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LungMAP2 URMC cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq) protocol

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

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We use this protocol and it's working

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Abstract

The LungMAP program has been working for over a decade to provide the lung community with a comprehensive resource for the lung research community, including reference data sets and protocols [1]. The University of Rochester has served as the Human Tissue Core and worked extensively with Research Center lab groups. We have established protocols for procuring human tissue including, but not limited to lung, and processing them for a variety of downstream analysis modalities including the generation of single cell suspensions [2, 3, 4]. We have successfully utilized singe cells suspensions to generate single cell CITESeq data utilizing the 10X Genomics Single Cell 3' (v3.1) RNA sequencing platform [5-7]. This protocol describes the steps necessary for preparing generating sequencing data following staining single cells with ADT-tagged antibodies (including library preparation, sequencing steps, and mapping steps).

References

1. **https://www.lungmap.net/about-lungmap/lungmap-objectives/**

2. 602.2 Donor Acceptance Criteria for URMC HTC SenNet Inclusion **dx.doi.org/10.17504/protocols.io.biz7kf9n**.

3. 603.3 & 604.5_URMC_HTC_Lung and Lobe Processing for SenNet
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4. 702.B.3 URMC HTC Lung Tissue Digestion SOP + Worksheet 052820
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5. 709.1 Staining of Dissociated Lung Cells for Cellular Indexing of Transcriptomes and Epitopes by Sequencing (CITE sequencing) V.2 **dx.doi.org/10.17504/protocols.io.e6nvw1jk7lmk/v2**

6. TotalSeq™-A Antibodies and Cell Hashing with 10x Single Cell
3' Reagent Kit v3 3.1 Protocol **dx.doi.org/10.17504/protocols.io.8aahsae**

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

Attachments

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) and TotalSeq™-A Antibodies and Cell Hashing with 10x Single Cell 3' Reagent Kit v3.1 (Dual Index) Protocol (https://www.biolegend.com/en-us/protocols/totalseq-a-dual-index-protocol).

Troubleshooting

GEM Generation and Barcoding

1 **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.

2 **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.

3 **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. Cell Loading

a.load 32,000 nuclei → target recovery 20,000

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.

4 **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.

5 **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.

6 **GEM-RT Incubation** 3d 0h 50m

1. Set up the cDNA reaction.

2. Incubate in a thermocycler to complete reverse transcription (53 °C 00:45:00 , 85 °C 00:05:00 , 4 °C HOLD).
3. 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.

7 **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.

8 **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.

9 **cDNA Amplification with the addition of ADT and HTO primers**

1. Prepare cDNA Amplification Mix on ice (see 10x User Guide for details and Step 16 of TotalSeq™-A Antibodies and Cell Hashing with 10x Single Cell 3' Reagent Kit v3.1

(Dual Index) Protocol dx.doi.org/10.17504/protocols.io.8aahsae). Vortex and centrifuge briefly.

2. Add 65 µL cDNA Amplification Reaction Mix including ADT and HTO primers 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

10 **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. ***Transfer 70 ul of supernatant to a new tube. Note: the ADT/HTO material will be in the supernatant and the cellular RNA expression pool will be on the SPRI beads. Library construction will be completed in parallel with 3' Gene Expression Library Construction***
4. Add 200 µL freshly prepared 80% ethanol, wait 30 sec and remove ethanol. Repeat.
5. Place on magent-HIGH until solution clears. Transfer 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.

11 **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

12 **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.

13 **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

14 **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.

15 **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)

16 **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.

17 **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.

18 **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

Amplify Dual-Indexed ADT Sequencing Library

19 Aliquot 22.5 uL of each sample (ADT cDNA) to a new, labeled 0.5-mL strip tube.

20 Add 25 uL of 2X Q5 HotStart HiFi Master Mix to each sample.

21 Add 2.5 uL of appropriate indexed primer-pair (10uM each primer) to each sample. Mix by pipetting and spin briefly.

22

Step	Temperature	Time
1	95°C	Hold
2	95°C	3:00
3	95°C	0:20
4	60°C	0:30
5	72°C	0:20
6	Complete 12 cycles	
7	72°C	5:00
8	4°C	Hold

23 Add 60 ul AMPure beads (1.2X ratio) to each sample and pipette to mix 10X

24 Incubate 5 minutes at room temperature.

25 Place tube on magnet and wait 1 minute until solution is clear.

26 Carefully remove and discard the supernatant.

27 Add 200 µl 80% Ethanol to the tube without disturbing the pellet and stand for 30 seconds (first Ethanol wash).

28 Carefully remove and discard the ethanol wash.

29 Add 200 µl 80% Ethanol to the tube without disturbing the pellet and stand for 30 seconds (second Ethanol wash).

30 Carefully remove and discard the ethanol wash.

31 Centrifuge tube briefly and return it to magnet.

32 Remove and discard any remaining ethanol and allow the beads to air dry for 2 minutes.

33 Resuspend beads in 32 µl EB.

34 Pipette mix vigorously and incubate at room temperature for 5 minutes.

35 Place tube on magnet and transfer 30 uL supernatant to low-bind tube.

36 Quantify library by standard methods (Qubit & BioAnalyzer). ADT library will be around 210 bp.

Sequencing

37 NovaSeq Sequencing (target - 100,000 reads per nucleus)

1. Paired End, Dual Indexing
    a. Read 1: 28 cycles
    b. i7 Index: 10 cycles
    c. i5 Index: 10 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

38 Create a feature reference file corresponding to the antibody panel and library mapping file corresponding to the file paths for the ADT and GEX libraries. Run cellranger count, incorporating both of these files and the combined Human+COVID reference

39 Create fastq from bcl files using cellranger mkfastq with default parameters

40 Insert 10X provided well indices into Clarity LIMS generated sample sheet.

41 Create combined human and COVID reference by downloading the Human 2020-A reference from https://www.10xgenomics.com/support/software/cell-ranger/latest/release-notes/cr-reference-release-notes#2020-a. Download the full COVID sequence from https://www.ncbi.nlm.nih.gov/nuccore/MT547814. Append the COVID fasta onto the human genome fasta. Create a record within the human GTF file corresponding to the full COVID sequence.

Protocol references

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6. TotalSeq™-A Antibodies and Cell Hashing with 10x Single Cell
3' Reagent Kit v3 3.1 Protocol **dx.doi.org/10.17504/protocols.io.8aahsae**

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

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