



Version 4

Aug 07, 2021

Protocol for nuclei isolation from fresh and frozen tissues using Salty-Ez10 buffer: compatible with snRNA-Seq and Multiome workflows from 10x Genomics V.4

Luciano G Martelotto¹¹Harvard Medical School

In Development



Share

dx.doi.org/10.17504/protocols.io.bw6qphdw

Human Cell Atlas Method Development Community

Single Cell Core, Harvard Medical School

1 more workspace



Luciano Martelotto
Harvard Medical School

DISCLAIMER

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

The protocol content here is for informational purposes only and does not constitute legal, medical, clinical, or safety advice, or otherwise; content added to protocols.io is not peer reviewed and may not have undergone a formal approval of any kind. Information presented in this protocol should not substitute for independent professional judgment, advice, diagnosis, or treatment. Any action you take or refrain from taking using or relying upon the information presented here is strictly at your own risk. You agree that neither the Company nor any of the authors, contributors, administrators, or anyone else associated with protocols.io, can be held responsible for your use of the information contained in or linked to this protocol or any of our Sites/Apps and Services.

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.

DOI

dx.doi.org/10.17504/protocols.io.bw6qphdw

EXTERNAL LINK

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


PROTOCOL CITATION

Luciano G Martelotto 2021. Protocol for nuclei isolation from fresh and frozen tissues using Salty-Ez10 buffer: compatible with snRNA-Seq and Multiome workflows from 10x Genomics. **protocols.io**
<https://dx.doi.org/10.17504/protocols.io.bw6qphdw>
Version created by Luciano Martelotto

KEYWORDS

snRNAseq, 10x, nuclei, isolation, FACS

LICENSE

 This is an open access protocol distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

CREATED

Aug 05, 2021

LAST MODIFIED

Aug 07, 2021

PROTOCOL INTEGER ID

52144

GUIDELINES

Use RNA techniques.

The use of RNase Inhibitor is HIGHLY recommended.

MATERIALS TEXT

Required Buffers and Reagents

1. Salty-Ez10 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 (Miltényi, 130-091-376)
- 1 mM DTT (Thermo)
- 10% 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 workflow. 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 MgCl₂·6H₂O: 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:

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

The protocol content here is for informational purposes only and does not constitute legal, medical, clinical, or safety advice, or otherwise; content added to protocols.io is not peer reviewed and may not have undergone a formal approval of any kind. Information presented in this protocol should not substitute for independent professional judgment, advice, diagnosis, or treatment. Any action you take or refrain from taking using or relying upon the information presented here is strictly at your own risk. You agree that neither the Company nor any of the authors, contributors, administrators, or anyone else associated with protocols.io, can be held responsible for your use of the information contained in or linked to this protocol or any of our Sites/Apps and Services.

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 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 Salty-Ez10 Lysis Buffer (supplemented with RNase Inhibitor 0.2-0.5 U/uL), mix gently^{5m} by pipetting using **wide-bore tips** and incubate on ice for  **00:05:00**. Repeat mixing 2-3 times during the incubation.

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 through 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.
- 7 **Optional:** add  **1 mL** of Salty-Ez10 Lysis Buffer (supplemented with RNase Inhibitor 0.2-0.5 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**.^{10m}

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 exchange, 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.

- 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 snRNA-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.