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

April 24th – 28th, 2017
Boston, MA
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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 entire 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
- Assay for mycoplasma contamination
- 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 and troubleshooting with course instructors

PRACTICAL LABORATORY SESSIONS
- Isolate and expand PBMCs for reprogramming
- Reprogramming of PBMCs: plate transduced PBMCs onto MEF feeders and observe morphological changes at early- and late-stages of reprogramming process
- iPSC cell culture propagation and maintenance on feeder (MEFs) and feeder-free (matrigel™) matrices
- Colony passaging by manual picking and cell dissociation solutions; removing (“cleaning”) differentiated cells
- Cryopreservation and thawing of hiPSCs
- 3D CX plating onto matrigel
- Hands-on introduction to flow cytometry (FACS)
- Observation of immunofluorescence-stained iPSC colonies using antibodies against stem cell surface markers
### iPSC Core

#### Course Schedule

**Monday**
- **9:00am**
  - Welcome (Jeff)
- **10:00am**
  - General iPSC culture methods (Jeff), Detailed description of culture, freezing, etc. (Selina) Intro
- **11:00am**
  - Blood donations (GM, GM, AW); 3 tubes each donor
  - Harvest PBMCs; pull off buffy coat (one each participant)
- **noon**
  - View expanded PBMCs; plate reprogrammed cells onto MEFs (Jeff)
  - View later (~48h) reprogrammed cells
- **1:00pm**
  - Plate post-MEFs
- **2:00pm**
  - Passage 1 and 2 (1:3 and 1:10 with collagenase)
  - Passage 1 and 2 (3:1 and 1:10 with collagenase)

**Tuesday**
- **9:00am**
  - ESC and the embryo (DK) Introduction to the embryo, ESCs, differentiation
- **10:00am**
  - Detailed description of freezing methodology (Jeff) Observe Monday cells
- **11:00am**
  - Freeze hiPSCs
- **noon**
  - Plate matrigel
  - Passage hiPSCs onto matrigel with RetsiSR
- **1:00pm**
  - Practice picking hiPSCs onto MEFs (2x) Picking hiPSCs onto MEFs (DK)
- **2:00pm**
  - Feed hiPSCs
  - Feed endobronchial with Dylan
- **3:00pm**
  - Feed hiPSCs
  - Feed endobronchial with Dylan

**Wednesday**
- **9:00am**
  - Gene Editing (GM)
- **10:00am**
  - CRISPR design (Katie)
  - Thaw hiPSCs from previous day
- **noon**
  - Lunch
- **1:00pm**
  - Plate matrigel
  - Passage hiPSCs onto matrigel with RetsiSR
- **2:00pm**
  - Practice picking hiPSCs onto MEFs (2x)
- **3:00pm**
  - Feed hiPSCs
  - Feed endobronchial with Dylan

**Thursday**
- **9:00am**
  - Directed Differentiation/Definitive Endoderm (Fin)
- **10:00am**
  - Mycoplasma (10/15) (Greg)
- **noon**
  - Lunch
- **1:00pm**
  - Plate definitive endoderm
  - Analyze cells by FACS
  - 3-D matrigel culture of lung progenitors (Dylan/Anjali/Leon)
- **2:00pm**
  - Adaptation; Passage hiPSCs from MEFs to matrigel using Monday's passaged iPSC for each student
  - Feed hiPSC

**Friday**
- **9:00am**
  - Characterization (DK)
- **10:00am**
  - Intro characterization (Mj)
- **noon**
  - Lunch
- **1:00pm**
  - If: DAPI/FDA/TO-90/SE4 (Mj)
  - If: observe all cells done during the week

**Q&A**
- **Beer hour, all CREM invited**
Workflow Diagram for iPSC Training Course

Isolate PBMCs from Blood Sample

- Cell Expansion
  - mycoplasma testing
  - gDNA extracted for DNA fingerprinting

Reprogramming

- Cells transduced with reprogramming factors
- Incubate 37°C 3-4 days

Plate cells onto feeder layer (MEFs)

- 20-30 days

iPSC colonies are picked and expanded

- 2-3 weeks

iPSCs characterized and banked

Directed Differentiation of iPSCs

Differentiation to endoderm

- FACS endoderm
- Differentiation to lung progenitors
- Lung progenitor characterization
- Re-plating lung progenitors in 3D matrigel

iPSC culture and maintenance

- Feeder-independent
  - Plating matrigel
  - Passaging hiPSCs using ReLeSR
  - Freezing hiPSCs
  - Thawing hiPSCs

- Feeder-dependent
  - Plating MEF feeder cells
  - Collagenase passaging
  - Picking to pass
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. Resuspend cell pellet in fetal bovine serum (FBS) containing 10% DMSO to freeze. Aliquot ~2x10⁶ 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 ~1x10⁶ cells/mL blood drawn; for 8 mLs blood drawn, should obtain 6-8x10⁶ cells for 3-4 frozen cryovials.

Generation of iPS Cells 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)

Day (-9) (Erythroblast expansion)
Collect PB into BD Vacutainer 4 mL cell preparation tubes (CPTs) with sodium citrate OR into EDTA or heparinized tubes and ficoll to extract MNCs. Alternatively, thaw previously frozen vial of MNCs.

Fresh cells collected into CPTs
- Draw 4 mLs of PB (or BM) into a CPT. Invert tube 8-10x and keep upright at room temperature (RT)
- Centrifuge 30 min at 1,800 RCF at RT (ideally within 2 hrs of collection)
- Use a sterile transfer pipette or 1 mL pipet tip to collect buffy coat into sterile 15 mL conical centrifuge tube
- Bring total volume to 10 mLs with sterile DPBS, invert several times
- Centrifuge 15 minutes at 300 RCF and aspirate supernatant
- Re-suspend pellet in 10 mL of sterile DPBS and perform cell count
- Transfer 1-2×10⁶ cells into sterile 15 mL conical centrifuge tube and centrifuge at 300 RCF, 10 min
- Re-suspend cell pellet in 2 mL of expansion medium (EM) and transfer to 1 well of a 12 well dish
- Incubate cells at 37°C
- Centrifuge remaining cells at 300 RCF for 10 min and freeze ~2×10⁶ cells/vial in 1 mL freezing medium (90% serum, 10% DMSO)

Frozen cells
- Thaw 1 vial of MNCs into 10 mL of QBSF and centrifuge at 300 RCF for 10 min
- Re-suspend pellet in 2 mL of EM and transfer to 1 well of a 12 well dish, incubate at 37°C
(After 3 days inoculate FG293 sentinel plate with supernatant; after 3 additional days, harvest gDNA; assay for mycoplasma)

<table>
<thead>
<tr>
<th>Expansion Medium (EM)*</th>
<th>Working Conc</th>
<th>Final Conc</th>
<th>Volume (5 mLs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>QBSF-60 (serum free medium)</td>
<td>500x</td>
<td>100 μg/ml</td>
<td>up to 5 mLs</td>
</tr>
<tr>
<td>Primocin</td>
<td>500x</td>
<td>100 μg/ml</td>
<td>10 μL</td>
</tr>
<tr>
<td>Ascorbic Acid (AA)</td>
<td>5 mg/mL</td>
<td>10 μL/mL</td>
<td>50 μL</td>
</tr>
</tbody>
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**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** | 5×10⁻⁵ M | 1 μM | 0.1 mL |

*EM = QBSF (base media) + Primocin + AA + growth factors
**Keep dexamethasone protected from light (discard every 2 weeks)
Generation of iPS Cells from Human Peripheral Blood (continued)

Day (-3) and Day (-6) (Feed cells)

- Transfer cells to sterile 15 mL conical tube and wash well 1x with 1 mL of QBSF to collect adherent cells (*d3, prior to washing cells, inoculate FG293 sentinel plate for mycoplasma assay*)
- Spin cells at 300 RCF for 10 min and resuspend in 2 mLs of fresh EM
- Continue to culture in 1 well of a 12 well dish

Day (-9)/Day 0 (Transduction)

Transduce cells with reprogramming vectors using the Sendai virus delivery and expression system (CytoTune iPS 2.0 Sendai Reprogramming Kit; Life Technologies, Inc., Cat# A16517) when cells noticeably dividing.

- Transfer cells from 12well dish to sterile 15 mL conical tube and wash well 1x with 1 mL of QBSF to collect adherent cells
- Spin cells at 300 RCF for 10 min and resuspend in 1 mL of fresh EM plus polybrene (5 μg/mL)
- Count cells and plate 1.0 – 2.0 x10⁵ cells into one well of a 12 well plate
- Add each viral vector at recommended MOI (titer of each virus is lot specific; see COA); gently rock plate to distribute virus

*Example:*

<table>
<thead>
<tr>
<th>CytoTune 2.0</th>
<th>Titer (CIU/mL)</th>
<th>MOI</th>
<th>Vol per 1.5x10⁵ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>hKOs</td>
<td>1.0x10⁸</td>
<td>5</td>
<td>7.5 ul</td>
</tr>
<tr>
<td>cMyc</td>
<td>0.84x10⁸</td>
<td>5</td>
<td>8.9 ul</td>
</tr>
<tr>
<td>Klf4</td>
<td>0.98x10⁸</td>
<td>3</td>
<td>4.6 ul</td>
</tr>
</tbody>
</table>

- Spinnoculation: Spin plate at 2250 rpm at 25°C x 90 min. After spin, incubate plate at 37°C
- At end of day, add an additional 1 mL of fresh EM to cells (for a total of 2 mLs of EM)

Day 1 (Wash cells to remove virus)

- Collect cells in a conical tube and spin at 300 RCF for 10 min; resuspend cell pellet in 2 mL fresh EM
- Continue to culture in 1 well of a 12 well dish

Day 2 or 3 (Plate MEFs)

- Plate inactive MEFs onto 3-6well gelatin(0.1%)-coated plates in MEF media (*for use on Day 4*)

<table>
<thead>
<tr>
<th>MEF media</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMDM</td>
<td>89%</td>
</tr>
<tr>
<td>(<em>plus Primocin™ 100 μg/mL and GlutaMAX (1X)</em></td>
<td></td>
</tr>
<tr>
<td>FBS</td>
<td>10%</td>
</tr>
<tr>
<td>NEAA (100x)</td>
<td>1%</td>
</tr>
<tr>
<td>Beta-mercaptoethanol (55mM)</td>
<td>0.1%</td>
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Generation of iPS Cells from Human Peripheral Blood (continued)

Day 4 (Plate cells onto MEFs)

• Collect cells into 15 ml conical tube, wash well with QBSF and spin at 300 RCF for 10 min. Resuspend cell pellet in 3 ml of MEF media supplemented with 10 ng/mL bFGF and AA/growth factors/cytokines (see EM above)
• Plate 1 mL of cells per 6-well MEF plate. Add additional 1.5 mL/well of MEF media with bFGF, AA, and growth factors for a total of 2.5 mL of media/well
• Spin plate at 500 rpm at 25 °C for 30 min

Day 6

• Feed cells every other day with 2.5 mL of MEF media with 10 ng/mL bFGF, and AA as above (no growth factors/cytokines)
• Aspirate and discard floating cells with each feed

~ Day 8 (small colonies)

• Once small colonies appear, feed cells daily with 2mLs of human embryonic stem cell (hESC) media with 10 ng/mL of bFGF

~ Day 18 (pick colonies)

• Each colony is picked into one well of a 12-well plate with MEFs, with 1 mL/well hESC media and 10 μM Rock Inhibitor (RI; 1:1000; 10 mM stock, STEMGENT, Cat# 04-0012)
• Feed cells daily thereafter with hESC (without RI)

References:

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
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

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

D. Kotton 8/17/2010 As learned from Robert Lafyatis, MD.
Human Fibroblast Reprogramming to make human iPS Cells using a Lentiviral Stem Cell Cassette**

1. Plate $1 \times 10^5$ 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 EF1a-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).

Preparing Feeder-Dependent Tissue Culture Plates

Inactive MEF feeder plates can be prepared several days prior to passaging of 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 1.5 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 µg/mL primocin) to each well and return to incubator.

4. Thaw frozen vial of Inactive MEFs 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 appropriate volume for seeding ~ 3x10^5 cells in ~ 500 µL volume per well (6-well). Add ~ 3x10^5 cells/500 µL DMEM Complete Medium to each well of previously prepared gelatin-coated dish (from steps 1-3).

7. Rock gently to evenly to 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 hiPS cell passaging.
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 1:10 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.

You will need:

- 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 2x 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.
Passaging hiPS Cells on Feeders using Collagenase IV (continued)

4. Wash well once with 2 mL of DPBS.

5. Add 1 mL of Collagenase 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 once with 2 mL of DPBS and once with 1 mL DMEM/F12.

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 three times (with a P1000 pipette tip set to 950 µL) to break the colonies into smaller clumps.

8. Transfer the desired dilution to wells of the new feeder plate (usually around 1:3 – 1:10 depending on the growth rate of the individual cell line). Rock gently to evenly distribute cell clumps.
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.

You will need:

- Collagenase Type IV, 1 mg/ml (StemCell Technologies 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), label them and place in a -20°C freezer.

2. Aspirate the medium from the hiPSC culture dish.

3. Wash once with 2 mL of 1x PBS.

4. Aspirate the PBS and add 1 mL of collagenase 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 2 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 cold cryovial.

11. Place the cryovial in a Freezing Container or in a Styrofoam rack at -80°C overnight and transfer to the -150°C the next day.
Thawing hiPSCs onto Feeders

You will need:

- DMEM/F12 (Life Technologies 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.
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).

You will need:

- Matrigel Corning™ Matrigel™ hESC-qualified Matrix (Corning Cat# 354277)
- mTeSR1™ Medium (STEMCELL Technologies, 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 ready to use.
Passaging hiPS Cells on Matrigel

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 1:20 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.

You will need:

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

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 2x 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 1:20) depending on growth rate of cells.
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 1:40 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.

You will need:

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

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 2x 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 minutes

6. Aspirate Gentle Cell Reagent and add 1 mL mTeSR forcefully to cells. If cells remain adherent to plate, tilt plate and use cell lifter to gently scrape cell aggregates together.

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 1:40) depending on growth rate of cells.
Freezing hiPS Cells growing on Matrigel

You will need:

- DPBS (Life Technologies Cat# 14190-144)
- DMEM/F12 (Life Technologies Cat# 11330-057)
- mTeSR (StemCell Technologies Cat# 05850)
- ReLeSR (StemCell Technologies Cat# 05782)
- 2x cold Freezing Medium (80% mTeSR/20% DMSO)
- 5 mL serological pipettes
- 15 mL conical tube
- Cryovials (Corning Cat# 430487)
- 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 with 2 mLs DMEM/F12.

3. 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.

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

5. Add 1 mL mTeSR down wall of well and gently tap or scrape with cell scraper to lift adherent colonies (take care to keep cell colonies as large as possible).

6. Transfer floating colonies to 15 mL conical tube using a 5 mL serological pipette.

7. Centrifuge 200 x g for 5 min and aspirate medium.

8. Add 0.5 mL mTeSR medium and gently tap to resuspend cell pellet.

9. Add 0.5 mL cold 2x Freezing Medium dropwise, while swirling tube to mix.

10. Transfer 1 mL of cell aggregates to each labeled cryovial.

11. Place the cryovial in a Freezing Container or in a Styrofoam rack at -80°C overnight and transfer to the -150°C freezer storage the following day.
Thawing hiPSC Cells growing on Matrigel

You will need:

- DPBS (Life Technologies 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 warmed 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 hiPSC cells evenly over well and return to incubator.
mTeSR1 Adaption of hiPSCs Cultured on Feeders

You will need:

- Matrigel Corning™ Matrigel™ hESC-qualified Matrix (Corning Cat# 354277)
- Collagenase Type IV, 1 mg/ml (StemCell Technologies Cat# 07909)
- ReLeSR (Stemcell Technologies Cat# 05872)
- Gentle Cell Dissociation Reagent (Stemcell Technologies Cat# 07174)
- mTeSR1 medium (StemCell Technologies Cat# 05850) at room temperature
- hESC medium
- DMEM/F12 (Life Technologies Cat# 11330-057)
- DPBS (Life Technologies 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 mLs mTeSR medium per well and return to incubator.

2. Aspirate media from iPSC well on feeders; rinse with 2x with 1 mL DPBS or DMEM/F12.

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 2x 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.
mTeSR1 Adaption of hiPSCs Cultured on Feeders (continued)

2. Choose appropriate dissociation reagent (ReLeSR or Gentle Cell) and passage cells according to manufacturer’s instructions.

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)
   - 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)
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 (provided in kit). Fluorophore-labeled goat anti-mouse IgM or anti-IgG can be used as secondary antibodies, appropriate to the isotype of the primary antibody.

You will need:
- ES Characterization Kit (EMD Millipore Cat# SCR001)
- 16% Paraformaldehyde (PFA) (EMS Cat# 15710)
- Wash buffer (1X TBST)
- DPBS (Life Technologies Cat# 14040-133)
- Normal goat serum (Vector, Cat# S-1000)
- Triton™ X-100 (Sigma, Cat# T8787)
- Alexa Fluor™ goat α-mouse AF488 or AF546 (Life Technologies, 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.
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 @ 4°C in DPBS. Cover in tin foil.
11. Images can be visualized with a fluorescence microscope.
**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**

<table>
<thead>
<tr>
<th>PRODUCT NAME</th>
<th>CATALOG #</th>
<th>SIZE</th>
<th>COMPONENTS</th>
</tr>
</thead>
</table>
| 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.

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

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

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™-EB™ (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 $2.1 \times 10^5$ per cm$^2$ (i.e. $2 \times 10^6$ 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.
Human Lung Directed Differentiation Protocol

INTRO
This protocol describes the methods to derive, sort and plate primordial lung progenitors from human iPSCs/ESCs.

REAGENTS
See associated excel file Human Lung Reagents_2017-1_13

PROTOCOL
Associated protocols:
STEMDiff Endoderm Kit 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 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 (“DS”= Dorsomorphin, Stemgent, 2μM, SB= SB431542, Tocris,10μM). Prepare “DS/SB + Y-27632” (Y=Y-27632, Tocris, 10μM). 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 1:3 to 1:8. Density affects NKX2-1 induction and should be optimized if NKX2-1 yields are low).
   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):
Prepare “CFKB” (Chir, FGF10, KGF, BMP4) media with and add freshly prepared retinoic acid (Ra) each time the cells are refed “CFKBra”. CFKBRA= CSFDM supplemented with 3μM CHIR99021 (Tocris), 10ng/ml rhFGF10, 10ng/ml rhKGF, 10ng/ml rhBMP4 (all from R&D Systems), 50-100nM Retinoic acid (Sigma) (Huang et al., Nature Biotech, 2014).
   A) Aspirate “DS/SB” media.
   B) Add 2ml/well of “CFKBra”.
   C) Refeed every 24-48 hours.
Human Lung Directed Differentiation Protocol (continued)

4) Sorting NKX2-1+/CD47+ Lung Progenitors and Expansion in 3-D Matrigel (Day 14-15):
   A. Prepare maturation media. Distal = “CFK+DCI”. Proximal=“FGF2+10” (see McCauley et al., Cell Stem Cell, March 2017).
   B. Prepare FACS buffer:
      1. Hank’s Balanced Salt Solution
      2. 2% FBS
      3. Primocin
      4. HEPES 25mM
      5. EDTA 2mM
   C. Aspirate “CFKBRa” 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 14-18 minutes
   F. Triturate (gently) until cell sheet is detached from plate and transfer to a 15ml conical
   G. Add an additional 1ml of trypsin. Manually shake, flick and roll the 15ml conical for 3-5 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).
   H. Centrifuge at 200G x 5min.
   I. Aspirate supernatant and re-suspend in CSFDM.
   J. Filter through a 40um filter x 2.
   K. Thaw matrigel (356231) on ice.
   L. Count cells then centrifuge single cell suspension samples at 300G for 5min.
   M. Aspirate supernatant and place Eppendorf or conical on ice for 2 min.
   N. Re-suspend each cell pellet at 250 to 500 cells per microliter of Matrigel using cold 200μl pipette tips. Take care to (a) avoid bubbles and (b) distribute the cells evenly in the Matrigel.
   O. Slowly pipette the matrigel + cells into the center of a 12 well plate (25-100μL/drops, typically 50μL).
   P. Place 12 well plate in the incubator for 20 min.
   Q. Add distal (CFK+DCI) or proximal (FGF2+10) media supplemented with 10μM Y-27632 media. See relevant protocols.
   R. Re-feed every 48 hours

References:
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, 200xg, 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. 100μl per tube.

b. Cell staining
   I. add the appropriate antibodies/isotypes (2μL) 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 (2μL)
      Isotype: Mouse IgG2a PE-conjugated, Stem Cell Technologies, Cat# 60108PE (2μL)
      CXCR4: Human CXCR4 R-PE conjugated, Stem Cell Technologies, Cat #60089PE (2μL)
      CKIT: APC anti-human CD117(CKIT), Biolegend, Cat# 313206 (2μL)
   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 200xg 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
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°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°C incubator.

9. Day 2: Cells are approximately 50% confluent in 15 cm plates, some areas 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°C and add DMEM Complete Medium to inactivate trypsin; spin down cells @ 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).
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):
   - 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 10µg/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
   - Typically trypsinize and inactive 3-5 MEF plates (15 cm) at one time:
Preparation of Inactive MEFs from Active MEFs (continued)

- Aspirate DCM from 15 cm MEF plates; take note of passage number
- Add 10 mLs/15 cm dish of 10µg/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
- 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) @ approx. 3.5 x 10^6 cells/cryovial (~ 4-5 vials/15 cm dish).
- Place cryovials in styrofoam or commercial freezing container for storage @ -80°C overnight and transfer cryovials to -150°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): CTTCWTCGACTTTYCAGACCCAAGGCAT
Myco280 (cb): ACACCATGGGAGYTGGTAAT

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<tr>
<th>Component</th>
<th>Concentration</th>
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<tr>
<td>10x PCR buffer</td>
<td>2.5</td>
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<tr>
<td>10mM dNTP</td>
<td>2</td>
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<tr>
<td>Primer mix (279/280)</td>
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</tr>
<tr>
<td>(10 µM each)</td>
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<tr>
<td>DNA</td>
<td>1</td>
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<tr>
<td>H₂O</td>
<td>18.25</td>
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<tr>
<td>Taq (commercial)</td>
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</table>
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 powerpoint file of the gel in the myco testing folder and add the details of the test to the myco log excel file.

Example 1:
hESC Medium

For 500 ml of hESC medium:

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

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<th>Reagents</th>
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<tr>
<td>APC anti-human CD117(CKIT)</td>
<td>Biolegend</td>
<td>313206</td>
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</tbody>
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# iPSC CORE Training Course Contact List

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List of Training Course Attendees

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