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Performance Characteristics of a PCR Assay for the Detection of KRAS Mutations in Formalin-Fixed Paraffin-Embedded Tissue Samples of Non-Small Cell Lung Cancer

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Abstract

Introduction: Approximately 25% of non-small cell lung cancer (NSCLC) tumors contain mutations in *KRAS*. These tumors are insensitive to therapy directed against the epidermal growth factor receptor and appear to be resistant to adjuvant chemotherapy. The current study demonstrates the performance of the cobas® *KRAS* Mutation Test (cobas test), a TaqMelt polymerase chain reaction (PCR) assay designed to detect 19 mutations in codons 12, 13, and 61.

Methods: To reflect real-world testing conditions, the study used formalin-fixed, paraffin-embedded tissue (FFPET) NSCLC samples for the predominant mutations. DNA blends of cell lines and plasmids were used where FFPET samples were not available.

Results: In the limit of detection study, a correct mutation call rate of ≥95% was obtained with approximately 5% mutant sequences using 3.1-50.0 ng DNA per PCR reaction. Mutation levels as low as 2.4% consistently yielded correct mutation calls when 50.0 ng DNA was used for testing. The cobas test performance was compared to Sanger sequencing in a method correlation using two cobas reagent lots and 194 specimens. After resolution of discordant results using 454 sequencing, the positive, negative, and overall percent agreement for mutations in codons 12/13 and 61 between the cobas test and sequencing ranged from 97.0% to 100%. Mutation detection was 100% reproducible and showed greater specificity than Sanger sequencing. Test performance was not impaired by the presence of interfering substances or clinically relevant microbes, and inclusivity testing demonstrated the kit's ability to detect rare mutations.

Conclusions: The cobas test is a robust, sensitive, and reproducible method for detecting *KRAS* mutations in FFPET tumor samples from NSCLC patients.

Keywords: Non-small cell lung cancer; Polymerase chain reaction; *KRAS*; Formalin-fixed; Paraffin-embedded

Introduction

KRAS is an intracellular signal transduction protein that has GTPase activity and functions downstream of the epidermal growth factor receptor (*EGFR*) [1]. It normally functions as a switch to control intracellular signaling. In non-small cell lung cancer (NSCLC), approximately 90% of *KRAS* activating mutations occur in codon 12, with codons 13, and 61 accounting for the next most common mutation sites [2]. Activating mutations in *KRAS* prevent GTP hydrolysis to GDP, thus inducing constitutive downstream signaling, and account for the gene's function as an oncogenic driver [3,4]. In NSCLC, *KRAS* mutations are found in approximately 30% of adenocarcinomas and 5% of squamous cell tumors, and they occur more frequently in Caucasians and current or former smokers [5,6].

Effective therapies that directly target mutated *KRAS* have proven elusive. Nonetheless, assessing the mutational status of *KRAS* is important for several reasons. As KRAS acts downstream of EGFR, tumors with *KRAS* activating mutations are resistant to EGFR inhibition. Thus the presence of *KRAS* mutations in lung tumors contraindicates treatment with anti-EGFR tyrosine kinase inhibitors (TKIs) [7]. Recent studies suggest that KRAS also confers resistance to adjuvant chemotherapy [6]. Moreover, promising clinical results have recently emerged demonstrating the clinical utility of targeting MEK, a signaling molecule that acts downstream of KRAS. In a randomized phase 2 study, Jänne and colleagues demonstrated an extension of progression-free survival (PFS) when the MEK inhibitor, selumetinib, was combined with docetaxel for second-line treatment of NSCLC [8]. The study evenly randomized 87 patients with mutated KRAS to receive docetaxel plus either selumetinib or placebo. Selumetinib added to docetaxel increased the overall response rate (ORR) to 37% versus 0% for placebo (*P*<0.001) and improved median PFS to 5.3 months versus 2.1 months for placebo (*P*=0`.014; HR, 0.58). Although not significant, median overall survival (OS) also improved in the selumetinib arm (9.4 months vs 5.2 months; HR, 0.80; *P*=0.21).With MEK established as an important target, other MEK inhibitors are being pursued for treating NSCLC. Trametinib is one such novel molecule that has shown promise in melanoma. It is currently approved for treating melanoma and has shown efficacy when treating NSCLC [9,10]. Cobimetinib (GDC-0973), which also inhibits MEK, has demonstrated efficacy and acceptable tolerability in a phase III trial in combination with vemurafenib in *BRAF* V600-mutated, treatment-naive, metastatic

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melanoma [11]. Cobimetinib is also being investigated in early phase trials in combination with several investigational agents in NSCLC and other tumor types, as well as in cancer patients with a mutant *KRAS* gene [12].

Given the importance of KRAS mutations in the mitogen-activated protein kinase (MAPK) pathway, there is a need for reliable testing methods to determine *KRAS* mutation status that could eventually predict response to MEK inhibitors or determine eligibility for drugs that target other molecules in the MAPK pathway that are upstream or downstream of KRAS. The current study was undertaken to evaluate the cobas® KRAS Mutation Test (cobas test) for its ability to detect common mutations in *KRAS* codons 12, 13, and 61 in formalin-fixed, paraffin-embedded tissue (FFPET) from NSCLC tumors. The cobas test uses TaqMelt real-time polymerase chain reaction (PCR) technology, a patented method that uses a dual-labelled probe to differentiate between mutated and wild-type DNA sequences with high sensitivity and specificity. To assess clinical utility, the cobas test was evaluated for limit of detection (LOD), repeatability, correlation to Sanger sequencing, inclusivity, and the effect of substances in the sample that could potentially interfere with performance.

Material and Methods

Tumor specimens

FFPET specimens from NSCLC tumors were purchased from commercial vendors (ABS Bio, Willington, DE, Asterand, Detroit, MI, Bioserve, Beltsville, MD, Conversant Bio, Huntsville, AL, Cureline Inc., Burlingame, CA, Cytomex, Lexington, MA, Discovery Life Sciences, Inc., Los Osos, CA, ILS Bio, Chestertown, MD, Indivumed Inc., Kensington, MD, and Protogenex, Culver City, CA) based on their origin of tissue and histological characteristics. Twelve adjacent 5-micron sections of each FFPET specimen were cut on a microtome. The first and the twelfth sections were stained with H&E and examined by a pathologist to confirm that they were NSCLC specimens and to assess the tumor content of each specimen. NSCLC cell lines with mutations in the KRAS gene (NCI-H23 with GGT>TGT in codon 12, NCI-H1734 with GGC>TGC in codon 13, and NCI-H460 with CAA>CAT in codon 61) were obtained from the American Type Culture Collection (www.atcc.org).

cobas® KRAS mutation test

The cobas test is a TaqMelt™-based PCR assay consisting of two major processes: (1) manual specimen preparation to obtain genomic DNA from a single 5-micron section from separate FFPET samples; and (2) PCR amplification and detection of target DNA using complementary primer pairs and two oligonucleotide probes labeled with a fluorescent dye. One probe is designed to detect the *KRAS* codon 12/13 sequence in exon 2, and the other probe is designed to detect the *KRAS* codon 61 sequence in exon 3 of the *KRAS* gene. Mutation detection is achieved by melting curve analysis using the cobas® z 480 analyzer. A mutant control, a negative control, and a calibrator are included in each run to confirm the validity of the run. The analysis of raw data and result reporting are fully automated. The DNA isolation, amplification/detection protocol, qPCR validation and result reporting can be performed in less than 8 hours and have been described previously [13].

Briefly, DNA is extracted from a single, deparaffinized 5 µm section of FFPET, quantified by Nanodrop (Thermo Scientific, Waltham, MA), and diluted with sample diluent to 2 ng/µL following the standard procedure described in the cobas test instructions for use [13]. The target DNA is amplified and detected on the cobas® z 480 analyzer using the amplification and detection reagents provided in the cobas test. Testing for this study was conducted with the cobas® 4800 System Application Software 2.0 software. Analysis was performed with the cobas® 4800 system KRAS Assay Specific Analysis Package v1.0.0.1113.

The 19 mutations detected by the kit are as follows, with the mutated nucleotide underlined:

G12A (GGT>GCT), G12C (GGT>TGT), G12D (GGT>GAT), G12R (GGT>CGT), G12S (GGT>AGT), G12V (GGT>GTT), G13A (GGC>GCC), G13C (GGC>TGC), G13D (GGC>GAC), G13R (GGC>CGC), G13S (GGC>AGC), G13V (GGC>GTC), Q61E (CAA>GAA), Q61H (CAA>CAT), Q61H (CAA>CAC), Q61K (CAA>AAA), Q61L (CAA>CTA), Q61P (CAA>CCA), and Q61R (CAA>CGA).

Sanger sequencing

DNA extracts from FFPET specimens using the same extraction method as described for the cobas test were sequenced by 2X bi-directional Sanger sequencing by a Clinical Laboratory Improvement Amendments (CLIA)-certified laboratory (SeqWright, Houston, TX) using an internally validated protocol.

Quantitative massively parallel pyrosequencing

Specimens which gave discordant results with the cobas test versus Sanger sequencing were retested using a quantitative massively parallel pyrosequencing method (454 GS Titanium, 454 Life Sciences, Branford, CT) by a CLIA-certified laboratory (SeqWright, Houston, TX) using a validated protocol [14]. Additionally, a randomly selected subset of specimens with concordant cobas test and Sanger sequencing results were tested by 454 sequencing to confirm the accuracy of mutation status. The validated LOD for the 454 *KRAS* protocol was 1%.

Limit of detection

Sixteen different point mutations in codons 12, 13 and 61 of the *KRAS* gene were examined for the LOD of the cobas test. Although the assay covers 19 mutations, three mutations with low frequency were not tested in our LOD study.

For the 5 predominant mutations in codons 12, 13 and 61 (one in codon 12, one in codon 13 and 3 in codon 61), FFPET specimens and cell lines were utilized. Testing was performed using three different lots of the cobas test and one lot of the cobas® DNA Sample Preparation Kit. DNA extracted from each mutant specimen was blended with genomic DNA extracted from a wild-type specimen to prepare various levels of mutant sequences (approximately 5% for FFPET specimens and 2.5%, 5%, and 10% for cell lines). 454 sequencing was used to assess and confirm the percentage of mutant sequences in DNA blends. The DNA blends were then serially diluted to input amounts ranging from 50.0 ng to 0.8 ng per PCR reaction. For FFPET samples, 5 different amounts of DNA were input into the reactions (50.0, 12.5, 3.1, 0.8 and 0.0 ng/PCR reaction) and were tested using 8 replicates and 3 reagent lots for each input level, yielding 24 results per DNA input level for each mutation tested. For cell line samples, 24 replicates for each of 5 DNA input levels (50.0, 12.5, 3.1, 0.8 and 0.0 ng per PCR reaction) for each of 3 reagent lots were tested, yielding a total of 72 results per DNA input level for each mutation and for each of 3 different percentages of mutated DNA sequences (2.5%, 5% and 10% mutant sequences). Sensitivity was determined as the lowest level of DNA input level that gave a correct mutation call rate with its one-sided upper limit of 95% confidence interval (CI) ≥ 95%.

In a separate analysis, the LOD was evaluated for 11 additional mutations in *KRAS* codons 12, 13, and 61. Due to the lack of naturally occurring specimens with 5% mutant sequences, FFPET DNA and plasmid blends at approximately 5% mutation (as assessed by 454 sequencing) were prepared including: seven FFPET specimens representing four *KRAS* codon 12 mutations, two FFPET specimens and three plasmids representing four *KRAS* codon 13 mutations, and one FFPET specimen and two plasmids representing three *KRAS* codon 61 mutations.

Before cobas testing, the mutation status and mutation percentage of the specimens were verified by 2X bi-directional Sanger sequencing and 454 sequencing, respectively.

Methods correlation with Sanger sequencing

Each of the two kit lots of the cobas test was used to test 194 NSCLC FFPET specimens for mutations in codons 12, 13, and 61. Positive, negative, and overall percent agreements (PPA, NPA and OPA) between the cobas test and 2X bi-directional Sanger test results were determined. For specimens that yielded discordant results for cobas testing and Sanger sequencing, discrepant analysis was performed by testing specimens with 454 sequencing for resolution. In addition, 54 randomly selected specimens with concordant Sanger and cobas test results were tested by 454 sequencing in order to confirm the accuracy of 454 sequencing as the resolution method.

Repeatability

The repeatability of the cobas test was evaluated across different reagent lots, days, operators and instruments with eight FFPET NSCLC specimens: two *KRAS* wild-type, two codon 12 mutants, two codon 13 mutants, and two codon 61 mutants. Three of the six *KRAS* mutants tested—one for each of codons 12, 13, and 61—had approximately 15% mutant sequences, which is 3 times the LOD of the cobas test, while the other three *KRAS* samples had greater than 15% mutant sequences. The materials used in the repeatability testing were: one **cobas**® DNA Sample Preparation Kit lot, two cobas test lots, two operators, and four **cobas**® 4800 System v.2.0.

Each operator performed two runs per day representing one run per reagent lot. The same test was conducted on each of 4 days using 4 instruments for a total of 8 runs with two replicates for each of the eight specimens. Therefore, the 16 results per run and 16 testing situations yielded a total of 256 analyses.

Interfering endogenous substances

The effects of endogenous substances, hemoglobin and triglycerides, on the performance of the cobas test were investigated in ten NSCLC FFPET specimens. Specimens were selected for mutation status based on Sanger and/or 454 sequencing. Three specimens were *KRAS* codon 12 mutation-positive, one was *KRAS* codon 13 mutation-positive, one was *KRAS* codon 61 mutation-positive, and five were wild-type. Four 5 µm sections were extracted for each specimen, one each in the following: Tissue Lysis Buffer + proteinase K (TLB/PK) (control), TLB/PK + saline (saline control), TLB/PK + 2 g/L hemoglobin in saline (Clinical and Laboratory Standards Institute (CLSI)-recommended test concentrations [15]), and TLB/PK + 37 mM triglycerides in saline (CLSI-recommended test concentrations [15]).

Hemoglobin and triglycerides were added during the lysis step of DNA extraction and were not subjected to deparaffinization. All DNA extracts were tested for interference of mutation detection using the cobas test.

Interference by common lung-related microorganisms

The effects of lung-related microorganisms on the performance of the cobas test were investigated in 10 FFPET NSCLC specimens. Three specimens were *KRAS* codon 12 mutation-positive, one was *KRAS* codon 13 mutation-positive, one was *KRAS* codon 61 mutation-positive, and five were wild-type. Approximately 2.5 x 10^5 colony forming units of *Streptococcus pneumoniae* and *Haemophilus influenzae* were added during the lysis step of DNA extraction. Phosphate-buffered saline control was also tested. One 5 µm section of each specimen was extracted and all DNA extracts were tested for interference of mutation detection using the cobas test.

Inclusivity

The intent of this study was to determine whether the configured cobas test could detect several additional rare mutations. In addition to the 16 single-base change mutations in codons 12, 13 and 61 tested for LOD studies, the inclusivity of the cobas test with a panel of rare *KRAS* mutations [16] in flanking sequences of codons 12, 13 and 61 (i.e., codons 11, 14, 15, 59, 60, 62, 63) and double or complex mutations in codons 12, 13 and 61 was also examined using plasmids and FFPET specimens that were available [16]. Twenty-three plasmids, each containing one of 23 possible mutations, were blended separately with wild-type genomic DNA from K562 cells to obtain 5% mutant/95% wild type sequences. Six different NSCLC FFPET specimens were also tested, including five with the G12F mutation (mutant allele frequencies ranging from 24% to 60%) and one with the A59T mutation (with its mutant allele frequency at 22%). All 29 samples were tested in 4 replicates by the cobas test and mutation detectability was calculated as the percentage of correct results. For the samples that did not give 100% detectability at 5% mutant sequences, further testing was carried out using DNA blends containing 10% and 15% mutant sequences in replicates of 4.

Results

Limit of detection

For the LOD analysis of the 5 predominant mutations in codons 12, 13, and 61, ≥ 95% correct mutation call rates were obtained across all specimen types with approximately ≥ 5% mutant sequences (as assessed by 454) at a DNA input of 0.8-12.5 ng per PCR reaction (Table 1). For all mutation levels examined that ranged from 2.4% to 12.1%, ≥ 95% correct mutation call rates were obtained at a DNA input of 50.0 ng per PCR reaction, which is the prescribed amount of DNA input for the cobas test, or less (Table 1). All of the 11 additional *KRAS* mutations in codons 12, 13, and 61 examined at approximately 5% mutant sequences were detected at rates whose one-sided upper limit of 95% CI were ≥ 95% correct mutation call rates at a DNA input of 50.0 ng per PCR reaction or less (Table 2).

Methods correlation

One of the 194 FFPET NSCLC specimens tested gave an invalid result by the cobas test kit Lot 2 and another specimen was positive for mutations in both codon 12 and codon 61 by Sanger sequencing. As a consequence, there were 195 valid results for cobas test kit Lot 1 and 194 valid results for cobas test kit Lot 2 for the comparison between the cobas test and Sanger sequencing (Table 3). Concordance rates between the cobas test and Sanger sequencing for Lot 1 and Lot 2 were 93.8% PPA for both lots, 92.9% NPA for Lot 1 and 90.7% NPA for Lot 2, and 93.3% OPA for Lot 1 and 92.3% OPA for Lot 2 (Table 3A and 3B). Sixteen specimens with discordant results by one or both lots were subjected

KRAS Mutation	Sample ID	Mutation (%)	Lowest DNA Input with Correct Call[a] (ng/PCR)
G12C, GGT > TGT	FFPET #1, DNA blend	6.2	12.5
	FFPET #2, DNA blend	6.0	3.1
	FFPET #3, DNA blend	5.6	3.1
	Cell line (NCI-H23), DNA blend #1	12.1	0.8
	Cell line (NCI-H23), DNA blend #2	6.1	3.1
	Cell line (NCI-H23), DNA blend #3	2.4	50.0
G13C, GGC > TGC	FFPET #4, DNA blend	6.2	3.1
	FFPET #5, DNA blend	4.4	3.1
	FFPET #6, DNA blend	4.4	3.1
	Cell line (NCI-H1734), DNA blend #4	7.8	0.8
	Cell line (NCI-H1734), DNA blend #5	4.9	3.1
	Cell line (NCI-H1734), DNA blend #6	2.8	3.1
Q61H, CAA > CAT	FFPET #7, DNA blend	4.5	3.1
	Cell line (NCI-H460), DNA blend #7	10.2	0.8
	Cell line (NCI-H460), DNA blend #8	6.1	0.8
	Cell line (NCI-H460), DNA blend #9	2.7	12.5
Q61H, CAA > CAC	FFPET #8, DNA blend	6.4	3.1
Q61L, CAA > CTA	FFPET #9, DNA blend	6.5	3.1

[a]Lowest amount of input DNA (ng/PCR) tested that yielded a correct mutation call rate of ≥ 95% (upper one-sided 95% CI). Each test used 72 replicates for cell line DNA blends and 24 replicates for FFPET DNA blends.

Table 1: Summary of LOD results for the predominant *KRAS* mutations in codons 12, 13 and 61.

KRAS Mutation	Sample ID	Mutation (%)	Lowest DNA Input with Correct Call (ng/PCR)[a]
G12A, GGT > GCT	FFPET #10, DNA blend	4.5	12.5
	FFPET #11, DNA blend	5.1	12.5
G12D, GGT > GAT	FFPET #12, DNA blend	5.0	12.5
	FFPET #13, DNA blend	5.2	12.5
G12S, GGT > AGT	FFPET #14, DNA blend	5.6	12.5
G12V, GGT > GTT	FFPET #15, DNA blend	2.9	12.5
	FFPET #16, DNA blend	5.7	12.5
G13A, GGC > GCC	Plasmid, DNA blend	3.7	50.0[b]
G13D, GGC > GAC	FFPET #17, DNA blend	4.8	3.1
	FFPET #18, DNA blend	6.8	3.1
G13S, GGC > AGC	Plasmid, DNA blend	1.6	50.0[b]
G13V, GGC > GTC	Plasmid, DNA blend	2.6	50.0[b]
Q61E, CAA > GAA	Plasmid, DNA blend	5.7	50.0[b]
Q61P, CAA > CCA	Plasmid, DNA blend	3.2	50.0[b]
Q61R, CAA > CGA	FFPET #19, DNA blend	4.8	50

[a] Lowest amount of input DNA (ng/PCR) tested with a correct mutation call rate of ≥95% (upper one-sided 95% CI) per 24 replicates. [b]Plasmid DNA blends were tested only at 50.0 ng per PCR reaction.

Table 2: Summary of LOD results for the additional 11 *KRAS* mutations in codons 12, 13 and 61.

to discrepant analysis by 454 sequencing and their results are shown in Table 4. For discrepant analysis, each specimen that produced different results with cobas testing versus Sanger sequencing was subjected to 454 sequencing directed at the area of the DNA sequence in question.

Based on the results from 454 sequencing, 12 (75%) of the 16 discordant specimens were correctly assessed by cobas test Lot 1, and 13 (87%) of the 15 discordant specimens were correctly assessed by cobas test Lot 2. The resulting PPA values for codon 12/13 were 97.0% for Lot 1 and 100.0% for Lot 2, the NPA values were 98.9% for both lots, and the OPA values were 97.9% for Lot 1 and 99.5% for Lot 2 (Tables 3C and 3D). It is noteworthy that each of the four specimens that showed discordant results after resolution by 454 sequencing had a mutation level below 5%, the LOD for the cobas test (Table 4). In addition to the 16 specimens with discordant results, 54 specimens with concordant cobas test and Sanger sequencing results were subjected to 454 sequencing. These included 19 specimens with mutations detected in codon 12/13 and 35 specimens with no mutation detected. The 454 sequencing results for all 54 specimens were concordant with the Sanger sequencing and cobas test results.

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(A)

		2X Bi-Directional Sanger Sequencing			
		MD Codon 12/13	**MD Codon 61**	**NMD**	**Total**
cobas *KRAS* Kit Lot 1	**MD Codon 12/13**	90	0	6	96
	MD Codon 61	0	1	1	2
	NMD	5	1	91	97
	Totals	95	2	98	195[a]

Positive agreement, 93.8% (95% CI, 87.2%–97.1%); Negative agreement, 92.9% (95% CI, 86.0%–96.5%); Overall agreement, 93.3% (95% CI, 88.9%–96.1%)

(B)

		2X Bi-Directional Sanger Sequencing			
		MD Codon 12/13	**MD Codon 61**	**NMD**	**Total**
cobas *KRAS* Kit Lot 2	**MD Codon 12/13**	90	0	8	98
	MD Codon 61	0	1	1	2
	NMD	5	1	88	94
	Totals	95	2	97	194[a]

Positive agreement, 93.8% (95% CI, 87.2%–97.1%); Negative agreement, 90.7% (95% CI, 83.0%–95.0%); Overall agreement, 92.3% (95% CI, 87.6%–95.3%)

(C)

		2X Bi-Directional Sanger Sequencing and Discordant Resolution			
		MD Codon 12/13	**MD Codon 61**	**NMD**	**Total**
cobas *KRAS* Kit Lot 1	**MD Codon 12/13**	96	0	0	96
	MD Codon 61	0	1	1*	2
	NMD	3*	0	94	97
	Totals	99	1	95	195[a]

Positive agreement, 97.0% (95% CI, 91.5%–99.0%); Negative agreement, 98.9% (95% CI, 94.3%–99.8%); Overall agreement, 97.9% (95% CI, 94.8%–99.2%); *All four samples with discordant results were at <5% mutation frequency.

(D)

		2X Bi-Directional Sanger Sequencing and Discordant Resolution			
		MD Codon 12/13	**MD Codon 61**	**NMD**	**Total**
cobas *KRAS* Kit Lot 2	**MD Codon 12/13**	98	0	0	98
	MD Codon 61	0	1	1*	2
	NMD	0	0	94	94
	Totals	98	1	95	194[a]

Positive agreement, 100.0% (95% CI, 96.3%–100.0%); Negative agreement, 98.9% (95% CI, 94.3%–99.8%); Overall agreement, 99.5% (95% CI, 97.1%–99.9%)

*The sample with a discordant result was at <5% mutation frequency. *MD* mutation detected, *NMD* no mutation detected—wild-type for codons 12/13 and 61 [a] N=194: One specimen is a double mutant (at codons 12 and 61) by Sanger sequencing. One of the 194 specimens produced an invalid result by cobas® Kit Lot 1.

Table 3: Method correlation between the cobas® *KRAS* Mutation Test and Sanger sequencing (Table 3A and 3B) and after discordant resolution with 454 sequencing (Tables 3C and 3D).

Sample	**Sanger Sequencing Results**	**cobas *KRAS* Test Results**		**454 Sequencing Results (% Mutation)**	**Concordance After 454 Resolution**	
		Lot 1	Lot 2		cobas *KRAS* Test Lot 1	cobas *KRAS* Test Lot 2
1	codon 12	NMD	NMD	NMD (<1%)	Yes	Yes
2	codon 12	NMD	NMD	NMD (<1%)	Yes	Yes
3	codon 12	NMD	NMD	NMD (<1%)	Yes	Yes
4	codon 12	NMD	NMD	NMD (<1%)	Yes	Yes
5	codon 12	NMD	NMD	NMD (<1%)	Yes	Yes
6	codons 12 & 61	codon 12/13	codon 12/13	codon 12 (23.7%)	Yes	Yes
7	NMD	codon 12/13	codon 12/13	codon 12 (59.2%)	Yes	Yes
8	NMD	codon 12/13	codon 12/13	codon 12 (13.3%)	Yes	Yes
9	NMD	codon 12/13	codon 12/13	codon 12 (4.9%)	Yes	Yes
10	NMD	codon 12/13	codon 12/13	codon 12 (9.7%)	Yes	Yes
11	NMD	codon 12/13	codon 12/13	codon 12 (39.3%)	Yes	Yes

12	NMD	codon 12/13	codon 12/13	codon 12 (3.0%)	Yes	Yes
13	NMD	NMD	codon 12/13	codon 12 (1.2%)	No	Yes
14	NMD	NMD	codon 12/13	codon 12 (3.7%)	No	Yes
15	NMD	codon 61	codon 61	codon 59 (2.8%)	No	No
16	NMD	NMD	invalid	codon 13 (1.8%)	No	N/A

NMD, no mutation detected

Table 4: Resolution of discordant results by 454 sequencing for method correlation

Test Day	Operator	Run	Reagent Kit Lot	Instrument ID	Results		Accuracy (%)
					Incorrect	Correct	
1	A	1	1	1	0	16	100
		2	2	2	0	16	100
	B	3	1	3	0	16	100
		4	2	4	0	16	100
2	A	5	1	3	0	16	100
		6	2	4	0	16	100
	B	7	1	1	0	16	100
		8	2	2	0	16	100
3	A	9	1	1	0	16	100
		10	2	2	0	16	100
	B	11	1	3	0	16	100
		12	2	4	0	16	100
4	A	13	1	3	0	16	100
		14	2	4	0	16	100
	B	15	1	1	0	16	100
		16	2	2	0	16	100

Table 5: Repeatability of the cobas® *KRAS* Mutation Test by day, operator, reagent kit lot and instrument.

Repeatability

Repeatability was assessed by testing tissue sections from 8 different NSCLC FFPET samples, with each sample tested twice for a given run. All 16 repeatability runs were valid, yielding an overall accuracy of 100%, representing 256 valid and correct results from 256 cobas test analyses across all specimens, operators, reagent kit lots, and instruments (Table 5 and 6).

Interfering substances

In an effort to simulate analysis of real-world samples, substances that could be present in NSCLC FFPET specimens were added prior to testing. No interference was observed for any of the 10 FFPET specimens tested for potential endogenous interfering substances at CLSI-recommended test concentrations of 2 g/L hemoglobin and 37 mM triglycerides [15].

None of the lung-related micro-organisms tested interfered with the cobas test. All five of the *KRAS* wild-type specimens spiked with micro-organisms gave valid results of mutation not detected, while all five *KRAS*-mutant specimens spiked with micro-organisms gave valid results of mutation detected for the correct codon. For both endogenous interfering substances and micro-organisms, all study controls gave valid and accurate mutation results (data not shown).

Inclusivity

Inclusivity testing was performed to determine the ability of the cobas test to detect rare mutations in the *KRAS* gene. *KRAS* mutations in codons 11, 14, 15, 59, 60, 62, and 63 were examined, as well as double or complex mutations in codons 12, 13 and 61. The cobas test detected 21 of the 23 mutations tested (Table 6). All plasmid samples were detected 100% of the time at the level of 5% mutant sequences, with the exceptions of p.G12F, p.G12L, p.G15S and c.30_31insGGA. The p.G12F and p.G15S were detected 100% of the time at the level of 10% and 15% mutant sequences. The remaining two mutations, p.G12L and c.30_31insGGA, were not detectable even at 15% mutant sequences. All of the six (6) FFPET specimens (five p.G12F and one p.A59T) were 100% detected.

Discussion

In keeping with the rapid development of targeted therapies, there is a continuing need for standardized testing of involved genes to rapidly and consistently determine mutation status. Accurate assignment of therapy to oncology patients, including the withholding of treatment from those who are unlikely to benefit, is essential for optimizing treatment. In NSCLC, KRAS plays a pivotal role as a driver oncogene; moreover, KRAS mutations predict a lack of benefit in responding to EGFR inhibitors and may reflect eligibility for treatment with inhibitors of MEK or direct inhibitors of mutant *KRAS* as they become available [5,8]. Thus reliable *KRAS* testing is imperative to ensure accurate treatment assignment for NSCLC patients with these new agents.

The current study was undertaken to evaluate the capabilities of the cobas test in detecting mutations in NSCLC FFPET specimens. FFPET specimens were chosen to represent the intention to diagnose population. Sanger sequencing was used as the standard for assessing the cobas test performance. In addition, the cobas test was rigorously analyzed for mutation detection capabilities, reproducibility, and robustness during various steps of the testing workflow likely to be encountered in the clinic.

To evaluate the LOD, artificial blends of DNA extracts from cell lines and FFPET samples were examined. The cobas test correctly called at least 95% of mutations in clinical samples containing as little

	Amino Acid Substitution	KRAS DNA Mutation	Detectability (%)
5% Mutant *KRAS* DNA on Plasmid	p.A11P	GCT > CCT	100
	p.G12F[a]	GGT > TTT	0[b]
	p.G12I	GGT > ATT	100
	p.G12L	GGT > CTT	0
	p.G12N	GGT > AAT	100
	p.G12Y	GGT > TAT	100
	p.G13I	GGC > ATC	100
	p.G13N	GGC > AAC	100
	p.V14I	GTA > ATA	100
	p.G15D	GGC > GAC	100
	p.G15S	GGC > AGC	0[c]
	p.A59E	GCA > GAA	100
	p.A59G	GCA > GGA	100
	p.A59S	GCA > TCA	100
	p.A59T[a]	GCA > ACA	100
	p.G60A	GGT > GCT	100
	p.E62D	GAG > GAT	100
	p.E63K	GAG > AAG	100
	p.G13D	GGC > GAC	100
	p.V14I	GTA > ATA	100
	DNA Insertions and Deletions	***KRAS* DNA Mutation**	**Detectability (%)**
	c.30_31insGGA	GGAGCT > GGAGGAGCT	0
	c.35delG	GGT>G^T	100
	c.36_37insGGT	GGTGGC > GGTGGTGGC	100
	c.187_189delGAG	GGAGT > G^^^T	100
NSCLC FFPET[d]	**Amino Acid Substitution**	***KRAS* DNA Mutation**	**Detectability (%)**
	p.G12F[a]	GGT > TTT	100
	p.A59T[a]	GCA > ACA	100

[a]These plasmids were tested in both the plasmid mixture and in NSCLC FFPET specimens. [b]The p.G12F plasmid blended at 5% with 95% wild-type genomic DNA was not detectable. However, the same plasmid blended at 10% or 15% was detected at 100%. [c]The p.G15S plasmid blended at 5% with 95% wild-type genomic DNA was not detectable. However, the same plasmid blended at 10% or 15% was detected at 100%. [d]Five different NSCLC FFPET specimens with the G12F mutation were tested and all 5 showed 100% detectability. One NSCLC FFPET specimen with the A59T mutation was tested and detected 100%.

Table 6: Inclusivity of the cobas® *KRAS* Mutation Test using 5% mutant *KRAS* DNA sequences blended with wild-type genomic DNA or FFPET NSCLC samples. The percent of *KRAS* mutant DNA in the FFPET samples is unknown.

as 2.4% mutated *KRAS* and containing at least 50 ng DNA, the amount stipulated by the manufacturer for accurate test results. Furthermore, the vast majority of samples were correctly called with less than 5 ng input DNA, simulating the ability to accurately detect mutations in highly damaged specimens. Correct calls were also obtained for 11 additional *KRAS* mutations when samples were tested with 50 ng DNA and approximately 5% mutant sequences. These conditions established by the cobas test enable the laboratory to perform molecular testing while preserving precious clinical material for additional biomarker testing.

Testing two lots of the cobas test against Sanger sequencing for detecting mutations in *KRAS* codons 12 and 13 yielded OPA values of 97.9% for lot 1 and 99.5% for lot 2 after resolution of discordant specimens via 454 sequencing. Repeatability was assessed by comparing results from cobas test kits from different lots, 2 different operators, and 4 different cobas® 4800 System instruments. Each run analyzed 8 different FFPET NSCLC tumor specimens that included 2 *KRAS* wild-type, and 2 each with mutations in either codon 12, 13, or 61. Each run correctly identified either wild-type or mutated *KRAS* with 100% accuracy. The results indicate that the cobas test can produce consistent and accurate results reliably, which is essential for a clinical diagnostic assay. Similarly, the cobas test yielded accurate results when lung FFPET samples were deliberately contaminated with substances of clinical interest. In the presence of triglycerides or hemoglobin at the CLSI-recommended levels, as well as in the presence of *Streptococcus pneumoniae* or *Haemophilus influenzae,* the cobas test correctly identified *KRAS* mutations. An earlier study demonstrated the cobas test specificity by showing a complete lack of cross-reactivity against *HRAS*, *NRAS*, and the pseudogene *KRASP1*, three sequences that are highly homologous to *KRAS* [17].

Previously, the cobas test identified 19 single-base change mutations in *KRAS* codons 12, 13, and 61 in colorectal cancer [17]. In this study of NSCLC, 16 of the 19 *KRAS* mutations in *KRAS* codons 12, 13, and 61 were detected at approximately 5% or below in mutant sequences. Furthermore, 23 additional mutations in codons 11, 14, 15, 60, 62, and 63 as well as double or complex mutations in codons 12, 13, and 61 were detected. In contrast, the *therascreen* KRAS test (Qiagen, Manchester, UK) identifies only 7 somatic mutations: 6 mutations in codon 12 and 1 mutation in codon 13 and none in codon 61. The additional 12 single-base change mutations in codons 12, 13, and 61 that are detected by the cobas test and not by the *therascreen* KRAS test represent more than 3% of NSCLC patients with *KRAS* mutations [18]. Furthermore, a recent report detailed the emergence and high frequency of codon 61 *KRAS* mutations, showing mutations in 16 (32%) of 50 plasma samples of colorectal cancer patients who became resistant to anti-*EGFR* antibodies; this finding underscores the importance of detecting codon 61 mutations in *KRAS* exon 3 [19].

In summary, the performance analysis presented in the current

study demonstrates that the cobas test is a robust, sensitive, and reproducible method for detecting *KRAS* mutations in FFPET tumor samples from NSCLC patients.

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