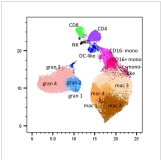

Mar 22, 2021

Processing of pediatric bronchoalveolar lavage samples for single cell analysis

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1 *Works for me* dx.doi.org/10.17504/protocols.io.btiankae

Respiratory Systems Immunology

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SUBMIT TO PLOS ONE

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).

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PROTOCOL CITATION

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KEYWORDS

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

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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:

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

MATERIALS TEXT

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**

SAFETY WARNINGS

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.

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

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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

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

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)

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

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

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)

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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:

Shivanthan Shanthikumar, Sarath C. Ranganathan, Richard Saffery, Melanie R. Neeland (2021). Application of high dimensional flow cytometry and unsupervised analysis to define the immune cell landscape of early childhood respiratory and blood compartments. bioRxiv.
https://doi.org/10.1101/2021.03.21.436363

Following processing of n=11 pediatric BAL samples as described in this protocol, we achieved a median viability of 78.9% (range 72-88.8%) (determined by live/dead staining using flow cytometry).

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 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.

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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

18 Discard the supernatant and resuspend the cell pellet 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

OPTION: Flow cytometry analysis of thawed BAL.

20 For flow cytometry analysis of cryopreserved BAL, please see our protocol published here:

Citation: Shivanthan Shanthikumar, Richard Saffery, Sarath C. Ranganathan, Melanie R Neeland (03/22/2021). Processing of pediatric bronchoalveolar lavage samples for single cell analysis. https://dx.doi.org/10.17504/protocols.io.btiankae

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

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