May 08, 2024

Processing of pediatric whole blood samples for single cell analysis

DOI
dx.doi.org/10.17504/protocols.io.ewov19y37lr2/v1

Liam Gubbels[1], Shivanthan Shanthikumar[1], Melanie R Neeland[1]

[1]Murdoch Children's Research Institute

earlyAIR

Liam Gubbels
Murdoch Children's Research Institute

OPEN ACCESS

DOI: dx.doi.org/10.17504/protocols.io.ewov19y37lr2/v1

Protocol Citation: Liam Gubbels, Shivanthan Shanthikumar, Melanie R Neeland 2024. Processing of pediatric whole blood samples for single cell analysis. **protocols.io https://dx.doi.org/10.17504/protocols.io.ewov19y37lr2/v1**

Protocol status: Working
We use this protocol and it's working

Created: April 01, 2024

Last Modified: May 08, 2024

Protocol Integer ID: 97621

Keywords: single-cell, flow cytometry, respiratory, Paediatric

Funders Acknowledgement:
Chan Zuckerberg Initiative
Grant ID: 2021-237883

Abstract

This protocol describes the collection, processing, and cryopreservation of whole blood samples for downstream single-cell analysis.

Guidelines

This is an experimental protocol for the processing of whole blood samples collected from children. Sample collection must have and be compliant with Human Ethics Committee approval.

Materials

- RPMI-1640 **Sigma Aldrich Catalog #**R5886
- Sample tube, EDTA K3E, 10 ml, cap red **Sarstedt Catalog #**26.358
- Fetal Bovine Serum
- 1X PBS (Phosphate-buffered saline)
- DMSO (dimethyl sulfoxide) **Sigma Aldrich Catalog #**D8418
- conical tubes, 50ml
- conical tubes, 15ml
- 1.5 mL Eppendorf tubes **Contributed by users**
- Screw cap micro tube, 2 mL **Sarstedt Catalog #**72.694.600
- Ficoll Paque PLUS **GE Healthcare Catalog #**17144003-500 ml
- 0.4% Trypan blue solution **Contributed by users**
- Cell Counting Slides for TC10™/TC20™ Cell Counter Dual-Chamber 5 x 30 slides 300 counts #1450015 **Bio-Rad Laboratories Catalog #**1450015
- FACS Tubes **Contributed by users**
- Brilliant stain buffer **Becton Dickinson (BD) Catalog #**563794
- Human TruStain FcX™ (Fc Receptor Blocking Solution) **BioLegend Catalog #**422301, 422302
- cryovial **Contributed by users**
- RNAlater **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**R0901-100ML

Flow antibodies: Flow cytometry panels.pdf 301KB

Equipment

Aurora - 5L Configuration

NAME

Spectral Flow Cytometer

TYPE

Cytek Aurora

BRAND

N/A

SKU

5L Configuration

SPECIFICATIONS

Equipment

TC20 Automated Cell Counter

NAME

automated cell counter

TYPE

BioRad

BRAND

1450102

SKU

http://www.bio-rad.com/en-us/product/tc20-automated-cell-counter?ID=M7FBG34VY&WT.mc_id=170125000484&WT.srch=1&WT.knsh_id=a8d381b8-4072-42a1-9b46-e12b739c51d1&gclid=CjwKCAjw6vvoBRBtEiwAZq-T1bzXM1YIstQFme2X7mMBPmH32OVGbPi9d7Gf5ZDnbv3PEjrzFqcwbxoCqM8QAvD_BwE

LINK

Safety warnings

Human samples should be processed in a laboratory with appropriate biosafety infrastructure and procedures

COLLECTION OF WHOLE BLOOD

1h 30m

1 Label an EDTA blood collection tube with study/patient ID.

2 After obtaining informed consent from family and/or patient, collect blood sample into EDTA tube.

3 Whole blood samples must remain at Room temperature and be processed in the laboratory within 00:30:00 to 01:00:00 of the procedure.

1h 30m

PROCESSING OF WHOLE BLOOD TO PBMC CELL SUSPENSION

47m

4 First, take 100µL whole blood and place into a pre-labelled tube for downstream single cell analysis, such as flow cytometry detailed herein from step 16.

5 Centrifuge the remaining whole blood sample at 700 x g, Room temperature, 00:07:00 to separate out plasma.

7m

6 Aspirate plasma layer and store in 2mL cryotubes, then transfer to -80 °C for long-term storage. Dilute the plasma-depleted blood to its initial volume using room temperature RPMI supplemented with 2% heat-inactivated fetal calf serum (referred to as RPMI 2% FCS).

7 Fill a 15mL tube with 2mL of Ficoll plaque plus and layer the diluted blood onto the surface of the Ficoll solution.

> **Note**
>
> Layer the cell suspension slowly to prevent the Ficoll solution from mixing with the cells.

8 Centrifuge the layered cell suspension at 400 x g, Room temperature, 00:30:00 , 4 Acceleration and NO brake .

45m

9 Once the spin is complete, carefully aspirate the mononuclear layer at the interface between the RPMI 2% FCS and the Ficoll solution into a new 15mL tube. Top up the cell suspension to 10mL using RPMI 2% FCS and centrifuge 400 x g, 4°C, 00:05:00 .

5m

Note

When collecting the cells, try to avoid Ficoll solution as much as possible.

10 There is an option here to harvest granulocytes for downstream analysis, including genomics, which we have detailed below.

10.1 While the mononuclear cells are spinning, harvest the granulocyte layer at the interphase between the erythrocytes and the Ficoll solution and place into a labelled cryovial. Add 1mL of RNAlater solution to the collected granulocytes and pipette mix thoroughly. Immediately transfer the granulocytes to -80 °C for long-term storage.

11 Discard supernatant and resuspend the cell pellet in 3mL RPMI 2% FCS.

12 Prepare cell suspension for cell counting. Here, we use trypan blue and a Bio-Rad TC-20 cell counter. Take 10µL of the cell suspension and place into a microcentrifuge tube for cell counting. Add 10µL of trypan blue to the microcentrifuge tube and mix thoroughly.

12.1 Load 10µL of stained cells onto a TC-20 slide (Bio-Rad) chamber and count. Record viability, total cell count, and live cell count.

Note

Cell counting can also be performed manually using a haemocytometer, or using other automated cell counters.

13 Top up the cell suspension to 10 mL with RPMI 2% FCS and centrifuge at 400 x g, Room temperature, 00:05:00 . 5m

CRYOPRESERVATION OF PBMC

14 Discard supernatant and resuspend cells at a ratio of 1:1 in RPMI 2% FCS and freeze solution (heat-inactivated FCS + 15% DMSO) such that cells are frozen between 1-10 million cells/mL. Transfer cells to cryogenic vial.

Note

If performing downstream single cell analysis on PBMC, prior to adding freeze solution is where you can allocate out the required number of fresh PBMCs.

15 Immediately place cryogenic vials into an isopropanol freezing container (e.g. Nalgene® Mr. Frosty) or Cool Cell (Corning) and transfer to -80 °C overnight.

16 For long-term storage, transfer the vials to liquid nitrogen.

PREPARATION OF WHOLE BLOOD CELLS FOR FLOW CYTOMETRY 1h 10m

17 Resuspend whole blood in 2mL red cell lysis buffer for 00:10:00 at Room Temperature. 15m

Centrifuge at 500 x g, 4°C, 00:05:00 and discard supernatant.

18 Resuspend cell suspension for fixable viability staining according to manufacturers' instructions (e.g. the Fixable UV Blue Stain from Invitrogen/ThermoFisher from Invitrogen/ThermoFisher). 5m

Following the required incubation, stop the reaction by the addition of 1mL staining buffer (2% heat-inactivated FCS in PBMS 2 mM EDTA, herein referred to as FACS buffer) and centrifuge at 400 x g, 4°C, 00:05:00

19 Resuspend cells in 25µL FACS buffer and add 15µL FC-block for 00:05:00 at Room temperature. 5m

20 The next steps will depend on the requirements for your specific panel. As an example, we have attached our 31-plex spectral cytometry panel that we routinely use on whole blood cells. All of the following steps are related to this panel.

tonsil_adenoid_blood_panel.pdf 127KB

21 Add 10µL of Brilliant Stain Buffer (Becton Dickinson) and then add 25µL of Cocktail 1A made up at 3X concentration and incubate for 00:10:00 In the dark at room temperature 10m

22 Then, directly add cockta made up at 2X concentration 1:1 with cells and incubate for 00:30:00 In the dark at room temperature 30m

23 Following staining, wash cells with 2mL FACS buffer and centrifuge at 400 x g, 4°C, 00:05:00 and resuspend cells in 100µL FACS buffer for acquisition on a flow cytometer (here, a Cytek 5L aurora). 5m

Note

Panels 1A and 1B were adapted from: "OMIP-069: Forty-Color Full Spectrum Flow Cytometry Panel for Deep Immunophenotyping of Major Cell Subsets in Human Peripheral Blood" and we thank the authors for their detailed methods.

CITATION

Park LM, Lannigan J, Jaimes MC (2020). OMIP-069: Forty-Color Full Spectrum Flow Cytometry Panel for Deep Immunophenotyping of Major Cell Subsets in Human Peripheral Blood..
LINK
https://doi.org/10.1002/cyto.a.24213