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JAX-Sen: Nuclei prep from frozen kidney tissue for single-nuclei RNA-seq

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Cellular Senescence Net...

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We use this protocol and it's working

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Abstract

We aim to study and characterize senescence in the C57BL/6J mouse kidney. This protocol describes the nuclei prep from frozen kidney tissue for single-nuclei RNA-seq.

Troubleshooting

Abstract

1 We aim to study and characterize senescence in the C57BL/6J mouse kidney. This protocol describes the nuclei prep from frozen kidney tissue for single-nuclei RNA-seq.

Reagents and Materials

2 **Nuclei Isolation:**

- Nuclei Extraction Buffer: 130-128-024 (Miltenyi)
- C Tubes: 130-093-237 (Miltenyi)
- MACS BSA Stock Solution: 130-091-376 (Miltenyi)
- DPBS: 14190144 (Thermo Fisher Scientific)
- ROCHE Protector RNase Inhibitor: 3335399001 (Millipore)
- Eppendorf™ DNA LoBind1.5 mL Microcentrifuge Tubes: 13-698-791 (Thomas Scientific)
- Lobind Eppendorf 2.0mL tubes: 13-698-792 (Thermo Fisher Scientific)
- Eppendorf 5.0mL tubes: 4011-9487 (USA Scientific)
- Eppendorf 15.0mL tubes: 4011-2208 (USA Scientific)
- BOApluriStrainer Mini 70 µm: 43-10070-50 (pluriSelect)
- BOApluriStrainer Mini 40 µm: 43-10040-50 (pluriSelect)
- Anti-Nucleus Microbeads: 130-132-997 (Miltenyi)
- LS Columns: 130-042-401 (Miltenyi)
- QuadroMACS Separator: 130-090-976 (Miltenyi)
- MultiStand: 130-042-303 (Miltenyi)

Cell Counting: LUNA FX7

- Acridine, Orange/Propidium Iodide Stain: F23001(New England BioGroup, LLC)
- Luna Slides:
- Ultra-low Fluorescent Slides: Fluorescent and Brightfield Mode (New England BioGroup, LLC)

Procedure

3 **Before Starting**

3.1 Pre-cool centrifuge, buffers, and consumables with sample contact (C Tube, tubes and Strainers, etc.) at 4 °C.

3.2 Prepare Buffers

3.3 1 kidney tissue ~140-240mg in weight. Keep on dry ice until ready to do the prep

3.4

Miltenyi Nucclei Extraction Buffer (Stock Solution)							
Item	**Stock**	**Final**	**1X**	**2X**	**4X**	**6X**	**8X**
Miltenyi Nuclei Extraction Buffer-**mL**	n/a	n/a	4.0	8.0	16.0	24.0	32.0
RNAse Inhibitor-**µL**	40U/µL	.2U/µL	20	40	80	120	160
Total			4mL	8mL	16mL	24mL	32mL
Nuclei Separation Buffer (NSB)							
Item	**Stock**	**Final**	1X	2X	4X	6X	8X
Miltenyi Nuclei Extraction Buffer-**mL**	n/a	14%	1.1	2.2	4.5	6.7	9.0
PBS pH 7.2-**mL**	1X	-	6.8	13.7	27.2	40.9	54.4
BSA-**µL**	10%	0.04%	32	64	128	192	256
RNAse Inhibitor-**µL**	40U/µL	.2U/µL	40	80	160	240	320
Total			8mL	16mL	32mL	48mL	64mL
For RINSING the column-No RNAse Inhibitor added							
Item	**Stock**	**Final**	1X	2X	4X	6X	8X
Miltenyi Nuclei Extraction Buffer-**mL**	n/a	14%	0.420	0.840	1.7	2.5	3.4
PBS pH 7.2-**mL**	1X	-	2.6	5.1	10.3	15.4	20.5
BSA-**µL**	10%	0.04%	12	24	48	72	96
Total			3mL	6mL	12mL	18mL	24mL
Resuspension Buffer (NRB)-for final resuspension							
Item	**Stock**	**Final**	1X	2X	4X	6X	8X
PBS pH 7.2-**µL**	1X	-	116	233	465	698	930
BSA-**µL**	10%	2%	30	60	120	180	240
RNAse Inhibitor-**µL**	40U/µL	1U/µL	4	8	15	23	30
Total			.150mL	.300mL	.600mL	.900mL	1.2mL

4 Miltenyi gentleMACS Procedure for Tissue Lysis and nuclei extraction

4.1 Add 2.0mL ice-cold Nuclei Extraction Buffer to each pre-cooled gentleMACS C Tubes on ice.

4.2 Transfer frozen tissue piece(s) into the gentleMACS C Tube containing lysis buffer and directly proceed with the following steps until samples are dissociated.

Note: Do not let frozen samples thaw before dissociation, as endogenous RNase might degrade RNA.

4.3 Close gentleMACS C Tube and place it on the gentleMACS Dissociator. Be sure the cap is closed completely.

4.4 Run the following gentleMACS Programs: (GentleMACs instrument either in 4^0C cooler or use cooling sleeves that are previously frozen at -20^0C.) Total lysis time is 28 minutes

-gentleMACS 4C_nuclei_1-5min
-Cus_4C_nuclei_10 program-15min
-5_Min_Mix-spin only
-3 min. on ice

4.5 A quick spin in a swinging bucket at 500 x g, 4°C for 10-15 sec may be necessary.

4.6 Pipette mix with wide bore tip and filter through pre-wet 70µm filter into a cold 5mL tube.

-Pre-wet filter with Nuclei Extractio Buffer.
-Add additional 2mL NEB to rinse the filter.

4.7 Spin down nuclei in swinging bucket at 500 x g, 4°C for 5 minutes

4.8 Remove supernatant and resuspend nuclei in 450µL Separation Buffer

4.9 Pass nuclei through 40µm strainer
Count nuclei using AOPI-Dilute nuclei 5 times (10µL nuclei + 40µL SB)

5 Miltenyi Anti-Nucleus Microbead Cleanup

5.1 Notes:

A. Keep cold and degas buffer before use, do not want bubbles in column.
B. Scale up Nuclei Separation Buffer if working with higher nucleus numbers.
C. Maximum nuclei per separation 2×10^7.
D. Use Refrigerator for labeling incubation.
E. Magnetic Labelling-Anti-Nucleus microbeads use 5mL or 15mL tubes.
F. Always wait until the column reservoir is empty before proceeding to the next step

5.2 Distribute 1M nuclei for each clean up into 5mL tube and add additional Separation Buffer so the total volume equals 450µL.

5.3 Add 50µl microbeads to nuclei, mix well and incubate 15 min in the refrigerator (2-8^{0}C) (not on ice)

5.4 Place column in the magnetic field of a suitable MACS Separator and prepare column by rinsing with 3 mL of nuclei separation buffer without RNase Inhibitor when 2-3 of the 15-minute incubation remains.

5.5 After the 15-minute incubation add an additional 2 mL nuclei separation buffer, mix well, and proceed to magnetic separation.

5.6 Apply nuclei suspension directly to the center of the column. Collect flowthrough containing debris in a 15mL tube.

5.7 Wash column 2-3 times with 1 mL of nuclei separation buffer. Add buffer as soon as the column is empty and rinse the inner column walls.

5.8 Remove column from the magnet and place it in a rack with a suitable collection tube depending on rack, 5mL tube is ideal.

5.9 Elute the Microbeads off the column by pipetting 2mL of Nuclei Separation Buffer onto the column, capture flowthrough in collection tube.

5.10 Immediately flush out the magnetically labeled nuclei by firmly pushing the plunger into the column.

5.11 Centrifuge 500xg for 5 minutes. Pipette off supernatant leaving ~20µL behind.

5.12 Add ~120 µL Nuclei Suspension Buffer (from Nuclei isolation) 1XPBS,2%BSA,1U/µL RNase Inhibitor
Optional-40µm strainer if cells are clumpy.
QC/Count Nuclei using AOPI.