



**User Guide | CG000527 | Rev D**

# **Chromium**

## **Fixed RNA Profiling Reagent Kits**

### **for Multiplexed Samples**

For use with:

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

Chromium Next GEM Chip Q Single Cell Kit  
*48 rxns PN-1000418 | 16 rxns PN-1000422*

Chromium Fixed RNA Kit, Human Transcriptome  
*4 rxns x 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*

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

# Notices

## Document Number

CG000527 | Rev D

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# Document Revision Summary

## Document Number

CG000527 | Rev D

## Title

Chromium Fixed RNA Profiling Reagent Kits for Multiplexed Samples

## Revision

Rev C to Rev D

## Revision Date

February 14, 2023

## Specific Changes

- Updated the number of tubes for Conc. Fixed & Perm Buffer, Conc. Quench Buffer, and Enhancer for Chromium Next GEM Single Cell Fixed RNA Sample Preparation Kit, 16 rxns PN-1000414.
- Updated the Sample Fixation Section to include guidance on Isolation of Cells from FFPE Tissue Sections.
- Removed  $-20^{\circ}\text{C}$  from storage of sample after Post-Hybridization Pool and Wash step.
- Updated guidance on the target concentration of Fixed RNA - Gene Expression Libraries.
- Updated Novaseq PhiX recommendations to 10%.

## General Changes

Updated for general minor consistency of language and terms throughout.

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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 Next GEM Single Cell Fixed RNA Sample Preparation Kit 16 rxns, PN-1000414 Store at -20°C			
		#	PN
●	Conc. Fix & Perm Buffer	3	2000517
●	Conc. Quench Buffer	6	2000516
●	Enhancer	1	2000482
10x GENOMICS			
















*\*The tube numbers have been updated in the 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

Chromium Next GEM Single Cell Fixed RNA Hybridization & Library Kit 4 rxns, PN-1000415 Store at -20°C				Chromium Next GEM Single Cell Fixed RNA Human Transcriptome Probe Kit 16 rxns, PN-1000420 Store at -20°C			
		#	PN			#	PN
	Hyb Buffer B	1	2000483		Human WTA Probes BC001	1	2000495
	Enhancer	1	2000482		Human WTA Probes BC002	1	2000496
	Conc. Post-Hyb Buffer	1	2000533		Human WTA Probes BC003	1	2000497
	Reducing Agent B	1	2000087		Human WTA Probes BC004	1	2000498
	GEM Enzyme Mix	1	2000490		Hyb Buffer B	1	2000485
	GEM Reagent Mix	1	2000491		Enhancer	2	2000482
	Pre-Amp Primers B	1	2000529		Conc. Post-Hyb Buffer	2	2000533
	Amp Mix	1	2000103				
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Chromium Next GEM Single Cell Fixed RNA Gel Bead Kit 4 rxns, PN-1000421 Store at -80°C		
	#	PN
Single Cell TL v1 Gel Beads (4 rxns)	1	2000538
10x GENOMICS		

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

Chromium Next GEM Single Cell Fixed RNA Hybridization & Library Kit 4 rxns, PN-1000415 Store at -20°C				Chromium Next GEM Single Cell Fixed RNA Mouse Transcriptome Probe Kit 16 rxns, PN-1000491 Store at -20°C			
		#	PN			#	PN
	Hyb Buffer B	1	2000483		Mouse WTA Probes BC001	1	2000703
	Enhancer	1	2000482		Mouse WTA Probes BC002	1	2000704
	Conc. Post-Hyb Buffer	1	2000533		Mouse WTA Probes BC003	1	2000705
	Reducing Agent B	1	2000087		Mouse WTA Probes BC004	1	2000706
	GEM Enzyme Mix	1	2000490		Hyb Buffer B	1	2000485
	GEM Reagent Mix	1	2000491		Enhancer	2	2000482
	Pre-Amp Primers B	1	2000529		Conc. Post-Hyb Buffer	2	2000533
	Amp Mix	1	2000103				
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Chromium Next GEM Single Cell Fixed RNA Gel Bead Kit 4 rxns, PN-1000421 Store at -80°C		
	#	PN
Single Cell TL v1 Gel Beads (4 rxns)	1	2000538
10x GENOMICS		



## Chromium Fixed RNA Kit, 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
 Reducing Agent B	1	2000087
 GEM Enzyme Mix	1	2000490
 GEM Reagent Mix	1	2000491
 Pre-Amp Primers B	1	2000529
 Amp Mix	1	2000103










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Chromium Next GEM  
Single Cell Fixed RNA Hybridization Kit  
64 rxns, PN-1000457  
Store at -20°C

	#	PN
 Hyb Buffer B	4	2000485
 Conc. Post-Hyb Buffer	8	2000533










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Chromium Next GEM  
Single Cell Fixed RNA Human  
Transcriptome Probe Kit  
64 rxns, Module 1 PN-1000456  
Store at -20°C

	#	PN
 Enhancer	4	2000482
 Human WTA Probes BC001	1	2000495
 Human WTA Probes BC002	1	2000496
 Human WTA Probes BC003	1	2000497
 Human WTA Probes BC004	1	2000498
 Human WTA Probes BC005	1	2000499
 Human WTA Probes BC006	1	2000500
 Human WTA Probes BC007	1	2000501
 Human WTA Probes BC008	1	2000502

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Chromium Next GEM  
Single Cell Fixed RNA Human  
Transcriptome Probe Kit  
64 rxns, Module 2 PN-1000456  
Store at -20°C

	#	PN
 Enhancer	4	2000482
 Human WTA Probes BC009	1	2000503
 Human WTA Probes BC010	1	2000504
 Human WTA Probes BC011	1	2000505
 Human WTA Probes BC012	1	2000506
 Human WTA Probes BC013	1	2000507
 Human WTA Probes BC014	1	2000508
 Human WTA Probes BC015	1	2000509
 Human WTA Probes BC016	1	2000510




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

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## 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
 Reducing Agent B	1	2000087
 GEM Enzyme Mix	1	2000490
 GEM Reagent Mix	1	2000491
 Pre-Amp Primers B	1	2000529
 Amp Mix	1	2000103










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Chromium Next GEM  
Single Cell Fixed RNA Hybridization Kit  
64 rxns, PN-1000457  
Store at -20°C

	#	PN
 Hyb Buffer B	4	2000485
 Conc. Post-Hyb Buffer	8	2000533










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Chromium Next GEM  
Single Cell Fixed RNA Mouse  
Transcriptome Probe Kit  
64 rxns, Module 1 PN-1000492  
Store at -20°C

	#	PN
 Enhancer	4	2000482
 Mouse WTA Probes BC001	1	2000703
 Mouse WTA Probes BC002	1	2000704
 Mouse WTA Probes BC003	1	2000705
 Mouse WTA Probes BC004	1	2000706
 Mouse WTA Probes BC005	1	2000707
 Mouse WTA Probes BC006	1	2000708
 Mouse WTA Probes BC007	1	2000709
 Mouse WTA Probes BC008	1	2000710

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Chromium Next GEM  
Single Cell Fixed RNA Mouse  
Transcriptome Probe Kit  
64 rxns, Module 2 PN-1000492  
Store at -20°C


	#	PN
 Enhancer	4	2000482
 Mouse WTA Probes BC009	1	2000711
 Mouse WTA Probes BC010	1	2000712
 Mouse WTA Probes BC011	1	2000713
 Mouse WTA Probes BC012	1	2000714
 Mouse WTA Probes BC013	1	2000715
 Mouse WTA Probes BC014	1	2000716
 Mouse WTA Probes BC015	1	2000717
 Mouse WTA Probes BC016	1	2000718


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Chromium Next GEM <b>Single Cell Fixed RNA Gel Bead Kit</b> 4 rxns, PN-1000421 <i>Store at -80°C</i>		
	#	PN
Single Cell TL v1 Gel Beads (4 rxns)	1	2000538
10x GENOMICS		

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

Chromium <b>Partitioning Oil</b> <i>Store at ambient temperature</i>			
	#	PN	
 Partitioning Oil	6	2000190	

Chromium <b>Recovery Agent</b> <i>Store at ambient temperature</i>			
	#	PN	
 Recovery Agent	6	220016	

Chromium Next GEM <b>Chip Q &amp; Gaskets</b> <i>Store at ambient temperature</i>			
	#	PN	
Next GEM Chip Q	6	2000518	
Chip Gasket, 6-pack	1	370017	



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

Chromium  
Partitioning Oil  
Store at ambient temperature

#

PN

Partitioning Oil

2

2000190

Chromium  
Recovery Agent  
Store at ambient temperature

#

PN

Recovery Agent

2

220016

Chromium Next GEM  
Chip Q & Gaskets  
Store at ambient temperature

#

PN

Next GEM Chip Q

2

2000518

Chip Gasket, 2-pack

1

3000072

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

Dual Index Kit TS Set A <i>Store at -20°C</i>		
	#	PN
Dual Index Plate TS Set A	1	3000511

## 10x Genomics Accessories

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

## Recommended Thermal Cyclers

The table below lists the thermal cyclers that have been validated by 10x Genomics. Thermal cyclers used must support uniform heating of 100 µl emulsion volumes

Supplier	Description	Part Number
BioRad	C1000 Touch Thermal Cycler with 96-Deep Well Reaction Module	1851197
Eppendorf	MasterCycler X50s	North America 6311000010
Analytik Jena	Biometra TAdvanced 96 SG with 96-well block (silver, 0.2 mL) and gradient function	846-x-070-241

If using thermal cyclers other than BioRad C1000, the ramp rates should be adjusted for all the steps as described below

- Eppendorf MasterCycler X50s: 3°C/sec heating and 2°C/sec cooling
- Analytik Jena Biometra TAdvanced: 2°C/sec heating and cooling

## Additional Kits, Reagents & Equipment

The listed items have been tested by 10x Genomics and perform optimally with the assay. Substituting materials may adversely affect system performance. This list does not include standard laboratory equipment, such as water baths, pH meters, freezers, etc.

Supplier	Description		Part Number
Plastics			
Eppendorf	DNA LoBind Tubes, 1.5 ml		022431021
	DNA LoBind Tubes, 5.0 ml		0030108310
	PCR Tubes 0.2 ml 8-tube strips	Choose either Eppendorf, USA Scientific or Thermo Fisher Scientific PCR 8-tube strips.	951010022
USA Scientific	TempAssure PCR 8-tube strip		1402-4700
Thermo Fisher Scientific	MicroAmp 8-Tube Strip, 0.2 ml		N8010580
	MicroAmp 8-Cap Strip, clear		N8010535
Corning	Corning Centrifuge Tubes with CentriStar Cap (15 ml), sterile		430790
	Self-Standing Polypropylene Centrifuge Tubes (50 ml), sterile		430921
Sysmex	Sterile single-pack CellTrics filters	Choose either Sysmex or Miltenyi Biotec filter.	04-004-2326
Miltenyi Biotec	Pre-Separation Filters (30 µm) <i>Alternative to Sysmex product</i>		130-041-407
Kits & Reagents			
Thermo Fisher Scientific	Nuclease-free Water		AM9937
	Tween 20 Surfact-Amps Detergent Solution (10% solution)		28320
Beckman Coulter	SPRIselect Reagent Kit		B23318
Millipore Sigma	Ethyl Alcohol, Pure (200 Proof, anhydrous)		E7023-500ML
	Glycerol for Molecular biology, ≥99.0%		G5516
Acros Organics	Glycerol, 99.5%, for molecular biology, DNase, RNase and Protease free <i>Alternative to Millipore Sigma product</i>		327255000
Qiagen	Qiagen Buffer EB		19086
Ricca Chemical Company	Glycerin (glycerol), 50% (v/v) Aqueous Solution		3290-32
Cell Counting			
Thermo Fisher Scientific	Ethidium Homodimer-1	Choose Ethidium Homodimer-1, Trypan Blue, or PI Staining Solution based on the presence of debris in the sample.	E1169
	Trypan Blue Stain (0.4%)		T10282
	Countess II FL Automated Cell Counter <i>Discontinued</i>		AMAQAF1000
	Countess II FL Automated Cell Counting Chamber Slides		C10228
Nexcelom Biosciences	Cellaca MX High-throughput Automated Cell Counter		MX-112-0127
	ViaStain PI Staining Solution		CS1-0109-5mL
	ViaStain AO/PI Staining Solution <i>Alternative to PI Staining Solution</i>		CS2-0106-5mL

Equipment		
VWR	VWR Mini Centrifuge <i>Or any equivalent mini centrifuge</i>	76269-064
	Divided Polystyrene Reservoirs	41428-958
	Vortex Mixer	10153-838
Eppendorf	Eppendorf ThermoMixer C	5382000023
	Eppendorf ThermoTop with condens.protect technology	5308000003
	Eppendorf SmartBlock 1.5 ml, Thermoblock for 24 reaction vessels	5360000038
	Eppendorf SmartBlock 2.0 ml, thermoblock for 24 reaction vessels	5362000035
Quantification & Quality Control		
Agilent	2100 Bioanalyzer Laptop Bundle	G2953CA
	High Sensitivity DNA Kit	5067-4626
	4200 TapeStation	G2991AA
	High Sensitivity D5000: ScreenTape/ Reagents	5067-5592/ 5067-5593
	High Sensitivity D1000: ScreenTape/ Reagents	5067-5584/ 5067-5585
PerkinElmer	LabChip GX Touch HT Nucleic Acid Analyzer	CLS137031
	DNA High Sensitivity Reagent Kit	CLS760672
KAPA Biosystems	KAPA Library Quantification Kit for Illumina Platforms	KK4824

This list does not include reagents required for sample fixation. See [Sample Preparation on page 27](#) for the key reagents needed for fixation and consult Demonstrated Protocols CG000478 and CG000553 for a complete list of reagents for sample fixation and storage.








## Recommended Pipette Tips

10x Genomics recommends using only validated emulsion-safe pipette tips for all Single Cell protocols. Rainin pipette tips have been extensively validated by 10x Genomics and are highly recommended for all single cell assays. If Rainin tips are unavailable, any of the listed alternate pipette tips validated by 10x Genomics may be used.

Supplier	Description	Part Number (US)
<b>Recommended Pipettes &amp; Pipette tips</b>		
Rainin	<b>Pipettes</b>	
	Pipet-Lite Multi Pipette L8-50XLS+	17013804
	Pipet-Lite Multi Pipette L8-200XLS+	17013805
	Pipet-Lite Multi Pipette L8-10XLS+	17013802
	Pipet-Lite Multi Pipette L8-20XLS+	17013803
	Pipet-Lite LTS Pipette L-2XLS+	17014393
	Pipet-Lite LTS Pipette L-10XLS+	17014388
	Pipet-Lite LTS Pipette L-20XLS+	17014392
	Pipet-Lite LTS Pipette L-100XLS+	17014384
	Pipet-Lite LTS Pipette L-200XLS+	17014391
	Pipet-Lite LTS Pipette L-1000XLS+	17014382
	<b>Pipette Tips</b>	
	Tips LTS 200UL Filter RT-L200FLR	30389240
	Tips LTS 1ML Filter RT-L1000FLR	30389213
	Tips LTS 20UL Filter RT-L10FLR	30389226
<b>Alternate Recommendations</b> (If Rainin pipette tips are unavailable, any of the listed pipette tips may be used)		
Eppendorf	<b>Pipettes</b>	
	Eppendorf Research Plus, 8-channel, epT.I.P.S. Box, 0.5 – 10 µl	3125000010
	Eppendorf Research Plus, 8-channel, epT.I.P.S. Box, 10 – 100 µl	3125000036
	Eppendorf Research Plus, 8-channel, epT.I.P.S. Box, 30 – 300 µl	3125000052
	Eppendorf Research Plus, 1-channel, epT.I.P.S.® Box, 0.1 – 2.5 µl	3123000012
	Eppendorf Research Plus, 1-channel, epT.I.P.S.® Box, 0.5 – 10 µl	3123000020
	Eppendorf Research Plus, 1-channel, epT.I.P.S.® Box, 2 – 20 µl	3123000039
	Eppendorf Research Plus, 1-channel, epT.I.P.S.® Box, 2 – 200 µl	3123000055
	Eppendorf Research Plus, 1-channel, epT.I.P.S.® Box, 100 – 1000 µl	3123000063

Supplier	Description	Part Number (US)
Labcon*	<b>Pipette Tips</b> (compatible with Eppendorf pipettes only)	
	ep Dualfilter T.I.P.S., 2-20 µl	0030078535
	ep Dualfilter T.I.P.S., 2-200 µl	0030078551
	ep Dualfilter T.I.P.S., 2-1,000 µl	0030078578
	ZAP SLIK 20 µL Low Retention Aerosol Filter Pipet Tips for Rainin LTS	4-1143-965-008
	ZAP SLIK 200 µL Low Retention Aerosol Filter Pipet Tips for Rainin LTS	4-1144-965-008
Biotix*	ZAP SLIK 1200 µL Low Retention Aerosol Filter Pipet Tips for Rainin LTS	4-1145-965-008
	xTIP4 Racked Pipette Tips, Rainin LTS Pipette Compatible, 0.1-20 µl	63300931
	xTIP4 Racked Pipette Tips, Rainin LTS Pipette Compatible, 200 µl	63300001
	xTIP4 Racked Pipette Tips, Rainin LTS Pipette Compatible, 1200 µl	63300004
*Compatible with Rainin pipettes		

## Protocol Steps & Timing

Steps	Timing	Stop & Store	
<b>Sample Fixation</b>	2-3 h		4°C ≤1 week/-80°C ≤6 months (fixed single cells/nuclei/tissue pieces/cells isolated from FFPE tissue sections)
<b>Step 1: Probe Hybridization (page 45)</b>			
1.1 Probe Hybridization (page 49)	16-24 h		
<b>Step 2: GEM Generation and Barcoding (page 51)</b>			
2.1 Post-Hybridization Pool & Wash (page 54)	60-90 min		-80°C ≤6 months
2.2 Prepare GEM Master Mix + Sample Dilution (page 61)	30 min		
2.3 Load Chromium Next GEM Chip Q (page 66)	10 min		
2.4 Run the Chromium X/iX (page 68)	5.5 min		
2.5 Transfer GEMs (page 69)	5 min		
2.6 GEM Incubation (page 70)	125 min		4°C ≤1 week (GEMs) -80°C ≤6 months (washed undiluted sample)
<b>Step 3: GEM Recovery and Pre-Amplification (page 71)</b>			
3.1 Post-GEM Incubation – Recovery (page 73)	10 min		
3.2 Pre-Amplification PCR (page 74)	55 min		4°C ≤72 h/-20°C ≤1 week
3.3 DNA Cleanup – SPRIselect (page 75)	30 min		4°C ≤72 h/-20°C ≤4 weeks
<b>Step 4: Library Construction (page 76)</b>			
4.1 Sample Index PCR (page 78)	40 min		4°C ≤72 h
4.2 Post Sample Index PCR Size Selection – SPRIselect (page 80)	30 min		4°C ≤72 h or -20°C long-term
4.3 Post Library Construction QC (page 81)	60 min		



## Stepwise Objectives

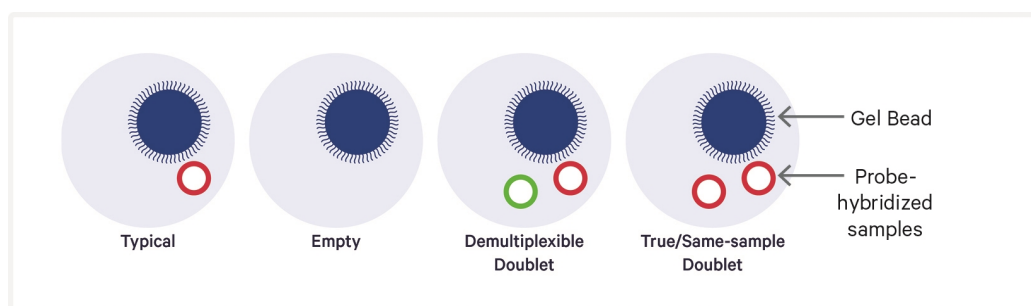
Chromium Fixed RNA Profiling offers comprehensive, scalable solutions to measure gene expression in formaldehyde fixed samples. Gene expression is measured using probe pairs designed to hybridize to mRNA specifically. Using a microfluidic chip, the fixed and probe-hybridized single cell and nuclei suspensions are 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.

### 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.

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.

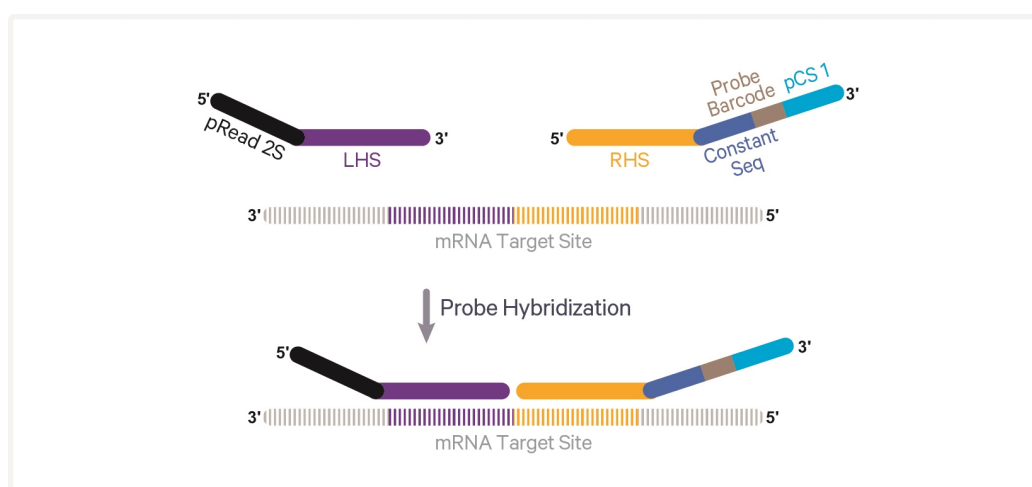
## Sample Fixation for Chromium Fixed RNA Profiling

Samples are fixed in a 4% formaldehyde fixative solution, as described in the Demonstrated Protocols CG000478 and CG000553 and using Chromium Next GEM Single Cell Fixed RNA Sample Preparation Kit (10x Genomics PN-1000414). Fixed samples are taken through a series of steps to generate Fixed RNA – Gene Expression libraries. A high-level overview of each step in the protocol, including gene expression library construction, is provided in the following sections.

### Step 1: Probe Hybridization

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

#### Probe Hybridization

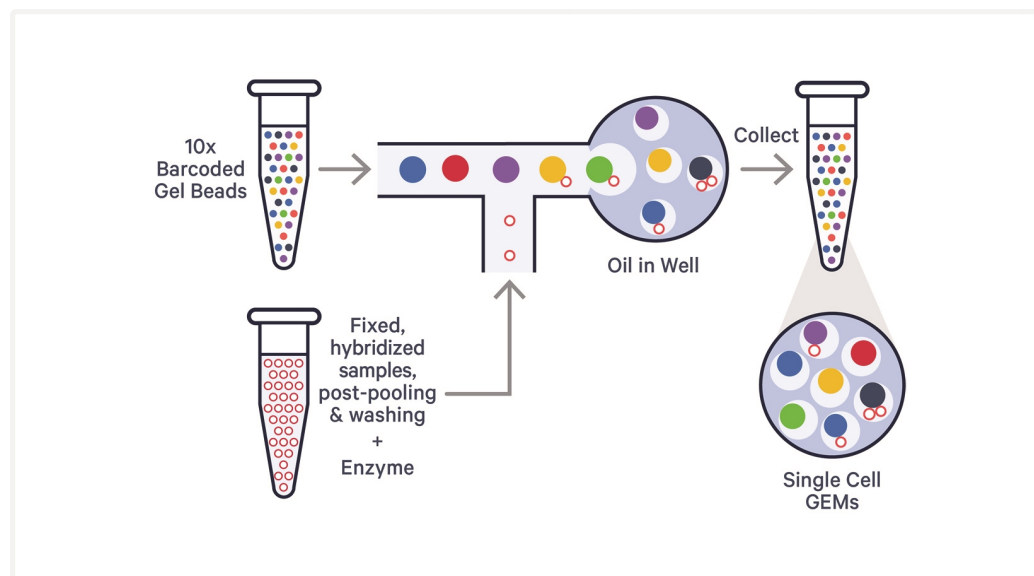


### Step 2: GEM Generation & Barcoding

After hybridization, the samples containing different Probe Barcodes are pooled together and the unbound probes are washed off. After pooling and

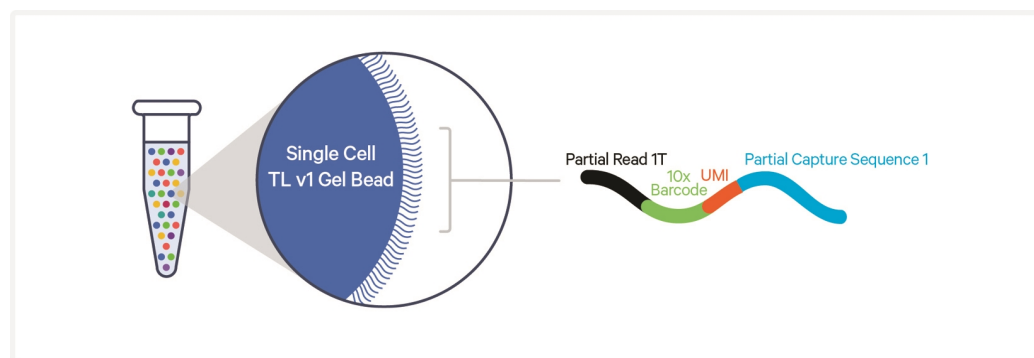
washing the samples, GEMs are generated by combining barcoded Gel Beads, a Master Mix containing pooled cells, and Partitioning Oil onto Chromium Next GEM Chip Q.

### 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 an Illumina TruSeq Read 1 sequence (Read 1T, read 1 sequencing primer), a 16 nt 10x GEM Barcode (or 10x Barcode), a 12 nt unique molecular identifier (UMI), and partial Capture Sequence 1 (sequence complementary to the probe).

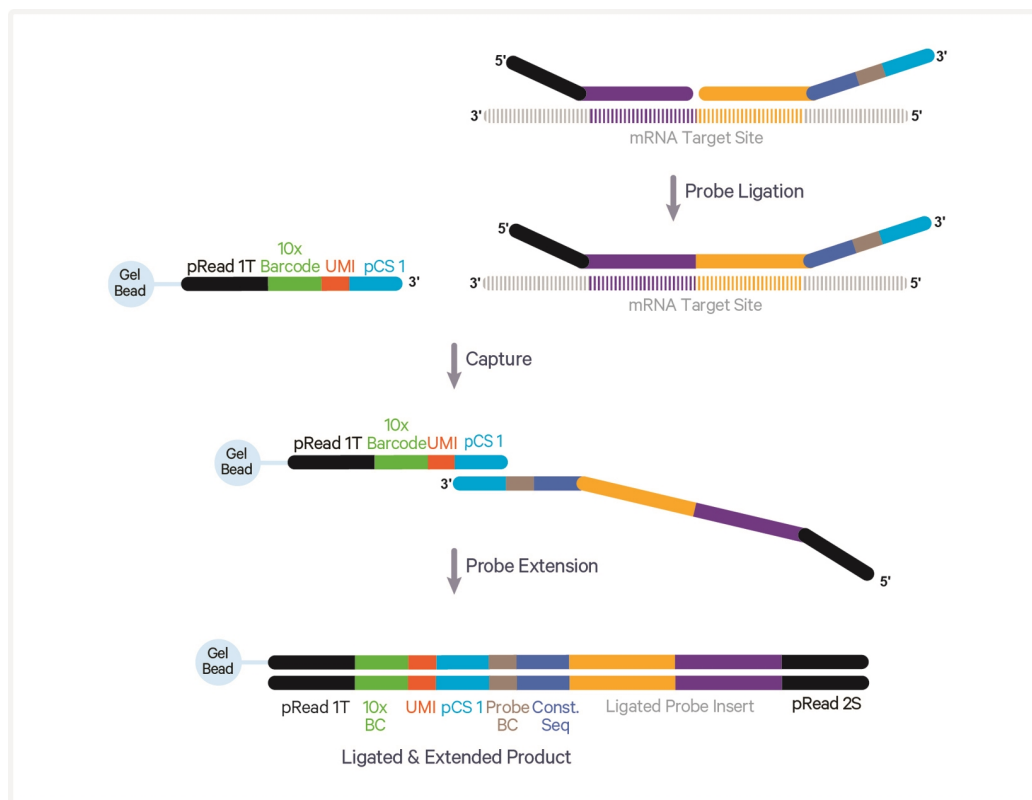
### Gel Bead



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

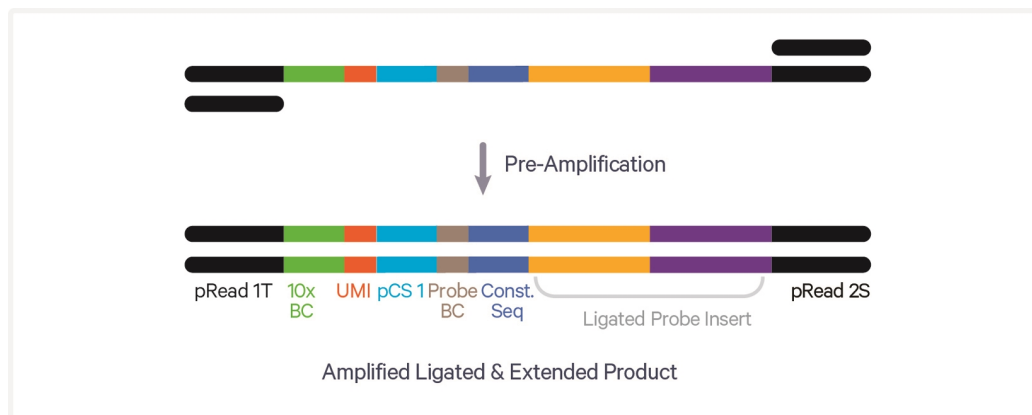
Finally, a heat denaturation step inactivates the enzymes in the GEM reaction.

#### Inside Individual GEMs



## Step 3: GEM Recovery & Pre-Amplification

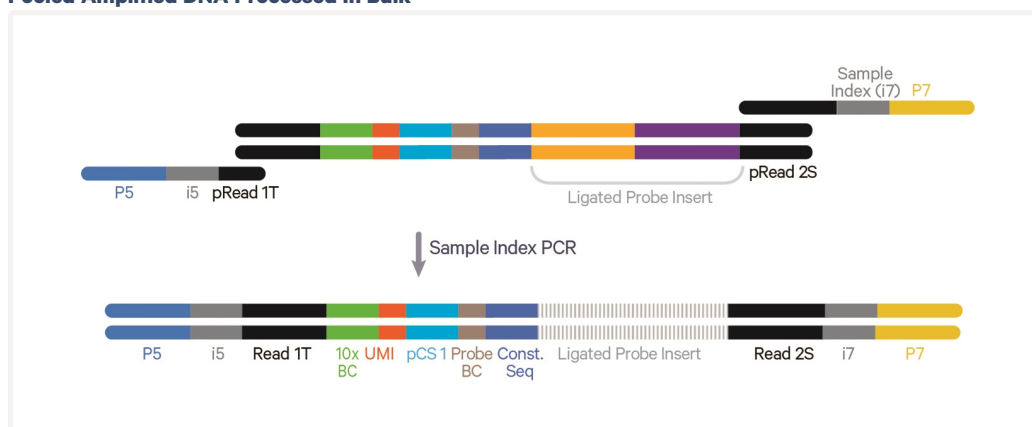
Once the ligation and barcoding steps are completed, the GEMs are broken by the addition of Recovery Agent, inverting the mixture, and removing the Recovery Agent. A PCR master mix is added directly to the post-GEM aqueous phase to pre-amplify the ligated product. The pre-amplified products are then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

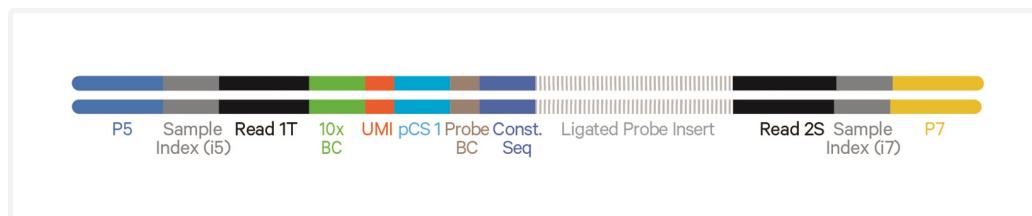
P5, P7, i5 and i7 sample indexes, and Illumina Small Read 2 (Read 2S) sequences are added via Sample Index PCR. The final libraries contain the P5 and P7 priming sites used in Illumina sequencers.

**Pooled Amplified DNA Processed in Bulk**

## Step 5: Sequencing

A Chromium Fixed RNA Profiling – Gene Expression library comprises standard Illumina paired-end constructs which begin and end with P5 and P7. The 16 bp 10x GEM Barcode and 12 bp UMI are encoded in Read 1T. Small RNA Read 2 (Read 2S) sequences the ligated probe insert, 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 single library contains reads derived from up to 16 samples, with cell barcodes composed of a Probe Barcode and a 10x GEM Barcode (10x Barcode).

See Appendix for [Oligonucleotide Sequences on page 99](#)

## Sample Preparation

Chromium Fixed RNA Profiling is compatible with fixed samples and cells derived from FFPE tissue sections.

### Sample Fixation

**Fixation of cells or nuclei suspensions:** Cells and nuclei suspensions are fixed in a 4% formaldehyde fixative solution and then quenched. Consult the Demonstrated Protocol Fixation of Cells & Nuclei for Chromium Fixed RNA Profiling (CG000478) for details.

**Fixation and dissociation of tissues:** Fresh or flash frozen tissue samples are first fixed and then dissociated. Consult the Demonstrated Protocol Tissue Fixation & Dissociation for Chromium Fixed RNA Profiling (CG000553) for details.

Chromium Next GEM Single Cell Fixed RNA Sample Preparation Kit (10x Genomics PN-1000414) is required for fixation. Fixed samples can be stored short-term at 4°C or long-term at -80°C.

#### Key Reagents for Sample Fixation

Vendor	Description	Part Number
10x Genomics	Chromium Next GEM Single Cell Fixed RNA Sample Preparation Kit, 16 rxns	1000414
Thermo Fisher Scientific	Formaldehyde (37% by Weight/Molecular Biology), Fisher BioReagents	BP531-500

*Consult the appropriate Demonstrated Protocols for a complete list of reagents.*

### Isolation of Cells from FFPE Tissue Sections

Cells isolated from formaldehyde fixed & paraffin embedded (FFPE) tissue sections can also be used for Chromium Fixed RNA Profiling. If using cells derived from the FFPE tissue sections, consult Isolation of Cells from FFPE Tissue Sections for Chromium Fixed RNA Profiling (Demonstrated Protocol CG000632). Fixed samples (dissociated cells resuspended in appropriate buffer) can be stored for short-term at 4°C or long-term at -80°C.

## 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. In addition, other configurations are also compatible.

- Sample Sub-pooling: Sub-pooling one sample over multiple Probe Barcodes will allow the capture of more cells from that sample, with a lower undetected multiplet rate
- See Appendix for other [Alternate Multiplexing Configurations & Pooling Strategies on page 95](#).



*This illustration provides an overview of the various multiplexing configurations using the human/mouse 4 rxns x 4 BC kits (PN-1000475 or PN-1000496). The same principle can be applied when using the human/mouse 4 rxns x 16 BC kits (PN-1000476 or PN-1000497).*



## 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 guidance



Signifies critical step requiring accurate execution



Troubleshooting section includes additional guidance



Chip Q specific steps

## Emulsion-safe Plastics

Use validated emulsion-safe plastic consumables when handling GEMs as some plastics can destabilize GEMs.

## 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 or 100,000 nuclei per hybridization and the maximum input is  $2 \times 10^6$  cells/nuclei per hybridization.
- During post-hybridization washing steps, some cell loss is expected. It is recommended to start the hybridization reaction with  $\sim 1 \times 10^6$  cells/nuclei per hybridization, if possible.

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

## Incubation Time

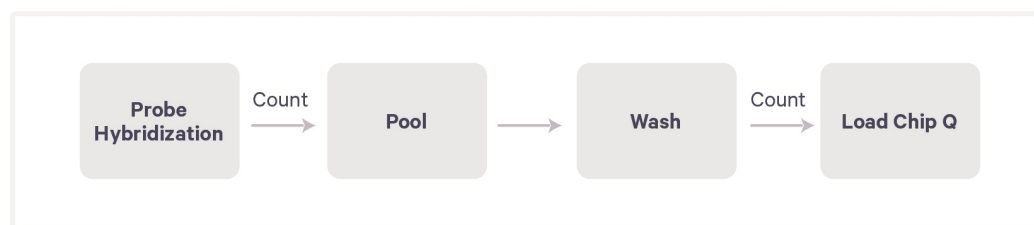
- Recommended incubation time for probe hybridization is 16-24 h.
- Incubation time should be of same length for all samples. DO NOT mix samples with different hybridization times in one experiment.

## 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**).

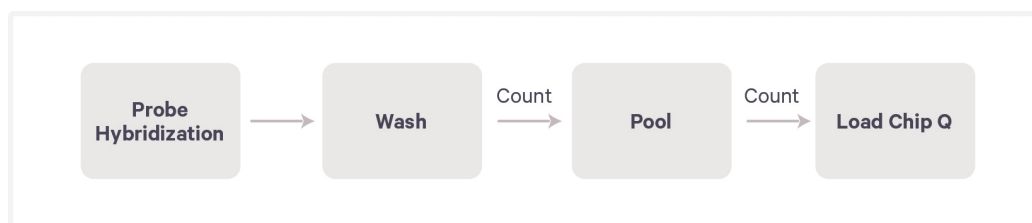
The Chromium Fixed RNA Profiling Reagent Kits contain sufficient buffer volumes to complete either workflow with no impact on the data quality.

### 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 54 for Pooled Wash workflow.

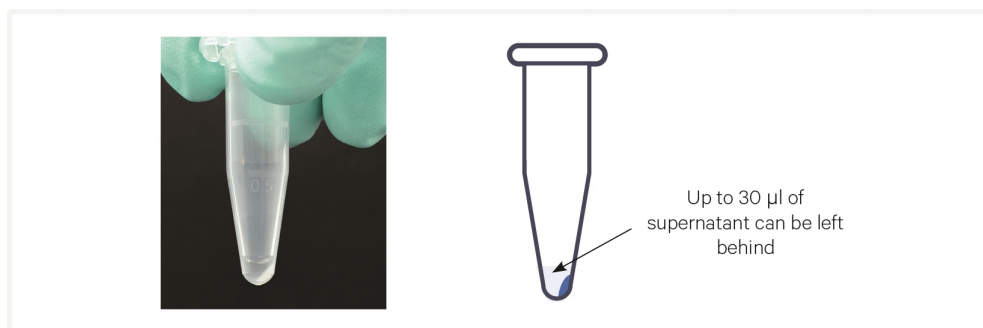
## Individual Wash Workflow



- 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 91.

## 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  $\mu$ 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 Multiplet Rate (%)	Cells Loaded/ Probe Barcode	Cells Recovered/ Probe Barcode	Cells Equally Distributed on 4 Probe Barcodes		Cells Equally Distributed on 16 Probe Barcodes	
			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.

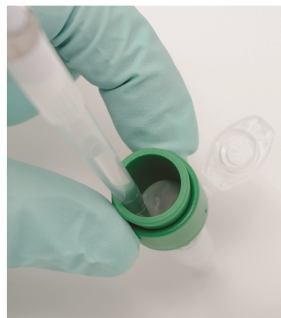
## Sample Filtration

- 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.

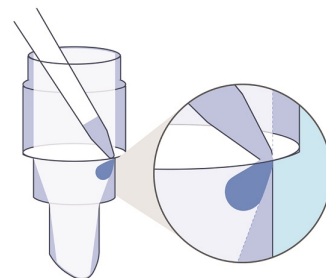


DO NOT use 40 μm Flowmi Tip Strainer for filtration.

- 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.

Filtration using 30  $\mu$ m FiltersPre-Separation Filters from  
Miltenyi Biotec

Celltrix Filters from Sysmex

Touch the filter membrane  
with the tip where the filter  
meets the wallPre-Separation Filters from  
Miltenyi Biotec

Celltrix Filters from Sysmex

## Cell Counting

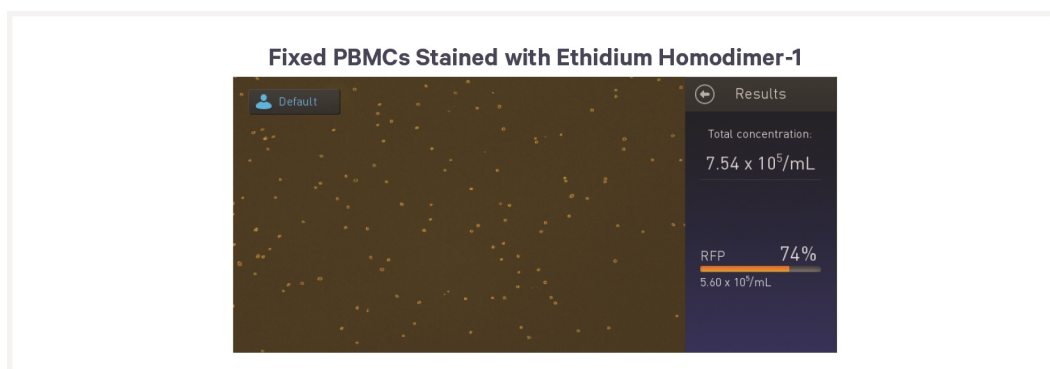
- Accurate counting is critical for optimal assay performance.
- It is strongly recommended that the sample be stained with a fluorescent dye such as Ethidium Homodimer-1 or PI staining solution and counted using an automated fluorescent cell counter (Countess II Automated Cell Counter or a Cellaca Counter).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

### Counting using Ethidium Homodimer-1

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

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





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

## Counting using PI Staining Solution

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

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

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

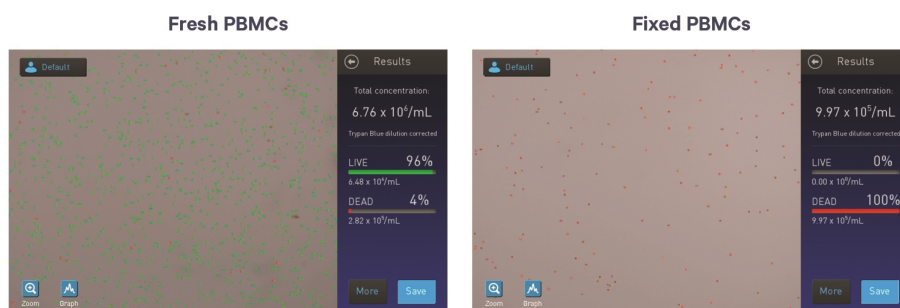
## Counting using Trypan Blue (Only for Debris-Free Samples)

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

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

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

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





## Chromium Next GEM Chip Handling

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

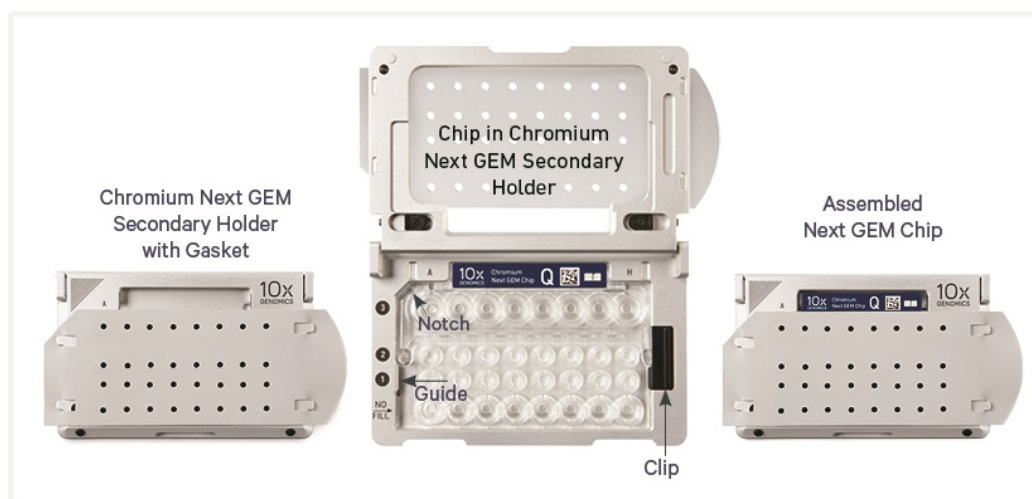
## Chromium Next GEM Secondary Holders



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

## Chromium Next GEM Chip & Holder Assembly with Gasket

- Chromium Next GEM Chip Q is only compatible with Chromium Next GEM Secondary Holder (PN-3000332). DO NOT use any other holder
- Close the holder lid. Attach the gasket by holding the tongue (curved end, to the right) and hook the gasket on the left-hand tabs of the holder. Gently pull the gasket toward the right and hook it on the two right-hand tabs.
- DO NOT touch the smooth side of the gasket.
- Open the chip holder.
- Align notch on the chip (upper left corner) and the open holder with the gasket attached.
- Slide the chip to the left until the chip is inserted under the guide on the holder. Depress the right hand side of the chip until the spring-loaded clip engages.
- Keep the assembled unit with the attached gasket until ready for dispensing reagents into the wells.





## Chromium Next GEM Chip Loading

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



- Refer to [2.3 Load Chromium Next GEM Chip Q](#) on page 66 for specific instructions.

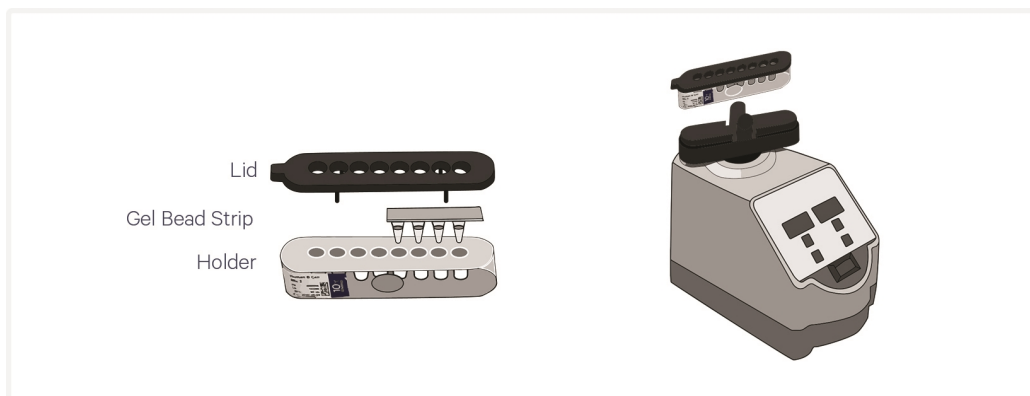
## 50% Glycerol Solution for Addition to Unused Chip Wells

- 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

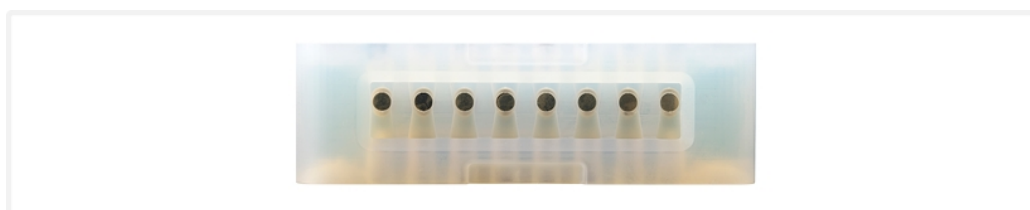
- Use nuclease-free water and molecular biology grade Glycerol from Millipore Sigma, PN-G5516, to prepare 50% glycerol solution as described previously. DO NOT use 50% glycerol solution from Ricca Chemical Company, Glycerin (glycerol), 50% (v/v) Aqueous Solution, PN-3290-32.

## 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.

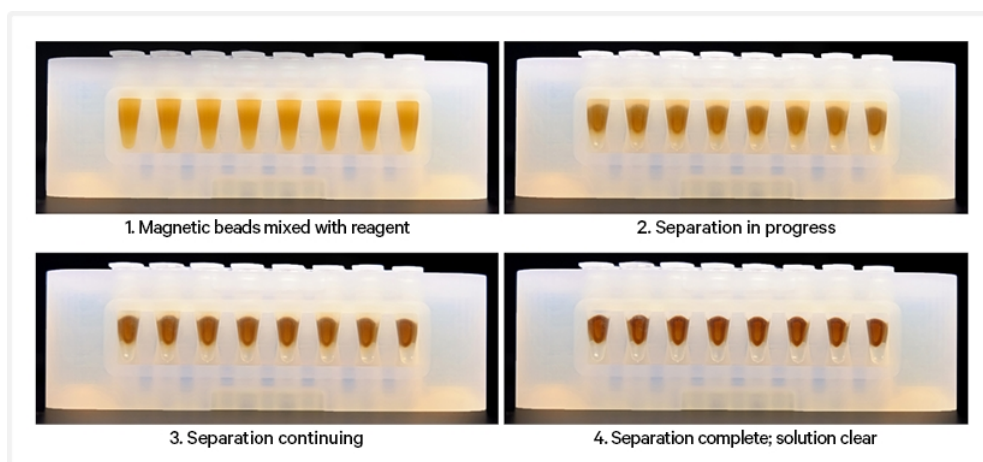
## 10x Magnetic Separator



- 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).

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

## Sample Indices in Sample Index PCR

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









# Step 1:

## Probe Hybridization

1.0 Get Started	46
Probe Hybridization Guidelines for Multiplexing	47
1.1 Probe Hybridization	49



## 1.0 Get Started

Action	Item	10x PN	Preparation & Handling	Storage
<b>Thaw &amp; Keep Warm</b>				
<input type="checkbox"/>	 <b>Hyb Buffer B</b>	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
<input type="checkbox"/>	 <b>Enhancer</b>	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
<b>Place on Ice</b>				
<input type="checkbox"/>	 <b>Fixed Cell Suspension</b>	—	Consult Demonstrated Protocols: • Fixation of Cells & Nuclei (CG000478) • Tissue Fixation & Dissociation (CG000553). • Isolation of Cells from FFPE Tissue Sections (CG000632)	—
<input type="checkbox"/>	 <b>Human WTA Probes BC001-BC016</b> OR <b>Mouse WTA Probes BC001-BC016</b>	2000495- 2000510  2000703- 2000718	Thaw on ice. Vortex and centrifuge briefly.	-20°C

## Probe Hybridization Guidelines for Multiplexing

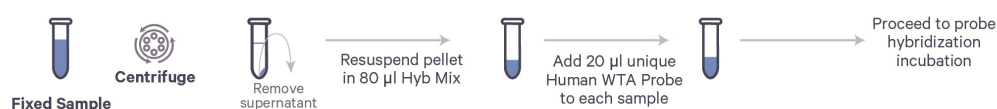
### Read these guidelines before starting probe hybridization

To generate Fixed RNA – Gene Expression libraries, fixed single cell/nuclei suspensions are mixed with probes, and hybridized overnight (16-24 h) at 42°C.

### Number of samples is 4 or 16

Add a unique sample to a unique Probe Barcode.

#### Unique Sample to Unique Probe Barcode

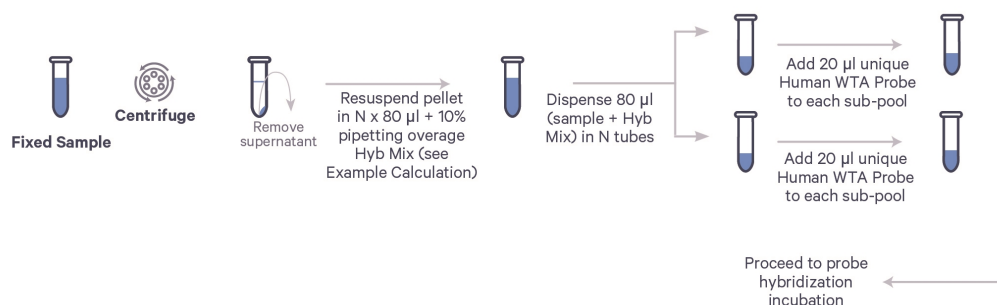


### Number of samples is <4 or <16

Choose one or more sample and divide the sample/s into up to 4 or 16 sub-pools by resuspending the pellet in an appropriate volume Hyb Mix ( $80 \mu\text{l} \times \text{Number of sub-pools (N)} + 10\%$ , see Example Calculation on the next page) and dispensing  $80 \mu\text{l}$  in N individual tubes. Each sub-pool is then hybridized with a unique Probe Barcode. These uniquely barcoded sub-pools can be bioinformatically combined to form a single sample in Cell Ranger analysis. This strategy allows an increase in the final cell load of a particular sample.

#### Same Sample across Multiple Probe Barcodes (Sample Sub-pooling)

Decide the number (N) of sub-pools needed for each sample



## Hyb Mix Volume When Sub-pooling

### Example Calculation of Hyb Mix when # of Sub-pools=2

Number of Sub-pools: **2**

#### Volume of Hyb Mix for N=2 Sub-pools

$$\begin{aligned} &= \text{Volume of Hyb Mix for 1 reaction} * \text{Number of sub-pools} \\ &= 80 * 2 = \mathbf{160\ \mu l} \end{aligned}$$

#### Overage Volume for 10% Excess

$$= \frac{160\ \mu l \times 10}{100} = \mathbf{16\ \mu l}$$

#### Final Volume of Hyb Mix for N=2 Sub-pools

$$= 160 + 16 = \mathbf{176\ \mu l}$$

### Example Calculation of Hyb Mix when # of Sub-pools=8

Number of Sub-pools: **8**

#### Volume of Hyb Mix for N=8 Sub-pools

$$\begin{aligned} &= \text{Volume of Hyb Mix for 1 reaction} * \text{Number of sub-pools} \\ &= 80 * 8 = \mathbf{640\ \mu l} \end{aligned}$$

#### Overage Volume for 10% Excess

$$= \frac{640\ \mu l \times 10}{100} = \mathbf{64\ \mu l}$$

#### Final Volume of Hyb Mix for N=8 Sub-pools

$$= 640 + 64 = \mathbf{704\ \mu l}$$

*Minimum number of cells needed for hybridization is 50,000 per hybridization reaction. If sub-pooling a sample, adjust the number of cells added for that sample. For example, 110,000 cells would be required if the number of sub-pools is 2 (2\*50,000 + 10%).*

## 1.1 Probe Hybridization





Before starting this protocol, ensure that samples have been appropriately fixed and quenched. Consult Fixation of Cells & Nuclei for Chromium Fixed RNA Profiling (Demonstrated Protocol CG000478) for details.

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

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

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

Hyb Mix	PN	1X* (µl)	1X* + 20% (µl)	4X* + 20% (µl)	16X* + 20% (µl)
<i>Add reagents in the order listed</i>					
 <b>Hyb Buffer B</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
 <b>Enhancer</b> 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
<b>Total</b>	-	<b>80.0</b>	<b>96.0</b>	<b>384.0</b>	<b>1536.0</b>

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

Sample Type	Recommended #
Fixed cells	<b>50,000-2 x 10<sup>6</sup></b>
Fixed nuclei	<b>100,000-2 x 10<sup>6</sup></b>
FFPE dissociated suspension	<b>100,000-2 x 10<sup>6</sup></b>



*DO NOT exceed  $2 \times 10^6$  cells in one hybridization reaction. Note that  $>2 \times 10^6$  cells can be centrifuged if planning to follow the sub-pooling guidelines and divide sample into  $<2 \times 10^6$  cells per 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  $\mu$ l of supernatant may be left behind to optimize cell recovery without significantly impacting assay performance.*



- f. Resuspend each pellet in **80  $\mu$ l** 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.

*\*If a single sample is to be run with multiple Probe Barcodes to increase the total number of cells loaded for that sample (Sample Sub-pooling), resuspend pellet in an appropriate volume Hyb Mix. See the [Probe Hybridization Guidelines for Multiplexing on page 47](#) for guidance on the volume of Hyb Mix needed when sub-pooling.*

- g. Add **20  $\mu$ l** unique single Human/Mouse WTA Probes BC001-BC016 (PN-2000495-2000510 or PN-2000703-2000718) to the **80  $\mu$ l** mixture of Hyb Mix and fixed sample and gently pipette mix 10x with pipette set at 80  $\mu$ l. 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. If sub-pooling the samples, dispense the 80  $\mu$ l sample + Hyb Mix to different tubes before adding the probes.*

- h. 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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## 2.0 Get Started



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

Action	Item	10x PN	Preparation & Handling	Storage
<b>Equilibrate to Room Temperature</b>				
<input type="checkbox"/>	<b>Single Cell TL v1 Gel Beads</b>	2000538	Equilibrate to room temperature 30 min before loading the chip.	–80°C
<input type="checkbox"/>	 <b>Reducing Agent B</b>	2000087	Vortex, verify no precipitate, centrifuge briefly.	–20°C
<b>Thaw &amp; Keep Warm</b>				
<input type="checkbox"/>	 <b>Enhancer</b>	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
<b>Place on Ice</b>				
<input type="checkbox"/>	 <b>Conc. Post-Hyb Buffer</b>	2000533	Thaw at room temperature and keep on ice.	–20°C
<input type="checkbox"/>	 <b>GEM Enzyme Mix</b>	2000490	Centrifuge briefly before adding to the mix.	–20°C
<input type="checkbox"/>	 <b>GEM Reagent Mix</b>	2000491	Thaw at room temperature. Vortex, verify no precipitate, centrifuge briefly. Keep on ice.	–20°C
<b>Obtain</b>				
<input type="checkbox"/>	 <b>Partitioning Oil</b>	2000190	—	Ambient
<input type="checkbox"/>	<b>Next GEM Chip Q</b>	2000518	See Tips & Best Practices.	Ambient
<input type="checkbox"/>	<b>Chromium Next GEM Secondary Chip Holder</b>	3000332	See Tips & Best Practices.	Ambient
<input type="checkbox"/>	<b>10x Gasket</b>	370017/ 3000072	See Tips & Best Practices.	Ambient



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

## 2.1 Post-Hybridization 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 32](#) 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 91](#).

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

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

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

*\*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 µl** 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 36](#). 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:* Nuclei samples or other 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 59](#) 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 µl** 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 36](#). 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:* Nuclei samples or other 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 59](#) 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 33](#).
- g. Resuspend cell pellet in **1 ml** Post-Hyb Wash Buffer and transfer to a 1.5-ml 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.
- l. 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 <i>Add reagents in the order listed</i>	PN	1 Pool + 10% ( $\mu$ l)	4 Pools + 10% ( $\mu$ l)
<b>Nuclease-free Water</b>	-	1567.5	6270.0
 <b>Conc. Post-Hyb Buffer</b>	2000533	82.5	330.0
<b>Total</b>	-	<b>1650.0</b>	<b>6600.0</b>

- 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 <sup>6</sup>	500
1 x 10 <sup>6</sup> -4 x 10 <sup>6</sup>	750
5 x 10 <sup>6</sup> -8 x 10 <sup>6</sup>	1,000
9 x 10 <sup>6</sup> -12 x 10 <sup>6</sup>	1,250
13 x 10 <sup>6</sup> -16 x 10 <sup>6</sup>	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 34](#) 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 36](#). 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 μl (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 61](#) for details.

### **Sample Storage**

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

### **Using Stored Samples**

- When ready to use samples stored at -80°C from this step, thaw at room temperature until no ice remains and then continue from **2.1q** (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 =  $\frac{\text{Cells per Sample Added to the Pool}}{\text{Post-Hybridization Cell Conc.}}$

### 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 Barcode	Post-Hybridization Cell Conc.	Total Cells in Hybridization	Cells per Sample Added to the Pool	Sample Volume to be Added (µl)
	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

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

GEM Master Mix		PN	1X* (μl)	4X* + 10% (μl)
Add reagents in the order listed				
	GEM Reagent Mix	2000491	20.9	92.1
	Reducing Agent B	2000087	1.7	7.3
	GEM Enzyme Mix	2000490	12.4	54.6
Total		–	35.0	154.0

\*1X = 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 70](#).*

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

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

# Cell Suspension Volume Calculator for Multiplexing 4 Samples

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

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

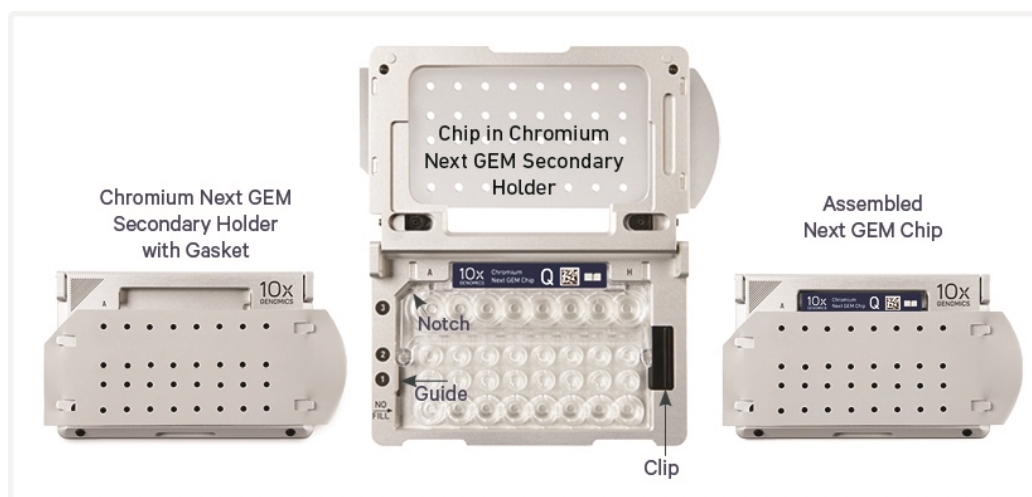
## Assemble Chromium Next GEM Chip Q



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



See [Tips & Best Practices on page 30](#) for chip handling instructions.



- 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.
- Remove the chip from the sealed bag. Use the chip within  $\leq 24$  h.
- 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.

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



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

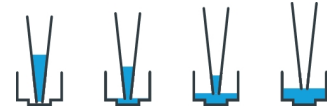


## 2.3 Load Chromium Next GEM Chip Q

Chip loading instructions are unique to Chip Q.

### TIPS

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



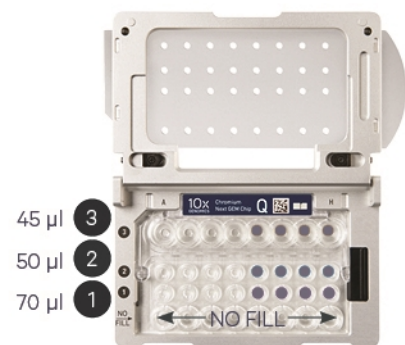
### a. Add 50% glycerol solution to each unused well

- **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.*

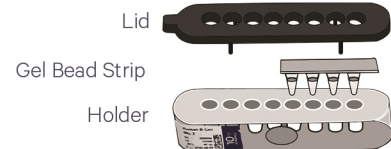
### Glycerol



### 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



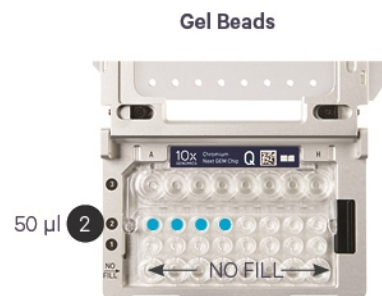
- With pipette set to 70 µl, gently **pipette mix** the GEM Master Mix + Sample 15x.
- Using the same pipette tips, dispense **70 µl** GEM Master Mix + Sample into the bottom center of wells in **row labeled 1** without introducing bubbles.

### Master Mix + Sample + Buffer



**d. Load Row Labeled 2**

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

**e. Load Row Labeled 3**

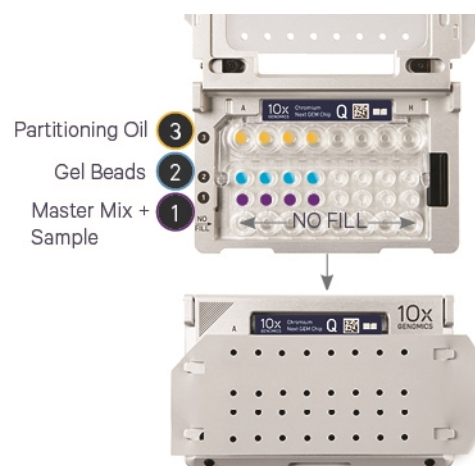
- Dispense **45  $\mu$ l** Partitioning Oil into the wells in **row labeled 3** from a reagent reservoir.

*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  $\mu$ 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  $\mu$ l but <100  $\mu$ l of GEMs. Though the cell recovery efficiency might be slightly reduced, it's recommended to carry forward with library preparation and sequencing to recover information from the rest of the sample.*



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



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

## 2.6 GEM Incubation

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

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

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

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



**DO NOT store the GEMs at -20°C.**

- c. Sample placed on ice at step [2.2 Prepare GEM Master Mix + Sample Dilution on page 61](#) can either be discarded or stored at -80°C for up to **6 months**. See Sample Storage below:

### Sample Storage

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

### Using Stored Samples

- When ready to use samples stored at -80°C from this step, thaw at room temperature until no ice remains and then continue from step **2.1q** of [2.1 Post-Hybridization Pool & Wash on page 54](#) 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	72
3.1 Post-GEM Incubation - Recovery	73
3.2 Pre-Amplification PCR	74
3.3 DNA Cleanup - SPRIselect	75



## 3.0 Get Started

Action	Item	10x PN	Preparation & Handling	Storage
<b>Equilibrate to Room Temperature</b>				
<input type="checkbox"/>	 <b>Reducing Agent B</b>	2000087	Thaw, vortex, verify no precipitate, centrifuge briefly.	-20°C
<input type="checkbox"/>	 <b>Pre-Amp Primers B</b>	2000529	Thaw, vortex, centrifuge briefly.	-20°C
<input type="checkbox"/>	<b>Beckman Coulter SPRIselect Reagent</b>	—	Manufacturer's recommendations.	Ambient
<b>Place on Ice</b>				
<input type="checkbox"/>	 <b>Amp Mix</b>	2000103	Vortex and centrifuge briefly.	-20°C
<b>Obtain</b>				
<input type="checkbox"/>	 <b>Recovery Agent</b>	220016	—	Ambient
<input type="checkbox"/>	<b>Qiagen Buffer EB</b>	—	Manufacturer's recommendations.	Ambient
<input type="checkbox"/>	<b>10% Tween 20</b>	—	Manufacturer's recommendations.	Ambient
<input type="checkbox"/>	<b>10x Magnetic Separator</b>	230003	—	Ambient
<input type="checkbox"/>	<b>Prepare 80% Ethanol</b> 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 ( $\mu$ l)	4X + 10% ( $\mu$ l)
<i>Add reagents in the order listed</i>				
	Amp Mix	2000103	25.0	110.0
	Pre-Amp Primers B	2000529	10.0	44.0
Total			35.0	154.0

- b. Add **35  $\mu$ l** Pre-Amplification Mix to sample (aqueous sample).
- 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 $\mu$ l	~30-45 min
Step	Temperature	Time hh:mm:ss
1	98°C	00:03:00
2	98°C	00:00:15
3	67°C	00:00:20
4	72°C	00:01:00
5	Go to Step 2, 7x (total 8 cycles)	
6	72°C	00:01:00
7	4°C	Hold



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


### 3.3 DNA Cleanup – SPRIselect

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

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

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

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

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

# Step 4:



## Library Construction

4.0 Get Started	77
4.1 Sample Index PCR	78
4.2 Post Sample Index PCR Size Selection - SPRIselect	80
4.3 Post Library Construction QC	81





## 4.0 Get Started

Action		Item	10x PN	Preparation & Handling	Storage
<b>Equilibrate to Room Temperature</b>					
<input type="checkbox"/>		<b>Dual Index Plate TS Set A</b> Verify name & PN. Use indicated plate only	3000511	Vortex and centrifuge briefly.	–20°C
<input type="checkbox"/>		<b>Beckman Coulter SPRIselect Reagent</b>	—	Manufacturer's recommendations.	—
<input type="checkbox"/>		<b>Agilent Bioanalyzer High Sensitivity Kit</b> If used for QC	—	Manufacturer's recommendations.	—
<input type="checkbox"/>		<b>Agilent TapeStation ScreenTape &amp; Reagents</b> If used for QC	—	Manufacturer's recommendations.	—
<b>Place on Ice</b>					
<input type="checkbox"/>		<b>Amp Mix</b>	2000103/2000047	Vortex and centrifuge briefly.	–20°C
<input type="checkbox"/>		<b>KAPA Library Quantification Kit for Illumina Platforms</b>	—	Manufacturer's recommendations.	—
<b>Obtain</b>					
<input type="checkbox"/>		<b>Qiagen Buffer EB</b>	—	Manufacturer's recommendations.	Ambient
<input type="checkbox"/>		<b>10x Magnetic Separator</b>	230003	See Tips & Best Practices.	Ambient
<input type="checkbox"/>		<b>Prepare 80% Ethanol</b> Prepare 2.5 ml for 4 GEM reactions.	—	Prepare fresh.	Ambient

## 4.1 Sample Index PCR

- a. Choose the appropriate sample index sets to ensure that no sample indices overlap in a multiplexed sequencing run. Record the 10x sample index name (PN-3000511 Dual Index Plate TS Set A well ID) used.
- b. Prepare Sample Index PCR Mix on ice.

Sample Index PCR Mix		PN	1X (μl)	1X + 10% (μl)	4X + 10% (μl)
Add reagents in the order listed					
○	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  $\mu$ l** sample from the step [DNA Cleanup – SPRIselect on page 75](#) to a new tube strip.
- d. Add **60  $\mu$ l** Sample Index PCR Mix to **20  $\mu$ l** sample.
- e. Add **20  $\mu$ l** of an individual Dual Index TS Set A to each sample. Pipette mix 5x (pipette set to 90  $\mu$ l). Centrifuge briefly.
- f. Incubate in a thermal cycler with the following protocol.

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

Targeted Cell Recovery	Total Cycles*			
	for Cell Lines	for PBMCs & Nuclei	for Cells from Fixed & Dissociated Tissues**	for Cells from FFPE Tissue Sections
500-2,000	12	16	15-16	17
2,000-4,000	11	15	14-15	16
4,000-7,000	10	14	13-14	15
7,000-12,000	9	13	12-13	14
12,000-25,000	8	12	11-12	13
25,000-50,000	7	11	10-11	12
50,000-128,000	6	10	9-10	11


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

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



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

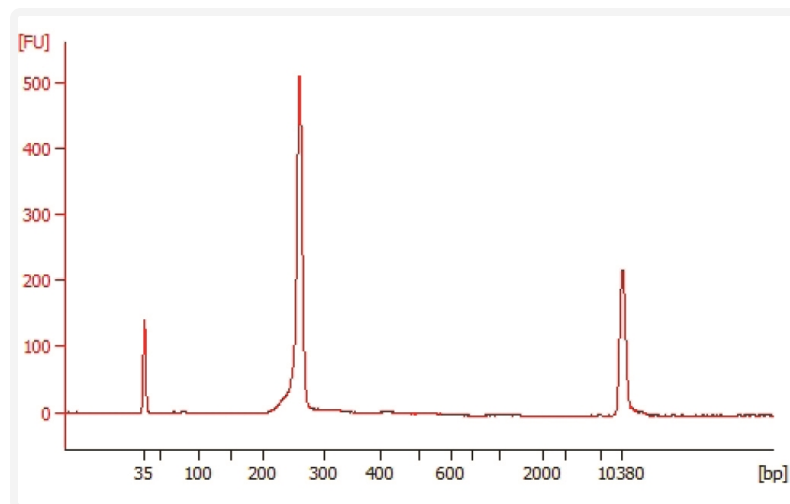
## 4.2 Post Sample Index PCR Size Selection – SPRIselect

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

## 4.3 Post Library Construction QC

Run **1  $\mu$ l** sample at **1:80** dilution on an Agilent Bioanalyzer High Sensitivity chip.

**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 97](#)
- [Agilent TapeStation Traces on page 98](#)
- [LabChip Traces on page 98](#)

# Step 5:

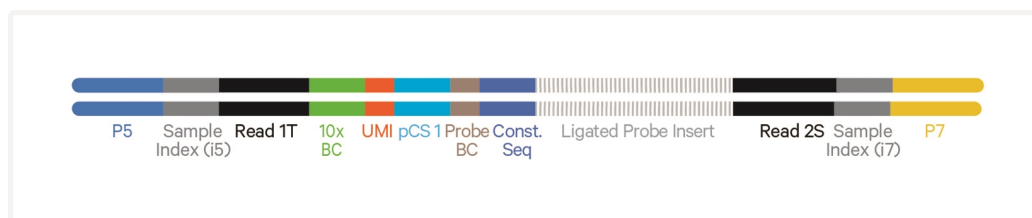
## Sequencing

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## Sequencing Libraries

Chromium Fixed RNA Profiling – Gene Expression libraries comprise standard Illumina paired-end constructs which begin with P5 and end with P7. These libraries include 16 bp 10x GEM Barcodes (10x Barcode) encoded at the start of TruSeq Read 1 (Read 1T). Sample index sequences are incorporated as the i5 and i7 index reads. TruSeq Read 1 (Read 1T) and Small RNA Read 2 (Read 2S) are used in paired-end 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. A single library contains reads derived from up to 16 samples, with cell barcodes composed of a Probe Barcode and a 10x GEM barcode (or 10x BC). Sequencing these libraries produces a standard Illumina BCL data output folder.

### Chromium Fixed RNA Profiling – Gene Expression Library



## Illumina Sequencer Compatibility

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

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

## Sample Indices

Each sample index in the or Dual Index Kit TS Set A (PN-1000251) is a mix of one unique i7 and one unique i5 sample index. If multiple samples are pooled in a flow cell lane, the sample index name (i.e. the Dual Index TS Set A plate well ID, SI-TS-) is needed in the sample sheet used for generating FASTQs with “cellranger mkfastq”. Samples utilizing the same sample index should

not be pooled together, or run on the same flow cell lane, as this would not enable correct sample demultiplexing.

## Fixed RNA – Gene Expression Library Sequencing Parameters

Parameter	Description
Sequencing Depth	Minimum 10,000 read pairs per cell
Sequencing Type	Paired-end, dual indexing
Sequencing Read	Recommended Number of Cycles
Read 1	28 cycles
i7 Index	10 cycles
i5 Index	10 cycles
Read 2	90 cycles (Minimum required Read 2 length is 76 bp)

## Library Loading

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.

### Library Loading

Instrument	Loading Concentration (pM)	PhiX (%)
MiSeq	12	5
NextSeq 500/550	1.6	5
NextSeq 1000/2000	650	5
NovaSeq	150*/300	10

\* Use 150 pM loading concentration for Illumina XP workflow.

## Library Pooling

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



## Data Analysis and Visualization

Sequencing data may be analyzed using Cell Ranger or 10x Genomics Cloud Analysis 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 Gene Expression data to align reads, generate Feature Barcode matrices and perform clustering and gene expression analysis.

- Input: Base call (BCL) and FASTQ
- Output: BAM, MEX, CSV, HDF5, Web Summary, .cloupe/.loupe
- Operating System: Linux

### Cloud Analysis

*Cloud Analysis is currently only available for US customers.*

Cloud Analysis allows users to run Cell Ranger analysis pipelines from a web browser while computation is handled in the cloud.

- Key features: scalable, highly secure, simple to set up and run
- Input: FASTQ
- Output: BAM, MEX, CSV, HDF5, Web Summary, .cloupe/.loupe

### Loupe Browser

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

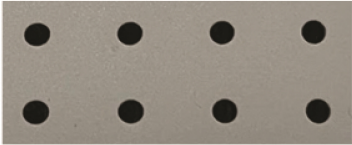
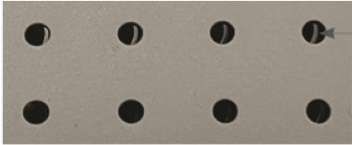


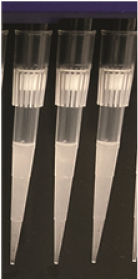
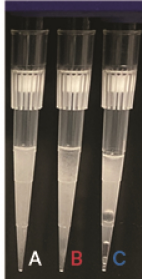
- Input: .cloupe
- Output: Data visualization, including t-SNE and UMAP projections, custom clusters, differentially expressed genes
- Operating System: MacOS, Windows

# Troubleshooting

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## GEM Generation & Barcoding

Step	Normal	Abnormal
After loading the Chip	 <p>The gasket holes are aligned with the wells.</p>	 <p>Misaligned gasket holes &amp; chip wells</p> <p>Gasket holes are misaligned with the sample and gel bead wells. Open and close the chip holder slowly once.</p>
After Chip Q is removed from X/iX and the wells are exposed	 <p>All recovery wells are similar in volume and opacity.</p>	 <p>Recovery well G indicates a reagent clog. Recovery well C and E indicates a wetting failure. Recovery wells B, D, and F are normal. Wells A and H contain 50% Glycerol Solution.</p>
Transfer GEMs	 <p>All liquid levels are similar in volume and opacity without air trapped in the pipette tips.</p>	 <p> <span style="color: blue;">○</span> Adequate emulsion volume (no clog or wetting failure)  <span style="color: red;">●</span> Wetting failure  <span style="color: blue;">●</span> Low emulsion volume (clog)         </p> <p>Pipette tip A shows normal GEM generation, pipette tip B indicates a wetting failure, and pipette tip C shows a clog and wetting failure.</p>

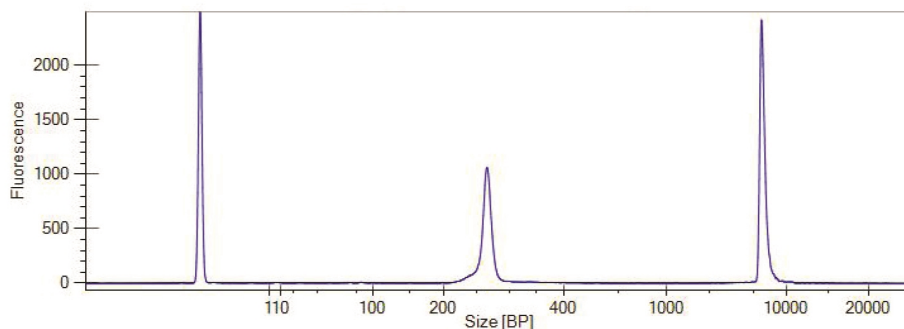


Consult Best Practices to Minimize Chromium Next GEM Chip Clogs and Wetting Failures (Technical Note CG000479) for more information. If a channel clogs or wetting failure occurs during GEM generation, it is recommended that the sample be remade. If any of the listed issues occur, take a picture and send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

## Post Library Construction QC

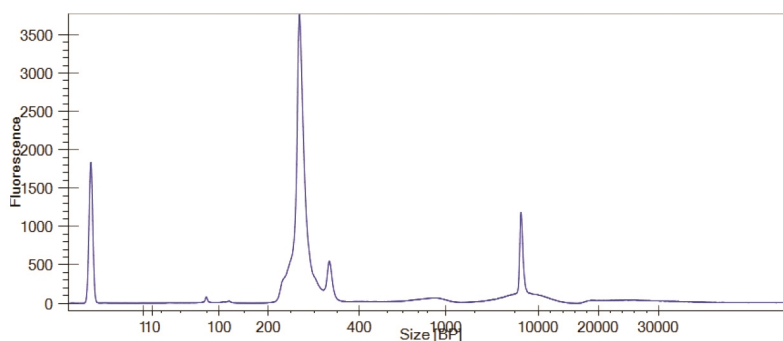
### Step

Fixed RNA – Gene  
Expression Library  
Correct Sample  
Index PCR cycling



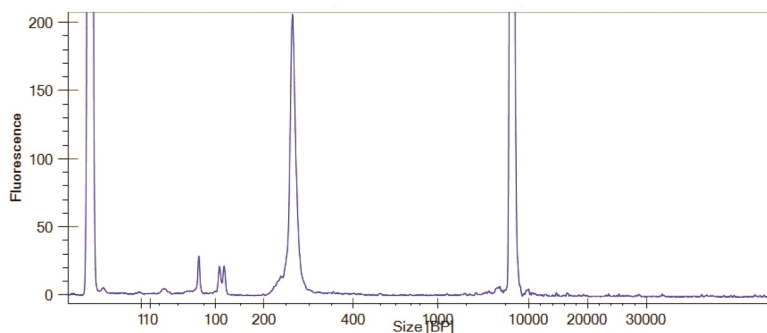
The ideal target library concentration is 50-200 nM. However, if the concentration is between 10-50 nM or between 200-500 nM and if the libraries do not contain low or high molecular weight peaks, sequencing can still be performed.

### Over cycling



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

### Under cycling



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

## Chromium X Series Errors

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

### There are two types of errors:

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

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

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

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



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

# Appendix

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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 32](#) before starting. This protocol is only supported with  $\geq 200,000$  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				
Add reagents in the order listed.	PN	1X* + 10% (ml)	4X* + 10% (ml)	16X* + 10% (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
<b>Total</b>	<b>-</b>	<b>2.2</b>	<b>8.8</b>	<b>35.2</b>

\*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  $\mu$ l** Post-Hyb Wash Buffer to each sample. Pipette mix 5x.  
*If the hybridization was performed in 8-tube strips, add 175  $\mu$ 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  $\mu$ 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.
- l. 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 <i>Add reagents in the order listed.</i>		PN	1X* + 10% (μl)	4X* + 10% (μl)	16X* + 10% (μl)
	Nuclease-free Water	-	522.5	2090.0	8360.0
	Conc. Post-Hyb Buffer	2000533	27.5	110.0	440.0
	<b>Total</b>	-	<b>550.0</b>	<b>2200.0</b>	<b>8800.0</b>

\*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 34](#) 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 36](#) 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 Barcode	Post-Hybridization Cell Conc.	Total Cells in Sample	Cells per Sample Added to the Pool	Sample Volume to be Added (μl)
	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 36](#).

*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 μl, centrifuge, then remove 300 μl of supernatant, and finally resuspend the cell pellet in the remaining 100 μl (400/100 = 4).*

- v.** Proceed **immediately** to [Prepare GEM Master Mix + Sample Dilution on page 61](#).

## 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  $10,000 \times$  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 ( $10,000 \times 3$ ).
- 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  $8,000 \times$  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 ( $8,000 \times 14$ ).



## 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 66](#) for details.

### Steps

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

- Load 70  $\mu$ l to row labeled 1
- Load 50  $\mu$ l to row labeled 2
- Load 45  $\mu$ l 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  $\mu$ l to row labeled 1

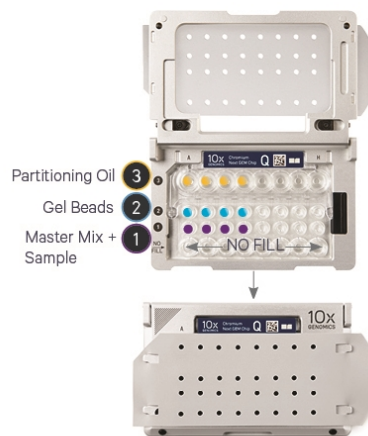
**d. Load Row Labeled 2**

- Aspirate Gel Beads
- Load 50  $\mu$ l to row labeled 2
- Wait 60 sec

**e. Load Row Labeled 3**

- Load 45  $\mu$ l Partitioning Oil to row labeled 3

**f. Close the lid and prepare for run.**



## Post Library Construction Quantification

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

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

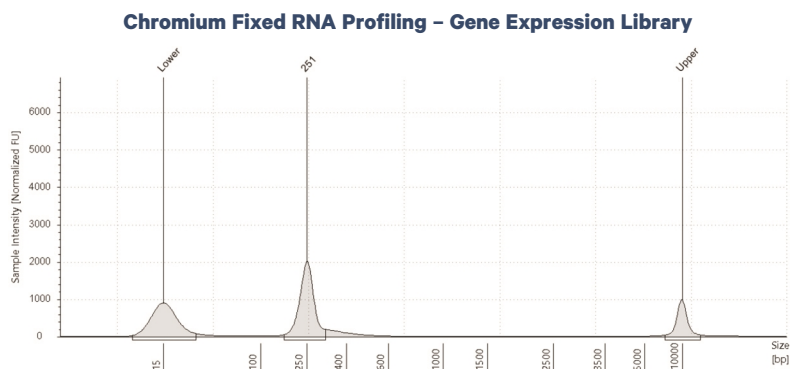
- d. Dispense **16 µl** Quantification Master Mix for sample dilutions and DNA Standards into a 96 well PCR plate.
- e. Add **4 µl** sample dilutions and **4 µl** DNA Standards to appropriate wells. Centrifuge briefly.
- f. Incubate in a thermal cycler with the following protocol.

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

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

## Agilent TapeStation Traces

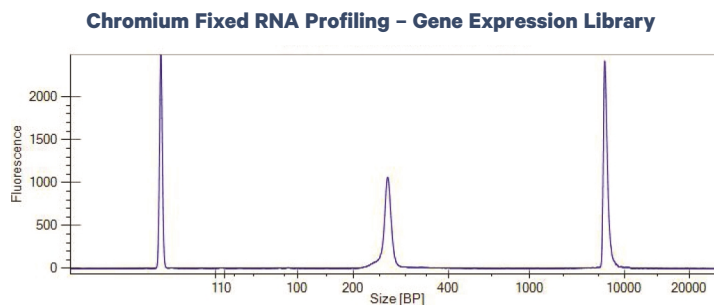
Agilent TapeStation High Sensitivity D5000 ScreenTape was used.



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

## LabChip Traces

DNA High Sensitivity Reagent Kit was used.



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

## Oligonucleotide Sequences

### Gel Bead Primer

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



### Chromium Fixed RNA Profiling – Gene Expression Library

5'-AATGATACGGCGACACGAGATCTACAC-N10-ACACTCTTCCCTACACGACGCTCTTCCGATCT-N16-N12-TTGCTAGGACCG-BC8-NN-TACGTGCTAACCGCGT-Ligated\_Probe\_Insert-TGGAATTCGGGTGCCAAGGAAGTCCAGTCAC-N10-ATCTCGTATGCCGCTCTTCTGCTTC-3'  
3'-TTACTATGCCGCTGGTGGCTCTAGATGTG-N10-TGTGAGAAAGGGATGTCTGCGAGAAGGCTAGA-N16-N12-AACGATCCTGGC-BC8-NN-ATGCACGATTGGCGCA-Ligated\_Probe\_Insert-ACCTTAAGAGCCACGGTTCCTTGAGGTCAGTG-N10-TAGAGCATACGGCAGAAGACGAAC-5'

