CG000377 Rev A

USER GUIDE

Targeted Gene Expression - Spatial

FOR USE WITH

Target Hybridization Kit, 16 rxns PN-1000248
Library Amplification Kit, 16 rxns PN-1000249
Human Gene Signature Panel, 16 rxns PN-1000245
Human Gene Signature Panel, 4 rxns PN-1000258
Human Immunology Panel, 16 rxns PN-1000246
Human Immunology Panel, 4 rxns PN-1000259
Human Pan-Cancer Panel, 16 rxns PN-1000247
Human Pan-Cancer Panel, 4 rxns PN-1000260
Human Neuroscience Panel, 16 rxns PN-1000278
Human Neuroscience Panel, 4 rxns PN-1000277

Notices

Document Number

CG000377 • Rev A

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

Email: support@10xgenomics.com

10x Genomics

6230 Stoneridge Mall Road

Pleasanton, CA 94588 USA

Document Revision Summary

Document Number	CG000377
Title	Targeted Gene Expression - Spatial User Guide
Revision	Rev A
Revision Date	October 2020

Table of Contents

Introduction

Targeted Gene Expression Reagent Kits
Chromium Accessories
Recommended Thermal Cyclers
Additional Kits, Reagents & Equipment
Protocol Steps & Timing
Stepwise Objectives

Targeted Gene Expression Reagent Kits

Refer to SDS for handling and disposal information.

Target Hybridization Kit
16 rxns PN-1000248 (store at –20°C)

Target Hybridization Kit

	#	PN
Universal Blockers	1	2000290
Human Cot DNA	1	3000478
Hyb Buffer	1	3000479
Hyb Enhancer	1	3000480
Conc. Equil. Buffer	1	2000319
Conc. Wash Buffer	1	2000320
Amp Mix	1	2000047
Library Amp Primers	1	2000291

10xGenomics.com 10x GENOMICS

Library Amplification Kit*
16 rxns PN-1000249 (store at –20°C)

Library Amplification Kit

	#	PN
Amp Mix	1	2000047
Library Amp Primers	1	2000291

10xGenomics.com 10x GENOMICS

*Optional - only if library reamplification is required.

Human Gene Signature Panel, 16 rxns PN-1000245 (store at -20°C)

	#	PN
Human Gene Signature Panel	1	2000285

Human Immunology Panel, 16 rxns PN-1000246 (store at -20°C)

	#	PN
Human Immunology Panel	1	2000286

Human Pan-Cancer Panel, 16 rxns PN-1000247 (store at -20°C)

	#	PN
Human Pan-Cancer Panel	1	2000287

Human Neuroscience Panel, 16 rxns PN-1000278 (store at -20°C)

	#	PN
Human Neuroscience Panel	1	2000366

Targeted Gene Expression Reagent Kits

Refer to SDS for handling and disposal information.

Human Gene Signature Panel, 4 rxns PN-1000258 (store at -20°C)

	#	PN
Human Gene Signature Panel	1	2000322

Human Immunology Panel, 4 rxns PN-1000259 (store at -20°C)

	#	PN
Human Immunology Panel	1	2000323

Human Pan-Cancer Panel, 4 rxns PN-1000260 (store at -20°C)

	#	PN
Human Pan-Cancer Panel	1	2000324

Human Neuroscience Panel, 4 rxns PN-1000277 (store at -20°C)

	#	PN
Human Neuroscience Panel	1	2000367

Chromium Accessories

Product	Part Number (Kit)	Part Number (Item)
10x Magnetic Separator	120250	230003

Recommended Thermal Cyclers

Thermal cyclers used must support uniform heating of 125 µl reaction volumes.

Supplier	Description	Part Number
BioRad	C1000 Touch Thermal Cycler with 96-Deep Well Reaction Module	1851197
Eppendorf	MasterCycler Pro (discontinued)	North America 950030010 International 6321 000.019
Thermo Fisher Scientific	Veriti 96-Well Thermal Cycler	4375786

Additional Kits, Reagents & Equipment

The items in the table below have been validated by 10x Genomics and are highly recommended for the Targeted Gene Expression protocol. Substituting materials may adversely affect system performance. This list may not include some standard laboratory equipment.

Supplier	Description		Part Number (US)
Plastics			
Eppendorf	PCR Tubes 0.2 ml 8-tube strips	Choose either Eppendorf, USA Scientific or Thermo Fisher Scientific PCR 8-tube strips.	951010022
USA Scientific	TempAssure PCR 8-tube strip		1402-4700
Thermo Fisher Scientific	MicroAmp 8-Tube Strip, 0.2 ml MicroAmp 8 -Cap Strip, clear		N8010580 N8010535
Rainin	Tips LTS 200UL Filter RT-L200FLR Tips LTS 1ML Filter RT-L1000FLR Tips LTS 20UL Filter RT-L10FLR		30389240 30389213 30389226
Kits & Reagents			
Thermo Fisher Scientific	Nuclease-free Water Dynabeads M-270 Streptavidin (required)		AM9937 65305
Millipore Sigma	Ethanol, Pure (200 Proof, anhydrous)		E7023-500ML
Beckman Coulter	SPRIselect Reagent		B23318
Qiagen	Qiagen Buffer EB		19086
Add-on Panel Reagents			
IDT	NGS Discovery Pool (<50 baits) NGS Discovery Pool (50-20,000 baits, 200 amol/bait/µl) NGS Discovery Pool (20,001-80,000 baits, 50 amol/bait/µl) xGen Lockdown Probe Pool (<50 baits) xGen Lockdown Probe Pool (50-4,000 baits, 400 amol/bait/µl) xGen Lockdown Probe Pool (4,001-20,000 baits, 200 amol/bait/µl)		- - - - - -
IDT	IDTE, pH 8.0 (1X TE Solution) (alternatively, use any nuclease-free 1X TE buffer: 10 mM Tris, pH 8.0, 0.1 mM EDTA)		11-05-01-13

Additional Kits, Reagents & Equipment

The items in the table below have been validated by 10x Genomics and are highly recommended for the Targeted Gene Expression protocol. Substituting materials may adversely affect system performance. This list may not include some standard laboratory equipment.

Supplier	Description	Part Number (US)
Equipment		
VWR	Vortex Mixer	10153-838
Thermo Fisher Scientific	MYFUGE 12 Mini Centrifuge (alternatively, use any equivalent mini centrifuge)	C1012
Eppendorf	Eppendorf ThermoMixer C Eppendorf SmartBlock 1.5 ml, Thermoblock for 24 reaction vessel (alternatively, use a temperature-controlled Heat Block) Eppendorf Vacufuge Plus Complete System (alternatively, use any equivalent vacuum centrifuge that accommodates 0.2 ml tubes) Rotor for Eppendorf Vacufuge Plus Concentrator Eppendorf PCR Tube Work Tray (alternatively, use any equivalent adapter that accommodates 0.2 ml tubes) Eppendorf Polycarbonate Work Tray Frame	5382000023 5360000038 022822993 022822241 951010031 951010049
Rainin	Pipet-Lite Multi Pipette L8-50XLS+ Pipet-Lite Multi Pipette L8-200XLS+ Pipet-Lite Multi Pipette L8-10XLS+ Pipet-Lite Multi Pipette L8-20XLS+ Pipet-Lite LTS Pipette L-2XLS+ Pipet-Lite LTS Pipette L-10XLS+ Pipet-Lite LTS Pipette L-20XLS+ Pipet-Lite LTS Pipette L-100XLS+ Pipet-Lite LTS Pipette L-200XLS+ Pipet-Lite LTS Pipette L-1000XLS+	17013804 17013805 17013802 17013803 17014393 17014388 17014392 17014384 17014391 17014382
Quantification & Quality Control		
Agilent	2100 Bioanalyzer Laptop Bundle High Sensitivity DNA Kit 4200 TapeStation High Sensitivity D1000 ScreenTape/Reagents High Sensitivity D5000 ScreenTape/Reagents (Choose Bioanalyzer, TapeStation, LabChip or Fragment Analyzer based on availability & preference.)	G2943CA 5067-4626 G2991AA 5067-5592/ 5067-5593 5067-5584/ 5067-5585
PerkinElmer	LabChip GX Touch HT Nucleic Acid Analyzer DNA High Sensitivity Reagent Kit	CLS137031 CLS760672
Advanced Analytical	Fragment Analyzer Automated CE System - 12 cap Fragment Analyzer Automated CE System - 48/96 cap High Sensitivity NGS Fragment Analysis Kit	FSv2-CE2F FSv2-CE10F DNF-474
KAPA Biosystems	KAPA Library Quantification Kit for Illumina Platforms	KK4824

Protocol Steps & Timing

Steps		Timing	Stop & Store
Step 1 – Pre Hybridization Pooling			
1.1	Library Pooling & Drying	Variable	STOP –20°C long term
Step 2 – Library Capture			
2.1	Library Hybridization	135 min†	
2.2	Capture Bead Preparation	15 min	
2.3	Bait Capture	10 min	
2.4	Post Capture Cleanup - Streptavidin Beads	30 min	STOP 4°C ≤24 h or –20°C long term
Step 3 – Post Capture Amplification			
3.1	Library Amplification	Variable	
3.2	Post Amplification Cleanup - SPRIselect	20 min	
3.3	Targeted Library QC	Variable	STOP 4°C ≤72 h or –20°C long term

*~4 h workflow, excluding library drying, library amplification, & QC steps. Does not account for overnight library hybridization if working with Visium Spatial Gene Expression libraries prepared from short cDNA.
†Library Hybridization is extended to overnight if working with Visium Spatial Gene Expression libraries prepared from short cDNA.

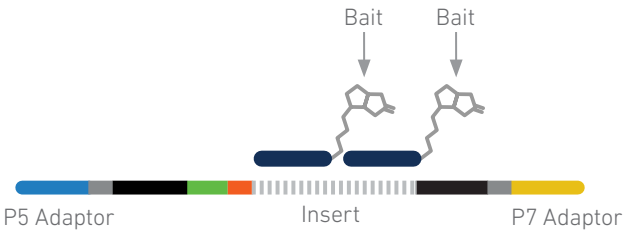
Stepwise Objectives

The Targeted Gene Expression product is a modular enrichment kit designed to enrich libraries for relevant genes, while decreasing sequencing requirements by up to 90%. Target enrichment is achieved by a hybrid capture workflow. Gene-specific, biotinylated baits are hybridized to their complement in the library, bound to streptavidin beads, and washed to remove non-targeted library molecules. The bead-bound, targeted library fragments are amplified to produce sequencing-ready libraries. Biotinylated baits are provided as pre-designed panels of genes. Add-on genes to pre-designed panels may be generated using the 10x Genomics Custom Panel Designer on the 10x Genomics website.

This document outlines the protocol for generating Targeted Gene Expression libraries from Visium Spatial Gene Expression libraries that have been generated previously using the Visium Spatial Gene Expression Reagent Kits User Guide (Document CG000239, Rev D and onward). For generating Targeted Gene Expression libraries from Chromium Single Cell 3' or 5' Gene Expression libraries, consult the Targeted Gene Expression Reagent Kits - Single Cell User Guide (Document CG000293).

Targeted Gene Expression Bait Design

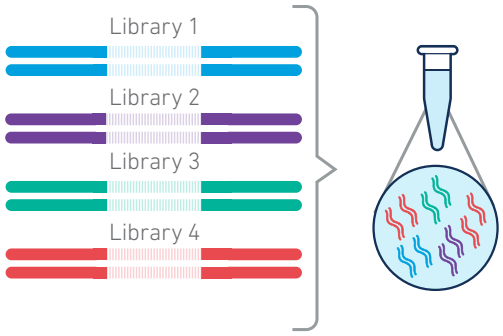
Targeted Gene Expression baits are single stranded, 120 nt DNA oligonucleotides with a 5' biotin modification. Each bait targets a unique library molecule. Baits span all mature mRNA sequences, including UTRs and all annotated isoforms.



Step 1 Pre Hybridization Pooling

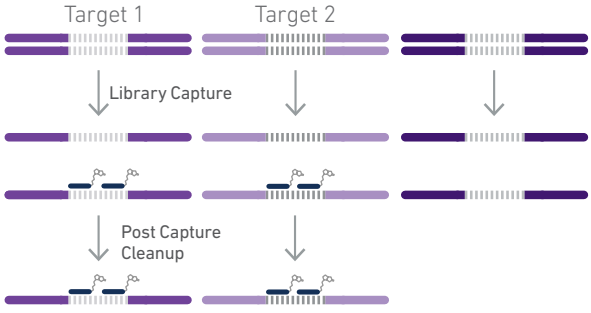
This workflow may be performed with a single library or a library pool in which up to eight libraries are multiplexed on a single baitset.

Prior to library capture, library pools or single libraries are mixed with Universal Blockers and Human Cot DNA and concentrated.



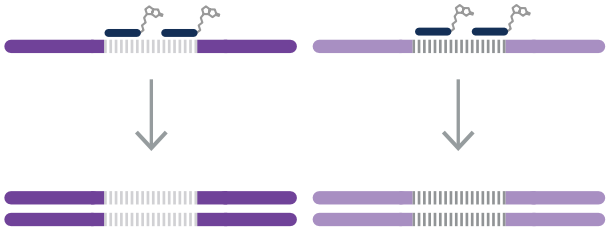
Step 2 Library Capture

Baits are added to the concentrated library for hybridization, followed by the addition of streptavidin beads. The mix is incubated to conjugate biotinylated baits to streptavidin beads. Subsequent washes remove non-hybridized library molecules.



Step 3 Post Capture Amplification

Hybridized library molecules bound to streptavidin beads are amplified with Illumina P5 and P7 primers prior to sequencing.



Step 4 Sequencing

Targeted libraries have structures identical to the libraries produced by the underlying gene expression assay. They are standard Illumina paired-end constructs that are flanked with P5/P7, necessary for binding to the Illumina flow cell. The 10x Barcode and UMI are encoded in Read 1, while Read 2 is used to sequence the cDNA fragment.

Illumina sequencer compatibility, sample indices, library loading and pooling for sequencing are summarized in the sequencing chapter of this User Guide. For sequencing parameter information for specific library types, consult the relevant User Guide.

Tips & Best Practices

Icons

Tips & Best Practices section includes additional guidance

Signifies critical step requiring accurate execution

Troubleshooting section includes additional guidance

General Reagent Handling

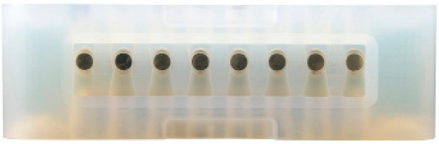
- Fully thaw and thoroughly mix reagents before use.
- Promptly move reagents back to the recommended storage after use.
- Calculate reagent volumes with 10% excess of 1 reaction values.
- Thoroughly mix samples with the beads during bead-based cleanup steps.
- Pulse vortexing instead of continuously vortexing minimizes reagent splashing.

Pipette Calibration

- Follow manufacturer's calibration and maintenance schedules.
- Pipette accuracy is particularly important when using SPRIselect reagents.

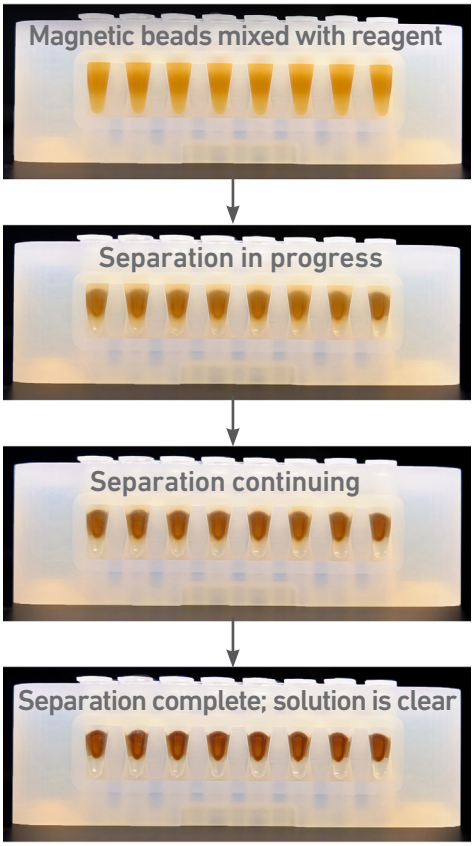
10x Magnetic Separator

- 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)** throughout the protocol.
- Keep 8-tube strip intact, even if only working with one sample. This prevents unintended tube strip movement while on the magnet.



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 adjacent panel for an example.
- The time needed for the solution to clear may vary based on specific step, reagents, volume of reagents etc.
- Magnetic beads settle quickly. Ensure that beads are fully resuspended before adding to samples.



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.

Step 1

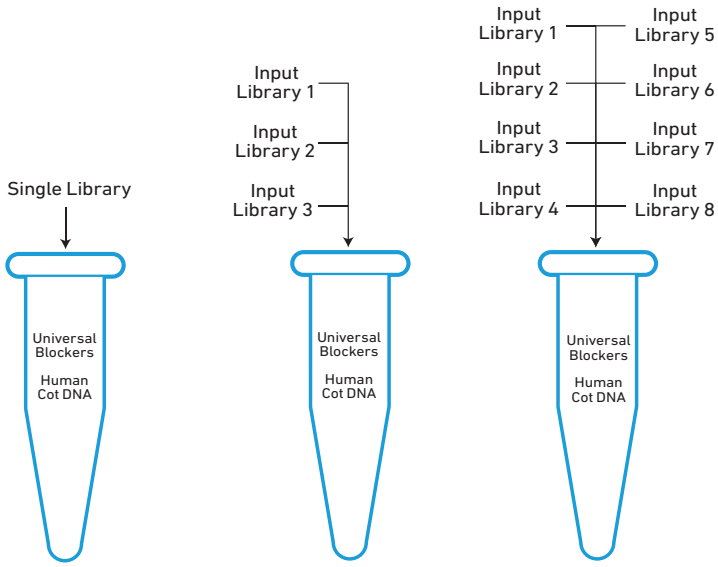
Pre Hybridization Pooling

1.1 Library Pooling and Drying

Workflow Overview

Targeted Gene Expression workflow

The Targeted Gene Expression workflow may be performed with a single library or a library pool, as shown in the following examples. Each library pool accommodates up to eight input libraries. A downloadable worksheet (Targeted Gene Expression Pooling Worksheet, Document CG000296) for calculations relevant to pooling is available on the 10x Genomics Support website. SPRIselect beads from the original library cleanup will negatively affect assay performance. Ensure that libraries are free from SPRIselect beads. Refer to Troubleshooting for more information.



Single Library

- Add library volume equivalent to 300 ng DNA/sample in each tube of an 8-tube strip and proceed to step 1.1.
- If the amount of input library required is greater than the amount available, the library can be reamplified as described in the Pre Pulldown Library Amplification section in the Appendix.

Library Pool

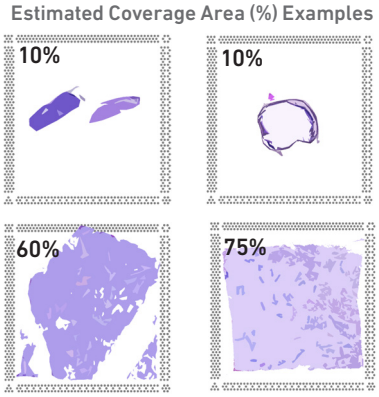
- Calculate input library volume based on the Calculation Overview on the next page.
- If less than the calculated required amount of DNA (~300 ng/library, depending on pooling strategy) is available, perform the amplification steps described in the Appendix.
- Do not pool Visium Spatial Gene Expression libraries with Chromium Single Cell 3' or 5' Gene Expression libraries. If targeting Chromium Single Cell libraries, consult the Targeted Gene Expression Reagent Kits - Single Cell User Guide (CG000293).
- Do not pool libraries with the same sample index.
- For optimal performance, pool libraries prepared from similar tissue types with similar expected numbers of tissue-covered spots.

Calculating Tissue-Covered Spots

When pooling Visium Spatial Gene Expression libraries, pooling calculations take into account the number of tissue-covered spots. Estimating the number of tissue-covered spots can be performed visually, or by using the Visium Manual Alignment Wizard in Loupe Browser for a more accurate measurement. If estimating visually, multiply the percent coverage by the total number of gene expression spots (4,992) to get the number of tissue-covered spots. If using Loupe Browser, the number of tissue-covered spots will be displayed during the "Identify Tissue" step. For more information, consult the 10x Genomics Support website.

Example: Estimate Coverage Area

- Estimate the approximate Capture Area (%) covered by the tissue section.
- **Example calculation for 60% coverage:** (0.60 x 4,992 total spots) = 2,995.2 tissue-covered spots.



Calculation Overview

Calculating library volumes needed for pooling

A downloadable worksheet (Targeted Gene Expression Pooling Worksheet, Document CG000296) for these calculations is available on the 10x Genomics Support website. Refer to the Appendix for a detailed example calculation.

These calculations assume 300 ng DNA from each input library will be added to the library pool. This number is adjusted based on spot number expected, sequencing depth, and library size. If less than the calculated required amount of DNA (~300 ng/library, depending on pooling strategy) is available, perform the amplification steps described in the Pre Pulldown Library Amplification section in the Appendix.

The following information is required for each input library: average library size (bp), library concentration (nM), tissue-covered spot number expected, and read pairs per tissue-covered spot desired.

10x Genomics recommends 5,000 to 10,000 read pairs per tissue-covered spot for pre-designed panels as a starting point, but this number may need optimization.

Library size is determined from a BioAnalyzer, TapeStation, or LabChip trace, while concentration is determined using qPCR. Refer to the Appendix for representative traces and quantification instructions.

The formula used to determine input library volume needed for a library pool is:

$$\text{Input Library Volume (µl)} = \frac{\text{Input Library Moles}}{\text{Input Library Concentration (nM)}}$$

Input Library Moles are calculated in (1b) in the Determine Moles of Input Library section, which provides example calculations with the following data:

Input Library	Tissue-Covered Spot Number Expected	Reads Pairs per Tissue-Covered Spot Desired	Input Library Size	Input Library Concentration
Library 1	4,400	5,000	454 bp	142.5 nM
Library 2	3,000	5,000	411 bp	168 nM

The calculated Input Library Volume for Library 1 is **8.8 µl.** The calculated Input Library Volume for Library 2 is **5.1 µl.** Example calculations are only provided for Library 1. If the calculated volume is less than **1 µl,** dilute input library to lower Input Library Concentration and increase the calculated Input Library Volume.

Unit conversions are not described in this Calculation Overview. Ensure that units are converted properly such that the final answer is given in the units described.

Determine Moles Input Library

Determine input library moles needed to calculate input library volume for addition to the library pool (refer to previous page). Step-by-step instructions along with an example are provided below.

Step	Instructions	Formulae	Library 1 Example (refer to values on previous page)
1a	Calculate input library reads by multiplying the number of tissue-covered spots expected for that library by the read pairs per tissue-covered spot desired.	Input Library Reads = Tissue-Covered Spot Number Expected x Reads Pairs per Tissue-Covered Spot Desired	8,800,000 = 4,400 x 5,000
1b	Divide input library reads by library pool reads, the sum of reads required for all input libraries.	Read Fraction = Input Library Reads / Library Pool Reads	0.59 = 22,000,000 / 37,000,000
1c	Multiply read fraction by library size to determine the weighted input library size.	Weighted Input Library Size (bp) = Read Fraction x Input Library Size (bp)	269.9 bp = 0.59 x 454 bp
1d	Sum all weighted library sizes in the library pool for the average library pool size.	Average Library Pool Size (bp) = Weighted Input Library Sizes Sum (bp)	436.6 bp = 269.9 bp + 166.6 bp
1e	Average library pool size is used to calculate library pool moles.	Library Pool Moles = Total Input Mass (300 ng x # Input Libraries) / Average Library Pool Size (bp) * Molar Mass Base Pair (650 g/mol per bp)	2.11×10^{-12} mol = 300×10^{-9} g x 2 / 436.6 bp x 650 g/mol per bp
1f	Use read fraction and library pool moles to calculate input library moles.	Input Library Moles = Read Fraction x Library Pool Moles	1.26×10^{-12} mol = 0.59 x 2.11×10^{-12} mol
1g	Use the solution of (1f) to calculate input library volume.	Input Library Volume (µl) = Input Library Moles / Input Library Concentration (nM)	**8.8 µl** = 1.26×10^{-12} mol / 142.5×10^{-9} M

Protocol Modifications for Visium Spatial Gene Expression Libraries Prepared from Short cDNA

If the average cDNA length for a Visium Spatial Gene Expression whole transcriptome analysis library is less than or equal to 700 bp, two modifications to the Targeted Gene Expression protocol are necessary. Failure to follow these protocol modifications will lead to a reduction in assay performance.

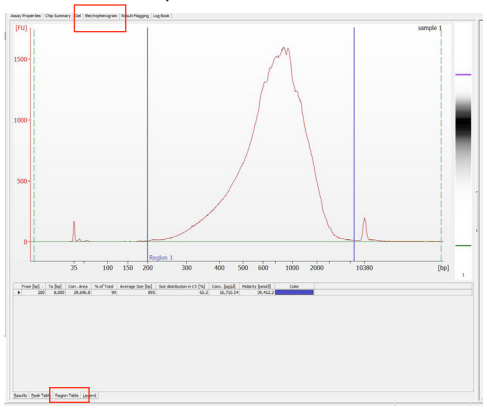
Refer to the 10x Genomics Support website for the latest User Guide for Visium Spatial Library preparation (Document CG000239). Visium Spatial Gene Expression libraries prepared using Rev A-C of the Visium Spatial Gene Expression Reagent Kits User Guide are not compatible with the Targeted Gene Expression assay.

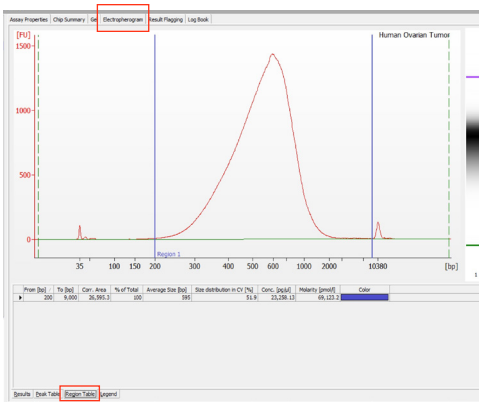
To calculate average cDNA size from a Bioanalyzer trace, follow the steps in the table below.

EXAMPLE CALCULATION

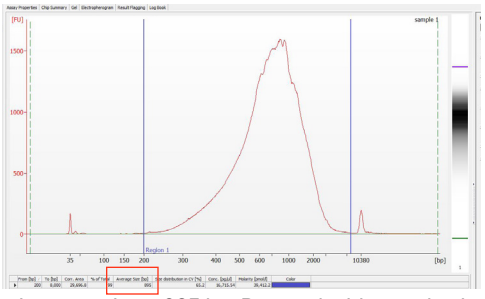
i. Select Region

Under the "Electropherogram" view choose the "Region Table". Manually select the region of ~200 – ~9000 bp.

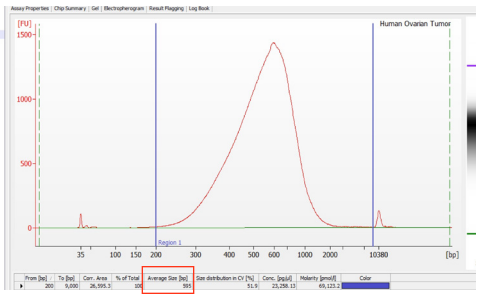




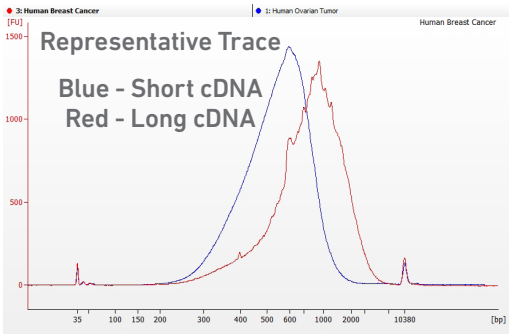
ii. Note Average size [bp]



Average size = 895 bp. Proceed with standard protocol.



Average size = 595 bp. Modify protocol with changes listed on page 24.



Protocol Modifications for Visium Spatial Gene Expression Libraries Prepared from Short cDNA

In Step 2.1, hybridization should be extended from **2 h** to overnight (**16-24 h**). Additionally, the temperature for bait capture and subsequent washes should be lowered to **60°C** from **65°C**.

Lid Temperature	Reaction Volume	Run Time
100°C	70 µl	~18-24 h
Step	**Temperature**	**Time**
Pre-equilibrate	95°C	Hold
Denaturation	95°C	00:00:30
Hybridization	65°C	Hold (overnight)
Hold for Bait Capture and Washes	60°C	Hold

If the library pool contains both short and long cDNA length libraries, perform the short cDNA protocol. The short cDNA protocol is not detrimental to libraries that were prepared from cDNA greater than 700 bp in length. To reduce batch effects, samples that will be compared to one another within the same project should be run with the same protocol.

1.0 Pre Hybridization Pooling

CHECKLIST – GET STARTED!
Refer to SDS for handling and disposal information.

Items	10x PN	Preparation & Handling	Storage
Equilibrate to room temperature			
☐ **Universal Blockers**	2000290	Thaw, centrifuge briefly.	-20°C
☐ **Human Cot DNA**	3000478	Thaw, centrifuge briefly.	-20°C

1.1 Library Pooling and Drying

Pipetting volumes of less than **1 µl** may result in high variability. Refer to Calculation Overview.

SPRIselect beads carried over from the original library preparation or cleanup will negatively affect assay performance. Ensure that libraries are free from SPRIselect beads. Refer to Troubleshooting for more information.

STOP

a. Add **20 µl** Human Cot DNA and **2 µl** Universal Blockers to one 0.2-ml tube in a tube strip for each sample being processed.

b. Add single library/library pool (see Workflow Overview) to each tube containing Human Cot DNA and Universal Blockers. Pipette mix 15x and centrifuge briefly.

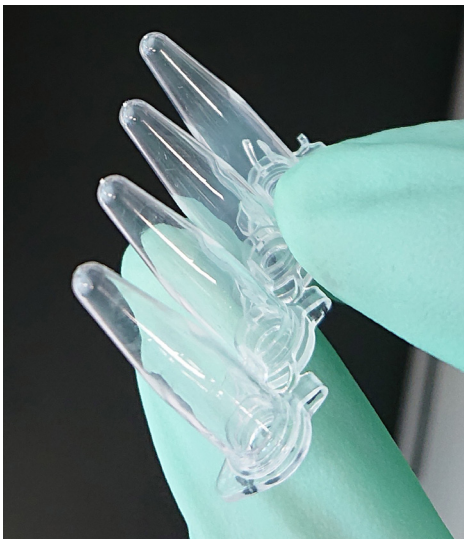
c. Uncap tubes and transfer tube strip to a vacuum centrifuge.

d. Run the vacuum centrifuge using manufacturer's recommended settings, up to **60°C**, until the samples are dry. Heating is optional.

e. Remove tube strip from vacuum centrifuge.

f. Store at **-20°C** for **long-term** storage, or proceed to the next step.

Library Pellet May Be Visible After Drying



Step 2

Library Capture

2.0 Library Capture

CHECKLIST – GET STARTED!
Refer to SDS for handling and disposal information.

Items	10x PN	Preparation & Handling	Storage
Select panel and equilibrate to room temperature			
☐ **Human Gene Signature Panel**	2000285/ 2000322	Thaw, centrifuge briefly.	-20°C
☐ **Human Immunology Panel**	2000286/ 2000323	Thaw, centrifuge briefly.	-20°C
☐ **Human Pan-Cancer Panel**	2000287/ 2000324	Thaw, centrifuge briefly.	-20°C
☐ **Human Neuroscience Panel**	2000366/ 2000367	Thaw, centrifuge briefly.	-20°C
⚠ **If adding genes to a pre-designed panel, proceed to relevant Panel Dilution Guidelines.**			
Equilibrate to room temperature			
☐ **Hyb Enhancer**	3000480	*Hazardous - refer to SDS* Thaw, centrifuge briefly.	-20°C
☐ **Conc. Wash Buffer**†	2000320	Thaw, centrifuge briefly.	-20°C
☐ **Conc. Equilibration Buffer**†	2000319	Thaw, centrifuge briefly.	-20°C
⚠ ☐ **Dynabeads M-270 Streptavidin**† Verify name & PN	-	Equilibrate unwashed beads **30 min** before performing washes. Vortex thoroughly.	4°C
Thaw at 65°C			
☐ **Hyb Buffer**	3000479	*Hazardous - refer to SDS* Thaw for **10 min** at **65°C** at maximum speed in a thermomixer. Verify no precipitate. Cool to room temperature.	-20°C
Obtain			
☐ **Nuclease-free Water**	-	Manufacturer's recommendations.	Ambient
☐ **IDTE, pH 8.0 (1X TE Solution)***	-	Manufacturer's recommendations.	Ambient

*If adding genes to a pre-designed panel.
†If working with Visium Spatial Gene Expression libraries prepared from short cDNA, equilibrate these reagents after the overnight hybridization and before Capture Bead Preparation.

2.0 Library Capture

CHECKLIST – GET STARTED!

Prepare

☐ **Equilibration Buffer†**

Equilibration Buffer Store at room temperature	Stock	Final	1X + 10% (µl)	4X+ 10% (µl)	8X+ 10% (µl)
Conc. Equilibration Buffer	4X	1X	82.5	330.0	660
Nuclease-free Water	-	-	247.5	990.0	1,980.0
Total	-	-	**330.0**	**1,320.0**	**2,640.0**

☐ **Wash Buffer†**

Wash Buffer Store at room temperature	Stock	Final	1X + 10% (µl)	4X+ 10% (µl)	8X + 10% (µl)
Conc. Wash Buffer	10X	1X	79.8	319.0	638.0
Nuclease-free Water	-	-	717.8	2,871.0	5,742.0
Total	-	-	**797.6**	**3,190.0**	**6,380.0**

†If working with Visium Spatial Gene Expression libraries prepared from short cDNA, prepare these reagents after the overnight hybridization and before Capture Bead Preparation.

Targeted Gene Expression Panel Selection

The Targeted Gene Expression workflow is performed with a panel of baits. 10x Genomics offers pre-designed panels that target over 1,000 genes per panel. Custom genes may be added to pre-designed panels with the 10x Genomics Custom Panel Designer tool. 10x Genomics supports the addition of 1-200 genes to pre-designed panels. The volume of bait pool required depends on the number of baits in the target pool. Fully Custom Panels are not supported for targeted libraries prepared from Visium Spatial libraries.

If working with pre-designed panels, proceed to step 2.1A. If adding genes to pre-designed panels, proceed to Panel Dilution Guidelines - Add-on Genes to Pre-designed Panels.

If working with Visium Spatial Gene Expression libraries prepared from short cDNA, some protocol modifications are necessary. Refer to Protocol Modifications for Visium Spatial Gene Expression libraries prepared from short cDNA for more information.

2.1A Library Hybridization - Pre-designed Panels

For Pre-designed Panels

Only if working with pre-designed panels. If adding genes to pre-designed panels, proceed to Panel Dilution Guidelines - Add-on Genes to Pre-designed Panels.

If working with Visium Spatial Gene Expression libraries prepared from short cDNA, some protocol modifications are necessary. Refer to Protocol Modifications for Visium Spatial Gene Expression Libraries Prepared from Short cDNA for more information.

a. Prepare the thermal cycler with the following Library Hybridization and Wash protocol and start the program.

	Standard Protocol		Short cDNA Protocol	
Lid Temperature	Reaction Volume	Run Time	Reaction Volume	Run Time
100°C	70 µl	~2 h 45 min	70 µl	Overnight
Step	**Temperature**	**Time**	**Temperature**	**Time**
Pre-equilibrate	95°C	Hold	95°C	Hold
Denaturation	95°C	00:00:30	95°C	00:00:30
Hybridization	65°C	02:00:00	65°C	Hold (Overnight)
Hold for Bait Capture and Washes	65°C	Hold	60°C	Hold

b. Prepare Hybridization Master Mix. Pipette mix 15x and centrifuge briefly. Maintain at **room temperature**.

Hybridization Master Mix *Add reagents in the order listed*	10x PN	1X (µl)	4X + 10% (µl)	8X + 10% (µl)
Hyb Buffer	3000479	8.5	37.4	74.8
Hyb Enhancer	3000480	2.7	11.9	23.8
Pre-designed Panel	-	4.0	17.6	35.2
Nuclease-free Water	-	1.8	7.9	15.8
Total	-	**17.0**	**74.8**	**149.6**

c. Add **17 µl** Hybridization Master Mix to each sample and pipette mix 15x to resuspend. Centrifuge briefly.

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

e. Transfer library tube strip to preheated thermal cycler and advance the protocol.

Ensure caps are tightly closed. Evaporation may result in reduced performance.

f. Proceed to step 2.2 **immediately** after hybridization if following the standard protocol, or after overnight hybridization if following the short cDNA protocol. Ensure that streptavidin beads are maintained at room temperature for at least **30 min** before proceeding to step 2.2a.

Panel Dilution Guidelines - Add-on Genes to Pre-designed Panels

For Add-on Genes to Pre-designed Panels

- Prepare add-on panel working dilutions according to the Tables 1 and 2 prior to proceeding to step 2.1B.
- Add-on panels with fewer than 50 baits are duplicated to meet the minimum order threshold due to synthesis limitations. For example, an add-on panel design with 18 baits would be duplicated three times, resulting in 54 baits at a 3-fold higher concentration. Due to the higher concentration of these baits, they must be diluted twice.
- If working with add-on panels with fewer than 50 baits, perform the dilution outlined in Table 1 by diluting **5 µl** of add-on panel according to the duplication factor used when ordering. For example, if baits were duplicated 3 times, combine **5 µl** of baits with **10 µl** IDTE pH 8.0. Carry this volume into the calculation in Table 2.
- If working with add-on panels with more than 50 baits, perform only the dilution outlined in Table 2.
- Table 2 working dilutions may be stored at **-20°C** for up to **one month** if combined with a pre-designed panel at a 1:2 ratio. For example, **2 µl** of an add-on panel at working dilution may be combined with **4 µl** of a pre-designed panel and stored with 10% overage.

Table 1. Dilution of add-on panels with less than 50 baits to account for duplication factor in bait synthesis.

	Volume (µl) for								
Duplication Factor	2	3	4	5	6	7	8	9	10
IDT Bait Pool*	5	5	5	5	5	5	5	5	5
IDTE, pH 8.0	5	10	15	20	25	30	35	40	45
Total	**10**	**15**	**20**	**25**	**30**	**35**	**40**	**45**	**50**

*Applicable to both IDT Discovery Pools and xGen Lockdown Pools

Table 2. Generation of add-on panel working dilutions (20 amol/bait/µl).

	Volume (µl) for		
	<50 baits	50-4,000 baits	4,001-20,000 baits
IDT NGS Discovery Pool	2*	2	2
IDTE, pH 8.0	18	18	18
Total	**20**	**20**	**20**
IDT xGen Lockdown Pool	2*	2	2
IDTE, pH 8.0	38	38	18
Total	**40**	**40**	**20**

*Volume from Table 1.

- Equilibrate pre-designed panels and add-on panels to **room temperature** and proceed to step 2.1B.

2.1B Library Hybridization - Add-on Genes to Pre-designed Panels

For Add-on Genes to Pre-designed Panels

Only if adding genes to pre-designed panels. If working with pre-designed panels, proceed to step 2.1A.

If working with Visium Spatial Gene Expression libraries prepared from short cDNA, some protocol modifications are necessary. Refer to Protocol Modifications for Visium Spatial Gene Expression Libraries Prepared from Short cDNA for more information.

a. Prepare the thermal cycler with the following Library Hybridization and Wash protocol and start the program.

	Standard Protocol		Short cDNA Protocol	
Lid Temperature	**Reaction Volume**	**Run Time**	**Reaction Volume**	**Run Time**
100°C	70 µl	~2 h 45 min	70 µl	Overnight
Step	**Temperature**	**Time**	**Temperature**	**Time**
Pre-equilibrate	95°C	Hold	95°C	Hold
Denaturation	95°C	00:00:30	95°C	00:00:30
Hybridization	65°C	02:00:00	65°C	Hold (Overnight)
Hold for Bait Capture and Washes	65°C	Hold	60°C	Hold

b. Prepare Hybridization Master Mix. Pipette mix 15x and centrifuge briefly. Maintain at room temperature.

Hybridization Master Mix *Add reagents in the order listed*	**10x PN**	**1X (µl)**	**4X + 10% (µl)**	**8X + 10% (µl)**
Hyb Buffer	3000479	8.5	37.4	74.8
Hyb Enhancer	3000480	2.7	11.9	23.8
Pre-designed Panel†	-	4.0	17.6	35.2
Add-on Panel Working Dilution†	-	2.0	8.8	17.6
Total	-	**17.2**	**75.7**	**151.4**

†If add-on panels were combined with pre-designed panels for storage, replace these reagents with an appropriate volume of the combined panel. For example, use **6 µl** of pre-designed panel + add on panel to replace **4 µl** of pre-designed panel and **2 µl** of add-on panel working dilution.

c. Add **17 µl** Hybridization Master Mix to each sample and pipette mix 15x to resuspend. Centrifuge briefly.

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

e. Transfer library tube strip to preheated thermal cycler and advance the protocol.

Ensure caps are tightly closed. Evaporation may result in reduced performance.

f. Proceed to step 2.2 **immediately** after hybridization if following the standard protocol, or after overnight hybridization if following the short cDNA protocol. Ensure that streptavidin beads are maintained at room temperature for at least **30 min** before proceeding to step 2.2a.

2.2 Capture Bead Preparation

a. Prepare Bead Resuspension Mix. Vortex and centrifuge briefly.

Bead Resuspension Mix	PN	1X (µl)	4X + 10% (µl)	8X + 10% (µl)
Hyb Buffer	3000479	8.5	37.4	74.8
Hyb Enhancer	3000480	2.7	11.9	23.8
Nuclease-free Water	-	5.8	25.5	51
Total	-	**17**	**74.8**	**149.6**

TIPS

b. Vortex to resuspend Dynabeads M-270 Streptavidin. Add **50 µl** streptavidin beads per sample in separate tubes in a new tube strip.

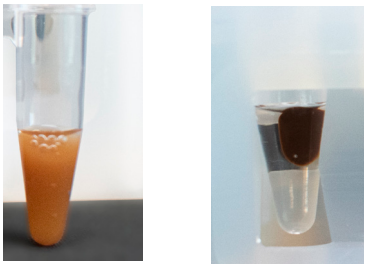
c. Add **100 µl** Equilibration Buffer to each tube. Pipette mix 15x.

d. Place on a 10x Magnetic Separator•**High** position (magnet•**High**) until the solution clears. Remove supernatant. Remove from the magnet.

e. **Repeat** steps c and d two more times for a total of 3 washes. Centrifuge briefly and place on the magnet•**Low**. Remove excess buffer.

f. Resuspend each streptavidin bead pellet in **17 µl** Bead Resuspension Mix. Pipette mix 15x. Maintain at **room temperature** during library hybridization and use within **2 h** of preparation.

Streptavidin Beads Before and After Separation



2.3 Bait Capture

a. Remove library tube strip from thermal cycler after Hybridization.

Ensure that the thermal cycler remains at **65°C** for subsequent washes. If working with Visium Spatial Gene Expression libraries prepared from short cDNA, advance the protocol to **60°C** for step 2.3c.

b. Add **17 µl** resuspended streptavidin beads to each sample. Pipette mix 15x (pipette set to 17 µl).

c. Transfer library tube strip back to thermal cycler set to **65°C**. Thermal cycler should be at **60°C** if working with Visium Spatial Gene Expression libraries prepared from short cDNA.

d. Incubate in thermal cycler for **5 min**.

2.4 Post Capture Cleanup - Streptavidin Beads

TIPS

a. Add **100 µl** Wash Buffer to each sample. Pipette mix 15x.

b. Place on the magnet•**High** for **1 min**.

c. Remove supernatant.

d. Remove from the magnet. Add **150 ul** Wash Buffer to each bead pellet.

e. Pipette mix 15x.

f. Incubate in thermal cycler set to **65°C** for **5 min**. Thermal cycler should be at **60°C** if working with Visium Spatial Gene Expression libraries prepared from short cDNA.

g. Remove tubes from thermal cycler.

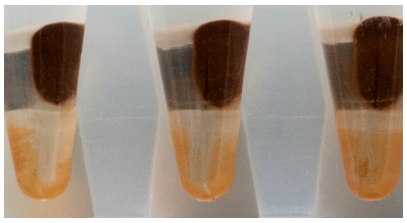
h. Centrifuge briefly. Place on the magnet•**High** for **1 min**.

i. Remove supernatant.

j. **Repeat** steps d–i three times for a total of 4 washes.

Avoid Splashing Liquid onto Cap

Streptavidin Bead Adherence to Tube Wall during Washes is Normal



Ensure that samples are incubated at **65°C** after each wash or at **60°C** if working with Visium Spatial Gene Expression libraries prepared from short cDNA.

k. Add **40 µl** nuclease-free water to each sample to generate Target-bound Streptavidin Beads.

l. Pipette mix 15x (pipette set to 35 µl).

m. Aliquot **20 µl** of each Target-bound Streptavidin Bead sample in each tube of a new tube strip for amplification.

STOP

n. Store remaining Target-bound Streptavidin Beads at **4°C ≤ 24 h** or at **-20°C** for **long-term** storage.

Step 3

Post Capture Amplification

3.0 Library Amplification

CHECKLIST – GET STARTED!
Refer to SDS for handling and disposal information.

Items	10x PN	Preparation & Handling	Storage
Thaw on ice			
☐ ◯ **Amp Mix**	2000047	Centrifuge briefly.	-20°C
☐ ● **Library Amp Primers**	2000291	Thaw, centrifuge briefly.	-20°C
Equilibrate to room temperature			
☐ **Beckman Coulter SPRIselect Reagent**	-	Manufacturer's recommendations.	-
☐ **Agilent TapeStation Screen Tape and Reagents** If used for QC	-	Manufacturer's recommendations.	-
☐ **Agilent Bioanalyzer High Sensitivity Kit** If used for QC	-	Manufacturer's recommendations.	-
☐ **DNA High Sensitivity Reagent Kit** If LabChip used for QC	-	Manufacturer's recommendations.	-
Place on ice			
☐ **KAPA Library Quantification Kit for Illumina Platforms**	-	Manufacturer's recommendations.	-
Obtain			
☐ **Qiagen Buffer EB**	-	Manufacturer's recommendations.	Ambient
☐ **80% Ethanol**	-	Prepare fresh.	Ambient
☐ **Nuclease-free Water**	-	Manufacturer's recommendations.	Ambient

3.1 Library Amplification

a. Prepare Library Amplification Mix on ice. Vortex and centrifuge briefly.

Library Amplification Mix *Add reagents in the order listed*	10x PN	1X (µl)	4X + 10% (µl)	8X + 10% (µl)
Nuclease-free Water	-	20	88	176
Library Amp Primers	2000291	10	44	88
Amp Mix	2000047	50	220	440
Total	-	**80**	**352**	**704**

b. Add **80 µl** Library Amplification Mix to each sample.

c. Pipette mix 15x (pipette set to 90 µl). Centrifuge briefly. If beads form a pellet, pipette mix 10x to resuspend.

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

Lid Temperature	Reaction Volume	Run Time
105°C	100 µl	~15-20 min
Step	**Temperature**	**Time**
1	98°C	00:00:45
2	98°C	00:00:20
3	60°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

Recommended starting points for cycle number optimization. If final library is below 10 nM, amplification step can be repeated using the 20 µl Target-bound Streptavidin Bead Samples stored in step 2.4 with additional cycles. Additional library amplification reagents are available in the optional Library Amplification Kit (PN-1000249).

Input Libraries	Total Cycles
1	12
2-3	11
4-6	10
7-8	9

3.2 Post Library Amplification Cleanup - SPRIselect

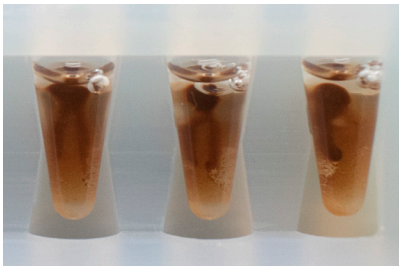
a. Pipette mix amplified final library to resuspend settled beads.

b. Place tube strip on the magnet•**High** for **2 min**. DO NOT discard supernatant.

c. Transfer **95 µl** supernatant from each sample to separate tubes in a new tube strip.

d. Vortex thoroughly to resuspend the SPRIselect reagent. Add **95 µl** SPRIselect reagent **(1.0X)** to each transferred supernatant and pipette mix 15x.

Streptavidin Bead Adherence to Tube Wall during PCR is Normal



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

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

g. Remove the supernatant.

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

i. Remove the ethanol.

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

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

l. Remove any remaining ethanol. Air dry for **2 min**. DO NOT exceed **2 min** as this will decrease elution efficiency.

m. Remove from magnet and add **35.5 µl** Buffer EB. Pipette mix 15x.

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

o. Place the tube strip on the magnet•**Low** until the solution clears.

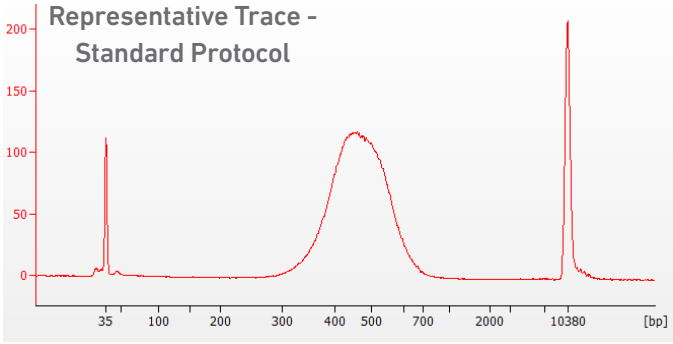
p. Transfer **35 µl** sample to a new tube strip.

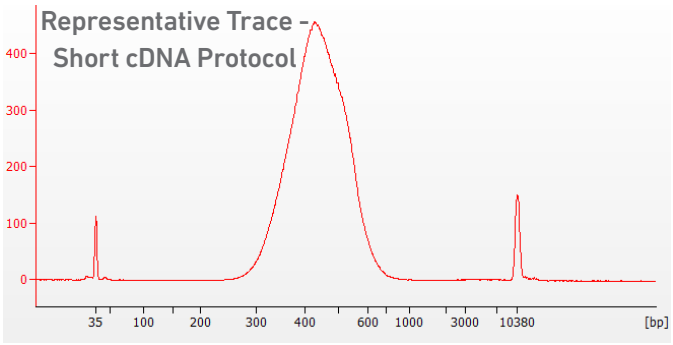
STOP

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

3.3 Targeted Library QC

Run **1 µl s**ample at **1:5 dilution** on an Agilent Bioanalyzer High Sensitivity chip.





Determine the average fragment size from the Bioanalyzer trace.

Alternate QC Method:

- Agilent TapeStation.
- LabChip.

See Appendix for representative traces

See Appendix for Post Library Construction Quantification

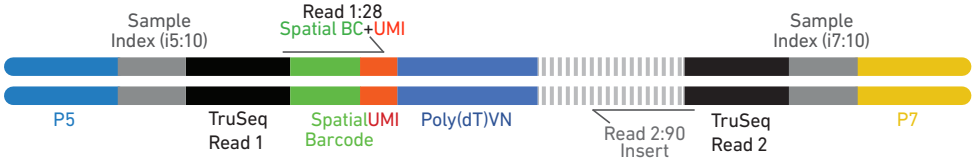
Sequencing

4

Sequencing Libraries

Targeted Gene Expression libraries generated from Visium Spatial Gene Expression libraries comprise standard Illumina paired-end constructs that are flanked with P5/P7, necessary for binding to the Illumina flow cell. TruSeq Read 1 is used for priming and sequencing the 16 bp Spatial Barcode and 12 bp UMI, and TruSeq Read 2 is used for priming and sequencing the cDNA insert. The two 10 bp sample indexes are sequenced in the i5 and i7 read respectively. Sequencing these libraries produces a standard Illumina BCL data output folder.

Visium Spatial 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.

- MiSeq
- NextSeq 500/550
- HiSeq 2500 (Rapid Run)
- HiSeq 3000/4000
- NovaSeq
- iSeq

Sample Indices and Library Pooling

The Targeted Gene Expression product is compatible with Illumina TruSeq single and dual indexed adaptors. Indexing should follow the requirements of the whole transcriptome library prepared prior to targeting.

Targeted libraries may be pooled for sequencing, taking into account the differences in number of tissue-covered spots and read depth requirements between each library. Targeted libraries generated from Single Cell 3' Gene Expression libraries, Single Cell 5' Gene Expression libraries, or Visium Spatial Gene Expression libraries may be pooled for sequencing if they share the same indexing configuration. Mixed single and dual indexed libraries introduce additional complexity due to varying read configurations and are not supported by Illumina.

The sample index name (i.e. the Dual Index plate well ID, SI-TT-) is needed in the sample sheet used for generating FASTQs with "spaceranger mkfastq". Libraries utilizing the same sample index should not be pooled for target enrichment or for sequencing on the same flow cell lane, as this would not enable correct sample demultiplexing. Do not pool whole transcriptome libraries and their derived targeted libraries together on the same lane as they will share the same sample index.

Library Sequencing Depth & Run Parameters

Sequencing depth requirements vary based on the complexity and gene expression profile of the sample, as well as the panel size and content. For pre-designed panels, 10x Genomics recommends 5,000-10,000* read pairs per tissue-covered spot.

If sequence data for a whole transcriptome library from a relevant sample type is available, it can be used to estimate the required sequencing depth for targeted libraries. The whole transcriptome analysis (WTA) data can be analyzed using the targeted-depth tool in Space Ranger by supplying the appropriate "target panel CSV" file for the pre-designed panel. The targeted-depth tool provides sequencing depth recommendations based on the fraction of reads in the WTA library that map to genes included in the panel. These recommendations take advantage of the efficiency enabled by targeting while sequencing enough to match the sensitivity of the WTA dataset. For more information, consult the Space Ranger section of the 10x Genomics Support website.

*The maximum supported sequencing depth for targeted libraries prepared from Visium Spatial Gene Expression libraries is 15,000 tissue-associated read pairs per tissue covered spot. If libraries are sequenced beyond this depth, the sequence data will be downsampled in Space Ranger.

Sequencing Depth	5,000-10,000 read pairs per tissue-covered spot
Sequencing Type	Paired-end, dual indexing

Sequencing Read	**Recommended Number of Cycles**			
	Read 1	**i7 Index**	**i5 index**	**Read 2**
Visium Spatial Gene Expression Library	28	10	10	90

Library Loading

Once quantified and normalized, Targeted Gene Expression 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.

Instrument	Loading Concentration (pM)	PhiX (%)
iSeq	1.8	1
MiSeq	11	1
NextSeq 500/550	1.8	1
HiSeq 2500 (RR)	11	1
HiSeq 4000	240	1
NovaSeq	150*/300	1

* Use 150 pM loading concentration for Illumina XP workflow.

Troubleshooting

5

STEP	NOTES
1.0 Not enough input mass	• If Visium Spatial Gene Expression assays routinely yield libraries of <300 ng, add an additional PCR cycle to the sample index PCR during library preparation. Otherwise, perform the amplification protocol in the Pre Pulldown Library Amplification section of the Appendix.
1.1 Carryover of SPRIselect beads	• Carryover of SPRIselect beads from the original library cleanup may result in low reads mapped to the targeted transcriptome. Ensure that libraries are placed on a 10x Magnetic Separator•**Low** position (magnet•**Low**) for **5 min** and transfered to a new tube prior to library pooling.
1.1a Omission of Universal Blockers	• Omission of Universal Blockers may result in very low Reads Mapped Confidently to the Targeted Transcriptome in targeted libraries. Ensure that Universal Blockers are added prior to proceeding to step 1.1b.
2.4h Inadequate supernatant removal	• Unintended transfer of bead supernatant during bead washing may result in very low Reads Mapped Confidently to the Targeted Transcriptome in targeted libraries. Ensure that no liquid is carried over between wash steps, inluding in tube strip caps.
3.1 Low targeted library yield (<10 nM)	• Increase total number of PCR cycles by 1-2 cycles. The remaining Target-bound Streptavidin Bead sample (**20 µl**) may be amplified with the optional Library Amplification Kit

Appendix

Input Library Volume Example Calculation
Pre Pulldown Library Amplification
Post Amplification Cleanup - SPRI Select
qPCR Library Quantification
Agilent TapeStation Traces

Input Library Volume Example Calculation

These example calculations use the following example data:

Input Library	Tissue-Covered Spot Number Expected	Reads Pairs per Tissue-Covered Spot Desired	Input Library Size	Input Library Concentration
Library 1	3,000	5,000	460 bp	110 nM
Library 2	3,000	5,000	439 bp	100 nM
Library 3	2,000	5,000	450 bp	67 nM
Library 4	4,000	10,000	455 bp	125 nM
Library 5	4,000	10,000	461 bp	45 nM
Library 6	2,500	5,000	454 bp	159 nM
Library 7	1,000	5,000	445 bp	120 nM
Library 8	1,000	5,000	450 bp	123 nM

Calculation 1: Determine per-sample read requirement and weighted average library size.

Multiply the number of tissue-covered spots expected for each input library by the read pairs per tissue-covered spot desired to get reads required. Sum the reads required for all input libraries to get the reads required for the library pool.

Component Library	Tissue-Covered Spot Number Expected		Reads Pairs per Tissue Covered Spot Desired		Reads Required
Library 1	3,000	x	5,000	=	15,000,000
Library 2	3,000	x	5,000	=	15,000,000
Library 3	2,000	x	5,000	=	10,000,000
Library 4	4,000	x	10,000	=	40,000,000
Library 5	4,000	x	10,000	=	40,000,000
Library 6	2,500	x	5,000	=	12,500,000
Library 7	1,000	x	5,000	=	5,000,000
Library 8	1,000	x	5,000	=	5,000,000
Total					**142,500,000**

Input Library Volume Example Calculation

Divide reads required for each input library by the total reads required for the library pool to determine read fraction. Finally, multiply read fraction by library size to get weighted average library size.

Input Library	Reads Required		Total Reads Required		Read Fraction		Size		Weighted Size
Library 1	16,000,000	/	148,000,000	=	0.11	x	460 bp	=	48.4 bp
Library 2	16,000,000	/	148,000,000	=	0.11	x	439 bp	=	46.2 bp
Library 3	16,000,000	/	148,000,000	=	0.11	x	450 bp	=	31.6 bp
Library 4	40,000,000	/	148,000,000	=	0.27	x	455 bp	=	127.7 bp
Library 5	40,000,000	/	148,000,000	=	0.27	x	461 bp	=	129.4 bp
Library 6	16,000,000	/	148,000,000	=	0.11	x	454 bp	=	39.8 bp
Library 7	2,000,000	/	148,000,000	=	0.01	x	445 bp	=	15.6 bp
Library 8	2,000,000	/	148,000,000	=	0.01	x	450 bp	=	15.8 bp
Total					**1.0**				**454.6 bp**

Calculation 2: Determine total moles of library in the library pool.

Use the molar mass of one base pair (650 g/mol) and the average weighted library size (the sum of all weighted sizes) to convert to library pool moles.

Library	Input Mass		Average Library Pool Size		Base Pair Molar Mass		Library Pool Moles
Pooled	2400 x 10^{-9} g	/	(**454.6 bp**	x	650 g/mol.bp)	=	**8.12 x 10^{-12} mol**

Input Library Volume Example Calculation

Pipetting volumes of less than **1 µl** may result in high variability. Refer to Calculation Overview.

Calculation 3: Determine volume of each component library needed for the pooled library.

Multiply input library read fractions by library pool moles to determine input library moles. Use the concentration of input libraries to calculate input library volume in microliters. The high input library volume required for Library 5 indicates that reamplification is required to obtain sufficient volume for this library pool.

Input Library	Read Fraction		Library Pool Moles	Input Library Moles	Library Concentration		Conversion Factor		Input Library Volume
Library 1	0.11	x	8.12×10^{-12} mol	= (8.78e-13 mol / 110 x 10^{-9} M)		x	1e6	=	**7.8 µl**
Library 2	0.11	x	8.12×10^{-12} mol	= (8.78e-13 mol / 100 x 10^{-9} M)		x	1e6	=	**8.6 µl**
Library 3	0.07	x	8.12×10^{-12} mol	= (8.78e-13 mol / 67 x 10^{-9} M)		x	1e6	=	**8.5 µl**
Library 4	0.28	x	8.12×10^{-12} mol	= (2.20e-12 mol / 125 x 10^{-9} M)		x	1e6	=	**18.2 µl**
Library 5	0.28	x	8.12×10^{-12} mol	= (2.20e-12 mol / 45 x 10^{-9} M)		x	1e6	=	**50.7 µl**
Library 6	0.09	x	8.12×10^{-12} mol	= (8.78e-13 mol / 159 x 10^{-9} M)		x	1e6	=	**4.5 µl**
Library 7	0.04	x	8.12×10^{-12} mol	= (1.10e-13 mol / 120 x 10^{-9} M)		x	1e6	=	**2.4 µl**
Library 8	0.04	x	8.12×10^{-12} mol	= (1.10e-13 mol / 123 x 10^{-9} M)		x	1e6	=	**2.3 µl**

Pre Pulldown Library Amplification

CHECKLIST – GET STARTED!
Refer to SDS for handling and disposal information.

Items	10x PN	Preparation & Handling	Storage
Thaw on ice			
☐ ◯ **Amp Mix**	2000047	Centrifuge briefly.	-20°C
☐ ● **Library Amp Primers**	2000291	Thaw, centrifuge briefly.	-20°C
Equilibrate to room temperature			
☐ **Beckman Coulter SPRIselect Reagent**	-	Manufacturer's recommendations.	-
☐ **Agilent TapeStation Screen Tape and Reagents** If used for QC	-	Manufacturer's recommendations.	-
☐ **Agilent Bioanalyzer High Sensitivity Kit** If used for QC	-	Manufacturer's recommendations.	-
☐ **DNA High Sensitivity Reagent Kit** If LabChip used for QC	-	Manufacturer's recommendations.	-
Place on ice			
☐ **KAPA Library Quantification Kit for Illumina Platforms**	-	Manufacturer's recommendations.	-
Obtain			
☐ **Qiagen Buffer EB**	-	Manufacturer's recommendations.	Ambient
☐ **80% Ethanol**	-	Prepare fresh.	Ambient
☐ **Nuclease-free Water**	-	Manufacturer's recommendations.	Ambient

Pre Pulldown Library Amplification

a. Prepare Library Amplification Mix on ice. Pipette mix thoroughly.

Library Amplification Reaction Mix *Add reagents in the order listed*	10x PN	1X (µl)	4X + 10% (µl)	8X + 10% (µl)
Nuclease-free Water	-	30	132	264
Library Amp Primers	2000291	10	44	88
Amp Mix	2000047	50	220	440
Total	-	**90**	**396**	**792**

b. Add **20 ng** library (**10 µl** pre-diluted to 2 ng/µl) to **90 µl** Library Amplification Mix.

c. Pipette mix 10x (pipette set to 90 µl). Centrifuge briefly.

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

Lid Temperature	Reaction Volume	Run Time
105°C	100 µl	~10 min
Step	**Temperature**	**Time**
1	98°C	00:00:45
2	98°C	00:00:20
3	60°C	00:00:30
5	72°C	00:00:20
6	Go to Step 2 five more times for a total of six cycles.*	
7	72°C	00:01:00
8	4°C	Hold

*If running multiple pulldowns on the same reamplified Visium Spatial Gene Expression library, seven cycles of amplification are recommended.

e. Proceed to Post Amplification Cleanup - SPRIselect after completion of thermal cycler protocol.

Post Amplification Cleanup - SPRIselect

a. Vortex to resuspend the SPRIselect reagent. Add **100 µl** SPRIselect reagent **(1.0X)** to the sample and pipette mix 15x.

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

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

d. Remove the supernatant.

e. 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. Air dry for **2 min**. DO NOT exceed **2 min** as this will decrease elution efficiency.

j. Remove from magnet and add **40.5 µl** Buffer EB. Pipette mix 15x.

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

l. Place the tube strip on the magnet•**Low** until the solution clears.

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

STOP

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

Refer to step 3.3 for QC instructions. This amplification protocol should generate **1-2 µg** library.

qPCR Library Quantification

a. Prepare SYBR Fast Master Mix + Primer before first use by combining **5 ml** KAPA SYBR FAST qPCR Master Mix and **1 ml** Primer Premix and mix thoroughly. ROX Low and ROX High are omitted.

b. Thaw KAPA Library Quantification Kit for Illumina Platforms.

c. Dilute **2 µl** sample with Buffer EB 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).

d. 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**

e. Dispense **16 µl** Quantification Master Mix for sample dilutions and DNA Standards into an appropriate qPCR plate.

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

g. Prepare a qPCR system with the following protocol. Insert the plate and start the program.

Lid Temperature	Reaction Volume	Run Time
-	20 µl	35 min
Step	**Temperature**	**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)	

h. 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

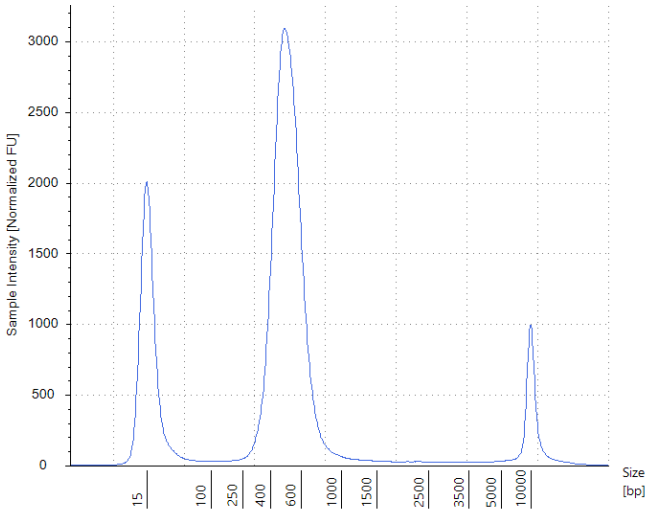
Agilent TapeStation Traces

Agilent TapeStation High Sensitivity D5000 ScreenTape was used.

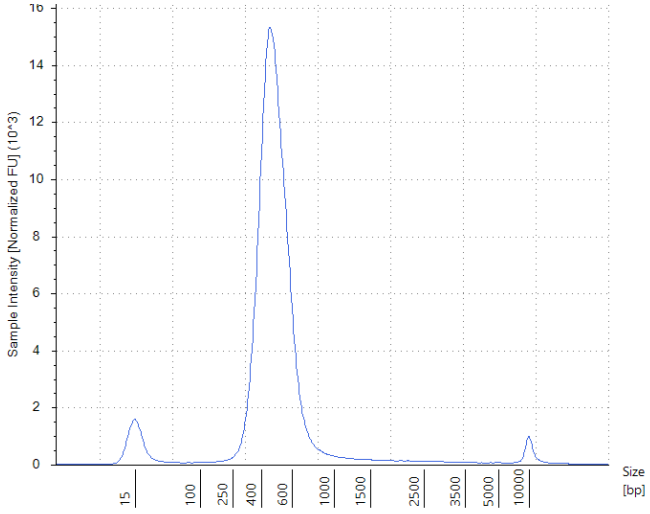
Protocol steps correspond to the Targeted Gene Expression - Spatial User Guide (CG000377).

Protocol Step 3.3 – Targeted Library QC

Representative Trace - Standard Protocol



Representative Trace - Short cDNA Protocol



Run 2 µl diluted sample (1:5 dilution) mixed with 2 µl loading buffer.

LabChip Traces

LabChip Traces

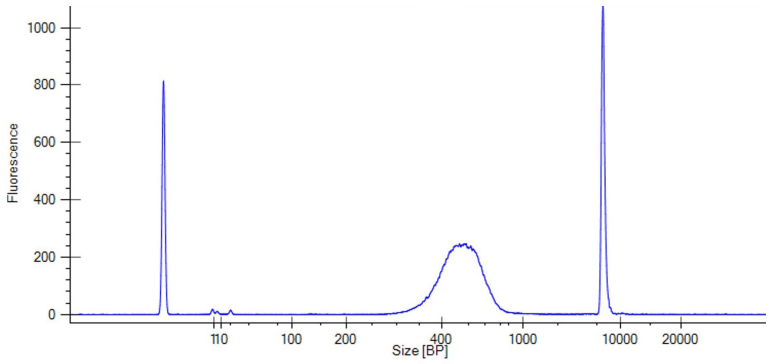
DNA High Sensitivity Reagent Kit was used.

Protocol steps correspond to the Targeted Gene Expressions - Spatial User Guide (CG000377).

Protocol Step 3.3 – Targeted Library QC

Run 10 µl diluted sample (1:10 dilution).

Representative Trace - Standard Protocol



Representative Trace - Short cDNA Protocol