Development of Highly Multiplexed, Whole Process Reference Materials for Monitoring Oncology RNA Fusions

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INTRODUCTION

Genomic structural alterations are increasingly actionable for targeted therapeutics and personalized medicine. Molecular diagnostics are rapidly being introduced for detection of fusion RNAs from FFPE material by highly multiplexed next-generation sequencing assay panels and these are replacing manual fluorescent in-situ hybridization (FISH) methods. However, commercial quality control materials for these assays are lacking, and validation materials for rare fusions are frequently not available. We developed Seraseq™ FFPE Fusion RNA Reference Material to fill this unmet need.

MATERIALS AND METHODS

Design of RNA Constructs: Highly multiplexed RNAs were designed which contain multiple fusion targets observed predominantly in solid tumors (Figure 1). Modifications were made to these constructs to improve intracellular stability.

RNA Fusion	Primary Cancer Tissue	5' Partner-Exon #	3' Partner-Exon #
EML4-ALK	Lung	EML4 Exon 13	ALK Exon 20
NPM1-ALK	Lymphoid	NPM1 exon 5	ALK Exon 20
KIF5B-RET	Lung	KIF5B Exon 24	Ret Exon 11
NCOA4-RET	Thyroid	NCOA4 Exon 8	RET exon 12
CD74-ROS1	Lung	CD74 Exon 6	Ros 1 Exon 34
SLC34A-ROS1	Lung, Stomach	SLC34A Exon 4	Ros 1 Exon 34
TPM3-NTRK1	Lung, Large Intestine	TPM3 Exon 8	NTRK1 Exon 10
TFG-NTRK1	Thyroid (rare)	TFG Exon 5	NTRK1 Exon 10
FGFR3-BAIAP2L1	Urinary tract (rare)	FGFR3 exon 18	BAIAP2L1 Exon 2
FGFR3-TACC3	Urinary tract, CNS	FGFR3 exon 18	TACC3 Exon 11
PAX-PPARG	Thyroid	Pax8 Exon 8	PPARG Exon 1
ETV6-NTRK3	Kidney, Breast, Soft Tissue	ETV6 Exon 5	NTRK3 Exon 13

Figure 1: Fusions contained in the Reference Material

Cell Engineering and Production of FFPE Curls: The RNA was introduced into GM24385 reference cell line (The 1000 Genomes Project, Coriell) and stabilized using proprietary methodologies. The engineered cells were fixed in formalin. Digital PCR with TaqMan® chemistry was used to determine the average number of synthetic RNA transcripts per cell. These engineered cells were then mixed with native GM24385 cells to achieve a consistent "low positive" formulation. The cell mixture was embedded in a paraffin block, and 10 micron sections were produced.

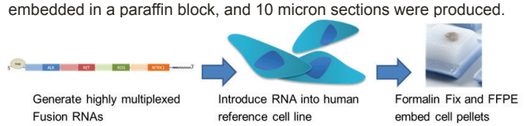


Figure 2: Workflow for production of reference materials

Quality Control Testing: Total nucleic acid was extracted from one or more FFPE curls using Agencourt® FormaPure® Kit and tested using the ArcherDx FusionPlex® Lung Thyroid Panel run on the Illumina MiSeq® instrument, the Ion AmpliSeq® RNA Fusion Lung Cancer Research Panel run on the Ion PGM®, or the Thermo Fisher Oncomine® Comprehensive Panel.

RESULTS AND DISCUSSION

Pre Analytical Information:

# of curls	Volume	Concentration (by Qubit® RNA HS)	Total Yield	260/280 Ratio (by NanoDrop®)
1 curl	35 µL	8.8 ng/µL	308 ng	1.88
1 curl	35 µL	6.6 ng/µL	231 ng	2.00
1 curl	35 µL	8.05 ng/µL	282 ng	1.98

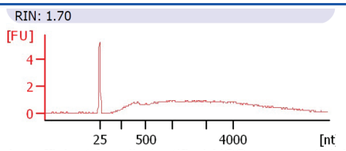
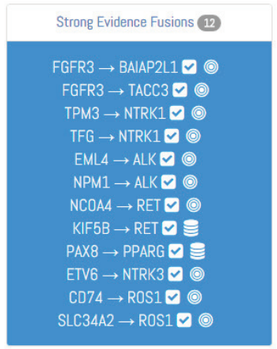


Figure 3: (Left) The average yield of total nucleic acid from one FFPE curl was 273 ng, which is sufficient for all identified NGS assays. (Right) Agilent Bioanalyzer trace showing that the RNA extracted from the FFPE is fragmented and lacks 18S and 28S peaks, similar to patient samples.

Results on ArcherDx FusionPlex Lung Thyroid Panel:



Fusion	Spanning Reads Lot 1	Spanning Reads Lot 2
EML4-ALK	90	118
NPM-ALK	136	108
KIF5B-RET	199	191
NCOA4-RET	341	226
CD74-ROS1	54	65
SLC34A2-ROS1	38	34
TPM3-NTRK1	127	143
TFG-NTRK1	124	115
FGFR3-BAIAP2L1	361	412
FGFR3-TACC3	396	328
Pax8-PPARG	152	179
ETV6-NTRK3	195	172

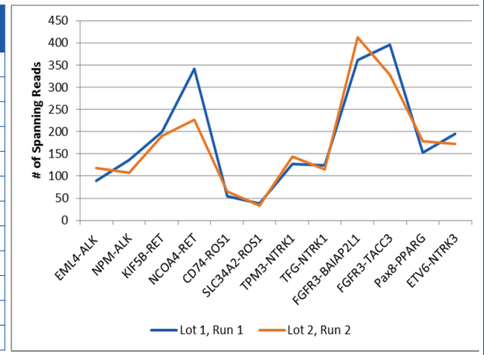


Figure 4. (Left) Summary from Archer FusionPlex Report. ◎ Symbol means the exact breakpoint is known in Archer's database. ☑ Symbol means all strong-evidence filters passed. ⛁ Symbol means these are known fusion partners in Archer's database. (Middle) Two runs were performed with independent lots of reference material and the reads spanning the junction are shown. All twelve (12) fusions present in the prototype were detected as high confidence calls on the ArcherDx Lung Thyroid panel. Note that the minimum spanning reads for a valid fusion call is five (5). (Right) Graphical representation of the two different lots tested on two different independent runs of ArcherDx FusionPlex Assay. The similarity in the number of spanning reads indicates Lot-to-Lot and Run-to-Run consistency.

Results on Oncomine and Ion AmpliSeq RNA Fusion Lung Cancer Research Panel:

Locus	Oncomine Variant Class	Genes	Read Counts
chr2: 42491871 - chr2: 29446394	Fusion	EML4(6) - ALK(20)	92
chr2: 42522656 - chr2: 29446394	Fusion	EML4(13) - ALK(20)	8380
chr10: 32306070 - chr10: 43609927	Fusion	KIF5B(24) - RET(11)	12561
chr10:51582939 - chr10: 43612031	Fusion	NCOA4(7) - RET(12)	2403
chr5: 149784242 - chr6: 117645578	Fusion	CD74(6) - ROS1(34)	513
chr4:25665952 - chr6:117645578	Fusion	SLC34A2(4) - ROS1(34)	410
chr1: 154142875 - chr1:156844362	Fusion	TPM3(7) - NTRK1(10)	14706
chr4:1808661 - chr7: 97991744	Fusion	FGFR3(17) - BAIAP2L(12)	3282
chr4: 1808661 - chr4: 1741428	Fusion	FGFR3(17) - TACC3(11)	22269
chr2: 113992970 - chr3: 12421202	Fusion	PAX8(9) - PPARG(2)	9346

Locus	Genes	Read Counts
chr2: 42522656 - chr2: 29446394	EML4(13) - ALK(20)	11328
chr10: 32306070 - chr10: 43609927	KIF5B(24) - RET(11)	15506
chr5: 149784242 - chr6: 117645578	CD74(6) - ROS1(34)	8126
chr4:25665952 - chr6:117645578	SLC34A2(4) - ROS1(34)	3600
chr1: 154142877 - chr1:156844362	TPM3(8) - NTRK1(10)	13728
chr3:100451516 – chr1: 156844362	TFG(5) - NTRK1(10)	7618

Figure 5: (Left) Report from Oncomine Comprehensive panel. This panel does not assay for NPM1-ALK, ETV6-NTRK3 orTFG-NTRK1, the remaining nine (9) mutations in the reference material were positively detected. (Right) Report from Ion AmpliSeq RNA Fusion Lung Cancer Research Panel, which assays for only six (6) of the fusions present in the reference material.

CONCLUSIONS

- Seraseq FFPE Fusion RNA Reference Material is a whole process control that monitors all steps from nucleic acid extraction, to library preparation, sequencing and data analysis.
- The planned format for the reference material is one FFPE curl per vial with multiple vials per kit. The average yield of total nucleic acid per curl was 273 ng. While the yield is limited, similar to patient samples, it is ample material to test by any of the available NGS panels.
- All twelve (12) fusions present in the Seraseq FFPE Fusion RNA Reference Material were detected as high-confidence calls on the ArcherDx Lung Thyroid panel.
- All fusions present in the reference material and assayed on the Oncomine Comprehensive panel were detected. All fusions present in the reference material and assayed on the more limited Ion AmpliSeq RNA Fusion Lung Cancer Research Panel were also detected.
- Embedded cell lines with genomic mutations give extremely high positive results. (In our hands, testing of a SLC34A2-ROS1 cell line embedded in FFPE gave greater than five thousand (>5,000) reads across the fusion junction on the Archer assay even after de-duplication.) Seraseq FFPE Fusion RNA Reference material gave low positive results, similar to patient samples.
- Two different lots of Seraseq FFPE Reference Material were prepared and tested on two different independent runs of ArcherDx Lung Fusion panel. The consistency of results from lot-to-lot and run-to-run shows the product's utility as a daily run control.
- Seraseq FFPE Fusion RNA Reference Material fulfills the need for highly multiplexed reference materials in FFPE format for quality control of NGS based detection of oncology RNA fusions. It will allow laboratories to challenge their assay system and verify performance at levels expected for patient samples.

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