Version 4

Mar 02, 2021

S.O.F protocol for nuclei isolation from fresh and frozen tissues using OptiPrep® discontinuous gradient V.4

Luciano G Martelotto[1]

[1]Harvard Medical School

1 Works for me dx.doi.org/10.17504/protocols.io.bsxhnfj6

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

Luciano Martelotto
Harvard Medical School

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 (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://singlecellcore.hms.harvard.edu/

DOI

dx.doi.org/10.17504/protocols.io.bsxhnfj6

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PROTOCOL CITATION

Luciano G Martelotto 2021. S.O.F protocol for nuclei isolation from fresh and frozen tissues using OptiPrep® discontinuous gradient . **protocols.io**
https://dx.doi.org/10.17504/protocols.io.bsxhnfj6
Version created by Luciano Martelotto

KEYWORDS

snRNAseq, 10x, nuclei, isolation, FACS

CREATED

Mar 02, 2021

LAST MODIFIED

Mar 02, 2021

PROTOCOL INTEGER ID

47817

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).

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 10m and incubate for **00:05:00** on ice. Then centrifuge the nuclei at **500 x g** for **00:05:00** at **4 °C** .

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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**.

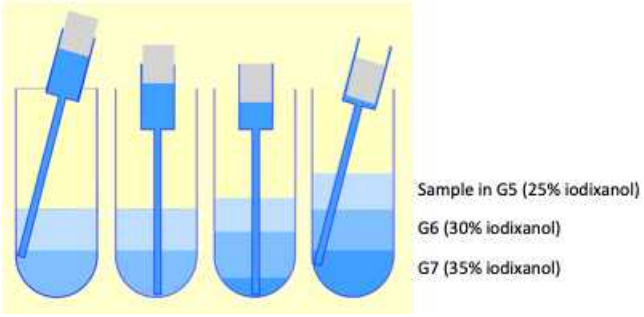
9 Centrifuge the nuclei at **500 x g** for **00:05:00** at **4 °C** .

10 5m
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** .

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.



Layering the gradient. Image adapted from *OptiPrep™ Application Sheet S10*.

13 20m
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).

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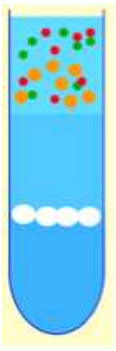

Position on nuclei pellet. Image adapted from *OptiPrep™ Application Sheet S10*.

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[5m] well but gently, and centrifuge the nuclei at **500 x g** for **00:05:00** at **4 °C** .

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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