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

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TST Nuclei Isolation with GentleMACS - 220301

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ABSTRACT

This protocol describes the process of nuclei isolation from frozen tissue. The protocol has been applied to frozen melanoma, breast, and lung metastases for the Human Tumor Atlas Network (HTAN) single-nuclei RNA-seq preparation.

MATERIALS

Name	Catalog #	Vendor
GentleMACS C Tubes	130-093-237	Miltenyi B
Tween-20	P-7949	Sigma-Aldrich
DNAse/RNAse Free Distilled Water	10977023	Thermo Fisher Scientific
MACS BSA Stock Solution (10%)	130091376	Miltenyi Biotec
Magnesium Chloride Solution for Molecular Biology (1.00 M)	M1028	Sigma-Aldrich
Falcon‰ Cell Strainers - Mesh size: 40um; blue	102095-532	VWR
5M NaCl Solution	AM9759	Thermo Fisher Scientific
UltraPure 1M Tris-HCl Buffer pH 7.5	15567027	Thermo Fisher Scientific
Calcium Chloride 1M Sterile	97062-820	VWR
INCYTO C-Chip Neubauer Improved Disposable Hemocytometers	22-600-100	VWR International Ltd
Falcon‰ 15 mL Conical Centrifuge Tubes	05-527-90	Fisher Scientific
Falcon‰ 50 mL Conical Centrifuge Tubes	14-432-22	Fisher Scientific
Falcon‰ Round-Bottom Polystyrene Test Tubes with Cell Strainer Snap Cap	08-771-23	Fisher Scientific
Protector RNase Inhibitor (40 U/µL)	RNAINH-RO	Roche Diagnostics
PBS (1X, pH 7.4) without Calcium and Magnesium	10010023	Fisher Scientific

1 Buffer Preparation

Prepare the necessary buffers and solutions as outlined below:

2x ST (50 mL stock solution can be prepared ahead of time and stored at room temperature)

Reagent	Stock Concentration	2X ST Buffer Concentration	Volume for 50 mL of 2X ST Buffer
NaCl	5 M	292 mM	2.92 mL
Tris	1 M	20 mM	1 mL
CaCl2	1 M	2 mM	100 µL
MgCl2	1 M	42 mM	2.1 mL

H2O	-	-	43.88 mL

TST (2 mL should be prepared for each tissue sample)
*10% Tween-20 can be prepared ahead of time and stored at 4ºC

Reagent	Volume for 2 mL Working Solution (per Sample)
2x ST stock solution	1000 µL
MACS BSA Stock Solution (10%)	2 µL (0.01% final concentration)
10% Tween-20*	6 µL
H2O	942 µL
Protector RNase Inhibitor (40 U/µL)	50 µL (1 U/µL final concentration)

1x ST (3.5 mL should be prepared for each tissue sample)

Reagent	Volume for 3.5 mL Working Solution (per Sample)
2x ST stock solution	1748 µL
H2O	1664.5 µL
Protector RNase Inhibitor (40 U/µL)	87.5 µL

PBS + BSA (1%) + RNase Inhibitors (1 mL should be prepared for each tissue sample)

Reagent	Volume for 3.5 mL Working Solution (per Sample)
PBS 1X	875 µL
MACS BSA Stock Solution (10%)	100 µL
Protector RNase Inhibitor (40 U/µL)	25 µL

2 Tissue Dissociation

Fill a GentleMACS C tube with **2 mL of TST buffer per sample**. Keep tubes on wet ice.'

Transfer a **~1x1x1 mm** piece of tissue directly into the buffer, making sure that the tissue is not stuck to any walls in the tube. Return tubes to ice immediately after transferring tissue.

- *If the tissue piece is significantly larger than the recommended size, a smaller piece should be cut on dry ice for nuclei isolation. Multiple pieces can be cut for tissue that looks heterogenous.*

Once all tissue pieces are transferred into buffer, invert the tubes with a firm flick.

Tissue should be floating freely in buffer and not caught on any walls of the tube or tube cap.

Secure the C tubes to the GentleMACS Dissociator and run the **m_spleen_01.01** program. After it

completes, repeat the program a second time for a total of two runs on each tissue sample.

3 First Centrifugation

Detach the tubes from the GentleMACS Dissociator.
Incubate the samples on **wet ice for 5 minutes (upside down).**

Centrifuge the tubes for **2 minutes at 500g at 4ºC** to collect the suspension and remove foam.
Set the acceleration to 10 and deceleration to 5.
Immediately following the centrifugation, resuspend the nuclei pellet in the supernatant within the same C tube.

4 First Filtration

Prepare a **15 mL Falcon tube on wet ice** with a a 40 um Falcon Cell Strainer. Wash the filter with **1 mL of 1x ST buffer with RNase inhibitors.**

Transfer the homogenized ~2 mL suspension of nuclei into the filter.

Wash the used C tube with **1 mL of 1x ST buffer with RNase inhibitors**, then transfer into the filter. Wash the filter with an **additional 1 mL of 1x ST with RNase inhibitors.**
The total volume of suspension should be about 5 mL.

5 Second Centrifugation

Centrifuge in a swinging bucket rotor for **10 minutes at 500 g at 4ºC.**
The longer centrifugation helps recover more nuclei in a pellet than 5 minutes.

If there is no pellet observed, transfer to a smaller tube and centrifuge again for 10 minutes. Then follow the steps below.

Carefully **transfer the supernatant** to a new, labeled 15 mL Falcon and set aside on wet ice.
If yield is low, more nuclei may be recovered by spinning down the supernatant again.

Resuspend the remaining nuclei pellet in **50-70 µL of PBS + BSA (1%) + RNase Inhibitors solution.**
Volume can be adjusted based on pellet size and desired concentration.

6 Second Filtration

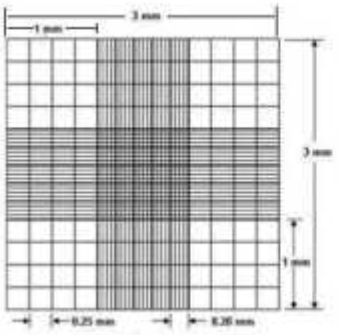
Prepare a **5 mL FACS tube with filter cup on wet ice.**

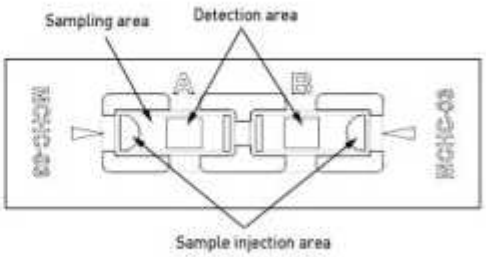
Carefully **transfer the nuclei suspension** through the new filter.
To pass a small volume through the filter, it can be helpful to hold the pipette tip against the membrane of the filter and apply gentle pressure while dispensing.

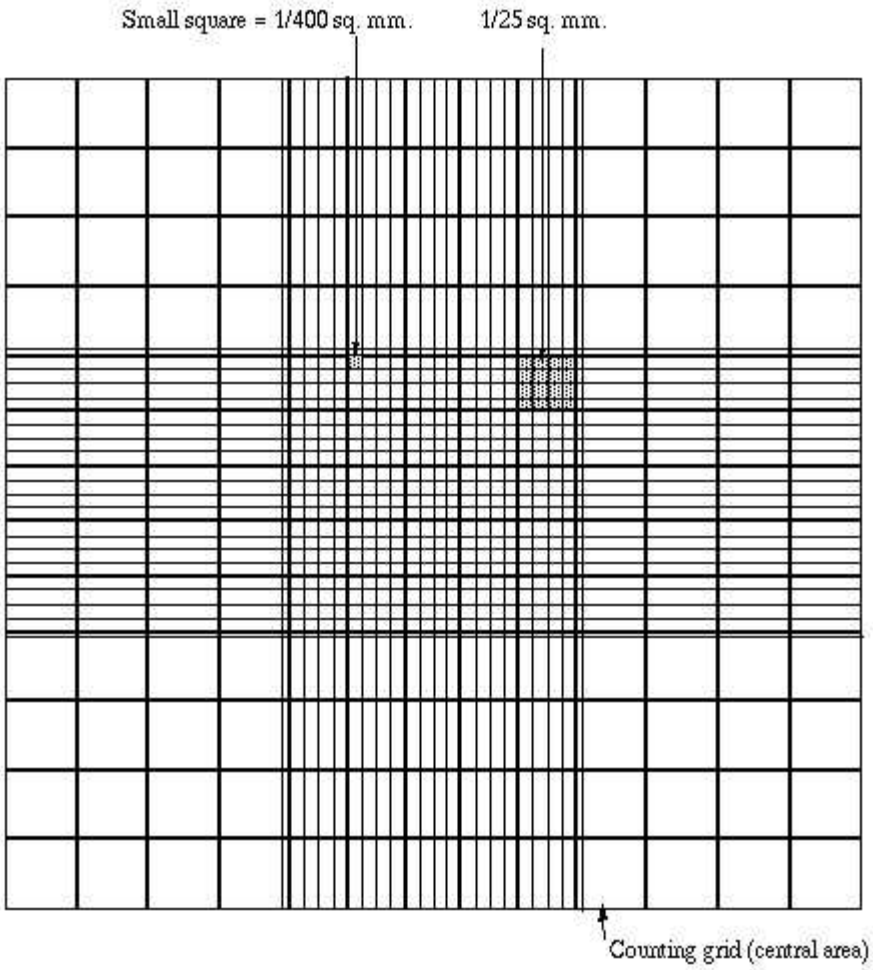
If necessary, the suspension can be carefully pulled through to the other side of the filter using a ***clean*** *pipette tip.*

7 Quality Control

Count nuclei using INCYTO C-Chip Neubauer Improved Disposable Hemocytometers and dilute to desired concentration if necessary.







8 Loading

Load sample on 10X (recommended to load between 8,000 -10,000 nuclei per 10X channel). For multiplexing of 4 samples, load 32,000- 40,000 per 10X channel.