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

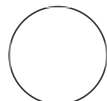
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Protocol to isolate and fix nuclei from flash frozen mouse adrenal glands for IGVF V.2

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ABSTRACT

This protocol describes isolation of nuclei from left **and** right 10 week old mouse adrenal glands from 8 founder strains (B6J, AJ, 129S1J, NZOJ, WSBJ, NODJ, PWKJ, and CASTJ), preparation of a single nucleus suspension, and fixation for single nucleus RNA-seq using Parse Biosciences. We process 1 rep from each strain per day; e.g. female rep 1 across all 8 strains. The main products we use are Parse Biosciences Nuclei Fixation Kit (v2) and Miltenyi Biotec's gentleMACS Octo Dissociator with accessories. This protocol takes about 3.5 hours from start to finish.

The results are 2 aliquots of fixed single-nucleus suspensions for Parse per each of the 8 samples at $\geq 2,500$ nuclei/ul.

The first part of the protocol describes tissue lysis and nuclei extraction using Miltenyi Biotec's gentleMACS Octo Dissociator with accessories. The second part describes nuclei fixation using Parse Biosciences Evercode Nuclei Fixation Kit with v2 reagents. Due to low nuclei recovery, we modify the original Parse Biosciences Evercode fixation protocol (attached) by using half volumes of all fixation reagents. We do not fix extra nuclei for other assays such as SHARE-seq.

ATTACHMENTS

[SO+10122022_Evercode+Fixation+v2.0.2+User+Manual.pdf](#)

GUIDELINES

1. We recommend using a 5 ml pipette for aspirations and resuspensions > 1 ml.
2. Record everything in the [IGVF spreadsheet](#), "Samples into experiment" tab.

MATERIALS

Name	Manufacturer	Cat. #

PROTOCOL integer ID:
90584

Keywords: Fixation, Nuclei fixation, Split-seq, Evercode, snRNA-seq, Parse Biosciences, Nuclei isolation, UCI, Mortazavi, IGVF, Mouse, Adrenal, Adrenal glands, Adrenals

Name	Manufacturer	Cat. #
Nuclei Fixation Kit v2	Parse Biosciences	ECF2003
Nuclei Extraction Buffer	Miltenyi Biotec	130-128-024
RNase Inhibitor, murine	New England Biolabs	M0314L
PBS	HyClone	SH30256.02
7.5% BSA	Life Technologies	15260037
gentleMACS C Tube	Miltenyi Biotec	130-093-237
gentleMACS Octo Dissociator	Miltenyi Biotec	130-095-937
MACS SmartStrainers (30 um)	Miltenyi Biotec	130-098-458
NucBlue Fixed Cell ReadyProbes	Thermo Fisher	R37606
Hemacytometer	Fisher Scientific	02-671-51B
Mr. Frosty	Sigma-Aldrich	635639

Reagents/equipment, manufacturer and catalog number

Name	Reagent	Volume (for 8 samples)	Final Concentration
Lysis buffer	Nuclei Extraction Buffer	35 ml	NA
	40 U/ul RNase inhibitor	175 ul	0.2 U/ul
NB-BSA + RNase inhibitor	Nuclei Buffer (Parse Biosciences)	3.15 ml	NA
	7.5% BSA	350 ul	0.75%
	RNase inhibitor (Parse Biosciences)	44.1 ul	

Buffers

Setup

- 1 Label tubes.

- 2 Pre-chill centrifuge to **4°C**.
- 3 Prepare ice buckets.
- 4 Prepare **35 ml lysis buffer** on ice in a 50 mL conical tube. Distribute **2 mL** into 8 gentleMACS C Tubes on ice. Add **175 µl RNase inhibitor** to the lysis buffer aliquot the day of the experiment.
- 5 Prepare **3.5 ml NB + BSA**. Add **44.1 µl RNase inhibitor** included in Parse Biosciences fixation kit the day of the experiment.
- 6 Prepare **1.5 ml nuclei buffer + RNase inhibitor** for final resuspension. Add **18.6 µl RNase inhibitor** to **1.5 ml nuclei buffer**.
- 7 Thaw components of **1 Parse Biosciences Nuclei Fixation v2 kit** at room temperature, then place on ice.
- 8 Distribute **20 µl NucBlue Fixed Cell ReadyProbes** into 16 PCR strip tubes for cell counting. Need 8 tubes for counting after nuclei extraction, and another 8 tubes for final fixed nuclei.

Tissue lysis and nuclei extraction

- 9 Keep flash frozen tissue samples on dry ice until lysis.

- 10 Drop whole frozen tissue into a chilled gentleMACS C Tube with **2 ml lysis buffer**. Close tubes firmly and invert immediately, ensuring tissue is not stuck to the bottom or side. Keep tubes on ice and proceed immediately to dissociation. There should be 2 adrenal glands.
- 11 Run the gentleMACS Program **4C_nuclei_1** on the Octo Dissociator (~**5 minutes**).
- 12 Remove tubes, ensuring tissue did not get stuck on the sides, and spin down in a 4C centrifuge for ~**10 seconds** to bring liquid to the bottom, then place tubes back on ice.
- 13 Filter nuclei suspension through **30 um** MACS SmartStrainer into a 5 ml tube. Fit a tube rack in ice for extra stability while filtering.
- 14 Wash **30 um** MACS SmartStrainer with **2 ml** additional lysis buffer. Add 2 ml to C tubes, cap, and swish to recover any nuclei stuck to the sides and cap of the C tubes, then wash the strainer.
- 15 Discard strainer and centrifuge the 4 ml nuclei suspension at **4C, 350g** for **5 minutes**.
- 16 Discard supernatant and resuspend nuclei pellet in **375 ul NB-BSA + RNase inhibitor** and filter through a **40 um** strainer (provided in Parse Biosciences kit) into a new 5 ml tube.
- 17 Count nuclei. Use **1:6** dilution factor, **4 ul + 20 ul dye**.

Parse Nuclei Fixation

- 18 Add **125 uL Nuclei Fixation Solution** to the filtered nuclei in 375 ul and mix 3 times. Do not over-mix.

- 19 Incubate nuclei for **10 minutes** on ice. Set 2 P200 pipettes to 40 ul and 125 ul.
- 20 Add **40 uL Nuclei Permeabilization Solution** and mix by pipetting 3 times with the P200 still set to 125 uL. Do not over-mix.
- 21 Incubate **3 minutes** with nuclei on ice.
- 22 Add **2 mL Nuclei Neutralization Solution** and invert the tube once to mix.
- 23 Centrifuge at **4C, 750g** for **10 minutes**.
- 24 Aspirate and discard supernatant.
- 25 Resuspend the samples in **150 ul Nuclei Buffer with RNase inhibitor** without BSA and filter through a 40 um filter into a labeled 1.5 ml tube. Do NOT filter, will lose too much volume.
- 26 Count nuclei. Use a **1:11** dilution factor, e.g. **2 ul + 20 ul dye**.

- 27** Add Nuclei DMSO: **2.5 ul** into 150 ul samples and gently flick tubes to mix. One minute later, add another **2.5 ul** and flick to mix, then after another minute add a final **2.5 ul** for a total volume of **7.5 ul**. Mix by gently pipetting 5x with a P200 set to 75 ul.
- 28** Split nuclei suspension into 2 labeled tubes, **75 ul** per tube.
- 29** Place tubes in a Mr. Frosty for storage at **-80C**. The next day, move tubes to boxes in -80C racks.

