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Nuclei isolation from snap-frozen human placental tissue for bulk ATACseq

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This protocol describes the isolation of nuclei from snap-frozen human placental tissue for bulk ATACseq. It is a modified version of a protocol (SCBL Protocols - 10x Multiome Nuclei Isolation) supplied by the Jackson Laboratory (JAX), which is itself modified from Sigma Aldrich's Nuclei Isolation Kit: Nuclei EZ Prep protocol, and 10x Genomics' Demonstrated Protocol – Nuclei Isolation for Single Cell Multiome ATAC + Gene Expression Sequencing. It is recommended to work with no more than four samples at a time so that all samples can be processed in a timely manner.

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Equipment:

Benchtop microfuge with cooling function
Fume hood
Laminar flow hood
Cordless motor for disposable pestles (Z359971-1EA)
Metal plate
Automated cell counter
Heating block
Set of pipettes
Microscope

Supplies/reagents:

60mm Petri dishes (Fisher, cat # FB0875713A)
LoBind Eppendorf tubes (Eppendorf, cat # 022431021)
#22 sterile disposable scalpels (Fisher, cat # 22-079-714)
Trizma hydrochloride solution, 1M, pH 7.4 (Sigma, cat # T2194-100ML)
Magnesium chloride solution, 1M (Sigma, cat # M1028-100ML)
Nuclei Isolation Kit: Nuclei EZ Prep (Sigma, cat # NUC101-1KT)
Magnesium acetate tetrahydrate (Sigma, cat # M2545-250G) - use to prepare 300mM solution, filter sterilize
N,N-Dimethylformamide (Sigma, cat # 227056-100ML)
Trypan Blue solution, 0.4% (Sigma, cat # T8154-20ML)
Nuclease-free water
Bel-Art Disposable Pestles, polypropylene, With 1.5 mL Tubes, pack of 100 (Sigma, cat # BAF199230000-100EA)
Single-use tweezers, angled,blue,sterile (Thomas Scientific, cat # 19A00N163)
Dithiothreitol (DTT), 0.1M Solution (Fisher, cat # 707265ML)
Pluriselect pluriStrainer Mini 40 UM, 100/Bag (Fisher, cat # NC1469671)
Pluriselect PluriStrainer Mini 20 µm Cell Strainer, 100/Pk, Sterile (Fisher, cat # NC1423042)
Digitonin, 5% (Fisher, cat # BN2006)
Tween-20, 10%
NaCl (5 M), RNase-free (Fisher, cat # AM9760G)
Bovine Serum Albumin solution, 10% in DPBS (Sigma, cat # A1595-50ML)
200ul wide bore filter tips (Fisher, cat # 2069GPK)
1000ul wide bore filter tips (Fisher, cat # 2079GPK)
Cell Counting Slides for TC10™/TC20™ Cell Counter, Dual-Chamber (Bio-Rad, cat # 1450011)
Tris base (Fisher, cat # BP152-500) - use to prepare 1M solution; adjust to pH 7.8 using glacial acetic acid, filter sterilize
Potassium acetate (Sigma, cat # P5708-500G) - use to prepare 3M solution, filter sterilize
Bucket of ice
Filter tips

Preparation

1 Pre-chill microfuge to **4 °C** .

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2 Prepare bucket of ice, and chill metal plate (allows for a flat surface to place Petri dish on top of - to keep tissue cool while cutting).

3 Dispense **200 µL Nuclei EZ Storage Buffer** into microfuge tube and place on ice

4 Thaw **110 µL** aliquot of **100 millimolar (mM) DTT** and place on ice.

Prior to starting this protocol, prepare and freeze **110 µL** aliquots of **100 millimolar (mM) DTT** from stock solution.

5 Thaw **500 µL** aliquot of tagmentation buffer and place on ice.

Prior to starting this protocol, prepare **10 mL** tagmentation buffer as follows:

330 µL 1M Tris-acetate, pH 7.8
220 µL 3M Potassium acetate
367 µL 300mM Magnesium acetate
1.6 mL Dimethylformamide
7.483 mL Nuclease-free water

Freeze **500 µL** aliquots of tagmentation buffer.

Take care when handling dimethylformamide. Wear appropriate personal protective equipment and work in a fume hood. Dispose of waste appropriately.

6 Pull snap-frozen placenta samples (~150-200 mg each) from -80 °C and place on dry ice until ready to process.

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This protocol describes nuclei isolation from 4 snap-frozen samples at a time. It is helpful to fill out a checklist (see below for example) to keep track of where each sample is in the protocol as you work through the various steps. Samples are staggered in such a way that the first sample is one step ahead of the second sample, which is one step ahead of the third sample, and so on.

ATACseq_sample_tracker.pdf

7 Label 4 microfuge tubes (the ones that are supplied with the disposable pestles) with sample names and pre-chill on ice.

8 Get the following materials ready; you won't have time to do this in between steps:

4 x #22 disposable scalpels
4 x 60mm Petri dishes
4 x disposable pestles/tubes, along with cordless motor
4 x disposable tweezers
4 x 40um filters
4 x 20um filters
4 x BioRad counting slides
0.4% Trypan Blue stain
200ul wide bore filter tips
1000ul wide bore filter tips

Make fresh buffers

9 Prepare **1 mL fresh lysis buffer** as detailed below, and keep on ice:

988 µL EZ lysis buffer
2 µL 5% Digitonin
10 µL 100mM DTT

Check carefully that there is no precipitate in the 5% digitonin stock solution. If a white precipitate is present, heat at **94 °C** **00:05:00** . Re-dissolved digitonin is good for 1 week at room temperature.

10 Prepare **8.5 mL wash buffer** as detailed below, and keep on ice:

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- **85 µL 1M Tris-Cl, pH 7.4**
- **17 µL 5M Sodium chloride**
- **25.5 µL 1M Magnesium chloride**
- **850 µL 10% Tween-20**
- **85 µL 10% BSA**
- **85 µL 100mM DTT**
- **7352.5 µL Nuclease-free water**

Dispense BSA solution in a laminar flow hood to maintain sterility of the stock solution.

Nuclei isolation 25m

11 Place a sterile Petri dish onto the chilled metal plate sitting on ice. Move tube containing first placenta sample from dry ice to wet ice for ~30 seconds, then tap slightly-thawed tissue into pre-chilled Petri dish.

12 Cut tissue into small pieces (~ 2 to 3mm in size) using a fresh disposable #22 scalpel. Use disposable tweezers to hold tissue in place while cutting. Transfer tissue into labeled pre-chilled microfuge tube.

Take care when cutting. If tissue is too hard and/or too much pressure is applied with the scalpel, tissue can fly out of the Petri dish. Cut cautiously and slowly to begin with - it is sometimes helpful to rock the blade from side to side to gradually split the frozen tissue. As the tissue thaws it gets a little easier to cut, but if it is allowed to thaw too much then it gets more difficult.

13 Add **125 µL lysis buffer** and grind tissue with a disposable pestle attached to a cordless motor.

It should only take a few 5 second pulses to achieve a "smoothie-like" consistency. Do not over-grind.

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14 Place ground-up tissue on ice for **00:05:00** . During this time you can start step 11 for the second placenta sample. 5m

15 Centrifuge sample **500 x g, 4°C, 00:05:00** . During this time you can start step 11 for the third placenta sample. 5m

16 Remove and discard supernatant, and resuspend pellet gently in **100 µL fresh lysis buffer** using 200ul wide bore filter tips. Incubate on ice for **00:05:00** . During this time you can start step 11 for the fourth placenta sample. 5m

17 Using a 1000ul wide bore filter tip, add **500 µL wash buffer** , mix gently, and centrifuge **500 x g, 4°C, 00:05:00** . 5m

18 Remove and discard supernatant and repeat wash twice, saving the last suspension on ice until all samples reach this step.

19 Filter each sample though a 40um filter into a fresh microfuge tube. To maximize recovery of nuclei, wash old microfuge tube with **200 µL fresh wash buffer** , and pass through the same 40um filter.

20 Repeat previous step, this time with a 20um filter. This filter will remove debris that wasn't removed in the previous step.

21 Centrifuge **500 x g, 4°C, 00:05:00** . 5m

22 Remove and discard supernatant carefully, and gently resuspend pellet in **50 µL tagmentation buffer** using 200ul wide bore filter tip. Clumps of nuclei should break up easily. Place resuspended nuclei on ice.

23 Take **5 µL** aliquot of resuspended nuclei; add **20 µL tagmentation buffer** and

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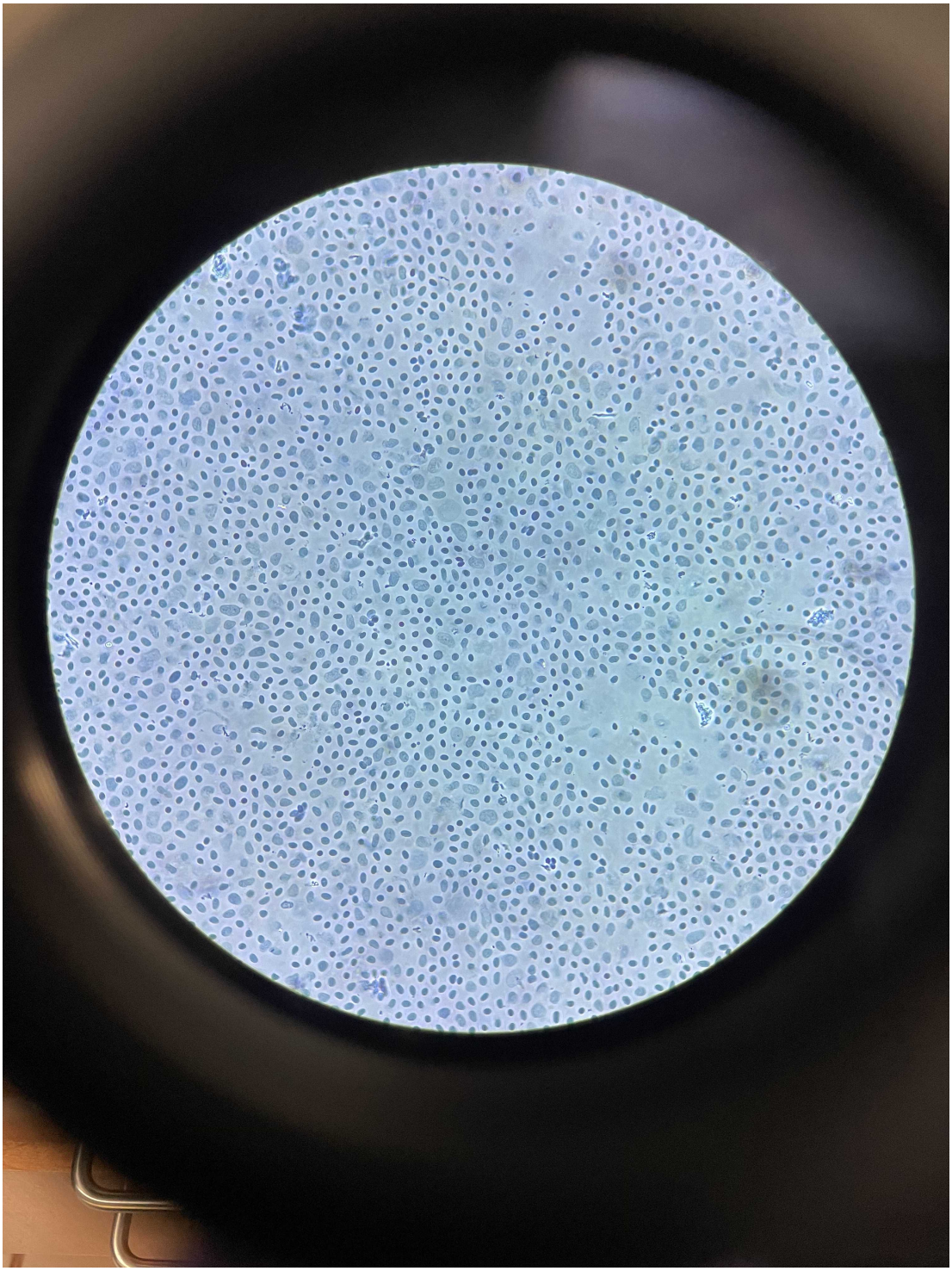
25 µL 0.4% Trypan blue stain . Flick to mix, then load **10 µL** to both sides of a BioRad counting slide. Determine nuclei concentration using an automated cell counter.

Nuclei should stain light blue; not too dark as that would indicate damage to the nuclear membrane. Nuclei should not be clumped together, and there should be little debris.



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24 Based on nuclei concentration, prepare **10 µL** of a 5,000 nuclei/ul solution (50,000 total nuclei), using tagmentation buffer for dilution. Proceed immediately to tagmentation and library preparation.

It helps to use a pre-formulated Excel spreadsheet to quickly determine the volume of nuclei and tagmentation buffer to use. Several intermediate dilutions are often required to ensure that pipetting volumes are sensible.

Post-steps 5m

25 Centrifuge remaining nuclei from step 22 **500 x g, 4°C, 00:05:00** . Remove and discard supernatant, and gently resuspend nuclei in **50 µL Nuclei EZ Storage Buffer** using 200ul wide bore filter tips, making sure to break up clumps of nuclei. Store nuclei at **-80 °C** for up to several months. 5m

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