ACCURATE AND REPRODUCIBLE DETECTION OF FUSIONS AND EXON SKIPPING EVENTS IN NSCLC-DERIVED SAMPLES USING A COMPREHENSIVE, TARGETED RNA-SEQ SYSTEM ACROSS MULTIPLE LABORATORIES

Abstract # 1764

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SUMMARY

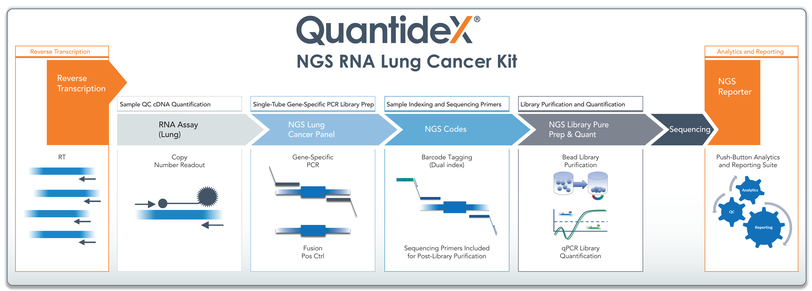
- Targeted RNA-Seq of expression markers in NSCLC FFPE tumors requires integrated reagents, protocols, and interpretive software that can ensure consistent results across laboratories.
- In this study, a comprehensive targeted NGS system, the QuantideX® NGS RNA Lung Cancer Kit (RUO)*, was evaluated in 5 laboratories to assess accuracy and reproducibility.
- The results demonstrated excellent agreement among laboratories for the detection of targeted fusions, 3′-5′ imbalances, and *MET* exon 14 skipping events, with actionable fusions reliably detected at <10 ng FFPE RNA.

INTRODUCTION

The reliable assessment of cancer-associated RNA fusions or exon skipping events in lung cancer requires optimized wetware, hardware, and software that can generate accurate results from one laboratory to the next. We evaluated a comprehensive system for targeted RNA-Seq that includes reagents for nucleic acid quantification, library prep, run controls, and companion bioinformatics software. The reproducibility of this system was evaluated in a multi-phase study design at 5 independent laboratories using a common set of cell-line and challenging FFPE tumor RNA samples.

MATERIALS AND METHODS

The QuantideX NGS RNA Lung Cancer Kit (RUO) was evaluated at Asuragen (Austin, TX), Jewish General Hospital (Montreal, CA), University of Kansas Medical Center (Kansas City, KS), Q[2] Solutions (Morrisville, NC), and one additional laboratory. A set of 30 total nucleic acid (TNA) samples derived from residual FFPE tumors and cancer cell lines along with two controls were used across the multiple sites.



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

Figure 1. Overview of QuantideX NGS RNA Lung Cancer Kit (RUO) from wet lab to dry bench analytics. The workflow is designed to minimize the number of steps, operator hands-on-time, and overall turn-around-time. The bundled QuantideX® Reporter (RUO) software package runs locally on a standard computer. The panel covers 107 recurrent gene fusions including *ALK*, *RET* and *ROS1*, *MET* ex14 skipping, 23 mRNA markers of prognostic and theranostic value and 3 internal control mRNA markers.

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

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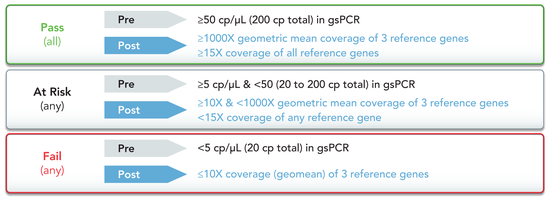

Figure 2. Criteria for assigning QC categories for libraries are integrated into the custom analytical pipeline. Results from the pre-analytical assessment of functional copies of a reference gene and post-NGS analytics of reference target coverage are combined for QC assignment.

	Sample ID	Test Set 1	Test Set 2	Test Set 3		Sample ID	Test Set 2
	N.Ctrl (NTC)	N.Ctrl (NTC)	N.Ctrl (NTC)	N.Ctrl (NTC)	N.Ctrl (NTC)	EVAL15	EVAL15
	P.Ctrl	P.Ctrl	P.Ctrl	P.Ctrl	P.Ctrl	EVAL16	EVAL16
Positive FFPE	EVAL01	EVAL01	EVAL01	EVAL01	EVAL01	EVAL17	EVAL17
Positive FFPE	EVAL02	EVAL02	EVAL02	EVAL02	EVAL02	EVAL18	EVAL18
	EVAL03	EVAL03	EVAL03	EVAL03	EVAL03	EVAL19	EVAL19
	EVAL04	EVAL04	EVAL04	EVAL04	EVAL04	EVAL20	EVAL20
	EVAL05	EVAL05	EVAL05	EVAL05	EVAL05	EVAL21	EVAL21
	EVAL06	EVAL06	EVAL06	EVAL06	EVAL06	EVAL22	EVAL22
Positive FFPE	EVAL07		EVAL07	EVAL07	EVAL07	EVAL23	EVAL23
Positive FFPE	EVAL08		EVAL08	EVAL08	EVAL08	EVAL24	EVAL24
Positive FFPE	EVAL09		EVAL09	EVAL09	EVAL09	EVAL25	EVAL25
	EVAL10		EVAL10	EVAL10	EVAL10	EVAL26	EVAL26
	EVAL11		EVAL11	EVAL11	EVAL11	EVAL27	EVAL27
Positive cell lines	EVAL12		EVAL12	EVAL12	EVAL12	EVAL28	EVAL28
Positive cell lines	EVAL13		EVAL13	EVAL13	EVAL13	EVAL29	EVAL29
Positive cell lines	EVAL14		EVAL14	EVAL14	EVAL14	EVAL30	EVAL30

Fusion positive FFPE input titration from 125 ng to 9 ng

Figure 3. Summary of the sample set and evaluation study design. The 30 evaluation samples comprised 13 fusion positives derived from 5 unique clinical FFPE specimens and 2 cell lines, including a 6-point series of a fusion-positive FFPE titrated down to 9 ng mass input, and a cell-line-derived *MET* exon 14 variant. Blinded samples were aliquoted and distributed to the independent laboratories. Test set 1 was evaluated with an on-site trainer (Asuragen), and all sites were trained in less than 2 days. Sites 2-4 ran all test sets, whereas site 1 only ran set 2, and site 5 only ran set 1 and a subset of set 2.

RESULTS

QC Summary

	Sample Library "Pass"	Sample Library "At Risk"	Sample Library "Fail"
Site 1	30/30	0/30	0/30
Site 2	56/58	0/58	2/58
Site 3	54/64	10/64	0/64
Site 4	59/64	5/64	0/64
Site 5	11/20	9/20	0/20
Total	210/236	24/236	2/236
PosCtrl	14/14	0/14	0/14
NegCtrl	0/14	0/14	14/14

Table 1. Summary of QC results assigned to NGS libraries across the 5 sites. Left) Number of libraries, excluding controls, assigned to each QC category. One failed library was associated with operator error. Another failed library was triggered by a reference gene dropout. **Bottom)** The 24 at-risk libraries were caused by low seeding densities on the MiSeq coupled with a large number of pooled libraries loaded onto the flow cell.

Runs with "At Risk" Library	Flow Cell	Cluster Density	# Libraries	Reads/Library	# At Risk
Site 3 Run 2	V3	375 K/mm²	32	202K	8
Site 3 Run 3	V3	563 K/mm²	32	279K	2
Site 4 Run 2	V3	430 K/mm²	32	283K	5
Site 5 Run 2	V2	N/A	18 + 5	155K	9

Targeted Fusions

		Reference: Pos	Reference: Neg
Evaluation	Pos	117	0(8†)
Evaluation	Neg	0	107

	Pass only	Including "At Risk"	All
Total Libraries	224	248	264
True Positives	117 (114)†	130 (126)†	131 (127)†
True Negatives	107 (102)†	118 (113)†	132 (127)†
Missed Calls	0	0	1
Unexpected Calls	0 (8)†	0 (9)†	0 (9)†

	Pass only	Including "At Risk"	All
Sensitivity	100.0%	100.0%	99.2%
Specificity	92.7%†	92.6%†	93.4%†
PPV	93.4%†	93.3%†	93.4%†
NPV	100.0%	100.0%	99.2%

Table 2. Analytical summary of fusion and splice variant calls. Calls across the 5 sites were in strong agreement with the reference results. One missed call was due to a failed library. Unexpected calls (†) were traced to neighboring well contamination (6/9) or stock tube contamination (3/9). Detected fusion calls across the 264 sample libraries were limited to variants that were known to be present in positive samples.

3′-5′ Imbalance

		Reference: Pos	Reference: Neg
Evaluation	Pos	84	0
Evaluation	Neg	0	140

	Pass only	Including "At Risk"	All
Total Libraries	224	248	264
True Positives	84	95	95
True Negatives	140	152	166
Missed Calls	0	1	2
Unexpected Targets	0	0	1

	Pass only	Including "At Risk"	All
Sensitivity	100.0%	99.0%	98.0%
Specificity	100.0%	100.0%	99.4%
PPV	100.0%	100.0%	99.0%
NPV	100.0%	99.3%	98.8%

Table 3. Analytical summary of fusion 3′-5′ imbalance calls. Calls were consistent with reference results, and resulted in perfect agreement for passing libraries. The two missed calls occurred in either an at-risk or failed library. The single unexpected call was observed in a failed library.

CONCLUSIONS

- Training to use the QuantideX NGS RNA Lung Cancer Kit (RUO) was achieved in less than two days.
- Both pre-analytical (amplifiable copy number) and post-analytic NGS-based QC criteria were integrated into the bioinformatic software to help assure reliable variant calls.
- "At-Risk" libraries were linked to underseeding of the NGS flow cell.
- A handful of unexpected fusion calls mapped to neighboring samples with high expression levels (cell lines) underscoring the importance of best practices for contamination control.
- Reproducible targeted fusion, 3′-5′ imbalance, and *MET* exon 14 skipping calls were observed across all 5 laboratories using a diverse sample set of cell-lines and FFPE tumor biopsies.

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