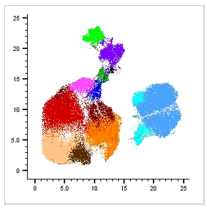

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May 18, 2022

Processing of pediatric bronchoalveolar lavage samples for single cell analysis V.2

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Human Cell Atlas Method Development Community earlyAIR team

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This protocol describes the collection, processing, cryopreservation and thawing of pediatric bronchoalveolar lavage (BAL) samples for downstream single cell analysis (including flow cytometry, cell sorting, and single cell transcriptomics).

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Shivanthan Shanthikumar, Richard Saffery, Sarath C. Ranganathan, Melanie R Neeland 2022. Processing of pediatric bronchoalveolar lavage samples for single cell analysis . **protocols.io** https://dx.doi.org/10.17504/protocols.io.36wgq4b9ovk5/v2 Melanie Neeland

single cell analysis, BAL, respiratory, pediatric, lung, flow cytometry

protocol ,

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

For guidelines on how to safely perform bronchoscopy and lavage in children, please see:

Faro A, Wood RE, Schechter MS, Leong AB, Wittkugel E, Abode K, Chmiel JF, Daines C, Davis S, Eber E, Huddleston C, Kilbaugh T, Kurland G, Midulla F, Molter D, Montgomery GS, Retsch-Bogart G, Rutter MJ, Visner G, Walczak SA, Ferkol TW, Michelson PH, American Thoracic Society Ad Hoc Committee on Flexible Airway Endoscopy in Children. (2015). Official American Thoracic Society technical standards: flexible airway endoscopy in children.. American journal of respiratory and critical care medicine.
https://doi.org/10.1164/rccm.201503-0474ST

RPMI-1640 **Sigma Aldrich Catalog #R5886**

Fetal Bovine Serum **Contributed by users**

1X PBS (Phosphate-buffered saline) **Contributed by users**

DMSO (dimethyl sulfoxide) **Sigma Aldrich Catalog #D8418**

conical tubes, 50ml **Contributed by users**

conical tubes, 15ml **Contributed by users**

Benzonase® Nuclease **Merck Millipore Catalog #E1014-25KU**

Corning® cell strainer **Corning Catalog #CLS431751-50EA**

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

COLLECTION OF BAL.

1 After obtaining informed consent from family and/or patient, obtain any excess BAL fluid collected at the time of clinically indicated bronchoscopy and lavage.

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2 For guidelines on how to safely perform bronchoscopy and lavage in children, please see:

> Faro A, Wood RE, Schechter MS, Leong AB, Wittkugel E, Abode K, Chmiel JF, Daines C, Davis S, Eber E, Huddleston C, Kilbaugh T, Kurland G, Midulla F, Molter D, Montgomery GS, Retsch-Bogart G, Rutter MJ, Visner G, Walczak SA, Ferkol TW, Michelson PH, American Thoracic Society Ad Hoc Committee on Flexible Airway Endoscopy in Children. (2015). Official American Thoracic Society technical standards: flexible airway endoscopy in children.. American journal of respiratory and critical care medicine.
> https://doi.org/10.1164/rccm.201503-0474ST

3 BAL samples must be placed on ice and processed in the laboratory within 30 minutes -1 hour of the procedure.

PROCESSING OF BAL TO RECOVER SINGLE CELLS. 30m

10m

4 Centrifuge BAL samples at **300 x g, 4°C, 00:10:00** .

5 Remove supernatant and resuspend cell pellet in 10mL of pre-chilled RPMI supplemented with 2% heat-inactivated fetal calf serum (herein referred to as RPMI 2% FCS).

> Cell-free BAL supernatant can be stored at **-80 °C** for future proteomic analysis (e.g. quantification of cytokines)

10m

6 Filter cell suspension through a 70-120µm cell strainer and centrifuge filtered cell suspension **300 x g, 4°C, 00:10:00** .

> In some cases, BAL samples may require a second filtering step to remove additional debris

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7 Discard supernatant and resuspend cell suspension in 2mL chilled RPMI 2% FCS. Remove 10μL for cell counting. Top up the cell suspension to 10mL RPMI 2% FCS and centrifuge **300 x g, 4°C, 00:10:00** while performing cell count. 10m

Cell counting can be performed manually using a haemocytometer, or using an automated cell counter (although the accuracy of some automated cell counters is limited by their inability to count large alveolar macrophages)

OPTION: Workflow for single cell analysis of fresh BAL samples.

8 If samples will be cryopreserved for storage, proceed to step 9 of this protocol.

If samples will be processed for immediate analysis (e.g. flow cytometry, cell sorting, or sc-RNAseq of live single cells) the following steps can be performed:

8.1 Resuspend cell suspension in chilled staining buffer for fixable viability staining according to manufacturers' instructions (e.g. the LIVE/DEAD™ Fixable Near-IR Stain from Invitrogen/ThermoFisher).

8.2 For a protocol detailing flow cytometry analysis of fresh BAL samples, please see our article:

Shanthikumar S, Ranganathan SC, Saffery R, Neeland MR (2021). Mapping Pulmonary and Systemic Inflammation in Preschool Aged Children With Cystic Fibrosis.. Frontiers in immunology. https://doi.org/10.3389/fimmu.2021.733217

CRYOPRESERVATION OF BAL.

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

This step should be done on ice. Add freeze solution to cell suspension in a drop-by-drop

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manner.

10 Immediately place cryogenic vials into an isopropanol freezing container (e.g. Nalgene® Mr. Frosty) and transfer to **-80 °C** overnight.

11 For long term storage, transfer the vials of frozen BAL cells to liquid nitrogen.

THAWING OF CRYOPRESERVED BAL FOR SINGLE CELL ANALYSIS. 12m

12 Warm thaw media (RPMI + 10% heat-inactivated FCS + 25U/mL Benzonase) to **37 °C** in a water bath.

For every sample to be thawed, place 8mL of warmed thaw media into a 15mL tube.

13 Remove cryopreserved BAL samples from liquid nitrogen and keep on dry ice for transport to the laboratory.

14 Place cryovials into the water bath for cell thawing, approximately **00:02:00** . 2m

15 Using a pasteur pipette, transfer cells from cryovial into the 15mL tube containing warmed thaw media.

16 Rinse cryovial with 1mL warmed thaw media to recover any remaining cells and transfer to the 15mL tube.

17 Centrifuge the cell suspension at **300 x g, 00:10:00** at room temperature. 10m

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18 Discard the supernatant and resuspend the cell pellet in 1mL RPMI 2%FCS for cell counting, followed by a final wash in 10mL RPMI 2%FCS and centrifuge at **300 x g, 00:10:00** at room temperature. 10m

19 Once the supernatant has been discarded, the cells are now ready to be resuspended at the required dilution for the first steps in your single cell experiment.

Following cryopreservation and thaw of n=21 pediatric BAL samples as described in this protocol, we achieved a median viability of 76.1% (range 61-90.7%) (determined by live/dead staining using flow cytometry).

Cryopreservation and thaw of BAL samples will result in the loss of some granulocyte populations. For a fresh vs thaw comparison of the immune cell profile of BAL, see our protocol published here:

> Shanthikumar S, Burton M, Saffery R, Ranganathan SC, Neeland MR (2020). Single-Cell Flow Cytometry Profiling of BAL in Children.. American journal of respiratory cell and molecular biology. https://doi.org/10.1165/rcmb.2019-0453MA

PREPARATION OF CELLS FOR FLOW CYTOMETRY 45m

20 Resuspend cell suspension for fixable viability staining according to manufacturers' instructions (e.g. the LIVE/DEAD™ Fixable Near-IR Stain from Invitrogen/ThermoFisher). 5m

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

21 Resuspend cells in human FC-block according to manufacturers' instructions for **00:05:00** at room temperature. 5m

22 Add required antibody cocktail made up at 2X concentration 1:1 with the cells and incubate for 30m

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00:30:00 on ice. For examples of relevant antibody stains for pediatric lung samples, please see our published work below.

> Shanthikumar S, Ranganathan SC, Saffery R, Neeland MR (2021). Mapping Pulmonary and Systemic Inflammation in Preschool Aged Children With Cystic Fibrosis.. Frontiers in immunology. https://doi.org/10.3389/fimmu.2021.733217

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

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