

User Guide | CG000673 | Rev A

Chromium Fixed RNA Profiling Reagent Kits

for Multiplexed Samples with Feature Barcode technology for Protein using Barcode Oligo Capture

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 4 BC PN-1000475 | 4 rxns x 16 BC PN-1000476

Chromium Fixed RNA Kit, Mouse Transcriptome 4 rxns x 4 BC PN-1000496 | 4 rxns x 16 BC PN-1000497

Fixed RNA Feature Barcode Multiplexing Kit 64 rxns PN-1000628

Dual Index Kit TS Set A 96 rxns PN-1000251

Dual Index Kit TN Set A 96 rxns PN-1000250

Notices

Document Number

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Document Revision Summary

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Chromium Fixed RNA Profiling Reagent Kits for Multiplexed Samples with Feature Barcode technology for Protein using Barcode Oligo Capture

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September 07, 2023

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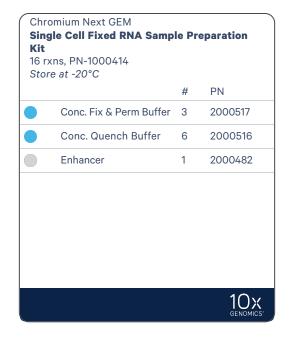
Introduction

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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 Fixed RNA Kit, Human Transcriptome, 4 rxns x 4 BC PN-1000475

Sing Libr 4 rx	omium Next GEM gle Cell Fixed RNA Hyb rary Kit ns, PN-1000415 re at -20°C	ridiza	tion &	Sing Prol 16 m	omium Next GEM gle Cell Fixed RNA Hum be Kit kns, PN-1000420 re at -20°C	an Tra	anscriptome
		#	PN			#	PN
	Hyb Buffer B	1	2000483		Human WTA Probes BC001	1	2000495
	Enhancer	1	2000482		Human WTA Probes	1	2000496
	Conc. Post-Hyb	1	2000533		BC002	'	2000490
	Buffer				Human WTA Probes	1	2000497
\bigcirc	Reducing Agent B	1	2000087		BC003		
•	GEM Enzyme Mix	1	2000490		Human WTA Probes BC004	1	2000498
	GEM Reagent Mix	1	2000491		Hyb Buffer B	1	2000485
	Pre-Amp Primers B*	1	2000529		Enhancer	2	2000482
\bigcirc	Amp Mix	1	2000103		Conc. Post-Hyb Buffer	2	2000533
			10x				10x

^{*}This tube is not used in this assay.

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

Chromium Fixed RNA Kit, Mouse Transcriptome, 4 rxns x 4 BC PN- 1000496

Sing Libr 4 rx	omium Next GEM gle Cell Fixed RNA Hyb rary Kit ns, PN-1000415 re at -20°C	ridiza	tion &	Chr Sing Pro 16 rs	gle be l xns,
		#	PN		
	Hyb Buffer B	1	2000483		N B
	Enhancer	1	2000482		N
	Conc. Post-Hyb Buffer	1	2000533		B
0	Reducing Agent B	1	2000087		В
	GEM Enzyme Mix	1	2000490		N B
	GEM Reagent Mix	1	2000491		Н
	Pre-Amp Primers B*	1	2000529		Е
0	Amp Mix	1	2000103		C
			10x		

Sing Pro	omium Next GEM gle Cell Fixed RNA Mous be Kit xns, PN-1000491 re at -20°C	e Tra	inscriptome
		#	PN
	Mouse WTA Probes BC001	1	2000703
	Mouse WTA Probes BC002	1	2000704
	Mouse WTA Probes BC003	1	2000705
	Mouse WTA Probes BC004	1	2000706
	Hyb Buffer B	1	2000485
	Enhancer	2	2000482
	Conc. Post-Hyb Buffer	2	2000533
			10x

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

^{*}This tube is not used in this assay.

Chromium Fixed RNA Kit, Human Transcriptome, 4 rxns x 16 BC PN-1000476

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				
0	Reducing Agent B	1	2000087				
	GEM Enzyme Mix	1	2000490				
	GEM Reagent Mix	1	2000491				
	Pre-Amp Primers B*	1	2000529				
0	Amp Mix	1	2000103				
			10x				

Sing 64 rx	mium Next GEM le Cell Fixed RNA Hyl cns, PN-1000457 e at -20°C	bridizat	ion Kit
		#	PN
	Hyb Buffer B	4	2000485
	Conc. Post-Hyb Buffer	8	2000533
			10x

^{*}This tube is not used in this assay.

Chromium Next GEM Single Cell Fixed RNA Human Transcriptome Probe Kit 64 rxns, Module 1 PN-1000456 Store at -20°C				Sing Tran 64 m	mium Next GEM le Cell Fixed RNA Hur scriptome Probe Kit kns, Module 2 PN-1000/ e at -20°C		
		#	PN			#	PN
	Enhancer	4	2000482		Enhancer	4	2000482
	Human WTA Probes BC001	1	2000495		Human WTA Probes BC009	1	2000503
	Human WTA Probes BC002	1	2000496		Human WTA Probes BC010	1	2000504
	Human WTA Probes BC003	1	2000497		Human WTA Probes BC011	1	2000505
	Human WTA Probes BC004	1	2000498		Human WTA Probes BC012	1	2000506
	Human WTA Probes BC005	1	2000499		Human WTA Probes BC013	1	2000507
	Human WTA Probes BC006	1	2000500		Human WTA Probes BC014	1	2000508
	Human WTA Probes BC007	1	2000501		Human WTA Probes BC015	1	2000509
	Human WTA Probes BC008	1	2000502		Human WTA Probes BC016	1	2000510
			10x GENOMICS				10x

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

Chromium Fixed RNA Kit, Mouse Transcriptome, 4 rxns x 16 BC PN-1000497

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				
0	Reducing Agent B	1	2000087				
	GEM Enzyme Mix	1	2000490				
	GEM Reagent Mix	1	2000491				
	Pre-Amp Primers B*	1	2000529				
0	Amp Mix	1	2000103				
			10x				

Chromium Next GEM Single Cell Fixed RNA Hybridization Kit 64 rxns, PN-1000457 Store at -20°C							
		#	PN				
	Hyb Buffer B	4	2000485				
	Conc. Post-Hyb Buffer	8	2000533				
			10x				

*This tube is not used in this assay.

Chromium Next GEM Single Cell Fixed RNA Mouse Transcriptome Probe Kit 64 rxns, Module 1 PN-1000492 Store at -20°C				Chromium Next GEM Single Cell Fixed RNA Mouse Transcriptom Probe Kit 64 rxns, Module 2 PN-1000492 Store at -20°C			
		#	PN			#	PN
	Enhancer	4	2000482		Enhancer	4	2000482
	Mouse WTA Probes BC001	1	2000703		Mouse WTA Probes BC009	1	2000711
	Mouse WTA Probes BC002	1	2000704		Mouse WTA Probes BC010	1	2000712
	Mouse WTA Probes BC003	1	2000705		Mouse WTA Probes BC011	1	2000713
	Mouse WTA Probes BC004	1	2000706		Mouse WTA Probes BC012	1	2000714
	Mouse WTA Probes BC005	1	2000707		Mouse WTA Probes BC013	1	2000715
	Mouse WTA Probes BC006	1	2000708		Mouse WTA Probes BC014	1	2000716
	Mouse WTA Probes BC007	1	2000709		Mouse WTA Probes BC015	1	2000717
	Mouse WTA Probes BC008	1	2000710		Mouse WTA Probes BC016	1	2000718
			10x				10x

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

Fixed RNA Feature Barcode Multiplexing Kit, 64 rxns, PN-1000628*

		#	PN			#	PN
Antibod Barcode	y Multiplexing AB001	1	2000917	•	Antibody Multiplexing Barcode AB009	1	20009
Antibod Barcode	y Multiplexing AB002	1	2000918	•	Antibody Multiplexing Barcode AB010	1	20009
Antibod Barcode	y Multiplexing AB003	1	2000919	•	Antibody Multiplexing Barcode AB011	1	20009
Antibod Barcode	y Multiplexing AB004	1	2000920	•	Antibody Multiplexing Barcode AB012	1	20009
Antibod Barcode	y Multiplexing AB005	1	2000921	•	Antibody Multiplexing Barcode AB013	1	20009
Antibod Barcode	y Multiplexing AB006	1	2000922	•	Antibody Multiplexing Barcode AB014	1	20009
Antibod Barcode	y Multiplexing AB007	1	2000923	•	Antibody Multiplexing Barcode AB015	1	20009
Antibod Barcode	y Multiplexing AB008	1	2000924	•	Antibody Multiplexing Barcode AB016	1	20009
Pre-Amp	Primers C	1	2000953				
Amp Mix	<	1	2000047				

^{*}Fixed RNA Feature Barcode Multiplexing Kit, 64 rxns, PN-1000628 will support 64 Probe Hybridization reactions and 16 Pre-Amplification PCRs using this User Guide.

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

	Chromiu Partitionin Store at ambient to	g Oil	ture	Red	-	n A gent mbient tempera	ture	
		#	PN				#	PN
	Partitioning Oil	6	2000190		Re	covery Agent	6	220016
Chip Q	um Next GEM & Gaskets : ambient temperature	e						
					#	PN		
Next GEN	M Chip Q				6	2000518		
Chip Gas	ket, 6-pack				1	370017		
								10x

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

Part	omium itioning Oil e at ambient temperat	ure			over	m y Agent ambient tempera	ture	
		#	PN				#	PN
	Partitioning Oil	2	2000190	0	Re	ecovery Agent	2	220016
Chip	nium Next GEM Q & Gaskets at ambient temperatu	re						
					#	PN		
Next G	EM Chip Q				2	2000518		
Chip G	asket, 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	

Dual Index Kit TN Set A, 96 rxns PN-1000250

"	
#	PN
Dual Index Plate TN Set A 1	3000510

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

mple Labeling & Fixation ep 1: Probe Hybridization (page 42) Probe Hybridization (page 45)	variable* *Refer to the a	ppropriate	4°C ≤1 week/-80°C ≤6 months Demonstrated Protocols for details.
		ppropriate	Demonstrated Protocols for details.
	16-24 h		
Probe Hybridization (page 45)	16-24 h		
1 Tobe Trybhalzation (page 40)			
ep 2: GEM Generation and Barcoding (page 47)			
Post-Hybridization Pool & Wash (page 50)	60-90 min	STOP	-80°C ≤6 months
Prepare GEM Master Mix + Sample Dilution (page 57)	30 min		
B Load Chromium Next GEM Chip Q (page 62)	10 min		
Run the Chromium X/iX (page 64)	5.5 min		
Transfer GEMs (page 65)	5 min		
GEM Incubation (page 66)	125 min	STOP	4°C ≤1 week (GEMs) -80°C ≤6 months (washed undiluted sample)
ep 3: GEM Recovery and Pre-Amplification (page 67)			
Post-GEM Incubation – Recovery (page 69)	10 min		
Pre-Amplification PCR (page 70)	55 min	STOP	4°C ≤72 h/-20°C ≤1 week
B DNA Cleanup – SPRIselect (page 71)	30 min	STOP	4°C ≤72 h/-20°C ≤4 weeks
ep 4: Fixed RNA – Gene Expression Library Construction (p	age 72)		
Sample Index PCR (page 74)	40 min	STOP	4°C ≤72 h
Post Sample Index PCR Size Selection - SPRIselect (page)	30 min	STOP	4°C ≤72 h/-20°C long term
Post Library Construction QC (page 77)	60 min		
ep 5: Fixed RNA – Protein Expression Library Construction	(page 78)		
Sample Index PCR (page 80)	40 min	STOP	4°C ≤72 h
Post Sample Index PCR Size Selection – SPRIselect (page	30 min	STOP	4°C ≤72 h/-20°C long term
Post Library Construction QC (page 83)	60 min		

Sample Preparation

This User Guide is compatible with intact, fresh cells that are labeled with TotalSeqTM-C antibody-oligonucleotide conjugates and then fixed. See below for the documents needed to prepare samples for this User Guide. Consult Chromium Fixed RNA Profiling - Protocol Planner (CG000528) for additional details.

Sample Preparation Document Resources

Sample Preparation	Description & Resource	
Step 1: Sample labeling	Label cells with appropriate antibody- oligonucleotide conjugates* (Demonstrated Protocol CG000529)	Cell Surface Protein Labeling Demonstrated Protocol CG000529
Step 2: Sample fixation	Fix labeled cells (Demonstrated Protocol CG000478)	Sample Fixation Demonstrated Protocol CG000478

^{*}Use TotalSeqTM-C style antibody-oligonucleotide conjugates

Stepwise Objectives

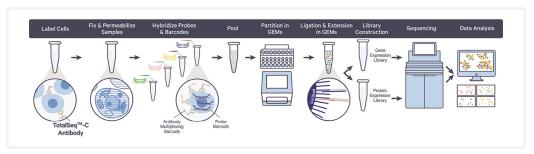
Chromium Fixed RNA Profiling offers comprehensive, scalable solutions to measure gene and protein expression in formaldehyde fixed samples that are labeled with antibody-oligonucleotide conjugates prior to fixation. Protein expression is measured by oligonucleotide conjugated antibodies, which are used to label cells prior to fixation. Gene expression is measured using probe pairs designed to hybridize to mRNA specifically.

For this workflow, labeled and fixed cell suspensions are first hybridized with Probe Barcodes and Antibody Muliplexing 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.

Similarly, Antibody Multiplexing Barcodes are attached to the Feature Barcode oligonucleotide and the 10x GEM Barcode is added inside the GEMs (barcode oligo capture). The ligated products are then pre-amplified in bulk, after which protein expression libraries are generated and sequenced.

Workflow Overview



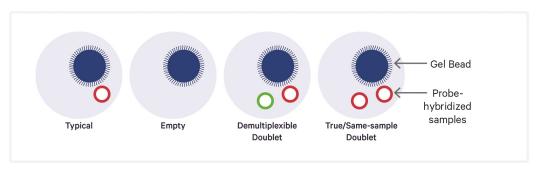
Sample Multiplexing

Chromium Fixed RNA Profiling for Multiplexed Samples provides an efficient and cost-effective way to further increase experiment size and cell number by enabling up to 4 or 16 samples to be run within a single GEM reaction. Multiplex-compatible Chromium Next GEM Single Cell Fixed RNA Human/Mouse Transcriptome Probe kits contain 4 or 16 probe sets, where each probe set includes a Probe Barcode that enables sample multiplexing and downstream demultiplexing. Multiplexing allows for up to 16 unique samples to be barcoded with 16 uniquely barcoded probe sets. Similarly, Fixed RNA

Feature Barcode Multiplexing Kit contains 16 unique Antibody Multiplexing Barcodes that allow sample multiplexing and downstream demultiplexing for protein expression.

To achieve single cell resolution, cells from any one of the multiplexed samples are delivered at a limiting dilution, such that only 1-10% of GEMs contain a cell hybridized with probes with a given Probe Barcode. The inclusion of the Probe Barcode in each probe pair allows the identification of GEMs containing more than one cell and to demultiplex the data generated from those cells, provided the cells have unique Probe Barcodes.

GEMs Generated During Multiplexing



When using the Chromium Fixed RNA Kit, Human/Mouse Transcriptome, 4 rxns x 4 BC PN-1000475/PN-1000495, up to 10,000 cells can be recovered and demultiplexed per sample, yielding a total of 40,000 cells recovered per GEM reaction. When using more than four Probe Barcodes, up to 8,000 cells can be recovered and demultiplexed per sample; thus with the Chromium Fixed RNA Kit, Human/Mouse Transcriptome, 4 rxns x 16 BC PN-1000476/PN-1000496 using 16 Probe Barcodes, 128,000 cells (16 * 8,000) can be recovered and demultiplexed.

128,000 cells recovered per GEM reaction represents the upper cell limit supported by the chip. Higher cell numbers may result in clogs or microfluidic failures.

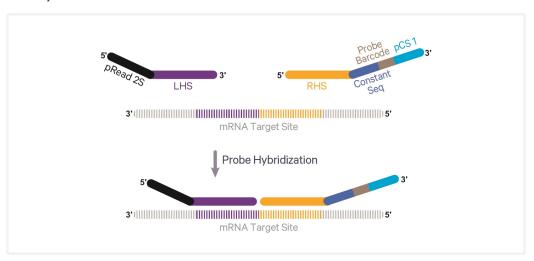
User Guide Steps

A high-level overview of each step in this User Guide, including gene and protein 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. Simultaneously, Antibody Multiplexing Barcodes are added to the labeled and fixed sample.

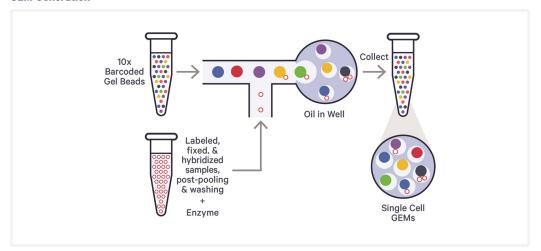
Probe Hybridization



Step 2: GEM Generation & Barcoding

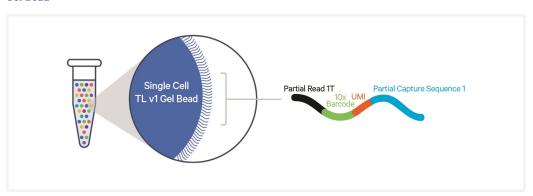
After hybridization, the samples containing different Probe Barcodes and Antibody Multiplexing Barcodes are pooled together and the unbound probes are washed off. After pooling and washing the samples, GEMs are generated by combining barcoded Gel Beads, a Master Mix containing pooled cells, and Partitioning Oil onto Chromium Next GEM Chip Q.

GEM Generation

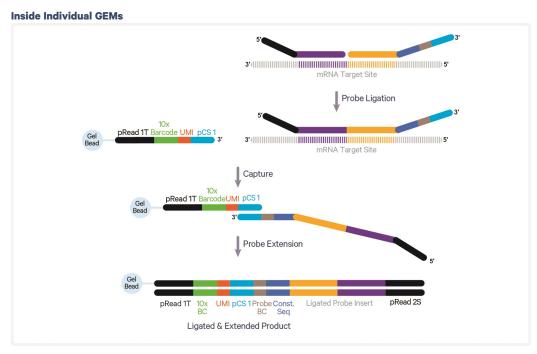


Immediately following GEM generation, the Gel Bead is dissolved, releasing the barcoded Gel Bead primers, and any co-partitioned cell is lysed. Gel Bead primers contain a partial TruSeq Read 1 sequence (partial Read 1T, read 1 sequencing primer), a 16 nt 10x GEM Barcode (or 10x Barcode), a 12 nt unique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

Gel Bead

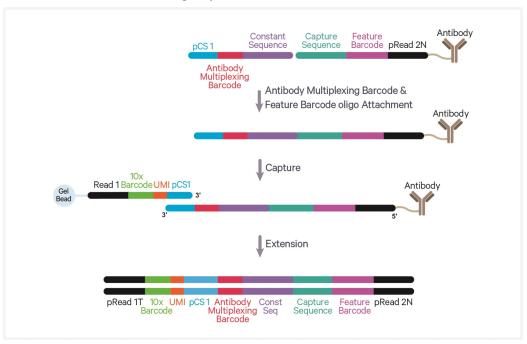


After GEM generation, the partitioned cells, Gel Beads, and Master Mix are placed in a thermal cycler and taken through several steps. First, a ligation step seals the nick between the left hand and right hand probe, while the probes remain hybridized to their target RNA. Second, the Gel Bead primer 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.



Simultaneously, the protein Feature Barcode oligonucleotide is attached to the Antibody Multiplexing Barcode. The Gel Bead primer hybridizes to the capture sequence on the Antibody Multiplexing Barcode that is bound to the protein Feature Barcode (barcode oligo capture) containing (i) a Nextera Read 2 (Read 2N), (ii) a 15 nt Feature Barcode, and (iii) Capture Sequence. Extension by polymerase, produces 10x Barcoded DNA from Antibody Multiplexing Barcode and the protein Feature Barcode.

Inside Individual GEMs - Barcode Oligo Capture

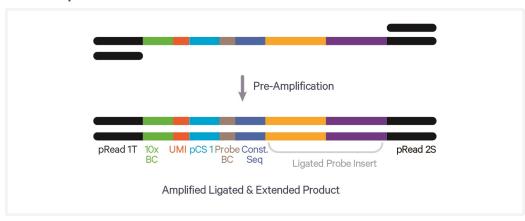


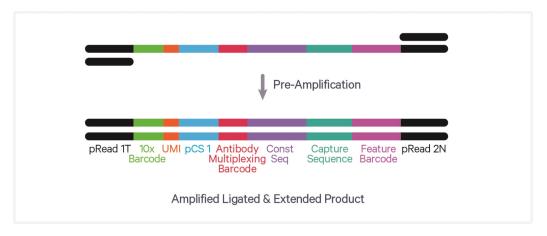
Finally, a heat denaturation step inactivates the enzymes in the GEM reaction.

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, along with barcoded Feature Barcodes identifying proteins. 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

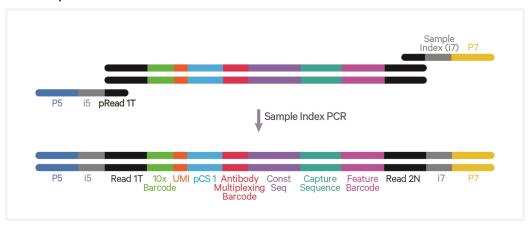


Step 5: Fixed RNA - Protein Expression Library Construction

The amplified DNA from protein Feature Barcode 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, an Illumina TruSeq Read 1 (Read 1T), and a Nextera Read 2 (Read 2N) are added via Sample Index PCR. The final libraries contain the P5 and P7 priming sites used in Illumina sequencers.

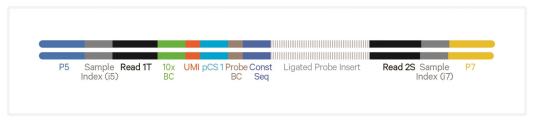
Pooled Amplified DNA Processed in Bulk



Step 6: 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, constant sequence, and the 8 bp Probe Barcode that identifies the probe set used to hybridize the sample.

Chromium Fixed RNA Profiling - Gene Expression Library



A Chromium Fixed RNA Profiling – Protein Expression library comprises standard Illumina paired-end constructs which begin and end with P5 and P7. The 16 bp 10x GEM Barcode (10x Barcode) and 12 bp UMI are encoded in Read 1T. Illumina Nextera Read 2 (Read 2N) sequences the Feature Barcode, constant sequence, and Antibody Multiplexing Barcode that identifies the sample.

Chromium Fixed RNA Profiling - Protein Expression Library



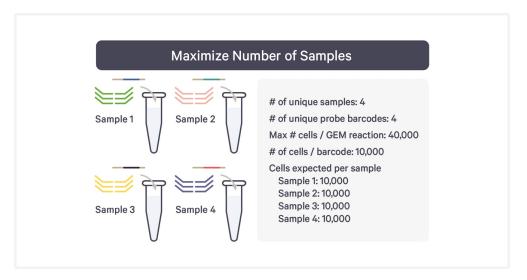
A single library contains reads derived from up to 16 samples, with cell barcodes composed of a Probe Barcode or an Antibody Multplexing Barcode and a 10x GEM Barcode (10x Barcode).

See Appendix for Oligonucleotide Sequences on page 104

Multiplexing Experiment Design

Chromium Fixed RNA Kit, Human/Mouse Transcriptome, 4 rxns x 4 BC PN-1000475/PN-1000496 and the Chromium Fixed RNA Kit, Human/Mouse Transcriptome, 4 rxns x 16 BC PN-1000476/PN-1000497 are compatible with and most efficiently used by hybridizing and pooling 4 or 16 samples, respectively.

• See Appendix for other Alternate Multiplexing Configurations & Pooling Strategies on page 98.



Pooling Samples with Different RNA Content

Due to the nature of a multiplexing pool, the sequencing reads for the pool will be distributed to different samples in proportion to their inherent RNA content and all the samples will have the same sequencing saturation. As a result, a sample with cells high in RNA will receive more reads per cell, whereas a sample with cells low in RNA will have proportionally fewer reads per cell, and sequencing saturation will be approximately the same for each sample. Because the distribution of reads across samples is determined by the composition of the pool, it is not possible to add reads to specific samples in the pool.

The recommendation therefore is to

- pool samples when comfortable with sequencing to the same percent saturation for each sample
- keep samples separate if sequencing one closer to saturation than the others is preferred

Tips & Best Practices



Icons



Tips & Best Practices section includes additional quidance



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

• When multiplexing 4 or 16 samples, the minimum input is 50,000 cells per hybridization and the maximum input is 2×10^6 cells 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 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.

Post-Hybridization Pooling & Washing Guidelines

Samples hybridized with unique probes can be pooled immediately after hybridization and washed as a pool (**Pooled Wash workflow**). Alternatively, samples can be washed individually and pooled after the washing is complete (**Individual Wash workflow**).

The Chromium Fixed RNA Profiling Reagent Kits contain sufficient buffer volumes to complete either workflow with no impact on the data quality.

Pooled Wash Workflow



- Recommended when using samples with low input cell numbers into probe hybridization.
- Count cells after probe hybridization to ensure equal representation of each sample in the final pool loaded on the microfluidic chip.
- After counting, adjust the volume of each sample such that the final
 washed pool contains an equal number of cells from each sample. A
 downloadable worksheet (Fixed RNA Profiling for Multiplexed Samples Pooling Workbook, Document CG000565) for calculations relevant to
 pooling is available on the 10x Genomics Support website.

After probe hybridization, follow Post-Hybridization Pool & Wash on page
 50 for Pooled Wash workflow.

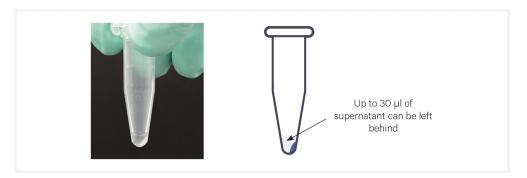
Individual Wash Workflow



- Not recommended with cell input numbers <200,000 into probe hybridization.
- Count cells from each sample immediately before pooling and loading on the microfluidic chip to achieve even representation of samples.
- After hybridization, see Appendix and follow Post-Hybridization Pool & Wash Individual Wash Workflow on page 94.

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 μ l 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 Probe Barcode with a per sample undetected multiplet rate of 0.4% to

- 8.0%. The solution can target up to 40,000 cells when multiplexing 4 samples and up to 128,000 cells when multiplexing 16 samples.
- When multiplexing 4 samples, the recommended starting point is to target ~16,000 total cells per GEM reaction (4,000 cells per Probe Barcode), and a multiplet rate of ~3.2%.
- When multiplexing 16 samples, the recommended starting point is to target ~64,000 total cells per GEM reaction (4,000 cells per Probe Barcode), and a multiplet rate of ~3.2%.
- The minimum cell input concentration to get maximum cell recovery is 1,650 cells/µl for multiplexing 4 samples and 5,280 cells/µl for multiplexing 16 samples.
- For each multiplexed sample, assuming 1 unique Probe Barcode is used per sample, the undetected (i.e. with same Probe Barcode) cell multiplet rate for Chip Q is approximately 0.8% multiplets per 1,000 cells recovered. Up to 128,000 cells can be recovered on Chip Q with a low multiplet rate any GEMs with multiplets derived from dissimilar Probe Barcodes can be demultiplexed.

Undetectable	Cells Loaded/	Cells Recovered/		ributed on 4 Probe odes		ibuted on 16 Probe odes
Multiplet Rate (%)	Probe Barcode	Probe Barcode	Cells Loaded/ Well	Cells Recovered/ Well	Cells Loaded/ Well	Cells Recovered/ Well
~0.4	825	500	3,300	2,000	13,200	8,000
~0.8	1,650	1,000	6,600	4,000	26,400	16,000
~1.6	3,300	2,000	13,200	8,000	52,800	32,000
~2.4	4,950	3,000	19,800	12,000	79,200	48,000
~3.2	6,600	4,000	26,400	16,000	105,600	64,000
~4.0	8,250	5,000	33,000	20,000	132,000	80,000
~4.8	9,900	6,000	39,600	24,000	158,400	96,000
~5.6	11,550	7,000	46,200	28,000	184,800	112,000
~6.4	13,200	8,000	52,800	32,000	211,200	128,000
~7.2	14,850	9,000	59,400	36,000	n/a*	n/a*
~8.0	16,500	10,000	66,000	40,000	n/a*	n/a*

^{*} These cell numbers are not supported.

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.





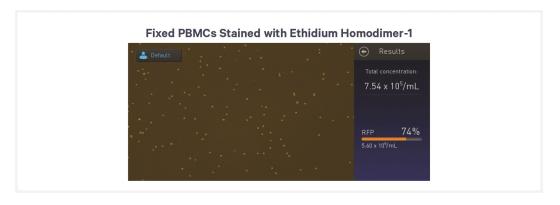
Cell Counting

- 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 subcellular debris. The optimal cell concentration for the Countess is 1,000-4,000 cells/ μ 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 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.



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/ μ l. 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 μ l fixed cell suspension to 15 μ l 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.

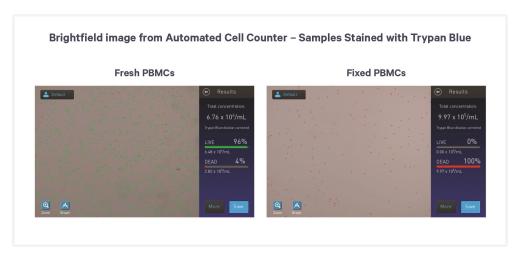
Samples stained with PI Staining Solution can also be counted using Countess II FL Automated Cell Counter. See manufacturer's instructions for details.

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

Debris-free samples 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 will be stained with trypan blue stain and appear non-viable.



Chip Q

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.



Chip Q

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 62 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.

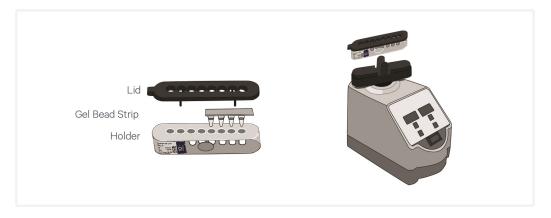
OR

- 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.

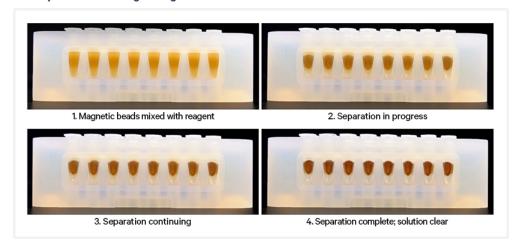
10x Magnetic Separator



- 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: = Volume of SPRIselect reagent added to the sample = 50 μ I = 0.5X Volume of DNA sample 100 μ I

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	43
Probe Hybridization Guidelines for Multiplexing	44
1.1 Probe Hybridization	45

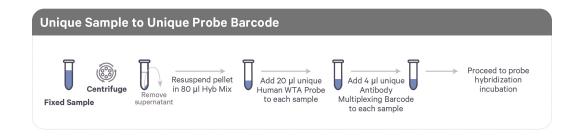
1.0 Get Started

Actio	n	Item	10x PN	Preparation & Handling	Storage
Thaw	& Keep W	/arm			
	•	Hyb Buffer B	2000485/2000483	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				
		Labeled & Fixed Cell Suspension	_	Consult Chromium Fixed RNA Profiling - Protocol Planner (CG000528) for details on applicable Demonstrated Protocols.	_
	•	Antibody Multiplexing Barcode AB001-AB016	2000917- 2000932	Thaw on ice. Vortex and centrifuge briefly.	-20°C
	•	Human WTA Probes BC001- BC016 OR Mouse WTA	2000495- 2000510	Thaw on ice. Vortex and centrifuge briefly.	-20°C
		Probes BC001- BC016	2000703-		

Probe Hybridization Guidelines for Multiplexing

Read these guidelines before starting probe hybridization

To generate Fixed RNA – Gene Expression & Protein Expression libraries, fixed single cell suspensions are mixed with probes (Probe Barcodes and Antibody Multiplexing Barcodes), and hybridized overnight (16-24 h) at 42°C.



1.1 Probe Hybridization



Before starting this protocol, ensure that samples have been appropriately labeled, fixed and quenched. Consult Fixed RNA profiling - Protocol Planner (CG000528) for details on the labeling and 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 μΙ	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)	16X* + 20% (μl)
	Hyb Buffer B Thaw at 42°C. Add warm to the mix and if appears milky keep it back on 42°C.	2000485/ 2000483	70.0	84.0	336.0	1344.0
	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	192.0
	Total	-	80.0	96.0	384.0	1536.0

^{*}1X = 1 fixed sample, 4X = 4 fixed samples, 16X = 16 fixed samples

- c. Incubate Hyb Mix at 42°C for 5 min.
- **d.** Centrifuge fixed cells 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 recommended during hybridization.

Sample Type	Recommended #
Fixed cells	50,000-2 x 10 ⁶



DO NOT exceed 2×10^6 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 μ l of supernatant may be left behind to optimize cell recovery without significantly impacting assay performance.



- **f.** Resuspend each pellet in **80 \mul** Hyb Mix and transfer to a tube strip or a new 1.5-ml microcentrifuge tube (only if a thermomixer with heated lid is available for incubation). Keep sample at **room temperature**. DO NOT place on ice.
- **g.** Add **20 μl** unique single Human/Mouse WTA Probes BC001-BC016 (PN-2000495-2000510 or PN-2000703-2000718) to the **80 μl** mixture of Hyb Mix and fixed sample and gently pipette mix 10x with pipette set at 80 μl. Record the Human/Mouse WTA Probes name and part number used for each sample.



Only one WTA Probe should be added to each tube containing sample + Hyb mix.

h. Add **4 \mul** of any Antibody Multiplexing Barcode (AB001-AB016) to each tube and gently pipette mix 10x with pipette set at 80 μ l. Record the Antibody Multiplexing Barcode name and part number used for each sample.



Only one Antibody Multiplexing Barcode should be added to each tube.

i. Incubate sample for 16-24 h at 42°C in a thermocycler. Alternatively, if working with 1.5-ml microcentrifuge tubes, incubate the tube for 16-24 h at 42°C in a thermomixer with heated lid and no shaking.



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

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Post-Hybridization Pooling Calculation	55
2.2 Prepare GEM Master Mix + Sample Dilution	57
2.3 Load Chromium Next GEM Chip Q	62
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2.6 GEM Incubation	66

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
Equilibrat	te to Rooi	m Temperature			
		Single Cell TL v1 Gel Beads	2000538	Equilibrate to room temperature 30 min before loading the chip.	-80°C
	0	Reducing Agent B	2000087	Vortex, verify no precipitate, centrifuge briefly.	-20°C
Thaw & K	eep Warı	n			
		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	lce				
		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/ 3000072	See Tips & Best Practices.	Ambient
		Sample Filters	_	Manufacturer's	Ambient

Action	Item	10x PN	Preparation & Handling	Storage
	Sysmex Sterile Single-pack CellTrics Filters/Miltenyi Biotec Pre-Separation Filters (30 µm)		recommendations.	
	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 Pool & Wash



Samples hybridized with unique Probe Barcodes can be pooled immediately after hybridization, and washed as a pool (Pooled Wash workflow). Alternatively, samples can be washed individually and pooled after the washing is complete (Individual Wash workflow). See Post-Hybridization Pooling & Washing Guidelines on page 29 for guidance on choosing the appropriate workflow. The following section provides instructions for Pooled Wash workflow. See Appendix for Post-Hybridization Pool & Wash -Individual Wash Workflow on page 94.

Pooled Wash Workflow



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

Volumes for 1 well/GEM reaction (includes 10% overage)

Post-Hyb Wash Buffer Add reagents in the order listed		PN	Pooling 4 samples (ml)*	Pooling 16 samples (ml)*
	Nuclease-free Water	-	4.95	13.86
	Conc. Post-Hyb Buffer	2000533	0.275	0.77
	Enhancer Heat at 65°C for 10 min. Vortex and verify no precipitate. Add warm to the mix.	2000482	0.275	0.77
	Total	-	5.5	15.40

Volumes for 4 wells/GEM reactions (includes 10% overage)

Post-l	Hyb Wash Buffer		Pooling 4	Pooling 16
Add reagents in the order listed		PN	samples (ml)*	samples (ml)*
	Nuclease-free Water	-	19.80	55.44
	Conc. Post-Hyb Buffer	2000533	1.10	3.08
	Enhancer Heat at 65°C for 10 min. Vortex and verify no precipitate. Add warm to the mix.	2000482	1.10	3.08
	Total	-	22.0	61.6

^{*}Volumes are in ml

b. Remove tubes from thermal cycler (8-tube strips) or thermomixer (1.5-ml microcentrifuge tubes) after overnight incubation.

c. If hybridization was performed in 8-tube strips:

i. Dilute each sample by adding 175 μ l Post-Hyb Wash Buffer and pipette mix 5x.



ii. Take 10 μl of diluted sample and determine cell concentration using a Countess II Automated Cell Counter, a Cellaca counter, or a hemocytometer. See Tips & Best Practices for Cell Counting on page 33. Calculate the total cell number present in the tube.



Accurate cell counting is critical for optimal assay performance. When using fluorescent dyes, signal to noise ratio may be lower because of the Conc. Post-Hyb Buffer composition. Increasing the dilution or a serial dilution into PBS before counting may improve the signal to noise ratio.

- iii. *Optional:* Sample types prone to clumping can be filtered with a 30 μ m filter (Sysmex CellTrics or Miltenyi Biotec Pre-Separation Filters) before pooling.
- **iv.** Pool an equal number of cells from different hybridization reactions into a 5-ml (for 4 pooling samples) or 15-ml (for pooling 16 samples) centrifuge tube. See Post-Hybridization Pooling Calculation on page 55 on how to calculate the volume of each sample to be added.
- v. Add 2.3 ml Post-Hyb Wash Buffer (if multiplexing 4 samples) or add
 9.2 ml Post-Hyb Wash Buffer (if multiplexing 16 samples). Mix by inverting 5x.
- vi. Proceed to step e.

d. If hybridization was performed in 1.5-ml microcentrifuge tubes:

i. Dilute each sample by adding **750 \mu l** Post-Hyb Wash Buffer and pipette mix 5x.



ii. Take 10 μl of diluted sample and determine cell concentration using a Countess II Automated Cell Counter, a Cellaca counter, or a hemocytometer. See Tips & Best Practices for Cell Counting on page 33. Calculate the total cell number present in the tube.



Accurate cell counting is critical for optimal assay performance. When using fluorescent dyes, signal to noise ratio may be lower because of the Conc. Post-Hyb Buffer composition. Increasing the dilution or a serial dilution into PBS before counting may improve the signal to noise ratio.

- iii. *Optional*: Sample types prone to clumping can be filtered with a 30 μm filter (Sysmex CellTrics or Miltenyi Biotec Pre-Separation Filters) before pooling.
- **iv.** Pool an equal number of cells from different hybridization reactions into a 5-ml (for 4 pooling samples) or 15-ml (for pooling 16 samples)

centrifuge tube. See Post-Hybridization Pooling Calculation on page 55 on how to calculate the volume of each sample to be added.

- v. Proceed to step e.
- e. Centrifuge each tube containing pooled samples 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 30.
- g. Resuspend cell pellet in 1 ml Post-Hyb Wash Buffer and transfer to a 1.5ml microcentrifuge tube.
- **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.
- k. Resuspend cell pellet in **0.5 ml** Post-Hyb Wash Buffer. Pipette mix 5x.
- 1. Incubate at 42°C for 10 min in a thermomixer or a heat block.
- m. Centrifuge at 850 rcf for 5 min at room temperature.
- **n.** Remove the supernatant without disturbing the pellet.
- o. Resuspend cell pellet in 0.5 ml Post-Hyb Wash Buffer. Pipette mix 5x.
- **p.** Incubate sample at **42°C** for **10 min** in a thermomixer or a heat block.
- q. Prepare Post-Hyb Resuspension Buffer. Pipette mix 10x and maintain at 4°C.

Post-Hyb Resuspension Buffer Add reagents in the order listed		PN	1 Pool + 10%	4 Pools + 10%	
			(μl)	(μl)	
	Nuclease-free Water	-	1567.5	6270.0	
	Conc. Post-Hyb Buffer	2000533	82.5	330.0	
	Total	-	1650.0	6600.0	

- **r.** Centrifuge the sample at **850 rcf** for **5 min** at **room temperature**.
- s. Remove the supernatant without disturbing the pellet.
- t. Resuspend cell pellet in an appropriate volume of chilled Post-Hyb Resuspension Buffer. The buffer volume will depend upon the starting number of cells in the pool (table below). Pipette mix 20x to resuspend and breakup any cell clumps and maintain on ice.

Final resuspension volume

Starting Total Cell Number in Pool	Post-Hyb Resuspension Buffer (μl)
<1 x 10 ⁶	500
1 x 10 ⁶ -4 x 10 ⁶	750
5 x 10 ⁶ -8 x 10 ⁶	1,000
9 x 10 ⁶ -12 x 10 ⁶	1,250
13 x 10 ⁶ -16 x 10 ⁶	1,500

u. Pass the sample through a 30 μm filter (Sysmex CellTrics or Miltenyi Biotec Pre-Separation Filters) into a new 1.5-ml/2-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.



See Sample Filtration on page 31 for details.

v. 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 33. As concentrated cell suspensions are required when targeting very high cell loads, 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 μ l (400/100 = 4).

• Recount to confirm final concentration.



w. 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 57 for details.

Sample Storage

- Add 0.1 volume Enhancer to sample in Post-Hyb Resuspension Buffer. For example, add 50 μ l Enhancer to 500 μ l 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 **2.1q** (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.

Post-Hybridization Pooling Calculation

This section provides calculation for pooling an equal number of cells from each sample. These calculations can be used at step 2.1c and 2.1d. Additionally, a downloadable worksheet (Fixed RNA Profiling for Multiplexed Samples - Pooling Workbook, Document CG000565) for calculations relevant to pooling is available on the 10x Genomics Support website.

- Count cells and determine Post-Hybridization Cell Concentrations
- Calculate Total Cells in Hybridization Total Cells in Hybridization = Post-Hybridization Cell Conc. * Sample Volume
- Calculate Cells per Sample Added to the Pool Cells per Sample Added to the Pool = Cell count of sample with lowest Total Cells in Hybridization
- Calculate Sample Volume to be Added for Each Sample Sample Volume to be Added for Each Sample = Cells per Sample Added to the Pool Post-Hybridization Cell Conc.

Example Calculations

If hybridization was done in 8-tube strips - example calculation for pooling an equal number of cells from each of 4 samples

Four fixed samples hybridized with unique Probe Barcodes for multiplexing, diluted, and counted post-hybridization

Probe Barcode	Post-Hybridization Cell Conc.	Total Cells in Hybridization	Cells per Sample Added to the Pool	Sample Volume to be Added (µl)
	Cells in 265 µl Post-Hyb Wash Buffer	Post-Hybridization Cell Conc. * 265	Cell Count of Sample with Lowest Concentration	Cells per Sample Added to the Pool/ Post-Hybridization Cell Conc.
BC001	1,200 cells/µl	318,000	238,500	238,500/1,200 = 198.75
BC002	2,300 cells/µl	609,500	238,500	238,500/2,300 = 103.69
BC003	900 cells/µl	238,500	238,500	238,500/900 = 265.0
BC004	2,800 cells/µl	742,000	238,500	238,500/2,800 = 85.18

If hybridization was done in 1.5-ml microcentrifuge tubes - example calculation for pooling an equal number of cells from each of 4 samples

Four fixed samples hybridized with unique Probe Barcodes for multiplexing, diluted, and counted post-hybridization

Probe	Post-Hybridization Cell Conc.	Total Cells in Hybridization	Cells per Sample Added to the Pool	Sample Volume to be Added (µl)
Barcode	Cells in 840 µl Post-Hyb Wash Buffer	Post-Hybridization Cell Conc. * 840	Cell Count of Sample with Lowest Concentration	Cells per Sample Added to the Pool/ Post-Hybridization Cell Conc.
BC001	388 cells/µl	325,920	244,440	244,440/388 = 630.0
BC002	744 cells/μl	624,960	244,440	244,440/744 = 328.5
BC003	291 cells/μl	244,440	244,440	244,440/291 = 840.0
BC004	905 cells/μl	760,200	244,440	244,440/905 = 270.1



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*	4X* + 10%	
		FN	(μl)	(μl)	
•	GEM Reagent Mix	2000491	20.9	92.1	
\bigcirc	Reducing Agent B	2000087	1.7	7.3	
•	GEM Enzyme Mix	2000490	12.4	54.6	
	Total	-	35.0	154.0	

^{*1}X = 1 well/GEM reaction, 4X = 4 wells/GEM reactions

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 66.

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 for Multiplexing 4 Samples

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

Cell Stock					Targete	d Cell R	ecovery				
Concentration (Cells/µl)	2000	4000	8000	12000	16000	20000	24000	28000	32000	36000	40000
500	6.6 33.4	13.2 26.8	26.4 13.6	39.6 0.4	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	4.4	8.8	17.6	26.4	35.2						
750	35.6	31.2	22.4	13.6	4.8	n/a	n/a	n/a	n/a	n/a	n/a
	3.3	6.6	13.2	19.8	26.4	33.0	39.6				
1000	36.7	33.4	26.8	20.2	13.6	7.0	0.4	n/a	n/a	n/a	n/a
4050	2.6	5.3	10.6	15.8	21.1	26.4	31.7	37.0	,	,	,
1250	37.4	34.7	29.4	24.2	18.9	13.6	8.3	3.0	n/a	n/a	n/a
1500	2.2	4.4	8.8	13.2	17.6	22.0	26.4	30.8	35.2	39.6	n/o
1500	37.8	35.6	31.2	26.8	22.4	18.0	13.6	9.2	4.8	0.4	n/a
1750	1.9	3.8	7.5	11.3	15.1	18.9	22.6	26.4	30.2	33.9	37.7
1750	38.1	36.2	32.5	28.7	24.9	21.1	17.4	13.6	9.8	6.1	2.3
2000	1.7	3.3	6.6	9.9	13.2	16.5	19.8	23.1	26.4	29.7	33.0
2500	38.4	36.7	33.4	30.1	26.8	23.5	20.2	16.9	13.6	10.3	7.0
2250	1.5	2.9	5.9	8.8	11.7	14.7	17.6	20.5	23.5	26.4	29.3
	38.5	37.1	34.1	31.2	28.3	25.3	22.4	19.5	16.5	13.6	10.7
2500	1.3	2.6	5.3	7.9	10.6	13.2	15.8	18.5	21.1	23.8	26.4
	38.7	37.4	34.7	32.1	29.4	26.8	24.2	21.5	18.9	16.2	13.6
2750	1.2	2.4	4.8	7.2	9.6	12.0	14.4	16.8	19.2	21.6	24.0
	38.8	37.6	35.2	32.8	30.4	28.0	25.6	23.2	20.8	18.4	16.0
3000	1.1	2.2	4.4	6.6	8.8	11.0	13.2	15.4	17.6	19.8	22.0
	38.9	37.8	35.6	33.4	31.2	29.0	26.8	24.6	22.4	20.2	18.0
3250	1.0	2.0	4.1	6.1	8.1	10.2	12.2	14.2	16.2	18.3	20.3
	39.0	38.0	35.9	33.9	31.9	29.8	27.8	25.8	23.8	21.7	19.7 18.9
3500	0.9 39.1	1.9 38.1	3.8 36.2	5.7 34.3	7.5 32.5	9.4 30.6	11.3 28.7	13.2 26.8	15.1 24.9	17.0 23.0	21.1
	0.9	1.8	3.5	5.3	7.0	8.8	10.6	12.3	14.1	15.8	17.6
3750	39.1	38.2	36.5	34.7	33.0	31.2	29.4	27.7	25.9	24.2	22.4
	0.8	1.7	3.3	5.0	6.6	8.3	9.9	11.6	13.2	14.9	16.5
4000	39.2	38.4	36.7	35.1	33.4	31.8	30.1	28.5	26.8	25.2	23.5
	0.8	1.6	3.1	4.7	6.2	7.8	9.3	10.9	12.4	14.0	15.5
4250	39.2	38.4	36.9	35.3	33.8	32.2	30.7	29.1	27.6	26.0	24.5
	0.7	1.5	2.9	4.4	5.9	7.3	8.8	10.3	11.7	13.2	14.7
4500	39.3	38.5	37.1	35.6	34.1	32.7	31.2	29.7	28.3	26.8	25.3
/750	0.7	1.4	2.8	4.2	5.6	6.9	8.3	9.7	11.1	12.5	13.9
4750	39.3	38.6	37.2	35.8	34.4	33.1	31.7	30.3	28.9	27.5	26.1
5000	0.7	1.3	2.6	4.0	5.3	6.6	7.9	9.2	10.6	11.9	13.2
5000	39.3	38.7	37.4	36.0	34.7	33.4	32.1	30.8	29.4	28.1	26.8
5250	0.6	1.3	2.5	3.8	5.0	6.3	7.5	8.8	10.1	11.3	12.6
3230	39.4	38.7	37.5	36.2	35.0	33.7	32.5	31.2	29.9	28.7	27.4
5500	0.6	1.2	2.4	3.6	4.8	6.0	7.2	8.4	9.6	10.8	12.0
2300	39.4	38.8	37.6	36.4	35.2	34.0	32.8	31.6	30.4	29.2	28.0
5750	0.6	1.1	2.3	3.4	4.6	5.7	6.9	8.0	9.2	10.3	11.5
2,00	39.4	38.9	37.7	36.6	35.4	34.3	33.1	32.0	30.8	29.7	28.5
6000	0.6	1.1	2.2	3.3	4.4	5.5	6.6	7.7	8.8	9.9	11.0
	39.5	38.9	37.8	36.7	35.6	34.5	33.4	32.3	31.2	30.1	29.0
Yellow boxes	Indicate a	low transfe	r volume t	nat may res	ult in highe	er cell load	variability				

Cell Suspension Volume Calculator for Multiplexing 16 Samples

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

Cell Stock	Targeted Cell Recovery								
Concentration (Cells/µI)	8000	16000	32000	48000	64000	80000	96000	112000	128000
500	26.4 13.6	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
1000	13.2 26.8	26.4 13.6	n/a	n/a	n/a	n/a	n/a	n/a	n/a
1500	8.8 31.2	17.6 22.4	35.2 4.8	n/a	n/a	n/a	n/a	n/a	n/a
2000	6.6 33.4	13.2 26.8	26.4 13.6	39.6 0.4	n/a	n/a	n/a	n/a	n/a
2500	5.3 34.7	10.6 29.4	21.1 18.9	31.7 8.3	n/a	n/a	n/a	n/a	n/a
3000	4.4 35.6	8.8 31.2	17.6 22.4	26.4 13.6	35.2 4.8	n/a	n/a	n/a	n/a
3500	3.8 36.2	7.5 32.5	15.1 24.9	22.6 17.4	30.2 9.8	37.7 2.3	n/a	n/a	n/a
4000	3.3 36.7	6.6 33.4	13.2 26.8	19.8 20.2	26.4 13.6	33.0 7.0	39.6 0.4	n/a	n/a
4500	2.9 37.1	5.9 34.1	11.7 28.3	17.6 22.4	23.5 16.5	29.3 10.7	35.2 4.8	n/a	n/a
5000	2.6 37.4	5.3 34.7	10.6 29.4	15.8 24.2	21.1 18.9	26.4 13.6	31.7 8.3	37.0 3.0	n/a
5500	2.4 37.6	4.8 35.2	9.6 30.4	14.4 25.6	19.2 20.8	24.0 16.0	28.8 11.2	33.6 6.4	38.4 1.6
6000	2.2 37.8	4.4 35.6	8.8 31.2	13.2 26.8	17.6 22.4	22.0 18.0	26.4 13.6	30.8 9.2	33.6 6.4
6500	2.0 38.0	4.1 35.9	8.1 31.9	12.2 27.8	16.2 23.8	20.3 19.7	24.4 15.6	28.4 11.6	32.5 7.5
7000	1.9 38.1	3.8 36.2	7.5 32.5	11.3 28.7	15.1 24.9	18.9 21.1	22.6 17.4	26.4 13.6	30.2 9.8
7500	1.8 38.2	3.5 36.5	7.0 33.0	10.6 29.4	14.1 25.9	17.6 22.4	21.1 18.9	24.6 15.4	28.2 11.8
8000	1.7 38.4	3.3 36.7	6.6 33.4	9.9 30.1	13.2 26.8	16.5 23.5	19.8 20.2	23.1 16.9	26.4 13.6
8500	1.6 38.4	3.1 36.9	6.2 33.8	9.3 30.7	12.4 27.6	15.5 24.5	18.6 21.4	21.7 18.3	24.8 15.2
9000	1.5 38.5	2.9 37.1	5.9 34.1	8.8 31.2	11.7 28.3	14.7 25.3	17.6 22.4	20.5 19.5	23.5 16.5
9500	1.4 38.6	2.8 37.2	5.6 34.4	8.3 31.7	11.1 28.9	13.9 26.1	16.7 23.3	19.5 20.5	22.2 17.8
10000	1.3 38.7	2.6 37.4	5.3 34.7	7.9 32.1	10.6 29.4	13.2 26.8	15.8 24.2	18.5 21.5	21.1 18.9
Yellow boxes	Indicate a lo	ow transfer v	olume that m	nay result in I	nigher cell lo	ad 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 27 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.

Prep Gel Beads



c. Load Row 1



- With pipette set to 70 μ l, 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.



Partitioning Oil

Gel Beads

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

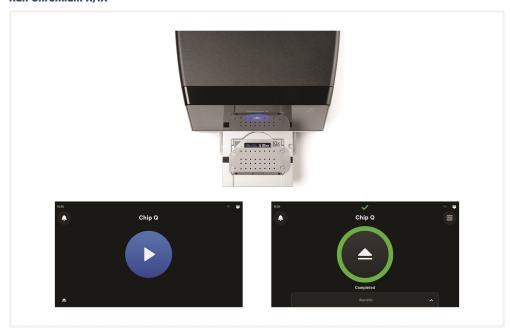
Consult the Chromium X Series (X/iX) User Guide (CG000396) for detailed instrument operation instructions and follow the instrument touchscreen prompts for execution. Run time for Chip Q is ~5.5 min.

- **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 μ l 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 μ l volume. A volume of 100 μ l 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 μΙ	~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 57 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 μ l Enhancer to 500 μ l 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 **2.1q** of **2.1** Post-Hybridization Pool & Wash on page 50 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	68
3.1 Post-GEM Incubation – Recovery	69
3.2 Pre-Amplification PCR	70
3.3 DNA Cleanup – SPRIselect	71



3.0 Get Started

Actio	n	Item	10x PN	Preparation & Handling	Storage
Equilil	orate to R	Room Temperature			
	0	Reducing Agent B	2000087	Thaw, vortex, verify no precipitate, centrifuge briefly.	-20°C
	•	Pre-Amp Primers C Verify name & PN Retrieve from Fixed RNA Feature Barcode Multiplexing Kit, 64 rxns, PN-1000628.	2000953	Thaw, vortex, centrifuge briefly.	-20°C
		Beckman Coulter SPRIselect Reagent	_	Manufacturer's recommendations.	Ambient
Place	on Ice				
	\bigcirc	Amp Mix	2000047	Vortex and centrifuge briefly.	-20°C
Obtair	1				
	0	Recovery Agent	220016	_	Ambient
		Qiagen Buffer EB	_	Manufacturer's recommendations.	Ambient
		10% Tween 20	_	Manufacturer's recommendations.	Ambient
		10x Magnetic Separator	230003	_	Ambient
		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

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

Pre-Amplification Mix		PN	1X	4X + 10%	
Add reagents i	in the order listed	FIN	(μl)	(μl)	
	Amp Mix	2000047	25.0	110.0	
	Pre-Amp Primers C	2000953	10.0	44.0	
	Total		35.0	154.0	

- **b.** Add **35 \mul** 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 μΙ	~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 (t	otal 8 cycles)
6	72°C	00:01:00
7	4°C	Hold

^{*}Revision-specific update



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		PN	1000 μΙ
	Buffer EB		980
	10% Tween 20	-	10
\circ	Reducing Agent B	2000087	10
	Total		1000

b. Centrifuge the sample (PCR product) for 30 sec in a microcentrifuge and transfer 70 μ l 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 μ l 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.
- 1. 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** μ **l** 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	73
4.1 Sample Index PCR	74
4.2 Post Sample Index PCR Size Selection – SPRIselect	76
4.3 Post Library Construction QC	77

4.0 Get Started

Action		Item	10x PN	Preparation & Handling	Storage
Equilib	rate to R	oom Temperature			
	A	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 o	n Ice				
	\bigcirc	Amp Mix	2000103/2000047	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	230003	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	1Χ (μl)	1X + 10% (μl)	4X + 10% (μl)
Amp Mix		2000103/2000047	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 71 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 μΙ	~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 to	otal # of cycles
6	72°C	00:01:00
7	4°C	Hold

Targeted Call December	Total Cycles*		
Targeted Cell Recovery	for Cell Lines	for PBMCs	
500-2,000	11	15	
2,000-4,000	10	14	
4,000-7,000	9	13	
7,000-12,000	8	12	
12,000-25,000	7	11	
25,000-50,000	6	10	
50,000-128,000	5	9	

^{*}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).



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 μ l 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.
- 1. Place on the magnet-Low until the solution clears.
- **m.** Transfer **40 μl** to a new tube strip.

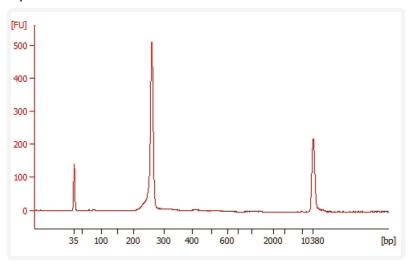


n. Store at 4°C for up to 72 h or at -20°C for long-term storage.

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 100
- Agilent TapeStation Traces on page 102
- LabChip Traces on page 102

Step 5:

Fixed RNA - Protein Expression Library Construction

5.0 Get Started	79
5.1 Sample Index PCR	80
5.2 Post Sample Index PCR Size Selection – SPRIselect	82
5.3 Post Library Construction QC	83



5.0 Get Started

Overview

Action	n	Item	10x PN	Preparation & Handling	Storage
Equilib	rate to R	Room Temperature			
	A	Dual Index Plate TN Set A Verify name & PN. Use indicated plate only	3000510	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 o	on Ice				
	\bigcirc	Amp Mix	2000047/ 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	230003	See Tips & Best Practices.	Ambient
		Prepare 80% Ethanol Prepare 2.5 ml for 4 GEM reactions.	_	Prepare fresh.	Ambient

5.1 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-3000510 Dual Index Plate TN Set A well ID) used.
- **b.** Prepare Sample Index PCR Mix.

Sample Ind	lex PCR Mix	PN	1X	1X + 10%	4X + 10%
Add reagents in the order listed			(μl)	(μl)	(μΙ)
\bigcirc	Amp Mix	2000047/ 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 (pre-amplified DNA) from the DNA Cleanup - SPRIselect on page 71 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 TN Set A to each sample and record the well ID used. 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 μΙ	~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

^{*}Optimization of cycle number may be needed based on target protein expression levels and number of antibodies used for labeling.

Targeted Cell Recovery	Total Cycles
500-2,000	14-15
2,000-4,000	13-14
4,000-7,000	12-13
7,000-12,000	11-12
12,000-25,000	10-11
25,000-50,000	9-10
50,000-128,000	8-9



g. Store at $4^{\circ}C$ for ≤ 72 h, or proceed to the next step.

5.2 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 μ l 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.
- 1. Place on the magnet-Low until the solution clears.
- **m.** Transfer **40 μl** to a new tube strip.

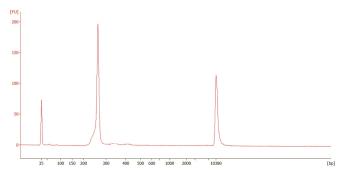


n. Store at 4°C for up to 72 h or at -20°C for long-term storage.

5.3 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 the average size of the library.

Representative Trace



Determine the average fragment size from the Bioanalyzer trace.

Alternate QC Method

Agilent TapeStation

LabChip

See Appendix for:

- Post Library Construction Quantification on page 100
- Agilent TapeStation Traces on page 102
- LabChip Traces on page 102

Step 6:

Sequencing

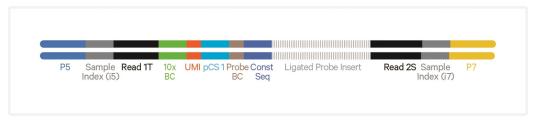
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Sequencing Libraries

Chromium Fixed RNA Profiling – Gene Expression and Protein 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 pairedend sequencing of Fixed RNA – Gene Expression libraries. Read 2 sequences the ligated probe sequence, constant sequence, and the 8 bp Probe Barcode that identifies the probe set used to hybridize the sample.

Chromium Fixed RNA Profiling - Protein Expression Library



TruSeq Read 1 (Read 1T) and Nextera Read 2 (Read 2N) are used for pairedend sequencing of Fixed RNA – Protein Expression library. Read 2 sequences the Feature Barcode sequence, constant sequence, and Antibody Multiplexing Barcode that identifies the sample.

A single library contains reads derived from up to 16 samples, with cell barcodes composed of a Probe Barcode or an Antibody Multplexing Barcode and a 10x GEM Barcode (10x Barcode).

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.

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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) or Dual Index Kit TN Set A (PN-1000250) 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 & Protein Expression Library Sequencing Parameters

Parameter	Description
Sequencing Depth	Minimum 10,000 read pairs per cell for Gene Expression library Minimum 5,000 read pairs per cell for Protein Expression library
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 76 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

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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	5
NextSeq 500/550	2.5	5
NextSeq 1000/2000	650	5
NovaSeq 6000 standard	100-150	10
NovaSeq 6000 Xp workflow	150-200	10

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

Library Pooling

Fixed RNA – Gene Expression and Protein Expression libraries may be pooled for sequencing, taking into account the differences in cell number and percell 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.

Library Pooling Example

Libraries	Sequencing Depth (read pairs per cell)	Library Pooling Ratio
Fixed RNA – Gene Expression library	10,000	2
Fixed RNA – Protein Expression library	5,000	1

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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, generate Feature Barcode matrices, 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.

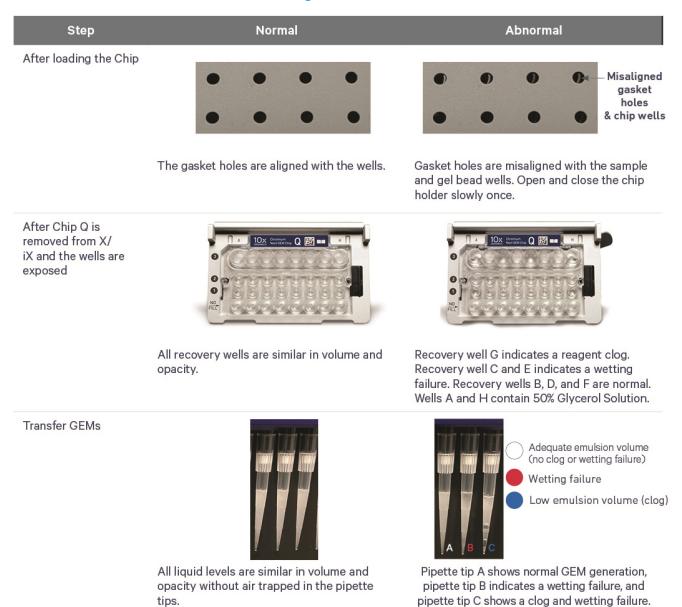
- Input: .cloupe
- Output: Data visualization, including t-SNE and UMAP projections, custom clusters, differentially expressed genes
- Operating System: MacOS, Windows

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Troubleshooting

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GEM Generation & Barcoding



-**Ö**-

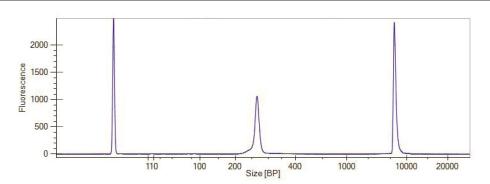
Consult Best Practices to Minimize Chromium Next GEM Chip Clogs and Wetting Failures (Technical Note CG000479) for more information. If a channel clogs or wetting failure occurs during GEM generation, it is recommended that the sample be remade. If any of the listed issues occur, take a picture and send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

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Post Library Construction QC

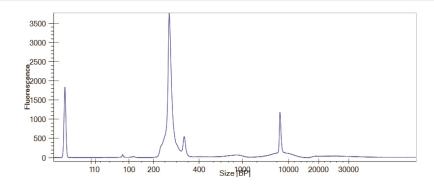
Step

Fixed RNA – Gene Expression Library Correct Sample Index PCR cycling



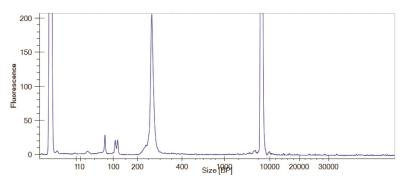
The ideal target library concentration is 50-200 nM. However, if the concentration is between 10-50 nM or between 200-500 nM and if the libraries do not contain low or high molecular weight peaks, sequencing can still be performed.

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.

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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.

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Post-Hybridization Pool & Wash - Individual Wash Workflow



This workflow will also allow an increased flexibility to store samples after washing and later change how samples are pooled before loading the chip.

See Post-Hybridization Pooling & Washing Guidelines on page 29 before starting. This protocol is only supported with ≥200,000 cell inputs into hybridization

a. Prepare Post-Hyb Wash Buffer. Pipette mix 10x and keep at **room temperature**. DO NOT keep at 4°C.

Post-Hyb Wash Buffer 1X* + 10%				4X* + 10%	16X* + 10%
Add re	agents in the order listed.	PN	(ml)	(ml)	(ml)
	Nuclease-free Water	-	1.98	7.92	31.68
	Conc. Post-Hyb Buffer	2000533	0.11	0.44	1.76
	Enhancer	2000482	0.11	0.44	1.76
	Total	-	2.2	8.8	35.2

^{*}1X = 1 sample, 4X = 4 samples, 16X = 16 samples

- **b.** Remove tubes from thermal cycler (8-tube strips) or thermomixer (1.5-ml microcentrifuge tubes) after overnight incubation.
- c. Add 900 µl Post-Hyb Wash Buffer to each sample. Pipette mix 5x.

If the hybridization was performed in 8-tube strips, add 175 μ l 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 μ l 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.
- **g.** Resuspend each cell pellet in **0.5 ml room temperature** Post-Hyb Wash Buffer. Pipette mix 5x.
- h. Incubate at 42°C for 10 min in thermomixer or a heat block.
- i. Centrifuge at 850 rcf for 5 min at room temperature.
- **j.** Remove the supernatant without disturbing the pellet.

- **k.** Resuspend each cell pellet in **0.5 ml room temperature** Post-Hyb Wash Buffer. Pipette mix 5x.
- 1. Incubate at 42°C for 10 min in 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)	4Χ* + 10% (μΙ)	16X* + 10% (µl)
Nuclease-free Water	-	522.5	2090.0	8360.0
Conc. Post-Hyb Buffer	2000533	27.5	110.0	440.0
Total	-	550.0	2200.0	8800.0

*1X = 1 sample, 4X = 4 samples, 16X = 16 samples

- **n.** Centrifuge sample at **850 rcf** for **5 min** at **room temperature**.
- **o.** Remove the supernatant without disturbing the pellet.
- **p.** Resuspend each cell pellet in **0.5 ml chilled** Post-Hyb Resuspension Buffer. Pipette mix 20x to resuspend and breakup any cell clumps and maintain on ice.
- **q.** Pass each sample through a 30 μm filter (Sysmex CellTrics or Miltenyi Biotec Pre-Separation Filters) into a new 1.5-ml microcentrifuge tube.



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 31 for details.

- **r.** Determine cell concentration of each sample using a Countess II Automated Cell Counter, a Cellaca counter, or a hemocytometer. See Cell Counting on page 33 for details.
- **s.** Store the sample after resuspending in appropriate reagents (see Sample Storage on the next page) or **immediately** proceed to next step.
- **t.** Pool an equal number of cells from different hybridization reactions into a 5-ml (for pooling 4 samples) or 15-ml (for pooling 4 or 16 samples) centrifuge tube. See table below on how to calculate the volume of each sample to be added.

Example calculation for pooling an equal number of cells from each of 4 samples

Four fixed samples hybridized with unique probes for multiplexing, washed, and counted post-hybridization wash

Probe	Post-Hybridization Cell Conc.	Total Cells in Sample	Cells per Sample Added to the Pool	Sample Volume to be Added (µl)
Barcode	Cells in 490 µl Post-Hyb Resuspension Buffer	Post-Hybridization Cell Conc. * 490	Cell Count of Sample with Lowest Concentration	Cells per Sample Added to the Pool/ Post-Hybridization Cell Conc.)
BC001	660 cells/µl	323,400	242,550	242,550/660 = 367.5
BC002	1,265 cells/µl	619,850	242,550	242,550/1,265 = 191.7
BC003	495 cells/µl	242,550	242,550	242,550/495 = 490.0
BC004	1,540 cells/µl	754,600	242,550	242,550/1,540 = 157.5

u. 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 33.

If the sample concentration is not sufficient to achieve the desired target cells 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. Recount to confirm final concentration. 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 μ l, centrifuge, then remove 300 μ l of supernatant, and finally resuspend the cell pellet in the remaining 100 μ l (400/100 = 4).

v. Proceed **immediately** to Prepare GEM Master Mix + Sample Dilution on page 57.

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 of 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 m of this section.

Alternate Multiplexing Configurations & Pooling Strategies

It is possible to pool fewer than 4 or 16 samples, for example if a sample is lost during hybridization or post-hybridization washing or if fewer than the maximum number of samples is desired. In such cases, pool the remaining samples post-hybridization and continue with the protocol. Consult Fixed RNA Profiling for Multiplexed Samples - Pooling Workbook (Document CG000565) for guidance on such alternate pooling strategies. This worksheet can also be used for calculating sample volumes for pooling.

When pooling less than 4 or 16 samples, the number of cells targeted per GEM well should be adjusted as explained below:

- When using the human/mouse 4 rxns x 4 BC kits (PN-1000475 or PN-1000496) to pool less than 4 samples per well, the total number of cells targeted should not exceed 10,000 cells per Probe Barcode (or 10,000 * number of Probe Barcodes used/per well). For example, if pooling 3 samples hybridized with 3 unique Probe Barcodes, the total number of cells targeted should not exceed 30,000 cells/well (10,000 * 3).
- When using the human/mouse 4 rxns x 16 BC kits (PN-1000476 or PN-1000497) to pool less than 16 samples per well, the total number of cells targeted should not exceed 8,000 cells per Probe Barcode (or 8,000 * number of Probe Barcodes used/per well). For example, if pooling 14 samples hybridized with 14 unique Probe Barcodes, the total number of cells targeted should not exceed 112,000 cells/well (8,000 * 14).



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 62 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 $2 \mu l$ 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	1Χ (μΙ)
SYBR Fast Master Mix + Primer	12
Water	4
Total	16

- **d.** Dispense **16** μ l Quantification Master Mix for sample dilutions and DNA Standards into a 96 well PCR plate.
- e. Add $4~\mu l$ sample dilutions and $4~\mu l$ 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.

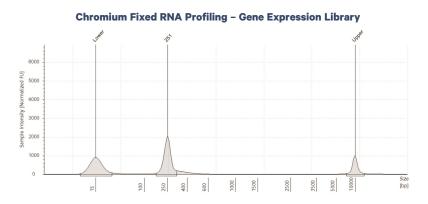
Alternate Sequencing Parameters

This alternative configuration reads the sequences required for sample demultiplexing in Read 1. This read scheme is utilized if the sequences required for sample demultiplexing do not occur in the same positions when read in Read 2.

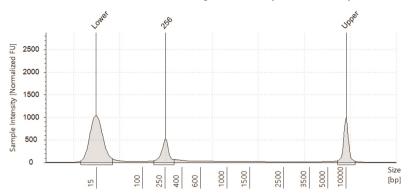
Parameter	Description
Sequencing Depth	Minimum 10,000 read pairs per cell for Gene Expression library Minimum 5,000 read pairs per cell for Protein Expression library
Sequencing Type	Paired-end, dual indexing
Sequencing Read	Recommended Number of Cycles
Read 1	48 cycles
i7 Index	10 cycles
i5 Index	10 cycles
Read 2	50 cycles

Agilent TapeStation Traces

Agilent TapeStation High Sensitivity D5000 ScreenTape was used.



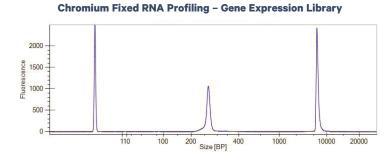
Chromium Fixed RNA Profiling - Protein Expression Library



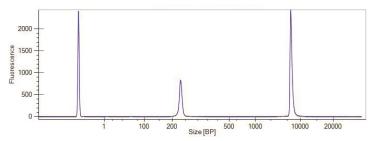
All traces are representative. Samples were run at 1:80 dilution.

LabChip Traces

DNA High Sensitivity Reagent Kit was used.



Chromium Fixed RNA Profiling - Protein Expression Library



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

Oligonucleotide Sequences

Gel Bead Primer

Gel Bead Primers

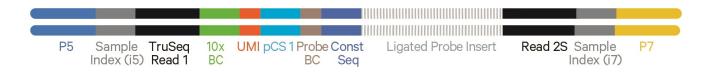
5'-CTACACGACGCTCTTCCGATCT-N16-N12-TTGCTAGGACCG-3'



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'



Protein Feature Barcode

5'-CGGAGATGTGTATAAGAGACAG-N10-N15-N9-CCCATATAAGAAA-3'



Chromium Fixed RNA - Protein Expression Library

5-AATGATACGGCGACCACCGAGATCTACAC-N10-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-N16-N12-TTGCTAGGACCG-AB8-N5-AACCGCGTATTCAGGT-TTTCTTATATGGG-N9-N15-N10-CTGTCTCTTATACACATCTCCGAGCCCACGAGAC-N10-ATCTCGTATGCCGTCTTCTGCTTG-3'

3-TTACTATGCCGCTGGTGGCTCTAGATGTG-N10-TGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA-N16-N12-AACGATCCTGGC-AB8-N5-TTGGCGCATAAGTCCA-AAAGAATATACCC-N9-N15-N10-GACAGAGAATATGTGTAGAGGCTCGGGTGCTCTG-N10-TAGAGCATACGGCAGAAGACGAAC-5'

