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

Introduction

10x Genomics Visium Spatial Gene Expression technology for FFPE enables 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 both 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 custom probe design, spike-in, and analysis for Visium Spatial Gene Expression for FFPE, Visium CytAssist Spatial Gene Expression, and Chromium Single Cell Gene Expression Flex for Singleplexed Samples (Fixed RNA Profiling). Additional optimization may be required. Performing a pilot experiment with these unsupported workflow modifications is recommended prior to committing to larger studies.

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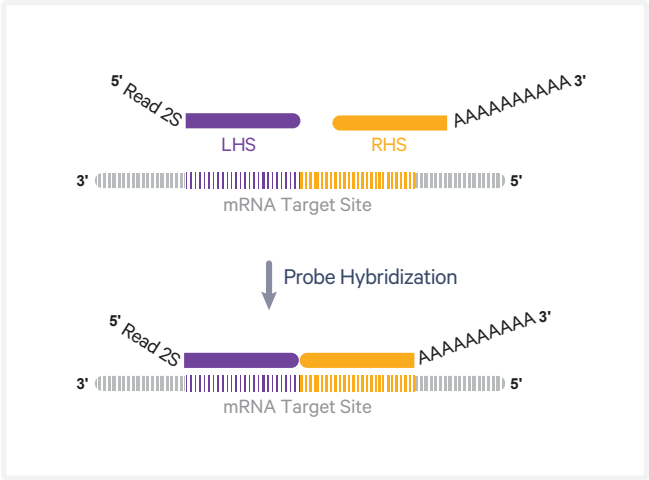

Figure 1. Probe design for the Visium Spatial Gene Expression assay. Probes in the Visium Assay include a LHS and RHS probe pair. The LHS probe contains a partial Read 2S as well as sequence complimentary to the mRNA target site. The RHS probe contains a phosphate on the 5' base for ligation, sequences complimentary to the target mRNA site, and a poly A tail.

Visium Spatial Gene Expression Probe Design

The following guidance applies to both the Visium Spatial Gene Expression for FFPE (Direct Placement) and Visium CytAssist Spatial Gene Expression (CytAssist-enabled) assays.

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

Species	Assay	Probe Set Version
Mouse	Direct Placement	Visium Mouse Transcriptome Kit v1
	CytAssist-enabled	
Human	Direct Placement	Visium Human Transcriptome Kit v1
	CytAssist-enabled	Visium Human Transcriptome Kit v2

Table 2. Probe panels used for either Direct Placement or CytAssist-enabled assays.

The Visium Human Transcriptome Kit v2 contains a panel with three pairs of probes for each target mRNA, with each probe containing 25 bps complimentary to the target mRNA sequence. Each probe is referred to as the left hand side (LHS) or right hand side (RHS) probe. The Visium Mouse and Human Transcriptome Kit v1 contain panels with one pair of probes for each target mRNA.

When designing custom probes for Visium Spatial Gene Expression for FFPE, 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 complementary nucleotide in the target RNA must be an A.

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

Table 1. Probe sequences Visium Spatial Gene Expression for FFPE probes. The target_LHS and target_RHS parts of each probe contain unique 25 bp sequences targeting the transcript of interest. The Visium Spatial Gene Expression for FFPE RHS probe follows its target sequence with a series of thirty adenines.

- 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, 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 aren't 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

Order custom probes from any oligo synthesis provider. Follow the guidance below:

- 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.

Using Custom Probes for Visium Spatial Gene Expression

To use custom probes, prepare a LHS spike-in pool containing 48 nM of each probe in nuclease-free water. Prepare a separate RHS spike-in pool also containing 48 nM of each probe in nuclease-free water. For example, a spike-in pool with 9 LHS probes would contain 48 nM of each of the 9 LHS probes (432 nM total LHS probe).

Add 5 µl of each probe pool to FFPE Hyb Buffer and the 10x Genomics probes, as shown in Table 3, to generate the modified probe hybridization mix. This results in a final concentration of 2.4 nM per probe. The modified probe hybridization mix will replace the original probe hyb mix from Step 1.1g (Visium Spatial Gene Expression for FFPE Reagent Kits User Guide, Document CG000407) or Step 1.1h (Visium CytAssist Spatial Gene Expression for FFPE Reagent Kits User Guide, Document CG000495).

If performing the CytAssist-enabled assay and working with 11 mm Capture Areas, double all volumes in the modified probe hybridization mix, resulting in a total volume of 200 µl.

Modified Probe Hybridization Mix *Add in order listed*	**10x PN**	**1X (µl)**
FFPE Hyb Buffer	2000423	70
Human WT Probes - RHS OR Mouse WT Probes – RHS	2000657/ 2000449 2000455	10
Human WT Probes - LHS OR Mouse WT Probes – LHS	2000658/ 2000450 2000456	10
LHS Custom Probes, each probe at 48 nM	-	5
RHS Custom Probes, each probe at 48 nM	-	5
Total		**100**

Table 3. Modified Probe Hybridization Mix for use in the Visium Spatial Gene Expression assay.

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 pre-built reference. Update the gene annotation file (GTF) with new targets using a text editor.
  - Follow the existing format of the Space Ranger GTF.
  - Ensure the gene name is unique.
- Modify the genome reference in FASTA format that contain 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 the 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 of any adapter, R2, or polyA 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 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, complimentary to the target gene.

Chromium Single Cell Gene Expression Flex for Singleplexed Samples Probe Design Overview

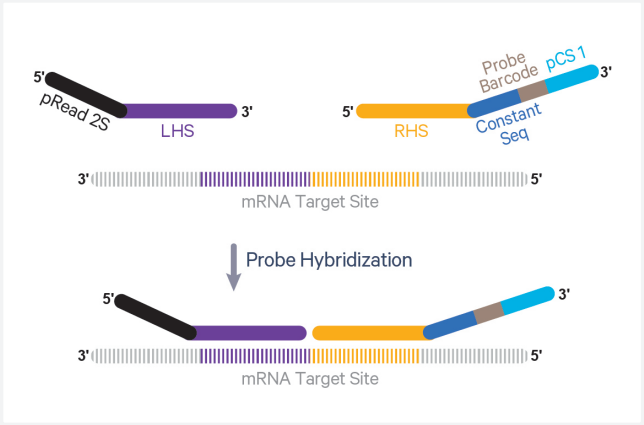

Figure 2. Probe design for Chromium Single Cell Gene Expression Flex. The left hand side (LHS) probe contains a partial Read 2S (pRead 2S) as well as complimentary sequences to the mRNA target site. The RHS probe contains a phosphate on the 5' base for ligation, sequences complimentary to the target, constant sequence, Probe Barcode, and a partial capture sequence 1 (pCS1).

Chromium Single Cell Gene Expression Flex for Singleplexed Samples Probe Design

This Technical Note provides guidance for singleplexed samples only.

10x Genomics probe panels consist of three pairs of probes for most target mRNAs, with each probe containing 25 bps complimentary to the target mRNA sequence. Each probe is referred to as the left hand side (LHS) or right hand side (RHS) probe. An overview of the composition of 10x Genomics probes for Single Cell Gene Expression Flex is provided in Figure 2, and the accompanying sequence is provided in Table 4.

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.
- The 25th nucleotide of the probe (3' most nucleotide of the LHS probe) must be a T. The complementary 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, 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 aren't 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.

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

Table 4. Sequences for Single Cell Gene Expression Flex probes. Each probe in the probe pair represent 25 bps of homology to the target transcript. The target_LHS and target_RHS parts of each probe contain unique 25 bp sequences targeting the transcript of interest.

Ordering Custom Probes

Custom probes can be ordered from any oligo synthesis provider. Follow the guidance below:

- 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.

Using Custom Probes for Chromium Single Cell Gene Expression Flex for Singleplexed Samples

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).

5 µl of these probes (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 (Step 1.1g in the Chromium Fixed RNA Profiling Reagent Kits for Singleplexed Samples with Feature Barcode technology for Cell Surface Protein User Guide (CG000477) as shown in Table 5.

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	2000474 2000495	20
Custom Probes, each probe at 40 nM (LHS and RHS combined)	-	5
Total		**105**

Table 5. Modified Probe Hybridization Mix for use in the Chromium Single Cell Gene Expression Flex for Singleplexed Samples assay.

Analysis

The use of custom probes requires the following file modifications for successful Cell Ranger (v7.1 or later) 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 pre-built reference. Update the gene annotation file (GTF) with new targets using a text editor.
  - Follow the existing format of the Cell Ranger GTF.
  - Ensure the gene name is unique.
- Modify the genome reference in FASTA format that contain additional contigs for new targets.
- Generate a new genome reference build with the new GTF and FASTA files created above using cellranger mkref. This build will be used for data analysis using cellranger multi.
  - Name the new reference and new probe CSV files so that they can be distinguished from the default files.
  - For more information, consult the Creating a Reference Package with cellranger mkref page in the Single Cell Gene Expression section of 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 Fixed RNA Profiling 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 adapter, 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, complimentary to the target gene.

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 for Singleplexed Samples assays. While no impact on assay performance is anticipated, the use of custom probes in these assays has not been tested 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. Chromium Fixed RNA Profiling Reagent Kits for Singleplexed Samples with Feature Barcode technology for Cell Surface Protein User Guide (CG000477).

Document Revision Summary

Document Number	CG000621
Title	Custom Probe Design for Visium Spatial Gene Expression and Chromium Single Cell Gene Expression Flex
Revision	Rev B
Revision Date	February 2023
Specific Changes	• Updated probe design guidance to include "The 25th nucleotide of the probe (3' most nucleotide of the LHS probe) must be a T. The complementary nucleotide in the RNA target must be an A." • Updated probe set reference csv section to include "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."

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