Version 2

Mar 12, 2021

Daughter of Frankenstein protocol for nuclei isolation from fresh and frozen tissues using OptiPrep® *continuous* gradient V.2

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1 *Works for me* dx.doi.org/10.17504/protocols.io.bs99nh96

Human Cell Atlas Method Development Community | Single Cell Core, Harvard Medical School

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SUBMIT TO PLOS ONE

ABSTRACT

This protocol is the result of the combination of various nuclei isolation protocols for single cell RNA-seq experiments using droplet-based methods, and is an extension of the Frankenstein (D.O.F means Daughter Of Frankenstein).

Developed to prepare nuclei isolates from fresh and frozen material of small-to-large sizes. The good thing is that it does not uses FACS but OptiPrep® ***continuous*** gradient to remove debris. It is the alternative protocol when FACS is not available.

This protocol works better with smaller samples. After centrifugation the nuclei will form a pellet and contaminating membranes will float at the top. This continuous gradient can be applied for as many times as needed to the same prep to continue cleaning up your nuclei suspensions.

EXTERNAL LINK

https://singlecellcore.hms.harvard.edu/

DOI

dx.doi.org/10.17504/protocols.io.bs99nh96

EXTERNAL LINK

https://singlecellcore.hms.harvard.edu/

PROTOCOL CITATION

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WHAT'S NEW

Gradient solutions were amended.

KEYWORDS

snRNAseq, 10x, nuclei, isolation, FACS

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Mar 12, 2021

PROTOCOL INTEGER ID

48161

GUIDELINES

Use RNA techniques.

The use of RNAse Inhibitor is HIGHLY recommended.

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MATERIALS TEXT

Required Buffers and Reagents

Nuclei EZ lysis buffer

1. **Sigma Catalog #EZ PREP NUC-101** (keep at 4°C)

2. Nuclei Wash and Resuspension Buffer (prepare fresh, keep at 4°C)

1x PBS
1.0% BSA (MACS® BSA Stock Solution, Miltenyi)
0.2-0.5 U/uL RNase Inhibitor (Protector RNA Inhibitor, Millipore Sigma)

3. Gradient Solutions

G60: OptiPrep®: 60% (w/v) solution of iodixanol in water.

GD: 150 mM KCl, 30 mM MgCl2, 120 mM, Tricine-KOH pH 7.8.

GW: 50% of iodixanol --> mix 5 volumes of G60 with 1 volume of GD (final: 50% iodixanol, 25 mM KCl, 5 mM MgCl2, 20 mM Tricine-KOH pH 7.8).

GH: 0.25 M Sucrose, 25 mM KCl, 5 mM MgCl2, 20 mM Tricine-KOH pH 7.8. (keep at 4°C).

G30: solutions of 30% --> mix 6 volumes of GW with 4 volumes of GH (final: 30% iodixanol, 25 mM KCl, 5 mM MgCl2, 20 mM Tricine-KOH pH 7.8). **Prepare fresh.**

4. Stock solutions and GD/GH Set-Up

-500 mM Tricine: 8.96 g in 100 ml water
-1 M KCl: 7.45 g in 100 ml water
-1 M MgCl2x6H2O: 20.3 g in 100 ml water

GD: To 50 ml water add 24 ml, 15 ml and 3 ml respectively of the Tricine, KCl and MgCl2•6H2O stock solutions (above); adjust to pH 7.8 with 1 M KOH and make up to 100 ml. Keep at RT.

GH: Dissolve 8.5 g of sucrose in 50 ml of water; add 4 ml, 2.5 ml and 0.5 ml respectively of the Tricine, KCl and MgCl2•6H2O stock solutions (above); adjust to pH 7.8 with 1 M KOH and make up to 100 ml. Keep at 4°C.

SAFETY WARNINGS

See SDS (Safety Data Sheet) for safety warnings and hazards.

DISCLAIMER:

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BEFORE STARTING

All samples and reagents are kept on ice or at 4 °C (wet ice).

Prepare all buffers and reagents as described in the "Materials" section.

Tissue Homogenization

1 Mince/chop tissue with a razor blade to small pieces. The tissue may be as small as a grain of rice.

> For mincing the tissue, you may take the tube out of ice, however, be quick and return it to ice.

2 Add **300 µl** of chilled Nuclei EZ Lysis Buffer (supplemented with RNAse Inhibitor 0.2-0.5 U/uL) to the tissue in 1.5 mL tube.

3 **Gently** homogenize the sample using a douncer by stroking 10-20 times. Keep nuclei suspension on ice at all times.

4 Add an extra **700 µl** of chilled Nuclei EZ Lysis Buffer (supplemented with RNAse Inhibitor 0.2-0.5 U/uL), mix gently by pipetting using **wide-bore tips** and incubate on ice for **00:05:00** . Repeat mixing 2-3 times during the incubation. 5m

Nuclei Isolation 10m

5 Filter homogenate using a 70 µm-strainer mesh to fit a 15 ml Falcon tube (e.g. pluriStrainer Mini 70 µm Cell Strainer).

6 Transfer flow though into a 1.5 mL LoBind tube and centrifuge the nuclei at **500 x g** for **00:05:00** at **4 °C** and remove supernatant leaving behind ~ **50 µl** .

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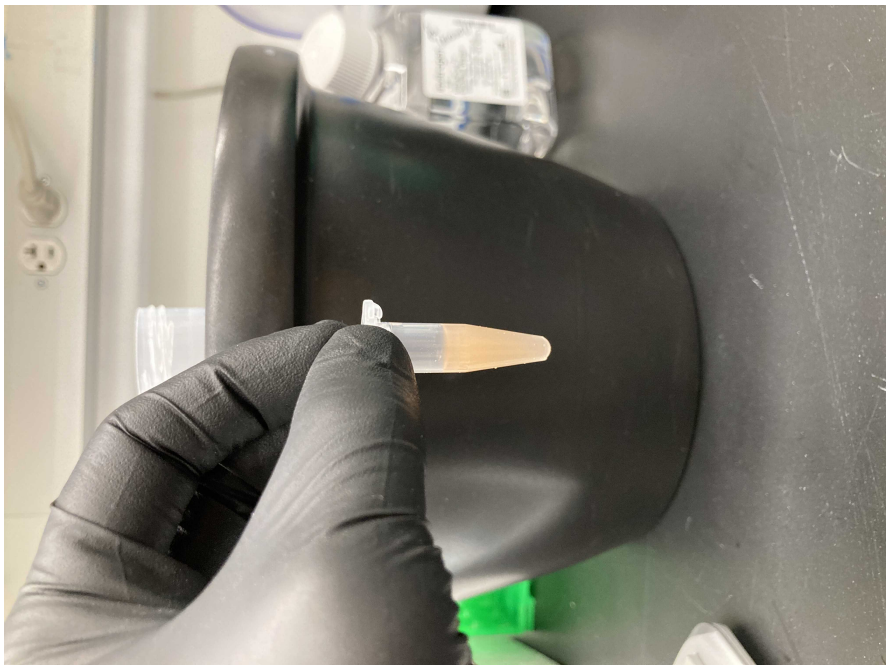

Homogenate after filtering.

Nuclei pellet.

7 **Optional:** add **1 mL** of EZ Lysis buffer (supplemented with RNAse Inhibitor 0.2-0.5 U/uL), gently resuspend pellet 10m
(optional: incubate for **00:05:00** on ice for additional lysis). Then centrifuge the nuclei at **500 x g** for **00:05:00** at **4 °C** .

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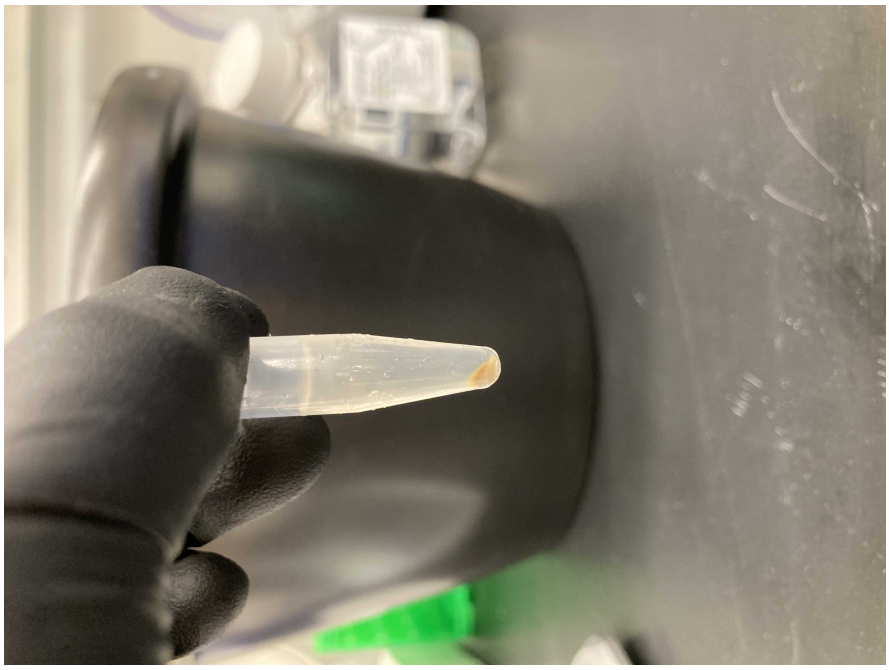

Homogenate after re-pelleting.

8 Remove supernatant as much as possible without disturbing pellet (if pellet looks loose leave ~ **50 µl** behind) and add **300 µl** of G30 buffer. Mix well but gently to resuspend the pellet.

9 ⚠

Carefully, **underlay** the sample with **1 mL** of the G30. A clear separation between homogenate and added G30 will form. Do not disturb.

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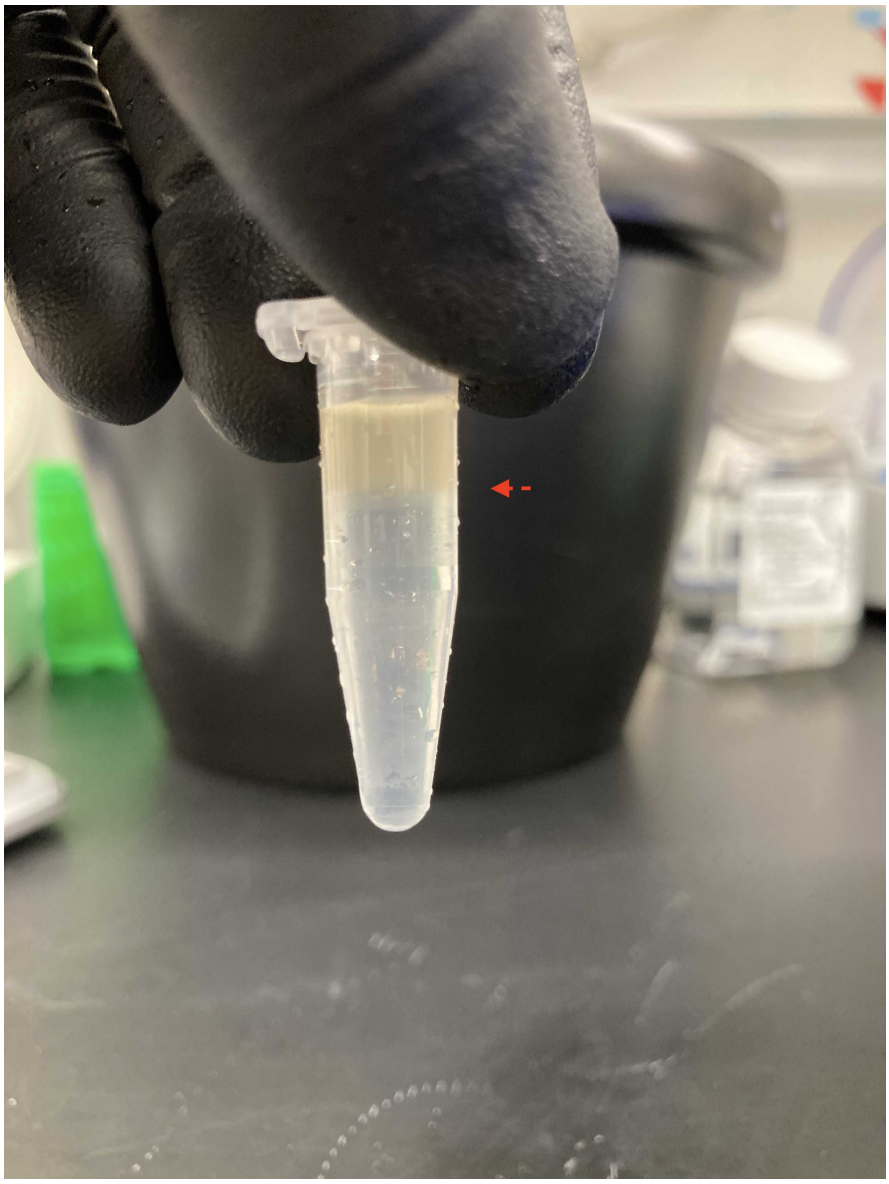

Homogenate after resuspending and underlaying G30. Arrow indicates the separation of both layers.

10 Centrifuge the nuclei at **8000 x g** for **00:20:00** at **4 °C** . After centrifugation the nuclei will form a pellet and 20m
contaminating membranes will float at the top. This continuous gradient can be applied for as many times as needed to the same prep to continue cleaning up your nuclei suspensions (Optional Step 11).

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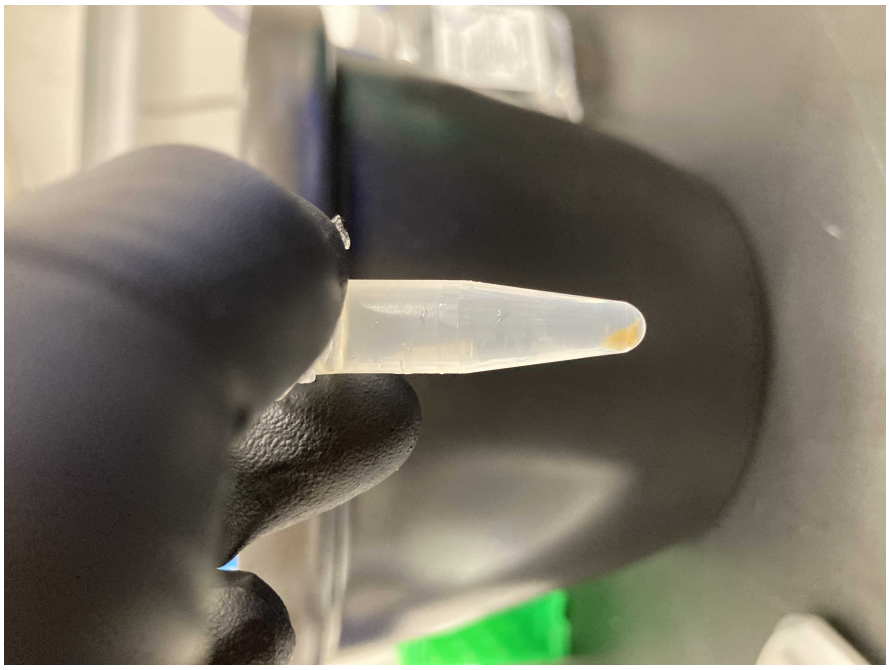

Clean pelleted nuclei.

11 Optional: repeat steps 8-10) for additional cleaning.

Nuclei Wash and Resuspension 10m

12 ⚠ 5m

After centrifugation, remove supernatant and add **1 mL** of ice-cold Nuclei Wash and Resuspension Buffer supplemented (supplemented with RNAse Inhibitor 0.2-0.5 U/uL) **without resuspending the nuclei.** Then centrifuge the nuclei at **500 x g** for **00:05:00** at **4 °C** .

13 Remove supernatant as much as possible without disturbing pellet (if pellet looks loose leave ~ **50 µl** behind) and resuspend the pellet in **1 mL** of ice-cold Nuclei Wash and Resuspension Buffer supplemented (supplemented with RNAse Inhibitor 0.2-0.5 U/uL). Then centrifuge the nuclei at **500 x g, 00:05:00** for **00:05:00** at **4 °C** . 5m

14 Remove supernatant and resuspend with **300 µl** of ice-cold Nuclei Wash and Resuspension Buffer (supplemented with RNAse Inhibitor 0.2-0.5 U/uL) and filter using 40-um Flowmi® Cell Strainer and transfer to a new LoBind 1.5 mL Eppendorf tube.

15 ⚠

Check integrity and purity under microscope and count manually or using an automatic counter. For automatic counter I recommend LUNA-FL™ Dual Fluorescence Cell Counter and Acridine Orange/Propidium Iodide (AO/PI) Cell Viability Kit

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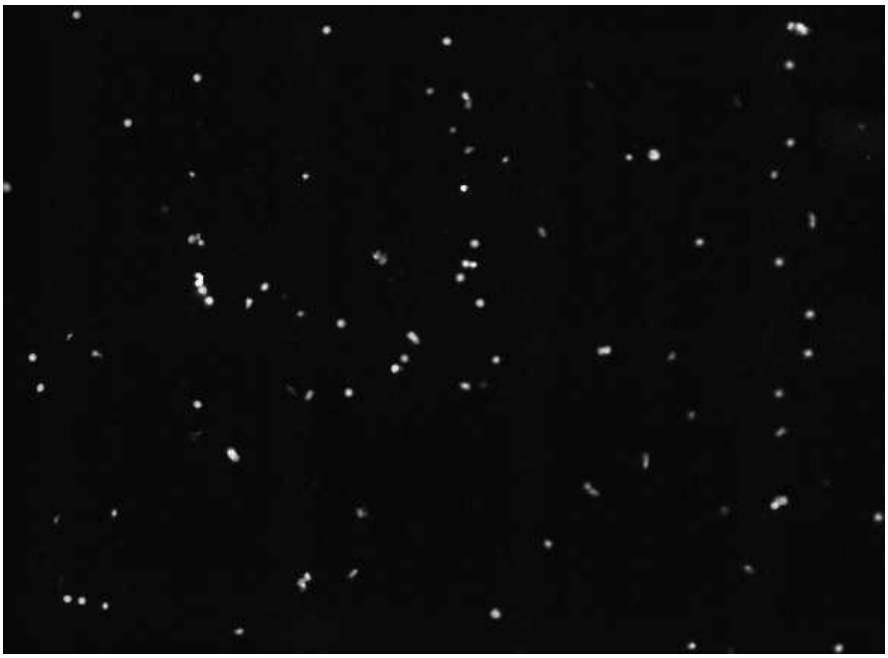
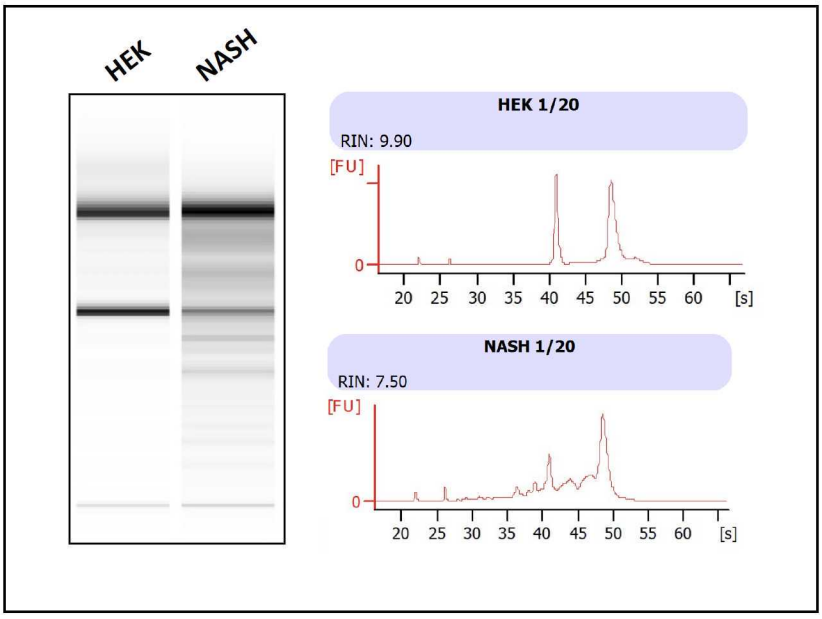


DAPI-stained nuclei post-clean up.



BioA traces of total RNA rom HEK293 cell line and RNA extracted from a representative nuclei suspension; in this case liver as an example. This sample was successfully run on the 10x Genomics Chromium using the 3' v3.1 Dual Index kit.

16 Proceed to your amazing snRNA-Seq experiment!

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