Custom Probe Design for GEM-X Flex v2

Introduction

GEM-X Flex v2 assay 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 may be designed for use with this assay using the guidance provided in this document. While no impact on assay performance is anticipated, the use of custom probes in these assays 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.

This Technical Note provides guidance for designing and using custom probes, including probe pooling and dilution for GEM-X Flex v2 assay. Additional optimization may be required. Performing a pilot experiment with these unsupported workflow modifications is recommended prior to larger studies.

Probe Design for GEM-X Flex v2

The following guidance outlines how to design custom probes for use with the GEM-X Flex v2 assay. Custom probes designed for GEM-X Flex v2 are not compatible with earlier versions of the Flex assay.

10x Genomics probe panels consist of three probe pairs for most target mRNAs. Each probe contains appropriate handle sequences and a 25 bp sequence that is the reverse complement of the target mRNA. Each probe is referred to as the left-hand side (LHS) or right-hand side (RHS) probe. An overview of 10x Genomics probe configuration for GEM-X Flex v2 assay is provided in Figure 1 along with the accompanying probe sequences in Table 1. Example probes to detect common fluorescent proteins (EGFP, RFP) are provided in Appendix B.

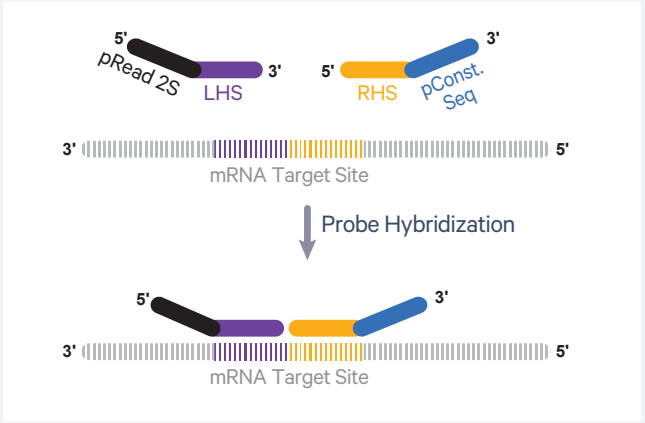


Figure 1. Probe design for GEM-X Flex v2. The left-hand side (LHS) probe contains a partial Read 2S (pRead 2S) as well as a 25 base pair sequence that is reverse complement of the target site. The right-hand side (RHS) probe contains a phosphate on the 5' base for ligation, sequences reverse complementary to the target, and a partial constant sequence.

When designing custom probes, consider the following:

- GC content should be between 44 – 72% for each 25 bp probe half.
- Avoid homopolymer repeats.
- Avoid overlap with annotated repeat or low complexity sequences.
- If possible, design probes for coding regions of mRNA as opposed to untranslated regions.
- It is recommended to have a TN ligation junction, where T is the 25th nucleotide of the probe (3' most nucleotide of the LHS probe). In this case, the opposing nucleotide in the target RNA would be an A. The 26th nucleotide (5' most base of the RHS probe) can be any base (N).
- While preferred, the TN motif is not always strictly required, and other nucleotide motifs can also function effectively. However, for a simplified and robust design process, 10x Genomics recommends using the T at the specified position.
- Avoid common single nucleotide polymorphisms (SNPs) and potential mismatches at the ligation junction. See the UCSC Genome Browser and the Single Nucleotide Polymorphism Database (dbSNP). If avoiding SNPs is not possible, SNPs and mismatches should be at least four bp away from the ligation junction.
- If probes can bind to sequences other than the target mRNA sequence, an off-target signal may be observed. To check for off-target homology, align the probe sequence to the reference transcriptome using the Basic Local Alignment Search Tool (BLAST). Matches to off-target genes should have at least five mismatches in at least one of the LHS or RHS probes to prevent efficient hybridization.
- Designing three probe pairs per target mRNA is recommended, especially for low-expressing genes. However, if the gene is not long enough or there are not enough specific 50 bp regions, fewer than three probe pairs is acceptable.
- Custom probe pairs should not overlap with each other or with WTA probes to avoid competition between probes for the same binding site in the target RNA.
- If running a singleplex Flex v2 experiment with GEM-X Flex v2 Human/Mouse 4 Samples Kit (PN-1000926/ 1000930), the RHS probe should have a partial Capture Sequence (pCS1) instead of partial Constant Seq. See 10x Genomics support site for sequencing recommendations in such cases.

GEM-X Flex v2 Custom Probe Sequence	
Singleplex & Multiplex Probes	
LHS Probe	**RHS Probe**
5'-CCTTGGCACCCGAGAATTCCA-target_LHS-3'	/5Phos/-target_RHS-CCCATATAAGAAA-3' partial Constant Sequence
Singleplex Probes using the GEM-X Flex v2 Human/Mouse 4 Samples Kit (PN-1000926/ 1000930)	
LHS Probe	**RHS Probe**
5'-CCTTGGCACCCGAGAATTCCA-target_LHS-3'	/5Phos/-target_RHS-CGGTCCTAGCAA-3' partial Capture Sequence 1 (pCS1)

Table 1. GEM-X Flex v2 probe configuration with sequences. Each probe in the probe pair represents 25 bp sequences that are reverse complement of the target transcript.

Ordering Custom 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 Guidance

- Probes should go through standard desalting.
- No HPLC purification is required.
- Probes should be resuspended in IDTE pH 8.0 (or low EDTA TE Buffer).
- RHS probes must be 5' phosphorylated.
- Ordering custom probes as an oPool at 50 pmol scale is preferred. This simplifies probe pooling and dilution upstream of probe hybridization. It is recommended to use 0.1 pmol (0.11 pmol to account for the 10% overage in Modified Probe Hyb Mix) of each custom probe per hybridization reaction.
- The table provides the number of reactions supported based on oPool scale:

oPool synthesis scale *(pmol per probe)*	**# of hyb reactions** *(probes added to Modified Probe Hybridization Mix)*	**# of hyb reactions** *(probes added to sample)*
1 (not recommended)	9	10
10	90	100
50 (preferred)	454	500

Table 2. Number of reactions supported based on oPool scale.

- LHS and RHS probes can be combined in the same oPool or ordered as separate pools.

Using Custom Probes

To use custom probes, prepare a spike-in pool containing 40 nM of each probe in IDTE, pH 8.0. For example, a spike-in pool with 9 probe pairs would contain 40 nM of each of the 9 LHS probes and 9 RHS probes (720 nM total probe).

Recommended dilution of custom probes upstream of probe hybridization depends on ordering format. Example dilutions for oPools, standard desalted, and Ultramer custom probes are provided in Appendix A.

Hybridization Set up

Follow step a if all samples will use the same custom probes. Alternatively, follow step b if custom probes will vary between samples.

a. Prepare a Modified Probe Hyb Mix by adding 2.75 µl of the custom probe spike-in pool (LHS + RHS probes combined) per sample to the Probe Hyb mix (Table 3). Add 52.5 µl of the Modified Probe Hyb Mix to each sample tube/well.

Modified Probe Hyb Mix *Add in order listed*	**10x PN**	**1X (µl) + 10% overage**
Hyb Buffer B	2000485	38.5
Enhancer	2000482	5.5
Additive A	220093	2.75
Human WTA Probes OR Mouse WTA Probes	2001259/2001492/ 2001490 2001275/2001493/ 2001491	8.25
Custom Probes, each probe at 40 nM (LHS and RHS combined)	-	2.75
Total		**57.5**

Table 3. Modified Probe Hyb Mix for the GEM-X Flex v2 assay.

OR

b. If different custom probes will be used in each sample, prepare the Probe Hyb Mix as described in the User Guide, then add 2.5 µl of the custom probe spike-in pool to the respective sample.

Analysis

The use of custom probes requires the following file modifications for successful Cell Ranger (v10 or later) analysis:

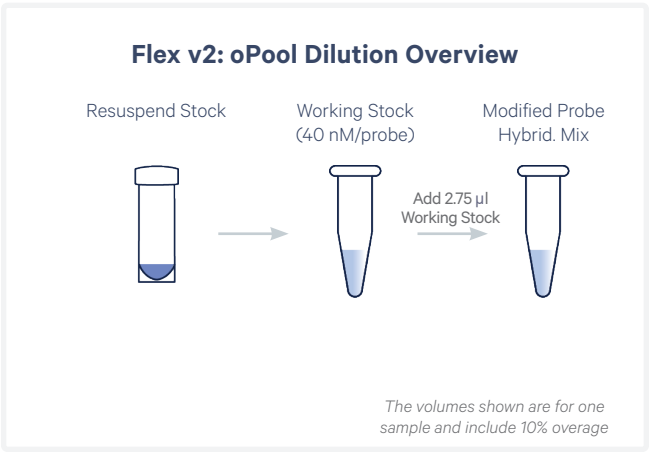
Probe Set Reference CSV

- Create a Probe Set Reference CSV file for just the custom probes. A description of the necessary columns can be found on the 10x Genomics website.
- Set the Metadata field #panel_type=custom
- If new genes are added and a new genome reference is created using the mkref pipeline, the #reference_genome and the #reference_ version in the header of the new probe set CSV file should be modified to match the name and version of the genome reference used for analysis.
- Note that starting with Cell Ranger v9.0, providing a transcriptome reference is optional when analyzing Flex Gene Expression and Antibody Capture libraries.
- When running Cell Ranger, include rows for both the Chromium Transcriptome Probe Set and the newly made Custom Probe Set in the multi config CSV file. An example can be found on the 10x Genomics website.

Appendix A: GEM-X Flex v2 Custom Probe Pooling & Dilution

Custom probe pooling and dilution, upstream of the probe hybridization step, is dependent on the format in which the custom probe is acquired and the number of probes being pooled. Example dilutions for oPools, standard desalted, and Ultramer custom probes are provided here.

See Hybridization Set up for guidance on preparing the Probe Hyb Mix. A general overview is provided below.



oPool Oligo Pools

For combining fewer than 20 oPools (50 pmol scale) or 4 oPools (10 pmol scale) in a single spike-in pool, follow the steps described below. If combining more than these numbers of oPools, contact support@10xgenomics.com. While the upper limit for the number of oligo sequences with oPools has not been determined, successful tests have been conducted with spike-in pools containing over 7,000 sequences.

Resuspend stock: Resuspend 50 pmol or 10 pmol oPool Oligos in 62.5 µl IDTE (10 mM Tris, 0.1 mM EDTA, pH 8.0). If using 1 pmol scale (not recommended), resuspend in 25 µl and proceed to step d. Store resuspended oligos at -20°C.
Example: Centrifuge oPool tube (50 pmol/oligo scale) briefly, add 62.5 µl IDTE (pH 8.0), and shake for 1–2 h or allow content to resuspend overnight at 4°C.

a. Prepare working stock: Using the resuspended stock, prepare the spike-in pool working stock containing 40 nM each of the LHS and RHS probes. Scale up volumes proportionally depending on the number of hybridizations needed.

If the spike-in pool contains fewer than 20 probes (i.e. 10 LHS & 10 RHS probes), the working stock (40 nM/probe) should be prepared fresh before use and any remaining solution should be discarded. If the spike-in pool contains more than 20 probes, the working stock (40 nM/probe) may be prepared ahead of time and stored at -20°C.

Spike-in Pool Working Stock *(16 hybridizations)*	**Volume (µl)** *(50 pmol scale)*	**Volume (µl)** *(10 pmol scale)*
IDTE, pH 8.0	41.8	33
Resuspended Stock*	2.2	11
Total	**44**	**44**

**If adding multiple resuspended oPool stocks, add volume indicated from each oPool stock and reduce the volume of IDTE pH 8.0 proportionally.*

b. Pipette mix 15x (pipette set to 30 µl), centrifuge briefly.

c. Proceed to Hybridization Set up as described on Page 3.

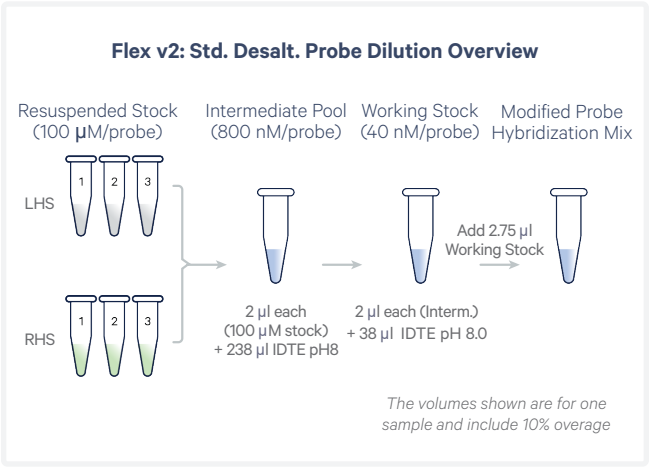
Standard Desalted or Ultramer

When ordering probes synthesized as individual oligos in tubes or plates, the recommended dilution scheme depends on the number of total custom probes being used in the experiment.

For ≤120 Total Custom Probes (≤60 LHS probes + ≤60 RHS probes)

a. Resuspend stock: Resuspend each oligo in IDTE (10 mM Tris, 0.1 mM EDTA, pH 8.0) for a stock concentration of 100 µM. Store resuspended stock at -20°C.

b. Prepare intermediate pool: Using the resuspended stock, prepare the intermediate pool by combining 2 µl from each 100 µM resuspended probe in IDTE (10 mM Tris, 0.1 mM EDTA, pH 8.0) for a total volume of 250 µl.



Example: *Intermediate pool for 6 custom probes*

Intermediate Pool (BC001) *800 nM/probe (100 µM stock)*	**Volume (µl)**
IDTE (pH 8.0)	238
LHS Custom Probe 1	2
LHS Custom Probe 2	2
LHS Custom Probe 3	2
RHS Custom Probe 1	2
RHS Custom Probe 2	2
RHS Custom Probe 3	2
Total	**250**

c. Vortex **30 sec**, centrifuge briefly. The intermediate stock may be stored at -20°C.

d. Prepare working stock: Using the intermediate pool, prepare a spike-in pool working stock.

Spike-in Pool Working Stock *(16 hybridizations)*	**Volume (µl)**
IDTE pH 8.0	41.8
Intermediate Pool* (800 nM/probe)	2.2
Total	**44**

**If adding multiple resuspended Intermediate Pool stocks, add 2 µl from each Intermediate Pool stock and reduce the volume of IDTE pH 8.0 proportionally.*

e. Pipette mix 15x (pipette set to 30 µl), centrifuge briefly.

f. Proceed to Hybridization Set up as described on Page 3.

For 120-2,500 Total Custom Probes (≤1,250 LHS probes + ≤1,250 RHS probes)

a. Resuspend stock:
Resuspend each oligo in IDTE (10 mM Tris, 0.1 mM EDTA, pH 8.0) for a stock concentration of 100 µM. Store resuspended stock at -20°C.

b. Prepare working stock:
Using the resuspended stock, prepare a spike-in pool working stock for a total volume of 5,000 µl.

Example: *pool containing a total of 300 custom probes or 150 custom probe pairs*

Spike-in Pool Working Stock (100 µM stock)	**Volume (µl)**
IDTE pH 8.0	4,400
LHS Custom Probes (1-150)	300 (2 µl each x 150)
RHS Custom Probes (1-150)	300 (2 µl each x 150)
Total	**5,000**

c. Vortex **30 sec**, centrifuge briefly.

d. Proceed to Hybridization Set up as described on Page 3.

For >2500 Total Custom Probes

Contact support@10xgenomics.com

Appendix B: Probe Design

The following sequences are example custom probe pairs designed to detect EGFP (Enhanced green fluorescent protein) and mRFP (monomeric red fluorescent protein, referred to as RFP in this document) reporter genes. The probes were designed based on reference sequences from Addgene (EGFP, RFP).

The target sequence included in the custom probes is a reverse complement of the common EGFP and RFP sequences. The LHS probes are listed in Table 4 and the RHS probes are listed in Table 5. If using the listed sequences, confirm that the EGFP or RFP constructs used in the experiment include the binding site for these probes.

Custom LHS Probes for GEM-X Flex v2 Assay	
Probe Configuration	**5'-CCTTGGCACCCGAGAATTCCA-target_LHS-3'**
EGFP-LHS-1	5'-CCTTGGCACCCGAGAATTCCAggtagtggtcggcgagctgcacgct-3'
EGFP-LHS-2	5'-CCTTGGCACCCGAGAATTCCAagggtgtcgccctcgaacttcacct-3'
EGFP-LHS-3	5'-CCTTGGCACCCGAGAATTCCAatggtgcgctcctggacgtagcctt-3'
RFP-LHS-1	5'-CCTTGGCACCCGAGAATTCCAtcggtcttgtaggcgccgggcagct-3'
RFP-LHS-2	5'-CCTTGGCACCCGAGAATTCCAaagttggtgccgcgcagcttcacct-3'
RFP-LHS-3	5'-CCTTGGCACCCGAGAATTCCAcctcgatctcgaactcgtggccgtt-3'

Table 4. LHS probe sequences for detection of EGFP and RFP reporter genes.

Custom RHS Probes for GEM-X Flex v2 Assay*	
Probe Configuration	**/5Phos/-target_RHS-CCCATATAAGAAA-3'**
EGFP-RHS-1	/5Phos/gccgtcctcgatgttgtggcggatcCCCATATAAGAAA-3'
EGFP-RHS-2	/5Phos/cggcgcgggtcttgtagttgccgtcCCCATATAAGAAA-3'
EGFP-RHS-3	/5Phos/cgggcatggcggacttgaagaagtcCCCATATAAGAAA-3'
RFP-RHS-1	/5Phos/gcacgggcttcttggccatgtaggtCCCATATAAGAAA-3'
RFP-RHS-2	/5Phos/tgtagatgaactcgccgtcctgcagCCCATATAAGAAA-3'
RFP-RHS-3	/5Phos/cacggagccctccatgcgcaccttgCCCATATAAGAAA-3'

Table 5. RHS probe sequences for detection of EGFP and RFP reporter genes.

**These RHS probes containing a partial Constant Sequence are for a multiplex experiment. For singleplex experiments using the GEM-X Flex v2 Human/Mouse 4 Samples Kit (PN-1000926/ 1000930), each of the RHS probes must be designed and synthesized with a partial Capture Sequence 1 (sequence listed in Table 1) as described in the Probe Design section.*

Conclusion

This Technical Note provides guidance on the design and use of custom probes with the GEM-X Flex v2 assay. While no impact on assay performance is anticipated, the use of custom probes in this assay has not been tested extensively and is not supported by 10x Genomics. Performing a pilot experiment with these unsupported workflow modifications is recommended prior to committing to larger studies.

References

1. GEM-X Flex v2 User Guide (CG000834)
2. GEM-X Flex v2 with Feature Barcode technology for Protein Expression User Guide (CG000835)
3. GEM-X Flex v2 for Singleplexed Samples User Guide (CG000841)

Document Revision Summary

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