HEAD-TO-HEAD COMPARISON OF TWO COMMERCIALLY AVAILABLE NEXT-GENERATION SEQUENCING TECHNOLOGIES THAT DETECT GENE FUSIONS IN NON-SMALL CELL LUNG CANCER

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SUMMARY

- The accurate detection of cancer-associated RNA fusions and other variants by next-generation sequencing requires reliable and integrated methods that can support a range of FFPE RNA inputs and quality.
- We compared two kits, the QuantideX® NGS RNA Lung Cancer Kit (RUO) (QX) and FusionPlex® ALK, RET, ROS1 v2 Kit (RUO) (FP), using 30-288 ng FFPE lung tumor RNA and admixtures down to ≤5% variant.
- Although both kits correctly identified all fusions when QC requirements were met, QX permitted >5-fold lower RNA inputs and achieved twice the rate of samples passing QC compared to FP.

INTRODUCTION

The reliable assessment of cancer-associated gene fusions by next-generation sequencing (NGS) is often challenged by low sample input quantity and quality, necessitating rigorous QC assessments to lend confidence to test results. Integration of these QC results with standardized reagents and bioinformatics is critical to assure consistent results from one laboratory to the next. Here we present a head-to-head comparison of two commercially available NGS kits that include reagents and software and are designed to detect non-small cell lung cancer (NSCLC)-related fusions.

MATERIALS AND METHODS

Total nucleic acid was isolated from 20 residual FFPE NSCLC biopsies using FormaPure™ FFPE Extraction Kit (Beckman Coulter), and RNA was quantified using the Qubit® RNA HS Assay Kit (Thermo Fisher Scientific). The isolates were processed into 24 unique samples, and NGS analysis was performed by an independent laboratory using the QuantideX® NGS RNA Lung Cancer Kit RUO (QX) (Asuragen) and the FusionPlex® ALK, RET, ROS1 v2 Kit (RUO) (FP) (using updated FusionPlex chemistries and protocols available Q4 2016, ArcherDX). Libraries were sequenced on the MiSeq® System (Illumina) and analyzed using each kit's bioinformatics software suite.

QuantideX NGS RNA Lung Cancer Kit (QX) Content

3' Fusion Genes	# of Fusions
ALK	53
ROS1	22
RET	12
FGFR3	7
NTRK3	3
NTRK1	4
NRG1	2
FGFR1	1
FGFR2	1
MBIP	1
PDGFRA	1

3'-5' imbalance
ALK
ROS1
RET
NTRK1
PDGFRA

Exon Skipping Event
MET e13:e14
MET e14:e15
MET e13:e15

mRNA Expression Targets
ABCB1
BRCA1
CD274 (PDL1)
CDKN2A
CTLA4
ERCC1
ESR1
FGFR1
FGFR2
IFNGR
ISG15
MET
MSLN
PDCD1
PDCD1LG2 (PDL2)
PTEN
RRM1
TDP1
TERT
TLE3
TOP1
TUBB3
TYMS
Endogenous Ctrls.

Archer FusionPlex ALK, RET, ROS1 v2 Kit (FP) Content

Fusion Driver Gene	Imbalance
ALK	*ALK*
ROS1	*ROS1*
RET	*RET*

Anchored Multiplex PCR does not require knowledge of fusion partner

Table 1. Content covered by the QuantideX NGS RNA Lung Cancer Kit (RUO) and the FusionPlex ALK, RET, ROS1 v2 Kit (RUO). The QX assay comprehensively covers known recurrent fusion breakpoints, whereas the FP assay is breakpoint agnostic and can detect novel fusions. The presence of a novel fusion can be detected with QX assay using 3'-5' imbalance markers, but information of the fusion partner will be lost. QX additionally covers *MET* exon 14 splice variants and expression data on 23 mRNA markers.

Sample ID	Qubit (ng/µL)	Input into RT (ng)	Annotation
NGS01	57.6	288	*ALK* Positive
NGS02	39.6	198	*ALK* Positive
NGS03	25.5	127.5	Fusion Negative
NGS04	15.0	75	Fusion Negative
NGS05	15.3	76.5	*ALK* Positive (NGS01) 15%†
NGS06	15.1	75.5	*ALK* Positive (NGS01) 5%†
NGS07	23.4	117	*ALK* Positive (NGS02) 15†
NGS08	23.1	115.5	*ALK* Positive (NGS02) 5%†
NGS09	31.8	159	None
NGS10	30.8	154	None
NGS11	12.7	63.5	None
NGS12	11.8	59	*ALK* Positive
NGS13	8.9	44.4	*ALK* Positive
NGS14	8.5	42.4	None
NGS15	6.1	30.5	None
NGS16	6.7	33.6	*ALK* Positive

Sample ID	Input into RT (ng)	Annotation
QC01	20	Pre-analytical QC only
QC02	20	Pre-analytical QC only
QC03	20	Pre-analytical QC only
QC04	20	Pre-analytical QC only
QC05	20	Pre-analytical QC only
QC06	20	Pre-analytical QC only
QC07	20	Pre-analytical QC only
QC08	20	Pre-analytical QC only

†Represents admixtures of fusion-positive FFPE into a fusion-negative FFPE.

Table 2. Sample set evaluated by both QX and FP assays. All samples met the minimum input requirements (>20 ng RNA input) as stated by each kit manufacturer. Sixteen FFPE samples were evaluated using inputs ranging from 30-288 ng RNA. Four of these 16 samples were admixtures of known fusion-positive and negative FFPE RNA formulated with 5-15% variant (measured by RNA mass) and 2-15% (measured by functional RNA copy number). An additional 8 FFPE samples were assessed by each kit's pre-analytical QC assay, but not NGS, using 20 ng RNA consistent with the minimum stated input requirements for both assays.

*Research Use Only – Not For Use In Diagnostic Procedures
Preliminary research data. The full performance characteristics of this assay have not yet been established.

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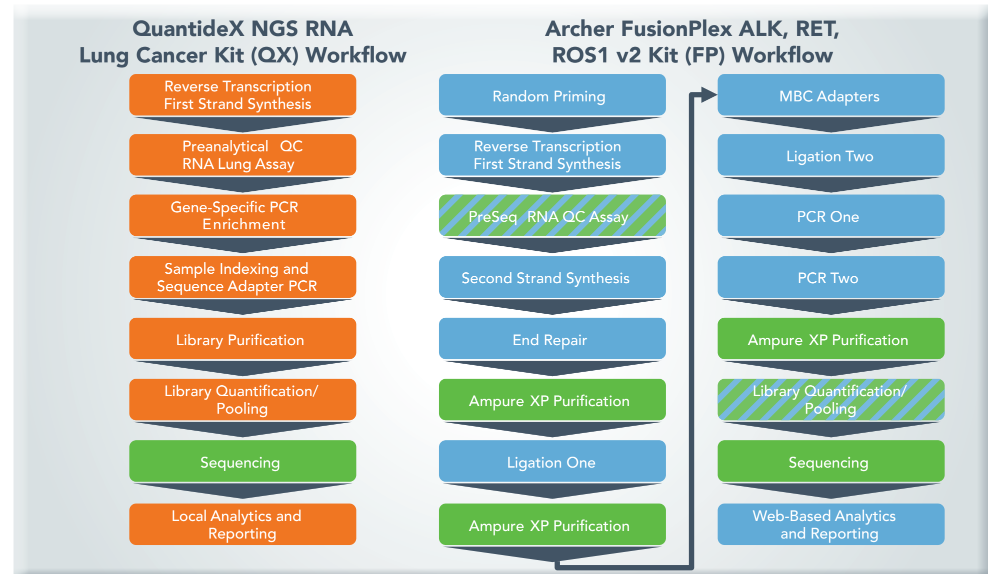

Figure 1. Workflow for both QX and FP assays. Due to the difference in enrichment strategies (targeted enrichment versus anchored multiplex PCR), the QX system requires fewer workflow steps (orange). However, the FP system provides components in premixed lyophilized form. Orange and blue fields represent steps with reagents provided in the respective kits, whereas green fields represent steps that require external reagents/ systems. Quantification steps in the FP workflow require 3rd party qPCR buffers to be purchased and are, therefore partially supplied (blue/green bands).

RESULTS

Sample ID	QuantideX NGS RNA Lung Cancer Kit			FusionPlex ALK, RET, ROS1 v2 Kit		
	Pre-analytical	Copies/µL	Post-analytical	Pre-analytical	PreSeq Cq	Post-analytical
NGS01	PASS	477	PASS	PASS	25.3	PASS
NGS02	PASS	426	PASS	PASS	25.9	PASS
NGS03	PASS	358	PASS	PASS	26.8	PASS
NGS04	PASS	285	PASS	PASS	28.2	PASS
NGS05	PASS	250	PASS	PASS	28.4	FAIL
NGS06	PASS	398	PASS	PASS	28.2	FAIL
NGS07	PASS	373	PASS	PASS	26.7	FAIL
NGS08	PASS	381	PASS	PASS	26.7	FAIL
NGS09	PASS	608	PASS	PASS	25.6	PASS
NGS10	PASS	717	PASS	PASS	25.1	PASS
NGS11	PASS	649	PASS	PASS	27.3	PASS
NGS12	PASS	152	PASS	PASS	29.2	FAIL
NGS13	PASS	112	PASS	PASS	28.9	FAIL
NGS14	PASS	118	PASS	PASS	30.2	FAIL
NGS15	PASS	92	PASS	PASS	30.2	FAIL
NGS16	PASS	285	PASS	PASS	27.9	PASS

Sample ID	QX Copies/µL	FP PreSeq Cq
QC01	177	30.1
QC02	139	30.7
QC03	164	31.1
QC04	61	32.0
QC05	142	29.9
QC06	197	29.4
QC07	60	32.2
QC08	89	32.0

Table 3. Pre- & post-analytical QC results for both QX and FP assays. The QX and FP pre-analytical QC results demonstrated a dose response agreement for sample quality. However, post-analytical QC pass rates were lower for FP than for QX. FP failed samples included samples with sufficiently high mass input and samples with pre-analytical QC scores of less than 28 Cq.

Sample ID	Reference Fusion	QuantideX NGS RNA Lung Cancer Kit		FusionPlex ALK, RET, ROS1 v2 Kit	
		Fusion Call	Imbalance	Strong Fusion Call	Weak Fusion Call
NGS01	*EML-ALK*	*EML-ALK*	*ALK*	*EML4-ALK*	None
NGS02	*EML-ALK*	*EML-ALK*	*ALK*	*EML4-ALK*	None
NGS03	None	None	-	None	None
NGS04	None	None	-	None	None
NGS05	*EML-ALK*	*EML-ALK*	Not Called (1910/0)*	None†	None†
NGS06	*EML-ALK*	*EML-ALK*	Not Called (1733/0)*	None†	None†
NGS07	*EML-ALK*	*EML-ALK*	Not Called (1612/0)*	None†	None†
NGS08	*EML-ALK*	*EML-ALK*	Not Called (359/37)*	None†	None†
NGS09	None	None	-	None	SPEG-*RET*
NGS10	None	None	-	None	LDLRAD2-*RET*
NGS11	None	None	-	None	None
NGS12	*EML-ALK*	*EML-ALK*	*ALK*	*EML4-ALK*†	None†
NGS13	*EML-ALK*	*EML-ALK*	*ALK*	None†	None†
NGS14	None	None	-	None†	None†
NGS15	None	None	-	None†	None†
NGS16	*EML-ALK*	*EML-ALK*	*ALK*	*EML4-ALK*	None

*Supporting coverage for imbalance shown as: (3' expression reads / 5' expression reads)
†Libraries failed post analytical QC

Imbalances not called due to threshold, but evidence of imbalance is present (imbalance calls not required to confirm fusion calls)

Missed calls in admixtures and in low input sample

Weak evidence calls noted as mispriming event

Table 4. Summary of NGS results using the QX and FP assays. The QX NGS results were 100% concordant with the reference results. False-positive and false-negative results are highlighted with ORANGE text. FP NGS results included five false-negative results, four of which occurred in the admixture samples. All missed calls were associated with libraries flagged as failed by post-analytical QC (marked with †). FP NGS also showed weak evidence flagged as mispriming for spurious fusions in two samples.

CONCLUSIONS

- Both kits included reagents for library prep, integrated multiple QC metrics to inform interpretations of results, and bioinformatics software for analysis.
- Both kits generated accurate calls when all QC criteria were satisfied.
- Using QX, all 16 sequenced sample libraries exceeded pre- and post-analytical QC requirements, and all fusions were correctly identified.
- For FP, only 8/16 samples passed post-analytical QC to generate reliable calls.
- Our results emphasize the importance of verifying minimum input requirements for each NGS technology and scrutinizing QC checkpoints to ensure reliable results.

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