A Simple and Versatile Next-Generation Sequencing Technology for Co-Detection of RNA Structural Variants and DNA Mutations in Lung Cancer

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Summary

- Non-small cell lung cancer (NSCLC) demonstrates remarkable molecular diversity, highlighting the need to detect both RNA and DNA markers for personalized medicine.
- We describe the detection of both RNA and DNA variants by combining QuantideX® NGS RNA Lung Cancer Kit* and QuantideX® NGS DNA Hotspot 21 Kit*† libraries on a single MiSeq flow cell.
- Two different library pooling methods were used to accommodate up to 24 DNA and 24 RNA libraries prepared from FFPE tumor samples. For both methods, all libraries produced variant calls consistent with the expected results using a streamlined workflow that required less than three days from sample-to-answer.

Introduction

Mutational categories associated with NSCLC initiation and progression include single-nucleotide variants (SNVs), DNA insertions and deletions (INDELs) and copy number variations (CNVs), as well as RNA fusions and post-transcriptional splicing variants. This diversity challenges profiling of cancer samples because not all genetic alterations are addressable by the same molecular methods. For instance, gene fusions may be assessed using fluorescence in situ hybridization (FISH) break-apart probes, immunohistochemistry (IHC) staining, and, more recently, next-generation sequencing (NGS), yet still different assays and technologies may be used to interrogate SNVs, INDELs, and/or CNVs. Therefore, comprehensive molecular analysis often requires the use of multiple test methods. Even if the same method is used, such as NGS, different workflows, detection modalities, and analyses are often required. This fragmentation is inefficient, creating additional material and labor costs, and erecting barriers to broader adoption. To address these issues, we describe a targeted NGS approach with system-level integration that can detect RNA and DNA variants using a single, unified workflow.

1A

QuantideX® NGS RNA Lung Cancer Kit*					
3' Fusion Genes	# of Fusions	3'/5' Imbalance		Exon Skipping Event	
ALK	53	*ALK*		*MET e13:e14*	
ROS1	22	*ROS1*		*MET e14:e15*	
RET	12	*RET*		*MET e13:e15*	
FGFR3	7	*NTRK1*			
NTRK1	4	mRNA Expression Targets			
NTRK3	3	*ABCB1*	*ESR1*	*MSLN*	*TERT*
NRG1	2	*BRCA1*	*FGFR1*	*PDCD1*	*TLE3*
FGFR1	1	*CD274*[1]	*FGFR2*	*PDCD1LG2*[2]	*TOP1*
FGFR2	1	*CDKN2A*	*IFNGR*	*PTEN*	*TUBB3*
MBIP	1	*CTLA4*	*ISG15*	*RRM1*	*TYMS*
PDGFRA	1	*ERCC1*	*MET*	*TDP1*	Controls

[1]PD-L1
[2]PD-L2

1B

QuantideX® NGS DNA Hotspot 21 Kit*†		
Gene Coverage		
ABL1	*FGFR1*	*KIT*
ALK1	*FGFR3*	*KRAS*
AKT1	*FLT3*	*MET*
AKT2	*HRAS*	*NRAS*
BRAF	*IDH1*	*PDGFRA*
EGFR	*IDH2*	*PIK3CA*
ERBB2	*JAK2*	*RET*

Table 1. QuantideX® NGS Panels. A) The QuantideX® NGS RNA Lung Cancer Kit interrogates fusions, exon skipping variants, and expression targets frequently observed in NSCLC. The panel targets 107 NSCLC-relevant fusions, *ALK* , *RET*, *ROS1* and *NTRK1* 3'/5' imbalances, *MET* exon 14 skipping events (e13:e15), 23 mRNA targets, and three endogenous control transcripts. **B)** The QuantideX® NGS DNA Hotspot 21 Kit interrogates 46 gene regions within 21 genes with high clinical significance and potentially actionable content in various human cancers. The panel reports >1,500 known COSMIC variants, including SNVs and small INDELs.

*RUO. Not for use in diagnostic procedures. †CE marked. For US export only. Conflict of Interest Disclosure: All authors have the financial relationship to disclose: Employment by Asuragen.
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Materials and Methods

Total nucleic acid (TNA) and DNA was isolated from formalin-fixed, paraffin-embedded (FFPE) NSCLC biopsies. Sequencing libraries were prepared by using the QuantideX® NGS RNA Lung Cancer Kit or the QuantideX® NGS DNA Hotspot 21 Kit, and the resultant libraries were combined following the library pooling strategies below. The final concentration value for both pooling method was 2.5 nM for Illumina MiSeq® System loading. Study 1 assessed equimolar input: Sixteen DNA Hotspot 21 libraries, eight RNA Lung Cancer libraries, and controls were mixed in equimolar amounts based on concentrations determined by Library Quant analysis (included in kit). The pool was loaded onto a single flow cell for sequencing analysis. Study 2 assessed weighted input: Twenty-two DNA Hotspot 21 libraries, twenty-two RNA Lung Cancer libraries, and a positive control (PC) and no template control (NTC) for each kit were mixed in separate RNA- and DNA-based pools. Each pool was quantified and then combined at a 3:1 ratio of RNA:DNA. The pool was loaded onto a single flow cell for sequencing analysis. Data analysis was performed using QuantideX® NGS Reporter, an integrated informatics processing and data reporting pipeline that incorporates pre-analytical QC data into the variant calling algorithm.

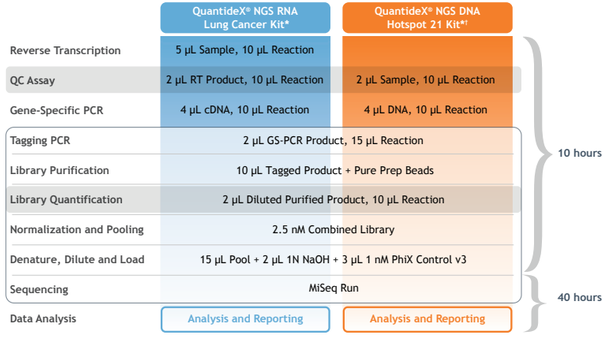


Figure 1. QuantideX® RNA and DNA Co-loading Workflow Overview.

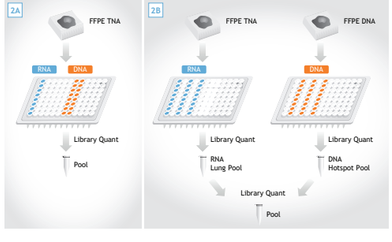
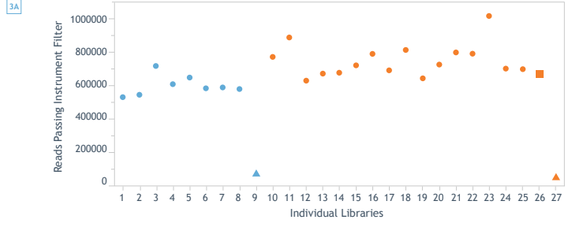


Figure 2. QuantideX® RNA and DNA Library Prep Workflow for Co-loading. A) Method 1: PCR for DNA and RNA libraries can be performed on the same plate and processed simultaneously. When 24 or fewer total libraries were analyzed, DNA and RNA library can be normalized and pooled into a single pool. **B)** Method 2: When up to 48 libraries are analyzed, the alternative strategy is to normalize RNA and DNA libraries separately. RNA and DNA pools can be combined in a 3:1 ratio to ensure optimal coverage for both RNA and DNA libraries.

Results

3A



3B

Figure 3. Reads Passing Instrument Filter. Paired-End Reads Passing Instrument Filter for RNA Lung Panel Libraries vs DNA Hotspot 21 Libraries. For illustrative purposes, NTC libraries were included; these demonstrated low coverage as expected. **A)** In study 1, the number of reads passing instrument filters was >500,000 for each of the eight RNA Lung Cancer libraries and the 16 DNA Hotspot 21 libraries as well as the DNA PC. **B)** In study 2, the average number of reads for the RNA Lung panel libraries was 383,819 compared to 129,840 for the DNA Hotspot 21 libraries. Coverage across libraries within each panel was uniform except for the RNA Lung Cancer PC. This PC showed elevated coverage due to a high library concentration and the minimum volume input requirement of 2 ul for pooling.

4A Study 1

RNA Lung Cancer Panel		
Library	Fusion or Variant	Imbalance
Library 1	*EML4-ALK*	*ALK*
Library 2	*MET 13-15*	-
Library 3	*CCDC6-RET*	*RET*
Library 4	*CD74-NRG1*	-
Library 5-8	Wild-type	-

DNA Hotspot 21 Panel	
Library	Variant
Library 9	*KRAS* p.G12V
Library 10	*PIK3CA* p.N1044K • *EGFR* p.E746_A750del
Library 11	*KRAS* p.G12C
Library 12	*EGFR* p.D770_N771insSVD
Library 13	*EGFR* p.L858R
Library 14	*PIK3CA* p.E542K
Library 15	*PIK3CA* p.E545K • *PIK3CA* p.H1047R
Library 16	*PIK3CA* p.E542K • *BRAF* p.V600E
Library 17	*KRAS* p.G12C • *PIK3CA* p.E545K
Library 18	*BRAF* p.V600E
Library 19	*NRAS* p.Q61K
Library 20	*KRAS* p.G12A
Library 21-24	Wild-type

4B Study 2

RNA Lung Cancer Panel		
Library	Fusion or Variant	Imbalance
Library 30	*EML4-ALK*	*ALK*
Library 31	*KIF58-RET*	*RET*
Library 32	*EML4-ALK*	-
Library 33	*MET 13-15*	-
Library 34	*MET 13-15*	-
Library 35	*CCDC6-RET*	*RET*
Library 36	*CD74-NRG1*	-
Library 37-52	Wild-type	-

DNA Hotspot 21 Panel			
Library	Variant	Library	Variant
Library 53	*EGFR* p.G719A	Library 64	*KRAS* p.G13D
Library 54	*KRAS* p.G12V, *IDH1* p.R132L *JAK2* p.V617F	Library 65	*KRAS* p.G12D *PIK3CA* p.E542K
Library 55	*KRAS* p.A146T	Library 66	*KRAS* p.G12V • *PIK3CA* p.E545K
Library 56	*EGFR* p.N771_P772insT	Library 67	*KRAS* p.G12D
Library 57	*KRAS* p.G12D • *PIK3CA* p.Q546K	Library 68	*KRAS* p.Q61H
Library 58	*KRAS* p.G12V	Library 69	*KRAS* p.G12C
Library 59	*KRAS* p.G12A	Library 70	*KRAS* p.G12D
Library 60	*KRAS* p.G12C	Library 71	*KRAS* p.G12V
Library 61	*KRAS* p.G12D	Library 72	*KRAS* p.G12A
Library 62	*KRAS* p.G12D	Library 73	*KRAS* p.G12C • *JAK2* p.V617F
Library 63	*KRAS* p.G12D • *PIK3CA* p.E545K	Library 74	*EGFR* p.H773_V774insAH

Table 2. Fusion, Imbalance and Variant Calls. All RNA Lung Cancer libraries and DNA Hotspot 21 libraries were 100% concordant with expected results (mutations in *EGFR*, *RAS*, *PIK3CA*, *JAK2*, *IDH1* and *BRAF*, along with fusions in *ALK*, *RET*, and *NRG1* and skipped *MET*ex14), including wild-type and controls (positive and no template). Variant calls were 100% concordant with independent results and included. **A)** In study 1, 16 DNA libraries and eight RNA libraries were analyzed along with controls. RNA fusions were called with 189 to 11,274 reads and DNA mutations were detected down to 5% allele frequency. No call was made for the four negative RNA samples or the three negative DNA samples. **B)** In study 2, 22 DNA libraries and 22 RNA libraries were analyzed along with controls. RNA fusions were called with 82 to 11,965 reads and DNA mutations were detected down to 5% allele frequency. No call was made for the 16 negative RNA samples.

Conclusions

- QuantideX® NGS RNA Lung Cancer and DNA Hotspot 21 libraries could be processed simultaneously and pooled sample-by-sample to generate accurate results when no more than 24 samples were tested. To support up to 48 libraries per flow cell, libraries from the two panels could be formulated in separate RNA- and DNA-based pools and then combined at a 3:1 ratio.
- The results demonstrate that QuantideX® NGS libraries can be combined to accurately detect multiple categories of FFPE RNA and DNA variants in less than three days.
- The simplicity and speed of the approach, coupled with the standardized workflow, has potential to increase the accessibility of NGS analysis and accelerate turnaround time for NSCLC molecular results.

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