SEP 18, 2023

OPEN ACCESS

DOI:
dx.doi.org/10.17504/protocols.io.kxygx3wmzg8j/v1

Protocol Citation: Sébastien Vigneau, Yashika_Rustagi 2023. 10X Multiome TST Nuclei Isolation. **protocols.io** https://dx.doi.org/10.17504/protocols.io.kxygx3wmzg8j/v1

Protocol status: Working
We use this protocol and it's working

Created: Sep 08, 2023

Last Modified: Sep 18, 2023

10X Multiome TST Nuclei Isolation

Sébastien Vigneau[1], Yashika_Rustagi[1]

[1]Dana-Farber Cancer Institute

HTAN DFCI/Broad/MGH

Allison Frangieh

ABSTRACT

This protocol describes the process of nuclei isolation from frozen tissue. The protocol has been slightly modified from the *Nature Medicine* RNA-Seq toolbox manuscript (Slyper et al., 2020) and applied to frozen melanoma and liver metastases for the Human Tumor Atlas Network (HTAN) single-nuclei RNA-seq Trans-Network Project (snRNAseq TNP).

MATERIALS

Preparation Materials

- 2 - 30µM filters
- 2+ Hemocytometers
- Scalpel
- 2 Paper rulers
- Spring scissors
- Forceps
- Petri dishes

Materials

	Name	Catalogue #	Vendor
	20x Nuclei Buffer*	2000153/2000207	10X Genomics
	Digitonin	BN2006	Thermo Fisher
	Trizma Hydrochloride Solution, pH 7.4	T2194	Sigma-Aldrich
	Sodium Chloride Solution 5M**	AM9759	Thermo Fisher
	Magnesium Chloride Solution, 1M	M1028	Sigma-Aldrich
	Calcium Chloride Solution 1M – 100 mL	21115	Sigma-Aldrich
	UltraPure Tris HCl, pH 7.5 – 1000mL	15567-027	Life Technologies
	Nonidet P40 Substitute	74385	Sigma-Aldrich

PROTOCOL integer ID:
87580

	Name	Catalogue #	Vendor
	Sigma Protector RNAse Inhibitor	335402001	Sigma-Aldrich
	DTT	646563	Sigma-Aldrich
	BSA (20mg/mL)	B9000S	New England BioLabs
	MACS BSA Stock Solution	130-091-376	Miltenyi Biotec
	MACS SmartStrainers (30um)	130-098-458	Miltenyi Biotec
	Tween-20***	1662404	Bio-Rad

*Provided in 10X Multiome Kit

History:
** Previously used: Sodium Chloride Solution 5M Sigma-Aldrich (59222C)
***Previously used: TWEEN-20 from Sigma-Aldrich (P7949)

PREPARATION

1 Clean off bench space with RNAse away, then 70% EtOH.

2 Set swing-bucket **centrifuge to 4°C.**

3 Prepare tissue culture hood by turning off UV light, opening the sash, and allowing for the 3-minute ventilation. Clean off work area with 70% EtOH and check for any supplies needed in the hood (i.e. Tips, tubes, serological tips, etc.).

4 Gather materials.

5 Clean spring scissors and forceps with 70% EtOH. Leave on Kim wipe to dry.

6 If working with OCT,prepare ice bucket with enzyme blocks and at least 50 mL PBS.

7 **Take out 10x Reagents.** Obtain the 20x Nuclei Buffer and ATAC Buffer from the 10x MO ATAC Kit in -20°. Obtain gel beads and Template Switch Oligo from -80°. Keep enzymes in freezer in kit until it is time to load.

7.1 See Materials section, Preparation Materials

Buffer Aliquot Preparation

8 Obtain aliquots from -20°C. Let thaw for approximately 20-30 minutes. Mix with P1000 once the last reagent is added. DO NOT vortex buffers. Once prepared, *keep all buffers on ice.* Always add Protective RNAse Inhibitor (PRI) last. DO NOT vortex PRI. See *Appendix A* for stock buffers. See *Appendix B* if making buffers the day of nuclei isolation.

8.1 **1X ST**

1. Obtain **1X ST buffer (w/o PRI)** aliquot of 2.925mL.
2. Prior to use add **75 µL of Protective RNAse Inihibitor.**
3. Keep on ice.

8.2 TST

1. Obtain **TST buffer (w/o BSA, PRI)** aliquot of 1.94mL.
2. Prior to use add **10 µL of 20mg/mL BSA.**
3. Add **50µL of Protective RNAse Inhibitor.**
4. Keep on ice.

8.3 Lysis Buffer

1. Obtain **Lysis Buffer (w/o DTT, BSA PRI)** aliquot of 874µL.
2. Prior to use add **1µL of DTT**.
3. **Aliquot 87.5µL** into a new tube.
4. Add **10µL of 10% BSA** to the tube from step 3.
5. Add **2.5µL of Protective RNAse Inhibitor** to the tube from step 3.
6. Keep on ice.

8.4 Wash Buffer

1. Obtain **Wash Buffer (w/o DTT, BSA, PRI)** aliquot of 874µL.
2. Prior to use add **1µL of DTT**
3. Add **100µL of 10% BSA**
4. Add **25µL Protector RNAse Inhibitor.**
5. Keep on Ice.

8.5 Diluted Nuclei Buffer

1. Obtain 20x Nuclei Buffer from 10x MO ATAC kit. Vortex and spin down the 20x Nuclei Buffer. Prepare the Diluted Nuclei Buffer using the following table:

	Reagent	Volume
	20x Nuclei Buffer (10x Kit)	50 µL
	DTT	1 µL
	UltraPure H2O	924 µL
	Protector RNAse Inhibitor	25 µL

2. Keep on ice.

MECHANICAL DISSOCIATION

9 *Avoid thawing tissue before dissociation. If tissue is large enough for excess, cut into smaller fragments about the size of a grain of rice on ice. Note percentage of tissue used, and properly store remaining tissue in original storage case (cassette, tube, etc.)*

10 **OCT Block:**

1. Place OCT block on dry petri dish on dry ice. Using a scalpel, scrape away thin pieces of OCT using the “parmesan cheese” method . Stop using the “parmesan cheese” method when the tissue is more visible through the OCT. Be careful not to cut away any tissue.
2. Add tissue to petri dish with ~at least 50 mL of **cold PBS** on ice (enough to submerge sample) and carefully pull away the remaining OCT using two forceps.
3. Transfer tissue to 1.5 mL tube on **wet ice**. Add **1 mL** of **TST buffer** and chop on ice with spring scissors for **10 minutes**. *Note: After 3-5 minutes, suspension may be homogenized enough to instead pipette with a P1000 for the remainder of the 10 minutes.*

11 **Cryoprep - (See COVARIS cryoPREP Protocol)**

1. Transfer 1mL of TST Buffer with pulverized tissue from tissue pouch into 1.5 mL tube on wet ice. Check pulverization quality to move on to chopping or pipetting.
2. Chop on ice with spring scissors for 5 minutes. *Note: After ~3 minutes, suspension may be homogenized enough to instead pipette with a P1000 for the remainder of the 5 minutes.*

FILTRATION

12 Attach a **30 µm filter** to a 15 mL Falcon tube on **wet ice**. Pass the homogenized ~1 mL nuclei suspension of nuclei through the filter.

13 Wash the previously used 1.5 mL tube with **an additional 1 mL of TST buffer,** then pass through the same filter.

14 Wash the filter with **3 mL** of **1X ST buffer.** The total volume of the suspension should be about 5 mL. Pull the suspension through the filter using a pipette if necessary.

15 Centrifuge for **10 minutes** at **500 g** at **4°C**. Set the stop break to 5.

16 Carefully aspirate the ~5 mL of supernatant to a new 15 mL Falcon tube on **wet ice**.

NUCLEI PERMEABILIZATION

17 Resuspend nuclei pellet in **100 µL** of **Lysis Buffer**. Pipette mix.

18 Incubate for **2 minutes** on **wet ice**.

19 Add **1 mL** of **Wash Buffer** and pipette mix.

20 Centrifuge for **10 minutes** at **500g** at **4°C.**

21 Remove supernatant. Keep supernatant in new tube on wet ice.

22 Resuspend pellet in **100-150 µL** of chilled **Diluted Nuclei Buffer**. Keep on **wet ice**.

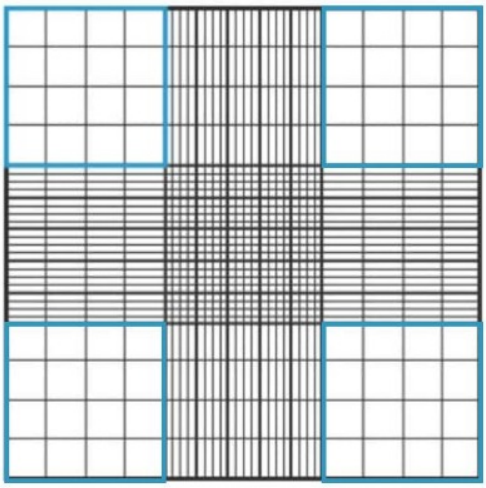
NUCLEI QUALITY ASSESSMENT AND COUNTING

23 Count nuclei and assess quality using a hemocytometer. Dilute to desired concentration with Diluted Nuclei Buffer for easier counting, if necessary. Make a 1:2 dilution with Trypan Blue (5 µL stock, 5 µL Trypan Blue) to count.

24 To count nuclei: Count all four corners** and calculate concentration for (nuclei/µL)

$$Concentration\ of\ nuclei\ stock\ = \frac{Total\ nuclei}{4} \times Dilution\ Factor\ \times 10$$

**Counting reference: count the 16 squares in each of the blue sections.



Note: If nuclei concentration is too low for downstream workflow, nuclei can be concentrated by centrifuging for 10 minutes at 500 g at 4°C, removing a fraction of the supernatant and resuspending in the remaining supernatant.

25 Using the table below, prepare the nuclei stock for loading.

Targeted Nuclei Recovery	Nuclei Stock Concentration (nuclei/µl)
500	160-400
1,000	320-810
2,000	650-1,610
3,000	970-2,420
4,000	1,290-3,230
5,000	1,610-4,030
6,000	1,940-4,840
7,000	2,260-5,650
8,000	2,580-6,450
9,000	2,900-7,260
10,000	3,230-8,060

LOADING THE CONTROLLER

26 Double check with Group Lead and sample sheet to determine how many nuclei to load.

27 **If stock concentration is higher,** create a dilution using Diluted Nuclei Buffer for optimal loading concentration.

28 **If stock concentration is lower**, assess volume needed to load optimal number/minimal number of nuclei. If needed, stock can be spun down and resuspended in Diluted Nuclei Buffer at a lower volume to create higher concentration for loading.

29 When creating dilution, always use **at least 5 µL** of nuclei stock to ensure accurate pipetting. If dilution factor is higher than 25, create a serial dilution.

29.1 *Example of dilution calculation:*

Concentration of stock: 60,000 nuclei/µL
Need a 1:12 dilution to get to 5,000 nuclei/µL
Create a 1:3 dilution from stock
5 µL of stock with 10 µL of Diluted Nuclei Buffer
Left with 20,000 nuclei/µL
From the 1:4 dilution, create a 1:4 dilution
5 µL of 1:3 dilution with 15 µL of Diluted Nuclei Buffer
Left with 5,000 nuclei/µL

30 **Once sample is prepared to be loaded, perform another quality assessment**. Using a new 1.5 mL tube, add 5µL of loading sample to 5 µL of Trypan blue. Using a new hemocytometer, count the Trypan-stained sample. This calculation is to make sure the loading sample is the correct concentration. Use the calculation below to determine the concentration of the loading sample prior to loading it on the controller:

$$\frac{Total\ nulcei\ count}{4} \times 2 \times 10 = concentration\ (nuclei/\mu L)\ of\ loading\ sample$$

31 Proceed to transposition steps as outlined in "Chromium Next GEM Single Cell Multiome ATAC + Gene Expression" User Guide (CG000338).

Appendix A: BUFFER STOCK PREPARATION

32 *Prepare the necessary buffers and solutions as outlined below for both nuclei isolation and permeabilization steps:*

32.1 **2X ST** Buffer Stock. (Can be stored at 4°C for up to a month.)

Reagent	Stock Concentration	2X ST Buffer Concentration	Volume
NaCl	5M	292 mM	**2.92 mL**
Tris (pH 7.5)	1M	20 mM	**1 mL**
$CaCl_2$	1M	2 mM	**0.1 mL**
$MgCl_2$	1M	42 mM	**2.1 mL**
UltraPure H_2O	-	-	**43.88 mL**
Total			50 mL

32.2 **1X ST** Buffer Stock. (w/o Protective RNAse Inhibitor).
Prep aliquots of **2.925mL.** Store at -20°C.

Reagent	Stock Concentration	1X ST Buffer Concentration	Volume
2X ST	2X	1X	**25 mL**
UltraPure H2O			**23.75 mL**
Protector RNAse Inhibitor	40 U/µL	1 U/µL	~~1.25 mL~~
Total			50 mL

32.3 **TST** Buffer Stock. (w/o Protective RNAse Inhibitor and BSA).
Prep aliquots of **1.94mL**. Store at -20°C.

Reagent	Stock Concentration	TST Buffer Concentration	Volume (mL)
2X ST	2X	1X	**25 mL**
Tween 20	10%	0.3%	**1.5 mL**
UltraPure H2O			**22 mL**
BSA (20mg/mL)	20 mg/mL	0.1 mg/mL	~~0.25 mL~~
Protector RNAse Inhibitor	40 U/µL	1 U/µL	~~1.25 mL~~
Total			50 mL

32.4 **Lysis Buffer** Stock. (w/o Protective RNAse Inhibitor, BSA, and DTT).

Before preparing the remaining buffers, place the digitonin on the thermomixer at **95°C for 5**

minutes at 1500 rpm.

Dilute **NP-40 substitute** to a **10%** solution before adding to Lysis Buffer.

Prep aliquots of **874µL**. Store at -20°C.

Reagent	Stock Concentration	Lysis Buffer Concentration	Volume
Tris-HCl (pH 7.4)	1 M	10 mM	**500 µL**
NaCl	5 M	10mM	**100 µL**
MgCl2	1 M	3 mM	**150 µL**
Tween 20	10%	0.01%	**50 µL**
NP-40 Substitute	10%	0.01%	**50 µL**
Digitonin	5%	0.001%	**10 µL**
UltraPure H2O			**42.840 mL**
DTT	1 M	1 mM	~~50 µL~~
BSA (10%)	10%	1%	~~5000 µL~~
Protector RNAse Inhibitor	40 U/µL	1 U/µL	~~1250 µL~~
	Total		50

32.5 **Wash Buffer** Stock. (w/o Protective RNAse Inhibitor, BSA, and DTT).

Prep aliquots of **874µL.** Store at -20°C.

Reagent	Stock Concentration	Wash Buffer Concentration	Volume
Tris-HCl (pH 7.4)	1 M	10 mM	**500 µL**
NaCl	5 M	10mM	**100 µL**
MgCl2	1 M	3 mM	**150 µL**
Tween 20	10%	0.1%	**500 µL**
UltraPure H2O			**42.450 mL**
DTT	1 M	1 mM	~~50 µL~~
BSA (10%)	10%	1%	~~5000 µL~~
Protector RNAse Inhibitor	40 U/µL	1 U/µL	~~1250 µL~~
	Total		50

Appendix B: BUFFER PREPARATION

33 *If no stock buffers are available, please use the following tables.*

33.1 **2X ST** Buffer. (Can be stored at 4°C for up to a month)

Reagent	Stock Concentration	2X ST Buffer Concentration	Volume (mL)
NaCl	5M	292 mM	2.92 mL
Tris (pH 7.5)	1M	20 mM	1 mL
$CaCl_2$	1M	2 mM	0.1 mL
$MgCl_2$	1M	42 mM	2.1 mL
UltraPure H_2O	-	-	43.88 mL
Total			50 mL

33.2 **TST Buffer**. Keep on ice.

Reagent	Volume
2X ST stock solution (from step 1)	1000 µL
BSA (20 mg/mL)	10 µL
10% Tween-20	60 µL
UltraPure H_2O	880 µL
Protector RNAse Inhibitor (40 U/µL)	50 µL
Total (per sample)	2000 µL

33.3 **1x ST buffer.** Keep on ice.

Reagent	Volume
2X ST stock solution (from step 1)	1500 µL
UltraPure H_2O	1425 µL
Protector RNAase Inhibitor (40 U/µL)	75 µL
Total (per sample)	3000 µL

33.4 **Lysis Buffer**.

Before preparing the 1X Lysis Buffer, place the Digitonin on the thermomixer at **95°C for 5 minutes at 1500 rpm**

Keep on ice.

Reagent	Final Concentration	Volume
Tris-HCl (pH 7.4)	10 mM	**10 µL**
NaCl	10 mM	**2 µL**
MgCl2	3 mM	**3 µL**
10% Tween-20	0.01%	**1 µL**
Diluted Nonidet P40 Substitute	0.01%	**1 µL**
Digitonin	0.001%	**0.2 µL**
UltraPure H_2O		**856.8 µL**
BSA (10%)	1%	**100 µL**
DTT	1 mM	**1 µL**
Protector RNAse Inhibitor	1 U/µL	**25 µL**
Total (per sample)		**1000 µL**

33.5 **Wash Buffer.** Keep on ice.

Reagent	Stock Concentration	Final Concentration	Volume
Tris-HCl (pH 7.4)	1 M	10 mM	**10 µL**
NaCl	5 M	10 mM	**2 µL**
MgCl2	1 M	3 mM	**3 µL**
BSA (10%)	10%	1%	**100 µL**
10% Tween-20	10%	0.10%	**10 µL**
DTT	1000 mM	1 mM	**1 µL**
UltraPure H_2O	-	-	**835 µL**
Protector RNAse Inhibitor	40 U/µL	1 U/µL	**25 µL**
Total (per sample)			**1000 µL**

33.6 **Diluted Nuclei Buffer.** Keep on ice.

Reagent	Stock Concentration	Final Concentration	Volume
20x Nuclei Buffer (from 10x Kit)	20X	1X	**50 µL**
DTT	1000 mM	1 mM	**1 µL**
UltraPure H_2O	-	-	**924 µL**
Protector RNAse Inhibitor	40 U/µL	1 U/µL	**25 µL**
Total (per sample)			**1000 µL**