User Guide | CG000527 | Rev B

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

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

Notices

Document Number

CG000527 | Rev B

Legal Notices

Instrument & Licensed Software Updates Warranties

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

Support

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Document Revision Summary

Document Number

CG000527 | Rev B

Title

Chromium Fixed RNA Profiling Reagent Kits for Multiplexed Samples

Revision

Rev A to Rev B

Revision Date

August 09, 2022

- Updated chip assembly guidance in Tips & Best Practices and step 2 - GEM Generation & Barcoding
- Updated Troubleshooting section to include guidance on gasket misalignment and GEM transfer
- Updated to include reference to pooling calculator
- Updated to include guidance on pooling samples with different RNA content cells

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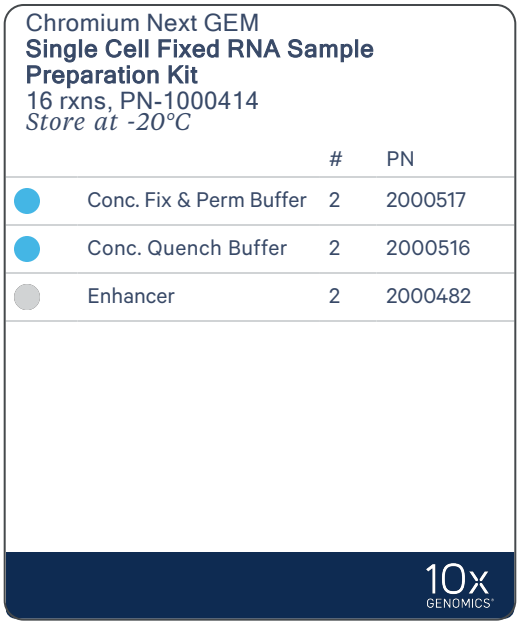
Chromium Fixed RNA Profiling Reagent Kits

Refer to SDS for handling and disposal information

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



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

Chromium Next GEM
Single Cell Fixed RNA Hybridization & Library Kit
4 rxns, PN-1000415
Store at -20°C

	#	PN
Hyb Buffer B	1	2000483
Enhancer	1	2000482
Conc. Post-Hyb Buffer	1	2000533
Reducing Agent B	1	2000087
GEM Enzyme Mix	1	2000490
GEM Reagent Mix	1	2000491
Pre-Amp Primers B	1	2000529
Amp Mix	1	2000103

10x GENOMICS

Chromium Next GEM
Single Cell Fixed RNA Human Transcriptome Probe Kit
16 rxns, PN-1000420
Store at -20°C

	#	PN
Human WTA Probes BC001	1	2000495
Human WTA Probes BC002	1	2000496
Human WTA Probes BC003	1	2000497
Human WTA Probes BC004	1	2000498
Hyb Buffer B	1	2000485
Enhancer	2	2000482
Conc. Post-Hyb Buffer	2	2000533

10x GENOMICS

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

10x GENOMICS

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

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

10x GENOMICS

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

10x GENOMICS

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

10x GENOMICS

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

Chromium
Partitioning Oil
Store at ambient temperature

		#	PN
○	Partitioning Oil	6	2000190

Chromium
Recovery Agent
Store at ambient temperature

		#	PN
○	Recovery Agent	6	220016

Chromium Next GEM
Chip Q & Gaskets
Store at ambient temperature

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

10x GENOMICS

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

Chromium
Partitioning Oil
Store at ambient temperature

		#	PN
○	Partitioning Oil	2	2000190

Chromium
Recovery Agent
Store at ambient temperature

		#	PN
○	Recovery Agent	2	220016

Chromium Next GEM
Chip Q & Gaskets
Store at ambient temperature

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

10x GENOMICS

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

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

	#	PN
Dual Index Plate TS Set A	1	3000511

10x Genomics Accessories

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

Recommended Thermal Cyclers

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 items in the table below have been validated by 10x Genomics and are highly recommended for 10x Genomics workflows, training, and system operations. Substituting materials may adversely affect system performance. This list does not include standard laboratory equipment, such as water baths, centrifuges, vortex mixers, 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	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	04-004-2326
Miltenyi Biotec	Pre-Separation Filters (30 µm) *Alternative to Sysmex product*	130-041-407
Kits & Reagents		
Thermo Fisher Scientific	Nuclease-free Water	AM9937
	Low TE Buffer (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA)	12090-015
	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	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	E1169
	Trypan Blue Stain (0.4%)	T10282
	Countess II FL Automated Cell Counter	AMAQAF1000
	Countess II FL Automated Cell Counting Chamber Slides	C10228
Nexcelom Biosciences	Cellaca MX High-throughput Automated Cell Counter	MX-112-0127
	ViaStain AO/PI Staining Solution	CS2-0106-5mL

Choose either Eppendorf, USA Scientific or Thermo Fisher Scientific PCR 8-tube strips.

Choose either Sysmex or Miltenyi Biotec filter.

Choose Ethidium Homodimer-1, Trypan Blue, or AO/PI Staining Solution based on the presence of debris in the sample.

Equipment			
VWR	VWR Mini Centrifuge *Or any equivalent mini centrifuge*		76269-064
	Divided Polystyrene Reservoirs		41428-958
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	Choose Bioanalyzer, TapeStation or LabChip based on availability & preference.	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 Fixation Guidelines on page 24 for the key reagents needed for fixation and consult Fixation of Cells & Nuclei for Chromium Fixed RNA Profiling (Demonstrated Protocol CG000478) 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)
Recommended Pipettes & Pipette tips		
Rainin	**Pipettes** 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
	Pipette Tips Tips LTS 200UL Filter RT-L200FLR	30389240
	Tips LTS 1ML Filter RT-L1000FLR	30389213
	Tips LTS 20UL Filter RT-L10FLR	30389226
Alternate Recommendations *(If Rainin pipette tips are unavailable, any of the listed pipette tips may be used)*		
Eppendorf	**Pipettes** 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)
	Pipette Tips (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
Labcon*	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
	ZAP SLIK 1200 µL Low Retention Aerosol Filter Pipet Tips for Rainin LTS	4-1145-965-008
Biotix*	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
Sample Fixation	2-3 h	STOP 4°C ≤1 week/-20°C or -80°C ≤3 months
Step 1: Probe Hybridization (page 42)		
1.1 Probe Hybridization (page 46)	16-24 h	
Step 2: GEM Generation and Barcoding (page 48)		
2.1 Post-Hybridization Pool & Wash (page 51)	60-90 min	
2.2 Prepare GEM Master Mix + Sample Dilution (page 57)	30 min	
2.3 Load Chromium Next GEM Chip Q (page 62)	10 min	
2.4 Run the Chromium X/iX (page 64)	5.5 min	
2.5 Transfer GEMs (page 65)	5 min	
2.6 GEM Incubation (page 66)	125 min	STOP 4°C ≤1 week (GEMs) STOP -20°C or -80°C ≤1 month (washed samples)
Step 3: GEM Recovery and Pre-Amplification (page 67)		
3.1 Post-GEM Incubation – Recovery (page 69)	10 min	
3.2 Pre-Amplification PCR (page 70)	55 min	STOP 4°C ≤72 h/-20°C ≤1 week
3.3 DNA Cleanup – SPRIselect (page 71)	30 min	STOP 4°C ≤72 h/-20°C ≤4 weeks
Step 4: Library Construction (page 72)		
4.1 Sample Index PCR (page 74)	40 min	STOP 4°C ≤72 h
4.2 Post Sample Index PCR Size Selection – SPRIselect (page 76)	30 min	STOP 4°C ≤72 h or -20°C long-term
4.3 Post Library Construction QC (page 77)	60 min	

Stepwise Objectives

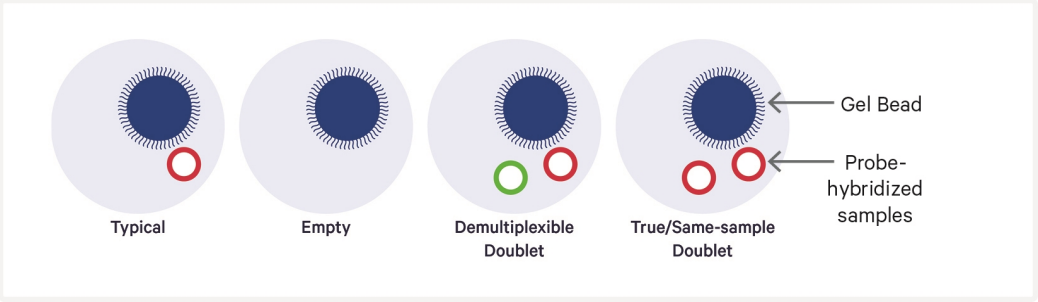
Chromium Fixed RNA Profiling offers comprehensive, scalable solutions to measure gene expression in formaldehyde fixed samples. Gene expression is measured using probe pairs designed to hybridize to mRNA specifically. Using a microfluidic chip, the fixed and probe-hybridized single cell and nuclei suspensions 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 Transcriptome Probe kits (PN-1000420/1000456) 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 Transcriptome, 4 rxns x 4 BC PN-1000475, 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 Transcriptome, 4 rxns x 16 BC PN-1000476 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.

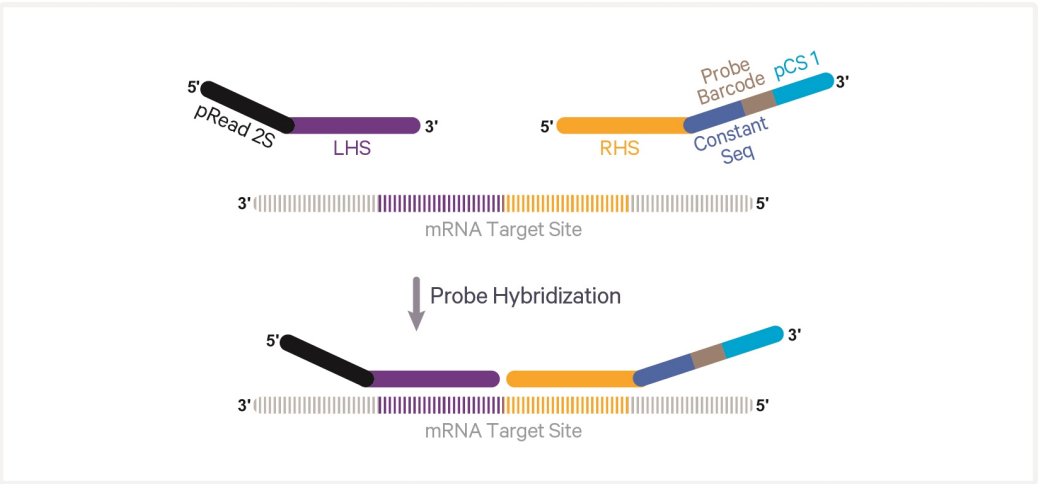
Sample Fixation for Chromium Fixed RNA Profiling

Samples are fixed in a 4% formaldehyde fixative solution, as described in the Fixation of Cells & Nuclei for Chromium Fixed RNA Profiling (Demonstrated Protocol CG000478) 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

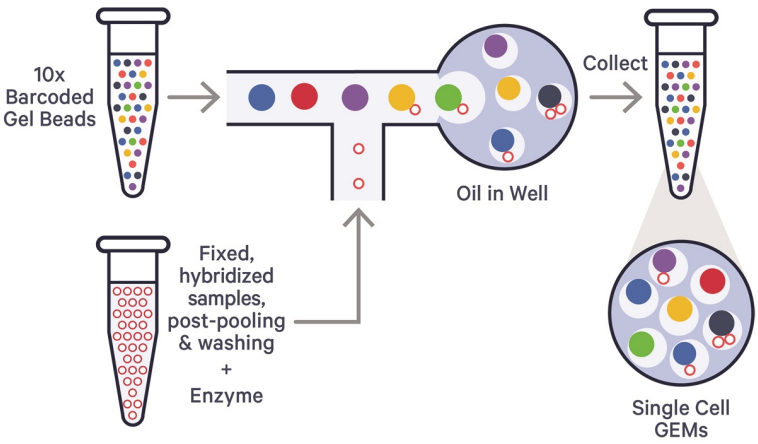


Step 2: GEM Generation & Barcoding

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,

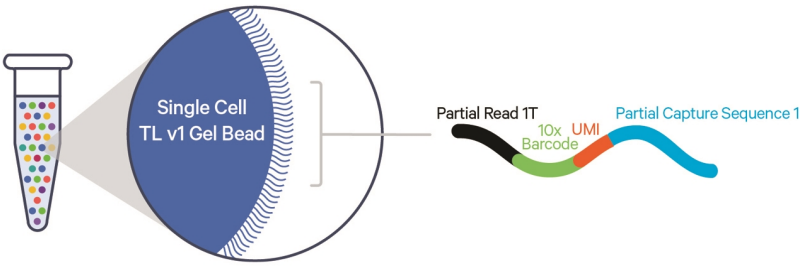
a Master Mix containing pooled cells, and Partitioning Oil onto Chromium Next GEM Chip Q.

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).

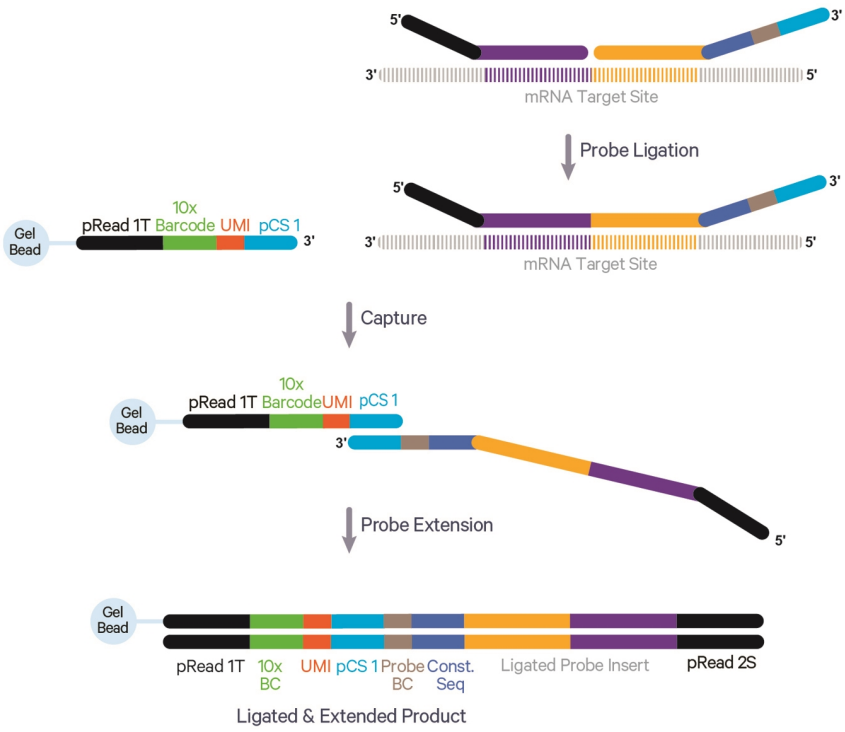
Gel Bead



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.

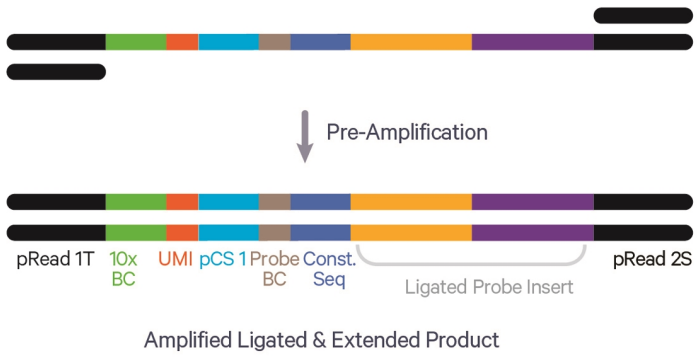
Inside Individual GEMs



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

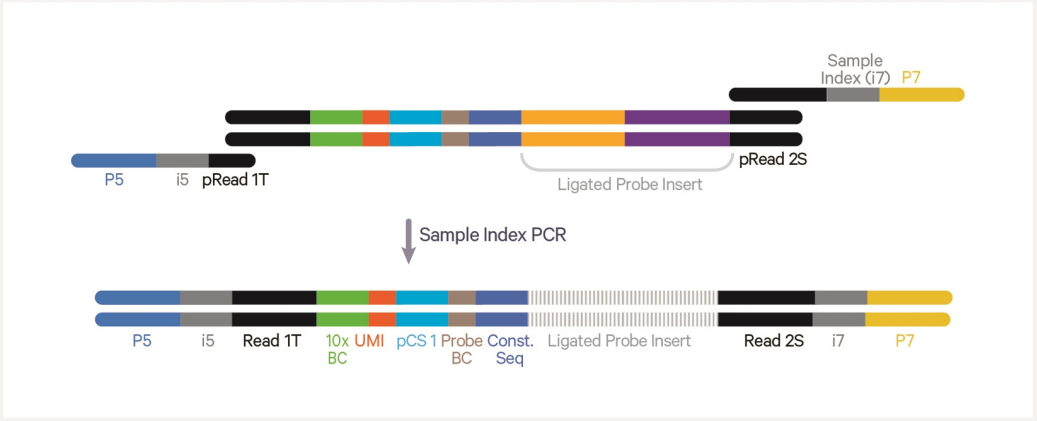


Step 4: Fixed RNA – Gene Expression Library Construction

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.

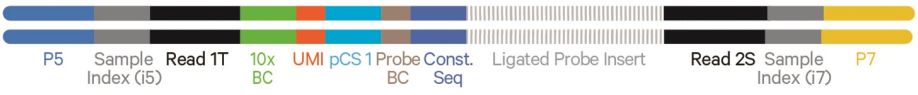
Pooled Amplified DNA Processed in Bulk



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 95

Sample Fixation Guidelines

Overview

Chromium Fixed RNA Profiling is only compatible with samples (single cell and nuclei suspensions) that are appropriately fixed. Single cell and nuclei suspensions are fixed in a 4% formaldehyde fixative solution and then quenched as described in the Fixation of Cells & Nuclei for Chromium Fixed RNA Profiling (Demonstrated Protocol CG000478) and using Chromium Next GEM Single Cell Fixed RNA Sample Preparation Kit (10x Genomics PN-1000414).

Fixed samples can be stored short-term at 4°C or long-term at −20°C or −80°C. Fixation and quenching protocols as well as the storage guidelines are described in detail in the Fixation of Cells & Nuclei for Chromium Fixed RNA Profiling (Demonstrated Protocol CG000478).

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 Fixation of Cells & Nuclei for Chromium Fixed RNA Profiling (Demonstrated Protocol CG000478) for a complete list of reagents for sample fixation.

Multiplexing Experiment Design

Chromium Fixed RNA Kit, Human Transcriptome, 4 rxns x 4 BC PN-1000475 and the Chromium Fixed RNA Kit, Human Transcriptome, 4 rxns x 16 BC PN-1000476 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 91.

This illustration provides an overview of the various multiplexing configurations using the Chromium Fixed RNA Kit, Human Transcriptome, 4 rxns x 4 BC PN-1000475. The same principle can be applied when using the Chromium Fixed RNA Kit, Human Transcriptome, 4 rxns x 16 BC PN-1000476.

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 x 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 ~1 x 10^6 cells/nuclei per hybridization, if possible.

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

Incubation Time

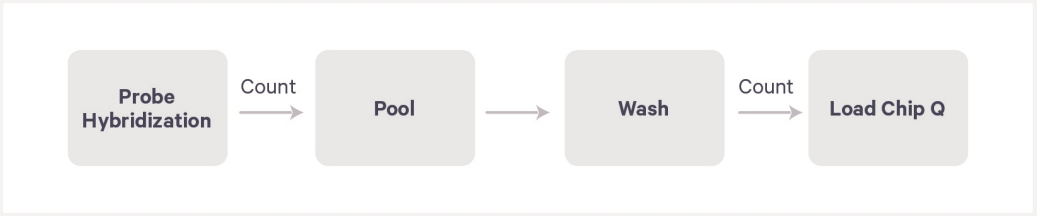
- Recommended incubation time for probe hybridization is 16-24 h.
- Incubation time should be of same length for all samples. DO NOT mix samples with different hybridization times in one experiment.

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**).

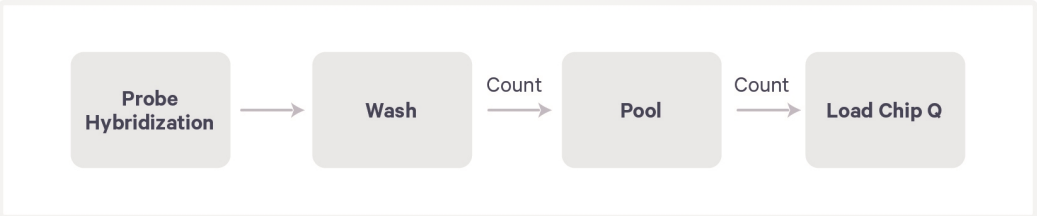
The Chromium Fixed RNA Profiling Reagent Kits contain sufficient buffer volumes to complete either workflow with no impact on the data quality.

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 51 for Pooled Wash workflow.

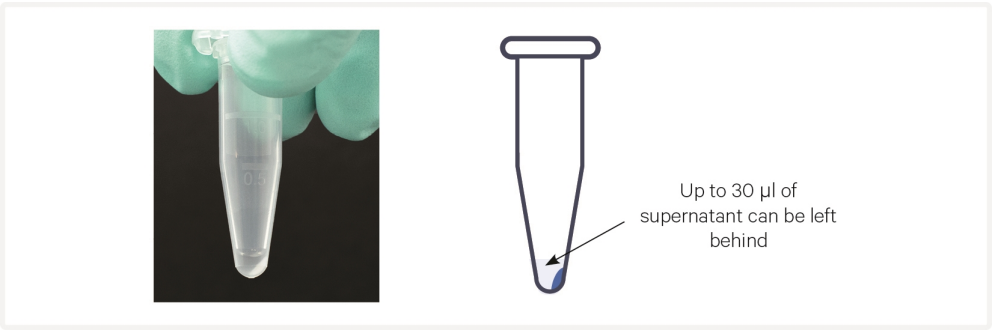
Individual Wash Workflow



- 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 87.

Sample Washing & Recovery

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



Cell Counts for Chip Loading

- The Chromium Fixed RNA Profiling is designed to target 500-10,000 cells per 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

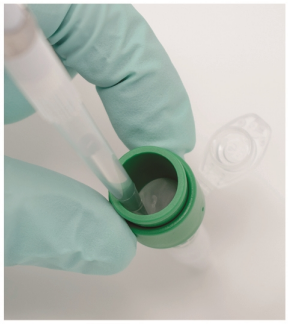
- After post-hybridization wash, pass the sample through a 30 µm filter (Sysmex CellTrics or Miltenyi Biotec Pre-Separation) into a new 1.5-ml microcentrifuge tube.

DO NOT use 40 µm Flowmi Tip Strainer for filtration.

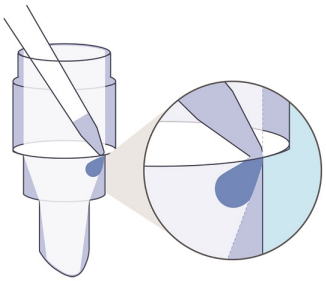
- Hold the pipette tip at an angle and touch the filter membrane where the filter meets the wall. Slowly pipette through the filter. Tap gently or centrifuge briefly if liquid remains at the end of the filter.
- To maximize recovery, residual volume can be pipetted from underneath the filter.

Filtration using 30 µm Filters

Pre-Separation Filters from Miltenyi Biotec



Celltrix Filters from Sysmex



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

Pre-Separation Filters from Miltenyi Biotec

Celltrix Filters from Sysmex

Cell Counting

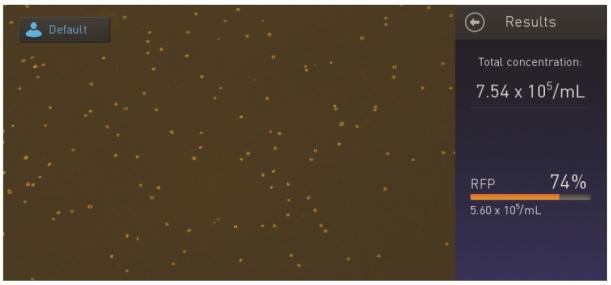
- Accurate counting is critical for optimal assay performance.
- It is strongly recommended that the sample be stained with a fluorescent dye such as Ethidium Homodimer-1 or AO/PI staining solution and counted using an automated fluorescent cell counter (Countess II Automated Cell Counter or a Cellaca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

Counting using Ethidium Homodimer-1

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

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

Fixed PBMCs Stained with Ethidium Homodimer-1



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

Counting using AO/PI Staining Solution

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

- Add 25 µl AO/PI Staining Solution into Mixing Row of Cellaca plate.
- Gently mix the sample. If the sample is too concentrated, a 1:1 dilution in PBS can also be prepared. For example, add 15 µl fixed cell suspension to 15 µl PBS.
- Add 25 µl sample to Mixing Row of plate containing AO/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 AO/PI Staining Solution can also be counted using Countess II FL Automated Cell Counter. See manufacturer's instructions for details.

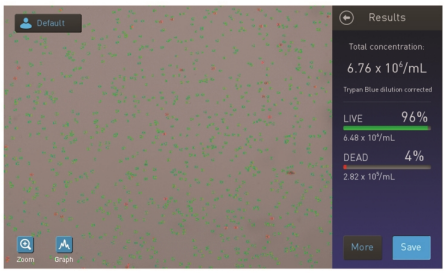
Counting using Trypan Blue (Only for Debris-Free Samples)

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

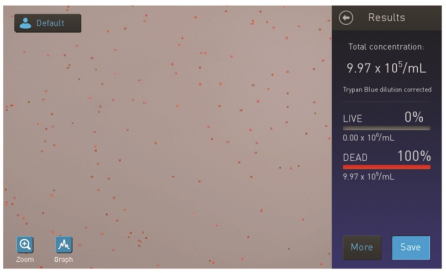
- Mix 1 part 0.4% trypan blue and 1 part sample.
- Transfer 10 µl sample to a Countess II Cell Counting Slide chamber or a hemocytometer.
- Insert the slide into the Countess II Cell Counter and determine the cell concentration. Or if using hemocytometer, count fixed cells by placing hemocytometer under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

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

Fresh PBMCs



Fixed PBMCs



Chromium Next GEM Chip Handling

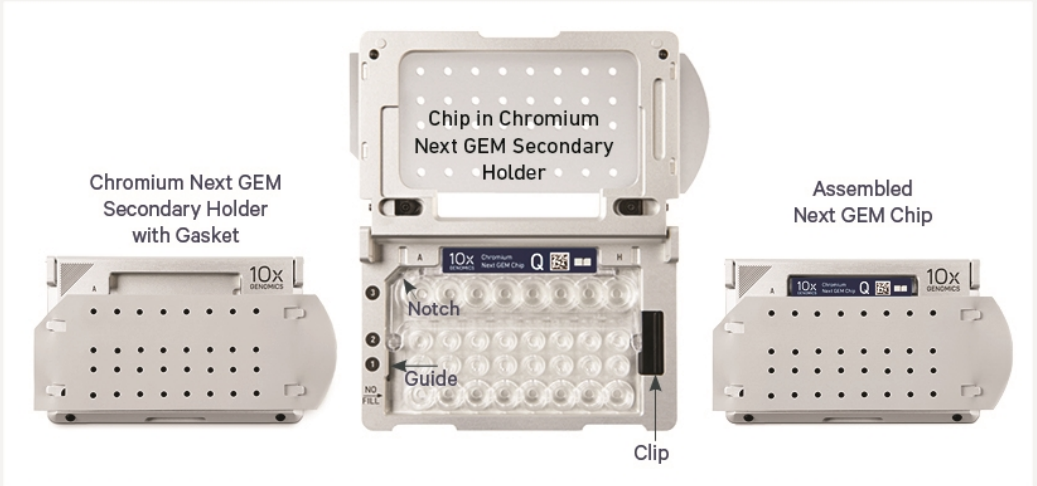
- Chromium Fixed RNA Profiling uses Chromium Chip Q.
- Minimize exposure of reagents, chips, and gaskets to sources of particles and fibers, laboratory wipes, frequently opened flip-cap tubes, clothing that sheds fibers, and dusty surfaces.
- After removing the chip from the sealed bag, use in **≤24 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 62 for specific instructions.

50% Glycerol Solution for Addition to Unused Chip Wells

- Purchase 50% glycerol solution from Ricca Chemical Company, Glycerin (glycerol), 50% (v/v) Aqueous Solution, PN-3290-32.

  OR

- Prepare 50% glycerol solution:
  - Mix an equal volume of water and ≥99% Glycerol, Molecular Biology Grade.
  - Filter through a 0.2 µm filter.
  - Store at **−20°C** in 1-ml LoBind tubes. 50% glycerol solution should be equilibrated to room temperature before use.
- Adding glycerol to non-sample chip wells is essential to avoid chip failure.

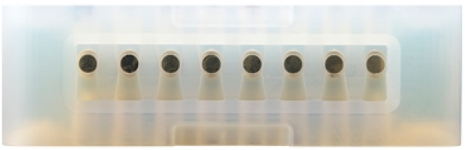
50% Glycerol Solution for Sample Storage

- Use nuclease-free water and molecular biology grade Glycerol from Millipore Sigma, PN-G5516, to prepare 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.

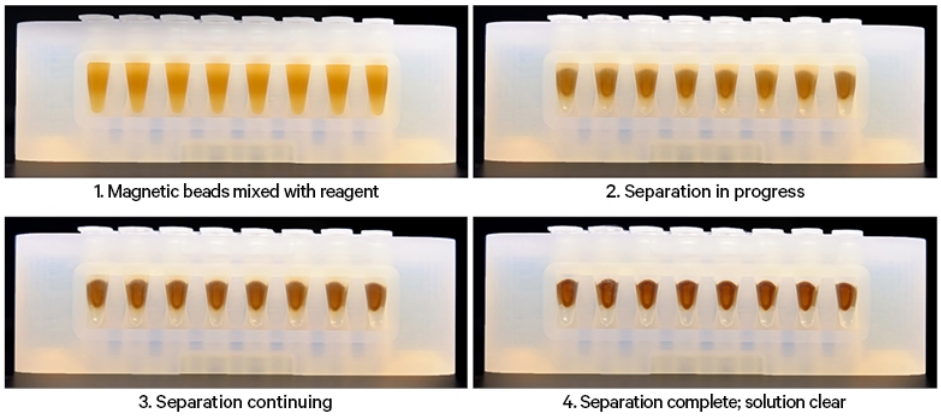
10x Magnetic Separator



- 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).

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

Sample Indices in Sample Index PCR

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

Step 1:

Probe Hybridization

1

1.0 Get Started

Action	Item	10x PN	Preparation & Handling	Storage
Thaw & Keep Warm				
☐	**Hyb Buffer B**	2000485/ 2000483	Thaw at 42°C. Vortex and centrifuge briefly. Keep warm and verify no precipitate before use. DO NOT keep the thawed buffer on ice, or the solution will precipitate. Thawed Hyb Buffer B can be kept at 42°C for up to 1 h.	–20°C
☐	**Enhancer**	2000482	Thaw for 10 min at 65°C. Vortex and centrifuge briefly. Keep warm and verify no precipitate before use. DO NOT keep the thawed reagent on ice, or the solution will precipitate. Once thawed, Enhancer can be kept at 42°C for up to 10 min.	–20°C
Place on Ice				
☐	**Fixed Cell Suspension**	—	Consult Demonstrated Protocol Fixation of Cells & Nuclei for Chromium Fixed RNA Profiling (CG000478).	—
☐	**Human WTA Probes BC001-BC016**	2000495-2000510	Thaw on ice. Vortex and centrifuge briefly.	–20°C

Probe Hybridization Guidelines for Multiplexing

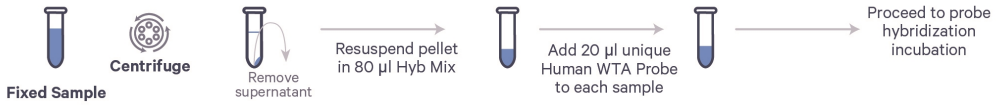
Read these guidelines before starting probe hybridization

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.

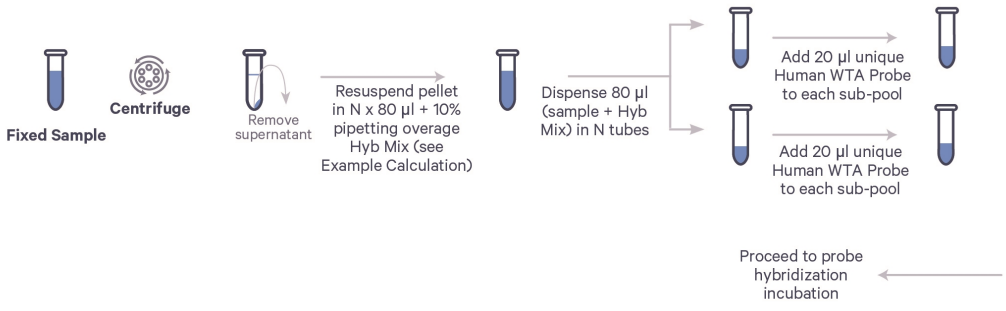
Unique Sample to Unique Probe Barcode



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 µl x Number of sub-pools (N) + 10%, see Example Calculation on the next page) and dispensing 80 µl 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

= Volume of Hyb Mix for 1 reaction * Number of sub-pools

= 80 * 2 = **160 µl**

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 = **176 µl**

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

Number of Sub-pools: **8**

Volume of Hyb Mix for N=8 Sub-pools

= Volume of Hyb Mix for 1 reaction * Number of sub-pools

= 80 * 8 = **640 µl**

Overage Volume for 10% Excess

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

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

= 640 + 64 = **704 µl**

*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 *Add reagents in the order listed*		PN	1X* (µl)	1X* + 20% (µl)	4X* + 20% (µl)	16X* + 20% (µl)
	Hyb Buffer B *Thaw at 42°C. Add warm to the mix and if appears milky keep it back on 42°C.*	2000485/ 2000483	70.0	84.0	336.0	1344.0
	Enhancer *Heat at 65°C for 10 min. Vortex and verify no precipitate. Add warm to the mix.*	2000482	10.0	12.0	48.0	192.0
	Total	-	**80.0**	**96.0**	**384.0**	**1536.0**

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

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

d. Centrifuge fixed cells/nuclei resuspended in Quenching Buffer/post-storage processing buffer (0.5X PBS + 0.02% BSA) at **850 rcf** for **5 min** at **4°C**. It is recommended to use between 50,000-2 x 10^6 fixed cells or 100,000-2 x 10^6 fixed nuclei per hybridization reaction.

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

f. Resuspend each pellet in **80 µl** Hyb Mix* 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 44 for guidance on the volume of Hyb Mix needed when sub-pooling.*

g. Add **20 µl** unique single Human WTA Probes BC001-BC016 (PN-2000495-2000510) to the **80 µl** mixture of Hyb Mix and fixed sample and gently pipette mix 10x with pipette set at 80 µl. Record the Human WTA Probes name and part number used for each sample.

Only one Human WTA Probe should be added to each tube containing sample + Hyb mix. If sub-pooling the samples, dispense the 80 µl 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

2.0 Get Started

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

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

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

2.1 Post-Hybridization Pool & Wash

TIPS

Samples hybridized with unique Probe Barcodes can be pooled immediately after hybridization, and washed as a pool (**Pooled Wash workflow**). Alternatively, samples can be washed individually and pooled after the washing is complete (**Individual Wash workflow**). See Post-Hybridization Pooling & Washing Guidelines on page 29 for guidance on choosing the appropriate workflow. The following section provides instructions for **Pooled Wash workflow**. See Appendix for Post-Hybridization Pool & Wash – Individual Wash Workflow on page 87.

Pooled Wash Workflow

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

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

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

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

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

**Volumes are in ml*

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

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

i. Dilute each sample by adding **175 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 33. Calculate the total cell number present in the tube.

Accurate cell counting is critical for optimal assay performance.

iii. Pool an equal number of cells from different hybridization reactions into a 5-ml (for 4 pooling samples) or 15-ml (for pooling 16 samples) centrifuge tube. See Post-Hybridization Pooling Calculation on page 55 on how to calculate the volume of each sample to be added.

iv. 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.

v. 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 33. Calculate the total cell number present in the tube.

Accurate cell counting is critical for optimal assay performance.

iii. Pool an equal number of cells from different hybridization reactions into a 5-ml (for 4 pooling samples) or 15-ml (for pooling 16 samples) centrifuge tube. See Post-Hybridization Pooling Calculation on page 55 on how to calculate the volume of each sample to be added.

iv. Proceed to step e.

e. Centrifuge each tube containing pooled samples at **850 rcf** for **5 min** at **room temperature**.

f. Remove the supernatant without disturbing the pellet. See Tips & Best Practices for Sample Washing & Recovery on page 30.

g. Resuspend cell pellet in **1 ml** Post-Hyb Wash Buffer and transfer to a 1.5-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 *Add reagents in the order listed*	**PN**	**1 Pool + 10% (µl)**	**4 Pools + 10% (µl)**
Nuclease-free Water	-	1567.5	6270.0
Conc. Post-Hyb Buffer	2000533	82.5	330.0
Total	**-**	**1650.0**	**6600.0**

r. Centrifuge the sample at **850 rcf** for **5 min** at **room temperature**.

s. Remove the supernatant without disturbing the pellet.

t. Resuspend cell pellet in an appropriate volume of **chilled** Post-Hyb Resuspension Buffer. The buffer volume will depend upon the starting number of cells in the pool (table below). Pipette mix 20x to resuspend and breakup any cell clumps and maintain on ice.

Final resuspension volume

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

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

DO NOT use 40 µm Flowmi Tip Strainer for filtration.

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

TIPS

See Sample Filtration on page 31 for details.

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

TIPS

See Tips & Best Practices for Cell Counting on page 33. As concentrated cell suspensions are required when targeting very high cell loads, a serial dilution may be needed to accurately determine cell concentration.

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

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

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

w. Proceed **immediately** to next step.

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

*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 from step 2.1 Post-Hybridization Pool & Wash on page 51 on ice. These samples can be stored later after GEM incubation. Guidelines for storage of remaining samples are provided in step 2.6 GEM Incubation on page 66.

c. Add **35 µl** of prepared GEM Master Mix into each tube containing diluted sample and **immediately** proceed to the next step.

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

Cell Suspension Volume Calculator for Multiplexing 4 Samples

Volume of Cell Suspension Stock per reaction (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										

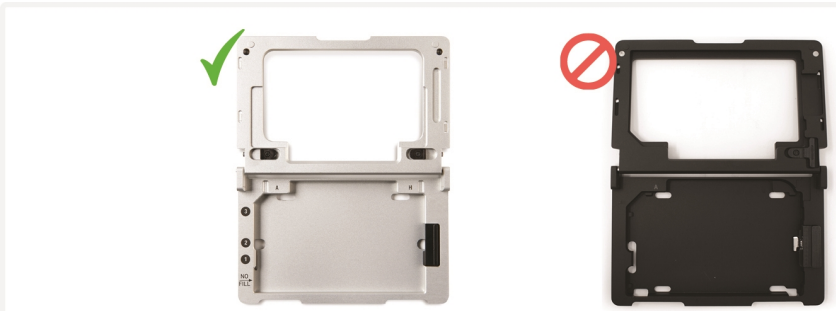
Cell Suspension Volume Calculator for Multiplexing 16 Samples

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

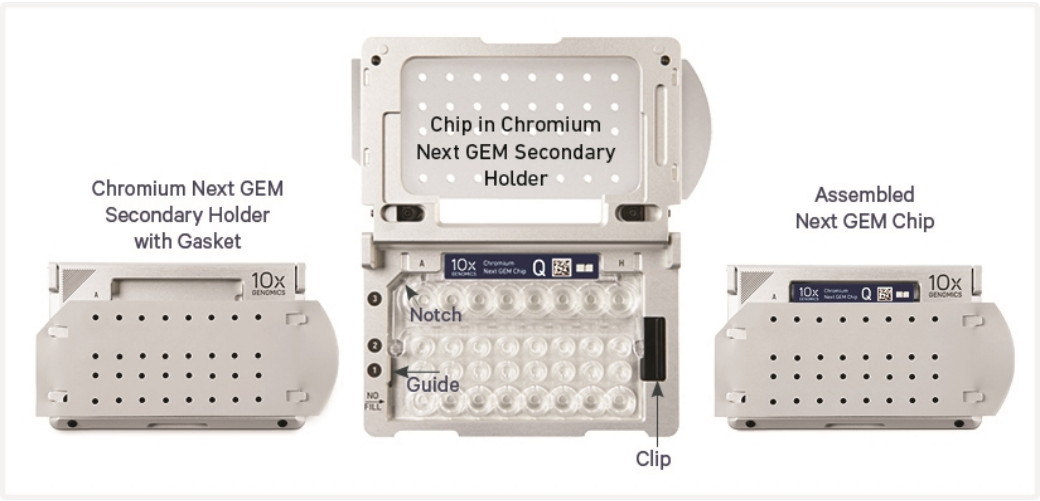
Cell Stock Concentration (Cells/µl)	Targeted Cell Recovery								
	8000	16000	32000	48000	64000	80000	96000	112000	128000
500	26.4 13.6	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
1000	13.2 26.8	26.4 13.6	n/a	n/a	n/a	n/a	n/a	n/a	n/a
1500	8.8 31.2	17.6 22.4	35.2 4.8	n/a	n/a	n/a	n/a	n/a	n/a
2000	6.6 33.4	13.2 26.8	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.



TIPS See Tips & Best Practices on page 27 for chip handling instructions.



a. Close the holder lid. Attach the gasket by holding the tongue (curved end, to the right) and hook the gasket on the left-hand tabs of the holder. Gently pull the gasket toward the right and hook it on the two right-hand tabs.

b. DO NOT touch the smooth side of the gasket.

c. Open the chip holder.

d. Remove the chip from the sealed bag. Use the chip within ≤ 24 h.

e. Align notch on the chip (upper left corner) and the open holder with the gasket attached.

f. Slide the chip to the left until the chip is inserted under the guide on the holder. Depress the right hand side of the chip until the spring-loaded clip

engages.

g. Keep the assembled unit with the attached gasket open until ready for and while dispensing reagents into the wells.

h. DO NOT touch the smooth side of the gasket.

i. The assembled chip is ready for loading the indicated reagents. Refer to 2.3 Load Chromium Next GEM Chip Q on the next page for reagent volumes and loading order.

j. After loading reagents, close the chip holder. DO NOT press down on the top of the gasket.

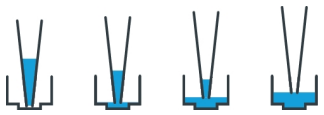
For GEM generation, load the indicated reagents only in the specified rows, starting from row labeled 1, followed by rows labeled 2 & 3.
DO NOT load reagents in the bottom row labeled NO FILL.

2.3 Load Chromium Next GEM Chip Q

Chip loading instructions are unique to Chip Q.

TIPS

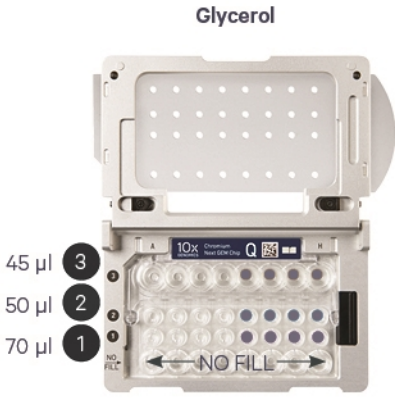
- After removing chip from the sealed bag, use in **≤24 h**.
- Open the lid (gasket attached) of the assembled chip and lay flat for loading.
- When loading the chip, raising and depressing the pipette plunger should each take **~5 sec**. When dispensing, raise the pipette tips at the same rate as the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



a. Add 50% glycerol solution to each unused well

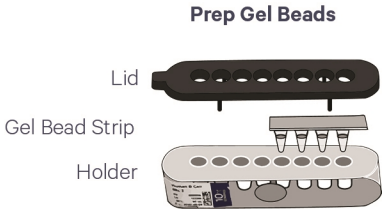
- **70 µl** in each unused well in row labeled 1
- **50 µl** in each unused well in row labeled 2
- **45 µl** in each unused well in row labeled 3

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



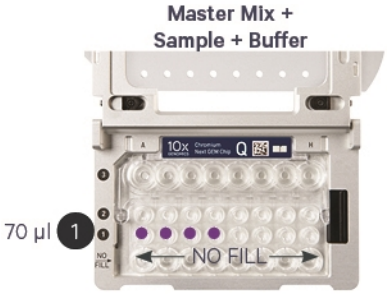
b. Prepare Gel Beads

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



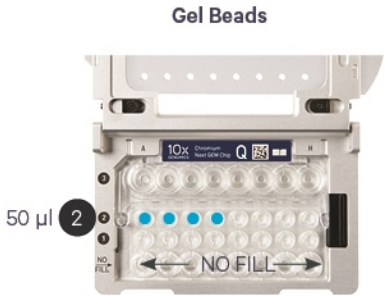
c. Load Row 1

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



d. Load Row Labeled 2

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



e. Load Row Labeled 3

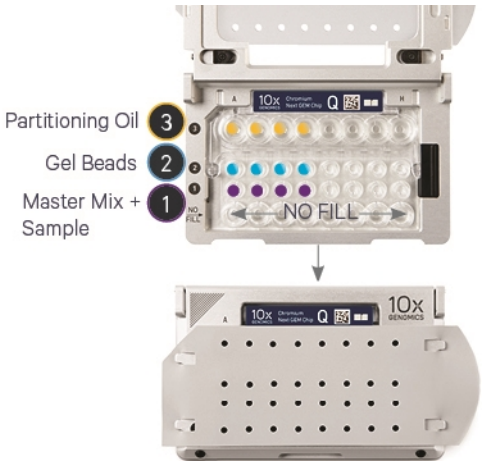
- Dispense **45 µl** Partitioning Oil into the wells in **row labeled 3** from a reagent reservoir.

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

f. Prepare for Run

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

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



2.4 Run the Chromium X/iX

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

a. Press the eject button on the Chromium X to eject the tray. If the eject button is not touched within **1 min**, tray will close automatically. System requires a few seconds before the tray can be ejected again.

b. Place the assembled chip with the gasket in the tray, ensuring that the chip stays horizontal. Press the button to retract the tray.

c. Press the play button.

d. At completion of the run **(~5.5 min)**, Chromium X/iX will chime. **Immediately** proceed to the next step.

Run Chromium X/iX

2.5 Transfer GEMs

a. Place a tube strip on ice.

b. Press the eject button of the Chromium X/iX and remove the chip.

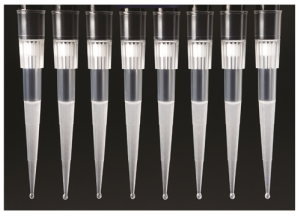
c. Discard the gasket. Open the chip holder. Fold the lid back until it clicks to expose the wells at 45 degrees.

d. Check the volume in rows labeled 1-2. Abnormally high volume in any well indicates a clog.

e. Slowly aspirate **100 µl** GEMs from the lowest points of the recovery wells in the top row labeled 3 without creating a seal between the tips and the bottom of the wells.

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

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



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

2.6 GEM Incubation

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

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

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

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

DO NOT store the GEMs at −20°C.

c. Sample placed on ice at step 2.2 Prepare GEM Master Mix + Sample Dilution on page 57 can either be discarded or stored at **−20°C** or **−80°C** for up to **1 month**. 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 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 **−20°C** or **−80°C** for up to **1 month**. For best results, storage at −80°C is strongly recommended.

Using Stored Samples

- When ready to use samples stored at −20°C or −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 51. Samples may undergo a color change during storage (e.g. black, light gray, or green), however this will not impact assay performance.

Step 3:

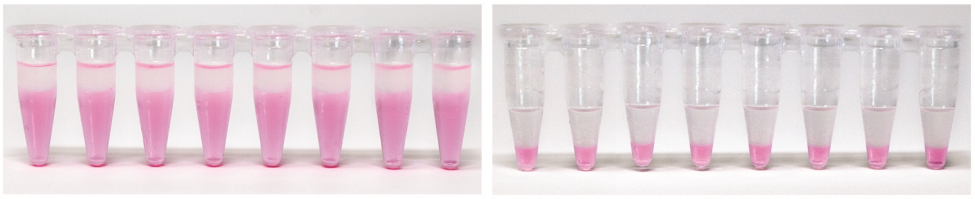
GEM Recovery and Pre-Amplification

3

3.0 Get Started

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

3.1 Post-GEM Incubation – Recovery



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

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

b. Firmly secure the cap on the tube strip, ensuring that no liquid is trapped between the cap and the tube rim. Mix by inverting the capped tube strip 5x.

DO NOT invert without firmly securing the caps.

c. Wait **2 min**.

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

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

d. Centrifuge briefly.

e. Slowly remove and discard **125 µl** Recovery Agent/Partitioning Oil (pink) from the bottom of the tube. DO NOT aspirate any aqueous sample.

f. Proceed directly to Pre-Amplification PCR. No cleanup step is required.

3.2 Pre-Amplification PCR

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

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

b. Add **35 µl** Pre-Amplification Mix to 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 µl	~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

STOP

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

3.3 DNA Cleanup – SPRIselect

a. Prepare Elution Solution. Vortex and centrifuge briefly.

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

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

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

c. Vortex to resuspend the SPRIselect reagent. Add **126 µl** SPRIselect reagent **(1.8X)** to each sample and pipette mix 15x (pipette set to 180 µl).

d. Incubate **5 min** at **room temperature**.

e. Place on the magnet•**High** until the solution clears.

f. Remove the supernatant. DO NOT discard any beads.

g. With the tube still in the magnet, add **200 µl** 80% ethanol to the pellet. Wait **30 sec**.

h. Remove the ethanol.

i. **Repeat** steps g and h for a total of 2 washes.

j. Centrifuge briefly and place on the magnet•**Low**.

k. Remove any remaining ethanol. DO NOT let the sample dry to ensure maximum elution efficiency.

l. Remove from the magnet. Add **101 µl** Elution Solution. Wait **1 min** before resuspending. Pipette mix 15x.

m. Incubate **2 min** at **room temperature**.

n. Place the tube strip on the magnet•**High** until the solution clears.

o. Transfer **100 µl** sample to a new tube strip.

STOP

p. Store at **4°C** for **≤72 h** or at **−20°C** for **≤4 weeks**, or proceed to the next step.

Step 4:

Library Construction

4

4.0 Get Started

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

4.1 Sample Index PCR

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

b. Prepare Sample Index PCR Mix on ice.

Sample Index PCR Mix *Add reagents in the order listed*		**PN**	**1X (µl)**	**1X + 10% (µl)**	**4X + 10% (µl)**
◯	**Amp Mix**	2000103/2000047	50.0	55.0	220.0
	Nuclease-free Water	—	10.0	11.0	44.0
	Total		**60.0**	**66.0**	**264.0**

c. Transfer **ONLY 20 µl** sample from the step DNA Cleanup – SPRIselect on page 71 to a new tube strip.

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

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

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

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

Targeted Cell Recovery	Total Cycles*	
	for Cell Lines	for PBMCs
500-2,000	12	16
2,000-4,000	11	15
4,000-7,000	10	14
7,000-12,000	9	13
12,000-25,000	8	12
25,000-50,000	7	11
50,000-128,000	6	10

**Optimization of cycle number may be needed based on the total RNA content of the sample. Target concentration is between 100 nM and 200 nM. If optimization is needed, additional Amp Mix can be obtained using the Library Amplification Kit (PN-1000249). 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. 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.

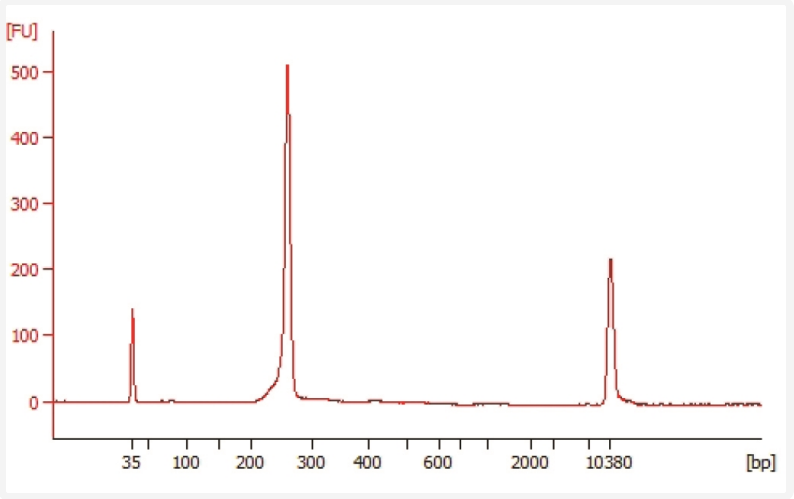
m. Transfer **40 µl** to a new tube strip.

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 µl** 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 93
- Agilent TapeStation Traces on page 94
- LabChip Traces on page 94

Step 5:

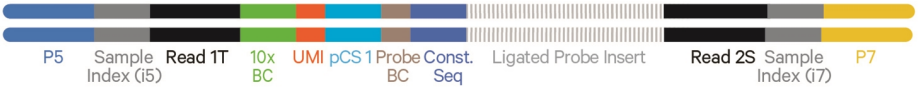
Sequencing

5

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 i7 Index i5 Index Read 2	28 cycles 10 cycles 10 cycles 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	5

** 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

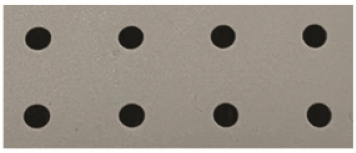
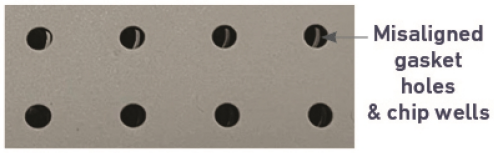
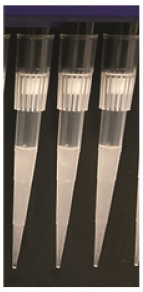
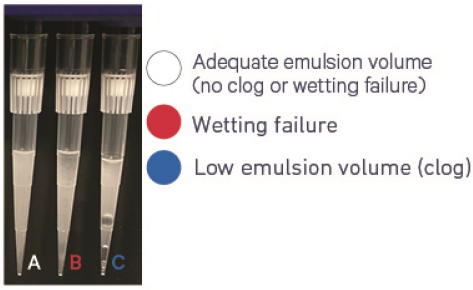
Loupe Browser is an interactive data visualization tool that requires no prior programming knowledge.

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

Troubleshooting

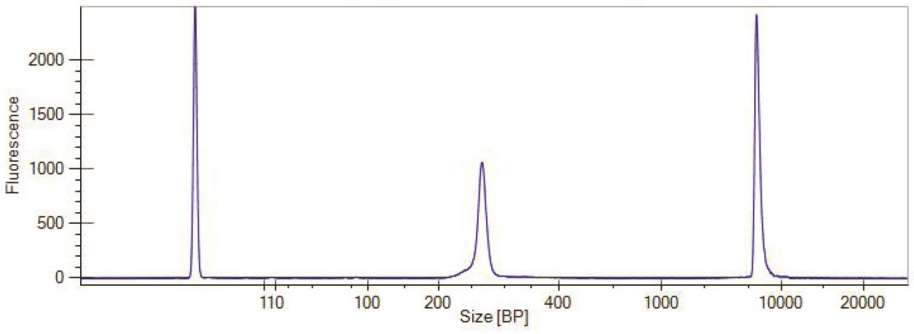
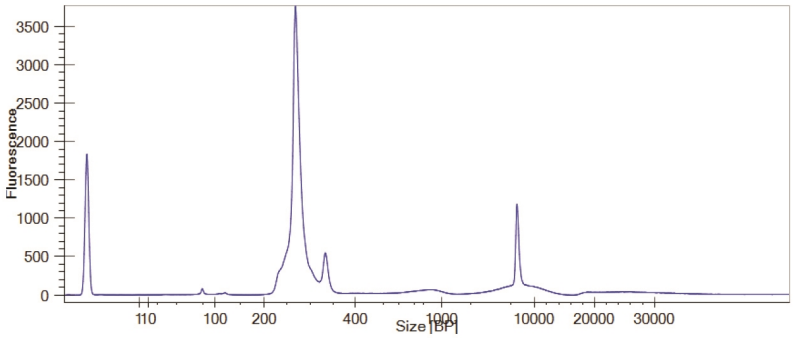
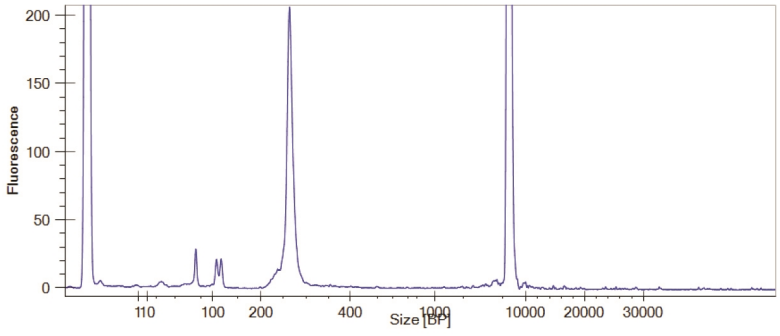
6

GEM Generation & Barcoding

Step	Normal	Abnormal
After loading the Chip		
	The gasket holes are aligned with the wells.	Gasket holes are misaligned with the sample and gel bead wells. Open and close the chip holder slowly once.
After Chip Q is removed from X/iX and the wells are exposed		
	All recovery wells are similar in volume and opacity.	Recovery well G indicates a reagent clog. Recovery well C and E indicates a wetting failure. Recovery wells B, D, and F are normal. Wells A and H contain 50% Glycerol Solution.
Transfer GEMs		
	All liquid levels are similar in volume and opacity without air trapped in the pipette tips.	Pipette tip A shows normal GEM generation, pipette tip B indicates a wetting failure, and pipette tip C shows a clog and wetting failure.

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

Post Library Construction QC

Step	
Fixed RNA – Gene Expression Library Correct Sample Index PCR cycling	 Fixed RNA – Gene Expression libraries should fall within the range of 100-200 nM. If the concentration falls below or above this range, sequencing data quality may be impacted, sample index PCR should be repeated with greater or fewer number of PCR cycles, respectively.
Over cycling	Additional higher molecular weight peaks present in the library trace indicate over cycling. 
Under cycling	Higher proportion of low molecular weight peaks present in the library trace indicate under cycling. 

Chromium X Series Errors

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

There are two types of errors:

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

a. System Error

b. Pressure Error

c. Chip Error

d. Run Error

e. Temperature Error

f. Software Error

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

a. Gasket Error

b. Tray Error

c. Chip Error

d. Unsupported Chip Error

e. Network Error

f. Update Error

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

Appendix

7

Post-Hybridization Pool & Wash – Individual Wash Workflow

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

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

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

Post-Hyb Wash Buffer *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
	Total	**-**	**2.2**	**8.8**	**35.2**

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

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

c. Add **900 µl** Post-Hyb Wash Buffer to each sample. Pipette mix 5x.

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

d. Incubate at **42°C** for **10 min** in a thermomixer or a heat block.

e. Centrifuge at **850 rcf** for **5 min** at **room temperature**.

f. Remove the supernatant without disturbing the pellet.

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 *Add reagents in the order listed*	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
	Total	**-**	**550.0**	**2200.0**	**8800.0**

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

n. Centrifuge sample at **850 rcf** for **5 min** at **room temperature**.

o. Remove the supernatant without disturbing the pellet.

p. Resuspend each cell pellet in **0.5 ml chilled** Post-Hyb Resuspension Buffer. Pipette mix 20x to resuspend and breakup any cell clumps and maintain on ice.

q. Pass each sample through a 30 µm filter (Sysmex CellTrics or Miltenyi Biotec Pre-Separation Filters) into a new 1.5-ml microcentrifuge tube.

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

TIPS

See Sample Filtration on page 31 for details.

r. Determine cell concentration of each sample using a Countess II Automated Cell Counter, a Cellaca counter, or a hemocytometer. See Cell Counting on page 33 for details.

s. Store the sample after resuspending in appropriate reagents (see Sample Storage on the next page) or **immediately** proceed to next step.

t. Pool an equal number of cells from different hybridization reactions into a 5-ml (for pooling 4 samples) or 15-ml (for pooling 4 or 16 samples) centrifuge tube. See table below on how to calculate the volume of each sample to be added.

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

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

Probe 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 33.

If the sample concentration is not sufficient to achieve the desired target cells recovery, concentrate the sample as follows:

- *Centrifuge a known volume of sample at 850 rcf for 5 min at room temperature.*
- *Carefully remove only a fraction of the supernatant, and pipette thoroughly to resuspend the cell pellet in the remaining volume. Recount to confirm final concentration. The amount of supernatant removed should be proportional to the desired increase in concentration.*

  For example, to increase the concentration 4-fold from a starting volume of 400 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 57.

Sample Storage

- Add 0.1 volume Enhancer to sample in Post-Hyb Resuspension Buffer. For example, add 50 µl Enhancer to 500 µl of sample in Post-Hyb Resuspension Buffer.
- Add 50% glycerol 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 **–20°C** or **–80°C** for up to **1 month**. For best results, storage at –80°C is strongly recommended.

Using Stored Samples

- When ready to use samples stored at –20°C or –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 Chromium Fixed RNA Kit, Human Transcriptome, 4 rxns x 4 BC PN-1000475 to pool less than 4 samples per well, the total number of cells targeted should not exceed 10,000 cells per Probe Barcode (or 10,000 * number of Probe Barcodes used/per well). For example, if pooling 3 samples hybridized with 3 unique Probe Barcodes, the total number of cells targeted should not exceed 30,000 cells/well (10,000 * 3).
- When using the Chromium Fixed RNA Kit, Human Transcriptome, 4 rxns x 16 BC PN-1000476 to pool less than 16 samples per well, the total number of cells targeted should not exceed 8,000 cells per Probe Barcode (or 8,000 * number of Probe Barcodes used/per well). For example, if pooling 14 samples hybridized with 14 unique Probe Barcodes, the total number of cells targeted should not exceed 112,000 cells/well (8,000 * 14).

Chromium Fixed RNA Profiling: Chip Loading Overview

This section provides a quick overview to the Chip Q loading and does not include detailed instructions. Refer to Load Chromium Next GEM Chip Q on page 62 for details.

Steps

a. Add 50% glycerol solution to each unused well

- Load 70 µl to row labeled 1
- Load 50 µl to row labeled 2
- Load 45 µl to row labeled 3

b. Prepare Gel Beads

- Vortex for 30 sec
- Centrifuge for 5 sec

c. Load Row Labeled 1

- Mix GEM Master Mix + Sample
- Load 70 µl to row labeled 1

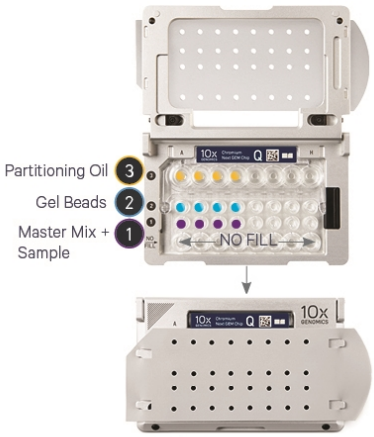
d. Load Row Labeled 2

- Aspirate Gel Beads
- Load 50 µl to row labeled 2
- Wait 60 sec

e. Load Row Labeled 3

- Load 45 µl Partitioning Oil to row labeled 3

f. Close the lid and prepare for run.



Post Library Construction Quantification

a. Thaw KAPA Library Quantification Kit for Illumina Platforms.

b. Dilute **2 µl** sample with deionized water to appropriate dilutions that fall within the linear detection range of the KAPA Library Quantification Kit for Illumina Platforms. (For more accurate quantification, make the dilution(s) in duplicate).

c. Make enough Quantification Master Mix for the DNA dilutions per sample and the DNA Standards (plus 10% excess) using the guidance for 1 reaction volume below.

Quantification Master Mix	1X (µl)
SYBR Fast Master Mix + Primer	12
Water	4
Total	**16**

d. Dispense **16 µl** Quantification Master Mix for sample dilutions and DNA Standards into a 96 well PCR plate.

e. Add **4 µl** sample dilutions and **4 µl** DNA Standards to appropriate wells. Centrifuge briefly.

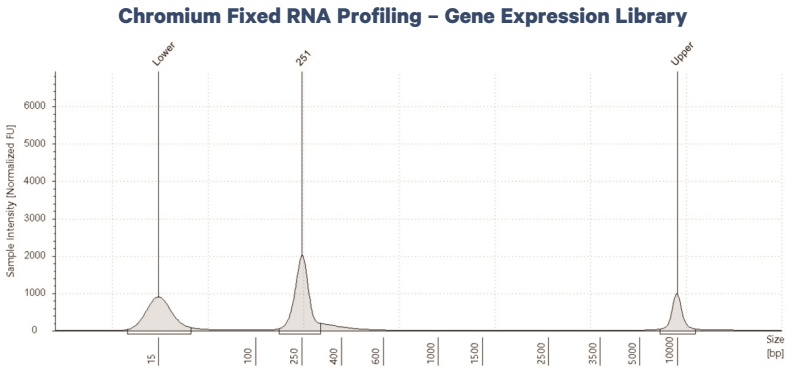
f. Incubate in a thermal cycler with the following protocol.

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

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

Agilent TapeStation Traces

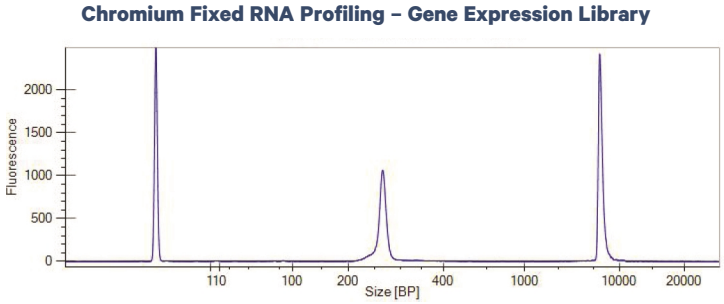
Agilent TapeStation High Sensitivity D5000 ScreenTape was used.



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

LabChip Traces

DNA High Sensitivity Reagent Kit was used.



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

Oligonucleotide Sequences

Gel Bead Primer

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

Chromium Fixed RNA Profiling – Gene Expression Library

5'-AATGATACGGCGACCACCGA-N10-GATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT-N16-N12-TTGCTAGGACCG-BC8-NN-TACGTGCTAACCGCGT-Ligated_Probe_Insert-TGGAATTCTCGGGTGCCAAGGAACTCCAGTCAC-N10-ATCTCGTATGCCGTCTTCTGCTTG-3'
3'-TTACTATGCCGCTGGTGGCT-N10-CTAGATGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA-N16-N12-AACGATCCTGGC-BC8-NN-ATGCACGATTGGCGCA- Ligated_Probe_Insert -ACCTTAAGAGCCCACGGTTCCTTGAGGTCAGTG-N10-TAGAGCATACGGCAGAAGACGAAC-5'