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SenNet URMC 10X Genomics Single-Nucleus RNA-Sequencing for Transcriptomic Profiling

Forked from 10X Genomics Single-Nucleus RNA-Sequencing for Transcriptomic Profiling of Adult Human Tissues

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We use this protocol and it's working

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Abstract

10X Genomics Single Cell 3' (v3.1) RNA sequencing is a microdroplet-based method that permits the effective capture and sequencing of the mRNA and pre-mRNA molecules from single nuclei [1]. RNA molecules are transcribed and processed within the nucleus before exporting to ER for translation into proteins. As such, nuclear RNA is a mixture of nascent transcripts, partially or fully processed mRNA, and various non-coding RNA molecules. The total RNA content within the nucleus is roughly 10% of the RNA content in a whole cell, but has been found to accurately represent whole cell expression values in adult human tissues [2,3] including the kidney [4]. Nuclei can be readily isolated from frozen tissues with a combination of chemical and physical treatments that can effectively circumvent the non-uniform or incomplete dissociation of solid tissues into single cells, as well as RNA degradation or artefacts (such as stress response) during dissociation. Here we present a modified version of the published 10X protocol [1] that we have adapted for the processing of adult human kidney nuclei.

References

1. Chromium Next GEM Single Cell 3’ Reagent Kits v3.1(Dual Index) User Guide. Document Number: CG000315. October 2022.
2. Lake et al. (2016). Science, doi:10.1126/science.aaf1204.
3. Lake et al. (2018). Nature Biotechnology, doi:10.1038/nbt.4038.
4. Lake et al. (2019). Nature Communications, doi:10.1038/s41467-019-10861-2.

Attachments

PDF

CG000315_ChromiumNex...

4.3MB

Guidelines

Full protocol is from 10X Genomics. All modifications are to the original protocol (Chromium Next GEM Single Cell 3' Reagent Kits v3.1 User Guide (Rev E) CG000315, support.10xgenomics.com)

Materials

MATERIALS

Chromium Next GEM Single Cell 3 Kit v3.1 **10x Genomics Catalog #**1000268

Chromium Next GEM Chip G Single Cell Kit, 48 rxns **10x Genomics Catalog #**1000120

Dual Index Kit TT Set A, 96 rxns **10x Genomics Catalog #**PN-1000215

Nuclease-free water
Ethanol (200 proof)
Dynabeads MyOne Silane (in Kit #1000268)
SPRI select reagent set (Beckman Coulter B23318)
50% glycerol
10% Tween 20
Low TE Buffer (10mM Tris-HCl, pH 8.0, 0.1 EDTA)
Qiagen buffer EB
Qubit dsDNA HS Assay Kit
PCR strip tubes with flat cap
LoBind 1.5 ml tubes
Qubit Assay tubes
10x Genomics recommends using only validated emulsion-safe pipette tips for all Single Cell protocols. (See 10x Protocol)

Isolate Nuclei

1 Prepare nuclei according to the protocol "Isolation of single nuclei from solid tissues" steps 1-14. **dx.doi.org/10.17504/protocols.io.bh26j8he**

2 Resuspend nuclei in 100 µL to 1 mL of PBS + 0.1% RNase Inhibitor (volume depends on target concentration)

3 Perform manual trypan blue nuclear exclusion counts on a hemacytometer.

4 Check nuclei integrity by light microscope concurrent with manual trypan exclusion counting. Nuclei should appear distinct, have rounded borders and the majority occurring as singlets. High clumping rates would indicate damaged nuclei and would require re-filtering using 30-µm CellTrics filter or exclusion.

5 Dilute nuclei stock to be in the 700-1200 nuclei per uL range in 50 µL total volume using the dilution guide

Note

If nuclei concentrations fall below target range, then centrifugation can be performed to increase nuclei concentrations to be within range
- Start with nuclei stock in PBS with 0.1% BSA
- Spin 500xg for 5 minutes
- Resusupend in appropriate volume PBS
- Use 10 uL to count; 50 uL for loading
-

QC cutoff: minimum of 10,000 nuclei (for 200 nuclei per uL in 50 uL total volume)

Safety information

Caution: BSA is necessary to avoid clumping and prevent nuclei loss from sticking to the tube during the spin down. DO NOT USE more than .01% BSA in nuclei stock

GEM Generation and Barcoding

6 Prepare Reagents for use

1. Equilibrate gel beads to room temperature for 00:30:00 .

2. Thaw, vortex, and centrifuge RT Reagent, Template Switch Oligo, and Reducing Agent B. Verify no precipitate in Reducing Agent B.
3. Centrifuge RT Enzyme C before adding to Master Mix.

7 Prepare Master Mix

1. Prepare on ice. Pipette mix 15x and centrifuge briefly.
2. Add 31.9 µL Master Mix to each tube of a PCR 8-tube strip on ice.

8 Load Chromium Single Cell G Chip

1. Assemble Next GEM Chromium Chip G in a 10X Chip Holder.
2. Must load chip in order according to row label for microfluidic channels to work properly: Row 1 → Row 2 → Row 3
3. Make sure NO bubbles are introduced while loading chip.
4. Dispense 50% glycerol solution into unused Chip Wells (if <8 samples used per chip)
   a. 75 µL into unused wells in Row 1
   b. 40 µL into unused wells in Row 2
   c. 45 µL into unused wells in Row 3

DO NOT add 50% glycerol solution to the bottom row of NO FILL wells. DO NOT use any substitute for 50% glycerol solution.

5. Use Cell Suspension Volume Calculator Table (see step 1.2 of Chromium Next GEM Single Cell 3' v3.1 protocol) to add the appropriate volume of nuclease-free water to Master Mix already in PCR 8-tube strip. Add corresponding volume of gently pipette-mixed single nuclei suspension to Master Mix. Total of 75 µL in each tube. Gently pipette mix, avoid introducing bubbles. DO NOT add nuclease free-water directly to single cell suspension, add instead to Master Mix.
6. Nuclei Loading
   a. Minimum: load 800 nuclei → target recovery 500 nuclei
   b. Maximum: load 1600 nuclei → target recovery 10000 nuclei
7. Load 70 µL Master Mix + Nuclei Suspension into the bottom center of each well in Row 1 without introducing bubbles.
8. Snap the Gel Bead strip into a 10X Vortex Adapter. Vortex 00:00:30. Remove the Gel Bead strip and flick sharply downward to ensure maximum recovery. Confirm there are no bubbles at the bottom of the tubes and liquid levels look even.
10. Load 50 µL gel beads into wells of Row 2. Only puncture the foil seal for gel bead tubes being used. Dispense slowly and without introducing bubbles.
11. Load 45 µL partitioning oil into each Row 3 by pipetting two aliquots of 140 µL.
12. Attach 10X gasket. Align the top-notch. Ensure gasket holes are aligned with the wells. Avoid touching the smooth gasket surface. Do not press down on the gasket. Run chip

immediately after loading the partitioning oil.

9 Run the Chromium Controller 18m

1. Press the eject button on the controller to eject tray.
2. Place the assembled chip with the gasket in the tray. Press the button to retract the tray.
3. Confirm the program on screen. Press the play button.
4. At completion of the run (~ 00:18:00), the controller will chime. Proceed immediately to the next step.

Firmware Version 4.0 or higher is required in the Chromium Controller or the Chromium Single Cell Controller used for this protocol.

10 Transfer GEMs

1. Chill strip tubes on ice.
2. Press the eject button to remove the chip.
3. Discard the gasket. Open the chip holder. Fold the lid back until it clicks to expose the wells at 45 degrees.
4. Check the volume in rows 1-2. Abnormally high volume in any well indicates a clog.
5. Slowly aspirate 100 uL GEMs from the lowest points of the Recovery Wells without creating a seal between the pipette tips and the wells.

Note

Inspect the GEMs in the pipette tip. GEMS should appear opaque and uniform across all channels. Excess partitioning oil (clear) in the pipette tips indicates a potential clog.

6. Slowly dispense (~ 00:00:20) GEMs into the tube strip on ice with the pipette tips against the sidewalls of the wells.

If multiple chips are run back-to-back, cap/cover the GEM-containing tube strip and place on ice for no more than 1 h.

11 GEM-RT Incubation 3d 0h 50m

1. Incubate in a thermocycler to complete reverse transcription (53 °C 00:45:00 , 85 °C 00:05:00 , 4 °C HOLD).
2. Store at 4 °C for up to 72:00:00 or at -20 °C for up to a week. Or proceed to the next step.

12 Post GEM-RT Cleanup & cDNA Amplification - Prepare Reagents for use (See 10X User Guide for details)

1. Thaw, vortex, and centrifuge a tube of Reducing Agent B and cDNA primers.
2. Maintain Amp Mix on ice after vortex and centrifuge
3. Thaw Cleanup Buffer at 65 °C for 00:10:00 with shaking at max rpm then cool to room temperature. Verify no visible crystals. Cool to RT.

13 Post GEM RT-Cleanup --Dynabead 17m

1. Add 125 µL Recovery Agent to each sample at room temperature. DO NOT MIX. Wait 00:02:00 .
2. Carefully aspirate 125 µL of pink oil phase from the bottom of the tube. DO NOT aspirate any aqueous sample.
3. Prepare Dynabeads Cleanup Mix (See 10X User Guide for detail)
4. Vortex and briefly centrifuge mixture. Add 200 µL to each sample. Pipette 10x to mix.
5. Incubate at room temperature for 00:10:00 ; pipette mix again 00:05:00 into incubation.
6. Prepare Elution Solution I (See User Guide for Detail). Vortex and centrifuge briefly.
7. Place sample on magnetic separator-HIGH until solution clears. Remove the supernatant (acqueous phase and Recovery Agent). Add 300 µL freshly prepared 80% ethanol. Wait 30 sec. Remove Ethanol. Repeat.Remove the ethanol.
8. Centrifuge briefly. Place on the magnet•Low. Remove remaining ethanol. Air dry for 1 min.
9. Remove from the magnet. Immediately add 35.5 µl Elution Solution I (prepared in step above). Pipette mix (pipette set to 30 µl) without introducing bubbles.
10. Incubate 2 min at room temperature.
11. Place on the magnet•Low until the solution clears. Transfer 35 µl sample to a new tube strip.

14 cDNA Amplification

1. Prepare cDNA Amplification Mix on ice (see 10x User Guide for details). Vortex and centrifuge briefly.
2. Add 65 µL cDNA Amplification Reaction Mix to 35 µL sample.
3. Pipette mix 15x to mix and centrifuge briefly.

4. Incubate in a thermocycler to amplify cDNA.

Note

- Use cycle number optimization table in 10x User Guide) for total number of cycles
- When using nuclei, increase the optimized number of cycles by one cycle
- Maximum: load 1600 nuclei - perform 12 cycles

15 cDNA Cleanup --SPRIselect

3d 0h 2m

1. Perform a 0.6X beads purification with SPRIselect reagent (60 µL)
2. Incubate x 5 minutes at RT.
3. Place on magent-HIGH until solution clears. Remove supernatent.
4. Add 200 µL freshly prepared 80% ethanol, wait 30 sec and remove ethanol. Repeat.
5. Centrifuge briefly. Place on magnet-LOW. Add 40.5 µL Qiagen Buffer EB. Pipette mix x 15x. Incubate 00:02:00 RT.
6. Place on magent-HIGH until solution clears. Transfer 40 µL to a new strip tube.
7. Store at 4 °C for up to 72:00:00 or at -20 °C for up to 4 weeks. Or proceed to the next step.

16 cDNA QC & Quantification

1. Run TapeStation to obtain concentration and size
2. Expected size range for amplified cDNA: 400 to 2500 bp
3. cDNA total yield range: 80 to 1000 ng

Note

QC Cutoff: Minimum cDNA total yield of 80 ng

3' Gene Expression Library Construction

17 Prepare Reagents for use

1. Thaw, vortex, and centrifuge Fragmentation Buffer, Adaptor Oligos, Ligation Buffer, SI Primer. Verify no precipitate.
2. Maintain on ice Fragmentation Enzyme, DNA Ligase, and Amp Mix.

18 Fragmentation, End Repair & A-tailing

1. Prepare Fragmentation Mix on ice (See User Manual for volume details). Pipette mix and centrifuge briefly.
2. Transfer 10 µL purified cDNA to a new strip tube

Note

Note that 10 uL (25%) cDNA sample is sufficient for generating 3' Gene Expression Library. The remaining 30 uL (75%) cDNA sample can be stored at 4 C for up to 72 hours or at -20 C for up to 4 weeks for generating additional 3' Gene Expression Libraries.

3. Add 25 µL Buffer EB to each sample
4. Add 15 µL Fragmentation Mix to each sample
5. Pipette mix, centrifuge briefly, and transfer to pre-cooled block. “SKIP” hold step to initiate the protocol

19 Post Fragmentation, End Repair & A-tailing Double Sided Size Selection --SPRIselect 12m

1. Vortex to resuspend the SPRIselect reagent. Add 30 µL SPRIselect reagent (0.6X) to each sample and pipette mix 15x.
2. Incubate 00:05:00 at room temperature.
3. Centrifuge briefly.
4. Place sample on magnetic separator-HIGH until solution clears. DO NOT discard supernatant.
5. Transfer 75 µL supernatant to a new tube strip tube.
6. Vortex to resuspend the SPRIselect reagent. Add 10 µL SPRIselect reagent (0.8X) to each transferred supernatent and pipette mix 15x.
7. Incubate 00:05:00 RT
8. Place sample on magnetic separator-HIGH until solution clears.
9. Remove 80 µL supernatant. DO NOT discard any beads.
10. Wash twice with 125 µL freshly prepared 80% ethanol.
11. Centrifuge briefly. Place on magent-LOW until solution clears. Remove remaining ethanol, do not overdry.

12. Remove from magnet. Add 50.5 µL Qiagen Buffer EB, pipette mix x 15. Incubate x 00:02:00 RT.
13. Place on magent-HIGH until clear. Transfer 50 µL to a new strip tube.

20 Adaptor Ligation 15m

1. Prepare Adaptor Ligation Mix on ice. Pipette mix and centrifuge briefly.
2. Add 50 µL Adaptor Ligation Mix to sample. Pipette mix 15x. Centrifuge briefly.
3. Incubate in a thermocycler to ligate adaptor. (20 °C x 00:15:00 , 4 °C Hold)

21 Post Ligation Cleanup --SPRIselect

1. Perform a 0.8X beads purification with SPRIselect reagent (add 80 µL reagent to each sample, pipette-mix x 15x, incubate 5 min at RT)
2. Place on magnet-HIGH until solution clears, remove supernatant. Wash twice with freshly prepared 80% ethanol (add 200 µL, wait 30 sec, remove ethanol and repeat)
3. Centrifuge briefly. Place on magent-LOW. Remove remaining ethanol and air dry up to 2 min.
4. Remove from magnet. To elute, add 30.5 µL Qiagen Buffer EB. Pipette mix x 15. Incubate 2 min at RT. Place on magent-LOW until solution clears.
5. Transfer 30 µL to a new strip tube.

22 Sample Index PCR

1. Choose the appropriate sample index sets to ensure that no sample indices overlap in a multiplexed sequencing run.
2. Prepare Sample Index PCR Mix on ice. Pipette mix and centrifuge briefly.
3. Add 50 µL Amp Mix to the 30 µL sample already in the new strip tube.
4. Add 20 µL of an individual Dual Index TT Set A to each sample. Record well assignment. Pipette-mix x5 and centrifuge briefly.
5. Incubate in a thermocycler to incorporate sample indices.

Note

Use cycle number recommendation table (see Chromium Next GEM Single Cell 3' v3.1(Dual Index) User Guide, Rev E) for total number of cycles; recommended number of cycles is based on cDNA input

6. Store at 4 °C for up to 72:00:00. Or proceed to the next step.

23 Post Sample Index PCR Double Sided Size Selection --SPRIselect

1. Vortex to resuspend the SPRIselect reagent. Add 60 µL SPRIselect reagent (0.6X) to each sample and pipette mix 15x.
2. Incubate 00:05:00 at room temperature.
3. Centrifuge briefly
4. Place sample on magnetic separator. DO NOT discard supernatant.
5. Transfer 150 µL supernatant to a new tube strip tube.
6. Vortex to resuspend the SPRIselect reagent. Add 20 µL SPRIselect reagent (0.8X) to each sample and pipette mix 15x.
7. Incubate 00:05:00 at room temperature.
8. Centrifuge briefly
9. Place sample on magnetic separator-HIGH until solution clears.
10. Remove 165 µL supernatant. DO NOT discard any beads.
11. With tube still on magnet, add 200 µL freshly prepared 80% ethanol to pellet. Wait 30 sec and then remove ethanol.
12. Repeat Step 11 for total of 2 washes then centrifuge briefly. Place on magnet-LOW. Remove remaining ethanol.
13. Remove from magnet. Add 35.5 µL Qiagen Buffer EB. Pipette-mix x 15)
14. Incubate 2 min at RT.
15. Place on magnet-LOW until solution clears then transfer 35 µL to a new strip tube.
16. Store at 4 °C for up to 72:00:00 or at -20 °C for long-term storage.

Note

QC Post Library Construction:

- **Quantify Library (e.g. using Qubit dsDNA HS Assay)**
- **Estimate Library size range (e.g. using TapeStation or BioAnalyzer) - expected size range of 300 to 800 bp, average: 475 bp**
- If additional peaks below 200 bp are observed, see User Guide v 3.1 for recommendations

Sequencing

24 MiSeq Sequencing - **QC for estimation of library quality and number of nuclei captured**

1. Paired End, Single Indexing
    a. Read 1: 28 cycles
    b. i7 Index: 8 cycles
    c. i5 Index: 0 cycles
    d. Read 2: 91 cycles
2. Library Loading
    a. 10X recommended Loading concentration: 11 pM
    b. Optional: 1% PhiX
3. Output
    a. 22-25 million reads

25 NovaSeq Sequencing (target - 25,000-50,000 reads per nucleus)

1. Paired End, Single Indexing
    a. Read 1: 28 cycles
    b. i7 Index: 8 cycles
    c. i5 Index: 0 cycles
    d. Read 2: 91 cycles
2. Library Loading
    a. 10X recommended Loading concentration: 300 pM
    b. Optional: 1% PhiX
3. Output
    a. SP: 650–800 million reads
    b. S1: 1.3–1.6 billion reads
    c. S2: 3.3 –4.1 billion reads

d. S4: 8-10 billion reads

Cell Ranger Mapping and Analysis Pipeline

26 Generate pre-mRNA reference

Note

IMPORTANT: for nuclei experiments, we need to use the reference files with pre-mrna in order to count intronic reads in the UMI counts

b. Generate Sample Sheet

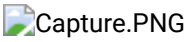
Note

Use sample sheet generator provided by 10X Genomics to generate a "SampleSheet.csv":**Sample Sheet Generator**

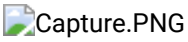
c. Generate fastq files

Note

Use **mkfastq** command



d. Run count for each sample



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5. HuBMAP Lung TMC UCSD & URMC Protocol Overall for Multimodal Single Cell/Nucleus Assays DOI **dx.doi.org/10.17504/protocols.io.4r3l27qpqg1y/v1**. **Gloria S Pryhuber**[1],**Elizabeth Duong**[2],**Blue Lake**[2],**Cory Poole**[1],**Heidie Huyck**[1],**Ravi Misra**[1],Xin Sun[2],**Jim Hagood**[3],**Dinh H Diep**[2], Kun Zhang[21] University of Rochester Medical Center; 2University of California San Diego; [3]University of North Carolina at Chapel Hill