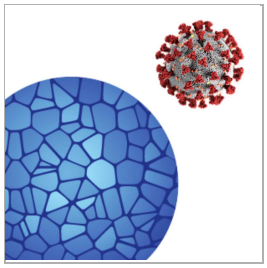

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CZI Pediatric Nasopharyngeal Swab Processing for 10X scRNA-seq (Illustrated Protocol)

Forked from Human Nasopharyngeal Swab Processing for Viable Single-Cell Suspension

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Human Cell Atlas Method Development Community | Coronavirus Method Development Community

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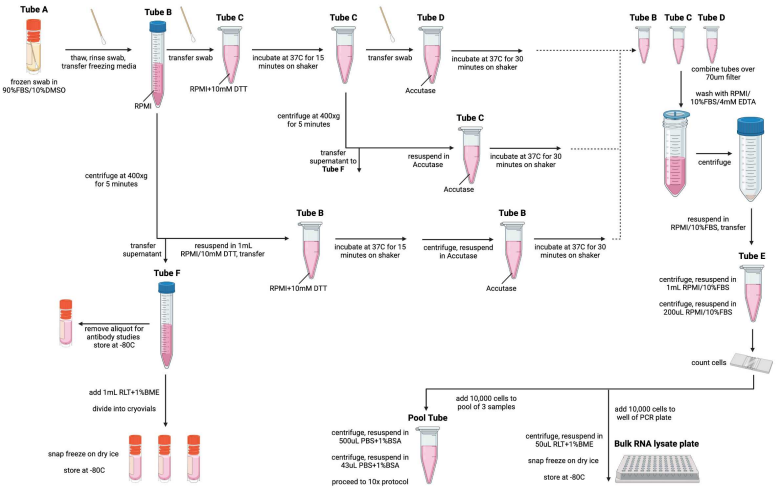
ABSTRACT

A protocol for recovering viable single cell suspensions from cryopreserved human nasopharyngeal swabs for pooled single-cell RNA-seq and individual bulk RNA-seq. The illustrated schematic below details the process.



ATTACHMENTS

Cell Isolation from Nasopharyngeal Swabs for scRNA.docx

IMAGE ATTRIBUTION

Created with BioRender

GUIDELINES

Samples should be collected by a trained medical professional using a nasal swab (FLOQSwabs, Copan flocked swabs) in accordance with the manufacturer's instructions. Briefly, the process was performed as follows. First, the patient's head was tilted back slightly, and the swab was inserted along the nasal septum, above the floor of the nasal passage to the nasopharynx until slight resistance was felt. The swab was then left in place for several seconds to absorb secretions and slowly removed while rotating. The swab was placed in a 1.7 mL cryovial containing 90% fetal bovine serum (FBS)/10% dimethyl sulfoxide (DMSO) and frozen using a slow-cooling device (Thermo Scientific Mr. Frosty Freezing Container) at -80 °C, and stored in liquid nitrogen.

MATERIALS TEXT

Materials & Reagents for 1 swab (with overages):

- 2.5 mL of RPMI + 10 mM Dithiothreitol (DTT) (made fresh)
- 3.5 mL of Accutase
- 6 mL RPMI
- 8 mL quenching buffer containing RPMI + 10% fetal bovine serum (FBS) + 4 mM EDTA
- 2 mL RPMI + 10% FBS
- 15 mL conical labeled **Tube B** containing 2 mL RPMI (previously 5 mL)
- 1.5 mL tube labeled **Tube B**, empty
- 1.5 mL tube labeled **Tube C**, with 1 mL RPMI + 10 mM DTT
- 1.5 mL tube labeled **Tube D**, with 1 mL Accutase
- 50 mL conical
- 70 µm cell strainer that fits 50 mL conical
- 1.5 mL tube labeled **Tube E**
- 1.5 mL tube for counting
- 1.5 mL tube for pooling cells (if doing 3 samples, need 1 tube)
- 10 µL trypan blue
- 1 mL PBS.+ 1% BSA
- 15 mL conical for collecting viral supernatant labeled **Tube F**
- 3 cryovials for viral supernatant lysate
- 3 96-well PCR plates or 3 cryovials for bulk RNAseq lysate
- RLT + 1% 2-mercaptoethanol (BME)

Equipment

- Forceps and scissors
- Thermomixer set to 37ºC, agitating at 300 rpm
- Hemocytometer slides

SAFETY WARNINGS

For hazard information and safety warnings regarding nasopharyngeal swabs or any listed materials, please refer to the SDS (Safety Data Sheet).

For samples obtained from individuals diagnosed with, or at any risk of, an infection (e.g. SARS-CoV-2), additional precautions should be taken in accordance with your institute's regulations on biosafety. These include elimination of aerosol generating steps where possible (e.g., no vacuum aspiration), all steps prior to cell lysis should be carried out in a biosafety cabinet, including thermomixing and centrifugation where possible. When necessary, samples should only be removed from the biosafety cabinet in decontaminated and sealed secondary containment. Personal protective equipment including a gown, two pairs of non-sterile gloves, a protective surgical or N95 mask, and a face shield should be worn during sample processing.

ATTACHMENTS

Cell Isolation from Nasopharyngeal Swabs for scRNA.docx

Before You Start

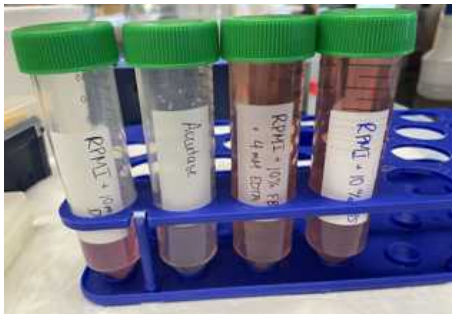
1 Record sample characteristics in Table 1

Site ID	Study ID	Case/Control	Virus	Date of Enrollm	Sex	Age	Sample ID	Batch	Pool

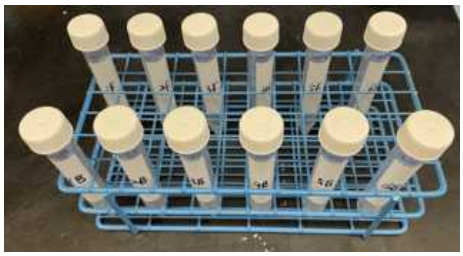
Site ID	Study ID	Case/Control	Virus	Date of Enrollm	Sex	Age	Sample ID	Batch	Pool

2 Prepare 50 mL conicals with necessary reagents:

- 1X RPMI + 10 mM DTT
- 1X Accutase
- RPMI + 10% FBS + 4 mM EDTA (quenching buffer)
- RPMI + 10% FBS



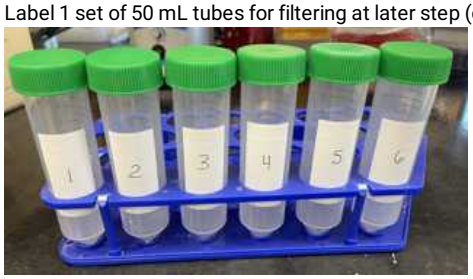
3 Label 2 sets of 15 mL conicals **(Tube B & Tube F).** Prepare **Tube B** with 2 mL RPMI .



Tube B and Tube F setup for 6 swabs

4 Label 4 sets of 1.5 mL tubes **(Tube B, Tube C, Tube D, Tube E).** Prepare **Tube C** with 1 mL RPMI + 10 mM DTT and **Tube D** with 1 mL Accutase

5 Label 1 set of 50 mL tubes for filtering at later step (can set aside for beginning of protocol)



Final Set-Up

6

Timing

7 Fill out timing chart at indicated steps of protocol

Step	Time
Start time:	
Time after preparation of single cell suspension:	
Time after counting:	
Time 10X controller run started:	
Time 2nd 10X controller run started (if applicable):	
Time lysis buffer added for bulk lysates:	

Tube A (the cryovial)

8 Rapidly (within 1-2 minutes) thaw cryovial (**Tube A**) in thermal block set to 37 °C.

Safety information

Carefully perform in accordance with your institute's safety guidelines. If handling potentially infectious material, inspect for cracks or leaks during warming

9 Remove swab from **Tube A** using clean forceps.

Step 9-11 - first swab transfer.mp4

10 Dip swab in **Tube B** (15 mL conical) once. Swirl briefly (approximately 3 seconds) to rinse swab.

11 Move swab from **Tube B** (15 mL conical) to **Tube C**. Trim swab handle using scissors if necessary so that it can fit inside a 1.5mL tube (see video). Proceed directly to step 27 for **Tube C**. In parallel, continue processing **Tube B**.

12 Transfer liquid in **Tube A** to **Tube B** (15 mL conical).

13 Using 1 mL RPMI from **Tube B** (15 mL conical), wash **Tube A**.

13.1 Collect washing from **Tube A** in **Tube B** (15 mL conical). Continue below with **Tube B**

14 Discard **Tube A**.

Tube B

15 Centrifuge **Tube B** (15 mL): 400 x g, 4°C, 00:05:00 .

16 Remove supernatant with serological pipette *or P1000*. Do **not** discard supernatant.

17 Transfer supernatant to **Tube F**.

18 Resuspend pellet in 1 mL RPMI + 10mM DTT .

19 Transfer suspended cells from **Tube B** (15 mL) to **Tube B** (1.5 mL). Discard empty 15 mL conical.

20 Place **Tube B** (1.5 mL) on thermomixer (37°C, 300 rpm).

21 Incubate for 00:15:00. In parallel, continue processing **Tube C.**

22 Centrifuge **Tube B** (1.5 mL): 400 x g, 4°C, 00:05:00.

23 Remove supernatant with P1000 pipette. Discard supernatant.

24 Resuspend pellet in 1 mL Accutase.

25 Place **Tube B** (1.5 mL) on thermomixer (37ºC, 300 rpm).

26 Incubate for 00:30:00.

Tube C

27 Place **Tube C** on thermomixer (37ºC, 300 rpm).

28 Incubate for 00:15:00.

29 Place swab in **Tube D**. Proceed directly to step 36 for **Tube D**.
Step 29 - second swab transfer.mp4

30 Centrifuge remaining liquid at 400 x g, 4°C, 00:05:00.

31 Remove supernatant with P1000 pipette. Do not discard supernatant.

32 Place supernatant in **Tube F.** Keep **Tube F** on ice until you proceed to Step 71.

33 Resuspend pellet in 1 mL Accutase.

34 Place **Tube C** on thermomixer (37ºC, 300 rpm).

35 Incubate for 00:30:00.

Tube D

36 Place **Tube D** on thermomixer (37ºC, 300 rpm).

37 Incubate for 00:30:00.

Note

Take 10X reagents out at this step so that they have time to equilibrate to the appropriate temperature.

After Tube B, C, and D's 30 minute Incubations

38 After **Tube B**, **Tube C**, and **Tube D** have each finished their 30 minute incubations:

Note

In practice, we wait until all tubes have finished their 30 minute incubation in Accutase to synchronize. We leave tubes on incubation for a maximum 50 minutes.

39 Place 70 µm filter in 50 mL conical.

40 Wet filter with 3 mL quenching buffer (RPMI + 10% FBS + 4 mM EDTA).

41 Pipette contents of **Tube B**, **Tube C**, and **Tube D** onto filter with P1000 pipette

Step 41 - adding Tubes B,C,D contents to filter.mp4

Note

Do not discard original tubes.

42 Use 1 mL quenching buffer to wash each **Tube B**, **Tube C**, and **Tube D**.

42.1 Manually agitate the swab in **Tube D** in the quenching media with forceps to ensure full rinse.

43 Add quenching buffer from washes to filter. Discard **Tubes B**, **C**, and **D**.

44 Wash filter with additional 2 mL quenching buffer.

45 Discard filter, cap 50 mL conical.

46 Centrifuge 50 mL conical 400 x g, 4°C, 00:10:00.

Note

It will likely be very challenging to see a pellet in the 50 mL conical at this point

47 Remove supernatant with serological pipette. Leave ~500 µL in the bottom of the tube.

Approximate residual volume to aim for

Note

Remove supernatant carefully at this step! You don't want to pipette up your cells but you will need to add the volume you leave in this 50 mL conical and 1 mL RPMI + 10% FBS to a 1.5 mL tube in the next steps, so try not to exceed 500 µL residual volume.

48 Add 500 µL RPMI + 10% FBS to the tube to resuspend cells.

49 Transfer resuspended cells (~ 1 mL) from 50 mL conical to **Tube E** (1.5 mL tube).

50 Wash 50 mL conical with 500 µL RPMI + 10% FBS.

50.1 Transfer washing from 50 mL conical to **Tube E**.

51 Centrifuge **Tube E** 400 x g, 4°C, 00:05:00.

52 Remove supernatant with P1000 pipette.

53 Resuspend pellet in 1 mL RPMI + 10% FBS.

Example pellet in Tube E after centrifuging

Note

At this point, you should be able to see a reasonably-sized pellet!

54 Centrifuge **Tube E** 400 x g, 4°C, 00:05:00 5m

55 Resuspend pellet in 200 µL RPMI + 10% FBS

Count cells from Tube E

56 In a new empty 1.5 mL tube, add 10 µL trypan blue.

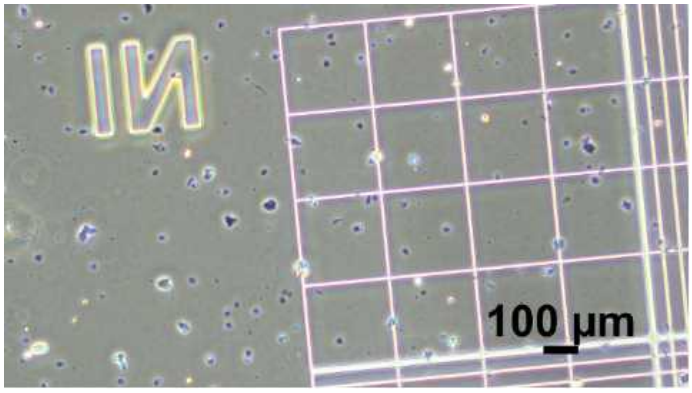
57 Add 10 µL cells from Tube E to 1.5 mL tube containing trypan blue.

58 Pipette to mix cells in trypan blue, transfer 10 µL to hemocytometer port.

59 Count viable cells across 8 quadrants. One quadrant is the corner of a hemocytometer grid, consisting of 16 smaller squares.

60 Record total cell number and calculate cell concentration using the "Cell Counts & 10X Volumes" sheet of the Calculation Tables template.
Calculation Tables template.xlsx

61 Take photo of cells at 4X. This can be done using a camera attached to the microscope or with a phone placed adjacent to the microscope lens.



Representative hemocytometer image at 4X

Prepare cells for 10X

62 Prepare 1.5 mL tube for each pool

63 For each sample, add "Volume Necessary for Desired Number of Cells" from "Cell Counts & 10X Volumes" sheet of the Calculation Tables template to correct pool.

64 Pipette cells to mix

65 If volume of pool is > 100 µL, centrifuge cells 400 x g, 4°C, 00:05:00 , then resuspend in 500 µL PBS + 1% BSA and proceed to next step. 5m

If volume of pool is < 100 µL, add 500 µL PBS + 1% BSA directly to pool and proceed to next step.

66 Centrifuge cells 400 x g, 4°C, 00:05:00 5m

67 Resuspend cells in 43.3 µL PBS + 1% BSA (Total volume of cell suspension + water on page 27 of 10X Protocol).

68 Add 43.3 µL cell suspension directly to 31.9 µL master mix (Step 1.2b of 10X Protocol)

69 Proceed with instructions in 10X Protocol to load the chip and run the controller (through Step 1.3)

70 At Step 1.4f, take a picture of the GEMs in the pipette tips.

Example photo of successful run

Tube F Processing (Viral Lysates)

71 Remove a 200uL aliquot from **Tube F** and transfer to a new, empty 1.5mL tube. Store at -80C for antibody studies.

72

Note

In **Tube F**, you should have 2 mL from original RPMI + 0.5-1 mL from swab cryopreservative + 1 mL from **Tube C** supernatant = ~4 mL total

Add 1 mL RLT + 1% BME to **Tube F.**

73 Distribute contents of **Tube F** into 3 cryovials per sample

Note

You can save time here by labeling cryovials/plates for viral and bulk lysates the day before!

74 Snap freeze on dry ice

75 Store at -80 °C

40m

Lysates for Bulk RNA-seq

76 For each lysate, add "Volume Necessary for Each Lysate" from "Bulk RNA Lysate Volumes" sheet of the Calculation Tables template to one well of a 96-well PCR plate according to the Lysate Storage Plate map.

Note

Typically, we aim to store 3 lysates per sample. To do so, we label 3 plates and fill 1 well per sample in each plate, if cell numbers allow. Make sure to record which well has which sample.

77 Seal plates and centrifuge 400 x g, 4°C, 00:05:00 5m

78 Aspirate media

> Note
>
> It may be difficult to see a pellet here, so it's okay to leave some residual volume before resuspending in lysis buffer in the next step

79 Resuspend cells in 50 µL RLT + 1% BME . Mix with pipette. Bubbles are okay here.

80 Seal plate with foil seal and spin down briefly.

81 Place plate on dry ice for 00:15:00 - 00:20:00 to snap-freeze lysate. 35m

82 Store at -80 °C until ready to use.