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Nuclei Isolation from Flash Frozen Intestinal Tissue

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We use this protocol and it's working

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Abstract

This protocol is for isolating, fixing, and freezing nuclei from flash frozen human intestinal full thickness samples for single nucleus RNA sequencing. Adapted from Invent Biotech Minute Detergent-Free Nuclei isolation Kit Protocol for Mammalian Tissues, and Parse Evercode Fixation v2 Protocol.

Obtain Reagents Before Starting

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Reagent	Vendor	Catalog Number	Storage	Notes
Minute Detergent-Free Single Nuclei Isolation Kit	Invent Biotech	NI-024	Some reagents 4°C, some -20°C	
Protector RNase inhibitor	Millipore Sigma	3335402001		
Parse Evercode Nuclei Fixation	Parse Biosciences			1 Parse Nuclei Fixation per 4 tissue samples
MACS BSA Stock Solution	Miltenyi	130-091-376	4°C	10% BSA Solution
Molecular Biology Grade Water	Corning	46-000-CI	Room Temperature	
Optional - RNAqueous Micro Total RNA Isolation Kit	ThermoFisher	AM1931	4°C	

Methods

10m

2 Thaw a Mr. Frosty freezing container at room temperature, or fill up to line with Isopropanol.

3 Cool centrifuge to 4°C.

4 Follow Parse fixation protocol for coating 15 mL conicals in 1% BSA prior to starting. Coat 2 conicals per sample you're isolating.
a. We coat each conical with 10 mL 1% BSA by pipetting 1 mL MACS BSA Stock Solution into 9 mL Molecular Biology Grade Water.
b. Cap the conicals and incubate for 30 minutes at room temperature. Aspirate the BSA out and leave uncapped in a biosafety cabinet for 30 minutes at room temperature.
c. Tubes can now be capped and proceed to be used in the protocol, or stored at 4°C for up to a month.

5 Thaw buffer B, then add RNase inhibitor (lab stock) at 1:200 dilution to buffers A and B (0.5 mL of each buffer needed per sample) and keep on ice.
a. We prepare buffer A and buffer B for one extra sample.

6 Get dry ice and precool 10 cm Petri dishes, forceps, and razor blades by placing directly on dry ice for 5-10 minutes.

7 Start to thaw the rest of the Parse fixation kit and keep on ice. Place the RNase inhibitor directly on ice, and the DMSO directly at room temperature.

8 Once Nuclei Buffer is thawed, add 63 µL RNase inhibitor (Parse stock) into the tube and mix thoroughly by pipetting up and down 5x with a p1000 set to 750 µL and store on ice.

9 Prepare Nuclei Buffer (plus RNase inhibitor) + BSA in a new tube for each sample and store on ice. Use the table below (adapted from Parse fixation protocol but altered).

Volume to add based on number of samples:

Number of Samples	1	2	3	4
Nuclei Buffer (plus RNase inhibitor)	750	1,500	2,250	3,000
MACS BSA Stock Solution	50	100	150	200
Total (uL)	**800**	**1,600**	**2,400**	**3,200**

10 Label two 1.5 mL tubes per sample – one for nuclei and one for RNA (RNA tube is optional). This step can also be done later on during spins.

11 Grab flash frozen tissue biopsy from -80°C and keep on dry ice.

12 Add tissue to cold Petri dish on dry ice and use cold razor blade to cut into small pieces, while holding with cold forceps.

13 Transfer tissue pieces to a Filter Cartridge in a Collection Tube (from Invent kit) on ice (not dry ice).

14 Add 200 µL cold buffer A with RNase inhibitor to the filter, grind the tissue for 2 minutes using the plastic rod provided (the plastic rod is reusable, clean by water and 70% ethanol).
a. Grinding motion: twist rod between fingers while gently pushing on bottom and sides of filter. After one minute, check tissue and if it's rising up the rod, push back down to the bottom of filter and continue grinding for next minute.

15 After grinding is complete and the liquid is now red with stringy tissue/fat chunks remaining, add 300 µL cold buffer A with RNase inhibitor to the same filter and incubate on ice for 5-10 minutes with the cap open.

16 Repeat for each sample.

17 Cap the tubes and resuspend the tissue homogenate by inverting the tube a few times.

18 Centrifuge in a tabletop microfuge at 15,000 x g for 30 seconds.

19 Discard the filter and resuspend the pellet by pipetting up and down with a p1000, then centrifuge at 500 x g for 2-3 minutes at 4°C.

20 Discard the supernatant and resuspend the pellet in 0.5 mL cold buffer B with RNase inhibitor, centrifuge at 600 x g for 8-10 minutes (to remove membrane debris) at 4°C. The pellet contains isolated nuclei.

21 Then move over to following the Parse Evercode Nuclei Fixation Protocol to fix the nuclei and freeze:

22 Aspirate supernatant and resuspend in 750 µL Nuclei Buffer + BSA prepared in step 9.

23 OPTIONAL: Take 150 µL Nuclei Buffer + BSA + nuclei from each sample tube and transfer to the labeled RNA 1.5 mL tubes (from step 10) and centrifuge these tubes at 600 x g for 8-10 minutes.

24 OPTIONAL: Aspirate RNA tubes and resuspend in 100 µL RNA Lysis buffer and put into -80°C.
a. RNA Lysis Buffer is the Lysis Buffer in the RNAqueous Micro Total RNA Isolation Kit.
b. Steps 23 and 24 are optional steps to see the RNA quality on the bioanalyzer before sending for sequencing.

25 Filter the remaining 600 µL of Nuclei Buffer + BSA + Nuclei through the Parse provided 40 um Falcon filters and into the BSA coated 15 mL tubes (prepared in step 4).
a. Press the tip of the pipette directly onto the filter and depress the plunger to ensure all the liquid passes through the strainer.

26 Then, continue rest of Parse Evercode Nuclei Fixation Protocol (starting at Step 7):

27 Add 250 µL of Nuclei Fixation Solution to the 15 mL tube for and mix immediately by pipetting up and down exactly 3x with a p1000 set to 250 µL. Return the tube to ice and repeat for each sample.

28 Incubate on ice for 10 minutes.

29 Add 80 µL Nuclei Permeabilization Solution to the 15 mL tube and mix thoroughly by pipetting up and down 3x with a p1000 set to 250 µL. Return the tube to ice and repeat for each sample.

30 Incubate on ice for 3 minutes.

31 Invert the Nuclei Neutralization Buffer tube 5x to mix.

32 Add 1 mL of Nuclei Neutralization Buffer to the 15 mL tube. Gently invert the 15 mL tube once to mix and return to ice. Repeat for each sample.

33 Take 50 µL from the tube and add into one well of a 96 well plate. Repeat for each sample and stain each well with 100µL prepared DAPI + PBS at 1:1000. Incubate for 20 mins are room temperature and image.
a. This step is to assess the quality of the nuclei before sequencing.

34 Centrifuge the 15 mL tubes in a swinging bucket rotor for 10 minutes at 200 x g at 4°C.

35 Remove and discard the supernatant. Fully resuspend the pellet in 150 µL cold Nuclei Buffer (without BSA but with RNase inhibitor added) with a p1000 set to 150 µL and return to ice. Repeat for each sample.

36 Pipette nuclei through a 40 µm strainer into a new labeled RNA 1.5 mL tube (from step 10) with a p1000 and store on ice. Repeat for each sample.

37 Add 2.5 µL DMSO and gently flick each tube 3x to mix. Repeat for each sample.

38 Incubate on ice for 1 minute.

39 Repeat steps 37 and 38 two more times for a total addition of 7.5 µL of DMSO.

40 Mix gently by pipetting up and down 5x with a p200 set to 75 µL to avoid creating bubbles.

41 Place tubes in Mr. Frosty and into -80°C.