university of groningen

University Medical Center Groningen

University of Groningen

Dutch National Round Robin Trial on Plasma-Derived Circulating Cell-Free DNA Extraction Methods Routinely Used in Clinical Pathology for Molecular Tumor Profiling

van der Leest, Paul; Ketelaar, Emma M; van Noesel, Carel J M; van den Broek, Daan; van Boerdonk, Robert A A; Deiman, Birgit; Rifaela, Naomi; van der Geize, Robert; Huijsmans, Cornelis J J; Speel, Ernst Jan M

Published in:
Clinical Chemistry

DOI:
10.1093/clinchem/hvac069

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2022

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
van der Leest, P., Ketelaar, E. M., van Noesel, C. J. M., van den Broek, D., van Boerdonk, R. A. A., Deiman, B., Rifaela, N., van der Geize, R., Huijsmans, C. J. J., Speel, E. J. M., Geerlings, M. J., van Schaik, R. H. N., Jansen, M. P. H. M., Dane-Vogelaar, R., Driehuis, E., Leers, M. P. G., Sidorenkov, G., Tamminga, M., van Kempen, L. C., & Schuuring, E. (2022). Dutch National Round Robin Trial on Plasma-Derived Circulating Cell-Free DNA Extraction Methods Routinely Used in Clinical Pathology for Molecular Tumor Profiling. *Clinical Chemistry*, *68*(7). https://doi.org/10.1093/clinchem/hvac069

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the “Taverne” license. More information can be found on the University of Groningen website: https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment.

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Download date: 20-05-2025

Clinical Chemistry 00:0
1–10 (2022)

Dutch National Round Robin Trial on Plasma-Derived Circulating Cell-Free DNA Extraction Methods Routinely Used in Clinical Pathology for Molecular Tumor Profiling

Paul van der Leest,[a] Emma M. Ketelaar,[a] Carel J.M. van Noesel,[b] Daan van den Broek,[c] Robert A.A. van Boerdonk,[d] Birgit Deiman,[e,f,g,h] Naomi Rifaela,[a] Robert van der Geize,[i] Cornelis J.J. Huijsmans,[j] Ernst Jan M. Speel,[k] Maartje J. Geerlings,[l] Ron H.N. van Schaik,[m] Maurice P.H.M. Jansen,[n] Ria Dane-Vogelaar,[o] Else Driehuis,[p] Mathie P.G. Leers,[q] Grigory Sidorenkov,[r] Menno Tamminga,[s] Léon C. van Kempen,[a] and Ed Schuuring[a,*]

BACKGROUND: Efficient recovery of circulating tumor DNA (ctDNA) depends on the quantity and quality of circulating cell-free DNA (ccfDNA). Here, we evaluated whether various ccfDNA extraction methods routinely applied in Dutch laboratories affect ccfDNA yield, ccfDNA integrity, and mutant ctDNA detection, using identical lung cancer patient–derived plasma samples.

METHODS: Aliquots of 4 high-volume diagnostic leukapheresis plasma samples and one artificial reference plasma sample with predetermined tumor-derived mutations were distributed among 14 Dutch laboratories. Extractions of ccfDNA were performed according to local routine standard operating procedures and were analyzed at a central reference laboratory for mutant detection and assessment of ccfDNA quantity and integrity.

RESULTS: Mutant molecule levels in extracted ccfDNA samples varied considerably between laboratories, but there was no indication of consistent above or below average performance. Compared to silica membrane–based methods, samples extracted with magnetic beads–based kits revealed an overall lower total ccfDNA yield (−29%; $P < 0.0001$) and recovered fewer mutant molecules (−41%; $P < 0.01$). The variant allelic frequency and sample integrity were similar. In samples with a higher-than-average total ccfDNA yield, an augmented recovery of mutant molecules was observed.

CONCLUSIONS: In the Netherlands, we encountered diversity in preanalytical workflows with potential consequences on mutant ctDNA detection in clinical practice. Silica membrane–based methodologies resulted in the highest total ccfDNA yield and are therefore preferred to detect low copy numbers of relevant mutations. Harmonization of the extraction workflow for accurate quantification and sensitive detection is required to prevent introduction of technical divergence in the preanalytical phase and reduce interlaboratory discrepancies.

Introduction

Liquid biopsies for the detection of tumor-derived variants have gained increasing interest in clinical diagnostics. Due to its minimally invasive nature, application of

[a]Department of Pathology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands; [b]Department of Pathology, Amsterdam University Medical Centres, University of Amsterdam, Amsterdam, The Netherlands; [c]Department of Laboratory Medicine, Netherlands Cancer Institute, Amsterdam, The Netherlands; [d]Department of Pathology, Amsterdam University Medical Centres, Vrije Universiteit Amsterdam, Amsterdam, The Netherlands; [e]Clinical Laboratory, Catharina Hospital Eindhoven, Eindhoven, The Netherlands; [f]Institute for Complex Molecular Systems, Laboratory of Chemical Biology, Eindhoven University of Technology, Eindhoven, The Netherlands; [g]Department of Biomedical Engineering, Laboratory of Chemical Biology, Eindhoven University of Technology, Eindhoven, The Netherlands; [h]Expert Center Clinical Chemistry Eindhoven, Eindhoven, The Netherlands; [i]Department of Molecular Pathology, Laboratorium Pathologie Oost-Nederland, Hengelo, The Netherlands; [j]Pathologie-DNA, Laboratory for Molecular Diagnostics, location Jeroen Bosch Hospital, 's-Hertogenbosch, The Netherlands; [k]Department of Pathology, GROW-School for Oncology and Developmental Biology, Maastricht University Medical Center, Maastricht, The Netherlands; [l]Department of Human Genetics, Radboud Institute for Molecular Life Sciences, Radboud University Medical Center, Nijmegen, The Netherlands; [m]Department of Clinical Chemistry, Erasmus MC University Medical Center, Rotterdam, The Netherlands; [n]Department of Medical Oncology, Laboratory of Translational Genomics, Erasmus MC University Medical Center, Rotterdam, The Netherlands; [o]Clinical Chemistry and Haematology, University Medical Center Utrecht, Utrecht, The Netherlands; [p]Department of Pathology, University Medical Center Utrecht, Utrecht, The Netherlands; [q]Department of Clinical Chemistry & Hematology, Zuyderland Medical Center, Sittard-Geleen/Heerlen, The Netherlands; [r]Department of Epidemiology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands; [s]Department of Pulmonary Medicine, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands.
*Address correspondence to this author at: Hanzeplein 1, 9713GZ Groningen, The Netherlands. Fax +31 50 361 99 12; E-mail: e.schuuring@umcg.nl.
Received December 16, 2021; accepted March 14, 2022.
https://doi.org/10.1093/clinchem/hvac069

© American Association for Clinical Chemistry 2022.
This is an Open Access article distributed under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs licence (https://creativecommons.org/licenses/by-nc-nd/4.0/), which permits non-commercial reproduction and distribution of the work, in any medium, provided the original work is not altered or transformed in any way, and that the work is properly cited. For commercial re-use, please contact journals.permissions@oup.com

Downloaded from https://academic.oup.com/clinchem/advance-article/doi/10.1093/clinchem/hvac069/6591828 by guest on 02 June 2022

liquid biopsy ranges from early screening and molecular target detection to treatment response prediction, minimal residual disease, and disease monitoring, including early detection of therapy resistance (1, 2). However, so far only 4 CE-IVD and Food and Drug Administration–approved commercial liquid biopsy tests are applicable in the clinical setting, being the Cobas EGFR Mutation Test v2 (Roche Diagnostics), Therascreen® PIK3CA RGQ PCR Kit (Qiagen), FoundationOne® Liquid CDx (Foundation Medicine), and Guardant 360® CDx (Guardant Health) (2, 3).

To date, (pre)analytical conditions vary substantially with respect to plasma collection and processing, storage conditions, circulating cell-free DNA (ccfDNA) extraction methods, elution volumes, and analytical input (4–6). Although these aspects have been demonstrated to drastically affect the ccfDNA quantity, quality, and mutant detection sensitivity (4, 7), standardization is lacking. The detection of tumor-derived mutant DNA [circulating tumor DNA (ctDNA)] in the ccfDNA in plasma of cancer patients is the most frequently applied liquid biopsy approach (1, 8–10). Previous studies have examined the differences between ccfDNA extraction methods using pooled purified human or artificial plasma with spiked-in (mutant) DNA (4, 11–14). In the majority of these studies, the QIAamp Circulating Nucleic Acid Kit yielded the most ccfDNA (4, 11, 12) and the most consistent results (13), whereas one study observed the highest yield using magnetic beads–based chemistry (i.e., QIAsymphony DSP Circulating DNA Kit) (14).

Within the framework of the CANCER-ID consortium (http://www.cancer-id.eu), an intra- and interlaboratory comparison of ccfDNA extraction methods with well-defined mutant spiked-in plasma samples demonstrated that extraction methods based on magnetic beads–based chemistry (i.e., Maxwell RSC LV ccfDNA Kit) resulted in a lower total yield of ccfDNA compared to silica membrane–based extraction methods (i.e., QIAamp Circulating Nucleic Acid Kit) (4). However, magnetic beads–based extraction methods displayed an increase in the short-to-long DNA fragments ratio, likely due to more efficient extraction of shorter ccfDNA fragments (4, 15). Similar results were observed with ccfDNA extracted from patient-derived plasma and high-volume diagnostic leukapheresis (DLA) plasma (7). It remains unclear whether the quantitative and qualitative differences of total ccfDNA extracted with different methods affect the detection and quantification of mutant ctDNA.

The aim of this study was to gain insight in the performance of ccfDNA extraction methods that are used in current diagnostic settings across laboratories in the Netherlands. Within the framework of the COIN consortium (www.cfdna.nl/coin), we performed a round robin ccfDNA extraction trial among 14 laboratories that are presently using ctDNA testing in clinical or research setting. The participating laboratories received an aliquot of 4 high-volume DLA plasma samples and one commercial spiked-in reference plasma sample for ccfDNA extraction. All extracted ccfDNA was sent to a central reference laboratory to analyze and compare the differences in detection of mutant ctDNA and ccfDNA quantity and integrity.

Material and Methods

SELECTION OF PARTICIPANTS

A survey was conducted among laboratories specialized in pathology, medical oncology, and laboratory medicine in the Netherlands to identify sites performing plasma-derived ctDNA analysis and willing to participate in this interlaboratory round robin trial (see the online Supplemental Appendix 1). Fourteen laboratories that indicated willingness to participate in this round robin trial received a more detailed survey on their applied standardized ccfDNA extraction method, plasma volume, and elution volume, among others (Supplemental Appendix 2).

PLASMA SAMPLE SELECTION AND QUANTITATIVE VALIDATION

DLA plasma samples from 4 patients with metastatic nonsmall cell lung cancer who were treated at the University Medical Center Groningen (UMCG; Groningen, The Netherlands) were retrieved from the UMCG DLA-Biobank. On-treatment DLA cell-free plasma samples were collected in citrate as described previously (16, 17) and stored as 50 mL fractions at −80°C within 30 min after withdrawal. All patients provided written informed consent for use of their samples for research and validation purposes. DLA samples were selected based on the presence of a driver mutation in the pretreatment tumor tissue biopsy and the availability of sufficient plasma (at least 50 mL). Droplet digital PCR (ddPCR) analysis was performed as reported previously (7, 18, 19) to select 4 DLA cases with detectable ctDNA levels, as well as sufficient ccfDNA yield using Qubit. After thawing, 50 mL of the 4 selected DLA cases were centrifuged at 1600 × *g* for 10 min to separate the plasma from the debris. Plasma was aliquoted in fractions of 1 or 2 mL and stored at −80°C until their shipment on dry ice to the participating laboratories.

In addition, artificial ccfDNA in plasma (40 ng/mL) spiked-in with the *EGFR* T790M mutation at 1% variant allelic frequency (VAF) was sent to each laboratory as reference material (Sample 5; purchased from SensID, Rostock, Germany).

Downloaded from https://academic.oup.com/clinchem/advance-article/doi/10.1093/clinchem/hvac069/6591828 by guest on 02 June 2022

All plasma processing steps were performed in a laboratory not used for any molecular testing to reduce contamination risk.

CCFDNA EXTRACTION BY THE PARTICIPANTS

All laboratories were requested to perform the ccfDNA extraction from 2 to 4 mL of plasma using their regular protocols (Supplemental Fig. 1). ccfDNA samples were stored at −20°C until sent on dry ice to the central laboratory (UMCG). The total volume of ccfDNA was returned to the UMCG and stored at −20°C until further analyses.

CENTRAL ASSESSMENT OF THE CCFDNA ELUATES EXTRACTED BY THE PARTICIPANTS

ccfDNA volumes were measured, and the concentration was determined using the Qubit dsDNA HS assay kit on a Qubit 2.0 fluorometer (Thermo Fischer Scientific) and LiquidIQ on the MassARRAY System (Agena Bioscience) as reported previously (7, 20). Integrity was determined through measuring the fragment size distribution of 137, 420, and 1950 bp fragments using the β-actin 1-tube 3-size ddPCR assay as described previously (7). Traces of ccfDNA fragment sizes were analyzed using the TapeStation (Agilent) according to the manufacturer's instructions. In all LiquidIQ and TapeStation analyses, Seraseq ctDNA Complete WT (Seracare Life Sciences) was used as reference material to compare inter-run variability.

MUTANT CTDNA ASSESSMENT USING DDPCR

Mutation-specific ddPCR analysis was performed as reported previously (7, 18). Primer and probe sequences are summarized in Supplemental Table 1. ddPCR assays were performed using the Bio-Rad QX200™ platform according to manufacturer's instructions and included mutant, wildtype, and no template controls. ddPCR analyses were performed with 2.5 to 8.8 µL of ccfDNA with different concentrations up to 0.67 ng/µL. Data were analyzed with the QuantaSoft™ analytical software version 1.7.4.0917 and QuantaSoft™ Analysis Pro 1.0.596 (Bio-Rad). Droplet counts were used to calculate the number of copies per initial volume of plasma input, as well as the VAF calculated by the QuantaSoft™ Analysis Pro 1.0.596 software. To determine the expected technical variation in mutant molecules and VAF of ddPCR analyses, a mutation-specific ddPCR analysis was performed in triplicate on a separate extraction of each sample performed at the UMCG. The expected variation was defined as the median ±CV, defined by the ratio of the SD to the mean.

All molecular testing was performed in the NEN-EN-ISO15189–accredited laboratory for molecular pathology at the UMCG. All standard precautions were taken to avoid contamination of amplification products using separate laboratories for pre- and post-PCR handling.

STATISTICAL ANALYSES

Statistical analyses were performed using Prism version 8.4.2. (GraphPad software). For statistical assessment between silica membrane– and magnetic beads–based kits, a Mann–Whitney *U*-test was performed. For statistical assessment of multiple groups, a 1-way ANOVA with nonparametric measures (Kruskal–Wallis test) was performed followed by Dunn's multiple comparison test. Multilevel linear regression analysis with measurements clustered on sample level was performed using a generalized linear mixed model in SPSS software (version 25) to test the association between the extraction methods on the one hand and 3 outcomes on the other hand, namely ccfDNA yield, ccfDNA integrity, and mutant ctDNA levels. The tested associations were adjusted for the potential confounders plasma volume and elution volume.

Results

CHARACTERIZATION OF DLA SAMPLES IN THE ROUND ROBIN TRIAL

Prior to shipment, ccfDNA and mutant ctDNA levels in an aliquot of the 4 DLA samples and the reference plasma sample were quantified by the central laboratory. ccfDNA was extracted with the silica membrane–based QIAamp Circulating Nucleic Acid Kit as reported previously (7). The number of copies as well as VAF of expected mutations were determined in triplicate (Table 1). The mean variation, expressed as CV of the triplicate analyses, was 40% and 33% for mutant molecules and VAF, respectively, which were slightly higher compared to the 31% CV established previously using reference material (21). Sample 3 demonstrated a low yield and low number of mutant molecules (Table 1). Therefore, Sample 3 was only included for the comparison of ccfDNA yield and integrity and excluded from mutation detection.

CCFDNA EXTRACTION METHODS

In total, 14 Dutch laboratories participated in this interlaboratory round robin trial, all receiving a 2 to 4 mL of each plasma sample. Table 2 describes the routine procedures for ccfDNA extraction of the participants. Six different extraction methods were used: the QIAamp Circulating Nucleic Acid Kit (Qiagen), Cobas cfDNA Sample Preparation Kit (Roche), Maxwell RSC LV ccfDNA Kit (Promega Benelux BV), QIAamp MinElute ccfDNA Mini Kit (Qiagen), QIAsymphony DNA Mini Kit (Qiagen), and the MagNa Pure 24 Total NA Isolation Kit (Roche). A broad range of

Downloaded from https://academic.oup.com/clinchem/advance-article/doi/10.1093/clinchem/hvac069/6591828 by guest on 02 June 2022

Table 1. Sample characteristics.

Sample ID	Tissue mutation	CcfDNA, ng/µL	VAF, ddPCR, %	Mutant molecules,[a] ddPCR
Sample 1	*KRAS* c.34G > T; p.(G12C)	0.97	1.7 ± 0.70	58 ± 23
Sample 2	*KRAS* c.34G > T; p.(G12C)	0.73	1.7 ± 0.55	47 ± 17
Sample 3	*TP53* c.818G > A; p.(R273H)	0.32	2.7 ± 0.71	17 ± 7.1
Sample 4	*TP53* c.536A > G; p.(H179R)	0.52	4.0 ± 1.5	23 ± 8.7
Sample 5	*EGFR* c.2369C > T; p.(T790M)	1.82	0.8 ± 0.18	74 ± 21
Reference[b]	*KRAS* c.35G > A; p.(G12D)	1.00	0.9 ± 0.28	10 ± 2.8

[a]Depicted as mutant copies/mL of plasma.
[b]Reference ccfDNA samples spiked-in with the *KRAS* p.(G12D) mutation.

Table 2. ccfDNA extraction methods used in this interlaboratory study.

Lab	Plasma volume, mL	Elution volume, µL	Extraction method	Chemistry	Execution	Remarks
1	4	39 (38–40)	CNA	Silica membrane	Manual	None
2	2	32 (26–32)	CNA	Silica membrane	Manual	Yes[a],b
3	2	52 (52–52)	CNA	Silica membrane	Manual	None
4	3	49 (48–51)	CNA	Silica membrane	Manual	Yes[c]
5	2	80 (78–80)	COB	Silica membrane	Manual	None
6	2	100 (100–100)	COB	Silica membrane	Manual	None
7	2	44 (40–46)	RSC	Magnetic beads	Automated	None
8	2	50 (46–57)	RSC	Magnetic beads	Automated	None
9	2	45 (42–48)	RSC	Magnetic beads	Automated	None
10	4	64 (62–66)	RSC	Magnetic beads	Automated	Yes[d]
11	3	41 (34–42)	ME	Magnetic beads	Manual	None
12	2	26 (24–27)	ME	Magnetic beads	Manual	Yes[b]
13	2	66 (65–69)	SYM	Magnetic beads	Automated	None
14	4	48 (43–50)	MNA	Magnetic beads	Automated	None

Elution volumes of ccfDNA extracted from the 5 plasma samples were measured upon arrival in the UMCG and are displayed as a mean (range).
RSC, Maxwell RSC LV ccfDNA Kit; CNA, QIAamp Circulating Nucleic Acid Kit; MNA, MagNa Pure 24 Total NA Isolation Kit; COB, Cobas ccfDNA Sample Preparation Kit; SYM, QIAsymphony DNA Mini Kit; ME, QIAamp MinElute ccfDNA Mini Kit.
[a]Applied a modified protocol (see Supplemental Appendix 1).
[b]ccfDNA samples were not frozen when received by the UMCG.
[c]Plasma samples thawed during transport to Lab 4 due to shipping delays. Upon arrival, the plasma was stored at 4°C.
[d]ccfDNA samples were stored at 4°C before they were sent to UMCG.

elution volumes was observed when the extracted ccfDNA arrived at the central reference laboratory at the UMCG (Table 2).

RECOVERY OF MUTANT CTDNA

Mutant-specific ddPCR assays were performed to quantify the expected mutations in the samples. In total, 56 ddPCR reactions were performed (Fig. 1, A; Supplemental Table 1). For each plasma sample, the median number of ctDNA copies per mL plasma was determined and variation calculated. In 12 of the 56 samples tested, more mutant molecules were detected than the median plus one CV. Eight of these (75%) were extracted with a silica membrane–based method. In 15 samples, fewer mutant molecules were detected, of which 11 were extracted with magnetic beads (74%) (Fig. 1, A). This suggested that ccfDNA extracted with a silica membrane–based method resulted in a higher recovery of mutant molecules. When mutant molecule levels were normalized against the median of each plasma sample and stratified for silica

Downloaded from https://academic.oup.com/clinchem/advance-article/doi/10.1093/clinchem/hvac069/6591828 by guest on 02 June 2022

membrane– and magnetic beads–based chemistry (Fig. 1, B), fewer mutant molecules were detected in ccfDNA extracted from plasma with a magnetic beads–based extraction method [0.76 vs 1.29 (−41%), $P < 0.01$] (Fig. 1, B), confirming the higher ctDNA recovery with a silica membrane–based extraction method. Multilevel linear regression analysis revealed that ccfDNA extraction using the Maxwell RSC LV ccfDNA Kit resulted in fewer mutant molecules compared with the QIAamp Circulating Nucleic Acid Kit ($P < 0.001$), but other factors were not found significantly different (Supplemental Table 2A). With respect to VAF, fewer samples with frequencies above or below the average were observed ($n = 17$), and no significant difference was found between the silica membrane– and magnetic beads–based extraction methods ($P = 0.70$) (Fig. 1, C and D).

YIELD OF TOTAL CCFDNA

To determine whether the lower detection of mutant molecules copies in ccfDNA extracted from plasma with a magnetic beads–based method is related to the yield of ccfDNA using different extraction methods, total ccfDNA was quantified with the fluorescence-based quantification assay Qubit (Fig. 2, A). The elution buffer of the MagNa Pure 24 Total NA Isolation Kit contains a component that quenches fluorescence (22); hence, the samples from Lab 14 could not be quantified with the Qubit assay and were excluded from this analysis. In total, 65 Qubit reactions were performed, and the observed overall CV was 40%. For each plasma sample, the median amount of ccfDNA (nanogram) per milliliter of plasma was determined. In 10 ccfDNA samples, a higher ccfDNA yield was detected than the calculated variation of the median, all extracted with a silica membrane–based method (Fig. 2, A). In 8 samples, a lower yield compared to the average was observed, of which 7 were extracted with magnetic beads (88%). When normalized against the median, a decreased yield was detected in ccfDNA extracted from plasma with a magnetic beads–based extraction method [1.21 vs 0.86 (−29%), $P < 0.0001$] (Fig. 2, B). Except for the QIAamp MinElute ccfDNA Mini Kit, all extraction methods yielded significantly less total ccfDNA compared with the QIAamp Circulating Nucleic Acid Kit method in multilevel linear regression analysis (Supplemental Table 2B). In addition, a plasma volume of 2 mL and a higher elution volume increased the total ccfDNA yield during extraction.

CCFDNA INTEGRITY

As a measure of integrity, fragment size distributions were determined using the β-actin 1-tube 3-size ddPCR assay as described previously (7). The β-actin 1-tube 3-size ddPCR assay can generate fragments of 137, 420, and 1950 bp (Supplemental Fig. 2). More fragments of each size were extracted with silica membrane–based methods compared to magnetic beads–based kits (Supplemental Fig. 3). A lower ratio of 1950 bp (long-sized fragments; i.e., genomic DNA) and 137 bp fragments (short-sized fragments; i.e., ccfDNA), indicates a lower abundance of genomic DNA (Fig. 3, A). Seventy ddPCR reactions were performed. For each plasma sample, the median ratio of long-to-short–sized ccfDNA fragments and variation thereof was determined. In 9 ccfDNA samples, a higher ratio of long-to-short–sized ccfDNA fragments was detected than the expected variation of the median, of which 6 were extracted with a magnetic beads–based method (67%). In 14 ccfDNA samples, a lower ratio than the expected variation of the median was observed, of which 8 were extracted with magnetic beads (57%). No difference in the long-to-short–sized ratio between silica membrane– and magnetic beads–based methods were observed after normalization to the median ($P = 0.81$) (Fig. 3, B). The ultrasonic fragmented DNA in the reference sample (Sample 5) barely showed long-sized fragments and was therefore excluded from the normalized analysis. Multilevel linear regression analysis revealed no significant differences, except for a lower long-to-short–sized ratio when using a 3-mL plasma input volume ($P < 0.05$) (Supplemental Table 2C).

EFFECT OF CCFDNA YIELD AND INTEGRITY ON MUTANT MOLECULE DETECTION

To determine whether the difference in the quantification of mutant molecule detection (i.e., higher or lower than the median ± 1 CV, respectively) was related to yield or integrity when using different ccfDNA extraction methods, all data were compared (Fig. 4). In general, most results below the median -1 CV, referred to as "lower-than-average," were observed when samples were extracted with magnetic beads–based methods (77%), while the majority of "higher-than-average" results were obtained with silica membrane–based extraction methods (65%). For the samples with a higher-than-average mutant molecule detection, only 27% and 25% had a higher-than-average yield and integrity, respectively. On the other hand, 21% of the samples with lower-than-average mutant molecule detection also had a lower-than-average yield. ccfDNA integrity of all samples was comparable according to the heatmap analysis. Based on these results, discrepant mutant molecule levels could not be attributed to difference in yield or integrity (Fig. 4). Regarding the absolute ccfDNA amount, an increased yield was observed in the samples with higher-than-average mutant molecule detection quantified with either Qubit (Supplemental Fig. 4, A)

Downloaded from https://academic.oup.com/clinchem/advance-article/doi/10.1093/clinchem/hvac069/6591828 by guest on 02 June 2022

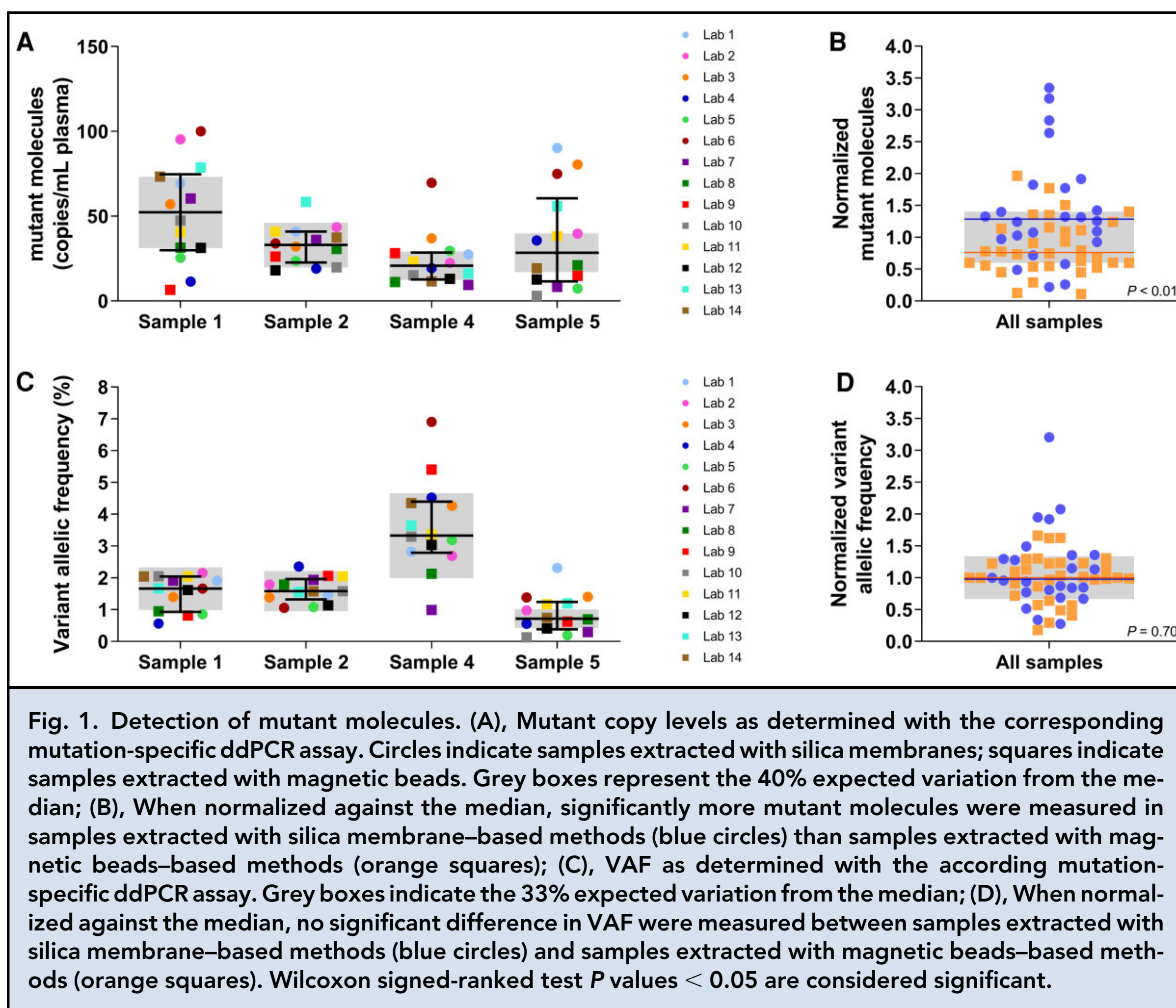

Fig. 1. Detection of mutant molecules. (A), Mutant copy levels as determined with the corresponding mutation-specific ddPCR assay. Circles indicate samples extracted with silica membranes; squares indicate samples extracted with magnetic beads. Grey boxes represent the 40% expected variation from the median; (B), When normalized against the median, significantly more mutant molecules were measured in samples extracted with silica membrane–based methods (blue circles) than samples extracted with magnetic beads–based methods (orange squares); (C), VAF as determined with the according mutation-specific ddPCR assay. Grey boxes indicate the 33% expected variation from the median; (D), When normalized against the median, no significant difference in VAF were measured between samples extracted with silica membrane–based methods (blue circles) and samples extracted with magnetic beads–based methods (orange squares). Wilcoxon signed-ranked test *P* values < 0.05 are considered significant.

or LiquidIQ (Supplemental Figure 4, B). Multilevel linear regression analysis confirmed the association between higher yield and increased mutant molecule levels (Supplemental Table 3A). In contrast, no discrepancies were determined in regard of sample integrity (Supplemental Fig. 4, C). In line with this observation, analysis of the TapeStation traces of the ccfDNA fragment size distribution did not reveal differences between samples with higher- or lower-than-average detected mutant molecules (Supplemental Fig. 5). As such, no association between integrity and mutant molecule levels was observed in multilevel linear regression analysis (Supplemental Table 3B).

Discussion

In this Dutch national round robin trial, we report on differences in ccfDNA yield and integrity with different ccfDNA extraction methods and the impact on ctDNA mutation detection. In general, extraction methods relying on silica membrane chemistry outperformed magnetic beads–based tests regarding ccfDNA yield and ctDNA recovery, while sample integrity was comparable. As such, yield seemed to affect mutant molecule detection more prominently than sample integrity.

According to current guidelines, cancer diagnostics rely on the availability of tumor tissue biopsy for accurate classification and (actionable) mutant detection. If liquid biopsy approaches reach sufficient analytical sensitivity regarding molecular tumor profiling in metastasized cancer, they harbor potential to overcome the limitations of conventional tumor tissue–based analyses (e.g., invasiveness, inability of repetition, tumor heterogeneity). To implement plasma-derived ccfDNA testing in the clinical setting, it is crucial to establish standardized guidelines and storage conditions (i.e., blood collection tubes, transport, storage time), as well as cell-free plasma processing (7). Previous studies have shown that selection of ccfDNA extraction methodology and plasma input and elution volumes drastically impact the yield and quality of the extracted material

Downloaded from https://academic.oup.com/clinchem/advance-article/doi/10.1093/clinchem/hvac069/6591828 by guest on 02 June 2022

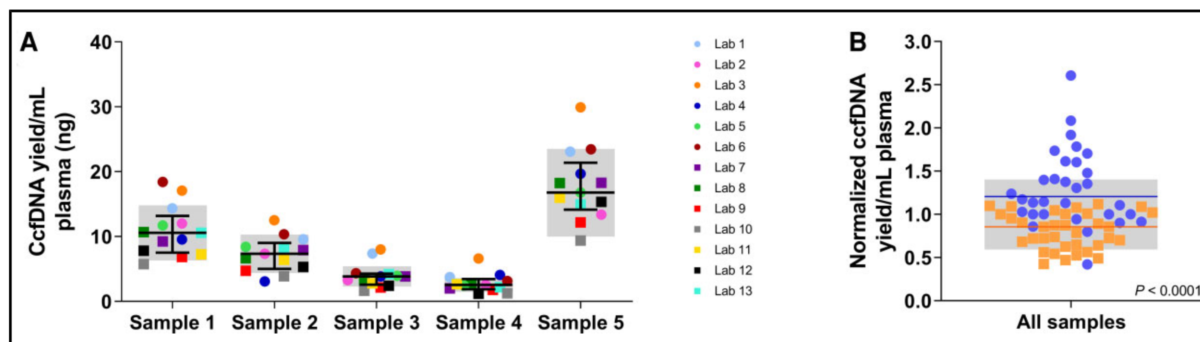

Fig. 2. Determination of ccfDNA yield. (A), Samples from each laboratory were quantified using Qubit. Circles indicate samples are extracted with silica membranes; squares samples are extracted with magnetic beads. Grey boxes represent the 40% expected variation from the median; (B), Normalization against the median reveals significantly more mutant molecules in samples extracted with silica membrane–based methods (blue circles) than samples extracted with magnetic beads–based methods (orange squares). Lab 14 was excluded from yield quantification due to fluorescence quenching reagents in the elution buffer (22). Wilcoxon signed-ranked test *P* values < 0.05 are considered significant.

(4, 21). Here, we collected the plasma processing procedures routinely applied by 14 clinical laboratories across the Netherlands to summarize its variety and ultimate effect on ccfDNA mutation detection, quantity, and quality using identical patient-derived plasma samples.

Plasma samples of lung cancer patients generally yield various amounts of ccfDNA, ranging from almost nonexistent values to micrograms per milliliter (9). To accurately quantify mutant molecule levels of ctDNA, it is crucial to recover the most ccfDNA possible for clinically relevant interpretation regarding treatment decision-making, as multigene predictive testing with next-generation sequencing relies on high input amounts for optimal and sensitive detection (21). In addition, single-target approaches, such as ddPCR, often require repetitive testing due to low ccfDNA concentration or multiple suspected targets, for which a lot of material is required. Here, we determined that extraction with silica membrane–based methods yields more total ccfDNA and ctDNA than magnetic beads–based methods, in agreement with previous reports using artificial spiked-in or patient-derived plasma (4, 7, 11, 12). This implies that ccfDNA extraction with silica membrane–based methods increases the chance of recovering

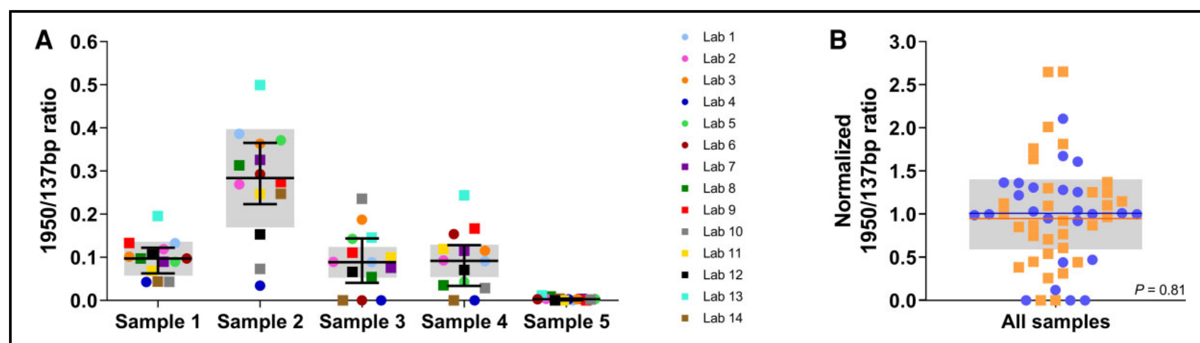

Fig. 3. Ratio of long-to-short-sized ccfDNA fragments. (A), The 1950/137 bp ratio was determined with the β-actin one-tube 3-size ddPCR assay. Circles indicate samples are extracted with silica membranes; squares samples are extracted with magnetic beads. Grey boxes represent the 40% expected variation from the median; (B), Normalization against the median reveals no differences between silica membrane–based methods (blue circles) than samples extracted with magnetic beads–based methods (orange squares). Sample 5 was excluded from the normalized data since the material was artificially fragmented. Wilcoxon signed-ranked test *P* values < 0.05 are considered significant.

Downloaded from https://academic.oup.com/clinchem/advance-article/doi/10.1093/clinchem/hvac069/6591828 by guest on 02 June 2022

mutant molecules from a plasma sample, especially when ccfDNA concentrations are low. However, the ctDNA recovery using samples extracted with either a silica membrane–based or magnetic beads–based method also revealed some intragroup variation, highlighting that processing conditions and the choice of extraction method can influence outcome. Larger series are required to elaborate on the performance of individual extraction methods. Overall, our results demonstrated that harmonization of the ccfDNA extraction workflow for accurate quantification and sensitive detection is required to prevent technical divergence in the preanalytical phase and reduce interlaboratory discrepancies.

As ccfDNA is highly fragmented, with peak sizes around 167 bp, and the ctDNA fraction is even shorter in length (23–25), preferential extraction of short-sized fragments in contrast to genomic DNA might augment the chance of detecting mutant molecules despite a potential lower yield. In this study, we confirmed previous observations showing increased detection of all fragment sizes using silica membrane–based methods compared to magnetic beads (4, 7, 15). However, these studies using artificially spiked-in plasma samples determined a relatively higher amount of short-sized ccfDNA resulting in a decreased long-to-short–sized fragments ratio, which seems not to be apparent in patient-derived plasma samples (5). Accordingly, no discrepancies between silica membrane– and magnetic beads–based extraction methods were observed regarding the VAF, in agreement with previous studies (4, 7, 26). Although Bos et al. recently established that measuring mutant molecule levels correlated strongly with VAF percentages (27), longitudinal studies showed that monitoring mutant molecules outperforms VAF regarding treatment response prognosis (18, 25, 28). In summary, even though mutant molecules were detectable in all the analyzed samples and the number of measured mutant molecules were mostly within the expected variation from the median, silica membrane–based methods seem preferable to yield the highest amounts of total ccfDNA and ctDNA without compromising on sample integrity.

As the laboratories were instructed to perform the ccfDNA extraction according to their routine standard operating procedures, various ccfDNA extraction methods with variable plasma and elution volumes were applied. This round robin trial was facilitated by availability of unique high-volume (>50 mL) patient-derived DLA plasma samples, which enabled distribution of aliquots of identical plasma sample to each participating laboratory, minimizing discrepancies

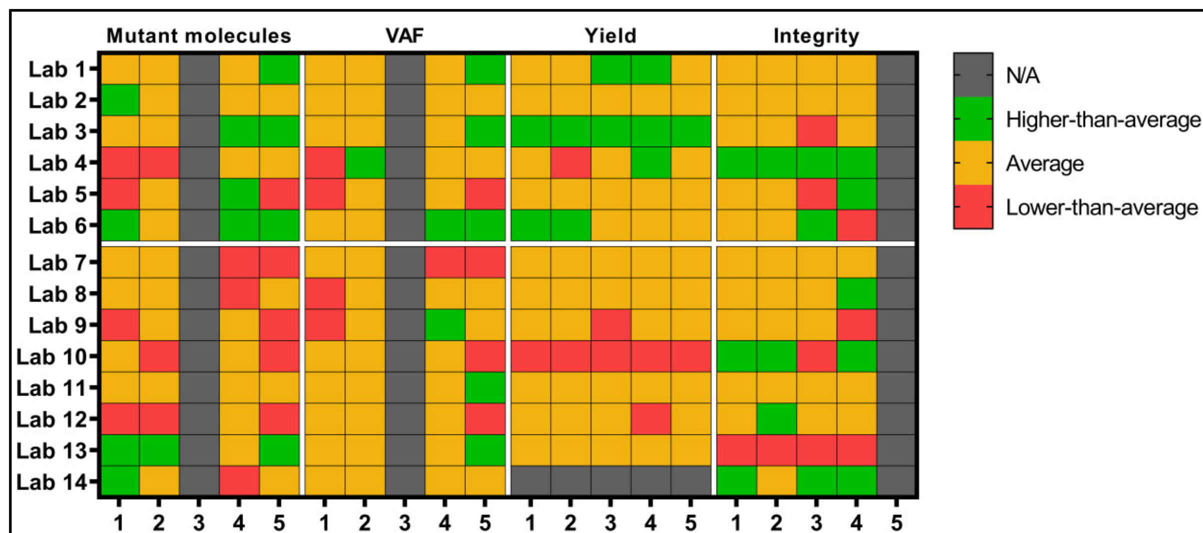

Fig. 4. Heatmap of ccfDNA characteristics. All samples for each laboratory were rated above average (green), average (within expected range from the median; yellow), or below average (red) based on mutant molecule levels, VAF, yield, and integrity. Plasma samples 1 to 5 are depicted on the x-axis per category. Variations in mutant molecule detection were not specifically attributable to yield or integrity. Sample 3 was excluded from mutant molecule detection since expected copy numbers and yield were considered insufficient. Lab 14 was excluded from yield quantification due to fluorescence quenching reagents in the elution buffer (22). Sample 5 was excluded from integrity analysis as this reference sample was artificially fragmented.

Downloaded from https://academic.oup.com/clinchem/advance-article/doi/10.1093/clinchem/hvac069/6591828 by guest on 02 June 2022

induced through plasma handling and hemolysis. As DLA is citrated plasma, it is not identical to plasma collected via conventional blood withdrawal; however, it has previously been proven to contain detectable ctDNA at similar VAFs as matched blood draws in Streck tubes (21). Due to limited DLA plasma volumes, distributed aliquots were maximized to 4 mL whereas 3 laboratories indicated the use of 8 mL of plasma in their routine practice. Transport conditions and sample handling (e.g., storage conditions, freeze–thaw cycles) also might have affected the quality of source and extracted material. To resolve these issues, we requested that the laboratories document all procedures adequately. Despite some divergence, these conditions seemed to have affected the ccfDNA minimally, except for thawing of the plasma during shipment. Compared to other laboratories using QIAamp Circulating Nucleic Acid Kit, Lab 4 showed poorer ccfDNA yield and mutant molecule levels, as well as DNA degradation resulting in fewer long-sized fragments. On the other hand, thawing of ccfDNA eluates seems not to have affected the quality of the material, consistent with previous observations (13).

Altogether, application of various ccfDNA extraction methods with variable plasma and elution volumes introduced considerable discrepancies regarding ccfDNA mutation detection and quantity. Based on this first Dutch national round robin trial, a silica membrane–based method outperformed magnetic beads–based chemistry regarding ctDNA recovery and total ccfDNA yield, increasing the chance of mutant molecule detection and reducing variation. To ensure accurate mutant molecule detection and quantification at every liquid biopsy institute, critical evaluation of appropriate preanalytical conditions should be pursued. It is key to consistently apply similar extraction conditions to prevent inaccurate quantitative analyses, especially during disease monitoring, interinstitutional studies, and interdisciplinary clinical practices. Collaborative initiatives, such as the Dutch national COIN consortium and the European Liquid Biopsy Society, should establish ranges for acceptable variation regarding performance specifications that cell-free plasma and ccfDNA extraction protocols must meet to be considered for implementation in standardized liquid biopsy workflows in clinical routine diagnostics.

Supplemental Material

Supplemental material is available at *Clinical Chemistry* online.

Nonstandard Abbreviations: ccfDNA, circulating cell-free DNA; ctDNA, circulating tumor DNA; DLA, diagnostic leukapheresis; UMCG, University Medical Center Groningen; ddPCR, droplet digital PCR; VAF, variant allelic frequency.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 4 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; (c) final approval of the published article; and (d) agreement to be accountable for all aspects of the article thus ensuring that questions related to the accuracy or integrity of any part of the article are appropriately investigated and resolved.*

Authors' Disclosures or Potential Conflicts of Interest: *Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest*:

Employment or Leadership: E. Schuuring, board member of the Dutch Society of Pathology.
Consultant or Advisory Role: E.J.M. Speel, AstraZeneca; D. van den Broek, Roche Diagnostics (paid to the institution); E. Schuuring, Agena Bioscience, Amgen, AstraZeneca, Bayer, Bristol Meyer Squibb, CC Diagnostics, Illumina, Janssen-Cilag (Johnson & Johnson), Lilly, MSD/Merck, Novartis, and Roche (paid to institution).
Stock Ownership: None declared.
Honoraria: D. van den Broek, Roche Diagnostics (paid to the institution); E. Schuuring, lectures for Agena Bioscience, Biocartis, Bio-Rad, Illumina, Lilly, Roche, and SeraCare (paid to institution).
Research Funding: The COIN consortium is funded by the ZonMw 'Personalised Medicine' programme (project number 848101011). E.J.M. Speel, research grants from AstraZeneca; D. van den Broek, funding from Roche Diagnostics (paid to the institution); E. Schuuring, research grants from Abbott, Agena Bioscience, AstraZeneca, Bayer, Biocartis, Bio-Rad, Boehringer Ingelheim, CC Diagnostics, Invitae/Archer, and Roche (all paid to the institution).
Expert Testimony: D. van den Broek, Roche Diagnostics (paid to the institution).
Patents: None declared.
Other Remuneration: E.J.M. Speel, non-financial support from Biocartis; E. Schuuring, support for travel expenses from Biocartis and Bio-Rad.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, preparation of manuscript, or final approval of manuscript.

Acknowledgments: We thank all contributing members of the COIN consortium, in particular E. de Jonge (Erasmus MC University Medical Center), A.J.C. van den Brule (Pathologie-DNA), O. Karademir (Laboratorium Pathologie Oost-Nederland), T.E.J. Theunissen and K. Vaes (both Maastricht University Medical Center), E.J. Kamping (Radboud University Medical Center) and M.J.L. Ligtenberg (Department of Human Genetics and Department of Pathology, Radboud University Medical Center), M. Bekker-Lettink (Vrije Universiteit Amsterdam), and B. Jongen (Zuyderland Medical Center), for their contribution to this study.

References

1. Heitzer E, Haque IS, Roberts CES, Speicher MR. Current and future perspectives of liquid biopsies in genomics-driven oncology. Nat Rev Genet 2019;20:71–88.
2. Heitzer E, van den Broek D, Denis MG, Hofman P, Hubank P, Mouliere F, et al.

Downloaded from https://academic.oup.com/clinchem/advance-article/doi/10.1093/clinchem/hvac069/6591828 by guest on 02 June 2022

Recommendations for a practical implementation of circulating tumour DNA mutation testing in metastatic non-small cell lung. ESMO Open 2022;7:100399.

3. Food and Drug Administration. List of cleared or approved companion diagnostic devices (in vitro and imaging tools). https://www.fda.gov/medical-devices/in-vitro-diagnostics/list-cleared-or-approved-companion-diagnostic-devices-in-vitro-and-imaging-tools (Accessed December 2021).
4. Lampignano R, Neumann MHD, Weber S, Kloten V, Herdean A, Voss T, et al. Multicenter evaluation of circulating cell-free DNA extraction and downstream analyses for the development of standardized (pre)analytical work flows. Clin Chem 2020;66:149–60.
5. Deans ZC, Williams H, Dequeker EMC, Keppens C, Normanno N, Schuuring E, et al. Review of the implementation of plasma ctDNA testing on behalf of IQN Path ASBL: a perspective from an EQA providers' survey. Virchows Arch 2017;471:809–13.
6. Godsey JH, Silvestro A, Barrett JC, Bramlett K, Chudova D, Deras I, et al. Generic protocols for the analytical validation of next-generation sequencing-based ctDNA assays: a joint consensus recommendation of the BloodPAC's Analytical Variables Working Group. Clin Chem 2020; 66:1156–66.
7. Leest PV, Boonstra PA, Elst AT, Kempen LCV, Tibbesma M, Koopmans J, et al. Comparison of circulating cell-free DNA extraction methods for downstream analysis in cancer patients. Cancers (Basel) 2020;12:1222.
8. Moding EJ, Diehn M, Wakelee HA. Circulating tumor DNA testing in advanced non-small cell lung cancer. Lung Cancer 2018;119:42–7.
9. Ossandon MR, Agrawal L, Bernhard EJ, Conley BA, Dey SM, Divi RL, et al. Circulating tumor DNA assays in clinical cancer research. J Natl Cancer Inst 2018; 110:929–34.
10. Aggarwal C, Thompson JC, Black TA, Katz SI, Fan R, Yee SS, et al. Clinical implications of plasma-based genotyping with the delivery of personalized therapy in metastatic non-small cell lung cancer. JAMA Oncol 2019;5:173–80.
11. Diefenbach RJ, Lee JH, Kefford RF, Rizos H. Evaluation of commercial kits for purification of circulating free DNA. Cancer Genet 2018;228-229:21–7.
12. van Dessel LF, Vitale SR, Helmijr JCA, Wilting SM, van der Vlugt-Daane M, Oomen-de Hoop E, et al. High-throughput isolation of circulating tumor DNA: a comparison of automated platforms. Mol Oncol 2019;13:392–402.
13. van Ginkel JH, van den Broek DA, van Kuik J, Linders D, de Weger R, Willems SM, Huibers MMH. Preanalytical blood sample workup for cell-free DNA analysis using Droplet Digital PCR for future molecular cancer diagnostics. Cancer Med 2017;6: 2297–307.
14. Warton K, Graham L-J, Yuwono N, Samimi G. Comparison of 4 commercial kits for the extraction of circulating DNA from plasma. Cancer Genet 2018:228-229:143–50.
15. Kloten V, Rüchel N, Brüchle NO, Gasthaus J, Freudenmacher N, Steib F, et al. Liquid biopsy in colon cancer: comparison of different circulating DNA extraction systems following absolute quantification of KRAS mutations using Intplex allele-specific PCR. Oncotarget 2017;8:86253–63.
16. Tamminga M, de Wit S, Hiltermann TJN, Timens W, Schuuring E, Terstappen LWMM, Groen HJM. Circulating tumor cells in advanced non-small cell lung cancer patients are associated with worse tumor response to checkpoint inhibitors. J Immunother Cancer 2019;7:173.
17. Tamminga M, Oomens L, Hiltermann TJN, Andree KC, Tibbe A, Broekmaat J, et al. Microsieves for the detection of circulating tumor cells in leukapheresis product in non-small cell lung cancer patients. Transl Lung Cancer Res 2020;9:1093–100.
18. van der Leest P, Hiddinga B, Miedema A, Aguirre Azpurua ML, Rifaela N, Elst AT, et al. Circulating tumor DNA as a biomarker for monitoring early treatment responses of patients with advanced lung adenocarcinoma receiving immune checkpoint inhibitors. Mol Oncol 2021;15: 2910–22.
19. Boonstra PA, Elst AT, Tibbesma M, Bosman LJ, Mathijssen R, Atrafi F, et al. A single digital droplet PCR assay to detect multiple KIT exon 11 mutations in tumor and plasma from patients with gastrointestinal stromal tumors. Oncotarget 2018;9: 13870–83.
20. Lamy P-J, van der Leest P, Lozano N, Becht C, Duboeuf F, Groen HJM, et al. Mass spectrometry as a highly sensitive method for specific circulating tumor DNA analysis in NSCLC: a comparison study. Cancers (Basel) 2020;12:3002.
21. Weber S, Spiegl B, Perakis SO, Ulz CM, Abuja PM, Kashofer K, et al. Technical evaluation of commercial mutation analysis platforms and reference materials for liquid biopsy profiling. Cancers (Basel) 2020;12:1588.
22. Roche LifeScience. MagNA Pure LC Total Nucleic Acid Isolation Kit. https://pim-eservices.roche.com/LifeScience/Document/7e146942-7113-eb11-fe90-005056a772fd (Accessed December 2021).
23. Sanchez C, Snyder MW, Tanos R, Shendure J, Thierry AR. New insights into structural features and optimal detection of circulating tumor DNA determined by single-strand DNA analysis. NPJ Genomic Med 2018;3:31.
24. Mouliere F, Chandrananda D, Piskorz AM, Moore EK, Morris J, Ahlborn LB, et al. Enhanced detection of circulating tumor DNA by fragment size analysis. Sci Transl Med 2018;10:eaat4921.
25. Weber S, van der Leest P, Donker HC, Schlange T, Timens W, Tamminga M, et al. Dynamic changes of circulating tumor DNA predict clinical outcome in patients with advanced non–small-cell lung cancer treated with immune checkpoint inhibitors. JCO Precis Oncol 2021;5: 1540–53.
26. Sorber L, Zwaenepoel K, Deschoolmeester V, Roeyen G, Lardon F, Rolfo C, Pauwels P. A comparison of cell-free DNA isolation kits: isolation and quantification of cell-free DNA in plasma. J Mol Diagn 2017;19: 162–8.
27. Bos MK, Nasserinejad K, Jansen MPHM, Angus L, Atmodimedjo PN, de Jonge E, et al. Comparison of variant allele frequency and number of mutant molecules as units of measurement for circulating tumor DNA. Mol Oncol 2021;15:57–66.
28. de Kock R, van den Borne B, Youssef-El Soud M, Belderbos H, Brunsveld L, Scharnhorst V, Deiman B. Therapy monitoring of EGFR-positive non-small-cell lung cancer patients using ddPCR multiplex assays. J Mol Diagn 2021;23:495–505.

Downloaded from https://academic.oup.com/clinchem/advance-article/doi/10.1093/clinchem/hvac069/6591828 by guest on 02 June 2022