Version 2

Jan 17, 2021

S.O.F protocol for nuclei isolation from fresh and frozen tissues using OptiPrep® discontinuous gradient V.2

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1 Works for me dx.doi.org/10.17504/protocols.io.brizm4f6

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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 (S.O.F means Son 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® discontinuous gradient to remove debris. It is the alternative protocol when FACS is not available.

EXTERNAL LINK

https://research.unimelb.edu.au/centre-for-cancer-research/our-research/single-cell-innovation-lab

ATTACHMENTS

___Frankenstein____protocol_for_nuclei_isolation_from_fresh_and_frozen_tissue_FINAL.pdf

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PROTOCOL CITATION

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Version created by Luciano Martelotto

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WHAT'S NEW

This version contains a correction in buffer G4, the rest is the same.

KEYWORDS

snRNAseq, 10x, nuclei, isolation, FACS

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ATTACHMENTS

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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 4°C)

2. Nuclei wash and resuspension buffer (prepare fresh, keep 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

G1: OptiPrep®: 60% (w/v) solution of iodixanol in water.

G2: 150 mM KCl, 30 mM MgCl2, 120 mM, Tricine-KOH pH 7.8

G3: solution containing 50% (w/v) of iodixanol --> mix 5 volumes of G1 with 1 volume of solution G2 (final: 50% iodixanol, 25 mM KCl, 5 mM MgCl2, 20 mM Tricine-KOH pH 7.8).

G4: 0.25 M Sucrose, 25 mM KCl, 5 mM MgCl2, 20 mM Tricine-KOH pH 7.8.

G5: solution containing 25% (w/v) of iodixanol --> mix equal volumes of G3 and G4 (final: 25% iodixanol, 25 mM KCl, 5 mM MgCl2, 20 mM Tricine-KOH pH 7.8).

G6: solutions of 30% --> mix 6 volumes of G3 with 4 volumes of G4 (final: 30% iodixanol, 25 mM KCl, 5 mM MgCl2, 20 mM Tricine-KOH pH 7.8).

G7: solutions of 35% --> mix 7 volumes of G3 with 3 volumes of G4 (final: 35% iodixanol, 25 mM KCl, 5 mM MgCl2, 20 mM Tricine-KOH pH 7.8).

4. Stock solutions and G2/G4 Set-Up

Keep the following Stock Solutions at 4°C:

-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

G2: 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.

G4: 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.

SAFETY WARNINGS

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

DISCLAIMER:

DISCLAIMER – FOR INFORMATIONAL PURPOSES ONLY; USE AT YOUR OWN RISK

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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 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 tube on ice at all times.

Nuclei Isolation 10m

4 Add an extra **900 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. RNAse inhibitor will now be in the range of 0.2-0.5 U/uL.

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

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pluriStrainer Mini 70 µm
Cell Strainer
pluriSelect 43-10070-40

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** .

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

8 Remove supernatant as much as possible without disturbing pellet (if pellet looks loose leave ~ **50 µl** behind) and add **1 mL** of Nuclei Wash and Resuspension Buffer (supplemented with RNAse Inhibitor 0.2-0.5 U/uL) **without resuspending**.

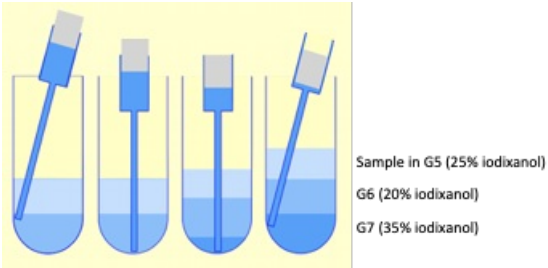
9 Centrifuge the nuclei at **500 x g** for **00:05:00** at **4 °C** .

10 Optional: Remove supernatant as much as possible without disturbing pellet (if pellet looks loose leave ~ **50 µl** behind) and add **500 µl** of Nuclei Wash and Resuspension Buffer (supplemented with RNAse Inhibitor 0.2-0.5 U/uL) **without resuspending**. Centrifuge the nuclei at **500 x g** for **00:05:00** at **4 °C** . 5m

11 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 G5 buffer and transfer onto a 10 mL LoBind centrifuge tube. Then add another mL of G5 (final volume 2 mL).

Debris Removal 10m

12 Carefully, underlayer the sample with **2 mL** of the 30% iodixanol and then **2 mL** of the 35% iodixanol, as shown in the picture below.



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13 Centrifuge the nuclei at **5000 x g** for **00:20:00** at **4 °C** using swinging bucket centrifuge with break set to OFF. (Note: using **10000 x g** is also possible). 20m

14 After centrifugation, a white-ish band of nuclei between the 30%-35% iodixanol interface should be visible. Aspirate the top layers down until the white nuclei band at the interphase of 29%-35%. Using 200 uL tip, collect the nuclei band and transfer to a fresh 1.5 mL LoBind tube.

15 Then top up to ~1.3 mL with Wash and Resuspension Buffer (supplemented with RNAse Inhibitor 0.2-0.5 U/uL), mix well but gently, and centrifuge the nuclei at **500 x g** for **00:05:00** at **4 °C** . 5m

16 Remove supernatant and repeat once more for a total of 2 washes.

17 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 (F23001).

18 Proceed to your amazing snRNA-Seq experiment!

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