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PBMC 10X Genomics Single Cell CUT&Tag Protocol

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ABSTRACT

This protocol is based on the bulk CUT&Tag protocol developed in the Henikoff laboratory (https://doi.org/10.1038/s41467-019-09982-5) and adapted for single nuclei input onto the 10X Genomics single cell ATAC-seq platform.Throughout this protocol the nuclei will be lost due to multiple washes and partial clumping, so to ensure enough single nuclei are retained, we set up to start with 1 million PBMC-isolated nuclei when starting the primary antibody incubation (B. CUT&Tag protocol, Step 11). To get 1 million PBMC-isolated nuclei aim to input double the amount of live PBMCs, since the nuclei isolation protocol is set up to yield single nuclei solutions by performing low relative centrifugal force (RCF) spins that will prevent nuclei clumping, but will not pellet all nuclei after multiple washes.

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Single Cell CUT&Tag, scC&T, PBMC

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MATERIALS TEXT

A. Nuclei isolation protocol

Buffers and Reagents:

1. Lysis buffer (keep at 4°C; Nuclei EZ Lysis Buffer, Millipore Sigma NUC-101 # N3408)

2. Wash Buffer (50 ml)
- 1 mL 1 M HEPES pH 7.5 (final concentration 20 mM; Sigma-Aldrich # H3375)
- 1.5 mL 5 M NaCl (final concentration 150 mM)
- 12.5 µL 2 M Spermidine (final concentration 0.5 mM; Sigma-Aldrich # S2501)
- 1 cOmplete, EDTA-free Protease Inhibitor Cocktail tablet (Sigma-Aldrich # 11873580001)
- water to 50 ml (Invitrogen # 10977015)

Use nuclease free stocks and water.
Make fresh for each experiment.
Store at 4°C over night after the primary antibody incubation at STEP 11.

B. CUT&Tag protocol

Buffers and Reagents:

1. Wash Buffer
The same Wash Buffer is used as in the **A. Nuclei isolation protocol**. Use nuclease free reagents.

2. Detergents
- 10% Nonidet P40 Substitute (NP40; dissolved in water; Thermo Scientific # J19628AP)
- 5% Digitonin (Millipore Sigma # 300410):
  Make fresh each time.
  Add boiling water to Digitonin powder.
  Incubate 15 min at 98°C.
  Cool on ice for 1 h before use.

3. Np40-Dig Wash Buffer (150 mM NaCl)
1 ml of Wash Buffer + 1 µl of 10% NP40 Substitute (final concentration 0.01%) + 2 µl of 5% Digitonin (final concentration 0.01%)

4. Medium-salt Wash Buffer (300 mM NaCl)
1 ml of NP40-Dig Wash Buffer + 30 µl 5 M NaCl

5. Primary Antibody Incubation Buffer
1 ml of Np40-Dig Wash Buffer + 4 µl of 0.5 M EDTA (final concentration 2 mM)

6. Tagmentation Buffer (10 mM $MgCl_2$)
990 µl Medium-salt Wash Buffer + 10 µl of 1 M $MgCl_2$

7. pA-Tn5 – MEDS Complex, already loaded
See: https://www.protocols.io/view/3xflag-patn5-protein-purification-and-meds-loading-8yrhxv6.
Alternatively pA/G-Tn5 (Protein A/G fused to Tn5) is commercially available and can be used with the following protocol: https://www.epicypher.com/content/documents/protocols/cutana-cut&tag-protocol.pdf.
Note that pA-Tn5 and pA/G-Tn5 may be available as different concentration stocks, and make sure to use these stocks according to their appropriate protocol-directed dilution factors!

8. Antibodies
1°:
Rabbit anti-$H_3K_4me_2$ (Millipore/Upstate # 07-030)
Rabbit anti-$H_3K_9me_3$ (Abcam # 8898)
Rabbit anti-$H_3K_{27}me_3$ (Cell Signaling Technology # 9733)
Rabbit anti-$H_3K_{27}ac$ (Millipore # MABE-647)
2°:
Guinea pig anti-rabbit (heavy & light chain; Antibodies Online # ABIN101961)

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A. Nuclei isolation protocol

1 NOTES:

1. All samples and reagents are kept on ice (wet ice) or at 4°C.
2. Use low retention tubes (Thermo Scientific # 3451) and pipette tips (Corning # 4140, 4138, 4136), and avoid over pipetting throughout the entire protocol.
3. All spins are at 500 RCF for 5 minutes at 4°C, using a swinging-bucket rotor.
4. After spins, remove the supernatant carefully, leaving about 50 µl on top of the pellet, and resuspend the pellet in the left-over buffer by tapping the tube.
5. If clumps persist, use a wider bore low retention pipet tip (P1000) to gently pipet up and down the nuclei after resuspension in the appropriate buffer. Keep the pipetting of the sample to a minimum that suffices for nuclei pellet dispersion.

2 In a 1.5 ml tube add 500 µL chilled Nuclei EZ Lysis Buffer to 2 million live PBMCs. Mix the sample by pipetting up and down gently, avoiding bubbles.

3 Incubate on ice for 5 minutes. Repeat mixing the sample 1-2 times during the incubation.

4 Filter homogenate using a 70 µm cell strainer (Millipore Sigma # BAH136800070) and transfer to a new 1.5 mL tube.

5 Centrifuge the nuclei at 500 RCF for 5 minutes at 4°C and remove supernatant leaving about 50 µL above the nuclei pellet.

6 Add 500 µL Wash Buffer and incubate 5 minutes without resuspending to allow buffer interchange. After incubation, add 1 mL of Wash Buffer and resuspend the nuclei.

7 Centrifuge the nuclei at 500 RCF for 5 minutes at 4°C, remove supernatant leaving behind about 50 µL and gently resuspend nuclei in 1 mL Wash Buffer.

8 Repeat STEP 7 and resuspend in 500 µL Wash Buffer. Collect all nuclei by washing off nuclei from the wall of the centrifuge tube.

9 Filter nuclei with a 40 µm cell strainer (Millipore Sigma # BAH136800040). Visually inspect nuclei integrity under a microscope and count the number of nuclei with a cell counter or hemocytometer.

B. CUT&Tag protocol

10 NOTES:

1. Use 1 million nuclei per sample.
2. Primary antibody: dilute 1:100 in 1ml
3. Secondary antibody: dilute 1:100 in 1ml
4. pA-Tn5 - MEDS: dilute 1:100 in 1ml
5. Use all buffers at room temperature (RT), unless other incubation temperatures are specified.

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6. Use low retention tubes and pipette tips and avoid over pipetting throughout the entire protocol.
7. All spins are at 600 RCF for 3 minutes at RT, unless otherwise specified, using a swinging-bucket rotor. After spins, remove the supernatant carefully, leaving about 50 µl on top of the pellet, and resuspend the pellet in the left-over buffer by tapping the tube.

11 Bind primary antibody:
Prepare 1:100 dilution of primary antibody in 1ml Primary Antibody Incubation Buffer and incubate with 1 million PBMC-isolated nuclei on rotator/nutator at 4°C overnight.

12 Wash:
Spin down nuclei and wash nuclei pellets 2X in 0.5 ml NP40-Dig Wash Buffer.

13 Bind secondary antibody:
Prepare 1:100 dilution of secondary antibody in NP40-Dig Wash Buffer. Add 1ml to each sample. Keep on rotator at RT for 45 minutes.

14 Transition to Medium-salt Wash Buffer from now on. Spin at 600 rcf for 3 minutes. Remove 500 µl supernatant after spin and add 500 µl Medium-salt Wash Buffer (300 mM NaCl).

15 Wash:
Spin down nuclei and wash nuclei pellets 1X with 0.5 ml Medium-salt Wash Buffer.

16 Bind pA-Tn5 - MEDS:
Prepare 1:100 dilution of pA-Tn5 - MEDS complex in Medium-salt Wash Buffer and add 1 ml to each sample. Place on rotator at RT for 1 hour.

17 Wash:
Spin down nuclei and wash nuclei pellets 2X with 0.5 ml in Medium-salt Wash Buffer.

18 Tagmentation:
Add 1 ml Tagmentation Buffer to the sample. Incubate at 37°C for 1 hour. After incubation, add 111 µl 10% BSA/H2O to the 1 ml nuclei in the Tagmentation Buffer (for a final 1% BSA) to prevent nuclei clumping during the following centrifugation step.

19 Transition to 1% BSA/PBS and 4°C from now on. Spin at 600 RCF for 3 minutes at 4°C. Remove 500 µl supernatant after spin and add 500 µl cold 1% BSA/PBS.

20 Wash:
Spin down nuclei at 600 RCF at 4°C and wash nuclei pellets 1X with 0.5 ml in cold 1% BSA/PBS.

21 Count/Strain nuclei:
Resuspend nuclei in a small amount of cold 1% BSA/PBS to aim for a concentration of at least 1100 nuclei/µl if you plan to target 10,000 nuclei recovery. Count the nuclei and inspect for clumps. Filter out clumps through 20-30 µm pore size strainer (Miltenyi # 130-101-812, 130-041-407).

22 Load the nuclei into the 10X Genomics scATAC chip H:
https://assets.ctfassets.net/an68im79xiti/7L2MU4QSWfrEgd2h13Efac/d5326fcdc6363aa04e4fdf11b2a1f2f8/CG00

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Follow the 10X Genomics protocol from Step 2, by mixing 15 µl nuclei (e.g. 1020 nuclei/µl for 10,000 targeted nuclei recovery, based on a recovery efficiency factor of 1.53) with 60 µl Barcoding master mix from step 2.1.

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