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SenNet URM C 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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Cellular Senescence Net...



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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



CG000315_ChromiumNe

X...

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 μ L 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- μ m CellTrics filter or exclusion.
- 5 Dilute nuclei stock to be in the 700-1200 nuclei per μ L range in  50 μ L 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
- Resuspend in appropriate volume PBS
- Use 10 μ L to count; 50 μ L for loading
-

QC cutoff: minimum of 10,000 nuclei (for 200 nuclei per μ L in 50 μ L 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 $\rightarrow$ Row 2 $\rightarrow$ 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 $\rightarrow$ target recovery 500 nuclei
- b. Maximum: load 1600 nuclei $\rightarrow$ 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 μ L Recovery Agent to each sample at room temperature. DO NOT MIX. Wait  00:02:00 .
 2. Carefully aspirate  125 μ L 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 μ L 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 (aqueous phase and Recovery Agent). Add  300 μ L 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 μ L Elution Solution I (prepared in step above). Pipette mix (pipette set to 30 μ L) without introducing bubbles.
 10. Incubate 2 min at room temperature.
 11. Place on the magnet•Low until the solution clears. Transfer 35 μ L 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 magnet-HIGH until solution clears. Remove supernatant.
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 magnet-HIGH until solution clears. Transfer  40 μL to a new strip tube.
7. Store at  4 $^{\circ}\text{C}$ for up to  72:00:00 or at  -20 $^{\circ}\text{C}$ 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 μL (25%) cDNA sample is sufficient for generating 3' Gene Expression Library. The remaining 30 μL (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
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 supernatant 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.

12m



10. Wash twice with  125 μL freshly prepared 80% ethanol.
11. Centrifuge briefly. Place on magnet-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 magnet-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 $^{\circ}\text{C}$ x  00:15:00 ,  4 $^{\circ}\text{C}$ 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 magnet-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 magnet-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 $^{\circ}\text{C}$ 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

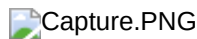
Note

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



 Capture.PNG

Protocol references

References

1. Chromium Next GEM Single Cell 3' Reagent Kits v3.1(Dual Index) User Guide. Document Number: CG000315. October 2022.
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