

USER GUIDE

Targeted Gene Expression Reagent Kits



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

Next GEM reagents are specific to Next GEM products and should not be used interchangeably with non-Next GEM reagents.

Notices

Document Number

CG000293 • Rev B

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Document Revision Summary

Document Number	CG000293
Title	Targeted Gene Expression Reagent Kits User Guide
Revision	Rev B
Revision Date	July 2020

Specific Changes:

- Added information on fully custom panels and add-on panels.

General Changes:

- Updated for general minor consistency of language and terms throughout.

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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

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

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

Library Amplification Kit


	#	PN
 Amp Mix	1	2000047
 Library Amp Primers	1	2000291

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*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

Targeted Gene Expression Reagent Kits


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

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	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
Fully Custom and 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	5382000023
	Eppendorf SmartBlock 1.5 ml, Thermoblock for 24 reaction vessel (alternatively, use a temperature-controlled Heat Block)	5360000038
	Eppendorf Vacufuge Plus Complete System (alternatively, use any equivalent vacuum centrifuge that accommodates 0.2 ml tubes)	022822993
	Rotor for Eppendorf Vacufuge Plus Concentrator	022822241
	Eppendorf PCR Tube Work Tray (alternatively, use any equivalent adapter that accommodates 0.2 ml tubes)	951010031
	Eppendorf Polycarbonate Work Tray Frame	951010049
Rainin	Pipet-Lite Multi Pipette L8-50XLS+	17013804
	Pipet-Lite Multi Pipette L8-200XLS+	17013805
	Pipet-Lite Multi Pipette L8-10XLS+	17013802
	Pipet-Lite Multi Pipette L8-20XLS+	17013803
	Pipet-Lite LTS Pipette L-2XLS+	17014393
	Pipet-Lite LTS Pipette L-10XLS+	17014388
	Pipet-Lite LTS Pipette L-20XLS+	17014392
	Pipet-Lite LTS Pipette L-100XLS+	17014384
	Pipet-Lite LTS Pipette L-200XLS+	17014391
	Pipet-Lite LTS Pipette L-1000XLS+	17014382
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	 –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	 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	 4°C ≤72 h or –20°C long term

*~4 h workflow, excluding library drying, library amplification, & QC steps

Stepwise Objectives



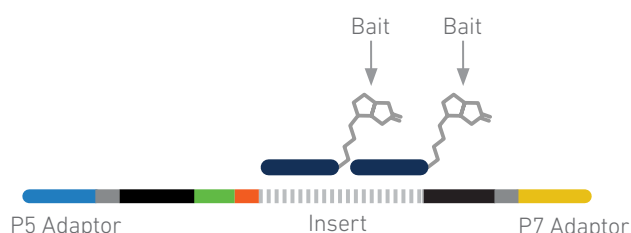
The Targeted Gene Expression product is a modular enrichment kit designed to enrich libraries for relevant genes, while decreasing sequencing requirements by up to 90%. This product is compatible with Chromium Single Cell 3' Gene Expression (v3, Single/Dual Index v3.1) and Chromium Single Cell 5' Gene Expression (v1, v1.1, v2) libraries.

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. Custom 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 Whole Transcriptome Analysis (WTA) libraries that have been generated previously using the relevant 10x Genomics User Guides.

Targeted Gene Expression Bait Design

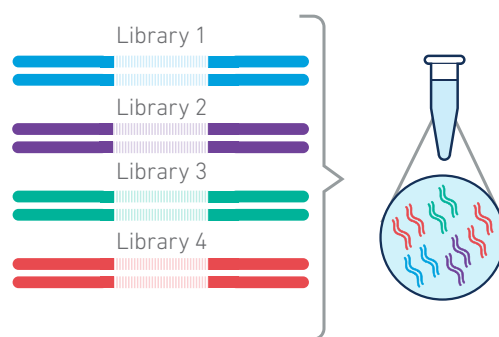
Targeted Gene Expression baits are single stranded, 120 nt DNA oligonucleotides with a 5' biotin modification. Each bait targets a unique library molecule. Baits span all mature mRNA sequences, including UTRs and all annotated isoforms.



Step 1 Pre Hybridization Pooling

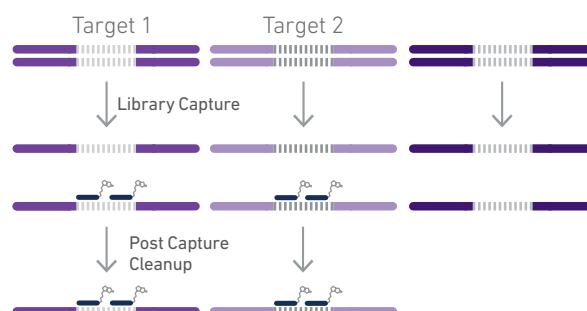
This workflow may be performed with a single library or a library pool in which up to eight libraries are multiplexed on a single baitset.

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 which begin with P5 and end with P7. 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



TIPS

Icons



Tips & Best Practices section includes additional guidance



Signifies critical step requiring accurate execution



Troubleshooting section includes additional guidance

General Reagent Handling

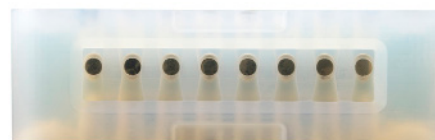
- Fully thaw and thoroughly mix reagents before use.
- Promptly move reagents back to the recommended storage after use.
- Calculate reagent volumes with 10% excess of 1 reaction values.
- Thoroughly mix samples with the beads during bead-based cleanup steps.
- Pulse vortexing instead of continuously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

Pipette Calibration

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

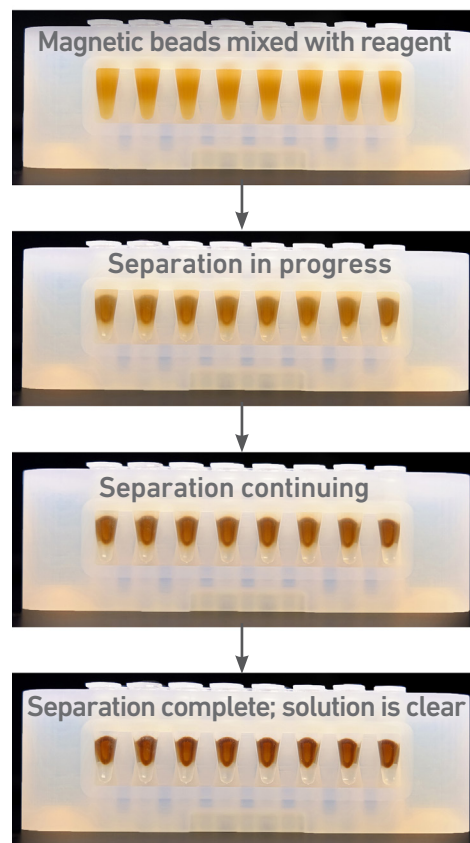
10x Magnetic Separator

- 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

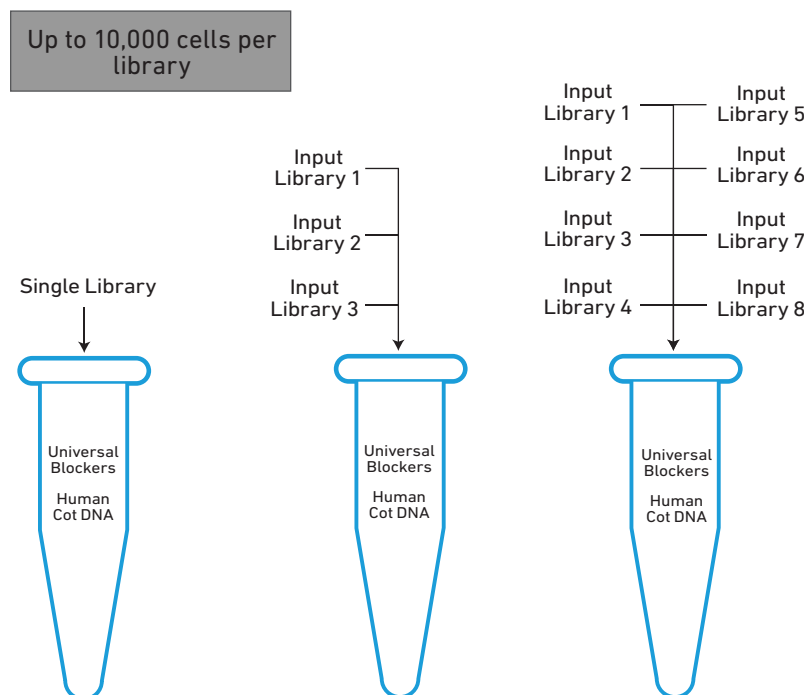
1.1 Library Pooling and Drying

1

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.



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 Appendix.

Library Pool

- Calculate input library volume based on the Calculation Overview.
- Amplification may also be performed for a library pool bait capture if less than 325 ng DNA is available.
- For optimal performance, pool similar input libraries according to library cell number expected, cell type, and product (Chromium Single Cell 3' or 5' Gene Expression).

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 cell 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 Appendix.

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

If the input library has already been sequenced, enter the Estimated Number of Cells. Otherwise, enter the expected number of recovered cells. 10x Genomics recommends 2,000 read pairs per cell 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:

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

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

Input Library	Cell Number Expected	Reads Pairs per Cell Desired	Input Library Size	Input Library Concentration
Library 1	4,400	2,000	454 bp	142.5 nM
Library 2	3,000	2,000	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 cell number expected for that library by the read pairs per cell desired.	$\text{Input Library Reads} = \text{Cell Number Expected} \times \text{Reads Pairs per Cell Desired}$	$8,800,000 = 4,400 \times 2,000$
1b	Divide input library reads by library pool reads, the sum of reads required for all input libraries.	$\text{Read Fraction} = \frac{\text{Input Library Reads}}{\text{Library Pool Reads}}$	$0.59 = \frac{8,800,000}{14,800,000}$
1c	Multiply read fraction by library size to determine the weighted input library size.	$\text{Weighted Input Library Size (bp)} = \text{Read Fraction} \times \text{Input Library Size (bp)}$	$269.9 \text{ bp} = 0.59 \times 454 \text{ bp}$
1d	Sum all weighted library sizes in the library pool for the average library pool size.	$\text{Average Library Pool Size (bp)} = \frac{\text{Weighted Input Library Sizes}}{\text{Sum (bp)}}$	$436.6 \text{ bp} = 269.9 \text{ bp} + 166.6 \text{ bp}$
1e	Average library pool size is used to calculate library pool moles.	$\text{Library Pool Moles} = \frac{\text{Total Input Mass (300 ng x \# Input Libraries)}}{\text{Average Library Pool Size (bp) * Molar Mass Base Pair (650 g/mol per bp)}}$	$2.11 \times 10^{-12} \text{ mol} = \frac{300 \times 10^{-9} \text{ g} \times 2}{436.6 \text{ bp} \times 650 \text{ g/mol per bp}}$
1f	Use read fraction and library pool moles to calculate input library moles.	$\text{Input Library Moles} = \text{Read Fraction} \times \text{Library Pool Moles}$	$1.26 \times 10^{-12} \text{ mol} = 0.59 \times 2.11 \times 10^{-12} \text{ mol}$
1g	Use the solution of (1f) to calculate input library volume.	$\text{Input Library Volume (}\mu\text{l)} = \frac{\text{Input Library Moles}}{\text{Input Library Concentration (nM)}}$	$8.8 \mu\text{l} = \frac{1.26 \times 10^{-12} \text{ mol}}{142.5 \times 10^{-9} \text{ M}}$

1.0
Pre Hybridization
Pooling

CHECKLIST – GET STARTED!			
Items	10x PN	Preparation & Handling	Storage
Equilibrate to room temperature			
<input type="checkbox"/> ● Universal Blockers	2000290	Thaw, centrifuge briefly.	-20°C
<input type="checkbox"/> ● 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.

- 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

- 2.1 Library Hybridization
- 2.2 Capture Bead Preparation
- 2.3 Bait Capture
- 2.4 Post Capture Cleanup - Streptavidin Beads



2.0 Library Capture

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

*If working with a fully custom panel or adding genes to a pre-designed panel

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. Fully custom panels may also be created with the Custom Panel Designer on the 10x Genomics website. For fully custom panels, 10x Genomics supports 10-1,500 target genes. 10x Genomics also 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.

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

2.1A Library Hybridization - Pre-designed Panels

For Pre-designed Panels

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

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

Lid Temperature	Reaction Volume	Run Time
100°C	70 µl	~2 h 45 min

Step	Temperature	Time
Pre-equilibrate	95°C	Hold
Denaturation	95°C	00:00:30
Hybridization	65°C	02:00:00
Hold for Washes	65°C	Hold

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

Hybridization Master Mix <i>Add reagents in the order listed</i>	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 **immediately** to step 2.2.

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 2.1C.
- 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.

Duplication Factor	Volume (µl) for								
	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 fully custom panels, proceed to Panel Dilution Guidelines - Fully Custom Panels.

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


Lid Temperature	Reaction Volume	Run Time
100°C	70 µl	~2 h 45 min

Step	Temperature	Time
Pre-equilibrate	95°C	Hold
Denaturation	95°C	00:00:30
Hybridization	65°C	02:00:00
Hold for Washes	65°C	Hold

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

Hybridization Master Mix <i>Add reagents in the order listed</i>	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	8.8	17.6
Total	-	17.2	75.7	151.4

*If add-on panels were combined with pre-designed panels for storage, replace both asterisked 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 immediately to step 2.2.

Panel Dilution Guidelines - Fully Custom Panels

For Fully Custom Panels

If using fully custom panels:

- Prepare fully custom panel working dilutions according to the dilutions in Table 1 prior to proceeding to step 2.1C.

Table 1. Dilution of IDT NGS Discovery and xGen Lockdown Probe Pools.

	Volume for		
	4,001-10,000 baits (μ l)	10,001-20,000 baits (μ l)	>20,000* baits (μ l)
IDT NGS Discovery Pools			
IDT NGS Discovery Pool	2	1	1
IDTE, pH 8.0	2	3	4
Total	4	4	5
IDT xGen Lockdown Probes			
IDT xGen Lockdown Probe Pool	2	1	N/A
IDTE, pH 8.0	2	3	N/A
Total	4	4	N/A

*panels containing up to ~60,000 baits have been tested by 10x Genomics.

- Fully custom panels with less than 4,000 baits require no dilution. Dilutions may be prepared in advance and stored at **-20°C** for up to **one month**.
- Fully custom panels require modified post-capture cycling conditions during step 3. Refer to Post-capture Cycling Conditions - Fully Custom Panels.
- Equilibrate fully custom panel to room temperature and proceed to step 2.1C.

2.1C Library Hybridization - Fully Custom Panels

For Fully Custom Panels

Only if working with fully custom panels. 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.

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

Lid Temperature	Reaction Volume	Run Time
100°C	70 µl	~2 h 45 min
Step	Temperature	Time
Pre-equilibrate	95°C	Hold
Denaturation	95°C	00:00:30
Hybridization	65°C	02:00:00
Hold for Washes	65°C	Hold

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

Hybridization Master Mix <i>Add reagents in the order listed</i>	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
Fully Custom Panel Working Dilution	-	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 **immediately** to step 2.2.

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.

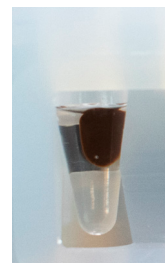
c. Add **100 μl** Equilibration Buffer to each tube. Pipette mix 15x.

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.

Streptavidin Beads Before and After Separation



2.3 Bait Capture



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

Ensure that thermal cycler remains at **65°C** for subsequent washes.

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**.

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

e. Remove library tube strip from thermal cycler. Proceed **immediately** to the next step.

2.4 Post Capture Cleanup - Streptavidin Beads

TIPS

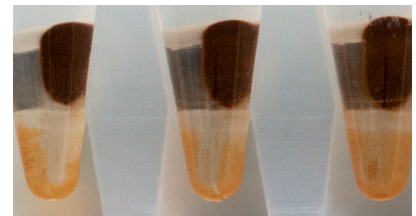
- a. Add **100 µl** Wash Buffer to each sample. Pipette mix 15x.
 - b. Place on the magnet•High for **1 min.**
 - c. Remove supernatant.
 - d. Remove from the magnet. Add **150 ul** Wash Buffer to each bead pellet.
 - e. Pipette mix 15x.
 - f. Incubate in thermal cycler set to **65°C** for **5 min.**
 - 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.
- ! Ensure that samples are incubated at **65°C** after each wash
- 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.
 - n. Store remaining Target-bound Streptavidin Beads at **4°C ≤ 24 h** or at **-20°C** for long-term storage.



Avoid Splashing Liquid onto Cap



Streptavidin Bead Adherence to Tube Wall during Washes is Normal





Step 3

Post Capture Amplification

- 3.1 Library Amplification
- 3.2 Post Library Amplification Cleanup - SPRIselect
- 3.3 Targeted Library QC



3

3.0 Library Amplification

CHECKLIST – GET STARTED!			
Items	10x PN	Preparation & Handling	Storage
Thaw on ice			
<input type="checkbox"/>  Amp Mix	2000047	Centrifuge briefly.	-20°C
<input type="checkbox"/>  Library Amp Primers	2000291	Thaw, centrifuge briefly.	-20°C
Equilibrate to room temperature			
<input type="checkbox"/> Beckman Coulter SPRIselect Reagent	-	Manufacturer's recommendations.	-
<input type="checkbox"/> Agilent TapeStation Screen Tape and Reagents <small>If used for QC</small>	-	Manufacturer's recommendations.	-
<input type="checkbox"/> Agilent Bioanalyzer High Sensitivity Kit <small>If used for QC</small>	-	Manufacturer's recommendations.	-
<input type="checkbox"/> DNA High Sensitivity Reagent Kit <small>If LabChip used for QC</small>	-	Manufacturer's recommendations.	-
Place on ice			
<input type="checkbox"/> KAPA Library Quantification Kit for Illumina Platforms	-	Manufacturer's recommendations.	-
Obtain			
<input type="checkbox"/> Qiagen Buffer EB	-	Manufacturer's recommendations.	Ambient
<input type="checkbox"/> 80% Ethanol	-	Prepare fresh.	Ambient
<input type="checkbox"/> 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 <i>Add reagents in the order listed</i>	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 using fully custom panels, refer to Post-capture Cycling Conditions - Fully Custom Panels. 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

Post-capture Cycling Conditions - Fully Custom Panels

Only Required for Fully Custom Panels

Table 1 provides post-capture PCR cycling recommendations for fully custom panels and should be used in place of the “recommended starting points for cycle number optimization” table from Step 3.1d.

To use this table, determine the expected expression level of the fully custom panel. If a representative WTA library is available, this value can be determined using the targeted-depth subcommand in Cell Ranger. Given an existing WTA dataset and a target panel .csv file, targeted-depth computes the fraction of reads mapped to targeted genes from the panel. For instructions on running this script, refer to the Targeted Depth section of the 10x Genomics Support website. Take the “Fraction of Reads from Targeted Genes” and find the corresponding number of cycles recommended based on the number of input libraries in the library pool.

If expected expression level is not known or cannot be calculated, the number of genes in the fully custom panel can be used instead. This assumes that expression levels of the genes in the fully custom panel will mirror the distribution of expression levels across all genes in the transcriptome.

Table 1. Recommended Number of PCR Cycles for Fully Custom Panels. Round to the nearest expression level.

Expected Panel Expression Level	Genes in Fully Custom Panel	PCR Cycles for:			
		1*	2-3*	4-6*	7-8*
<0.01%	<10	18	18	18	18
0.01%	10-200	17	17	16	15
0.05%		15	15	14	13
0.10%		15	14	13	12
0.25-1%		14	12	11	10
2-4%	200-500	13	12	11	10
5-8%	500-1500	12	11	10	9

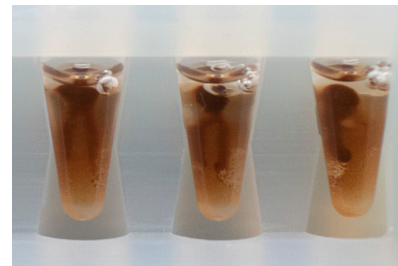
*number of input libraries

If the final library yield is 300 nM or greater, Step 3 - Post Capture Amplification of the Targeted Gene Expression User Guide should be repeated using the remaining volume of Target-bound Streptavidin Beads with a reduced number of cycles. Remove one PCR cycle for every 2-fold reduction required to achieve a 20-100 nM final library. For example, if ten total cycles resulted in a 300 nM final library, removing three cycles reduces the final library yield by 8-fold ($2^3 = 8$ fold) to approximately 40 nM.

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

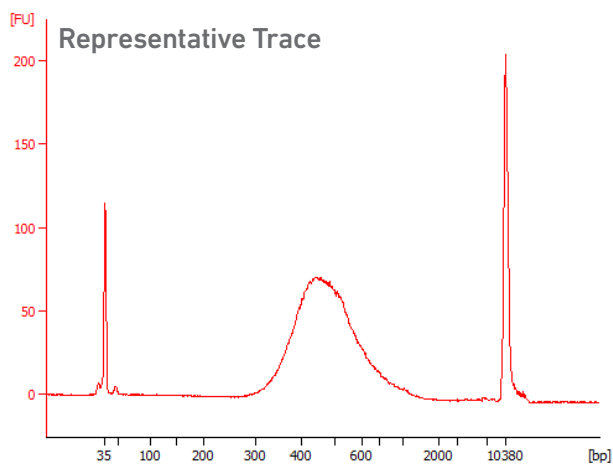


- e. Incubate **5 min** at **room temperature**.
- f. Place on the magnet•**High** until the solution clears.
- g. Remove the supernatant.
- h. Add **200 µl** 80% ethanol to the pellet. Wait **30 sec**.
- i. Remove the ethanol.
- j. **Repeat** steps h and i for a total of 2 washes.
- k. Centrifuge briefly 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.
- 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 μ l sample at 1:10 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

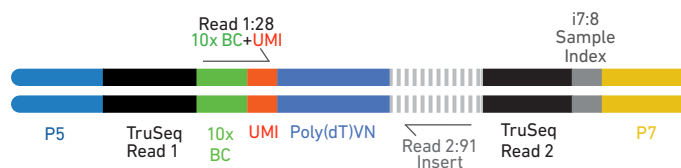
Sequencing Libraries

The Targeted Gene Expression product is compatible with single or dual indexed libraries. These include libraries generated with the Single Cell 3' Gene Expression and Single Cell Immune Profiling 5' Gene Expression solutions.

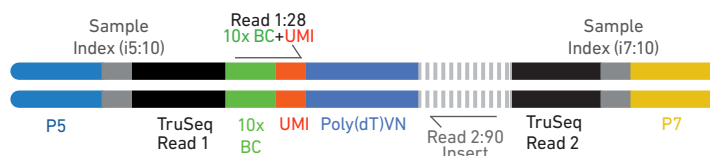
10x Genomics Single Cell Gene Expression libraries comprise standard Illumina paired-end constructs which begin with P5 and end with P7. For single indexed libraries, an 8 bp sample index sequence is incorporated as the i7 sample index read. For dual indexed libraries, 10 bp sample index sequences are incorporated as the i7 and i5 sample index reads. TruSeq Read 1 and Read 2 are standard Illumina sequencing primer sites used in paired-end sequencing. TruSeq Read 1 is used to sequence 16 bp 10x Barcodes and 12 bp UMI for 3' Single Cell Gene Expression libraries or a 10 bp UMI for 5' Single Cell Gene Expression libraries.

Sequencing these libraries produces a standard Illumina BCL data output folder.

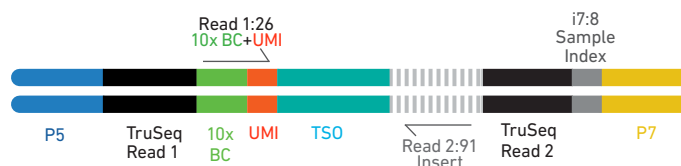
Chromium Single Cell 3' Gene Expression Single Index Library



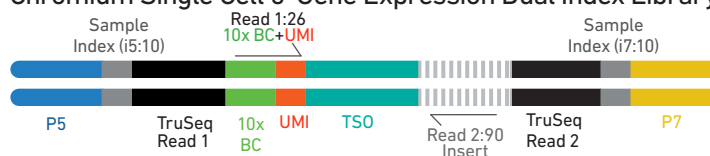
Chromium Single Cell 3' Gene Expression Dual Index Library



Chromium Single Cell 5' Gene Expression Single Index Library



Chromium Single Cell 5' Gene Expression Dual Index 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 cell number and per-cell read depth requirements between each library. When pooling libraries for target enrichment or for sequencing, ensure that indices are unique for each input library.

Targeted libraries generated from Single Cell 3' and 5' libraries may be pooled for sequencing if they share the same indexing configuration and if their indices are unique. Mixed single and dual indexed libraries introduce additional complexity due to varying read configurations and are not supported by Illumina. Do not pool whole transcriptome libraries and their derived targeted libraries together on the same lane as they will share the same sample index.

The sample index name (i.e. the Single Index plate well ID, SI-GA-) 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.

Library Sequencing Depth & Run Parameters

The recommended sequencing depth for pre-designed panels is dependent on the complexity and gene expression profile of the sample, as well as the panel chosen.

Generally, 10-20% of the sequencing depth required for an equivalent whole transcriptome library is recommended. For pre-designed panels, this is typically between 2,000-10,000 read pairs per cell. However, sequencing depth may require optimization depending on cellular RNA content. The expression level of targeted genes will vary by cell type and by the proportion of different cell types within a sample. This dictates what fraction of molecules in a whole transcriptome library will be from the targeted genes of interest (the library fraction on target).

To compare sequencing requirements between a whole transcriptome library and its derived targeted library, multiply the fraction on target for the whole transcriptome library by the number of reads required to achieve sufficient depth on the genes of interest in the whole transcriptome library. Sequence two-fold more reads in the targeted library than the number of on target reads in the whole transcriptome library.

For example, a whole transcriptome library sequenced to 30,000 read pairs per cell, where 5% reads are for the genes on the targeted panel, would have a recommended sequencing depth for its targeted library of 3,000 read pairs per cell ($30,000 \times 0.05 \times 2$). 10x Genomics recommends 2,000-10,000 read pairs per cell as a starting sequencing depth for targeted libraries where the sequencing depth for the associated whole transcriptome library is not known or if WTA profiling has not been performed.

For custom panels, depth requirements are likely to differ from pre-designed panels. If WTA data are available for a relevant sample type, the targeted-depth subcommand in Cell Ranger can help estimate sequencing depth recommendations. Given an existing WTA dataset and a target panel .csv file, targeted-depth computes the fraction of reads mapped to targeted genes from the panel and provides sequencing depth recommendations that take advantage of the efficiency enabled by targeting. For more information, consult the 10x Genomics Support website.

Sequencing Depth	Minimum 2,000 read pairs per cell			
Sequencing Type	Consult Relevant User Guide			
Sequencing Read	Recommended Number of Cycles			
	Read 1	i7 Index	i5 index	Read 2
3' Gene Expression Library (v3.1 Dual Index)	28	10	10	90
3' Gene Expression Library (v3/v3.1)	28	8	0	91
5' Gene Expression Library (v2 Dual index)	26	10	10	90
5' Gene Expression Library (v1/v1.1)	26	8	0	91

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	<ul style="list-style-type: none"> If Chromium Single Cell 3' Gene Expression assays routinely yield libraries of <300 ng, add an additional PCR cycle to the sample index PCR during Single Cell 3' Gene Expression library preparation. Otherwise, perform the amplification protocol in the Pre Pulldown Library Amplification section of the Appendix.
1.1a Omission of Universal Blockers	<ul style="list-style-type: none"> 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	<ul style="list-style-type: none"> 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, including in tube strip caps.
3.1 Low targeted library yield (<10 nM)	<ul style="list-style-type: none"> 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	Cell Number Expected	Reads Pairs per Cell Desired	Input Library Size	Input Library Concentration
Library 1	8,000	2,000	460 bp	110 nM
Library 2	8,000	2,000	439 bp	100 nM
Library 3	8,000	2,000	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	8,000	2,000	454 bp	159 nM
Library 7	1,000	2,000	445 bp	120 nM
Library 8	1,000	2,000	450 bp	123 nM

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

Multiply the number of cells expected for each input library by the read pairs per cell desired to get reads required. Sum the reads required for all input libraries to get the reads required for the library pool.

Component Library	Cell Number Expected		Reads Pairs per Cell Desired		Reads Required
Library 1	8,000	x	2,000	=	16,000,000
Library 2	8,000	x	2,000	=	16,000,000
Library 3	8,000	x	2,000	=	16,000,000
Library 4	4,000	x	10,000	=	40,000,000
Library 5	4,000	x	10,000	=	40,000,000
Library 6	8,000	x	2,000	=	16,000,000
Library 7	1,000	x	2,000	=	2,000,000
Library 8	1,000	x	2,000	=	2,000,000
Total					148,000,000

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	=	49.7 bp
Library 2	16,000,000	/	148,000,000	=	0.11	x	439 bp	=	47.5 bp
Library 3	16,000,000	/	148,000,000	=	0.11	x	450 bp	=	48.6 bp
Library 4	40,000,000	/	148,000,000	=	0.27	x	455 bp	=	123.0 bp
Library 5	40,000,000	/	148,000,000	=	0.27	x	461 bp	=	124.6 bp
Library 6	16,000,000	/	148,000,000	=	0.11	x	454 bp	=	49.1 bp
Library 7	2,000,000	/	148,000,000	=	0.01	x	445 bp	=	6.0 bp
Library 8	2,000,000	/	148,000,000	=	0.01	x	450 bp	=	6.1 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	$= (8.78\text{e-}13 \text{ mol} / 110 \times 10^{-9} \text{ M})$	x	1e6	= 8.0 µl
Library 2	0.11	x	8.12×10^{-12} mol	$= (8.78\text{e-}13 \text{ mol} / 100 \times 10^{-9} \text{ M})$	x	1e6	= 8.8 µl
Library 3	0.11	x	8.12×10^{-12} mol	$= (8.78\text{e-}13 \text{ mol} / 67 \times 10^{-9} \text{ M})$	x	1e6	= 13.1 µl
Library 4	0.27	x	8.12×10^{-12} mol	$= (2.20\text{e-}12 \text{ mol} / 125 \times 10^{-9} \text{ M})$	x	1e6	= 17.6 µl
Library 5	0.27	x	8.12×10^{-12} mol	$= (2.20\text{e-}12 \text{ mol} / 45 \times 10^{-9} \text{ M})$	x	1e6	= 48.8 µl
Library 6	0.11	x	8.12×10^{-12} mol	$= (8.78\text{e-}13 \text{ mol} / 159 \times 10^{-9} \text{ M})$	x	1e6	= 5.5 µl
Library 7	0.01	x	8.12×10^{-12} mol	$= (1.10\text{e-}13 \text{ mol} / 120 \times 10^{-9} \text{ M})$	x	1e6	= 0.9 µl
Library 8	0.01	x	8.12×10^{-12} mol	$= (1.10\text{e-}13 \text{ mol} / 123 \times 10^{-9} \text{ M})$	x	1e6	= 0.9 µl

Pre Pulldown Library Amplification

CHECKLIST – GET STARTED!			
Items	10x PN	Preparation & Handling	Storage
Thaw on ice			
<input type="checkbox"/>  Amp Mix	2000047	Centrifuge briefly.	-20°C
<input type="checkbox"/>  Library Amp Primers	2000291	Thaw, centrifuge briefly.	-20°C
Equilibrate to room temperature			
<input type="checkbox"/> Beckman Coulter SPRIselect Reagent	-	Manufacturer's recommendations.	-
<input type="checkbox"/> Agilent TapeStation Screen Tape and Reagents <small>If used for QC</small>	-	Manufacturer's recommendations.	-
<input type="checkbox"/> Agilent Bioanalyzer High Sensitivity Kit <small>If used for QC</small>	-	Manufacturer's recommendations.	-
<input type="checkbox"/> DNA High Sensitivity Reagent Kit <small>If LabChip used for QC</small>	-	Manufacturer's recommendations.	-
Place on ice			
<input type="checkbox"/> KAPA Library Quantification Kit for Illumina Platforms	-	Manufacturer's recommendations.	-
Obtain			
<input type="checkbox"/> Qiagen Buffer EB	-	Manufacturer's recommendations.	Ambient
<input type="checkbox"/> 80% Ethanol	-	Prepare fresh.	Ambient
<input type="checkbox"/> 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 <i>Add reagents in the order listed</i>	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

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 μ l** 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 μ l** 80% ethanol to the pellet. Wait **30 sec**.
- f. Remove the ethanol.
- g. **Repeat** steps e and f for a total of 2 washes.
- h. Centrifuge briefly and place on the magnet•**Low**.
- i. Remove any remaining ethanol. Air dry for **2 min**. DO NOT exceed **2 min** as this will decrease elution efficiency.
- j. Remove from magnet and add **40.5 μ l** 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 μ l** from sample to a new tube strip.
- 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 μ g** library.

- qPCR Library Quantification**
- 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.
 - Thaw KAPA Library Quantification Kit for Illumina Platforms.
 - 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).
 - 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

- Dispense **16 µl** Quantification Master Mix for sample dilutions and DNA Standards into an appropriate qPCR plate.
- Add **4 µl** sample dilutions and **4 µl** DNA Standards to appropriate wells. Centrifuge briefly.
- 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	00:00:30
	 Read signal	
4	Go to Step 2, 29X (Total 30 cycles)	

- 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

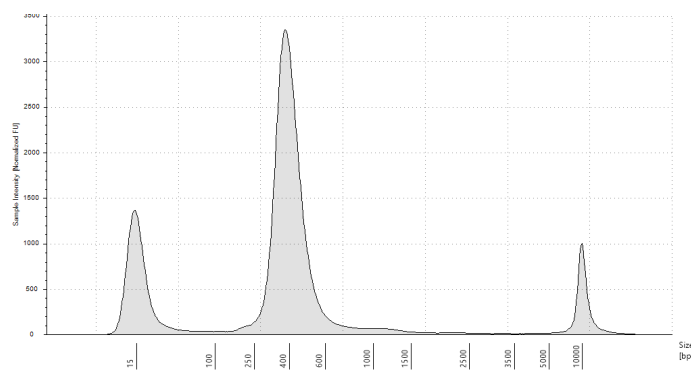
Agilent TapeStation Traces

Agilent TapeStation High Sensitivity D5000 ScreenTape was used.

Protocol steps correspond to the Targeted Gene Expression Reagent Kits User Guide (CG000293).

Appendix – Pre Pulldown Library Amplification

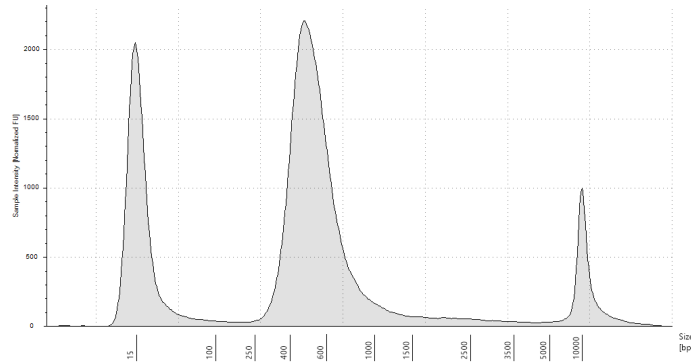
Representative Trace



Run 2 μ l diluted sample (1:10 dilution) mixed with 2 μ l loading buffer.

Protocol Step 3.3 – Targeted Library QC

Representative Trace



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

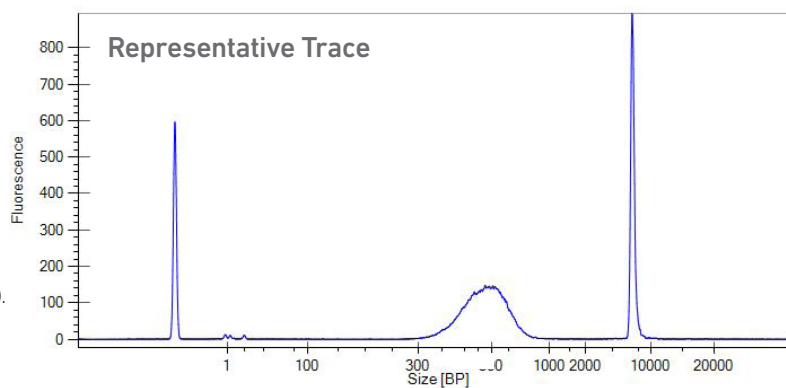
LabChip Traces

LabChip Traces

DNA High Sensitivity Reagent Kit was used.

Protocol steps correspond to the Targeted Gene Expression Reagent Kits User Guide (CG000293).

Appendix – Pre Pulldown Library Amplification



Protocol Step 3.3 – Targeted Library QC

