CRISPR Screening with GEM-X Flex Gene Expression

Introduction

This Technical Note describes designing and using custom probes for CRISPR guide capture with the GEM-X Flex assay. This document also provides guidance on workflow modifications required when performing CRISPR Screening with the GEM-X Flex assay. Guidance on analysis of CRISPR screening and Gene Expression Flex data in Cell Ranger is also provided here.

GEM-X Flex Gene Expression offers comprehensive, scalable solutions to measure gene expression in fixed samples. For this assay, 10x Genomics provides a predesigned whole transcriptome panel of probes for target hybridization. Custom probes to enable CRISPR screening may be designed for use with the assay using the guidance provided in this document. While no impact on assay performance is anticipated, the use of custom probes is not supported or validated by 10x Genomics. 10x Genomics cannot guarantee that custom probes will yield data comparable to that from the whole transcriptome panel.

The guidance provided in this document is for the GEM-X Flex assay. It can still be applied to the Next GEM Flex assay if the spike-in volume is increased to 5 µl and the targeted cell recovery is reduced as specified in the Next GEM assay.

Contents

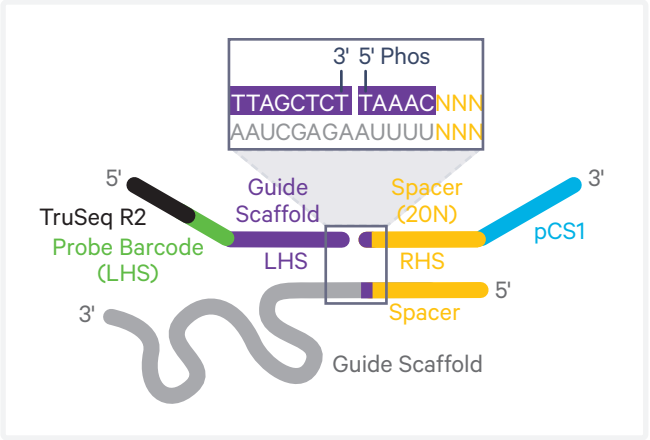


Figure 1. CRISPR guide capture using custom probes for GEM-X Flex Gene Expression.

CRISPR Guide Probe Design

An overview of the CRISPR guide probe design for GEM-X Flex Gene Expression is provided in Figure 1.

CRISPR Guide Capture Custom Probe Sequences — Multiplex	
LHS Probe	
5'-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-XXXXXXXX-GCTATGCTGTTTCCAGCTTAGCTCT-3'	
TruSeq R2	**Probe Barcode (multiplex)** **Guide Scaffold**
RHS Probe	
/5Phos/-TAAAC-NNNNNNNNNNNNNNNNNNNN-CGGTCCTAGCAA-3'	
Spacer (20N)	pCS1

Table 1. CRISPR custom probe sequences for GEM-X Flex Gene Expression.

The sequences for these probe designs are provided in Table 1. CRISPR LHS Probe Barcode sequences (CR001-CR016) used are listed in Table 2.

To design custom probes for CRISPR guide capture for use with the GEM-X Flex assay, the guide scaffold and spacer sequences must be known in advance. Custom probe design for CRISPR guide capture is based on the probe design principles provided in Technical Note Custom Probe Design for Visium Spatial Gene Expression and Chromium Single Cell Gene Expression Flex (CG000621), with the following modifications:

- LHS probe has a TruSeq R2, a Probe Barcode and a 25-bp sequence that is the reverse-complement of the guide scaffold. *The TruSeq handle allows for separate indexing of the CRISPR library.*
- CRISPR LHS Probe Barcode sequences are different from those used in the WTA probes. See Table 2.
- RHS probe contains a 5' phosphate (/5Phos/), a 5 bp sequence that is the reverse complement of the guide scaffold, a 20 bp sequence that is the reverse complement of the targeted spacer, and a partial Capture Sequence 1 (pCS1).

Guide scaffold sequences vary between vectors. The LHS and RHS probe sequence should be changed to match the guide scaffold used. See Guide RNA Compatibility.

For multiplex experiments, each LHS probe must have a unique eight base Probe Barcode (CR001-CR016; sequences in Table 2). Furthermore, in a multiplex experiment, each hybridization reaction should have a unique pair of Probe Barcodes for CRISPR and WTA probes, i.e., the same CRISPR Probe Barcode cannot be used with multiple WTA Probe Barcodes. For singleplex experiments, the Probe Barcode may be omitted.

LHS Probe *for multiplexing*	**Sequence**
CR001*	ACCGTCCA
CR002*	CAACCTGT
CR003*	GTTAGATG
CR004*	TGGTAGAC
CR005	ACTGTGCG
CR006	CGACAAGC
CR007	TTGTCCAT
CR008	AATCAGGC
CR009	CGGAGTCT
CR010	AGCTGAAG
CR011	CAGCTCCA
CR012	TTAGAGGC
CR013	CCATAACT
CR014	TTTCGCGG
CR015	AGACCGCT
CR016	GCTTATTC

Table 2. Unique Probe Barcode sequences for integrating in the LHS probe for CRISPR multiplexing experiments using the GEM-X Flex assay. *The first four Probe Barcodes (CR001-CR004) are base-balanced and are recommended when performing a 4-plex experiment.

Guide RNA Compatibility

The secondary structure formed by the gRNA (hybridized crRNA:tracrRNA) may impact the binding affinity of custom LHS spike-in probes and therefore CRISPR application performance. The preferred scaffold sequence for a CRISPR screening experiment is shown below:

5'-NNNNNNNNNNNNNNNNNNNNGTTTAA-GAGCTAAGCTGGAAACAGCATAGCAAGTTTA-AATAAGGCTAGTCCGTTATCAACTTgaaaAAGT-GGCACCGAGTCGGTGC...-3'

Other scaffolds can work, but may require some degree of optimization. For example, another commonly used guide scaffold has an A>T base substitution (Figure 2), which strengthens the secondary structure of the gRNA and inhibits probe binding. In such cases, the addition of locked nucleic acids (LNAs) to the LHS probe may improve binding affinity to overcome the stronger secondary structure of the gRNA.

Several LHS probes containing LNAs in different positions and longer lengths were tested (see Appendix), one of which was able to significantly improve binding. For this guide scaffold containing the A>T base substitution, the recommended LHS probe sequence is shown in Table 3.

Ordering CRISPR Guide Capture Probes

Custom probes can be ordered from any oligonucleotide synthesis provider. 10x Genomics has tested custom probes in various formats available from IDT, including DNA oligos (standard desalted), Ultramer DNA Oligonucleotides, and oPool Oligo Pools. In limited testing, comparable results were observed with all formats.

Key Guidelines

- Probes should go through standard desalting.
- No HPLC purification is required.
- Probes should be resuspended in IDTE (or low EDTA TE Buffer).
- RHS probes must be 5' phosphorylated.
- LHS barcoded probes should be ordered individually, in standard desalted format.
- RHS probes may be ordered individually in standard desalted format or as an oPool.

Using CRISPR Guide Capture Probes

To use CRISPR guide capture probes, a combined spike-in pool containing the RHS probes, each at a working concentration of 40 nM, and the LHS CRISPR barcoded probe at 400 nM is first prepared. 2.5 µl of this spike-in pool is then added to the sample after adding the 10x Genomics human/mouse WTA probes to the resuspended cell pellet.

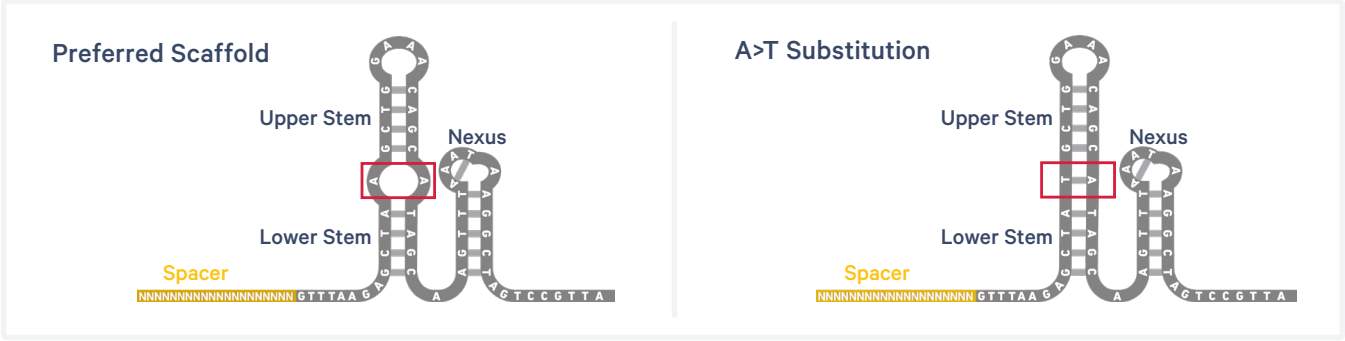


Figure 2. Comparison of guide scaffold secondary structure

CRISPR Guide Capture LHS Probe Design for A>T Base Substitution
LHS Probe Sequence for Guide Scaffold with A>T base substitution
5'-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-XXXXXXXX-CTTGCTATGC+TGT+TT+CC+AGCATAGCTCT-3'
TruSeq R2 Probe Barcode (multiplex) Guide Scaffold

Table 3. Modified CRISPR guide capture LHS probe design for A>T base substitution. The modified LHS probe has a TruSeq R2, a Probe Barcode, and a 28-bp sequence that contains four locked nucleic acids (LNAs) and is the reverse-complement of the guide scaffold. *+ denotes LNA modified oligonucleotide and the base substitution highlighted in red rectangle in Figure 2 is shown here (bold, red).*

Modified Probe Hybridization Mix *Add in order listed*	**10x PN**	**1X (µl)**
Hyb Buffer B	2001312	35
Enhancer	2000482	5
Human WTA Probes OR Mouse WTA Probes	2001259-2001274 2001275-2001290	10
Combined spike-in pool (RHS probes at 40 nM* and LHS CRISPR probe at 400 nM*)	-	2.5
Total		**52.5**

**These are the recommended concentrations based on the guide scaffold sequences tested. If using a different guide scaffold sequence, further increasing the probe concentration may be beneficial to optimize performance (see Appendix).*

Table 4. Modified Probe Hybridization Mix for use in the GEM-X Flex Gene Expression assay.

Probe Pooling & Dilution

a. Standard Desalted or Ultramer LHS

- Resuspend each LHS probe in IDTE (10 mM Tris, 0.1 mM EDTA, pH 8.0) for a stock concentration of **100 µM**.
- Dilute each LHS CRISPR probe to **8 µM**. Store resuspended probes at -20°C.
  Example: Dilute 8 µl of 100 µM LHS CRISPR probe stock in 92 µl IDTE pH 8.0.

The following sections provide guidance for preparing both standard and oPool RHS probes. Choose either b or c and proceed to step d.

b. Standard Desalted or Ultramer RHS

- Resuspend each RHS probe in IDTE (10 mM Tris, 0.1 mM EDTA, pH 8.0) for a stock concentration of **100 µM**. Store resuspended probes at -20°C.
- Create a pool of RHS CRISPR probes, each at **800 nM** per probe.

RHS CRISPR Probe Pool *800 nM/probe (100 µM stock)*	**1X (µl)**
IDTE (pH 8.0)	242
RHS Custom CRISPR Probe 1	2
RHS Custom CRISPR Probe 2	2
RHS Custom CRISPR Probe 3	2
RHS Custom CRISPR Probe 4	2
Total	**250**

Table 5. Example of RHS CRISPR probe pool.

- Proceed to **step d**.

c. oPool RHS Probes

- Resuspend RHS oPool probes in IDTE (10 mM Tris, 0.1 mM EDTA, pH 8.0) for a stock concentration of **800 nM/probe**. Store resuspended probes at -20°C.
  Resuspension volume for oPool is based only on the oPool scale and not on the number of oligos in the oPool. To determine resuspension volume, first convert the oPool scale (provided in pmol/oligo) to nmol per oligo and then calculate the volume of IDTE needed for 800 nM/oligo concentration. Example: Centrifuge oPool tube (50 pmol/oligo scale) briefly, add 62.5 µl IDTE (pH 8.0), and resuspend for a concentration of 800 nM/oligo.
- Proceed to **step d**.

d. Create a spike-in working stock of LHS and RHS CRISPR probes, at **400 nM** LHS and **40 nM** RHS. Create one working stock for each of the LHS Probe Barcodes.

Spike-in Pool Working Stock, CR001	**1X (µl)**
IDTE (pH 8.0)	36
LHS CRISPR, CR001 (8 µM)**	2
RHS CRISPR Pool* (800 nM/probe from Step b or c)	2
Total	**40**

**If adding probes from multiple oPools, add 2 µl from each oPool and reduce volume of nuclease-free water proportionally.*
*** When performing a multiplexing experiment, a stock of RHS probes may be pooled and split across multiple tubes prior to adding the LHS probe.*

Table 6. Example of a spike-in working stock for LHS Probe Barcode CR001.
See Figure 3 for an example of a 4-plex reaction.

e. Add **2.5 µl** from the spike-in working stock to the Modified Probe Hybridization Mix.

f. Store Spike-in Pool Working Stock at -20°C.

Pooling RHS Probes for Multiplexing

- Prepare a spike-in working stock of RHS probes.

RHS Spike-in Pool Master Mix	1X (µl)	4X + 10% (µl)
Nuclease-free water	36	158.4
RHS CRISPR Pool (800 nM/probe from step b/c)	2	8.8
Total	**38**	**167.2**

- Add **38 µl** RHS Spike-in Pool Master Mix to **4** individual tubes, labeled CR001 - CR004. These tubes will become the Spike-in Pool Working Stocks.
- Add **2 µl** LHS CRISPR probe to each corresponding tube. Pipette mix with pipette set to 30 µl. Centrifuge briefly.

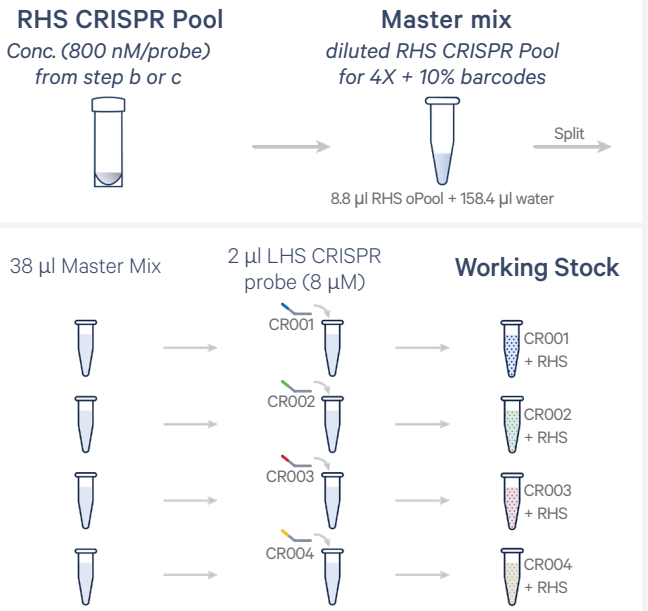


Figure 3. Example: 4-plex reaction for pooling RHS probes.

Sample Prep Considerations

Assay Cell Load

100–200 cells are recommended per targeting sgRNA to ensure enough statistical power to determine the significance of the perturbation. For nontargeting sgRNAs, which are critical for providing a baseline for calculating perturbations, 500–1,000 cells are recommended.

Chromium Chip

Cells recovery per GEM well

When multiplexing with all 16 probes, up to 320,000 cells per well may be recovered using the GEM-X Flex assay.

Perturbations measured using a single well

When loading the maximum number of cells (320,000) in a single well of a GEM-X FX chip, a total of 1,600–3,200 sgRNAs, including nontargeting sgRNAs may be profiled.

Perturbations measured using a single chip

When using all 8 wells in a chip (targeted recovery of ~2.5 x 10^6 cells), a total of 12,000–25,000 sgRNAs may be tested including nontargeting sgRNAs.

CRISPR screens can be scaled further using the 10x Genomics GEM-X Flex n-plex kit. See Appendix for examples.

# of Guides	Min. # of Cells *200 cells/guide*	# of GEM Wells	# of Cells Targeted per GEM Well	# of Cells/ Hybridiz. Rxn	# of Cells into Fixation
1,000	200,000	1	**200,000**	100,000	2 x 10^6
2,500	500,000	2	**250,000**	100,000	2 x 10^6
5,000	1 x 10^6	4	**250,000**	150,000	3 x 10^6
10,000	2 x 10^6	8	**250,000**	250,000	5 x 10^6
12,500	2.5 x 10^6	8	**320,000**	300,000	6 x 10^6
25,000	5 x 10^6	16	**320,000**	600,000*	12 x 10^6

**For 600,000 cells in hybridization, double the volumes of all hybridization reaction components.*

Table 7. CRISPR screening recommendations when using 16 probes.

GEM-X Flex + CRISPR Workflow

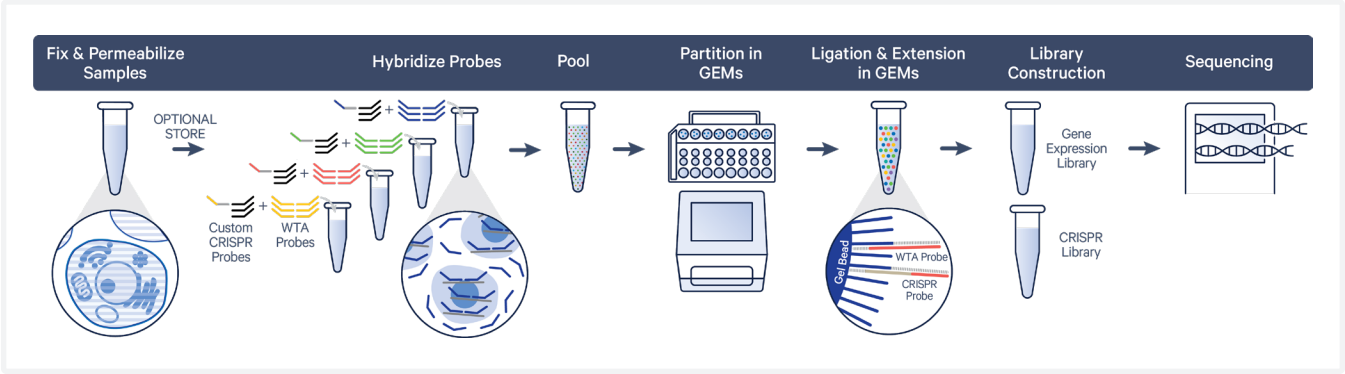


Figure 4. Fixed samples are hybridized with custom CRISPR and WTA barcoded probes, pooled, then partitioned in the Chromium X instrument. Resulting GEMs are transferred and incubated for probe ligation and 10x GEM Barcode addition. Gene expression and CRISPR screening libraries are constructed and then sequenced.

Additional Reagents

To perform CRISPR screening with the GEM-X Flex assay, additional 10x Genomics reagents are required beyond the GEM-X Flex kit. These include:

- Dual Index Kit TT Set A 96 rxns, PN-1000215
- Amp Mix. This can be obtained from Chromium GEM-X Single Cell 5' Feature Barcode Kit* v3, PN-1000703.
  **Primers from this kit are not needed.*
- Optional: Fixed RNA Feature Barcode Multiplexing Kit, PN-1000628, if performing an experiment with protein expression.
- User-supplied TruSeq Primers:
  Fwd primer, TruSeq Read 1: CTACACGACGCTCTTCCGATCT
  Rev primer, TruSeq Read 2: GTGACTGGAGTTCAGACGTGTG
  Primers should be ordered as standard desalted.

Protocol Modifications

To perform CRISPR screening, the following assay steps have been modified and/or added:

- Step 1: Probe Hybridization (*Modified*)
- Step 4: Pre-Amplification PCR (*Modified*)
- New Step: Feature PCR
- New Step: CRISPR Screening Library Construction

The following sections only provide guidance on steps that are updated or new from the original gene expression workflow. See the relevant User Guide for the complete workflow steps as well as best practices for GEM-X Flex assay.

Step 1. Probe Hybridization

Add 2.5 µl of CRISPR guide capture probes (combined spike-in pool containing the RHS CRISPR probes and the LHS barcoded probe) to the Modified Hybridization mix as shown in Table 8.

Modified Probe Hybridization Mix *Add in order listed*	**10x PN**	**1X (µl)**
Hyb Buffer B	2001312	35
Enhancer	2000482	5
Human WTA Probes OR Mouse WTA Probes	2001259-2001274 2001275-2001290	10
Combined spike-in pool (RHS CRISPR probes at 40 nM and LHS barcoded probe at 400 nM)	-	2.5
Total		**52.5**

Table 8. Modified Probe Hybridization Mix.

If performing an experiment with protein expression, the Modified Probe Hybridization Mix would additionally contain 2 µl of Antibody Multiplexing Barcode.

Perform hybridization in a thermal cycler with the following protocol:

Lid Temperature	Reaction Volume	Run Time
42°C	50 µl	Overnight
Step	**Temperature**	**Time** *hh:mm:ss*
1	60°C with -1°C/cycle*	00:05:00
2	Go to Step 1, 17x (Total 18 cycles)	
3	42°C	Hold

**This slow ramp used for Flex+CRISPR involves a gradual temperature decrease to facilitate the binding of probes to the guide RNA scaffold. The samples are hybridized at 60°C for 5 min, followed by decreasing the temperature by 1°C every 5 min until reaching 42°C and then holding at 42°C overnight. This slow ramp is designed to favor the unfolding of the guide scaffold, allowing better probe binding.*

Table 9. Thermal cycler protocol for Probe Hybridization.

After Probe Hybridization, perform the Post-Hybridization Washing (Step 2) and GEM Generation and Barcoding (Step 3) as described in the User Guide.

Step 4. Pre-Amplification PCR

Perform the Pre-Amplification PCR step as indicated in the User Guide with the modified Pre-Amplification PCR Mix outlined below. Note, the modified Pre-Amplification PCR must include a TruSeq Read 2 primer. The choice of primers depends on the Flex workflow being performed.

CRISPR Pre-Amp Mix	PN	1X (µl)
Amp Mix C	2001311	25
Primers *See table below for the appropriate primers*	-	10
Total		**35**

Table 10. Pre-Amplification PCR mix for CRISPR.

Workflow	Primer
Singleplex (CG000786) & Multiplex (CG000787)	Pre-Amp Primers B (PN-2000529) supplemented with a user-supplied TruSeq R2 primer*
Singleplex (CG000788) & Multiplex (CG000789) with Feature Barcode technology for Protein	Pre-Amp Primers C (PN-2000953) supplemented with a user-supplied TruSeq R2 primer*.

**When supplementing the Pre-Amp PCR with a user-supplied TruSeq Read 2 primer, add 2 µl of 50 µM TruSeq Read 2 primer to each pre-amplification reaction.*

Table 11. Primers for Pre-Amplification PCR.

After Pre-amplification PCR, perform DNA Cleanup – SPRIselect as indicated in the User Guide.

New Step. Feature PCR

Following DNA Cleanup – SPRIselect, perform an additional PCR (CRISPR Feature PCR) using TruSeq primers to amplify CRISPR material prior to indexing.

a. Prepare Feature PCR Mix on ice. Vortex and centrifuge briefly.

Feature PCR Mix	PN	1X (µl)
Amp Mix	2000047	50
TruSeq R1 Primer (50 µM)	*User supplied***	2
TruSeq R2 Primer (50 µM)	*User supplied***	2
Nuclease-free Water	-	26
Total		**80**

**User-supplied TruSeq Primer Sequences:

- *Fwd primer, TruSeq Read 1: CTACACGACGCTCTTCCGATCT*
- *Rev primer, TruSeq Read 2: GTGACTGGAGTTCAGACGTGTG*

Table 12. Feature PCR Mix.

b. Transfer **ONLY 20 µl** sample from DNA Cleanup – SPRIselect to a new tube strip.

c. Add **80 µl** Feature PCR Mix to **20 µl** sample. Pipette mix **5x** (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	~25 min
Step	**Temperature**	**Time** *hh:mm:ss*
1	98°C	00:00:45
2	98°C	00:00:20
3	63°C	00:00:30
4	72°C	00:00:20
5	Go to Step 2, 7x (Total 8 cycles)	
6	72°C	00:01:00
7	4°C	Hold

Table 13. Thermal cycler protocol for Feature PCR.

e. Perform 1.8X SPRI cleanup (**180 µl** SPRIselect Reagent) and elute in **101 µl** EB + 0.1% Tween-20 (made by combining 990 µl EB + 10 µl of 10% Tween-20). Transfer **100 µl** to a new tube. See Appendix for detailed SPRI cleanup steps.

New Step. CRISPR Screening Library Construction

Before starting, determine sample index PCR cycle numbers using one of the following methods:

- Using qPCR (Recommended): See Optional: CRISPR Screening Library Cycle Number Determination Using qPCR in Appendix.
- Using Targeted cell recovery: Use the same cycle number used for Gene Expression Library Construction.

a. Prepare Sample Index PCR Mix on ice.

Sample index PCR Mix	PN	1X (µl)
Amp Mix C or Amp Mix	2001311 or 2000047	50
Nuclease-free Water	-	10
Total		**60**

Table 14. Thermal cycler protocol for Sample Index PCR.

b. Transfer **ONLY 20 µl** sample from the cleaned up Feature PCR step to a new tube strip. The remaining sample can be stored at −20°C for up to 4 weeks, for generating additional libraries.

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

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

e. Incubate in a thermal cycler using the same PCR program used for Gene Expression Library Construction (**Step 5.1g of the User Guide CG000786 – CG000789**). Use the cycle number determined at the beginning.

f. Perform a 1.0X SPRI cleanup (**100 µl** SPRIselect Reagent) and elute in **41 µl** EB. Transfer **40 µl** to a new tube. See Appendix for detailed SPRI cleanup steps.

Library Sequencing

- Pooling GEM-X Flex CRISPR Screening libraries with Gene Expression libraries is recommended to maintain nucleotide diversity during sequencing.
- Use the following PhiX input for sequencing GEM-X Flex libraries:
  - 1% PhiX for Singleplex
  - 5% PhiX for Multiplex (use 10% for NovaSeq)
- Minimum recommended sequencing depth is 10,000 read pairs/cells for GEM-X Flex Gene Expression Library and 5,000 read pairs/cell for GEM-X Flex CRISPR Screening Library
- The default sequencing configuration for GEM-X Flex libraries is Read 2 (Table 15). However, in some cases, a modified Read 1 configuration (Table 16) is preferred.

Sequencing Read	Number of Cycles
Read 2 Sequencing Configuration	
Read 1	28 cycles
i7 Index	10 cycles
i5 Index	10 cycles
Read 2	90 cycles*
**Minimum required Read 2 length is 76 bp*	

Table 15. Read 2 sequencing configuration.

Sequencing Read	Number of Cycles
Modified Read 1 Sequencing Configuration	
Read 1	48 cycles
i7 Index	10 cycles
i5 Index	10 cycles
Read 2	See below for recommended cycles
Cases where Modified Read 1 is Needed	**Number of Cycles for Read 2**
Protein expression with mixed PTG and BioLegend antibodies	58
CRISPR with A>T substitution scaffold and protein expression with mixed PTG and BioLegend antibodies	61
Custom probes without a constant sequence to supplement WTA gene expression	58
See the Technical Note Sequencing Metrics & Base Composition of Chromium Flex Libraries (CG000677) for more information.	

Table 16. Read 1 sequencing configuration with modified read 2 cycles.

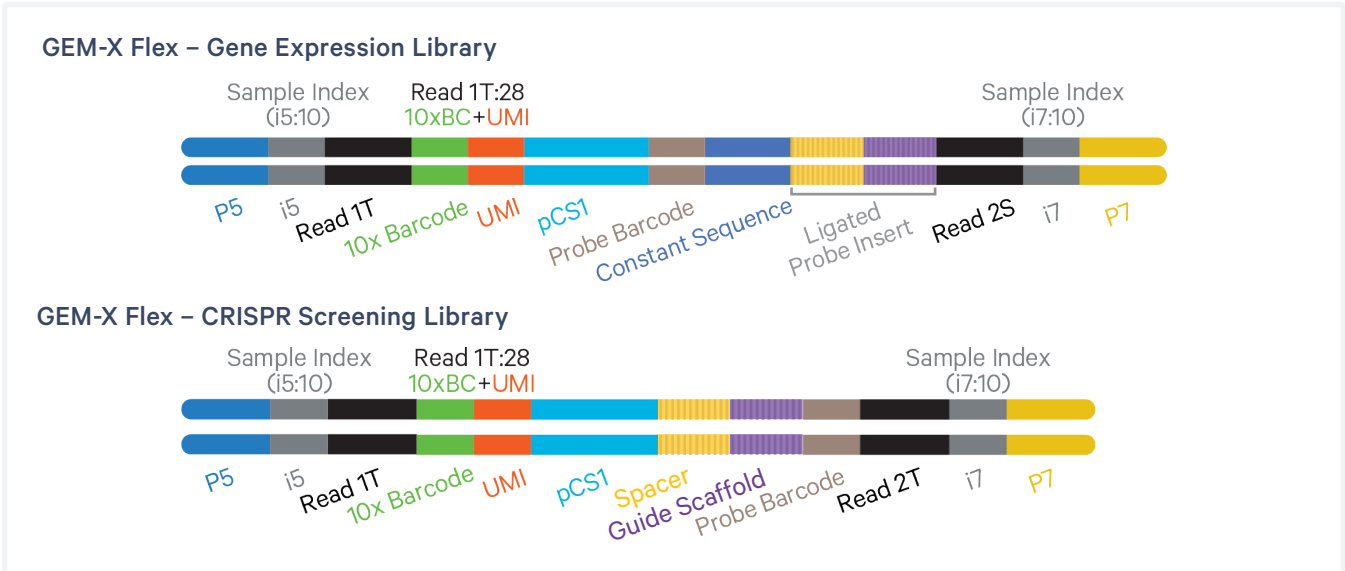


Figure 5. GEM-X Flex Gene Expression and CRISPR Screening libraries.

CRISPR + GEM-X Flex Data Analysis

The analysis of singleplex and multiplex Flex + CRISPR data is supported in Cell Ranger. For analyzing multiplex experiments, the Multi Config CSV needs to specify Probe Barcodes for both WTA and CRISPR, see 10x Genomics Support website for details. For additional information and examples of Multi Config CSVs for Flex+CRISPR experiments, see 10x Genomics Support website. Similar to the current on-market CRISPR workflows, a feature reference file will be needed to identify the different protospacers. Follow the Feature Reference example for 5' CRISPR Guide Capture.

Data Highlight

Experimental Design

A human K562 cell line, stably expressing KRAB-dCas9, was transduced with a pooled lentiviral CRISPRi lncRNA and protein coding library containing ~6,903 sgRNAs (Millipore PN-CRISPRIS10-1KT). The CRISPRi library consisted of 4,045 sgRNAs targeting 280 lncRNAs and 567 protein coding genes plus 177 nontargeting sgRNA controls. The target genes for the remaining 2,681 sgRNAs were unknown and set to "ignore".

To enable a double positive selection strategy, sgRNA vectors included antibiotic resistance to puromycin and fluorescent reporter BFP (Figure 6). Following lentiviral transduction, cells were allowed to recover for 48 hours, then grown in the presence of puromycin for four days. On day 6, analysis by flow cytometry confirmed 95% of the cells expressed BFP. Given the high purity of the CRISPRi cells, the cell sorting step was omitted and cells were directly cryopreserved and stored in liquid nitrogen.

GEM-X Flex: Sample Preparation & Library Construction

Cryopreserved cells were thawed, counted, and fixed for 24 hours at 4°C, following the Demonstrated Protocol Fixation of Cells & Nuclei for GEM-X Flex Gene Expression (CG000782). The Gene Expression and matching CRISPR libraries were generated as described in the GEM-X Flex Gene Expression Reagent Kit for Multiplex Samples (CG000787) with the workflow modifications described in this document. The fixed cells were run as a 16-plex multiplexed experiment, where the sample was evenly split across 16 separate hybridization reactions, each using a unique Probe Barcode (sample sub-pooling).

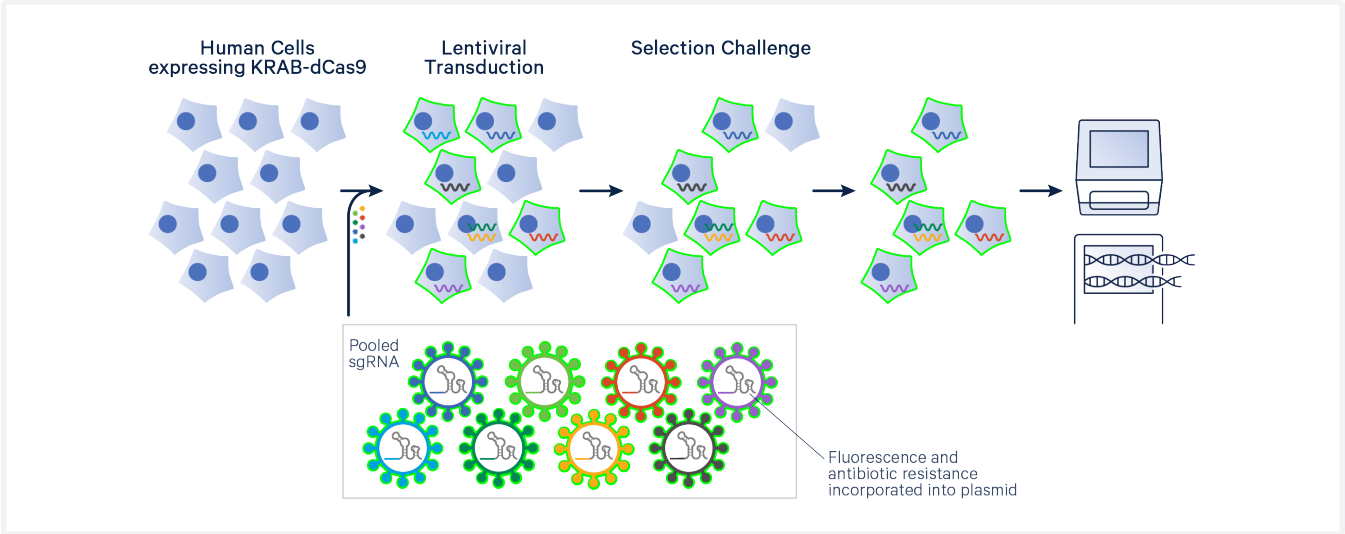


Figure 6. CRISPR experimental design.

To capture the noncoding RNAs targeted by CRISPRi, an additional 2,558 custom probe pairs were added to supplement the WTA probes in the hybridization reaction. Additionally, to capture the CRISPRi sgRNAs, 6,903 custom RHS probes and one of the 16 barcoded LHS probes were added to the hybridization reaction. The 16-plex libraries were pooled and sequenced on an Illumina NovaSeq X at a read depth of 10,000 read pairs/cell for Gene Expression libraries and 5,000 read pairs/cell for CRISPR libraries. A total of 1,233,385 cells were captured across four GEM wells.

GEM-X 5' v3: Sample Preparation & Library Construction

To generate a matching 5' v3 CRISPR Screening dataset, cryopreserved cells were thawed, counted, and loaded onto the Chromium X. Libraries were generated as described in the Chromium GEM-X Single Cell 5' Reagent Kits v3 with Feature Barcode technology for CRISPR Screening User Guide (CG000735). Libraries were pooled and sequenced on an Illumina NovaSeq 6000 to a read depth of approximately 20,000 mean read pairs/cell for gene expression libraries and 5,000 mean read pairs/cell for CRISPR screening libraries. A total of 83,943 cells were captured across four GEM wells.

Data Analysis

Cell Ranger 9.0.0 was used to analyze the data from each GEM well (4 for GEM-X Flex and 4 for GEM-X 5' v3). For each assay, the resulting 4 outputs were then aggregated together using the cellranger aggr pipeline.

See the 10x Genomics support website for the GEM-X Flex and GEM-X 5' v3 datasets.

Results

To assess CRISPR screening assay performance, CRISPR application metrics from the GEM-X Flex assay were compared against GEM-X 5' v3 (Table 17). Similar results were observed across most metrics, notably Fraction Reads with Putative Protospacer Sequence, Guide Reads in Cells, Cells with ≥1 or ≥2 protospacers detected and Median UMIs per Cell at 1,000 Guide Reads Usable per Cell.

CRISPR Application	GEM-X Flex	GEM-X 5' v3
Fraction Reads with Putative Protospacer Sequence	95.70%	96.10%
Fracture Guide Reads	95.20%	81.2%
Fraction Guides Reads Usable	84.90%	76.2%
Mean Guide Reads Usable per Cell	3,855	3,563
Guide Reads in Cells	91.00%	95.9%
Cells with 1 or more protospacers detected	92.30%	84.8%
Cells with 2 or more protospacers detected	31.20%	25.7%
Median UMIs per Cell	551	658
Median UMIs per Cell at 1k Guide Reads Usable per Cell	365	318

Table 17. CRISPR Application metrics comparison of GEM-X Flex and GEM-X 5' v3

Cells containing sgRNAs against the same gene target were combined and imported into Loupe. Figure 7 shows CRISPR-based cell clustering Aggr UMAPs for GEM-X Flex (7A) and GEM-X 5' v3 (7B), split by gene target. Each sgRNA and targeted gene has a higher cell representation in GEM-X Flex made possible with the higher cell recovery per GEM well.

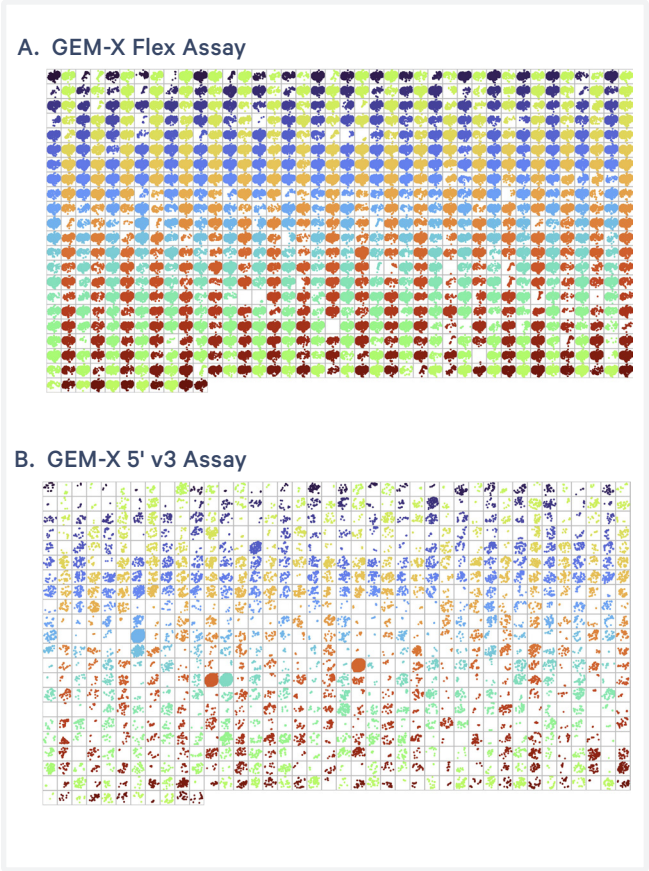


Figure 7. CRISPR-based cell clustering colored and split by gene target on (A) the GEM-X Flex and (B) the GEM-X 5' v3 assay.

Quality Control Data

The distribution of guide RNA assignments across both GEM-X Flex and 5' v3 assays is shown in Figure 8. Both assays had a similar distribution of guide RNA assignments, with 60% of cells containing 1 protospacer, 20% of cells with 2 protospacers, and 10% of cells containing 3 or greater.

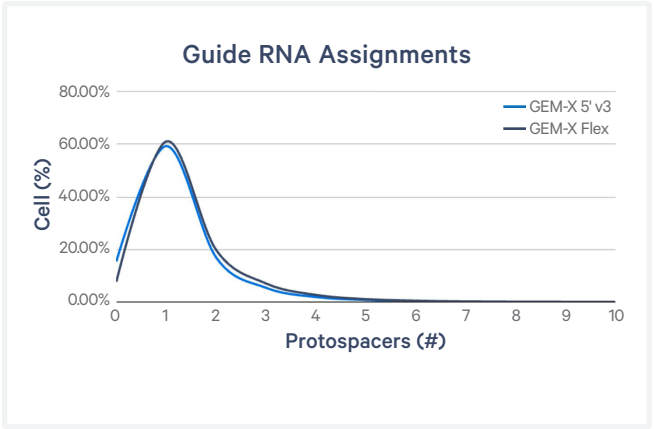


Figure 8. Protospacer assignments in K562 cells transduced with lentiviral pool of 6,093 sgRNAs are similarly distributed between GEM-X Flex and GEM-X 5' v3.

Correlation of Guide Distribution

The percentage of cells with guides for a given target (Figure 9) is well correlated between 5' v3 and Flex, which is expected given that the same sample was used as input upstream of the two assays. The similarity in the observed distribution of cells per target between assays gives confidence that the target identification by Flex CRISPR is accurate. One target, BANP was excluded due to a specificity issue with the probes used in the GEM-X Flex assay.

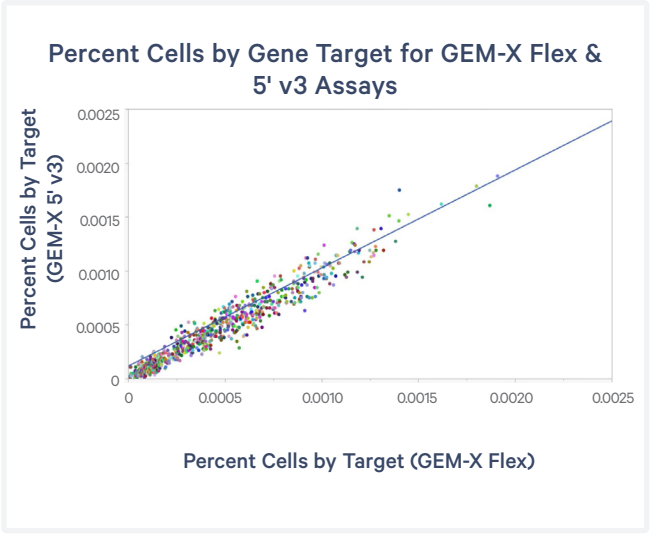


Figure 9. Correlation plot showing cell percentage by gene target between GEM-X Flex and GEM-X 5' v3 (R^2=0.939).

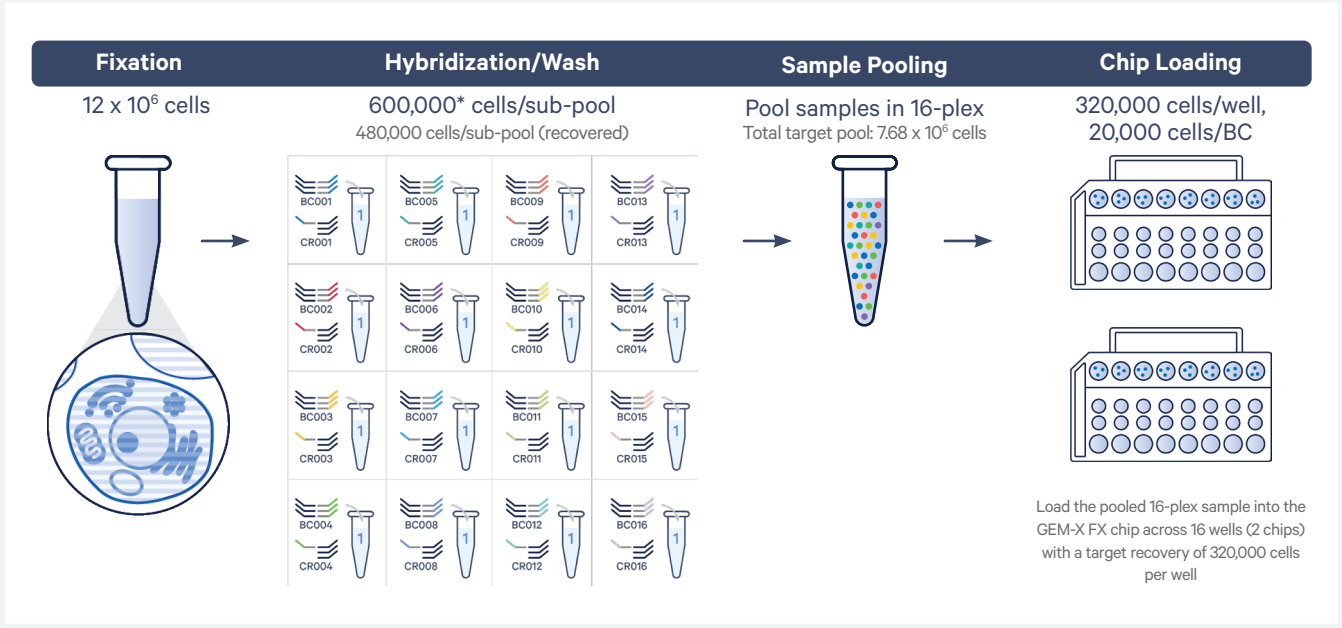
Appendix

Appendix A1. Scaling of CRISPR Screens with GEM-X Flex n-plex Kit

Example 1 – One Sample, 5 x 10^6 Cells

Kits used:

- GEM-X Flex Gene Expression Human/Mouse n-plex, 64 samples PN-1000829/1000831 (1 kit)
- Chromium GEM-X Single Cell 5' Feature Barcode Kit v3, PN-1000703 (1 kit)



A minimum cell concentration of 14,000 cells/µl is required to achieve a targeted cell recovery of 320,000 cells per GEM well.

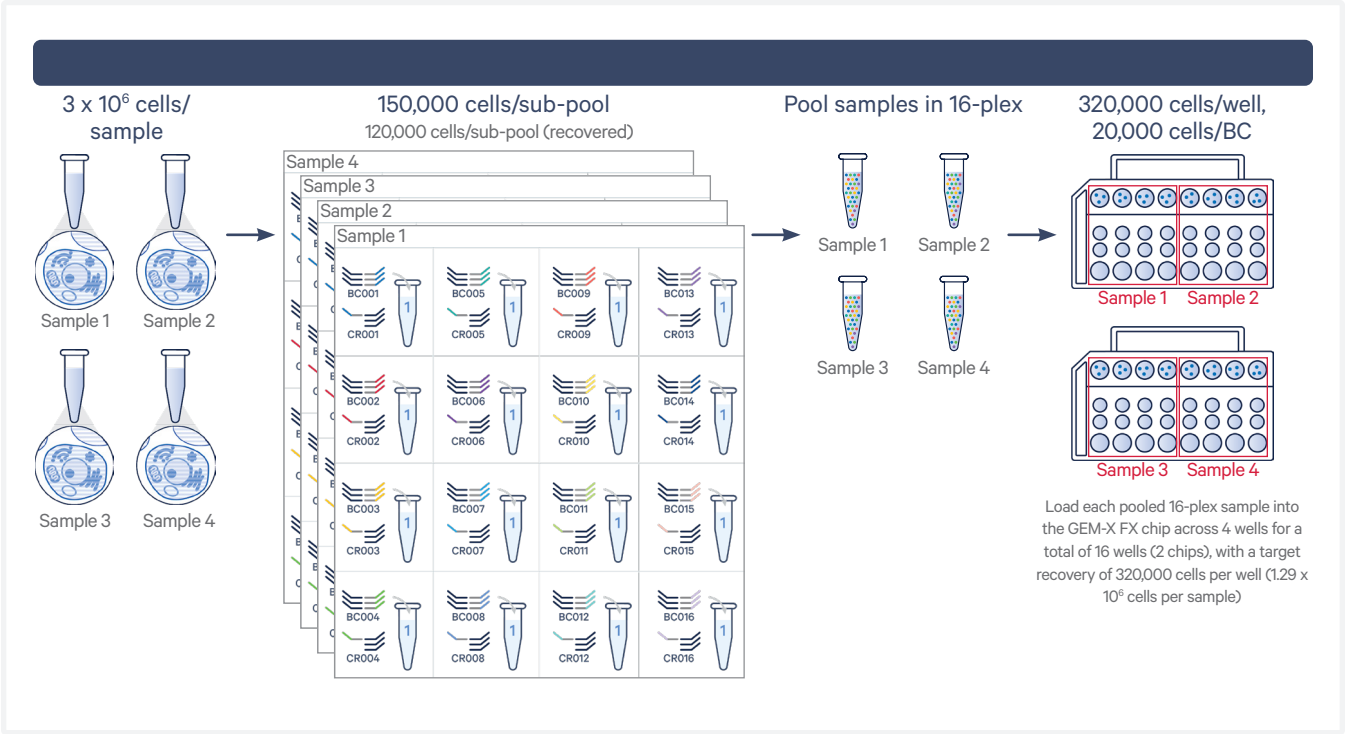
**For 600,000 cells in hybridization, double the volumes of all hybridization reaction components.*

Appendix A1. Scaling of CRISPR Screens with GEM-X Flex n-plex Kit *contd.*

Example 2 – Four Samples, 1.25 x 10^6 cells each, 5 x 10^6 cells total

Kits used:

- GEM-X Flex Gene Expression Human/Mouse n-plex, 64 samples (1 kit) (1000829/ 1000831)
- Chromium GEM-X Single Cell 5' Feature Barcode Kit v3, PN-1000703 (1 kit)



A minimum cell concentration of 14,000 cells/µl is required to achieve a targeted cell recovery of 320,000 cells per GEM lane.

Appendix A2. SPRI cleanup

Follow the instructions below for 1.8X or 1.0X SPRI cleanup steps. ▲ denotes volumes for 1.8X SPRI and ■ denotes volumes for 1.0X SPRI.

a. Vortex to resuspend the SPRIselect reagent.

b. Add **▲180 µl** (1.8X) or **■ 100 µl** (1.0X) SPRIselect Reagent to each sample. Pipette mix 15x.

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

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

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

f. With the tube still in the magnet, add **▲300 µl** or **■ 200 µl** 80% ethanol to the pellet. Wait 30 sec.

g. Remove the ethanol.

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

i. Remove the ethanol.

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

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

l. Remove from the magnet.

m. Add **▲101 µl** Buffer EB + 0.1% Tween-20 or **■41 µl** Buffer EB. Wait **1 min** before resuspending. Pipette mix 15x.

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

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

p. Transfer **▲100 µl** or **■40 µl** to a new tube strip.

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

Appendix A3. Optional: CRISPR Screening Library Cycle Number Determination Using qPCR

a. Prepare 1 µM stock of primers TruSeq R1 and TruSeq R2 by diluting in nuclease-free water.

b. Perform the optional qPCR step for determining library cycle number as indicated in the User Guide, with the modified qPCR mix outlined below.

qPCR Mix *Add in order listed*	**Stock**	**Final**	**1X (µl)**	**2X + 10% (µl)**	**5X + 10% (µl)**
Amp Mix C/Amp Mix 10x PN: 2001311/2000047 *Up to 8 qPCR reactions can be performed using the amount provided in the reagent tube.*	-	-	5.0	11.0	27.5
TruSeq R1 primer	1 µM	0.125 µM	1.25	2.75	6.87
TruSeq R2 primer	1 µM	0.125 µM	1.25	2.75	6.87
EvaGreen, EvaGreenPlus (Biotium, 31000-T or 31077T) *Minimize light exposure*	20X	1X	0.5	1.10	2.75
Nuclease-free Water	-	-	1.0	2.2	5.5
Total			**9.0**	**19.8**	**49.5**

Table 18. qPCR Mix preparation.

Appendix A4. Optimizing Probe Concentrations

The recommended working concentrations of RHS probes and LHS CRISPR barcoded probe provided in this document are based on the guide scaffold sequences tested (see Guide RNA Compatibility). If using a guide scaffold with a different sequence, further increasing the LHS probe concentration may be beneficial to optimize CRISPR application metrics (Figure 10). It is recommended to test a working concentration range of 40 nM - 1200 nM (2 nM - 60 nM final concentrations in hybridization reaction) and evaluate at which concentration the CRISPR median UMIs per cell plateaus (after normalizing the read depth). It may also be possible to increase the RHS probe concentration.

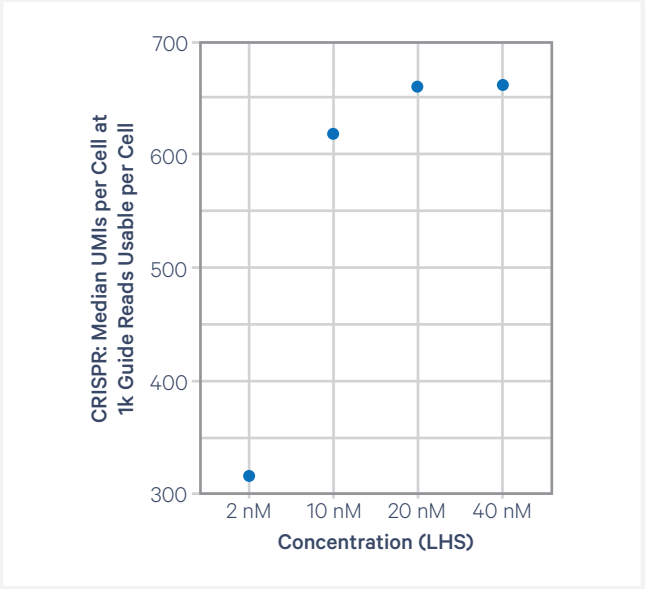


Figure 10. Effect of LHS probe concentration on CRISPR application metrics. A range of final LHS probe concentrations between 2 nM to 40 nM (spike-in working pool concentration range of 40 nM - 800 nM, respectively) was tested while keeping the RHS probe concentration constant at a final concentration of 2 nM (working concentration of 40 nM). The Median UMIs per Cell at 1k Guide Reads Usable per Cell did not improve when more than 20 nM of LHS barcoded probe was used.

Appendix A5. Probe Design Optimization for Difficult Guide Scaffolds

When optimizing the CRISPR Screening + Flex assay on a sample that uses guide scaffolds with an A>T base substitution, higher concentration of a LHS probe without LNA modifications is unlikely to improve CRISPR application metrics relative to 5' v3. Using the modified slow ramp for hybridization showed a very slight improvement in median

Flex + CRISPR						CRISPR: Cells with ≥1 protospacers detected*	CRISPR: Cells with ≥2 protospacers detected*	CRISPR: Median UMIs/Cell at 1000 Guide Reads Usable/Cell*
Probe	**LHS probe, guide targeting sequence** *(LNA base)*	**LHS length (nt)**	**LHS in hyb**	**RHS in hyb**	**Hyb**			
No LNA	GCTATGCTGTTTCCAGC**A**TAGCTCT	25	40 nM	10 nM	42°C	7.8% 11.0%	0.2% 0.7%	*not determined* 1
No LNA	GCTATGCTGTTTCCAGC**A**TAGCTCT	25	40 nM	10 nM	60–42°C ramp	32.3% 34.2%	2.8% 3.2%	3 3
LNA Probe A	CTTGCTATGC**T**GTTT**C**CAGC**A**TAG**C**TCT	28	60 nM	10 nM	60–42°C ramp	88.4% 87.8%	19.8% 20.0%	42.5 41
LNA Probe B	CTTGCTATGC**T**GT**T**T**C**C**A**GC**A**TAGCTCT	28	60 nM	10 nM	60–42°C ramp	94.2% 93.3%	23.5% 23.0%	116 116
5' v3						81.0% 86.0%	20.0% 23.0%	404 402

Table 19. Effect of locked nucleic acid modifications (LNAs) to LHS probes on CRISPR screening application metrics. LHS probes with and without LNA modifications were tested on samples with a guide scaffold with an A>T base substitution, with and without the modified hybridization program and compared to 5' v3 CRISPR screening metrics. *replicate values are shown.

CRISPR UMIs per cell, suggesting that the change in temperature may help to unfold the guide scaffold secondary structure to allow probe binding.

By using the slow ramp, the number of cells with 1 or more protospacers detected improved but was still less than half that detected by 5' v3 (Table 19). Several different LHS probes containing LNAs and longer guide targeting regions (28 bp instead of 25 bp) were tested, and the data for two of these probes are shown. LNA probe B has more than twice the sensitivity in terms of UMIs compared to LNA probe A, despite having the same length and number of LNA modifications. This demonstrates that LNA modifications may improve LHS probe binding to the guide scaffold, and thus sensitivity. While several general guidelines for incorporating LNAs are provided below (as described on Qiagen website, see reference 15), these data also demonstrate that the optimal placement and number of LNA modified bases in the probe sequence must be determined empirically.

Some key guidelines for incorporating LNAs include:

- LNA will bind tightly to other LNA residues. Avoid self-complementarity and cross-hybridization to other LNA-containing oligonucleotides.
- Keep the GC content between 30–60%
- Avoid stretches of more than 4 LNA bases

References

GEM-X Flex User Guides

3. GEM-X Flex Gene Expression Reagent Kits For Singleplexed Samples User Guide (CG000786)
4. GEM-X Flex Gene Expression Reagent Kits For Multiplexed Samples User Guide (CG000787)
5. GEM-X Flex Gene Expression Reagent Kits For Singleplexed Samples with Feature Barcode technology for Protein Expression User Guide (CG000788)
6. GEM-X Flex Gene Expression Reagent Kits For Multiplexed Samples with Feature Barcode technology for Protein Expression User Guide (CG000789)

GEM-X Flex Demonstrated Protocols

7. Fixation of Cells & Nuclei for GEM-X Flex Gene Expression Demonstrated Protocol (CG000782)
8. Tissue Fixation & Dissociation for GEM-X Flex Gene Expression Demonstrated Protocol (CG000783)
9. Sample Preparation from FFPE Tissue Sections for GEM-X Flex Gene Expression Demonstrated Protocol (CG000784)
10. Cell Surface & Intracellular Protein Labeling for GEM-X Flex Gene Expression Demonstrated Protocol (CG000781)

Datasets

11. GEM-X Flex
12. GEM-X 5' v3

Technical Notes

13. Technical Note Custom Probe Design for Visium Spatial Gene Expression and Chromium Single Cell Gene Expression Flex (CG000621)

Others

14. Liu SJ et al. CRISPRi-based genome-scale identification of functional long noncoding RNA loci in human cells. *Science* 355: 6320, 2017
15. QIAGEN Custom LNA Oligonucleotide Design and Applications

Document Revision Summary

Document Number	CG000814
Title	CRISPR Screening with GEM-X Flex Gene Expression
Revision	Rev A
Revision Date	April 2025
Description of Changes	• N/A

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