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

A) cSFDM (complete serum free differentiation media):

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

B) Stemdiff DE Kit, StemCell Technologies 05110

C) DS/SB Media
   cSFDM Base
   10uM SB43152 (Tocris 1614)
   2uM Dorsomorphin (Stemgent 04-0024)

D) CBRa Media
   cSFDM Base
   3uM CHIR99021 (Tocris 4423)
   10ng/ml rhBMP4 (R&D 314-BP-050)
   100nM Retinoic Acid (Sigma R2625)

E) Other Reagents
   Rho-associated kinase inhibitor (Y-27632, Tocris 1254)
   Gentle Cell Dissociation Reagent (StemCell Technologies 07174)
   Matrigel Basement Membrane Matrix (hESC-Qualified) (Corning 354277)
   Growth Factor-Reduced Matrigel (Corning 356231)
   Calcein blue (LifeTechnologies C1429)

PROTOCOL

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

A) Definitive endoderm induction (StemDiff Endoderm kit: 4 days)
   1. Grow PSCs in mTeSR1 for differentiation according to the instructions in the StemDiff Endoderm kit
   2. Coat wells in matrigel (dilute according to Corning manufacturer's instructions) (Corning 354277)
   3. Dissociate 2 x 10^6 PSCs in Gentle Cell Dissociation Reagent (GCDR) and plate them onto 1 well of a matrigel-coated 6 well plate in mTeSR1+ 10uM Y-27632
4. 24 hours later, “Day 0,” change media to StemDiff Endoderm Kit Base media + supplement MR and CJ (Previously supplement A + B)
5. 24 hours later, “Day 1,” change media to StemDiff Endoderm Kit Base media + supplement CJ only (Previously supplement B)
6. 24 hours later, “Day 2,” change media to StemDiff Endoderm Kit Base media + supplement CJ only
   a. Note: these day numbers differ from the StemDiff Endoderm Kit, which refers to them as Day 1-3. In total cells should be in supplements A+B for 24 hours and B only for 48 hours before the next step.
   b. You can check definitive endoderm efficiency at this point by performing FACS for C-KIT and CXCR4. The majority of the cells should be positive for both markers.

B) Anterior Foregut Endoderm Induction (DS/SB media: day 3 to 6)

2. Add 1ml GCDR to each well of Day 3 endoderm, incubate at room temperature for 2 minutes
3. Aspirate GCDR and add 1ml DS/SB media + 10uM Y-27632 to each well. Use cell scraper to detach cells from the plate. Gently pipet up and down once. (Cells should be passaged in clumps).
   a. If performing FACS for C-KIT and CXCR4 at this stage, you can leave cells around the very edge of the well when scraping and after passing the well, pipette the few remaining cells to single cell and stain to check endoderm efficiency.
4. Passage each well between 1:2 to 1:10 into previously coated Matrigel plates in DS/SB media + 10uM Y-27632
   a. Cell density affects NKX2-1 induction and should be optimized for each PSC line. We typically start with 1:6.
5. 24 hours later, change media to DS/SB without 10uM Y-27632
6. Leave plates in DS/SB media for 48 hours

C) NKX2-1 Lung Progenitor Induction (CBRa media: Day 6 to 15)

1. Prepare “CB” media and add freshly prepared retinoic acid each time the cells are refed “CBRa”.
2. Aspirate “DS/SB” media and wash once with DMEM or other empty media.
3. Add 2ml/well of “CBRa”.
4. Refeed every 48 hours. (As cells grow they may need additional feeding every 24 hours)
5. Within 3-5 days NKX2-1+ cells begin to specify
   a. Specification percentage may vary based on the cell density plated at day 3. Typically, NKX2-1 percentage peaks between days 14-16.

D) Sorting NKX2-1+/CD47+ Lung Progenitors (Day 14-15):

1. Prepare maturation media. Distal = “CK+DCI”. Proximal = “FGF2+10” (see relevant protocols)
2. Prepare FACS buffer:
   1. Hank’s Balanced Salt Solution
   2. 2% FBS
   3. Primocin 100 µg/ml
   4. HEPES 25mM
   5. EDTA 2mM
   6. ROCK inhibitor (10 µM Y-27632)
3. Aspirate “CBRa” media and wash once with empty media.
4. Add 1ml warm 0.05% trypsin per well and use a 10ul pipette tip to etch many scrapes across the monolayer in a crosshatch pattern. Place in incubator for 5-7 minutes.
5. After 5-7 minutes, pipette up and down gently 1-2 times to detach cells from plate, then return to the incubator for a further 5-7 minutes
   a. Cells are sensitive to over-pipetting at this stage, so leave them in trypsin long enough for most of the dissociation to be enzymatic
6. Pipette (gently) to detach cell sheet from plate and transfer to a 15ml conical
7. Allow clumps to settle for 20-30 sec. Transfer cloudy supernatant to a separate 15ml conical containing FBS to inactivate trypsin. Add 1ml warm trypsin to conical with clumps and shake, flick and roll the
remaining clumps until mostly dissociated. Then transfer the remaining cells to the conical containing FBS to inactivate trypsin. (Total time in trypsin should be no more than 15 minutes)

(If using a PSC line without an NKX2-1 knock-in reporter, proceed to CD47/CD26 staining protocol at this point).

For PSC lines with NKX2-1 knock-in reporter:

8. Centrifuge at 300g x 5 min.
10. Filter through a 40um filter x 2 and transfer sample to FACS tube.
11. Add live/dead stain to FACS sample and prepare collection tubes with FACS buffer.
12. Sort live/NKX2-1+ cells.
13. While sorting, thaw Matrigel (Corning 356231) on ice and make either distal (CK+DCI) or proximal (FGF2+10+DCI) media supplemented with 10uM Y-27632.
14. Once sorted, follow protocol for either distal or proximal cell differentiation.

VERSION HISTORY

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<tr>
<th>Date</th>
<th>Description</th>
<th>Author</th>
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<tr>
<td>2017-01-13</td>
<td>Updated to reflect CBRa media as developed by Finn Hawkins (FJH)</td>
<td>FJH</td>
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<tr>
<td>2017-03-22</td>
<td>Update to new CReM protocol formatting</td>
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<tr>
<td>2017-10-06</td>
<td>Additional passaging step (optional) added</td>
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<td>2017-11-22</td>
<td>Updated passaging step</td>
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<td>2018-04-03</td>
<td>Updated plating density day 3</td>
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<td>2022-01-20</td>
<td>Updated for website, removed day 8-10 passaging step</td>
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