



# SINGLE CELL

Workshop  
**COLOMBIA 2024**



**MISTI** Massachusetts Institute of Technology

Universidad de los Andes  
Colombia

CeMeP  
Grupo de Investigación celular y molecular  
de Microorganismos Patógenos

**SHALEK LAB**  
from cells to systems

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## 2024 Single Cell Workshop Colombia



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## PBMC Cell Isolation Protocol

Author: Tyler Dao and Evelyn Tong

Sources: Stem cell Technologies, Eppendorf and Shalek Lab

### Introduction

Isolation of peripheral blood mononuclear cells (PBMC) is performed through density gradient centrifugation by exploiting differences in density between various leukocytes and the density gradient medium. Due to their higher density, granulocytes, erythrocytes and dead cells pass through the density gradient medium layer whereas lymphocytes and monocytes accumulate at the plasma-gradient boundary due to their lower density. After centrifugation, there should be 5 layers: plasma (top, yellowish), PBMC cells (middle, cloudy), gradienty medium (middle, clear), granulocytes/erythrocytes (bottom, red). Protocol adapted from Stem Cell and Eppendorf. Isolation of untouched human T cells from peripheral blood mononuclear cells (PBMC) is done by depleting B cells, NK cells, monocytes, platelets, dendritic cells, granulocytes and erythrocytes. This is done by using a mixture of IgG antibodies against non-T cells. (credit to Iris and Ben Allsup)

### Materials

- Whole blood sample
- Lymphoprep/ Ficoll-Paque (Density gradient medium)
- Culture Media/ PBS + 2% FBS
- RBC Lysis buffer

### Procedure

1. Dilute blood sample with culture media or PBS + 2% FBS at a 1:1 volume ratio
2. In a fresh tube, add in the density gradient medium at the specified volume
3. Gently layer the diluted blood on top of the density gradient medium (try not to mix the two layers)

#### Mixture Recommendations

Blood (mL)	1	2	3	4	5	10	15
PBS + 2% FBS (mL)	1	2	3	4	5	10	15
Ficoll-Paque PLUS or Lymphoprep (mL)	1.5	3	3	4	10	15	15

4. Centrifuge the samples at 800 x g for 20-30min with the **brakes OFF** (aka no deceleration)
5. Harvest PBMCs by inserting the pipette tip directly through the upper plasma layer to the mononuclear cells at the interface  
Alternative: Remove upper plasma layer supernatant first, then collect cells in the middle layers

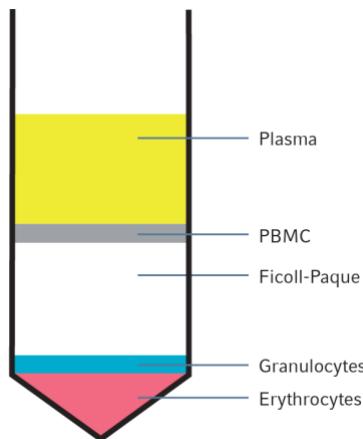


Figure 1. Density gradient of a buffy coat with Ficoll-Paque PLUS (schematic)

6. Wash harvested cells with 5 mL of PBS

### RBC Lysis (Optional)

1. Resuspend pellet in 10 mL of RBC lysis buffer
2. Incubate for 10 min at RT
3. Stop the reaction by adding 20-30 mL of PBS
4. Centrifuge cells at 350 x g for 10 min
5. Resuspend pellet and counts (See Appendix 1)

# Protocol for Dissociation of Fresh Tissue for Single-Cell RNA Sequencing

Author: Sarah Ingabire

Sources: Stem Cell Technologies, ThermoFischer and Shalek Lab

## General principles/rules:

1. Keep all tissues undissociated and on ice until ready
2. Keep all cells on ice unless actively dissociating
3. Never vortex digestion enzymes
4. Always save at least 1 M cells at each step

MOST IMPORTANTLY: These is a general protocol for the purpose of the workshop. Tissue dissociation protocol are tailored to the cell type and study of interest (noted where appropriate), it's critical to know what cell type you're going after (Is it rare? What percent of the tissue is your cell type/types of interest? Does this cell type survive freeze/thaw well? Do you care about multiple different rare cell types? Do you care about relative proportions of different cell types? What type of tissue are you getting?). For some reference protocols consult <http://www.worthington-biochem.com/tissuedissociation/>

## Materials

- Fresh tissue to be dissociated in either sterile medium or PBS.
- Sterile RPMI or DMEM medium
- Sterile PBS
- Sterile pipettes and tips
- Sterile 15 mL and 50 mL conical tubes
- Sterile 100 um cell strainer
- Hemocytometers (or any other type of cell counter)
- Plastic petri dishes
- Scalpels/ scissors
- Forceps
- RBC Lysis buffer

*Preparation Notes: Begin by thawing a vial of dissociation medium and 0.25% Trypsin at RT or using a hot bead bath 15 min prior to use*

## Protocol

1. Obtain the fresh tissue sample (ie mouse spleens) and transfer it to a sterile culture petri dish on ice containing 1-2 mL of media (DMEM or RPMI).
2. Using sterile forceps and scissors/scalpels, cut the tissue into small pieces about 1 – 2mm in size. For optimal results, make sure to mince the cells into a homogeneous paste.
3. Carefully add 2 mL of Trypsin-EDTA (0.25%) (Enzymes needed for dissociation variate from tissue) into the dish containing the minced tissue and mix well by pipetting up and down several times. *P.s. Use wide bore tip to allow larger pieces of tissue to pass through the tip and mix more thoroughly with the dissociation mixture.*

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4. Transfer the tissue pieces with the tissue dissociation media into a sterile 15 mL conical tube. Incubate for 15 min at 37°C. For best results, it is recommended to check and gently agitate the mixture every 5 min. *P.S. Use wide bore tip if preferred and mix thoroughly.*
5. Add 5 mL of sterile RPMI/DMEM medium and mix gently by inversion.
6. Filter the cell suspension through a 100 um cell strainer into a new sterile 50 mL conical tube. Rinse the strainer with sterile RPMI/DMEM medium to ensure that all cells are captured.
7. Centrifuge the cell suspension at 300 x g for 10 minutes at 4°C.
8. Discard the supernatant and resuspend the cell pellet in 5-10 mL of sterile PBS. Centrifuge the tube again at 300 x g for 10 minutes at 4°C.
9. Resuspend the cell pellet in 2-5 mL of 1x RBC lysis buffer.
10. Incubate the suspension for 5 minutes on ice.
11. Wash the cell suspension with 10-20 mL of PBS.
12. Centrifuge the cells at 400-600 x g for 5 minutes at 4°C and discard the supernatant.
13. Resuspend the cell pellet in 1 mL of PBS.
14. Using a hemocytometer, count the cells and adjust the cell concentration as needed for downstream applications. (See Appendix 1)

## Protocol for Dissociation of Fresh Frozen Tissue for Single-Nuclei RNA Sequencing

(Adapted from Slyper\*, Porter\*, Ashenbergs\*, et al., *Nature Medicine* (2020) doi:10.1038/s41591-020-0844-1)

**2X ST:** (*filter before using, prep in RNase-clean hood, store in 4C TC fridge*)

Reagent	Stock	2X Solution Concentrations	250mL 2X stock
NaCl	5 M	292 mM	14.6 mL
Tris	1 M	20 mM	5 mL
CaCl <sub>2</sub>	1 M	2 mM	500 µL
MgCl <sub>2</sub>	1 M	42 mM	10.5 mL
H <sub>2</sub> O	—	—	219.4 mL

### Buffers (make fresh day of and chill on ice)

**Note:** Need to adjust RNase inhibitor concentration if doing multi-omic

**1X ST + RNase inhibitors (2.5mL per sample):**

- 1.25mL 2X ST
- 1.25mL H<sub>2</sub>O (molecular grade, RNase DNase free)
- 12.5 µL of Protector RNase Inhibitor (0.2 U/uL final concentration)

*Nuclei Extraction Buffer - TST with RNase inhibitors (2mL per sample, more if no gentleMACS used, see below):*

- 1 mL 2X ST (protocol to make 2X stock at start of protocol)
- 10 µL 2% BSA
- 60 µL 1% Tween-20 [990µL H<sub>2</sub>O + 10µL Tween-20]
- 950 µL H<sub>2</sub>O (molecular grade, RNase DNase free)
- 10 µL of Protector RNase Inhibitor (0.2 U/uL final concentration)

*PBS + BSA + RNase Inhibitor (1.5mL per sample)*

- 1342.5µL PBS
- 150µL of 10% BSA (desired final concentration is 1% BSA)
- 7.5µL of Protector RNase Inhibitor (0.2 U/uL final concentration)

### Protocol: Nuclei Isolation with Miltenyi gentleMACS Tissue Dissociator (preferred)

**Note:** For all steps pre-lysis: work in RNase-free environment – ethanol and zap down all surfaces that enter the hood.

**Note:** Minimizing time interval between dissociation and sample lysis is critical. Less than 90 minutes from thawing to sample lysis leads to highest quality data.

1. Keep frozen tissue sample on dry ice
2. Prepare C-tube with 2 mL of Nuclei Extraction Buffer, keep on wet ice
3. Obtain ethanol cleaned forceps, “chip”/“pluck” off flash frozen tissue piece from inside of cryovial, place in C-tube containing Nuclei Extraction Buffer (do not pre-thaw tissue piece, place it frozen into the C-tube)
  - a. *Cut tissue into smaller chunks using a scalpel on a petri dish on dry ice*
  - b. Note: Samples should be in nuclei extraction buffer for ~10min total. If you’re doing 2 samples and are efficient, you may be faster than that, so flick tubes down by hand and wait for 5 min
4. Invert C-tube onto gentleMACS tissue dissociator
5. Homogenize (program: m\_spleen\_01, C-tube), lasts 1 minute
6. Detach C-tube after program ends, inspect for remaining tissue chunks/visible pieces, if they exist (often are stuck up near the cap). Homogenize for an additional run of m\_spleen\_01.
7. [during homogenization] Prepare 50 mL conical containing 40 µm filter on ice, pre-wet filter with 1 mL of 1X ST with RNase inhibitors
8. Optional (to try to increase nuclei yield): Spin gentleMACS tube for 2 min at 500g (break set to 10). Resuspend pellet with nuclei extraction buffer already in the tube
9. Place C-tube on ice
10. Pipette contents of C-tube to wetted filter.
  - a. If not all solution passes through filter, do not repeatedly pipette up and down – we do not want to shear nuclei and increase ambient soup/contamination. As long as the majority of the solution makes it through the filter, proceed with the protocol. If needed, make extra 1X ST with RNase inhibitor and wash (subject to concern that this will take extra time).
11. Wash original C-tube with 1 mL of 1X ST with RNase inhibitors, transfer washings to the 40 µm filter
12. Discard filter and C-tube, and transfer solution from 50mL tube to 15mL Falcon
13. Centrifuge 15 mL conical: 500 g x 10 minutes at 4°C, brake on 5 (*15 min total*)
14. Optional (to try to remove additional debris): Do not disturb the pellet, and aspirate and discard supernatant. Without disturbing the pellet, add 500µL of ST with RNase inhibitors. Spin for 5min at 500g with brake at 5.
15. After centrifugation, aspirate and discard supernatant. Attempt to remove as much supernatant as possible without disturbing pellet. Resuspend pellet in ~500µL of PBS + 1% BSA + RNase inhibitor. Note that it is not recommended to centrifuge again after this point

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- a. Volume for resuspension varies by input frozen tissue, often between 200  $\mu\text{L}$  and 3 mL is sufficient, start with 500  $\mu\text{L}$  and modify after looking at debris and nuclei concentration under microscope
  - b. If working with smaller tissue pieces (e.g., human clinical samples), you may want to resuspend in small volume. Liat mentioned 50 $\mu\text{L}$  to 200 $\mu\text{L}$
16. Pre-wet a 35um filter on FACS tube with 500 $\mu\text{L}$  of PBS + 1% BSA + RNase inhibitor, then transfer resuspended pellet to 35  $\mu\text{m}$  filter top on FACS tube using P200, pipette by gently pushing down on filter so full volume goes through filter
- a. If not all solution passes through filter, do not repeatedly pipette up and down – we do not want to shear nuclei and increase ambient soup/contamination. As long as some solution makes it through the filter, then we will be fine – most protocols only require  $\sim 10^4$  nuclei, and this protocol should give hundreds of thousands to millions of nuclei. As context, I have worked with clinical biopsies that were ~0.5mm in diameter and ~2mm long; I routinely got out hundreds of thousands of nuclei.
17. Count nuclei using hemocytometer
- a. 10  $\mu\text{L}$  nuclei in solution with RNase inhibitors directly into hemocytometer port. Collaborators have noted that trypan can cause components of the extraction or ST buffer to crash out and create debris. If possible, DAPI staining provides easier counts.
  - b. Look for: shiny, round nuclei, minimal debris
  - c. Do not trust counts coming out of an automated counter
18. Dilute an appropriate number of nuclei in an appropriate volume for your single-cell processing technology of choice.
- a. Honeycomb recommends cells be in 0.1%BSA/PBS
  - b. 10X fixation recommends 0.04% BSA/PBS

If interested in freezing excess nuclei (untested) from either protocol:

Recommended freezing media:

- 597  $\mu\text{L}$  nuclease-free water
- 100 $\mu\text{L}$  10X PBS (1X final concentration)
- 100 $\mu\text{L}$  DMSO (10% final concentration)
- 200 $\mu\text{L}$  10% BSA (2% final concentration)
- 3 $\mu\text{L}$  RNase inhibitor (Protector or RNAsin Plus)

### Protocol: Nuclei Isolation without gentleMACS Tissue Dissociator

Adapted from: Ruiz Daniels, et al. A versatile nuclei extraction protocol for single nucleus sequencing in non-model species-Optimization in various Atlantic salmon tissues. PLoS One. 2023.

**Note:** This protocol requires more 1xST buffer than the gentleMACS protocol, please prepare buffers accordingly

**Note:** For all steps pre-lysis: work in RNase-free environment – ethanol and zap down all surfaces that enter the hood.

**Note:** Minimizing time interval between dissociation and sample lysis is critical. Less than 90 minutes from thawing to sample lysis leads to highest quality data.

19. Place a 6-well tissue culture plate on ice and add 1ml of TST to one well, add frozen tissue sample
20. Keeping the culture plate on ice, mince tissue initially using Tungsten Carbide scissors for 30 seconds
21. Mince for up to 5 minutes with Noyes Spring Scissors
22. Pipette up and down with P1000 for up to 5 minutes (with low retention filter tip)
23. Pass lysate through a 40µm strainer
24. Wash the cell strainer with 1ml TST
25. Add 3ml of chilled 1xST buffer to the lysate to stop the reaction.
26. Move the 5ml of lysate to a labelled 15ml falcon tube on ice
27. Centrifuge at 4°C for 5-10 minutes at 500g in a swinging bucket centrifuge
28. Discard the supernatant with a P1000 pipette, and gently resuspend the pellet in PBS-BSA (100uL - 1mL depending on expected yield, start with 500uL)
29. Transfer resuspended pellet to 35 µm filter top on FACS tube using P200, pipette by gently pushing down on filter so full volume goes through filter
30. Count nuclei using hemocytometer
  - a. *10 µL nuclei in solution with RNase inhibitors directly into hemocytometer port. Collaborators have noted that trypan can cause components of the extraction or ST buffer to crash out and create debris. If possible, DAPI staining provides easier counts.*
  - b. *Look for: shiny, round nuclei, minimal debris*
  - c. *Do not trust counts coming out of an automated counter*
31. Dilute an appropriate number of nuclei in an appropriate volume for your single-cell processing technology of choice.
  - a. Honeycomb recommends cells be in 0.1%BSA/PBS
  - b. 10X fixation recommends 0.04% BSA/PBS

## Appendix 1. Cell counting using a hemocytometer.

1. Add an equal volume of Trypan Blue (dilution factor =2) and mix by gentle pipetting.
2. Load 10  $\mu\text{L}$  of single-cell suspension into hemocytometer port Dilute some of your sample if needed for accurate counting: 200-2,000 cells/ $\mu\text{L}$
3. Count cells in four quadrants of hemocytometer (orange), 16 boxes in each quadrant Do Count all cells fully inside each box, touching the top, and/or left sides of each box Do Notes:
  - Not Count any cells touching the bottom and/or right sides of each box (see Figure 1)
  - Count the number of viable (seen as bright cells) and non-viable cells (stained blue). Ideally >100 cells should be counted in order to increase the accuracy of the cell count (see notes below)

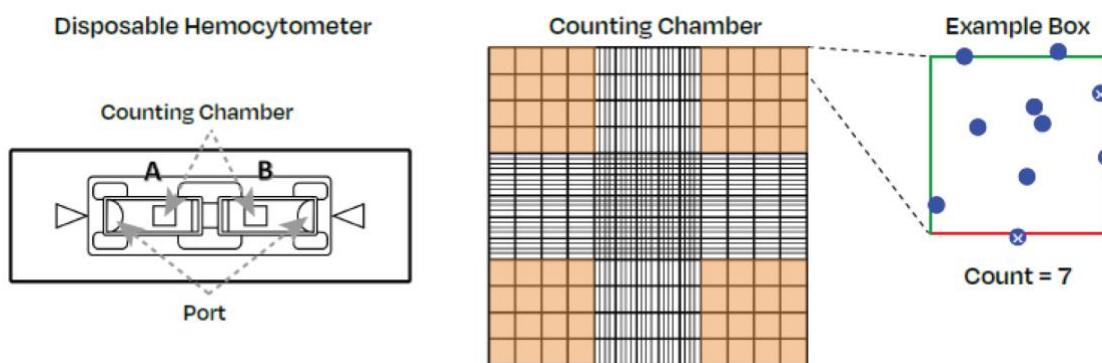


Figure 1. Cell counting example (Adapted from Honeycomb Biotechnologies, Inc.)

4. Calculate average cell count
5. Calculate sample concentration (cells/ $\mu\text{L}$ )
6. Cells per  $\mu\text{L}$  = Average Count x Volume Factor (always 10) x Dilution Factor (ex. 10 for 1:10)

Sample	Quadrant 1	Quadrant 2	Quadrant 3	Quadrant 4	Concentration

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### Appendix 2. Fixation of Cells & Nuclei for Chromium Fixed RNA Profiling



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### Appendix 3. Chromium Fixed RNA Profiling RevA



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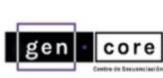
### Appendix 4. HIVE CLX™ scRNASeq Sample Capture



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### Appendix 5. HIVE CLX™ scRNASeq Transcriptome Recovery & Library Preparation

NOTES.

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# Fixation of Cells & Nuclei for Chromium Fixed RNA Profiling

## Introduction

Chromium Fixed RNA Profiling (Gene Expression Flex) offers comprehensive scalable solutions to measure gene expression in single cell and nuclei suspensions that are fixed with formaldehyde. This protocol outlines how to perform fixation on single cell and nuclei suspensions for use with the Chromium Fixed RNA Profiling workflow. This protocol also provides guidance on storage of fixed cells and post-storage processing.

Prior to fixation, samples can also be labeled using a specific protein binding molecule, such as an antibody conjugated to a Feature Barcode oligonucleotide.

**Consult Demonstrated Protocol Cell Surface Protein Labeling for Chromium Fixed RNA Profiling with Feature Barcode technology (CG000529) for guidance.**

## Additional Guidance

This protocol was demonstrated using primary cells (including peripheral blood mononuclear cells - PBMCs), dissociated tumor cells (DTCs), cell lines (including Jurkat, K562, 293T), and nuclei suspensions. Optimize this protocol (e.g., centrifugation conditions) based on sample type.



Cells carry potentially hazardous pathogens. Follow material supplier recommendations and local laboratory procedures and regulations for the safe handling, storage, and disposal of biological materials.

## Specific Reagents & Consumables

Vendor	Item	PN
<b>For Cell Thaw &amp; Sample Fixation</b>		
10x Genomics	Chromium Next GEM Single Cell Fixed RNA Sample Preparation Kit	1000414 <i>Kit components are listed on the next page</i>
<i>Millipore Sigma</i>		
Millipore Sigma	Bovine Serum Albumin In DPBS (10%) Alternative to Thermo Fisher product	A1595
Thermo Fisher Scientific	UltraPure Bovine Serum Albumin (BSA, 50 mg/mL) Alternative to Millipore Sigma product	AM2616
	Formaldehyde (37% by Weight/Molecular Biology), Fisher BioReagents	BP531-25
	Nuclease-free Water (not DEPC-Treated)	AM9937
Miltenyi Biotec	MACS BSA Stock Solution Alternative to Thermo Fisher product	130-091-376
Corning	Phosphate-Buffered Saline, 1X without Calcium and Magnesium	21-040-CV
	*Corning RPMI 1640 1X with L-Glutamine	10-040-CM
VWR	*Seradigm Premium Grade Fetal Bovine Serum (FBS)	97068-085
<i>*Only needed for cell thawing.</i>		
<b>For Cell Counting</b>		
Nexcelom Biosciences	ViaStain PI Staining Solution	CS1-0109-5mL
	ViaStain AOPI Staining Solution	CS2-0106-5mL <i>Alternative to PI Staining Solution.</i>
	<sup>†</sup> Cellaca MX High-throughput Automated Cell Counter	MX-112-0127
<b>For Sample Filtration</b>		
Sysmex	Sterile Single-Pack CellTrics Filters	04-004-2326

Vendor	Item	PN
Miltenyi Biotec	Pre-Separation Filters (30 µm)	130-041-407
<i>Choose either Sysmex or Miltenyi Biotec filter.</i>		
Thermo Fisher Scientific	<sup>†</sup> Countess II FL Automated Cell Counter <i>Discontinued</i>	AMAQAF1000
	Countess Automated Cell Counting Chamber Slides	C10228
	Countess 3 FL Automated Cell Counter	AMQAF2000
	Trypan Blue Stain (0.4%)	T10282
	Ethidium Homodimer-1	E1169

<sup>†</sup>Choose either Countess II/3 or Cellaca.

For Storage & Post-Storage Processing		
Acros Organics	Glycerol, 99.5%, for molecular biology, DNase, RNase and Protease free, Alternative to Millipore Sigma product	327255000
Millipore Sigma	Glycerol for molecular biology, ≥99.0%, Alternative to Acros Organics product	G5516-100ML
	Albumin, Bovine Serum, 10% Aqueous Solution, Nuclease-Free Alternative to Thermo Fisher product	126615
	Protector RNase Inhibitor	3335402001
Thermo Fisher Scientific	UltraPure BSA (50 mg/mL) Alternative to Millipore Sigma product	AM2616
Additional Materials		
Eppendorf	DNA LoBind Tubes 2.0 ml	022431048
	ThermoMixer C	5382000023
VWR	Vortex Mixer	10153-838

This list may not include some standard laboratory equipment.

## Chromium Next GEM Single Cell Fixed RNA Sample Preparation Kit, 16 rxns PN-1000414

Chromium Single Cell Fixed RNA Sample Preparation Kit
16 rxns, PN-1000414
Store at -20°C # PN
● Conc. Fix & Perm Buffer 3 2000517
● Conc. Quench Buffer 6 2000516
● Enhancer 1 2000482

10x  
GENOMICS

## Preparation - Buffers

Buffers for Fixation - Prepare fresh			
Fixation Buffer	Stock	Final	Per Sample (µl)
Maintain at room temperature			
Nuclease-free Water	-	-	791.9
Conc. Fix & Perm Buffer (10x Genomics PN-2000517)	10X	1X	100
<i>Thaw at room temperature. Vortex, check for precipitation, and centrifuge briefly.</i>			
Formaldehyde*	37%	4%	108.1

Quenching Buffer	Stock	Final	Per Sample (µl)
Maintain at 4°C			
Nuclease-free Water	-	-	875.0
Conc. Quench Buffer (10x Genomics PN-2000516)	8X	1X	125.0
<i>Thaw at room temperature. Vortex and centrifuge briefly.</i>			

Additional Buffers
PBS + 0.04% BSA (maintain at 4°C)
*Formaldehyde should always be used with adequate ventilation, preferably in a fume hood. Follow appropriate regulations.

Buffers for Storage of Fixed Samples - Prepare fresh				
50% Glycerol Solution	Stock	Final	For 1 Sample	For 4 samples + 10% (µl)
Mix an equal volume of nuclease-free water and 99% Glycerol, Molecular Biology Grade.				
Filter through a 0.2 µm filter.				
Store at room temperature in 2-ml LoBind tubes.				

Additional Buffers				
0.5X PBS + 0.02% BSA	Stock	Final	For 1 Sample	For 4 samples + 10% (µl)
Prepare fresh & maintain at 4°C				
Nuclease-free Water	-	-	493.0	2169.2
1X PBS	1X	0.5X	500.0	2200.0
RNase-free BSA*	10.0%	0.02%	2.0	8.8
RNase Inhibitor	40.0	0.2	5.0	22.0

\*Adjust the volume of BSA based on the stock percentage so that the final BSA concentration is 0.02%.

All buffer preparations should be fresh.

## Tips & Best Practices

The following recommendations are critical for optimal performance of the Chromium Fixed RNA Profiling assay.

### Sample Quality

- Use high-quality single cell or nuclei suspensions that can withstand the fixation steps.
- Perform pilot experiments to determine if the sample type is suitable for the fixation.
- Highly viable single cell or nuclei suspensions (>80%) will have the greatest sensitivity and cell recovery. However, the Chromium Fixed RNA Profiling assay is robust to samples at much lower viability, with successful results demonstrated even with low viability samples (50% or lower). Low viability samples may have more variable cell calling and lower sensitivity.
- Samples should have minimal debris for best results; debris can have associated RNA that can contribute to noncell background.

### Centrifugation & Pellet Resuspension

- Use a swinging-bucket rotor for higher cell/nuclei recovery.
- Centrifugation speed and time may need optimization depending upon the sample type.
- When working with samples with low cell numbers (i.e. <500,000 cells), complete removal of the supernatant is not required. Up to 30 µl supernatant may be left behind to optimize cell recovery without significantly impacting assay performance.



- After each buffer addition step, gently mix cells/nuclei 5x, or until the pellet is completely resuspended, without introducing bubbles.

### Fixation Conditions

- Fixation temperature and time depend upon the subsequent use of the fixed sample.

Fixed Sample Use	Fixation Time & Temperature
Fixed sample to be processed immediately	1 h at room temperature (20°C)
Fixed sample to be stored subsequently	16-24 h at 4°C

*DO NOT mix samples with different fixation times in one experiment.*

- If planning to store the fixed samples, it is strongly recommended to perform a 16-24 h fixation at 4°C and store the fixed samples at -80°C for best results.

### Fixed Cell Counting

- Accurate sample counting is critical for optimal assay performance.
- It is recommended that the sample be stained with a fluorescent dye such as Ethidium Homodimer-1 or PI Staining Solution and counted using an automated fluorescent cell counter (Countess II Automated Cell Counter or a Cellaca counter).
- See Appendix for details on fixed cell counting.

### Fixed Sample Storage

- Fixed cell or nuclei suspensions can be stored at 4°C for up to 1 week or at -80°C for up to 6 months after resuspending in appropriate reagents.
- Sample storage and post-storage guidelines are provided in the Appendix.

## Cell/Nuclei Number Recommendation

- For sample fixation, the recommended minimum number is 300,000 cells or 500,000 nuclei to ensure there are enough cells/nuclei for the downstream workflow.
- The recommended maximum number for fixation is  $10 \times 10^6$  per 1 ml Fixation Buffer.
- It may be possible to use less than the recommended minimum numbers. The lower cell numbers during fixation will impact the cell input number during hybridization and may affect the data quality.
- Some cell loss is expected during the fixation steps depending up on the sample type, cell type, and user experience.

For Fixation	Input # per Hybridization	
	Singleplex	Multiplex
<b>Optimal Cell Number</b>		
300,000– $10 \times 10^6$ cells	200,000– $2 \times 10^6$ cells	50,000– $2 \times 10^6$ cells
500,000– $10 \times 10^6$ nuclei	400,000– $2 \times 10^6$ nuclei	100,000– $2 \times 10^6$ nuclei
<b>Low Cell Number</b>		
100,000 cells/nuclei	50,000 cells/nuclei	25,000 cells/nuclei

### Important Considerations

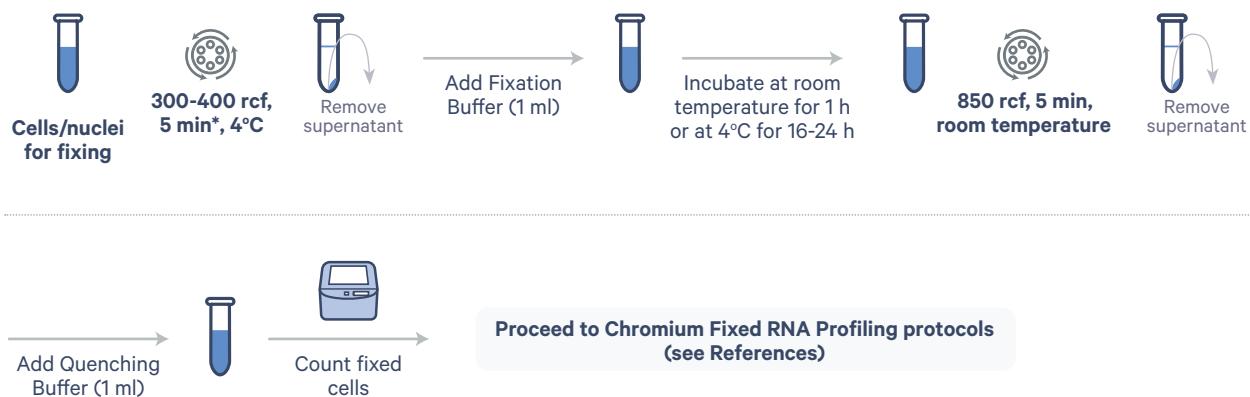
Using low sample input (less than 300,000 cells or less than 500,000 nuclei) for fixation may lead to:

- Loss of pellet
- Difficulty in pooling samples in equal number when multiplexing
- Not enough cells left after washing to target maximum cell load (8,000–10,000 cells/Probe Barcode)
- Drop in usable data and complexity (10% – 20%)

### Mitigation Strategies

- Follow better sample preparation practices including use of a swinging bucket rotor and leaving up to 30  $\mu$ l supernatant behind to avoid losing cell/nuclei pellet
- Consult Chromium Fixed RNA Profiling for Multiplexed Samples - Pooling Workbook (Document CG000565) for guidance on alternative pooling strategies that help in maximizing pellet size and in pooling samples in equal number when multiplexing

## Fix Sample



\*For dissociated tumor cells, centrifuge at 150 rcf for 10 min at 4°C.

## Fixation Protocol

Chromium Next GEM Single Cell Fixed RNA Sample Preparation Kit, 16 rxns (PN-1000414) was used for sample fixation. This protocol is compatible with both cell and nuclei suspensions.

This protocol has been demonstrated using 300,000-10x10<sup>6</sup> cells or 500,000-10x10<sup>6</sup> nuclei. If cell/nuclei number exceeds this recommendation, additional Fixation Buffer will be needed.

### Optional

Label cells with TotalSeq antibody. Refer to Demonstrated Protocol Cell Surface Protein Labeling for Chromium Fixed RNA Profiling with Feature Barcode technology (CG000529) for details. Optional cell surface protein labeling must be completed prior to sample fixation.

### Thaw Cells

If using fresh cells or nuclei suspensions, directly proceed to Sample Fixation.

- a. Pre-warm **10 ml** media (RPMI + 10% FBS) for cell thawing.
- b. Remove cryovials containing cryopreserved cells from storage, thaw in the water bath at **37°C** for **1-2 min**. Remove from the water bath when a tiny ice crystal remains.
- c. Add **1 ml** pre-warmed media (RPMI + 10% FBS) to the thawed cell vial.
- d. Pipette mix the cells and transfer to a 15-ml conical tube containing **9 ml** pre-warmed media (RPMI + 10% FBS).
- e. Centrifuge at **300-400 rcf for 5 min** (PBMCs/cell lines) or **150 rcf for 10 min** (dissociated tumor cells) at **4°C**.

*Use of a swinging-bucket rotor is recommended for higher cell recovery. Centrifugation speed and time may need optimization depending upon the sample type.*

- f. Remove the supernatant without disturbing the pellet.

*Transfer pipettes may be used to remove supernatant after centrifugation to minimize disturbance to the pellet.*

- g. Resuspend the pellet in **1 ml** chilled PBS + 0.04% BSA. Gently pipette mix and transfer to a 1.5-ml microcentrifuge tube.
- h. Determine and record cell concentration and viability of the sample using an Automated Cell Counter (Countess II/3/Cellaca MX) or hemocytometer. If cell debris and large clumps are present, pass the sample through a Cell Strainer.
- i. Proceed to Sample Fixation

### Sample Fixation

- a. Centrifuge sample at **300-400 rcf for 5 min** (PBMCs/cell lines) or **150 rcf for 10 min** (dissociated tumor cells) at **4°C**.
- b. Remove the supernatant without disturbing the pellet.
- c. Add **1 ml** room temperature Fixation Buffer to the sample pellet and pipette mix 5x.
- d. Incubate for **1 h at room temperature (20°C)** or for **16-24 h at 4°C**. If planning to store fixed samples, a **16-24 h** fixation at **4°C** is recommended.



*DO NOT agitate or mix the sample during incubation.*

*To minimize variability for room temperature fixations, incubation at controlled 20°C temperature (e.g. with a thermomixer, heat block, or water bath) is recommended. Fixation time and temperature should be consistent across all samples in an experiment.*

- e. Centrifuge at **850 rcf for 5 min at room temperature**.
- f. Remove the supernatant without disturbing the pellet.
- g. Add **1 ml chilled** Quenching Buffer to the sample pellet and pipette mix 5x and keep on ice.
- h. Determine cell concentration of the fixed sample using an Automated Cell Counter (Countess II/3 or Cellaca MX) or hemocytometer. See Appendix for Fixed Cell/Nuclei Counting.

*For accurate cell counting, it is strongly recommended that the cell/nuclei suspension be stained with a fluorescent dye such as Ethidium Homodimer-1 or AO/PI Staining Solution and counted using an automated fluorescent cell counter.*

- i. Proceed **immediately** to the appropriate Chromium Fixed RNA Profiling protocols (see References) or store the sample after resuspending in appropriate reagents.

 Samples can be stored at 4°C for up to 1 week or at -80°C for up to 6 months, depending upon the reagents used for storage. See Appendix for guidance on storage and post-storage processing.

## Appendix

### Fixed Sample Storage Guidance

Fixed samples can be stored for short or long-term.

#### Short-term Storage at 4°C

- a. Thaw Enhancer (10x Genomics PN-2000482) for **10 min at 65°C**. Vortex and centrifuge briefly. Keep warm and verify no precipitate before use.

 DO NOT keep the thawed reagent on ice, or the solution will precipitate. Once thawed, Enhancer can be kept at 42°C for up to 10 min.

- b. Add **0.1 volume** pre-warmed Enhancer to fixed sample in Quenching Buffer. For example, add 100 µl Enhancer to 1,000 µl fixed sample in Quenching Buffer. Pipette mix. Alternatively, to conserve the Enhancer volume, centrifuge cells at 850 rcf, remove 500 µl Quenching Buffer, and add 50 µl Enhancer to the sample.
- c. Store sample at **4°C** for up to **1 week**.

#### Long-term Storage at -80°C

- a. Thaw Enhancer (10x Genomics PN-2000482) for **10 min at 65°C**. Vortex and centrifuge briefly. Keep warm and verify no precipitate before use.

 DO NOT keep the thawed reagent on ice, or the solution will precipitate. Once thawed, Enhancer can be kept at 42°C for up to 10 min.

- b. Add **0.1 volume** pre-warmed Enhancer to fixed sample in Quenching Buffer. For example, add 100 µl Enhancer to 1,000 µl fixed sample in Quenching Buffer. Pipette mix. Alternatively, to conserve the Enhancer volume, centrifuge cells at 850 rcf, remove 500 µl Quenching Buffer, and add 50 µl Enhancer to

the sample.

- c. Add 50% glycerol for a final concentration of 10%. For example: add 275 µl 50% glycerol to 1,100 µl fixed sample in Quenching Buffer and Enhancer. Pipette mix.

- d. Store at **-80°C** for up to **6 months**.

If planning to store the fixed samples, it is strongly recommended to perform a 16-24 h fixation at 4°C during the fixation step and store the fixed samples at -80°C for best results.

### Post-Storage Processing

Samples may undergo a color change during storage (e.g. black, light gray, or green), however this will not impact assay performance.

If samples were stored at -80°C, thaw at room temperature until no ice is present.

- a. Centrifuge sample at **850 rcf for 5 min at room temperature**.
- b. Remove the supernatant without disturbing the pellet.
- c. Resuspend cell pellet in **1 ml 0.5X PBS + 0.02% BSA\*** (optionally supplemented with 0.2 U/µl RNase Inhibitor) or Quenching Buffer and keep on ice.

\*Use RNase-free BSA at this step. See Specific Reagents & Consumables for details.

- d. Determine cell concentration of the fixed sample using an Automated Cell Counter (Countess II/Cellaca MX) or hemocytometer. See Fixed Cell/Nuclei Counting.
- e. Proceed **immediately** to the appropriate Chromium Fixed RNA Profiling protocols (see References).

### Fixed Sample Shipping Guidance

- a. Fixed samples resuspended in Quenching Buffer supplemented with Enhancer can be shipped with a cold pack. See Short-term Storage for details.
- b. Fixed samples resuspended in Quenching Buffer supplemented with Enhancer and Glycerol can be shipped on dry ice. See Long-term Storage for details.

## Fixed Cell/Nuclei Counting

- Accurate sample counting is critical for optimal assay performance.
- It is strongly recommended that the fixed sample be stained with a fluorescent dye such as Ethidium Homodimer-1 or PI staining solution and counted using an automated fluorescent cell counter (Countess II Automated Cell Counter or a Cellaca counter).

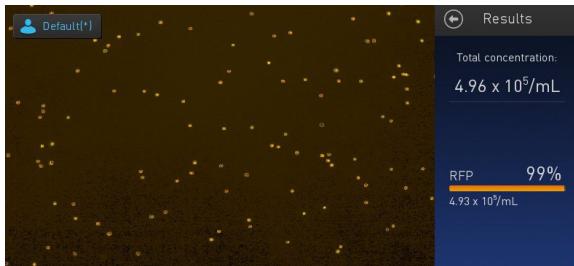
### Counting using Ethidium Homodimer-1

This protocol provides instructions for counting samples using Ethidium Homodimer-1 and the Countess II FL Automated Cell Counter (with RFP light cube) to enable accurate quantification even in the presence of subcellular debris. The optimal cell concentration for the Countess is 1,000-4,000 cells/ $\mu$ L. Refer to manufacturer's instructions for details on operations.

- Vortex Ethidium Homodimer-1, centrifuge briefly, and dilute the concentrated stock as per manufacturer's instructions (~1:100 dilution).
- Aliquot **10  $\mu$ L** diluted Ethidium Homodimer-1 in a tube.
- Gently mix the sample. Immediately add **10  $\mu$ L** sample to **10  $\mu$ L** diluted Ethidium Homodimer-1. Gently pipette mix 10x.
- Transfer **10  $\mu$ L** sample to a Countess II Cell Counting Slide chamber.
- Insert the slide into the Countess II FL Cell Counter. Image the sample using the RFP setting for fluorescent illumination and filtering. Optimize focus and exposure settings.
- Confirm the absence of large clumps using the brightfield mode. Make sure the cell counter is circling RFP positive cells. Note the RFP-positive concentration. Multiply by dilution factor 2 to determine cell concentration.

Samples stained with Ethidium Homodimer-1 can also be counted using the Cellaca counter. Refer to manufacturer's instructions for details.

### Fixed PBMCs Stained with Ethidium Homodimer-1



### Counting using PI Staining Solution

This protocol provides instructions for counting samples using PI staining solution and the Cellaca Counter to enable accurate quantification even in the presence of subcellular debris. The optimal cell concentration for the Cellaca Counter is 100-10,000 cells/ $\mu$ L. Refer to manufacturer's instructions for details on operations.

- Add **25  $\mu$ L** PI Staining Solution into Mixing Row of Cellaca plate
- Gently mix the sample. If the sample is too concentrated, a 1:1 dilution in PBS can also be prepared. For example, add 15  $\mu$ L fixed cell suspension to 15  $\mu$ L PBS.
- Add **25  $\mu$ L** sample to Mixing Row of plate containing PI Staining Solution. Gently pipette mix 8x.
- Transfer stained sample to Loading Row of Cellaca plate.
- For counting fixed samples, only use the PI (Propidium Iodide) channel. Refer to manufacturer's instructions for details.

Samples stained with PI staining solution can also be counted using the Countess II FL Automated Cell Counter. Refer to manufacturer's instructions for details.

## Counting using Trypan Blue (Only for Debris-free Samples)

Debris-free samples (cells or nuclei suspensions) can also be counted using trypan blue. This protocol provides instructions for counting sample using trypan blue and a hemocytometer or Countess II Automated Cell Counter.

- Mix **1 part** 0.4% trypan blue and **1 part** sample.
- Transfer **10  $\mu$ L** sample to a Countess II Cell Counting Slide chamber or a hemocytometer.
- Insert the slide into the Countess II Cell Counter and determine the cell concentration. Or if using a hemocytometer, count fixed cells by placing the hemocytometer under the microscope.
- The majority of fixed cells or nuclei suspensions will be stained with trypan blue stain and appear nonviable.

**Brightfield image from Automated Cell Counter – Samples Stained with Trypan Blue**



## References

1. Chromium Fixed RNA Profiling Reagent Kits for Singleplexed Samples (CG000691)
2. Chromium Fixed RNA Profiling Reagent Kits for Singleplexed Samples with Feature Barcode technology for Protein using Barcode Oligo Capture (CG000674)
3. Chromium Fixed RNA Profiling Reagent Kits for Multiplexed Samples with Feature Barcode technology for Protein using Barcode Oligo Capture (CG000673)
4. Chromium Fixed RNA Profiling Reagent Kits User Guide for Multiplexed Samples (CG000527)
5. Chromium Fixed RNA Profiling Reagent Kits User Guide for Singleplexed Samples with Feature Barcode technology for Protein (CG000477)

## Document Revision Summary

<b>Document Number</b>	CG000478
<b>Title</b>	Fixation of Cells & Nuclei for Chromium Fixed RNA Profiling
<b>Revision</b>	Rev C to Rev D
<b>Revision Date</b>	January 2024
<b>Specific Changes</b>	<p>Updated the section Recommended Cell/Nuclei Number on page 4</p> <p>Added individual components of the Chromium Next GEM Single Cell Fixed RNA Sample Preparation Kit, 16 rxns PN-1000414 on page 2</p> <p>Updated the Reference section to include additional User Guides on page 9</p>
<b>General Changes</b>	Updated for general minor consistency of language and terms throughout.

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Pleasanton, CA 94588 USA



## 2024 Single Cell Workshop Colombia

### Appendix 3. Chromium Fixed RNA Profiling RevA



User Guide | CG000691 | Rev A

# Chromium

# Fixed RNA Profiling Reagent Kits

## For Singleplexed Samples

For use with:

Chromium Next GEM Single Cell Fixed RNA Sample Preparation Kit

16 rxns PN-1000414

Chromium Next GEM Chip Q Single Cell Kit

48 rxns PN-1000418 / 16 rxns PN-1000422

Chromium Fixed RNA Kit, Human Transcriptome,

4 rxns x 1 BC PN-1000474

Chromium Fixed RNA Kit, Mouse Transcriptome,

4 rxns x 1 BC PN-1000495

Dual Index Kit TS Set A 96 rxns PN-1000251

# Notices

## Document Number

CG000691 | Rev A

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## Support

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Pleasanton, CA

# Document Revision Summary

## Document Number

CG000691 | Rev A

## Title

Chromium Fixed RNA Profiling Reagent Kits For Singleplexed Samples

## Revision

Rev A

## Revision Date

September 07, 2023

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# Chromium Fixed RNA Profiling Reagent Kits

*Refer to SDS for handling and disposal information*

## Chromium Next GEM Single Cell Fixed RNA Sample Preparation Kit, 16 rxns PN-1000414

Chromium Next GEM Single Cell Fixed RNA Sample Preparation Kit			
16 rxns, PN-1000414 Store at -20°C			
#	PN		
●	Conc. Fix & Perm Buffer	3	2000517
●	Conc. Quench Buffer	6	2000516
●	Enhancer	1	2000482

10x  
GENOMICS®

## Chromium Fixed RNA Kit, Human Transcriptome, 4 rxns x 1 BC PN-1000474

Chromium Next GEM Single Cell Fixed RNA Hybridization & Library Kit			
4 rxns, PN-1000415 Store at -20°C			
#	PN		
●	Hyb Buffer B	1	2000483
●	Enhancer	1	2000482
●	Conc. Post-Hyb Buffer	1	2000533
○	Reducing Agent B	1	2000087
●	GEM Enzyme Mix	1	2000490
●	GEM Reagent Mix	1	2000491
●	Pre-Amp Primers B	1	2000529
○	Amp Mix	1	2000103

10x  
GENOMICS®

Chromium Next GEM Single Cell Fixed RNA Human Transcriptome Probe Kit			
4 rxns, PN-1000423 Store at -20°C			
#	PN		
●	Human WTA Probes BC001	1	2000495

10x  
GENOMICS®

Chromium Next GEM <b>Single Cell Fixed RNA Gel Bead Kit</b> 4 rxns, PN-1000421 Store at -80°C		
	#	PN
Single Cell TL v1 Gel Beads (4 rxns)	1	2000538

10x  
GENOMICS®

## Chromium Fixed RNA Kit, Mouse Transcriptome, 4 rxns x 1 BC PN-1000495

Chromium Next GEM <b>Single Cell Fixed RNA Hybridization &amp; Library Kit</b> 4 rxns, PN-1000415 Store at -20°C		
	#	PN
Hyb Buffer B	1	2000483
Enhancer	1	2000482
Conc. Post-Hyb Buffer	1	2000533
Reducing Agent B	1	2000087
GEM Enzyme Mix	1	2000490
GEM Reagent Mix	1	2000491
Pre-Amp Primers B	1	2000529
Amp Mix	1	2000103

Chromium Next GEM <b>Single Cell Fixed RNA Mouse Transcriptome Probe Kit</b> 4 rxns, PN-1000490 Store at -20°C		
	#	PN
Mouse WTA Probes BC001	1	2000703

Chromium Next GEM <b>Single Cell Fixed RNA Gel Bead Kit</b> 4 rxns, PN-1000421 Store at -80°C		
	#	PN
Single Cell TL v1 Gel Beads (4 rxns)	1	2000538

10x  
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## Chromium Next GEM Chip Q Single Cell Kit, 48 rxns PN-1000418

<p><b>Chromium Partitioning Oil</b> Store at ambient temperature</p> <table border="1"> <thead> <tr> <th></th><th>#</th><th>PN</th></tr> </thead> <tbody> <tr> <td><input checked="" type="radio"/></td><td>Partitioning Oil</td><td>6</td><td>2000190</td></tr> </tbody> </table>		#	PN	<input checked="" type="radio"/>	Partitioning Oil	6	2000190	<p><b>Chromium Recovery Agent</b> Store at ambient temperature</p> <table border="1"> <thead> <tr> <th></th><th>#</th><th>PN</th></tr> </thead> <tbody> <tr> <td><input type="radio"/></td><td>Recovery Agent</td><td>6</td><td>220016</td></tr> </tbody> </table>		#	PN	<input type="radio"/>	Recovery Agent	6	220016
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## Chromium Next GEM Chip Q Single Cell Kit, 16 rxns PN-1000422

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## Dual Index Kit TS Set A, 96 rxns PN-1000251

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Dual Index Plate TS Set A	1	3000511				

## 10x Genomics Accessories

Product	Part Number (Kit)	Part Number (Item)
10x Vortex Adapter	120251	330002
10x Magnetic Separator	120250	230003
Chromium Next GEM Secondary Holder	1000142	3000332

## Third-Party Items

Successful execution of Chromium Fixed RNA Profiling workflow requires third-party reagents, kits, and equipment in addition to those provided by 10x Genomics. All third-party reagents and consumables should be obtained prior to starting this library construction workflow.

Refer to the [Chromium Fixed RNA profiling - Protocol Planner \(CG000528\)](#) for a detailed list of the following third-party items:

- Additional reagents, kits, and equipment
- Recommended pipette tips
- Recommended thermal cyclers



10x Genomics has tested all items listed in the Protocol Planner. These items perform optimally with the assay. Substituting materials may adversely affect assay performance.

## Protocol Steps & Timing

Steps	Timing	Stop & Store
<b>Sample Fixation</b>	variable*	 4°C ≤1 week/-80°C ≤6 months
*Refer to the appropriate Demonstrated Protocols for details.		
<b>Step 1: Probe Hybridization (page 33)</b>		
1.1 Probe Hybridization (page 35)	16-24 h	
<b>Step 2: GEM Generation and Barcoding (page 37)</b>		
2.1 Post-Hybridization Wash (page 40)	60 min	 -80°C ≤6 months
2.2 Prepare GEM Master Mix + Sample Dilution (page 43)	30 min	
2.3 Load Chromium Next GEM Chip Q (page 47)	10 min	
2.4 Run the Chromium X/iX (page 49)	5.5 min	
2.5 Transfer GEMs (page 50)	5 min	
2.6 GEM Incubation (page 51)	125 min	 4°C ≤1 week (GEMs) -80°C ≤6 months (washed undiluted sample)
<b>Step 3: GEM Recovery and Pre-Amplification (page 52)</b>		
3.1 Post-GEM Incubation – Recovery (page 54)	10 min	
3.2 Pre-Amplification PCR (page 55)	55 min	 4°C ≤72 h/-20°C ≤1 week
3.3 DNA Cleanup – SPRIselect (page 56)	30 min	 4°C ≤72 h/-20°C ≤4 weeks
<b>Step 4: Fixed RNA – Gene Expression Library Construction (page 57)</b>		
4.1 Sample Index PCR (page 59)	40 min	 4°C ≤72 h
4.2 Post Sample Index PCR Size Selection – SPRIselect (page 61)	30 min	 4°C ≤72 h/-20°C long term
4.3 Post Library Construction QC (page 62)	60 min	

## Sample Preparation

This User Guide is compatible with fixed cells, fixed nuclei, cells derived from fixed and dissociated tissue, and cells derived from FFPE tissue sections.

### Sample Preparation Document Resources

Sample Preparation	Description & Resource	
<b>Step 1: Sample fixation</b>  <i>Choose appropriate protocol for fixation/isolation depending upon the sample type.</i>	Fix single cell & nuclei suspensions ( <a href="#">Demonstrated Protocol CG000478</a> )	<b>Sample Fixation</b>  Demonstrated Protocol CG000478
	Fix and dissociate tissues ( <a href="#">Demonstrated Protocol CG000553</a> )	<b>Tissue Fixation</b>  Demonstrated Protocol CG000553
	Isolate cells from FFPE sections ( <a href="#">Demonstrated Protocol CG000632</a> )	<b>FFPE Samples - Cell Isolation</b>  Demonstrated Protocol CG000632

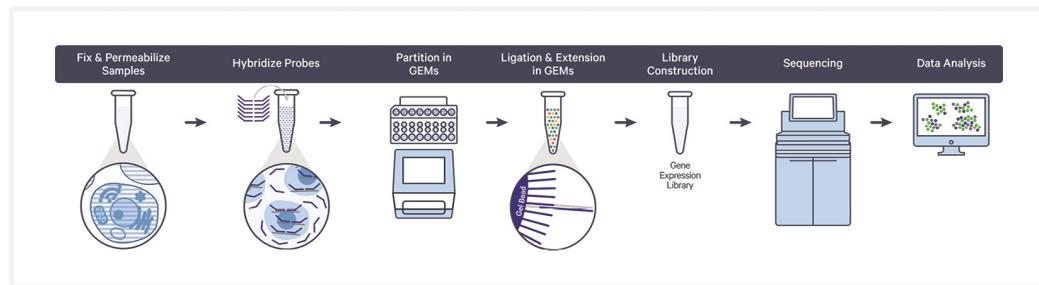
## Stepwise Objectives

Chromium Fixed RNA Profiling offers comprehensive, scalable solutions to measure gene expression in formaldehyde fixed samples. Gene expression is measured using probe pairs designed to hybridize to mRNA specifically.

For this workflow, fixed samples are first hybridized with Probe Barcodes. Using a microfluidic chip, the hybridized samples are then partitioned into nanoliter-scale Gel Beads-in-emulsion (GEMs). A pool of ~737,000 10x GEM Barcodes (also referred to as 10x Barcodes) is sampled separately to index the contents of each partition.

Inside the GEMs, probes are ligated and the 10x GEM Barcode is added, and all ligated probes within a GEM share a common 10x GEM Barcode. Barcoded and ligated probes are then pre-amplified in bulk, after which gene expression libraries are generated and sequenced.

### Workflow Overview

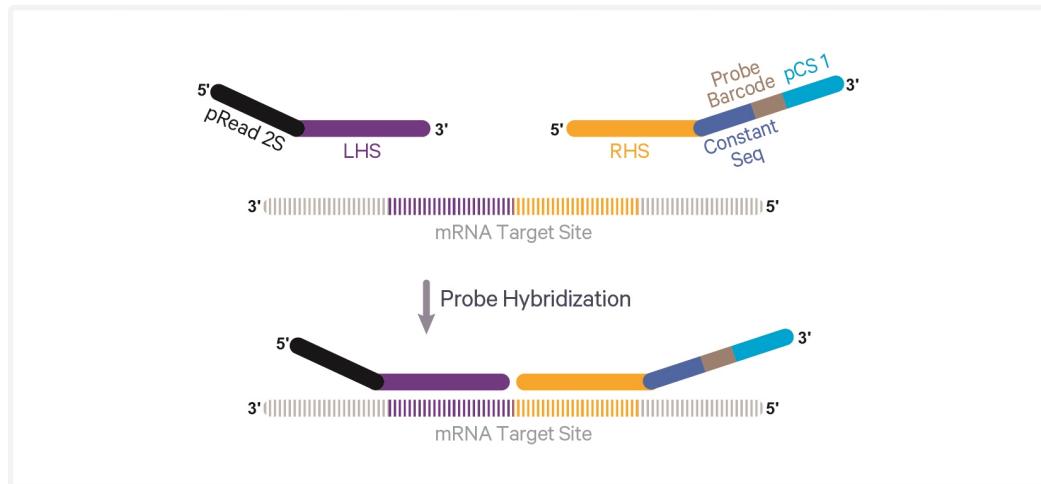


## User Guide Steps

A high-level overview of each step in this User Guide, including gene expression library construction is provided in the following sections.

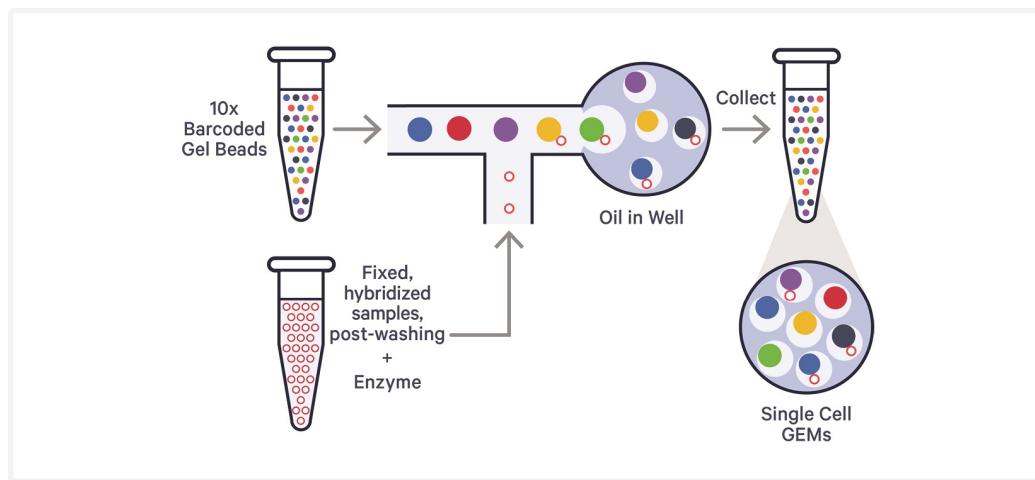
## Step 1: Probe Hybridization

The whole transcriptome probe pairs, consisting of a left hand side (LHS) and a right hand side (RHS) for each targeted gene, are added to the fixed sample. Together, probe pairs hybridize to their complementary target RNA in an overnight incubation.

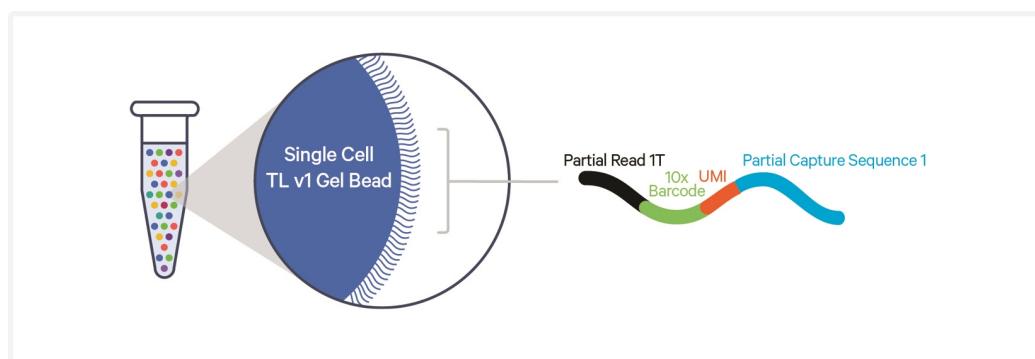


## Step 2: GEM Generation & Barcoding

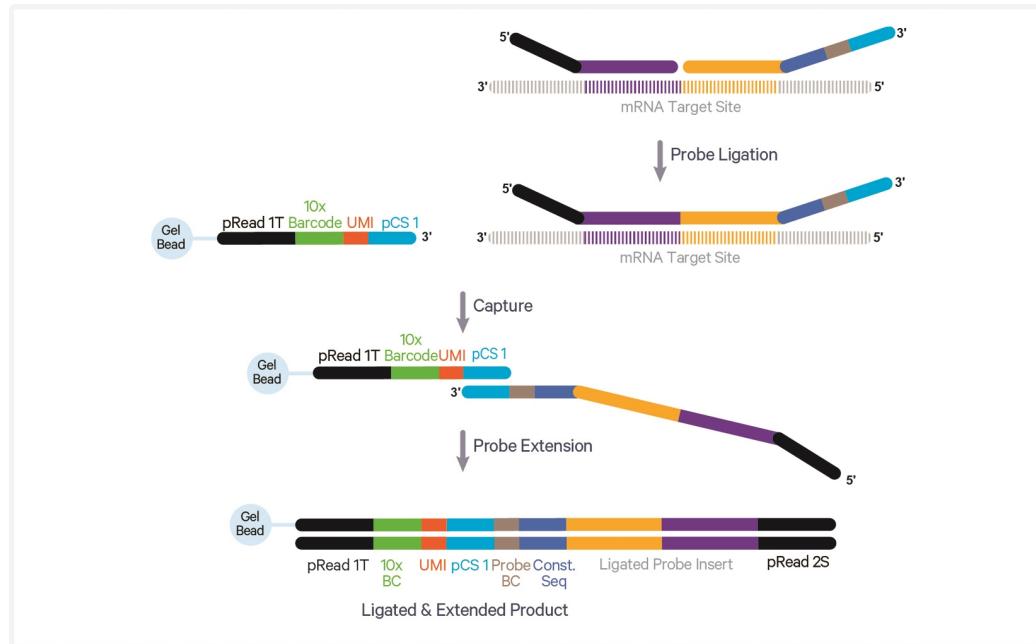
After hybridization, the unbound probes are washed off. GEMs are generated by combining barcoded Gel Beads, a Master Mix containing cells, and Partitioning Oil onto Chromium Next GEM Chip Q.

**GEM Generation**

Immediately following GEM generation, the Gel Bead is dissolved, releasing the barcoded Gel Bead primers, and any co-partitioned cell is lysed. Gel Bead primers contain a partial TruSeq Read 1 sequence (partial Read 1T, read 1 sequencing primer), a 16 nt 10x GEM Barcode (or 10x Barcode), a 12 nt unique molecular identifier (UMI), and partial Capture Sequence 1 (sequence complementary to the probe).

**Gel Bead**

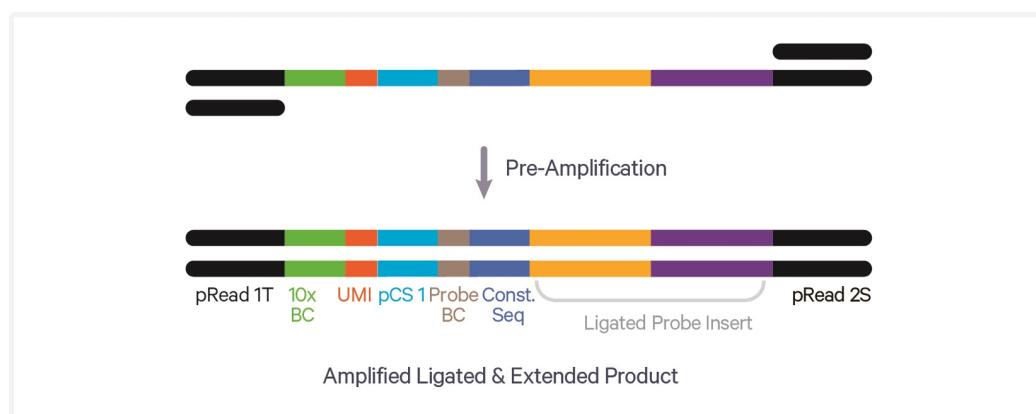
After GEM generation, the partitioned cells, Gel Beads, and Master Mix are placed in a thermal cycler and taken through several steps. First, a ligation step seals the nick between the left hand and right hand probe, while the probes remain hybridized to their target RNA. Second, the Gel Bead primer hybridizes to the capture sequence on the ligated probe pair and is extended by a polymerase to add the UMI, 10x GEM Barcode, and partial Read 1T.

**Inside Individual GEMs**

Finally, a heat denaturation step inactivates the enzymes in the GEM reaction.

## Step 3: GEM Recovery & Pre-Amplification

Once the ligation and barcoding steps are completed, the GEMs are broken by the addition of Recovery Agent, inverting the mixture, and removing the Recovery Agent. A PCR master mix is added directly to the post-GEM aqueous phase to pre-amplify the ligated products. The pre-amplified products are then cleaned up by SPRIselect.

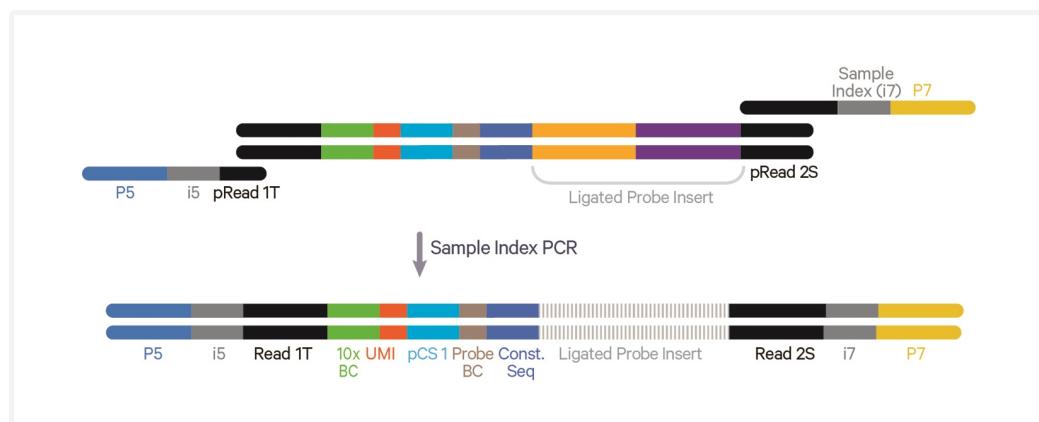
**DNA Pre-Amplification**

## Step 4: Fixed RNA – Gene Expression Library Construction

The 10x barcoded, ligated probe products undergo indexing via Sample Index PCR. This, in turn, generates final library molecules that are cleaned up by SPRIselect, assessed on a bioanalyzer or a similar instrument, quantified, and then sequenced.

P5, P7, i5 and i7 sample indexes, and Illumina TruSeq Read 1 sequence (Read 1T) and Small Read 2 (Read 2S) sequences are added via Sample Index PCR. The final libraries contain the P5 and P7 priming sites used in Illumina sequencers.

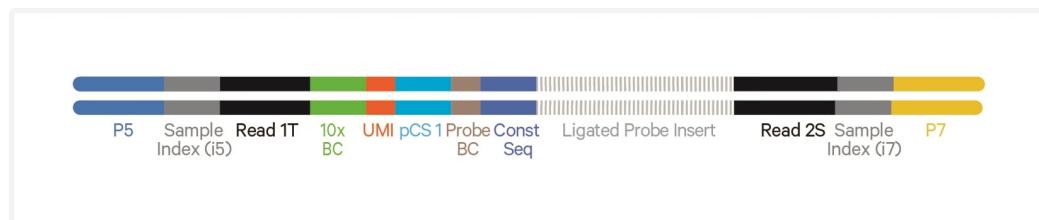
### Pooled Amplified DNA Processed in Bulk



## Step 5: Sequencing

A Chromium Fixed RNA Profiling – Gene Expression library comprises standard Illumina paired-end constructs which begin and end with P5 and P7. The 16 bp 10x GEM Barcode and 12 bp UMI are encoded in Read 1T. Small RNA Read 2 (Read 2S) sequences the ligated probe insert.

**Chromium Fixed RNA Profiling – Gene Expression Library**



See Appendix for Oligonucleotide Sequences on page 75

# Tips & Best Practices



## Icons



Tips & Best Practices section includes additional guidance



Signifies critical step requiring accurate execution



Troubleshooting section includes additional guidance



Chip Q specific steps

## Emulsion-safe Plastics

- Use validated emulsion-safe plastics and other consumables when handling GEMs as some plastics can destabilize GEMs.
- Consult [Fixed RNA Profiling - Protocol Planner \(CG000528\)](#) for a detailed list of plastics and other consumables.

## General Reagent Handling

- Fully thaw the reagents at indicated temperatures. Thoroughly mix reagents before use.
- Keep all enzymes and Master Mixes at indicated temperatures during setup and use. Promptly move reagents back to the recommended storage.
- Calculate reagent volumes with indicated % excess of 1 reaction values.
- Cover Partitioning Oil tubes and reservoirs to minimize evaporation.
- Thoroughly mix samples with the beads during bead-based cleanup steps.

## Pipette Calibration

- Follow manufacturer's calibration and maintenance schedules.
- Pipette accuracy is particularly important when using SPRIselect reagents.

## Probe Hybridization

### Cell Counts

- The minimum input is 200,000 cells or 400,000 nuclei per hybridization and the maximum input is  $2 \times 10^6$  for cells/nuclei per hybridization.
- During post-hybridization washing steps, some cell loss is expected. It is recommended to start the hybridization reaction with  $\sim 1 \times 10^6$  cells/nuclei

per hybridization, if possible.

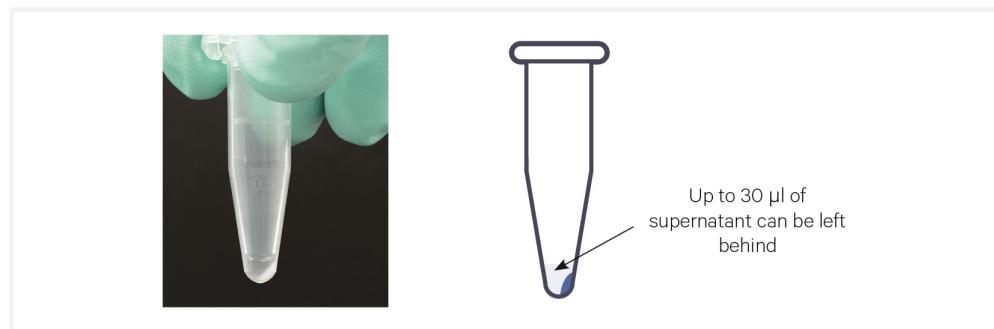
- If proceeding with <500,000 fixed cells in a hybridization reaction, use a swinging bucket rotor for centrifugation and carefully remove the supernatant without disturbing the pellet. In such cases, complete removal of the supernatant is not required. Up to 15 µl of supernatant may be left behind prior to resuspending the cell pellet in the Hyb Mix to optimize cell recovery without significantly impacting assay performance.

### Incubation Time

- Recommended incubation time for probe hybridization is 16-24 h.
- Incubation time should be of same length for all samples. DO NOT mix samples with different hybridization times in one experiment.

## Sample Washing & Recovery

- Swinging bucket centrifuge can increase cell recovery during washing.
- When performing post-hybridization washing with low cell numbers (i.e. <500,000 cells), complete removal of the supernatant is not required. Up to 30 µl of supernatant may be left behind to optimize cell recovery without significantly impacting assay performance.



## Cell Counts for Chip Loading

- The Chromium Fixed RNA Profiling is designed to target 500-10,000 cells per sample with a per sample undetected multiplet rate of 0.4% to 8.0%.
- Recommended starting point is to target ~4,000 cells, and a multiplet rate of ~3.2%.
- The minimum cell input concentration to get maximum cell recovery is 413 cells/µl.

Undetectable Multiplet Rate (%)	# of Cells Loaded	# of Cells Recovered
~0.4	825	500
~0.8	1,650	1,000
~1.6	3,300	2,000
~2.4	4,950	3,000
~3.2	6,600	4,000
~4.0	8,250	5,000
~4.8	9,900	6,000
~5.6	11,550	7,000
~6.4	13,200	8,000
~7.2	14,850	9,000
~8.0	16,500	10,000

## Sample Filtration

- After post-hybridization wash, pass the sample through a 30 µm filter (Sysmex CellTrics or Miltenyi Biotec Pre-Separation) into a new 1.5-ml microcentrifuge tube.



DO NOT use 40 µm Flowmi Tip Strainer for filtration.

- Hold the pipette tip at an angle and touch the filter membrane where the filter meets the wall. Slowly pipette through the filter. Tap gently or centrifuge briefly if liquid remains at the end of the filter.
- To maximize recovery, residual volume can be pipetted from underneath the filter.

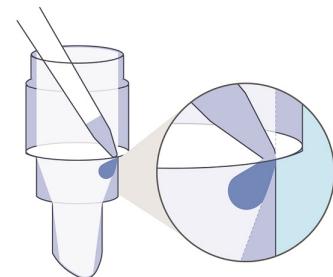
Filtration using 30 µm Filters



Pre-Separation Filters from  
Miltenyi Biotec



Celltrix Filters from Sysmex



Touch the filter membrane  
with the tip where the filter  
meets the wall



Pre-Separation Filters from  
Miltenyi Biotec



Celltrix Filters from Sysmex

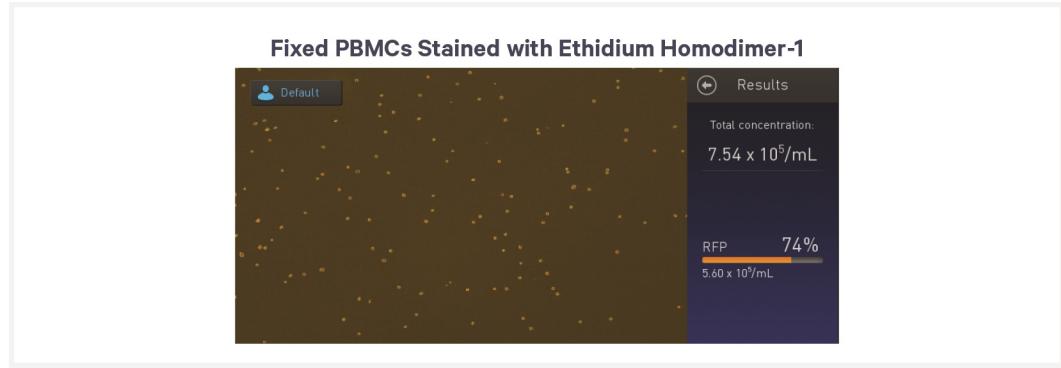
## Cell Counting

- Accurate counting is critical for optimal assay performance.
- It is strongly recommended that the sample be stained with a fluorescent dye such as Ethidium Homodimer-1 or PI staining solution and counted using an automated fluorescent cell counter (Countess II Automated Cell Counter or a Cellaca Counter).
- If using an automated cell counter, ensure that the cells are being circled correctly. The settings of the automated cell counters may need to be adjusted for optimal cell-detection accuracy

### Counting using Ethidium Homodimer-1

This protocol provides instructions for counting sample using Ethidium Homodimer-1 and the Countess II FL Automated Cell Counter (with RFP light cube) to enable accurate quantification even in the presence of sub-cellular debris. The optimal cell concentration for the Countess is 1,000–4,000 cells/ $\mu$ l. Refer to manufacturer's instructions for details on operations.

- Vortex Ethidium Homodimer-1, centrifuge briefly, and dilute the concentrated stock as per manufacturer's instructions (~1:100 dilution).
- Aliquot 10  $\mu$ l diluted Ethidium Homodimer-1 in each tube.
- Gently mix the sample. Immediately add 10  $\mu$ l sample to 10  $\mu$ l diluted Ethidium Homodimer-1. Gently pipette mix 10x.
- Transfer 10  $\mu$ l sample to a Countess II Cell Counting Slide chamber.
- Insert the slide into the Countess II FL Cell Counter. Image the sample using the RFP setting for fluorescent illumination and filtering. Optimize focus and exposure settings and confirm the absence of large clumps using the bright-field mode. Make sure the Countess is circling RFP positive cells. Note the RFP-positive concentration. Multiply by dilution factor 2 to determine cell concentration.



Samples stained with Ethidium Homodimer-1 can also be counted using Cellaca counter. See manufacturer's instructions for details.

## Counting using PI Staining Solution

This protocol provides instructions for counting sample using PI staining solution and the Cellaca Counter to enable accurate quantification even in the presence of sub-cellular debris. The optimal cell concentration for the Cellaca Counter is 100-10,000 cells/ $\mu$ L. Refer to manufacturer's instructions for details on operations.

- Add 25  $\mu$ L PI Staining Solution into Mixing Row of Cellaca plate.
- Gently mix the sample. If the sample is too concentrated, a 1:1 dilution in PBS can also be prepared. For example, add 15  $\mu$ L fixed cell suspension to 15  $\mu$ L PBS.
- Add 25  $\mu$ L sample to Mixing Row of plate containing PI Staining Solution. Gently pipette mix 8x.
- Transfer stained sample to Loading Row of Cellaca plate.
- For counting fixed samples, only use the PI (Propidium Iodide) channel. Refer to manufacturer's instructions for details.

Samples stained with PI Staining Solution can also be counted using Countess II FL Automated Cell Counter. See manufacturer's instructions for details.

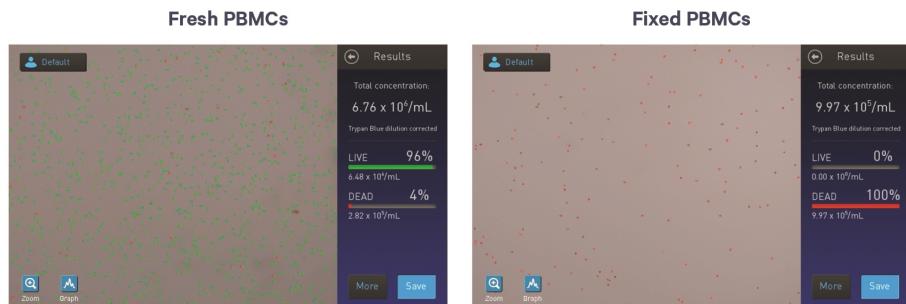
## Counting using Trypan Blue (Only for Debris-Free Samples)

Debris-free samples (cells or nuclei suspensions) can also be counted using trypan blue. This protocol provides instructions for counting sample using trypan blue and a hemocytometer or Countess II Automated Cell Counter.

- Mix 1 part 0.4% trypan blue and 1 part sample.

- Transfer 10  $\mu$ L sample to a Countess II Cell Counting Slide chamber or a hemocytometer.
- Insert the slide into the Countess II Cell Counter and determine the cell concentration. Or if using hemocytometer, count fixed cells by placing hemocytometer under the microscope.
- The majority of fixed cells or nuclei suspensions will be stained with trypan blue stain and appear non-viable.

Brightfield image from Automated Cell Counter – Samples Stained with Trypan Blue





## Chromium Next GEM Chip Handling

- Chromium Fixed RNA Profiling uses Chromium Chip Q.
- Minimize exposure of reagents, chips, and gaskets to sources of particles and fibers, laboratory wipes, frequently opened flip-cap tubes, clothing that sheds fibers, and dusty surfaces.
- After removing the chip from the sealed bag, use in **≤24 h**.
- Execute steps without pause or delay, unless indicated. When using multiple chips, load, run, and collect the content from one chip before loading the next.
- Fill all unused input wells in rows labeled 1, 2, and 3 on a chip with an appropriate volume of 50% glycerol solution before loading the used wells. **DO NOT** add glycerol to the wells in the bottom NO FILL row.
- Avoid contacting the bottom surface of the chip with gloved hands and other surfaces. Frictional charging can lead to inadequate priming of the channels, potentially leading to either clogs or wetting failures.
- Minimize the distance that a loaded chip is moved to reach the Chromium X/iX.
- Keep the chip horizontal to prevent wetting the gasket with oil, which depletes the input volume and may adversely affect the quality of the resulting emulsion.

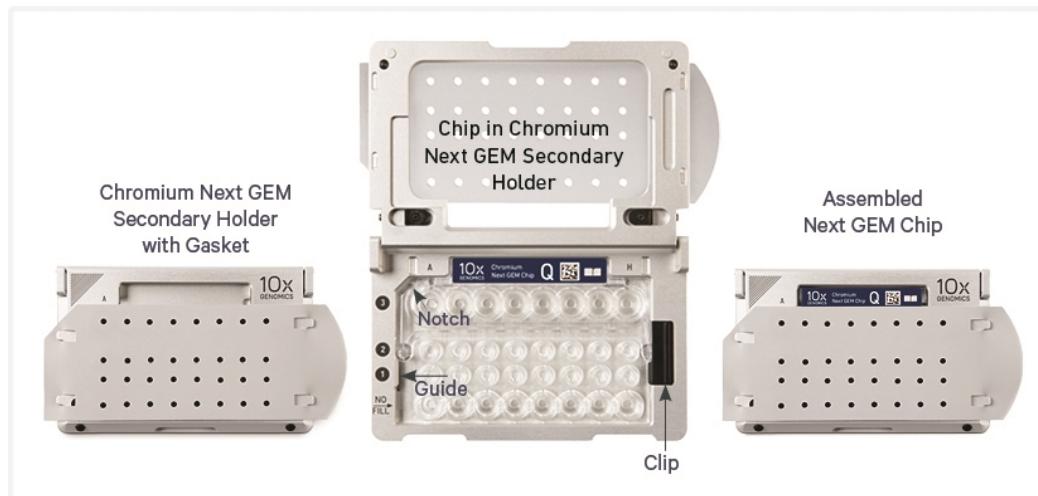
## Chromium Next GEM Secondary Holders



- Chromium Next GEM Secondary Chip Holders encase Chromium Next GEM Chips.
- The holder lid flips over to become a stand, holding the chip at 45 degrees for optimal recovery from each well.
- Squeeze the black sliders on the back side of the holder together to unlock the lid and return the holder to a flat position.

## Chromium Next GEM Chip & Holder Assembly with Gasket

- Chromium Next GEM Chip Q is only compatible with Chromium Next GEM Secondary Holder (PN-3000332). DO NOT use any other holder.
- 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.
- DO NOT touch the smooth side of the gasket.
- Open the chip holder.
- Align notch on the chip (upper left corner) and the open holder with the gasket attached.
- Slide the chip to the left until the chip is inserted under the guide on the holder. Depress the right hand side of the chip until the spring-loaded clip engages.
- Keep the assembled unit with the attached gasket until ready for dispensing reagents into the wells.





## Chromium Next GEM Chip Loading

- Place the assembled chip and holder flat (gasket attached) on the bench with the lid open.
- Dispense at the bottom of the wells without introducing bubbles.
- When dispensing Gel Beads into the chip, wait for the remainder to drain into the bottom of the pipette tips and dispense again to ensure complete transfer.



- Refer to [2.3 Load Chromium Next GEM Chip Q](#) on page 47 for specific instructions.

## 50% Glycerol Solution for Addition to Unused Chip Wells

- Purchase 50% glycerol solution from Ricca Chemical Company, Glycerin (glycerol), 50% (v/v) Aqueous Solution, PN-3290-32.  
OR
- Prepare 50% glycerol solution:
  - Mix an equal volume of water and ≥99% Glycerol, Molecular Biology Grade.
  - Filter through a 0.2 µm filter.
  - Store at **-20°C** in 1-ml LoBind tubes. 50% glycerol solution should be equilibrated to room temperature before use.
- Adding glycerol to non-sample chip wells is essential to avoid chip failure.

## 50% Glycerol Solution for Sample Storage

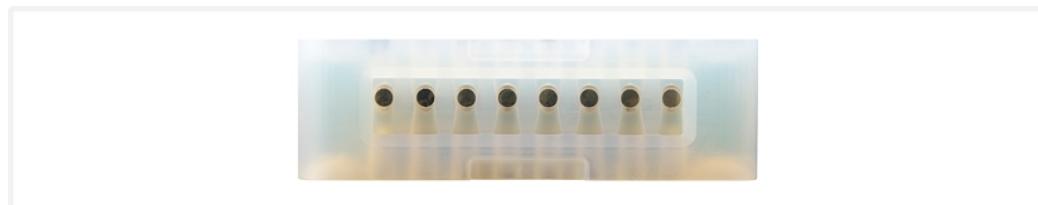
- Use nuclease-free water and molecular biology grade Glycerol from Millipore Sigma, PN-G5516, to prepare fresh 50% glycerol solution as described previously. DO NOT use 50% glycerol solution from Ricca Chemical Company, Glycerin (glycerol), 50% (v/v) Aqueous Solution, PN-3290-32.

## Gel Bead Handling



- Use one tube of Gel Beads per sample. DO NOT puncture the foil seals of tubes not used at the time.
- After removing the Gel Bead strip from the packaging, equilibrate the Gel Bead strip to **room temperature** for at least **30 min** before use.
- Store unused Gel Beads at **-80°C** and avoid more than 12 freeze-thaw cycles. DO NOT store Gel Beads at **-20°C**.
- Snap the tube strip holder with the Gel Bead strip into a 10x Vortex Adapter. Vortex **30 sec**.
- Centrifuge the Gel Bead strip for **~5 sec** after removing from the holder. Confirm there are no bubbles at the bottom of tubes and the liquid levels look even. Place Gel Bead strip back in the holder and secure the holder lid.

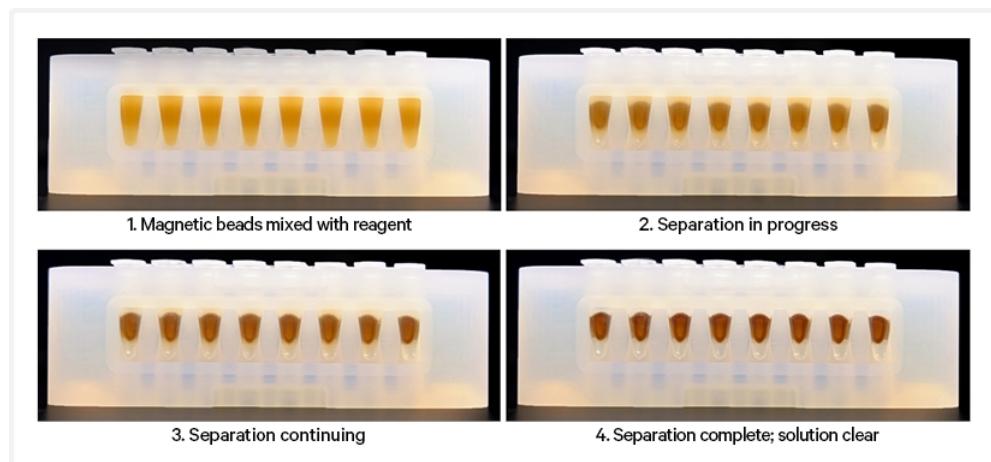
## 10x Magnetic Separator



- Offers two positions of the magnets (high and low) relative to a tube, depending on its orientation. Flip the magnetic separator over to switch between high (magnet•**High**) or low (magnet•**Low**) positions.
- If using MicroAmp 8-Tube Strips, use the high position (magnet•**High**) only throughout the protocol.

## Magnetic Bead Cleanup Steps

- During magnetic bead based cleanup steps that specify waiting “until the solution clears”, visually confirm clearing of solution before proceeding to the next step. See panel below for an example.
- The time needed for the solution to clear may vary based on specific step, reagents, volume of reagents etc.
- **Visually Confirm Clearing of Magnetic Bead Solution**



## SPRIselect Cleanup & Size Selection

- After aspirating the desired volume of SPRIselect reagent, examine the pipette tips before dispensing to ensure the correct volume is transferred.
- Pipette mix thoroughly as insufficient mixing of sample and SPRIselect reagent will lead to inconsistent results.
- Use fresh preparations of 80% Ethanol.

### Tutorial — SPRIselect Reagent:DNA Sample Ratios

SPRI beads selectively bind DNA according to the ratio of SPRIselect reagent (beads).

Example Ratio:  $\frac{\text{Volume of SPRIselect reagent added to the sample}}{\text{Volume of DNA sample}} = \frac{50 \mu\text{l}}{100 \mu\text{l}} = 0.5X$

## Sample Indices in Sample Index PCR

- Choose the appropriate sample index sets to ensure that no sample indices overlap in a multiplexed sequencing run.
- Verify and use the specified index plate only. DO NOT use the plates interchangeably.
- Each well in the Dual Index Plate contains a unique i7 and a unique i5 oligonucleotide.

# Step 1:

## Probe Hybridization

1.0 Get Started	34
1.1 Probe Hybridization	35

## 1.0 Get Started

Action	Item	10x PN	Preparation & Handling	Storage
<b>Thaw &amp; Keep Warm</b>				
<input type="checkbox"/>	 <b>Hyb Buffer B</b>	2000483	<p>Thaw at 42°C. Vortex and centrifuge briefly. Keep warm and verify no precipitate before use.</p> <p> DO NOT keep the thawed buffer on ice, or the solution will precipitate.</p> <p>Thawed Hyb Buffer B can be kept at 42°C for up to 1 h.</p>	-20°C
<input type="checkbox"/>	 <b>Enhancer</b>	2000482	<p>Thaw for 10 min at 65°C. Vortex and centrifuge briefly. Keep warm and verify no precipitate before use.</p> <p> DO NOT keep the thawed reagent on ice, or the solution will precipitate. Once thawed, Enhancer can be kept at 42°C for up to 10 min.</p>	-20°C
<b>Place on Ice</b>				
<input type="checkbox"/>	 <b>Fixed Cell Suspension</b>	—	Consult <a href="#">Chromium Fixed RNA Profiling - Protocol Planner (CG000528)</a> for details on applicable Demonstrated Protocols.	—
<input type="checkbox"/>	 <b>Human WTA Probes BC001</b> OR <b>Mouse WTA Probes BC001</b>	2000495 2000703	Thaw on ice. Vortex and centrifuge briefly.	-20°C

## 1.1 Probe Hybridization



Before starting this protocol, ensure that samples have been appropriately fixed and quenched. Consult Fixed RNA profiling - Protocol Planner (CG000528) for details on the fixation protocols to use.

- Set a thermomixer with heated lid to 42°C or prepare a thermal cycler with the following incubation protocol and start the program.

Lid Temperature	Reaction Volume	Run Time
42°C	100 µl	Overnight
Step	Temperature	Time
Pre-equilibrate	42°C	Hold
Probe Hybridization	42°C	16-24 h

- Prepare Hyb Mix at **room temperature**. Pipette mix 10x.

Hyb Mix	PN	1X* (µl)	1X* + 20% (µl)	4X* + 20% (µl)
<i>Add reagents in the order listed</i>				
● <b>Hyb Buffer B</b> <i>Thaw at 42°C. Add warm to the mix and if appears milky keep it back on 42°C.</i>	2000483	70.0	84.0	336.0
● <b>Enhancer</b> <i>Heat at 65°C for 10 min. Vortex and verify no precipitate. Add warm to the mix.</i>	2000482	10.0	12.0	48.0
<b>Total</b>	-	<b>80.0</b>	<b>96.0</b>	<b>384.0</b>

\*1X = 1 fixed sample, 4X = 4 fixed samples

- Incubate Hyb Mix at **42°C** for **5 min**.
- Centrifuge fixed cells/nuclei resuspended in Quenching Buffer/post-storage processing buffer (0.5X PBS + 0.02% BSA) at **850 rcf** for **5 min** at **4°C**. The following table provides guidelines for number of cells/nuclei recommended during hybridization.

Sample Type	Recommended #
Fixed cells	<b>200,000-2 x 10<sup>6</sup></b>
Fixed nuclei	<b>400,000-2 x 10<sup>6</sup></b>
FFPE dissociated suspension	<b>400,000-2 x 10<sup>6</sup></b>



*DO NOT exceed 2 x 10<sup>6</sup> cells in one hybridization reaction.*

- e. Remove the supernatant.

*If proceeding with <500,000 fixed cells, use a swinging bucket rotor for centrifugation and carefully remove the supernatant without disturbing the pellet. In such cases, complete removal of the supernatant is not required. Up to 15 µl of supernatant may be left behind to optimize cell recovery without significantly impacting assay performance.*



- f. Resuspend each pellet in **80 µl** Hyb Mix. Keep sample at **room temperature**. DO NOT place on ice.
- g. Add **20 µl** Human/Mouse WTA Probes BC001 to the **80 µl** mixture of Hyb Mix and fixed sample and gently pipette mix 10x with pipette set at 80 µl.
- h. Incubate sample for **16-24 h** at **42°C** in a thermomixer with heated lid and no shaking. If a thermomixer with heated lid is not available, samples can be transferred into 8-tube strips and incubated in a thermocycler.



*Incubation for less than 16 h is not recommended. Incubation time should be consistent across all samples in an experiment.*

# Step 2:

## GEM Generation and Barcoding

2.0 Get Started	38
2.1 Post-Hybridization Wash	40
2.2 Prepare GEM Master Mix + Sample Dilution	43
2.3 Load Chromium Next GEM Chip Q	47
2.4 Run the Chromium X/iX	49
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2.6 GEM Incubation	51

2

## 2.0 Get Started



Firmware Version 1.1.0 or higher is required in the Chromium X/iX used for this Chromium Fixed RNA Profiling protocol.

Action	Item	10x PN	Preparation & Handling	Storage
<b>Equilibrate to Room Temperature</b>				
<input type="checkbox"/>	<b>Single Cell TL v1 Gel Beads</b>	2000538	Equilibrate to room temperature 30 min before loading the chip.	-80°C
<input type="checkbox"/>	<input checked="" type="radio"/> <b>Reducing Agent B</b>	2000087	Vortex, verify no precipitate, centrifuge briefly.	-20°C
<b>Thaw &amp; Keep Warm</b>				
<input type="checkbox"/>	<input checked="" type="radio"/> <b>Enhancer</b>	2000482	Thaw for 10 min at 65°C. Vortex and centrifuge briefly. Keep warm and verify no precipitate before use.	-20°C
				DO NOT keep the thawed reagent on ice, or the solution will precipitate. Once thawed, Enhancer can be kept at 42°C for up to 10 min.
<b>Place on Ice</b>				
<input type="checkbox"/>	<input checked="" type="radio"/> <b>Conc. Post-Hyb Buffer</b>	2000533	Thaw at room temperature and keep on ice.	-20°C
<input type="checkbox"/>	<input checked="" type="radio"/> <b>GEM Enzyme Mix</b>	2000490	Centrifuge briefly before adding to the mix.	-20°C
<input type="checkbox"/>	<input checked="" type="radio"/> <b>GEM Reagent Mix</b>	2000491	Thaw at room temperature. Vortex, verify no precipitate, centrifuge briefly. Keep on ice.	-20°C
<b>Obtain</b>				
<input type="checkbox"/>	<input checked="" type="radio"/> <b>Partitioning Oil</b>	2000190	—	Ambient
<input type="checkbox"/>	<b>Next GEM Chip Q</b>	2000518	See Tips & Best Practices.	Ambient
<input type="checkbox"/>	<b>Chromium Next GEM Secondary Chip Holder</b>	3000332	See Tips & Best Practices.	Ambient
<input type="checkbox"/>	<b>10x Gasket</b>	370017/3000072	See Tips & Best Practices.	Ambient
<input type="checkbox"/>	<b>Sample Filters</b>	—	Manufacturer's	Ambient

Action	Item	10x PN	Preparation & Handling	Storage
	<b>Sysmex Sterile Single-pack CellTrics Filters/Miltenyi Biotez Pre-Separation Filters (30 µm)</b>		recommendations.	
<input type="checkbox"/>	<b>10x Vortex Adapter</b>	330002	See Tips & Best Practices.	Ambient
<input type="checkbox"/>	<b>50% glycerol solution</b> for adding to unused wells	—	See Tips & Best Practices.	
<input type="checkbox"/>	<b>Glycerol for molecular biology, ≥99%</b> Prepare fresh 50% glycerol solution for sample storage	—	See Tips & Best Practices.	—

## 2.1 Post-Hybridization Wash



- a.** Prepare Post-Hyb Wash Buffer. Vortex briefly and keep at **room temperature**. DO NOT keep at 4°C.

Post-Hyb Wash Buffer	PN	1X + 10% (ml)*	4X + 10% (ml)*
<i>Add reagents in the order listed</i>			
<b>Nuclease-free Water</b>	-	1.98	7.92
<b>Conc. Post-Hyb Buffer</b>	2000533	0.11	0.44
<b>Enhancer</b> <i>Heat at 65°C for 10 min. Vortex and verify no precipitate. Add warm to the mix.</i>	2000482	0.11	0.44
<b>Total</b>	-	<b>2.2</b>	<b>8.8</b>

\*Volumes are in ml

- b.** Remove tubes from thermomixer (1.5-ml microcentrifuge tubes) after overnight incubation. If hybridization was performed in 8-tube strips, remove tubes from thermal cycler.
- c.** Add **900 µl** Post-Hyb Wash Buffer to the sample in 1.5-ml microcentrifuge tube. Pipette mix 5x.

*If the hybridization was performed in 8-tube strips, add 175 µl Post-Hyb Wash Buffer to the sample, gently pipette mix, and transfer to a 1.5-ml microcentrifuge tube. Wash the tube strips with additional Post-Hyb Wash Buffer, transfer to the microcentrifuge tube, and add the remaining volume of Post-Hyb Wash Buffer for a total of 900 µl Post-Hyb Wash Buffer to the sample.*

- d.** Incubate at **42°C** for **10 min** in a thermomixer or a heat block.
- e.** Centrifuge at **850 rcf** for **5 min** at **room temperature**.
- f.** Remove the supernatant without disturbing the pellet.



*See Tips & Best Practices for Sample Washing & Recovery on page 21.*

- g.** Resuspend cell pellet in **0.5 ml** Post-Hyb Wash Buffer. Pipette mix 5x.
- h.** Incubate at **42°C** for **10 min** in a thermomixer or a heat block.
- i.** Centrifuge at **850 rcf** for **5 min** at **room temperature**.
- j.** Remove the supernatant without disturbing the pellet.

*When using cells derived from FFPE tissue sections, perform one extra 0.5 ml wash by repeating steps g-j one more time*

- k.** Resuspend cell pellet in **0.5 ml** Post-Hyb Wash Buffer. Pipette mix 5x.
- l.** Incubate sample at **42°C** for **10 min** in a thermomixer or a heat block.
- m.** Prepare Post-Hyb Resuspension Buffer. Pipette mix 10x and maintain at **4°C**.

Post-Hyb Resuspension Buffer	PN	1X + 10% ( $\mu$ l)	4X + 10% ( $\mu$ l)
<i>Add reagents in the order listed</i>			
<b>Nuclease-free Water</b>	-	522.5	2090.0
 <b>Conc. Post-Hyb Buffer</b>	2000533	27.5	110.0
<b>Total</b>	-	<b>550.0</b>	<b>2200.0</b>

- n.** Centrifuge the sample at **850 rcf** for **5 min** at **room temperature**.
- o.** Remove the supernatant without disturbing the pellet.
- p.** Resuspend cell pellet in **500  $\mu$ l chilled** Post-Hyb Resuspension Buffer. Pipette mix 20x to resuspend and breakup any cell clumps and maintain on ice.
- q.** Pass the sample through a 30  $\mu$ m filter (Sysmex CellTrics or Miltenyi Biotec Pre-Separation Filters) into a new 1.5-ml microcentrifuge tube and **place on ice**.

DO NOT use 40  $\mu$ m Flowmi Tip Strainer for filtration.



*Filtration is essential for optimal microfluidic performance. Hold the pipette tip at an angle and touch the filter membrane where the filter meets the wall. Slowly pipette through the filter. Tap gently or centrifuge briefly if liquid remains at the end of the filter. To maximize recovery, residual volume can be pipetted from underneath the filter.*



See [Sample Filtration on page 23](#) for details.



- r.** Determine cell concentration of the sample using a Countess II Automated Cell Counter, a Cellaca counter, or a hemocytometer.

See Tips & Best Practices for [Cell Counting on page 24](#). A serial dilution may be needed to accurately determine cell concentration.

*If the sample concentration is not sufficient to achieve the desired targeted cell recovery, concentrate the sample as follows:*

- *Centrifuge a known volume of sample at 850 rcf for 5 min at room temperature.*
- *Carefully remove only a fraction of the supernatant, and pipette thoroughly to resuspend the cell pellet in the remaining volume. The*

*amount of supernatant removed should be proportional to the desired increase in concentration.*

*For example, to increase the concentration 4-fold from a starting volume of 400 µl, centrifuge, then remove 300 µl supernatant, and finally resuspend the cell pellet in the remaining 100 µl (400/100 = 4).*

- Recount to confirm final concentration.



- s. Store the sample (see Sample Storage below) at **-80°C** for up to **6 months** or proceed **immediately** to the next step. If directly proceeding with next step, the undiluted samples can be placed on ice and then stored later after GEM Incubation. See [Prepare GEM Master Mix + Sample Dilution](#) on page 43 for details.

### Sample Storage

- Add 0.1 volume Enhancer to sample in Post-Hyb Resuspension Buffer. For example, add 50 µl Enhancer to 500 µl of sample in Post-Hyb Resuspension Buffer.
- Add 50% glycerol (freshly prepared) for a final concentration of 10%. For example, add 137.5 µl 50% glycerol to 550 µl sample in Post-Hyb Resuspension Buffer and Enhancer.
- Store at **-80°C** for up to **6 months**.

### Using Stored Samples

- When ready to use samples stored at -80°C from this step, thaw at room temperature until no ice remains and then continue from **2.1m** (Prepare Post-Hyb Resuspension Buffer) to wash the sample once before proceeding to the step 2.2. Samples may undergo a color change during storage (e.g. black, light gray, or green), however this will not impact assay performance.



## 2.2 Prepare GEM Master Mix + Sample Dilution

Before preparing GEM Master Mix, ensure that the Gel Beads are properly thawed and ready to use.

- Prepare Master Mix on ice. Pipette mix 15x and centrifuge briefly.

GEM Master Mix		PN	1X ( $\mu$ L)	4X + 10% ( $\mu$ L)
<i>Add reagents in the order listed</i>				
<input checked="" type="radio"/>	GEM Reagent Mix	2000491	20.9	92.1
<input type="radio"/>	Reducing Agent B	2000087	1.7	7.3
<input checked="" type="radio"/>	GEM Enzyme Mix	2000490	12.4	54.6
<b>Total</b>		-	<b>35.0</b>	<b>154.0</b>

- Add the appropriate volume of Post-Hyb Resuspension Buffer to the appropriate volume of sample into each tube of a PCR 8-tube strip on ice. Refer to the Cell Suspension Volume Calculator on the next page for the volumes.

Use the Post-Hyb Resuspension Buffer prepared at the previous step (2.1) for sample dilution. Additional buffer can be prepared using the buffer preparation table in step 2.1.



*Place remaining undiluted sample on ice. These samples can be stored later after GEM incubation. Guidelines for storage of remaining samples are provided in step 2.6 [GEM Incubation on page 51](#).*

- Add **35  $\mu$ L** of prepared GEM Master Mix into each tube containing diluted sample and **immediately** proceed to the next step.

*Pipette mixing at this step is not required, and will be performed prior to loading into the chip.*

## Cell Suspension Volume Calculator

Volume of Cell Suspension Stock per reaction ( $\mu$ l) | Volume of Post-Hyb Resuspension Buffer per reaction ( $\mu$ l)

Cell Stock Concentration (Cells/ $\mu$ l)	Targeted Cell Recovery										
	500	1000	2000	3000	4000	5000	6000	7000	8000	9000	10000
400	2.1 37.9	4.1 35.9	8.3 31.8	12.4 27.6	16.5 23.5	20.6 19.4	24.8 15.3	28.9 11.1	33.0 7.0	37.1 2.9	n/a
600	1.4 38.6	2.8 37.3	5.5 34.5	8.3 31.8	11.0 29.0	13.8 26.3	16.5 23.5	19.3 20.8	22.0 18.0	24.8 15.3	27.5 12.5
800	1.0 39.0	2.1 37.9	4.1 35.9	6.2 33.8	8.3 31.8	10.3 29.7	12.4 27.6	14.4 25.6	16.5 23.5	18.6 21.4	20.6 19.4
1000	0.8 39.2	1.7 38.4	3.3 36.7	5.0 35.1	6.6 33.4	8.3 31.8	9.9 30.1	11.6 28.5	13.2 26.8	14.9 25.2	16.5 23.5
1200	0.7 39.3	1.4 38.6	2.8 37.3	4.1 35.9	5.5 34.5	6.9 33.1	8.3 31.8	9.6 30.4	11.0 29.0	12.4 27.6	13.8 26.3
1400	0.6 39.4	1.2 38.8	2.4 37.6	3.5 36.5	4.7 35.3	5.9 34.1	7.1 32.9	8.3 31.8	9.4 30.6	10.6 29.4	11.8 28.2
1600	0.5 39.5	1.0 39.0	2.1 37.9	3.1 36.9	4.1 35.9	5.2 34.8	6.2 33.8	7.2 32.8	8.3 31.8	9.3 30.7	10.3 29.7
1800	0.5 39.5	0.9 39.1	1.8 38.2	2.8 37.3	3.7 36.3	4.6 35.4	5.5 34.5	6.4 33.6	7.3 32.7	8.3 31.8	9.2 30.8
2000	0.4 39.6	0.8 39.2	1.7 38.4	2.5 37.5	3.3 36.7	4.1 35.9	5.0 35.1	5.8 34.2	6.6 33.4	7.4 32.6	8.3 31.8
2200	0.4 39.6	0.8 39.3	1.5 38.5	2.3 37.8	3.0 37.0	3.8 36.3	4.5 35.5	5.3 34.8	6.0 34.0	6.8 33.3	7.5 32.5
2400	0.3 39.7	0.7 39.3	1.4 38.6	2.1 37.9	2.8 37.3	3.4 36.6	4.1 35.9	4.8 35.2	5.5 34.5	6.2 33.8	6.9 33.1
2600	0.3 39.7	0.6 39.4	1.3 38.7	1.9 38.1	2.5 37.5	3.2 36.8	3.8 36.2	4.4 35.6	5.1 34.9	5.7 34.3	6.3 33.7
2800	0.3 39.7	0.6 39.4	1.2 38.8	1.8 38.2	2.4 37.6	2.9 37.1	3.5 36.5	4.1 35.9	4.7 35.3	5.3 34.7	5.9 34.1
3000	0.3 39.7	0.6 39.5	1.1 38.9	1.7 38.4	2.2 37.8	2.8 37.3	3.3 36.7	3.9 36.2	4.4 35.6	5.0 35.1	5.5 34.5
3200	0.3 39.7	0.5 39.5	1.0 39.0	1.5 38.5	2.1 37.9	2.6 37.4	3.1 36.9	3.6 36.4	4.1 35.9	4.6 35.4	5.2 34.8
3400	0.2 39.8	0.5 39.5	1.0 39.0	1.5 38.5	1.9 38.1	2.4 37.6	2.9 37.1	3.4 36.6	3.9 36.1	4.4 35.6	4.9 35.1
3600	0.2 39.8	0.5 39.5	0.9 39.1	1.4 38.6	1.8 38.2	2.3 37.7	2.8 37.3	3.2 36.8	3.7 36.3	4.1 35.9	4.6 35.4
3800	0.2 39.8	0.4 39.6	0.9 39.1	1.3 38.7	1.7 38.3	2.2 37.8	2.6 37.4	3.0 37.0	3.5 36.5	3.9 36.1	4.3 35.7
4000	0.2 39.8	0.4 39.6	0.8 39.2	1.2 38.8	1.7 38.4	2.1 37.9	2.5 37.5	2.9 37.1	3.3 36.7	3.7 36.3	4.1 35.9
Yellow boxes		Indicate a low transfer volume that may result in higher cell load variability									

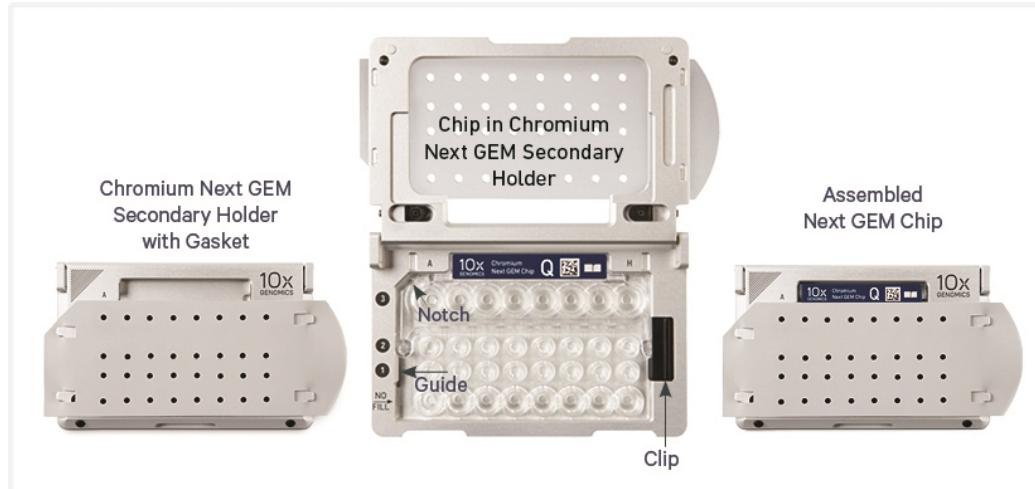
## Assemble Chromium Next GEM Chip Q



Chromium Next GEM Chip Q is only compatible with Chromium Next GEM Secondary Holder (PN-3000332). DO NOT use any other holder.



See [Tips & Best Practices on page 19](#) for chip handling instructions.



- a. 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.
- b. DO NOT touch the smooth side of the gasket.
- c. Open the chip holder.
- d. Remove the chip from the sealed bag. Use the chip within ≤ 24 h.
- e. Align notch on the chip (upper left corner) and the open holder with the gasket attached.
- f. Slide the chip to the left until the chip is inserted under the guide on the holder. Depress the right hand side of the chip until the spring-loaded clip

engages.

- g. Keep the assembled unit with the attached gasket open until ready for and while dispensing reagents into the wells.
- h. DO NOT touch the smooth side of the gasket.
- i. The assembled chip is ready for loading the indicated reagents. Refer to [2.3 Load Chromium Next GEM Chip Q on the next page](#) for reagent volumes and loading order.
- j. After loading reagents, close the chip holder. DO NOT press down on the top of the gasket.



For GEM generation, load the indicated reagents only in the specified rows, starting from row labeled 1, followed by rows labeled 2 & 3.  
DO NOT load reagents in the bottom row labeled NO FILL.

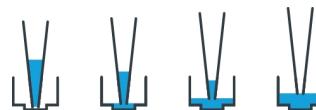


## 2.3 Load Chromium Next GEM Chip Q

Chip loading instructions are unique to Chip Q.



- After removing chip from the sealed bag, use in **≤24 h**.
- Open the lid (gasket attached) of the assembled chip and lay flat for loading.
- When loading the chip, raising and depressing the pipette plunger should each take **~5 sec**. When dispensing, raise the pipette tips at the same rate as the liquid is rising, keeping the tips slightly submerged.

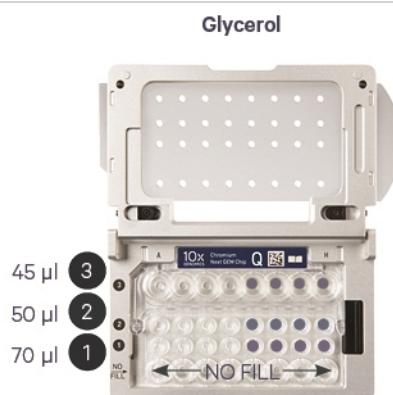


### a. Add 50% glycerol solution to each unused well

- 70 µl** in each unused well in row labeled 1
- 50 µl** in each unused well in row labeled 2
- 45 µl** in each unused well in row labeled 3



**DO NOT** add 50% glycerol solution to the bottom row of NO FILL wells. **DO NOT** use any substitute for 50% glycerol solution.



### b. Prepare Gel Beads

- Snap the tube strip holder with the Gel Bead strip into a 10x Vortex Adapter. Vortex **30 sec**.
- Centrifuge the Gel Bead strip for **~5 sec**. Confirm there are no bubbles at the bottom of the tubes & the liquid levels are even.
- Place the Gel Bead strip back in the holder. Secure the holder lid.



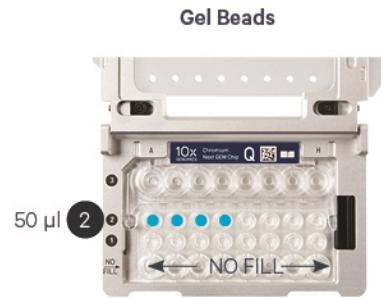
### c. Load Row 1

- ⚠️
- With pipette set to 70 µl, gently **pipette mix** the GEM Master Mix + Sample 15x.
  - Using the same pipette tips, dispense **70 µl** GEM Master Mix + Sample into the bottom center of wells in **row labeled 1** without introducing bubbles.



#### d. Load Row Labeled 2

- Puncture the foil seal of the Gel Bead tubes. Slowly aspirate **50 µl** Gel Beads.
- Dispense into the wells in **row labeled 2** without introducing bubbles.
- Wait **60 sec.**



#### e. Load Row Labeled 3

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

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



#### f. Prepare for Run

- Close the lid (gasket already attached). DO NOT touch the smooth side of the gasket. DO NOT press down on the top of the gasket.

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





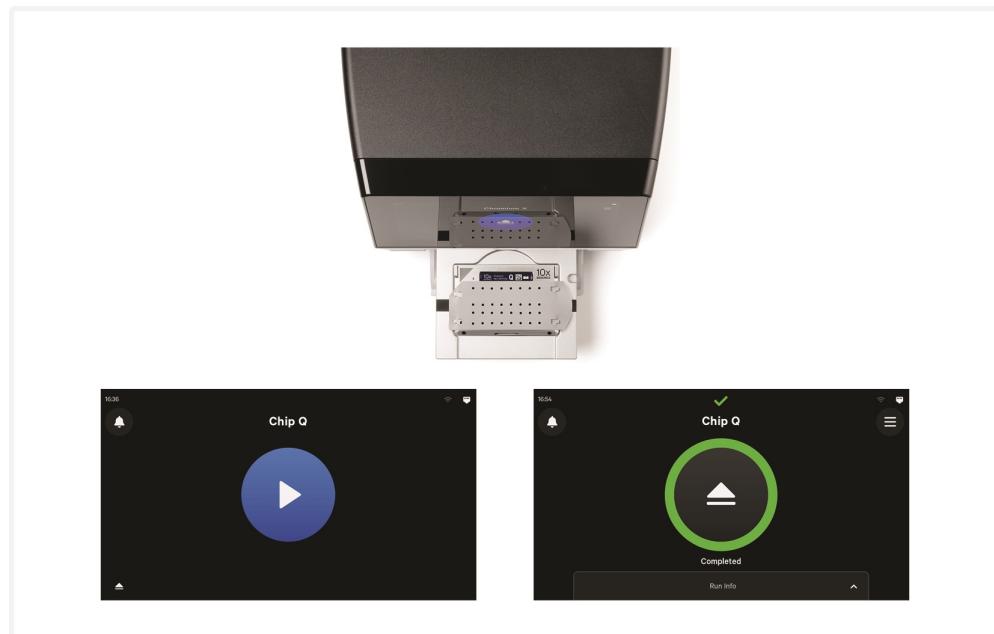
## 2.4 Run the Chromium X/iX

Consult the Chromium X Series (X/iX) User Guide (CG000396) for detailed instrument operation instructions and follow the instrument touchscreen prompts for execution. Run time for Chip Q is ~5.5 min.

- a. Press the eject button on the Chromium X to eject the tray.  
If the eject button is not touched within **1 min**, tray will close automatically. System requires a few seconds before the tray can be ejected again.
- b. Place the assembled chip with the gasket in the tray, ensuring that the chip stays horizontal. Press the button to retract the tray.
- c. Press the play button.
- d. At completion of the run (**~5.5 min**), Chromium X/iX will chime.  
**Immediately** proceed to the next step.



### Run Chromium X/iX

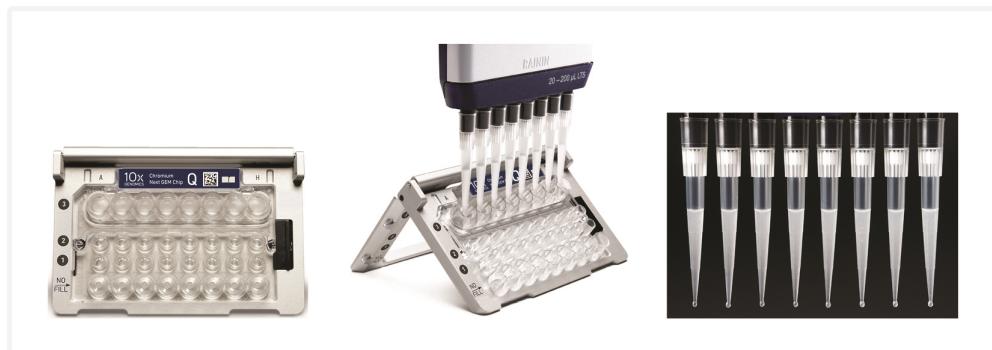


## 2.5 Transfer GEMs

- a. Place a tube strip on ice.
- b. Press the eject button of the Chromium X/iX and remove the chip.
- c. Discard the gasket. Open the chip holder. Fold the lid back until it clicks to expose the wells at 45 degrees.
-  d. Check the volume in rows labeled 1-2. Abnormally high volume in any well indicates a clog.
- e. 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.

*In some cases, minor clogs may result in recovery of >90 µl but <100 µl of GEMs. Though the cell recovery efficiency might be slightly reduced, it's recommended to carry forward with library preparation and sequencing to recover information from the rest of the sample.*

-  f. Withdraw pipette tips from the wells. GEMs should appear opaque and uniform across all channels.



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

## 2.6 GEM Incubation

Use a thermal cycler that can accommodate at least 100 µl volume. A volume of 100 µl is the preferred setting on Bio-Rad C1000 Touch. In alternate thermal cyclers, use highest reaction volume setting.

- Incubate in a thermal cycler with the following protocol.

Lid Temperature	Reaction Volume	Run Time
80°C	100 µl	~125 min
Step	Temperature	Time hh:mm:ss
1	25°C	00:60:00
2	60°C	00:45:00
3	80°C	00:20:00
Hold	4°C	Hold

- Store at **4°C** for up to **a week**, or proceed to the next step.



**DO NOT store the GEMs at -20°C.**

- Sample placed on ice at step [2.2 Prepare GEM Master Mix + Sample Dilution on page 43](#) can either be discarded or stored at **-80°C** for up to **6 months**. See Sample Storage below:

### Sample Storage

- Add 0.1 volume Enhancer to sample in Post-Hyb Resuspension Buffer. For example, add 50 µl Enhancer to 500 µl of sample in Post-Hyb Resuspension Buffer.
- Add 50% glycerol (freshly prepared) for a final concentration of 10%. For example, add 137.5 µl 50% glycerol to 550 µl sample in Post-Hyb Resuspension Buffer and Enhancer.
- Store at **-80°C** for up to **6 months**.

### Using Stored Samples

- When ready to use samples stored at -80°C from this step, thaw at room temperature until no ice remains and then continue from step **2.1m** of [2.1 Post-Hybridization Wash on page 40](#) step to wash the sample once before proceeding to the step 2.2. Samples may undergo a color change during storage (e.g. black, light gray, or green), however this will not impact assay performance.

# Step 3:

## GEM Recovery and Pre-Amplification

3.0 Get Started	53
3.1 Post-GEM Incubation – Recovery	54
3.2 Pre-Amplification PCR	55
3.3 DNA Cleanup – SPRIselect	56

3

## 3.0 Get Started

Action	Item	10x PN	Preparation & Handling	Storage
<b>Equilibrate to Room Temperature</b>				
<input type="checkbox"/>	<input checked="" type="radio"/> Reducing Agent B	2000087	Thaw, vortex, verify no precipitate, centrifuge briefly.	-20°C
<input type="checkbox"/>	<input checked="" type="radio"/> Pre-Amp Primers B Verify name & PN	2000529	Thaw, vortex, centrifuge briefly.	-20°C
<input type="checkbox"/>	Beckman Coulter SPRiselect Reagent	—	Manufacturer's recommendations.	Ambient
<b>Place on Ice</b>				
<input type="checkbox"/>	<input checked="" type="radio"/> Amp Mix	2000103	Vortex and centrifuge briefly.	-20°C
<b>Obtain</b>				
<input type="checkbox"/>	<input checked="" type="radio"/> Recovery Agent	220016	—	Ambient
<input type="checkbox"/>	Qiagen Buffer EB	—	Manufacturer's recommendations.	Ambient
<input type="checkbox"/>	10% Tween 20	—	Manufacturer's recommendations.	Ambient
<input type="checkbox"/>	10x Magnetic Separator	230003	—	Ambient
<input type="checkbox"/>	Prepare 80% Ethanol Prepare 2.5 ml for 4 GEM reactions.	—	Prepare fresh.	—



## 3.1 Post-GEM Incubation – Recovery



- a. Add **125 µl** Recovery Agent to each sample at **room temperature**. DO NOT pipette mix or vortex the biphasic mixture.
- b. 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.



DO NOT invert without firmly securing the caps.

- c. Wait **2 min**.

*The resulting biphasic mixture contains Recovery Agent/Partitioning Oil (pink) and aqueous phase (translucent/opaque).*

*A smaller aqueous phase volume indicates a clog during GEM generation.*



- d. Centrifuge briefly.
- e. Slowly remove and discard **125 µl** Recovery Agent/Partitioning Oil (pink) from the bottom of the tube. DO NOT aspirate any aqueous sample.
- f. Proceed directly to Pre-Amplification PCR. No cleanup step is required.

## 3.2 Pre-Amplification PCR

- Prepare Pre-Amplification Mix on ice. Vortex and centrifuge briefly.

Pre-Amplification Mix		PN	1X ( $\mu$ L)	4X + 10% ( $\mu$ L)
<i>Add reagents in the order listed</i>				
<input type="radio"/>	<b>Amp Mix</b>	2000103	25.0	110.0
<input checked="" type="radio"/>	<b>Pre-Amp Primers B</b>	2000529	10.0	44.0
<b>Total</b>			<b>35.0</b>	<b>154.0</b>

- Add **35  $\mu$ l** Pre-Amplification Mix to aqueous sample from step 3.1f.
- Cap firmly and invert 8x to mix. Centrifuge briefly.
- Incubate in a thermal cycler with the following protocol.

Lid Temperature	Reaction Volume	Run Time
105°C	100 $\mu$ l	~30-45 min
Step	Temperature	Time hh:mm:ss
1	98°C	00:03:00
2	98°C	00:00:15
3	63°C*	00:00:20
4	72°C	00:01:00
5	Go to Step 2, 7x (total 8 cycles)	
6	72°C	00:01:00
7	4°C	Hold

\*Revision-specific update



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

### 3.3 DNA Cleanup – SPRIselect

- a. Prepare Elution Solution. Vortex and centrifuge briefly.

Elution Solution	PN	1000 µl
<i>Add reagents in the order listed</i>		
Buffer EB		980
10% Tween 20	-	10
○ Reducing Agent B	2000087	10
<b>Total</b>		<b>1000</b>

- b. Centrifuge the sample (PCR product) for 30 sec in a microcentrifuge and transfer **70 µl** of the upper layer to a new tube.

*Presence of a cloudy precipitate at the interface between phases is normal. Avoid transferring the precipitate when transferring 70 µl at this step.*

- c. Vortex to resuspend the SPRIselect reagent. Add **126 µl** SPRIselect reagent (**1.8X**) to each sample and pipette mix 15x (pipette set to 180 µl).
- d. Incubate **5 min at room temperature**.
- e. Place on the magnet•**High** until the solution clears.
- f. Remove the supernatant. DO NOT discard any beads.
- g. With the tube still in the magnet, add **200 µl** 80% ethanol to the pellet. Wait **30 sec**.
- h. Remove the ethanol.
- i. **Repeat** steps g and h for a total of 2 washes.
- j. Centrifuge briefly and place on the magnet•**Low**.
- k. Remove any remaining ethanol. DO NOT let the sample dry to ensure maximum elution efficiency.
- l. Remove from the magnet. Add **101 µl** Elution Solution. Wait **1 min** before resuspending. Pipette mix 15x.
- m. Incubate **2 min at room temperature**.
- n. Place the tube strip on the magnet•**High** until the solution clears.
- o. Transfer **100 µl** sample to a new tube strip.
- p.  Store at **4°C** for **≤72 h** or at **-20°C** for **≤4 weeks**, or proceed to the next step.

# **Step 4:**

## **Fixed RNA – Gene Expression Library Construction**

4.0 Get Started	58
4.1 Sample Index PCR	59
4.2 Post Sample Index PCR Size Selection – SPRIselect	61
4.3 Post Library Construction QC	62



## 4.0 Get Started

Action	Item	10x PN	Preparation & Handling	Storage
<b>Equilibrate to Room Temperature</b>				
<input type="checkbox"/>	 <b>Dual Index Plate TS Set A</b> Verify name & PN. Use indicated plate only	3000511	Vortex and centrifuge briefly.	-20°C
<b>Place on Ice</b>				
<input type="checkbox"/>	 <b>Amp Mix</b>	2000103	Vortex and centrifuge briefly.	-20°C
<input type="checkbox"/>	<b>KAPA Library Quantification Kit for Illumina Platforms</b>	—	Manufacturer's recommendations.	—
<b>Obtain</b>				
<input type="checkbox"/>	<b>Qiagen Buffer EB</b>	—	Manufacturer's recommendations.	Ambient
<input type="checkbox"/>	<b>10x Magnetic Separator</b>	230003	See Tips & Best Practices.	Ambient
<input type="checkbox"/>	<b>Prepare 80% Ethanol</b> Prepare 2.5 ml for 4 GEM reactions.	—	Prepare fresh.	Ambient

## 4.1 Sample Index PCR

- a. 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-3000511 Dual Index Plate TS Set A well ID) used.
- b. Prepare Sample Index PCR Mix on ice.

Sample Index PCR Mix	PN	1X ( $\mu$ L)	1X + 10% ( $\mu$ L)	4X + 10% ( $\mu$ L)
<i>Add reagents in the order listed</i>				
○ Amp Mix	2000103	50.0	55.0	220.0
Nuclease-free Water	—	10.0	11.0	44.0
Total		60.0	66.0	264.0

- c. Transfer **ONLY 20  $\mu$ l** sample from the step [DNA Cleanup – SPRIselect](#) on page 56 to a new tube strip.
- d. Add **60  $\mu$ l** Sample Index PCR Mix to **20  $\mu$ l** sample.
- e. Add **20  $\mu$ l** of an individual Dual Index TS Set A to each sample. Pipette mix 5x (pipette set to 90  $\mu$ l). Centrifuge briefly.
- f. Incubate in a thermal cycler with the following protocol.

Lid Temperature	Reaction Volume	Run Time
105°C	100 $\mu$ l	~25-40 min
Step	Temperature	Time hh:mm:ss
1	98°C	00:00:45
2	98°C	00:00:20
3	54°C	00:00:30
4	72°C	00:00:20
5	Go to step 2, see table below for total # of cycles	
6	72°C	00:01:00
7	4°C	Hold

Targeted Cell Recovery	for Cell Lines	Total Cycles*		
		for PBMCs & Nuclei	for Cells from Fixed & Dissociated Tissues**	for Cells from FFPE Tissue Sections
500-2,000	11	15	14-15	16
2,000-4,000	10	14	13-14	15
4,000-7,000	9	13	12-13	14
7,000-10,000	8	12	11-12	13

\*Optimization of cycle number may be needed based on the total RNA content of the sample. The ideal target library concentration is 50 - 200 nM. However, if the concentration is between 10-50 nM or between 200-500 nM and if the libraries do not contain low or high molecular weight peaks, sequencing can still be performed. If optimization is needed, additional Amp Mix can be obtained using the Fixed RNA Feature Barcode Kit (PN-1000419). For dissociated tumor cells, cycle numbers for cell lines can be used as a starting point. For dissociated primary cells, cycle numbers for PBMCs can be used as a starting point.

\*\*For cells derived from the fixed and dissociated tissue samples, the cycle number will depend on the RNA expression level of the tissue and on overall quality of the tissue prior to fixation. Additional optimization may be required.

- g. Store at **4°C** for **≤72 h**, or proceed to the next step.



## 4.2 Post Sample Index PCR Size Selection – SPRIselect

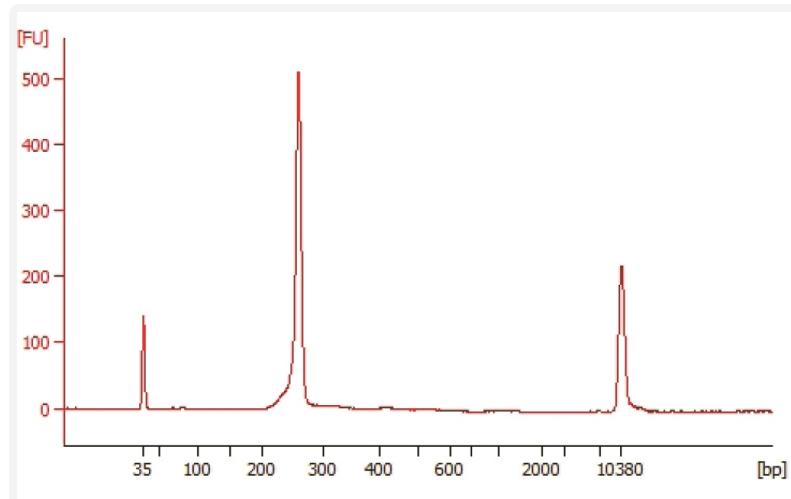
- a. Vortex to resuspend the SPRIselect reagent. Add **100 µl** SPRIselect Reagent (**1.0X**) to each sample. Pipette mix 15x (pipette set to 180 µl).
- b. Incubate **5 min** at **room temperature**.
- c. Place on the magnet•**High** until the solution clears.
- d. Remove the supernatant. DO NOT discard any beads.
- e. With the tube still in the magnet, add **200 µl** 80% ethanol to the pellet. Wait **30 sec**.
- f. Remove the ethanol.
- g. **Repeat** steps e and f for a total of 2 washes.
- h. Centrifuge briefly and place on the magnet•**Low**.
- i. Remove any remaining ethanol. DO NOT let the sample dry to ensure maximum elution efficiency.
- j. Remove from the magnet. Add **41 µl** Buffer EB. Pipette mix 15x.
- k. Incubate **2 min** at **room temperature**.
- l. Place on the magnet•**Low** until the solution clears.
- m. Transfer **40 µl** to a new tube strip.
- n. Store at **4°C** for up to **72 h** or at **-20°C** for **long-term** storage.



## 4.3 Post Library Construction QC

Run **1  $\mu$ l** sample at **1:80** dilution on an Agilent Bioanalyzer High Sensitivity chip. Select the region between 150-300 bp to determine average size of the library.

**Representative Trace**



### Alternate QC Method

Agilent TapeStation

LabChip

See Appendix for:

- Post Library Construction Quantification on page 73
- Agilent TapeStation Traces on page 74
- LabChip Traces on page 74

# Step 5:

## Sequencing

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## Sequencing Libraries

Chromium Fixed RNA Profiling – Gene Expression libraries comprise standard Illumina paired-end constructs which begin with P5 and end with P7. These libraries include 16 bp 10x GEM Barcodes (10x Barcode) encoded at the start of TruSeq Read 1 (Read 1T). Sample index sequences are incorporated as the i5 and i7 index reads.

### Chromium Fixed RNA Profiling – Gene Expression Library



TruSeq Read 1 (Read 1T) and Small RNA Read 2 (Read 2S) are used in paired-end sequencing of Fixed RNA – Gene Expression libraries.

Sequencing these libraries produces a standard Illumina BCL data output folder.

## Illumina Sequencer Compatibility

The compatibility of the listed sequencers has been verified by 10x Genomics. Some variation in assay performance is expected based on sequencer choice. For more information about performance variation, visit the 10x Genomics Support website.

- MiSeq
- NextSeq 500/550
- NextSeq 1000/2000
- NovaSeq 6000

## Sample Indices

Each sample index in the Dual Index Kit TS Set A (PN-1000251) is a mix of one unique i7 and one unique i5 sample index. If multiple samples are pooled in a flow cell lane, the sample index name (i.e. the Dual Index TS Set A plate well ID, SI-TS-) is needed in the sample sheet used for generating FASTQs with “cellranger mkfastq”. Samples utilizing the same sample index should not be pooled together, or run on the same flow cell lane, as this would not enable correct sample demultiplexing.

## Fixed RNA – Gene Expression Library Sequencing Parameters

Parameter	Description
Sequencing Depth	Minimum 10,000 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*

\*Minimum required Read 2 length is 50 bp

## Library Loading

Library quantification should be done with the KAPA DNA Quantification Kit using the average insert size determined by Agilent Bioanalyzer, Perkin Elmer LabChip, or Agilent TapeStation QC. Alternate methods to KAPA qPCR for final library quantification may result in under quantification, and consequently overloading.

Once quantified and normalized, the libraries should be denatured and diluted as recommended for Illumina sequencing platforms. Refer to Illumina documentation for denaturing and diluting libraries. Refer to the 10x Genomics Support website for more information.

The following table provides library loading concentrations that are recommended as general guidelines based on internal testing. Libraries might need to be titrated for optimal performance.

### Library Loading

Instrument	Loading Concentration (pM)	PhiX (%)
MiSeq	12	1
NextSeq 500/550	2.5	1
NextSeq 1000/2000	650	1
NovaSeq 6000 standard	100-150	1
NovaSeq 6000 Xp workflow	150-200	1

*These recommendations are based on qPCR quantification. Alternative quantification methods may affect optimal loading concentration.*

## Library Pooling

Fixed RNA – Gene Expression libraries may be pooled for sequencing, taking into account the differences in cell number and per-cell read depth requirements between each library. Samples utilizing the same sample index should not be pooled together or run on the same flow cell lane, as this would not enable correct sample demultiplexing.

## Data Analysis and Visualization

Sequencing data may be analyzed using Cell Ranger or 10x Genomics Cloud Analysis (see supported pipelines and products webpage) and visualized using Loupe Browser. Key features for these tools are listed below. For detailed product-specific information, visit the 10x Genomics Support website.

### Cell Ranger

Cell Ranger is a set of analysis pipelines that processes Chromium Single Cell Gene Expression and Gene Expression Flex data to align reads, and perform clustering and gene expression analysis.

- Input: Base call (BCL) to generate FASTQ files
- Output: BAM, MEX, CSV, HDF5, Web Summary, .cloupe
- Operating System: Linux

### Loupe Browser

Loupe Browser is an interactive data visualization tool that requires no prior programming knowledge.

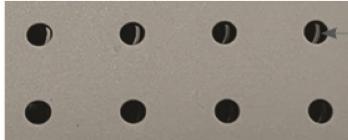
- Input: .cloupe
- Output: Data visualization, including t-SNE and UMAP projections, custom clusters, differentially expressed genes
- Operating System: MacOS, Windows

# Troubleshooting

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## GEM Generation & Barcoding

Step	Normal	Abnormal
After loading the Chip		
	The gasket holes are aligned with the wells.	Gasket holes are misaligned with the sample and gel bead wells. Open and close the chip holder slowly once.
After Chip Q is removed from X/iX and the wells are exposed		
	All recovery wells are similar in volume and opacity.	Recovery well G indicates a reagent clog. Recovery well C and E indicates a wetting failure. Recovery wells B, D, and F are normal. Wells A and H contain 50% Glycerol Solution.
Transfer GEMs		 <ul style="list-style-type: none"><li>○ Adequate emulsion volume (no clog or wetting failure)</li><li>● Wetting failure</li><li>● Low emulsion volume (clog)</li></ul>
	All liquid levels are similar in volume and opacity without air trapped in the pipette tips.	Pipette tip A shows normal GEM generation, pipette tip B indicates a wetting failure, and pipette tip C shows a clog and wetting failure.

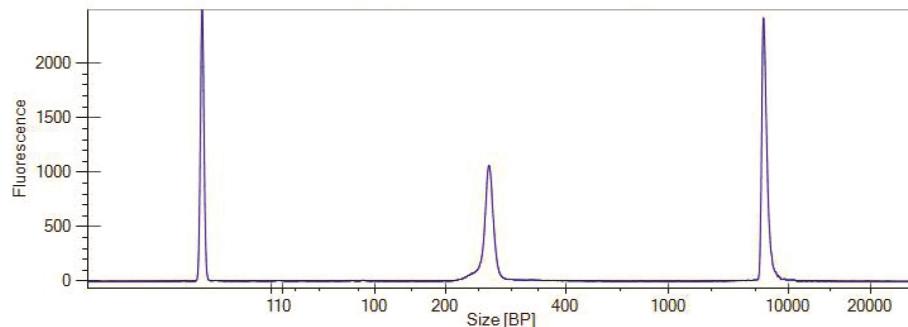


Consult Best Practices to Minimize Chromium Next GEM Chip Clogs and Wetting Failures (Technical Note CG000479) for more information. If a channel clogs or wetting failure occurs during GEM generation, it is recommended that the sample be remade. If any of the listed issues occur, take a picture and send it to [support@10xgenomics.com](mailto:support@10xgenomics.com) for further assistance.

## Post Library Construction QC

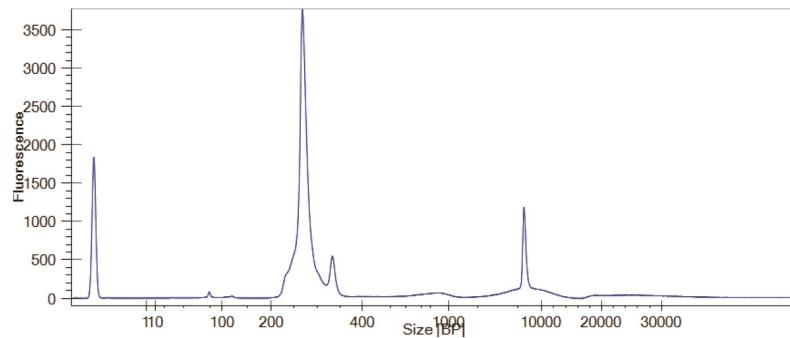
### Step

Fixed RNA – Gene Expression Library  
Correct Sample Index PCR cycling



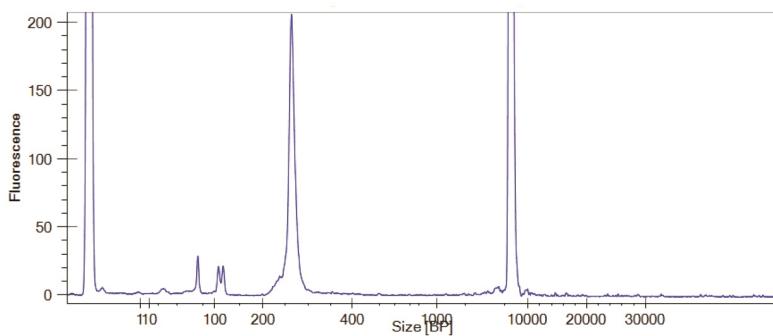
The ideal target library concentration is 50-200 nM. However, if the concentration is between 10-50 nM or between 200-500 nM and if the libraries do not contain low or high molecular weight peaks, sequencing can still be performed.

Over cycling



Additional higher molecular weight peaks present in the library trace indicate over cycling.

Under cycling



Higher proportion of low molecular weight peaks present in the library trace indicate under cycling.

## Chromium X Series Errors

The Chromium X touchscreen will guide the user through recoverable errors. If the error continues, or if the instrument has seen critical or intermediate errors, email [support@10xgenomics.com](mailto:support@10xgenomics.com) with the displayed error code. Support will request a troubleshooting package. Upload pertinent logs to 10x Genomics by navigating to the Logs menu option on screen.

### There are two types of errors:

**Critical Errors** – When the instrument has seen a critical error, the run will immediately abort. Do not proceed with any further runs. Contact [support@10xgenomics.com](mailto:support@10xgenomics.com) with the error code.

- a. System Error
- b. Pressure Error
- c. Chip Error
- d. Run Error
- e. Temperature Error
- f. Software Error

**User Recoverable Errors** – Follow error handling instructions through the touchscreen and continue the run.

- a. Gasket Error
- b. Tray Error
- c. Chip Error
- d. Unsupported Chip Error
- e. Network Error
- f. Update Error



*Consult the Chromium X Series (X/iX) User Guide (CG000396) for additional information and follow the Chromium X touchscreen prompts for execution. The Chromium X touchscreen will guide the user through recoverable errors.*

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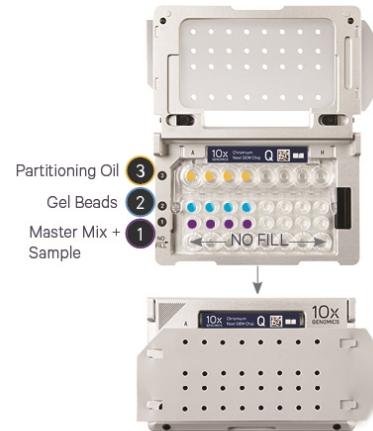


## Chromium Fixed RNA Profiling: Chip Loading Overview

This section provides a quick overview to the Chip Q loading and does not include detailed instructions. Refer to [Load Chromium Next GEM Chip Q](#) on page 47 for details.

### Steps

- a. Add 50% glycerol solution to each unused well
  - Load 70 µl to row labeled 1
  - Load 50 µl to row labeled 2
  - Load 45 µl to row labeled 3
- b. Prepare Gel Beads
  - Vortex for 30 sec
  - Centrifuge for 5 sec
- c. Load Row Labeled 1
  - Mix GEM Master Mix + Sample
  - Load 70 µl to row labeled 1
- d. Load Row Labeled 2
  - Aspirate Gel Beads
  - Load 50 µl to row labeled 2
  - Wait 60 sec
- e. Load Row Labeled 3
  - Load 45 µl Partitioning Oil to row labeled 3
- f. Close the lid and prepare for run.



## Post Library Construction Quantification

- a. Thaw KAPA Library Quantification Kit for Illumina Platforms.
- b. Dilute **2 µl** sample with deionized water to appropriate dilutions that fall within the linear detection range of the KAPA Library Quantification Kit for Illumina Platforms. (For more accurate quantification, make the dilution(s) in duplicate).
- c. Make enough Quantification Master Mix for the DNA dilutions per sample and the DNA Standards (plus 10% excess) using the guidance for 1 reaction volume below.

Quantification Master Mix	1X (µl)
SYBR Fast Master Mix + Primer	12
Water	4
<b>Total</b>	<b>16</b>

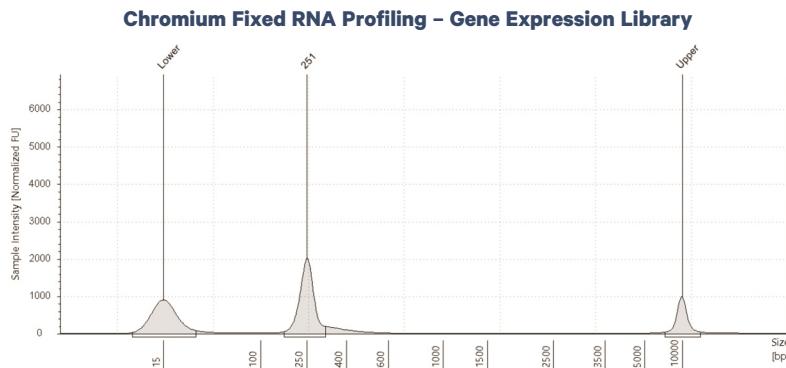
- d. Dispense **16 µl** Quantification Master Mix for sample dilutions and DNA Standards into a 96 well PCR plate.
- e. Add **4 µl** sample dilutions and **4 µl** DNA Standards to appropriate wells. Centrifuge briefly.
- f. Incubate in a thermal cycler with the following protocol.

Step	Temperature	Run Time
1	95°C	00:03:00
2	95°C	00:00:05
3	67°C  Read Signal	00:00:30
4	Go to Step 2, 29X (Total 30 cycles)	

- g. Follow the manufacturer's recommendations for qPCR-based quantification. For library quantification for sequencer clustering, determine the concentration based on insert size derived from the Bioanalyzer/TapeStation trace.

## Agilent TapeStation Traces

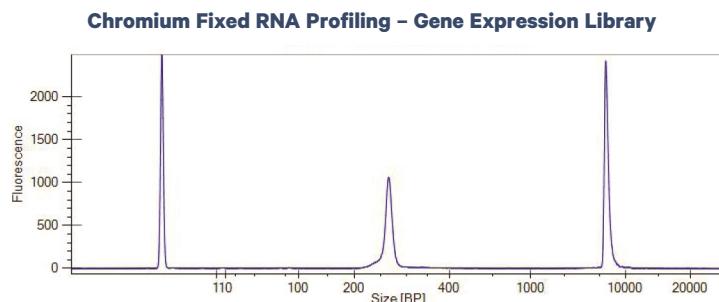
Agilent TapeStation High Sensitivity D5000 ScreenTape was used.



*All traces are representative. Samples were run at 1:80 dilution.*

## LabChip Traces

DNA High Sensitivity Reagent Kit was used.



*All traces are representative. Samples were run at 1:80 dilution.*

## Oligonucleotide Sequences

### Gel Bead Primer

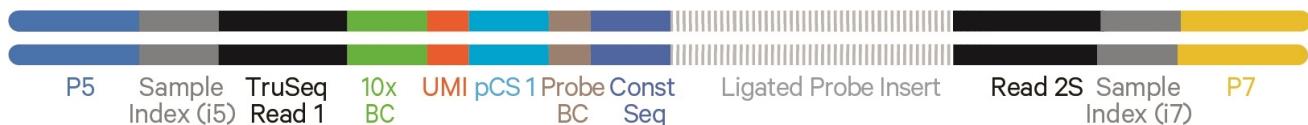
Gel Bead Primers



### Chromium Fixed RNA – Gene Expression Library

5'-AATGATAACGGCGACCACCGAGATCTACAC-N10-ACACTCTTCCCTACAGACGCTTCCGATCT-N16-N12-TTGCTAGGACCG-BC8-NN-TACGTGCTAACCGCGT-Ligated\_Probe\_Insert-TGGAATTCTCGGTGCCAGGAACCTCCAGTCAC-N10-ATCTCGTATGCCGTCTCTGTTG-3'

3'-TTACTATGCCGCTGGGGCTAGATGTG-N10-TGTGAGAAAGGGATGTGCTGGAGAAGGCTAGA-N16-N12-AACGATCCTGGC-BC8-NN-ATCCACGATTGGCGCA-Ligated\_Probe\_Insert-ACCTTAAGAGCCCACGGTCCCTGAGGTAGTG-N10-TAGAGCATACGGCAGAACGAAAC-5'



## 2024 Single Cell Workshop Colombia

### Appendix 4. HIVE CLX™ scRNASeq Sample Capture



HONEYCOMB

# HIVE CLX™ scRNAseq Sample Capture

## User Protocol

Version 1.0 | May 2023

Any cell. Any where. Any time.™





## **This product is for research use only. Not for use in diagnostic procedures.**

This manual is proprietary to Honeycomb Biotechnologies, Inc. and licensed for distribution by Revvity and intended only for customer use in connection with the product(s) described herein and for no other purpose.

This document and its contents shall not be used or distributed for any other purpose without the prior written consent of Honeycomb Biotechnologies, Inc. Follow the protocol associated with the kit.

## **Revision History**

<b>Version</b>	<b>Date</b>	<b>Description</b>
Version 1.0	May 2023	CLX Product Launch

# HIVE CLX™ scRNAseq Sample Capture

---

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# GENERAL INFORMATION

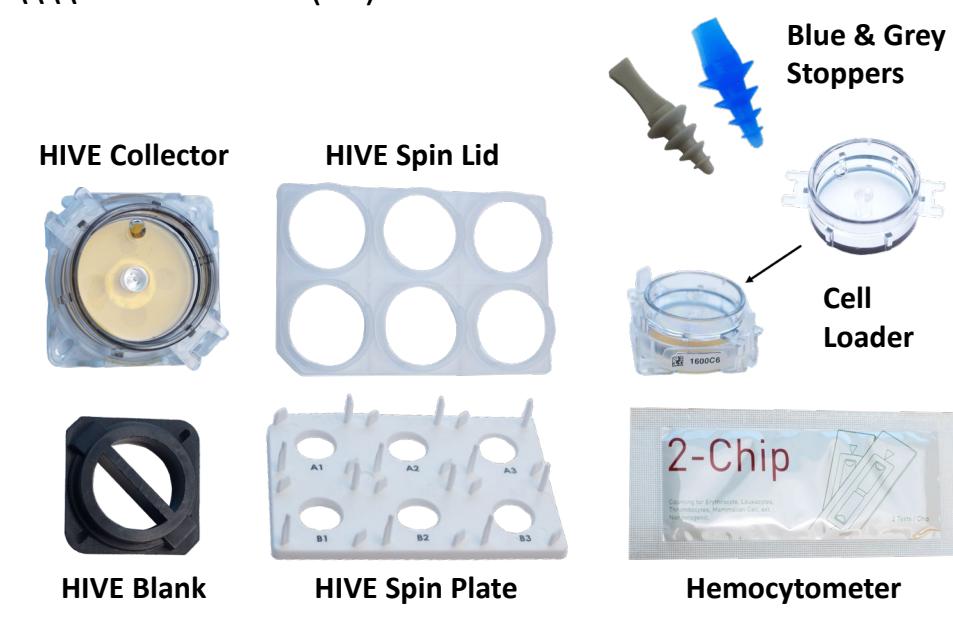
## PRODUCT OVERVIEW

HIVE CLX scRNAseq is a complete solution to create NGS libraries from single cells. This Sample Capture Kit includes handheld, single-use HIVE Collectors for gentle cell capture and scalable sample processing. Each HIVE contains a picowell array that is pre-loaded with barcoded capture beads for 3'-transcripts.

The HIVE CLX scRNAseq Sample Capture product contains enough parts and reagents for 8 samples. Loaded HIVE Collectors can be stably stored and shipped for seamless library prep and sequencing.

## KIT CONTENTS & STORAGE

- //// HIVE Collectors stored at -20°C (x8)
- \|\| Blue Stoppers (x8)
- //// CLX Sample Wash Solution (50 mL)
- \|\| CLX Cell Preservation Solution (20 mL)
- //// Disposable Hemocytometers (x8)
- \|\| HIVE Spin Plates (x2)
- //// HIVE Spin Lids (x2)
- \|\| HIVE Blanks (x4)



**NOTE**

We recommend using PBS + 0.1% BSA as loading media for maximum cell recovery. Use an alternative cell media if your specific sample type is incompatible with PBS + 0.1% BSA.

**NOTE**

Page 10 offers protocols for loading samples with a centrifuge or by gravity in a refrigerator.

**NOTE**

Follow your institution's biosafety protocols for sample handling, which may include using a biosafety cabinet.

**NOTE**

Example of compatible centrifuge: Eppendorf 5810™ with S-4-104 rotor and MTP/Flex buckets.

**NOTE**

If laboratory equipment is limited to the perpendicular orientation, then use positions A2 and B2 only on the HIVE Spin Plate.

**USER-SUPPLIED MATERIALS**

- //// Cell Media (PBS recommended)
- \\$\\$\\$ Cell Media + 1% FBS or 0.1% BSA (PBS + 0.1% BSA recommended)
- //// P1000 (1 mL) pipette & tips
- \\$\\$\\$ P20 (20 µL) pipette & tips
- //// Absorbent paper towels
- \\$\\$\\$ Shipping materials (optional): Large resealable plastic bag, dry ice (30 lb for 3 days in shipping), Styrofoam box, cardboard box, tape, labels

**REQUIRED EQUIPMENT**

- //// -20°C freezer
- \\$\\$\\$ Centrifuge or 4°C refrigerator
- //// Brightfield microscope to count cells

**OPTIONAL EQUIPMENT**

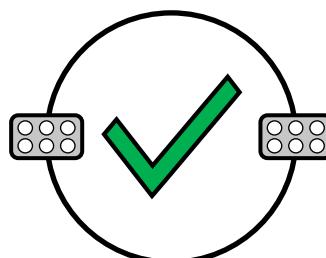
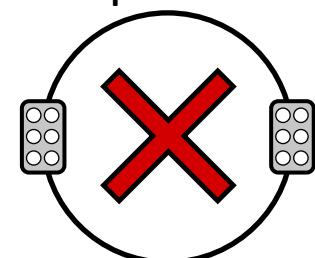
- //// -80°C freezer or dry ice for shipping
- \\$\\$\\$ Biosafety cabinet for sample handling
- //// Nunc™ Square Bioassay Dishes for HIVE handling
- \\$\\$\\$ Vacuum aspirator

**CENTRIFUGE SPECIFICATIONS**

- //// 30 RCF spin speed
- \\$\\$\\$ Swinging-bucket centrifuge
- //// Compatible with deep-well plates
- \\$\\$\\$ Radial plate orientation (see diagram below)

**Radial Orientation**

*Plates must be loaded into the centrifuge in a radial orientation*

**Perpendicular**

# SAMPLE PREPARATION

## IMPORTANT!

$\geq 90\%$  cell viability gives best data.  
Poor viability causes poor data quality.

## NOTE

We recommend washing the cells once with cell media (supplemented with protein) prior to loading.

## IMPORTANT!

Cells will settle quickly.  
Mix the sample immediately before any transfer.

## NOTE

20–200 cells/quadrant  
 $= 200\text{--}2,000 \text{ cells}/\mu\text{L}$

## NOTE

Diluting 50  $\mu\text{L}$  sample to 500  $\mu\text{L}$  is a 10 $\times$  dilution factor. Record dilution factors on next page.

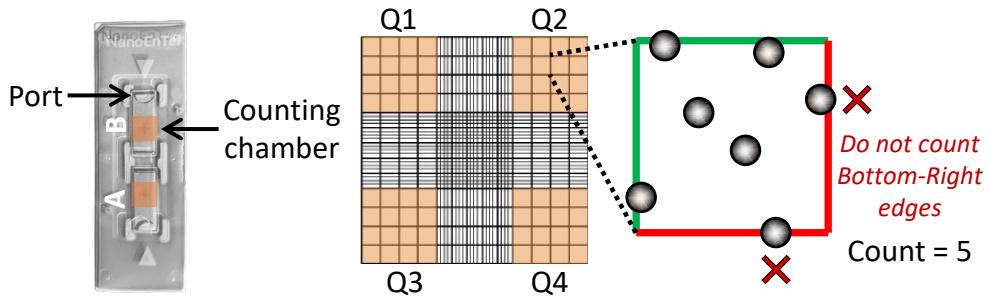
## SAMPLE REQUIREMENTS & RECOMMENDATIONS

- //// Sample must be a single cell suspension in media
- \\\ Media must contain at least 1% FBS or 0.1% BSA
- /// See Appendix 2 for loading recommendations

## MEASURE CELL CONCENTRATIONS

1. Follow user-defined protocols to create single cell suspensions.
2. Open disposable hemocytometer package and place one hemocytometer onto a flat surface.
3. Mix sample with gentle pipetting, then pipet 10  $\mu\text{L}$  into Chamber A of the disposable hemocytometer.
4. Mount the hemocytometer to a microscope, then observe each quadrant of the counting chamber.
  - Count all 4 quadrants (16 boxes/quadrant).
  - Count cells fully inside a box or that touch the top or left sides of a box. Do not count cells touching the bottom or right sides.
  - For accurate counting, you may need to dilute a portion of the sample to make 20–200 cells per quadrant, then repeat Steps 2–4 using Chamber B.
5. Complete tables on next page; then use results to dilute samples with cell media containing protein.
  - We recommend using PBS + 0.1% BSA for dilutions.

### Hemocytometer      Counting Chamber      Example Box





## CALCULATE SAMPLE CELL CONCENTRATIONS

Sample ID	Counts in Hemocytometer Quadrant				Average Cell Count (cells)	Dilution Factor	Sample Concentration (cells/ $\mu$ L)
	Q1	Q2	Q3	Q4			
Example	3	5	5	7	5	10	500

**Dilution Factor** = Diluted Volume / Sample Volume

**Sample Concentration (cells/ $\mu$ L)** = Average Cell Count  $\times$  Dilution Factor  $\times$  10

## CALCULATE DILUTION VOLUMES FOR SAMPLE LOADING

Sample ID	Sample Concentration (cells/ $\mu$ L)	# of HIVE Collectors	Cells Loaded per HIVE (cells)	Loading Volume ( $\mu$ L)	Sample Volume ( $\mu$ L)	Cell Media Volume with Protein ( $\mu$ L)
Example	500	1	30,000	1,000	66	1034

**Loading Volume** recommended at 1,000  $\mu$ L per HIVE

**Cells Loaded per HIVE** recommended at 30,000 cells per HIVE (See Appendix 2)

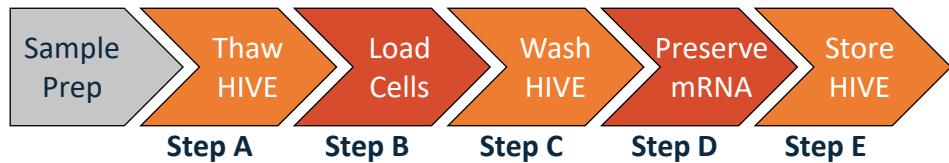
**Sample Volume ( $\mu$ L)** = 1.1  $\times$  (# of HIVE Collectors  $\times$  Cells Loaded) / Sample Concentration

**Media Volume with Protein ( $\mu$ L)** = (1.1  $\times$  Loading Volume) – Sample Volume

**NOTE** Calculations for Sample Volume and Media Volume include a 10% excess volume for loading

# CLX CAPTURE PROTOCOL

## CLX SAMPLE CAPTURE WORKFLOW



## HOW TO HANDLE THE HIVE COLLECTORS

*Handle the HIVE Collector along the edges of the device. Keep port at 12 o'clock position.*

### IMPORTANT!

Pipette along inside of the Cell Loader wall. Avoid pipetting directly onto the yellow HIVE Array.

### NOTE

We recommend a vacuum aspirator to easily remove liquids and bubbles that may appear after spinning (see Troubleshooting on Page 16).

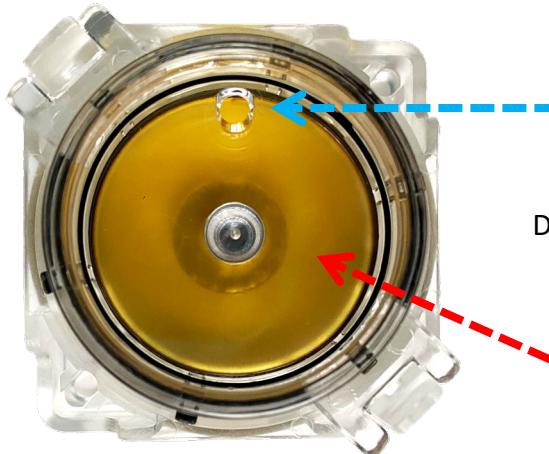
### IMPORTANT!

Always keep HIVE Collectors flat during transportation and incubation. Only tilt when directed to do so.

### NOTE

Keep the port at 12 o'clock with the port edge farthest from you.

### HIVE Collector



#### Port for Pipetting

Insert tip through port and gently pipette at an angle. Dispense along Cell Loader wall.

#### Yellow HIVE Array

Array of picowells pre-loaded with barcoded beads

#### Tilt Towards You



When adding liquid into the HIVE Collector

#### Keep HIVE Flat



When transporting and incubating

#### Tilt Away From You



When removing liquid from the HIVE Collector





Video Protocol

### NOTE

HIVE Collectors may be thawed during sample prep (see Page 6).

### IMPORTANT!

Always keep HIVE Collectors flat. Only tilt when directed to do so.

### IMPORTANT!

If loading samples by gravity, skip to the Alternate Step B on Page 10.

### IMPORTANT!

**Do Steps 5–8 one HIVE at a time!**

### IMPORTANT!

Cells will settle quickly. Thoroughly mix the sample immediately before any transfer.

### IMPORTANT!

Do not touch HIVE Array surface! Hold the pipette tip at an angle and aspirate along the Cell Loader wall (see picture on Page 8)

### NOTE

If using the recommended loading volume of 1 mL sample, dispense ~3 mL media (without added protein).

## STEP A: PREPARE HIVE COLLECTORS AND SAMPLES

1. Remove HIVE Collectors from -20°C freezer.
2. Remove packaging and thaw at room temperature for **30 minutes**. Keep HIVE Collectors **flat**.
3. Label white sticker on the HIVE Collector with sample name and record HIVE serial number in the sample tracking table in Appendix 1.
4. Remove Grey Stopper from sample port and set aside for future use.

## STEP B: LOAD SAMPLES BY CENTRIFUGATION

5. **Tilt the HIVE Collector away from you.** Use a pipette or vacuum aspirator to remove the thawed storage liquid (~1 mL) through the port.
6. **Tilt the HIVE Collector towards you.** Mix the sample that was prepared on Page 6 and dispense the entire Loading Volume through the port.
  - If media bubbles up through the port, remove excess media from the surface, pat the lid dry with a paper towel, and try loading again with a fresh pipette tip.
7. **Tilt the HIVE Collector towards you.** Use a P1000 pipette to dispense cell media (without added protein) through the port to fill the HIVE Collector up to 4 mL total volume.
  - **Tilt the HIVE towards you** to prevent blockages and overflow due to the large liquid volume.
8. **Place the HIVE Collector flat.** Re-insert the Grey Stopper into the sample port.
  - Some liquid may overflow when inserting the Grey Stopper. Pipet excess liquid or pat dry with a paper towel.



**NOTE**

Spin lid does not need to be used in this spin.

**IMPORTANT!**

Load Spin Plates in a radial orientation, not perpendicular (see Page 5).

**NOTE**

Use this Alternative Step if you do not have a suitable centrifuge.

If you use a centrifuge in Step B, DO NOT use this Alternate Step.

9. Put HIVE Collectors onto a HIVE Spin Plate. Align open corners of the HIVE Collectors with the raised pins on the HIVE Spin Plate.

10. Place loaded HIVE Spin Plates into centrifuge.

- Balance centrifuge with additional HIVE Spin Plate and HIVE Blanks, if needed.

11. Centrifuge Spin Plates at 30 RCF for **3 minutes**. Proceed to Step 12.

**ALTERNATE STEP B: LOAD SAMPLES BY GRAVITY**

5Alt. **Tilt the HIVE Collector away from you.** Use a pipette or vacuum aspirator to remove the thawed storage liquid (~1 mL) through the port.

6Alt. **Tilt the HIVE Collector towards you.** Mix the sample that was prepared on Page 6 and dispense the entire sample loading volume through the port. Swirl HIVE Collector to fully cover the array surface. Re-insert the grey stopper into the sample port.

- If media bubbles up through the port, remove excess media from the surface, pat the lid dry with a paper towel, and try loading again with a fresh pipette tip.

7Alt. Place HIVE Collectors in a 4°C refrigerator. Incubate for **30 minutes**. Proceed to Step 12.

- Place all HIVE Collectors flat in the refrigerator.



**IMPORTANT!**

**Do Steps 12–15 one HIVE at a time!**

**NOTE**

DO NOT shake the HIVE vigorously.

**NOTE**

Repeat Steps 14–15 to wash twice if the cell loading media contains higher concentrations of protein (>1% FBS or >0.1% BSA).

**IMPORTANT!**

**Do Steps 16–19 one HIVE at a time!**

**NOTE**

Use the Blue Stopper to indicate that a HIVE Collector is loaded with cells and ready for Transcriptome Recovery.

**IMPORTANT!**

Always keep HIVE Collectors flat until fully frozen at -80°C or on dry ice (see Page 12).

**STOP POINT****STEP C: WASHING LOADED HIVE COLLECTORS**

12. Remove Grey Stopper and discard.
13. **Tilt the HIVE Collector away from you.** Remove all media by aspiration or pipetting.
  - After removing 2 mL media, **gently** shake the HIVE Collector from side-to-side to pop any large bubbles, then remove the rest of the media.
14. **Tilt the HIVE Collector towards you.** Use a P1000 pipette to dispense 2 mL CLX Sample Wash Solution through the port.
15. **Place the HIVE Collector flat.** Gently swirl the HIVE Collector to cover the entire HIVE Array.

**STEP D: MOLECULAR PRESERVATION**

16. **Tilt the HIVE Collector away from you.** Remove CLX Sample Wash Solution by aspiration or pipetting.
17. **Tilt the HIVE Collector towards you.** Use a P1000 pipette to dispense 2 mL CLX Cell Preservation Solution through the port.
18. **Place the HIVE Collector flat.** Gently swirl the HIVE Collector to cover the entire HIVE Array.
19. Insert the Blue Stopper into the sample port.

**STEP E: STORAGE**

Place loaded HIVE Collectors in the original packaging. Store frozen at **-80°C** until ready to proceed with the HIVE CLX scRNAseq Transcriptome Recovery.

- If continuing directly to Transcriptome Recovery, incubate in the Cell Preservation Solution at room temperature for at least 30 minutes first.
- If a -80°C freezer is unavailable, store at -20°C.



# SHIPPING & RECEIVING

## NOTE

Ship loaded HIVE Collectors according to IATA instructions. Consult your institution's guidelines.

## IMPORTANT!

HIVE Collectors will NOT be completely frozen at -20°C.

## SHIPPING INSTRUCTIONS

1. Place HIVE Collectors in original packaging and cover with absorbent paper towels. Tape HIVE box closed and place in a resealable plastic bag.
  - Biological Substances, Category B (UN 3373) shipments require triple waterproof packaging.
2. If stored at -20°C, freeze box for **at least 30 minutes**, either at **-80°C** or under 1–2 inches of **dry ice** in a Styrofoam box.
3. For quality control, we recommend unsealing the HIVE box and photographing the frozen HIVE Collectors before shipping.
4. Place absorbent material between the HIVE box and Styrofoam box. Use enough material to absorb all box contents.
  - Biological Substances, Category B (UN 3373) shipments require absorbent material.
5. Place HIVE box in Styrofoam box and add 30 lb dry ice for up to 3 days in shipping.
  - The HIVE Styrofoam box (16  $\frac{3}{4}$ "  $\times$  16  $\frac{3}{4}$ "  $\times$  15") is 85% full after adding 30 lb dry ice.
  - Do not tape or seal the Styrofoam box. The courier must be able to open Styrofoam box to replenish dry ice during shipment, if needed.
6. Place the Styrofoam box inside of a cardboard box for pickup by carrier.
  - If reusing a box, remove all markings and labels.



**NOTE**

For calculating volume in package, there is 2 mL per HIVE Collector.

7. Insert packing list with descriptions, volumes, and quantities.
  - Example description: “Watertight sample container with leukocytes from healthy human blood, filled with 2 mL cell preservation solution categorized as non-dangerous goods.”
8. Seal box and label outside of package:
  - Recommended labels: dry ice label, dry ice weight, red up arrows, fragile label, and “Biological Substance Category B UN3373” label.
9. Ship packages.
  - We recommend shipping overnight on a Monday to avoid weekend delays.
  - We recommend using a courier service to ensure dry-ice replenishment and correct documentation, especially for international shipments.

**RECEIVING INSTRUCTIONS**

1. Once received, unpackage and retrieve the box of HIVE Collectors.
2. Check that the package contains dry ice and that any temperature sensors were not activated.
3. Place the unpackaged HIVE box on dry ice.
4. Check the HIVE Collectors for Blue Stoppers. Ensure that the frozen liquid is white in color and completely covers the yellow HIVE Array surface.
5. For quality control, photograph the frozen HIVE Collectors before and after shipping.
6. Immediately put the box in -80°C storage to ensure samples remain frozen.



## QUALITY CONTROL FOR SHIPPING & RECEIVING

### IMPORTANT!

For quality control, photograph the frozen HIVE Collectors before and after shipping.



### Good shipping

*Frozen liquid is white and completely covers the HIVE Array.*

*Blue Stopper remains in place.*



### Bad shipping

*Frozen liquid is white, but the liquid does not cover the right side (the yellow HIVE Array is visible). The HIVE was tilted during the freezing process.*

*Blue Stopper is missing.*



### Bad shipping

*Frozen liquid is clear. CLX Cell Preservation Solution was not used.*





## APPENDIX 1: SAMPLE TRACKING TABLE

Sample ID	HIVE Serial #	Concentration (cells/ $\mu$ L)	Number cells loaded	Viability	Loading media	Experimental Condition
<i>Example</i>	1437A6	900	30,000	93%	PBS+ 0.1% BSA	Wild Type



## APPENDIX 2: LOADING RECOMMENDATIONS

### CELL RECOMMENDATIONS

The HIVE CLX scRNAseq Solution is compatible with cell inputs from 500-60,000 cells. For new users, we recommend a cell input of 30,000 cells in order to maximize cell recovery and minimize doublet formation. As the number of cells loaded per HIVE collector increases, doublet rate and sequencing cost also increase. Use the desired number of single cells recovered to determine how many cells to load, based on the table below. We recommend loading based on the number of live cells in the sample.

Single-Cell Recovery†	Cell Input	Doublet Rate†	Recommended Reads/Sample (million)‡	# of HIVEs per Novaseq SP flowcell*
220	500	0%	7	>>8
850	2,000	2%	27	>8
3,000	7,500	5%	100	8
6,000	15,000	9%	200	4
11,000	30,000	14%	400	2
17,000	60,000	36%	800	1

†Performance metrics estimated from experimental data using human PBMCs.

‡Recommendations for reads/sample balance the amount of biological information gathered with sequencing costs and should achieve 80% recovery of cells, genes, and transcripts. These are recommended starting points for most applications, but you may need to tailor sequencing depths for your specific experiments.

\*The nominal NovaSeq SP flow cell offers 800 M reads.

### MEDIA RECOMMENDATIONS

We recommend using PBS + 0.1% BSA as loading media (cell media with protein) for maximum cell recovery. Use an alternative cell media if your specific sample type is incompatible with PBS + 0.1% BSA. Loading media must contain at least 1% FBS or 0.1% BSA.



## APPENDIX 3: QUICK PROTOCOL FOR CAPTURE

### HIVE CLX scRNASeq Sample Capture

1. Thaw HIVE Collectors for **30 minutes**.
2. During thaw, count cells and determine viability (recommended).
3. Remove thawed liquid.
4. Add 1 mL cell suspension.
5. Add 3 mL cell media (without protein). Place HIVE Collectors on Spin Plate. Spin at 30 RCF for **3 minutes**.
  - Gravity Loading: Place HIVE Collectors in 4°C refrigerator **for 30 minutes**.
6. Remove media.
7. Wash with 2 mL CLX Sample Wash Solution.
8. Add 2 mL CLX Cell Preservation Solution.
9. Insert Blue Stopper.
10. Freeze at -80°C.



# TROUBLESHOOTING

Problem	Possible Causes and Suggested Solutions
<b>The sample does not spread across the HIVE Array</b>  <i>When dispensed into the HIVE Collector, the sample beads on the surface and will not spread across the entire HIVE Array. The surface appears hydrophobic.</i>	<b>Make sure to dilute cells with media containing added protein</b>  The cell suspension was most likely diluted with media without added protein. Protein (1% FBS or 0.1% BSA) helps wet the HIVE Array.
<b>Bubbles appear after spinning</b>  <i>After the spin, many bubbles appear in the HIVE Collector, and they are difficult to remove with a P1000 pipette.</i>	<b>Media with high protein content creates bubbles</b>  This is normal and will not affect cell capture. Bubble removal is easier with a vacuum aspirator than a P1000 pipette. Alternatively, use gravity loading for high protein samples.
<b>Poor data quality after Transcriptome Recovery</b>  <i>Despite there being sufficient reads, the scRNAseq data contained fewer cells than expected, fewer genes than expected, and/or high mitochondrial reads.</i>	<b>Poor sample quality causes poor data quality</b>  Poor cell viability and/or poor sample purity is the most common reason for poor data quality. We recommend using a dead cell removal kit to improve sample viability. If these sample features cannot be improved, increase the number of cells loaded per HIVE to compensate for dead and non-nucleated cells.  We highly recommend the Nexcelom Cellometer® K2 Fluorescent Cell Counter for sample QC, which measures cell viability and sample purity faster and more accurately than trypan blue. <sup>1</sup>

## REFERENCES FOR TROUBLESHOOTING

1. [Honeycomb™ Appnote. Best Practices: The Cellometer® K2 for HIVE scRNAseq](https://honeycomb.bio/resources/the-cellometer-k2-for-hive-scrnaseq/?resource_id=48ab2f9b45957ab574cf005eb8a76760)  
[https://honeycomb.bio/resources/the-cellometer-k2-for-hive-scrnaseq/?resource\\_id=48ab2f9b45957ab574cf005eb8a76760](https://honeycomb.bio/resources/the-cellometer-k2-for-hive-scrnaseq/?resource_id=48ab2f9b45957ab574cf005eb8a76760)
2. [Honeycomb™ Troubleshooting Guide](https://honeycombbio.zendesk.com/hc/en-us/articles/14881004938907-HIVE-CLX-Troubleshooting-Guide)  
<https://honeycombbio.zendesk.com/hc/en-us/articles/14881004938907-HIVE-CLX-Troubleshooting-Guide>

## NOTES



Talk to a HIVE™ expert  
[support@honeycomb.bio](mailto:support@honeycomb.bio)



# HONEYCOMB

## MORE INFORMATION

Download protocols & example data: [www.honeycomb.bio](http://www.honeycomb.bio)

Contact HIVE technical support: [support@honeycomb.bio](mailto:support@honeycomb.bio)

Speak with a HIVE expert: [sales@honeycomb.bio](mailto:sales@honeycomb.bio)

Ready for quotes & ordering: [NGS@revvity.com](mailto:NGS@revvity.com)



Any cell. Any where. Any time.™

## 2024 Single Cell Workshop Colombia

Appendix 5. HIVE CLX™ scRNASeq Transcriptome Recovery & Library Preparation



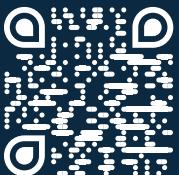
# HIVE CLX™ scRNAseq

## Transcriptome Recovery & Library Preparation

### User Protocol

Version 1.0 | May 2023

Any cell. Any where. Any time.™





# HONEYCOMB

## **This product is for research use only. Not for use in diagnostic procedures.**

This manual is proprietary to Honeycomb Biotechnologies, Inc. and licensed for distribution by Revvity and intended only for customer use in connection with the product(s) described herein and for no other purpose.

This document and its contents shall not be used or distributed for any other purpose without the prior written consent of Honeycomb Biotechnologies, Inc. Follow the protocol associated with the kit.

## **Revision History**

<b>Version</b>	<b>Date</b>	<b>Description</b>
Version 1.0	May 2023	CLX Product Launch

# HIVE CLX™ scRNAseq Transcriptome Recovery and Library Preparation

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# HIVE CLX™ scRNAseq Transcriptome Recovery and Library Preparation

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## TROUBLESHOOTING

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# GENERAL INFORMATION

## NOTE

New users require a HIVE UDI Plate and the HIVE CLX Starter Bundle, which includes:

- //// Demo Parts
- \\\\\\ Assembled Vacuum
- //// HIVE Plates and Seals
- \\\\\\ Lysis Boxes
- //// HIVE Accessories

## PRODUCT OVERVIEW

HIVE CLX scRNASeq is a complete solution to create NGS libraries from single cells. Use the HIVE CLX Sample Capture protocol to capture single cells with barcoded capture beads in HIVE Collectors. Use this Transcriptome Recovery and Library Preparation protocol to convert loaded HIVE Collectors into sequencing libraries for scRNASeq.

The HIVE CLX scRNASeq Transcriptome Recovery product contains Spin Parts and HIVE Parts & Reagents for 8 samples. The Library Preparation reagents are included in the HIVE CLX scRNASeq Library Preparation product.

## TRANSCRIPTOME RECOVERY PARTS & REAGENTS

- //// HIVE Top with Protective Cover (x8)
- \\\\\\ Bead Collector (x8)
- //// Drying Cap (x8)
- \\\\\\ Spin Plate (x2), Spin Lid (x2), HIVE Blanks (x8)
- //// HIVE Transcriptome Recovery Reagents (x8): CLX Storage Wash Solution, CLX Lysis Stock, CLX Hybridization Buffer, CLX Bead Recovery Solution, CLX Wash A, CLX SPRI Beads, Water

**Bead Collector**



**Drying Cap**



**HIVE Blank**



**Spin Lid**



Talk to a HIVE™ expert  
[support@honeycomb.bio](mailto:support@honeycomb.bio)

**HIVE Top with Protective cover**



**Red Stopper**



**Spin Plate**



## LIBRARY PREP REAGENTS

Reagents (x8 reactions)	Cap Color
CLX Reducing Solution	White
CLX 1 <sup>st</sup> Strand Wash	Pink
CLX 1 <sup>st</sup> Synthesis Buffer	Blue
CLX 1 <sup>st</sup> Strand Enzyme	Blue
CLX Clean-Up Buffer	Green
CLX Clean-Up Enzyme	Green
CLX 1 <sup>st</sup> Strand Control	Clear
10× NaOH (1M)	Clear
CLX 2 <sup>nd</sup> Synthesis Buffer	Violet
CLX 2 <sup>nd</sup> Strand Oligo	Violet
CLX 2 <sup>nd</sup> Strand Enzyme	Violet
CLX WTA Oligo	Orange
CLX PCR Enzyme (x2)	Orange
CLX Read 1 Seq Primer	Red
CLX Read 2 Seq Primer	Red
CLX Index 1 Seq Primer	Red
CLX Index 2 Seq Primer	Red

### NOTE

Store all Library Prep reagents at -20°C.

## USER-SUPPLIED MATERIALS FOR LIBRARY PREP

//// The Starter Bundle includes one set of plates, sealing films, and foils. Refer to this list to purchase more materials.

\\\ The Assembled Vacuum includes the 96-well manifold and vacuum trap system. The vacuum pump/line is sold separately.

Material	Recommended Product
96-well filter plates	Millipore MultiScreen™ HTS 96 HV, opaque CAT# MSHVN4B10
96-well deep well storage plate	Abgene™, 0.8 mL, polypropylene CAT# AB0765
96-well PCR plate	Thermo Scientific, semi-skirted, flat deck CAT# AB1400L
Adhesive PCR sealing film	Bio-Rad Microseal™ 'B', optical CAT# MSB1001
Adhesive PCR sealing foil	Excel Scientific eXTReMe™ FoilSeal™ CAT# XTR-FOIL-100
96-well vacuum manifold	Millipore Multiscreen™ CAT# MAVM0960R
Vacuum trap system	VWR Vactrap™ 2 HDPE, 2+1 L bottle assembly with tray, 0.2 µm vent filter, not autoclavable CAT# 76207-602
Vacuum pump/line (optional)	Cole-Parmer Air Admiral®, 0.37 cfm CAT# EW-79202-00

### NOTE

You may use an existing vacuum pump that pulls ≤15 inches Hg (≤381 mm Hg) and fits tubing with an inner diameter of 0.25 inches (0.63 cm).



### IMPORTANT!

If using the Transcriptome Recovery Kit without the Library Prep Kit, you must supply Reducing Solution (1 M DTT).

### NOTE

//// Recommended thermocycler:  
Eppendorf Mastercycler® X50  
(CAT# 2231000923)

\|\| Recommended bar magnet:  
Invitrogen DynaMag™-96 Side Skirted  
(CAT# 12027)

/// Recommended for DNA  
quantification:  
Thermo Scientific Qubit™ 4  
Fluorometer (CAT# Q33238)

\|\| Recommended for fragment  
analysis:

LabChip GX Touch™, TapeStation™, or  
Bioanalyzer™ systems

/// Recommended TapeStation Kit:  
D5000 ScreenTape Assay and Reagents

## ADDITIONAL USER-SUPPLIED MATERIALS

- //// Molecular biology grade ethanol (absolute)
- \|\| Wescodyne® (bleach alternative)
- //// Single-channel P1000 (1,000 µL) pipette & tips
- \|\| Single-channel P200 (200 µL) pipette & tips
- //// Single-channel P10 (10 µL) pipette & tips
- //// 15 mL centrifuge tubes, RNase-/DNase-free
- \|\| 1.5 mL microfuge tubes, RNase-/DNase-free
- \|\| Absorbent paper towels
- \|\| 8-channel P200 & P10 pipettes (optional)
- //// 5–25 mL serological pipettes (optional)
- \|\| 25–50 mL reagent reservoirs (optional),  
RNase-/DNase-free
- //// PCR tube strips (optional), RNase-/DNase-free

## REQUIRED EQUIPMENT

- //// -20°C freezer, 4°C refrigerator, and ice bucket
- \|\| 37°C oven or Filter Plate Adapter (see Page 8)
- //// Thermocycler for 96-well plates
- \|\| Bar magnet for 96-well plates
- //// Benchtop vortex mixer
- \|\| Centrifuge
- //// Instrument for DNA quantification
- \|\| Instrument for DNA fragment analysis with  
analysis kit for >1,000 bp DNA smears



**NOTE**

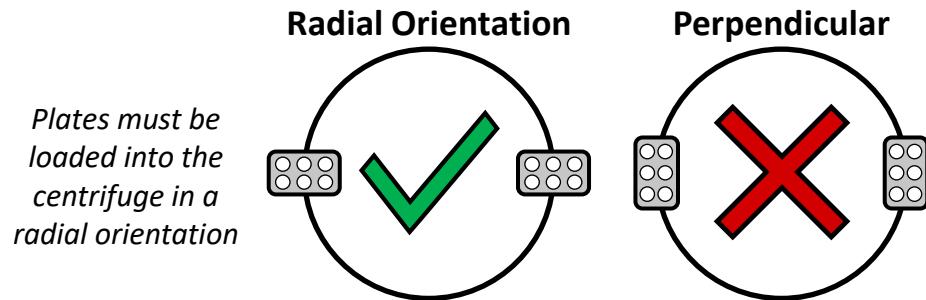
Example of compatible centrifuge:  
Eppendorf 5810™ with S-4-104 rotor  
and MTP/Flex buckets.

**NOTE**

The radial plate orientation prevents smearing of the pellet when collecting beads. If laboratory equipment is limited to the perpendicular orientation, then use positions A2 and B2 only on the HIVE Spin Plate.

**CENTRIFUGE SPECIFICATIONS**

- //// Minimum 1800 RCF spin speed
- \|\| Swinging-bucket centrifuge
- /// Compatible with deep-well plates
- \|\| Radial plate orientation (see diagram below)

**HIVE ACCESSORIES**

New users will require the HIVE CLX scRNASeq Starter Bundle, which includes HIVE Accessories such as the Closure Tool and Filter Plate Adapter.

Use the Closure Tool to attach the HIVE Top and (optionally) the Bead Collector onto the HIVE Base.

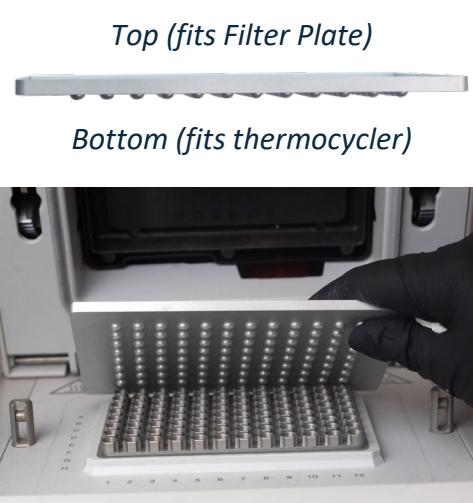
The Filter Plate Adapter can convert thermocycler blocks into 37°C incubators for filter plates. Check lid clearance and fit in the heating block before use.

**WARNING!**

The Closure Tool has a pinch point.  
Keep hands clear during operation.

**NOTE**

The Filter Plate Adapter reduces clearance height between the heating block and lid.

**Closure Tool****Filter Plate Adapter**

# CLX TRANSCRIPTOME RECOVERY PROTOCOL

## CLX TRANSCRIPTOME RECOVERY WORKFLOW



## HOW TO HANDLE THE HIVE COLLECTORS

### IMPORTANT!

Pipette along inside of the Cell Loader wall. Avoid pipetting directly onto the yellow HIVE Array.

### NOTE

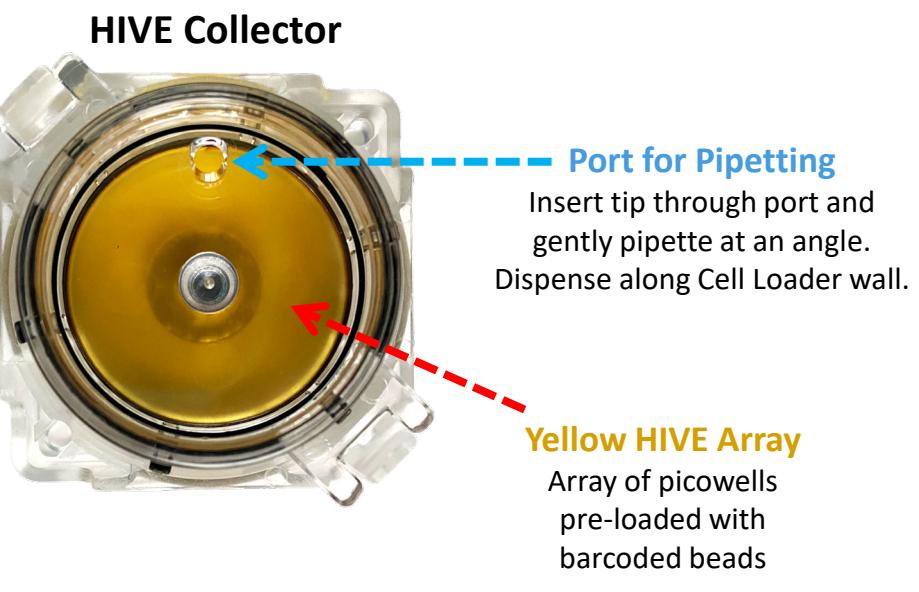
We recommend a vacuum aspirator to easily remove liquids and bubbles that may appear after spinning (see Troubleshooting on Page 62).

### IMPORTANT!

Always keep HIVE Collectors flat during transportation and incubation. Only tilt when directed to do so.

### NOTE

Keep the port at 12 o'clock with the port edge farthest from you.



### Tilt Towards You



### Keep HIVE Flat



### Tilt Away From You



When adding liquid into the HIVE Collector

When transporting and incubating

When removing liquid from the HIVE Collector



Talk to a HIVE™ expert  
[support@honeycomb.bio](mailto:support@honeycomb.bio)

## QUALITY CONTROL FOR SHIPPING & RECEIVING

### IMPORTANT!

For quality control, photograph the frozen HIVE Collectors before and after shipping.



### Good shipping

*Frozen liquid is white and completely covers the HIVE Array.*

*Blue Stopper remains in place.*



### Bad shipping

*Frozen liquid is white, but the liquid does not cover the right side (the yellow HIVE Array is visible). The HIVE was tilted during the freezing process.*

*Blue Stopper is missing.*



### Bad shipping

*Frozen liquid is clear, which indicates CLX Cell Preservation Solution was not used.*

*Non-frozen liquid indicates that sample thawed during shipping.*





### IMPORTANT!

Always keep HIVE Collectors flat.  
Only tilt when directed to do so.

### IMPORTANT!

For CLX Cell Preservation Solution  
and CLX Storage Wash Solution,  
**DO NOT MIX WITH BLEACH!**

## STEP A: SEAL CELL-LOADED HIVE

### Materials required

- Loaded HIVE Collectors
- HIVE Tops with Protective Covers
- Drying Caps
- Spin Plate and HIVE Blanks

1. Prepare the **Closure Tool** workstation with **HIVE Tops with Protective Covers and Drying Caps**.
2. Remove **HIVE Collectors** from freezer.
3. Fully thaw HIVE Collectors at **37°C for ~15 minutes** or at room temperature for ~60 minutes.
4. Remove **Blue Stopper** from ports. Discard.
5. Wash each HIVE by performing the following steps, **one HIVE at a time**.
  - a. **Tilt the HIVE Collector away from you.**  
Remove all liquid (~2 mL) by aspiration or pipetting.
  - b. **Tilt the HIVE Collector towards you.** Dispense 2 mL of **CLX Storage Wash Solution** through the port.
  - c. **Place the HIVE Collector flat.** Gently swirl the HIVE Collector to cover the entire HIVE Array.
  - d. Repeat Step 5a-c for each remaining HIVE, **one at a time**.



6. At **Closure Tool** workstation, squeeze **HIVE Tops** and **Protective Covers** together until the assembly fully clicks into place.



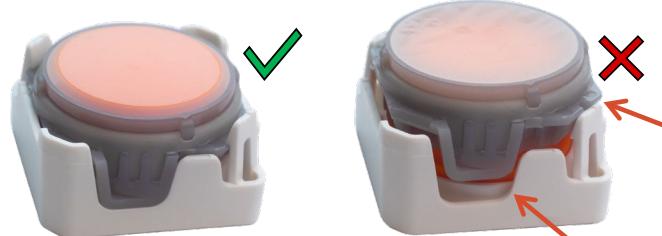
7. Bend out the black arms of the **Protective Cover**, lift to remove and discard. Place the **HIVE Top** on the benchtop with membrane facing up.



8. Ensure that the **HIVE Top** is fully clicked into place.

- If assembly was squeezed correctly, the white arms of the HIVE Top will grip the gray membrane frame.
- If not, press down on edge of gray membrane frame to \*click\* in the frame.

#### Inspect HIVE Top



Gray frame is snapped into white housing

White arms do not fully snap over gray frame



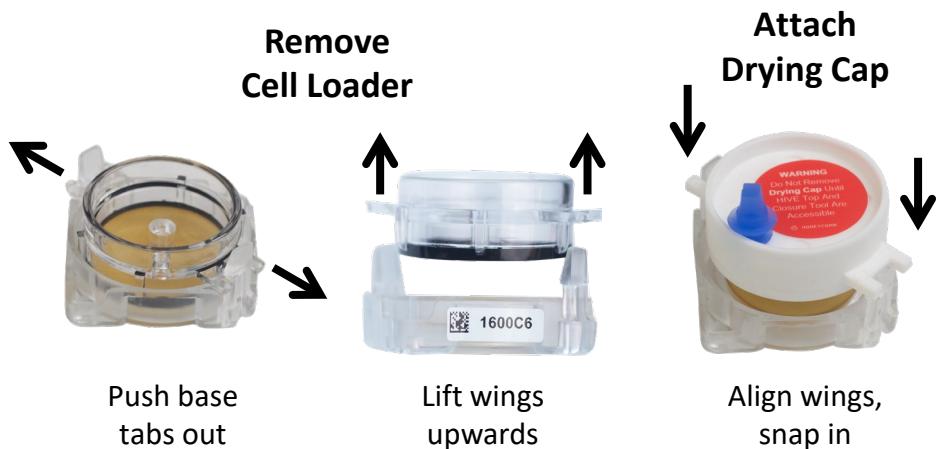
### IMPORTANT!

Do not dry out the HIVE array! Handle HIVE Collectors one at a time, and **immediately** attach a Drying Cap.

9. **Tilt the HIVE Collectors away from you.** Remove all **CLX Storage Wash Solution** by aspiration or pipetting.

10. Remove the **Cell Loader** from the **HIVE Base** and **immediately attach a Drying Cap** to the Base.

- To remove the Cell Loader: Push outwards on the HIVE Base tabs while lifting upwards on the wings of the Cell Loader. Discard the Cell Loader.
- To attach a Drying Cap: Align wings of the Drying Cap to HIVE Base tabs. Snap into the HIVE Base. The red sticker on the Drying Cap should face upwards.



11. Place **HIVE Base with Drying Cap** in the **Spin Plate**.

Align open corners in the HIVE Base with the pins of the Spin Plate.

- Split HIVEs evenly between two spin plates. Balance plates with HIVE Blanks, if needed.

12. Place assembled plates in centrifuge. Set centrifuge to **400 RCF** and spin. Stop the spin once the centrifuge reaches 400 RCF.

- Set centrifuge to maximum acceleration and maximum braking.

13. Take assembled plates to Closure Tool workstation.

### IMPORTANT!

HIVEs should be sealed (Steps 13-17) within 5 minutes of centrifugation.



Talk to a HIVE™ expert  
[support@honeycomb.bio](mailto:support@honeycomb.bio)

14. Remove the centrifuged HIVE Base assemblies from the Spin Plate. Remove the **Blue Stopper** from the Drying Cap Port.
15. With the Drying Cap Port at the 6 o'clock position, **tilt the HIVE Base assembly towards you** and gently tap to pool liquid at the Drying Cap Port. Remove remaining liquid by aspiration or pipetting.



- IMPORTANT!**  
Complete this step one HIVE at a time to avoid drying out the HIVE array!  
Avoid touching the membrane or array surface.
16. **In less than 5 seconds per HIVE, quickly exchange a Drying Cap with a HIVE Top as follows:**
  - a. Remove Drying Cap: Gently push out on HIVE Base tabs and lift up on Drying Cap wings. Dispose of the Drying Cap.
  - b. Pick up and flip over the HIVE Top: Membrane will be facing down.
  - c. Align open corners of the HIVE Top and Base: Align and assemble HIVE Top and Base but DO NOT squeeze or click closed.
  - d. Use Closure Tool: Slide assembly into Closure Tool and use lever. Close Tool slowly and press through the resistance to \*click\* together. Compress only once.
  - e. Repeat 16a-d with remaining HIVEs.
17. Incubate the closed HIVE Base and Top assemblies at room temperature for **30 minutes** to seal the membrane onto the HIVE Array.





### NOTE

If continuing with the Library Prep, prepare 37°C incubation setup to thaw reagents ahead of time (see Step 11).

### IMPORTANT!

All calculations for reaction volumes already include excess volumes. DO NOT ADJUST CALCULATIONS.

### NOTE

HIVE Serial # is on the sticker on the HIVE Base.

Spin Plates are not pre-labeled. Mark Plate with a unique Plate ID.

## STEP B: LYSIS & HYBRIDIZATION

### Materials required

- CLX Lysis Stock
- CLX Hybridization Buffer
- CLX Reducing Solution (white cap)
- HIVE Spin Plate
- Lysis boxes from Starter Bundle (optional)

1. Thaw **CLX Reducing Solution** (white cap) at room temperature. Vortex Solution if white pellet does not dissolve during thaw. Spin down.
2. Prepare **Lysis Solution** by pipetting **CLX Lysis Stock** and **CLX Reducing Solution** into sterile tube. Follow table below.

Lysis Solution Component	1 sample	8 samples	N Samples
CLX Lysis Stock	1,000 µL	8,000 µL	
CLX Reducing Solution	100 µL	800 µL	
<b>Total Volume</b>	<b>1,100 µL</b>	<b>8,800 µL</b>	
Volume Used in STEP B	1,000 µL	8,800 µL	
<i>Excess Volume</i>	<i>100 µL</i>	<i>800 µL</i>	

3. Use the **Sample Tracking Table** (Appendix 1) to record the Serial # and Sample ID of each HIVE. Record the Spin Plate ID and Plate Position of each assembly in the next steps.
4. Place the sealed **HIVE assemblies** on the **Spin Plate** by aligning the HIVE open corners over Spin Plate pins. Do not push down until the next step.



5. Lyse each HIVE by performing the following steps, **one HIVE at a time.**

- a. Remove white/orange top: Gently press down on the open corners of a HIVE assembly to pop off the white/orange top. Discard the top.
  - If the white/orange top does not pop off, check that the HIVE is aligned on Spin Plate pins, repeat compression, and lift off the orange top.

- b. Immediately dispense 1 mL Lysis Solution and swirl plate: Pipet directly onto the exposed membrane and swirl to cover the array. Do not dry out the HIVE array!



Align assembly onto Spin Plate  
Press down on open corners

Remove and discard orange top

6. Incubate HIVEs on the Spin Plate with **Lysis Solution** at room temperature for **5 minutes**.
7. **Tilt the Spin Plate towards you.** Remove **Lysis Solution** from all membranes by aspiration or pipetting.
  - Pipet from the bottom edge of the gray frames. Be careful not to damage membranes.
8. Dispense 1 mL **CLX Hybridization Buffer** onto all membranes. Swirl plate to cover the array.



**IMPORTANT!**

For CLX Hybridization Buffer,  
**DO NOT MIX WITH BLEACH!**

**NOTE**

If needed, turn valve clockwise to increase vacuum or counterclockwise to decrease.

9. **Tilt the Spin Plate towards you.** Remove **CLX Hybridization Buffer** from all membranes by aspiration or pipetting.
  - Pipet from the bottom edge of the gray frames. Be careful not to damage membranes.
10. Again, dispense 1 mL **CLX Hybridization Buffer** onto all membranes. Swirl plate. Incubate at room temperature for **15 minutes**.
11. If continuing with **Library Preparation**, during the 15-minute incubation in Step 10, prepare for STEP D:
  - a. Thaw one tube of **CLX 1<sup>st</sup> Strand Control** (clear cap) at room temperature.
  - b. Thaw **CLX 1<sup>st</sup> Strand Wash** (pink cap) and **CLX 1<sup>st</sup> Synthesis Buffer** (blue cap) at 37°C.
  - c. Retrieve **CLX 1<sup>st</sup> Strand Enzyme** (blue cap) and place on ice.
  - d. Vortex reagents and spin down. Keep reagents on ice until use.
  - e. Prepare Vacuum setup:
    - Cover a 96-well Filter Plate with a foil seal. Be careful to seal all wells, and DO NOT remove the clear plastic bracket at the bottom of the Filter Plate.
    - Place sealed Filter Plate on vacuum manifold.
    - Plug in pump to an electrical outlet and turn on.
    - Press down firmly on the 96-well Filter Plate and check the vacuum gauge. The vacuum should be between 5–15 inch-Hg (127–381 mmHg).
    - Turn off pump and let vacuum gauge return to 0 before removing Filter Plate.





### IMPORTANT!

Perform Steps 1–3 one HIVE at a time.  
See Page 19 for illustrations.

### IMPORTANT!

CLX Hybridization Buffer may contain  
trace lysis buffer, so  
**DO NOT MIX WITH BLEACH!**

### IMPORTANT!

If both tabs do not \*click\* together,  
the HIVE will leak in the centrifuge.

### WARNING!

The Closure Tool has a pinch point.  
Keep hands clear during operation.

## STEP C: BEAD RECOVERY

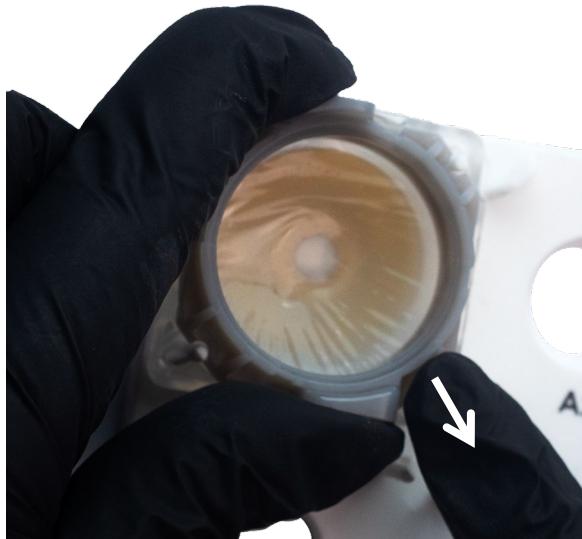
### Materials required

- CLX Bead Recovery Solution
- Bead Collectors
- Red Stoppers
- Filter Plate (Plate Kit)
- Vacuum setup (Vacuum Kit)
- Spin Plate, Spin Lid, and HIVE Blanks

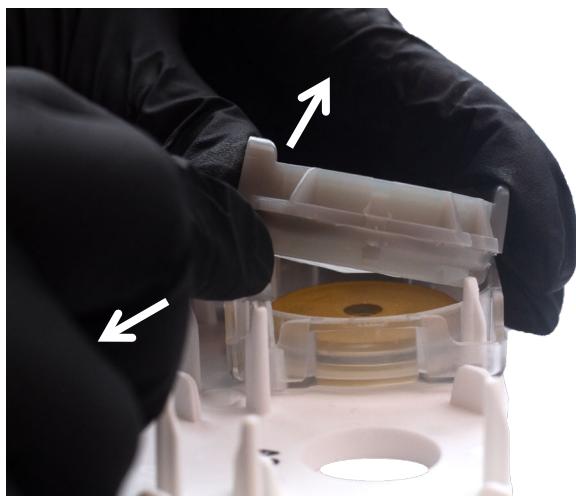
1. **Tilt the Spin Plate towards you** and remove **CLX Hybridization Buffer** from one membrane.
  - Pipet from the bottom edge of the gray frames. Be careful not to damage membranes.
2. Remove the **membrane** from the **HIVE base** by performing the following steps:
  - a. Use your right hand to pull the lower right HIVE Base tab towards you.
  - b. Use your left hand to gently peel the membrane away from you.
  - c. Discard the membrane with the attached gray frame.
3. Place a **Bead Collector** onto a **HIVE Base** and compress until you hear **\*2 CLICKS\*** (one per tab).
  - Align Bead Collector wings with the Base tabs.
  - If it is too difficult to compress by hand, put Spin Plate in the Closure Tool and close slowly and firmly until you hear **\*2 CLICKS\***.
4. Repeat Steps 1–3 for each HIVE.



### Step 2: Peel off membrane



Place left hand on the grey frame  
Use right index finger to pull lower right  
HIVE Base tab towards you



Use left hand to peel membrane from  
bottom right to top left,  
removing membrane completely

### Step 3: Compress Bead Collector

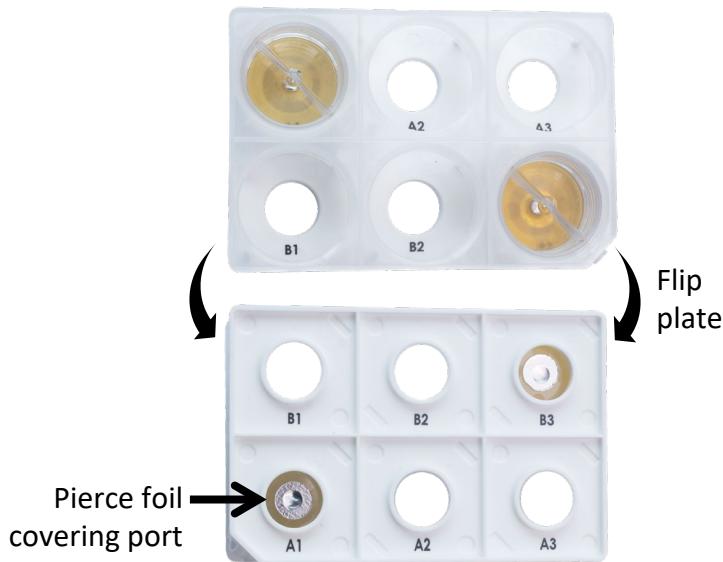


*Listen for \*2 CLICKS\**



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[support@honeycomb.bio](mailto:support@honeycomb.bio)

5. Balance **Spin Plate(s)** with **HIVE Blanks**, if needed.
6. Place **Spin Lid** on **Spin Plate**. Flip Spin Plate and Lid assembly upside down. Place on benchtop.
  - Firmly grasp Spin Plate and Lid with both hands as you flip the assembly upside down.



7. Use 1 mL pipette tip to pierce foil and completely open the **HIVE Base Ports** (see picture above).
8. Use pipette to quickly dispense 1 mL **CLX Bead Recovery Solution** into the Port. Repeat 2 more times for a total of 3 mL.
  - Hold the pipette straight up and at the Port opening while dispensing liquid.
9. Insert **Red Stoppers** into the **HIVE Base Ports**.
10. Use pipette or absorbent paper towel to remove any residual solution around the **Red Stopper**.
11. Repeat Steps 5–10 for all **Spin Plates**.
12. Place the **Spin Plate(s)** in centrifuge with the **Red Stoppers** facing upwards. Spin at max speed ( $\leq 3,000$  RCF) for **5 minutes**.

### TROUBLESHOOTING TIP

If converting RPM to g-force (RCF), confirm the radius of the rotor first.



**IMPORTANT!**

Use the Sample Tracking Table (Appendix 1) to keep track of the Spin Plate position and Filter Plate well for each sample.

**NOTE**

You do not need to add any liquid to the Bead Collector after Step 14.

12. Assign an unused well of the Filter Plate for the samples and the **CLX 1<sup>st</sup> Strand Control** (use the **Sample Tracking Table** in Appendix 1).
13. Remove the foil seals from the assigned wells of the Filter Plate.
  - Do NOT remove the bracket at the bottom of the Filter Plate.
14. Set pipette to 300 µL. For each sample, transfer bead pellet to assigned Filter Plate well:
  - a. Remove **Red Stopper**. Set aside.
  - b. Gently touch the pipette tip to the bottom of the **Bead Collector** and remove bead pellet.
  - c. Transfer each bead pellet to the assigned well on the Filter Plate.
  - d. Mix beads in well by gently pipetting up and down 5 times to break up any clumps.
  - e. Re-insert the **Red Stopper**.
  - f. Repeat Step 14 for each sample.
15. Place **Spin Plate(s)** in centrifuge with **Red Stoppers** facing upwards. Spin at max speed ( $\leq$ 3,000 RCF) for **1 minute**.
  - Balance the Spin Plate with HIVE Blanks, if needed.
16. Place Filter Plate on the vacuum manifold. Turn on pump and press down on Filter Plate so liquid flows out. Turn off the pump when liquid stops. Wait ~30 seconds for vacuum gauge to read 0 then remove Filter Plate from vacuum manifold.



**IMPORTANT!**

Use the Sample Tracking Table (Appendix 1) to keep track of the Spin Plate position and Filter Plate well for each sample.

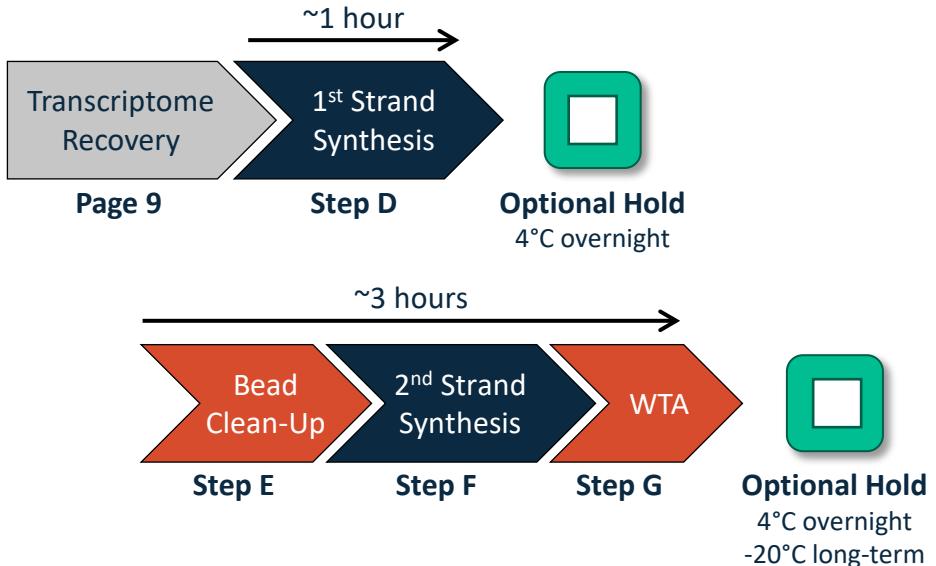
17. Remove the **Spin Plate(s)** from the centrifuge.
18. Perform second bead pellet transfer by repeating step 14:
  - a. Remove **Red Stopper**. Set aside.
  - b. Remove bead pellet from the **Bead Collector**.
  - c. Transfer each bead pellet to the same assigned well from step 14.
  - d. Mix beads in well by gently pipetting up and down 5 times to break up any clumps.
19. Immediately proceed to STEP D if using the **Library Preparation** product.
  - You have now completed Transcriptome Recovery.



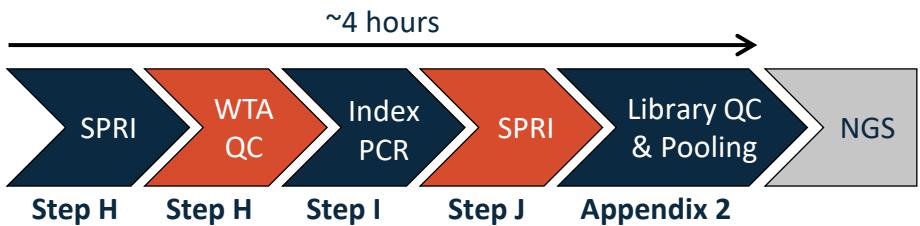
# CLX LIBRARY PREP PROTOCOL

## CLX LIBRARY PREP WORKFLOW

### Processing Day 1



### Processing Day 2



You may stop after any step  
in Processing Day 2



**Optional Hold**  
4°C overnight  
-20°C long-term



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## STEP D: 1<sup>ST</sup> STRAND SYNTHESIS

### Materials required

- CLX 1<sup>st</sup> Strand Wash (**pink cap**)
- CLX 1<sup>st</sup> Synthesis Buffer (**blue cap**)
- CLX 1<sup>st</sup> Strand Enzyme (**blue cap**)
- CLX 1<sup>st</sup> Strand Control (clear cap)
- CLX Bead Recovery Solution
- Clear PCR plate seals (Plate Kit)
- Filter Plate adaptor (optional)
- Vacuum setup (Vacuum Kit)

1. Set incubation oven or thermocycler to **37°C**. If using thermocycler with Filter Plate Adaptor, set lid temperature to **50°C** to prevent condensation.
2. Dispense 250 µL of **CLX Bead Recovery Solution** into the thawed tube of **CLX 1<sup>st</sup> Strand Control**. Pipet to resuspend.
3. Rapidly pipet up-and-down 10 times to mix the **CLX 1<sup>st</sup> Strand Control** beads, and **immediately** transfer the entire tube contents to the assigned well on the Filter Plate.
4. Prepare **1<sup>st</sup> Strand Reaction Mix** with table below. Pipet to mix. Keep on ice until use.
  - **Count the CLX 1<sup>st</sup> Strand Control as a sample!**

1 <sup>st</sup> Strand Reaction Mix Component	1 sample	8 samples	N Samples
CLX 1 <sup>st</sup> Synthesis Buffer	175 µL	1,400 µL	
CLX 1 <sup>st</sup> Strand Enzyme	15 µL	120 µL	
<b>Total Volume</b>	<b>190 µL</b>	<b>1,520 µL</b>	
Volume Used in STEP D	175 µL	1,400 µL	
<i>Excess Volume</i>	<i>15 µL</i>	<i>120 µL</i>	

### IMPORTANT!

All calculations for reaction volumes already include excess volumes.  
DO NOT ADJUST CALCULATIONS.



5. Place Filter Plate on the vacuum manifold. Turn on pump and press down on Filter Plate so liquid flows out. Turn off the pump when liquid stops. Wait ~30 seconds for vacuum gauge to read 0.
6. Dispense 175  $\mu$ L **CLX 1<sup>st</sup> Strand Wash (pink cap)** into each Filter Plate well with sample beads.
7. Tilt the Filter Plate. Gently pipet 5 times to mix. Pipet slowly and try not to make bubbles.
8. Use the vacuum manifold to remove liquid from Filter Plate as previously described in Step 5.
9. Pat dry the bottom of the Filter Plate with an absorbent paper towel.
10. Use a clear adhesive PCR plate film to seal the Filter Plate bottom.
11. Dispense 175  $\mu$ L **1<sup>st</sup> Strand Reaction Mix (Step 4)** into each Filter Plate well with sample beads.
12. Tilt the Filter Plate. Gently pipet 5 times to mix. Pipet slowly and try not to make bubbles.
13. Use scissors to cut a strip of clear adhesive PCR plate film that fits over the Filter Plate wells. Seal the sample wells.
14. Incubate Filter Plate at **37°C for 60 minutes**.
15. If not pausing overnight, proceed to STEP E and prepare the **Clean-Up Reaction Mix**.



**IMPORTANT!**

Only perform the washes in Step 16 if pausing overnight. If continuing with Library Prep, proceed to STEP E.

**PAUSE POINT**

16. To pause the protocol and store the Filter Plate overnight, complete the following washes after the 60-minute incubation in Step 14:
  - a. Carefully remove the bottom and top films from the Filter Plate.
  - b. Use the vacuum manifold to remove liquid from the plate as previously described in Step 5.
  - c. Dispense 175  $\mu$ L **CLX Wash A** into each sample well.
  - d. Use the vacuum manifold to remove liquid from the plate as previously described in Step 5.
  - e. Pat dry the plate bottom with an absorbent paper towel.
  - f. Use a clear adhesive film to seal the plate bottom.
  - g. Dispense 175  $\mu$ L **CLX Wash A** into each sample well.
  - h. Seal the top of the sample wells with adhesive film.
  - i. Store the sealed Filter Plate overnight at 4°C.





## STEP E: BEAD CLEAN-UP

### Materials required

- CLX Clean-Up Buffer (**green cap**)
- CLX Clean-Up Enzyme (**green cap**)
- CLX Wash A
- Clear PCR plate seals (Plate Kit)
- Vacuum setup (Vacuum Kit)
- Filter Plate adaptor (optional)

1. Set incubation oven or thermocycler to **37°C**. If using thermocycler with Filter Plate Adaptor, set lid temperature to **50°C** to prevent condensation.
2. Thaw **CLX Clean-Up Buffer (green cap)** at **37°C**. Vortex, spin down, and keep on ice until use. Retrieve **CLX Clean-Up Enzyme (green cap)**, spin down, and keep on ice until use.
3. Prepare **Clean-Up Reaction Mix** with table below. Pipet to mix. Keep on ice until use.
  - **Count the CLX 1<sup>st</sup> Strand Control as a sample!**

Clean-Up Reaction Mix Component	1 sample	8 samples	N samples
CLX Clean-Up Buffer	175 µL	1,400 µL	
CLX Clean-Up Enzyme	10 µL	80 µL	
<b>Total Volume</b>	<b>185 µL</b>	<b>1,480 µL</b>	
<b>Volume Used in STEP E</b>	<b>175 µL</b>	<b>1,400 µL</b>	
<i>Excess Volume</i>	<i>10 µL</i>	<i>80 µL</i>	

### IMPORTANT!

All calculations for reaction volumes already include excess volumes. DO NOT ADJUST CALCULATIONS.

### NOTE

There may be some liquid on the bottom seal.

4. Carefully remove the adhesive film from the bottom, then top of the Filter Plate.



5. Place Filter Plate on the vacuum manifold. Turn on pump and press down on Filter Plate so liquid flows out. Turn off the pump when liquid stops. Wait ~30 seconds for vacuum gauge to read 0.
6. Dispense 175 µL **CLX Wash A** into each Filter Plate well with sample beads.
7. Use the vacuum manifold to remove liquid from Filter Plate as previously described in Step 5.
8. Pat dry the bottom of the Filter Plate with an absorbent paper towel.
9. Use a clear adhesive PCR plate film to seal the Filter Plate bottom.
10. Dispense 175 µL **Clean-Up Reaction Mix** (Step 3) into each Filter Plate well with sample beads.
11. Tilt the Filter Plate. Gently pipet 5 times to mix. Pipet slowly and try not to make bubbles.
12. Use a clear adhesive PCR plate film to seal the sample wells.
13. Incubate Filter Plate at **37°C for 45 minutes**.





## STEP F: 2<sup>nd</sup> STRAND SYNTHESIS

### Materials required

- CLX 2<sup>nd</sup> Synthesis Buffer (**violet cap**)
- CLX 2<sup>nd</sup> Strand Oligo (**violet cap**)
- CLX 2<sup>nd</sup> Strand Enzyme (**violet cap**)
- 10× NaOH (clear cap)
- CLX Wash A
- Water
- Clear PCR plate seals (Plate Kit)
- Vacuum setup (Vacuum Kit)
- Filter Plate adaptor (optional)
- Reagent reservoirs (optional)

1. Set incubation oven or thermocycler to **37°C**. If using thermocycler with Filter Plate Adaptor, set lid temperature to **50°C** to prevent condensation.
2. Thaw **CLX 2<sup>nd</sup> Synthesis Buffer (violet cap)**, **CLX 2<sup>nd</sup> Strand Oligo (violet cap)**, and **10× NaOH** (clear cap) at room temperature for **15 minutes**. Vortex solutions if pellets do not dissolve during thaw. Spin down and transfer to ice until use.
3. Retrieve **CLX 2<sup>nd</sup> Strand Enzyme (violet cap)**, spin down, and keep on ice until use.



## TROUBLESHOOTING TIP

The stock 10× NaOH remains stable for 10 freeze-thaw cycles. Mark the vial to keep record of each freeze-thaw cycle.

### IMPORTANT!

All calculations for reaction volumes already include excess volumes.  
DO NOT ADJUST CALCULATIONS.

### IMPORTANT!

All calculations for reaction volumes already include excess volumes.  
DO NOT ADJUST CALCULATIONS.

### NOTE

There may be some liquid on the bottom seal.

4. Dilute **10× NaOH** (clear cap) with **Water** to make **1× NaOH**. Keep at room temperature until use.
  - Prepare fresh **1× NaOH** for each experiment.
  - Immediately return stock **10× NaOH** (clear cap) to the -20°C freezer.
  - **Count the CLX 1<sup>st</sup> Strand Control as a sample!**

Dilution Component	1 sample	8 samples	N samples
Water	180 µL	1,440 µL	
10× NaOH	20 µL	160 µL	
<b>Total Volume</b>	<b>200 µL</b>	<b>1,600 µL</b>	
Volume Used in STEP F	175 µL	1,400 µL	
<i>Excess Volume</i>	25 µL	200 µL	

5. Prepare **2<sup>nd</sup> Strand Reaction Mix** with table below. Pipet to mix. Keep on ice until use.
  - **Count the CLX 1<sup>st</sup> Strand Control as a sample!**

2 <sup>nd</sup> Strand Reaction Mix Component	1 sample	8 samples	N samples
CLX 2 <sup>nd</sup> Synthesis Buffer	175 µL	1,400 µL	
CLX 2 <sup>nd</sup> Strand Oligo	10 µL	80 µL	
CLX 2 <sup>nd</sup> Strand Enzyme	5 µL	40 µL	
<b>Total Volume</b>	<b>190 µL</b>	<b>1,520 µL</b>	
Volume Used in STEP F	175 µL	1,400 µL	
<i>Excess Volume</i>	15 µL	120 µL	

6. Carefully remove the PCR plate film covering the bottom, then top of the Filter Plate.



7. Place Filter Plate on the vacuum manifold. Turn on pump and press down on Filter Plate so liquid flows out. Turn off the pump when liquid stops. Wait ~30 seconds for vacuum gauge to read 0.
8. Dispense 175  $\mu\text{L}$  **CLX Wash A** into each Filter Plate well with sample beads.
9. Use the vacuum manifold to remove liquid from Filter Plate as previously described in Step 7.
10. Pat dry the bottom of the Filter Plate with an absorbent paper towel.
11. Place Filter Plate onto the vacuum manifold but **do not turn on pump**.
12. Dispense 175  $\mu\text{L}$  **1x NaOH** (Step 4) into each Filter Plate well with sample beads.
13. Tilt Filter Plate. Gently pipet 5 times to mix. Pipet slowly and try not to make bubbles.
14. Incubate for **exactly 5 minutes**.
15. Turn on pump. Press down on the Filter Plate so liquid flows out. Leave the pump on for Step 16.
16. Wash the Filter Plate with CLX Wash A:
  - a. Dispense 175  $\mu\text{L}$  **CLX Wash A** into each sample well of the Filter Plate.
  - b. Repeat Step 16a 2 more times.
17. Turn off the pump when liquid stops. Wait ~30 seconds for vacuum gauge to read 0. Leave the Filter Plate on the vacuum manifold.



18. Remove the Filter Plate from the vacuum manifold. Pat dry the bottom of the Filter Plate with an absorbent paper towel.
19. Use a clear adhesive PCR plate film to seal the Filter Plate bottom.
20. Dispense 175 µL **2<sup>nd</sup> Strand Reaction Mix** (Step 5) into each Filter Plate well with sample beads.
21. Tilt the Filter Plate. Gently pipet 5 times to mix. Pipet slowly and try not to make bubbles.
22. Use a clear adhesive PCR plate film to seal the sample wells.
23. Incubate Filter Plate at **37°C for 30 minutes**.
24. During the 30-minute incubation, prepare reagents for the WTA Reaction in STEP G.





## STEP G: WHOLE TRANSCRIPTOME AMPLIFICATION (WTA)

### Materials required

- CLX PCR Enzyme (**orange cap**)
- CLX WTA Oligo (**orange cap**)
- CLX Wash A
- Water
- Clear PCR plate seals (Plate Kit)
- Vacuum setup (Vacuum Kit)
- Full-height PCR Plate
- Deep Well Plate
- Filter Plate adaptor (optional)
- Multichannel pipettes (optional)
- Reagent reservoirs (optional)

1. Thaw **CLX PCR Enzyme (orange cap)** and **CLX WTA Oligo (orange cap)** at room temperature. Transfer to ice until use.
2. Set up **WTA Reaction Program** on thermocycler:

Step	Temperature (°C)	Time	# Cycles
1	95	5 minutes	1
2	98	20 seconds	20
3	60	45 seconds	
4	70	60 seconds	
5	72	2 minutes	1
6	4	hold	—



3. Prepare **WTA Reaction Mix** with table below.  
Pipet to mix. Keep on ice until use.
  - Count the CLX 1<sup>st</sup> Strand Control as a sample!

WTA Reaction Mix Component	1 sample	8 samples	N samples
CLX PCR Enzyme	200 µL	1,600 µL	
CLX WTA Oligo	40 µL	320 µL	
Water	180 µL	1,440 µL	
<b>Total Volume</b>	<b>420 µL</b>	<b>3,360 µL</b>	
Volume Used in STEP G	400 µL	3,200 µL	
<i>Excess Volume</i>	20 µL	160 µL	

### IMPORTANT!

All calculations for reaction volumes already include excess volumes.  
DO NOT ADJUST CALCULATIONS.

### NOTE

There may be some liquid on the bottom seal.

4. Carefully remove the PCR plate film covering the bottom, then top of the Filter Plate.
5. Place Filter Plate on the vacuum manifold. Turn on pump. Press down on the Filter Plate so liquid flows out. Leave the pump on for Step 6.
6. Wash the Filter Plate with **CLX Wash A**:
  - a. Dispense 175 µL **CLX Wash A** into each Filter Plate sample well.
  - b. Repeat Step 6a 2 more times.
7. Turn off the pump when liquid stops. Wait ~30 seconds for vacuum gauge to read 0. Leave the Filter Plate on the vacuum manifold.
8. Remove the Filter Plate. Pat dry the bottom of the Filter Plate with an absorbent paper towel.
9. Use a clear adhesive PCR plate film to seal the Filter Plate bottom.



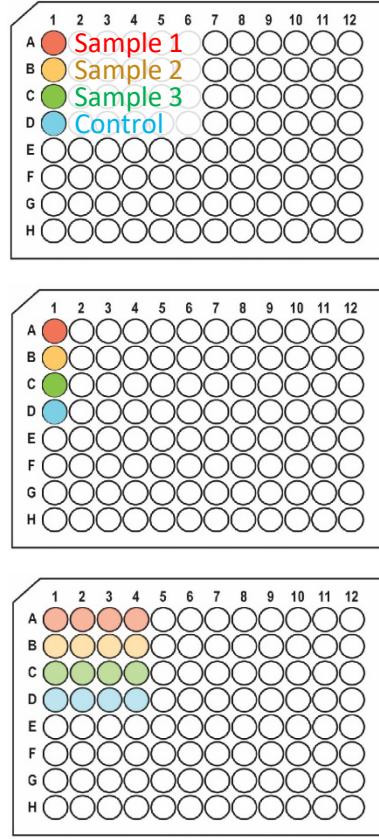
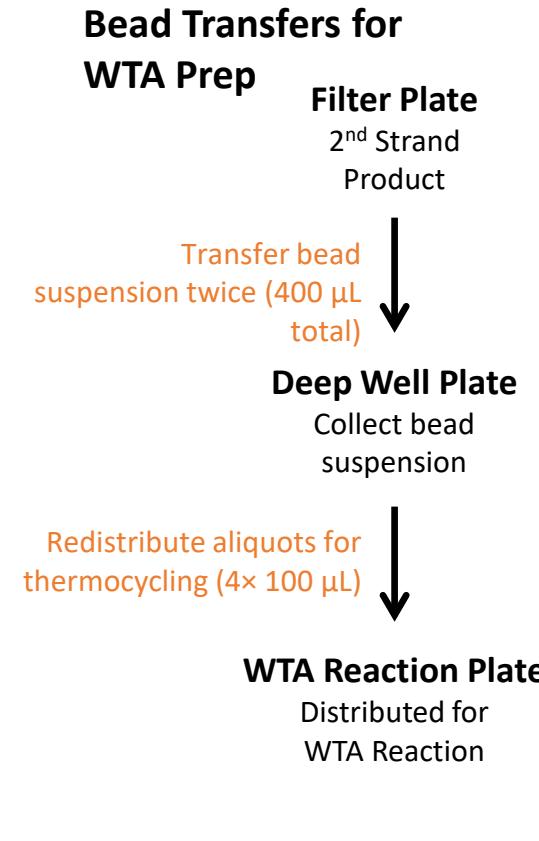
## IMPORTANT!

Ensure all wells are clean and unused.

## IMPORTANT!

Use the Sample Tracking Table (Appendix 1) to keep track of the well positions for each sample.

10. Assign 1 well in the Deep Well Plate and 4 wells in a **WTA Reaction Plate** for each sample using the **Sample Tracking Table** (Appendix 1).



11. Transfer beads from the Filter Plate to the Deep Well Plate, one sample at a time:
  - a. Tilt the Filter Plate and dispense 200  $\mu\text{L}$  **WTA Reaction Mix** (Step 3) into each Filter Plate sample well.
  - b. Rapidly pipet 10 times to flush sample beads to the right edge of the Filter Plate well.
  - c. Immediately transfer the bead suspension to the assigned well on the Deep Well Plate.
12. Repeat Step 11 with a clean pipette tip and another 200  $\mu\text{L}$  **WTA Reaction Mix**. Combine the transfers for a total volume of 400  $\mu\text{L}$  in the assigned well of the Deep Well Plate.



**IMPORTANT!**

Beads settle quickly.  
Fully resuspend beads before  
each 100 µL transfer.

**IMPORTANT!**

Use the Sample Tracking Table  
(Appendix 1) to keep track of the  
well positions for each sample.

**STOP POINT**

13. Set single- or multi-channel pipette to 100 µL. Redistribute beads from Deep Well Plate to the **WTA Reaction Plate**, one sample at a time:
  - a. Rapidly pipet 10 times to resuspend beads.
  - b. Immediately transfer 100 µL bead suspension to one of the assigned wells on the plate.
  - c. Repeat Steps 13a–b 3 more times to transfer remaining beads to 3 additional wells in the plate. This step will result in 4× 100 µL reactions for WTA.
14. Seal the **WTA Reaction Plate** with a clear adhesive PCR film.
15. Place the plate on the thermocycler and run the **WTA Reaction Program** (~1 hour) from Step 2.
16. Store **WTA Reaction Plate** overnight at 4°C or long-term at -20°C.





### IMPORTANT!

All calculations for reaction volumes already include excess volumes.  
DO NOT ADJUST CALCULATIONS.

### NOTE

The Purified WTA Product Plate wells will contain 100 µL after combining the WTA Reaction wells (25 µL each).

## STEP H: WTA SPRI CLEAN-UP

### Materials required

- CLX SPRI Beads
- CLX Wash A
- Molecular biology grade ethanol (absolute)
- 96-well plate bar magnet
- 1.5 mL microfuge tubes
- PCR Plate or strip tubes
- Multichannel pipettes (optional)
- Reagent reservoirs (optional)

1. Dilute absolute ethanol with **Water** to make an **80% Ethanol** solution. Follow table below.
  - **Count the CLX 1<sup>st</sup> Strand Control as a sample!**

80% Ethanol Component	1 sample	8 samples	N samples
Absolute ethanol	400 µL	3,200 µL	
Water	100 µL	800 µL	
<b>Total Volume</b>	<b>500 µL</b>	<b>4,000 µL</b>	
Volume Used in STEP H	350 µL	2,800 µL	
<i>Excess Volume</i>	<i>150 µL</i>	<i>1,200 µL</i>	

2. Label a new PCR Plate as “Purified WTA Product”. Use the **Sample Tracking Table** (Appendix 1) to **assign 2 wells for each sample** in the **Purified WTA Product Plate**.
3. Carefully remove the film sealing the **WTA Reaction Plate** from STEP G.
4. For each sample, combine 25 µL from the 4 wells in the **WTA Reaction Plate** into **1 new well** assigned on the **Purified WTA Product Plate**.
5. Seal the **WTA Reaction Plate** with a clear adhesive PCR film and store at -20°C for future experiments or troubleshooting.



6. Vortex the **CLX SPRI Beads** to resuspend. Dispense 90 µL into each well with WTA product. Pipet up and down 15 times to mix thoroughly. Repeat with a fresh pipette tip for each sample.
7. Incubate at room temperature for **3 minutes**.
8. Place the **Purified WTA Product Plate** onto the magnet for **2 minutes**. The liquid will turn clear as the magnet draws the SPRI beads out of solution.
9. Remove the supernatant and discard.
10. Remove the plate from the magnet.
11. Dispense 50 µL **CLX Wash A** to each well with WTA product. Pipet up and down 15 times to resuspend SPRI beads. Repeat with a fresh pipette tip for each sample.
12. Incubate at room temperature for **3 minutes** to elute DNA.
13. Place the plate onto the magnet for **2 minutes**. The liquid will turn clear as the magnet draws the SPRI beads out of solution.
14. For each sample, transfer the supernatant to the second well assigned in the **Sample Tracking Table** (Appendix 1).
  - **DO NOT DISCARD THE SUPERNATANT!**
15. Remove the plate from the magnet.
16. Vortex the **CLX SPRI Beads** to resuspend. Dispense 45 µL to each well with transferred supernatant. Pipet up and down 15 times to mix. Repeat with a fresh pipette tip for each sample.

**IMPORTANT!**

The supernatant in Step 14 contains the WTA product.

**IMPORTANT!**

Use the Sample Tracking Table (Appendix 1) to keep track of the well positions for each sample.



17. Incubate at room temperature for **3 minutes**.
18. Place the plate onto the magnet for **2 minutes**.  
The liquid will turn clear as the magnet draws the SPRI beads out of solution.
19. Remove the supernatant and discard.
20. Wash the SPRI beads with **80% Ethanol**:
  - a. Dispense 175 µL of **80% Ethanol** (Step 1) into each well containing WTA product.
  - b. Move the entire plate over the bar magnet 6 times to pull the beads through the ethanol solution.
  - c. Place the plate onto the magnet for **30 seconds**.
  - d. Remove ethanol and discard. Avoid the pellet.
  - e. Repeat Steps 20a–d one more time.
22. Use a 10 µL pipette to remove any residual ethanol from the bead pellet. Avoid the pellet.
23. Leave the plate on the magnet for **5 minutes** to air dry.
24. Dispense 20 µL **CLX Wash A** to each well with WTA product. Remove the plate from the magnet.
25. Adjust pipette volume to 10 µL and pipet up and down 10 times to mix. Repeat with a fresh pipette tip for each sample.

**NOTE**

If using a different style of magnet than the bar type, pipet up and down several times to wash the beads. They will not resuspend in the 80% Ethanol.

**IMPORTANT!**

Removing the plate from the magnet would cause beads to enter solution and sample loss when pipetting.

**NOTE**

If environment is humid, increase drying time to 10 minutes.



**IMPORTANT!**

The supernatant in Step 27 contains the purified WTA product.

**NOTE**

We recommend using the Qubit™ to quantify DNA concentrations.

**NOTE**

WTA yield varies with the sample type and the number of cells loaded.

Expect >2 ng/µL WTA product for the CLX 1<sup>st</sup> Strand control and 1–5 ng/µL for an input of 30,000 PBMCs .

26. Incubate at room temperature for **3 minutes** to elute DNA.
27. Place the plate onto the magnet for **2 minutes**.  
The liquid will turn clear as the magnet draws the SPRI beads out of solution.
  - **DO NOT DISCARD THE SUPERNATANT!**
  - Beads in the Purified WTA Product will not interfere with the downstream application, but you may still choose to transfer product to a clean PCR plate or strip tube. If beads remain in the well, be sure to return the plate to the magnet when withdrawing liquid.
28. Measure and record the DNA concentration of each sample (and the **CLX 1<sup>st</sup> Strand Control**) in the **Purified WTA Product Plate**.

Sample ID	Purified WTA Product Wells	DNA Concentration (ng/µL)
CLX 1 <sup>st</sup> Strand Control		

**IMPORTANT!**

If the WTA Product is <0.2 ng/µL and the sample contained ≥2,000 cells, see Troubleshooting on Page 63.

29. If the purified WTA product is ≥0.2 ng/µL for all samples, proceed to Index PCR in STEP I.

- If the WTA product is <0.2 ng/µL and the sample contained <2,000 cells, then perform WTA Reamplification (Appendix 5).



**NOTE**

- HIVE UDI Plate: clear plate
- HIVE CDI Plate: blue plate

Each well in the Index Plate is a unique i7+i5 oligo index (see Appendix 4). We recommend using the HIVE UDI plate because both i7 (index 1) and i5 (index 2) are unique.

**STEP I: INDEX PCR****Materials required**

- CLX PCR Enzyme (**orange cap**)
- Index Plate
- 96-well PCR Plate
- 96-well plate bar magnet
- Thermocycler

1. Thaw **CLX PCR Enzyme (orange cap)** and **Index Plate** (HIVE UDI Plate or HIVE CDI Plate) at room temperature for **15 minutes**. Transfer to ice until use.
2. Set up **Index PCR Program** on thermocycler:

Step	Temperature (°C)	Time	# Cycles
1	95	5 minutes	1
2	95	30 seconds	9
3	60	30 seconds	
4	72	60 seconds	
5	72	2 minutes	1
6	4	hold	—

**NOTE**

Use one unique Index per sample to multiplex libraries for sequencing.

3. Label a new PCR Plate as “Index Reaction Plate”. Using the **Sample Tracking Table** (Appendix 1), assign an **Index Reaction Plate** well and an Index Plate ID for each sample.



4. Copy DNA Concentrations from Page 40 into the table below. Calculate the volumes of Purified WTA Product needed for 5 ng of DNA for the Index Reaction. Add **Water** (if needed) to bring the volume up to 5  $\mu\text{L}$ .
- If the WTA Product concentration is <1 ng/ $\mu\text{L}$ , then add 5  $\mu\text{L}$  WTA Product to the Index Reaction.
  - If WTA Product concentration is >10 ng/ $\mu\text{L}$ , dilute 1  $\mu\text{L}$  of the sample to 1 ng/ $\mu\text{L}$  and add 5  $\mu\text{L}$  into the Index Reaction.

Sample ID	DNA Concentration (ng/ $\mu\text{L}$ )	Volume Product for up to 5 ng DNA ( $\mu\text{L}$ )	Volume Water ( $\mu\text{L}$ )
Example	3.2	$5/3.2=1.6 \mu\text{L}$	$5-1.6=3.4 \mu\text{L}$
Example	0.8	5 $\mu\text{L}$	0 $\mu\text{L}$
CLX 1 <sup>st</sup> Strand Control			

$$\text{Volume for 5 ng } (\mu\text{L}) = 5 \text{ ng} / \text{DNA Concentration (ng}/\mu\text{L})$$

$$\text{Volume Water } (\mu\text{L}) = 5 \mu\text{L} - \text{Volume for 5 ng } (\mu\text{L})$$

### IMPORTANT!

Use the Sample Tracking Table (Appendix 1) to keep track of the sample position and Index ID while preparing the Index Reaction Plate.

5. Dispense 25  $\mu\text{L}$  of **CLX PCR Enzyme (orange cap)** into all assigned wells in the **Index Reaction Plate**.
6. Using the table above, dispense the calculated volume of Purified WTA Product and **Water** into the assigned wells in the **Index Reaction Plate** with **CLX PCR Enzyme**.



**NOTE**

Each well in the Index Plate is a unique i7+i5 oligo index (see Appendix 4). Record the Index ID!

**STOP POINT**

7. For each sample, use pipette tip to pierce the foil seal on the **Index Plate** well. Dispense 20 µL of Index into the assigned **Index Reaction Plate** well.
8. Seal the **Index Reaction Plate** with a clear adhesive PCR film. Place on thermocycler and run **Index PCR Program** from Step 2.
9. If not pausing overnight, proceed to STEP J and prepare solutions for SPRI Clean-Up.
10. After the Index PCR Reaction, you may leave the **Index Reaction Plate** holding overnight at 4°C in the thermocycler or store long-term at -20°C.





### IMPORTANT!

All calculations for reaction volumes already include excess volumes.  
DO NOT ADJUST CALCULATIONS.

## STEP J: INDEX SPRI CLEAN-UP

### Materials required

- CLX SPRI Beads
- CLX Wash A
- Molecular biology grade ethanol (absolute)
- 96-well plate bar magnet
- 1.5 mL microfuge tubes
- PCR Plate or strip tubes
- Multichannel pipettes (optional)
- Reagent reservoirs (optional)

1. Dilute absolute ethanol with **Water** to make an **80% Ethanol** solution. Follow table below.
  - **Count the CLX 1<sup>st</sup> Strand Control as a sample!**

80% Ethanol Component	1 sample	8 samples	N samples
Absolute ethanol	400 µL	3,200 µL	
Water	100 µL	800 µL	
<b>Total Volume</b>	<b>500 µL</b>	<b>4,000 µL</b>	
Volume Used in STEP J	350 µL	2,800 µL	
<i>Excess Volume</i>	<i>150 µL</i>	<i>1,200 µL</i>	

2. Assign a well in the same **Index Reaction Plate** from Step I for the Purified Index PCR Product using the **Sample Tracking Table** (Appendix 1).
  - To prevent transfer errors, choose an adjacent column or row and maintain order of the samples.
  - You may also transfer purified product into a strip of PCR tubes (with lid).
3. Carefully remove the film sealing the **Index Reaction Plate** from STEP I.



4. Vortex the **CLX SPRI Beads** to resuspend. Dispense 40 µL to each sample well. Pipet up and down 15 times to mix. Repeat with a fresh pipette tip for each sample.
  - Pipet the CLX SPRI Beads into the reaction wells, not the wells assigned for purified product.
5. Incubate at room temperature for **3 minutes**.
6. Place the plate onto the magnet for **2 minutes**. The liquid will turn clear as the magnet draws the SPRI beads out of solution.
7. Remove the supernatant and discard.
8. Remove the plate from the magnet.
9. Dispense 50 µL **CLX Wash A** to each sample well. Pipet up and down 15 times to resuspend SPRI beads. Repeat with a fresh pipette tip for each sample.
10. Incubate at room temperature for **3 minutes to elute DNA**.
11. Place the plate onto the magnet for **2 minutes**. The liquid will turn clear as the magnet draws the SPRI beads out of solution.
12. For each sample, transfer the supernatant to the well assigned in the **Sample Tracking Table** (Appendix 1).
  - **DO NOT DISCARD THE SUPERNATANT!**
13. Remove the plate from the magnet.

### IMPORTANT!

The supernatant in Step 12 contains the purified Index PCR product.

### IMPORTANT!

Use the Sample Tracking Table (Appendix 1) to keep track of the well positions for each sample.



14. Vortex the **CLX SPRI Beads** to resuspend. Dispense 40 µL to a well with transferred supernatant. Pipet up and down 15 times to mix. Repeat with a fresh pipette tip for each sample.
15. Incubate at room temperature for **3 minutes**.
16. Place the plate onto the magnet for **2 minutes**. The liquid will turn clear as the magnet draws the SPRI beads out of solution.
17. Remove the supernatant and discard.
18. Wash the SPRI beads with **80% Ethanol**:
  - a. Dispense 175 µL of **80% Ethanol** (Step 1) into every well containing WTA product.
  - b. Move the entire plate over the 96-well bar magnet 6 times to pull the beads through the ethanol solution.
  - c. Place the plate onto the magnet for **30 seconds**.
  - d. Remove ethanol and discard. Avoid the pellet.
  - e. Repeat Steps 18a–d one more time.
19. Use a 10 µL pipette to remove any residual ethanol from the bead pellet. Avoid the pellet.

**NOTE**

If using a different style of magnet than the bar type, pipet up and down several times to wash the beads. They will not resuspend in the 80% Ethanol.

**IMPORTANT!**

Removing the plate from the magnet would cause beads to enter solution and sample loss when pipetting.



**NOTE**

If environment is humid, increase drying time to 10 minutes.

**IMPORTANT!**

The supernatant in Step 25 contains the purified Index PCR product.

**IMPORTANT!**

Use the Sample Tracking Table (Appendix 1) to keep track of the well positions for each sample.

**IMPORTANT!**

**CUSTOM PRIMERS ARE REQUIRED (Appendix 3).**

**STOP POINT**

21. Leave the plate on the magnet for **5 minutes** to air dry.
22. Remove the plate from the magnet.
23. Dispense 30 µL **CLX Wash A** to a well with Index Reaction product. **Pipet up and down 10 times** to mix. Repeat with a fresh pipette tip for each sample.
24. Incubate at room temperature for **3 minutes** to elute DNA.
25. Place the plate onto the magnet for **1 minute**. The liquid will turn clear as the magnet draws the SPRI beads out of solution.
  - **DO NOT DISCARD THE SUPERNATANT!**
26. Transfer 25 µL **Purified Index PCR Product** to the assigned well or PCR tube assigned on the **Sample Tracking Table** (Appendix 1).
27. Store **Purified Index PCR Product** at -20°C.
  - a. See **Appendix 2** for recommendations for library QC and pooling.
  - b. See **Appendix 3** for recommendations for sequencing setup and information on the required custom primers.



## APPENDIX 1: SAMPLE TRACKING TABLE

Sample ID	HIVE Serial #	Spin Plate Position/ID	Filter Plate Well	Deep Well Plate Position	WTA Reaction Wells	Purified WTA Product Wells	Index Reaction Plate Well	Index Type (UDI/CDI)	Index Plate Well ID	Index Plate Purified Product Well
<i>Example</i>	1437A6	A1/Plate-1	A1	A1	A1-A4	A1	A1	UDI	A1	A4



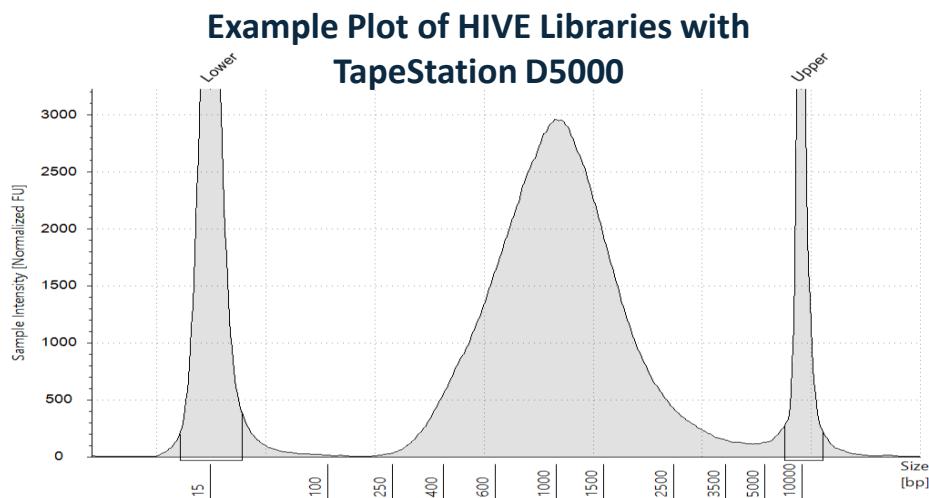


## APPENDIX 2: LIBRARY QC & POOLING

### LIBRARY QC

We recommend using the Qubit™ to quantify DNA concentrations for QC and for normalization when pooling HIVE libraries. The expected library yield is 1–50 ng/µL, but yield will vary with the sample type and the number of cells loaded.

We recommend using the LabChip GX Touch™, TapeStation™, or Bioanalyzer™ systems and kits suited for sizing >1,000 bp DNA fragments to evaluate library quality and size distributions (recommended TapeStation Kit: D5000 ScreenTape Assay and Reagents). HIVE libraries are expected to have a broad smear of DNA sizes with a peak between 500–1,200 bp.



### LIBRARY POOLING

Pooling is required to sequence multiple libraries in a single run. Check with your sequencing provider or core facility before pooling, because each facility will have submission requirements for minimum library volumes and concentrations.

Illumina® provides instructions for normalizing and pooling libraries<sup>1</sup> and for converting mass concentrations (ng/µL) to molar concentrations (nM).<sup>2</sup> For HIVE libraries, we recommend using the universal fragment size (750 bp) for conversion instead of the peak size from the fragment analyzer.

Prior to sequencing, we highly recommend quantifying the final pooled sample with qPCR using the KAPA Library Quantification Kit™ to ensure accurate loading into the sequencer and optimizing cluster density.<sup>3</sup>



## APPENDIX 2: LIBRARY QC & POOLING

### OTHER CONSIDERATIONS WHEN POOLING

We recommend pooling libraries with proportions that reflect the number of cells in each sample. For example, if 3 samples contained 10,000, 5,000, and 5,000 cells and were pooled for sequencing, we would pool libraries at 50%, 25%, and 25% proportions, respectively. These proportions would allocate an even read depth per cell across all samples.

Samples with the same cell type can be pooled, but we do not recommend pooling two different cell types with different transcript levels. For example, we would not advise pooling a cell line sample and a primary cell sample. If this type of pooling is necessary, you may need to empirically adjust the sample proportions to achieve the desired coverage and read depth per cell.

### REFERENCES FOR APPENDIX 2

1. Illumina® Best practices for manually normalizing library concentrations.  
<https://support.illumina.com/bulletins/2017/03/best-practices-for-manually-normalizing-library-concentrations.html>
2. Illumina® Converting ng/µl to nM when calculating dsDNA library concentration.  
<https://support.illumina.com/bulletins/2016/11/converting-ngrl-to-nm-when-calculating-dsdna-library-concentration-.html>
3. Optimizing cluster density on Illumina® sequencing systems.  
<https://assets.illumina.com/content/dam/illumina-marketing/documents/products/other/miseq-overclustering-primer-770-2014-038.pdf>



# APPENDIX 3: SEQUENCING RECOMMENDATIONS

## SEQUENCING SETUP

HIVE libraries require paired-end, dual-indexed sequencing. We do not recommend spiking in PhiX controls.

	Read 1	Index 1 (i7)	Index 2 (i5)	Read 2
Primers	CLX Read 1 Seq Primer	CLX Index 1 Seq Primer	CLX Index 2 Seq Primer	CLX Read 2 Seq Primer
Target	Cell Barcode	I7 index	I5 index	Insert
Cycles	25	8	8	50

## COMPATIBLE SEQUENCERS

Illumina® NextSeq™ 500/550

Illumina® NextSeq™ 2000

Illumina® NovaSeq™ 6000

## CUSTOM PRIMERS

Custom CLX sequencing primers (**red caps**) are provided at 100 µM in the Library Prep Reagents. HIVE sequencing requires dual-indexing and is compatible with the Illumina® NextSeq™ 500/550 75-cycle kits, NextSeq™ 2000 P1/P2/P3 100-cycle kits, and NovaSeq™ v1.5 100-cycle kits. Custom sequencing primers should be used alone, and we do not recommend spike-in with Illumina® standard sequencing primers. For custom primers, please refer to the Illumina® instructions<sup>1–3</sup> and/or consult with sequencing facility staff.



## APPENDIX 3: SEQUENCING RECOMMENDATIONS

### READS PER SAMPLE

Consult the table below to determine the optimal number of reads per sample. For example, if we loaded 2 HIVE Collectors with 30,000 cells each, we could assume an average recovery of 12,000 cells per HIVE. We would then pool the 2 HIVE Collectors on a single NovaSeq™SP flow cell using both lanes (~800M reads per flow cell) to expect ~33,000 reads/cell.

Single-Cell Recovery <sup>†</sup>	Cell Input	Recommended Reads/Sample (million) <sup>‡</sup>	# of HIVEs per Novaseq SP flowcell*	# of HIVEs per NextSeq 550 HO flowcell <sup>^</sup>
220	500	7	na	na
850	2,000	27	na	na
3,000	7,500	100	8	4
6,000	15,000	200	4	2
11,000	30,000	400	2	1
17,000	60,000	800	1	na

<sup>†</sup>Performance metrics estimated from experimental data using human PBMCs.

<sup>‡</sup>Recommendations for reads/sample balance the amount of biological information gathered with sequencing costs and should achieve 80% recovery of cells, genes, and transcripts. These are recommended starting points for most applications, but you may need to tailor sequencing depths for your specific experiments.

\*The nominal NovaSeq SP flow cell offers 800 M reads.

<sup>^</sup>The nominal NextSeq 550 HO flow cell offers 400 M reads.

### REFERENCES FOR APPENDIX 3

1. Illumina® NovaSeq™ Series Custom Primers Guide.  
<https://support.illumina.com/downloads/novaseq-custom-primers-guide-1000000022266.html>
2. Illumina® NextSeq™ 500/550 Series Custom Primers Guide.  
<https://support.illumina.com/downloads/nextseq-500-custom-primers-guide-15057456.html>
3. Illumina® NextSeq™ 2000 Series Custom Primers Guide.  
<https://support.illumina.com/downloads/nextseq-1000-2000-custom-primers-guide.html>



# APPENDIX 4: INDEX PLATE LAYOUT

## HIVE UDI INDEX PLATE LAYOUT (PN 10922, clear plate)

Each well of the Unique Dual Index Plate contains a unique i7+i5 index.

Well ID	i7 Index 1	i5 Index 2	
	Sequencing Primer	Forward Strand Workflow	Reverse Complement Workflow
A01	AATCGTTA	AATAACGT	ACGTTATT
B01	AATCGGCG	AATATTGA	TCAATATT
C01	AATCCGTT	AATATGCT	AGCATATT
D01	AAGATACA	AATATCTG	CAGATATT
E01	AAGACGAA	AATAGATT	AATCTATT
F01	AAGTAAGT	AATAGTCC	GGACTATT
G01	AAGTTATC	AATAGCAA	TTGCTATT
H01	AAGTTGGA	AATACAGG	CCTGTATT
A02	GTCTACAT	TTCTTGAA	TTCAAGAA
B02	TCGCCCGA	GTATACCG	CGGTATAC
C02	TGCGTACA	TTCTCATA	TATGAGAA
D02	GTCGCTGT	TTATATCA	TGATATAA
E02	TTATTATG	TTAGCGCA	TGCGCTAA
F02	TGACTGAA	TTAGACGT	ACGTCTAA
G02	GTACAGCT	TGATCGGT	ACCGATCA
H02	GGACAAACG	TGATGGCC	GGCCATCA
A03	CGCTGCTC	GGCAGATC	GATCTGCC
B03	CTGGCCTC	GATCCAAC	GTGGATC
C03	GAATCAAT	TCTGTGAT	ATCACAGA
D03	TCGGATGT	CTGCGGAT	ATCCGCAG
E03	CGCTTATA	GCGGCCGT	ACGGCCGC
F03	AAGACTGT	GTGGACTA	TAGTCCAC
G03	CAACTGCT	AGTAGTAT	ATACTACT
H03	TTCGAACC	TGTCACCT	AGGTGACA
A04	GATCAACA	CTATGTTA	TAACATAG
B04	GAACTTAT	AGATACGC	GCGTATCT
C04	TGAGTCAG	CCGAACTT	AAGTCGG
D04	CGAGCCGG	GCGGCTTG	CAAGCCGC
E04	TCTATCAG	CAGTAACC	GGTTACTG
F04	CAATGATG	CACGGACG	CGTCCGTG
G04	CATGATGA	GTAGAGG	CCTCTAAC
H04	CAGACCAC	GCTTCGGC	GCCGAAGC
A05	CGAAGGAC	GTGACGC	GCGTCAAC
B05	CGTATTGG	GGTATCTT	AAGATACC
C05	GAATGCTC	GTCTAAC	TGTTAGAC
D05	CGATTATC	GAGTTGAT	ATCAACTC
E05	CGGTGGTA	GCCTAGTA	TACTAGGC
F05	CACAGTAA	CACTAGAG	CTCTAGTG
G05	TGACTACT	CCTTACAG	CTGTAAGG
H05	TTCTGGTG	CCAGTGGT	ACCACTGG
A06	GATGCCGG	ATCTACGA	TCGTAGAT
B06	GAAGCACA	CCTCTGGC	GCCAGAGG
C06	GAATATCC	GACGCCAT	ATGGCGTC
D06	TCGAAGCT	GCACGTAG	CTCAGTGC
E06	TCACCAAT	CACGGCGC	GCGCCGTG
F06	TGGTCATT	GCAGATGG	CCATCTGC
G06	CAGAAGAT	GTACATTG	CAATGTAC
H06	CAATCGAA	GCACACGC	GCGTGTGC

Well ID	i7 Index 1	i5 Index 2	
	Sequencing Primer	Forward Strand Chemistry	Reverse Complement Workflow
A07	CTACGAAG	CTCGACAG	CTGTCGAG
B07	CTTAATAC	CCATTGTG	CACAATGG
C07	CTTATGAA	GCCAATGT	ACATTGGC
D07	CTATCATT	GACCACCT	AGGTGGTC
E07	CTGGAAGC	GGTGCAGA	TCTGCACC
F07	CAACCGTG	CTCTCACG	CGTGAGAG
G07	TGAGGCGC	GGAGACCA	TGGTCTCC
H07	AAGTACAG	GTCACGTC	GACGTGAC
A08	GATGCGTC	GAGGCTGC	GCAGCCTC
B08	GAAGTCTT	ACTACGGT	ACCGTAGT
C08	TCGGCACC	CCAACGTC	GACGTTGG
D08	CGCGCAA	TGGCTAGG	CCTAGCCA
E08	TCCTCGAT	GTAACTGC	GCAGTTAC
F08	TGGTGCAC	GGAATCAC	GTGATTCC
G08	CAGGTTCC	CGAACACC	GGTGTTCG
H08	CCGTGCCA	GCAGCTCC	GGAGCTGC
A09	CTACGGCA	CCTCGTAG	CTACGAGG
B09	GAAGAGGC	AAGTGTCA	TAGCACTT
C09	AAGAACCG	GTAGATAA	TTATCTAC
D09	CGAACGGA	CCTACGG	CCGGTAGG
E09	AAGAGAGC	CAGCCAGT	ACTGGCTG
F09	CCACAATG	CGTTGACG	CGTCAACG
G09	TGAACAGG	GAGAACAA	TTGTTCTC
H09	CATTGCAC	CATGCAGC	GCTGCATG
A10	GATTCCCT	CATAGGCA	TGCCTATG
B10	CGGATAAC	GCCGAACG	CGTTCGGC
C10	CTCACGAT	CTTACGGC	GCCGTAAG
D10	CTACTGAC	GGAGGATG	CATCCTCC
E10	TCAACGAG	CGTCAACC	GGTTGACG
F10	TGTGTGCC	CATCAGGT	ACCTGATG
G10	CAGTGTGG	TGTGAATC	GATTACAA
H10	TTACCTGG	ACGATTGC	GCAATCGT
A11	CTACTCGA	AGATGAAC	GTTCATCT
B11	GAATCTGG	TGTCCACG	CGTGGACA
C11	TCGGTCGA	CCAAGTGC	GCACTTGG
D11	TCTTAAGT	CGCTGAAT	ATTCAGCG
E11	TGCGAGAC	GCCGGCGA	TCGCCGGC
F11	CACCAACG	CGTTGAA	TTACAACG
G11	TTCCACCA	GGTTAAGG	CCTTAACC
H11	CTGCAACG	GACATTGCG	CGAATGTC
A12	GATTGAG	CCGAGTAT	ATACTCGG
B12	CTGATTGA	GACACACT	AGTGTGTC
C12	TCGGAAG	CTAACTCA	TGAGTTAG
D12	TTAGAGTC	TGTGACGA	TCGTCACA
E12	CCTGGTGT	GCCTCCGG	CCGGAGGC
F12	TGTGTTAA	GGCACGGT	ACCGTGCC
G12	CCGCTGTT	AGACCGCA	TGCGGTCT
H12	TACTGTTA	GCGAATAC	GTATTGCG

### Download the HIVE UDI Index Plate Layout

<https://honeycombbio.zendesk.com/hc/en-us/articles/14283947472923-HIVE-UDI-Index-Plate>



## APPENDIX 4: INDEX PLATE LAYOUT

### HIVE CDI INDEX PLATE LAYOUT (PN 10271, blue plate)

Each well of the Combined Dual Index Plate contains an i7+i5 combination index.

#### I7 INDEX IDs

Row	Sequencing Primer
A	TAAGGCGA
B	CGTACTAG
C	AGGCAGAA
D	TCCTGAGC
E	GGACTCCT
F	TAGGCATG
G	CTCTCTAC
H	CAGAGAGG

#### I5 INDEX IDs

Row	Forward Strand Workflow	Reverse Complement Workflow
1	TAGATCGC	GCGATCTA
2	CTCTCTAT	ATAGAGAG
3	TATCCTCT	AGAGGATA
4	AGAGTAGA	TCTACTCT
5	GTAAGGAG	CTCCTTAC
6	ACTGCATA	TATGCAGT
7	AAGGAGTA	TACTCCTT
8	CTAACGCCT	AGGCTTAG
9	CGTCTAAT	ATTAGACG
10	TCTCTCCG	CGGAGAGA
11	TCGACTAG	CTAGTCGA
12	TTCTAGCT	AGCTAGAA

#### Download the HIVE CDI Index Plate Layout

<https://honeycombbio.zendesk.com/hc/en-us/articles/14284034410779-HIVE-CDI-Index-Plate>



## APPENDIX 5: WTA REAMPLIFICATION

### WTA REAMPLIFICATION USING PURIFIED WTA PRODUCT

1. Thaw **CLX PCR Enzyme** (orange cap) and **CLX WTA Oligo** (orange cap) at room temperature. Transfer to ice until use.
2. Set up **WTA Re-Amp Reaction Program** on thermocycler:

Step	Temperature (°C)	Time	# Cycles
1	95	5 minutes	1
2	98	20 seconds	
3	60	45 seconds	8
4	70	60 seconds	
5	72	2 minutes	1
6	4	hold	—

3. Prepare **WTA Re-Amp Reaction Mix** with table below. Pipet to mix. Keep on ice until use.

- **Count the CLX 1<sup>st</sup> Strand Control as a sample!**

WTA Reaction Mix Component	1 sample	8 samples	N samples
CLX PCR Enzyme	50 µL	400 µL	
CLX WTA Oligo	10 µL	80 µL	
Nuclease-free Water	40 µL	320 µL	
<b>Total Volume</b>	<b>100 µL</b>	<b>800 µL</b>	
Volume Used	95 µL	760 µL	
<i>Excess Volume</i>	5 µL	40 µL	

#### IMPORTANT!

All calculations for reaction volumes already include excess volumes.  
DO NOT ADJUST CALCULATIONS.



## APPENDIX 5: WTA REAMPLIFICATION

4. Copy information from the **Sample Tracking Table** (Appendix 1) into the table below. Label a new PCR Plate as “**WTA Re-Amp Reaction Plate**”. For each sample, assign a Re-Amp Reaction Plate well.

Sample ID	WTA Reaction Wells	WTA Re-Amp Reaction Wells
CLX 1 <sup>st</sup> Strand Control		

5. Aliquot 95 µL of the **WTA Re-Amp Reaction Mix** to the wells in the **WTA Re-Amp Reaction Plate**.
6. Place the **Purified WTA Product Plate** onto the magnet for **1 minute**. The liquid will turn clear as the magnet draws the SPRI beads out of solution.
7. Dispense 5 µL of purified WTA product (STEP H) to the assigned well of the **WTA Re-Amp Reaction Plate**. Pipet up and down to mix. Repeat with a fresh pipette tip for all samples.
8. Seal the **WTA Re-Amp Reaction Plate** with a clear adhesive PCR film. Place on thermocycler and run **WTA Re-Amp Reaction Program** from Step 2.
9. After the WTA Re-Amp Reaction, you can leave the **WTA Re-Amp Reaction Plate** holding overnight at 4°C in the thermocycler or store long-term at -20°C.
10. Again, proceed to STEP H for WTA SPRI Clean-Up.





# APPENDIX 6: QUICK PROTOCOL FOR TRANSCRIPTOME RECOVERY

## HIVE CLX scRNASeq Transcriptome Recovery & Library Preparation

### Step A: Prepare HIVE Collectors

1. Thaw HIVEs at **37°C for 15 minutes** or at room temperature for 60 minutes.
2. Remove Storage Liquid.
3. Add 2 mL **CLX Storage Wash Solution**.
4. Prepare **HIVE Top**.
5. Remove liquid and immediately add **Drying Cap**.
6. Briefly centrifuge at 400 RCF then stop spin.
7. Remove liquid from HIVE through Drying Cap port.
8. Remove **Drying Cap** and immediately use **Closure Tool** to add **HIVE Top**.
9. Incubate at room temperature for **30 minutes**.

### Step B: Lysis & Hybridization

1. Prepare **Lysis Solution** (1 mL **CLX Lysis Stock** + 100 µL **CLX Reducing Solution**).
2. Place **HIVE Collectors** on **Spin Plate**. Push down to remove plungers.
3. Add 1 mL **Lysis Solution** and incubate for **5 minutes**, then remove.
4. Wash with 1 mL **CLX Hybridization Buffer**.
5. Add 1 mL **CLX Hybridization Buffer** and incubate for **15 minutes**.



# APPENDIX 6: QUICK PROTOCOL FOR TRANSCRIPTOME RECOVERY

## Step C: Bead Recovery

1. Remove liquid.
2. Peel off membrane.
3. Add **Bead Collector**.
4. Add **Spin Lid** and flip assembly upside down.
5. Add 3 mL **CLX Bead Recovery Solution**.
6. Insert **Bead Recovery Stopper**.
7. Spin at max speed for **5 minutes**.
8. Set pipette to 300 µL and remove bead pellet.
9. Transfer bead pellet to **Filter Plate** well.
10. Pipet up and down to break up clumps.
11. Spin **HIVE Collectors** at max speed for **1 minute**.
12. Place **Filter Plate** on the vacuum and remove liquid.
13. Remove bead pellet and transfer to the same well in the **Filter Plate**.
14. Pipet up and down to break up clumps.



## APPENDIX 7: QUICK PROTOCOL FOR LIBRARY PREP

### Step D: 1<sup>st</sup> Strand Synthesis

1. Add 250 µL of **CLX Bead Recovery Solution** to the **CLX 1<sup>st</sup> Strand Control**.
2. Mix control and add to a **Filter Plate** well.
3. Prepare **1<sup>st</sup> Strand Reaction Mix** (175 µL **CLX 1<sup>st</sup> Strand Buffer** + 15 µL **CLX 1<sup>st</sup> Strand Enzyme**).
4. Place **Filter Plate** on the vacuum and remove liquid.
5. Add 175 µL **CLX 1<sup>st</sup> Strand Wash** and pipet to mix.
6. Use vacuum to remove liquid.
7. Dry bottom of **Filter Plate** and seal.
8. Add 175 µL of **1<sup>st</sup> Strand Reaction Mix** and pipet to mix.
9. Incubate at **37°C for 60 minutes**.

### Step E: Bead Clean-Up

1. Prepare **Clean-Up Reaction Mix** (175 µL **CLX Clean-Up Buffer** + 10 µL **CLX Clean-Up Enzyme**).
2. Place **Filter Plate** on the vacuum and remove liquid.
3. Wash with 175 µL **CLX Wash A**.
4. Use vacuum to remove liquid.
5. Dry bottom of **Filter Plate** and seal.
6. Add 175 µL of **Clean-Up Reaction Mix** and pipet to mix.
7. Incubate at **37°C for 45 minutes**.



## APPENDIX 7: QUICK PROTOCOL FOR LIBRARY PREP

### Step F: 2<sup>nd</sup> Strand Synthesis

1. Dilute **10× NaOH** to make **1× NaOH** (180 µL Water + 20 µL **10× NaOH**)
2. Prepare **2<sup>nd</sup> Strand Reaction Mix** (175 µL **CLX 2<sup>nd</sup> Strand Buffer** + 10 µL **CLX 2<sup>nd</sup> Strand Oligo** + 5 µL **CLX 2<sup>nd</sup> Strand Enzyme**).
3. Place **Filter Plate** on the vacuum and remove liquid.
4. Wash with 175 µL **CLX Wash A**.
5. Use vacuum to remove liquid.
6. Dry bottom of **Filter Plate**. Return Filter Plate to manifold but do not turn on.
7. Add 175 µL **1× NaOH** and pipet to mix.
8. Incubate **5 minutes**.
9. Turn pump on to remove liquid.
10. Wash 3 times with 175 µL **CLX Wash A**.
11. Dry bottom of **Filter Plate** and seal.
12. Add 175 µL of **2<sup>nd</sup> Strand Reaction Mix** and pipet to mix.
13. Incubate at **37°C for 30 minutes**.

### Step G: Whole Transcriptome Amplification (WTA)

1. Prepare **WTA Reaction Mix** (200 µL **CLX PCR Enzyme** + 40 µL **CLX WTA Oligo** + 180 µL **Water**).
2. Place **Filter Plate** on the vacuum and remove liquid.
3. Wash 3 times with 175 µL **CLX Wash A**.
4. Dry bottom of **Filter Plate** and seal.
5. Add 200 µL of **WTA Reaction Mix** to the **Filter Plate** and pipet to mix.
6. Transfer beads to the **Deep Well Plate**.
7. Repeat transfer with another 200 µL of **WTA Reaction Mix**.
8. Redistribute WTA bead suspension into 4 reactions (100 µL each) in a PCR plate
9. Run **WTA Reaction Program** on thermocycler (95°C 5 min, [98°C 20 sec, 60°C 45 sec, 70°C 60 sec] for 20 cycles, 72°C 2 min, 4°C hold).



## APPENDIX 7: QUICK PROTOCOL FOR LIBRARY PREP

### Step H: WTA SPRI Clean-Up

1. Prepare **80% Ethanol**, 400 µL per sample.
2. Combine 25 µL from each well containing the WTA reaction into 1 new well.
3. Add 90 µL **SPRI Beads** to each well. Incubate for **3 minutes**.
4. Place plate on magnet. Incubate for **2 minutes**.
5. Remove and discard supernatant. Remove plate from magnet.
6. Add 50 µL **CLX Wash A** and pipet to mix. Incubate for **3 minutes**.
7. Place plate on magnet. Incubate for **2 minutes**.
8. Transfer supernatant to a new well.
9. Add 45 µL **SPRI Beads** to each well. Incubate for **3 minutes**.
10. Place plate on magnet. Incubate for **2 minutes**.
11. Remove and discard supernatant
12. Add 175 µL **80% Ethanol**.
13. Move plate across bar magnet multiple times to mix.
14. Discard ethanol and repeat wash.
15. Dry pellet on magnet for **5 minutes**.
16. Add 20 µL **CLX Wash A**. Incubate for **3 minutes**.
17. Place plate on magnet and remove 2 µL for quantification.
18. If the purified WTA Product is  $\geq 0.2$  ng/µL, proceed to Step I.



## APPENDIX 7: QUICK PROTOCOL FOR LIBRARY PREP

### Step I: Index PCR

1. Calculate the volume for 5 ng of purified WTA product. Use **Water** to bring volume up to 5  $\mu\text{L}$ .
2. Add 25  $\mu\text{L}$  of **CLX PCR Enzyme** to one well per sample.
3. Add 5  $\mu\text{L}$  (containing 5 ng) of purified WTA product.
4. Add 20  $\mu\text{L}$  **Index**.
5. Run **Index PCR Program** on thermocycler (95°C 5 min, [95°C 30 sec, 60°C 30 sec, 73°C 60 sec] for 9 cycles, 72°C 2 min, 4°C hold).

### Step J: Index PCR SPRI Clean-Up

1. Prepare **80% Ethanol** (400  $\mu\text{L}$  per sample).
2. Add 40  $\mu\text{L}$  **SPRI Beads** to each well. Incubate for **3 minutes**.
3. Place plate on magnet. Incubate for **2 minutes**.
4. Remove and discard supernatant. Remove plate from magnet.
5. Add 50  $\mu\text{L}$  **CLX Wash A** and pipet to mix. Incubate **3 minutes**.
6. Place plate on magnet. Incubate for **2 minutes**.
7. Transfer supernatant to a new well.
8. Add 40  $\mu\text{L}$  **SPRI Beads** to each well. Incubate **3 minutes**.
9. Place plate on magnet. Incubate for **2 minutes**.
10. Remove and discard supernatant.
11. Add 175  $\mu\text{L}$  **80% Ethanol**.
12. Move plate across bar magnet multiple times to mix.
13. Discard ethanol and repeat wash.
14. Dry pellet on magnet for **5 minutes**.
15. Add 30  $\mu\text{L}$  **CLX Wash A**. Incubate for **3 minutes**.
16. Place plate on magnet. Incubate for **1 minute**.
17. Move supernatant to a new well.



# APPENDIX 8: FOLLOW THE MOLECULE

## FOLLOW THE MOLECULE

Bead Oligos

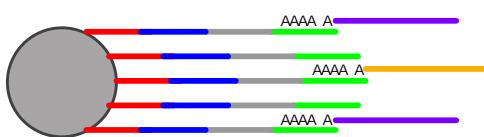
Universal Primer Sequence (UPS)

Cell Barcode

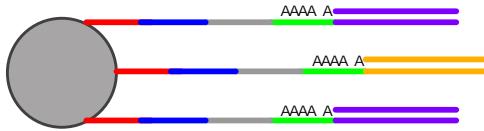
Random Linker Sequence

Transcript Capture Sequence - poly(dT)

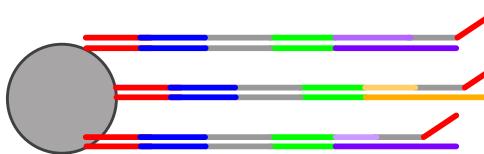
**1) Hybridization:** Capture poly-A transcripts



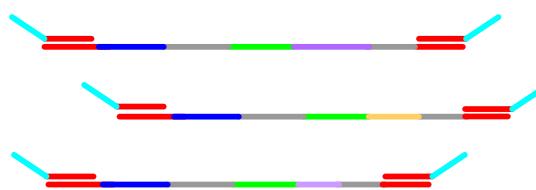
**3) Bead Clean-Up:** Remove any bead oligos without 1st strand cDNA



**5) 2nd Strand Synthesis:** Randomly prime synthesis of 2nd strand cDNA



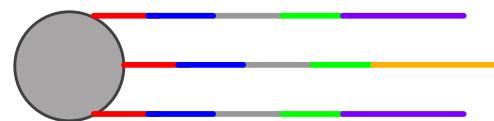
**7) Index PCR:** Add P5+ i5 and P 7+i7 to WTA product with UPS primers, for library multiplexing and Illumina sequencing



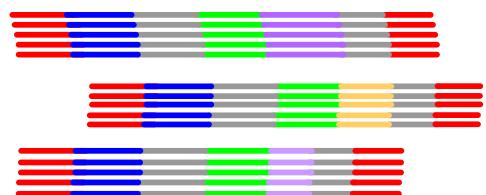
**2) 1st Strand Synthesis:** Bead oligos acts as primer for making 1st-strand cDNA



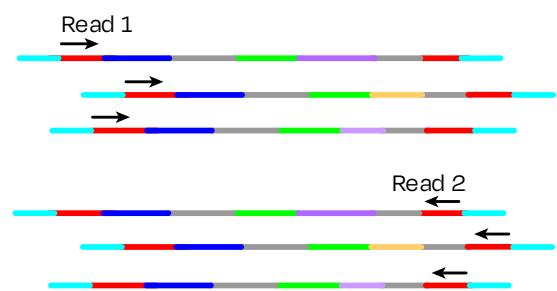
**4) NaOH denaturation:** Makes 1st strand cDNA single-stranded



**6) WTA:** Amplify 2nd strand cDNA with UPS primers



**8) Sequencing:** Read 1 for cell barcode, and Read 2 for transcript identity





# TROUBLESHOOTING

Problem	Possible Causes and Suggested Solutions
<p><b>WTA Product is low (&lt;0.2 ng/<math>\mu</math>L) despite loading <math>\geq</math>2,000 cells</b></p> <p><i>Even though there were sufficient cells loaded into the HIVE Collector, the first WTA reaction failed to produce sufficient DNA to proceed.</i></p>	<p><b>Double-check calculations for sample loading</b></p> <p><b>Poor sample quality may prohibit analysis</b></p> <p>If DNA concentration of the WTA Product is low before reamplification when you loaded sufficient cells, the sample may be compromised. Proceeding with WTA reamplification may not be worthwhile.</p>
<p><b>WTA Product is low (&lt;0.2 ng/<math>\mu</math>L) after WTA reamplification</b></p> <p><i>After performing WTA Reamplification in Appendix 5 then SPRI Clean-Up in STEP H, the DNA concentration of the WTA Product remains too low to proceed.</i></p>	<p><b>Contact Technical Support</b></p> <p>Email: <a href="mailto:support@honeycomb.bio">support@honeycomb.bio</a></p>



Problem	Possible Causes and Suggested Solutions
<b>Few reads in sequencing data</b>  <i>Even though there was sufficient WTA Product for scRNAseq, the sequencing data contained few reads.</i>	<b>HIVE sequencing requires custom primers</b>  Check to make sure you used custom primers for sequencing (see Appendix 3).
<b>Poor sequencing data quality</b>  <i>Despite there being sufficient reads, the scRNAseq data contained fewer cells than expected, fewer genes than expected, and/or high mitochondrial reads.</i>	<b>Poor sample quality causes poor data quality</b>  Poor cell viability and/or poor sample purity is the most common reason for poor data quality. If these sample features cannot be improved, measure cell viability and sample purity and increase the number of cells loaded per HIVE to compensate for dead and non-nucleated cells.  We highly recommend the Nexcelom Cellometer® K2 Fluorescent Cell Counter for sample QC, which measures cell viability and sample purity faster and more accurately than trypan blue. <sup>1</sup>

## REFERENCES FOR TROUBLESHOOTING

1. [Honeycomb™ Appnote. Best Practices: The Cellometer® K2 for HIVE scRNAseq](https://honeycomb.bio/resources/the-cellometer-k2-for-hive-scrnaseq/?resource_id=48ab2f9b45957ab574cf005eb8a76760)  
[https://honeycomb.bio/resources/the-cellometer-k2-for-hive-scrnaseq/?resource\\_id=48ab2f9b45957ab574cf005eb8a76760](https://honeycomb.bio/resources/the-cellometer-k2-for-hive-scrnaseq/?resource_id=48ab2f9b45957ab574cf005eb8a76760)
2. [Honeycomb™ Troubleshooting Guide](https://honeycombbio.zendesk.com/hc/en-us/articles/14881004938907-HIVE-CLX-Troubleshooting-Guide)  
<https://honeycombbio.zendesk.com/hc/en-us/articles/14881004938907-HIVE-CLX-Troubleshooting-Guide>

## NOTES



# HIVE™ CLX scRNaseq Transcriptome Recovery & Library Preparation



Talk to a HIVE™ expert  
[support@honeycomb.bio](mailto:support@honeycomb.bio)



# HONEYCOMB

## MORE INFORMATION

Download protocols & example data: [www.honeycomb.bio](http://www.honeycomb.bio)

Contact HIVE technical support: [support@honeycomb.bio](mailto:support@honeycomb.bio)

Speak with a HIVE expert: [sales@honeycomb.bio](mailto:sales@honeycomb.bio)

Ready for quotes & ordering: [NGS@revvity.com](mailto:NGS@revvity.com)



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