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External Quality Assessment Schemes for Biomarker Testing in Oncology

International Quality Network for Pathology ctDNA Working Group and the European Society of Pathology Foundation Assessors; Van Casteren, Kaat; Keppens, Cleo; Schuuring, Ed; Deans, Zandra C; Normanno, Nicola; Patton, Simon J; Dequeker, Elisabeth M C

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Comparison of Performance between Formalin-Fixed, Paraffin-Embedded—Tissue and Cell-Free Tumor DNA in Plasma

Kaat Van Casteren,*†‡ Cleo Keppens,* Ed Schuuring,§ Zandra C. Deans,¶ Nicola Normanno,‖ Simon J. Patton,** and Elisabeth M.C. Dequeker* in collaboration with the International Quality Network for Pathology ctDNA Working Group and the European Society of Pathology Foundation Assessors

From the Biomedical Quality Assurance Research Unit,* Department of Public Health and Primary Care, University of Leuven, Leuven, Belgium; the Center for Oncological Research Antwerp,† University of Antwerp, Wilrijk, Belgium; the Laboratory of Pathological Anatomy,‡ Antwerp University Hospital, Edegem, Belgium; the Department of Pathology,§ University of Groningen, University Medical Center of Groningen, Groningen, the Netherlands; the UK National External Quality Assessment Service for Molecular Genetics/Genomics Quality Assessment,¶ Department of Laboratory Medicine, Royal Infirmary of Edinburgh, Edinburgh, United Kingdom; the Cell Biology and Biotherapy Unit,‖ Instituto Nazionale Tumori "Fondazione G. Pascale," Istituto di Ricovero e Cura a Carattere Scientifico, Naples, Italy; and the European Molecular Quality Network,** Manchester Center for Genomic Medicine, St. Mary's Hospital, Manchester, United Kingdom

Liquid biopsies have emerged as a useful addition to tissue biopsies in molecular pathology. Literature has shown lower laboratory performances when a new method of variant analysis is introduced. This study evaluated the differences in variant analysis between tissue and plasma samples after the introduction of liquid biopsy in molecular analysis. Data from a pilot external quality assessment scheme for the detection of molecular variants in plasma samples and from external quality assessment schemes for the detection of molecular variants in tissue samples were collected. Laboratory performance and error rates by sample were compared between matrices for variants present in both scheme types. Results showed lower overall performance [65.6% ($n = 276$) versus 89.2% ($n = 1607$)] and higher error rates [21.0% to 43.5% ($n = 138$) versus 8.7% to 16.7% ($n = 234$ to 689)] for the detection of variants in plasma compared to tissue, respectively. In the plasma samples, performance was decreased for variants with an allele frequency of 1% compared to 5% [56.5% ($n = 138$) versus 74.6% ($n = 138$)]. The implementation of liquid biopsy in the detection of circulating tumor DNA in

plasma was associated with poor laboratory performance. It is important both to apply optimal detection methods and to extensively validate new methods for testing circulating tumor DNA before treatment decisions are made. *(J Mol Diagn 2020, 22: 736−747; https://doi.org/10.1016/j.jmoldx.2020.02.011)*

Molecular biomarker testing is essential for treatment decisions, mainly in non−small-cell lung cancer (NSCLC), metastatic colorectal cancer, melanoma, and gastrointestinal stromal tumors, and is currently widely accepted as a standard of patient care using tissue biopsy material.[1,2] Molecular predictive testing of epidermal growth factor receptor (*EGFR*), KRAS proto-oncogene, GTPase (*KRAS*), BRAF proto-oncogene (*BRAF*), human epidermal growth factor receptor 2 (*HER2*; alias *ERBB2*), anaplastic lymphoma receptor tyrosine kinase (*ALK*), Ret proto-oncogene, ROS proto-oncogene 1 (*ROS1*), neurotrophic tyrosine kinase receptor (NTRKs), and Met-skipping as well as programed cell death ligand 1 (*CD274*; alias *PDL1*) staining are common practice in tissue biopsies in NSCLC.[1,3−9] For metastatic colorectal cancer, *KRAS*, NRAS proto-oncogene, GTPase (*NRAS*), and *BRAF* variant testing and microsatellite instability analysis are performed before the selection of patients for anti-*EGFR* treatment.[2,10−13] Patients with gastrointestinal stromal tumors are tested for variants in Kit proto-oncogene and platelet-derived growth factor receptor α (*PDGFRA*),[14] and melanoma, for *BRAF*.[15]

According to the ISO 15189 standard, good quality management is required for diagnostic laboratories performing routine analysis on samples from cancer patients, to ensure the reporting of only correct outcomes in all patients.[16] Therefore, laboratories participate in yearly external quality assessment (EQA) schemes that evaluate their quality of analysis. Until recently, EQA schemes assessed the performance only on patient material from tumor-resection specimens of formalin-fixed, paraffin-embedded (FFPE) tissue blocks or, in some cases, from cell lines with specific variants.[17,18] The use of plasma samples for predictive testing through the detection of variants in circulating cell-free (cf) DNA has recently emerged as a promising noninvasive method, especially in patients in whom no appropriate tissue is available.[1,19−22] However, today, the clinical validity and clinical utility of most circulating cell-free tumor (ct) DNA testing methods are lacking.[23,24] On the other hand, the findings from an international survey in 2016 demonstrated that many laboratories are using cfDNA testing in routine practice.[22,23,25−28] The only US Food and Drug Administration (FDA)-approved cfDNA-based test with clinical utility in lung cancer is the Cobas *EGFR* Mutation Test version 2 (Roche Diagnostics, Pleasanton, CA) for the detection of *EGFR* variants in cfDNA from patients with NSCLC.[29−31] This test can be used on cfDNA from plasma samples for first-line predictive *EGFR* testing or for detecting the *EGFR* c.2369C>T p.(Thr790Met) variant associated with progression on first- and second-line EGFR-targeted tyrosine kinase inhibitors,

in cases in which not enough tissue or no tissue is available for biopsy.[1,23,32] This indication led to the development of other variant-detection kits specialized for use in plasma samples, and to their accelerated approval by the European Medicines Agency and the FDA.[30] Now that plasma samples have emerged as a new sample type for predictive testing, the need for quality assessment of performance using plasma samples has risen. To meet the needs of this new field, ctDNA pilot EQA schemes are being organized by several EQA providers, such as the European Society of Pathology (ESP) Foundation[33] and the Qualitätssicherungs-Initiative Pathologie.[34] In both programs, samples with *EGFR*, *KRAS*, and *BRAF* variants were included. The International Quality Network for Pathology (IQN Path; *www.iqnpath.org*, last accessed July 22, 2019) organized a first joint ctDNA pilot scheme in 2017 as a collaboration between different EQA providers.[28] The four participating EQA providers were the Association Italiana di Oncologica Medica, the European Molecular Genetics Quality Network, the ESP Foundation, and the UK National EQA service. Results from this pilot EQA scheme were recently published[33] and showed an overall error rate of 20.1% on both *EGFR* and *RAS* mutational analysis ($n = 268$) using spiked plasma samples. *RAS* testing held the largest share of these errors, 29.8% ($n = 114$), compared to 13.0% ($n = 154$) for *EGFR* testing. In addition, a higher error rate was observed in the samples with a variant allele frequency (VAF) of 1% compared to 5%.[33]

Results from previous EQA schemes on tissue resections indicated that laboratories report more errors when a new technique or method has emerged,[18,35,36] but also that repeated EQA participation improves laboratory performance over the years.[17,18] Is this also the case for the implementation of plasma samples in molecular pathology, or is the performance in these plasma samples similar to the performance in FFPE tissue samples? In the present study, performance levels and error rates on sample level were compared between laboratories analyzing plasma samples and FFPE tissue samples. Samples were analyzed for common variants important in treatment decisions using different molecular techniques, which has previously not yet been performed in other European EQA schemes.

Materials and Methods

Analysis results from the IQN Path cfDNA 2017 pilot scheme were compared to analysis results from lung and colon tissue EQA schemes organized by the ESP Foundation, in close collaboration with the University of Leuven
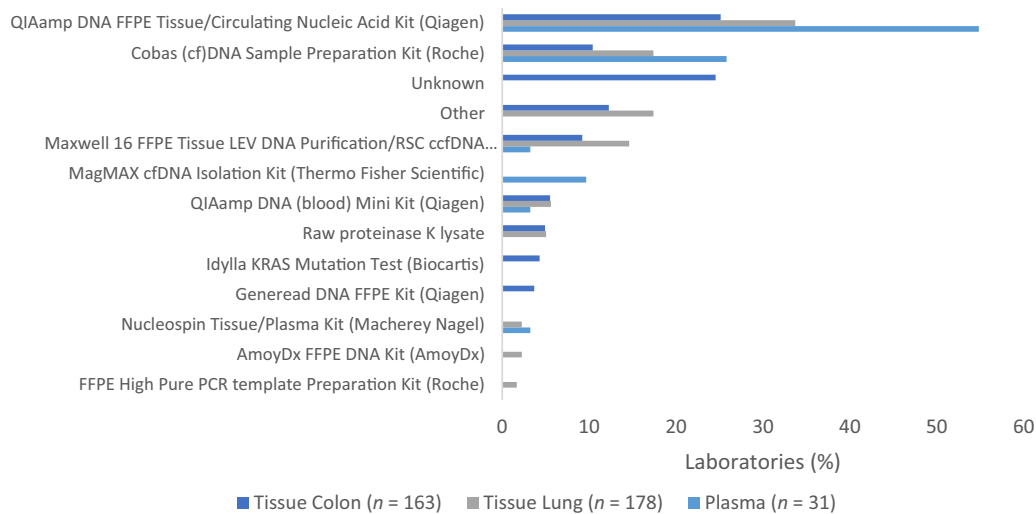
DNA extraction methods

Figure 1 Overview of the used DNA extraction methods. Representation of the DNA extraction methods that were used by all unique participants during the International Quality Network for Pathology circulating cell-free (cf) DNA 2017 pilot scheme[33] and the European Society of Pathology external quality assessment lung and colon schemes. For the laboratories participating to the lung and colon schemes, the last known DNA extraction method was used for this analysis. A switch to a different method was observed for 19 participants in the lung schemes and seven participants in the colon schemes. Qiagen, Hilden, Germany; Roche Diagnostics, Pleasanton, CA; Thermo Fisher Scientific, Schwerte, Germany; Biocartis, Mechelen, Belgium; Macherey Nagel, Düren, Germany; AmoyDx, Xiamen, China. ccf, circulating cell-free; FFPE, formalin-fixed, paraffin embedded.

(Leuven, Belgium), between 2015 and 2017. For the pilot plasma scheme, 32 laboratories were selected to participate based on their diagnostic testing procedures and the number of clinical samples tested in routine, as reported recently (laboratories with the highest number of plasma samples analyzed in routine practice were selected).[28] Although

samples were sent to all 32, one of the participants did not report any results. Ten spiked plasma samples harboring previously validated *EGFR*, *KRAS*, and *NRAS* gene variants were sent to the participating laboratories for analysis using their routine protocol, starting with cfDNA extraction (Figure 1). The ESP Foundation tissue EQA schemes

Table 1 Detailed Description of the Different Scores that Were Used for the Assessment of All Participants from the IQN Path ctDNA 2017 Pilot Scheme and the ESP Foundation Lung and Colon EQA Schemes

Score	Subcategory	Explanation
Correct	True correct results	Correct variant identified
	Correct but unspecified	Variant reported as "exon 19 deletion" in *EGFR* gene instead of exact nucleotide sequence
Incorrect	False-positive results	Additional variant reported
	False-negative results	
	VAF < LOD	Wild-type result reported but used detection method could not identify the variant because sensitivity is too low
	VAF > LOD	Wild-type result reported although sensitivity of method is sufficient to detect it = true false-negative
	Completely incorrect results	Variant not detected, but another variant reported in the same or a different gene
Minor errors	Almost correct result with an error that has no potential clinical implication for treatment indications (eg, different variant in codon 12 of the *KRAS* gene[5,39,40] or mispositioning of exon 19 deletion in the *EGFR* gene with a few nucleotides)	
Analytical failure	No test outcome given due to random failure of technical equipment or insufficient DNA content	

ctDNA, circulating cell-free tumor DNA; *EGFR*, epidermal growth factor receptor; EQA, external quality assessment; ESP, European Society of Pathology; IQN Path, International Quality Network for Pathology; *KRAS*, KRAS proto-oncogene, GTPase; LOD, limit of detection; VAF, variant allele frequency.

(*https://www.esp-foundation.org/activities/eqaschemes*, last accessed July 22, 2019) assessed the entire analytical process, from genotyping to reporting of laboratory findings, using a set of predefined score criteria. Laboratories started their analysis of the EQA samples with DNA extraction according to routine practice (Figure 1). The scope of the schemes organized by the ESP Foundation includes variants in the *EGFR*, *KRAS*, *BRAF*, and *NRAS* genes for NSCLC and metastatic colorectal cancer. The scheme organization is compliant with the predefined standards for EQA providers, ISO 17043.[37,38] For the past 10 years, the organization of these yearly EQA schemes has led to an extensive database of EQA results. The detailed setup, organization, and methods of result collection for both schemes have been previously described.[17,18]

To ensure uniformity across data from these different EQA schemes, results from all samples were reassessed (Table 1). Results from each laboratory were classified as either correct, minor error without impact, incorrect with potential implications on therapy decisions, or analytical failure for which no test outcome could be provided. Correct and incorrect classifications were subdivided using more detail (Table 1). For example, incorrect results were subclassified as false-positive, false-negative, or completely incorrect. These included also the laboratories that could not detect the variant due to insufficient sensitivity of their detection methods (Table 1).

For tissue, 125, 123, and 105 laboratories participated in the ESP Foundation colon EQA schemes, and 106, 97, and 114 laboratories participated in the ESP Foundation lung EQA schemes, in 2015, 2016, and 2017, respectively. Based on the 10 samples in the IQN Path cfDNA 2017 pilot EQA scheme, five different groups were formed to analyze laboratory performance (ie, percentage of laboratories that identified the correct variant) and error rates (ie, percentage of errors made on the total amount of samples) of molecular analysis in plasma samples compared to FFPE samples, one for each variant that was included in both the plasma and tissue schemes (Table 2). These variants were: for colon cancer, *NRAS* c.182A>G p.(Gln61Arg), and for lung cancer, *EGFR* c.2235_2249del p.(Glu746_Ala750del), c.2573T>G p.(Leu858Arg), and c.2369C>T p.(Thr790Met). The *KRAS* c.35G>C p.(Gly12Val) variant was tested for both lung and colon cancers. Each variant was present in one plasma sample with a VAF of 5% and one with a VAF of 1%. The *EGFR* variants c.2573T>G p.(Leu858Arg) and c.2369C>T p.(Thr790Met) were simultaneously present in the same sample from the IQN Path cfDNA 2017 pilot EQA scheme, but were assessed independently from one another for this analysis. In addition, the two samples containing wild-type DNA offered in

Table 2 Overview of the Used Variants within the IQN Path ctDNA 2017 Pilot Scheme and the Corresponding ESP Foundation Lung and Colon EQA Schemes Forming the Five Comparison Groups Used for Analysis

Comparison group (one for each variant)	Plasma samples in 2017 IQN path ctDNA scheme			Tissue samples in ESP Foundation EQA schemes 2015—2017			
	No. samples	VAF, %	Laboratory performance, %	No. samples	VAF, %	Laboratory performance, %	ESP Foundation EQA scheme type and year
KRAS: c.35G>C	24	5	67	56	18	91	Lung 2017
p.(Gly12Val)	24	1	42	105	42	96	Colon 2017
NRAS: c.182A>G	21	5	71	108	39	94	Colon 2015
p.(Gln61Arg)	21	1	43	15	37	93	Colon 2016
EGFR: c.2235_2249del	31	5	74	114	73	81	Lung 2015
p.(Glu746_Ala750del)	31	1	68	43	30	93	Lung 2016
EGFR: c.2573T>G	31	5	94	114	18	85	Lung 2015
p.(Leu858Arg)				114	38	89	
	31	1	58	96	24	91	Lung 2016
				105	20	95	Lung 2017
				107	35	98	
				107	18	95	
				104	19	84	
EGFR: c.2369C>T	31	5	84	114	23	78	Lung 2015
p.(Thr790Met)	31	1	65	42	19	95	Lung 2016
				53	5	77	
				107	35	81	Lung 2017
				103	20	94	

ctDNA, circulating cell-free tumor DNA; *EGFR*, epidermal growth factor receptor; EQA, external quality assessment; ESP, European Society of Pathology; IQN Path, International Quality Network for Pathology; *KRAS*, KRAS proto-oncogene, GTPase; LOD, limit of detection; *NRAS*, NRAS proto-oncogene, GTPase; VAF, variant allele frequency.

the IQN Path cfDNA 2017 pilot EQA scheme were not used for the comparison. All samples that were used in the ESP Foundation lung and colon EQA schemes from the previous three years harboring these five variants were also grouped together. The VAFs of these FFPE samples varied between 5% and 73% (Table 2).

Laboratory performance and error rates on sample level of all participants in either the IQN Path plasma scheme or the ESP Foundation tissue schemes of the previous three years were compared. In both analyses, laboratory performance in plasma and tissue samples was calculated on an overall level for all variants combined and for the detection of each variant independently. Additionally, the amount and types of errors for each scheme and for each variant separately were analyzed. Laboratory performance was calculated based on the amount of correct results and the total amount of all submitted results by the participating laboratories. The error rates were calculated based on the total amount of errors and the total amount of samples. Results of the analysis, for each comparison group and for each of the variants, were displayed in separate panels. Finally, the performance scores for each of the methods used for variant detection were analyzed.

Results

The four classifications of laboratory performance used in this analysis are shown in Table 1, and the results from the comparisons between plasma samples and FFPE tissue samples are shown in Table 2. The VAF of the tissue
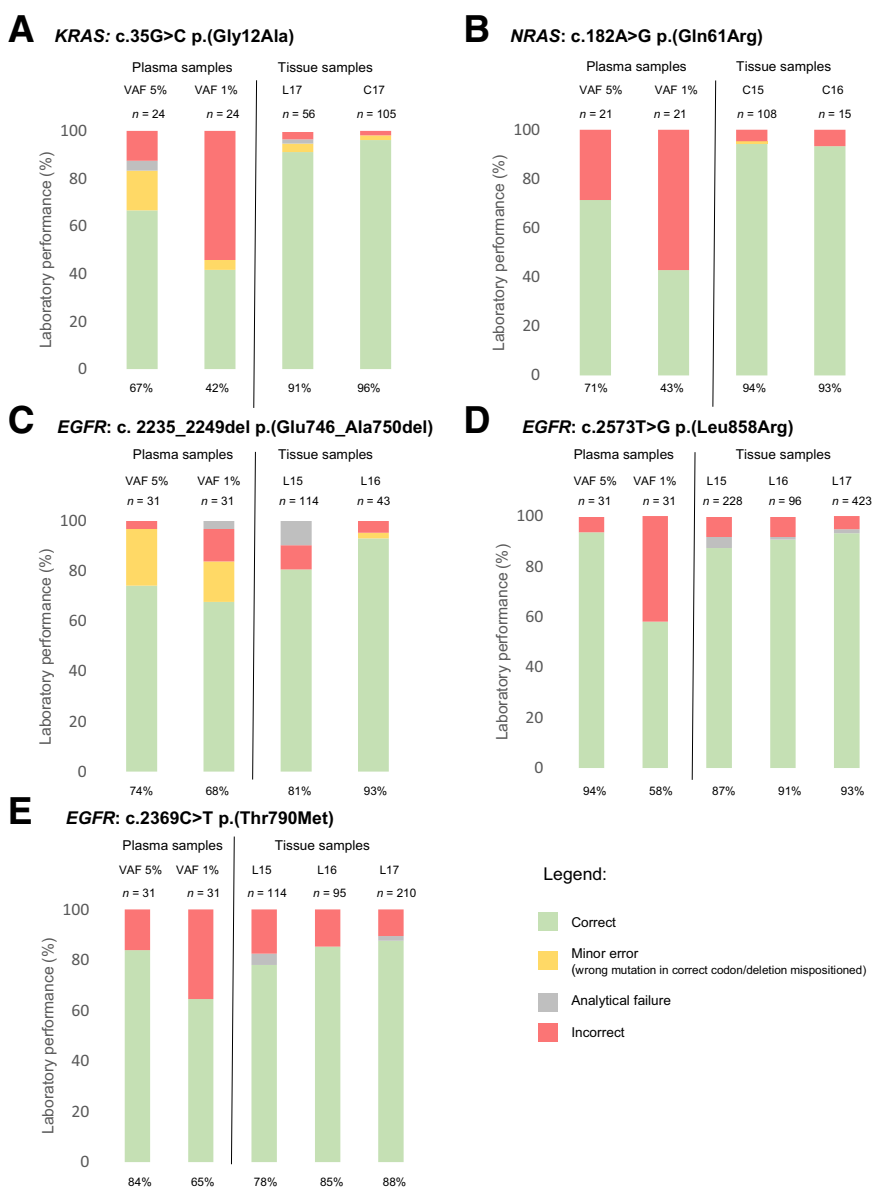
Figure 2 Comparison of the laboratory performances on variant testing between plasma and formalin-fixed, paraffin embedded (FFPE) tissue samples. Each panel consists of all performance data for each variant group: **A:** *KRAS*: c.35G>C p.(Gly12Ala); **B:** *NRAS*: c.182A>G p.(Gln61Arg); **C:** *EGFR*: c.2235_2249del p.(Glu746_Ala750del); **D:** *EGFR*: c.2573T>G p.(Leu858Arg); **E:** *EGFR*: c.2369C>T p.(Thr790Met). In each panel the laboratory performance for the two plasma samples [1% and 5% variant allele frequency (VAF)] separately are compared with the performance for the FFPE tissue samples separated per external quality assessment (EQA) scheme. C15, European Society of Pathology (ESP) EQA colon scheme 2015; C16, ESP EQA colon scheme 2016; C17, ESP EQA colon scheme 2017; L15, ESP EQA lung scheme 2015; L16, ESP EQA lung scheme 2016; L17, ESP EQA lung scheme 2017.

samples varied between 5% and 73%, and the laboratory performances reached at least 77.4%, with a maximum of 98.1% (Table 2). Because there was no correlation between VAF and performance for tissue samples, VAF was not considered during further analysis. The difference in laboratory performance between plasma samples with VAFs of 5% and 1% were more substantial [74.6% ($n = 138$) versus 56.5% ($n = 138$)] (Figure 2) and were considered as different groups in further analysis of the plasma samples.

The scores from each variant group (Table 2) were visualized in more detail in Figure 2, with each group displayed in a separate panel. For each of the five variants (Figure 2), a lower laboratory performance was observed for the plasma samples when compared to the FFPE tissue samples [mean correct samples: plasma, 65.6% ($n = 276$) versus tissue, 89.2% ($n = 1607$)]. Furthermore, the analysis of the plasma samples showed overall lower performance on the detection of *KRAS/NRAS* variants compared to *EGFR* variants (55.6% [$n = 90$] versus 73.7% [$n = 186$], respectively). This finding was less obvious on the laboratory performance in detection in FFPE tissue samples [*RAS* variants, 94.4% ($n = 284$); *EGFR* variants, 88.1% ($n = 1323$)].

Analytical failures were reported 2 times (0.7%, $n = 276$) by two independent laboratories participating in the plasma scheme compared to 39 times (2.4%, $n = 1607$) by laboratories participating in the tissue scheme. Minor

errors were reported 17 times (6.2%, $n = 276$) in the plasma scheme compared to 6 times (0.4%, $n = 1607$) in the tissue scheme, with the majority presenting in the detection of the *EGFR* c.2235_2249del p.(Glu746_Ala750del) variant.

To investigate specific types of reported errors (eg, false-negative, false-positive, incorrect, minor error, or analytical failure) (Table 1), the error rates were compared between plasma and tissue or within variant groups, and visualized by EQA scheme (Figure 3A) and by variant (Figure 3B). For this analysis, only incorrect results were considered, with 100% representing all errors made within an EQA scheme (Figure 3A) and within the variant groups (Figure 3B). The error rates for the combined detection of the three investigated genes, *EGFR*, *KRAS*, and *NRAS*, in the ESP Foundation lung EQA schemes using FFPE tissue samples remained relatively low [2015, 16.7% ($n = 456$); 2016, 11.1% ($n = 234$); and 2017, 8.7% ($n = 689$)]. The error rates in the IQN Path cfDNA 2017 pilot EQA scheme using plasma samples were significantly higher compared to these error rates for FFPE-tissue samples (VAF 5%: 21.0% ($n = 138$); VAF 1%, 43.5% ($n = 138$)] (Figure 3A). In the plasma scheme, more errors were reported in the detection of the *KRAS* and *NRAS* variants [45.8% ($n = 48$) and 42.9% ($n = 42$), respectively] compared to the *EGFR* variants (29.0%, 24.2%, and 25.8% ($n = 62$), respectively). In the tissue scheme, this finding was not observed, with a higher error rate for the detection of the *EGFR* variants
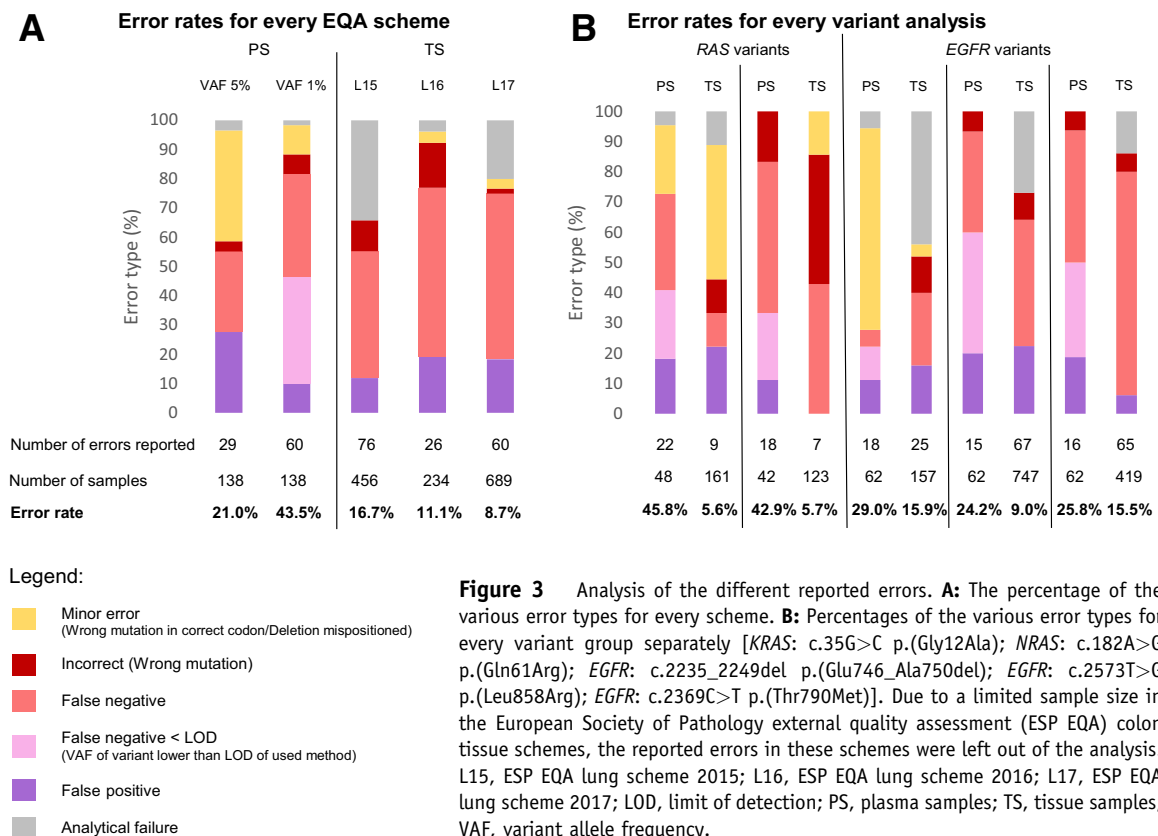
Figure 3 Analysis of the different reported errors. **A:** The percentage of the various error types for every scheme. **B:** Percentages of the various error types for every variant group separately [*KRAS*: c.35G>C p.(Gly12Ala); *NRAS*: c.182A>G p.(Gln61Arg); *EGFR*: c.2235_2249del p.(Glu746_Ala750del); *EGFR*: c.2573T>G p.(Leu858Arg); *EGFR*: c.2369C>T p.(Thr790Met)]. Due to a limited sample size in the European Society of Pathology external quality assessment (ESP EQA) colon tissue schemes, the reported errors in these schemes were left out of the analysis. L15, ESP EQA lung scheme 2015; L16, ESP EQA lung scheme 2016; L17, ESP EQA lung scheme 2017; LOD, limit of detection; PS, plasma samples; TS, tissue samples; VAF, variant allele frequency.

[15.9% ($n = 157$), 9% ($n = 747$), and 15.5% ($n = 419$)] compared to the *KRAS/NRAS* variants [5.6% ($n = 161$) and 5.7% ($n = 123$)] (Figure 3B).

Of all reported errors, false-negative was the most frequently reported error type in both the plasma and FFPE tissue schemes [32.6% ($n = 29/89$) and 50.6% ($n = 82/162$), respectively] (Figure 3A). Additionally, a large percentage of the reported errors consisted of false-positive results (plasma scheme, 15.7% ($n = 14/89$); tissue scheme, 15.4% ($n = 25/162$)] (Figure 3). Included in these false-positive results were the data from all of the laboratories that reported the correct variant but also an additional variant that was not present in the sample. The most frequently detected false-positive result was the *EGFR* c.2369C>T p.(Thr790Met) variant, followed by the *EGFR* c.2361G>A p.(Gln787 =) and c.2573T>G p.(Leu858Arg) variants. Two other *KRAS* variants, one in codon 12 and one in codon 13, were also frequently detected as false positive (data not shown). The false-positives were not correlated with a specific scheme type, year, case, or method. They were, however, mainly reported by the same laboratory. Analytical failures were reported more often on analysis of tissue samples compared to plasma samples [24.1% ($n = 162$) versus 2.2% ($n = 89$)]. Minor errors, on the other hand, were reported more often with the plasma scheme compared to the tissue scheme [19.1% ($n = 89$) versus 1.9% ($n = 162$)] (Figure 3).

The variant-detection methods used by participants in the IQN Path cfDNA 2017 pilot EQA scheme were reported previously.[33] Next-generation sequencing (NGS)-based methods were used most frequently (*KRAS*, 58% ($n = 48$); *NRAS*, 67% ($n = 42$); and *EGFR*, 39% ($n = 186$)] (Figure 4). For the *EGFR* variants, a large percentage of samples were also frequently analyzed using either droplet digital PCR (ddPCR; 23%, $n = 42$) and the FDA-approved

Cobas *EGFR* Mutation Test version 2 (Roche Diagnostics; 26%, $n = 48$) (Figure 4). Most errors were observed when non−NGS-based, laboratory-developed tests were used [*KRAS*, 100% ($n = 2$); *EGFR*, 50% ($n = 6$)]. Frequent errors were also observed when an NGS-based technique was used [*KRAS*, 32% ($n = 28$); *NRAS*, 50% ($n = 28$); and *EGFR*, 29% ($n = 72$)]. Error rates were lower when ddPCR (*NRAS*, 17% ($n = 6$); *EGFR*, 21% ($n = 42$)] and PCR-based commercial kits (FDA approved or not) [*NRAS*, 33% ($n = 6$); *EGFR*, 5% ($n = 66$)] were used. Error rates for *KRAS* variants detected using ddPCR or PCR-based commercial kits were more comparable to the performance of the NGS-based methods [commercial kits, 38% ($n = 8$); ddPCR, 25% ($n = 8$)] (Figure 4).

The variant-detection methods used by participants in the ESP Foundation lung and colon EQA schemes were reported previously.[33] In tissue samples, analysis of method performance in the detection of the variants showed that commercial kits were used most frequently [*KRAS*, 41% ($n = 161$); *NRAS*, 44% ($n = 123$); and *EGFR*, 53% ($n = 1323$)] (Figure 5). For *KRAS* and *EGFR* variants, these methods were followed closely by NGS-based techniques [*KRAS*, 36% ($n = 161$); *EGFR*, 29% ($n = 1323$)] (Figure 5). Most errors were observed when laboratories used a noncommercial method for the detection of *KRAS* and *EGFR* variants [6% ($n = 37$) and 15% ($n = 233$), respectively], whereas most errors in *NRAS* variants were observed when a commercial kit was used (7% [$n = 54$]) (Figure 5).

Discussion

The use of plasma samples for molecular analysis offers a noninvasive addition to the use of tissue samples. Because ctDNA is only a very small fraction of total cfDNA in

Figure 4 Method performance analysis for each of the three genes analyzed in plasma samples. **A−C:** Performance scores for each of the investigated variants were analyzed and grouped together based on the detection method that was used. All three *EGFR* variants were analyzed together. The commercial kit (Cobas *EGFR* mutation test version 2; Roche) approved by the US Food and Drug Administration was grouped together to visualize its performance separate from other commercial kits. BEAMing, beads, emulsion, amplification, and magnetics; ddPCR, droplet digital PCR; LDT, laboratory developed test; NGS, next-generation sequencing.

plasma, variant-detection assays with a much higher analytical sensitivity than detection assays currently used in tissue testing are required for the same variants in ctDNA testing. Due to a lack of optimization and validation of this matrix, currently plasma samples are used in only a very specific setting in NSCLC patients, whereas tissue samples are recommended for use in molecular diagnostics in multiple cancer types and multiple biomarkers.[1,2] This study showed higher error rates (Figure 3), and lower laboratory performances (Figure 2), in detecting clinically relevant variants in plasma samples compared to FFPE tissue samples. These results show that the setup of testing for plasma samples is not optimal, which can partially be explained by the fact that some of the methods used lack sufficient analytical sensitivity for the analysis of plasma samples. Currently, only one method with FDA approval is available for the detection of *EGFR* variants in plasma samples. This method has been shown to perform better compared to the other frequently used detection methods, but only 26% of the plasma samples in our study were analyzed for *EGFR* variants using the FDA-approved Cobas *EGFR* Mutation Test version 2 (Roche Diagnostics) (Figure 4C). This limited use of FDA-approved methods for plasma sample analysis may be an explanation for the lower performances and higher error rates that were observed during this first plasma-based EQA scheme (Figures 2–4). For the detection of *NRAS* and *KRAS* variants in plasma samples, no FDA-approved methods are currently available,

resulting in higher error rates across the different methods available for the detection of *RAS* variants (Figure 4, A and B).

In this study, laboratory performance using an NGS-based technique was lower in plasma samples compared to tissue samples (*KRAS*, 32% (*n* = 28) versus 1.7% (*n* = 58), respectively; *NRAS*, 50% (*n* = 28) versus 0% (*n* = 20); *EGFR*, 29% (*n* = 72) versus 9.6% (*n* = 385)] (Figures 4 and 5). Many laboratories use the same NGS technique for both tissue analysis and plasma analysis even though the VAF is most often lower in plasma samples. Laboratories not using NGS have most often switched to a plasma-specific technique such as ddPCR, reducing the number of NGS users within plasma compared to tissue schemes. The first EQA data using plasma samples (Figures 2 and 4) also showed that the lack of sensitivity of many currently available variant-detection methods, mostly NGS-based techniques, may lead to the lower laboratory performance and higher error rates for samples with a VAF of 1% or 5%. The importance of the VAF of the detected variants was also observed in a first German EQA initiative for plasma testing, which evaluated laboratory performance in the detection of the *EGFR* c.2369C>T p.(Thr790Met) variant in spiked plasma samples. The use of samples with a higher VAF in this EQA scheme yielded an overall laboratory performance of 83.3% (*n* = 42).[34] These findings indicate a need for EQA schemes that represent a more clinically relevant situation with samples with a lower VAF.
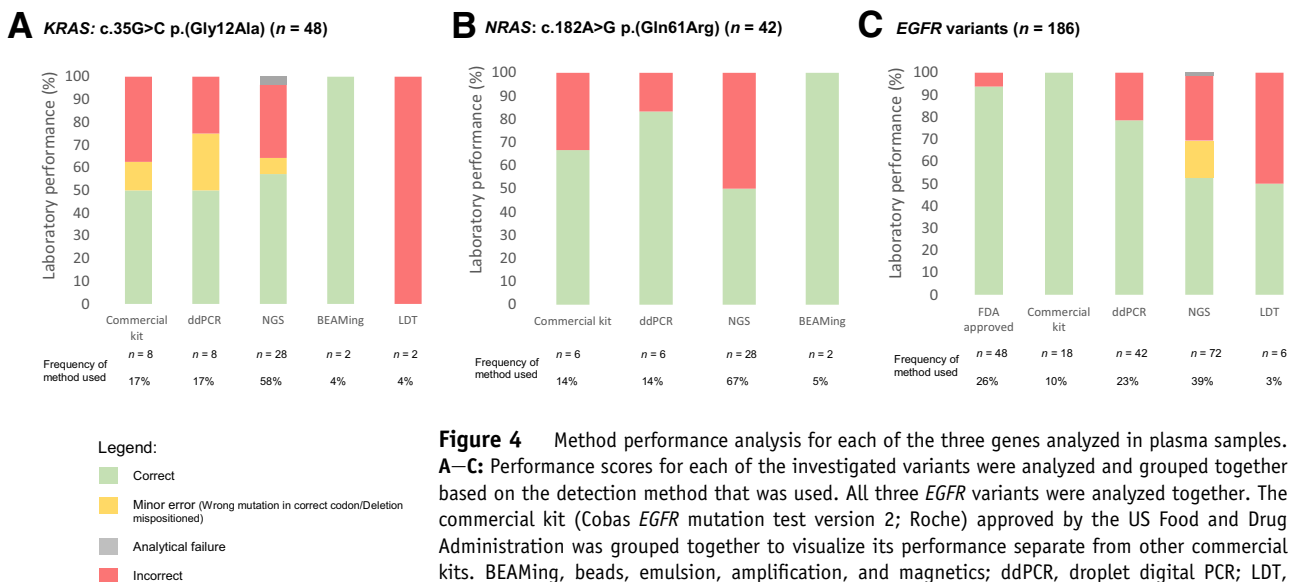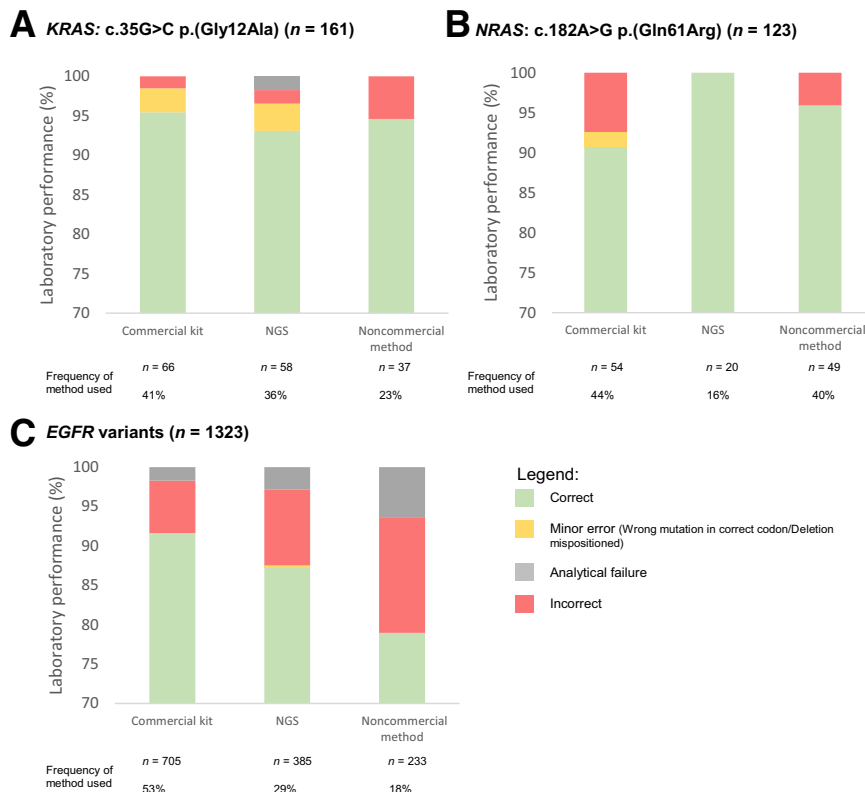

Figure 5 Method performance analysis for each of the three genes analyzed in tissue samples. **A–C:** Performance scores for each of the investigated variants were analyzed and grouped together based on the detection method that was used. All three *EGFR* variants were analyzed together. Due to the higher laboratory performance for tissue samples, the *y* axis was compressed slightly to a minimum of 70% for a better representation of the results. NGS, next-generation sequencing.

A significant percentage of errors reported on analysis of plasma samples were classified as false-negative < limit of detection, indicating that the VAF used in this EQA scheme was smaller than the currently used limit of detection of the variant-detection methods (Figure 3). These findings indicate a need for the development of improved variant-detection methods capable of detecting variants with a lower VAF (<1%), since these variants represent the clinically relevant variants present in patient plasma samples. Therefore, an improvement in sensitivity of the detection of variants in plasma samples is needed for reaching the same quality of analysis as for tissue samples. Recently, variant-detection assays with a limit of detection of close to 0.1% have been reported, such as ddPCR, BEAMing (beads, emulsion, amplification and magnetics), and some NGS assays.[41−43] With these recent developments, it is expected that, in the near future, more methods with a higher sensitivity, and that are able to detect more variants and other biomarkers in plasma samples, will be developed, providing a larger clinical application for the use of plasma samples in routine clinical practice. For the analysis of FFPE tissue samples, sensitivity is less problematic because in routine molecular diagnostics using tissue biopsy, in general a minimum of 20% neoplastic cells (∼10% VAF) is required, and most variant-detection assays used on tissue with limits of detection of 1% to 5% are suitable for this purpose. This is in agreement with the relative high performance of tissue-sample analysis, independent of the VAF varying between 5% and 73% in EQA tissue samples. Aside from the low sensitivity, current variant-detection methods for use on plasma samples are often unable to specify the exact nucleotide sequence of the variant. This is observed from the higher occurrence of minor errors in the analysis of plasma samples compared to tissue samples [19.1% (n = 17/89) versus 1.9% (n = 3/162)]. These findings represent the inability of some detection methods to distinguish between different variants and indicate a need for techniques that are able to genotype the observed variants. Additionally, a significant percentage of these errors are represented by false-positive results (10.0% to 27.6%), although not all with an equally important clinical impact (Figure 3). The false-positive error rate imposes a greater risk in lung cancer patients. These patients will receive a potentially toxic and expensive treatment that will lack any therapeutic benefit. It should be noted, however, that about one-third of all false positives were reported by a single laboratory that used an NGS detection method and reported all detected variants with a possible clinical relevance, some with a low frequency (data not shown).

The results from the present study also showed higher laboratory performance and lower error rates in the detection of *EGFR* variants compared to *RAS* variants in plasma samples (Figures 2 and 3). The higher scores for the detection of *EGFR* variants in plasma samples can be explained by the availability of companion diagnostics for these variants and not for *RAS* variants [eg, the detection of the *EGFR* c.2369C>T p.(Thr790Met) variant in liquid biopsy samples].[30] Since the implementation of the detection of molecular biomarkers in ctDNA extracted from plasma samples, the development of methods to detect *EGFR* variants in lung cancer was a higher priority because of its high clinical importance in these patients, especially in patients whose disease progressed on first- and second-line tyrosine kinase inhibitors and with no availability of tissue biopsy samples.[23,25,29] This better availability of methods focused on the detection of *EGFR* variants has been confirmed by the analysis of performance based on the detection method used. In this analysis, higher error rates were equally spread over the different *KRAS* variant (and *NRAS* variant) detection methods compared to *EGFR* detection methods (Figure 4). This is in contrast with the higher laboratory performance and lower error rates observed in the detection of *RAS* variants compared to *EGFR* variants in tissue samples (Figures 2, 3, and 5). These results are in line with the previously mentioned results from the IQN Path cfDNA 2017 pilot EQA scheme.[33] Based on results from this scheme[33,44] and a discussion-based workshop, a consensus opinion on good practices for plasma testing was drafted.[44]

It should also be mentioned that our study had some important limitations. The IQN Path cfDNA 2017 pilot EQA scheme was organized using spiked plasma samples instead of real patient material like in the ESP Foundation tissue EQA schemes. This may be an explanation for the lower laboratory performances and higher error rates in the detection of variants in plasma samples compared to tissue samples. Unfortunately, acquiring such high amounts of patient plasma samples for EQA purposes is currently not possible. To mimic clinical practice as closely as possible, spiked plasma samples with a relatively low VAF were used instead.

Despite these limitations, it was observed that when a new sample type was introduced into routine practice, laboratories had lower initial performance when using the newer technique. The main reason for this lower performance may have been the need for a method with sufficient sensitivity and specificity for application in plasma analysis. More research and more experience are necessary for achieving the same performance level on variant analysis using plasma samples as for the gold standard using tissue samples. Similar lower laboratory performance was previously observed with the implementation of *ROS1* testing in NSCLC patients and the expansion of the *RAS* testing scope. In those situations, EQA schemes had already proved to increase laboratory performance of participants over the years.[17,18,36] Quality assessment of plasma samples using EQA schemes can thus contribute to the approval of this matrix as clinical routine. The limitations of plasma testing should be closely monitored to avoid administering unnecessary harmful treatments in nonresponsive patients as well as to avoid missing a potentially beneficial therapeutic effect in other patients. With regard to the limitations of analyzing plasma samples, it is essential to ensure high sensitivity and

specificity of variant-detection methods to achieve high-quality testing. Based on the development of new variant-detection assays with both higher sensitivity and specificity,[41–43] significant improvement is expected in following ctDNA EQA schemes. These EQA schemes should represent clinical practice as closely as possible, although the use of patient samples is not possible due to practical and ethical considerations. The spiked or artificial plasma samples used for EQA schemes should represent clinically relevant patient samples with a low VAF and an amount of input DNA that will challenge the limit of detection of the methods of a laboratory.

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Author Contributions

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