Nuclei Isolation and Immunoprecipitation for 10X Sequencing V.1

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VERSION 1

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Protocol status: In development
We are still developing and optimizing this protocol.

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ABSTRACT

This protocol is for assessing the MIT AAVs from pooled injections.

GUIDELINES

Keep tissue/nuclei on ice as much as possible.

MATERIALS

Dynabeads - 4C
anti-GFP - 4C (roche)
Triton X 100 - 4C
DTT - 4C
RNAsin -20C

Prepare Stock Solutions

1 Make 20 mL **10% BSA** by combining 2 mL of BSA with 18 mL of MilliQ water in a 50 mL falcon tube. (4°C - 2 weeks)

2 Make 20 mL **10% Triton X-100** by combining 18 mL MilliQ water with 2 mL Triton X-100 in a 50 mL tube. Vortex and then incubate at room temperature for 20 minutes. Filter it through a 0.22 µm filter with a syringe into a clean 50 mL tube. (4°C - 1 month)

3 Make 250 mL **Nuclear Isolation Media** by filling a 250 mL bottle with 200 mL of MilliQ water and then adding 2.5 mL 1M Tris, 6.26 mL 1M KCl, 1.25 mL 1M MgCl2, and 21.45 g Sucrose. Shake until sucrose is dissolved then fill to 250 mL with MilliQ water. (4°C - 2 weeks)

4 Make 50 mL **Citrate-Phosphate Buffer** (pH 5) by adding 0.48 g Citric Acid and 0.91 g Dibasic Sodium Phosphate Dihydrate to a falcon tube and then filling it to 50 mL with MilliQ water. Titrate the pH with NaOH using the pH meter. (4°C - 1 month)

Prepare Fresh Solutions

5 Make 3 mL **Homogenization Buffer** per sample by adding 2.895 mL Nuclear Isolation Media (filtered via syringe) to a 5 mL eppendorf. Then add 3 µL 100 mM DTT and 30 µL 10% Triton X-100. Add 15 uL RNAsin and invert to mix. Store on ice.

6 Make 200 uL **Blocking Buffer** per sample by dividing your total desired volume of blocking buffer by 10 to get the amount of 10% BSA in uL. Add this amount to a tube and then fill the remainder with 1X PBS.

Homogenization

7 Clean dounce, scalpel, and forceps using MilliQ water, ethanol, RNase Zap, then MilliQ again. The red-tape forceps are for unfixed tissue.

8 Get tissue sample from -80°C freezer and place on dry ice. Weigh it on a sterile, tared weigh boat.

9 Add tissue to dounce and push it to the bottom using 1 mL of Homegenization Buffer and the pestle. Homogenize the tissue without creating bubbles. Then add the remaining 2 mL of the

Homogenization Buffer and continue to dounce until homogenized.

10 Pass all of the nuclei suspension through three FlowMi filters, 1000 µL at a time into a new 5 mL eppendorf.

11 Centrifuge at 900 g/rcf for 10 minutes at 4°C.

12 Discard the supernatant and resuspend the pellet in 210 µL Blocking Buffer. Incubate for 10 minutes on ice.

Cell Count

13 Add 9 uL of sample to a PCR tube and then add 1 uL of acridine orange.
If sample is very concentrated, instead add 2 uL sample to 2 uL of acridine orange and 16 uL 1X PBS.

14 Pipette mix and then add the total volume to a three-chamber cell counting chip and make note of the channels used (A, B, or C).

15 On the cell counter, select Fluorescence Cell Counting -> Cell Lines -> Advanced -> Protocol -> and then choose "Nuclei" from the list of protocols.

Then go to Settings and choose the 3 Channel option. Select all of the channels you will be using.

Then hit "Count" and then "Start Count."

16 When the cell count is complete, you will get a reading in cells/mL. Convert this to cells/uL by dividing this number by 1,000.

17 Save approximately 40,000 nuclei from the original sample to use as our unpurified population

for sequencing. (If the concentration was 1,000 cells/uL, then save 40 uL).

18 If there are channels left on the cell counter chip, mark the used channels on the back and place it back in the drawer for future use.

Immunoprecipitation

19 Vortex stock of Dynabeads Protein G and then add 5 uL to a 1.5 mL eppendorf. Place it on the mag rack for a minute and discard the supernatant remaining at the bottom of the tube without disturbing the beads.

20 Remove the tube from the magnet and resuspend the beads with 500 uL of Citrate Phosphate Buffer. Place the tube back on the mag rack for a minute and discard the supernatant. Repeat this step once more.

21 Add 100 uL of 1X PBS and 2 uL anti-GFP to the washed Dynabeads. Add this to the rotator in the 4°C fridge and let it incubate for 1 hour.

22 After incubating, place the tube on the magnet for 2 minutes and discard the supernatant without disturbing the beads.

23 Wash with 100 uL 1X PBS Buffer, enough to submerge the beads while they are on the rack. Discard the supernatant.

24 Add the total volume of nuclei sample to the beads. Incubate on the rotator in the 4°C fridge for 1 hour.

25 After incubation, place the samples on the mag rack for 2 minutes. Collect the supernatant in appropriately labeled eppendorf tubes. This will be our supernatant population for sequencing.

26 Wash the bead tubes with enough 1X PBS to submerge the beads (don't resuspend, gently) and then discard the supernatant.

27 Remove the tubes from the rack and then resuspend the beads in 50 uL 1X PBS.

Cell Count

28 Take another cell count of the supernatant and bead-bound samples. Reverse pipette to record their volumes.

29 Dilute all of the samples (unpurified, supernatant, and bead-bound) to 1,000 cells/uL. The bead-bound population likely won't need dilution.

30 Follow the 10X Chromium manual for the rest of the protocol to proceed with library prep and sequencing.