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Cell dissociation from nasal, bronchial and tracheal brushings with cold-active protease for single-cell RNA-seq

Forked from Cell dissociation from nasal and bronchial brushings with cold-active protease for single-cell RNA-seq

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

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SUBMIT TO PLOS ONE

ABSTRACT

This modified protocol provides details on the cell dissociation that should be performed to obtain single-cell suspensions from nasal, tracheal and bronchial epithelium brushings.

Cell dissociation is performed at 4°C to avoid gene expression alterations and maximize viability for scRNAseq.

The typical cell number recovery is 200,000-300,000 per nasal brushing and 10,000-30,000 per tracheal/bronchial brushing .Cell suspensions are suitable for single-cell RNA-sequencing protocols with viability generally >70%.

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Yoshida, M., Worlock, K. B., Huang, N., Lindeboom, R. G. H., Butler, C. R., Kumasaka, N., ... Meyer, K. B. (2021). The local and systemic response to SARS-CoV-2 infection in children and adults. MedRxiv, 2021.03.09.21253012. https://doi.org/10.1101/2021.03.09.21253012

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PROTOCOL CITATION

Kaylee B Worlock, Masahiro Yoshida, Kerstin Meyer, Marko Z. Nikolić 2021. Cell dissociation from nasal, bronchial and tracheal brushings with cold-active protease for single-cell RNA-seq. **protocols.io** https://dx.doi.org/10.17504/protocols.io.btpunmnw

MANUSCRIPT CITATION please remember to cite the following publication along with this protocol

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FORK NOTE

The main modifications include 1) the inclusion of tracheal brushes 2) the use of transport media for sample collection 3) the method for dislodging the cells from these brushes 4) the method of red cell lysis 5) altered wash and filtering steps and 6) an extra centrifuge step prior to resuspension and cell count to remove residue liquid. The use of transport media allowed us to transport and store the brushes for 2-3 hours before processing where necessary (e.g when multiple samples were collected).

FORK FROM

Forked from Cell dissociation from nasal and bronchial brushings with cold-active protease for single-cell RNA-seq, Laure-Emmanuelle Zaragosi

Citation: Kaylee B Worlock, Masahiro Yoshida, Kerstin Meyer, Marko Z. NikoliÃÂ (04/01/2021). Cell dissociation from nasal, bronchial and tracheal brushings withÃÂ cold-active protease for single-cell RNA-seq. https://dx.doi.org/10.17504/protocols.io.btpunmnw

KEYWORDS

brushing, bronchail epithelium, nasal epithelium, single-cell, dissociation, cold-active protease, tracheal epithelial

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GUIDELINES

Storage Conditions of Reagents

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A	B
Reagent	**Storage Condition**
HBSS	4°C
Hypothermosol	4°C
20 mM EDTA	room temperature
BSA (Sigma, A8806)	4°C
Protease from *Bacillus Licheniformis* (Sigma, P5380)	Store 100 µL aliquots (100 mg/mL) in DPBS at -80°C
Pen Strep (Gibco; 15070)(5,000 units/mL Penicillin and 5,000 µg/mL Streptomycin)	Store in 5mL aliquots at -20 °C
Amphotericin B (Fisher Scientific; 10746254)	Stored in 0.5 mL aliqyots at -20°C
Gentamicin (Gibco; 15710)	4°C
1x RBC lysis buffer (Invitrogen, #004333-57)	4°C
αMEM (Gibco; 22561-01)	4°C
Trypan blue (Thermo Fisher; 15250061)	room tempature
Hoechst 33342 (10 mg/mL)	4°C
NucGreen™ Dead 488 ReadyProbes™ Reagent	room temperature

Equipment for counting (optional)

Equipment	Supplier	Catalog no.
Countess II FL automated cell counter	Thermo Fisher Scientific	AMQAF1000

The protocol workflow is as follows:

1. Perform brushing of the epithelium of the nasal cavity/trachea/ bronchi
2. Transport if necessary or store on ice
3. Dissociation: triturate on ice

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4. Remove red blood cells if necessary
5. Prepare cells for Chromium

All steps should be performed on ice or at 4°C

MATERIALS TEXT

MATERIALS

EDTA **Contributed by users**

23G Needles **Contributed by users Catalog #4657667**

Protease from Bacillus Licheniformis **Sigma Catalog #P5380**

HypoThermosol® FRS 100 mL **Stemcell Technologies Catalog #7935**

Quick-Read 10 Chamber Slide **Globe Scientific Catalog #3805**

Countess™ Cell Counting Chamber Slides **Contributed by users Catalog #C10314**

DPBS no calcium, no magnesium **Invitrogen - Thermo Fisher Catalog #14190136**

21G needle **VWR international Ltd Catalog #BD-305165**

HBSS **Gibco - Thermo Fischer Catalog #14060040**

STEP MATERIALS

Quick-Read 10 Chamber Slide **Globe Scientific Catalog #3805**

Flowmi cell strainer **Contributed by users Catalog #H13680-0040** Step 22

Hoechst 33342, Trihydrochloride, Trihydrate - 10 mg/mL Solution in Water **Thermo Fisher Scientific Catalog #H3570**

NucGreen™ Dead 488 ReadyProbes™ Reagent **Thermo Fisher Scientific Catalog #R37109**

Ammonium Chloride Solution 100 mL **Stemcell Technologies Catalog #7800**

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SAFETY WARNINGS

Samples coming from patients with undetermined viral status should be process in cell culture rooms with the appropriate safety level and modification to the protocol (e.g. blunt needles).

BEFORE STARTING

Prepare *Bacillus Licheniformis* enzyme mix just prior to starting dissociation:

Volume (µl)	Reagent	Final concentration
850	Hypothermosol	1X
50	20 mM EDTA	0.5 mM
100	Protease from *B. Licheniformis* (100 mg/mL)	10 mg/mL

Preparation of Transport Media:

Supplement aMEM media bottle with:
- 5 mL Pen Strep (Finial concentration: 50 units/mL Penicillin and 50 µg/mL Streptomycin)
- 0.5 mL Gentamicin (Finial concentration; 10 ng/mL)
- 0.5 mL Amphotericin B (Finial concentration; 250 ng/mL

Prepare Inactivation buffer*:*

Make stock of 10% BSA in HBSS and store at -20 °C.
To make HBSS/BSA 2% aliquot 40 mL of HBSS in 50 mL conical and pipet in 10 mL of 10% BSA stock.

Prepare Wash buffer*:*

To make HBSS/BSA 1% aliquot 20 mL of HBSS in 50 mL conical and pipet in 20 mL of HBSS/BSA 2%.

Prepare Resuspension buffer*:*

To make HBSS/BSA 0.05% aliquot 1 mL of HBSS/BSA 2% in 50 mL conical and pipet in 39 mL of HBSS.

(Optional) Prepare cell staining reagent:

- HBSS: 500 µL
- Hoechst 33342 (10 mg/mL): 1 µL
- NucGreen™ Dead 488 ReadyProbes™ Reagent: 1 drop

Preparation of reagents and tubes (before starting):

1

PREPARATION OF DISSOCIATION MIX (Fresh at each experiment)

Ingredients:
- Hypothermosol
- Protease from Bacillus Licheniformis (100 mg/mL stock solution in PBS)
- EDTA 10 mM

Prepare 1 mL per sample:
- 850 microlitres of Hypothermosol

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- 100 microlitres of protease (Final concentration:10 mg/mL)
- 50 microlitres of EDTA (Final concentration: 0.5 mM)

Aliquote 1 mL of dissociation mix in a 15 mL falcon tube for every sample (**Dissociation tube**).

PREPARATION OF TRANSPORT MEDIA (STORED FOR UP TO ONE MONTH)

Ingredients:
- αMEM (Gibco; 22561-01)
- Pen Strep (Gibco; 15070)(5,000 units/mL Penicillin and 5,000 µg/mL Streptomycin)
- Amphotericin B (Fisher Scientific; 10746254) (250 µg/mL)
- Gentamicin (Gibco; 15710) (10mg/mL)

For Transport Media supplement aMEM media bottle with:
- 5 mL Pen Strep (Finial concentration: 50 units/mL Penicillin and 50 µg/mL Streptomycin)
- 0.5 mL Gentamicin (Finial concentration; 10 ng/mL)
- 0.5 mL Amphotericin B (Finial concentration; 250 ng/mL)

Aliquote 4 mL of Transport media in a 15 mL falcon tube for every sample (**Tube A**).

PREPARATION of INACTIVATION, WASH and RESUSPENSION BUFFERS:

Prepare stocks of **10% BSA** in HBSS and aliquot and store at -20 °C (filtered; 22mm filter).

Prepare **inactivation buffer**; 2% BSA/HBSS (aliquot and store @ -20 °C or @ 4°C for 1 week)
-40 mL HBSS+10 mL 10% BSA stock.

Prepare **wash buffer**; 1% BSA/HBSS (aliquot and store @ -20 °C or @ 4°C for 1 week)
- 20 mL HBSS + 20 mL 2% BSA/HBSS.

Prepare **resuspension buffer**; 0.05% BSA/HBSS (aliquot and store @ -20 °C or @ 4°C for 1 week)
-1 mL 2% BSA/HBSS + 39 mL HBSS

Sample Collection and processing 5m

2 Perform nasal brush biopsy in the nasal cavity in the inferior nasal conca zone (UBERON:0005922) using cytological sampling brushes (CY1050). Tracheal and bronchial brush biopsies were collected using single-use cytology brushes (BC-202D-2010) from the tracheal mucosa (UBERON:0000379) and bronchial mucosa (UBERON:0000410). Brushings were performed by a medical doctor.

Single-use Cytology Brush

Olympus **BC-202D-2010**
Single-use Cytology Brushes (2mm by 10mm) for Tracheal and Bronchial brushes

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Cytological Sampling Brush

Scientific Laboratory Supplies **CYT1050**

cytology brush

3 Carefully cut the cytology brush and place straight into a 15mL falcon tube (**Tube A**) containing 4 mL of cold transport media and store at 4 degrees Celsius.

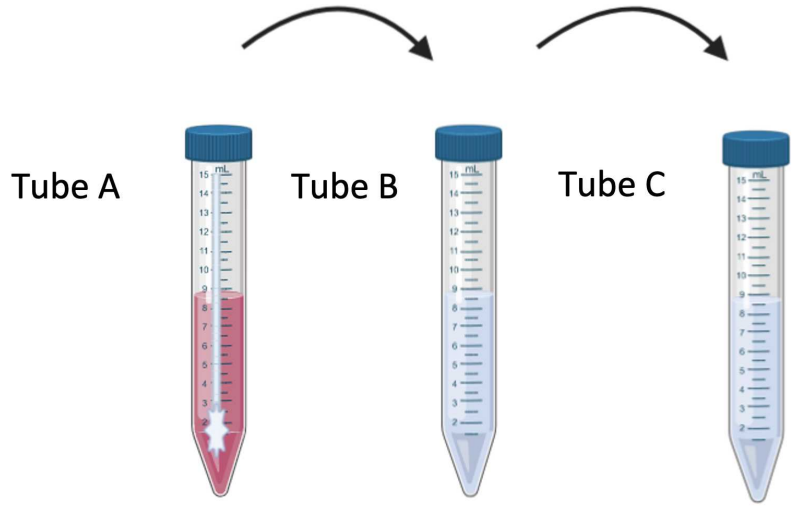
4 mL

Where possible samples should be processed immediately after collection. However, where multiple samples were collected, brushes were stored at 4 degrees Celsius for up to 2-3 hours to allow them to be processed together.

Collecting multiple brushes from the same donor

If multiple tracheal or bronchial brushes were collected from the same donor at this point they were collected in the same 15 mL falcon tube due to size. Whereas if two nasal brushes from the same donor were collected (one from each nostril) these were placed in their own 15 mL falcon tube and the cells collected together later in step 8.

4 Working in a CL2 hood on ice shake the 15 mL falcon tube (**Tube A**) containing the cytology brush vigorously (~30 seconds) to help dislodge cells and mucous. Place the tube back on ice for 1 min, allowing the aerosols to settle, before using a sterile set of tweezers to carefully transfer the brush from Tube A into a new 15 mL falcon tube (**Tube B**) containing 4 mL HBSS. Repeat shaking step, transferring the brush to a new 15 mL flacon tube containing 4 mL HBSS each time (e.g **Tube C** and **Tube D** etc) until no mucus or cells can be seen on the brush and/or to be coming off into the HBSS.



Collection of cells from cytology brush.

If samples from multiple different patients are being processed at the same time be sure to wash the

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tweezers with 70% ethanol and dry throughly between samples to help avoid cross contamination.

5 Transfer brush (or brushes if multiple tracheal or bronchial brushes were taken) into a new 15 mL falcon tube containing 1 mL of dissociation mix (**Dissociation tube**) and leave on ice to help collect any remaining cells still attached to the brush.

1 mL

4 °C

6 Spin all 15 mL falcon tubes used in Step 4. (**Tube A, B , C** and **D** ect.) for 5 mins at 400g. 5m

00:05:00 Spin at 400xg (4 °C)

7 Carefully remove supernatant from each tube, leaving ~0.5-1 mL.

8 Gentle re-suspend the cell pellet in remaining supernatant in each 15 mL falcon tube and transfer into **Tube A**.

If multiple nasal brushes from the same patient were collected (one from each nostril) at this point the cells were all pooled into one tube (Tube A).

9 5m

Spin **Tube A** and **Dissociation tube** (with brush/brushes still inside) for 5 mins at 400g.

00:05:00 Spin at 400xg (4 °C)

10 Discard brush/brushes from **Dissociation Tube** and carefully remove the supernatant from **Tube A**.

Try to remove as much of the supernatant as you can from Tube A, but sometime if the sample contains a lot of mucous this can be difficult, particularly if some of the mucous is seen to be floating. If it is particularly challenging you can try briefly re-spinning the sample at 400g for 2 mins at 4 °C and leaving slightly more supernatant in the tube.

11 Re-suspend any cells that might have collected first in the bottom of the **Dissociation tube** in the dissociation mix

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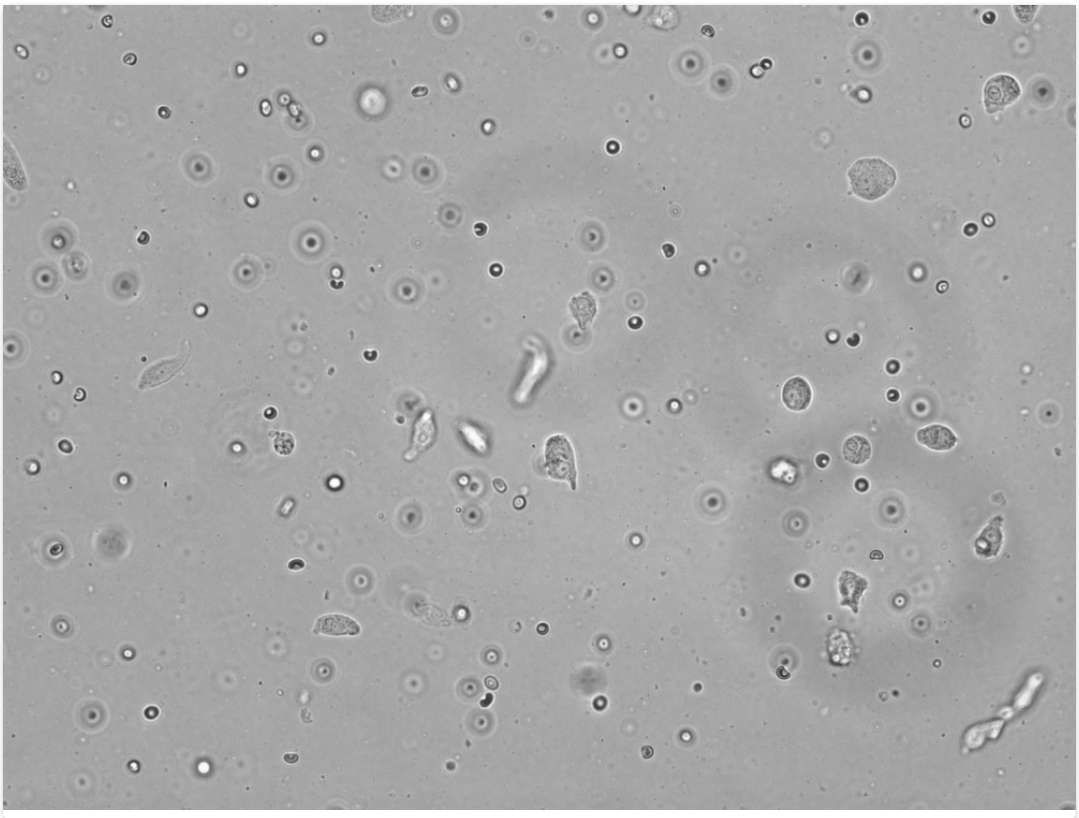
before transferring it to **Tube A** using a P100 pipette and gentle re-suspending the cell pellet.

12 Incubate cells on ice for 30 min, with gentle trituration with needles 5 times every 5 min. Use needle with decreasing sizes from 21G to 23G.

00:30:00 Incubation

00:05:00 Trituration



4 °C

13 Inactivate 1 mL of dissociation mix by adding 200 µL of **Inactivation buffer** (HBSS containing 2% BSA)

200 µl Inactivation buffer

14 Spin at 400g for 5 min at 4°C

5m

00:05:00 Spin at 400xg (4 °C)

15 Discard supernatant leaving 200 µL of residual liquid on the pellet.

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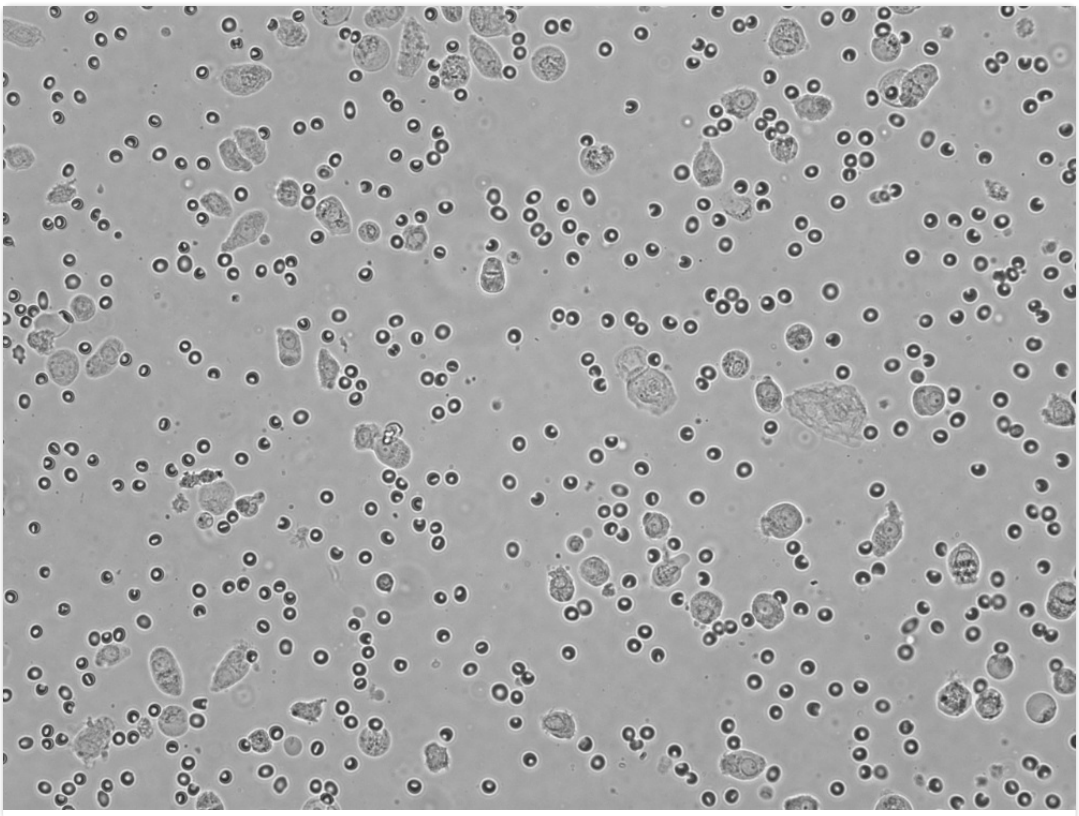
16 If RBC content appears lower than 50% or the pellet is hardly visible, go directly to step 21.

If the cell pellet appears bloody by eye (>40 % red), and/or under the microscope > 50% of the cells observed are RBCs perform RBC lysis: add 1mL of 1x **RBC lysis buffer** (Invitrogen, #004333-57) and gently re-suspend the cell pellet .

1 mL RBC lysis buffer at RT

Observe cells under an inverted microscope to evaluate red blood cells (RBC) content.
RBC content is better evaluated using an automated cell counter such as Countess, after addition of Hoechst 33342 to an aliquot of the cell suspension to discriminate nucleated cells from non-nucleated cells.



17 Incubate at room temperature (RT) for 3 min.

00:03:00 RBC lysis

Room temperature

WARNING: Be careful not to exceed 3 min or you will start to lyse your non-RBCs.

18 Add 9 mL of Inactivation buffer

9 mL PBS

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19 Spin at 400g for 5 min at 4°C 5m

00:05:00 Spin at 400xg (4 °C)

20 Discard supernatant leaving 10 µL of residual liquid on the pellet.

21 Resuspend in 1 mL of **wash buffer**

1 mL wash buffer

22 Filter 500 µL of cell suspension through Flowmi cell strainer (repeat)

Flowmi cell strainer **Contributed by users Catalog #H13680-0040**

https://www.youtube.com/watch?v=taS1BuTnvds

We filter using 2 x 500 µL steps and try to avoid taking up any obvious solid mucous clumps to remove the risk of blockages in the Flowmi and losing cells.

23 Spin at 400g for 5 min at 4°C 5m

00:05:00 Spin at 400xg (4 °C)

24 Discard supernatant leaving 10 µL of residual liquid on the pellet.

25 Briefly re-spin tubes again to collect any residual liquid left on the side of the tubes (to ensure volume is accurate) and discard supernatant leaving 10 µL of residual liquid on the pellet. 1m

00:01:00 Spin at 400xg (4 °C)

26 Resuspend in 20-50 µL of resuspension buffer depending on the size of the pellet (HBSS + 0.05% BSA).

50 µl Resuspension buffer

27 Preform cell count and calculate viability using trypan blue (Thermo Fisher; 15250061) and a hemocytometer.

Viability ideally > 70% for use with 10x

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Viability ideally > 70% for use with 10x.

28 Adjust concentration to 1000 cells/µL (with resuspension buffer) for 10X Chromium. Monitor final cell concentration.

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