT (in the hood). Observe the cells under the microscope. Some of the colonies will start to curl up at the edges.

6. Aspirate the collagenase and wash twice with 1 mL of DPBS.

7. Add 1 mL fresh hESC medium and scrape the cells from the bottom of the well with a cell scraper until the colonies are all free-floating. Pipette up and down 1-5x (with a P1000 pipette tip) to break the colonies into smaller clumps.

8. Transfer the desired dilution to fresh wells of the new feeder plate (usually around 1:3 – 1:20 depending on the growth rate of the individual cell line). Rock gently to evenly distribute cell clumps.

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Freezing hiPS Cells Growing on Feeders

Ideally, hiPS cells to be frozen should be between 60-80% confluent and most of the colonies in the well should be large and healthy. Usually, 1-2 wells of a 6-well plate are frozen per vial.

Materials and reagents:

- Collagenase Type IV, 1 mg/ml (StemCell Tech. Cat# 07909)
- DPBS (Life Technologies Cat# 14190-144)
- Cell scraper (Corning Cat# 3010)
- Cold hESC medium
- Cold 2x Freezing medium (60% FBS, 20% hESC medium, 20% DMSO)
- Cryovials (Corning Cat# 430487)

1. Determine the number of cryovials needed (usually 1 vial per well of a 6-well plate), adhere label and place in a -20C freezer to chill.
2. Aspirate the medium from the hiPSC culture dish.
3. Wash twice with 1-2 mL of DPBS.
4. Aspirate the DPBS and add 1 mL of collagenase (warm) per well of a 6-well plate and incubate the cells for 3 – 5 minutes at RT (in the hood).
5. Observe the cells under the microscope. Some of the colonies will start to curl up on the edges.
6. Aspirate the collagenase and wash twice with 1 mL of DPBS.
7. Add 0.5 mL of cold hESC medium.
8. Scrape the cells from the bottom of the well until the colonies are all floating. ***Do not pipette up and down when freezing hiPSCs!***
9. Add 0.5 mL of cold 2x freezing medium dropwise while swirling the plate to mix.
10. Place the 1 mL total volume in a chilled cryovial.
11. Place the cryovial in a freezing container or in a styrofoam rack at -80^{o}C overnight and transfer to the -150^{o}C the next day.

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Thawing hiPSCs onto Feeders

Reagents and materials:

- DMEM/F12 (Life Tech. Cat# 11330-057)
- Cold hESC medium
- Feeder plate
- Rock Inhibitor (Stemgent Cat# 04-0012-02)
- Dry ice
- 37°C water bath
- 5 mL serological pipettes
- 50 mL conical tube
- Centrifuge

1. Prepare 2.5 mL of hESC medium + 10 μM Rock Inhibitor.
2. Aspirate the medium from a well of a 6-well feeder plate, wash once with DMEM/F12, and add 1 mL of hESC medium + 10 μM Rock Inhibitor and place the plate in the incubator.
3. Bring the frozen cell cryovial on dry ice to the tissue culture room.
4. Quickly thaw the hiPSCs in a 37°C water bath by gently shaking the cryovial continuously until only a small frozen pellet remains.
5. Wipe the cryovial with a kimwipe sprayed with 70% ethanol and place in the hood.
6. Use a 5 mL serological pipette to transfer the cells to a 50 mL conical tube.
7. Add 5 mL of cold hESC medium dropwise while swirling the conical tube with the cells.
8. Centrifuge the cells for 5 minutes at 200 *x g*, 4°C.
9. Aspirate off the medium and add 1 mL of hESC medium + Rock Inhibitor (10 μM).
10. Gently flick the conical tube to resuspend the cells and transfer them to the well of the feeder plate using a 5 mL serological pipette.
11. Distribute hiPS cells evenly over feeders and return dish to incubator.

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Preparing Feeder-Independent Tissue Culture Dishes

hiPS cells may be maintained on feeder-free tissue culture dishes coated with Matrigel™, a reconstituted basement membrane extract that gels at room temperature. Matrigel is optimized for the culture of hESCs and used in combination with mTeSR growth medium. The concentration of Matrigel is lot specific and should be diluted and aliquoted for one time use according to the dilution factor provided with the certificate of analysis (CoA) for each lot. In general, each aliquot of Matrigel (270-350 μL) is diluted in 24mL of DMEM/F12 and is sufficient for coating four 6-well dishes (1 mL/well).

Reagents and materials:

- Matrigel Corning™ Matrigel™ hESC-qualified Matrix (Corning Cat# 354277)
- mTeSR1™ Medium (STEMCELL Tech. Cat# 05850)
- DMEM/F12 (Life Technologies Cat# 11330-057)
- 6-well tissue culture plates

1. To coat one 6-well tissue culture plate, thaw appropriate aliquot of Matrigel on ice. Once thawed add to 6 mLs cold (4°C) DMEM/F12 in a 15 mL conical centrifuge tube. Pipet to mix without introducing air bubbles.

2. Working quickly, add 1 mL of Matrigel-DMEM/F12 solution to each well of a 6-well tissue culture plate and rock gently to cover surface.

3. Incubate at room temperature (RT, 15-25°C) for at least an hour before use.

4. Aspirate the remaining liquid and wash 1x with 1 mL DMEM/F12.

5. Add 2 mLs mTeSR medium to each well and place in 37°C incubator until use.

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Passaging hiPS Cells on Matrigel using ReLeSR Dissociation Reagent

In general, hiPS cells should be split when the colonies become large and before they start touching each other. hiPS cells maintained on Matrigel-coated surfaces in the defined, feeder-independent media, mTeSR are typically passaged approximately every five to seven days at a 1:3 to 1:30 split ratio. For hiPS cells growing on Matrigel-coated dishes, cells may be passaged as clumps or aggregates using various non-enzymatic dissociation reagents such as ReLeSR or Gentle Cell Dissociation Reagent.

Reagents and materials:

- ReLeSR (StemCell Tech. Cat# 05782; alternatively, Gentle Cell Dissociation Reagent, Cat# 07174)
- mTeSR (StemCell Tech. Cat# 05850) supplemented with primocin (invivogen ant-pm-2)
- DMEM/F12 (Life Tech, Cat# 11330-057)
- DPBS (Life Tech. Cat# 14190-144)
- Matrigel-coated plates
- Cell scrapers (Corning Cat# 3010)

1. Aspirate Matrigel from a freshly plated well, wash once with 1 mL DMEM/F12 and add 2 mL of mTeSR medium per well of a 6-well plate (1 mL per well of a 12-well plate); return plate to the incubator.

2. Aspirate the medium from the hiPSC culture dish, removing the regions of differentiation with a pipette tip.

3. Wash twice with 1 mL of DPBS.

4. Add 1 mL of ReLeSR per well of a 6-well plate (500 μL per well of a 12-well plate) and aspirate within 1 minute.

5. Return plate to 37°C incubator for 5-7 min (~ 5 min 15 sec).

6. Remove plate from incubator, and add 1 mL mTeSR down side of wall of each well. Colonies will lift from the plate and differentiated colonies will remain adherent to plate. Gently tap plate to break up colonies. If colonies remain large, transfer floating colonies to a 50 mL conical tube or eppendorf tube and pipet up and down once very slowly.

7. Transfer desired dilution to prepared matrigel-coated wells (usually around 1:3 to 1:30) depending on growth rate of cells.

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Passaging hiPS Cells on Matrigel using Gentle Cell Dissociation Reagent

In general, hiPS cells should be split when the colonies become large and before they start touching each other. hiPS cells maintained on Matrigel-coated surfaces in the defined, feeder-independent media, mTeSR are typically passaged approximately every five to seven days at a 1:3 to 1:30 split ratio. For hiPS cells growing on Matrigel-coated dishes, cells may be passaged as clumps or aggregates using various non-enzymatic dissociation reagents such as ReLeSR or Gentle Cell Dissociation Reagent.

Reagents and materials:

- Gentle Cell Dissociation Reagent (StemCell Tech. Cat# 07174)
- mTeSR (StemCell Tech. Cat# 05850) supplemented with primocin (invivogen ant-pm-2)
- DMEM/F12 (Life Tech. Cat# 11330-057)
- DPBS (Life Tech. Cat# 14190-144)
- Matrigel-coated plates
- Cell scrapers (Corning Cat# 3010)

1. Aspirate Matrigel from a freshly plated well, wash once with 1 mL DMEM/F12 and add 2 mL of mTeSR medium per well of a 6-well plate (1 mL per well of a 12-well plate); return plate to the incubator
2. Aspirate the medium from the hiPSC culture dish, removing the regions of differentiation with a pipette tip.
3. Wash twice with 1 mL of DPBS.
4. Add 1 mL of Gentle Cell Dissociation Reagent (per well of a 6-well dish)
5. Incubate at RT for 4-6 min
6. Aspirate Gentle Cell Dissociation Reagent and slowly add 1 mL DPBS down side of wall to remove any floating cells.
7. Aspirate DPBS and add 1 mL mTeSR to cells. Using a cell lifter or cell scraper gently remove cell colonies from plate surface and tilt plate to gather floating cell aggregates.
7. Transfer cell aggregates to 50 mL falcon tube using 5 mL serological pipette. Pipet 1-3x slowly using a 5 mL serological pipette. If cell aggregates remain large, pipet cells slowly using a P1000 tip.
8. Transfer desired dilution to prepared Matrigel-coated wells (usually around 1:3 to 1:30) depending on growth rate of cells.

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Freezing hiPS Cells growing on Matrigel

Materials and reagents:

- DPBS (Life Tech. Cat# 14190-144)
- DMEM/F12 (Life Tech. Cat# 11330-057)
- mTeSR (StemCell Tech. Cat# 05850)
- Gentle Cell Dissociation Reagent (StemCell Tech. Cat# 07174)
- 2x cold Freezing Medium (80% mTeSR/20% DMSO)
- 5 mL serological pipettes
- 15 mL conical tube
- Cryovials (Corning Cat# 430487)
- Cell scrapers (Corning Cat# 3010)
- Freezing container

1. Remove differentiated colonies by scraping with a pipette tip.
2. Aspirate medium from wells of a 6-well tissue culture plate and rinse twice with 1 mL DPBS.
3. Add 1 mL of Gentle Cell Dissociation Reagent per well of a 6-well plate (500 μL per well of a 12-well plate)
4. Incubate at RT for 4-6 min
5. Aspirate Gentle Cell Dissociation Reagent and slowly add 1 mL DPBS down side of wall to remove any floating cells.
6. Aspirate DPBS and add 0.5 mL mTeSR to cells. Using a cell lifter or cell scraper gently detach cell colonies from plate surface. ***Do not pipette up and down when freezing hiPSCs!***
7. Add 0.5 mL of cold 2x Freezing Medium dropwise while swirling the plate to mix.
8. Place the 1 mL total volume hiPSCs into a chilled cryovial.
9. Place the cryovial in a freezing container or in a styrofoam rack at -80^{o}C overnight and transfer to the -150^{o}C the next day.

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Thawing hiPS Cells growing on Matrigel

Materials and reagents:

- DPBS (Life Tech. Cat# 14190-144)
- Warmed mTeSR medium (15°C -25°C)
- Matrigel-coated plate
- Rock Inhibitor (Stemgent Cat# 04-0012-02)
- Dry ice
- 37°C water bath
- 5 mL serological pipettes
- 50 mL conical tube

1. Prepare 2.5 mL of mTeSR medium + 10 μM Rock Inhibitor.
2. Aspirate Matrigel from a freshly coated 6-well dish, wash once with 1 mL DMEM/F12 and add 1 mL of mTeSR medium + 10 μM Rock Inhibitor and return plate to incubator.
3. Bring the frozen hiPS cell cryovial on dry ice to the tissue culture room.
4. Quickly thaw the hiPSCs in a 37°C water bath by gently shaking the cryovial continuously until only a small frozen pellet remains.
5. Wipe the cryovial with a kimwipe sprayed with 70% ethanol and place in the hood.
6. Use a 5 mL serological pipette to transfer the cells to a 50 mL conical tube.
7. Add 5 mL of mTeSR medium dropwise to cells while gently swirling the conical tube.
8. Centrifuge the cells for 5 minutes at 200 *x g*, RT.
9. Aspirate off the medium and add 1 mL of mTeSR medium + Rock Inhibitor (10 μM).
10. Gently flick the conical tube to resuspend the cells and transfer cell clumps using a 5 mL serological pipette to the newly-coated Matrigel well with 1 mL mTeSR + 10 μM Rock Inhibitor for a total volume of 2 mLs
11. Distribute hiPS cells evenly over well surface and return plate to incubator.

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mTeSR1 Adaption of hiPSCs Cultured on Feeders

Materials and reagents:

- Matrigel Corning™ Matrigel™ hESC-qualified Matrix (Corning Cat# 354277)
- Collagenase Type IV, 1 mg/ml (STEMCELL Tech. Cat# 07909)
- ReLeSR (STEMCELL Tech. Cat# 05872)
- Gentle Cell Dissociation Reagent (STEMCELL Tech. Cat# 07174)
- mTeSR1 medium (STEMCELL Tech. Cat# 05850) at room temperature
- hESC medium
- DMEM/F12 (Life Tech. Cat# 11330-057)
- DPBS (Life Tech. Cat# 14190-144)
- Rock Inhibitor (Stemgent Cat# 04-0012-02)

Passage hiPSCs (feeder-dependent) onto Matrigel coated-dish

1. Prepare Matrigel-coated plate (6-well dish) according to protocol “Preparing Feeder-Independent Tissue Culture Dishes”. Add 2 mL mTeSR medium per well and return to incubator.

2. Aspirate media from iPSC well on feeders; rinse twice with 1 mL DPBS.

3. Add 1 mL Collagenase Type IV (warmed to 37°C) to each well of a 6-well plate and incubate for 3 – 5 minutes at RT (in the hood). *Depending on the density of colonies you may need to passage two hiPSC wells into a single Matrigel-coated well.*

4. Aspirate collagenase and wash twice with 1 mL of DPBS, DMEM/F12 or mTeSR1 media.

5. Add 1 mL room temperature mTeSR1 medium or hESC/mTeSR1 mixture and gently scrape the cells from the bottom of the well with a cell scraper until the colonies are all free-floating. Pipette up and down 1-5x (with a P1000 pipette tip set to 950 mL) to break up colonies into small clumps. Do not over-pipette!

6. Add total volume of cell suspension to each well of a Matrigel-coated plate already containing 2 mL mTeSR media. Distribute cell colonies by rocking plate side-to-side before returning to incubator.

First passage from Matrigel to Matrigel

1. Prepare 4 Matrigel-coated wells (of a 12-well plate) for each cell line to be adapted.

2. Choose appropriate dissociation reagent (ReLeSR or Gentle Cell) and passage cells according to standard protocols.

3. Passage from a single 6-well plate into 4 wells of a 12-well plate as follows:
   Well 1: 1:2 dilution
   Well 2: 1:4 dilution (with Rock Inhibitor if the 6 well is 60% confluent or less)

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mTeSR1 Adaption of hiPSCs Cultured on Feeders (continued)

Well 3: 1:8 dilution (with Rock Inhibitor)
Well 4: 1:8 dilution

4. Repeat passaging in the above manner for 4-6 passages
   a. When possible, only carry forward wells that were not exposed to Rock Inhibitor
   b. Avoid picking-to-pass if possible because this will lengthen the adaptation process
   c. When 1:8 dilution without rock inhibitor begins to survive well, then split back into a 6-well plate and consider your adaptation process complete

Reference: *Derek Liberti (Kotton Lab)*

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Immunostaining of hiPS Cells for Pluripotency Markers

hiPS cells are plated on a feeder layer in 12-well tissue culture dishes and grown for 5 days. Most of the colonies in the well should be medium size at the time of fixation. The cell-surface-antigen expression of cultured cells can be analyzed by using immunofluorescence techniques. The following primary monoclonal antibodies are used to detect surface-antigen expression: anti-SSEA-1; anti-SSEA-4; TRA-1-60 and TRA-1-81. Fluorophore-labeled goat anti-mouse IgM or anti-IgG can be used as secondary antibodies, appropriate to the isotype of the primary antibody.

Materials and reagents:

- ES Characterization Kit (EMD Millipore Cat# SCR001)
- 16% Paraformaldehyde (PFA) (EMS Cat# 15710)
- Wash buffer (1X TBST)
- DPBS (Life Tech. Cat# 14040-133)
- Normal goat serum (Vector, Cat# S-1000)
- Triton™ X-100 (Sigma, Cat# T8787)
- Alexa Fluor™ goat a-mouse AF488 or AF546 (Life Tech. Cat# A21042; A11003)
- DAPI stain (Invitrogen D1306)
-

1. Fix cultured ES cells in 4% paraformaldehyde/DPBS for 20 minutes at room temperature (RT), rocking.
2. Wash 3x (5 minutes each) with wash buffer.
3. Permeabilize cells with 0.1% Triton X-100/DPBS for 10 minutes at RT.
4. Wash 3x (5-10 minutes each) with wash buffer, rocking.
5. Apply a Blocking solution (e.g. 4% normal goat serum/DPBS) for 30 minutes at RT, rocking.
6. Dilute primary antibodies to working concentrations in blocking solution (SSEA-1, SSEA-4, TRA-1-60 and TRA-1-81 at 1:50). Incubate primary antibodies for 1 hour at RT, rocking.
7. Wash 3x (5 minutes each) with wash buffer, rocking.
8. Dilute secondary antibodies in DPBS (1:250) just prior to use use. Incubate secondary antibodies for 30-60 minutes at RT, rocking. Cover plate with aluminum foil.
9. Wash 3x (5 minutes each) with wash buffer, rocking.
10. Add DAPI stain, 1:2000/DPBS, 5 min. Quick rinse in DPBS and store at 4°C in DPBS. Cover in tin foil.
11. Images can be visualized with a fluorescence microscope.

STEMdiff™ Definitive Endoderm Kit

Defined Animal Component-Free Medium for the Differentiation of Human ES and iPS Cells to Definitive Endoderm

Catalog #05110 1 Kit
Catalog #05115 1 Kit

STEMCELL™ TECHNOLOGIES
Scientists Helping Scientists™ | WWW.STEMCELL.COM
TOLL FREE PHONE 1 800 667 0322 • PHONE +1 604 877 0713
INFO@STEMCELL.COM • TECHSUPPORT@STEMCELL.COM
FOR GLOBAL CONTACT DETAILS VISIT OUR WEBSITE

Product Description

STEMdiff™ Definitive Endoderm Kit is a serum-free and animal component-free combination of a basal medium and supplements for the differentiation of human embryonic stem (ES) cells and induced pluripotent stem (iPS) cells to definitive endoderm. Cells differentiated to definitive endoderm using this kit can be used to generate more specified cells of endodermal lineage, including hepatocytes and pancreatic progenitors.

The purity of definitive endoderm cells (CXCR4+SOX17+ or CXCR4+c-Kit+) obtained with the STEMdiff™ Definitive Endoderm Kit is typically in the range of 75 - 99%.

STEMdiff™ Definitive Endoderm Kit (Catalog #05110) and STEMdiff™ Definitive Endoderm Kit (TeSR™-E8™ Optimized; Catalog #05115) have been optimized for the differentiation of human ES and iPS cells cultured in mTeSR™1 and TeSR™-E8™, respectively.

Product Information

PRODUCT NAME	CATALOG #	SIZE	COMPONENTS
STEMdiff™ Definitive Endoderm Kit	05110	1 Kit	• STEMdiff™ Definitive Endoderm Basal Medium (100 mL) • STEMdiff™ Definitive Endoderm Supplement A (100X; 0.35 mL) • STEMdiff™ Definitive Endoderm Supplement B (100X; 1.1 mL)
STEMdiff™ Definitive Endoderm Kit (TeSR™-E8™ Optimized)	05115	1 Kit	• STEMdiff™ Definitive Endoderm Basal Medium (100 mL) • STEMdiff™ Definitive Endoderm Supplement A (100X; 0.35 mL) • STEMdiff™ Definitive Endoderm Supplement B (100X; 1.1 mL) • STEMdiff™ Definitive Endoderm TeSR™-E8™ Supplement (20X; 7 mL)

Component Storage and Stability

The following components are sold as part of the STEMdiff™ Definitive Endoderm Kits (see Product Information) and are not available for individual sale.

COMPONENT NAME	COMPONENT #	STORAGE	SHELF LIFE
STEMdiff™ Definitive Endoderm Basal Medium	05111	Store at -20°C.	Stable until expiry date (EXP) on label.
STEMdiff™ Definitive Endoderm Supplement A (100X)	05112	Store at -20°C.	Stable for 12 months from date of manufacture (MFG) on label.
STEMdiff™ Definitive Endoderm Supplement B (100X)	05113	Store at -20°C.	Stable for 12 months from date of manufacture (MFG) on label.
STEMdiff™ Definitive Endoderm TeSR™-E8™ Supplement (20X)	05116	Store at 2 - 8°C.	Stable for 12 months from date of manufacture (MFG) on label.

Handling Frozen Components

05111 STEMdiff™ Definitive Endoderm Basal Medium

- Thaw entire bottle at room temperature (15 - 25°C) or overnight at 2 - 8°C, and mix thoroughly. Once thawed, use immediately or store at 2 - 8°C for up to 2 months. Alternatively, aliquot and store at -20°C until the expiry date as indicated on the label. After thawing the aliquots, use immediately or store at 2 - 8°C for up to 2 weeks. Do not re-freeze.

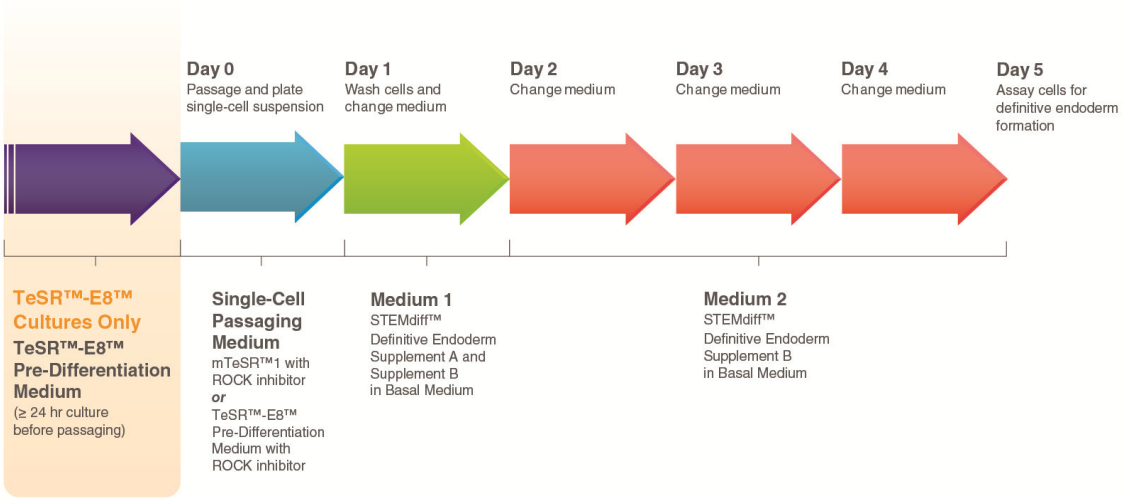
05112 STEMdiff™ Definitive Endoderm Supplement A (100X) OR **05113 STEMdiff™ Definitive Endoderm Supplement B (100X)**

- Thaw on ice and mix thoroughly. Once thawed, use immediately or aliquot and store at -20°C for up to 12 months from the date of manufacture as indicated on the label. After thawing the aliquots, use immediately. Do not re-freeze.

Materials Required But Not Included

PRODUCT NAME	CATALOG #
mTeSR™1 OR TeSR™-E8™	05850 OR 05940
Corning® Matrigel® hESC-qualified matrix OR Vitronectin XF™	Corning 354277 OR 07180
DMEM/F12 with 15 mM HEPES	36254
Gentle Cell Dissociation Reagent	07174
D-PBS Without Ca++ and Mg++ (PBS)	37350
Y-27632 (Dihydrochloride)	72302

Schematic of STEMdiff™ Definitive Endoderm Kit Procedure



Directions for Use

Please read the entire protocol before proceeding.

NOTE: For complete instructions on coating plates with Corning® Matrigel® or Vitronectin XF™, and maintaining high quality human ES and iPS cells for use in differentiation, please refer to the Technical Manual: Maintenance of Human Pluripotent Stem Cells in mTeSR™1 (Document #29106) or TeSR™-E8™ (Document #29267) available on our website at www.stemcell.com, or contact us to request a copy. Matrix-coated plates should be prepared in advance and be brought to room temperature (15 - 25°C) for at least 30 minutes prior to use.

Use sterile techniques when performing the following protocols. The following are instructions for use with 6-well plates. Indicated volumes are for a single well. If using alternative cultureware, adjust volumes accordingly.

1. Passaging Cells for Definitive Endoderm Induction

For optimal product performance, passage human ES or iPS cells using the specific passaging protocols for cells cultured in mTeSR™1 or TeSR™-E8™ as outlined in this section, before proceeding with differentiation to definitive endoderm (section 2).

NOTE: Human ES and iPS cells are ready for passage when cultures are approximately 70% confluent.

mTeSR™1 Cultures

This protocol is specific to human ES and iPS cells cultured in mTeSR™1 medium.

1. On Day 0, warm (15 - 25°C) sufficient volumes of mTeSR™1, DMEM/F12, and Gentle Cell Dissociation Reagent for passaging. Prepare Single-Cell Passaging Medium by adding Y-27632 (Dihydrochloride) to mTeSR™1 to reach a final concentration of 10 µM.
2. Wash the well to be passaged with 1 mL of D-PBS Without Ca++ and Mg++.
3. Aspirate wash medium and add 1 mL of Gentle Cell Dissociation Reagent.
4. Incubate at 37°C for 8 - 10 minutes.
5. Dislodge cells by pipetting up and down 1 - 3 times using a pipette with a p1000 tip. Ensure all remaining cell aggregates are broken up into single cells.
6. Immediately transfer cells to a tube containing an equal volume of DMEM/F12. Wash the well once with 1 mL of DMEM/F12 to collect any remaining cells and transfer to the tube. Centrifuge the tube at 300 x *g* for 5 minutes.
7. Resuspend cells in 1 mL of Single-Cell Passaging Medium and count the number of live cells using a hemocytometer.
8. Plate cells at a density of 2.1 x 10^5 per cm^2 (i.e. 2 x 10^6 cells per well) onto pre-coated plates. Adjust density if necessary, so that the cells are approximately 90 - 100% confluent on Day 1.
9. Incubate at 37°C for 24 hours.
10. Continue to section 2 (Differentiating Monolayer Cultures to Definitive Endoderm).

TeSR™-E8™ Cultures

This protocol is specific to human ES and iPS cells cultured in TeSR™-E8™ medium.

1. Follow a standard passaging protocol to passage TeSR™-E8™ cultures into one well of a 6-well plate, and perform daily medium changes for four days.

   NOTE: Refer to the Technical Manual: Maintenance of Human Pluripotent Stem Cells in TeSR™-E8™ (Document #29267) for recommended passaging protocols using TeSR™-E8™.
2. Four days after passaging TeSR™-E8™ cultures, prepare complete TeSR™-E8™ Pre-Differentiation Medium by diluting cold (2 - 8°C) STEMdiff™ Definitive Endoderm TeSR™-E8™ Supplement 1 in 20 in cold (2 - 8°C) TeSR™-E8™ medium (e.g. add 1 mL of Supplement to 19 mL of TeSR™-E8™). Prepare sufficient complete TeSR™-E8™ Pre-Differentiation Medium to be used until step 6 (i.e. at least 4 mL per well).

   NOTE: Complete Pre-Differentiation Medium can be stored at 2 - 8°C for up to 2 weeks.
3. Warm (15 - 25°C) only the volume of complete TeSR™-E8™ Pre-Differentiation Medium required on this day (i.e. 2 mL per well). Store remaining medium at 2 - 8°C.
4. Aspirate medium from the culture well and add 2 mL of complete TeSR™-E8™ Pre-Differentiation Medium.
5. Incubate at 37°C and perform daily medium changes (steps 3 and 4) until cultures are approximately 70% confluent, and are ready to be passaged.

   NOTE: For optimal differentiation performance, cells must be exposed to complete TeSR™-E8™ Pre-Differentiation Medium for at least 24 hours before the next passaging step.
6. Passage cells (Day 0):
   i. Warm (15 - 25°C) sufficient volumes of complete TeSR™-E8™ Pre-Differentiation Medium, DMEM/F12, and Gentle Cell Dissociation Reagent for passaging. Prepare Single-Cell Passaging Medium by adding Y-27632 (Dihydrochloride) to TeSR™-E8™ Pre-Differentiation Medium to reach a final concentration of 10 µM.
   ii. Wash the well to be passaged with 1 mL of D-PBS Without Ca++ and Mg++.
   iii. Aspirate wash medium and add 1 mL of Gentle Cell Dissociation Reagent.
   iv. Incubate at 37°C for 8 - 10 minutes.
   v. Dislodge cells by pipetting up and down 1 - 3 times using a pipette with a p1000 tip. Ensure all remaining cell aggregates are broken up into single cells.
   vi. Immediately transfer cells to a tube containing an equal volume of DMEM/F12. Wash the well once with 1 mL of DMEM/F12 to collect any remaining cells and transfer to the tube. Centrifuge the tube at 300 x *g* for 5 minutes.
   vii. Resuspend cells in 1 mL of Single-Cell Passaging Medium and count the number of live cells using a hemocytometer.

viii. Plate cells at a density of 2.1 x 10^5 per cm^2 (i.e. 2 x 10^6 cells per well) onto pre-coated plates. Adjust density if necessary, so that the cells are approximately 90 - 100% confluent on Day 1.

ix. Incubate at 37°C for 24 hours.

x. Continue to section 2 (Differentiating Monolayer Cultures to Definitive Endoderm).

2. Differentiating Monolayer Cultures to Definitive Endoderm

1. On Day 1, warm (37°C) sufficient volumes of DMEM/F12 and STEMdiff™ Definitive Endoderm Basal Medium for Day 1 use.
2. Prepare Medium 1 by diluting both STEMdiff™ Definitive Endoderm Supplement A and STEMdiff™ Definitive Endoderm Supplement B 1 in 100 in STEMdiff™ Definitive Endoderm Basal Medium (e.g. add 10 µL of Supplement A and 10 µL of Supplement B to 980 µL of Basal Medium).

   NOTE: Supplements should be thawed on ice and kept cold until added to STEMdiff™ Definitive Endoderm Basal Medium.
3. Aspirate medium and wash with 1 mL DMEM/F12.
4. Aspirate wash medium and replace with 2 mL of Medium 1.
5. Incubate at 37°C for 24 hours.
6. On Day 2, prepare Medium 2 by diluting STEMdiff™ Definitive Endoderm Supplement B 1 in 100 in STEMdiff™ Definitive Endoderm Basal Medium (e.g. add 10 µL of Supplement B to 990 µL of Basal Medium). Prepare sufficient Medium 2 to be used on Days 2, 3 and 4 (i.e. 6 mL per well).

   NOTE: STEMdiff™ Definitive Endoderm Supplement B should be thawed on ice and added to cold (2 - 8°C) STEMdiff™ Definitive Endoderm Basal Medium.
7. Warm (37°C) only the volume of Medium 2 required for Day 2 use (i.e. 2 mL per well). Store remaining Medium 2 at 2 - 8°C.
8. Aspirate medium from the well and add 2 mL of Medium 2.

   NOTE: A wash step with DMEM/F12 is not required at this step or during subsequent medium changes.
9. Incubate at 37°C for 24 hours.
10. On Day 3, warm (37°C) only the volume of Medium 2 required for Day 3 use (i.e. 2 mL per well). Store remaining Medium 2 at 2 - 8°C.
11. Aspirate medium from the well and add 2 mL of Medium 2.
12. Incubate at 37°C for 24 hours.
13. On Day 4, warm (37°C) only the volume of Medium 2 required for the Day 4 media change (i.e. 2 mL per well).
14. Aspirate medium from the well and add 2 mL of Medium 2.
15. Incubate at 37°C for 24 hours.
16. On Day 5, cells are ready to be assayed for the formation of definitive endoderm or carried forward into more specialized lineage differentiation protocols.

    NOTE: Expression of definitive endoderm markers may peak by Day 4 in some cell lines.

Assessment of Definitive Endoderm Cells

Purity of definitive endoderm cells can be measured by flow cytometry after labeling with fluorochrome-conjugated anti-CXCR4 (e.g. Anti-Human CD184 [CXCR4] Antibody, Clone 12G5, Catalog #60089) and anti-c-Kit (e.g. Anti-Human CD117 [c-Kit] Antibody, Clone 104D2, Catalog #60087) or anti-SOX17 antibodies. Results may vary depending on cell line used.

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CENTER FOR REGENERATIVE MEDICINE
BOSTON UNIVERSITY BOSTON MEDICAL CENTER

670 ALBANY STREET
2ND FLOOR
BOSTON, MA 02118
TEL: 617-638-6572
HTTP://WWW.BUMC.BU.EDU/STEMCELLS/

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Human CKIT/CXCR4 FACS

a. Preparation of cells for staining (all steps on ice)
   I. Monodisperse cells using a P1000 pipette after 4min in GCDR (gentle cell dissociation reagent)
   II. inactivate with stop media (DMEM +10% FBS), spin down (5 minutes, 200x*g*, 4°C) and wash once with 5 ml IMDM or DMEM/F12
   III. resuspend in 500 µl/condition of PBS^{+} (PBS+1-2% FBS) and divide between 5 Eppendorf tubes (unstained, isotypes, Cxcr4, cKit, Cxcr4/cKit double) e.e. 100uL per tube.

b. Cell staining
   I. add the appropriate antibodies/isotypes (2uL) per tube (e.g. no antibodies in the "unstained" tube, both antibodies in the "double" tube)

      Antibodies used for this protocol as of 4/16/16:
      Isotype: Mouse IgG1 APC-conjugated, Invitrogen, Cat# MG105 (2uL)
      Isotype: Mouse IgG2a PE-conjugated, Stem Cell Technologies, Cat# 60108PE (2uL)
      CXCR4: Human CXCR4 R-PE conjugated, Stem Cell Technologies, Cat #60089PE (2uL)
      CKIT: APC anti-human CD117(CKIT), Biolegend, Cat# 313206 (2uL)
   II. vortex briefly and transfer to ice for 30 min (cover with aluminum foil, vortex once again at 15 min)
   III. add 1 ml PBS^{+} per tube, spin at 200x*g* for 5 min in a tabletop centrifuge, carefully aspirate supernatant . Repeat.
   IV. Resuspend pellet in 350 µl PBS^{+}
   V. transfer to FACS polystyrene tubes with the cell strainer cap
   VI. take cells to LSRII for analysis

Finn Hawkins 4/16/16

670 ALBANY STREET, 2ND FLOOR
BOSTON, MA 02118
TEL: 617-414-2969
HTTP://WWW.BUMC.BU.EDU/STEMCELLS/

Human Lung Directed Differentiation Protocol (Progenitor Stage) – Kotton Laboratory

INTRO

This protocol describes the methods to derive, sort and plate primordial lung progenitors from human iPSCs/ESCs. There are separate protocols for subsequent alveolar and airway differentiations. See Hawkins et al., JCI 2017 for more details including characterization of the NKX2-1+ progenitors.

REAGENTS

See associated excel file Human Lung Reagents_2017-1_13

PROTOCOL

Associated protocols:
STEMDiff Endoderm Kit protocol
CD47/CD26 staining protocol

1) Definitive endoderm induction (Day 0 to Day 3-4):

A. Generate definitive endoderm following the stem cell technologies STEMdiff Definitive Endoderm kit protocol (see protocol) in a 6 well plate. Check C-kit/CXCR4 co-expression to assess sufficient definitive endoderm induction (see C-kit/CXCR4 staining protocol). This needs to be assessed for each iPSC/ESC and can change over time. Typically the majority of cells are C-kit+/CXCR4+ between 72 and 96 hours (Please note that in the STEMdiff protocol, day 1 is the day that supplements A+B are added. In the Kotton Lab protocol, this time-point is referred to as “Day 0”).

2) Anterior foregut endoderm (typically 72 hours):

A. Prepare “DS/SB” media. Prepare “DS/SB + Y-27632”. Prepare fresh matrigel coated plate (see Stem Cell technologies mTeSR1 protocol).
B. Aspirate definitive endoderm media and gently wash with 1ml of CSFDM. Add 1ml/well of Gentle Cell Dissociation Reagent. Wait 2-3 min. Aspirate. Add 1ml of room temperature “DS/SB + Y-27632” directly to the well. Gently triturate. The cells should very easily detach from the tissue culture plate as clumps. Plate the cells at a density of 20K to 40K cells/cm2 in “DS/SB + Y-27632” on freshly coated Matrigel plates (*These densities reflect replating within a range of 1:5 to 1:10. Density affects NKX2-1 induction and should be optimized if NKX2-1 yields are low. We typically start with 1:6).*
C. The following day change the media to “DS/SB”. The duration of “DS/SB” may need to be optimized for NKX2-1 induction. In our experience, 48 hours is insufficient. For most iPSC/ESCs we use 72 hours.

3) NKX2-1 Induction (Day 6 to 15):

A) Prepare “CB” media with and add freshly prepared retinoic acid each time the cells are refed “CBRa”.
B) Aspirate “DS/SB” media.
C) Add 2ml/well of “CBRa”.
D) Refeed every 24-48 hours.

4) Sorting NKX2-1+/CD47+ Lung Progenitors and Expansion in 3-D Matrigel (Day 14-15):

A. Prepare maturation media. Distal = “CK+DCI”. Proximal=”FGF2+10” (see relevant protocols)
B. Prepare FACS buffer:
    1. Hank’s Balanced Salt Solution
    2. 2% FBS
    3. Primocin
    4. HEPES 25mM
    5. EDTA 2mM
C. Aspirate “CBRa” media and wash X2 with CSFDM
D. Use a 10ul pipette to etch many scrapes across the monolayer
E. Add 1ml warm 0.05% trypsin and place in incubator for 4-5 minutes.

F. After 4-5 minutes, pipette up and down to detach cells from plate, then return to the incubator for a further 10 minutes (total trypsin time to this point is 14-15 minutes).
G. Triturate (gently) until cell sheet is detached from plate and transfer to a 15ml conical
H. Add an additional 1ml of trypsin. Manually shake, flick and roll the 15ml conical for 1-5 minutes. Stop occasionally and allow clumps to settle for 20-30 sec. Transfer cloudy supernatant to a separate 15ml conical containing “STOP Media”. Continue shaking, flicking and rolling the remaining clumps until mostly dissociated and then transfer the remaining cells to the conical containing “STOP Media” (proceed to CD47 staining protocol at this point).
I. Centrifuge at 200G x 5min.
J. Aspirate supernatant and re-suspend in FACS buffer
K. Filter through a 40um filter x 2.
L. Add Y-27632 (final concentration 10uM) and Propidium Iodide (500x) or Calcein Blue (1000X) to FACS sample and prepare collection tubes with FACS buffer supplemented with Y-27632 (final concentration 10uM).
M. Thaw Matrigel (356231) on ice
N. Sort NKX2-1+/PI negative or NKX2-1+/Calcein Blue+ cells.
O. Spin collected samples at 300G for 7min, resuspend in desired volume in FACS buffer and transfer to an Eppendorf. Spin 300g for 5 min.
P. Aspirate supernatant and place eppendorf or conical on ice for 2 min.
Q. Re-suspend each cell pellet at 250 to 1000 cells per microliter of matrigel using cold 200ul pipette tips. Take care to (a) avoid bubbles and (b) distribute the cells evenly in the matrigel.
R. Slowly pipette the matrigel + cells into the center of a 12 well plate (25-100uL/drops, typically 50uL).
S. Place 12 well plate in the incubator for 15-20 min.
T. Add distal (CK+DCI) or proximal (FGF2+10+DCI) media supplemented with 10uM Y-27632 media. See relevant protocols.
U. Re-feed every 48 to 72 hours

670 ALBANY STREET, 2ND FLOOR
BOSTON, MA 02118
TEL: 617-414-2969
HTTP://WWW.BUMC.BU.EDU/STEMCELLS/

CD47^{hi}/CD26^{lo} Sorting to Purify NKX2-1^{+} Cells Protocol

Required Reagents:

FACS Buffer

- Hank's Balanced Salt Solution—Life Technologies (Cat#14175145)
- Y-27632 dihydrochloride (Selective p160ROCK inhibitor)—R&D Systems (Cat#1254-50)
- HEPES Buffer Solution—Sigma Aldrich (Cat#H0887-100)
- EDTA
- FBS
- Primocin

Antibodies

- Anti-human CD47 PerCP/Cy5.5 conjugate, Biolegend (Cat#B191878)
- Anti-human CD26 PE conjugate, Biolegend (Cat#302706)
- PE mouse IgG1 isotype, Biolegend (Cat#400113)
- PerCP/Cy5-5 mouse IgG1 isotype—Biolegend (Cat#400149)
- Anti-TTF1 antibody [EP1584Y]—Abcam (Cat#:ab76013)
- Rabbit IgG, Monoclonal [EPR25A]-Isotype Control (Cat#: ab172730)
- Calcein blue, Life Technologies (C1429)
  Secondary antibody (for intracellular NKX2-1 FACS) based on your flow cytometer (we use Jackson ImmuoResearch Alexa 488 or Cy5 Donkey anti Rabbit IgG(H+L)).

CD47 Staining and Sorting to Purify NKX2-1^{+} Cells

Harvest and Dissociate Cells

I. Prepare FACS buffer:
   a. Hank's Balanced Salt Solution
   b. 2% FBS
   c. Primocin 100µg/mL
   d. HEPES 25mM
   e. EDTA 2mM.
II. Wash cells with DMEM/F12.
III. Aspirate wash media.
IV. Use a 10uL pipette to etch many scrapes across the monolayer of cells (**Figure 1**)—this step assists in the trypsin dissociation of the monolayer.
V. Add 1ml (per well of a 6-well plate) of 37ºC 0.05% trypsin and place in incubator for 14-18 minutes.
VI. Triturate (gently—2 or 3 times) until cell sheet is detached from plate and observe under the microscope. If many large clumps of cells remain then continue in tryspin for 3-5 additional minutes then triturate again 2-3 times. If mostly small clumps and single cells are visualized transfer to a 15ml or 50ml conical.
VII. Wash the well with an additional 1 mL of 0.05% trypsin and add this wash to the 15 mL conical tube. Manually shake and flick the conical for 3-5 minutes then pipette up and down slowly 2-3 times until the majority of clumps have dissociated.
VIII. Add 10 mL "Stop Media" (DMEM/F12 with 10% FBS). Pipette up and down with a 5-10ml pipette to mix the cell suspension. and filter through a 40µM filter

Stain Samples

IX. Perform a cell count by preferred method: Countess II, Luna Cell Counter, Hemocytometer—this count will be used to determine the staining volume.
X. Centrifuge 15 mL conical tube at 200G x 5min.
XI. Resuspend the cell pellet at 100 µL per 1 million cells in FACS buffer+10 µM Y-27632
XII. Aliquot cells into separate Eppendorfs for controls and staining (6 samples total):
    a. Unstained
    b. $CD47^{PerCP/Cy5.5}$ only
    c. $CD26^{PE}$ only
    d. $mIgG1^{PerCP/Cy5.5}$, $mIgG2^{PE}$ isotypes
    e. Calcein blue only
    f. $CD47^{PerCP/Cy5.5}/CD26^{PE}$
XIII. Add 0.5 µL antibody per 100µL staining volume
XIV. Stain the samples for 30 minutes on ice, protect from light
XV. Wash with FACS buffer to remove excess antibody, centrifuge 200xG for 5 minutes
XVI. Aspirate supernatant and re-suspend in FACS buffer+Y-27632 (10 µM) calcein blue(10 µM).
XVII. Filter again through a 40µm filter.
XVIII. Transfer filtered cell suspension to appropriate container for the sorting instrument that you are going to use and bring to sorter on ice, protect from light

Sort for $NKX2\text{-}1^{+}$ Cells

XIX. Sort $CD47^{hi}/CD26^{lo}$/calcein $blue^{+}$

There is variable efficiency of NKX2-1 induction in any given differentiation. Examples of unstained and isotype controls are shown below (**Figure 3**). We select the CD47hi/CD26 lo population (**Figures 4 ,5 and 6**). We also sort the CD47lo population and pre-sort samples as controls for subsequent NKX2-1 staining by intracellular FACS.

XX. Spin collected samples at 300G for 7min

Figures

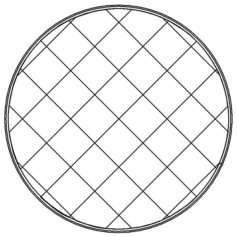

Figure 1: schematic of cross-hatch pattern using a p10 pipettor to facilitate trypsin dissociation in 6 well plate

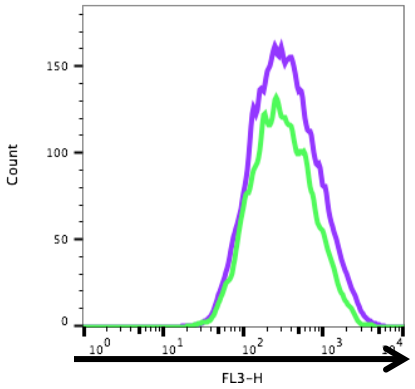

Figure 2: CD47 titration of 1 x 10^6 cells vs 1x10^5 cells with 0.5uL ab/100 uL staining volume

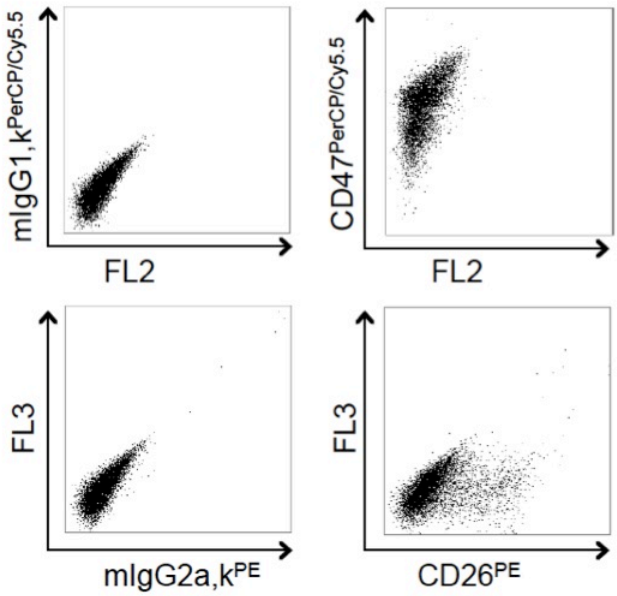

Figure 3: staining FACS analysis of isotypes vs. CD47 & CD26 on day 15 of directed differentiation

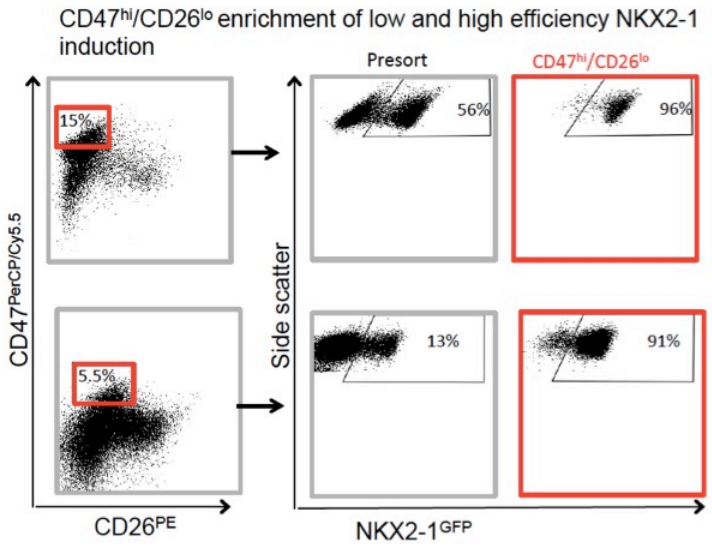

Figure 4: Representative CD47^{hi}/CD26^{lo} sorting strategy for NKX2-1 enrichment using the NKX2-1GFP reporter iPSC line

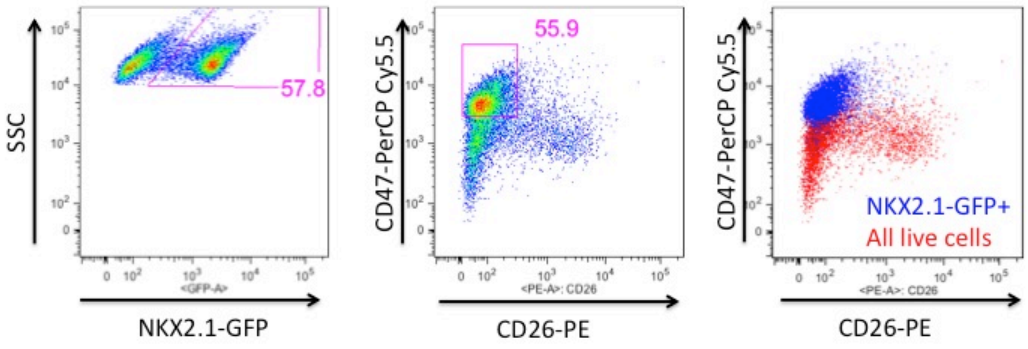

Figure 5: Using NKX2-1-GFP iPSC to identify the gating strategy for sorting NKX2-1+ cells using the CD47/CD26 staining.

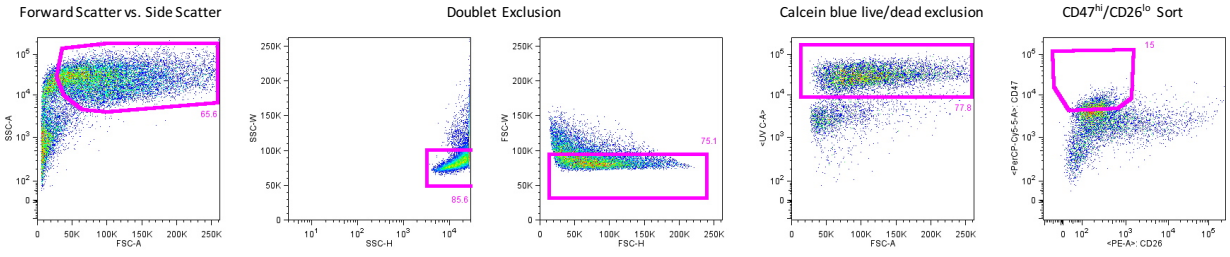

Figure 6: CD47^{hi}/CD26^{lo} sorting strategy

NKX2-1 Intracellular FACS

We highly recommend performing NKX2-1 intracellular FACS to quantify the percentage enrichment by the $CD47^{hi}/CD26^{lo}$ sort. See Figure 6 for representative FACS plots.

Samples:

- Day 0 iPSC
  - Unstained
  - Isotype + secondary
  - NKX2-1 +secondary
- Presort
  - Unstained
  - Isotype + secondary
  - NKX2-1 +secondary
- $CD47^{hi}/CD26^{lo}$
  - Unstained
  - Isotype + secondary
  - NKX2-1 +secondary
- $CD47^{lo}$
  - Unstained
  - Isotype + secondary
  - NKX2-1 +secondary

PFA:

- Dilute vial of 16% PFA 1:10 in 1x PBS.
- Make fresh daily.

"**Staining buffer"**

- Prepare in hood to maintain sterility of BSA
- Wear gloves when working with sodium azide (highly toxic)

Buffer Components	*Make from:*	*Dilution*	*For 50 ml*	*Location*
1x PBS	1x PBS	To final vol	46.2 ml	
0.5% BSA	7.5% BSA	1:15 dilution	3.33 ml	Media fridge
0.02% NaN_3	2% NaN_3	1:100 dilution	0.5 ml	Maria's fridge

XXI. Fix Cells

a. Harvest cells and count. Ensure cells are monodispersed prior to fixation. Ideally, you need more than 50K cells per condition.
b. Fix cells in 1 ml of 1.6% PFA at 37ºC for 30 min on rocker.
c. Wash twice with staining buffer - Dispose of PFA in appropriate waste container.
d. Resuspend in staining buffer at about 1 million per ml – store in fridge for two weeks+ at this stage.
    i. Use buffer with no NaN_3 if running samples same day or proceed straight into saponin buffer.

XXII. Wash Cells
   a. Spin down cells in tubes (500g for 4 min), aspirate the supernatant.
      i. Resuspend in 1 ml 1x Saponin buffer (Permeability Wash Buffer 10X, Biolegend cat#421002, diluted 1:10 with millipore water) and transfer samples to 1.7 ml Eppendorf tubes.

      ii. Split samples into 3 Eppendorfs (unstained, isotype control and stained):
   b. Wash cells twice with 1x Saponin Buffer. Then add 100ul of 4% Normal donkey serum in Saponin buffer for 30min. Wash x1 with saponin buffer, centrifuge at 300G x 5min and aspirate supernatant.

XXIII. Primary antibody stain
   a. Incubate with 1:100 dilution of TTF-1 primary antibody in 100 µl 1x Saponin Buffer to the "stained" sample and 1:100 dilution of the isoptye control to the "isotype sample and place all samples on rocker for 30 min at RT
   b. Wash twice with 1x Saponin Buffer.

XXIV. Secondary Antibody Stain
   a. Incubate with 2° antibody in 100 µl 1x saponin buffer at RT on rocker for 30 min (protect from light).
   b. Use 1:300 dilution of the 2° antibody in 1x saponin buffer or antibody solution.
   c. Wash twice with 1x Saponin Buffer.

XXV. Prep for Flow
   a. Resuspend cells in 300 µl FACS buffer (with no NaN_3)
   b. Filter through a 40µm filter and transfer to FACS test tubes.
   c. Bring to FACS machine (on ice) for flow.
   d. Samples can also be stored overnight in the fridge (block from light) until read on flow.

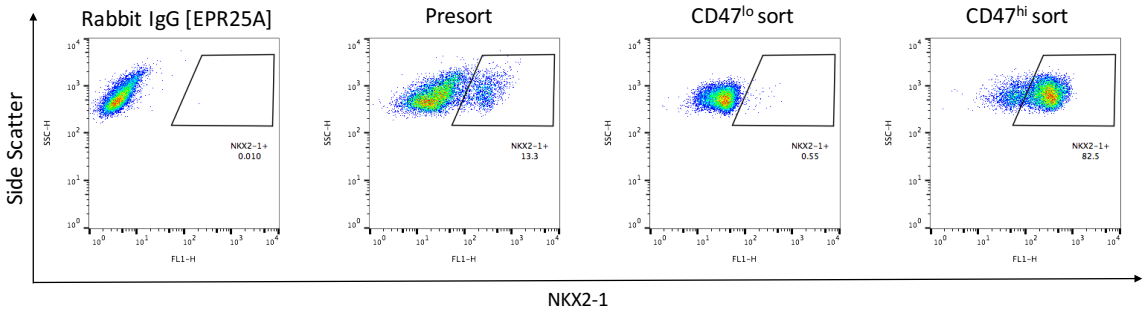

Figure 6: Intracellular FACS, NKX2-1 before & after $CD47^{hi}/CD26^{lo}$ sort

VERSION HISTORY

2017-08-29	Update to CReM template	FJH

Human Alveolosphere Directed Differentiation Protocol – Kotton Laboratory

INTRO

This protocol describes an approach to establish three-dimensional (3D) culture of purified PSC-derived lung and drive them towards SFTPC+ alveolar cells as published in: Jacob et al. Cell Stem Cell. 2017.

REAGENTS

A) cSFDM (complete serum free differentiation media):

Volume for 500 ml	Final concentration	Reference
375 ml IMDM	75%	ThermoFisher 12440053
125 ml Ham's F12	25%	Cellgro 10-080-CV
5 ml B-27 (with RA) supplement	1%	Invitrogen 17504-44
2.5 ml N-2 supplement	0.5%	Invitrogen 17502-048
3.3 ml BSA (7.5% stock)	0.05%	Invitrogen 15260-037
1 ml Primocin (100 µg/ml stock)	200 ng/ml	Invivogen NC9141851
5 ml Glutamax 100X	1X	ThermoFisher 35050-061
500 µl Ascorbic Acid (50 mg/ml stock)	50 µg/ml	Sigma A4544
1.5 ml MTG (from 26 µl in 2 ml IMDM)	$4.5x10^{-4}$M	Sigma M6145

B) CK+DCI Media

cSFDM Base
3uM CHIR99021 (Tocris 4423)
10ng/ml rhKGF (R&D 251-KG-010)
50nM Dexamethasone (Sigma D4902)
0.1mM 8BrcAMP (Sigma B7880)
0.1 mM IBMX (Sigma I5879)

C) Other Reagents

Rho-associated kinase inhibitor (Y-27632, Tocris 1254)
Growth Factor-Reduced Matrigel (Corning 356230)
Dispase (ThermoFisher 354235)

PROTOCOL

Begin this protocol after generating hPSC-derived NKX2-1+ lung progenitors and purifying by flow cytometry (related protocols: Human Lung Differentiation, CD47/CD26 sorting of lung progenitors)

A) Replating NKX2-1+ Lung Progenitors and Expansion in 3D Matrigel as Alveolospheres (Day 15 to 20+)

1. Thaw 3D matrigel and prepare CK+DCI media during sort.
2. Spin down sorted lung progenitor cells at 300g x 5min.
3. Resuspend 400 cells/ul in undiluted matrigel in droplets ranging from 20ul (96 well plate) to 1ml (10cm dish).
    a. *Again, the replating density can influence percent SFTPC$^{tdTomato+}$ cells at later timepoints as well. Try replating at lower densities if the efficiency is low.*
4. Place in 37C incubator for 20-30 minutes *without* adding media to allow the matrigel to solidify.
5. Add warm CK+DCI media + 10 µM Y-27632
6. After 48 hours, change media to CK+DCI *without* 10 µM Y-27632

7. Change media every 3 days, or as necessary. Be careful when aspirating to avoid dislodging the matrigel drop. At later timepoints, change media more frequently.
8. You should begin to see SFTPC$^{tdTomato+}$ cells within 2-5 days.
    a. Passage alveolospheres when they become confluent within the matrigel drop, otherwise efficiency will *decrease.*

B) Alveolosphere dissociation and passaging method

1. Aspirate media from matrigel drop.
2. Add 1 ml dispase (2mg/ml), leave at 37C for 1 hour, pipetting up and down once after ~30 mins.
3. Transfer 1ml dissociated organoids in dispase into a 15ml conical, add 10ml DMEM to wash.
4. Centrifuge 200g x 4min, aspirate supernatant, repeat once more
    a. *If a clear haze is seen above the pellet, the dispase has not totally dissolved and more dispase can be added to the pellet for another 20 minutes at 37C.*
5. Aspirate supernatant, aspirate leftovers with a P200 tip, leaving as little supernatant as possible.
    a. *It is important to remove all dispase since it will dissolve matrigel in culture as well.*
6. At this point you have 3 options:
    a. ***Option 1: Replate whole alveolospheres***
        i. Resuspend cells in 3D matrigel at desired concentration (1:3 to 1:10 is reasonable)
        ii. Place in 37C incubator for 20-30 minutes *without* adding media to allow the matrigel to solidify.
        iii. Add 1ml CK+DCI+ 10 μM Y-27632 media over matrigel drop
    b. ***Option 2: Replate single cells***
        i. Resuspend cells in 1ml trypsin/ well, incubate at 37C for 10-12 minutes, until pipetting only 3-5 times results in single cell suspension

            Dissociate cells in a tissue culture plate so that you can visualize them in a microscope. Cells are sensitive to over-pipetting at this stage, so leave them in trypsin long enough for most of the dissociation to be enzymatic. If alveolospheres have not dissociated into single cells by 12 minutes, spin down, add fresh trypsin, and leave another 5 mintues
        ii. Stop with FBS-containing media, spin at 300g x 5min, wash once with 10ml DMEM.
        iii. Resuspend cells in 3D matrigel at desired concentration (1:3 to 1:10 is reasonable)
        iv. Place in 37C incubator for 20-30 minutes *without* adding media to allow the matrigel to solidify.
        v. Add 1ml CK+DCI+ 10 μM Y-27632 media over matrigel drop (see below for recipe).
    c. ***Option 3: Sort alveolosphere cells***
        i. Follow protocol in section Fb to dissociate
        ii. Follow protocol on section D4-7 to sort
        iii. Follow protocol in section E to replate

C) CHIR Withdrawal and Addback (Day 30-40+)

1. Prepare alveolosphere cells for sorting as described above
2. Sort and replate NKX2-1^{GFP+} cells in 3D matrigel culture using reporter line as described above
3. After 24 hours in CK+DCI+10 μM Y-27632, change media to K+DCI media *(NO CHIR99021)*
    a. *You should see that the cells do not grow as fast as they do in CK+DCI media.*
4. After 5 days in K+DCI, change media back to CK+DCI media
    a. *You should see an increase in the size of the alveolospheres over this time and a SFTPC+ percent close to 50%.*

D) Alveolosphere Freezing Method

1. Dissociate alveolospheres into single cell suspension
    a. Aspirate media from matrigel drop
    b. Add 1 ml dispase (2mg/ml), leave at 37C for 1 hour, pipetting up and down once after ~30 mins.
    c. Transfer 1ml dissociated organoids in dispase into a 15ml conical, add 10ml DMEM to wash.
    d. Centrifuge 200g x 4min, aspirate supernatant, repeat once more
    e. Resuspend cells in 1ml trypsin/ well, incubate at 37C for 5-10 minutes
    f. Pipet up and down 5-10 times, stop with FBS-containing media
    g. Spin at 300g x 5min, wash once with 10ml DMEM.
2. Resuspend in 1ml Freeze Media/well (90% FBS, 10%DMSO) and freeze in a cryovial (first at -80C overnight, then long term storage at -150C.)

VERSION HISTORY

2017-07-10	Drafted by Anjali Jacob.	AJ
2017-08-09	RA concentration changed from 50nM to 100nM	LS
2018-04-03	Removed progenitor stage from protocol – see independent protocol to avoid confusion	FH

Generation and passaging of airway epithelial organoids from hPSC-derived lung progenitors – Kotton Lab

INTRO

This protocol describes an approach to establish three-dimensional (3D) culture of purified PSC-derived lung and drive them towards an airway epithelial phenotype. Cells are resuspended in 3D Matrigel matrix and cultured in media driving activation of FGF signaling via FGF2 and FGF10 and containing corticosteroids and cyclic-AMP drives cells to form epithelial spheres containing differentiated airway cell types.

REAGENTS

- Growth factor-reduced Matrigel matrix (Corning, cat. no. 356234)
- Complete Serum Free Differentiation Medium (cSFDM)
  - 375 mL 1x Iscove's Modified Dulbecco's Medium (IMDM, Invitrogen, cat. no. 12200-036)
  - 125 mL Ham's F12 (e.g. Invitrogen, cat. no. 11765-054)
  - 5 mL B27 with retinoic acid (ThermoFisher, cat. no. 17504044)
  - 2.5 mL N2 (ThermoFisher, cat. no. 17502048)
  - 500 µL ascorbic acid, 50 mg/mL
    - Ascorbic acid can be prepared by diluting stock powder (e.g. Sigma A4544-25G) in sterile, tissue-culture grade water to a final concenvration of 50 mg/mL
  - 19.5 µL monothioglycerol, 500 µg/mL (Sigma, cat. no. M6145-25ML)
  - 3.75 mL Bovine Albumin Fraction V, 7.5% solution (Gibco, cat. no. 15260-037)
  - 5 mL Glutamax (Gibco, cat. no. 35050)
  - 500 µL primocin (Invivogen, cat. no. ant-pm-2)
  - Filter sterilize (for example, with Millipore Steritop Sterile Vacuum Bottle-Top Filters, cat. no. SCGPS01RE) and store for up to one month at 4°C, kept away from light.

- 10x cAMP/IBMX Stock
  - 50 mL cSFDM base
  - 21.5 mg 8-Bromoadenosine 3',5'-cyclic monophosphate sodium salt (cAMP, Sigma-Aldrich, cat. no. B7880-100MG)
  - 500 uL 0.1 M IBMX (3-isobutyl-1-methylxanthine, Sigma, cat. no. I5879)
  - Filter sterilize and store at 4°C for up to 1 month

- 250 µg/mL recombinant human FGF2
  - 1 mg recombinant human FGF basic protein (R&D Systems cat. no. 233-FB-025)
  - 4 mL 0.1% BSA
    - Prepare 0.1% BSA in PBS by diluting 13 µL Bovine Albumin Fraction V, 7.5% solution in 1 mL PBS.
  - Filter sterilize.
  - Aliquot and store at -80°C for up to 1 year.
  - After thawing individual aliquots, store at 4°C for up to 1 week.
  -

- 10 µg/mL recombinant human FGF10
  - 25 µg recombinant human FGF10 protein (R&D Systems cat. no. 345-FG-025)
  - 2.5 mL 0.1% BSA
    - Prepare 0.1% BSA in PBS by diluting 13 µL Bovine Albumin Fraction V, 7.5% solution in 1 mL PBS.
  - Filter sterilize.
  - Aliquot and store at -80°C for up to 1 year.
  - After thawing individual aliquots, store at 4°C for up to 1 week.

• 100 µM dexamethasone
  — Prepare 1 mM stock:
    ○ Dexamethasone powder (Sigma, cat. no. D4902-25MG)
    ○ 63.7 mL molecular biology grade ethanol
    ○ Store at -20°C for up to 2 years.
  — Prepare 100 µM stock:
    ○ 500 µL 1 mM dexamethasone stock
    ○ 49.5 mL molecular biology grade ethanol
    ○ Aliquot and store at -20°C for up to 1 year. This is the working concentration.

• 10 mM Y-27632 (Y)
  — 10 mg Y-27632 dihydrochloride (Tocris, cat. no. 1254)
  — 3.1 mL sterile, tissue-culture grade water
  — Filter sterilize (for example, with EMD Millipore Sterile Disposable Vacuum Filter Units, cat. no. SCGP00525).
  — Aliquot and store at -80°C for up to 1 year.
  — After thawing individual aliquots, store at 4°C for up to 1 month.

• Airway Differentiation Medium:
  — 45 mL cSFDM base
  — 5 mL 10x cyclic AMP/IBMX stock
  — 50 µL 250 µg/mL rhFGF2
  — 500 µL 10 µg/mL rhFGF10
  — 25 µL dexamethasone
  — 50 µL 10 mM ROCK inhibitor

• 2 mg/mL dispase II
  — 100 mg Dispase II, powder (ThermoFisher, cat no. 17105041)
  — 50 mL DMEM
  — Dissolve and filter sterilize. Store at 4°C for up to 2 weeks or aliquot and freeze at -20°C for up to 6 months.

• 0.05% Trypsin-EDTA (e.g. Gibco, cat. no 25-300-062)
• Fetal bovine serum (FBS, e.g. Gibco, cat. no. 10082139)
• DMEM (Gibco cat. no. 11995-065)

PROTOCOL

Begin this protocol after generating hPSC-derived NKX2-1+ lung progenitors and purifying by flow cytometry (related protocols: Human Lung Differentiation, CD47/CD26 sorting of lung progenitors)

A) Establishing three-dimensional airway epithelial organoid culture

1. Spin down single cells post-sort for 5 minutes at 4°C and 300 x g.
2. Resuspend cells at a concentration of 400 cells/µL in undiluted Matrigel matrix and replate in Matrigel drops (size can vary but typically 20-25uL per well of a 12 well plate or 50 – 100 µL per well of 6 well plate).

Prior to resuspending cells, Matrigel should be thawed and kept cold (on ice) to prevent polymerization. For ease of thawing, it is convenient to aliquot the Matrigel to be used in 3D culture into 500 µL – 1 mL aliquots. Take care when resuspending cells not to introduce bubbles into the Matrigel and to efficiently disperse the cell pellet into single cells distributed throughout the Matrigel.

Smaller Matrigel drops can be used but are not as robust at generating organoids as cells can settle through the Matrigel and attach to the bottom of the plate.

If more cells are required downstream, cells can also be replated in several small (50 – 100 µL) drops in a 6-well plate or a p100 dish.

Cells should be plated in one drop per well of a 12-well plate.

3. Allow drops to solidify for 15-20 minutes at 37°C.
4. After drops have fully polymerized, add Airway Differentiation Medium carefully to wells.

Add enough media to ensure that drops are fully covered, typically 1 – 2 mL per well of a 12 well plate.

5. Cells will begin to form epithelial spheres ("organoids") after several days to one week of culture and will continue to proliferate and expand until the drop is filled with cells.

B) Passaging airway epithelial organoids

1. Aspirate media and add 2 mg/mL dispase to well to cover droplet (typically 1 mL/well) and incubate at 37°C for 20 minutes to 1 hour, until Matrigel is fully dissolved.

Dislodging the Matrigel pellet with a pipette prior to incubation and gentle pipetting 3-5 times after 10 min can facilitate dissocation.

2. Using a p1000 pipette, transfer dissociated organoids to a new 15-mL conical tube and add an equivalent volume of DMEM.
3. Spin down spheres for 1-2 minutes at 4°C, 300 x g.

If organoids have not formed a pellet after this time, spin for an additional 1-2 minutes.

If organoids are particularly large, they can be allowed to settle to the bottom of the conical instead of this centrifugation step. This is particuarily useful if there is a lot of debris in the Matrigel drop, as this will not settle and is aspirated with the supernatant.

4. Aspirate supernatant and add 1 – 2 mL 0.05% trypsin per dissociated drop.

For example, if 3 drops were originally dissociated, add 3 mL trypsin.

5. Transfer trypsin and cells to a well of a 6-well plate and incubate for 8 – 10 minutes at 37°C.

Cells will begin to visibly dissociate from the spheres. Allow cells to incubate with trypsin until they are entirely dissociated; they will not survive being mechanically dissociated by pipetting so most of the dissociation should be enzymatic. If cells have not dissociated after 12 minutes in trypsin, collect the cells, spin them down, and resuspend in fresh trypsin for an additional 3 – 4 minutes.

6. While cells are dissociating, prepare "stop medium" by adding 50 mL FBS to 450 mL DMEM.
7. Collect dissociated cells in a new 15 mL conical tube and add an equivalent volume of stop medium.
8. Spin down cells for 5 minutes at 4°C and 300 x g.
9. Resuspend cells in 1 mL DMEM and count using an automated cell counter.
10. Spin down cells and resuspend at a concentration of 400 cells/µL in undiluted Matrigel matrix and replate in 50 – 100 µL drops, allow drops to solidify for 20 minutes to 1 hour at 37°C, and add Airway Differentiation Medium carefully to wells.

This protocol also is effective to prepare a viable single cell solution of cells for flow cytometry or sorting from airway organoids. For this approach, spin down cells and resuspend in FACS buffer for staining or other downstream analysis.

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Preparation of Mouse Embryonic Fibroblasts

1. Order three timed-pregnant CF-1 mouse (Charles River Laboratories).

2. Day 1: On embryonic day 13.5, sacrifice the pregnant female and sterilize with 70% alcohol. Dissect out the uterus of the pregnant female using sterile tools and place it in a 50 mL conical containing DPBS and 1% Pen/Strep.

3. From this step forward, all steps should be performed using strict sterile conditions in a tissue culture biosafety cabinet. Transfer the uterus into a 10 cm dish containing 10 mLs of DPBS with 1% Pen/Strep. Dissect out the embryos (~ 33 total) from the uterus and remove any extra uterine or placental tissue, leaving just the embryos.

4. Transfer the embryos into a fresh 15 cm dish containing 2 mLs of 0.05% trypsin (warmed) per 3 embryos. Finely mince the embryos using a sterile razor blade.

5. Incubate the dish in a 37^{o}C incubator for 5 minutes.

6. Add an equal volume of DMEM complete media (10% FBS DMEM with 1% P/S/ L-Glut) to neutralize the trypsin.

7. Dispense ~ 10 mLs chopped embryos to each of 6 x 15 cm dishes (try to evenly distribute embryo tissues; add additional 10% DMEM Complete medium to increase volume to ~ 25 mLs medium.

8. Place each 15 cm dish flat (not stacked) in 37^{o}C incubator.

9. Day 2: Cells are approximately 50% confluent in 15 cm plates, some areas may be more dense due to cell aggregates.
   - Pass cells ~ 1:3 for ~ 18-20 x 15 cm plates (6 x 15 cm plates to 18-20 x 15 cm plates):
   - Trypsinize cells with 0.25% trypsin, 2-3 min 37^{o}C and add DMEM Complete Medium to inactivate trypsin; spin down cells at 300 *x g*, 5 min
   - Resuspend in DMEM Complete Medium (remove aliquot of cells for testing of mycoplasma)

10. Day 3: Add more 10% DMEM Complete Medium to plates.

11. Day 4. If cells confluent, freeze cells at passage 1, ~ 3-4 vials per 15 cm plate or proceed directly to protocol for preparing Active MEFs to Inactive MEFs (below).

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Preparation of Inactive MEFs from Active MEFs

1. Day 0: Thaw frozen vial of active MEFs P0 into 15 mL conical tube with 9 mLs 10% FBS DMEM Complete Medium (DCM).
   - Spin 300 x g, 5 min to remove DMSO

2. Resuspend cells in 1 mL DCM and add to 10 cm plate with 12 mLs pre-warmed DCM for a total of 13 mLs. Distribute cells by gentle rocking and place in 37°C incubator.

3. Day 1: Pass following day ~ 1:3 into one 15 cm plate (with 22 mLs pre-warmed DCM):
   It is necessary for cell cultures to be at least 90% confluent at time of passage for cells to continue to proliferate
   - Rinse 10 cm plate 2x with warm DPBS
   - Trypsinize with pre-warmed 0.25% Trypsin for ~ 3 min, 37°C; may be necessary to use cell lifter to loosen all cells from surface
   - Stop reaction with 7 mLs of DCM
   - Spin 300 x g, 5 min
   - Resuspend in ~ 3 mLs DCM and add to 15 cm plate; rock to distribute cells and return to 37°C incubator

4. Day 4: If cells confluent, pass 1:3: 1 x 15 cm plate into 3 x 15 cm plates following the trypsinization and spinning procedure described above.

5. Day 6: If cells confluent, pass 1:3: 3 x 15 cm plate into 9 x 15 cm plates as described above.

6. Day 8: If cells confluent, pass 1:3, 9 x 15 cm to 18 x 15 cm plates (as above).

7. Day 9: Prepare for cell harvest:
   - Thaw 100 mLs 0.25% Trypsin (place at 4°C overnight to thaw)
   - Prepare 500 mLs DCM
   - Prepare 200 mLs DCM with Mitomycin C (MitoC; Fisher Scientific; Cat# BP25312); resuspend MitoC vial (2 mg) in 1 mL DCM and add to 200 mLs DCM for a final concentration of 10*m*g/mL MitoC in DMC
   - Store all media and reagents at 4°C overnight

8. Day 10: Harvest CELLS
   Ideally you would like to start in afternoon for increased cell numbers; MEFs should appear confluent
   - Label cryovials and store in -20 °C until use
   - Trypsinize and inactive 3-5 MEF plates (15 cm) at one time:
     - Aspirate DCM from 15 cm MEF plates; take note of passage number
     - Add 10 mLs/15 cm dish of 10 μg/mL MitoC/DCM (2 mg vial/200 mLs DCM)
     - Incubate 37°C, ~ 2hr (between 2-3 hours; not longer than 3 hours)
     - STOP reaction: Remove MitoC/DCM to waste container and dispose properly
     - Wash twice in 10-12 mLs DPBS
     - Add ~ 3 mLs 0.25% trypsin and return to incubator for ~ 2-3 min.

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Preparation of Inactive MEFs from Active MEFs (continued)

- Add ~ 7 mLs DCM to plate and pipet up and down to break up clumps; transfer to 50 mL conical tube; rinse each plate with 2 additional mLs of DCM and add to conical tube
- remove aliquot of cells to count
- Spin 300 *x g*, 5 min and aspirate DCM
- Resuspend cell pellets in cold 1x Freezing Medium (90% FBS/10% DMSO) at approx. 3.5 x 10^6 cells/cryovial (~ 4-5 vials/15 cm dish).
- Place cryovials in styrofoam or commercial freezing container for storage at -80^{o}C overnight and transfer cryovials to -150^{o}C long-term storage freezer the following day

(B) Mycoplasma Testing Protocol: PCR of gDNA

1) Collect cell pellet

For cells in culture or inoculated surveillance plates the recommended method of harvesting cells for gDNA extraction is using a cell scraper. Avoid excessively large cell pellets (aim for less than 10^6 cells). Avoid using trypsin or EDTA to harvest cells as they may disrupt mycoplasmas. Spin down cell pellet in a clearly labeled eppendorf, aspirate the supernatant leaving just the cell pellet and store at -20 to -80 degrees until tested.

2a) Extract DNA (Tail Buffer Method)

-make tail buffer*
-thaw and spin down cells
-wash in 1x HEPES
-resuspend pellet in 0.5 ml tail buffer* supplemented with 400 µg/ml proteinase K
-incubate 1 hour at 37°C
-wash pellet with 70% EtOH
-resuspend in TE (~100 µl) overnight at 50°

***tail buffer**

100 mM Tris-HCL pH 8.0
5 mM EDTA pH 8.0
0.2% SDS
200 mM NaCl

Adjust DNA to ~50 ng/µg

2b) Extract DNA (Qiagen)

Alternatively, DNA may be extracted using a Qiagen DNA Mini Kit (cat#: 51304).

Adjust DNA to ~50 ng/µg

3) PCR

Mycoplasma PCR uses a primer set referred to as either “2/11” or “279/280” depending on the CReM lab. From here on referred to as 279/280. Include a GAPDH control for each sample. Each test should include (1) a positive control (2) a negative control/previously tested sample (3) a water sample.

Myco279 (cb): CTTCWTCGACTTYCAGACCCAAGGCAT
Myco280 (cb): ACACCATGGGAGYTGGTAAT

10x PCR buffer	2.5
10mM dNTP	2
Primer mix (279/280) (10 µM each)	1
DNA	1
H_2O	18.25
Taq (commercial)	0.25

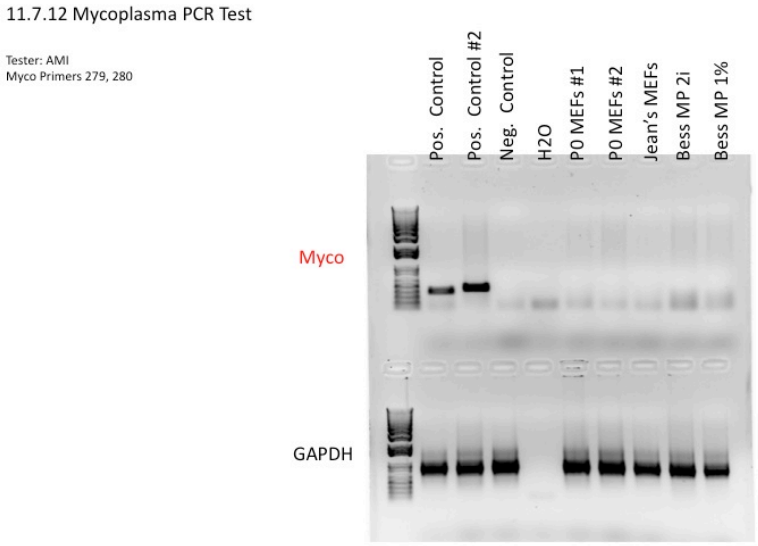
Program: 32 cycles
95°C; 3min

95°C; 30 sec
55°C; 30 sec
72°C; 1:30 min

72°C; 5 min

Analyze PCR products on a 1.5% agarose gel. Mycoplasma positive product size: approx. 450 bp. Ensure that each sample except water has a detectable GAPDH band. See examples below. Store a labeled, dated powepoint file of the gel in the myco testing folder and add the details of the test to the myco log excel file.

Example 1:



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hESC Medium

For 500 ml of hESC medium:

	stock	final	volume
• Knockout™ Serum Replacement (Life Tech. Cat# 10828-028)	100%	20%	100 mL
• GlutaMAX™ (Life Tech. Cat# 25030-081)	100x	1x	5 mL
• Non-Essential Amino Acids (Life Tech. Cat# 11140-050)	100x	1x	5 mL
• bFGF (R&D Systems Cat# 233-FB)	250 μg/mL	10 ng/mL	20 μL
• beta-Mercaptoethanol (Life Tech. Cat# 21985-023)	55 mM	100 μM	1 mL
• Primocin™ (Invivogen Cat# ant-pm-2)	50 mg/mL	100 μg/mL	1 mL
• DMEM/F12 medium (Life Tech. Cat# 11330-032)			388 mL

➢ Mix all the reagents in the top cup of a 500 ml filter system (0.22um) and filter. hESC medium can be stored at 4°C for two weeks.

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List of Reagents

Reagents	Company	Cat#
DMEM/F12	Life Tech	11330-057
Knockout™ SR	Life Tech	10828-028
L-Glutamine	Life Tech	25030-081
MEM NEAA	Life Tech	11140-050
2-Mercaptoethanol	Life Tech	21985-023
bFGF	R&D Systems	233-FB-001MG/CF
Collagenase Type IV	STEMCELL Technologies	07909
DMSO	Sigma	D2650
Defined FBS (w/o Heat Inactivation)	HyClone	SH30070.03
EmbryoMax® 0.1% Gelatin Solution	EMD Millipore	ES-006-B
mTeSR	STEMCELL Technologies	05850
ReLeSR	STEMCELL Technologies	05872
mFreSR	STEMCELL Technologies	05855
hESC-qualified Matrix, 5 ml *LDEV-Free	Corning	354277
ReproTeSR	STEMCELL Technologies	05920
Y27632/ROCK Inhibitor	STEMGENT	04-0012
Mitomycin C	Fisher Scientific	BP25312
Primocin	Invivogen	ant-pm-2

CytoTune™-iPS 2.0 Sendai Reprogramming Kit	Life Tech	A16517
Ascorbic Acid	Sigma	A4544
human Stem Cell Factor (SCF)	R&D Systems	255-SC
IL3	R&D Systems	203-IL
IGF1	R&D Systems	291-G1-01M
Erythropoietin (EPO)	R&D Systems	287-TC
Dexamethasone	Sigma	D4902
QBSF-60 Stem Cell Medium	Fisher	160-204-101

STEMdiff™ Definitive Endoderm Kit	STEMCELL Technologies	05110
Gentle Cell Dissociation Reagent	STEMCELL Technologies	07174
0.05% Trypsin	Life Tech	25300054
Mouse IgG1 APC-conjugated	Invitrogen	MG105
Mouse IgG2a PE-conjugated	STEMCELL Technologies	60108PE
Human CXCR4 R-PE conjugated	STEMCELL Technologies	60089PE
APC anti-human CD117(CKIT)	Biolegend	313206

Microsart RESEARCH Mycoplasma Kit	Sartorius	SMB95-1006

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CENTER FOR REGENERATIVE MEDICINE
BOSTON UNIVERSITY BOSTON MEDICAL CENTER

670 ALBANY STREET
2ND FLOOR
BOSTON, MA 02118
TEL: 617-638-6572
HTTP://WWW.BUMC.BU.EDU/STEMCELLS/

List of Human Lung Reagents

Reagents	Vendor	Catalog #	Stock	Final Conc.
Ascorbic Acid	Sigma	A4544-25G	50mg/ml	50ug/ml
B27 +RA	GIBCO	12587-044		0.5X
BSA 7.5%	GIBCO	15260-037	0.5g/ml	
Chirr99021	Stemgent	04-0004	3mm	3uM
Dorsomorphin	Stemgent	04-0024	2mM	2uM
EDTA	SIGMA	028K8710		
Gentle cell dissociation reagent	Stem Cell Technologies	7174		
Glutamax	GIBCO	35050		
Ham's F12	Corning Cellgro	10-080-CV		
rhBMP4	R&D systems	314-BP-050	10ug/ml	10ng/ml
HBSS (Hank's buffered saline solution)	Gibco	14175-079		
rhFGF10	R&D systems	345-FG	10ug/ml	10ng/ml
rhFGF7/KGF	R&D systems	251-KG-050	10ug/ml	10ng/ml
IBMX	SIGMA	I5879	1mM	
IMDM	GIBCO	12440-053		
Matrigel (2-D)	Corning	354277		
Matrigel (3-D)	Corning	356231		
Monothioglycerol	Sigma	M6145-25ml	3uL/ml	
N2	GIBCO	17502-048		
Primocin				
Retinoic Acid	Sigma	R2625	5mM	100nM
SB431542	Sigma	S4317-5MG	10mM	10uM
STEM diff Endoderm kit	Stem Cell Technologies	5110		
Y-27632	Tocris	1254		
HEPES	GIBCO	15630-080		
cyclic AMP (8-Br-cAMP)	SIGMA	b7880	1mM	0.1mM
Dexamthasone	SIGMA	D4902	100uM	50nM
CXCR4 Mouse Anti-Human mAb (PE)	Invitrogen	MHCXCR404		
Isotype Mouse IgG2a, (PE)	Invitrogen	MG2a04		
c-Kit (CD117) Mouse Anti-Human mAb (APC)	Invitrogen	CD11705		
Isotype Mouse IgG1, (APC)	Invitrogen	MG105		

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HTTP://WWW.BUMC.BU.EDU/STEMCELLS/

CReM/iPSC CORE Training Course Contact List

Name	Email
Dr. Marianne James iPSC Core Manager Center for Regenerative Medicine (CReM)	mfjames@bu.edu
Dr. Darrell Kotton Professor of Medicine and Pathology Director, Center for Regenerative Medicine (CReM)	dkotton@bu.edu
Dr. Gustavo Mostoslavsky Associate Professor of Medicine, Gastroenterology Section Co-Director, Center for Regenerative Medicine (CReM)	gmostosl@bu.edu
Áine R. Russell Research Study Technician, iPSC Core Lab Manager Center for Regenerative Medicine (CReM)	aineruss@bu.edu
Dr. Finn Hawkins Assistant Professor of Medicine, Pulmonary Section Center for Regenerative Medicine (CReM)	hawk@bu.edu
Dr. Andrew Wilson Associate Professor of Medicine Center for Regenerative Medicine (CReM)	awilson@bu.edu
Dr. George Kwong Boston University, Kotton Lab Center for Regenerative Medicine (CReM)	gkwong11@bu.edu
Nora J. Lee Research Study Technician, BU, Kotton Lab Center for Regenerative Medicine (CReM)	noralee@bu.edu
Dr. Rhiannon B. Werder Mostoslavsky Lab Manager Center for Regenerative Medicine (CReM)	rwerder@bu.edu
Mary Lou Beermann Research Study Technician, Finn Hawkin's Lb Center for Regenerative Medicine (CReM)	mlbee@bu.edu
Dr. Kostas Alysandratos BUSM, Department of Medicine, Assistant Professor Center for Regenerative Medicine (CReM)	kalysandratos@gmail.com
Dr. Andrew Berical BUSM, Department of Medicine, Pulmonary Fellow Center for Regenerative Medicine (CReM)	a.berical@bu.edu
Leon Sun MD PhD Candidate, BUSM, Kotton Lab Center for Regenerative Medicine (CReM)	ysun35@bu.edu
Dr. Kristy Abo BUSM, Department of Medicine, Pulmonary Fellow Center for Regenerative Medicine	kabo@bu.edu
David Roberts Research Study Technician, Kotton Lab Manager, Kotton Lab Center for Regenerative Medicine (CReM)	dar815@bu.edu
Dr. Greg Miller CReM Lab Manager Center for Regenerative Medicine (CReM)	milllergr@bu.edu

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CENTER FOR REGENERATIVE MEDICINE
BOSTON UNIVERSITY BOSTON MEDICAL CENTER

670 ALBANY STREET
2ND FLOOR
BOSTON, MA 02118
TEL: 617-638-6572
HTTP://WWW.BUMC.BU.EDU/STEMCELLS/

IPSC CORE

CReM/iPSC CORE Training Course Contact List (Continued)

Dr. Jyh-Chang Jean Assistant Professor of Medicine, Pulmonary Section, Center for Regenerative Medicine (CReM)	jjean@bu.edu
Becky Smeltzer Administrative Assistant Center for Regenerative Medicine (CReM)	rsmeltze@bu.edu
Meenakshi Lakshminarayanan Administrative Director Center for Regenerative Medicine (CReM)	mnarayan@bu.edu

List of Training Course Attendees

Name	Email

CReM
CENTER FOR REGENERATIVE MEDICINE
BOSTON UNIVERSITY BOSTON MEDICAL CENTER

670 ALBANY STREET
2ND FLOOR
BOSTON, MA 02118
TEL: 617-638-6572
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Suggested Reading

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