Sequencing Metrics & Base Composition of Visium Spatial Gene Expression for FFPE Libraries

Introduction

The Visium Spatial Gene Expression for FFPE Reagent Kits workflow produces Visium Spatial Gene Expression sequencing-ready libraries for measuring RNA in intact formalin fixed paraffin embedded (FFPE) tissue sections. This Technical Note presents a comparison of sequencing metrics for pooled Visium Spatial Gene Expression for FFPE libraries across Illumina platforms. The expected base percentage profiles and Phred quality scores (Q scores) based on a control library are described to provide general guidance on the expected range of sequencing metrics on Illumina platforms. Individual results may vary depending on the specific sequencing instrument and/or particular sample and loading characteristics.

Introduction

Visium Spatial Gene Expression dual index libraries (schematic shown in Figure 1) are generated from formalin fixed paraffin embedded (FFPE) tissue sections using Visium Spatial Gene Expression for FFPE reagents and protocols (see References).

Visium Spatial Gene Expression for FFPE libraries comprise standard Illumina paired-end constructs that are flanked with P5/P7, necessary for binding to the Illumina flow cell. TruSeq Read 1 is used for priming and sequencing the 16 bp Spatial Barcode and 12 bp UMI, and TruSeq Read 2S is used for priming and sequencing the 50 bp probe insert. The two 10 bp sample indexes are sequenced in the i5 and i7 reads respectively.

Tables 1-3 show representative plots and sequencing data derived from Visium Spatial Gene Expression for FFPE libraries sequenced alone or in combination with another library type. The sequencing configuration & run parameters are indicated for each dataset.

Figure 1. Schematic showing Visium Spatial Gene Expression dual index library for FFPE tissue.

Methods Overview

Four Visium Spatial Gene Expression libraries were generated from two Hematoxylin & Eosin (H&E) stained human lung tissue samples and two H&E stained mouse brain tissue samples as described in the Visium Spatial Gene Expression for FFPE - Deparaffinization, H&E Staining, Imaging & Decrosslinking Demonstrated Protocol (Document CG000409) and the Visium Spatial Gene Expression Reagents Kits for FFPE User Guide (Document CG000407). The libraries were pooled and sequenced at the indicated run parameters to generate the % Base and % ≥Q30 plots shown in Tables 1-2. These same libraries were used to illustrate sequencer compatibility. All libraries were quantified with the KAPA DNA Quantification Kit and sequenced with 1% PhiX.

Additionally, the same four Visium Spatial Gene Expression libraries were pooled with one Chromium Single Cell 3' v3.1 Gene Expression dual index library (pooled 40:60 ratio). Sequencing was performed at the indicated run parameters (Table 3) to generate the % Base and % ≥Q30 plots.

Results

The representative data shown in Tables 1-2 demonstrates compatibility of Visium Spatial Gene Expression - FFPE libraries with multiple sequencers.

The data also shows that the number of Read 2 sequencing cycles impacts the metrics. Limiting the Read 2 cycles to 50 bp (Table 1) in the probe insert results in high Q30 scores. Observing a decline in Q30 score at the ligation junction is expected due to probe design. Extending Read 2 to 90 bp (Table 2) leads to a drop in quality scores after the first 50 cycles of Read 2 due to sequencing through the poly-adenylated (poly(A)) sequence. However, this does not affect assay performance once it is trimmed by the pipeline.

Sequencing Visium Spatial Gene Expression - FFPE libraries alone results in a drop in quality scores after the first 25 cycles of Read 2 due to sequence preference in probe design. However, this does not affect assay performance. This skewed base distribution can be normalized by pooling Visium Spatial Gene Expression for Fresh Frozen or Chromium Single Cell 3' v3.1 Gene Expression dual index libraries with Visium Spatial Gene Expression - FFPE libraries (Table 3).

Conclusions

In summary, % Base by cycle and % ≥Q30 Quality Score distribution showed highly consistent profiles for all sequencing platforms tested. The data serve as guidelines for assessing the quality of Visium Spatial Gene Expression library sequencing.

Additional factors that may contribute to overall success of a sequencing run and impact downstream application performance metrics include:

- Starting with a high quality tissue block.
- Generating sequencing data from sections that remain adhered to the slide.
- Reliable and accurate library quantification using the KAPA DNA Quantification Kit based on the average insert size determined by Agilent Bioanalyzer QC.
- Sequencing platform loading concentration.

Visium Gene Expression - FFPE Library

Sequencing: R1-28; i7-10; i5-10; R2-50 cycles

Four Visium Spatial Gene Expression - FFPE dual index libraries were pooled and sequenced on indicated Illumina sequencers. 'Data by Cycle' plots from the Illumina SAV software displaying the percentage of base calls and Q30 quality scores along with additional metrics are shown in Table 1.

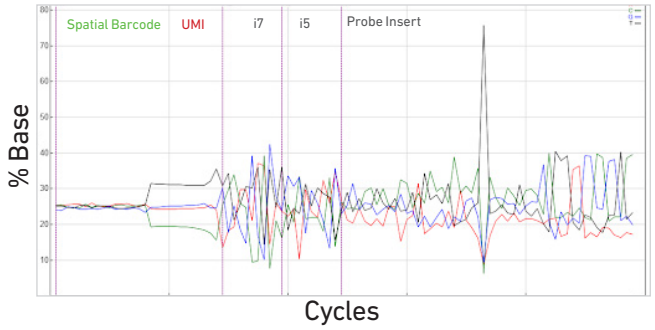
Sequencing configuration & run parameters:

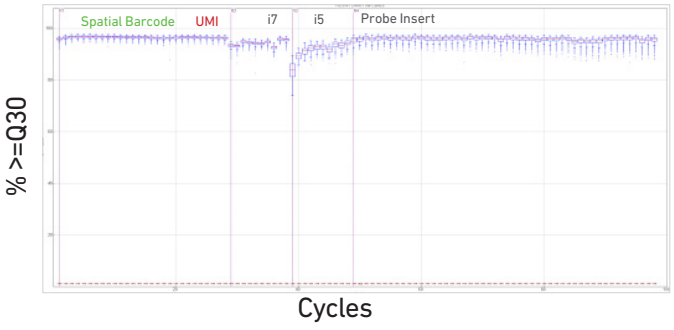
Minimum sequencing depth 25,000 read pairs/tissue covered spot.
Paired-end, dual indexing: Read 1: 28 cycles; i7 Index: 10 cycles; i5 Index: 10 cycles; Read 2: 50 cycles.

When limiting Read 2 cycles to 50 bp in the probe insert, the Q30 scores are high but can be impacted by the probe design.

Table 1: Representative Plots and Data *(Sequencing parameters: R1-28; i7-10; i5-10; R2-50 cycles)*

Plots shown are from a pool of four Visium Gene Expression - FFPE libraries sequenced on a NovaSeq SP flow cell.





Sequencer	Loading Conc. (pM) Cluster Density/%PF PhiX (%): 1	% ≥Q30				Yield per Lane (Gb)		Mapped Read (%)	
		R1	i7	i5	R2	R 1	R 2	Human	Mouse
iSeq	Loading Conc. (pM): 50 Cluster Density: 75 K/mm^2 PhiX (%): 1	96.0	93.1	86.5	87.4	0.16	0.28	72	86
MiSeq	Loading Conc. (pM): 10 Cluster Density: 90 K/mm^2 PhiX (%): 1	97.4	96.7	94	89.4	0.76	1.4	69	83
NextSeq 550	Loading Conc. (pM): 1.8 Cluster Density: 89 K/mm^2 PhiX (%): 1	96.1	94.4	93	91.7	13.52	24.75	71	85
NextSeq 2000	Loading Conc. (pM): 650 % PF*: 82 PhiX (%): 1	96.5	97.9	96.2	91.9	14.74	26.98	72	85
NovaSeq (SP flow cell)	Loading Conc. (pM): 300 % PF*: 83 PhiX (%): 1	96.3	96.3	93.4	93.5	28.61	52.39	72	85

*Percent Pass Filter (% PF) is reported for NovaSeq and NextSeq 2000 instead of cluster density due to the patterned flow cell

Visium Gene Expression - FFPE Library

Sequencing: R1-28; i7-10; i5-10; R2-90 cycles

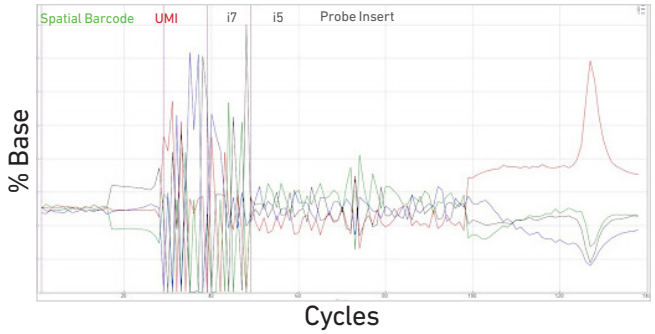
Four Visium Gene Expression - FFPE (dual index) libraries were pooled and sequenced on indicated Illumina sequencers. 'Data by Cycle' plots from the Illumina SAV software displaying the percentage of base calls and Q30 quality scores along with additional metrics are shown in Table 2.

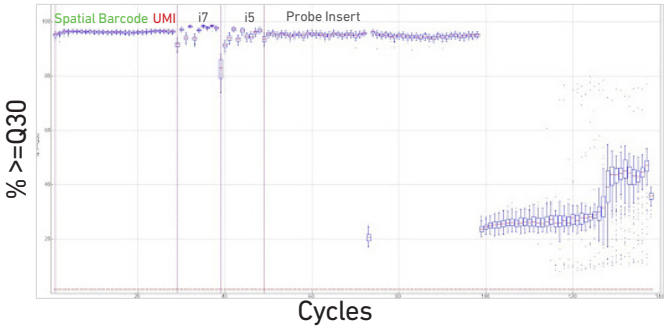
Sequencing configuration & run parameters:
Minimum sequencing depth 25,000 read pairs/tissue covered spot.
Paired-end, dual indexing: Read 1: 28 cycles; i7 Index: 10 cycles; i5 Index: 10 cycles; Read 2: 90 cycles.

Sequencing Read 2 to 90 bp leads to a drop in quality scores after the first 50 cycles due to sequencing through poly(A). However, this does not impact assay performance once it is trimmed by the analysis pipeline.

Table 2: Representative Plots and Data *(Sequencing parameters: R1-28; i7-10; i5-10; R2-90 cycles)*

Plots shown are from a pool of four Visium Gene Expression - FFPE libraries sequenced on a NovaSeq SP flow cell.





Sequencer	Loading Conc. (pM) Cluster Density/%PF PhiX (%)	% ≥Q30					Yield per Lane (Gb)		Mapped Read (%)	
		R1	i7	i5	R2	R2**	R 1	R 2	Human	Mouse
iSeq	Loading Conc (pM): 50 Cluster Density: 75 K/mm^2 PhiX (%): 1	96.0	93.1	86.5	77.2	96.2	0.16	0.51	72	86
MiSeq	Loading Conc. (pM): 10 Cluster Density: 90 K/mm^2 PhiX (%): 1	97.4	96.7	94	77.5	83.3	0.76	2.52	69	83
NextSeq 550	Loading Conc. (pM): 1.8 Cluster Density: 89 K/mm^2 PhiX (%): 1	96.1	94.4	93	55.4	92.8	13.52	44.56	71	85
NextSeq 2000	Loading Conc. (pM): 650 % PF*: 82 PhiX (%): 1	96.5	97.9	96.2	66.6	95.1	14.74	48.58	72	85
NovaSeq (SP flow cell)	Loading Conc. (pM): 300 % PF*: 83 PhiX (%): 1	96.3	96.3	93.4	66.1	93.9	28.61	94.31	72	85

*Percent Pass Filter (% PF) is reported for NovaSeq and NextSeq 2000 instead of cluster density due to the patterned flow cell
** Q30 scores post-pipeline trimming

Gene Expression Library Pooling: Visium - FFPE + Chromium Single Cell 3'

Sequencing: R1-28; i7-10; i5-10; R2-90 cycles

Sequencing Visium Spatial Gene Expression - FFPE libraries alone results in a drop in quality scores after the first 25 cycles of Read 2 due to sequence preference in probe design. However, this does not affect assay performance. This skewed base distribution can be normalized by pooling Visium libraries from fresh frozen tissue or Chromium Single Cell 3' v3.1 Gene Expression dual index libraries with Visium Spatial Gene Expression - FFPE libraries (see Table 3).

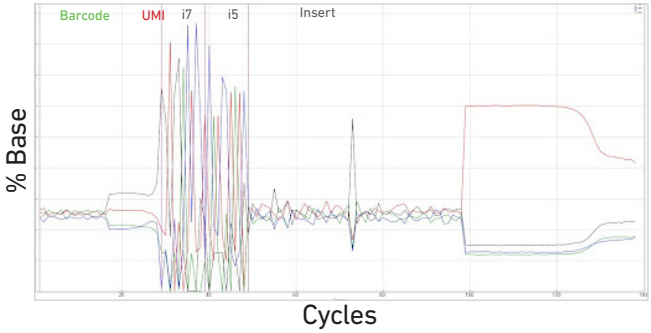
Four Visium Spatial Gene Expression - FFPE (dual index) libraries were pooled with one Chromium Single Cell 3' v3.1 Gene Expression library (40:60) and sequenced on indicated Illumina sequencers. 'Data by Cycle' plots from the Illumina SAV software displaying the percentage of base calls and Q30 quality scores along with additional metrics on NovaSeq are shown in Table 3.

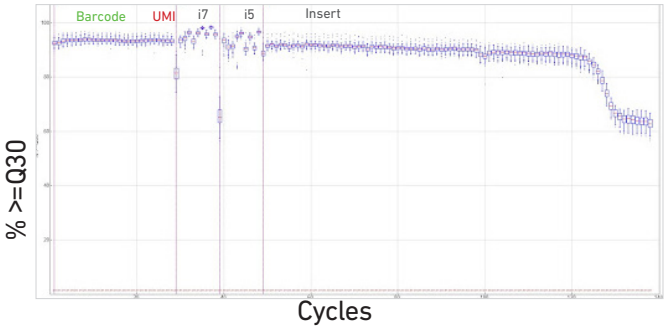
A maximum of 40% of the pool should consist of Visium Spatial Gene Expression - FFPE libraries when pooling with another library type. Higher fractions of the pool consisting of Visium Spatial Gene Expression - FFPE libraries could have a negative impact on the metrics of the other library type and is not supported.

Sequencing configuration & run parameters:
Minimum sequencing depth 25,000 read pairs/tissue covered spot.
Paired-end, dual indexing: Read 1: 28 cycles; i7 Index: 10 cycles; i5 Index: 10 cycles; Read 2: 90 cycles.

Table 3: Representative Plots *(Sequencing parameters: R1-28; i7-10; i5-10; R2-90 cycles)*

Plots shown are from a pool of four Visium Spatial Gene Expression - FFPE libraries with one Chromium Single Cell 3' v3.1 Gene Expression dual index library (40:60) sequenced on a NovaSeq SP flow cell.





Sequencer	Loading Conc. (pM) Cluster Density/%PF PhiX (%)	% ≥Q30					Yield per Lane (Gb)		Mapped Read (%) Human	Reads Mapped to Transcript.
		R1	i7	i5	R2	R2**	R 1	R 2		
NovaSeq (SP flow cell) 	Loading Conc. (pM): 300 % PF*: 81 PhiX (%): 1	95.8	96.5	95	90	96.3	28	92.3	87	63.5

*Percent Pass Filter (% PF) is reported for NovaSeq instead of cluster density due to the patterned flow cell
**Q30 scores post-pipeline trimming

References

1. Visium Spatial Gene Expression for FFPE – Deparaffinization, H&E Staining, Imaging & Decrosslinking (CG000409)
2. Visium Spatial Gene Expression Reagent Kits for FFPE User Guide (CG000407)

Document Revision Summary

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Contact:
support@10xgenomics.com
10x Genomics
6230 Stoneridge Mall Road
Pleasanton, CA 94588 USA