Human Lung Directed Differentiation Protocol

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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 attached) in a 6 well plate. Check C-kit/CXCR4 co-expression to assess sufficient definitive endoderm induction. 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.

2) Anterior foregut endoderm (typically 72 hours):

A. Prepare 12ml of “DS/SB” media per well of definitive endoderm. Divide equally between two conicals and add Y-27632 (ROCK inhibitor) to one (final concentration of 10uM). Place “DS/SB + Y-27632” at room temp. Label and place the other conical of “DS/SB” at 4 degrees celcius. Prepare fresh matrigel coated plate (see Stem Cell technologies E8 or 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 “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 200K cells/cm2 in “DS/SB + Y-27632” on freshly coated matrigel plates.
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/ESC we use 72 hours.

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

A) Prepare “CFKBRa” media with freshly prepared retinoic acid.
B) Aspirate “DS/SB” media.
C) Add 2ml/well of “CFKBRa”.
D) Refeed every 24-48 hours.

4) Expansion (Day 15-22):

A) Re-plating unsorted cells on matrigel:

I. Prepare fresh matrigel coated plates (see Stem Cell technologies E8 or mTeSR1 protocol).
II. Prepare “CFK media”
III. Aspirate “CFKBRa” media and wash X2 with CSFDM.
IV. Add warm 0.05% trypsin and place the tissue culture plate in the incubator for 2-4 minutes.
V. Aspirate trypsin and wash X 1 with STOP media (DMEM +10% FBS) and X1 with CSFDM.

VI. Add 1ml of “CFK” and triturate gently until the cell sheet has been dissociated into small clumps.
VII. Transfer to a 15 ml conical. Dilute to a 1:3 split ratio i.e. 1 well of a 6 well plate should be re-suspended with 6 ml of “CFK” and supplemented with Y-27632 (final concentration 10uM) and divided between three wells of freshly coated 6 well plate.
VIII. Replace media every 24-48 hours.

B) Re-plating unsorted cells *in* 3-D matrigel drops:

I. Thaw matrigel (356231) on ice
II. Repeat steps I to V above.
III. Add 1ml of CSFDM/well and triturate gently until the cell sheet has been dissociated into small clumps.
IV. Divide the dissociated 1ml sample between 1.5ml eppendorfs (approx. 50ul per eppendorf). Add 1ml CSFDM per eppendorf and centrifuge at 200G for 5 min.
V. Aspirate as much of the supernatant as possible without displacing the cell pellet (to avoid diluting the matrigel).
VI. Place eppendorf on ice for 2 min.
VII. Re-suspend each cell pellet in 50ul of matrigel using cold 200ul pipette tips. Take care to (a) avoid bubbles and (b) distribute the clumps evenly in the matrigel.
VIII. Slowly pipette the matrigel + cells into the center of a 12 well plate (50uL/drop).
IX. Place 12 well plate in the incubator for 20 min.
X. Add CFK + 10uM Y-27632 media.
XI. Re-feed with CFK every 24-48 hours.

C) Re-plating sorted NXX2-1+ cells in 3-D matrigel drops:

I. Prepare FACS buffer:
   - Hank’s Balanced Salt Solution
   - 2% FBS
   - Primocin
   - HEPES 25mM
   - EDTA 2mM

I. Wash day 15 cells with CSFDM
II. Use a 10ul pipette to etch many scrapes across the monolayer
III. Add 1ml warm 0.05% trypsin and place in incubator for 14-18 minutes
IV. Triturate (gently) until cell sheet is detached from plate and transfer to a 15ml conical
V. 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”.
VI. Centrifuge at 200G x 5min.

VII. Aspirate supernatant and re-suspend in FACS buffer
VIII. Filter through a 40um filter x 2.
IX. Add Y-27632 (final concentration 10uM) and Propidium Iodide to FACS sample and prepare collection tubes with FACS buffer supplemented with Y-27632 (final concentration 10uM).
X. Sort NKX2-1+/PI negative
XI. Spin collected samples at 300G for 7min
XII. Repeat steps B(V)-B(XI) above.

5) Maturation (Day 22+):

I. On day 22 prepare "CFK+DCI" media
II. Change media every 24-48 hours