Human Alveolosphere Directed Differentiation Protocol – Kotton Laboratory

INTRO
This protocol describes the methods to derive, sort and plate SFTPC+ cells from human iPSCs/ESCs. As published in: Jacob et al. Cell Stem Cell. 2017.

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) supplement</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 (100 µg/ml stock)</td>
<td>200 ng/ml</td>
<td>Invovgen 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^-6M</td>
<td>Sigma M6145</td>
</tr>
</tbody>
</table>

B) Stemdiff DE Kit, StemCell Technologies 05210

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) 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)

F) Other Reagents
   Rho-associated kinase inhibitor (Y-27632, Tocris 1254)
   Gentle Cell Dissociation Reagent (StemCell Technologies 07174)
   Growth Factor-Reduced Matrigel (Corning 356230)
   Calcein blue (LifeTechnologies C1429)
   Dispase (ThermoFisher 354235)

PROTOCOL

A) Definitive Endoderm Induction (StemDiff Endoderm kit: 4 days)
1. Plate PSCs in mTeSR1 for differentiation according to the instructions in the StemDiff Endoderm kit
2. Coat wells in dilute matrigel (dilute according to Corning manufacturer’s instructions) and wash with DMEM/F12 after 1 hour incubation.
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 A and B
5. 24 hours later, “Day 1,” change media to StemDiff Endoderm Kit Base media + supplement B only
6. 24 hours later, “Day 2,” change media to StemDiff Endoderm Kit Base media + supplement B 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)
1. Coat 3-4 wells per well of endoderm with dilute matrigel in a new 6 well plate and wash with DMEM/F12 after 1 hour incubation.
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, pipet up and down 3-5 times until all cells come off the plate
4. Passage each well 1:3 or 1:4 into previously coated plates in DS/SB media + 10uM Y-27632
   a. Cell density should be around 200K cells/cm², but the optimal cell density at this point varies from line to line.
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. After 72 hours total of AFE induction, wash plates with DMEM/F12 and change media to CBRa media
2. Refeed every 48 hours
3. Within 3-5 days NKX2-1+ cells begin to specify
   a. Specification percentage may vary based on the cell density plated at day 3. If cells are confluent by Day 8-9, this may decrease the yield of NKX2-1+ cells. Consider passaging cells on Day 9 1:3 again if they are too confluent or passaging less densely on day 3 (1:6 or 1:10). The appropriate passaging density on day 3 varies from PSC line to line.

D) Sorting NKX2-1+ Lung Progenitors (Day 15)
1. Prepare FACS buffer:
   a. 1x HBSS
   b. 2% ESC-qualified FBS
   c. ROCK inhibitor (10 µM Y-27632)
   d. Calcein blue
2. Aspirate CBRA media from each well and add 1ml warm 0.05% trypsin
3. Incubate at 37C for 10-15 minutes, until pipetting only 3-5 times results in single cell suspension
   a. Dissociate cells in a tissue culture plate so that you can visualize them in a microscope.
   b. Cells are sensitive to over-pipetting at this stage, so leave them in trypsin long enough for most of the dissociation to be enzymatic
   c. If cells have not dissociated into single cells by 15 minutes, spin down, add fresh trypsin, and leave another 5 minutes
4. Pipet trypsinized cells into 15ml conical, add 10ml FBS-containing media to inactivate trypsin.
5. Spin at 300g x 5min, resuspend each well in 1ml FACS buffer.
6. If using a PSC line with an NKX2-1 knockin reporter you are ready to sort: filter through a 40uM filter, transfer to polypropylene (white top) FACS tubes and prepare collection tubes with FACS buffer supplemented with ROCK inhibitor.
7. If using a PSC line without an NKX2-1 knockin reporter, first stain cells with CD47 and CD26 antibodies for 30min on ice protected from light.
   a. Add 0.5ul CD47 and 0.5ul CD26 per 1 x 10^6 cells in 100ul staining volume.
b. Use unstained, isotype, and single color controls to set compensation.
c. Wash with PBS and spin at 300g x 5min, resuspend in FACS buffer equivalent to 1ml/well.
d. You are ready to sort CD47hi/CD26lo lung progenitor cells- see step 6.

E) 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 20-50 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 SFTPCtdTomato+ 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 SFTPCtdTomato+ cells within 2-5 days.
   a. Passage alveolospheres when they become confluent within the matrigel drop, otherwise efficiency will decrease.

F) 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

G) 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%.

H) 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**

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<tr>
<th>Date</th>
<th>Action Description</th>
<th>Version Number</th>
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<td>2017-07-10</td>
<td>Drafted by Anjali Jacob.</td>
<td>AJ</td>
</tr>
<tr>
<td>2017-08-09</td>
<td>RA concentration changed from 50nM to 100nM</td>
<td>LS</td>
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