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Detection of *EGFR* Variants in Plasma

A Multilaboratory Comparison of a Real-Time PCR EGFR *Mutation Test in Europe*

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Molecular testing of *EGFR* is required to predict the response likelihood to targeted therapy in non—small cell lung cancer. Analysis of circulating tumor DNA in plasma may complement limitations of tumor tissue. This study evaluated the interlaboratory performance and reproducibility of a real-time PCR *EGFR* mutation test (cobas EGFR Mutation Test v2) to detect *EGFR* variants in plasma. Fourteen laboratories received two identical panels of 27 single-blinded plasma samples. Samples were wild type or spiked with plasmid DNA to contain seven common *EGFR* variants at six predefined concentrations from 50 to 5000 copies per milliliter. The circulating tumor DNA was extracted by a cell-free circulating DNA sample preparation kit (cobas cfDNA Sample Preparation Kit), followed by duplicate analysis with the real-time PCR *EGFR* mutation test (Roche Molecular Systems, Pleasanton, CA). Lowest sensitivities were obtained for the c.2156G>C p.(Gly719Ala) and c.2573T>G p.(Leu858Arg) variants for the lowest

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target copies. For all other variants, sensitivities varied between 96.3% and 100.0%. All specificities were 98.8% to 100.0%. Coefficients of variation indicated good intralaboratory and interlaboratory repeatability and reproducibility but increased for decreasing concentrations. Prediction models revealed a significant correlation for all variants between the predefined copy number and the observed semiquantitative index values, which reflect the samples' plasma mutation load. This study demonstrates an overall robust performance of the real-time PCR *EGFR* mutation test kit in plasma. Prediction models may be applied to estimate the plasma mutation load for diagnostic or research purposes.
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Lung cancer is the leading cause of cancer-related mortality worldwide, with most being attributable to non−small cell lung cancer (NSCLC) as its most common subtype.[1] Approximately 10% to 20% of non-Asian patients with advanced nonsquamous NSCLC present with variants in the kinase domain of the *EGFR* gene.[2] Tyrosine kinase inhibitors (TKIs) that target activating variants in *EGFR* have demonstrated strong effectiveness in the treatment of patients with NSCLC.[3,4] Therefore, molecular testing of *EGFR* in patients with NSCLC is required by the European Medicines Agency (*http://www.ema.europa.eu*, last accessed May 1, 2017) and the US Food and Drug Administration (*http://www.fda.gov*, last accessed May 1, 2017) and recommended by both the European Society of Medical Oncology[5] and the National Comprehensive Cancer Network guidelines to predict therapeutic response to first- (gefitinib and erlotinib), second- (afatinib), and third- (osimertinib) generation TKIs.[6] As a result, diagnostic laboratories are challenged to meet these requirements, using reliable methods and processes to ensure that patients receive a timely and accurate report. To date, genotyping of formalin-fixed, paraffin-embedded (FFPE) tumor tissue for *EGFR* alterations has become routine practice.[7] Although routinely performed, biopsy of tumor tissue is an invasive procedure and presents substantial challenges, such as limited tissue availability or intratumor and intertumor heterogeneity.[8,9] To overcome these limitations, less invasive techniques capable of capturing tumor heterogeneity and the molecular changes cancer cells undergo when they are exposed to therapy are desirable.[10] During the last decade, testing of liquid biopsy samples has shown promising results to overcome some of these barriers. Liquid biopsy samples can consist of almost all body fluids (blood, serum, plasma, urine, pleural effusion, ascites) and allow the extraction of tumor DNA from circulating tumor cells, exosomes, and cell-free circulating DNA (cfDNA). A variety of clinical indications have been hypothesized for different solid tumor types, including the prediction of treatment response and the evaluation of prognosis, early detection of disease recurrence, or the development of acquired resistance.[11] Although the amount and quality of cfDNA obtained from plasma samples can be challenging to work with, its included cell-free tumor DNA (ctDNA) appears to be an extremely effective and advantageous biomarker. Recent technical developments have improved the diagnostic accuracy of variants identified in ctDNA, and techniques are available that allow monitoring of tumor-associated genetic aberrations in plasma, such as real-time PCR, next-generation sequencing (NGS), droplet-digital PCR (ddPCR), or beads, emulsion, amplification, magnetics (BEAMing) PCR.[12] The variation in *EGFR* detection rates observed in prior studies with these methods highlights the need for a sensitive, standardized method for blood-based testing.[13−17] For the available platforms for ctDNA analysis, several key questions still need to be addressed, including the sensitivity and specificity as well as clinical features that may affect the sensitivity of *EGFR* analysis in plasma. In June 2016, a real-time PCR *EGFR* mutation test (cobas EGFR Mutation Test v2; Roche Molecular Systems Inc., Pleasanton, CA), hereafter referred to as the EGFRv2 test, was approved by the US Food and Drug Administration as a companion diagnostic for the detection of *EGFR* variants in plasma (*https://www.accessdata.fda.gov/cdrh_docs/pdf15/P150047b.pdf* and *https://www.accessdata.fda.gov/cdrh_docs/pdf15/P150047a.pdf*, last accessed March 5, 2018). Several individual studies on patient samples have demonstrated the feasibility of this assay, a strong overall agreement in *EGFR* results between plasma and FFPE tissue, and a high concordance of plasma results with other technologies, such as BEAMing and NGS.[13−17] Our aims were to evaluate the performance and reproducibility of the kit to detect common *EGFR* variants in contrived plasma samples among multiple test sites and to assess the correlation of those results with the copy numbers of the respective *EGFR* variants.

Material and Methods

Fourteen laboratories from 10 European countries were included in the study based on their previous experience with the EGFRv1, availability of necessary facilities, and a preceding survey on laboratory characteristics. To ensure harmonization, test sites received a 2-day hands-on training for cfDNA extraction and *EGFR* variant detection before the study. Two obligatory proficiency samples needed to be correctly identified, after which an operator qualification letter was issued that allowed the laboratory to proceed with testing.

Table 1 Overview of the 27 Samples Included in the Plasma Panel, the *EGFR* Variants, and Their Respective Copy Numbers

Panel member	*EGFR* variant	Copy numbers (target copies/mL)
1	c.2235_2249del p.(Glu746_Ala750del)	100
	c.2369C>T p.(Thr790Met)	
2	Wild-type	0
3	c.2235_2249del p.(Glu746_Ala750del)	5000
	c.2369C>T p.(Thr790Met)	
4	c.2582T>A p.(Leu861Gln)	500
	c.2307_2308ins p.(Val769_Asp770ins)	
5	c.2573T>G p.(Leu858Arg)	50
	c.2369C>T p.(Thr790Met)	
6	c.2235_2249del p.(Glu746_Ala750del)	250
	c.2369C>T p.(Thr790Met)	
7	c.2303G>T p.(Ser768Ile)	100
	c.2156G>C p.(Gly719Ala)	
8	c.2573T>G p.(Leu858Arg)	1000
	c.2369C>T p.(Thr790Met)	
9	c.2573T>G p.(Leu858Arg)	500
	c.2369C>T p.(Thr790Met)	
10	Wild-type	0
11	c.2582T>A p.(Leu861Gln)	5000
	c.2307_2308ins p.(Val769_Asp770ins)	
12	c.2573T>G p.(Leu858Arg)	100
	c.2369C>T p.(Thr790Met)	
13	c.2573T>G p.(Leu858Arg)	5000
	c.2369C>T p.(Thr790Met)	
14	c.2573T>G p.(Leu858Arg)	250
	c.2369C>T p.(Thr790Met)	
15	c.2303G>T p.(Ser768Ile)	50
	c.2156G>C p.(Gly719Ala)	
16	Wild-type	0
17	c.2235_2249del p.(Glu746_Ala750del)	1000
	c.2369C>T p.(Thr790Met)	
18	c.2582T>A p.(Leu861Gln)	50
	c.2307_2308ins p.(Val769_Asp770ins)	
19	c.2582T>A p.(Leu861Gln)	250
	c.2307_2308ins p.(Val769_Asp770ins)	
20	c.2235_2249del p.(Glu746_Ala750del)	50
	c.2369C>T p.(Thr790Met)	
21	c.2582T>A p.(Leu861Gln)	100
	c.2307_2308ins p.(Val769_Asp770ins)	
22	c.2303G>T p.(Ser768Ile)	5000
	c.2156G>C p.(Gly719Ala)	
23	c.2303G>T p.(Ser768Ile)	500
	c.2156G>C p.(Gly719Ala)	
24	c.2303G>T p.(Ser768Ile)	250
	c.2156G>C p.(Gly719Ala)	
25	c.2303G>T p.(Ser768Ile)	1000
	c.2156G>C p.(Gly719Ala)	
26	c.2582T>A p.(Leu861Gln)	1000
	c.2307_2308ins p.(Val769_Asp770ins)	
27	c.2235_2249del p.(Glu746_Ala750del)	500
	c.2369C>T p.(Thr790Met)	

Each test site received two identical plasma panels. Plasma samples are wild-type or contained a combination of two *EGFR* variants. Note that the c.2369C>T p.(Thr790Met) variant is present in two combinations, once with the exon 19 deletion c.2235_2249del p.(Glu746_Ala750del) variant (panel members 1, 3, 6, 17, 20, and 27) and once with the c.2573T>G p.(Leu858Arg) variant (panel members 5, 8, 9, 12, 13, and 14). For each panel member, two runs (A and B) were performed. Samples were generated and pretested with next-generation sequencing by Roche Molecular Systems (Pleasanton, CA).

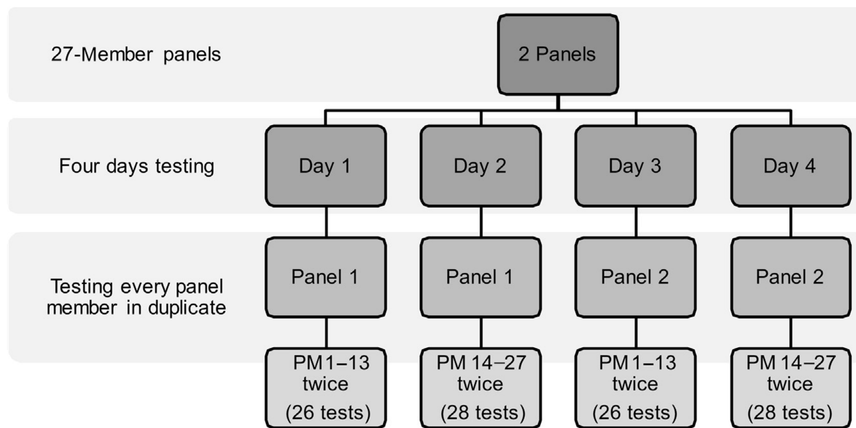

Figure 1 Overview of the testing algorithm followed by the laboratories to analyze two identical 27-plasma member (PM) panels in duplicate, yielding a total of four runs per PM.

Between January and March 2016, the test sites received two identical panels of 27 single-blinded samples that contained 4.2 mL of plasma each. Sample generation and subsequent confirmation of the copy numbers were performed by Roche Molecular Systems. Samples were generated by using normal (healthy donor) plasma in K2 EDTA tubes that were pretested by the EGFRv2 kit to be negative for any of the *EGFR* variants, after which nonsheared, linearized plasmids with an approximate size of 3.2 kb were spiked in. Copy number determination was performed by optical density testing (J.F.P., P.M.W., S.S., N.N., J.H.v.K., A.S, F.F., D.G.d.C., S.H., I.K., F.L-R., M.D.L., A.M., P.H., E.S., U.S., B.S., P.T., M.T., H.V., and E.M.C.D.) and confirmed by comparing to the EGFRv2 standard curve and NGS (in-house developed panel on MiSeq platform, Illumina, San Diego, CA). The limit of detection (LOD) of the EGFRv2 kit has previously been defined by the manufacturer.

Samples were shipped on dry ice and stored at −80°C before further analyses. The panels included three *EGFR* wild-type cases along with 24 cases of human plasma, spiked with plasmid DNA that contained seven common *EGFR* variant*s* at six predefined target copies per milliliter (Table 1).

The cfDNA was extracted from the plasma by the a cell-free circulating DNA sample preparation kit (cobas cfDNA Sample Preparation Kit, Roche Molecular Systems) according to the manufacturer's protocol (*https://www.*

Table 2 Analytical Sensitivity and Specificity of the EGFRv2 Test to Identify Seven *EGFR* Variants in Cell-Free Tumor DNA Derived From Plasma

Copy number (copies/mL)	c.2235_2249del p.(Glu746_Ala750del)	Number of tests	c.2369C>T p.(Thr790Met)	Number of tests	c.2573T>G p.(Leu858Arg)	Number of tests
Sensitivity						
50	100.0 (93.6–100.0)	56	96.3 (90.9–99.0)	109	88.7 (77.0–95.7)	53
<u>100</u>	<u>100.0 (93.5–100.0)</u>	<u>55</u>	<u>100.0 (96.6–100.0)</u>	<u>108</u>	<u>98.1 (89.9–100.0)</u>	<u>53</u>
250	100.0 (93.6–100.0)	56	99.1 (95.0–100.0)	110	98.1 (90.1–100.0)	54
500	100.0 (93.6–100.0)	56	100.0 (96.8–100.0)	112	98.2 (90.4–100.0)	56
1000	100.0 (93.6–100.0)	56	100.0 (96.8–100.0)	112	98.2 (90.4–100.0)	56
5000	100.0 (93.6–100.0)	56	100.0 (96.8–100.0)	112	98.2 (90.4–100.0)	56
Mean	100.0 (98.9–100.0)	335	99.2 (98.2–99.8)	663	96.6 (94.1–98.3)	328
Specificity						
0	100.0 (97.8–100.0)	166	100.0 (98.9–100.0)	332	100.0 (97.8–100.0)	166
50	100.0 (97.8–100.0)	164	100.0 (98.9–100.0)	331	100.0 (97.8–100.0)	167
100	100.0 (97.8–100.0)	165	100.0 (98.9–100.0)	332	100.0 (97.8–100.0)	167
250	99.4 (96.7–100.0)	165	100.0 (98.9–100.0)	332	100.0 (97.8–100.0)	167
500	100.0 (97.8–100.0)	167	100.0 (98.9–100.0)	334	100.0 (97.8–100.0)	167
1000	100.0 (97.8–100.0)	167	100.0 (98.9–100.0)	334	100.0 (97.8–100.0)	167
5000	100.0 (97.8–100.0)	168	100.0 (98.9–100.0)	336	100.0 (97.8–100.0)	168
Mean	99.9 (99.5–100.0)	1162	100.0 (99.8–100.0)	2331	100.0 (99.8–100.0)	1169

(*table continues*)

The exact 95% CIs are given in parenthesis. Underlined values are those observed at the kit's limit of detection. The number of tests represent the four tests performed (duplicate analysis of two identical panels) for a given variant by all 14 test sites, excluding runs during which a technical failure or protocol deviation occurred. Number of runs are higher for the c.2369C>T p.(Thr790Met) variant because of its presence in two combinations of *EGFR* variants included in the plasma panel.

accessdata.fda.gov/cdrh_docs/pdf15/p150047c.pdf, last accessed March 5, 2018). Subsequently, each sample was subjected to duplicate analysis with the EGFRv2 test according to the manufacturer's instructions. The EGFRv2 assay applies real-time PCR for the detection of 42 *EGFR* variants in exons 18, 19, 20, and 21 in both plasma and FFPE tissue[18] with a LOD of at least 100 copies/mL of plasma (Supplemental Table S1).

To determine the LOD (*https://www.accessdata.fda.gov/cdrh_docs/pdf15/p150047c.pdf*, last accessed March 5, 2018), sheared cell line DNA that contained each of the seven mutation classes detected by the test were added to healthy donor K2 EDTA plasma that was wild-type for *EGFR*. Serial dilutions were prepared and 24 replicates of each panel member were tested, using each of three EGFRv2 kit lots. The LOD was determined for each of the seven mutation classes detected by the test as the lowest concentration of DNA yielding an *EGFR* mutation detected rate of at least 95% for the targeted mutation. This study demonstrated that the EGFRv2 kit can detect mutations in *EGFR* exons 18, 19, 20, and 21 with $\leq$100 copies/mL of mutant DNA using the standard input of 25 μL of DNA stock per reaction well. The mean size of the sheared DNA was 220 bp, in line with the size of the DNA found in plasma.

Analyses were performed across four subsequent test days (Figure 1). Samples were analyzed automatically by the *EGFR* Plasma Analysis Package Software version 1.0 (Roche Molecular Systems) in combination with the cobas z480 analyzer with software version 2.1 (Roche Molecular Systems), after which an automated report was generated. Laboratories could request an additional plasma panel for testing with an alternate method, such as ddPCR.

In case an *EGFR* variant was detected in the sample, the software automatically reports a corresponding semi-quantitative index (SQI) value. This SQI is determined by using the observed Ct and a proprietary, unique algorithm for that specific variant. The SQI value correlates with the target copies per milliliter and is independent of the amount of background DNA, as opposed to the mutant allele frequencies.

An electronic database was set up to collect all results, that allowed the reporting of protocol deviations and the uploading of the automated reports, and included questions on additional testing variables. These data were collected and analyzed independently by the Biomedical Quality Assurance Research Unit (University of Leuven, Leuven, Belgium). Statistical analyses were performed with SAS version 9.4 (SAS Institute Inc., Cary, NC) in collaboration with the Leuven Biostatistics and Statistical Bioinformatics Center (University of Leuven). Graphs were generated using GraphPad Prism version 7.0 (GraphPad Software Inc., La Jolla, CA).

Results

Of the 14 test sites included in the study, nine of the laboratories were located in a university hospital, two in a community hospital, two in a private laboratory, and one in a

Table 2 (*continued*)

c.2156G>C p.(Gly719Ala)	Number of tests	c.2303G>T p.(Ser768Ile)	Number of tests	c.2582T>A p.(Leu861Gln)	Number of tests	c.2307_2308ins p.(Val769_Asp770ins)	Number of tests
80.4 (67.6–89.8)	56	100.0 (93.6–100.0)	56	100.0 (93.5–100.0)	55	100.0 (93.5–100.0)	55
69.6 (55.9–81.2)	56	100.0 (93.6–100.0)	56	100.0 (93.6–100.0)	56	100.0 (93.6–100.0)	56
89.1 (77.8–95.9)	55	100.0 (93.5–100.0)	55	100.0 (93.6–100.0)	56	100.0 (93.6–100.0)	56
98.2 (90.4–100.0)	56	100.0 (93.6–100.0)	56	100.0 (93.5–100.0)	56	100.0 (93.5–100.0)	56
98.2 (90.3–100.0)	55	100.0 (93.5–100.0)	55	100.0 (93.6–100.0)	55	100.0 (93.6–100.0)	55
100.0 (93.6–100.0)	56	100.0 (93.6–100.0)	56	100.0 (93.6–100.0)	56	100.0 (93.6–100.0)	56
89.2 (85.4–92.3)	334	100.0 (98.9–100.0)	334	100.0 (98.9–100.0)	334	100.0 (98.9–100.0)	334
100.0 (97.8–100.0)	166	100.0 (97.8–100.0)	166	100.0 (97.8–100.0)	166	100.0 (97.8–100.0)	166
100.0 (97.8–100.0)	164	100.0 (97.8–100.0)	164	100.0 (97.8–100.0)	165	100.0 (97.8–100.0)	165
98.8 (95.7–99.9)	164	100.0 (97.8–100.0)	164	100.0 (97.8–100.0)	164	100.0 (97.8–100.0)	164
100.0 (97.8–100.0)	166	100.0 (97.8–100.0)	166	100.0 (97.8–100.0)	165	100.0 (97.8–100.0)	165
100.0 (97.8–100.0)	167	100.0 (97.8–100.0)	167	100.0 (97.8–100.0)	168	100.0 (97.8–100.0)	168
100.0 (97.8–100.0)	168	99.4 (96.7–100.0)	168	100.0 (97.8–100.0)	167	100.0 (97.8–100.0)	167
100.0 (97.8–100.0)	168	100.0 (97.8–100.0)	168	98.8 (95.8–99.9)	168	100.0 (97.8–100.0)	168
99.8 (99.4–100.0)	1163	99.9 (99.5–100.0)	1163	99.8 (99.4–100.0)	1163	100.0 (99.8–100.0)	1163

cancer research institute. Ten of the 14 laboratories were also performing research besides diagnostic *EGFR* analyses. At the time of the survey, seven of the 14 test sites were accredited for molecular pathology by conforming to ISO15189 or equivalent national standards, and for five others accreditation was under way. However, analysis of liquid biopsy samples was not yet required to attain accreditation. Ten test sites reported having previous experience with ctDNA analysis, two in a diagnostic setting, three in a research setting, and five in both. The other four laboratories were planning to implement ctDNA analysis of plasma samples in the near future. Eleven of the 14 test sites were able to correctly identify both the *EGFR* c.2303G>T p.(Ser768Ile) and c.2156G>C p.(Gly719Ala) variants present in the two proficiency samples on the first run. Three other test sites succeeded after requesting a second set of samples (data not shown).

For the two plasma panels included in the study, runs were considered valid if the mutant and negative control included in the kit were valid. Invalid sample results were flagged by the kit's software. Test sites were asked to report any deviation from the manufacturer's protocol, which may lead to a result that is not considered reliable. Results displayed no failed overall runs; however, on an individual sample level, 3 of 1512 technical failures (0.2%) were observed, meaning that no information on the *EGFR* status could be obtained for this sample. Protocol deviations occurred in 12 of 1512 tests (0.8%). Samples for which a technical failure or protocol deviation occurred were excluded from further statistical analyses.

To estimate the analytical sensitivity and specificity of the kit, all four runs from each of the 14 laboratories were taken into account for a given panel member, with the exclusion of the results mentioned above. The sensitivity was calculated as the proportion of correctly identified variants (ie, the number of test samples in which the variant was detected divided by the number of test samples with the variant present). Specificity was defined as the proportion of correctly identified wild-type samples (ie, the number of test samples in which the variant was not detected divided by the number of test samples without the variant). Calculated sensitivities and specificities are represented in Table 2. Taking into account all seven variants and predefined copy numbers, our results showed a sensitivity of 98.0% (95% CI, 97.4%-98.5%) and a specificity of 99.9% (95% CI, 99.9%-100.0%). In 52 of the 2662 tests (2.0%), a false-negative result was reported. A large fraction (40.4%) of those false-negative results were observed at the lowest copy number of 50 copies/mL for all *EGFR* variants combined. The number of false-negative results decreased for increasing copy numbers and hence resulted in an increased sensitivity for higher copy numbers. In addition, most false-negative results occurred for the c.2156G>C p.(Gly719Ala) (69.2%) and c.2573T>G p.(Leu858Arg) (21.2%) variants. No correlation was observed between the percentage of false-negative results and a specific plasma sample or test site. In contrast to false-negative results, a false-positive result occurred in only 6 of the 9314 tests performed (0.06%). Moreover, none of these results were reported for any of the wild-type samples but were detected as an additional variant in a case for which an *EGFR* variant was already correctly identified. There was no correlation between the panel member, test site, copy number, or variant and the false-positive results.

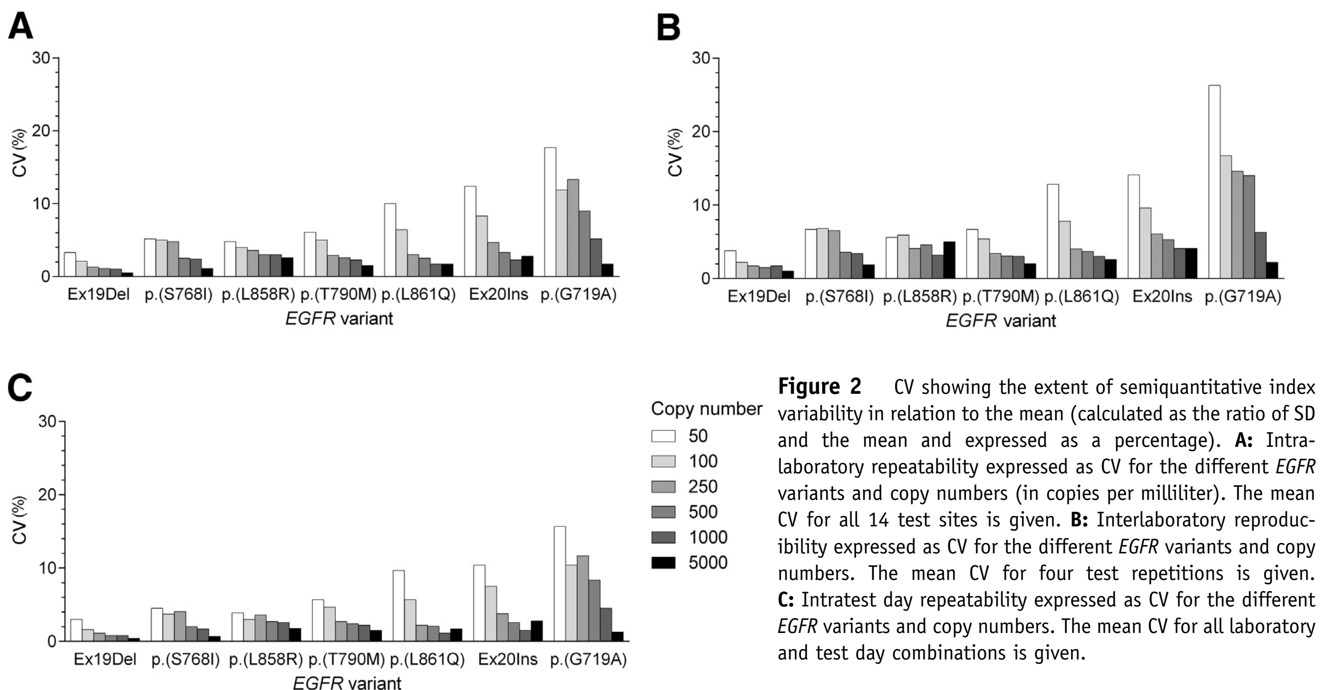

Figure 2 CV showing the extent of semiquantitative index variability in relation to the mean (calculated as the ratio of SD and the mean and expressed as a percentage). **A:** Intralaboratory repeatability expressed as CV for the different *EGFR* variants and copy numbers (in copies per milliliter). The mean CV for all 14 test sites is given. **B:** Interlaboratory reproducibility expressed as CV for the different *EGFR* variants and copy numbers. The mean CV for four test repetitions is given. **C:** Intratest day repeatability expressed as CV for the different *EGFR* variants and copy numbers. The mean CV for all laboratory and test day combinations is given.

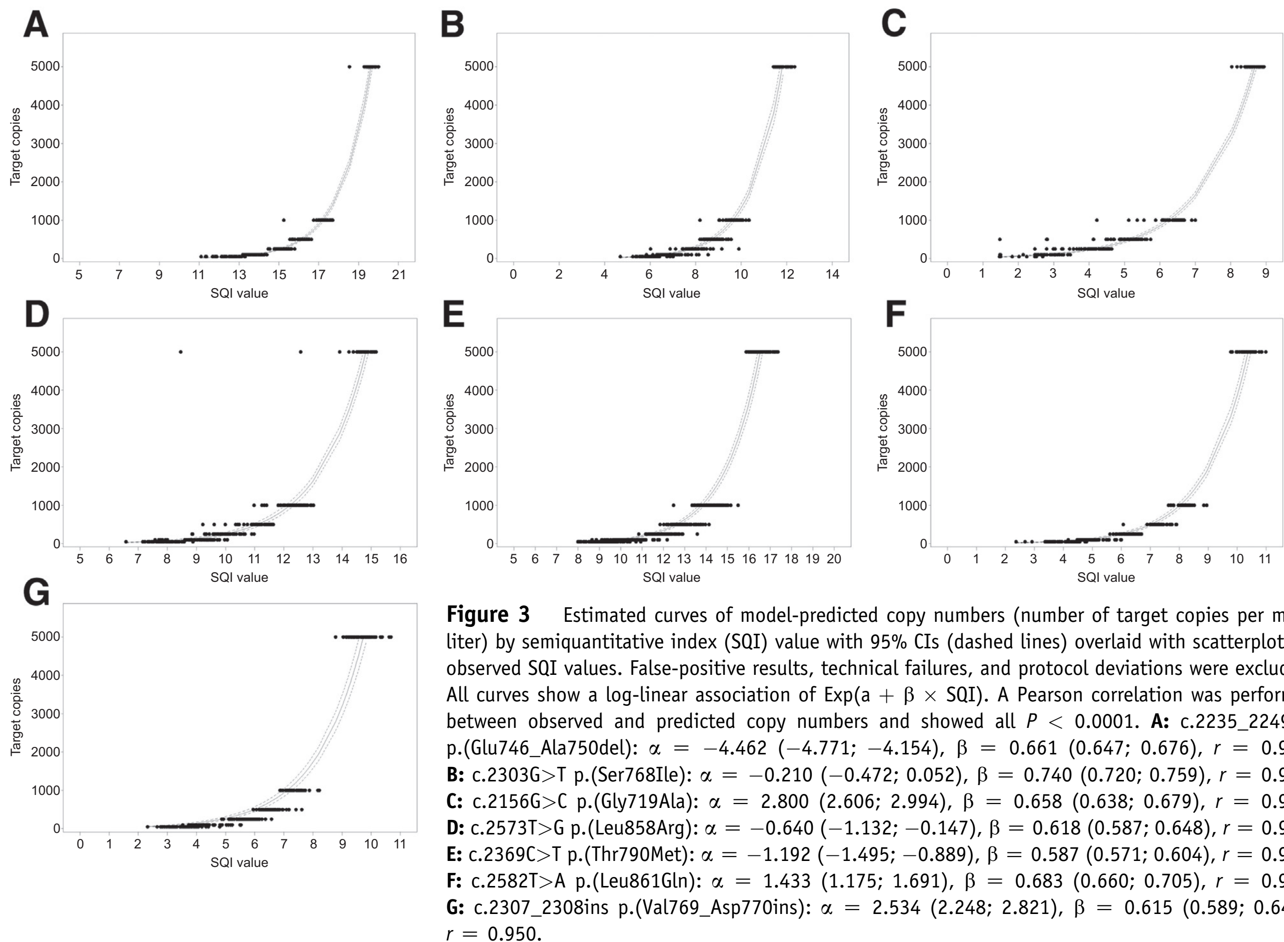

Figure 3 Estimated curves of model-predicted copy numbers (number of target copies per milliliter) by semiquantitative index (SQI) value with 95% CIs (dashed lines) overlaid with scatterplots of observed SQI values. False-positive results, technical failures, and protocol deviations were excluded. All curves show a log-linear association of Exp($a + \beta \times$ SQI). A Pearson correlation was performed between observed and predicted copy numbers and showed all $P < 0.0001$. **A:** c.2235_2249del p.(Glu746_Ala750del): $\alpha = -4.462$ (-4.771; -4.154), $\beta = 0.661$ (0.647; 0.676), $r = 0.989$. **B:** c.2303G>T p.(Ser768Ile): $\alpha = -0.210$ (-0.472; 0.052), $\beta = 0.740$ (0.720; 0.759), $r = 0.977$. **C:** c.2156G>C p.(Gly719Ala): $\alpha = 2.800$ (2.606; 2.994), $\beta = 0.658$ (0.638; 0.679), $r = 0.988$. **D:** c.2573T>G p.(Leu858Arg): $\alpha = -0.640$ (-1.132; -0.147), $\beta = 0.618$ (0.587; 0.648), $r = 0.966$. **E:** c.2369C>T p.(Thr790Met): $\alpha = -1.192$ (-1.495; -0.889), $\beta = 0.587$ (0.571; 0.604), $r = 0.965$. **F:** c.2582T>A p.(Leu861Gln): $\alpha = 1.433$ (1.175; 1.691), $\beta = 0.683$ (0.660; 0.705), $r = 0.951$. **G:** c.2307_2308ins p.(Val769_Asp770ins): $\alpha = 2.534$ (2.248; 2.821), $\beta = 0.615$ (0.589; 0.641), $r = 0.950$.

Sensitivities were the lowest for c.2369C>T p.(Thr790Met), c.2573T>G p.(Leu858Arg), and c.2156G>C p.(Gly719Ala) variants at 50 copies/mL (Table 2), which is below the kit's LOD (Supplemental Table S1). For c.2156G>C p.(Gly719Ala), sensitivities were lower at 100 and 250 copies/mL compared with samples with higher copy numbers for that variant. Specificities all exceeded 98.8% for all variants and copy numbers tested.

For every *EGFR* variant detected by the kit software, a corresponding SQI value is reported, which is specific for a given variant and reflects the potential mutation load in the sample. To evaluate the concordance of the SQI value within and among test sites, the extent of variability was defined in relation to the mean and expressed in percentage. This value, the CV, is represented in Figure 2 for every variant and copy number. The intralaboratory repeatability is defined as the CV among the four tests obtained within a single laboratory for that sample. The mean CV for all laboratories is presented in Figure 2A. In contrast, the interlaboratory reproducibility is quantified as the CV in test results obtained among the different test sites. The mean CV for the four tests is presented in Figure 2B. These results demonstrated overall low CVs for all variants, although increasing for lower copy numbers. Intralaboratory CVs were all lower compared with interlaboratory CVs because those tests were performed by the same operator. For intralaboratory repeatability and interlaboratory reproducibility, CVs were higher at all copy numbers for the c.2156G>C p.(Gly719Ala) variant compared with other variants, with a maximum value of 26.3% at 50 copies/mL. To calculate the intralaboratory and interlaboratory CVs, the four tests were considered as measurements under equal conditions. However, for every panel member, the first two runs (panel 1) were performed on the same test day, whereas the last two runs (panel 2) were performed on another test day (Figure 1). To explore whether the test day or panel adds additional variability, the intralaboratory repeatability was recalculated per laboratory and per test day, and the mean for all laboratory and test day combinations is represented in Figure 2C. The intralaboratory CVs were slightly lower compared with those in Figure 2A.

To evaluate the correlation between the SQI value and the corresponding copy number of a variant, log-linear models were used for the prediction of the target copy numbers based on SQI values. A random intercept by laboratory was used to account for clustering. The strength of the model was quantified by the Pearson correlation coefficient between the

Table 3 Model Residual Variability and Prediction Bias

EGFR variant	Residual variability (*P* value)	Prediction bias (*P* value)	Mean bias (95% CI)					
			50 copies/mL	100 copies/mL	250 copies/mL	500 copies/mL	1000 copies/mL	5000 copies/mL
c.2235_2249del p.(Glu746_Ala750del)	**0.0235**	0.1298	—	—	—	—	—	—
c.2303G>T p.(Ser768Ile)	**<0.0001**	0.0518	—	—	—	—	—	—
c.2156G>C p.(Gly719Ala)	**<0.0001**	**0.0074**	−10.0 (−79.0 to 59.0) $P = 0.7748$	1.1 (−71.2 to 73.4) $P = 0.9767$	17.2 (−48.9 to 83.4) $P = 0.6082$	16.5 (−46.0 to 78.9) $P = 0.6043$	**−102.6 (−165.6 to −39.6) $P = 0.0015$**	**72.2 (10.3 to 134.0) $P = 0.0223$**
c.2573T>G p.(Leu858Arg)	**<0.0001**	**0.0005**	−23.2 (−138.7 to 92.3) $P = 0.6929$	−45.1 (−154.8 to 64.7) $P = 0.4198$	−20.6 (−129.3 to 88.1) $P = 0.7098$	−45.8 (−152.5 to 60.9) $P = 0.3991$	**−124.6 (−231.3 to −17.8) $P = 0.0223$**	**217.3 (110.5 to 324.0) $P < 0.0001$**
c.2582T>A p.(Leu861Gln)	**0.0004**	**<0.0001**	−19.7 (−141.4 to 102.1) $P = 0.7507$	−32.9 (−153.5 to 87.8) $P = 0.5922$	−62.2 (−182.9 to 58.5) $P = 0.3113$	−112.8 (−234.5 to 9.0) $P = 0.0693$	**−129.5 (−250.1 to −8.8) $P = 0.0355$**	**304.5 (185.9 to 423.0) $P < 0.0001$**
c.2369C>T p.(Thr790Met)	**<0.0001**	**<0.0001**	−20.6 (−86.5 to 45.2) $P = 0.5385$	−32.9 (−97.9 to 32.0) $P = 0.3199$	**−86.1 (−150.7 to −21.4) $P = 0.0091$**	**−106.5 (−170.2 to −42.7) $P = 0.0011$**	**−228.6 (−292.4 to −164.8) $P < 0.0001$**	**180.6 (116.8 to 244.4) $P < 0.0001$**
c.2307_2308ins p.(Val769_Asp770ins)	**<0.0001**	**<0.0001**	−63.6 (−165.8 to 38.7) $P = 0.2223$	−90.5 (−191.9 to 10.8) $P = 0.0798$	**−173.7 (−275.0 to −72.3) $P = 0.0008$**	**−220.6 (−322.9 to −118.3) $P = <0.0001$**	**−191.5 (−292.9 to −90.2) $P = 0.0002$**	**225.7 (124.3 to 327.0) $P < 0.0001$**

Column 2: Levene's test to evaluate model residual variability of prediction error depending on concentration. Significance level $\alpha = 0.05$. For all variants, more variability of prediction error was observed. Variability was higher for increasing copy numbers (data not shown). Column 3: Analysis of variance to indicate systematic prediction bias depending on concentration. Significance level $\alpha = 0.05$. Columns 4 through 7: Amount and direction of bias predicted in column 3 for different copy numbers with 95% CIs. $P < 0.05$ indicates whether bias if different from zero. Mean bias <0 indicates overestimation. Mean bias >0 indicates underestimation. Significant results are shown in bold. Blank cells (—) represent copy numbers for which the direction of bias was not further evaluated because no bias was detected.

predefined copy numbers and the model-predicted values. Results revealed a nonlinear association of the following form:

$\exp(\alpha + \beta \times \text{SQI})$ (Figure 3), meaning that for a 1-unit increase in the observed SQI value, the predicted copy number in the sample will multiply by the exponential factor β. In addition, there was a significant (all $P < 0.0001$) correlation between observed and predicted concentration for all seven variants, with correlation coefficients that exceeded $r = 0.950$, indicating that copy numbers can be estimated based on the observed SQI values. In addition to those findings, an analysis of variance was applied to the model residuals to check the presence of a systematic prediction error (overestimation or underestimation) by copy number. The bias amount, direction, and corresponding *P* values are represented in Table 3 and displayed a significant prediction bias for five of seven *EGFR* variants included in the study. For those variants, an overestimation of the predicted copy numbers was apparent at 1000 copies/mL compared with a systematic underestimation at 5000 copies/mL. For the c.2369C>T p.(Thr790Met) and c.2307_2308ins p.(Val769_Asp770ins) variants, an overestimation at 250 and 500 copies/mL was also observed. No bias was detected at 50 and 100 copies/mL, respectively. Finally, Levene's test was used to test for differences in variability of prediction error by copy number (Table 3). Graphs for each of the seven variants demonstrate a higher model residual variability (more prediction uncertainty or error) for increasing copy numbers for all variants (Supplemental Figure S1).

Besides the EGFRv2 test, the test sites were allowed to request an additional plasma panel for analysis by a method of choice. One laboratory analyzed all 27 plasma members using Sanger sequencing, and two others performed ddPCR on a subset of plasma samples. Allelic frequencies detected by ddPCR all correlated well with predefined target copies (all $P < 0.0001$, Pearson $r > 0.93$) but to a lesser extent with the EGFRv2 SQI values reported by the same operator (all $P < 0.05$, Pearson $r > 0.80$). Because of the limited amount of data, results of these tests compared with the EGFRv2 test results are represented in Supplemental Tables S2, S3, and S4.

Discussion

Accurate identification of *EGFR* variants within an acceptable timeframe is critical to select appropriate therapy options for patients with advanced nonsquamous NSCLC. Although analysis of FFPE tissue currently remains the gold standard, the use of ctDNA in plasma as a surrogate sample type has shown promising results, and many laboratories are currently implementing this technique into research or diagnostic setting.[19] The aim of this study was threefold: i) to evaluate the performance of the EGFRv2 test to detect common *EGFR* variants in plasma

samples, ii) to evaluate the assay reproducibility among multiple test sites, and iii) to assess the correlation of those results with the copy numbers of the respective *EGFR* variants.

Results for the first goal demonstrate an overall robust performance of the EGFRv2 test to correctly detect the seven *EGFR* variants at predefined target copies in plasma, with an analytical sensitivity of 98.0% and specificity of 99.9%. Previously, several groups have reported an overall sensitivity and specificity of >72% and >96% to correctly identify *EGFR* variants in plasma.[13−15] However, it must be kept in mind that these were individual studies using patient samples and that the mass of cfDNA is variable.[17] In this study, linearized plasmids were spiked into plasma to assess the sensitivity and specificity at various ranges of target copy numbers. Although the size of the DNA present in the panels might differ from patient samples, this set-up allowed us to include an exact amount of amplifiable mutant copy numbers. Namely, shearing the DNA to be more representative of clinical samples would physically damage the DNA and alter the amplifiable DNA and thus also the amplifiable copy numbers. This set-up resulted in the ability not only to evaluate the kit's performance and reproducibility at specific levels of target copies but also to correlate these specific target copies to the obtained SQI values. It might be advisable to compare sensitivities of this study with those measured in patient samples; however, this set-up was favorable to assess the reproducibility of the kit between and within multiple test sites, which might be more difficult to reproduce with patient material, which is limited.

Not surprisingly, most false-negative results occurred at the lowest concentrations of 50 and 100 copies/mL. This finding corresponds with the LOD defined by the manufacturer of at least 100 copies/mL of mutant DNA (when using the standard input of 25 μL of DNA stock per reaction well). On a variant level, most false-negative results were observed for the c.2156G>C p.(Gly719Ala), which is not surprising because its LOD has previously been defined to be higher compared with the other variants (Table 3). Previously, lower sensitivities have also been reported in the literature for the detection of c.2156G>C p.(Gly719Ala), c.2573T>G p.(Leu858Arg), and c.2369C>T p.(Thr790Met) in patient samples.[13−16] Nevertheless, in this study, the overall results were acceptable, with mean sensitivities of 89.2%, 96.6%, and 99.2% and mean specificities of 99.8%, 100.0%, and 100% for these three variants, respectively, disregarding the copy numbers. In addition, detection of the other *EGFR* variants at the LOD showed an acceptable sensitivity and specificity, and no correlation was detected between the error percentage and a specific plasma sample or test site. Detection of the c.2369C>T p.(Thr790Met) variant at the lowest target copies of 50 copies/mL yielded a sensitivity of 96.3%. Detection of this variant at even low copies is important because it has been described to occur at low frequencies, confers acquired resistance to first- and second-generation EGFR TKI treatment, and is required for testing before administration of a third-generation TKI, such as osimertinib.[20,21] In addition, detection of high copy numbers demonstrated the performance of the kit without the risk of saturation of the PCR.

Besides false-negative results, false-positive results are of equal importance because both can have implications on treatment selection and patient outcome. In this study, only 0.06% of false-positive results were observed of which none were in wild-type samples but only as an additional variant in a sample that already contained two *EGFR* variants. In addition, none of the false-positive results included the c.2369C>T p.(Thr790Met) variant, so clinical indications would not be compromised. The fact that few false-positive results were observed could be explained by the kit's selective amplification process using the AmpErase (uracil-*N*-glycosylase enzyme) enzyme, reducing the possibility of PCR contamination.[18]

The second aim of this study was to evaluate the intralaboratory repeatability among the SQI values detected during the four different runs for every variant and concentration, as well as the interlaboratory reproducibility of this value among the 14 different test sites. This value correlates with the mutation load in a sample so that sequentially collected samples can be used to monitor changes in ctDNA during a specified period.[18] Results revealed that the SQI variability is acceptable but increases for lower copy numbers for all *EGFR* variants. Therefore, it may be useful to perform duplicate tests during validation to reduce the mean variation. Not surprisingly, interlaboratory CVs are higher compared with intralaboratory CVs because tests are performed by different operators. The small difference with the intratest day CV suggests that the test day or panel accounts for only limited additional variability among the SQI values reported by the same test operator. Strikingly, CVs are higher for the c.2156G>C p.(Gly719Ala) variant compared with all other variants. Although detection of this variant seems to be more difficult compared with others, CVs are within an acceptable range,[22] and the variant was always detected in at least one of the four runs.

The third aim of this study looked at the correlation of the SQI values with the predefined copy numbers, which revealed three interesting findings. First, there was a strong nonlinear association between the reported SQI values and the predefined target copies (Figure 3). Although this study used contrived plasma samples, Pearson correlation coefficients between the SQI value and the percentage of *EGFR* variant measured by NGS were previously reported for patient samples for c.2235_2249del p.(Glu746_Ala750del), c.2573T>G p.(Leu858Arg), and c.2369C>T p.(Thr790Met)[15] and were of the same order as results observed during this study. The resulting curves can be applied by future kit users to determine the copy number of

EGFR variants in plasma samples, which could have benefits in both diagnostics and research.

For instance, the ratio of the resistance and the sensitizing variants could help in selecting those patients who have a higher probability of benefiting from treatment with drugs targeting c.2369C>T p.(Thr790Met).[21,23] In addition, methods that allow estimating the mutation load in a sample could contribute to understanding the link between mutation load and tumor progression or treatment response to TKIs. However, the reproducibility and correlation to the SQI values should be validated in real patient material before using the data for interpretation of clinical samples.

Second, scatterplots of model residuals by concentration (Supplemental Figure S1) showed that all variants display a higher variability of prediction error for increasing concentrations (Table 3). This means that for higher copy numbers, the difference between the observed and predicted concentration becomes larger. On the basis of the intrinsic properties of an exponential association, an identical increase in SQI value will result in a larger difference between predicted copy numbers for higher observed SQI values.

Third, for all variants besides the c.2235_2249del p.(Glu746_Ala750del) and c.2303G>T p.(Ser768Ile), there was a systematic prediction bias that resulted in an underestimation at 1000 copies/mL and overestimation of the mutation load in the samples at 5000 copies/mL. This finding is not surprising because the inclusion of the 5000 copies/mL has skewed the curve in its direction. Therefore, it is important to take into account these systematic errors in Table 2 when calculating the predicted mutation load in the sample based on the curves and formula represented in Figure 2. The amount of ctDNA obtained in real patient samples is highly variable because it is linked to the patient's treatment and underlying biology and the exact ranges in which variants tend to occur still remain to be determined. Although the exact mutation load might be important for applications such as the early detection of resistance mechanisms, the qualitative detection of *EGFR* variants is currently the only requirement to inform the clinician about the likelihood of response to TKI therapy.

Finally, *EGFR* variants can be detected in ctDNA using a variety of methods, including real-time PCR, NGS, and ddPCR.[21,23] Because ctDNA is present at low concentrations, its detection requires highly sensitive techniques. One of the main advantages of the EGFRv2 test is that it is an efficient process to detect *EGFR* variants in plasma and to guide appropriate therapy options in patients with NSCLC, within an acceptable turnaround time. The fact that only a few technical errors occurred and that 14 test sites with different characteristics were able to successfully pass proficiency testing suggests the ease of implementing this method in the laboratory, although keeping in mind the previous working experience of the test sites with the EGFRv1 kit. Both in the IQNPath 2016 ctDNA pilot EQA scheme and the ESP 2016 Lung EQA scheme, adequacy of the EGFRv2 kit was also shown in both plasma and tissue material, with only a small percentage of false-positive or false-negative results (<3%) and no technical failures (unpublished data). Although commercial kits have the disadvantage that they are not able to determine the variant allele frequency, the curves described above allow users to estimate the mutation load in the plasma based on the SQI value. Although the variant allele frequency is dependent on the amount of background DNA in the sample, this is not the case for a sample's mutation load. However, to date, reporting of the SQI value is only available in the EGFRv2 kit with a European in vitro diagnostic label. In the United States, SQI values are provided for research use only but not for the US in vitro diagnostic labeled kit.

Although targeted kits, such as the EGFRv2, detect only known variants by using exact probes and their multiplexing capability is limited compared with NGS, their analytical sensitivity is higher, and turnaround time is significantly faster. In this study, a high concordance was observed between the EGFRv2 kit and ddPCR but to a lesser extent with Sanger on testing of an additional plasma panel (Supplemental Tables S1, S2, and S3). Previously, a high concordance has also been described between the EGFRv2 kit and BEAMing[14] or ultradeep NGS[15] for the c.2235_2249del p.(Glu746_Ala750del), c.2573T>G p.(Leu858Arg), and c.2369C>T p.(Thr790Met) variants. Not surprisingly, many samples had false-negative results by Sanger sequencing because of the low sensitivity of 20% to detect *EGFR* variants in ctDNA.

Conclusion

A number of prior studies have investigated the use of ctDNA to define the *EGFR* status with varying results.[13–17] This study demonstrates that the EGFRv2 test in combination with the cfDNA Sample Preparation Kit is a reliable and reproducible method for ctDNA analysis in plasma, with good analytical sensitivities and specificities as well as the possibility of evaluating the mutational load in plasma for diagnostic and research purposes. Although tissue is currently the preferred first sample choice for *EGFR* testing, the EGFRv2 test could be a valuable and convenient method in situations in which ctDNA analysis is recommended, especially for repeated longitudinal testing.

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Supplemental Data

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References

1. Stuart BW, Wild CP: World Cancer Report, World Health Organization (WHO). 2014. Available at http://publications.iarc.fr/Non-Series-Publications/World-Cancer-Reports/World-Cancer-Report-2014 (accessed July 28, 2016)
2. Li T, Kung HJ, Mack PC, Gandara DR: Genotyping and genomic profiling of non-small-cell lung cancer: implications for current and future therapies. J Clin Oncol 2013, 31:1039−1049
3. Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, Harris PL, Haserlat SM, Supko JG, Haluska FG, Louis DN, Christiani DC, Settleman J, Haber DA: Activating variants in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med 2004, 350: 2129−2139
4. Mok TS, Wu YL, Thongprasert S, Yang CH, Chu DT, Saijo N, Sunpaweravong P, Han B, Margono B, Ichinose Y, Nishiwaki Y, Ohe Y, Yang JJ, Chewaskulyong B, Jiang H, Duffield EL, Watkins CL, Armour AA, Fukuoka M: Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma. N Engl J Med 2009, 361: 947−957
5. Novello S, Barlesi F, Califano R, Cufer T, Ekman S, Levra MG, Kerr K, Popat S, Reck M, Senan S, Simo GV, Vansteenkiste J, Peters S; ESMO Guidelines Committee: Metastatic non-small-cell lung cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol 2016, 27(Suppl 5):v1−v27
6. Ettinger DS, Wood DE, Aisner DL, Akerley W, Bauman J, Chirieac LR, D’Amico TA, DeCamp MM, Dilling TJ, Dobelbower M, Doebele RC, Govindan R, Gubens MA, Hennon M, Horn L, Komaki R, Lackner RP, Lanuti M, Leal TA, Leisch LJ, Lilenbaum R, Lin J, Loo BW Jr, Martins R, Otterson GA, Reckamp K, Riely GJ, Schild SE, Shapiro TA, Stevenson J, Swanson SJ, Tauer K, Yang SC, Gregory K, Hughes M: Non-Small Cell Lung Cancer, Version 5.2017, NCCN Clinical Practice Guidelines in Oncology. J Natl Compr Canc Netw 2017, 15:504−535
7. Cree IA, Deans ZC, Ligtenberg MJ, Normanno N, Edsjö A, Rouleau E, Solé F, Thunnissen E, Timens W, Schuuring E, Dequeker E, Murray S, Dietel M, Groenen P, Van Krieken JH; European Society of Pathology Task Force on Quality Assurance in Molecular Pathology; Royal College of Pathologists: Guidance for laboratories performing molecular pathology for cancer patients. J Clin Pathol 2014, 67:923−931
8. O’Brien CP, Taylor SE, O’Leary JJ, Finn SP: Molecular testing in oncology: problems, pitfalls and progress. Lung Cancer 2014, 83: 309−315
9. Normanno N, Rachiglio AM, Roma C, Fenizia F, Esposito C, Pasquale R, La Porta ML, Iannaccone A, Micheli F, Santangelo M, Bergantino F, Costantini S, De Luca A: Molecular diagnostics and personalized medicine in oncology: challenges and opportunities. J Cell Biochem 2013, 114:514−524
10. Diaz LA, Bardelli A: Liquid biopsies: genotyping circulating tumor DNA. J Clin Oncol 2014, 32:579−586
11. Crowley E, Di Nicolantonio F, Loupakis F, Bardelli A: Liquid biopsy: monitoring cancer-genetics in the blood. Nat Rev Clin Oncol 2013, 10: 472−484
12. Rolfo C, Castiglia M, Hong D, Alessandro R, Mertens I, Baggerman G, Zwaenepoel K, Gil-Bazo I, Passiglia F, Carreca AP, Taverna S, Vento R, Santini D, Peeters M, Russo A, Pauwels P: Liquid biopsies in lung cancer: the new ambrosia of researchers. Biochim Piophys Acta 2014, 1846:539−546
13. Karlovich C, Goldman JW, Sun JM, Mann E, Sequist LV, Konopa K, Wen W, Angenendt P, Horn L, Spigel D, Soria JC, Solomon B, Camidge DR, Gadgeel S, Paweletz C, Wu L, Chien S, O’Donnell P, Matheny S, Despain D, Rolfe L, Raponi M, Allen AR, Park K, Wakelee H: Assessment of EGFR variant status in matched plasma and tumor tissue of NSCLC patients from a Phase I Study of Rociletinib (CO-1686). Clin Cancer Res 2016, 22:2386−2395
14. Thress KS, Brant R, Carr TH, Dearden S, Jenkins S, Brown H, Hammett T, Cantarini M, Barrett JC: EGFR variant detection in ctDNA from NSCLC patient plasma: a cross-platform comparison of leading technologies to support the clinical development of AZD9291. Lung Cancer 2015, 90:509−515
15. Marchetti A, Palma JF, Felicioni L, De Pas TM, Chiari R, Del Grammastro M, Filice G, Ludovini V, Brandes AA, Chella A, Malorgio F, Guglielmi F, De Tursi M, Santoro A, Crinò L, Buttitta F: Early prediction of response to tyrosine kinase inhibitors by quantification of EGFR mutations in plasma of NSCLC patients. J Thorac Oncol 2015, 10:1437−1443
16. Mok T, Wu YL, Lee JS, Yu CJ, Sriuranpong V, Sandoval-Tan J, Ladrera G, Thongprasert S, Srimuninnimit V, Liao M, Zhu Y, Zhou C, Fuerte F, Margono B, Wen W, Tsai J, Truman M, Klughammer B, Shames DS, Wu L: Detection and dynamic changes of EGFR variants from circulating tumor DNA as a predictor of survival outcomes in NSCLC patients treated with first-line intercalated erlotinib and chemotherapy. Clin Cancer Res 2015, 21:3196−3203
17. Weber B, Meldgaard P, Hager H, Wu L, Wei W, Tsai J, Khalil A, Nexo E, Sorensen BS: Detection of EGFR variants in plasma and biopsies from non-small cell lung cancer patients by allele-specific PCR assays. BMC Cancer 2014, 14:294
18. Malapelle U, Sirera R, Jantus-Lewintre E, Reclusa P, Calabuig-Fariñas S, Blasco A, Pisapia P, Rolfo C, Camps C: Profile of the Roche cobas® EGFR Mutation Test v2 for non-small cell lung cancer. Expert Rev Mol Diagn 2017, 17:209−215
19. Deans Z, Williams H, Dequeker E, Keppens C, Normanno N, Schuuring E, Patton S, Cheetham M, Butler R, Hall J; IQN Path ASBL: Review of the implementation of plasma ctDNA testing on behalf of IQN Path ASBL: a perspective from an EQA providers’ survey. Virchows Arch 2017, 471:809−813
20. Huang Z, Wang Z, Bai H, Wu M, An T, Zhao J, Yang L, Duan J, Zhuo M, Wang Y, Wang S, Wang J: The detection of EGFR variant status in plasma is reproducible and can dynamically predict the efficacy of EGFR-TKI. Thorac Cancer 2012, 3:334−340

21. Normanno N, Denis MG, Thress KS, Ratcliffe M, Reck M: Guide to detecting epidermal growth factor receptor (EGFR) mutations in ctDNA of patients with advanced non-small-cell lung cancer. Oncotarget 2017, 8:12501–12516
22. Reed GF, Lynn F, Meade BD: Use of coefficient of variation in assessing variability of quantitative assays. Clin Diagn Lab Immunol 2002, 9:1235–1239
23. Molina-Vila MA, Mayo-de-Las-Casas C, Giménez-Capitán A, Jordana-Ariza N, Garzón M, Balada A, Villatoro S, Teixidó C, García-Peláez B, Aguado C, Catalán MJ, Campos R, Pérez-Rosado A, Bertran-Alamillo J, Martínez-Bueno A, Gil Md, González-Cao M, González X, Morales-Espinosa D, Viteri S, Karachaliou N, Rosell R: Liquid biopsy in non-small cell lung cancer. Front Med (Lausanne) 2016, 3:69