**Single nuclei RNA sequencing (snRNA-seq) of frozen human lung tissue and hPCLS**

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**Abstract**

Single-cell RNA sequencing (scRNA-seq) has become an essential tool for delineating cellular diversity in normal tissues and alterations in disease states. This technique requires the dissociation of tissue specimens into cell suspensions. However, the isolation of intact cells can be challenging due to factors such as fragility, large size and tight interconnections. Additionally, single-nuclei isolation can be performed on frozen tissue, enabling the analysis of biobanked samples in a single batch. This protocol for single-nuclei RNA sequencing (snRNA-seq) provides an alternative approach to scRNA-seq, overcoming these limitations to generate high-quality transcriptomic data.

The analysis of gene expression at the cellular level has proven to be a powerful tool for understanding various aspects of lung biology and disease, particularly the process of lung aging. Aging affects different cell types within the human lung heterogeneously, leading to a range of associated changes in their function. Examining the patterns of gene expression in the numerous lung cell types provides insights into the aging and disease processes that contribute to cellular dysfunction. By analyzing these changes at the single-cell level, we can delineate the complex cellular diversity in the human lung and track alterations in molecular pathways involved in the dynamic process of lung aging. Understanding these gene expression patterns will offer opportunities for timely interventions and the identification of biomarker for early prognosis and personalized treatment therapies.

This protocol describes the process of single nuclei RNA sequencing from nuclei isolation from frozen tissue (whole, or from PCLS), to barcoding, to library construction, to sequencing.

**Materials:**

* **Chromium Nuclei Isolation Kit with RNase Inhibitor PN-1000494**
* **Chromium Next GEM Single Cell 3’ GEM Kit v3.1 16 rxns PN-1000123**
* **Library Construction Kit 16 rxns PN-1000190**
* **Chromium Next GEM Single Cell 3’ Gel Bead Kit v3.1, 16 rxns PN-1000122**
* **Dynabeads™ MyOne™ SILANE PN-2000048**
* **Dual Index Kit TT Set A, 96 rxns PN-1000215**

**Notes:**

* *If provided Lysis Reagent and Debris Removal Buffers appear cloudy or contain precipitate, warm the tubes to* **40°C** *and swirl until the buffers become clear again.*

**Before start:**

* **Pre-chill centrifuge to 4oC**
* **Thaw Reducing Agent B** – Thaw to room temperature.
* **Vortex –** Vortex, verify no precipitate, and centrifuge briefly all Lysis and Debris Removal reagents,
* **RNase Inhibitor –** Centrifuge briefly.
* **Buffer Preparation: Lysis Buffer & Debris Removal Buffer -** Prepare the following Lysis and Debris Removal Buffers on ice shortly before starting the Nuclei Isolation protocol. Prepare large volumes in a 15-ml or 50-ml conical tube. Vortex briefly before use.

|  |  |  |  |
| --- | --- | --- | --- |
| **Lysis Buffer (500** μ**l/rxn)**Add reagents in the order listed. | **1X+10% (**μ**l)** | **4X+10% (**μ**l)** | **8X+10% (**μ**l)** |
| **Lysis Reagent**  | **550** | 2,200 | 4,400 |
| **Reducing Agent B** | **0.55** | 2.2 | 4.4 |
| **Surfactant A** | **5.5** | 22 | 44 |
| **Total** | **556.05** | 2,224.2 | 4,448.4 |

|  |  |  |  |
| --- | --- | --- | --- |
| **Debris Removal Buffer (500** μ**l/rxn)**Add reagents in the order listed. | **1X+10% (**μ**l)** | **4X+10% (**μ**l)** | **8X+10% (**μ**l)** |
| **Debris Removal Reagent**  | **550** | 2,200 | 4,400 |
| **Reducing Agent B** | **0.55** | 2.2 | 4.4 |
| **Total** | **550.55** | 2,202.2 | 4,404.4 |

* **Buffer Preparation: Wash and Resuspension Buffer -** Prepare the following Wash and Resuspension Buffer on ice shortly before starting the Nuclei Isolation protocol. Prepare large volumes in a 15-ml or 50-ml conical tube. Vortex briefly before use.

|  |  |  |  |
| --- | --- | --- | --- |
| **Wash and Resuspension Buffer (3** m**l/rxn)**Add reagents in the order listed. | **1X+10% (**μ**l)** | **4X+10% (**μ**l)** | **8X+10% (**μ**l)** |
| **1X PBS**  | **2,887.5** | 11,550 | 23,100 |
| **10% BSA** | **330** | 1,320 | 2,640 |
| **RNase Inhibitor** | **82.5** | 330 | 660 |
| **Total** | **3,300** | 13,200 | 26,400 |

* **Place reagents and tubes on ice –** Label tops and sides of tubes, as well as tops of spin columns, before placing on ice and starting protocol.
* **Place Tissue and sample dissociation tubes on dry ice –** Pre-chill on dry ice.

**Nuclei Isolation from Frozen Tissue**

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

1. Transfer frozen tissue **(~50 mg;** use 2 slices if isolating from PCLS**)** to pre-chilled Sample Dissociation Tube (2000564) and place on wet ice.
2. Add Lysis Buffer **(200** μ**l)** & dissociate with pestle until homogeneous while on ice.

*Perform tissue dissociation on ice. Use one pestle per sample. DO NOT discard pestles until nuclei isolation process is complete.*

1. Add Lysis Buffer **(300** μ**l)** and pipette mix 10X. If not homogeneous, continue to dissociate with the pestle until able to pipette mix
2. Incubate on ice for **10 min**
3. Pipette dissociated tissue onto assembled and pre-chilled Nuclei Isolation Column and Collection Tube (2000562 & 2000563)
4. Centrifuge at 16,000 rcf for 20 sec, at 4°C
5. Discard column
6. Vortex flowthrough in Collection Tube for **10 sec** at 3,200 rpm minimumto resuspend nuclei
7. Centrifuge at 500 rcf for 3 min, at 4°C
8. Remove supernatant (s/n)
9. Resuspend pellet with Debris Removal Buffer **(500** μ**l)**
10. Centrifuge at 700 rcf for 10 min, at 4°C
11. Remove supernatant (s/n)
12. Resuspend nuclei in **1 ml** Wash and Resuspension Buffer
13. Centrifuge at 500 rcf for 5 min, at 4°C
14. Remove supernatant (s/n)
15. Repeat 14-15
16. Resuspend nuclei pellet in **50–500** μ**l** Wash and Resuspension Buffer
17. Vortex nuclei for **3 sec** and determine final nuclei concentration using AOPI or Ethidium Homodimer-1 fluorescent staining dyes and dilute if necessary for target nuclei load. Adjust nuclei concentration as necessary for intended downstream assay.
18. Vortex nuclei for **3 sec** and keep samples on ice.

**Proceed immediately to 10x Genomics Single GEM Generation and Barcoding**

**GEM Generation and Barcoding**

**Before start:**

* **Equilibrate to room temperature (RT) -** Single Cell 3’ v3.1 Gel Beads (2000164), RT Reagent B (2000165) and Template Switch Oligo (3000228)
* **Place on ice –** RT Enzyme C (20000085/2000102) and Cells suspension
* **Prepare Master Mix -** Prepare on ice.
1. Prepare Master Mix on ice. Pipette mix 15x and centrifuge briefly.

|  |  |  |  |
| --- | --- | --- | --- |
| **Master Mix (MM)**Add reagents in the order listed. | **1X (**μ**l)** | **4X+10% (**μ**l)** | **8X+10% (**μ**l)** |
| **RT Reagent B**  | **18.8** | 82.7 | 165.4 |
| **Template Switch Oligo** | **2.4** | 10.6 | 21.1 |
| **Reducing Agent B** | **2.0** | 8.8 | 17.6 |
| **RT Enzyme C** | **8.7** | 38.3 | 76.6 |
| **Total** | **31.9** | 140.4 | 280.7 |

1. Add 31.9 µl Master Mix into each tube of a PCR 8-tube strip on ice
2. Assemble Chromium Next GEM Chip

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* 1. Close the holder lid. Attach the gasket by holding the tongue (curved end, to the right) and hook the gasket on the left-hand tabs of the holder. Gently pull the gasket toward the right and hook it on the two right-hand tabs
	2. Remove the chip from the sealed bag, and use it within ≤ 24 hr
	3. Align notch on the chip (upper left corner) and the open holder with the gasket
	4. Slide the chip to the left until it is inserted under the guide on the holder. Depress the right hand side of the chip until the spring-loaded clip engages
	5. Keep the assembled unit open until all reagents have been loaded. Then close the chip holder
1. Load Chromium NextGEM Chip G
	1. Add 50% glycerol solution to each unused well (if processing <8 samples/chip)
		1. 70 µl in each unused well in row labeled 1
		2. 50 µl in each unused well in row labeled 2
		3. 45 µl in each unused well in row labeled 3
2. Prepare Master Mix + Cell suspension:
	1. Refer to the Cell Suspension Volume Calculator Table (refer to Chromium Next GEM Single Cell 3ʹ user Guide)
	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
	3. Gently pipette mix the cell suspension before adding to the Master Mix
3. Load Row Labeled 1
	1. Gently pipette mix the Master Mix + Cell Suspension
	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
4. Prepare Gel Beads
	1. Snap the tube strip holder with the Gel Bead strip into a 10x Vortex Adapter. Vortex 30 sec.
	2. Centrifuge the Gel Bead strip for ~5 sec.
	3. Confirm there are no bubbles at the bottom of the tubes and the liquid levels are even
	4. Place the Gel Bead strip back in the holder. Secure the holder lid
5. Load Row Labeled 2
	1. Puncture the foil seal of the Gel Bead tubes
	2. Slowly aspirate 50 μl Gel Beads
	3. Dispense into the wells in row labeled 2 without introducing bubbles
	4. Wait 30 sec.
6. Load Row Labeled 3
	1. Dispense 45 μl Partitioning Oil into the wells in row labeled 3 from a reagent reservoir and close lid

*Failure to add Partitioning Oil to the top row labeled 3 will prevent GEM generation and can damage the Chromium Controller or X/iX.*

*Run the chip in the Chromium Controller or X/iX immediately after loading the Partitioning Oil*

1. Run the Chromium Controller or X/iX
	1. Press the eject button on the Controller to eject the tray
	2. Place the assembled chip with the gasket in the tray, ensuring that the chip stays horizontal. Press the button to retract the tray
	3. Press the play button
	4. At completion of the run (~18 min), the Controller will chime. Immediately proceed to the next step
2. Transfer GEMs
	1. Place a tube strip on ice
	2. Press the eject button of the Controller
	3. Discard the gasket. Open the chip holder. Fold the lid back until it clicks to expose the wells at 45 degrees

Visually compare the remaining volume in rows labeled 1-2. Abnormally high volume in one well relative to other wells may indicate a clog

* 1. 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
	2. Over the course of ~20 sec, dispense GEMs into the tube strip on ice with the pipette tips against the sidewalls of the tubes
1. GEM-RT Incubation
	1. Incubate in a thermal cycler with the following protocol:

|  |  |  |
| --- | --- | --- |
| Lid Temperature | Reaction Volume | Run Time |
| 53oC | 125 µl | ~55 min |
| Step | **Temperature** | **Time** |
| 1 | 53oC | 45 min |
| 2 | 85oC | 5 min |
| 3 | 4oC | Hold |

* 1. 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**

**Before start:**

* **Equilibrate to room temperature (RT) –** Reducing Agent B (2000087), cDNA Primers (2000089) and Dynabeads MyOne SILANE (2000048)
* **Place on ice –** Amp Mix (2000047/2000103)
* **Thaw at 65oC -** Cleanup Buffer (2000088)
1. Dynabeads
	1. Add 125 μl Recovery Agent to each sample at room temperature. DO NOT pipette mix or vortex the biphasic mixture and wait 2 min

The resulting biphasic mixture contains Recovery Agent/Partitioning Oil (pink) and aqueous phase (clear), with no persisting emulsion (opaque).

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.

* 1. Slowly remove and discard 125 µl Recovery Agent/Partitioning Oil (pink) from the bottom of the tube. DO NOT aspirate any aqueous sample.
	2. Prepare Dynabeads Cleanup Mix:

|  |  |  |  |
| --- | --- | --- | --- |
| **Dynabeads Cleanup Mix**Add reagents in the order listed | **1X (**μ**l)** | **4X+10% (**μ**l)** | **8X+10% (**μ**l)** |
| **Cleanup Buffer** | 182 | 801 | 1602 |
| **Dynabeads MyOne SILANE** | 8 | 35 | 70 |
| **Reducing Agent B** | 5 | 22 | 44 |
| **Nuclease-free Water** | 5 | 22 | 44 |
| **Total** | 200 | 880 | 1760 |

* 1. Vortex and add 200 µl to each sample. Pipette mix 10x (pipette set to 200 µl).
	2. Incubate 10 min at room temperature (keep caps open). Pipette mix again at ~5 min after start of incubation to resuspend settled beads
	3. Prepare Elution Solution I. Vortex and centrifuge briefly.

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

* 1. At the end of 10 min incubation, place on a 10x Magnetic Separator•High position (magnet•High) until the solution clears.
	2. Remove the supernatant (aqueous phase and Recovery Agent).
	3. Add 300 µl 80% ethanol to the pellet while on the magnet. Wait 30 sec.
	4. Remove the ethanol.
	5. Add 200 µl 80% ethanol to pellet. Wait 30 sec.
	6. Remove the ethanol.
	7. Centrifuge briefly. Place on the magnet•Low.
	8. Remove remaining ethanol. Air dry for 1 min.
	9. Remove from the magnet. Immediately add 35.5 µl Elution Solution I.
	10. Pipette mix (pipette set to 30 µl) without introducing bubbles.
	11. Incubate 2 min at room temperature.
	12. Place on the magnet•Low until the solution clears
	13. Transfer 35 µl sample to a new tube strip.
1. cDNA amplification
	1. Prepare cDNA Amplification Mix on ice. Add reagents in the order listed. Vortex and centrifuge briefly.

|  |  |  |  |
| --- | --- | --- | --- |
| **cDNA Amplification Reaction Mix**Add reagents in the order listed | **1X (**μ**l)** | **4X+10% (**μ**l)** | **8X+10% (**μ**l)** |
| **Amp Mix** | 50 | 220 | 440 |
| **cDNA Primers** | 15 | 66 | 132 |
| **Total** | 65 | 286 | 572 |

* 1. Add 65 µl cDNA Amplification Reaction Mix to 35 µl sample.
	2. Pipette mix 15x (pipette set to 90 µl). Centrifuge briefly.
	3. Incubate in a thermal cycler with the following protocol:

|  |  |  |
| --- | --- | --- |
| Lid Temperature | Reaction Volume | Run Time |
| 105oC | 100 µl | ~30-45 min |
| Step | **Temperature** | **Time** |
| 1 | 98oC | 3 min |
| 2 | 98oC | 15 sec |
| 3 | 63 oC | 20 sec |
| 4 | 72 oC | 1 min |
| 5 | Go to Step 2 – 11 cycles |
| 6 | 72 oC | 1 min |
| 7 | 4oC | Hold |

* 1. Store at 4°C for up to 72 h or −20°C for ≤1 week or proceed to the next step.
1. cDNA Cleanup – SPRIselect:
	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).
	2. Incubate 5 min at room temperature.
	3. Place on the magnet•High until the solution clears.
	4. Remove the supernatant.
	5. Add 200 µl 80% ethanol to the pellet. Wait 30 sec.
	6. Remove the ethanol.
	7. Repeat steps e and f for a total of 2 washes.
	8. Centrifuge briefly and place on the magnet•Low.
	9. Remove any remaining ethanol. Air dry for 2 min. DO NOT exceed 2 min as this will decrease elution efficiency.
	10. Remove from magnet. Add 40.5 µl Buffer EB. Pipette mix 15x
	11. Incubate for 2 min at room temperature
	12. Place the tube strip on the magnet•High until the solution clears.
	13. Transfer 40 µl sample to a new tube strip.
	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.
2. cDNA Quantification
	1. Run 1 µl sample (Dilution Factor 1:10) on an Agilent Bioanalyzer High Sensitivity chip.

**Gene Expression Dual Index Library Construction**

**Before start:**

* **Equilibrate to room temperature (RT) –** Fragmentation Buffer (2000091), Adaptor Oligos (2000094), Ligation Buffer (2000092) and Dual Index Plate TT Set A (3000431)
* **Place on ice –** Fragmentation Enzyme (2000090/2000104), DNA Ligase (220110/220131) and Amp Mix (2000047/2000103)
* **Thaw at 65oC -** Cleanup Buffer (2000088)
1. Fragmentation, End Repair & A-tailing
	1. Prepare a thermal cycler with the following incubation protocol:

|  |  |  |
| --- | --- | --- |
| Lid Temperature | Reaction Volume | Run Time |
| 65oC | 50 µl | ~35 min |
| Step | **Temperature** | **Time** |
| Pre-cool block | 4oC | Hold |
| Fragmentation | 32oC | 5 min |
| End Repair & A-tailing | 65 oC | 30 min |
| Hold | 72 oC | Hold |

* 1. Vortex Fragmentation Buffer. Verify there is no precipitate.
	2. Prepare Fragmentation Mix on ice. Prepare Fragmentation Mix on ice. Pipette mix and centrifuge briefly. Pipette mix and centrifuge briefly.

|  |  |  |  |
| --- | --- | --- | --- |
| **Fragmentation Mix**Add reagents in the order listed | **1X (**μ**l)** | **4X+10% (**μ**l)** | **8X+10% (**μ**l)** |
| **Fragmentation Buffer** | 5 | 22 | 44 |
| **Fragmentation Enzyme** | 10 | 44 | 88 |
| **Total** | 15 | 66 | 132 |

* 1. 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 3ʹ Gene Expression libraries.

* 1. Add 25 µl Buffer EB to each sample.
	2. Add 15 µl Fragmentation Mix to each sample.
	3. Pipette mix 15x (pipette set to 35 µl) on ice. Centrifuge briefly.
	4. Transfer into the pre-cooled thermal cycler (4°C) and press “SKIP” to initiate the protocol.
1. Post Fragmentation,End Repair & A-tailing Double Sided Size Selection – SPRIselect:
	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)
	2. Incubate 5 min at room temperature.
	3. Place on the magnet•High until the solution clears. DO NOT discard supernatant.
	4. Transfer 75 µl supernatant to a new tube strip.
	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).
	6. Incubate 5 min at room temperature.
	7. Place on the magnet•High until the solution clears.
	8. Remove 80 µl supernatant. DO NOT discard any beads.
	9. Add 125 µl 80% ethanol to the pellet. Wait 30 sec.
	10. Remove the ethanol.
	11. Repeat steps i and j for a total of 2 washes.
	12. Centrifuge briefly. Place on the magnet•Low until the solution clears. Remove remaining ethanol.
	13. Remove from the magnet. Add 50.5 µl Buffer EB to each sample. Pipette mix 15x (pipette set to 45 µl).
	14. Incubate 2 min at room temperature.
	15. Place on the magnet•High until the solution clears.
	16. Transfer 50 µlsample to a new tube strip.
2. Adaptor Ligation
	1. Prepare Adaptor Ligation Mix. Pipette mix and centrifuge briefly.

|  |  |  |  |
| --- | --- | --- | --- |
| **Adaptor Ligation Mix**Add reagents in the order listed | **1X (**μ**l)** | **4X+10% (**μ**l)** | **8X+10% (**μ**l)** |
| **Ligation Buffer** |  20 | 88 | 176 |
| **DNA Ligase** | 10 | 44 | 88 |
| **Adaptor Oligos** | 20 | 88 | 179 |
| **Total** | 50 | 220 | 440 |

* 1. Add 50 µl Adaptor Ligation Mix to 50 µl sample. Pipette mix 15x (pipette set to 90 µl). Centrifuge briefly
	2. Incubate in a thermal cycler with the following protocol:

|  |  |  |
| --- | --- | --- |
| Lid Temperature | Reaction Volume | Run Time |
| 30oC | 100 µl | 15 min |
| Step | **Temperature** | **Time** |
| 1 | 20oC | 15 min |
| 2 | 4oC | Hold |

1. Post Ligation Cleanup – SPRIselect:
	1. Vortex to resuspend SPRIselect reagent. Add 80 µl SPRIselect (0.8X) reagent to each sample. Pipette mix 15x (pipette set to 75 µl).
	2. Incubate 5 min at room temperature.
	3. Place on the magnet•High until the solution clears.
	4. Remove the supernatant.
	5. Add 200 µl 80% ethanol to the pellet. Wait 30 sec.
	6. Remove the ethanol.
	7. Repeat steps e and f for a total of 2 washes
	8. Centrifuge briefly. Place on the magnet•Low until the solution clears.
	9. Remove remaining ethanol. Air dry for 2 min.
	10. Remove from the magnet. Add 30.5 µl Buffer EB to each sample. Pipette mix 15x.
	11. Incubate 2 min at room temperature.
	12. Place on the magnet•Low until the solution clears.
	13. Transfer 30 µl sample to a new tube strip.
2. Sample Index PCR
	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.
	2. Add 50 µl Amp Mix to 30 µl sample.
	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.
	4. Incubate in a thermal cycler with the following protocol:

|  |  |  |
| --- | --- | --- |
| Lid Temperature | Reaction Volume | Run Time |
| 105oC | 100 µl | ~25-40 min |
| Step | **Temperature** | **Time** |
| 1 | 98oC | 45 sec |
| 2 | 98oC | 20 sec |
| 3 | 54 oC | 30 sec |
| 4 | 72 oC | 20 sec |
| 5 | Go to Step 2 – \* # cycles calculated below |
| 6 | 72 oC | 1 min |
| 7 | 4oC | Hold |

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

|  |  |
| --- | --- |
| **cDNA Input** | **Total cycles** |
| 0.25-25 ng | 14-16 |
| 25-150 ng | 12-14 |
| 150-500 ng | 10-12 |
| 500-1,000 ng | 8-10 |
| 1,000-1,500 ng | 6-8 |
| >1,500 ng | 5 |

* 1. Store at 4°C for up to 72 h or proceed to the next step.
1. Post Sample Index PCR Double Sided Size Selection – SPRIselect
	1. Vortex to resuspend SPRIselect reagent. Add 60 µl SPRIselect (0.6X) reagent to each sample. Pipette mix 15x (pipette set to 150 µl).
	2. Incubate 5 min at room temperature.
	3. Place on the magnet•High until the solution clears. DO NOT discard supernatant.
	4. Transfer 150 µl supernatant to a new tube strip.
	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).
	6. Incubate 5 min at room temperature.
	7. Place on the magnet•High until the solution clears.
	8. Remove 165 µl supernatant. DO NOT discard any beads.
	9. Add 200 µl 80% ethanol to the pellet. Wait 30 sec.
	10. Remove the ethanol.
	11. Repeat steps i and j for a total of 2 washes.
	12. Centrifuge briefly. Place on the magnet•Low until the solution clears. Remove remaining ethanol.
	13. Remove from the magnet. Add 35.5 µl Buffer EB to each sample. Pipette mix 15x (pipette set to 35 µl).
	14. Incubate 2 min at room temperature.
	15. Place on the magnet•Low until the solution clears.
	16. Transfer 35 µlsample to a new tube strip.
	17. Store at 4°C for up to 72 h or at −20°C for long-term storage.
2. Post Library Construction QC
	1. Run 1 µl sample at 1:10 dilution on an Agilent Bioanalyzer High Sensitivity chip.
	2. Determine the average fragment size from the Bioanalyzer trace. This will be used as the insert size for library quantification.

**Sequencing**

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

|  |  |
| --- | --- |
| Sequencing Depth | Minimum 20,000 read pairs per cell |
| Sequencing Tyoe | Pair-end, dual indexing |
| Sequencing Read | Recommended Number of cycles |
| Read 1i7 Indexi5 IndexRead 2 | 28 cycles10 cycles10 cycles90 cycles |

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