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Processing of pediatric bronchoalveolar lavage samples for single cell analysis v 3

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We use this protocol and it's working

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Abstract

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).

Guidelines

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:

CITATION

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.

LINK

https://doi.org/10.1164/rccm.201503-0474ST

Materials

- RPMI-1640 **Sigma Aldrich Catalog #**R5886
- Fetal Bovine Serum
- 1X PBS (Phosphate-buffered saline)
- DMSO (dimethyl sulfoxide) **Sigma Aldrich Catalog #**D8418
- conical tubes, 50ml
- conical tubes, 15ml
- Benzonase® Nuclease **Merck Millipore Catalog #**E1014-25KU
- Corning® cell strainer **Corning Catalog #**CLS431751-50EA

Safety warnings

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

COLLECTION OF BRONCHOALVEOLAR LAVAGE (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.

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

CITATION

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.

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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 SINGLE CELL SUSPENSION

30m

4 Centrifuge BAL samples at 300 x g, 4°C, 00:07:00 . 7m

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).

Note

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

6 Filter cell suspension through a 70-120µm cell strainer and centrifuge filtered cell suspension 300 x g, 4°C, 00:07:00 . 7m

Note

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

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

8 Prepare cell suspension for cell counting. Here, we use AO/PI and the LUNA FL counter. Remove 18 µL for cell counting into a microcentrifuge tube. Add 2µL of AO/PI to the count tube and mix well.

8.1 Load 10µL of stained cells onto a Luna fluorescent counting slide and count. Record viability, total cell count, and live cell count.

Note

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

9 If choosing to run flow cytometry or other single cell assays on fresh cells, here is where you can allocate the required number of cells for downstream processing. For flow cytometry, described below, we allocate 200-300,000 cells prior to proceeding to cryopreservation for remaining cells.

CRYOPRESERVATION OF BAL MONONUCLEAR CELLS

10 Top up remaining cell suspension to 8mL with RPMI 2% FCS.

11 Fill a 15mL tube with 2mL of Ficoll plaque plus and layer the cell suspension onto the surface of the Ficoll solution.

Note

We choose to use a Ficoll gradient for cryopreservation. This is to remove fragile granulocytes that may affect viability of the sample upon thaw. We observe higher viability upon thaw following Ficoll preparation when compared to cryopreservation of whole BAL.

Note

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

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

13 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 with 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.

14 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. Transfer cells to cryogenic vial.

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

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

PREPARATION OF FRESH CELLS FOR FLOW CYTOMETRY

17 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

18 Resuspend cells in 25µL of FC-block for 00:05:00 at Room temperature . 5m

19 The next steps will depend on the requirements for your specific panel. As an example, we have attached our 17-plex spectral cytometry panel that we routinely use on paediatric airway samples, as well as a publication describing the application of this panel. All of the following steps are related to this panel.

nasal_bronchial_BAL_panel.pdf 114KB

CITATION

Neeland MR, Gubbels L, Wong ATC, Walker H, Ranganathan SC, Shanthikumar S (2024). Pulmonary immune profiling reveals common inflammatory endotypes of childhood wheeze and suppurative lung disease..
LINK
https://doi.org/pii:S1933-0219(24)00020-5.10.1016/j.mucimm.2024.03.001

20 Add 25µL of antibody cocktail made up at 2X concentration and incubate for 00:30:00 On ice . 30m

21 Following staining, wash cells with 2mL FACS buffer, centrifuge at 400 x g, 4°C, 00:05:00 and resuspend cells in 200µL FACS buffer for acquisition on a flow cytometer (here, a Cytek 5L Aurora). 5m

22 Immediately before running the sample, filter the cell suspension through a 35 µm cell strainer (Falcon™ 352235).

OPTION: THAWING OF CRYOPRESERVED BAL CELLS FOR SINGLE CELL ANALYSIS. 12m

23 If it is not possible to run single cell assays on fresh cells on the day of bronchoscopy, herein is a protocol for single cell analysis on cryopreserved and thawed BAL cells.

Noting this will not enable efficient analysis of granulocytes, which were removed during the Ficoll gradient steps above.

Granulocytes are unlikely to survive a cryopreservation and thaw process even if whole BAL was cryopreserved.

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

Note

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

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

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

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

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

29 Centrifuge the cell suspension at 300 x g, 00:07:00 at room temperature. 7m

30 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, 00:07:00 at room temperature. 7m

31 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.

Citations

Step 19

Neeland MR, Gubbels L, Wong ATC, Walker H, Ranganathan SC, Shanthikumar S. Pulmonary immune profiling reveals common inflammatory endotypes of childhood wheeze and suppurative lung disease.
https://doi.org/pii:S1933-0219(24)00020-5.10.1016/j.mucimm.2024.03.001