


# Significant Improvement in Detecting *BRAF*, *KRAS*, and *EGFR* Mutations Using Next-Generation Sequencing as Compared with FDA-Cleared Kits

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## Abstract

**Introduction** We compared mutations detected in *EGFR*, *KRAS*, and *BRAF* genes using next-generation sequencing (NGS) and confirmed by Sanger sequencing with mutations that could be detected by FDA-cleared testing kits.

**Methods** Paraffin-embedded tissue from 822 patients was tested for mutations in *EGFR*, *KRAS*, and *BRAF* by NGS. Sanger sequencing of hot spots was used with locked nucleic acid to increase sensitivity for specific hot-spot mutations. This included 442 (54%) lung cancers, 168 (20%) colorectal cancers, 29 (4%) brain tumors, 33 (4%) melanomas, 14 (2%) thyroid cancers, and 16% others (pancreas, head and neck, and cancer of unknown origin). Results were compared with the approved list of detectable mutations in FDA kits for *EGFR*, *KRAS*, and *BRAF*.

**Results** Of the 101 patients with *EGFR* abnormalities as detected by NGS, only 58 (57%) were detectable by cobas v2 and only 35 (35%) by theascreen. Therefore, 42 and 65%, respectively, more mutations were detected by NGS, including two patients with *EGFR* amplification. Of the 117 patients with *BRAF* mutation detected by NGS, 62 (53%) mutations were within codon 600, detectable by commercial kits, but 55 (47%) of the mutations were outside codon V600, detected by NGS only. Of the 321 patients with mutations in *KRAS* detected by NGS, 284 (88.5%) had mutations detectable by theascreen and 300 (93.5%) had mutations detectable by cobas. Therefore,

11.5 and 6.5% additional *KRAS* mutations were detected by NGS, respectively.

**Conclusion** NGS provides significantly more comprehensive testing for mutations as compared with FDA-cleared kits currently available commercially.

## Key Points

Significantly more mutations in *EGFR*, *BRAF*, and *KRAS* genes are detected when next-generation sequencing is used for analyzing tumors.

Significant improvement in mutation detection technology renders FDA-cleared kits inadequate for routine clinical testing.

Detected mutations that were missed by FDA-cleared tests may have significant impact on the clinical decision to treat or not to treat.

## 1 Introduction

Current advances in analyzing molecular abnormalities are accruing rapidly, making it difficult for FDA-approved testing kits to keep pace and remain the standard in patient care, especially in rapidly advancing fields like oncology. The advantage of these targeted tests (e.g., qPCR or IHC) for a small laboratory is the relative simplicity and limited sample requirements needed to achieve high sensitivity, precision, fast turnaround time, and robustness [1–3]. The advantage of these tests for ordering clinicians are that

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results are rapidly available (<24 h), require very little interpretation, and insurance carriers recognize many tests as medically necessary. The disadvantage of these tests for the patient is that they are limited in scope and could fail to be clinically informative; many times they become outdated quickly in a rapidly advancing field like oncology [4]. For example, the companion diagnostic (CDx) test for treating colorectal cancer with anti-EGFR (epidermal growth factor receptor) monoclonal antibodies (cetuximab and panitumumab) was originally standardized with testing for codon 12 and 13 in *KRAS*; however, currently, the American Society of Clinical Oncology (ASCO) provisional clinical opinion (PCO) recommendation calls for extended testing in codons 12, 13, 61, 117, and 149, not only in *KRAS*, but the *NRAS* gene as well [5, 6]. This means that some patients given anti-EGFR antibody therapy will not respond to this therapy because they have mutations in *KRAS* or *NRAS* not detected by the CDx. In fact, some data suggest that they may be harmed with this treatment [5, 6]. Similarly for *EGFR* mutations in non-small cell lung cancer (NSCLC), *EGFR*-activating mutations are treated with *EGFR*-tyrosine kinase inhibitors (TKIs). The FDA-approved *EGFR* test (cobas, Roche Diagnostic US, Indianapolis, IN, USA) does not cover many activating mutations in the tyrosine kinase domain [3, 7] that predict potential benefit from TKI therapy. In addition, NSCLC tumors insensitive to *EGFR* TKIs are those driven by the *KRAS* and *MET* oncogenes that are not fully profiled by cobas and theascreen (Qiagen, Hilden, Germany) tests. In contrast, next-generation sequencing (NGS) offers sensitivity and flexibility in mutation detection not found in other modalities. However, detection of mutations that were not included in the original drug efficacy studies raises a clinical dilemma for the treating physicians. In principle, one does not want to miss the opportunity of giving a patient a successful therapy, but also making a clinical decision to treat or not to treat based on a new abnormality not in the original FDA approval of the drug is difficult. Using computer software such as SIFT (<http://sift.jcvi.org>) or PROVEAN (Protein Variation Effect Analyzer) (<http://provean.jcvi.org>), which predicts the functional importance of the detected mutation based on the degree of conservation of amino acid residues in the sequence, is very helpful, but at this time, this is not accepted as a standard for basing clinical decisions. While this remains an issue, it is important to provide comprehensive data to the treating physician and to the scientific community.

Here we report the mutation profile and results of testing for mutations in *EGFR*, *BRAF*, and *KRAS* using a clinically validated Laboratory Developed Test (LDT) that combines NGS with confirmatory testing by Sanger sequencing on a large number of clinical samples and compare results with

the list of mutations that can be detected by two FDA-cleared kits for these three genes: cobas v2 and theascreen.

## 2 Methods

### 2.1 Patient Samples

A total of 822 consecutive paraffin-embedded cancer samples were tested using NGS and Sanger sequencing for mutations in *KRAS*, *BRAF*, and *EGFR* as routine molecular testing. These included 442 from lung, 168 from colorectal and stomach, 29 from brain tumors, 33 from melanoma, 14 from thyroid tissue, and 136 samples from various other tissue including pancreas, head and neck, as well as cancer of unknown origin (CUP). The study was performed after obtaining approval from the Institutional Review Board (IRB).

Formalin 7  $\mu\text{m}$  fixed paraffin embedded sections were examined by a US-certified pathologist for tumor content. Tumor content was noted and circled for each patient and then four to six consecutive sections were scrapped by a trained licensed clinical technologist. Only samples with >20% tumor were used for testing.

### 2.2 DNA Extraction

DNA was extracted using the QIAcube (Qiagen, Valencia, CA, USA) automated DNA extraction machine and DNA QIAamp FFPE tissue kit (Qiagen; Venlo, Netherlands) according to manufacturer's instructions. Extracted DNA was then quantified using a Nanodrop 2000 (Thermo Fisher Scientific; Waltham, MA, USA) instrument and adjusted to approximately 50–100 ng/ $\mu\text{L}$  with elution buffer (Qiagen, AE Buffer).

### 2.3 Sanger Sequencing

Targeted bi-directional Sanger sequencing was performed on all samples for *BRAF*, *KRAS*, and *EGFR*. We sequenced exons 18, 19, 20, and 21 of the *EGFR* gene; exon 2, exon 3, and exon 4 of the *KRAS* gene, and exon 15 of the *BRAF* gene.

Targets were amplified according to the polymerase manufacturer's protocol. In brief, genomic DNA was added to target-specific master mixes containing primers and polymerase enzyme and then amplified using an Applied Biosystems Veriti thermal cycler (ThermoFisher). The amplified products were filter-purified by Multiscreen PCR plates (Millipore, Billerica, MA, USA) and sequenced in both directions using the BigDye Terminator v3.1 Cycle Sequencing Kit with detection by an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA,

USA). Sequence data were then base-called, assembled, and analyzed by ABI Prism SeqScape software (Applied Biosystems). Lock nucleic acids (LNA) were used for blocking the wild-type alleles to increase the sensitivity of the Sanger sequencing. In brief, LNA oligomers (10–13 bp) are used to suppress or mask amplification of the wild-type alleles around codons 12, 13, and 61 of *KRAS*, codon 600 of *BRAF*, and codon 790 of *EGFR*.

## 2.4 Next-Generation Sequencing Protocol

DNA was sequenced using Illumina NGS protocols, including Illumina TruSeq library preparation, Illumina sample indexing, and Illumina synthesis by sequencing (SBS) protocols as recommended by the Illumina (San Diego, CA, USA). In brief, tumor DNA was amplified using either TruSeq kit or custom primers, and amplification products were confirmed with gel electrophoresis using a 2% agarose E-gel (ThermoFisher, Carlsbad, CA, USA). Samples were indexed and pooled. Libraries were then loaded on to a Illumina MiSeq (Illumina, San Diego, CA, USA) or Nextseq Instrument for SBS using 150 × 150 bp Illumina sequencing kit with Illumina mid-output flow cells. An experiment sheet was generated using Illumina Experiment Manager for each sequencing run. MiSeq Reporter was used for alignment and variant calling using the proper panel bed/manifest file. Exons 2, 3, and 4 of *KRAS* were sequenced. For *EGFR*, we sequenced exons 3, 7, 15, and 18–21. Exons 11 and 15 of *BRAF* were sequenced. The primers for targeted sequencing covered approximately 50 nucleotides from each side of each exon. Variants were annotated and filtered using Illumina Variant Studio. In brief, variants passed quality and annotation filters if they contained >3% allele frequency, and non-synonymous AA changes, insertions, or deletions. However, mutations that are well characterized (codons 12, 13, 61 in *KRAS*, V600 in *BRAF*, etc.) were accepted at variant allele frequency (VAF) of 1%. Each variant was then reviewed for common single nucleotide polymorphism (SNP) criteria including representation in the EVS, dbSNP, and 1000G databases. Each variant was then assessed for predicted functional effects of the mutation using PolyPhen, SIFT, and PROVEAN. For initial confirmation of variant calling, alignments were visualized using Integrated Genome Viewer (IGV). Variants were not reported if found in a SNP database. By performing functional bioinformatics, we obtained information on the biological significance of the detected mutation. Gene amplifications were identified using read coverage plots. For indel detection in *EGFR*, PRISM and PINDEL software were used. The detection limit of NGS was validated at 5% for new mutations and at 1% for well characterized mutations. In

addition, LNA was used for detecting T790 mutation, increasing sensitivity of NGS for this mutation to 0.01%.

Sequencing and library quality were assessed for every run using MiSeq reporter, which calculates amplicon read coverage per sample and uniformity of coverage. Positive and negative control samples were also sequenced in parallel with each run to confirm the sensitivity and specificity of each run. Overall sequencing quality was also assessed with MiSeq Reporter software. Average sequencing coverage across the entire coding regions was 10,000 in 94% of the sequenced amplicons. The sensitivity of the NGS testing was determined during validation to be at VAF of 3%.

The mutations detected by sequencing were compared with mutations covered by the FDA-cleared kits. These kits use polymerase chain reaction (PCR): the ARMS (Allele Refractory Mutation System) or scorpion technology is used in the thetascreen kit and real-time PCR is used in the cobas system.

## 2.5 Statistical Analysis

The Wilcoxon rank sum test or Kruskal–Wallis test was used to compare the categorical variables. *p* values <0.05 were considered statistically significant.

## 3 Results

### 3.1 *EGFR* Mutation Profile

Of the 822 consecutive patient samples, *EGFR* protein coding mutations were detected in 99 samples (12.0%) and *EGFR* gene amplification in two additional samples (Table 1) using our targeted NGS panel. The sensitivity of the NGS testing was determined during validation to be at VAF of 3%. Mutations detected with allele frequency >40 were explored for possibility of being germline mutations by testing normal tissue. Almost all mutations originated from lung cancer samples. All but four mutations were identified in the protein kinase domain (Fig. 1). Most mutations were discovered in exons 19 and 20, were single nucleotide variants (SNVs) resulting in changes to amino acid or early termination of the protein, and found in important motifs, such as the nucleotide binding site. While 88.9% (8/9) in-frame insertion and deletion (indel) mutations could be identified by the cobas kit, only 50.0% (5/10) of indel mutations resulting in protein coding frame-shifts and early protein termination would have been picked up by the cobas kit. Mutations were also tested using Sanger sequencing for confirmation. Except for low level variant allele frequency (VAF <10%), there was no discrepancy between NGS and Sanger sequencing in detecting mutations. As shown in Table 1 and Fig. 1, 42%

**Table 1** A list of all *EGFR* mutations detected by next-generation sequencing

Amino acid	Nucleotide change	Detectable by cobas	Not detectable by cobas
NP_005219.2:p.Phe712Ser	NM_005228.3:c.2135T>C	1	
NP_005219.2:p.Gly719Asp	NM_005228.3:c.2156G>A	1	
NP_005219.2:p.Leu747Ser	NM_005228.3:c.2240T>C	1	
NP_005219.2:p.Ser768Ile	NM_005228.3:c.2303G>T	1	
NP_005219.2:p.Gly719Cys	NM_005228.3:c.2155G>T	1	
NP_005219.2:p.Gly719Ala	NM_005228.3:c.2156G>C	1	
NP_005219.2:p.Glu746IlefsTer16	NM_005228.3:c.2235_2236delGG	1	
NP_005219.2:p.Glu746_Ala750del	NM_005228.3:c.2235_2249delGGAATTAAGAGAAGC	1	
NP_005219.2:p.Glu746IlefsTer16	NM_005228.3:c.2236_2237delGA	1	
NP_005219.2:p.Leu747LysfsTer17	NM_005228.3:c.2236_2242delGAATTAA	1	
NP_005219.2:p.Glu746_Arg748delinsdel	NM_005228.3:c.2236_2244delGAATTAAGA	1	
NP_005219.2:p.Glu746_Ala750del	NM_005228.3:c.2236_2250delGAATTAAGAGAAGCA	1	
NP_005219.2:p.Glu746ValfsTer11	NM_005228.3:c.2237_2253delAATTAAGAGAAGCAACA	1	
NP_005219.2:p.Leu747TyrfsTer15	NM_005228.3:c.2240_2252delTAAGAGAAGCAAC	1	
NP_005219.2:p.Leu747_Pro753delinsSer	NM_005228.3:c.2240_2257delTAAGAGAAGCAACATCTC	1	
NP_005219.2:p.Ser768Ile	NM_005228.3:c.2303G>T	1	
NP_005219.2:p.Asp770_Asn771insAsnProHis	NM_005228.3:c.2308_2309insACAACCCCC	3	
NP_005219.2:p.Asp770_Asn771insGly	NM_005228.3:c.2310_2311insGGG	1	
NP_005219.2:p.Asn771_Pro772insHis	NM_005228.3:c.2311_2312insACC	1	
NP_005219.2:p.Thr790Met	NM_005228.3:c.2369C>T	6	
NP_005219.2:p.Leu858Arg	NM_005228.3:c.2573T>G	27	
NP_005219.2:p.Leu861Gln	NM_005228.3:c.2582T>A	5	
<b>NP_005219.2:p.Glu709Ala</b>	<b>NM_005228.3:c.2126A&gt;C</b>		<b>1</b>
<b>NP_005219.2:p.Lys714Glu</b>	<b>NM_005228.3:c.2140A&gt;G</b>		<b>1</b>
<b>NP_005219.2:p.Leu718Val</b>	<b>NM_005228.3:c.2152C&gt;G</b>		<b>1</b>
<b>NP_005219.2:p.Gly719AlafsTer29</b>	<b>NM_005228.3:c.2154delG</b>		<b>1</b>
NP_005219.2:p.Gly719Ser	NM_005228.3:c.2155G>A		1
NP_005219.2:p.Val726Leu	NM_005228.3:c.2176G>T		1
NP_005219.2:p.Trp731Ter	NM_005228.3:c.2192G>A		1
NP_005219.2:p.Pro733Ser	NM_005228.3:c.2197C>T		1
NP_005219.2:p.Arg748Lys	NM_005228.3:c.2243G>A		1
NP_005219.2:p.Pro753ArgfsTer13	NM_005228.3:c.2257delC		1
NP_005219.2:p.Asp761Asn	NM_005228.3:c.2281G>A		1
NP_005219.2:p.Asp761Tyr	NM_005228.3:c.2281G>T		2
NP_005219.2:p.Met766_Ala767insAlaSerVal	NM_005228.3:c.2296_2297insTGGCCAGCG		1
NP_005219.2:p.Asn771GlnfsTer126	NM_005228.3:c.2309_2310insC		1
NP_005219.2:p.Pro772delinsHisThr	NM_005228.3:c.2314_2315insACA		1
NP_005219.2:p.Pro772His	NM_005228.3:c.2315C>A		1
NP_005219.2:p.His773Leu	NM_005228.3:c.2318A>T		1
NP_005219.2:p.Cys775ProfsTer121	NM_005228.3:c.2320_2321delGT		1
NP_005219.2:p.Arg776His	NM_005228.3:c.2327G>A		2
NP_005219.2:p.Gly779Ser	NM_005228.3:c.2335G>A		2
NP_005219.2:p.Gly779Cys	NM_005228.3:c.2335G>T		1
NP_005219.2:p.Gly779Val	NM_005228.3:c.2336G>T		1
NP_005219.2:p.Ile780Ser	NM_005228.3:c.2339T>G		1
NP_005219.2:p.Leu815Pro	NM_005228.3:c.2444T>C		1
NP_005219.2:p.Ala859Ser	NM_005228.3:c.2575G>T		2
NP_005219.2:p.Lys860Asn	NM_005228.3:c.2580A>T		1

**Table 1** continued

Amino acid	Nucleotide change	Detectable by cobas	Not detectable by cobas
<b>NP_005219.2:p.Glu1062Ter</b>	<b>NM_005228.3:c.3184G&gt;T</b>		<b>1</b>
<b>NP_005219.2:p.Arg108Lys</b>	<b>NM_005228.3:c.323G&gt;A</b>		<b>2</b>
<b>NP_005219.2:p.Tyr117Cys</b>	<b>NM_005228.3:c.350A&gt;G</b>		<b>2</b>
<b>NP_005219.2:p.Gly288ValfsTer5</b>	<b>NM_005228.3:c.862delG</b>		<b>1</b>
<b>NP_005219.2:p.Gly288Ser</b>	<b>NM_005228.3:c.862G&gt;A</b>		<b>1</b>
<b>NP_005219.2:p.Gly288Val</b>	<b>NM_005228.3:c.863G&gt;T</b>		<b>1</b>
<b>NP_005219.2:p.Ala289Thr</b>	<b>NM_005228.3:c.865G&gt;A</b>		<b>2</b>
<b>GENE amplification</b>			<b>2</b>
	<b>Total = 101</b>	<b>58%</b>	<b>42%</b>

Mutations listed in bold type are the unique mutations detected by NGS only and not by any of the commercial kits. The number of cases is shown

NGS next-generation sequencing

of the mutations would have been missed if the cobas v2 was used for testing, 64% of the mutations would have been missed if version 1 of cobas was used. Similarly, if the thescreen kit was used, 65% of mutations would have been missed. Furthermore, using an NGS panel with many genomic targets allows detection of *EGFR* gene amplification, which is not possible using cobas or thescreen.

### 3.2 BRAF Mutation Profile

Of the 117 *BRAF* mutations detected (Table 2) using NGS, 115 resulted in protein-coding changes (defined here as somatic with allele frequency <40%) and two were gene amplifications. Sanger sequencing was used for confirmation, with no discrepancies noted. Of the detected mutations, 62 (53%) were in codon V600 and the remainder were outside codon 600 (Fig. 1). Therefore, 47% of the detected mutations would have been missed if the cobas or thescreen kits were used, including the two gene amplifications. Four out of five indels resulting in in-frame protein coding insertion/deletion and one out of two indels resulting in a frameshift in the protein coding reading frame would be missed by cobas version 2. As shown in Fig. 1, 31 of the 55 mutations not detected by cobas are between 540–599 and 601–604, many of them in the same hydrophobic pocket adjacent to V600 (Fig. 1).

### 3.3 KRAS Mutation Profile

*KRAS* mutations were detected using NGS in 320 patient samples, and one sample showed amplification (total 321) (Table 3). Samples were re-tested using Sanger sequencing for confirmation, and no discrepancy was noted. Of the 321 detected mutations, 93.5% (300) were in codons 12, 13,

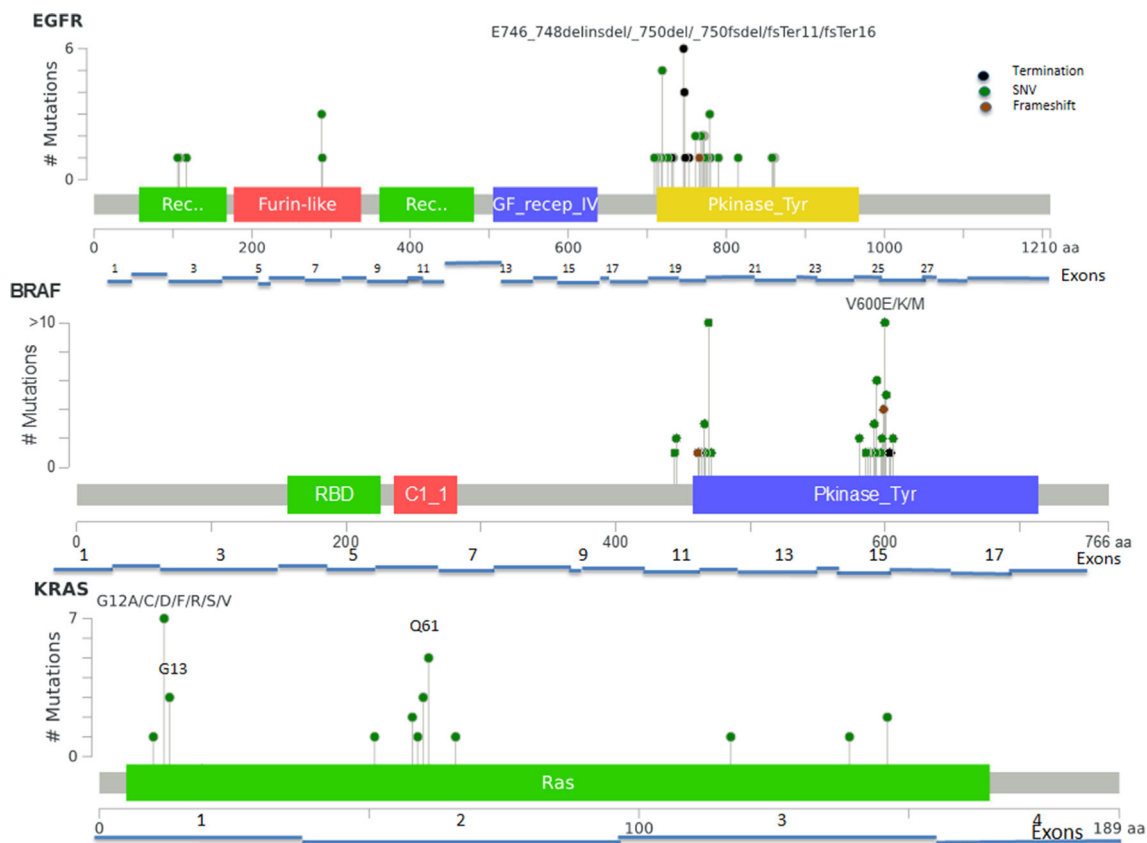
and 61, which could be detected by cobas v2. All but two of the mutations resulted in amino acid substitution events, two were indels, and one was a gene amplification. However, thescreen does not detect the mutations NGS uncovered in codons 58–60 (20 mutations), 117 (1 mutation), and 61 (16 mutations), which are in the NTP binding domain [8]; therefore, 11.5% of mutations would have been missed if the thescreen kit was used for detection. In addition, the amplification cannot be detected by Sanger or the thescreen/cobas tests; only the NGS panel could detect the *KRAS* amplifications.

## 4 Discussion

Molecular characterization of cancer is becoming essential for patient care and precision medicine. Companion testing is defined as the testing for specific abnormalities in order to be eligible for treatment with a specific drug. Giving a drug to a patient who may not respond to therapy is not only a waste of precious time, but could be harmful and may allow the tumor burden to grow and become less manageable [9]. On the other hand, failure to identify a potential drug therapy for a patient who may respond to a specific therapy is a waste of an opportunity that is very precious for the patient and the patient's family.

Therefore, using proper biomarkers for precise selection of patients for a specific therapy is critical and is now the basis of precision medicine. However, molecular testing and precision medicine are fields that are advancing at a very rapid pace. When a test is designed for a clinical trial using a state-of-the-art technology, it is very likely to be no longer state-of-the-art by the time this clinical trial is ended.





**Fig. 1** Schematic presentation of the protein structure and various functional domains of *EGFR*, *BRAF*, and *KRAS*. The sites and frequency of detection of the detected mutations are indicated with a relative scale shown on the left. The coding exons are indicated and numbered below each protein. *CI\_1* phorbol esters/diacylglycerol

Currently, multiple testing kits are on the market, some of which are FDA-cleared, but with the advent of new technology and NGS, some of these kits are no longer adequate for patient care. One of the currently approved kits for *EGFR* mutation detection misses 65% of the mutations; while the most recently approved FDA-cleared kit for *EGFR* testing (cobas v2) misses 42% of the *EGFR* mutations (Table 4). This means that almost half the patients who may benefit from this very effective therapy for lung cancer are potentially deprived of the opportunity of being treated. Other kits and tests based on similar PCR technology suffer from the same limitations. The recent advances in molecular technology and NGS make most of these tests inadequate by today's standard.

One may argue that there is a lack of data on drug response to an uncharacterized mutation by sequencing. In contrast, the mutations detected by the FDA-cleared test were considered in the clinical trial and proven to be associated with response to *EGFR* inhibitors, especially when this testing is not reimbursed. However, missing an opportunity of giving a patient a successful therapy should also be considered. While more data on the clinical

binding domain (*C1* domain), *GF recep IV* growth factor receptor domain IV, *Pkinase* protein kinase domain, *Pkinase Tyr* protein tyrosine kinase, *RBD* Raf-like Ras-binding kinase, *Rec* receptor L domain, *SNV* single nucleotide variant

relevance of these mutations and drug response is needed, it is important to note that most of the detected mutations (Fig. 1) cluster around the common mutations in the same functional domain and are predicted (by SIFT, PROVEAN, and Polyphen) to have the same biological impact. With this information, it is likely patients with mutations in exons 18, 19, and 21 will respond to *EGFR* inhibitor therapy [10, 11]. If these patients were only assessed with one of the approved kits, critical diagnostic information would be missed and no therapy could be recommended. If a patient has a mutation not previously reported in these exons, this patient deserves to be considered carefully for such therapy, especially when other options are limited. Of course, the cost of testing also might be a factor in testing fewer hot spots for mutations. However, the cost to the healthcare system will be significantly less when a targeted and effective therapy is used for treating patients and this may make up for more comprehensive testing costs.

Similarly, the current FDA-cleared kits test for *BRAF* mutations only in codon 600, constituting only 53% of the *BRAF* mutations detected in our patients. Recent studies

**Table 2** A list of all *BRAF* mutations detected by next-generation sequencing

Amino acid	Nucleotide change	Codon 600	Outside codon 600
NP_004324.2:p.Val600Lys	NM_004333.4:c.1798-1799GT>AA	1	
NP_004324.2:p.Val600Met	NM_004333.4:c.1798G>A	6	
NP_004324.2:p.Val600Glu	NM_004333.4:c.1799T>A	52	
NP_004324.2:p.Val600Glu	NM_004333.4:c.1799TG>AA	2	
NP_004324.2:p.Val600Lys	NM_004333.4:c.GT1798-1799TAA	1	
<b>NP_004324.2:p.Arg444Trp</b>	<b>NM_004333.4:c.1330C&gt;T</b>		<b>1</b>
<b>NP_004324.2:p.Asp445Tyr</b>	<b>NM_004333.4:c.1333G&gt;T</b>		<b>2</b>
<b>NP_004324.2:p.Gln461del</b>	<b>NM_004333.4:c.1381_1383delCAA</b>		<b>1</b>
<b>NP_004324.2:p.Arg462AsnfsTer17</b>	<b>NM_004333.4:c.1383_1384delAA</b>		<b>1</b>
NP_004324.2:p.Gly464Val	NM_004333.4:c.1391G>T		1
NP_004324.2:p.Gly466Glu	NM_004333.4:c.1397G>A		2
NP_004324.2:p.Gly466Ala	NM_004333.4:c.1397G>C		1
NP_004324.2:p.Ser467Leu	NM_004333.4:c.1400C>T		1
NP_004324.2:p.Gly469del	NM_004333.4:c.1405_1407delGGA		1
NP_004324.2:p.Gly469Arg	NM_004333.4:c.1405G>A		2
NP_004324.2:p.Gly469Glu	NM_004333.4:c.1406G>A		1
NP_004324.2:p.Gly469Ala	NM_004333.4:c.1406G>C		4
NP_004324.2:p.Gly469Val	NM_004333.4:c.1406G>T		3
NP_004324.2:p.Val471Ile	NM_004333.4:c.1411G>A		1
NP_004324.2:p.Asn581Ser	NM_004333.4:c.1742A>G		1
NP_004324.2:p.Asn581Ile	NM_004333.4:c.1742A>T		1
NP_004324.2:p.Glu586Lys	NM_004333.4:c.1756G>A		1
NP_004324.2:p.Asp587Glu	NM_004333.4:c.1761C>A		1
NP_004324.2:p.Thr589Lys	NM_004333.4:c.1766C>A		1
NP_004324.2:p.Ile592Val	NM_004333.4:c.1774A>G		1
NP_004324.2:p.Ile592Ter	NM_004333.4:c.1774delA		1
NP_004324.2:p.Ile592Met	NM_004333.4:c.1776A>G		1
NP_004324.2:p.Gly593Asp	NM_004333.4:c.1778G>A		1
NP_004324.2:p.Asp594IlefsTer4	NM_004333.4:c.1780delG		1
NP_004324.2:p.Asp594Asn	NM_004333.4:c.1780G>A		1
NP_004324.2:p.Asp594Gly	NM_004333.4:c.1781A>G		4
NP_004324.2:p.Leu597Gln	NM_004333.4:c.1790T>A		1
NP_004324.2:p.Ala598Thr	NM_004333.4:c.1792G>A		1
NP_004324.2:p.Ala598Val	NM_004333.4:c.1793C>T		1
NP_004324.2:p.Thr599_Val600insThr	NM_004333.4:c.1794_1796dupTAC		1
NP_004324.2:p.Thr599dup	NM_004333.4:c.1795_1797dupACA		1
NP_004324.2:p.Thr599Ile	NM_004333.4:c.1796C>T		2
NP_004324.2:p.Lys601Glu	NM_004333.4:c.1801A>G		3
NP_004324.2:p.Lys601Arg	NM_004333.4:c.1802A>G		2
NP_004324.2:p.Arg603Ter	NM_004333.4:c.1807C>T		1
NP_004324.2:p.Trp604Ter	NM_004333.4:c.1811G>A		1
NP_004324.2:p.Gly606Arg	NM_004333.4:c.1816G>A		2
<b>BRAF amplification</b>			<b>2</b>
		62	55
		53%	47%
	Total = 117		

Mutations listed in bold type are the unique mutations detected by NGS only and not by any of the commercial kits  
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**Table 3** A list of all *KRAS* mutations detected by next-generation sequencing

Amino acid	Nucleotide change	Detected by cobas	Not detected by cobas
NP_203524.1:p.Gly12Ser	NM_033360.2:c.34G>A	10	
NP_203524.1:p.Gly12Arg	NM_033360.2:c.34G>C	7	
NP_203524.1:p.Gly12Cys	NM_033360.2:c.34G>T	110	
NP_203524.1:p.Gly12Asp	NM_033360.2:c.35G>A	56	
NP_203524.1:p.Gly12Ala	NM_033360.2:c.35G>C	9	
NP_203524.1:p.Gly12Val	NM_033360.2:c.35G>T	60	
NP_203524.1:p.Gly13Ser	NM_033360.2:c.37G>A	3	
NP_203524.1:p.Gly13Cys	NM_033360.2:c.37G>T	4	
NP_203524.1:p.Gly13Asp	NM_033360.2:c.38G>A	24	
NP_203524.1:p.Gln61Lys	NM_033360.2:c.181C>A	4	
NP_203524.1:p.Gln61Arg	NM_033360.2:c.182A>G	1	
NP_203524.1:p.Gln61Leu	NM_033360.2:c.182A>T	1	
NP_203524.1:p.Gln61His	NM_033360.2:c.183A>C	4	
NP_203524.1:p.Gln61His	NM_033360.2:c.183A>T	6	
NP_203524.1:p.Gly12Phe	NM_033360.2:c.34-35GG>TT	1	
<b>NP_203524.1:p.Lys117Asn</b>	<b>NM_033360.2:c.351A&gt;T</b>		<b>1</b>
<b>NP_203524.1:p.Cys51Tyr</b>	<b>NM_033360.2:c.152G&gt;A</b>		<b>1</b>
<b>NP_203524.1:p.Thr58Lys</b>	<b>NM_033360.2:c.173C&gt;A</b>		<b>1</b>
<b>NP_203524.1:p.Thr58Ile</b>	<b>NM_033360.2:c.173C&gt;T</b>		<b>1</b>
<b>NP_203524.1:p.Ala59Thr</b>	<b>NM_033360.2:c.175G&gt;A</b>		<b>1</b>
<b>NP_203524.1:p.Gly60Ser</b>	<b>NM_033360.2:c.178G&gt;A</b>		<b>1</b>
<b>NP_203524.1:p.Gly60Asp</b>	<b>NM_033360.2:c.179G&gt;A</b>		<b>1</b>
<b>NP_203524.1:p.Gly60Val</b>	<b>NM_033360.2:c.179G&gt;T</b>		<b>1</b>
<b>NP_203524.1:p.Ala66Thr</b>	<b>NM_033360.2:c.196G&gt;A</b>		<b>1</b>
<b>NP_203524.1:p.Gly10Glu</b>	<b>NM_033360.2:c.29G&gt;A</b>		<b>1</b>
<b>NP_203524.1:p.Lys104ArgfsTer10</b>	<b>NM_033360.2:c.311delA</b>		<b>1</b>
<b>NP_203524.1:p.Ile139Asn</b>	<b>NM_033360.2:c.416T&gt;A</b>		<b>1</b>
<b>NP_203524.1:p.Ala146Thr</b>	<b>NM_033360.2:c.436G&gt;A</b>		<b>6</b>
<b>NP_203524.1:p.Ala146Val</b>	<b>NM_033360.2:c.437C&gt;T</b>		<b>1</b>
<b>NP_203524.1:p.Leu19Phe</b>	<b>NM_033360.2:c.57G&gt;T</b>		<b>1</b>
<b>Amplification</b>			<b>1</b>
		300	<b>21</b>
320 cases	Total = 321	93.5%	6.5%

Mutations listed in bold type are the unique mutations detected by NGS only and not by any of the commercial kits  
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suggest that mutations outside codon 600 behave similarly to those in codon 600 and may respond to BRAF and MEK inhibitors [12, 13]. Considering these patients with mutations outside the codon 600 for such therapy is a very viable option that should not be ignored.

As for *KRAS* mutations, the original companion testing included only testing codons 12 and 13, but the recent recommendation by the ASCO Provisional Clinical Opinion groups calls for extended testing to include testing in codons 12, 13, 61, 117, and 149, as well as similar codons in the *NRAS* gene [14]. This is very important because patients with mutations in *KRAS* or *NRAS* will not respond and may be harmed if treated with anti-EGFR antibodies. Based on our

data, 11.5% of the mutations will be missed in *KRAS* alone using the old FDA-cleared kit for *KRAS* and 6.5% of the mutations would be missed if the new improved *KRAS* kit is used. This is significant, because 42% of colorectal cancers show *KRAS* codon 12/13 mutations [14]. Based on our data, approximately 5% of all colorectal patients are receiving therapy that is expensive and harmful.

While this study confirms that NGS can detect significantly more mutations in the tested genes compared with various FDA-cleared kits, this study is limited by not demonstrating the clinical value for detecting these additional mutations. Studies and documentation of clinical value for this extended detection of mutation in the form of



**Table 4** Summary of detected mutations

Genes	Detected mutations by NGS	Missed cases by FDA-cleared kits		
		cobas	cobas v2	therascreen
<i>EGFR</i>	101	65 (64%)	42 (42%)	66 (65%)
<i>BRAF</i>	117	55 (47%)		55 (47%)
<i>KRAS</i>	321		21 (6.5%)	37 (11.5%)

The difference between NGS and other methods was statistically significant with  $p < 0.0001$  (Wilcoxon matched pairs test)

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increased response rate or prolonged survival need to be performed.

In summary, NGS provides a reliable means for comprehensive testing covering all possible mutations as well as indel and amplification. The current NGS technology is very reliable and easily adaptable for clinical testing and should be considered the gold standard for testing for mutations in *EGFR*, *KRAS*, and *BRAF*. An additional advantage of NGS is its built-in ability to multiplex and test multiple genes with only a minimal amount of DNA.

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#### Compliance with Ethical Standards

**Conflict of interest** All authors (WM, SB, SA, VAF and MA) are employed by a diagnostic company that offers clinical testing (NeoGenomics Laboratories).

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**Ethical approval and informed consent** Work was performed after obtaining IRB approval.

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