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Single Cell RNA sequencing (scRNAseq) of fresh human lung cell suspension

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ABSTRACT

Gene expression analysis is a molecular biology approach that allows, among other things, to evaluate the differential expression of genes involved in the development of a certain disease and to identify possible therapeutic targets. The lung is a complex organ composed of more than 52 different cell types, which makes it necessary to implement cutting-edge technologies such as single cell RNA seq analysis for its study. This approach allows to identify genes potentially associated with the development and/or progression of age-related diseases as the idiopathic pulmonary fibrosis, and to identify genes associated with the senescence process and to correlate their expression in certain cell types and their involvement in those diseases. This protocol allows the dissociation of a human lung specimen to a cell suspension and the generation of single cell 3´gene expression dual index libraries from single cells.

DOI

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PROTOCOL CITATION

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KEYWORDS

Single-cell RNA-seq, Human lung cells, TriState SenNET

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67943

GUIDELINES

N/A

MATERIALS TEXT

DNAse I **Worthington Biochemical Corporation Catalog #LS002139**

Ham's F12 Nutrition Mix **Gibco - Thermo Fischer Catalog #11765070**

DMEM high glucosa **Gibco - Thermo Fischer Catalog #11965118**

Liberase DL **Roche Catalog #5466202001**

Phosphate-buffered saline (PBS) **Gibco - Thermo Fischer Catalog #10010023**

Fetal Bovine Serum **Gibco - Thermo Fischer Catalog #16140071**

RBC Lysis Buffer **BioLegend Catalog #420302**

Distilled Water **Thermo Fisher Catalog #15230162**

Chromium Next GEM Single Cell 3' Kit v3.1 16 rxns **10x Genomics Catalog #PN-1000268**

Chromium Next GEM Chip G Single Cell Kit 16 rxns **10x Genomics Catalog #PN-1000127**

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

Nuclease-free Water **Invitrogen - Thermo Fisher Catalog #AM9937**

Low TE Buffer **Invitrogen - Thermo Fisher Catalog #12090-015**

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☒ Buffer EB Elution buffer **Qiagen Catalog #1014609**

☒ Ethanol Pure **Decon Labs Catalog #3916EA**

☒ SPRIselect Reagent Kit **Beckman Coulter Catalog #B23317**

☒ 10% Tween 20 **BIO-RAD Catalog #1662404**

☒ Glycerin (glycerol) 50% (v/v) Aqueous Solution **Ricca Chemical Company Catalog #19086**

☒ DNA LoBind Tubes 1.5 ml **Eppendorf Catalog #022431021**

☒ DNA LoBind Tubes 2.0 ml **Eppendorf Catalog #022431048**

☒ Tips LTS 200UL Filter RT-L200FLR **Rainin Catalog #30389240**

☒ Tips LTS 1ML Filter RT-L1000FLR **Contributed by users Catalog #30389213**

☒ Tips LTS 20UL Filter RT-L10FLR **Rainin Catalog #30389226**

☒ TempAssure PCR 8-tube strip **USA Scientific Catalog #1402-4700**

☒ 10x Vortex Adapter **10x Genomics Catalog #120251/ 330002**

☒ 10x Magnetic Separator **10x Genomics Catalog #120250/ 230003**

☒ Chromium Next GEM Secondary Holder **10x Genomics Catalog #1000142/ 3000332**

SAFETY WARNINGS

N/A

BEFORE STARTING

N/A

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Tissue Dissociation

1 Collect 15 to 30 g of lung tissue.

2 Soak the lung pieces in PBS (3x) to remove red blood cells.

3 Compress with a sterile gauze pad to remove excess liquid.

4 Carefully remove the parietal pleura.

5 Dissect the tissue into 1-cm3 pieces and transfer to a 50 ml conical tube (15 g of tissue without pleura per tube) containing the enzyme cocktail (1 mg/ml liberase DL, DNase I, DMEM).

6 Allow the sample to digest for 2 hours at 37°C.

7 Inactive the digestion buffer (collagenolytic and proteolytic activity) with 2 ml of cold FBS and leave the sample on ice for 5 minutes.

8 Serially filter the suspension through 300-µm, 100-µm, 70-µm and strainers.

9 After straining, centrifuge 500g for 7 minutes.

10 Remove supernatant, add 1x RBC lysis to pellet, re-suspend pellet.

Incubate in 4 degree C fridge for 7 minutes.

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11

12 Add PBS (10% FBS), centrifuge 500g for 7 minutes.

13 Remove supernatant, re-suspend pellet in 10ml PBS.

14 Filter through 40-µm strainer one more time to remove clumped, dead cells.

15 Count cells and viability using countess automatic cell counter, confirm reading under themicroscope.

16 Prepare a vial with a cell stock concentration of 1000 cells/ul and keep on ice.

17 Proceed to the GEM generation and barcoding step

GEM generation and barcoding

18 Prepare Master Mix on ice: add reagents in the order listed. Pipette mix 15x and centrifuge briefly.

Reagent	1X (ul)	2X (ul)	3X (ul)	4X (ul)	5X (ul)	6X (ul)	7X (ul)	8X (ul)
RT Reagent B	18.8	41.4	62.0	82.7	103.4	124.1	144.8	165.4
Template Switch Oligo	2.4	5.3	7.9	10.6	13.2	15.8	18.5	21.1
Reducing Agent B	2.0	4.4	6.6	8.8	11.0	13.2	15.4	17.6
RT Enzyme C	8.7	19.1	28.7	38.3	47.9	57.4	67.0	76.6
Total	31.9	70.2	105.3	140.4	175.5	210.5	245.6	280.7

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19 Add 31.9 µl Master Mix into each tube of a PCR 8-tube strip on ice.

20 Assemble Chromium Next GEM Chip G:

20.1 After removing the chip from the sealed bag, use the chip in ≤ 24 h.

20.2 Align notch on the chip (upper left corner) and the holder.

20.3 Insert the left-hand side of the chip under the guide. Depress the righthand side of the chip until the spring-loaded clip engages.

20.4 Close the lid before dispensing reagents into the wells.

21 Load Chromium NextGEM Chip G:

21.1 a)Add 50% glycerol solution to each unused well (if processing <8 samples/chip).
i)70 µl in each unused well in row labeled 1.
ii)50 µl in each unused well in row labeled 2.
iii)45 µl in each unused well in row labeled 3.

22 Prepare Master Mix + Cell suspension:

22.1 Refer to the Cell Suspension Volume Calculator Table

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22.2 Add the appropriate volume of nuclease-free water to Master Mix. Pipette mix 5x. Add corresponding volume of single cell suspension to Master Mix. Total of 75 µl in each tube.

22.3 Gently pipette mix the cell suspension before adding to the Master Mix.

23 Load Row Labeled 1:

23.1 Gently pipette mix the Master Mix + Cell Suspension.

23.2 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.

24 Prepare Gel Beads:

24.1 Snap the tube strip holder with the Gel Bead strip into a 10x Vortex Adapter. Vortex 30 sec.

24.2 Centrifuge the Gel Bead strip for ~5 sec.

24.3 Confirm there are no bubbles at the bottom of the tubes and the liquid levels are even.

24.4 Place the Gel Bead strip back in the holder. Secure the holder lid.

25 Load Row Labeled 2:

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25.1 Puncture the foil seal of the Gel Bead tubes.

25.2 Slowly aspirate 50 µl Gel Beads.

25.3 Dispense into the wells in row labeled 2 without introducing bubbles.

26 Load Row Labeled 3:

26.1 Dispense 45 µl Partitioning Oil into the wells in row labeled 3 from a reagent reservoir.

27 Attach GEM Gasket

27.1 Align the notch with the top left-hand corner.

27.2 Ensure the gasket holes are aligned with the wells.

28 Run the Chromium Controller:

28.1 Press the eject button on the Controller to eject the tray.

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28.2 Place the assembled chip with the gasket in the tray, ensuring that the chip stays horizontal. Press the button to retract the tray

28.3 Confirm the Chromium Chip G program on screen. Press the play button.

28.4 At completion of the run (~18 min), the Controller will chime. Immediately proceed to the next step.

29 Transfer GEMs:

29.1 Place a tube strip on ice.

29.2 Press the eject button of the Controller.

29.3 Discard the gasket. Open the chip holder. Fold the lid back until it clicks to expose the wells at 45 degrees.

29.4 Slowly aspirate 100 µl GEMs from the lowest points of the recovery wells in the top row labeled 3 without creating a seal between the tips and the bottom of the wells.

29.5 Over the course of ~20 sec, dispense GEMs into the tube strip on ice with the pipette tips against the sidewalls of the tubes.

30 GEM-RT incubation:

30.1 a)Incubate in a thermal cycler with the following protocol:

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Lid Temperature	Reaction Volume	Run time
53°C	125 ul	~55 min
Step	Temperature	Time
1	53°C	0:45:00
2	85°C	0:05:00
3	4°C	Hold

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

Post GEM–RT Cleanup & cDNA Amplification

31 Post GEM-RT Cleanup – Dynabeads:

31.1 a)Add 125 µl Recovery Agent to each sampleat room temperature. DO NOT pipette mix or vortex the biphasic mixture. Wait 2 min.
i) The resulting biphasic mixture contains Recovery Agent/Partitioning Oil (pink) and aqueous phase (clear), with no persisting emulsion (opaque).
ii) If biphasic separation is incomplete: Firmly secure the cap on the tube strip, ensuring that no liquid is trapped between the cap and the tube rim. Mix by inverting the capped tube strip 5x, centrifuge briefly, and proceed to step b.

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

31.3 a)Prepare Dynabeads Cleanup Mix:

Reagent	**1X (ul)**	**2X (ul)**	**3X (ul)**	**4X (ul)**	**5X (ul)**	**6X (ul)**	**7X (ul)**	**8X (ul)**
Cleanup buffer	182	400	601	801	1001	1201	1401	1602
Dynabeads MyOne SILANE	8	18	26	35	44	53	62	70
Reducing Agent B	5	11	17	22	28	33	39	44
Nuclease-free Water	5	11	17	22	28	33	39	44
Total	200	440	660	880	1100	1320	1540	1760

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31.4 Vortex and add 200 µl to each sample. Pipette mix 10x (pipette set to 200 µl).

31.5 Incubate 10 min at room temperature (keep caps open). Pipette mix again at ~5 min after start of incubation to resuspend settled beads

31.6 a)Prepare Elution Solution I. Add reagents in the order listed. Vortex and centrifuge briefly.

Reagent	2X (ul)	4X (ul)	6X (ul)	8X (ul)
Buffer EB	98	196	294	392
10% Tween 20	1	2	3	4
Reducing Agent B	1	2	3	4
Total	100	200	300	400

31.7 At the end of 10 min incubation, place on a 10x Magnetic Separator•High position (magnet•High) until the solution clears.

31.8 Remove the supernatant (aqueous phase and Recovery Agent).

31.9 Add 300 µl 80% ethanol to the pellet while on the magnet. Wait 30 sec.

31.10 Remove the ethanol.

31.11 Add 200 µl 80% ethanol to pellet. Wait 30 sec.

31.12 Remove the ethanol.

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31.13 Centrifuge briefly. Place on the magnet•Low.

31.14 Remove remaining ethanol. Air dry for 1 min.

31.15 Remove from the magnet. Immediately add 35.5 µl Elution Solution I.

31.16 Pipette mix (pipette set to 30 µl) without introducing bubbles.

31.17 Incubate 2 min at room temperature.

31.18 Place on the magnet•Low until the solution clears

31.19 Transfer 35 µl sample to a new tube strip.

32 cDNA amplification:

32.1 a)Prepare cDNA Amplification Mix on ice. Add reagents in the order listed. Vortex and centrifuge briefly.

Reagent	1X (ul)	2X (ul)	3X (ul)	4X (ul)	5X (ul)	6X (ul)	7X (ul)	8X (ul)
Amp Mix	50	110	165	220	275	330	385	440
cDNA Primers	15	33	50	66	83	99	116	132
Total	65	143	215	286	358	429	501	572

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32.2 Add 65 µl cDNA Amplification Reaction Mix to 35 µl sample.

32.3 Pipette mix 15x (pipette set to 90 µl). Centrifuge briefly.

32.4 a)Incubate in a thermal cycler with the following protocol:

Lid Temperature	Reaction Volume	Run time
105°C	100 ul	~30-45 min
Step	Temperature	Time
1	98°C	0:03:00
2	98°C	0:00:15
3	63°C	0:00:20
4	72°C	0:01:00
5	11 Cycles *	
6	72°C	0:01:00
7	4°C	Hold
* Targeted cell recovery >6,000		

32.5 Store at 4°C for up to 72 h or −20°C for ≤1 week or proceed to the next step.

33 cDNA Cleanup – SPRIselect:

33.1 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).

33.2 Incubate 5 min at room temperature.

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33.3 Place on the magnet•High until the solution clears.

33.4 Remove the supernatant.

33.5 Add 200 µl 80% ethanol to the pellet. Wait 30 sec.

33.6 Remove the ethanol.

33.7 Repeat steps 5 and 6 for a total of 2 washes.

33.8 Centrifuge briefly and place on the magnet•Low.

33.9 Remove any remaining ethanol. Air dry for 2 min. DO NOT exceed 2 min as this will decrease elution efficiency.

33.10 Remove any remaining ethanol. Air dry for 2 min. DO NOT exceed 2 min as this will decrease elution efficiency.

33.11 Incubate 2 min at room temperature.

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

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33.13 Transfer 40 µl sample to a new tube strip.

33.14 Store at 4°C for up to 72 h or at −20°C for up to 4 weeks, or proceed to the next step.

34 cDNA Quantification:

34.1 Run 2 µl sample with the Qubit dsDNA HS Assay Kit.

34.2 Multiply the cDNA concentration reported via the Qubit Fluorometer by the elution volume (40 µl) to obtain the total cDNA yield in ng.

3’ Gene Expression Dual Index Library Construction

35 Fragmentation,End Repair & A-tailing:

35.1 a)Prepare a thermal cycler with the following incubation protocol:

Lid Temperature	**Reaction Volume**	**Run time**
65°C	50 ul	~35 min
Step	Temperature	Time
Pre-cool block	4°C	Hold
Fragmentation	32°C	0:05:00
End Repair & A-tailing	65°C	0:30:00
Hold	4°C	Hold

35.2 Vortex Fragmentation Buffer. Verify there is no precipitate.

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35.3 Prepare Fragmentation Mix on ice. Prepare Fragmentation Mix on ice. Pipette mix and centrifuge briefly. Pipette mix and centrifuge briefly.

Reagent	1X (ul)	2X (ul)	3X (ul)	4X (ul)	5X (ul)	6X (ul)	7X (ul)	8X (ul)
Fragmentation Buffer	5	11	17	22	28	33	39	44
Fragmentation Buffer	10	22	33	44	55	66	77	88
Total	15	33	50	66	83	99	116	132

35.4 Transfer ONLY 10 µl purified cDNA sample from Pellet Cleanup to a tube strip. 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.

35.5 Add 25 µl Buffer EB to each sample.

35.6 Add 15 µl Fragmentation Mix to each sample.

35.7 Pipette mix 15x (pipette set to 35 µl) on ice. Centrifuge briefly.

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

36 Post Fragmentation,End Repair & A-tailing Double Sided Size Selection – SPRIselect:

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

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36.2 Incubate 5 min at room temperature.

36.3 Place on the magnet•High until the solution clears. DO NOT discard supernatant.

36.4 Transfer 75 µl supernatant to a new tube strip.

36.5 Vortex to resuspend SPRIselect reagent. Add 10 µl SPRIselect reagent (0.8X)to each transferred supernatant. Pipette mix 15x (pipette set to 80 µl).

36.6 Incubate 5 min at room temperature.

36.7 Place on the magnet•High until the solution clears.

36.8 Remove 80 µl supernatant. DO NOT discard any beads.

36.9 Add 125 µl 80% ethanol to the pellet. Wait 30 sec.

36.10 Remove the ethanol.

36.11 Repeat steps 9 and 10 for a total of 2 washes.

36.12 Centrifuge briefly. Place on the magnet•Low until the solution clears. Remove remaining ethanol.

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36.13 Remove from the magnet. Add 50.5 µl Buffer EB to each sample. Pipette mix 15x (pipette set to 45 µl).

36.14 Incubate 2 min at room temperature.

36.15 Place on the magnet•High until the solution clears.

36.16 Transfer 50 µlsample to a new tube strip.

37 Adaptor Ligation:

37.1 a)Prepare Adaptor Ligation Mix. Add reagents in the order listed. Pipette mix and centrifuge briefly.

A	B	C	D	E	F	G	H	I
Reagent	**1X (ul)**	**2X (ul)**	**3X (ul)**	**4X (ul)**	**5X (ul)**	**6X (ul)**	**7X (ul)**	**8X (ul)**
Ligation Buffer	20	44	66	88	110	132	154	176
b)	Add 50 µl Adaptor Ligation Mix to 50 µl sample. Pipette mix 15x (pipette set to 90 µl). Centrifuge briefly	22	33	44	55	66	77	88
Adaptor Oligos	20	44	66	88	110	132	154	176
Total	50	110	165	220	275	330	385	440

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37.2 Add 50 µl Adaptor Ligation Mix to 50 µl sample. Pipette mix 15x (pipette set to 90 µl). Centrifuge briefly

37.3 a)Incubate in a thermal cycler with the following protocol:

Lid Temperature	**Reaction Volume**	**Run time**
30°C	100 ul	~15 min
Step	Temperature	Time
1	20°C	0:15:00
2	4°C	Hold

38 Post Ligation Cleanup – SPRIselect:

38.1 Vortex to resuspend SPRIselect reagent. Add 80 µl SPRIselect (0.8X) reagent to each sample. Pipette mix 15x (pipette set to 75 µl).

38.2 Incubate 5 min at room temperature.

38.3 Place on the magnet•High until the solution clears.

38.4 Remove the supernatant.

38.5 Add 200 µl 80% ethanol to the pellet. Wait 30 sec.

38.6 Remove the ethanol.

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38.7 Repeat steps 5 and 6 for a total of 2 washes.

38.8 f)Centrifuge briefly. Place on the magnet•Low until the solution clears.

38.9 Remove remaining ethanol. Air dry for 2 min.

38.10 Remove from the magnet. Add 30.5 µl Buffer EB to each sample. Pipette mix 15x.

38.11 Incubate 2 min at room temperature.

38.12 Place on the magnet•Low until the solution clears.

38.13 Transfer 30 µlsample to a new tube strip.

39 Sample Index PCR:

39.1 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.

39.2 Add 50 µl Amp Mix to 30 µl sample.

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39.3 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.

39.4 a)Incubate in a thermal cycler with the following protocol:

Lid Temperature	**Reaction Volume**	**Run time**
105°C	100 ul	~ 25-40 min
Step	Temperature	Time
1	98°C	0:00:45
2	98°C	0:00:20
3	54°C	0:00:30
4	72°C	0:00:20
5	# Cycles *	
6	72°C	0:01:00
7	4°C	Hold

* The total cycles should be optimized based on 25% carry forward cDNA yield/input calculated during cDNA Quantification

Sequencing Depth	**Total Cycles**
Sequencing Depth	14 - 16
Sequencing Depth	12 - 14
Sequencing Depth	10 - 12
Sequencing Depth	8 - 10
Sequencing Depth	6 - 8
Sequencing Depth	5

39.5 Store at 4°C for up to 72 h or proceed to the next step.

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40 Post Sample Index PCR Double Sided Size Selection – SPRIselect:

40.1 Vortex to resuspend SPRIselect reagent. Add 60 µl SPRIselect (0.6X) reagent to each sample. Pipette mix 15x (pipette set to 150 µl).

40.2 Incubate 5 min at room temperature.

40.3 Place on the magnet•High until the solution clears. DO NOT discard supernatant.

40.4 Transfer 150 µl supernatant to a new tube strip.

40.5 Vortex to resuspend SPRIselect reagent. Add 20 µl SPRIselect reagent (0.8X)to each transferred supernatant. Pipette mix 15x (pipette set to 150 µl).

40.6 Incubate 5 min at room temperature.

40.7 Place on the magnet•High until the solution clears.

40.8 Remove 165 µl supernatant. DO NOT discard any beads.

40.9 Add 200 µl 80% ethanol to the pellet. Wait 30 sec.

40.10 Remove the ethanol.

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40.11 Repeat steps 9 and 10 for a total of 2 washes.

40.12 Centrifuge briefly. Place on the magnet•Low until the solution clears. Remove remaining ethanol.

40.13 Remove from the magnet. Add 35.5 µl Buffer EB to each sample. Pipette mix 15x (pipette set to 35 µl).

40.14 Incubate 2 min at room temperature.

40.15 Place on the magnet•Low until the solution clears.

40.16 Transfer 35 µlsample to a new tube strip.

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

41 Post Library Construction QC:

41.1 Run 1 µl sample at 1:10 dilution on an Agilent Bioanalyzer High Sensitivity chip.

41.2 Determine the average fragment size from the Bioanalyzer trace. This will be used as the insert size for library quantification.

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Sequencing

42 1. 3’ Gene Expression Library Sequencing Depth & Run Parameters:

A	B
Sequencing Depth	Minimum 20,000 read pairs per cell
Sequencing Type	Paired-end, dual indexing
Sequencing Read	Recommended Number of Cycles
Read 1	28 cycles
i7 index	10 cycles
i5 index	10 cycles
Read 2	90 cycles

43 Once quantified and normalized, the 3’ Gene Expression libraries should be denatured and diluted as recommended for Illumina sequencing platforms.

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