CD47hi/CD26lo 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 37°C 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 trypsin 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\textsuperscript{PerCP/Cy5.5} only
   c. CD26\textsuperscript{PE} only
   d. mlgG\textsubscript{1}\textsuperscript{PerCP/Cy5.5}, mlgG\textsubscript{2}\textsuperscript{PE} isotypes
   e. Calcein blue only
   f. CD47\textsuperscript{PerCP/Cy5.5}/CD26\textsuperscript{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 NKX2-1\textsuperscript{+} Cells

XIX. Sort CD47\textsuperscript{hi}/CD26\textsuperscript{lo}/calcein blue\textsuperscript{+}

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 CD47\textsuperscript{hi}/CD26\textsuperscript{lo} population (Figures 4, 5 and 6). We also sort the CD47\textsuperscript{lo} 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 $1 \times 10^6$ cells vs $1 \times 10^5$ cells with 0.5uL ab/100 uL staining volume

- High cell concentration
- Lower cell concentration

Figure 3: staining FACS analysis of isotypes vs. CD47 & CD26 on day 15 of directed differentiation
Figure 4: Representative CD47\textsuperscript{hi}/CD26\textsuperscript{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\textsuperscript{hi}/CD26\textsuperscript{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)

<table>
<thead>
<tr>
<th>Buffer Components</th>
<th>Make from:</th>
<th>Dilution</th>
<th>For 50 ml</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>1x PBS</td>
<td>1x PBS</td>
<td>To final vol</td>
<td>46.2 ml</td>
<td>Media fridge</td>
</tr>
<tr>
<td>0.5% BSA</td>
<td>7.5% BSA</td>
<td>1:15 dilution</td>
<td>3.33 ml</td>
<td>Media fridge</td>
</tr>
<tr>
<td>0.02% NaN(_3)</td>
<td>2% NaN(_3)</td>
<td>1:100 dilution</td>
<td>0.5 ml</td>
<td>Maria’s fridge</td>
</tr>
</tbody>
</table>

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 37\(^\circ\)C 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 isotype 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 NaN₃)
   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<sup>hi</sup>/CD26<sup>lo</sup> sort