Custom Probe Design for Visium Spatial Gene Expression and Chromium Single Cell Gene Expression Flex

Introduction

10x Genomics Visium Spatial Gene Expression technology provides spatial transcriptomic insights by analyzing mRNA in tissue sections derived from fixed tissue samples. Chromium Single Cell Gene Expression Flex offers comprehensive, scalable solutions to measure gene expression in fixed samples.

For these assays, 10x Genomics provides a pre-designed whole transcriptome panel of probes for target hybridization. Custom probes may be designed for use with either 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 Visium Spatial Gene Expression for FFPE, Visium CytAssist-enabled applications, and Chromium Single Cell Gene Expression Flex singleplexed and multiplexed experiments. The document also includes data highlighting custom probe specificity. Additional optimization may be required. Performing a pilot experiment with these unsupported workflow modifications is recommended prior to larger studies.

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Figure 1. Probe design for the Visium Spatial Gene Expression assay. The left-hand side (LHS) probe contains a partial Read 2S as well as a 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 poly(A) tail.

The following guidance applies to the Visium Spatial Gene Expression for FFPE (Visium v1), Visium CytAssist Spatial Gene Expression (Visium v2), and Visium HD Spatial Gene Expression assays.

An overview of the 10x Genomics probe configuration for Visium Spatial Gene Expression is provided in Figure 1. The sequences for these probe designs are provided in Table 1. Probe sets used are described in Table 2.

Species	Assay	Probe Set Version
Mouse	Visium v1, v2	Visium Mouse Transcriptome Kit v1
	Visium HD	Visium Mouse Transcriptome Kit v2
Human	Visium v1	Visium Human Transcriptome Kit v1
	Visium v2, HD	Visium Human Transcriptome Kit v2

Table 2. Probe panels used for Visium assays.

Visium Mouse and Human Transcriptome Kit v2 contain panels with three pairs of probes for each target mRNA, with each probe containing 25 bp sequence that is reverse complement of the target mRNA sequence. Each probe is referred to as the left-hand side (LHS) or right-hand side (RHS) probe. Visium Mouse and Human Transcriptome Kit v1 contain panels with one pair of probes for each target mRNA.

When designing custom probes for Visium, 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.

Visium Spatial Gene Expression Probe Sequence	
LHS Probe	**RHS Probe**
5'-CCTTGGCACCCGAGAATTCCA-target_LHS-3'	/5Phos/-target_RHS-AAAAAAAAAAAAAAAAAAAAAAAAAAAAAA-3'

Table 1. Probe sequences for Visium Spatial Gene Expression probe-based applications. The LHS and RHS probes include target_LHS and target_RHS parts respectively that each contains unique 25 bp sequences homologous to the target transcript. The target sequence of RHS probes is followed with a series of thirty adenines.

- The 25th nucleotide of the probe (3' most nucleotide of the LHS probe) must be a T. The opposing nucleotide in the target RNA must be an A.
- Avoid common single nucleotide polymorphisms (SNPs) and potential mismatches at the ligation junction. Refer to 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.
- Probe pairs should not overlap to avoid competition between probes for the same binding site in the target RNA.
- Add new probe sequences to the probe set reference CSV file. Refer to the Analysis section for more information.

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 Guidelines

- Probes should go through standard desalting.
- No HPLC purification is required.
- Probes should be supplied in IDTE (or low EDTA TE Buffer).
- RHS probes must be 5' phosphorylated.
- Ordering the custom probes as an oPool is preferred as it simplifies probe pooling and dilution upstream of the Probe Hybridization step (see Appendix A).
- 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 combined spike-in pool (LHS and RHS) containing 24 nM of each probe in nuclease-free water. For example, a spike-in pool with 9 probe pairs would contain 24 nM of each of the 9 LHS and 9 RHS probes (432 nM total probe).

Add 10 µl of the spike-in pool (LHS and RHS combined) to FFPE Hyb Buffer and the 10x Genomics probes, as shown in Table 3-4, to generate the Modified Probe Hybridization Mix. The Modified Probe Hybridization Mix will replace the original Probe Hybridization Mix in the Probe Hybridization step of Visium Spatial Gene Expression for FFPE Reagent Kits User Guide (CG000407), Visium CytAssist Spatial Gene Expression for FFPE Reagent Kits User Guide (CG000495), or Visium HD Spatial Gene Expression User Guide (CG000685).

6.5 mm Gaskets (Visium v2 and Visium HD) OR Visium v1			
Modified Probe Hybridization Mix *Add in order listed*	**10x PN**	**1X (µl)**	**2X + 10% (µl)**
FFPE Hyb Buffer	2000423	70	154
RHS Probes		10	22
Human WT OR Mouse WT	2000657/ 2000449 2000455		
LHS Probes		10	22
Human WT OR Mouse WT	2000658/ 2000450 2000456		
Custom Probes, each probe at 24 nM (LHS and RHS combined)	-	10	22
Total		**100**	**220**

Table 3. Modified Probe Hybridization Mix for Visium v1 or Visium v2/HD (6.5 mm gaskets).

11 mm Gaskets (Visium v2)			
Modified Probe Hybridization Mix *Add in order listed*	**10x PN**	**1X (µl)**	**2X + 10% (µl)**
FFPE Hyb Buffer	2000423	140	308
RHS Probes		20	44
Human WT OR Mouse WT	2000657/ 2000449 2000455		
LHS Probes		120	44
Human WT OR Mouse WT	2000658/ 2000450 2000456		
Custom Probes, each probe at 24 nM (LHS and RHS combined)	-	20	44
Total		**200**	**440**

Table 4. Modified Probe Hybridization Mix for Visium v2 (11 mm gaskets).

Analysis

The use of custom probes requires the following file modifications for successful Space Ranger analysis:

Genome Reference

- The following steps for modifying genome reference are required if the custom probes are targeting genes that are not already in the prebuilt reference. Update the gene annotation file (GTF) with new targets using a text editor.
  - Follow the existing format of Space Ranger GTF.
  - Ensure the gene name is unique.
- Modify the genome reference in FASTA format that contains additional contigs for new targets.
- Generate a new reference using Space Ranger mkref, which uses the modified GTF and FASTA files. This build will be used for data analysis using Space Ranger.
  - Name the new reference and new probe CSV files so that they can be distinguished from the default files.
  - For more information, consult "Creating a Reference Package with spaceranger mkref" page in the Spatial Gene Expression section on the 10x Genomics Support website.

Probe Set Reference CSV

- Find the appropriate probe set CSV on the Descriptions of Probe Set Reference CSV and Supporting Files page in the Spatial Gene Expression for FFPE section of the 10x Genomics Support website.
- Update the appropriate probe set CSV by appending the new custom probe information, depending on the probe set:
- **Human or Mouse WT Probes v1:**
  - 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.

- gene_id: the ID of the mRNA target (any identifier)
- probe_seq: combined LHS and RHS sequence trimmed to not include any adaptor, R2, or poly(A) sequences. Target mRNA sequence only.
- probe_id: pipe-separated gene_id|gene_name|7 character hash (any combination of letters and numbers)
- included: TRUE (will include in Space Ranger analysis)

- **Human or Mouse Probes v2 (same as above, with one additional edit)**
  - region: spliced or unspliced
    - spliced: the combined LHS and RHS sequence spans a splice junction
    - unspliced: the combined LHS and RHS sequence does not span a splice junction. For example, the sequence sits entirely within a single exon of the target gene.

Data Highlight

Custom probe pairs were designed to detect EGFP (Enhanced green fluorescent protein) and mRFP (monomeric red fluorescent protein, referred to as RFP in this document) reporter genes in samples processed as per the Visium v2 (CG000495) workflow. The probes were designed based on reference sequences from Addgene (EGFP, https://www.addgene.org/13031/; RFP, https://www.addgene.org/13032/).

Probe Design

The target sequence included in the custom probes is a reverse complement of the common EGFP and RFP sequences being detected in this experiment. The LHS probes are listed in Table 5 and the RHS probes are listed in Table 6. If using the listed sequences, confirm that the EGFP or RFP constructs used in an experiment include the binding site for these probes.

Custom LHS Probes for Visium Spatial Gene Expression Assay	
Probe Configuration	**5'-CCTTGGCACCCGAGAATTCCA-target_LHS-3'**
EGFP-LHS-1	CCTTGGCACCCGAGAATTCCAggtagtggtcggcgagctgcacgct
EGFP-LHS-2	CCTTGGCACCCGAGAATTCCAagggtgtcgccctcgaacttcacct
EGFP-LHS-3	CCTTGGCACCCGAGAATTCCAatggtgcgctcctggacgtagcctt
RFP-LHS-1	CCTTGGCACCCGAGAATTCCAtcggtcttgtaggcgccgggcagct
RFP-LHS-2	CCTTGGCACCCGAGAATTCCAaagttggtgccgcgcagcttcacct
RFP-LHS-3	CCTTGGCACCCGAGAATTCCAcctcgatctcgaactcgtggccgtt

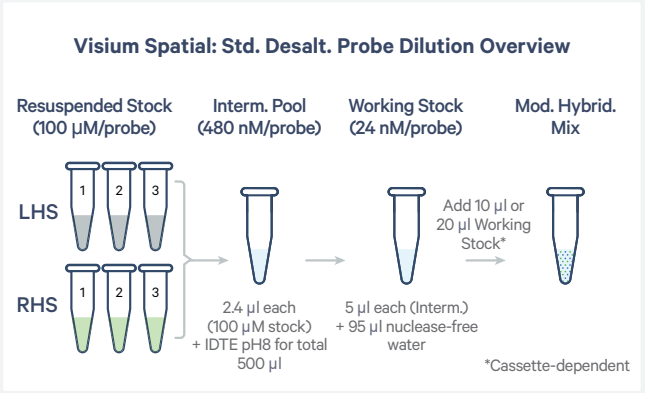
Table 5. LHS probe sequences for detection of EGFP and RFP reporter genes.

Custom RHS Probes for Visium Spatial Gene Expression Assay	
Probe Configuration	**/5Phos/-target_RHS-AAAAAAAAAAAAAAAAAAAAAAAAAAAAAA-3'**
EGFP-RHS-1	/5Phos/gccgtcctcgatgttgtggcggatc-AAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
EGFP-RHS-2	/5Phos/cggcgcgggtcttgtagttgccgtc-AAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
EGFP-RHS-3	/5Phos/cgggcatggcggacttgaagaagtc-AAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
RFP-RHS-1	/5Phos/gcacgggcttcttggccatgtaggt-AAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
RFP-RHS-2	/5Phos/tgtagatgaactcgccgtcctgcag-AAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
RFP-RHS-3	/5Phos/cacggagccctccatgcgcaccttg-AAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

Table 6. RHS probe sequences for detection of EGFP and RFP reporter genes.

Probe Dilutions

Custom probes used in this study were ordered as standard desalted probes (100 µM stock) that were diluted as illustrated in the overview (see Appendix A for additional guidance, including dilution of oPool Oligo Pools).



Experiment Overview & Results

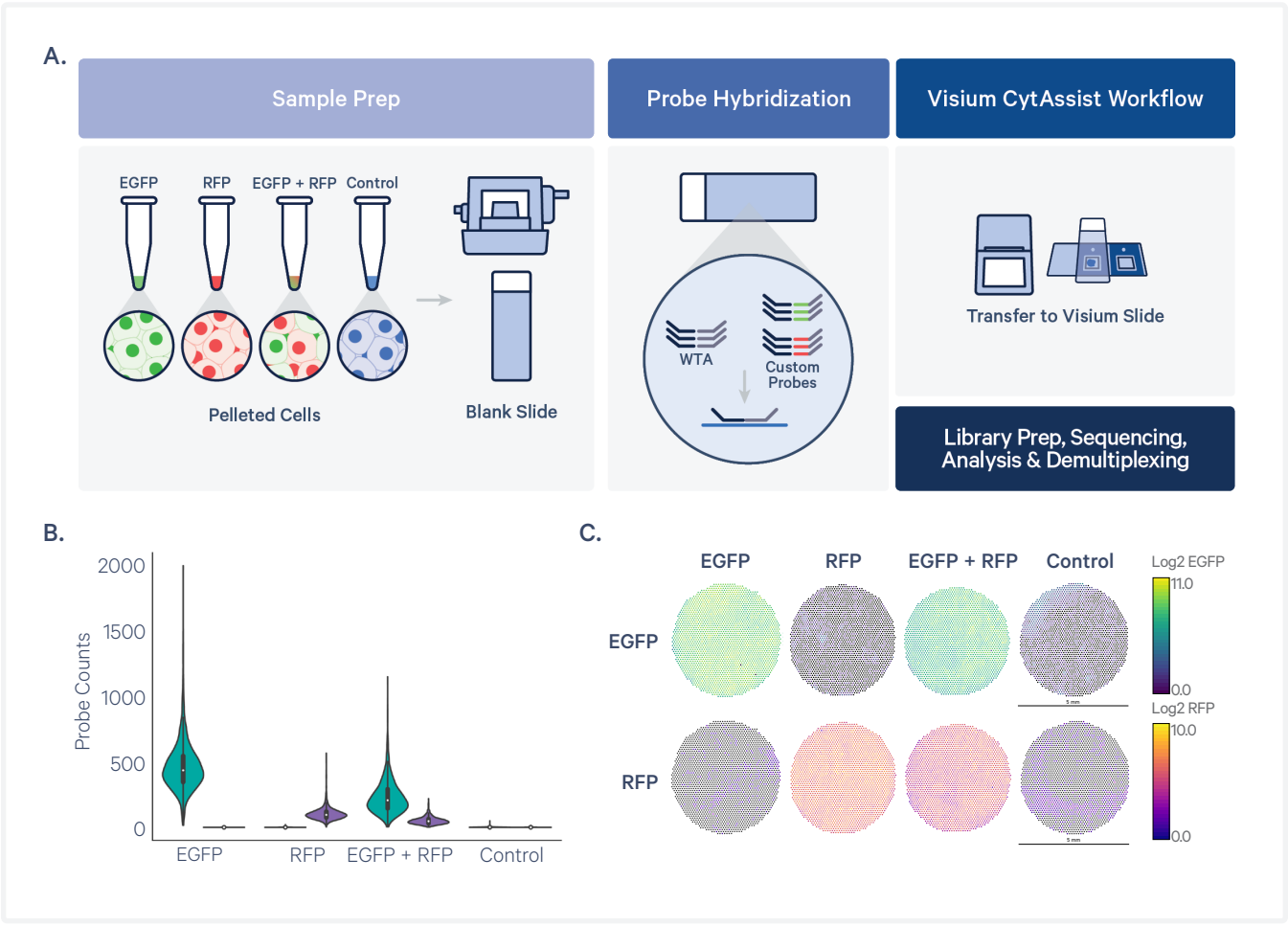


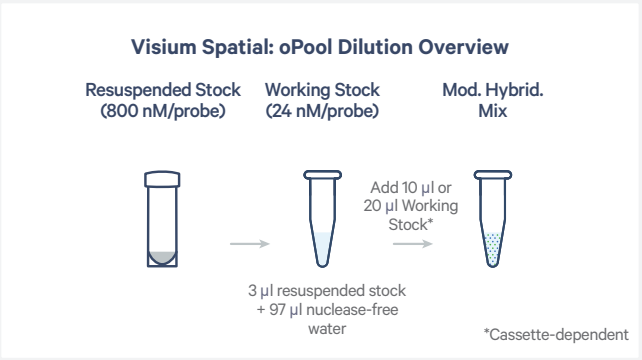
Figure 2. Custom probe specificity in detection of EGFP and RFP in a Visium Spatial Gene Expression experiment. In the experiment performed, all 4 cell pellets (GFP, RFP, GFP/RFP, neg. control) were embedded in a single FFPE block as a 2x2 array, sectioned onto a slide, then incubated with the modified hybridization mix, containing both the EGFP and RFP probes (A) and processed and analyzed per the Visium v2 (CG000495) workflow. EGFP is detected only in cells expressing EGFP but not in RFP-expressing cells. Similarly, RFP is only detected in cells expressing RFP but not in EGFP-expressing cells demonstrating the specificity of the custom probes (B-C).

Appendix A: Visium Spatial 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.

oPool Oligo Pools

For combining fewer than 30 oPools in a single spike-in pool, follow the steps described below. If combining more than 30 oPools, contact support@10xgenomics.com.



a. Resuspend stock:
Resuspend oPool Oligos in IDTE (10 mM Tris, 0.1 mM EDTA, pH 8.0) for a stock concentration of 800 nM/probe. Store resuspended oligos at -20°C.

TIPS *Resuspension volume 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 to a concentration of 800 nM/oligo.

b. Prepare working stock:
Using the resuspended stock, prepare the spike-in pool working stock containing 24 nM each of the LHS and RHS probes.

If the spike-in pool contains fewer than 34 probes, the working stock (24 nM/probe) should be prepared fresh before use and any remaining solution should be discarded.
If the spike-in pool contains 34 or more probes, the working stock (24 nM/probe) may be prepared ahead of time and stored at -20°C.

Spike-in Pool Working Stock *(24 nM/probe)*	**Volume (µl)**
Nuclease-free water	97
Resuspended Stock* (800 nM/probe)	3
Total	**100**

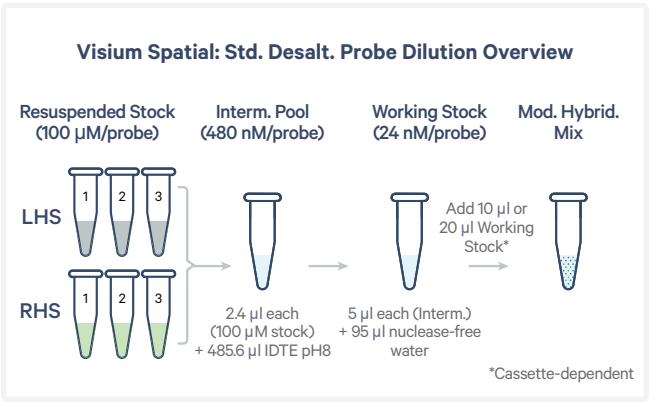
**If adding multiple resuspended oPool stocks, add 3 µl from each oPool stock and reduce the volume of nuclease-free water proportionally.*

c. Pipette mix 15X (pipette set to 90 µl), centrifuge briefly.

d. Add **10 µl** or **20 µl** (cassette-dependent) spike-in pool working stock to the Modified Probe Hybridization Mix (see Table 3-4).

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 ≤200 Total Custom Probes (≤100 LHS probes + ≤100 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 2.4 µl from each 100 µM resuspended probe in IDTE (10 mM Tris, 0.1 mM EDTA, pH 8.0) for a total volume of 500 µl.

Example: intermediate pool containing a total of 6 custom probes

Intermediate Pool *480 nM/probe (100 µM stock)*	**Volume (µl)**
IDTE (pH 8.0)	485.6
LHS Custom Probe 1	2.4
LHS Custom Probe 2	2.4
LHS Custom Probe 3	2.4
RHS Custom Probe 1	2.4
RHS Custom Probe 2	2.4
RHS Custom Probe 3	2.4
Total	**500**

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.

If the spike-in pool contains fewer than 34 probes, the working stock (24 nM/probe) should be prepared fresh before use and any remaining solution should be discarded. If the spike-in pool contains 34 or more probes, the working stock (24 nM/probe) may be prepared ahead of time and stored at -20°C.

Spike-in Pool Working Stock *(24 nM/probe)*	**Volume (µl)**
Nuclease-free water	95
Intermediate Pool* (480 nM/probe)	5
Total	**100**

e. Pipette mix 15X (pipette set to 90 µl), centrifuge briefly.

f. Add **10 µl** or **20 µl** (cassette-dependent) spike-in pool working stock to the Modified Probe Hybridization mix (Table 3-4).

For 200–4,160 Total Custom Probes (≤2,080 LHS probes + ≤2,080 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 10,000 µl.

Example: pool for 300 custom probes or 150 custom probe pairs

Spike-in Pool Working Stock *(100 µM stock)*	**Volume (µl)**
Nuclease-free water	9,280
LHS Custom Probes 1-150	360 (2.4 µl each X 150)
RHS Custom Probes 1-150	360 (2.4 µl each X 150)
Total	**10,000**

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

d. Add **10 µl** or **20 µl** (cassette-dependent) spike-in pool working stock to the Modified Probe Hybridization mix (Table 3-4).

TIPS *Spike-in pool working stock may be prepared ahead and aliquots can be stored at -20°C to minimize freeze-thaw.*

For >4,160 Total Custom Probes

Contact support@10xgenomics.com

Chromium Single Cell Gene Expression Flex

Probe Design

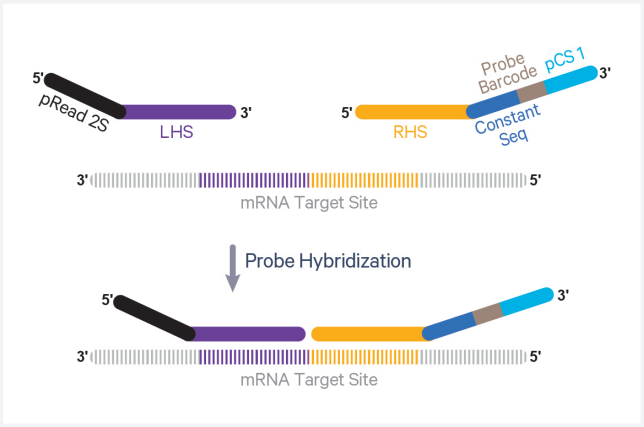


Figure 3. Probe design for Chromium Single Cell Gene Expression Flex. The left-hand side (LHS) probe contains a partial Read 2S (pRead 2S) as well as a 25 bp sequence that is reverse complement of the target site. The right-hand side (RHS) probe contains a phosphate on the 5' base for ligation, sequences reverse complementary to the target, Constant Sequence, Probe Barcode, and a partial capture sequence 1 (pCS1).

The following guidance outlines how to design custom probes for both singleplex and multiplexed experiments using the Chromium Next GEM Fixed RNA Profiling and GEM-X Flex Gene Expression assays.

10x Genomics probe panels consist of three probe pairs for most target mRNAs, with each probe containing 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 Single Cell Gene Expression Flex is provided in Figure 3 along with the accompanying probe sequences for singleplex and multiplex experiments in Table 7.

When designing probes for Chromium Flex experiments, the configuration presented in Table 7 is strongly preferred. Alternatively, a RHS probe configuration where the Constant Sequence and NN are omitted may be used to reduce synthesis cost. However, when using this alternate RHS probe configuration, the sequencing read parameters must be changed to Read 1–48 cycles, i7 index–10 cycles, i5 index–10 cycles, Read 2–50 cycles. If these parameters are not used, the Probe Barcode will not be detected correctly by Cell Ranger.

Single Cell Gene Expression Flex Custom Probe Sequence	
Singleplex Probe	
LHS Probe	**RHS Probe**
5'-CCTTGGCACCCGAGAATTCCA-target_LHS-3'	/5Phos/-target_RHS-ACGCGGTTAGCACGTA-NN-ACTTTAGG-CGGTCCTAGCAA-3' Constant Sequence / Probe Barcode
Multiplex Probe	
LHS Probe	**RHS Probe**
5'-CCTTGGCACCCGAGAATTCCA-target_LHS-3'	/5Phos/-target_RHS-ACGCGGTTAGCACGTA-NN-XXXXXXXX-CGGTCCTAGCAA-3' Constant Sequence / Probe Barcode

Table 7. Single Cell Gene Expression Flex probe configuration with sequences. Each probe in the probe pair represents 25 bp sequences homologous to the target transcript. The LHS probe sequence is the same for both singleplex and multiplex probes. The RHS probe for singleplex experiments includes Probe Barcode BC001 sequence ACTTTAGG while for multiplex experiments, each of the RHS probes must be designed and synthesized with a unique Probe Barcode BC001-BC016 (sequences listed in Table 8). All RHS probes also include an NN sequence to recover sequencing quality after reading the Constant Sequence before reading the Probe Barcode.

For singleplex experiments, the Probe Barcode BC001 sequence "ACTTTAGG" is included in the RHS probe (Table 7).

For multiplex experiments, each RHS probe must be designed and synthesized with a unique eight base Probe Barcode (BC001-BC016; sequences listed in Table 8). During the downstream Probe Hybridization step, when adding custom probes to the hybridization mix, the Probe Barcode on the custom probe must match the Probe Barcode of the corresponding Human/Mouse WTA Probe added to the mix. See illustrated examples of correct and incorrect Probe Barcode pairings in Table 9.

Probe Barcode in RHS probe *(for multiplexing)*	Sequence
BC001	ACTTTAGG
BC002	AACGGGAA
BC003	AGTAGGCT
BC004	ATGTTGAC
BC005	ACAGACCT
BC006	ATCCCAAC
BC007	AAGTAGAG
BC008	AGCTGTGA
BC009	ACAGTCTG
BC010	AGTGAGTG
BC011	AGAGGCAA
BC012	ACTACTCA
BC013	ATACGTCA
BC014	ATCATGTG
BC015	AACGCCGA
BC016	ATTCGGTT

Table 8. Unique Probe Barcode sequences for integrating in the RHS probe for multiplexing experiments using the Single Cell Gene Expression Flex assay.

The Probe Barcode sequences in the WTA probe sets are a mix of four distinct barcode sequences, which ensures balanced base composition during sequencing. For the purposes of a custom probe spike-in experiment, a single Probe Barcode sequence from each mixture is sufficient. For simplicity, the sequence beginning with A from each mixture is recommended.

Pairing	WTA Probe	Custom Probe
✓	BC001 	BC001 
✓	BC003 	BC003 
⊘	BC001 	BC002 
⊘	BC003 	BC004 

Table 9. Examples of correct and incorrect Probe Barcode pairings between WTA and custom probes for multiplexing experiments.

When designing custom probes for either singleplex or multiplex experiments, 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.
- The 25th nucleotide of the probe (3' most nucleotide of the LHS probe) must be a T. The opposing nucleotide in the target RNA must be an A.
- Avoid common single nucleotide polymorphisms (SNPs) and potential mismatches at the ligation junction. Refer to 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.
- Probe pairs should not overlap to avoid competition between probes for the same binding site in the target RNA.
- Add new probe sequences to the probe set reference CSV file. Refer to the Analysis section for more information.

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 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.
- Ordering custom probes as an oPool is preferred as it simplifies probe pooling and dilution upstream of Probe Hybridization (see Appendix B).
- LHS and RHS probes can be combined in the same oPools or ordered as separate pools.
- When ordering oPools for multiplexing experiments, RHS probes with different Probe Barcodes must be ordered as separate pools. LHS probes may be ordered as a separate oPool or included with each of the oPools for the differently barcoded RHS probes.

Using Custom Probes

To use custom probes, prepare a spike-in pool containing 40 nM of each probe in nuclease-free water. 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).

For multiplexed samples, all RHS probes within a pool should have the same Probe Barcode. For example, when performing a multiplex experiment using four Probe Barcodes, four spike-in pools, one for each Probe Barcode, should be prepared.

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 B.

2.5 µl or 5 µl (assay-dependent) of the custom probe spike-in pool (LHS and RHS probes combined) are added to the sample after the 10x Genomics Human/Mouse WTA Probes are added to the resuspended cell pellet as shown in Tables 10-11.

Chromium Next GEM Fixed RNA Profiling **Modified Probe Hybridization Mix** *Add in order listed*	**10x PN**	**1X (µl)**
Hyb Buffer	2000483	70
Enhancer	2000482	10
Human WTA Probes OR Mouse WTA Probes	2000495-2000510 2000703-2000718	20
Custom Probes, each probe at 40 nM (LHS and RHS combined)	-	5
Total		**105**

Table 10. Modified Probe Hybridization Mix for use in the Chromium NextGEM Fixed RNA Profiling assay.

GEM-X Flex Gene Expression **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	2001249-2001274 2001275-2001290	10
Custom Probes, each probe at 40 nM (LHS and RHS combined)	-	2.5
Total		**52.5**

Table 11. Modified Probe Hybridization Mix for use in the GEM-X Flex Gene Expression assay.

When adding custom probes to the Modified Probe Hybridization Mix, the Probe Barcode for the spike-in custom probe pool must match the Probe Barcode for the WTA Probes, as illustrated in Table 8.

Analysis

The use of custom probes requires the following file modifications for successful Cell Ranger (v7.2 or later) analysis:

Probe Set Reference CSV

- Find the appropriate probe set CSV on the Descriptions of Probe Set Reference CSV and Supporting Files page in the Single Cell Gene Expression Flex section of the 10x Genomics Support website.
- Update the appropriate probe set reference CSV file by appending the new custom probe information in the following columns:
  - 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.
  - gene_id: the ID of the mRNA target (any identifier)
  - probe_seq: combined LHS and RHS sequence trimmed of any adaptor, R2, or partial capture sequences. Target mRNA sequence only.
  - probe_id: Pipe-separated gene_id|gene_name|7 character hash (any combination of letters and numbers)
  - included: TRUE (will include in Cell Ranger analysis)
  - region: spliced or unspliced
    - spliced: the combined LHS and RHS sequence spans a splice junction
    - unspliced: the combined LHS and RHS sequence does not span a splice junction. For example, the sequence sits entirely within a single exon of the target gene

Data Highlight

Custom probe pairs were designed to detect EGFP (Enhanced green fluorescent protein) and mRFP (monomeric red fluorescent protein, referred to as RFP in this document) reporter genes in cell lines. Samples were fixed and processed as per the Single Cell Gene Expression Flex singleplex (CG000691) and multiplex (CG000527) workflows (see References). The probes were designed based on reference sequences from Addgene (EGFP, https://www.addgene.org/13031/; RFP, https://www.addgene.org/13032/).

Probe Design

The target sequence included in the custom probes is a reverse complement of the common EGFP and RFP sequences being detected in this experiment. The LHS probes are listed in Table 12 and the RHS probes are listed in Table 13. If using the listed sequences, confirm that the EGFP or RFP constructs used in your experiment include the binding site for these probes.

Custom LHS Probes for Single Cell Gene Expression Flex Assay	
Probe Configuration	**5'-CCTTGGCACCCGAGAATTCCA-target_LHS-3'**
EGFP-LHS-1	CCTTGGCACCCGAGAATTCCAggtagtggtcggcgagctgcacgct
EGFP-LHS-2	CCTTGGCACCCGAGAATTCCAagggtgtcgccctcgaacttcacct
EGFP-LHS-3	CCTTGGCACCCGAGAATTCCAatggtgcgctcctggacgtagcctt
RFP-LHS-1	CCTTGGCACCCGAGAATTCCAtcggtcttgtaggcgccgggcagct
RFP-LHS-2	CCTTGGCACCCGAGAATTCCAaagttggtgccgcgcagcttcacct
RFP-LHS-3	CCTTGGCACCCGAGAATTCCAcctcgatctcgaactcgtggccgtt

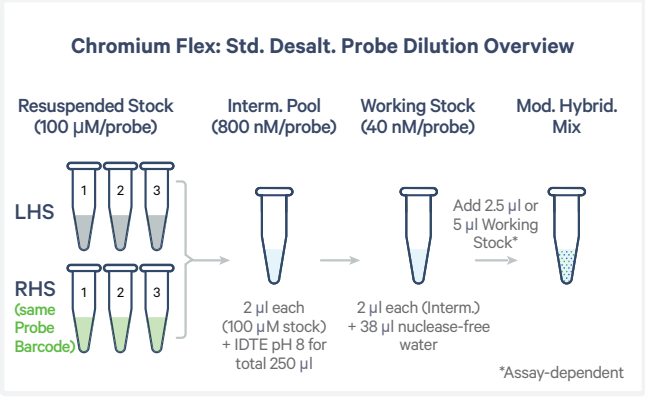
Table 12. LHS probe sequences for detection of EGFP and RFP reporter genes. These LHS probes may be paired with RHS probes (Probe Barcode BC001) for singleplex experiments or with RHS probes (Probe Barcodes BC001-BC016 listed in Table 8) for multiplex experiments.

Custom RHS Probes for Single Cell Gene Expression Flex Assay	
Probe Configuration	**/5Phos/-target_RHS-ACGCGGTTAGCACGTA-NN-ACTTTAGG-CGGTCCTAGCAA-3'**
EGFP-RHS-1	/5Phos/gccgtcctcgatgttgtggcggatcACGCGGTTAGCACGTANNACTTTAGGCGGTCCTAGCAA
EGFP-RHS-2	/5Phos/cggcgcgggtcttgtagttgccgtcACGCGGTTAGCACGTANNACTTTAGGCGGTCCTAGCAA
EGFP-RHS-3	/5Phos/cgggcatggcggacttgaagaagtcACGCGGTTAGCACGTANNACTTTAGGCGGTCCTAGCAA
RFP-RHS-1	/5Phos/gcacgggcttcttggccatgtaggtACGCGGTTAGCACGTANNACTTTAGGCGGTCCTAGCAA
RFP-RHS-2	/5Phos/tgtagatgaactcgccgtcctgcagACGCGGTTAGCACGTANNACTTTAGGCGGTCCTAGCAA
RFP-RHS-3	/5Phos/cacggagccctccatgcgcaccttgACGCGGTTAGCACGTANNACTTTAGGCGGTCCTAGCAA

Table 13. RHS probe sequences for detection of EGFP and RFP reporter genes. These RHS probes containing Probe Barcode BC001 are for a singleplex experiment. For multiplex experiments, each of the RHS probes must be designed and synthesized with a unique Probe Barcode BC001-BC0016 (sequences listed in Table 8) as described in the Probe Design section.

Probe Dilutions

Custom probes used in this study were ordered as standard desalted probes (100 µM stock) and were diluted as illustrated in the overview (see Appendix B for additional guidance, including dilution of oPool Oligo Pools). When preparing the dilutions for a multiplexing experiment, only RHS probes with the same probe barcode were added to each spike-in pool.



Chromium Flex Singleplex Experiment Overview & Results

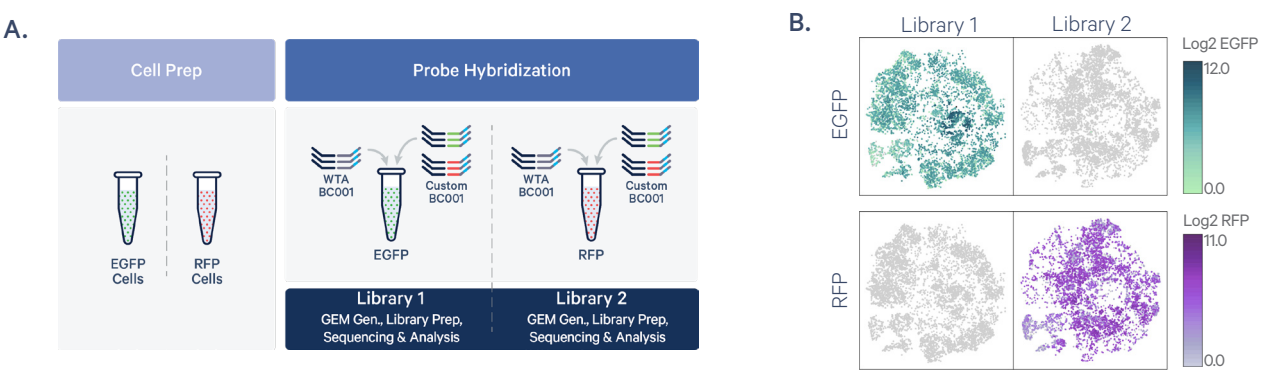


Figure 4. Custom probe specificity in detection of EGFP and RFP in a Single Cell Gene Expression Flex singleplex experiment. A mix of custom probes for detecting EGFP and RFP were added to individual hybridization reactions containing either EGFP- or RFP-expressing cells (A) and processed and analyzed as per the Chromium Fixed RNA Profiling Reagent Kits for Singleplexed Samples (CG000691) workflow. EGFP is detected only in cells expressing EGFP but not in RFP-expressing cells. Similarly, RFP is only detected in cells expressing RFP but not in GFP-expressing cells demonstrating the specificity of the custom probes (B).

Chromium Flex Multiplex Experiment Overview & Results

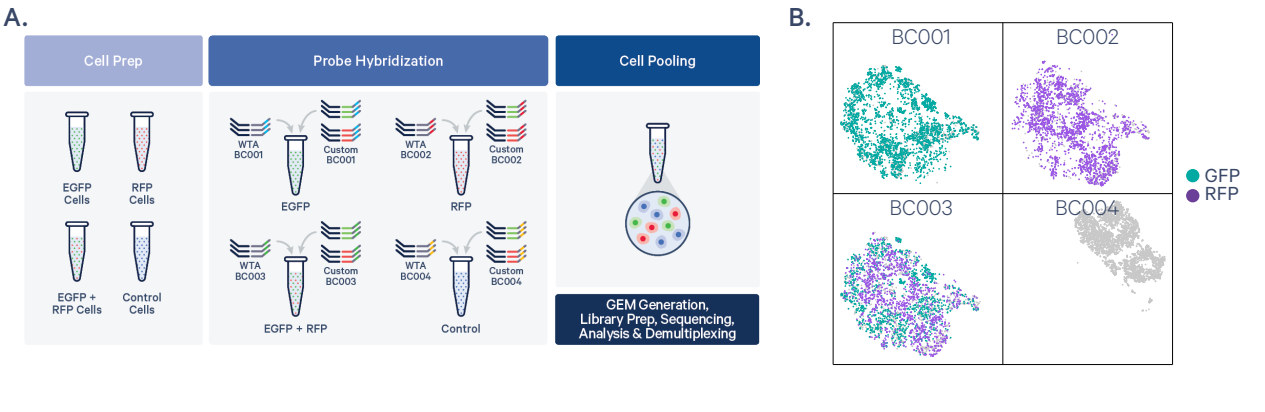


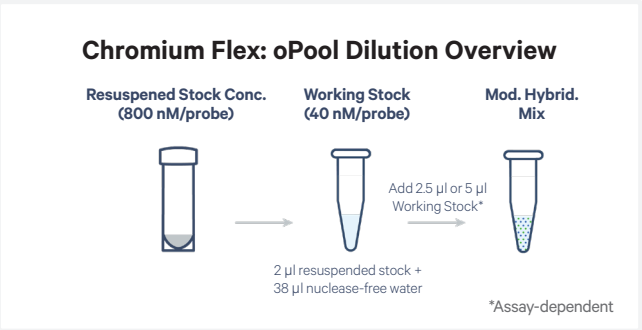
Figure 5. Custom probe specificity in detection of EGFP and RFP in a Single Cell Gene Expression Flex multiplex experiment. In a 4-sample multiplex experiment, a mix of custom probes for detecting EGFP and RFP were added to individual hybridization reactions containing either EGFP cells, RFP cells, a 50:50 mix of EGFP:RFP cells, or negative control cells (A). After hybridization, samples were pooled in equal proportion, washed, and loaded into a single GEM lane and were processed and analyzed as per the Chromium Fixed RNA Profiling Reagent Kits for Multiplexed Samples (CG000527) workflow. Using a UMI threshold of 1 or greater, EGFP is detected only in samples containing EGFP cells (BC001 and BC003) but not in RFP-only (BC002) or in negative control (BC004) cells. Similarly, RFP is only detected in samples expressing the RFP reporter (BC002 and BC003) but not in the EGFP-only sample or in the negative control (B).

Appendix B: Chromium Flex 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.

oPool Oligo Pools

For combining fewer than 20 oPools in a single spike-in pool, follow the steps described below. If combining more than 20 oPools, contact support@10xgenomics.com.



a. Resuspend stock:
Resuspend oPool Oligos in IDTE (10 mM Tris, 0.1 mM EDTA, pH 8.0) for a stock concentration of 800 nM/probe. Store resuspended oligos at -20°C.

TIPS *Resuspension volume 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.

b. Prepare working stock:
Using the resuspended stock, prepare the spike-in pool working stock containing 40 nM each of the LHS and RHS probes.

If the spike-in pool contains fewer than 20 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	Volume (µl)
Nuclease-free water	38
Resuspended Stock* (800 nM/probe)	2
Total	**40**

**If adding multiple resuspended oPool stocks, add 2 µl from each oPool stock and reduce the volume of nuclease-free water proportionally.*

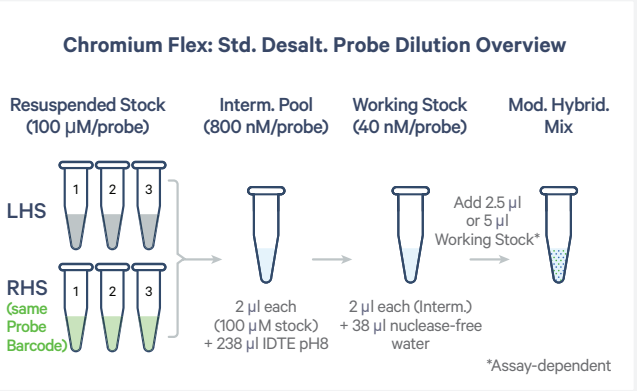
c. Pipette mix 15X (pipette set to 30 µl), centrifuge briefly.

d. Add **2.5 µl** or **5 µl** (assay-dependent) spike-in pool working stock to the Modified Probe Hybridization mix (see Tables 10-11).

All RHS probes in a spike-in pool working stock should have the same Probe Barcode. For example, when performing a multiplex experiment using four Probe Barcodes, four spike-in pools, one for each Probe Barcode, should be prepared.
When adding custom probes to the Modified Probe Hybridization Mix, the Probe Barcode for the spike-in custom probe pool must match the Probe Barcode of the WTA Probes used in the probe hybridization reaction.

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.

All of the RHS probes within a pool should have the same Probe Barcode. For example, when performing a multiplex experiment using four Probe Barcodes, four intermediate pools, one for each Probe Barcode, should be prepared.

Example: intermediate pool for Probe Barcode BC001 containing a total of 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, BC001	2
RHS Custom Probe 2, BC001	2
RHS Custom Probe 3, BC001	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 (BC001)	**Volume (µl)**
Nuclease-free water	38
Intermediate Pool* (800 nM/probe)	2
Total	**40**

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

f. Add **2.5 µl** or **5 µl** (assay-dependent) spike-in pool working stock to the Modified Probe Hybridization mix (see Tables 10-11).

When adding custom probes to the Modified Probe Hybridization Mix, the Probe Barcode for the spike-in custom probe pool must match the Probe Barcode of the WTA Probes used in the probe hybridization reaction.

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 for Probe Barcode BC001 containing a total of 300 custom probes or 150 custom probe pairs

Spike-in Pool Working Stock (BC001) (100 µM stock)	Volume (µl)
Nuclease-free water	4,400
LHS Custom Probes (1 -150)	300 (2 µl each X 150)
RHS Custom Probes BC001 (1-150)	300 (2 µl each X 150)
Total	**5,000**

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

d. Add **2.5 µl** or **5 µl** (assay-dependent) spike-in pool working stock to the Modified Probe Hybridization mix (see Tables 10-11).

All RHS probes in a spike-in pool working stock should have the same Probe Barcode. For example, when performing a multiplex experiment using four Probe Barcodes, four spike-in pools, one for each Probe Barcode, should be prepared.
When adding custom probes to the Modified Probe Hybridization Mix, the Probe Barcode for the spike-in custom probe pool must match the Probe Barcode of the WTA Probes used in the probe hybridization reaction.

For >2500 Total Custom Probes

Contact support@10xgenomics.com

Conclusion

This Technical Note provides guidance on the design and use of custom probes with the Visium Spatial Gene Expression and Chromium Single Cell Gene Expression Flex (singleplexed and multiplexed) assays. While no impact on assay performance is anticipated, the use of custom probes in these assays 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. Visium Spatial Gene Expression for FFPE Reagent Kits User Guide (CG000407).
2. Visium CytAssist Spatial Gene Expression Reagent Kits User Guide (CG000495).
3. Visium HD Spatial Gene Expression Reagent Kits User Guide (CG000685).
4. Chromium Fixed RNA Profiling Reagent Kits User Guide for Singleplexed Samples with Feature Barcode technology for Protein (CG000477).
5. Chromium Fixed RNA Profiling Reagent Kits User Guide for Singleplexed Samples (CG000691).
6. Chromium Fixed RNA Profiling Reagent Kits User Guide for Singleplexed Samples with Feature Barcode technology for Protein using Barcode Oligo Capture (CG000674).
7. Chromium Fixed RNA Profiling Reagent Kits User Guide for Multiplexed Samples (CG000527).
8. Chromium Fixed RNA Profiling Reagent Kits User Guide for Multiplexed Samples with Feature Barcode technology for Protein using Barcode Oligo Capture (CG000673).
9. GEM-X Flex Gene Expression Reagent Kit for Singleplex samples (CG000786)
10. GEM-X Flex Gene Expression Reagent Kit for Multiplex samples (CG000787)
11. GEM-X Flex Gene Expression Reagent Kit for Singleplex samples with Feature Barcode technology for Protein Expression (CG000788)
12. GEM-X Flex Gene Expression Reagent Kit for Multiplex samples with Feature Barcode technology for Protein Expression (CG000789)

Document Revision Summary

Document Number	CG000621
Title	Custom Probe Design for Visium Spatial Gene Expression and Chromium Single Cell Gene Expression Flex
Revision	Rev D
Revision Date	October 2024

Specific Changes:

- Updated Modified Probe Hybridization Mix table (page 4)
- Added references to Visium HD Spatial Gene Expression (page 2-4)
- Added references to GEM-X Flex Gene Expression and provided appropriate tables for the modified probe hybridization mix (page 11, 13-14)

General Changes:

Updated for general minor consistency of language, terms, and format throughout.

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