Version 5

Sep 13, 2021

Protocol for nuclei isolation from fresh and frozen tissues using Salty-Ez10 or Salty-Ez50 buffer: compatible with snRNA-Seq and Multiome workflows from 10x Genomics V.5

Luciano G Martelotto[1]

[1]Harvard Medical School

In Development Share

dx.doi.org/10.17504/protocols.io.bx64prgw

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

1 more workspace

Luciano Martelotto
Harvard Medical School

DISCLAIMER

ABSTRACT

This is a protocol in development, which means it has not yet tested/challenged with multiple samples. So, please make sure you take it for a test drive before committing to it. Once you do please share your experience with me via email, Twitter or as a comment.

In this version I labelled DTT as **optional** since after further testing I have not seen any difference with or without in snRNA-Seq workflow. Also, I recommend WRB1 for snRNA-Seq workflow only (although still works fine for Multiome) and WRB2 for both snRNA-Seq and Multiome workflows. Since WRB2 is 10x Genomics' recommendation for Multiome I have adopted this for this workflow.

I include a discussion about cycling during cDNA amp.

SaltyEz50 option: this has been particularly useful for samples where lysis in SaltyEz10 showed to be suboptimal. In my hands, those were breast, liver and pancreas.

DOI

dx.doi.org/10.17504/protocols.io.bx64prgw

EXTERNAL LINK

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

Citation: Luciano G Martelotto (09/13/2021). Protocol for nuclei isolation from fresh and frozen tissues using Salty-Ez10 or Salty-Ez50 buffer: compatible with snRNA-Seq and Multiome workflows from 10x Genomics. https://dx.doi.org/10.17504/protocols.io.bx64prgw

PROTOCOL CITATION

Luciano G Martelotto 2021. Protocol for nuclei isolation from fresh and frozen tissues using Salty-Ez10 or Salty-Ez50 buffer: compatible with snRNA-Seq and Multiome workflows from 10x Genomics. **protocols.io** https://dx.doi.org/10.17504/protocols.io.bx64prgw
Version created by Luciano Martelotto

WHAT'S NEW

Salty-Ez50 as an alternative when lysis is incomplete in Salty-Ez10.

KEYWORDS

snRNAseq, 10x, nuclei, isolation, FACS

CREATED

Sep 13, 2021

LAST MODIFIED

Sep 13, 2021

PROTOCOL INTEGER ID

53180

GUIDELINES

Use RNA techniques.

The use of RNAse Inhibitor is HIGHLY recommended.

MATERIALS TEXT

Required Buffers and Reagents

1. Salty-Ez10 or Salty-Ez50 Lysis Buffer

-10 mM Tris-HCl pH 7.5
-146 mM NaCl
-1 mM CaCl2
-21 mM MgCl2
-0.03% Tween-20 (Sigma Aldrich, P9416-50ML)
-0.01% BSA (Miltenyi, 130-091-376)
-1 mM DTT (Thermo)
-10% Ez Lysis Buffer **OR** 50% Ez Lysis Buffer (Sigma Aldrich)
-0.2-1 U/uL Protector RNAse Inhibitor (**yes, use this one!**)

2. Wash and Resuspension Buffers

Wash and Resuspension Buffer 1 (WRB1) for snRNA-Seq workflow

-1x PBS
-1% BSA (Molecular Grade)
-1 mM DTT (ThermoScientifics) (*Optional for snRNAseq workflow)
-0.2-1 U/uL Protector RNAse Inhibitor

Wash and Resuspension Buffer 2 (WRB2) snRNA-Seq and Multiome workflow

-10 mM Tris-HCl pH 7.5
-10 mM NaCl
-3 mM MgCl2
-1% BSA
-1 mM DTT (ThermoScientifics) (*Optional for snRNAseq worflow. I include it for Multiome)
-0.2-1 U/uL Protector RNAse Inhibitor

IMPORTANT: for Multiome or ATAC kits (10x Genomics) supplement WRB2 with 0.01% Digitonin (Thermo, BN2006) and 0.1% Tween-20.

***After further tests, I have not seen any difference with and without DTT in snRNAseq workflows, hence we consider this component optional. However, for Multiome I recommend it.**

For cell counting: Acridine Orange/Propidium Iodide (AO/PI) Cell Viability Kit (F23001)

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:

Citation: Luciano G Martelotto (09/13/2021). Protocol for nuclei isolation from fresh and frozen tissues using Salty-Ez10 or Salty-Ez50 buffer: compatible with snRNA-Seq and Multiome workflows from 10x Genomics. https://dx.doi.org/10.17504/protocols.io.bx64prgw

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 Salty-Ez10 or Salty-Ez50 Lysis Buffer (supplemented with RNAse Inhibitor 0.2-0.5 U/uL) to the tissue in 1.5 mL tube.

SaltyEz50 option has been particularly useful for samples where lysis in SaltyEz10 showed to be suboptimal. In my hands, those were breast, liver and pancreas. I recommend to check which one works best for your sample type. If trial is not possible I recommend SaltyEz50.

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 Salty-Ez10 or Salty-Ez50 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

Lysis Optimization can be done using the Luna-FL™Dual Fluorescence Cell Counter (this is the best automatic counter to me and makes Countess look like a Random Number Generator!) and by taking aliquots at different times until you get less than 5% viability using Acridine Orange/PI dual staining.

Nuclei Isolation 10m

5 Filter homogenate using a 70 µm-strainer mesh to fit a **pre-cooled** 15 ml Falcon tube (e.g. pluriStrainer Mini 70 µm Cell Strainer). This step is to remove undigested tissue or fat prior to centrifugation.

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** (these 5' count as lysis time too!). Remove supernatant leaving behind ~ **50 µl** if the pellet seems loose.

Citation: Luciano G Martelotto (09/13/2021). Protocol for nuclei isolation from fresh and frozen tissues using Salty-Ez10 or Salty-Ez50 buffer: compatible with snRNA-Seq and Multiome workflows from 10x Genomics. https://dx.doi.org/10.17504/protocols.io.bx64prgw

7 10m

Optional: add **1 mL** of Salty-Ez10 or Salty-Ez50 Lysis Buffer (supplemented with RNAse Inhibitor 0.2-if pellet is loose0.5 U/uL), gently resuspend pellet (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** .

Nuclei Wash and Resuspension 10m

8 ⚠

After removing the supernatant, add **500 µl** of WRB1 (supplemented with RNAse Inhibitor 0.2-0.5 U/uL) **without disturbing the pellet**. Let sit for 5' on ice and then gently resuspend the pellet (this incubation is important to avoid clumping).

--> ALTERNATIVELY, resuspend the pellet in WRB2 (supplemented with RNAse Inhibitor 0.2-0.5 U/uL). The 5' incubation is optional for this buffer. **WRB2 is my preferred choice when we don't use OptiPrep for cleaning up nucs (like in case of cell lines which produce very little debris, if any) because for some samples I have seen nucs clumping when resuspended directly in WRB1 directly after Salty-Ez10.** If you do OptiPrep then either WRB is fine.

Warning: some nucs preps clump when you add WRB1 (this is a known phenomenon with Tris to PBS enchange, hence 5' on ice helps but not always! It's sample dependent). In that case use WRB2. Both WRBs are compatible with all 10x kits.

Important: for 10x Multiome (10x Genomics) add 0.01% Digitonin in the WRB buffers (supplemented with RNAse Inhibitor 0.2-0.5 U/uL and 0.1% Tween-20), not during lysis step itself. In this way the time the nuclei is leaky (due to holes caused by Digitonin) is shorter, 5' on ice is enough to permeabilize nuclei. **Then replace it by the 1x Nuclei Buffer (NB) as indicated in the user guide** (this buffer is essential for ATAC or Multiome kit).

9 If cell debris and large clumps are observed, pass through a cell strainer. For low volume, use a 40 µm Flowmi Cell Strainer to minimize volume loss.

If high amounts of debris or micelles (very round droplets of fat, see attached photo) is present you may perform OptiPrep® ***continuous*** gradient as done in DOF (dx.doi.org/10.17504/protocols.io.bs99nh96, steps 9-12). Use WRBs for washes and resuspension for snRNA-Seq. For Multiome use WRBs for washes and resuspension 1x NB for resuspension. Pass through a cell strainer (e.g., 40 µm Flowmi Cell Strainer).

10 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. This instrument also provides information about size and multiplets which is very useful.

Citation: Luciano G Martelotto (09/13/2021). Protocol for nuclei isolation from fresh and frozen tissues using Salty-Ez10 or Salty-Ez50 buffer: compatible with snRNA-Seq and Multiome workflows from 10x Genomics. https://dx.doi.org/10.17504/protocols.io.bx64prgw

11 Proceed to your amazing snRNA-Seq or Multiome experiment!

Note on cycle number for cDNA amplification

There is not a rule of thumb for what how many extra cycles one need to add to get sufficient material for library prep. 10x Genomics indicates that one could make a library with just 1 ng, which I tested and it does, but we have to ramp up the cycles during SI-PCR. However, the libraries are less complex (less UMI and genes per nucs) and saturation is reached early on. Arguably, I prefer to have an input of >20 ng when possible so for that I add at least 2-4 cycles to start with (note the range of cells input is quite wide for cell input, e.g. 200-6000 --> 12 cycles for 3' v3.1 and 2000-6000 --> 14 cycles for low RNA content cells in 5' v2). Data so far has been great with significantly complex libraries and not nearly as saturated in 3' v3.1 chemistry. For 5' v2 chemistry, we have used the same approach. It is indeed challenging to determine then number of cycles to use for samples of unknown cell composition. Since 10x Genomics kits do not come with enough reagents to run extra cycles in case you need to increase the yield, I rather do a few more cycles upfront and reduce the cycling at SI-PCR. If you have any suggestions or comment I am welcome to hear about it.

Quoting 10x Genomics: *"The optimal number of cycles is a trade-off between generating sufficient final mass for library construction and minimizing PCR amplification artifacts."*

Interesting, for Multiome kit the total number of cycles for the RNA workflow ends up being 4 cycles more than standard snNA-Seq alone, e.g. 7 cycles pre-amp + 9 cDNA amp (16) for 2000 nucs versus 12 for standard scRNA-Seq (whole cell recommendation) so it seems 4 cycles more is within the range 10x Genomics recommends.

Citation: Luciano G Martelotto (09/13/2021). Protocol for nuclei isolation from fresh and frozen tissues using Salty-Ez10 or Salty-Ez50 buffer: compatible with snRNA-Seq and Multiome workflows from 10x Genomics. https://dx.doi.org/10.17504/protocols.io.bx64prgw