External Quality Assessment on Molecular Tumor Profiling with Circulating Tumor DNA-Based Methodologies Routinely Used in Clinical Pathology within the COIN Consortium

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BACKGROUND: Identification of tumor-derived variants in circulating tumor DNA (ctDNA) has potential as a sensitive and reliable surrogate for tumor tissue-based routine diagnostic testing. However, variations in pre(analytical) procedures affect the efficiency of ctDNA recovery. Here, an external quality assessment (EQA) was performed to determine the performance of ctDNA mutation detection work flows that are used in current diagnostic settings across laboratories within the Dutch COIN consortium (ctDNA on the road to implementation in The Netherlands).

METHODS: Aliquots of 3 high-volume diagnostic leukapheresis (DLA) plasma samples and 3 artificial reference plasma samples with predetermined mutations were distributed among 16 Dutch laboratories. Participating laboratories were requested to perform ctDNA analysis for *BRAF* exon 15, *EGFR* exon 18–21, and *KRAS* exon 2–3 using their regular circulating cell-free DNA (ccfDNA) analysis work flow. Laboratories were assessed based on adherence to the study protocol, overall detection rate, and overall genotyping performance.

RESULTS: A broad range of preanalytical conditions (e.g., plasma volume, elution volume, and extraction methods) and analytical methodologies (e.g., droplet digital PCR [ddPCR], small-panel PCR assays, and next-generation sequencing [NGS]) were used. Six laboratories (38%) had a performance score of >0.90; all other laboratories scored between 0.26 and 0.80. Although 13 laboratories (81%) reached a 100% overall detection rate, the therapeutically relevant *EGFR* p.(S752_I759del) (69%), *EGFR* p.(N771_H773dup) (50%), and *KRAS* p.(G12C) (48%) mutations were frequently not genotyped accurately.

CONCLUSIONS: Divergent (pre)analytical protocols could lead to discrepant clinical outcomes when using the same plasma samples. Standardization of (pre)analytical work flows can facilitate the implementation of reproducible liquid biopsy testing in the clinical routine.

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Introduction

Liquid biopsy approaches, particularly the detection of tumor-derived variants in circulating tumor DNA (ctDNA) in the plasma of cancer patients, have gained increasing interest in oncological diagnostics (1–4). Circulating cell-free DNA (ccfDNA)—which includes ctDNA—can easily be collected using a minimally invasive blood withdrawal. Liquid biopsy applications include early screening, molecular target detection, treatment response prediction, early detection of therapy resistance, and minimal residual disease (MRD) and disease monitoring (1, 5). However, clinical application of liquid biopsy remains limited due to the absence of harmonized (pre) analytical work flows, the lack of data showing clinical utility, regulation, and standardization (6, 7).

The COIN consortium (ctDNA on the road to implementation in The Netherlands; www.cfdna.nl/coin) is an endeavor to implement ctDNA as a biomarker in the Netherlands, which requires harmonization of methods. Current preanalytical protocols are very diverse concerning blood collection tubes and the processing thereof, storage conditions, ccfDNA extraction methods, and elution volumes (8–10). While ccfDNA quantity, quality, and mutation detection sensitivity can be dramatically impacted by preanalytical processes (11), standardization of these processes is lacking. A previous round-robin trial within the framework of the COIN consortium demonstrated that, indeed, preanalytical conditions vary significantly across laboratories in the Netherlands, and that the choice of ccfDNA extraction method affects the overall ccfDNA yield and ctDNA abundance (6). Other studies confirm that plasma volume, extraction method, and automation have an impact on the ccfDNA fraction that can be extracted from (artificial) plasma (8, 12–14), which was reviewed recently (15).

Because only a few plasma-based ctDNA detection assays have received FDA approval and/or CE marking required for in vitro diagnostic (IVD) devices for use in the clinical setting, many analyses are performed with research-use-only tests (5, 16). In addition, methodologies vary, from single-target PCR-based approaches through small, targeted panels, to broad next-generation sequencing (NGS) strategies. Each method has particular value for specific applications in clinical diagnostics: single-target approaches are suitable for tumor-informed monitoring or ctDNA dynamics, while expanded panel analyses are applicable to identify tumor-derived variants at primary detection or resistance mechanisms at progression to assist in treatment decision-making. However, other considerations (e.g., turnaround time, costs, reimbursement, and demand for expertise) frequently affect the choice of testing. Studies to compare the effect of different (pre)analytical ctDNA testing work flows on analyte detection are required to demonstrate a possible impact of the method of liquid biopsy testing in clinical practice.

The aim of this study was to gain insight into the performance of ctDNA mutation detection work flows that are used in current diagnostic settings across laboratories within the Dutch COIN consortium. An external quality assessment (EQA) was performed for the detection of tumor-derived driver mutations in ccfDNA involving 16 laboratories that are presently using ctDNA testing in clinical or research settings. The participating laboratories received aliquots of 3 commercial spiked-in reference plasma samples and 3 patient-derived high-volume diagnostic leukapheresis (DLA) plasma samples with a pathogenic mutation in either *EGFR* or *KRAS* for their analysis. Molecular reports were collected and assessed by a central reference laboratory.

Materials and Methods

RECRUITMENT OF PARTICIPANTS

A survey was conducted among laboratories specialized in pathology, medical oncology, and clinical chemistry within the Dutch COIN consortium to identify sites performing plasma-derived ctDNA analysis. Laboratories were invited to participate in this interlaboratory EQA. Seventeen laboratories indicated their willingness to participate in the EQA. All laboratories were requested to track their proceedings and report the results according to a standardized form (online Supplemental Appendix 1). One laboratory failed to report its results before the deadline and was excluded; hence, 16 laboratories were evaluated in this EQA.

PLASMA SAMPLE SELECTION AND CHARACTERIZATION

Aliquots (4 mL each) of 6 high-volume plasma samples were distributed among the participating laboratories. Three samples constituted of artificial ccfDNA (25 ng/mL) in plasma either spiked-in or not spiked-in with several clinically relevant mutations (purchased from LGC Clinical Diagnostics; online Supplemental Table 1). DLA plasma samples from 3 patients with metastatic non-small cell lung cancer (NSCLC) who were treated at the University Medical Center Groningen (UMCG, Groningen, the Netherlands) were retrieved from the UMCG DLA-Biobank. Patients provided written informed consent for the use of their samples for research and validation purposes. DLA plasma samples were selected based on (*a*) the availability of at least 100 mL of plasma, (*b*) identification of a common pathogenic mutation in *BRAF* exon 15, *EGFR* exon 18–21 or *KRAS* exon

2–3 during tumor tissue NGS analysis, and (*c*) that the identified common pathogenic mutation is also detectable in the DLA plasma. On-treatment cell-free DLA plasma samples were collected in citrate as described previously (17, 18) and stored as 50 mL fractions at −80°C within 30 min after withdrawal. After thawing, 2 DLA fractions of 50 mL were pooled and centrifuged at 1600*g* for 10 min to separate the plasma from the debris. Plasma was aliquoted to 1 mL volumes and stored at −80°C until their shipment on dry ice to the participating laboratories. Prior to distribution, plasma samples were analyzed for the presence of all mutations in *BRAF* exon 15, *EGFR* exon 18–21, and *KRAS* exon 2–3 in an independent analysis using the NGS-based Avenio ctDNA Expanded Kit (Roche). ccfDNA was extracted from 2 mL of DLA plasma with the silica membrane-based QIAamp Circulating Nucleic Acid Kit and quantified using the Qubit dsDNA HS assay kit (Thermo Fischer Scientific) as reported previously (11). ccfDNA concentration and ctDNA NGS-based variant allele frequencies (VAFs) detected in the plasma samples are described in Table 1.

ctDNA ANALYSIS BY THE PARTICIPANTS

All laboratories received 4 mL aliquots of each sample and were requested to perform ctDNA analysis for *BRAF* exon 15, *EGFR* exon 18–21, and *KRAS* exon 2–3 using their regular ccfDNA analysis work flow. Procedures from ccfDNA extraction to molecular profiling were documented and reported to the central reference laboratory (UMCG). Laboratories were assessed based on the execution of the study protocol (analysis of the requested loci), overall sensitivity of mutation detection, and overall performance according to custom performance score criteria (online Supplemental Table 2).

STATISTICAL ANALYSES

Descriptive statistics were used to characterize data. A performance score was calculated using custom marking criteria (Supplemental Table 2) by addition of all points and deductions awarded and division by the total number of variants tested for. Figures were generated with Prism version 9.1.0. (GraphPad Software).

Results

(PRE)ANALYTICAL WORK FLOW PROCEDURES

Table 2 describes the different procedures for ccfDNA extraction and quantification by the 16 participants. A broad range of plasma input and elution volume was used for the ccfDNA extraction; 8 different extraction methods were used. Eleven laboratories quantified the ccfDNA prior to analysis, most frequently using fluorescence-based methods (i.e., Qubit, Nanodrop, TapeStation).

Mutation analyses were performed with a great variety of methodologies (online Supplemental Appendix 2). Laboratories used either single-target analysis for a set of mutations using droplet digital PCR (ddPCR), small-panel PCR assays (i.e., Cobas), in-house–developed or commercially available NGS approaches (i.e., Oncomine, Avenio), or a combination of techniques to determine ctDNA mutations (Fig. 1). Some laboratories did not analyze all requested exons, which was considered during the overall detection rate and performance score analysis.

OVERALL DETECTION RATE AND PERFORMANCE SCORE

The reference samples (Samples 1 and 3) each contained 9 mutations and the clinical samples each had one actionable variant within the loci of interest (i.e., *BRAF* exon 15, *EGFR* exon 18–21, and *KRAS* exon 2–3; Table 1). The detection rate for the 21 variants across 5 samples was analyzed (Fig. 2; Supplemental Appendix 2). Several laboratories did not apply an assay that can detect every mutation; these mutations were removed from the total number of analyzed mutations for these laboratories to determine the overall detection rate, defined as the number of mutations identified (either specifically or nonspecifically genotyped) divided by the number of analyzed mutations (see "overall detection rate" in online Supplemental Table 3). Although 13 laboratories reported all mutations tested for, some clinically relevant mutations were not accurately genotyped (Fig. 2; Supplemental Table 3). The therapeutically relevant *EGFR* p.(S752_I759del) and p.(N771_H773dup) mutations were not identified by 11 (69%) and 8 (50%) of the participating laboratories, respectively (Fig. 2). Furthermore, only 33 out of a possible 64 calls (52%) specific for *KRAS* p.(G12C) were reported, which, in contrast to other codon 12/13 mutations, could render a patient eligible for targeted therapy. Lack of detection of these actionable mutations was primarily due to the inability of the applied assay to correctly genotype the expected variant.

The performance score was determined for each laboratory, considering the accuracy of detection and false-negative and false-positive rates (online Supplemental Tables 3 and 4). Six laboratories had a performance score of >0.90 (Fig. 3; Supplemental Table 3), of which 5 used an NGS approach and one used ddPCR (Fig. 1). The 9 laboratories with a performance score between 0.56 and 0.80 mostly applied ddPCR and Cobas approaches to determine the requested variants. Laboratory 4, which used an NGS approach, had a low performance score due to many nonspecific and false-negative calls (Supplemental Table 3). For each participating laboratory, the coverage

Table 1. Sample characteristics.

Sample type	Sample ID	Mutations in loci of interest[a]	ccfDNA, ng/μL	Input, ng	VAF	Mutant molecules[b]
Reference plasma samples	Sample 1[c]	*BRAF* c.1799T > A; p.(V600E)	1.34	25.0	0.54%	53
		EGFR c.2235_2249del; p.(E746_A750del)			0.38%	38
		EGFR c.2240_2257del; p.(L747_P753delinsS)			0.21%	21
		EGFR c.2254_2277del; p.(S752_I759del)			0.17%	16
		EGFR c.2369C > T; p.(T790M)			0.42%	41
		EGFR c.2573T > G; p.(L858R)			0.40%	40
		KRAS c.34G > T; p.(G12C)			0.46%	45
		KRAS c.35G > A; p.(G12D)			0.51%	50
		KRAS c.183A > C; p.(Q61H)			0.59%	58
	Sample 2[d]	—	1.22	25.0	—	—
	Sample 3[e]	*BRAF* c.1799T > A; p.(V600E)	1.51	25.0	1.14%	132
		EGFR c.2235_2249del; p.(E746_A750del)			1.10%	127
		EGFR c.2240_2257del; p.(L747_P753delinsS)			1.43%	166
		EGFR c.2254_2277del; p.(S752_I759del)			1.15%	133
		EGFR c.2369C > T; p.(T790M)			0.89%	103
		EGFR c.2573T > G; p.(L858R)			0.85%	99
		KRAS c.34G > T; p.(G12C)			0.95%	110
		KRAS c.35G > A; p.(G12D)			0.72%	84
		KRAS c.183A > C; p.(Q61H)			0.72%	83
Clinical plasma samples	Sample 4	*EGFR* c.2311_2319dup; p.(N771_H773dup)	0.392	15.2	9.3%	278
	Sample 5	*KRAS* c.34G > T; p.(G12C)	0.545	11.8	4.5%	100
	Sample 6	*KRAS* c.34G > T; p.(G12C)	0.878	36.3	1.6%	109

[a]Loci of interest encompass *BRAF* exon 15, *EGFR* exon 18–21, and *KRAS* exon 2–3. Mutations were determined in an independent run using the Avenio ctDNA Expended Kit at the central analysis laboratory (University Medical Center Groningen [UMCG]).
[b]Depicted as mutant copies/mL of plasma.
[c]SeraCare ctDNA Complete™ Reference Material VAF0.5% (see Supplemental Table 1).
[d]SeraCare ctDNA Complete™ Reference Material WT (see Supplemental Table 1).
[e]SeraCare ctDNA Complete™ Reference Material VAF1% (see Supplemental Table 1).

of requested exons, overall detection rate, and performance score are depicted in Fig. 3.

Discussion

Previous studies have determined the effect of various facets in the (pre)analytical work flow on the quantity and composition of ccfDNA, which resulted in overviews of the critical factors in liquid biopsy testing (15, 20, 21). However, whether these variables eventually affect the overall detection rate of tumor-derived mutations in ctDNA remained largely unclear. Here, the impact of divergent (pre)analytical protocols on the detection of clinically relevant mutations in cell-free plasma was evaluated across 16 laboratories within the Dutch COIN consortium.

Since (inter)national harmonization of (pre)analytics has not yet been achieved (22, 23), most laboratories develop their local liquid biopsy work flows and implement them for research and clinical purposes. Particularly in the preanalytical phase, variations that affect the quantity and quality of ccfDNA could be induced due to many different commercially available products (15, 21). To gain insight into the use of (pre)analytical products in the Netherlands, a general survey was conducted among laboratories contributing to the COIN consortium. Laboratories willing to participate in an interlaboratory EQA reported a wide variety of (pre)analytical work flows for liquid biopsy applications. As expected, differences in plasma use, ccfDNA extraction methods and conditions, and mutation detection assays were applied by the laboratories participating in this EQA. Variations in preanalytical conditions can considerably affect the quantity and quality

Table 2. Laboratory preanalytical conditions.

Lab	Plasma volume, mL	Elution volume, µL	Extraction method[a]	ccfDNA quantification method	Remarks
1	4	100	CNA	None	None
2	4	40	CNA	Qubit	None
3	4 (2 × 2)	120 (2 × 60)	DSP	Qubit	None
4	3	80	ME	None	None
5	4 (2 × 2)	200 (2 × 100)	COB	Qubit	None
6	4 (2 × 2)	160 (2 × 80)	COB	None	None
7	3	50	CNA	None	None
8	4	75	RSC	Nanodrop	None
9	4 (2 × 2)	200 (2 × 100)	COB	None	None
10	4	55	Custom[b]	ddPCR	None
11	2	52	CNA	Qubit	None
12	4	60	AVE	TapeStation	Yes[c]
13	4	25	MMA	Qubit	None
14	3	50	CNA	Qubit	None
15	4	50	CNA	Qubit	None
16	4	70	RSC	Qubit	None

[a]Abbreviations: CNA, QIAamp Circulating Nucleic Acid Kit; DSP, QIAsymphony DSP Circulating DNA Kit; ME, QIAamp MinElute ccfDNA Mini Kit; COB, Cobas ccfDNA Sample Preparation Kit; RSC, Maxwell RSC LV ccfDNA Kit; AVE, AVENIO cfDNA Isolation Kit; MMA, MagMAX Cell-Free Total Nucleic Acid Kit.
[b]Applied a modified protocol (see Supplemental Appendix 1).
[c]Plasma Samples 4 to 6 were nearly thawed upon arrival at the site.

of the ccfDNA, which ultimately can hamper the sensitivity and accuracy of mutation detection (6, 15). Moreover, the choice of an analytical method to detect tumor-derived mutations affects the detection rate as well (24, 25); 10 different assays were used in this EQA. Previous EQAs have shown highly discrepant detection rates among laboratories for plasma samples with mutations spiked-in at 1% VAF (1, 26). To this end, 3 commercially available reference plasma samples spiked-in with clinically relevant mutations at 1%, 0.5%, and 0% VAF were distributed. Biological features of clinical patient-derived plasma samples, however, might affect the detection of ctDNA in the plasma (20). Therefore, 3 patient-derived plasma samples with proven actionable mutations were included as well. Since the VAFs of these variants were quite high (>1%), which should be above the analytical sensitivity of the applied molecular profiling methods, the patient samples were also included in the performance analysis.

Laboratories were scored based on whether the requested loci of interest were tested, and on the overall detection rate of the expected variants. Molecular profiling was performed by 11/16 laboratories (69%) for at least one variant in all requested exons, of which 5 laboratories (31%) applied a test that could detect all variants present across the samples. Considering only variants for which analysis was performed, 13/16 laboratories (81%) scored an overall detection rate of 100% for the expected variants with or without accurate genotyping (i.e., specific amino acid change), implicating a high sensitivity of the (screening) assay used. However, reporting of a variant without the specific amino acid change (e.g., *KRAS* p.(G12/G13)) cannot support clinical decision-making if an actionable mutation (i.e., *KRAS* p.(G12C) is present. In current routine diagnostics, accurate characterization of the molecular profile of tumors is essential to treat NSCLC patients appropriately. Identification of the precise nucleic acid change could therefore determine whether a patient is considered eligible for targeted treatment or will receive nonspecific therapy. To this end, the participating laboratories were graded on performance based on custom marking criteria as well, considering the therapeutic implications of the molecular report and false-positive calls in the exons analyzed. In general, laboratories using screening assays (i.e., ddPCR screening assays, Cobas mutations tests) had a lower performance score as the methods were unable to distinguish the specific variant from other alterations at that codon. In contrast, NGS-based methodologies can define specific variants more accurately, but are more prone to false-positive calls since a wide range of genomic loci are analyzed simultaneously. Laboratory 16 had a performance score of 1 while using ddPCR but only tested for 57% of the variants, highlighting that—

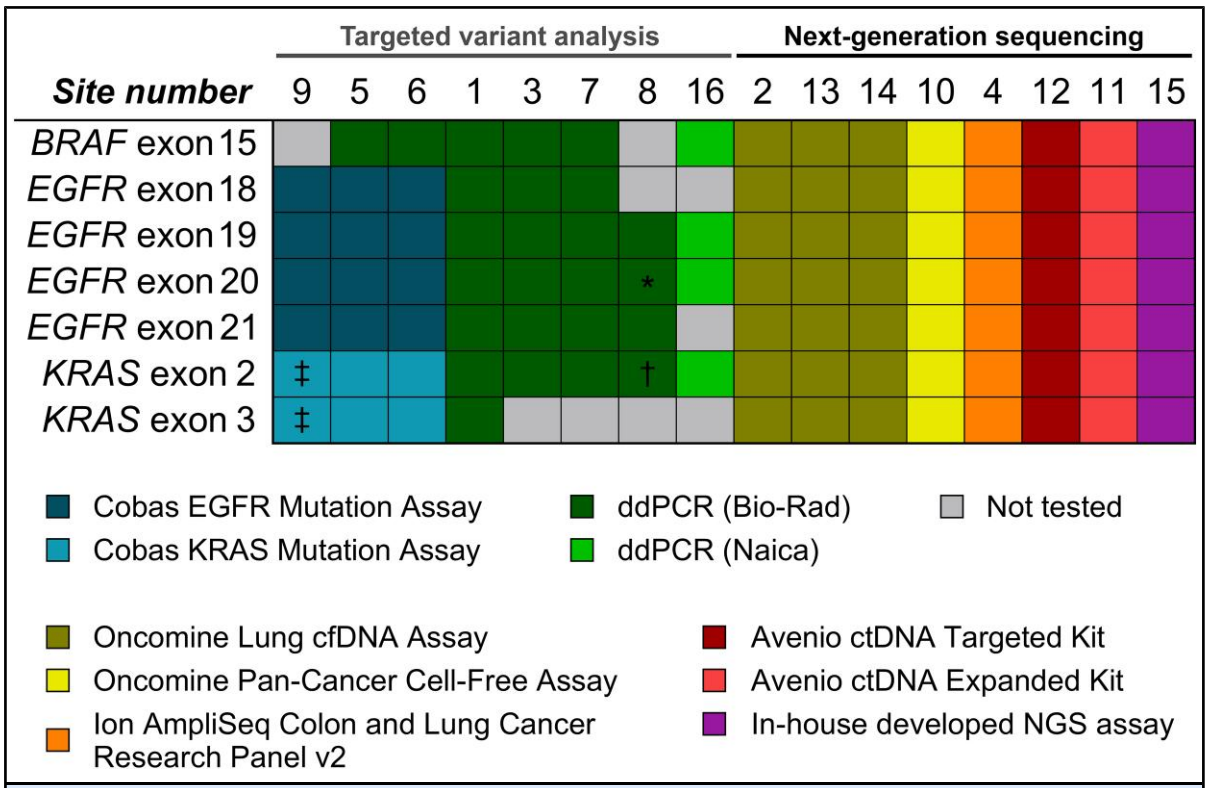

Fig. 1. Analyses performed by site to test for requested exons. Laboratory 15 applied an in-house developed NGS assay as reported previously (19). *Only performed on Sample 1 and Sample 3. †Only performed on Sample 4, Sample 5, and Sample 6. ‡*KRAS* analysis on Sample 4 was invalid.

although single-target testing can be sensitive and specific —a large number of sequential or parallel single-target tests will be required to analyze all loci of interest. Most laboratories that did not apply an NGS-based methodology were particularly unable to appropriately characterize the *KRAS* p.(G12C) and *EGFR* p.(N771_H773dup) mutations, which have clinical significance regarding treatment decision-making. *KRAS* p.(G12C)-mutant NSCLC patients with progressive disease following first-line (chemo-)immunotherapy are considered eligible for subsequent treatment with sotorasib or adagrasib according to international guidelines (https://www.nccn.org/professionals/physician_gls/PDF/nscl.pdf, accessed on November 6, 2023), while no targeted therapies are available for other *KRAS* mutations. Upon detection of most common *EGFR* mutations, patients are treated with osimertinib in first-line. However, *EGFR* exon 20 insertions are negative predictors for osimertinib treatment and, therefore, these patients generally receive amivantamab-targeted antibodies subsequent to (chemo-)immunotherapy. NSCLC patients with missense mutations at codon 600 of *BRAF* are preferably treated with first-line dabrafenib/trametinib combination therapy (27).

For this study, gathering plasma through the DLA procedure enabled collection and distribution of aliquots of identical clinical samples to the participating laboratories which would not have been feasible with regular blood draws. However, the VAFs in the patient-derived DLA samples were excessively high (>1%), while plasma ctDNA generally represents a low fraction of the total ccfDNA, particularly in early stages of disease (28). Detection with high sensitivity and specificity of ctDNA variants with low abundance in plasma can be challenging because of a possible low signal-to-noise ratio. Therefore, sequencing artifacts and clonal hematopoiesis of indeterminate potential (CHIP) will be more difficult to discriminate from true tumor-derived mutations (29, 30), especially for clinical applications in which low quantities of ctDNA are expected (e.g., screening, MRD monitoring). Novel technologies have been developed to resolve these issues (e.g., unique molecular identifiers [UMIs], patient-specific tumor-informed sequencing panels, and bioinformatics approaches), however remain to be validated on clinical samples with low plasma tumor fractions (31). Here, only Sample 1 contained variants with <0.5% (Supplemental Table 1). These were detected by all participants who performed

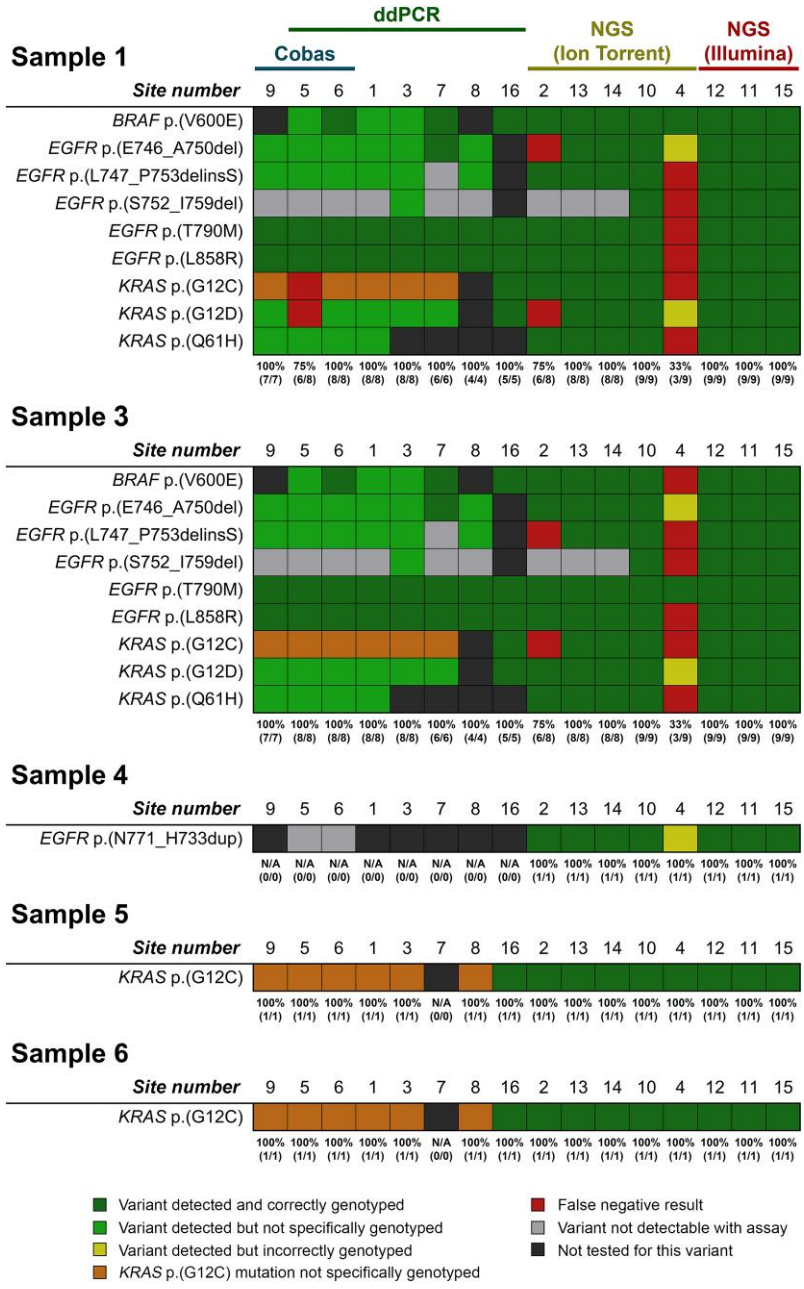

Fig. 2. Variant detection rate. Detectable variants in the requested exons (i.e., *BRAF* exon 15, *EGFR* exon 18–21, and *KRAS* exon 2–3) are shown for each sample. Percentages underneath columns represent the sensitivity of the detection of all variants tested for in a sample. Variants identified specifically or using a screening assay without specific genotyping are indicated seperately. Some variants were detected but incorrectly genotyped; this did not affect the molecular interpretation. Detection of a *KRAS* G12 mutation without specifically genotyping the *KRAS* p.(G12C) mutation was considered inaccurate for performance analysis. When a variant was detectable with the assay but not reported by the laboratory, it was considered a false negative. Whether the applied assay was unable to detect a certain variant or if no test was performed at all to identify a variant is highlighted as well. Detailed data are depicted in Supplemental Appendix 1 and performance is assessed in Supplemental Table 3. Abbreviation: N/A, not applicable.

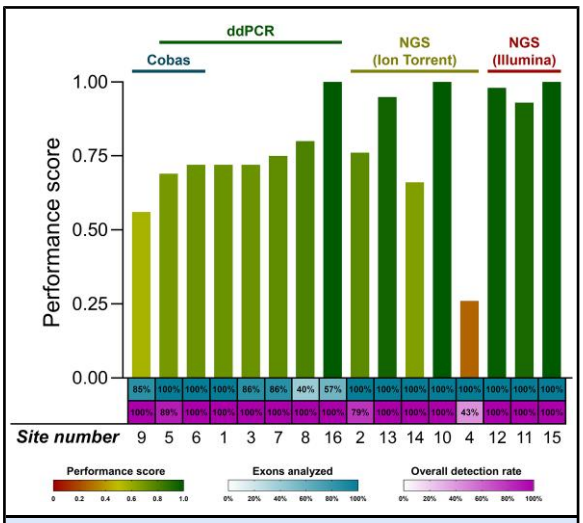

Fig. 3. Performance score, exon coverage, and overall detection rate. Performance was assessed using custom marking criteria (Supplemental Table 2). The percentage of analyzed exons is depicted based on the coverage of the applied assays. The percentage of identified variants detectable by the applied assays is expressed as the overall detection rate (also see Supplemental Table 2).

a test that should identify the particular variants, with Laboratory 4 as an exception. However, this was a commercial reference plasma sample containing high-quality spiked-in DNA and, therefore, does not reflect clinical samples. As such, the current study did not address in detail the accuracy of the detection of variants with low VAF in clinical samples.

This interlaboratory EQA exemplifies how divergent (pre)analytical protocols could lead to discrepant clinical outcomes when using the same plasma samples. Preanalytical work flows affect the quality of the material analyzed, whereas the choice of an analytical assay determines which variants could be identified. Although the sensitivity and specificity of single-target approaches or small panels are sufficient (8, 24), they are inadequate to cover all required genomic loci according to guidelines, rendering them inappropriate for primary screening purposes. National and international consortia should pursue the standardization of (pre)analytical work flows to facilitate the implementation of liquid biopsy testing in the clinical routine.

Supplemental Material

Supplemental material is available at *Clinical Chemistry* online.

Nonstandard Abbreviations: ctDNA, circulating tumor DNA; EQA, external quality assessment; COIN, ctDNA on the road to implementation in The Netherlands; DLA, diagnostic leukapheresis; ccfDNA, circulating cell-free DNA; ddPCR, droplet digital PCR; NGS, next-generation sequencing; IVD, in vitro diagnostic; NSCLC, non-small cell lung cancer; VAF, variant allele frequency.
Human Genes: *BRAF*, B-Raf proto-oncogene, serine/threonine kinase; *EGFR*, epidermal growth factor receptor; *KRAS*, KRAS proto-oncogene, GTPase.

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