Analytical Validation Of The QuantideX® NGS DNA Hotspot 21 Kit, A Diagnostic NGS System for the Detection of Actionable Mutations in FFPE Tumors

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Summary

- The QuantideX® NGS DNA Hotspot 21 Kit*,** interrogates 46 cancer hotspot regions and >1500 COSMIC mutations within 21 genes identified as clinically actionable in NCCN and ESMO guidelines.
- This kit was validated with residual formalin-fixed, paraffin-embedded (FFPE) DNA samples from lung, breast, colorectal cancer and melanoma using an NGS-in-a-Box™ workflow, reagents, and software that span pre-analytical, analytical, and post-analytical assay phases.
- The data demonstrate that the QuantideX NGS DNA Hotspot 21 Kit is an accurate *in vitro* diagnostic system with high sensitivity and specificity for the detection of FFPE tumor biopsy DNA using the fully integrated data analysis and reporting software, QuantideX NGS Reporter.

Introduction

Accurate multi-gene molecular diagnostics are critical for effective precision medicine in cancer. We describe analytical validation studies of the QuantideX NGS DNA Hotspot 21 Kit*,** designed for targeted sequencing of cancer-related mutation hotspots in 21 oncogenes (*ABL1, AKT1, AKT2, ALK1, BRAF, EGFR, ERBB2, FGFR1, FGFR3, FLT3, HRAS, IDH1, IDH2, JAK2, KIT, KRAS, MET, NRAS, PDGFRA, PIK3CA and RET*) using FFPE tumor DNA. This assay integrates reagents and bioinformatics software to report clinically-actionable variants and aid in the molecular diagnosis of cancer patients to predict sensitivity to a growing number of therapeutic agents.

Gene	Transcript ID	Codons Covered	Gene	Transcript ID	Codons Covered
ABL1	ENST00000318560	248-258	*HRAS*	ENST00000397596	9-20
		304-318			59-76
AKT1	ENST00000349310	17-27			113-121
AKT2	ENST00000392038	17-26	*IDH1*	ENST00000345146	122-134
ALK	ENST00000389048	1174-1195	*IDH2*	ENST00000330062	138-145
		1272-1278			163-174
BRAF	ENST00000288602	591-612	*JAK2*	ENST00000381652	607-618
		465-474	*KIT*	ENST00000288135	557-579
EGFR	ENST00000275493	709-721			815-826
		767-798	*KRAS*	ENST00000311936	4-15
		849-861			55-65
		737-761			137-148
		486-493			104-118
ERBB2	ENST00000269571	755-769	*MET*	ENST00000318493	1245-1256
		774-788	*NRAS*	ENST00000369535	9-20
		839-847			55-67
		877-883			110-119
FGFR1	ENST00000447712	250-262			144-150
		123-136	*PDGFRA*	ENST00000257290	560-572
FGFR3	ENST00000440486	248-260			840-852
		638-653	*PIK3CA*	ENST00000263967	540-551
		362-374			1038-1049
FLT3	ENST00000241453	829-840	*RET*	ENST00000355710	916-926

Table 1. QuantideX NGS DNA Hotspot 21 Panel Gene and Codon Coverage.

Materials and Methods

Residual clinical FFPE tumor biopsies and commercially available reference materials were used to assess assay performance. Samples were processed with QuantideX NGS DNA Hotspot 21 Kit reagents, including initial determination of amplifiable DNA copy number using the included DNA Assay (a qPCR-based QC assay) to guide inputs for library preparation and inform the variant analysis pipeline using sample-specific data. The single-tube target enrichment of 4 to 48 samples was performed on multiple thermal cycler platforms and libraries were quantified on multiple qPCR platforms. Pooled libraries were sequenced on the MiSeq® or MiSeqDx® systems (Illumina®) and raw FASTQ files were analyzed using QuantideX NGS Reporter software to identify SNVs and INDELs. Mutation status was determined by the Oncomine™ Focus Assay (Thermo Fisher Scientific Inc). Analytical validation studies were designed in compliance with CLSI and ISO guidelines.

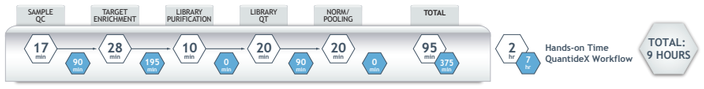

Figure 1. Overview of QuantideX NGS DNA Hotspot 21 Workflow. The workflow is designed to minimize the number of steps, operator hands-on time, and overall turn-around-time.

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Results

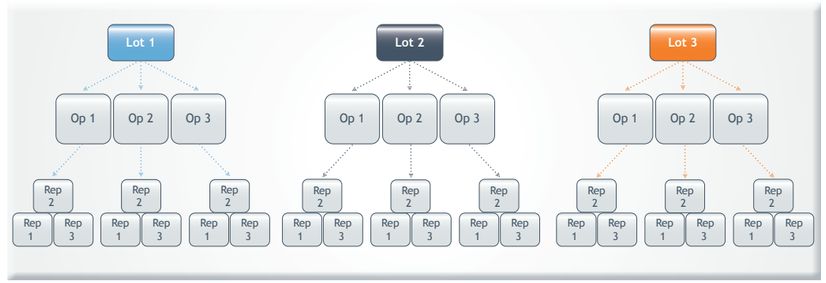

Figure 2. Precision Study. Precision of the device was assessed by testing six variant positive FFPE DNA samples in triplicate using three reagent lots by three operators on three MiSeqDx instruments.

Variant Group	PPA	95% CI	
		Upper Limit	Lower Limit
SNV	100%	100%	98.25%
INDEL	100%	100%	93.36%

Table 2. Precision Study. The point estimation of positive percent agreement (PPA) is 100% for SNVs and 100% for INDELs. The lower confidence interval of PPA estimate is 98.25% for SNVs and 93.36% for INDELs. High precision in average read depth as well as amplicon coverage uniformity across three manufactured lots was observed (data not shown).

Sample	n	True Positive	False Positive	True Negative	False Negative	OPA (%)	NPA (%)	NPV (%)
S0161773	20	0	0	20	0	100	100	100
S0161774	20	0	0	20	0	100	100	100
S0161775	20	0	0	20	0	100	100	100

Table 3. Limit of Blank (LoB) Study. Three wild-type FFPE DNA samples were tested by a single operator using nominal copy number input (approximately 6,000 copies) and a single lot of reagents, with twenty library replicates generated from each DNA sample. There were no positive mutation calls, resulting in a 0% false positive rate and a LoB of zero in wild-type ("blank") FFPE DNA samples.

Variant	Variant Class	Target VAF (OFA)	Mean VAF	Positive Call (n=20)	Percent Positive	LoD (VAF)
KRAS p.G12D	SNV	0.109	0.127	20	100	0.049
		0.082	0.086	20	100	
		0.055	0.058	20	100	
		0.038	0.045	15	75	
BRAF p.V600E	SNV	0.124	0.130	20	100	0.048
		0.093	0.098	20	100	
		0.062	0.056	20	100	
		0.043	0.045	19	95	
EGFR p.L858R	SNV	0.069	0.111	20	100	0.037
		0.052	0.085	20	100	
		0.035	0.051	20	100	
		0.024	0.033	17	85	
PIK3CA p.E542K	SNV	0.096	0.124	20	100	0.064
		0.072	0.087	20	100	
		0.048	0.052	17	85	
		0.034	0.041	16	80	
EGFR p.E746_A750del	Deletion	0.217	0.243	20	100	0.0616
		0.145	0.159	20	100	
		0.109	0.086	20	100	
		0.072	0.061	20	100	
EGFR p.K745insIPVAIK	Insertion	0.159	0.210	20	100	0.0482
		0.106	0.130	20	100	
		0.080	0.072	20	100	
		0.053	0.049	20	100	

Table 4. Limit of Detection (LoD) Study. Four FFPE DNA samples containing 4 SNVs, 1 insertion, and 1 deletion were titrated to 4 VAF levels using functional DNA copy number inputs near the lowest recommended input (250–650 copies). Each titrated sample was tested 20 times across 5 MiSeq runs by one operator using a single lot of reagents on 3 different MiSeqDx machines. The resultant LoD values ranged from 3.7% to 6.4% for the tested SNVs, 6.2% for the deletion, and 4.8% for the insertion.

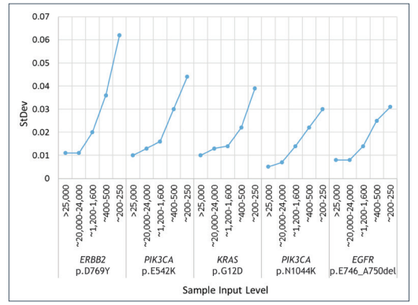

Figure 3. Measuring Range Study. Three variant-positive clinical FFPE DNA samples were tested by two operators using three reagent lots at five input levels incorporating DNA inputs within the nominal input range (400, 1,600 and 24,000 copies), less than the nominal input range (200 copies), and more than the nominal input range (25,000 copies). All of the replicates generated the correct mutation call within the DNA input range of 400 copies to 24,000 copies (data not shown), and standard deviations are less than 0.05 within the recommended DNA input range.

SNV						
	Comparator Method (+)	Comparator Method (-)	PPA (95% CI)		NPA (95% CI)	
Hotspot 21 (+)	77	1	95.06%	87.98%-98.06%	99.99%	99.99%-100%
Hotspot 21 (-)	4	45,132				
Total SNVs		45,214				
INDEL						
	Comparator Method (+)	**Comparator Method (-)**	**PPA (95% CI)**		**NPA (95% CI)**	
Hotspot 21 (+)	4	2	100.0%	51.01%-100%	99.99%	99.96%-100%
Hotspot 21 (-)	0	19,234				
Total INDELs		19,240				
All MOIs						
	Comparator Method (+)	**Comparator Method (-)**	**PPA (95% CI)**		**NPA (95% CI)**	
Hotspot 21 (+)	81	3	95.29%	88.52%-98.15%	99.99%	99.99%-100%
Hotspot 21 (-)	4	64,366				
Total MOIs		64,454				

Table 5. Accuracy Study. Fifty-seven residual clinical FFPE DNA samples and 20 contrived FFPE DNA samples were analyzed in singleton. Variant call results were analyzed against the comparator method results. In FFPE DNA samples, Positive Percent Agreement (PPA) was 95.06% for SNVs and 100% for small INDELs; Negative Percent Agreement (NPA) was 99.99% for both SNVs and small INDELs.

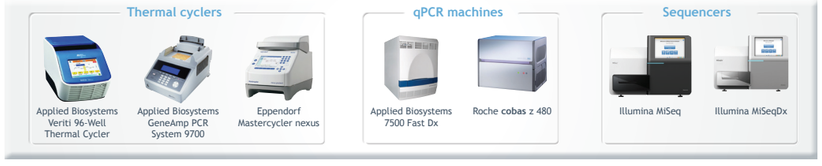

Figure 4. Equivalency Studies. The kit exhibited accurate and reproducible MiSeq data from library preparation across three common laboratory thermal cyclers (Applied Biosystems™ GeneAmp® PCR System 9700 and Veriti™ 96-Well Thermal Cyclers, and the Eppendorf Mastercycler® nexus). The qPCR assays are compatible with multiple commercially available qPCR machines (Applied Biosystems 7500 Fast Dx and Roche **cobas** z 480 system). The kit was validated on the Illumina MiSeq and MiSeqDx Systems.

Conclusions

- We describe a complete NGS diagnostic system that can detect >1500 COSMIC mutations, including SNVs and INDELs, located within hotspot regions of 21 oncogenes.
- The kit enables "sample to sequencer" in less than 9 hours, and the workflow ports directly into sequence analysis via the QuantideX NGS Reporter software.
- QuantideX NGS DNA Hotspot 21 validation studies exhibit high PPA for precision and accuracy, high sensitivity for SNVs (5%) and INDELs (10%), an LoB of zero for blank samples, robustness across a large DNA input range, and reproducibility across multiple thermal cyclers, qPCR machines, and sequencers.

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