Jan 27, 2026

Methods to generate single-nucleus RNA-seq libraries from lung cancer samples

DOI
https://dx.doi.org/10.17504/protocols.io.36wgqpk9yvk5/v1

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Protocol Citation: Jixiang Zhang, Sean K. Simmons, Nathan Haywood, Xian Adiconis, Joshua Z. Levin 2026. Methods to generate single-nucleus RNA-seq libraries from lung cancer samples. **protocols.io** https://dx.doi.org/10.17504/protocols.io.36wgqpk9yvk5/v1

Protocol status: Working
We use this protocol and it's working

Created: November 05, 2025

Last Modified: January 27, 2026

Protocol Integer ID: 231590

Keywords: single-nucleus RNA-seq, lung, Nuclei isolation, chromium nuclei isolation kit cg000505, seq libraries from fresh frozen lung cancer sample, seq libraries from lung cancer sample, nucleus rna, nuclei isolation part, fresh frozen lung cancer sample, 10x genomic, lung cancer sample, cell rna, seq library, seq library part, seq platform, user guide of chromium

Funders Acknowledgements:
NIH/National Cancer Institute
Grant ID: 1U2CCA233238-01

Abstract

This protocol describes the generation of single-nucleus RNA-seq libraries from fresh frozen lung cancer samples using the 10x Genomics single-cell RNA-seq platform. It is based primarily on vendor-provided protocols with minor modifications. The nuclei isolation part is based on "User Guide for the Chromium Nuclei Isolation Kit CG000505 Rev A" (10x Genomics) and single-nucleus RNA-seq library part is based on "User guide of Chromium Next GEM Single Cell 3' Reagent Kits v3.1 (Dual Index) (CG000315 Rev F)"

Guidelines

Tissue collection for this protocol requires prior approval by the users' Institutional Ethics Board or equivalent ethics committee.

Materials

Chromium Nuclei Isolation Kit with RNase Inhibitor (#1000494, 10x Genomics)
Chromium Next GEM Chip G Single Cell Kit (#1000120, 10x Genomics)
Dual Index Kit TT Set A (#1000215, 10x Genomics)
Chromium™ Next GEM Single Cell 3' Kit v3.1 (#1000268, 10x Genomics)
Library Construction Kit (#1000190, 10x Genomics)
10x Vortex Adapter (#120251, 10x Genomics)
Chromium Next GEM Secondary Holder (#1000195, 10x Genomics)
10x Magnetic Separator (#120250, 10x Genomics)
Bio-Rad T100 Thermal Cycler
TempAssure PCR 8-tube strip (#1402-4700, USA Scientific)
SPRIselect Reagent Kit (B23318, Beckman Coulter Life Sciences)
10% Tween 20 (#1662404, Bio-Rad)
Buffer EB (#19086, Qiagen)
High Sensitivity DNA Kit (#5067-4626, Agilent)
BSA (#A2153, Sigma-Aldrich)
AOPI (#CS2-0106, Nexcelom)
CHT4 Counting Chambers (#CHT4-SD100-002, Revvity)
Koptec Pure Ethanol-200 Proof (#V1016, Decon Labs)
10x PBS (#AM9625, Ambion)

Safety warnings

For hazard information and safety warnings, please refer to the MSDSs (Material Safety Data Sheets).

Before start

Pre-chill centrifuge to 4 °C and place reagents and tubes on ice

Part 1: Nuclei isolation

2h 7m 23s

1 Pre-chill centrifuge to 4 °C and place reagents and tubes on ice as indicated in the Get Started guide. Label tops and sides of tubes, as well as tops of spin columns, before starting protocol. 80%ethanol should be prepared fresh each time. 10m

1.1 *Perform all protocol steps on ice and centrifugation steps at* 4 °C .

2 Prepare buffers according to Buffer Preparation section and place On ice . 20m

Lysis Buffer

Lysis Buffer (500 µl/rxn) *Add reagents in the order listed*	PN	1X+10% (µl)	4X + 10% (µl)	8X + 10% (µl)
Lysis Reagent	2000558	550	2,200	4,400
Reducing Agent B	2000087	0.55	2.2	4.4
Surfactant A	2000559	5.5	22	44
Total	–	556.05	2,224.2	4,448.4

Debris Removal Buffer

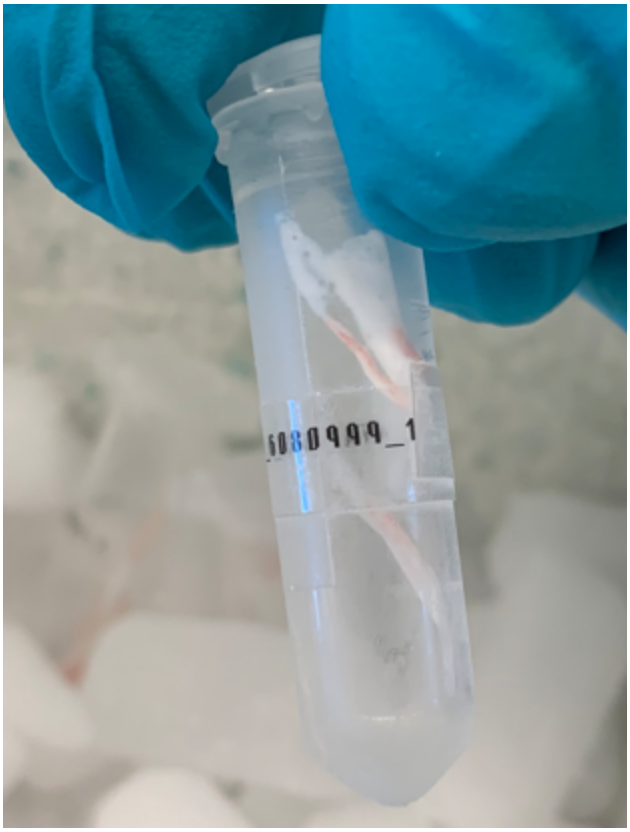
Debris Removal Buffer (500 µl/rxn) *Add reagents in the order listed*	PN	1X+10% (µl)	4X + 10% (µl)	8X + 10% (µl)
Debris Removal Reagent	2000560	550	2,200	4,400
Reducing Agent B	2000087	0.55	2.2	4.4
Total	–	550.55	2,202.2	4,404.4

Wash and Resuspension Buffer

Wash and Resuspension Buffer (3 ml/rxn) *Add reagents in the order listed*	PN	1X+10% (µl)	4X + 10% (µl)	8X + 10% (µl)
1X PBS (not provided)	-	2,887.5	11,550	23,100
10% BSA (not provided)	-	330	1,320	2,640
RNase Inhibitor	2000565	82.5	330	660
Total	–	3,300	13,200	26,400

3 Place Sample Dissociation Tube(s) On ice 5m

4 Obtain frozen tissue and place immediately on dry ice. 5m
Example of sliced lung sample as input. The tissue was frozen fresh and not fixed. The weight was around 3 mg.



Sample information

5 Transfer frozen tissue to pre-chilled Sample Dissociation Tube. 10m

6 Transfer Sample Dissociation Tubes(s) to wet ice. Add 200 µL Lysis Buffer to Sample Dissociation Tube. Dissociate tissue with plastic pestle until homogeneous. For multiple samples, add Lysis Buffer to each sample and then proceed to dissociate one at a time.
Perform tissue dissociation on ice. Use one pestle per sample. DO NOT discard pestles until nuclei isolation process is complete. 10m

7 Add 300 µL Lysis Buffer. Pipette mix 10x. If pipette tip clogs with unhomogenized tissue, continue to dissociate tissue with the pestle until able to pipette mix. 2m

8 Incubate on ice for 00:10:00 . 10m

9 Pipette dissociated tissue into pre-chilled Nuclei Isolation Column assembled with Collection Tube using pipette set to 500 µL . Transfer all liquid from Dissociation Tube to Nuclei Isolation Column to avoid nuclei loss. 3m

10 Centrifuge at 16000 rcf, 4°C, 00:00:20 . 20s

11 Discard column. Flowthrough in the Collection Tube will contain nuclei. Vortex 00:00:10 at 3200 rpm or max speed to resuspend nuclei. Flowthrough may appear opaque or cloudy. This is normal and it is safe to proceed. 5m

12 Centrifuge Collection Tube for 500 rcf, 4°C, 00:03:00 . Carefully discard supernatant using a pipette without disturbing nuclei pellet. Leave behind a small fraction (~ 200 µL) of supernatant if nuclei pellet is not apparent. 3m

13 Resuspend nuclei pellet in 500 µL Debris Removal Buffer. Gently pipette mix at least 15x, continuing until no pellet can be visualized. 2m

14 Centrifuge at 700 rcf, 4°C, 00:10:00 . Carefully discard supernatant using a pipette without disturbing nuclei pellet. Leave behind a small fraction (~ 200 µL) of 10m

supernatant if nuclei pellet is not apparent.

15 Resuspend nuclei pellet in 1 mL of Wash Buffer. 1m

16 Centrifuge at 500 rcf, 4°C, 00:05:00 . Carefully discard supernatant using a pipette without disturbing nuclei pellet. Leave behind a small fraction (~ 200 µL) of supernatant if nuclei pellet is not apparent. 5m

17 Centrifuge at 500 rcf, 4°C, 00:05:00 . Carefully discard as much supernatant as possible using a pipette without disturbing nuclei pellet. Leave behind a small remaining volume if the pellet is not visible. 5m

18 Resuspend nuclei pellet in 80 µL Resuspension Buffer, depending on expected recovery for input tissue type and mass. Gently pipette mix 15x using an appropriate pipette for resuspension volume.
Resuspend in a low volume if nuclei yield is expected to be low or is unknown. DO NOT resuspend in a volume less than 50 µL . 1m

19 Vortex nuclei for 00:00:03 at 3200 rpm or max speed immediately prior to counting to ensure accurate nuclei count. 3s

20 Determine nuclei concentration using AOPI and Cellometer K2 cell counter. 20m

Part 2: GEM generation and cDNA amplification 1h 55m

21 Prepare Master Mix On ice . Pipette mix 15x and centrifuge briefly. 10m

Master Mix *Add reagents in the order listed*	PN	1X (µl)	4X + 10% (µl)	8X + 10% (µl)
RT Reagent B	2000165	18.8	82.7	165.4
Template Switch Oligo	3000228	2.4	10.6	21.1
Reducing Agent B	2000087	2.0	8.8	17.6
RT Enzyme C	2000085/ 2000102	8.7	38.3	76.6
Total	-	31.9	140.4	280.7

22 Add corresponding volume of single cell suspension to Master Mix (to reach the number of 20,000 nuclei). Total of 75 µL in each tube. 10m

23 Gently pipette mix the cell suspension before adding to the Master Mix. 2m

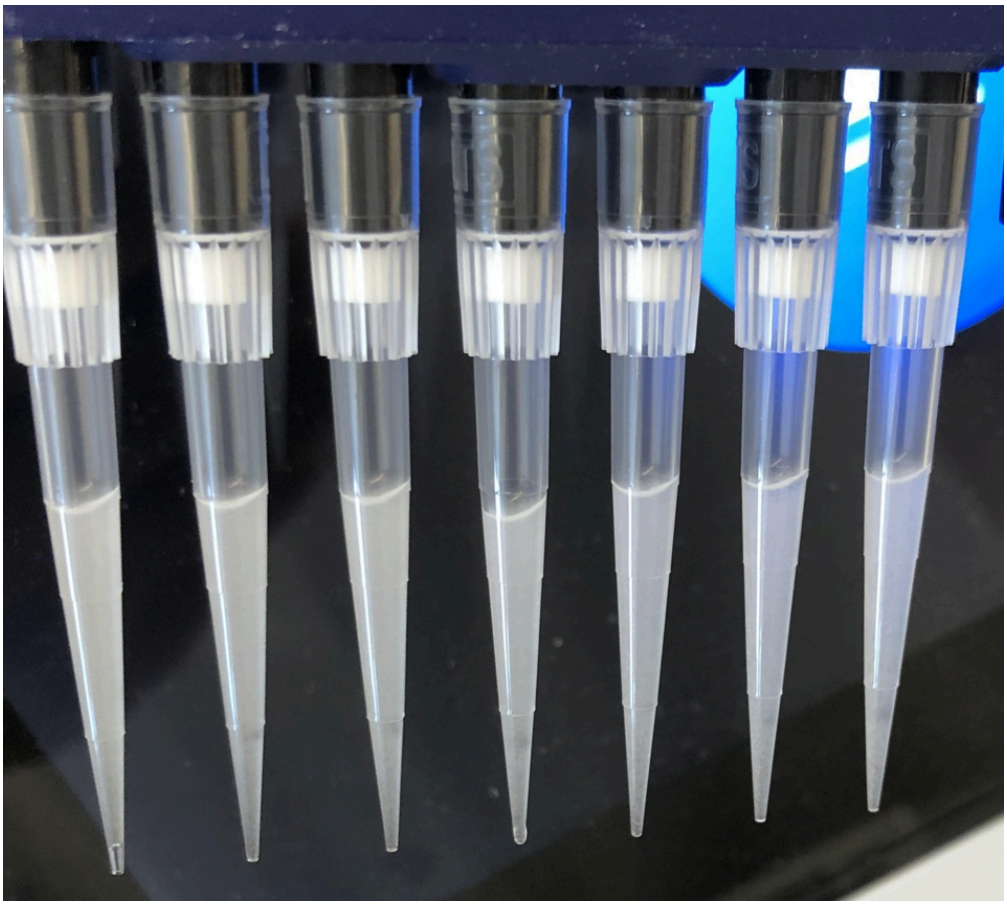
24 Gently pipette mix the Master Mix + Cell Suspension. Using the same pipette tip, dispense 70 µL Master Mix + Cell Suspension into the bottom center of each well in row labeled **1** without introducing bubbles. 2m

25 Load 50 µL Gel Beads, and 45 µL Partitioning Oil in row labeled **2** and **3** of Chip G, separately. 5m

26 Run the chip in the Chromium Controller to generate Gel Bead-in-emulsion (GEM). 30m

27 Transfer the GEMs into a PCR tube, 1m

Representative GEM generation:



GEM generation

28 Incubate in a thermal cycler with the following protocol. 55m
Lid Temperature: 53 °C , Reaction Volume: 125 µL , Run Time: 00:55:00
Step 1: 53 °C for 00:45:00
Step 2: 85 °C for 00:05:00
Step 3: 4 °C Hold

29 Store at 4 °C for up to 72 h or at -20 °C for up to a week or proceed to the next step.

30 Add 125 µL Recovery Agent to each sample at room temperature. DO NOT pipette mix or vortex the biphasic mixture. Wait 00:02:00 . 2m

31 Slowly remove and discard 125 µL Recovery Agent/Partitioning Oil (pink) from the bottom of the tube. DO NOT aspirate any aqueous sample. 1m

32 Prepare Dynabeads Cleanup Mix. 3m

Dynabeads Cleanup Mix	PN	1X (µl)	4X + 10% (µl)	8X + 10% (µl)
Cleanup Buffer	2000088	182	801	1602
Dynabeads MyOne SILANE	2000048	8	35	70
Reducing Agent B	2000087	5	22	44
Nuclease-free Water		5	22	44
Total		200	880	1760

33 Vortex and add 200 µL to each sample. Pipette mix 10x (pipette set to 200 µL). 2m

34 Incubate 00:10:00 at room temperature (keep caps open). Pipette mix again at ~ 00:05:00 after start of incubation to resuspend settled beads. 15m

35 Prepare Elution Solution I. Vortex and centrifuge briefly. 3m

Elution Solution I *Add reagents in the order listed*	PN	1X (µl)	10X (µl)
Buffer EB	-	98	980
10% Tween 20	-	1	10
○ Reducing Agent B	2000087	1	10
Total	-	100	1000

36 At the end of 00:10:00 incubation, place on a 10x Magnetic Separator•High position (magnet•High) until the solution clears. 10m

37 Remove the supernatant (aqueous phase and Recovery Agent). 2m

38 Wash with 300 µL 80% ethanol and followed with 200 µL 80% ethanol. 5m
Note: 80% ethanol should be prepared fresh each time.

39 Elute with 35.5 µL Elution Solution I. 5m

40 Prepare cDNA Amplification Mix on ice. Vortex and centrifuge briefly. 5m

cDNA Amplification Reaction Mix *Add reagents in the order listed*	PN	1X (µl)	4X + 10% (µl)	8X + 10% (µl)
○ **Amp Mix** Retrieve from Single Cell 3' GEM Kit	2000047/ 2000103	50	220	440
● **cDNA Primers**	2000089	15	66	132
Total	-	**65**	**286**	**572**

41 Add 65 µL cDNA Amplification Reaction Mix to 35 µL sample. 1m

42 Pipette mix 15x (pipette set to 90 µL). Centrifuge briefly. 1m

43 Incubate in a thermal cycler with the following protocol (12 PCR cycles). 50m 35s
Lid Temperature: 105 °C, Reaction Volume: 100 µL, Run Time: ~ 00:45:00
Step 1: 98 °C for 00:03:00
Step 2: 98 °C for 00:00:15
Step 3: 63 °C for 00:00:20
Step 4: 72 °C for 00:01:00
Step 5: Go to Step 2, for total 12 of cycles
Step 6: 72 °C for 00:01:00
Step 7: 4 °C Hold

44 Store at 4 °C for up to 72:00:00 or -20 °C for ≤1 week, or proceed to the next step.

45 cDNA Cleanup - SPRIselect

45.1 Vortex to resuspend the SPRIselect reagent. Add 60 µL SPRIselect reagent (0.6X) to each sample and pipette mix 15x (pipette set to 150 µL). 1m

45.2 Incubate 00:05:00 at room temperature 5m

45.3 Place on the magnet•High until the solution clears 1m

45.4 Remove the supernatant.

45.5 Add 200 µL 80% ethanol to the pellet. Wait 00:00:30. 30s

45.6 Remove the ethanol.

45.7 Repeat steps 45.5 and 45.6 for a total of 2 washes. 3m

45.8 Centrifuge briefly and place on the magnet•Low.

45.9 Remove any remaining ethanol. Air dry for 00:02:00. DO NOT exceed 00:02:00 as this will decrease elution efficiency. 2m

45.10 Remove from the magnet. Add 40.5 µL Buffer EB. Pipette mix 15x (pipette set to 35 µL).

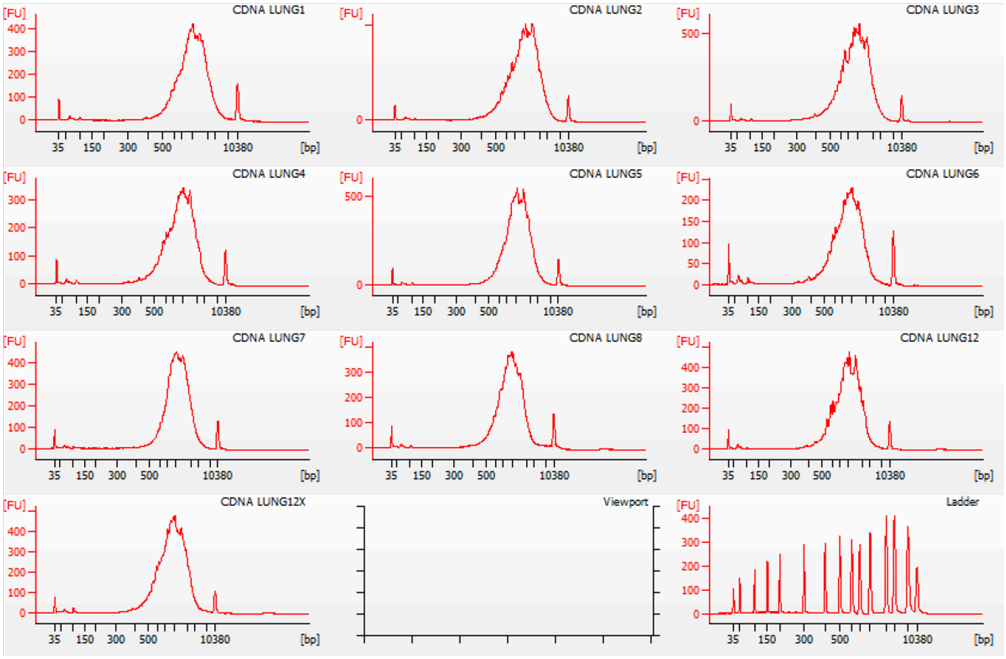
45.11 Incubate 00:02:00 at room temperature. 2m

45.12 Place the tube strip on the magnet•High until the solution clears.

45.13 Transfer 40 µL sample to a new tube strip.

45.14 Store at 4 °C for up to 72:00:00 or at -20 °C for up to 672:00:00, or proceed to the next step.

46 cDNA QC and Quantification: Run 1 µL sample on an Agilent Bioanalyzer High Sensitivity chip. 1h
Representative cDNA traces



cDNA traces

Part3: Gene Expression Dual Index Library Construction 6h 2m

47 **Fragmentation, End Repair & A-tailing**

47.1 Prepare a thermal cycler with the following incubation protocol. 2m
Lid Temperature: 65 °C, Reaction Volume: 50 µL, Run Time: ~ 00:35:00
Step 1: 4 °C for Hold
Step 2: 32 °C for 00:05:00
Step 3: 65 °C for 00:30:00
Step 4: 4 °C for Hold

47.2 Vortex Fragmentation Buffer. Verify there is no precipitate 2m

47.3 Prepare Fragmentation Mix on ice. Pipette mix and centrifuge briefly 2m

Fragmentation Mix *Add reagents in the order listed*	PN	1X (µl)	4X + 10% (µl)	8X + 10% (µl)
Fragmentation Buffer	2000091	5	22	44
Fragmentation Enzyme	2000090/ 2000104	10	44	88
Total	-	15	66	132

47.4 Transfer ONLY 10 µL purified cDNA sample from Pellet Cleanup to a tube strip. Note that only 10 µL (25%) cDNA sample transfer 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 h or at 20 °C for up to 4 weeks for generating additional 3' Gene Expression libraries. 1m

47.5 Add 25 µL Buffer EB to each sample. 1m

47.6 Add 15 µL Fragmentation Mix to each sample 1m

47.7 Pipette mix 15x (pipette set to 35 µL) on ice. Centrifuge briefly. 1m

47.8 Transfer into the pre-cooled thermal cycler (4 °C) and press “SKIP” to initiate the protocol. 40m

48 Post Fragmentation, End Repair & A-tailing Double Sided Size Selection – SPRIselect 40m

48.1 Vortex to resuspend SPRIselect reagent. Add 30 µL SPRIselect (0.6X) reagent to each
sample. Pipette mix 15x (pipette set to 75 µL).

48.2 Incubate 00:05:00 at room temperature 5m

48.3 Place on the magnet•High until the solution clears. DO NOT discard supernatant. 2m

48.4 Transfer 75 µL supernatant to a new tube strip. 1m

48.5 Vortex to resuspend SPRIselect reagent. Add 10 µL SPRIselect reagent (0.8X) to each transferred supernatant. Pipette mix 15x (pipette set to 80 µL). 1m

48.6 Incubate 00:05:00 at room temperature. 5m

48.7 Place on the magnet•High until the solution clears. 2m

48.8 Remove 80 µL supernatant. DO NOT discard any beads. 1m

48.9 Add 125 µL 80% ethanol to the pellet. Wait 00:00:30 . 30s

48.10 Remove the ethanol.

48.11 Repeat steps 48.9 and 48.10 for a total of 2 washes. 5m

48.12 Centrifuge briefly. Place on the magnet•Low until the solution clears. Remove remaining ethanol. DO NOT over dry to ensure maximum elution efficiency. 1m

48.13 Remove from the magnet. Add 50.5 µL Buffer EB to each sample. Pipette mix 15x (pipette set to 45 µL). 1m

48.14 Incubate 00:02:00 at room temperature. 2m

48.15 Place on the magnet•High until the solution clears. 2m

48.16 Transfer 50 µL sample to a new tube strip. 1m

49 Adaptor Ligation 20m

49.1 Prepare Adaptor Ligation Mix. Pipette mix and centrifuge briefly. 3m

Adaptor Ligation Mix *Add reagents in the order listed*	PN	1X (µl)	4X + 10% (µl)	8X + 10% (µl)
Ligation Buffer	2000092	20	88	176
DNA Ligase	220110/ 220131	10	44	88
Adaptor Oligos	2000094	20	88	176
Total	-	**50**	**220**	**440**

49.2 Add 50 µL Adaptor Ligation Mix to 50 µL sample. Pipette mix 15x (pipette set to 90 µL). Centrifuge briefly. 1m

49.3 Incubate in a thermal cycler with the following protocol. 15m
Lid Temperature: 30 °C , Reaction Volume: 100 µL , Run Time: ~ 00:15:00
Step 1: 20 °C for 00:15:00
Step 2: 4 °C for Hold

50 Post Ligation Cleanup – SPRIselect 20m

50.1 Vortex to resuspend SPRIselect Reagent. Add 80 µL SPRIselect Reagent (0.8X) to each 1m
sample. Pipette mix 15x (pipette set to 150 µL).

50.2 Incubate 00:05:00 at room temperature. 5m

50.3 Place on the magnet•High until the solution clears. 2m

50.4 Remove the supernatant.

50.5 Add 200 µL 80% ethanol to the pellet. Wait 00:00:30 . 30s

50.6 Remove the ethanol.

50.7 Repeat steps 50.5 and 50.6 for a total of 2 washes. 3m

50.8 Centrifuge briefly. Place on the magnet•Low.

50.9 Remove any remaining ethanol. Air dry for 00:02:00 . 2m
DO NOT exceed 2 min as this will decrease elution efficiency.

50.10 Remove from the magnet. Add 30.5 µL Buffer EB. Pipette mix 15x. 1m

50.11 Incubate 00:02:00 at room temperature. 2m

50.12 Place on the magnet•Low until the solution clears. 1m

50.13 Transfer 30 µL sample to a new tube strip. 1m

51 Sample Index PCR (Dual index) 1h

51.1 Choose the appropriate sample index sets to ensure that no sample indices overlap in a multiplexed sequencing run. Record the 10x Sample Index name (PN-3000431 **Dual Index Plate TT Set A** well ID) used. 2m

51.2 Add 50 µL Amp Mix (PN-2000047/2000103) to 30 µL sample. 2m

51.3 Add 20 µL of an individual Dual Index TT Set A to each sample and record the well ID used. Pipette mix 5x (pipette set to 90 µL). Centrifuge briefly. 2m

51.4 Incubate in a thermal cycler with the following protocol . 40m
Lid Temperature: 105 °C , Reaction Volume: 100 µL , Run Time: ~ 00:40:00
Step 1: 98 °C for 00:00:45
Step 2: 98 °C for 00:00:20
Step 3: 54 °C for 00:00:30
Step 4: 72 °C for 00:00:20
Step 5: Go to Step 2, for total 12 of cycles
Step 6: 72 °C for 00:01:00
Step 7: 4 °C Hold

51.5 Store at 4 °C for up to 72 h or at -20 °C for long-term storage.

52 Post Sample Index PCR Double Sided Size Selection – SPRIselect 30m

52.1 Vortex to resuspend the SPRIselect reagent. Add 60 µL SPRIselect Reagent (0.6X) to each sample. Pipette mix 15x (pipette set to 150 µL).

52.2 Incubate 00:05:00 at room temperature. 5m

52.3 Place the magnet•High until the solution clears. DO NOT discard supernatant. 3m

52.4 Transfer 150 µL supernatant to a new tube strip.

52.5 Vortex to resuspend the SPRIselect reagent. Add 20 µL SPRIselect Reagent (0.8X) to each transferred supernatant. Pipette mix 15x (pipette set to 150 µL). 1m

52.6 Incubate 00:05:00 at room temperature. 5m

52.7 Place the magnet•High until the solution clears. 2m

52.8 Remove 165 µL supernatant. DO NOT discard any beads.

52.9 With the tube still in the magnet, add 200 µL 80% ethanol to the pellet. Wait 00:00:30. 30s

52.10 Remove the ethanol. 30s

52.11 Repeat steps 52.9 and 52.10 for a total of 2 washes. 5m

52.12 Centrifuge briefly. Place on the magnet•Low. Remove remaining ethanol. 1m

52.13 Remove from the magnet. Add 35.5 µL Buffer EB. Pipette mix 15x (pipette set to 35 µL). 1m

52.14 Incubate 00:02:00 at room temperature. 2m

52.15 Place on the magnet•Low until the solution clears. 2m

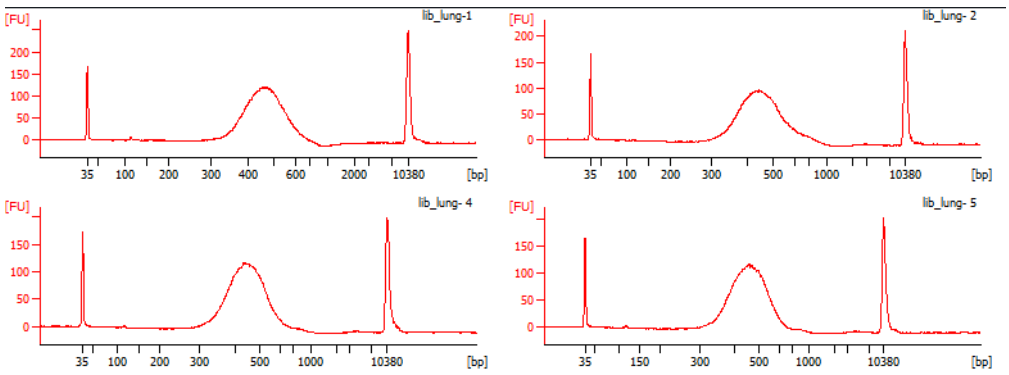
52.16 Transfer 35 µL to a new tube strip. 1m

52.17 Store at 4 °C for up to 72:00:00 or at -20 °C for long-term storage.

53 **Post Library Construction QC** 1h

53.1 Run 1 µL sample at 1:10 dilution on an Agilent Bioanalyzer High Sensitivity chip. 1h

Representative final library traces



Final library

Sequencing

3d

54 Sequencing type: Paired end
Sequencing Depth: Minimum 20,000 read pairs per cell
Sequencing Read: Read1 28 cycles; i7 Index 10 cycles; i5 Index 10 cycles; Read 2 90 cycles.
Sequencing platform: NovaSeq X25B 300 cycles.

3d

Example of QC data from CellRanger.

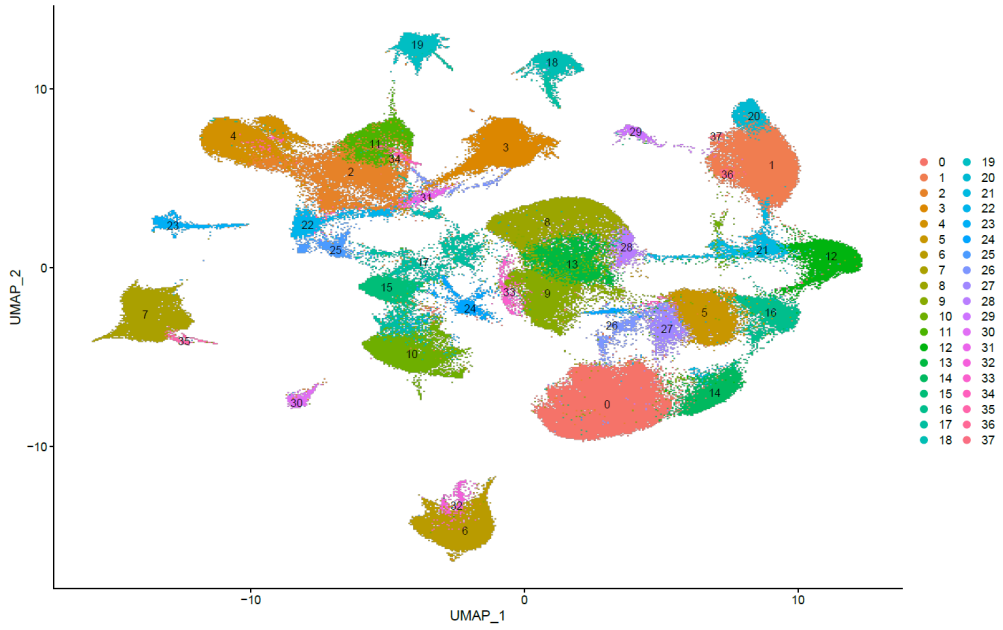
	Sample	Estimated Number of Cells	Mean Reads per Cell	Median Genes per Cell	Number of Reads	Valid Barcodes	Sequencing Saturation	Q30 Bases in Barcode	Q30 Bases in RNA Read	Q30 Bases in UMI	Reads Mapped to Genome	Reads Mapped Confidently to Genome	Reads Mapped Confidently to Intergenic Regions	Reads Mapped Confidently to Intronic Regions	Reads Mapped Confidently to Exonic Regions	Reads Mapped Confidently to Transcriptome	Reads Mapped Antisense to Gene	Fraction Reads in Cells	Total Genes Detected	Median UMI Counts per Cell
	1	11,194	23,838	1,617	266,843,473	93.30%	51.20%	97.40%	96.80%	97.70%	97.20%	90.50%	5.80%	56.80%	28.00%	57.20%	26.90%	77.60%	31,467	3,228
	2	12	19,	1,3	23	93.	52.	97.	96.	97.	97.	90.	5.0	55.	30.	56.	28.	70.	30	2,

		,451	063	58	7,358,866	50%	70%	00%	80%	50%	20%	70%	0%	30%	40%	70%	50%	00%	,733	88
	3	10,749	23,723	1,174	255,002,498	93.550%	63.220%	97.220%	96.880%	97.760%	97.220%	90.220%	4.50%	52.110%	33.770%	56.640%	28.880%	66.990%	30,114	2,009
	4	121,121	21,464	1,877	260,167,621	94.430%	55.500%	97.000%	96.880%	97.750%	97.110%	89.550%	5.20%	57.770%	26.660%	65.110%	18.440%	84.770%	30,799	3,760
	6	13,866	20,037	1,744	277,837,501	93.440%	47.660%	97.330%	97.000%	97.750%	97.880%	91.550%	4.50%	53.440%	33.660%	58.660%	27.880%	79.550%	30,883	3,317
	7	8,990	25,8778	1,370	232,641,973	92.110%	53.220%	97.220%	96.990%	97.760%	96.880%	88.660%	5.220%	56.770%	26.770%	55.880%	27.700%	78.660%	30,197	2,462
	8	10,839	25,930	2,300	281,058,313	94.440%	52.000%	97.220%	96.990%	97.760%	97.330%	92.110%	6.440%	58.000%	27.770%	61.330%	23.880%	74.330%	30,799	4,490

	9	12,505	22,453	1,506	280,772,363	94.20%	58.30%	97.10%	96.60%	97.60%	97.00%	90.90%	4.90%	54.10%	32.00%	60.90%	24.50%	70.80%	30,946	2,605

QC from CellRanger

Example of clustering data.



Cluster data

Protocol references

User Guide for the Chromium Nuclei Isolation Kit CG000505 Rev A

User guide of Chromium Next GEM Single Cell 3' Reagent Kits v3.1 (Dual Index) (CG000315 Rev F)

Acknowledgements

We thank Erin Kane and Sarah Mazzilli for providing sections from fresh frozen lung samples for these experiments.