10x GENOMICS

User Guide | CG000691 | Rev B

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 B

Legal Notices

Instrument & Licensed Software Updates Warranties

Updates to existing Instruments and Licensed Software may be required to enable customers to use new or existing products. In the event of an Instrument failure resulting from an update, such failed Instrument will be replaced or repaired in accordance with the 10x Limited Warranty, Assurance Plan or service agreement, only if such Instrument is covered by any of the foregoing at the time of such failure. Instruments not covered under a current 10x Limited Warranty, Assurance Plan or service agreement will not be replaced or repaired.

Support

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Document Revision Summary

Document Number

CG000691 | Rev B

Title

Chromium Fixed RNA Profiling Reagent Kits For Singleplexed Samples

Revision

Rev A to Rev B

Revision Date

June 10, 2024

Specific Changes

- Updated to add 10x Magnetic Separator B (PN-2001212) in 10x Genomics Accessories on page 10
- Updated the volume of 50% glycerol solution to be added to row labeled 3 in Load Chromium Next GEM Chip Q on page 47

General Changes

Updated for general minor consistency of language and terms throughout.

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Introduction

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

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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

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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

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Chromium Next GEM
Single Cell Fixed RNA Gel Bead Kit
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
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

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Chromium Next GEM
Single Cell Fixed RNA Mouse Transcriptome Probe Kit
4 rxns, PN-1000490
Store at -20°C

	#	PN
Mouse WTA Probes BC001	1	2000703

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Chromium Next GEM
Single Cell Fixed RNA Gel Bead Kit
4 rxns, PN-1000421
Store at -80°C

	#	PN
Single Cell TL v1 Gel Beads (4 rxns)	1	2000538

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

Chromium
Partitioning Oil
Store at ambient temperature

	#	PN
Partitioning Oil	6	2000190

Chromium
Recovery Agent
Store at ambient temperature

	#	PN
Recovery Agent	6	220016

Chromium Next GEM
Chip Q & Gaskets
Store at ambient temperature

	#	PN
Next GEM Chip Q	6	2000518
Chip Gasket, 6-pack	1	370017

10x GENOMICS

Chromium Next GEM Chip Q Single Cell Kit, 16 rxns PN-1000422

Chromium
Partitioning Oil
Store at ambient temperature

	#	PN
Partitioning Oil	2	2000190

Chromium
Recovery Agent
Store at ambient temperature

	#	PN
Recovery Agent	2	220016

Chromium Next GEM
Chip Q & Gaskets
Store at ambient temperature

	#	PN
Next GEM Chip Q	2	2000518
Chip Gasket, 2-pack	1	3000072

10x GENOMICS

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

Dual Index Kit TS Set A
Store at -20°C

	#	PN
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
10x Magnetic Separator B*	1000709 (Chromium X/iX Accessory Kit)/1000707 (GEM-X Transition Kit)	2001212
Chromium Next GEM Secondary Holder	1000142	3000332

*10x Magnetic Separator (PN-230003) and 10x Magnetic Separator B (PN-2001212) can be used interchangeably.

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
Sample Fixation	variable*	STOP 4°C ≤1 week/-80°C ≤6 months
	*Refer to the appropriate Demonstrated Protocols for details.	
Step 1: Probe Hybridization (page 33)		
1.1 Probe Hybridization (page 35)	16-24 h	
Step 2: GEM Generation and Barcoding (page 37)		
2.1 Post-Hybridization Wash (page 40)	60 min	STOP -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	STOP 4°C ≤1 week (GEMs) -80°C ≤6 months (washed undiluted sample)
Step 3: GEM Recovery and Pre-Amplification (page 52)		
3.1 Post-GEM Incubation – Recovery (page 54)	10 min	
3.2 Pre-Amplification PCR (page 55)	55 min	STOP 4°C ≤72 h/-20°C ≤1 week
3.3 DNA Cleanup – SPRIselect (page 56)	30 min	STOP 4°C ≤72 h/-20°C ≤4 weeks
Step 4: Fixed RNA – Gene Expression Library Construction (page 57)		
4.1 Sample Index PCR (page 59)	40 min	STOP 4°C ≤72 h
4.2 Post Sample Index PCR Size Selection – SPRIselect (page 61)	30 min	STOP 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	
Step 1: Sample fixation *Choose appropriate protocol for fixation/isolation depending upon the sample type.*	Fix single cell & nuclei suspensions (Demonstrated Protocol CG000478)	**Sample Fixation** Demonstrated Protocol CG000478
	Fix and dissociate tissues (Demonstrated Protocol CG000553)	**Tissue Fixation** Demonstrated Protocol CG000553
	Isolate cells from FFPE sections (Demonstrated Protocol CG000632)	**FFPE Samples - Cell Isolation** Demonstrated Protocol CG000632

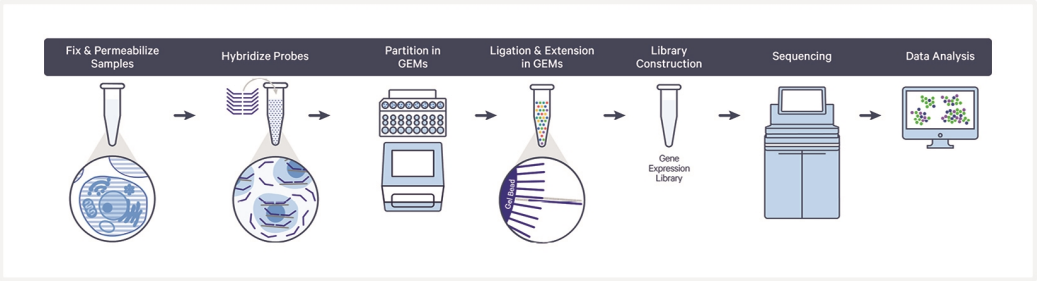
Stepwise Objectives

Chromium Fixed RNA Profiling (Gene Expression Flex) 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

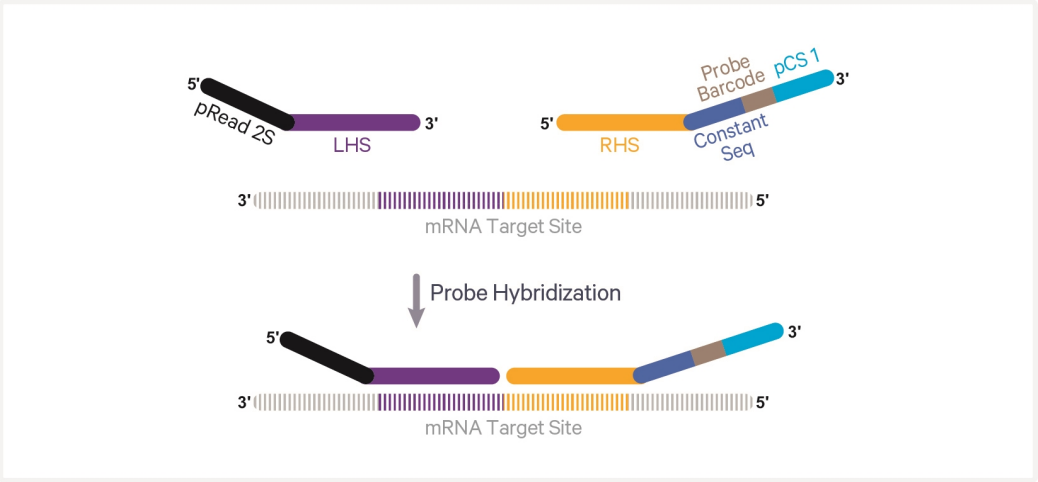


User Guide Steps

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

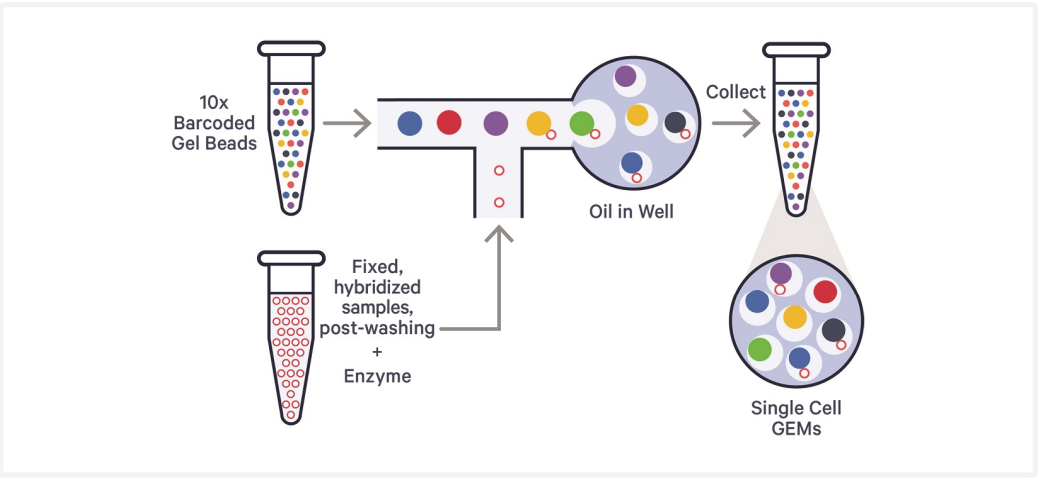
The whole transcriptome probe pairs, consisting of a left hand side (LHS) and a right hand side (RHS) for each targeted gene, are added to the fixed sample. Together, probe pairs hybridize to their complementary target RNA in an overnight incubation.



Step 2: GEM Generation & Barcoding

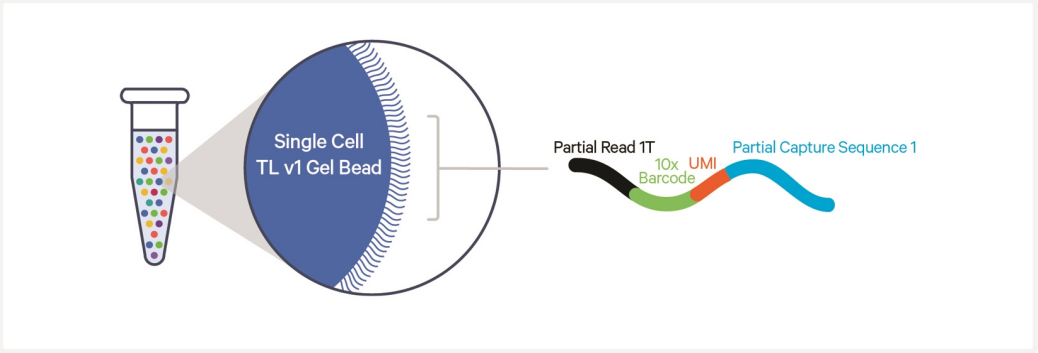
After hybridization, the unbound probes are washed off. GEMs are generated by combining barcoded Gel Beads, a Master Mix containing cells, and Partitioning Oil onto Chromium Next GEM Chip Q.

GEM Generation



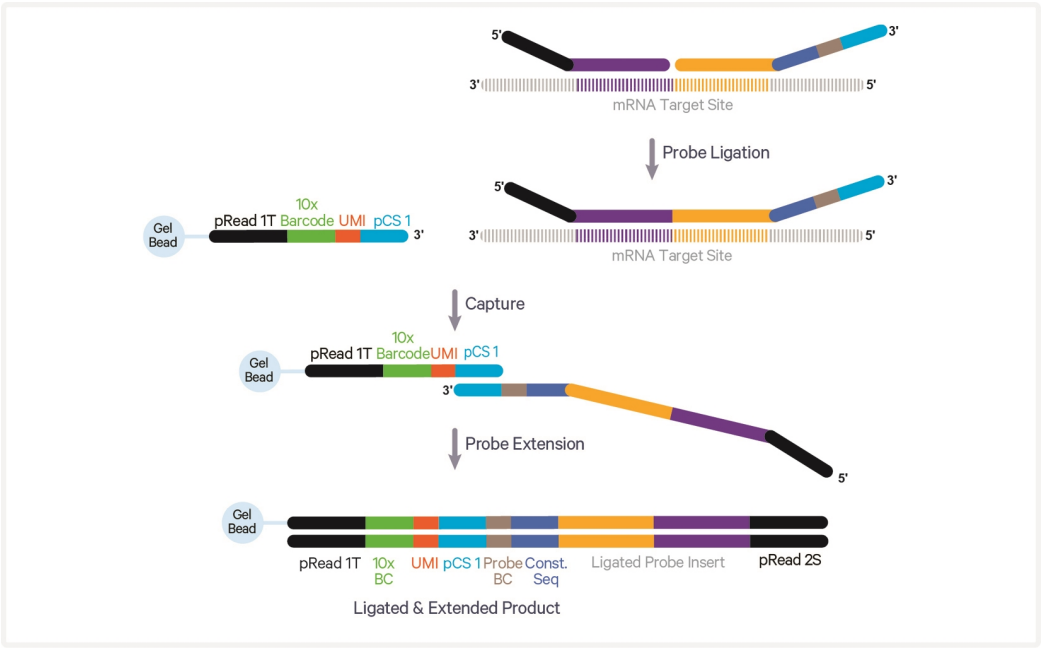
Immediately following GEM generation, the Gel Bead is dissolved, releasing the barcoded Gel Bead primers, and any co-partitioned cell is lysed. Gel Bead primers contain a partial TruSeq Read 1 sequence (partial Read 1T, read 1 sequencing primer), a 16 nt 10x GEM Barcode (or 10x Barcode), a 12 nt unique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

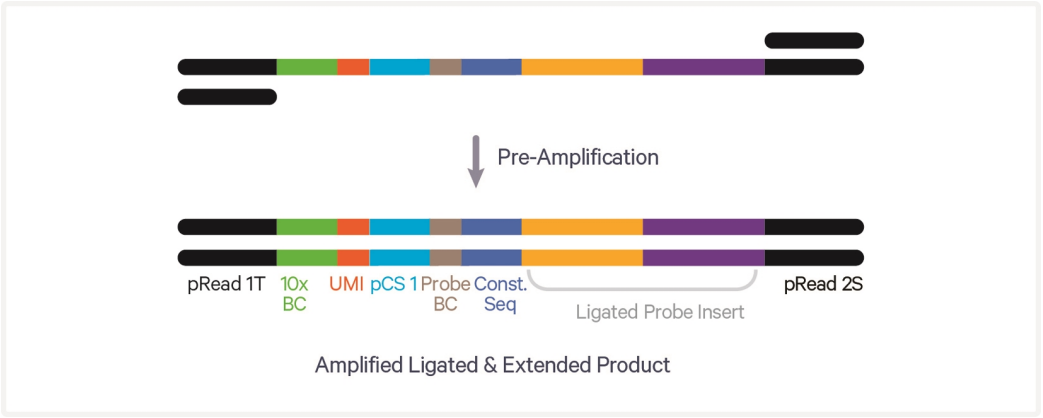


Finally, a heat denaturation step inactivates the enzymes in the GEM reaction.

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

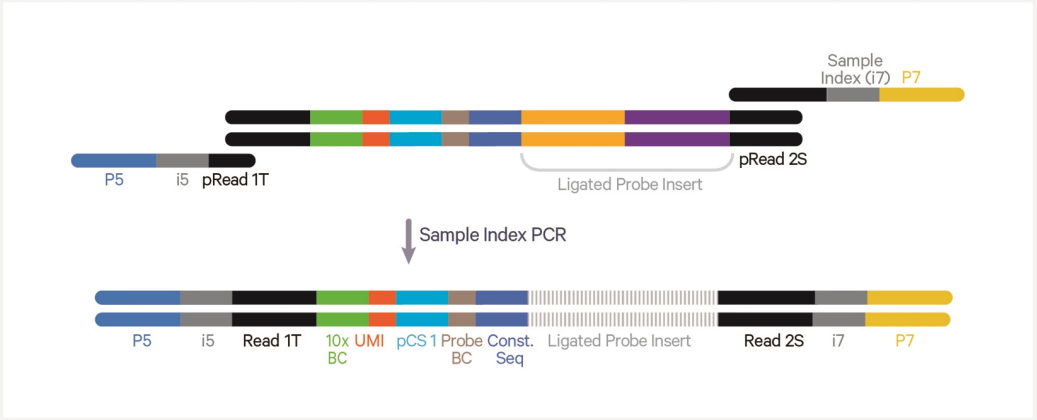


Step 4: Fixed RNA – Gene Expression Library Construction

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.

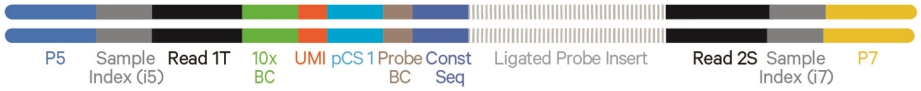
Pooled Amplified DNA Processed in Bulk



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 x 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 ~1 x 10^6 cells/nuclei

per hybridization, if possible.

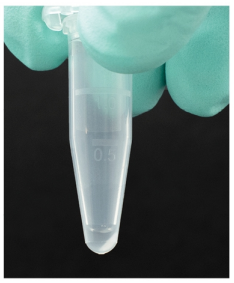
- If proceeding with <500,000 fixed cells in a hybridization reaction, use a swinging bucket rotor for centrifugation and carefully remove the supernatant without disturbing the pellet. In such cases, complete removal of the supernatant is not required. Up to 15 µl of supernatant may be left behind prior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

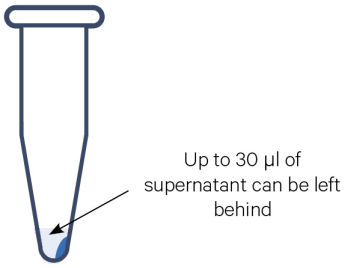
Incubation Time

- 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

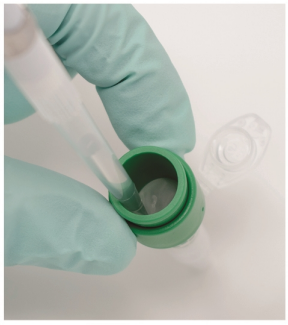
- After post-hybridization wash, pass the sample through a 30 µm filter (Sysmex CellTrics or Miltenyi Biotec Pre-Separation) into a new 1.5-ml microcentrifuge tube.

DO NOT use 40 µm Flowmi Tip Strainer for filtration.

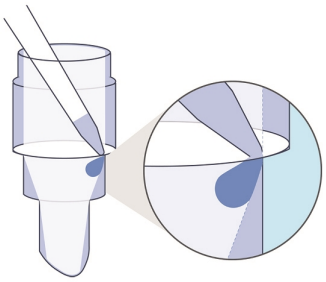
- Hold the pipette tip at an angle and touch the filter membrane where the filter meets the wall. Slowly pipette through the filter. Tap gently or centrifuge briefly if liquid remains at the end of the filter.
- To maximize recovery, residual volume can be pipetted from underneath the filter.

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

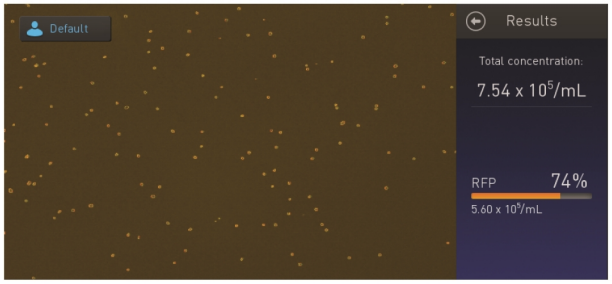
- Accurate counting is critical for optimal assay performance.
- It is strongly recommended that the sample be stained with a fluorescent dye such as Ethidium Homodimer-1 or PI staining solution and counted using an automated fluorescent cell counter (Countess II Automated Cell Counter or a Cellaca Counter).
- 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/µl. 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 µl diluted Ethidium Homodimer-1 in each tube.
- Gently mix the sample. Immediately add 10 µl sample to 10 µl diluted Ethidium Homodimer-1. Gently pipette mix 10x.
- Transfer 10 µl 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.

Fixed PBMCs Stained with Ethidium Homodimer-1



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/µl. Refer to manufacturer's instructions for details on operations.

- Add 25 µl 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 µl fixed cell suspension to 15 µl PBS.
- Add 25 µl 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 µl 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

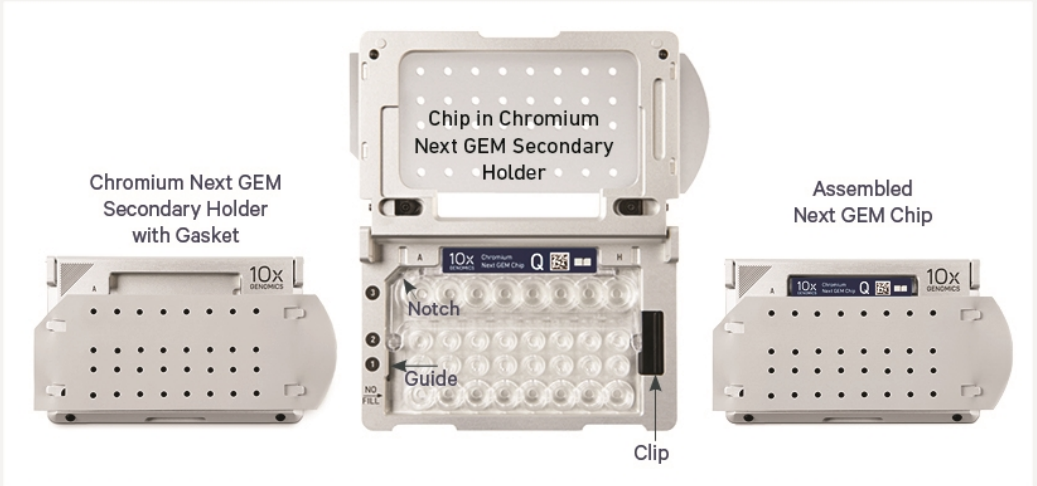
- Chromium Fixed RNA Profiling uses Chromium Chip Q.
- Minimize exposure of reagents, chips, and gaskets to sources of particles and fibers, laboratory wipes, frequently opened flip-cap tubes, clothing that sheds fibers, and dusty surfaces.
- After removing the chip from the sealed bag, use in **≤24 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.

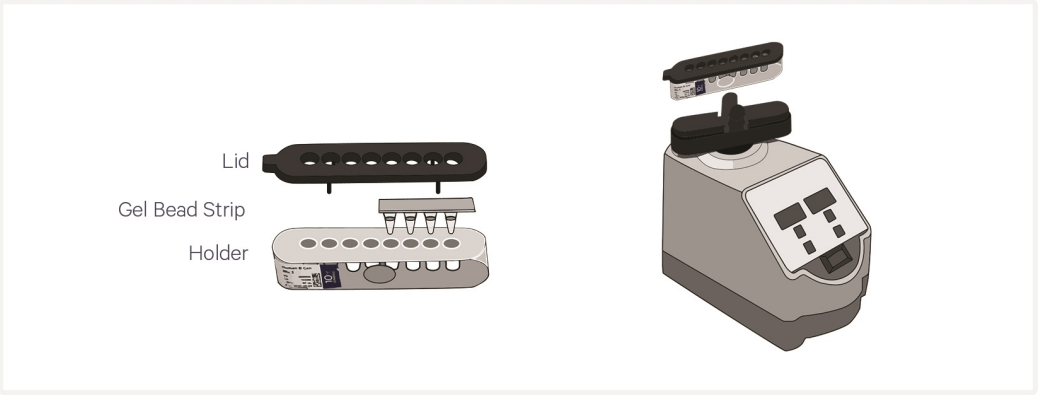
  OR

- 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

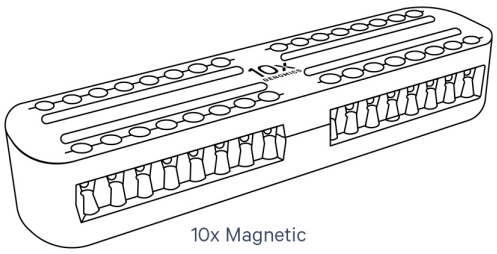


- Use one tube of Gel Beads per sample. DO NOT puncture the foil seals of tubes not used at the time.
- After removing the Gel Bead strip from the packaging, equilibrate the Gel Bead strip to **room temperature** for at least **30 min** before use.
- Store unused Gel Beads at **−80°C** and avoid more 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.

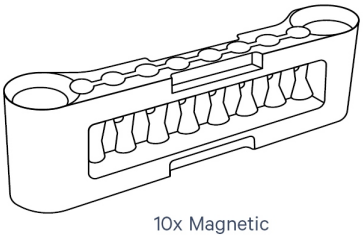
10x Magnetic Separator

- Images below are illustrative - actual appearance of magnetic separator may vary. Guidance applies to all 10x Magnetic Separators.
- 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.



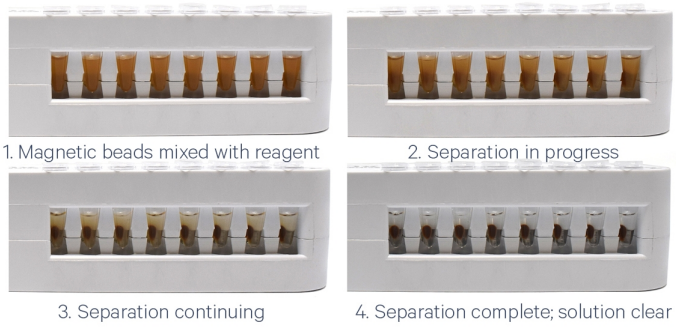
10x Magnetic Separator B



10x Magnetic Separator

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.
- Images below are representative - actual color of magnetic separator may vary. Guidance applies to all 10x Magnetic Separators.
- **Visually Confirm Clearing of Magnetic Bead Solution**



1. Magnetic beads mixed with reagent
2. Separation in progress
3. Separation continuing
4. Separation complete; solution clear

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).

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

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

1.0 Get Started

Action	Item	10x PN	Preparation & Handling	Storage
Thaw & Keep Warm				
☐	**Hyb Buffer B**	2000483	Thaw at 42°C. Vortex and centrifuge briefly. Keep warm and verify no precipitate before use. DO NOT keep the thawed buffer on ice, or the solution will precipitate. Thawed Hyb Buffer B can be kept at 42°C for up to 1 h.	–20°C
☐	**Enhancer**	2000482	Thaw 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.	–20°C
Place on Ice				
☐	**Fixed Cell Suspension**	—	Consult Chromium Fixed RNA Profiling - Protocol Planner (CG000528) for details on applicable Demonstrated Protocols.	—
☐	**Human WTA Probes BC001** OR **Mouse WTA Probes BC001**	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.

a. 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

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

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

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

c. Incubate Hyb Mix at **42°C** for **5 min**.

d. 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	**200,000-2 x 10^6**
Fixed nuclei	**400,000-2 x 10^6**
FFPE dissociated suspension	**400,000-2 x 10^6**

DO NOT exceed 2 x 10^6 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

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
Equilibrate to Room Temperature				
☐	**Single Cell TL v1 Gel Beads**	2000538	Equilibrate to room temperature 30 min before loading the chip.	–80°C
☐	**Reducing Agent B**	2000087	Vortex, verify no precipitate, centrifuge briefly.	–20°C
Thaw & Keep Warm				
☐	**Enhancer**	2000482	Thaw 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.	–20°C
Place on Ice				
☐	**Conc. Post-Hyb Buffer**	2000533	Thaw at room temperature and keep on ice.	–20°C
☐	**GEM Enzyme Mix**	2000490	Centrifuge briefly before adding to the mix.	–20°C
☐	**GEM Reagent Mix**	2000491	Thaw at room temperature. Vortex, verify no precipitate, centrifuge briefly. Keep on ice.	–20°C
Obtain				
☐	**Partitioning Oil**	2000190	—	Ambient
☐	**Next GEM Chip Q**	2000518	See Tips & Best Practices.	Ambient
☐	**Chromium Next GEM Secondary Chip Holder**	3000332	See Tips & Best Practices.	Ambient
☐	**10x Gasket**	370017/	See Tips & Best Practices.	Ambient

Action	Item	10x PN	Preparation & Handling	Storage
		3000072		
☐	**Sample Filters Sysmex Sterile Single-pack CellTrics Filters/Miltenyi Biotec Pre-Separation Filters (30 µm)**	—	Manufacturer's recommendations.	Ambient
☐	**10x Vortex Adapter**	330002	See Tips & Best Practices.	Ambient
☐	**50% glycerol solution** for adding to unused wells	—	See Tips & Best Practices.	
☐	**Glycerol for molecular biology, ≥99%** 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 *Add reagents in the order listed*	PN	1X + 10% (ml)*	4X + 10% (ml)*
Nuclease-free Water	-	1.98	7.92
Conc. Post-Hyb Buffer	2000533	0.11	0.44
Enhancer *Heat at 65°C for 10 min. Vortex and verify no precipitate.* *Add warm to the mix.*	2000482	0.11	0.44
Total	-	**2.2**	**8.8**

**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.

TIPS

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 *Add reagents in the order listed*	PN	1X + 10% (µl)	4X + 10% (µl)
Nuclease-free Water	-	522.5	2090.0
Conc. Post-Hyb Buffer	2000533	27.5	110.0
Total	**-**	**550.0**	**2200.0**

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 µl 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 µm 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 µm 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.

TIPS *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.

TIPS 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.*

STOP

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.

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

GEM Master Mix *Add reagents in the order listed*		PN	1X (µl)	4X + 10% (µl)
●	GEM Reagent Mix	2000491	20.9	92.1
○	Reducing Agent B	2000087	1.7	7.3
●	GEM Enzyme Mix	2000490	12.4	54.6
	Total	–	**35.0**	**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**.*

c. Add **35 µl** 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.

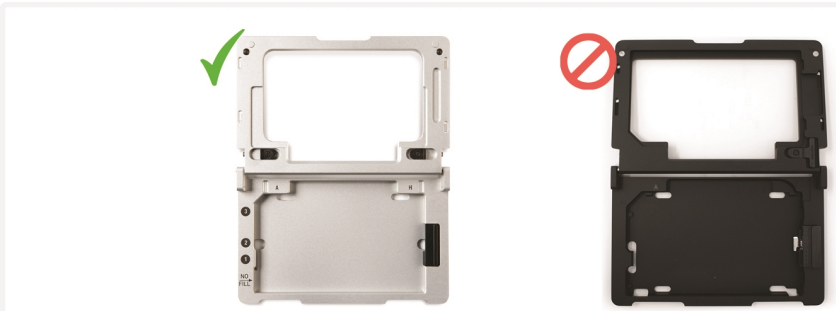
Cell Suspension Volume Calculator

Volume of Cell Suspension Stock per reaction (µl) | Volume of Post-Hyb Resuspension Buffer per reaction (µl)

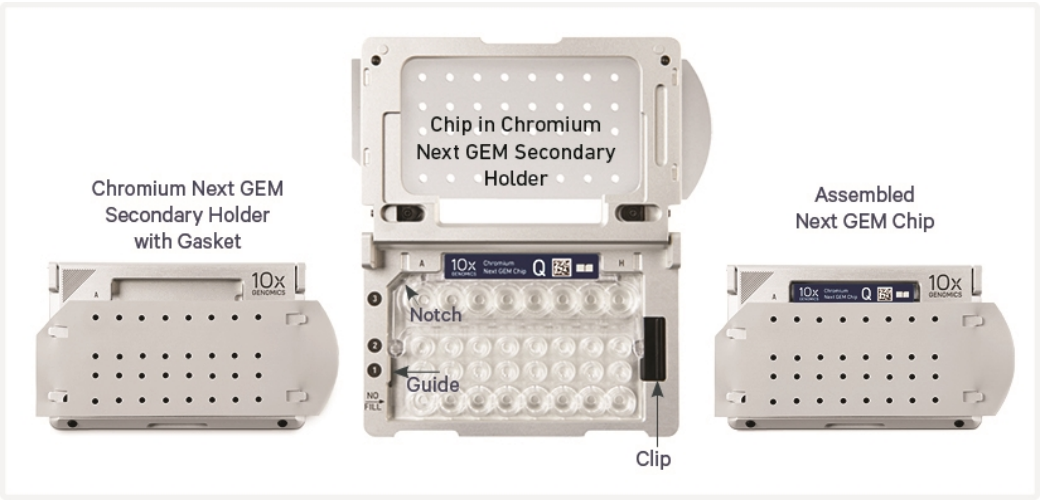
Cell Stock Concentration (Cells/µl)	Targeted Cell Recovery										
	500	1000	2000	3000	4000	5000	6000	7000	8000	9000	10000
400	2.1 37.9	4.1 35.9	8.3 31.8	12.4 27.6	16.5 23.5	20.6 19.4	24.8 15.3	28.9 11.1	33.0 7.0	37.1 2.9	n/a
600	1.4 38.6	2.8 37.3	5.5 34.5	8.3 31.8	11.0 29.0	13.8 26.3	16.5 23.5	19.3 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.



TIPS 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.

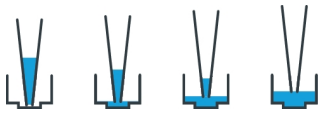
For GEM generation, load the indicated reagents only in the specified rows, starting from row labeled 1, followed by rows labeled 2 & 3.
DO NOT load reagents in the bottom row labeled NO FILL.

2.3 Load Chromium Next GEM Chip Q

Chip loading instructions are unique to Chip Q.

TIPS

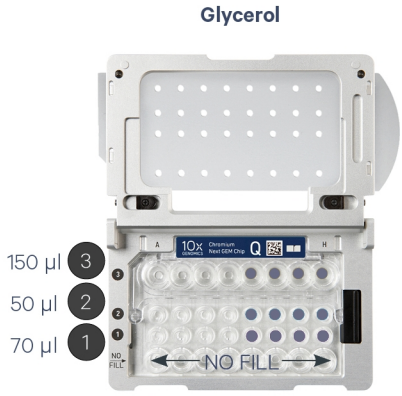
- After removing chip from the sealed bag, use in **≤24 h**.
- Open the lid (gasket attached) of the assembled chip and lay flat for loading.
- When loading the chip, raising and depressing the pipette plunger should each take **~5 sec**. When dispensing, raise the pipette tips at the same rate as the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



a. Add 50% glycerol solution to each unused well

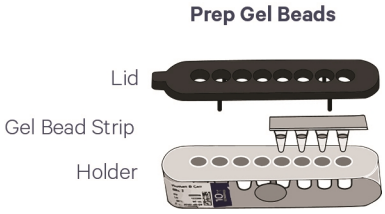
- **70 µl** in each unused well in row labeled 1
- **50 µl** in each unused well in row labeled 2
- **150 µl** in each unused well in row labeled 3

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.

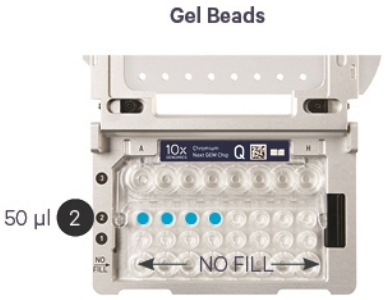


c. Load Row 1

- 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

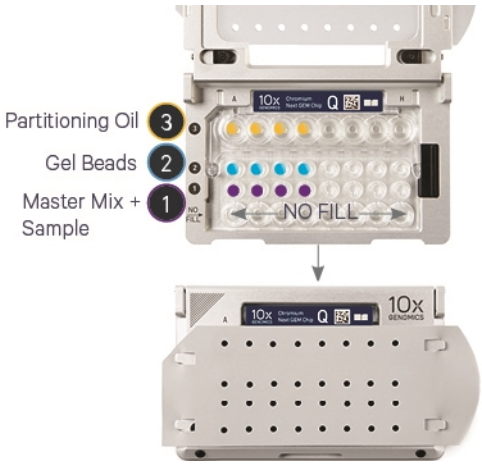
- Dispense **45 µl** Partitioning Oil into the wells in **row labeled 3** from a reagent reservoir.

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.

a. 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

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

DO NOT store the GEMs at −20°C.

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:

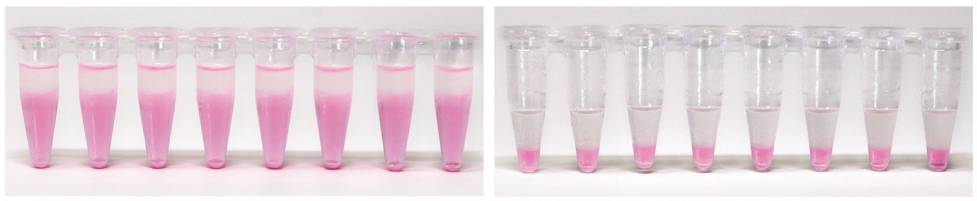
GEM Recovery and Pre-Amplification

3

3.0 Get Started

Action	Item	10x PN	Preparation & Handling	Storage
Equilibrate to Room Temperature				
☐ ◯	**Reducing Agent B**	2000087	Thaw, vortex, verify no precipitate, centrifuge briefly.	−20°C
☐ ●	**Pre-Amp Primers B** Verify name & PN	2000529	Thaw, vortex, centrifuge briefly.	−20°C
☐	**Beckman Coulter SPRIselect Reagent**	—	Manufacturer's recommendations.	Ambient
Place on Ice				
☐ ◯	**Amp Mix**	2000103	Vortex and centrifuge briefly.	−20°C
Obtain				
☐ ◯	**Recovery Agent**	220016	—	Ambient
☐	**Qiagen Buffer EB**	—	Manufacturer's recommendations.	Ambient
☐	**10% Tween 20**	—	Manufacturer's recommendations.	Ambient
☐	**10x Magnetic Separator/10x Magnetic Separator B**	230003/ 2001212	—	Ambient
☐	**Prepare 80% Ethanol** Prepare 2.5 ml for 4 GEM reactions.	—	Prepare fresh.	—

3.1 Post-GEM Incubation – Recovery



Only 4 reactions can be run per kit. Image shows 8 reactions derived from two kits.

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

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

Pre-Amplification Mix *Add reagents in the order listed*		PN	1X (µl)	4X + 10% (µl)
○	**Amp Mix**	2000103	25.0	110.0
●	**Pre-Amp Primers B**	2000529	10.0	44.0
	Total		**35.0**	**154.0**

b. Add **35 µl** Pre-Amplification Mix to aqueous sample from step 3.1f.

c. Cap firmly and invert 8x to mix. Centrifuge briefly.

d. Incubate in a thermal cycler with the following protocol.

Lid Temperature	Reaction Volume	Run Time
105°C	100 µl	~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

e. 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 *Add reagents in the order listed*		PN	1000 µl
	Buffer EB		980
	10% Tween 20	-	10
◯	**Reducing Agent B**	2000087	10
	Total		**1000**

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.

STOP **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

4.0 Get Started

Action	Item	10x PN	Preparation & Handling	Storage
Equilibrate to Room Temperature				
☐ ⚠	**Dual Index Plate TS Set A** Verify name & PN. Use indicated plate only	3000511	Vortex and centrifuge briefly.	–20°C
☐	**Beckman Coulter SPRIselect Reagent**	—	Manufacturer's recommendations.	—
☐	**Agilent Bioanalyzer High Sensitivity Kit** If used for QC	—	Manufacturer's recommendations.	—
☐	**Agilent TapeStation ScreenTape & Reagents** If used for QC	—	Manufacturer's recommendations.	—
Place on Ice				
☐ ◯	**Amp Mix**	2000103	Vortex and centrifuge briefly.	–20°C
☐	**KAPA Library Quantification Kit for Illumina Platforms**	—	Manufacturer's recommendations.	—
Obtain				
☐	**Qiagen Buffer EB**	—	Manufacturer's recommendations.	Ambient
☐	**10x Magnetic Separator/ 10x Magnetic Separator B**	230003/ 2001212	See Tips & Best Practices.	Ambient
☐	**Prepare 80% Ethanol** 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 *Add reagents in the order listed*		PN	1X (µl)	1X + 10% (µl)	4X + 10% (µl)
◯	**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 µl** sample from the step DNA Cleanup – SPRIselect on page 56 to a new tube strip.

d. Add **60 µl** Sample Index PCR Mix to **20 µl** sample.

e. Add **20 µl** of an individual Dual Index TS Set A to each sample. Pipette mix 5x (pipette set to 90 µl). Centrifuge briefly.

f. Incubate in a thermal cycler with the following protocol.

Lid Temperature	Reaction Volume	Run Time
105°C	100 µl	~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	Total Cycles* for Cell Lines	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.*

STOP

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.

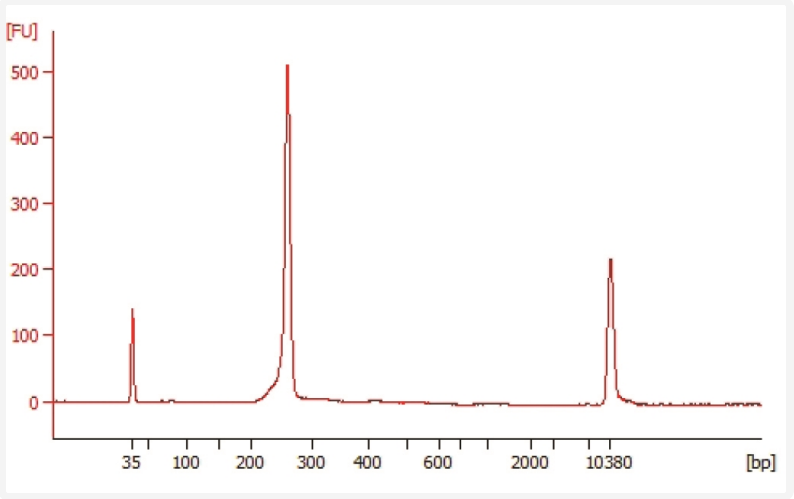
m. Transfer **40 µl** to a new tube strip.

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 µl** 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

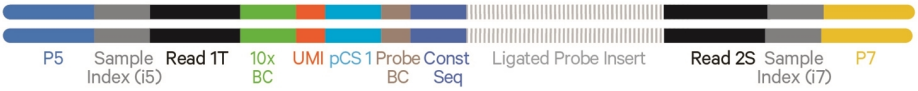
Step 5:

Sequencing

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 i7 Index i5 Index Read 2	28 cycles 10 cycles 10 cycles 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.

*The NovaSeq 6000 standard workflow permits loading one library pool across all lanes of the flow cell; whereas the Xp workflow enables sequencing various library pools in each lane of the NovaSeq 6000 flow cell.

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

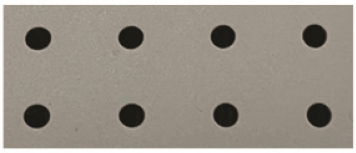
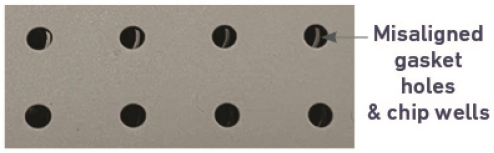
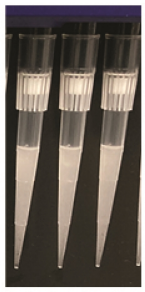
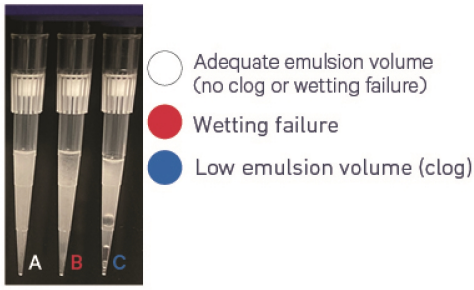
Loupe Browser is an interactive data visualization tool that requires no prior programming knowledge.

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

Troubleshooting

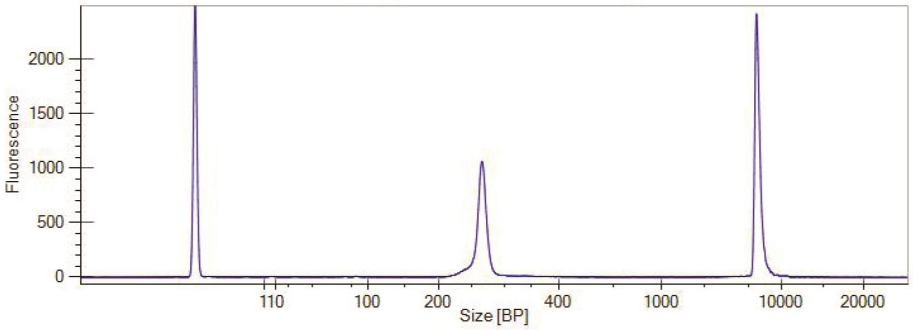
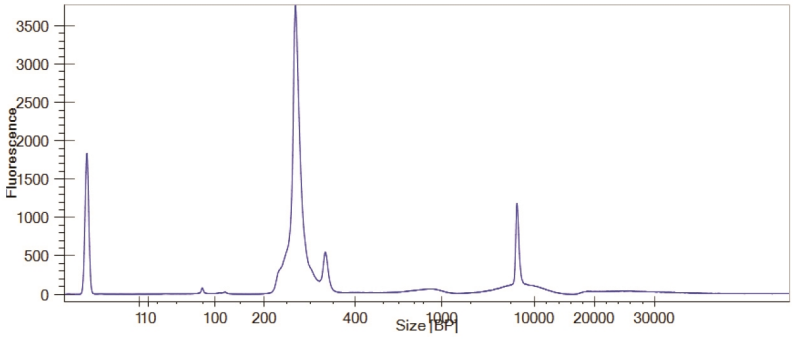
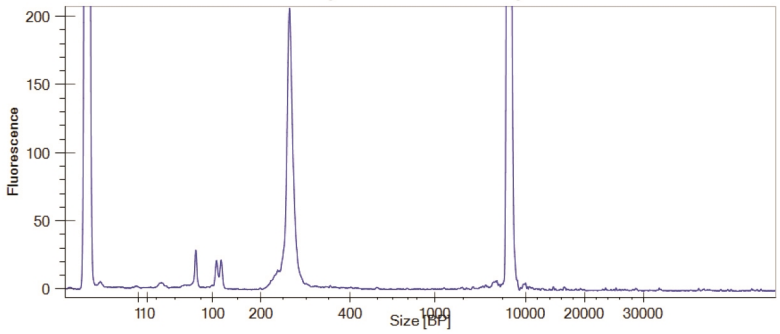
6

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		
	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 for further assistance. Replacement reagents and chips may be provided for properly documented clogs or wetting failures if they are associated with runs of unexpired reagents and chips, and are reported within 30 days of the expiration date.

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 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 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.

Appendix

7

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 150 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

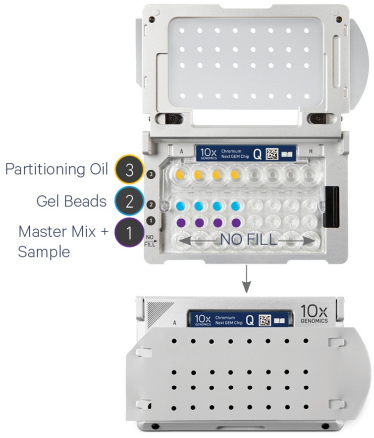
d. Load Row Labeled 2

- 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
Total	**16**

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.

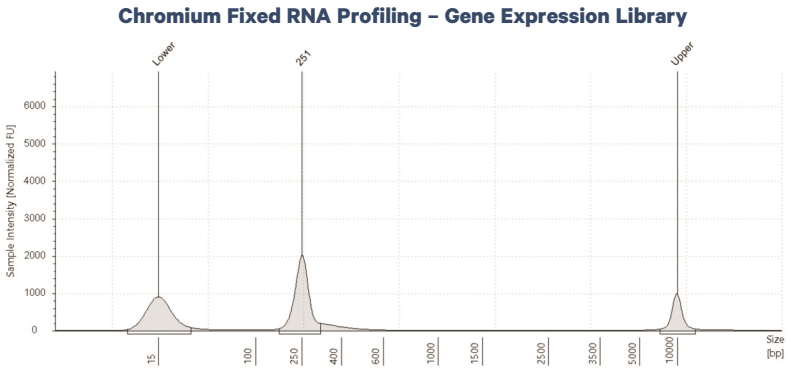
f. Incubate in a thermal cycler with the following protocol.

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

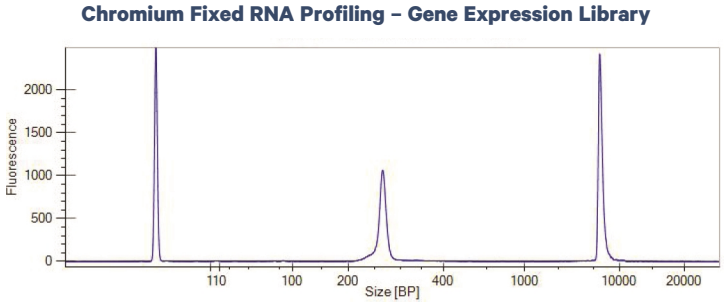
Agilent TapeStation High Sensitivity D5000 ScreenTape was used.



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

5'-CTACACGACGCTCTTCCGATCT-N16-N12-TTGCTAGGACCG-3'

Chromium Fixed RNA – Gene Expression Library

5'-AATGATACGGCGACCACCGAGATCTACAC-N10-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-N16-N12-TTGCTAGGACCG-BC8-NN-TACGTGCTAACCGCGT-Ligated_Probe_insert-TGGAATTCTCGGGTGCCAAGGAACTCCAGTCAC-N10-ATCTCGTATGCCGTCTTCTGCTTG-3'

3'-TTACTATGCCGCTGGTGGCTCTAGATGTG-N10-TGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA-N16-N12-AACGATCCTGGC-BC8-NN-ATGCACGATTGGCGCA-Ligated_Probe_insert-ACCTTAAGAGCCCACGGTTCCTTGAGGTCAGTG-N10-TAGAGCATACGGCAGAAGACGAAC-5'