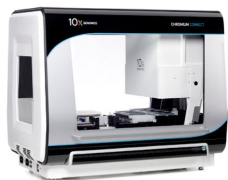
CG000656 Rev A



USER GUIDE

Automated Fixed RNA Profiling Library Construction

FOR USE WITH

Chromium Automated Library Construction Kit-B, 24 rxns PN-1000581

Chromium Automated Library Construction Kit-B, 4 rxns PN-1000597

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

Dual Index Kit TT Set A, 96 rxns PN-1000215

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Notices

Document Number

CG000656 • Rev A

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Introduction

Workflow Overview

Automated Library Construction Kit

Additional Kits, Reagents & Equipment

Recommended Thermal Cyclers

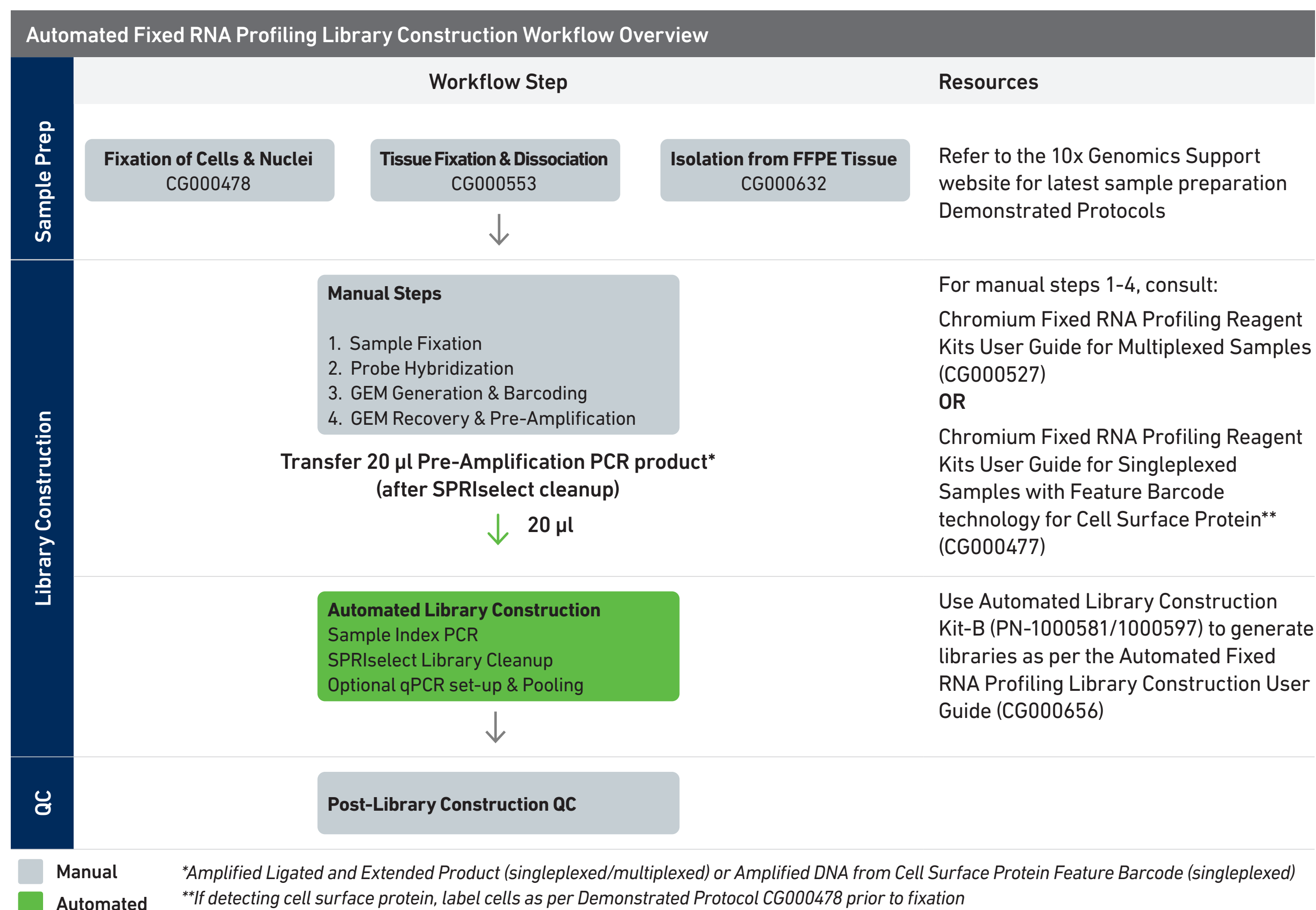
Protocol Steps & Timing

Workflow Overview

This User Guide provides an overview of the Automated Fixed RNA Profiling Library Construction workflow to generate Gene Expression (singleplexed/multiplexed) or Cell Surface Protein (Feature Barcode; singleplexed) libraries. These libraries can be generated on Chromium Connect from manually prepared Amplified Ligated and Extended Product (singleplexed/multiplexed) or Amplified DNA from Cell Surface Protein Feature Barcode (singleplexed) respectively. Consult the Chromium Fixed RNA Profiling Reagent Kits User Guide for Multiplexed Samples (CG000527) or the Chromium Fixed RNA Profiling Reagent Kits User Guide for Singleplexed Samples with Feature Barcode technology for Cell Surface Protein (CG000477) for details of the manual steps.

SPRIselect cleaned Pre-Amplification PCR product (20 µl), prepared as per the manual workflow, is the starting material for the automated Sample Index PCR and final Library Construction steps on Chromium Connect. During Automated Fixed RNA Profiling Library Construction, the Sample Index PCR cycle for a specific library type can be selected through the Chromium Connect User Interface as per recommendations provided in this User Guide (also specified in the relevant manual user guides).

For instrument operation, refer to the Chromium Connect Instrument User Guide (CG000180).



**Amplified Ligated and Extended Product (singleplexed/multiplexed) or Amplified DNA from Cell Surface Protein Feature Barcode (singleplexed)*
***If detecting cell surface protein, label cells as per Demonstrated Protocol CG000478 prior to fixation*

Automated Fixed RNA Profiling Library Construction Kit

Refer to SDS for handling and disposal information

Chromium Automated Library Construction Kit-B, 24 rxns PN-1000581

Reagent volumes and colors are different in each of the module types, not all module tubes contain reagents.

Automated Library Construction Kit-B, Module 1, 24 rxns PN-1000579 (store at 4°C)

Automated Library Construction Kit-B
Module 1, 24 rxns

	#
Module 1	24 tube strips

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Automated Library Construction Kit-B, Module 1

Automated Library Construction Kit-B, Module 2, 24 rxns PN-1000580 (store at -20°C)

Automated Library Construction Kit-B
Module 2, 24 rxns

	#
Module 2	24 tube strips

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Automated Library Construction Kit-B, Module 2

Chromium Automated Library Construction Kit-B, 4 rxns PN-1000597

Reagent volumes and colors are different in each of the module types, not all module tubes contain reagents.

Automated Library Construction Kit-B, Module 1, 4 rxns PN-1000595 (store at 4°C)

Automated Library Construction Kit-B
Module 1, 4 rxns

	#
Module 1	4 tube strips

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Automated Library Construction Kit-B, Module 1

Automated Library Construction Kit-B, Module 2, 4 rxns PN-1000596 (store at -20°C)

Automated Library Construction Kit-B
Module 2, 4 rxns

	#
Module 2	4 tube strips

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Automated Library Construction Kit-B, Module 2

Dual Index Kit TS Set A, 96 rxns PN-1000251 (store at −20°C)
for Fixed RNA Profiling Gene Expression library construction

Dual Index Kit TS Set A

	#	PN
Dual Index Plate TS Set A	1	3000511

Dual Index Kit TT Set A, 96 rxns PN-1000215 (store at −20°C)
for Fixed RNA Profiling Cell Surface Protein library construction

Dual Index Kit TT Set A

	#	PN
Dual Index Plate TT Set A	1	3000431

Additional Kits, Reagents & Equipment

The items in the table below have been validated by 10x Genomics and are required for the Chromium Connect Automated Fixed RNA Library Construction protocol. DO NOT substitute any of the listed materials.

Supplier	Description	Part Number (US)
Plastics		
Hamilton	CO-RE/CO-RE IITips 50 µl Filtered Tips* CO-RE/CO-RE II Tips 300 µl Filtered Tips* 60 ml Reagent Reservoir Self-Standing Hamilton PCR ComfortLid	235948 235903 194051 814300
	**CO-RE pipette tips will be phased out and replaced by new CO-RE II pipette tips (same part number as CO-RE tips) in 2022/2023. CO-RE II tips include a new sealing surface to interface with the CO-RE II stop disk. Geometry that interfaces with the current CO-RE stop disk is identical between the two tip designs and performance remains unaffected.*	
Eppendorf	96-well Full-Skirted Plate 96-well Semi-Skirted Plate *(Blue color listed; other colors are acceptable)*	951020460 951020362
Thermo Fisher Scientific	MicroAmp 8-Tube Strip, 0.2 ml MicroAmp 8-Cap Strip, clear	N8010580 4323032
Kits & Reagents		
Thermo Fisher Scientific	Nuclease-free Water	AM9937
Millipore Sigma	Ethanol, Pure (200 Proof, anhydrous)	E7023-500ML
Qiagen	Qiagen Buffer EB	19086
Equipment		
10x Genomics	10x Vortex Adapter Benchtop Vortex Benchtop Centrifuge Plate Centrifuge Benchtop Thermal Cycler *(optional)*	330002 standard lab equipment standard lab equipment standard lab equipment standard lab equipment
Additional materials ONLY for optional assays – qPCR and pooling		
Bio-Rad	10% Tween 20 96-well PCR Plates	1662404 HSP9665
Thermo Fisher Scientific	2 ml-Screw-cap Tubes, NonKnurl 0.5 ml-Screw-cap Tubes, NonKnurl	3488NK 3472NK
KAPA Biosystems	KAPA Library Quantification Kit for Illumina Platforms	KK4824
Qiagen	Qiagen Buffer EB	19086
Additional materials for Chromium Connect maintenance Use only indicated cleaning agents. DO NOT use bleach or organic oxidizers		
Thor Labs	Lens tissues	MC-5
VWR	Microcide SQ Broad Spectrum Disinfectant	25099
Contec	70% Isopropanol (alternative to VWR disinfectant)	SB167030IR

Additional Kits, Reagents & Equipment

Supplier	Description	Part Number (US)
Quantification & Quality Control		
Agilent *Choose Bioanalyzer, TapeStation, LabChip, or Qubit based on availability & preference.*	2100 Bioanalyzer Laptop Bundle *(discontinued)* (Replacement 2100 Bioanalyzer Instrument/ 2100 Expert Laptop Bundle) High Sensitivity DNA Kit 4200 TapeStation High Sensitivity D1000 ScreenTape/Reagents High Sensitivity D5000 ScreenTape/Reagents	G2943CA G2939BA/G2953CA 5067-4626 G2991AA 5067-5592/ 5067-5593 5067-5584/ 5067-5585
Thermo Fisher Scientific	Qubit 4.0 Fluorometer Qubit dsDNA HS Assay Kit	Q33238 Q32854
PerkinElmer	LabChip GX Touch HT Nucleic Acid Analyzer DNA High Sensitivity Reagent Kit	CLS137031 CLS760672

Protocol Steps & Timing

Steps		Timing
MANUAL	**Sample Preparation** *Prepare single cell or nuclei suspension for Fixed RNA Profiling workflows (multiplex workflow or singleplex workflow with optional Cell Surface Protein Labeling prior to fixation)*	Dependent on sample
	Sample Fixation	2-3 h
	Probe Hybridization	16-24 h
	GEM Generation & Barcoding Post-hybridization wash Prepare GEM Master Mix + Sample Dilution Load Chip Q Run Chromium X/iX Transfer GEMs GEM Incubation	**~4 h**
	GEM Recovery and Pre-Amplification Post-GEM Incubation - Recovery Pre-Amplification PCR DNA Cleanup - SPRIselect	**1.5 h**

Use only 20 µl pre-amplified, cleaned-up DNA product (derived from the multiplex workflow or the singleplex workflow with optional Cell Surface Protein Labeling) for Automated Fixed RNA Profiling Library Construction.

AUTOMATED	**Fixed RNA Profiling - Library Construction** • Sample Index PCR • SPRIselect Library Cleanup • Optional qPCR set-up & Pooling	~2.5 h Walk-away time
MANUAL	Post Library Construction QC	~60 min

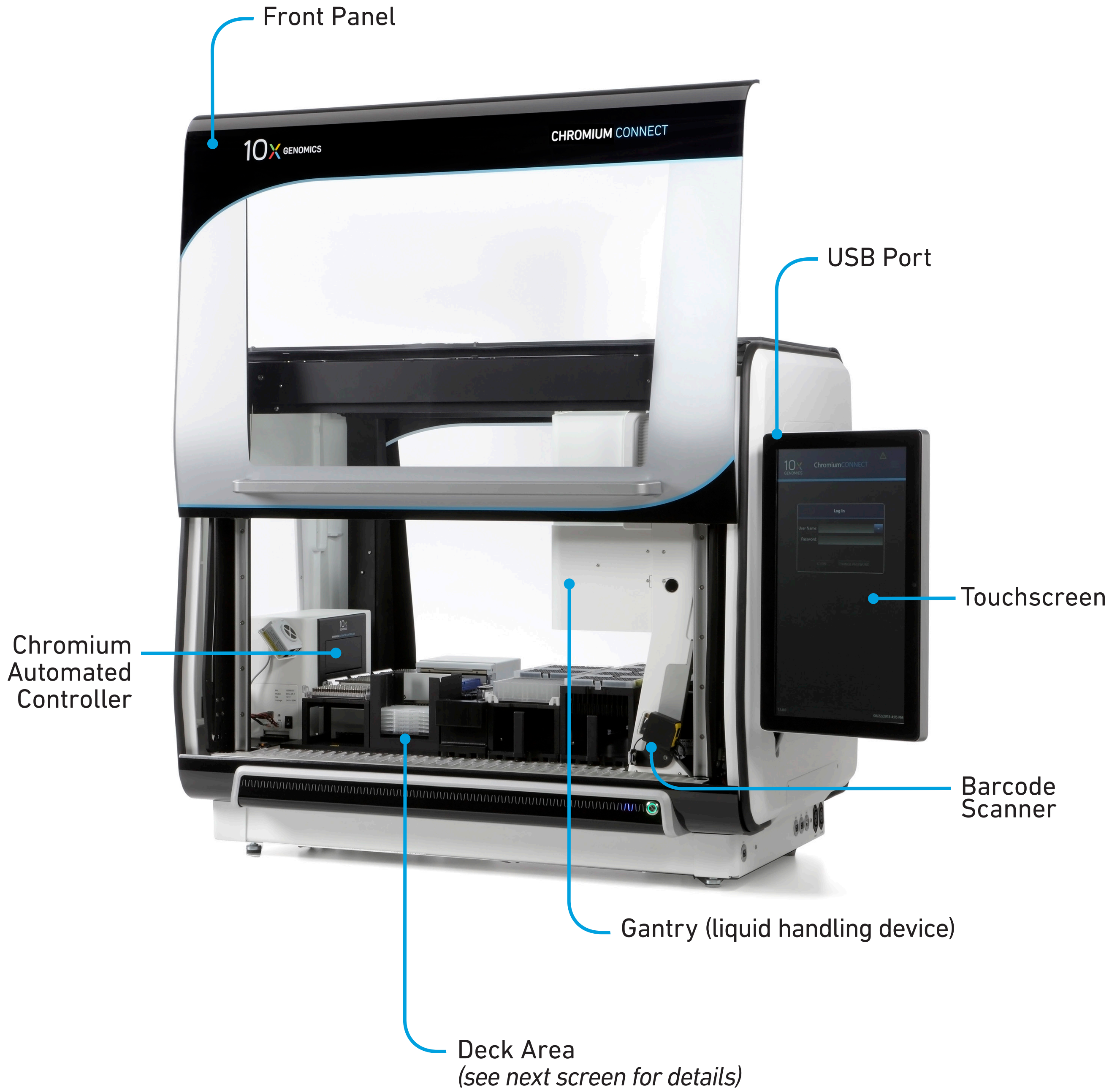
Chromium Connect

Instrument Orientation

Deck Orientation

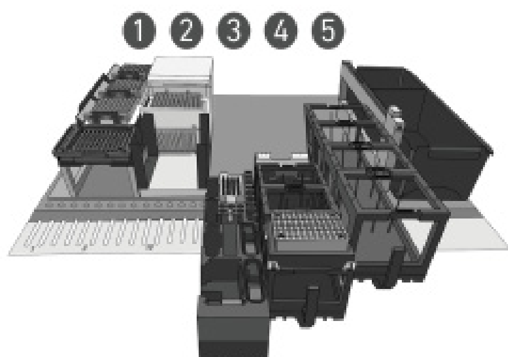
CSV Setup

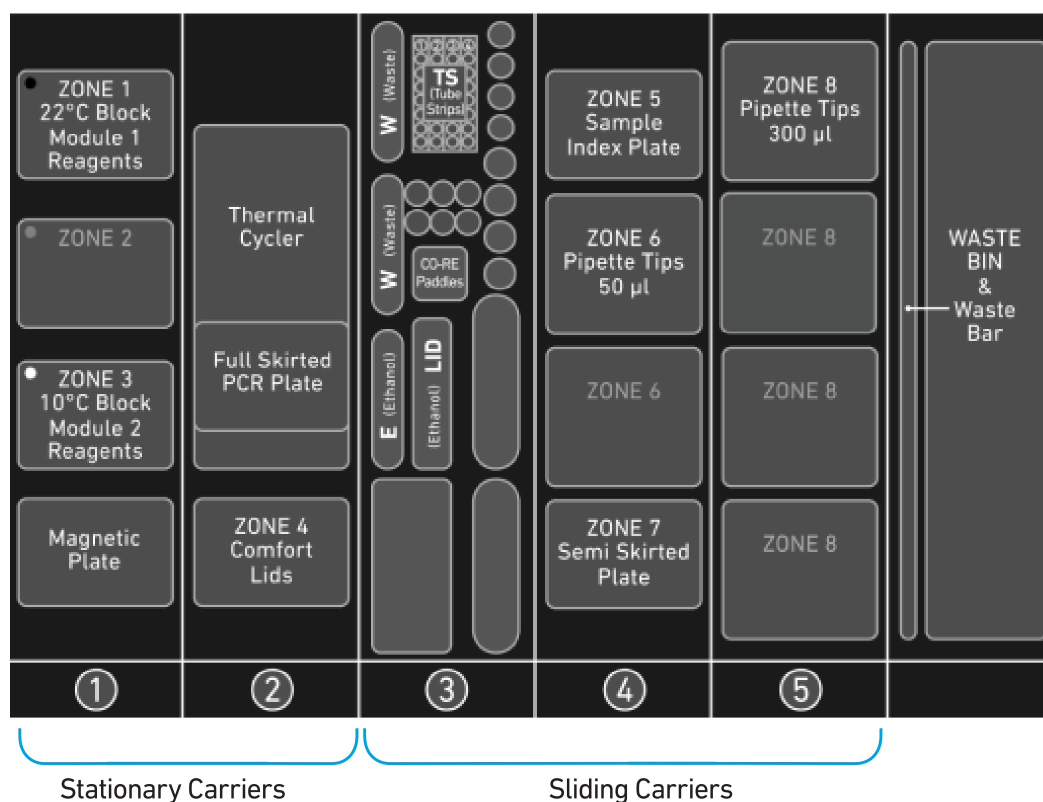
Instrument Orientation



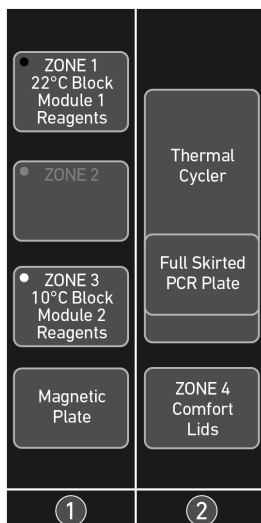
Refer to the Chromium Connect Instrument User Guide (CG000180) and Quick Reference Cards (CG000256) for more information.

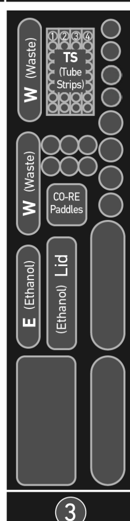
Deck Orientation





Refer to the Chromium Connect Instrument User Guide (CG000180) and Quick Reference Cards (CG000256) for more information.





Deck Layout Reagents/Consumables

Automated Single Cell Fixed RNA Profiling Library Construction

Carrier	Zone	Item
1 Stationary	Zone 1 (Black)	22°C Block, Reagent Strips, Module 1
	Zone 3 (White)	10°C Block, Reagent Strips, Module 2
	-	Magnetic Plate
2 Stationary	-	Thermal Cycler
	-	Full Skirted PCR Plate (within Thermal Cycler)
	Zone 4	ComfortLids (2)
3* Sliding Deck Rails: 15-18 Number of Lights: 4 *Assay choices determine items loaded in Carrier 3	Position W	Waste Reservoirs
	Position TS	Tube Strip (position 4)
	Position CP	CO-RE Paddles
	Position E	Ethanol Reservoir
	Position Lid	Lid for Ethanol Reservoir
4 Sliding Deck Rails: 19-24 Number of Lights: 6	Zone 5	Sample Index Plate TS or TT Set A Verify name & PN
	Zone 6	Pipette Tips 50 µl
	Zone 7	Semi Skirted Plate
5 Sliding Deck Rails: 25-30 Number of Lights: 6	Zone 8	Pipette Tips 300 µl

CSV Setup

Sample information can also be uploaded using a CSV file at the run set-up screen. Use the folder icon to search a network file system or USB drive. Navigate to the appropriate folder with CSV file and click "SELECT".

The Chromium Connect Input Sample Template (CG000309) is available on the 10x Genomics support website.

Run Set-up Screen

Sample CSV File

A sample CSV file is shown below. The columns highlighted in blue are mandatory to start a run. Any missing fields/corrections can be added during sample information setup. Final selections will be recorded in the final run report CSV file.

SAMPLEPARAMETERS	SAMPLENAME	SIINDEX	VOLUME	CellCount	Expression	CYCLES	USERDEFINED1	USERDEFINED2	USERDEFINED3	USERDEFINED4
ID1	aaa	A1								
ID2	bbb	B1								
ID3	ccc	C1								
ID4	ddd	D1								
ID5	eee	E1								
ID6	fff	F1								
ID7	ggg	G1								
ID8	hhh	H1								
RUNPARAMETERS	SELECTION									
runName	Sample Run									
Instruction Level	Standard									
Sample Input Type?	GEX Singleplex									
qPCR Setup?	No									
Pooling?	No									
SI Cycles	14									
Notes										

Uploading Sample Information Using a CSV File

The following tables provide specific guidelines on sample entry in the CSV file.

Sample Parameters	Information
Sample Name	Alphanumeric and up to 32 characters
Sample Index	Location on dual index plate to be used for each sample during SI PCR

Up to four user-defined fields (LIMS data) can be passed through the instrument for additional sample tracking.

Run Parameters	Information
Run Name	Alphanumeric and up to 32 characters
Instruction Level	Standard, Advanced, Expert Refer to the Chromium Connect Instrument User Guide (CG000180) for details
Sample Input Type	GEX Multiplex, GEX Singleplex, FB Singleplex
qPCR Setup	Opt-in for optional assay step: Yes/No
Pooling	Opt-in for optional assay step: Yes/No
SI Cycles	User defined field. Refer to appropriate section in this User Guide for guidance on optimal cycles.

Items & Reagents

Tips & Best Practices

Reagent Handling

- Fully thaw and thoroughly mix reagents before use.
- Ensure that there are no bubbles at the bottoms of modules and plate wells.
- Follow the prompts on the instrument touchscreen for handling modules during setup and use.
- Prepare and dispense 80% ethanol off-deck to avoid spilling on consumables.

Gather Items & Reagents for Fixed RNA Profiling Library Construction

Follow prompts on the Chromium Connect touchscreen to gather the listed items and reagents for loading the Deck Carriers. Gather the quantities specified for each of the items and reagents.

Item	Qty
Nuclease-free Water	10 ml
Ethanol, Pure (200 Proof, anhydrous)	40 ml
Hamilton	
ComfortLids	2
50 µl CO-RE/CO-RE II Pipette Tips, with filter (Black, Conductive)	1 rack
300 µl CO-RE/CO-RE II Pipette Tips, with filter (Black, Conductive)	1 rack
Reagent Reservoir, 60 ml	3
Eppendorf	
96-well Semi Skirted Plate	1
96-well Full Skirted Plate	1
Thermo Fisher Scientific	
MicroAmp 8-Tube Strip, 0.2 ml	1
10x Genomics	
Automated Fixed RNA Profiling Library Construction Kit	
Automated Library Construction Kit-B, Module 1 (stored at 4°C) *Black tube strip*	1 tube strip/sample
Automated Library Construction Kit-B, Module 2 (stored at -20°C) *White tube strip*	1 tube strip/sample
Dual Index Plate TS or TT Set A (stored at -20°C) *Verify name & PN*	1 plate

See Additional Kits, Reagents & Equipment list for performing optional assays and/or QC.

Thaw & Prep Reagents for Fixed RNA Profiling Library Construction

Follow prompts on the touchscreen to thaw and prepare reagents. Some important guidelines are highlighted below.

ACTION	GUIDELINES *Follow touchscreen prompts for specifics and timing*
Thaw Reagents	• Thaw reagents as indicated on the touchscreen. Verify no precipitate is present. • Ensure that the correct thawing locations and temperatures are used. • During reagent thaw load the consumables following touchscreen prompts.
Prepare Ethanol	• Prepare **50 ml** 80% Ethanol in Nuclease-free water and dispense in Ethanol Reservoir when prompted.
Automated Fixed RNA Profiling Library Construction Modules	• Thaw Modules as prompted on the touchscreen. • Confirm that there are no bubbles at the bottoms of any module tubes.
Dual Index Plate TS or TT, Set A	• Use the indicated plate. Verify name & PN. Vortex Dual Index Plate for **15 sec** at maximum speed and centrifuge at **300 rcf** for **1 min**.

Confirm that there are no bubbles at the bottoms of any module tubes and Dual Index Plate wells.

Quick Planner Card

Gather the listed items & reagents before running the assay. Follow the touchscreen prompts for detailed information.

Gather indicated items prior to running the assay	
☐ Nuclease free water – 10 ml	☐ Semi skirted plate, 96 well – 1 per run
☐ Ethanol, Pure (200 Proof, anhydrous) – 40 ml Combine 40 ml EtOH and 10 ml nuclease free water to prepare 80% EtOH	☐ Full skirted plate, 96 well – 1 per run
☐ ComfortLids – 2 per run	☐ 50 µl Black CO-RE/CO-RE II Pipette Tips, with filter • 1 rack
☐ Micro Amp 8-tube strips, 0.2 ml – 1 per run	
☐ Reagent reservoirs, 60 ml – 3 per run	☐ 300 µl Black CO-RE/CO-RE II Pipette Tips, with filter • 1 rack

10x Reagents	Storage	Preparation & Handling
☐ Reagent Module 1 (black tube strip) • 1 tube strip per sample	4°C	Maintain at room temperature for 30 min. Vortex, centrifuge at 300 rcf for 1 min.
☐ Reagent Module 2 (white tube strip) • 1 tube strip per sample	-20°C	Thaw at 4°C or on ice. Maintain chilled until ready to load. Before loading, invert mix (DO NOT vortex), centrifuge at 300 rcf for 1 min.
☐ Dual Index Plate TS or TT, Set A • 1 tube strip per sample	-20°C	Vortex for 15 sec at maximum speed, centrifuge at 300 rcf for 1 min.

Carrier Loading Guidelines

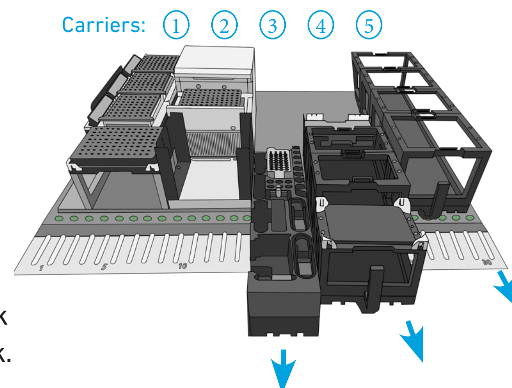
Carrier Loading Guidelines

Follow the instructions on the touchscreen to load the carriers.

Carriers

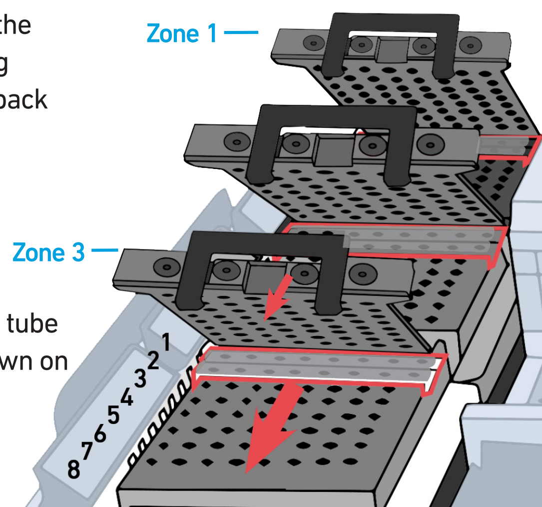
- Handle the carriers as prompted.
- Ensure that Carriers 3, 4, and 5 are completely slid out and placed on an off-deck workspace before loading.

Align the carriers to the corresponding Deck Rails when sliding them in or out of the deck.



Modules

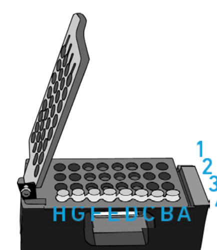
- Load one tube strip/sample of each of the indicated modules in the corresponding positions on the Carrier, starting from back to front row.
- DO NOT skip any rows when loading.
- Use pinhole alignment to place module tube strips in the correct orientation (as shown on the touchscreen).



Label Tube Strip Orientation

- The tube strip to receive the final libraries should be placed in Position 4 of the Tube Strip Holder.
- Label tube strip orientation for collecting final libraries.

Tube Strip (TS) Holder



Consult the Chromium Connect User Guide (CG000180) for more information.

Fixed RNA Profiling Library Construction Guidelines

Sample Index PCR

Post Sample Index PCR & Size Selection – SPRIselect

Post Library Construction QC

DNA Input

Follow the guidelines in the relevant manual workflow user guides (listed below) to prepare and cleanup pre-amplification DNA product as input for Automated Fixed RNA Profiling Library Construction.

- Chromium Fixed RNA Profiling Reagent Kits for Singleplexed Samples with Feature Barcode technology for Cell Surface Protein (CG000477).
- Chromium Fixed RNA Profiling Reagent Kits for Multiplexed Samples (CG000527).

The pellet formed after centrifugation may vary between different samples.
Ensure only the supernatant is transferred without disturbing the pellet.

Transfer DNA Input

a. Centrifuge **100 µl** cleaned-up pre-amplification DNA (prepared as per the manual workflow) at **500 rcf for 3 min** at **room temperature.**

Representative pre-amplification DNA

Example 1 *(before centrifuging)*

Example 1 *(after centrifuging)*

Example 2 *(before centrifuging)*

Example 2 *(after centrifuging)*

b. Transfer **20 µl** supernatant (without disturbing the pellet) to column 1 of a full skirted plate.
After transferring 20 µl, the remaining volume of pre-amplified DNA may be stored at 4°C for ≤72 h or at –20°C for ≤4 weeks.

Always use only **20 µl** cleaned-up pre-amplification DNA product as input for Automated Fixed RNA Profiling Library Construction.

c. Load onto the Chromium Connect deck, following the prompts on the instrument touchscreen.

Sample Information: Enter the sample information (sample name, singleplex or multiplex information, targeted cell recovery, etc.) in the "Sample ID" field on the instrument touchscreen.

Sample Index PCR

- Enter the applicable SI-PCR cycle number through the Chromium Connect User Interface as per recommendations provided below for specific library types (also specified in the manual workflow user guides).
- Cycle number selected will apply to all the samples in the run.

Chromium Fixed RNA Profiling – Gene Expression Library (multiplexed)

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

Chromium Fixed RNA Profiling – Gene Expression Library (singleplexed)

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

;

Chromium Fixed RNA Profiling – Cell Surface Protein Library (singleplexed)

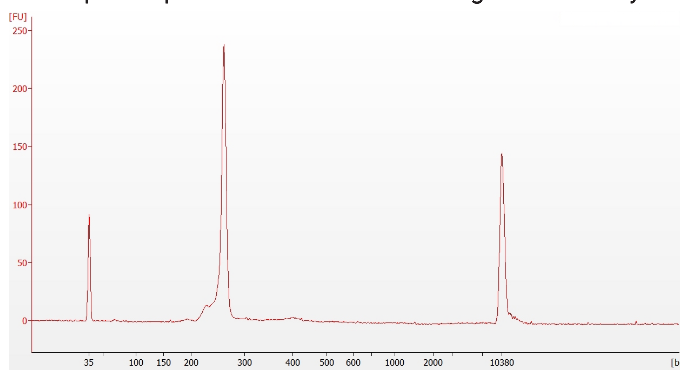
Targeted Cell Recovery	Total Cycles
500-2,000	14-15
2,000-4,000	13-14
4,000-10,000	12-13

**Optimization of cycle number may be needed based on the total RNA content of the sample. The ideal target library concentration is 50 - 200 nM. However, if the concentration is between 10-50 nM or between 200-500 nM and if the libraries do not contain low or high molecular weight peaks, sequencing can still be performed. For dissociated tumor cells, cycle numbers for cell lines can be used as a starting point. For dissociated primary cells, cycle numbers for PBMCs can be used as a starting point.*
***For cells derived from the fixed and dissociated tissue samples, the cycle number will depend on the RNA expression level of the tissue and on overall quality of the tissue prior to fixation. Additional optimization may be required.*

Post Library Construction QC

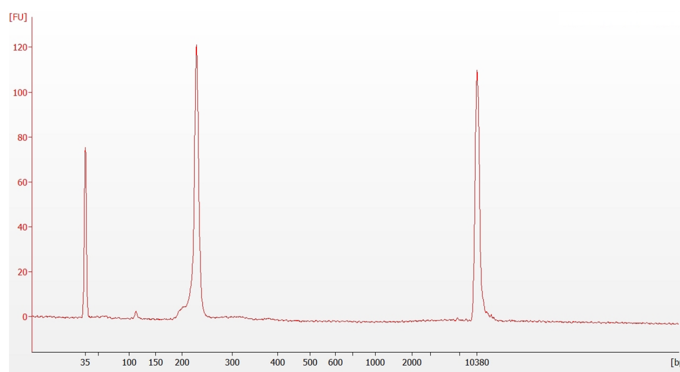
Representative Trace - Gene Expression Library

Run 1 µl sample at 1:40 dilution on an Agilent Bioanalyzer High Sensitivity chip.



Representative Traces - Cell Surface Protein Library

Run 1 µl sample at 1:20 dilution on an Agilent Bioanalyzer High Sensitivity chip.



The number of distinct peaks present may vary. An additional peak (~115 bp) may be present due to carryover adapters/primer dimers and can negatively impact sequencing performance. To reduce the additional peak, performing a SPRIselect (0.9X) cleanup before sequencing the library is recommended. See Appendix for representative traces and Post Library Construction Cleanup - SPRIselect steps. Note that ~40% of material may be lost when performing the SPRIselect cleanup.

Manually select the analysis region of ~150-300 bp and determine the average fragment size from the Bioanalyzer trace. This will be used as the insert size for library quantification.

Alternate QC Methods

- Agilent TapeStation
- PerkinElmer LabChip

See Appendix for representative traces

Post Library Construction Quantification & Pooling

Deck Orientation – Library Quantification

Post Library Construction Quantification

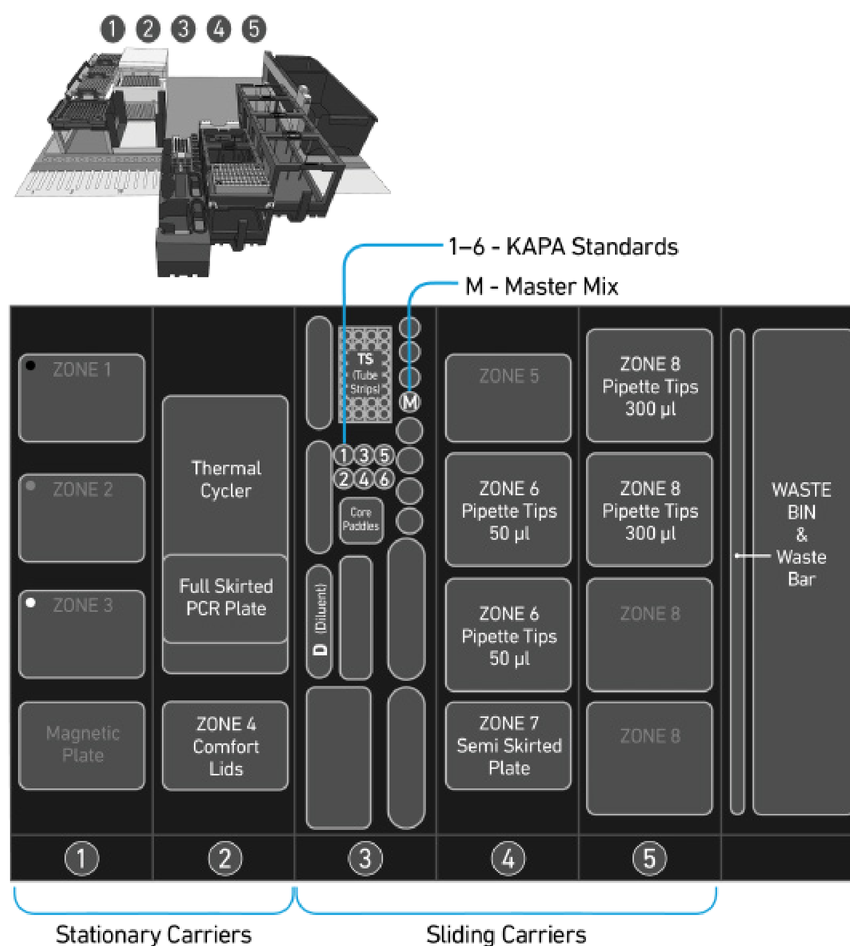
Deck Orientation – Library Pooling

Library Pooling

Deck Orientation – Library Quantification

Library quantification using qPCR is recommended for accurate pooling and loading on sequencers. If the option is selected during run-setup, automated qPCR plate set-up can be run directly on Chromium Connect after library generation and final library QC. Alternatively, the option can be selected from the instrument home screen, at the user's convenience. Up to 8 samples can be quantified on a 96 well reaction plate, including duplicates for each sample. The minimum sample volume required is **25 µl**. Only **6 µl** of the sample will be used for qPCR plate setup.

The Chromium Connect deck layout for Library Quantification setup is shown below.



Gather Items & Reagents

Follow prompts on the Chromium Connect touchscreen to gather the listed items and reagents for loading the Deck Carriers for Library Quantification.

Gather the quantities specified for each of the items and reagents.

Item	Qty
Hamilton	
ComfortLid	1
50 µl CO-RE/CO-RE II Pipette Tips, with filter (Black, Conductive)	1-2 racks
300 µl CO-RE/CO-RE II Pipette Tips, with filter (Black, Conductive)	1-2 racks
60-ml Reagent Reservoir	1
Eppendorf	
96-well Semi Skirted Plate	1
Thermo Fisher Scientific	
2-ml Tube with Screw Cap	6
Bio-Rad	
96-well Hard-Shell Full Skirted Plate	1
Reagent	**Qty**
Qiagen Buffer EB	50 ml
Nuclease-free Water	1 ml
10% Tween-20	250 µl
Libraries (in up to four 8-tube strips)	up to 8 libraries
KAPA Library Quantification Kit, thawed	
SYBR FAST Master Mix Primer Mix Standards	5 ml 1 ml 6

Post Library Construction Quantification

- Prepare reagents as prompted on the touchscreen.
- Vortex and centrifuge KAPA standards and libraries before use.
- Retrieve previously prepared Master Mix + Primer Mix
  OR
  Add **1 ml** Primer Mix to **5 ml** SYBR FAST Master Mix.
- Prepare specified Quantification Master Mix in the 2-ml tube using the guidance below.

# Sample	Master Mix + Primer Mix (µl)	Water (µl)	Total Vol (µl)
8	1305	435	1740
7	1200	400	1600
6	1095	365	1460
5	990	330	1320
4	885	295	1180
3	780	260	1040
2	675	225	900
1	570	190	760

Volumes listed take into account volume for 6 standards

See Tips & Best Practices for handling instructions. Load the chip in the order listed below. See dispensing instructions and illustration for each row. Always dispense slowly without introducing bubbles. Raising and depressing the pipette plunger should each take ~5 sec. Raise the pipette tips at the same rate as the liquid is rising, keeping the tips slightly submerged.

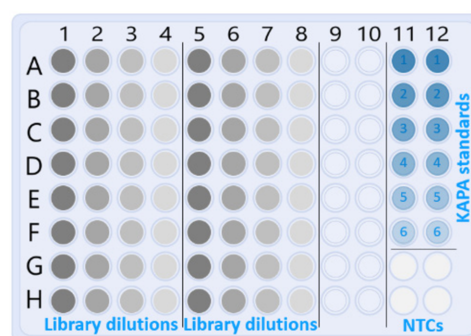
Post Library Construction Quantification

- Follow the touchscreen prompts for loading, scanning, and executing the run.
- During the run, the following steps will be executed by the instrument:
  -KAPA Master Mix transfer to the 96-well Hard Shell Full Skirted Plate (layout below)
  -Diluent transfer to dilution plate
  -Serial dilutions of libraries
  -Addition of library dilutions, KAPA Standards, and negative controls to the plate

Total reaction volume (20 µl)=
16 µl Master Mix
+
4 µl Library Dilution/
KAPA Standard/
Negative Control (NTC)

Dilutions:

1:12,500
1:62,500
1:312,500
1:1,562,500



- After the run is completed, follow the unloading instructions on the touchscreen.
- Cap and store libraries at **4°C ≤72 h** or **-20°C ≤4 weeks**.
- Remove Full Skirted Plate. Seal plate and centrifuge at **300 rcf** for **1 min** at **22°C**.
- Follow the manufacturer's recommendations for qPCR-based quantification. For library quantification for sequencer clustering, determine the concentration based on average size (bp) derived from the Bioanalyzer/TapeStation trace.

Step	Temperature	Run Time
1	95°C	00:05:00
2	95°C	00:00:30
3	60°C	00:00:45
4	Go to Step 2, 29X (Total 30 cycles)	

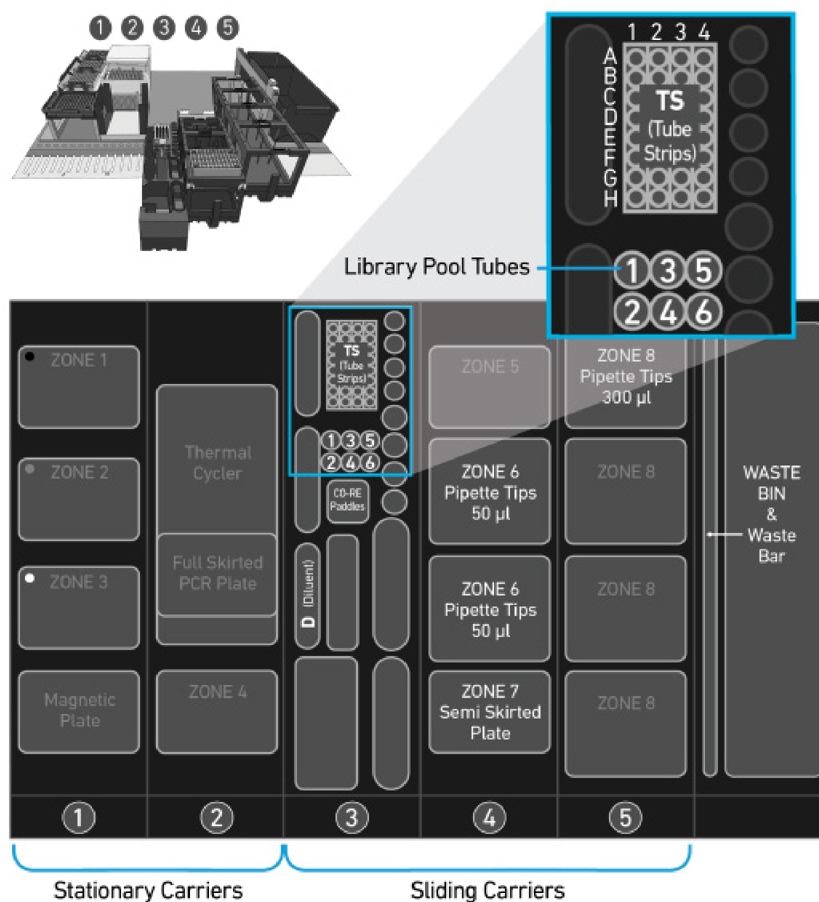
Resource:

Use the Chromium Connect Library Quantification Worksheet (CG000157) provided on the 10x Genomics Support website for calculating library concentrations.

Deck Orientation – Library Pooling

The libraries may be pooled on the Chromium Connect instrument and used for sequencing, taking into account the preferred cell numbers and per-cell read depth requirements for each library. Samples utilizing the same sample index should not be pooled together, or run on the same flow cell lane, as this would prevent correct sample demultiplexing.

The Chromium Connect deck layout for Library Pooling is shown below.



Gather Items & Reagents

Follow prompts on the Chromium Connect touchscreen to gather the listed items and reagents for loading the Deck Carriers for Library Pooling.

Gather the quantities specified for each of the items and reagents.

Item	Qty
Hamilton	
50 µl CO-RE/CO-RE II Pipette Tips, with filter (Black, Conductive)	1-2 racks
300 µl CO-RE/CO-RE II Pipette Tips, with filter (Black, Conductive)	1-2 racks
Reagent Reservoir, 60 ml	1
Eppendorf	
96-well Semi Skirted Plate	1
Thermo Fisher Scientific	
0.5-ml Tube with Screw Cap	6
Reagent	**Qty**
Qiagen Buffer EB	50 ml
Libraries (in up to four 8-tube strips)	up to 32 libraries

Library Pooling

- Follow the touchscreen prompts for loading, scanning, and executing the run.
- Briefly vortex and centrifuge libraries in the 8-tube strips.
- Ensure a minimum **25 µl** library volume is available in the tubes.
- After run is complete, follow touchscreen prompts to unload and store the libraries.
- Unload remaining items and clean as prompted on the touchscreen.

Resource:

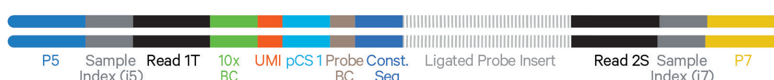
Use the Chromium Connect Library Pooling Worksheet (CG000466) provided on the 10x Genomics Support website to calculate volumes to be pooled. The calculated volumes can be input into the instrument either manually, or via the CSV file generated from this workbook.

Sequencing

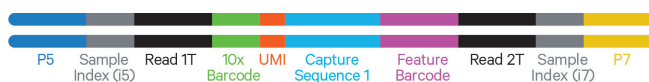
Sequencing Libraries

Chromium Fixed RNA Profiling – Gene Expression and Cell Surface Protein 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. TruSeq Read 1 (Read 1T) and TruSeq Read 2 (Read 2T) are used for paired-end sequencing of Fixed RNA – Cell Surface Protein library. Sequencing these libraries produces a standard Illumina BCL data output folder.

Chromium Fixed RNA Profiling – Gene Expression Library



Chromium Fixed RNA Profiling – Cell Surface Protein 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 Dual Index Kit TT Set A (PN-1000215) 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

Sequencing Depth	Minimum 10,000 read pairs per cell
Sequencing Type	Paired-end, dual indexing
Sequencing Read	Read 1: 28 cycles i7 Index: 10 cycles i5 Index: 10 cycles Read 2: 90 cycles *(minimum required Read 2 length is 50 bp)*

Fixed RNA – Cell Surface Protein Library Sequencing Parameters

Pooling Fixed RNA – Gene Expression & Cell Surface Protein libraries is recommended for sequencing to maintain nucleotide diversity.

Sequencing Depth	Minimum 5,000 read pairs per cell
Sequencing Type	Paired-end, dual indexing
Sequencing Read	Read 1: 28 cycles i7 Index: 10 cycles i5 Index: 10 cycles Read 2: 90 cycles *(minimum required Read 2 length is 25 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.

Fixed RNA – Gene Expression alone or in combination with Cell Surface Protein libraries

Library Loading:

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

* Use 150 pM loading concentration for Illumina XP workflow.

Library Pooling

Fixed RNA – Gene Expression and Cell Surface Protein 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.

Library Pooling Examples:

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

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

Appendix

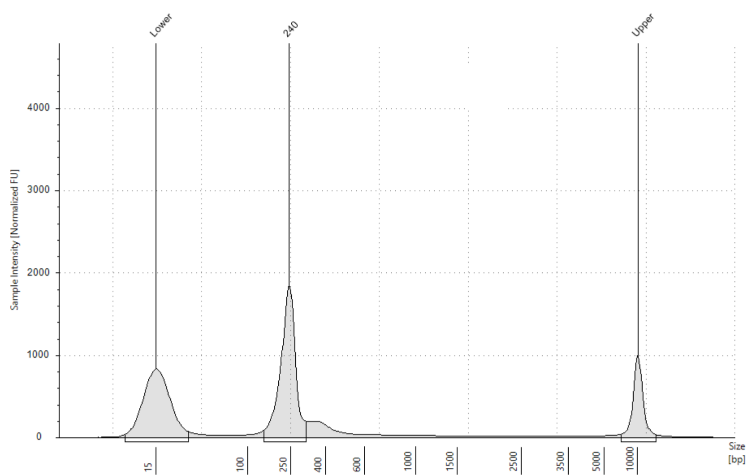
Agilent TapeStation Traces

LabChip Traces

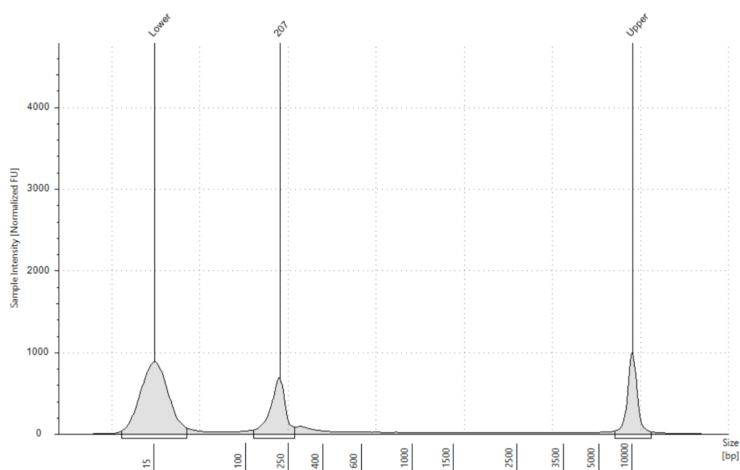
Agilent TapeStation Traces

Agilent TapeStation High Sensitivity D5000 ScreenTape was used.

Chromium Fixed RNA Profiling – Gene Expression Library



Chromium Fixed RNA Profiling – Cell Surface Protein Library

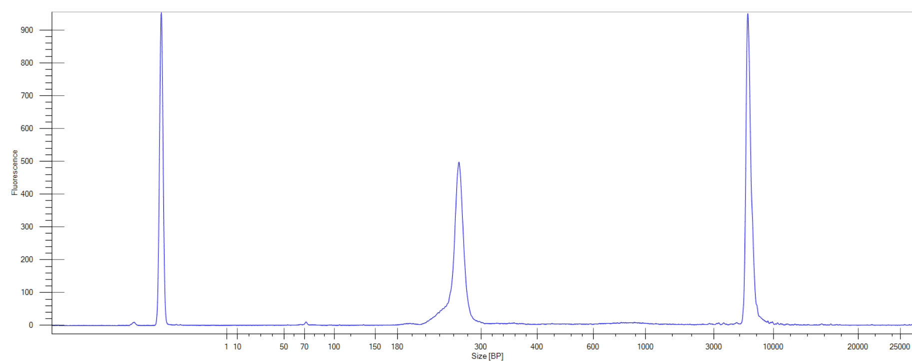


All traces are representative. Samples were run at 1:40 dilution.

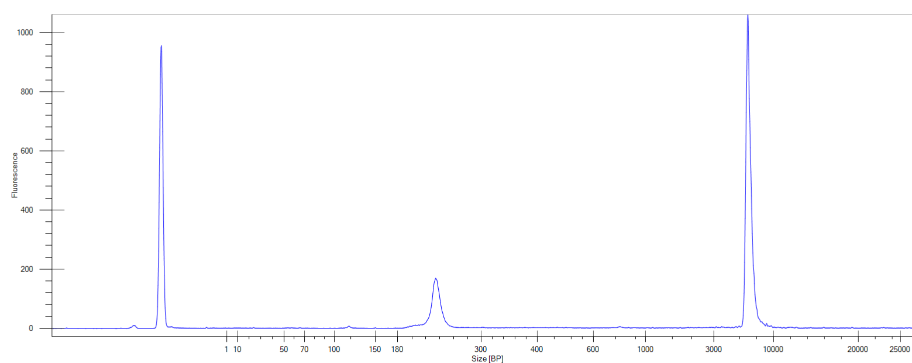
LabChip Traces

DNA High Sensitivity Reagent Kit was used.

Chromium Fixed RNA Profiling – Gene Expression Library



Chromium Fixed RNA Profiling – Cell Surface Protein Library



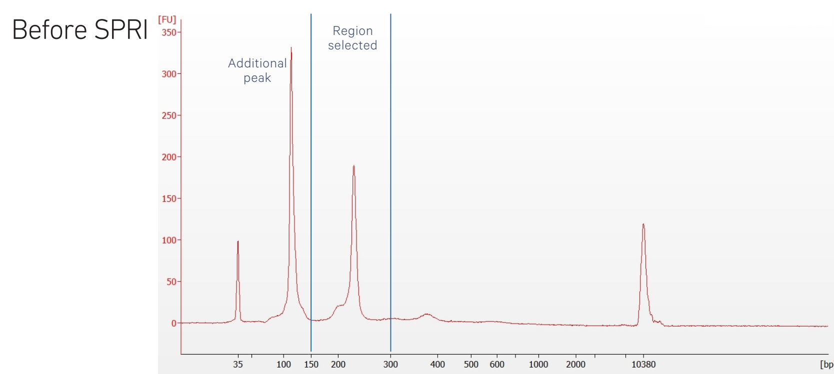
All traces are representative. Samples were run at 1:20 dilution.

Post Library Construction Cleanup – SPRIselect

	Item	10x PN	Preparation & Handling	Storage
Equilibrate to Room Temperature				
☐	**Beckman Coulter SPRIselect Reagent**	-	Manufacturer's recommendations.	-
Obtain				
☐	**Qiagen Buffer EB**	-	-	Ambient
☐	**10x Magnetic Separator**	230003	-.	Ambient
☐	**Prepare 80% Ethanol** Prepare 15 ml for 8 reactions	-	Prepare fresh.	Ambient
Retrieve				
☐	**Chromium Fixed RNA Profiling – Cell Surface Protein Library** QC trace shows additional peak (~115 bp)			4°C or –20°C

a. Retrieve **35 µl** undiluted library that shows an additional peak (~115 bp) during QC (representative trace below).

Before SPRI



b. Vortex to resuspend SPRIselect Reagent. Add **31 µl** SPRIselect Reagent **(0.9X)** to each sample/library. Pipette mix 15x (pipette set to 55 µl).

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

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

e. Remove the supernatant.

f. Add **200 µl** 80% ethanol to the pellet. Wait **30 sec**.

g. Remove the ethanol.

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

Post Library Construction Cleanup – SPRIselect

i. Centrifuge briefly. Place on the magnet•**Low**.

j. Remove any remaining ethanol. Air dry for **2 min**.

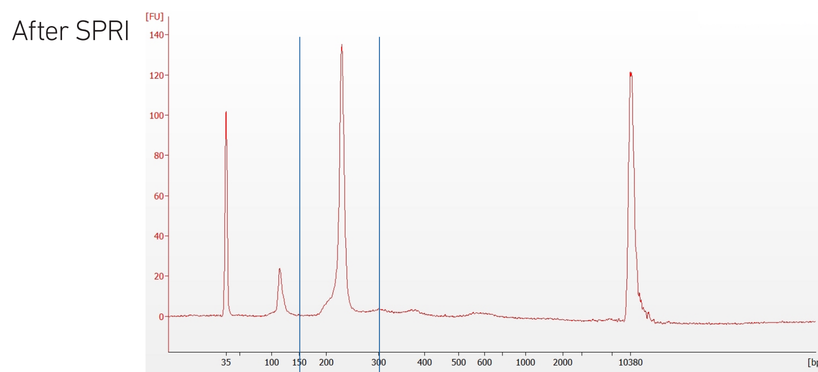
k. Remove from the magnet. Add **35.5 µl** Buffer EB. Pipette mix 15x. If beads still appear clumpy, continue pipette mixing until fully resuspended.

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

m. Place on the magnet•**Low** until the solution clears.

n. Transfer **35 µl** sample to a new tube. Perform Post Library Construction QC (representative trace below).

After SPRI



Manually select the analysis region of ~150-300 bp and determine the average fragment size from the Bioanalyzer trace. This will be used as the insert size for library quantification.