

Functional Characterization and a Real-World Clinical Laboratory Pilot of the Foundation for the National Institutes of Health Circulating Tumor DNA Quality Control Materials

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ABSTRACT

PURPOSE We previously developed quality control materials (QCMs) to aid in the development of circulating tumor DNA (ctDNA) assays. In this study, we further characterize the performance of the QCMs relative to clinical samples.

METHODS QCMs were provided by three manufacturers. To functionally characterize the QCMs, we (1) evaluated *EGFR* L858R and ex19del (range, 0.5%–5.0% variant allele frequency [VAF]) in QCMs compared with clinical samples by droplet digital polymerase chain reaction (ddPCR), targeted-amplicon sequencing (Tag-seq), and hybrid capture next-generation sequencing (NGS); and (2) evaluated the QCMs and clinical samples near the Tag-seq limit of detection. A clinical pilot was also conducted in 11 clinical laboratories spanning four continents.

RESULTS For functional characterization, part 1, QCM VAFs for hybrid capture were similar to ddPCR for *EGFR* L858R but lower for ex19del. By contrast, hybrid capture results for *EGFR* L858R clinical samples showed a positive trend compared with ddPCR. For amplicon NGS, QCMs performed similarly to clinical samples for both variants. For part 2, observed hit rates approximated expected values. In the clinical pilot, median ex19del VAF was higher for Tag-seq than hybrid capture for both 1.0% and 0.5% QCM formulations. Median QCM L858R VAFs were similar for Tag-seq and hybrid capture, with greatest interlaboratory differences observed for Thermo Fisher Scientific QCMs. For non-*EGFR* variants, we observed assay and QCM-dependent trends, with no particular QCM or assay driving these trends.

CONCLUSION This project revealed unexpected differences in performance of both assays and QCMs. These findings highlight the need for further validation across diverse alteration types and merit consideration by laboratories that rely on QCMs to develop and perform ctDNA assays for diagnostic applications.

ACCOMPANYING CONTENT

 [Data Supplement](#)

 [Visual Abstract](#)

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INTRODUCTION

In patients with advanced cancer, circulating tumor DNA (ctDNA) is now being primarily used to identify clinically actionable alterations for treatment selection, with several novel applications being developed, including the determination of minimal residual disease,¹ therapy response monitoring,² emergence of resistance,³ and early detection of cancer.⁴ A primary challenge for ctDNA assay development for noninvasive genotyping is the acquisition of clinical

specimens in suitable quantity for analytical validation. Reference materials or quality control materials (QCMs) fulfill a critical need for validation studies such as limit of detection (LOD) because pools of variants can be formulated in large quantities at precise levels. Ideally, these QCMs would have performance characteristics similar to clinical samples.

These QCMs could help address the discordance that has been observed previously when comparing between different

CONTEXT

Key Objective

To evaluate the performance of commercially available quality control materials (QCMs) from three manufacturers by comparing them directly with clinical circulating tumor DNA (ctDNA) samples across multiple assay platforms from different vendors, addressing the need for reliable and scalable validation materials for noninvasive cancer genotyping using liquid biopsies.

Knowledge Generated

The study demonstrates that the herein described QCMs can closely approximate the performance of clinical ctDNA samples. This comparison revealed assay- and laboratory-specific sources of variability, emphasizing the utility of QCMs in rigorous analytical validation.

Relevance

As ctDNA testing becomes more widely adopted in clinical oncology, the scarcity and heterogeneity of clinical specimens present significant challenges to assay validation. This work supports the broader use of standardized reference materials for assay development, validation, and quality assurance, facilitating more robust and reproducible ctDNA testing across laboratories and platforms.

ctDNA assays.^{5,6} A joint review from the ASCO and the College of American Pathologists highlighted the need for a standardized set of reference materials to facilitate cross-assay comparisons of analytical validation studies.⁷ In recognition of this need, several groups, including ours,⁸ have characterized commercially available standards that could potentially be used by all laboratories.^{9–12} In the initial phase of our project, we worked with three manufacturers of reference materials to manufacture a set of QCMs having variants

shared by each, which we designated as the FNIH QCMs (Table 1).⁸ We confirmed fit for purpose of FNIH QCMs in four core laboratories using three next-generation sequencing (NGS)–targeted panels and two droplet digital polymerase chain reaction (ddPCR) assays.⁸

Here, we report on a 2-part functional characterization study comparing the performance of the FNIH QCMs to clinical samples both near the LOD and within the quantitative range

TABLE 1. FNIH QCM Variant List

Gene	Variant	Variant Type	COSMIC ID	Manufacturer
<i>AKT1</i>	E17K	SNV	33765	HD; SC; TFS
<i>ALK</i>	G1202R	SNV	144250	SC; TFS
<i>EML4-ALK</i>	EML4-ALKv1	Translocation	COSF463	SC
	EML4-ALKv3	Translocation	COSF474	HD; TFS
<i>BRAF</i>	V600E	SNV	476	HD; SC; TFS
<i>BRCA1</i>	K654fs*47	del fs	1383519	SC; TFS
<i>BRCA2</i>	R2645fs*3	del fs	1738242	SC; TFS
<i>EGFR</i>	L858R	SNV	6224	HD; SC; TFS
<i>EGFR</i>	T790M	SNV	6240	HD; SC; TFS
<i>EGFR</i>	E746_A750 del	del in frame	6223	SC; TFS
	E746_A750 del	del in frame	6225	HD
<i>ERBB2</i>	A775_G776insYVMA	ins in frame	20959	HD; SC; TFS
<i>ERBB2</i>	Amplification	CNV	NA	HD; SC; TFS
<i>KRAS</i>	G12D	SNV	521	HD; SC; TFS
<i>CD74-ROS1</i>	CD74-ROS1	Translocation	COSF1201	HD; SC; TFS
<i>PIK3CA</i>	H1047R	SNV	775	HD; SC; TFS

Abbreviations: CNV, copynumber variation; COSMIC, Catalogue of Somatic Mutations in Cancer; del, deletion; FNIH, Foundation for the National Institutes of Health; fs, frameshift; HD, Horizon Discovery; ID, identifier; ins, insertion; NA, not applicable; QCM, quality control material; SC, SeraCare; SNV, single-nucleotide variant; TFS, Thermo Fisher Scientific.

by ddPCR and NGS, with a focus on single-nucleotide variant (SNV) and insertion-deletion (indel) variant classes. We also report a real-world assessment of the FNIH QCMs by 11 clinical laboratories spanning four continents.

METHODS

Clinical samples were obtained from fully consented patients enrolled in TIGER-X (ClinicalTrials.gov identifier: [NCT01526928](#)). Variant allele frequencies (VAFs) were confirmed by ddPCR at the National Institute of Standards and Technology (NIST). QCMs were provided by Revvity Mimix (formerly Horizon Discovery; HD), Thermo Fisher Scientific (TFS), and LGC Clinical Diagnostics (SeraCare; SC), and were formulated as cell-free DNA (cfDNA) in buffer for functional characterization and in either healthy donor plasma (TFS) or synthetic plasma (HD, SC) for the clinical pilot. QIASymphony DSP Circulating DNA Kit (Qiagen, Germantown, MD) was used for sample extraction.

Functional Characterization, Part 1: Quantitative Range Comparison

Nineteen unique clinical samples were separately prepared for *EGFR* L858R and ex19del. *EGFR* ex19del samples were from four isoforms (COSM6223, 6225, 12370, and 12384). Samples were diluted in pooled healthy donor cfDNA to 0.5%–5.0% VAFs. Additionally, one unique sample for each variant type was serially diluted to 5%, 2.5%, 1%, and 0.5% VAF, and four replicates of each dilution were used for the serial dilution testing. Samples were distributed to Dana-Farber Cancer Institute (DFCI) for Genexus-Oncomine and ddPCR for *EGFR* L858R and ex19del,¹³ NIST for *EGFR* L858R and ex19del ddPCR, and the Molecular Characterization Laboratory at Frederick National Laboratory for Cancer Research (FNLRCR) for TruSight Oncology 500 (TSO500) ctDNA (Data Supplement).

Functional Characterization, Part 2: LOD Testing

Clinical samples were diluted in healthy donor cfDNA and QCMs in wild-type QCMs, provided by the respective QCM vendors, down to LOD₉₅ (approximately 0.25% VAF), LOD₇₀, LOD₅₀, and LOD₂₀. Samples were distributed to DFCI for Genexus-Oncomine and to NIST for *EGFR* L858R and ex19del ddPCR (Data Supplement).

Clinical Pilot

Anonymized cfDNA from *EGFR* L858R and *EGFR* ex19del clinical samples were diluted in healthy donor cfDNA to 0.5% or 1.0% VAF. QCM samples in healthy donor plasma (TFS) or synthetic plasma (HD, SC) were provided prediluted by the vendors to 0.5% or 1.0%. Ten replicates of each sample type were provided to all pilot sites. Pilot sites were blinded to sample type (QCM v clinical) and the VAF of the samples. Clinical pilot sites using ddPCR were notified of the ex19del isoform present in the QCMs and clinical samples to ensure

they used a relevant ddPCR assay. Each clinical pilot testing site used its respective extraction (for QCMs) protocols and assays (Data Supplement).

RESULTS

Functional Characterization Study Overview

The aim of the functional characterization study was to determine how similarly the QCMs perform relative to clinical samples. We evaluated *EGFR* L858R and *EGFR* ex19del as representatives of SNVs and indels, respectively, and conducted the study in two parts. We first compared QCMs with clinical samples from 0.5% to 5.0% VAF by TSO500 ctDNA, a hybrid capture NGS assay, Genexus-Oncomine, a targeted-amplicon sequencing (Tag-seq) assay, and ddPCR. Informed by these data, we chose Genexus-Oncomine for a second functional characterization study near the LOD.

Functional Characterization From 0.5%–5.0% VAFs

Results for *EGFR* L858R are shown in [Figure 1A](#) and the Data Supplement (Table S1). For TSO500, VAFs were similar to ddPCR for HD and SC QCMs, with TFS QCM VAFs trending slightly lower compared with ddPCR. By contrast, for the clinical sample dilution series, VAFs as determined by TSO500 were reproducibly higher than ddPCR. For Genexus-Oncomine, the clinical sample dilution series performed more similarly to the QCM dilution series, with TFS QCMs having the greatest similarity.

Results for *EGFR* ex19del are shown in [Figure 1B](#) and the Data Supplement (Table S2). For TSO500, VAFs for the QCMs were all lower than observed by ddPCR. By contrast, a positive trend was observed for the clinical sample dilution series, where VAFs as determined by TSO500 were reproducibly higher than for ddPCR. For Genexus-Oncomine, the clinical sample dilution series of *EGFR* ex19del performed similarly to the QCM dilution series for the HD and SC QCMs. Although a slight negative trend was seen in the TFS QCM dilution series compared with the clinical sample dilution series, ddPCR and Genexus-Oncomine data showed greatest similarity for TFS QCMs. The trends observed for both the *EGFR* L858R and ex19del dilution series were confirmed in a separate experiment, in which a total of 38 clinical samples harboring *EGFR* L858R and ex19del variants between 0.5% and 5.0% VAFs were tested (Data Supplement, Fig S1).

We further analyzed the size distribution of sequenced mutant and wild-type fragments from samples in the functional characterization study on an exploratory basis (Data Supplement, Figs S2, S3, and S4). No gross differences between clinical samples and QCMs were identified, which might have explained some of the trends observed in the functional characterization study (eg, mutant fragments of a single size in some materials v mutant fragments having a distribution of sizes in clinical samples).

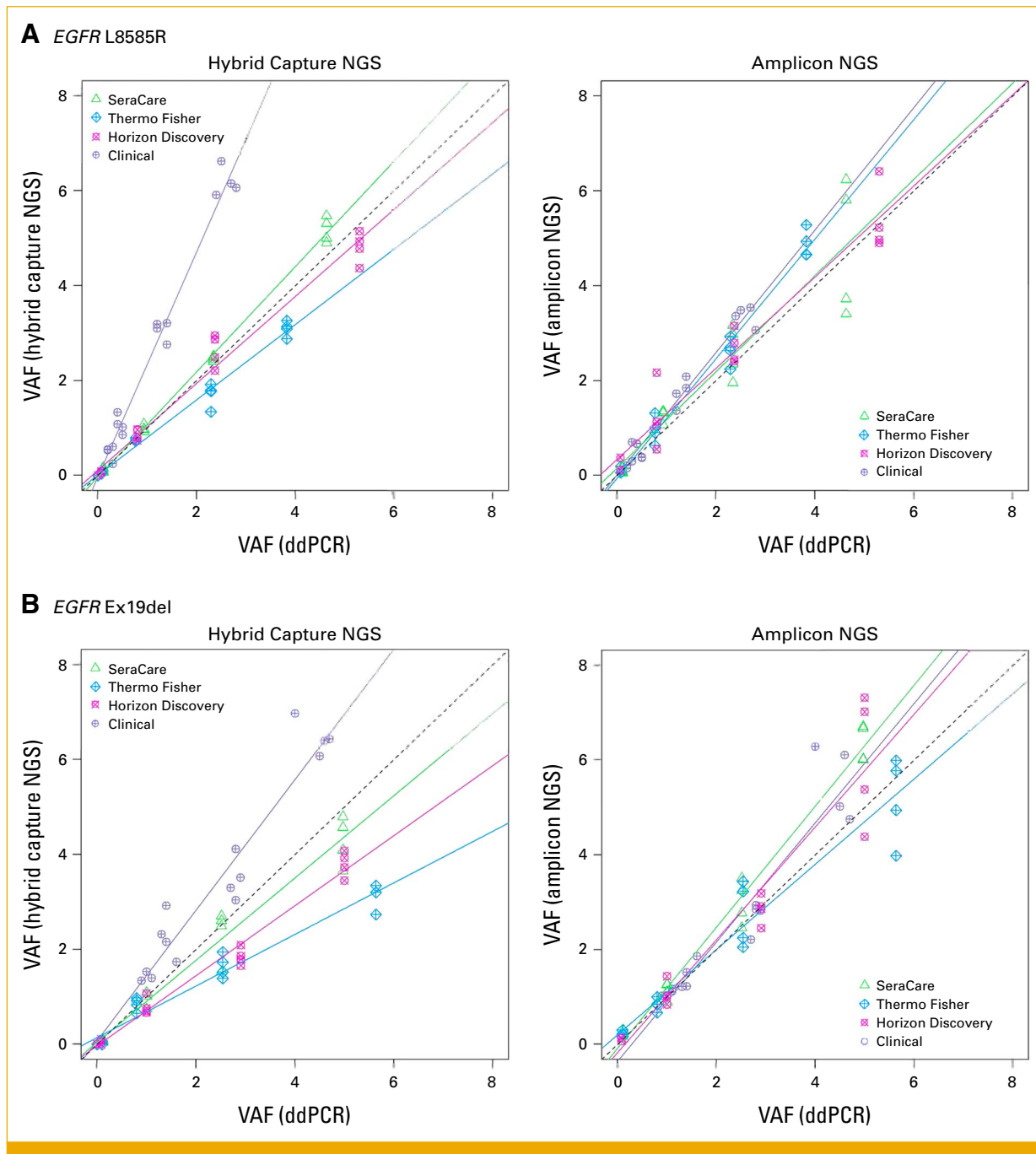


FIG 1. Functional characterization from 0.5% to 5.0% VAFs. (A) Results for *EGFR* L858R, the representative SNV profiled in the study. (B) Results for *EGFR* ex19del, the representative indel. The dashed black line in each graph represents the identity line where ddPCR and NGS VAFs are equal. Clinical in each graph legend refers to a dilution series made from a single clinical sample in healthy donor cfDNA. cfDNA, cell-free DNA; ddPCR, droplet digital polymerase chain reaction; indel, insertion/deletion; NGS, next-generation sequencing; SNV, single-nucleotide variant; Thermo Fisher, Thermo Fisher Scientific; VAF, variant allele fraction.

Functional Characterization Near Assay LOD

On the basis of the similarity in performance between ddPCR and Tag-seq, we chose Genexus-Oncomine to further characterize the QCMs near the assay LOD (Data Supplement). For the LOD assessment, we made four dilutions, starting at approximately 0.25% VAF (LOD95, dilution A),

the presumed LOD95 for the assay, and extending down to what we expected would be LOD70 (dilution B), LOD50 (dilution C), and LOD20 (dilution D) for each clinical sample and the QCMs. LOD95 represents the highest VAF at which at least 95% of replicates tested are expected to be detected. The hit rates are shown in Figure 2A and the Data Supplement (Table S3) for *EGFR* L858R. Probit analyses of the data

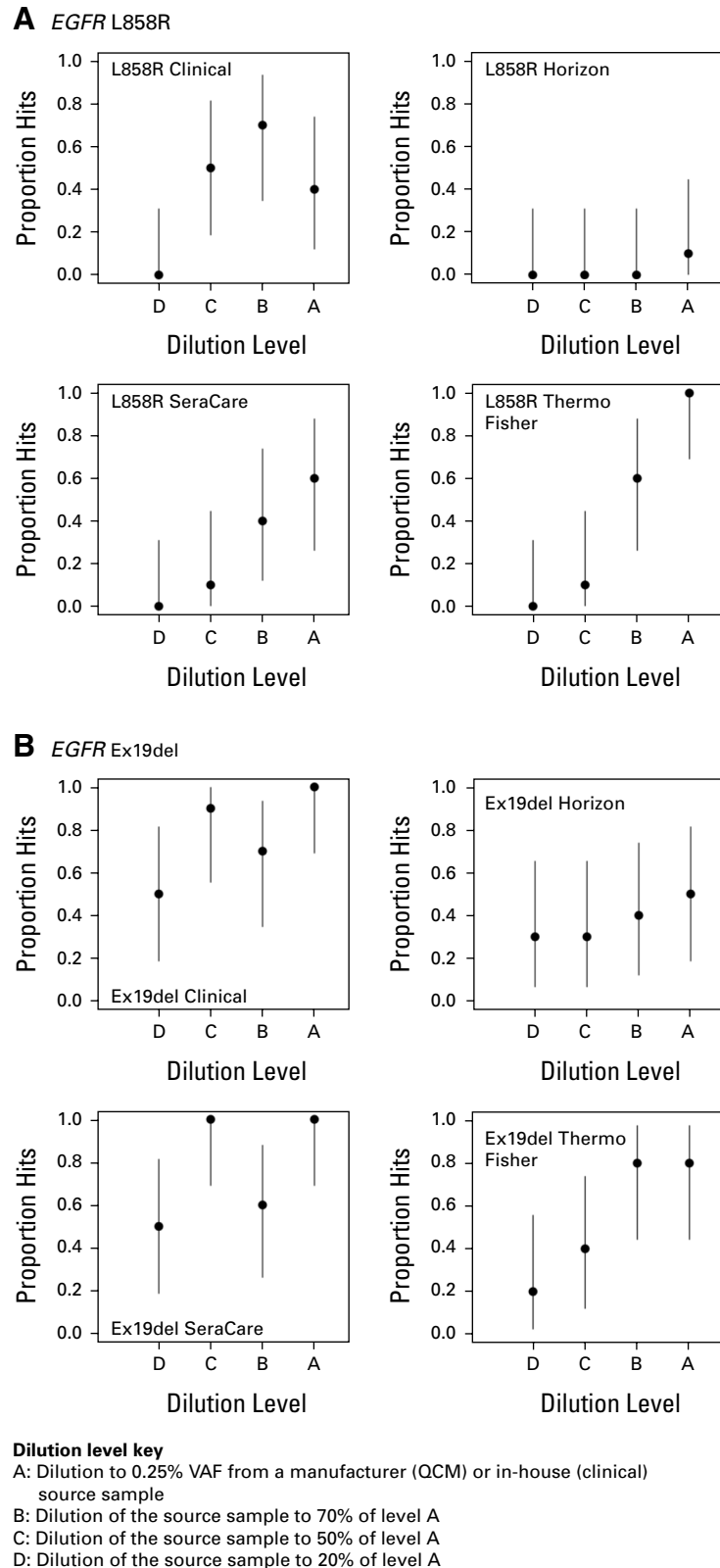


FIG 2. Functional characterization near assay LOD. (A) Dilution series hit rates for *EGFR* L858R. (B) Dilution series hit rates for *EGFR* Ex19del. Results were generated with the amplicon NGS platform. In each graph, points represent the proportion of 10 replicates with tested variant reported by the assay data analysis pipeline. Vertical bars represent Clopper-Pearson two-sided 95% CIs. The starting dilution, dilution A, was prepared at 0.25%, (continued on following page)

FIG 2. (Continued). the presumed LOD95 of the amplicon NGS assay. Horizon, Horizon Discovery; LOD, limit of detection; NGS, next-generation sequencing; QCM, quality control material; Thermo Fisher, Thermo Fisher Scientific; VAF, variant allele fraction.

are shown in the Data Supplement (Fig S5). Notably, the HD L858R hit rate at dilution A was 10% (95% CI, 0% to 45%), which was much lower than expected. Additionally, the dilution A hit rate for the L858R clinical sample did not trend with the other dilutions in the series. A separate reformulation of dilution A, not presented in Figure 2A, showed close agreement with the expected higher hit rate (data not shown). This strongly suggests a formulation error was responsible for the lower-than-expected hit rate seen with the original formulation of dilution A.

Hit rates for *EGFR* ex19del are shown in Figure 2B and the Data Supplement (Table S3), and probit analyses of the data are shown in the Data Supplement (Fig S6). Notably, the dilution C hit rate for both the ex19del clinical sample and SC QCMs did not trend with the other dilutions in their respective series. To gain insight into the SC ex19del results, we looked at hit rates for other variants in the SC QCM pool and observed that all but one variant had progressively lower hit rates with each successive dilution, as expected (Data Supplement, Fig S7). We therefore believe the higher-than-expected hit rate for SC dilution C, and likely for dilution C of the clinical sample, to be a chance outcome associated with the wide CI of testing only 10 replicates at each dilution.

A Real-World Clinical Pilot of the FNIH QCMs

To understand the real-world applicability of the QCMs, we enlisted 11 laboratories across four continents to compare QCM performance with clinical samples across a variety of assays. Among these, three used targeted hybrid capture NGS, five used ddPCR, and three used Tag-seq (assays 1-12; Data Supplement, Fig S8). Seven laboratories were affiliated with commercial diagnostic companies and four were within academic institutions. Laboratories were asked to report on any of the 14 variants present in the QCMs and given the opportunity to analyze contrived clinical specimens containing *EGFR* L858R or ex19del at VAF of 1.0% or 0.5% (Data Supplement, Table S4). Each laboratory was provided enough material to run 10 replicates each.

We first sought to characterize the variability of *EGFR* L858R and *EGFR* ex19del between QCMs and clinical specimens. For *EGFR* L858R ddPCR, VAF tracked similarly for the QCMs and clinical materials, with the median VAF for clinical samples reported as 0.53% (n = 58; IQR, 0.38-0.69) and 1.06% (n = 60; IQR, 0.71-1.35) for 0.5% and 1.0% formulations, respectively. This compared with the reported median *EGFR* L858R ddPCR VAF for QCMs formulated by HD of 0.55% (n = 13; IQR, 0.49-0.64) and 1.1% (n = 14; IQR, 0.67-1.15), for

SC of 0.6% (n = 18; IQR, 0.46-0.69) and 1.22% (n = 17; IQR, 1.0-1.28), and for TFS of 0.51% (n = 25; IQR, 0.36-0.7) and 1.0% (n = 26; IQR, 0.65-1.2; Fig 3; Data Supplement, Tables S5 and S6).

The variability in the ddPCR cohort is largely driven by one assay (assay 10). Similarly, the median VAF and IQR for Tag-seq were 0.51% (n = 9; IQR, 0.39-0.55) and 0.85% (n = 10; IQR, 0.71-1.00) for clinical samples, 0.39% (n = 11; IQR, 0.33-0.51) and 0.89% (n = 11; IQR, 0.67-0.98) for HD QCMs, 0.47% (n = 13; IQR, 0.41-0.59) and 0.76% (n = 13; IQR, 0.67-0.83) for SC QCMs, and 1.12% (n = 14; IQR, 0.49-1.67) and 1.93% (n = 14; IQR, 1.03-2.25) for TFS, at 0.5% and 1.0%, respectively. Of note, the variability observed in the TFS specimens was driven solely by assay 9, which reported VAF roughly twice as high. For hybrid capture NGS, median VAF and IQR were 0.61% (n = 15; IQR, 0.49-0.66) and 0.85% (n = 13; IQR, 0.71-1.00) for clinical samples, 0.47% (n = 30; IQR, 0.40-0.54) and 1.10% (n = 30; IQR, 1.00-1.35) for HD QCMs, 0.59% (n = 30; IQR, 0.52-0.67) and 1.05% (n = 30; IQR, 0.89-1.10) for SC QCMs, and 0.76% (n = 21; IQR, 0.59-0.89) and 1.56% (n = 20; IQR, 1.30-1.77) for TFS, at 0.5% and 1.0%, respectively (Data Supplement, Tables S5 and S6).

Similar to our *EGFR* L858R observations, VAF tracked similarly between QCMs and clinical materials for *EGFR* ex19del (Fig 3; Data Supplement, Tables S5 and S6), with the only assay-dependent variations for SC-formulated QCMs (n = 28, median VAF 1.05%, IQR 0.65-2.13; n = 30, median VAF 1.76%, IQR 1.33-2.32, at 0.5% and 1.0%) contributed by assay 10 (ddPCR) and for TFS QCMs contributed by assay 9 (Tag-seq; n = 14, median VAF, 1.06%, IQR, 0.45-1.56; n = 14, median VAF, 2.75%, IQR, 1.06-3.34, at 0.5% and 1.0%). Of note, a negative QCM-dependent trend was observed for HD QCMs (n = 26, median VAF, 0.36%, IQR, 0.30-0.46; n = 30, median VAF, 0.65, IQR, 0.42-0.90, at 0.5% and 1.0%) for all hybrid capture NGS assays (Fig 3). Assay 1 did not detect either *EGFR* alteration at any VAF in the TFS QCMs. Notably, all laboratories used an allele-specific assay targeting the specific *EGFR* ex19del present in the QCMs. One laboratory additionally used an *EGFR* drop-off assay in conjunction with the allele-specific assay, but only for clinical samples. No differences in performance were observed between the drop-off and allele-specific assays.

We next explored the variance of non-*EGFR*-activating alterations. Of the 3,523 results, 483 were by ddPCR (SNVs only), 509 by Tag-seq (493 SNVs and 16 insertions), and 1,355 by hybrid capture NGS (919 SNVs, 120 insertions, 40

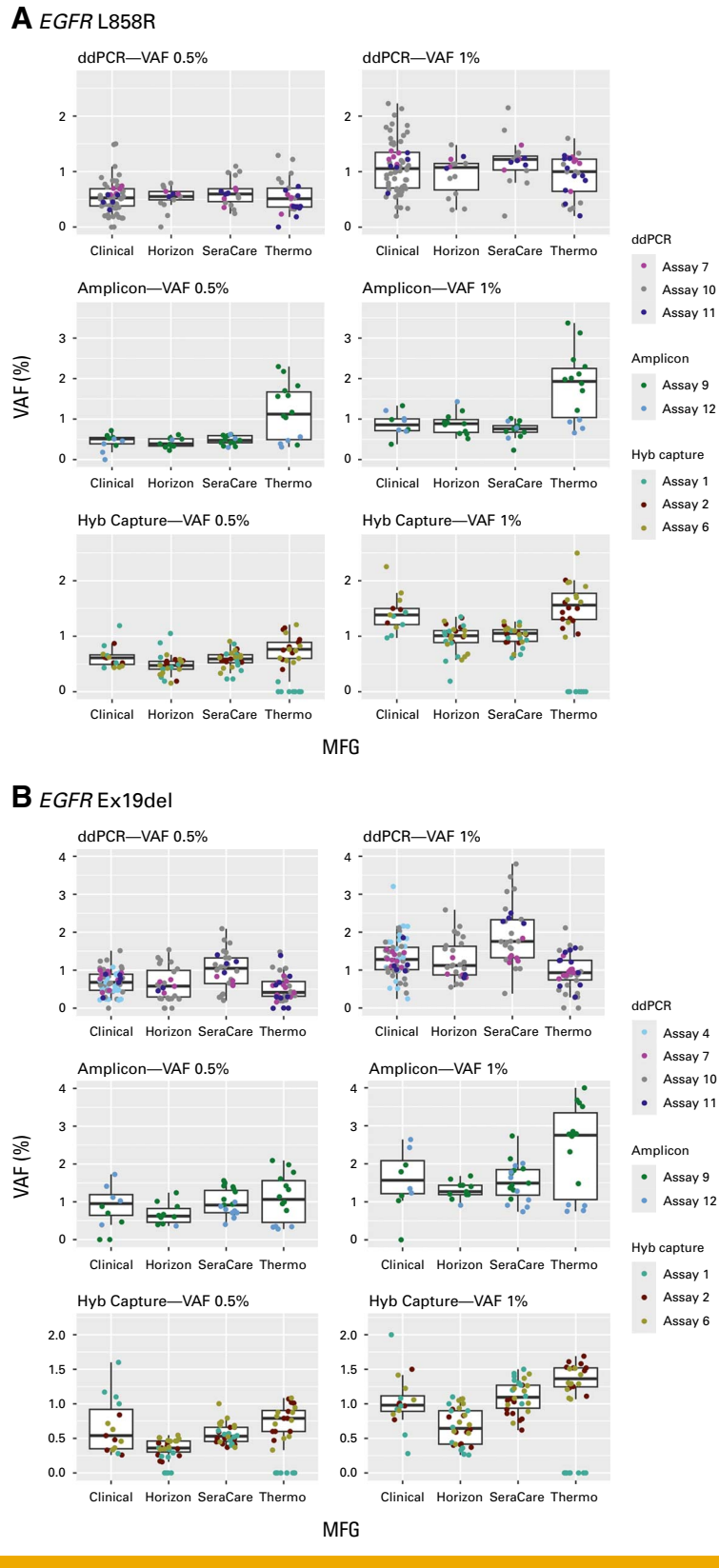


FIG 3. Clinical pilot study results for *EGFR* L858R and ex19del. (A) *EGFR* L858R results for clinical sample and QCMs. (B) *EGFR* ex19del results for clinical sample and QCMs. Results for the eight evaluable assays among 11 laboratory clinical pilot participants are shown. QCMs were formulated at expected VAFs of 0.5% and 1.0%. In each graph, boxes represent the IQR of (continued on following page)

FIG 3. (Continued). the distribution of reported variants from 25% to 75%. The horizontal line within each box represents the median. Vertical lines represent data points outside the IQR but $<1.5 \times$ IQR. ddPCR, droplet digital polymerase chain reaction; Horizon, Horizon Discovery; Hyb, hybrid; MFG, manufacturer; QCM, quality control material; Thermo, Thermo Fisher Scientific; VAF, variant allele fraction.

frameshifts, 216 translocations, and 60 CNVs; Data Supplement, Fig S8). As with *EGFR* L858R and ex19del, assay 1 detected none of the TFS QCMs at either VAF. Overall, we observed assay- and QCM-dependent trends across the data, with no particular QCM or assay being the sole source for the variance (Figs 4A and 4B). Notably, all assays reported *BRAF* V600E for HD at twice the expected VAF, and all assays reported *KRAS* G12D for TFS at half the expected VAF. *ERBB2* (p.A775_G776ins YVMA) was reported at twice the VAF by hybrid capture NGS assays for TFS (Assay 6) and HD (assays 1 and 6), but not for SC-manufactured QCMs (Fig 4; Data Supplement, Table S6). Clear positive trends were observed for assay 6 for *ROS* and *ALK* translocations for both SC and HD QCMs. Translocations in the TFS materials were not assessed (Fig 4C).

DISCUSSION

A European Society for Medical Oncology working group recently concluded that sufficient evidence now exists to support the use of ctDNA assays in routine clinical practice to direct patients with late-stage disease to molecularly targeted therapies.¹⁴ With that in mind, the FNIH Biomarkers Consortium set out to develop a source of well-vetted QCMs that can be used for the rapid development and analytical validation of ctDNA assays for therapy selection in patients with advanced cancer.

In our phase 1 study, we characterized QCMs for cfDNA from three manufacturers in four core laboratories.⁸ Herein, we extended our characterization of these materials by comparing them with clinical samples in both a functional characterization study and a real-world clinical pilot. Many aspects of the functional characterization study design were modeled after Clinical and Laboratory Standards Institute guidelines for functional characterization.¹⁵

An important overall finding was that we observed significant discrepancies in the performance of different assay platforms on both clinical specimens and the QCMs. For example, in the functional characterization study, we observed higher VAFs in clinical samples bearing *EGFR* ex19del and L858R mutations by hybrid capture NGS compared with ddPCR. Interestingly, this trend seemed unique to our hybrid capture assay because in the clinical pilot, the performance of the three hybrid capture NGS assays on clinical samples harboring those same mutations was more similar to ddPCR. Future studies should address the root cause of these differences and at which step(s) of the workflow of our hybrid capture NGS assay they may have

been introduced (eg, library preparation or bioinformatics analysis).

Notably, some trends seen in the functional characterization study were also observed in clinical pilot laboratories. For example, our hybrid capture NGS assay tended to undercall *EGFR* ex19del mutations in the QCMs compared with ddPCR and amplicon NGS, a trend reproduced in the clinical pilot. We postulate this finding may be related to the size-dependent higher amplification efficiency that is achieved with amplicon-based tests such as ddPCR or amplicon NGS on deletion-bearing fragments compared with matched wild-type from the same sample.

A strength of our study was that we tested our QCMs in real-world laboratories and observed unexpected laboratory-specific differences in assay performance. In a notable example, the two amplicon NGS assays consistently identified the same variants in the TFS QCMs at different VAFs. Our analysis of the sizing profile of sequenced mutant and wild-type fragments identified no obvious difference between the TFS QCMs and QCMs from the other manufacturers, which might have explained this discrepancy. Instead, we postulate that differences in primer design and amplicon size may be responsible.

One surprising finding of the clinical pilot was the heterogeneity and variance between laboratories, assays, and QCMs. Although some variance could clearly be attributed to a specific assay (eg, assay 9 for *EGFR* L858R), other trends were QCM-specific (eg, *BRAF* V600E for HD), while others still were unique to an assay and a particular QCM (eg, assay 1 detected no variants for any TFS QCMs). These observations suggest that QCMs are not yet identical to cfDNA isolated from plasma and that laboratories should not rely solely on these materials for assay development.

Other groups have reported on reference materials for cfDNA.^{9,10,12} As part of the Blood Profiling Atlas in Cancer (BLOODPAC) project, Hernandez et al¹⁶ characterized reference materials developed by SC and HD, two of the same vendors described herein. The authors concluded that their materials performed similarly when tested at nine independent BLOODPAC laboratories by ddPCR. Similarly, He et al¹⁰ evaluated a reference set from SC that contained 40 genetic alterations and showed consistent quantification across laboratories, with reliable detection down to 0.125% VAF. Importantly, He et al mainly focused on analytical validation of the assays, while we focused on fit for purpose of the QCMs.

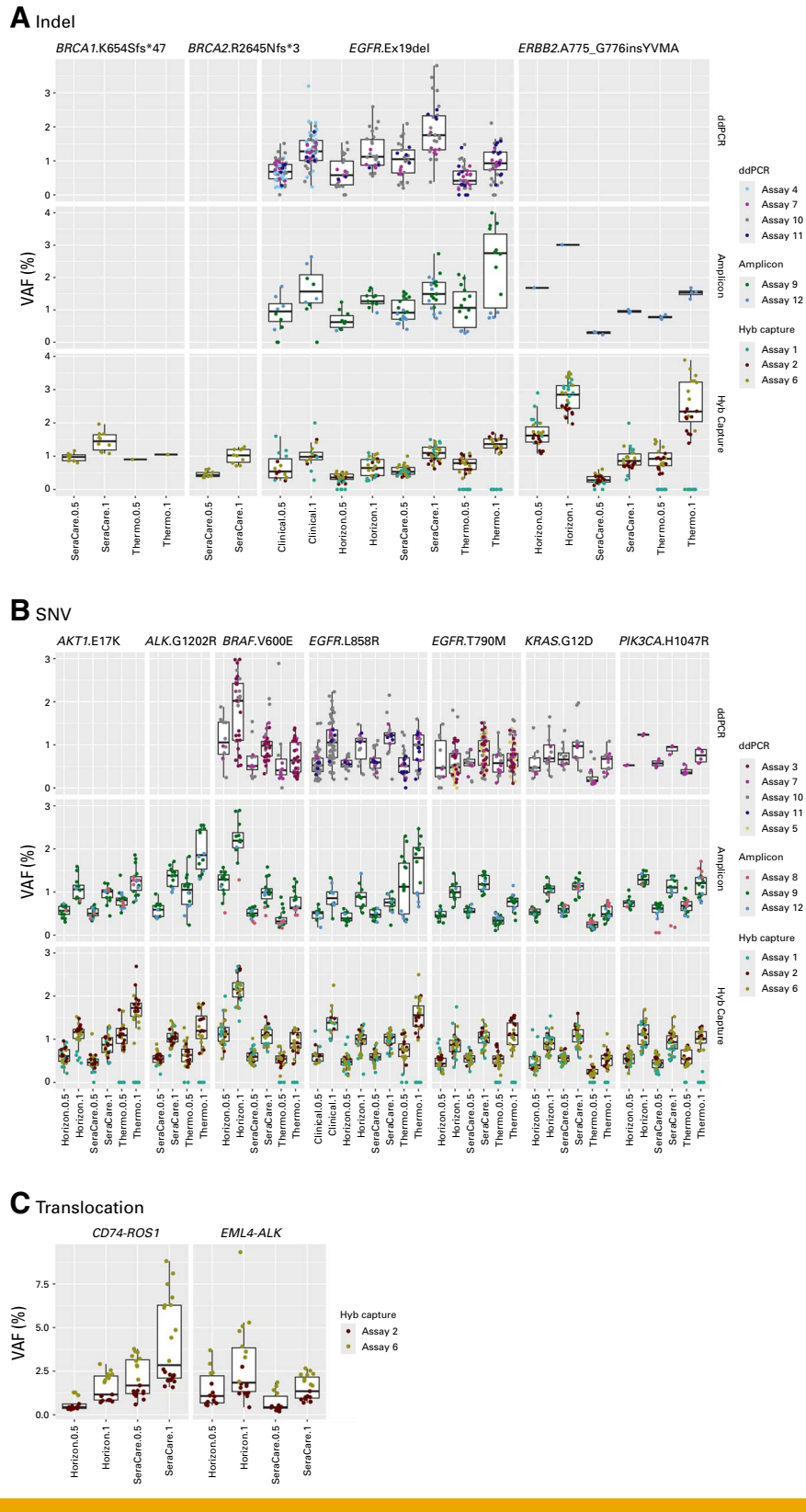


FIG 4. Clinical pilot study results for all variants assessed. (A) Indels reported by eight laboratories that tested at least one indel. Assay 4 was an *EGFR* drop-off assay, whereas assays 7, 10, and 11 were allele-specific. (B) SNVs reported by 11 laboratories that tested at least 1 SNV. (C) Fusions reported by the two laboratories that reported fusion results. QCMs were formulated at expected VAFs of 0.5% and 1.0%. In each graph, (continued on following page)

FIG 4. (Continued). boxes represent the IQR of the distribution of reported variants from 25% to 75%. The horizontal line within each box represents the median. Vertical lines represent data points outside the IQR but $< 1.5 \times$ IQR. ddPCR, droplet digital polymerase chain reaction; Horizon, Horizon Discovery; Hyb, hybrid; indel, insertion/deletion; QCM, quality control material; SNV, single-nucleotide variant; Thermo, Thermo Fisher Scientific; VAF, variant allele fraction.

Our study had several limitations. Challenges in sourcing CNVs and translocations in sufficient quantities limited our functional characterization study to SNVs and indels, the two most prevalent variant types observed in cancer genomes. By focusing our evaluation on *EGFR* L858R and *EGFR* ex19del, we did not comprehensively evaluate performance in homopolymer regions and other challenging genomic contexts. Additionally, because of resource limitations, we only tested 10 replicates in the functional characterization LOD study. This led to wider CIs in the hit rates and at least two instances of an aberrant result that was likely due to chance. We strongly recommend that future LOD studies be

performed with a substantially larger number of replicates (eg, 20).⁹

Going forward, we believe the major stakeholders in the clinical application of liquid biopsies—manufacturers, laboratorians, clinicians, regulators, and payers—should develop a consensus approach for the development, validation, and implementation of QCMs for cfDNA. Such an approach could include the materials characterized herein. Indeed, a standard set(s) of well-vetted QCMs adopted for use by all laboratories would greatly facilitate cross-laboratory comparisons and regulatory review.

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DISCLAIMER

Certain commercial equipment, instruments, and materials are identified to specify the experimental procedure. In no case does such identification imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment are necessarily the best available for the purpose.

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REFERENCES

1. Tie J, Wang Y, Tomasetti C, et al: Circulating tumor DNA analysis detects minimal residual disease and predicts recurrence in patients with stage II Colon cancer. *Sci Transl Med* 8:346ra92, 2016
2. Vega DM, Nishimura KK, Zariffa N, et al: Changes in circulating tumor DNA reflect clinical benefit across multiple studies of patients with non-small-cell lung cancer treated with immune checkpoint inhibitors. *JCO Precis Oncol* 10.1200/PO.21.00372
3. Thress KS, Paweletz CP, Felip E, et al: Acquired *EGFR* C797S mutation mediates resistance to AZD9291 in non-small cell lung cancer harboring *EGFR* T790M. *Nat Med* 21:560-562, 2015
4. Liu MC, Oxnard GR, Klein EA, et al: Sensitive and specific multi-cancer detection and localization using methylation signatures in cell-free DNA. *Ann Oncol* 31:745-759, 2020
5. Stetson D, Ahmed A, Xu X, et al: Orthogonal comparison of four plasma NGS tests with tumor suggests technical factors are a major source of assay discordance. *JCO Precis Oncol* 10.1200/PO.18.00191
6. Torga G, Pienta KJ: Patient-paired sample congruence between 2 commercial liquid biopsy tests. *JAMA Oncol* 4:868-870, 2018
7. Merker JD, Oxnard GR, Compton C, et al: Circulating tumor DNA analysis in patients with cancer: American Society of Clinical Oncology and College of American Pathologists joint review. *J Clin Oncol* 36:1631-1641, 2018
8. Williams PM, Forbes T, Lund SP, et al: Validation of ctDNA quality control materials through a precompetitive collaboration of the Foundation for the National Institutes of Health. *JCO Precis Oncol* 10.1200/PO.20.00528
9. Godsey JH, Silvestro A, Barrett JC, 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* 66:1156-1166, 2020
10. He HJ, Stein EV, Konigshofer Y, et al: Multilaboratory assessment of a new reference material for quality assurance of cell-free tumor DNA measurements. *J Mol Diagn* 21:658-676, 2019
11. Lindeman NI, Cagle PT, Aisner DL, et al: Updated molecular testing guideline for the selection of lung cancer patients for treatment with targeted tyrosine kinase inhibitors: Guideline from the College of American Pathologists, the International Association for the Study of Lung Cancer, and the Association for Molecular Pathology. *Arch Pathol Lab Med* 142:321-346, 2018
12. Deveson IW, Gong B, Lai K, et al: Evaluating the analytical validity of circulating tumor DNA sequencing assays for precision oncology. *Nat Biotechnol* 39:1115-1128, 2021
13. Oxnard GR, Paweletz CP, Kuang Y, et al: Noninvasive detection of response and resistance in *EGFR*-mutant lung cancer using quantitative next-generation genotyping of cell-free plasma DNA. *Clin Cancer Res* 20:1698-1705, 2014
14. Pascual J, Attard G, Bidard FC, et al: ESMO recommendations on the use of circulating tumour DNA assays for patients with cancer: A report from the ESMO Precision Medicine Working Group. *Ann Oncol* 33:750-768, 2022
15. Vore KD, Agrawal Y, Alspach TD, et al: Evaluation of Commutability of Processed Samples; Approved Guideline—Third Edition. CLSI document EP14-A3. Wayne, PA, Clinical and Laboratory Standards Institute, 2014
16. Hernandez KM, Bramlett KS, Agius P, et al: Contrived materials and a data set for the evaluation of liquid biopsy tests: A Blood Profiling Atlas in Cancer (BLOODPAC) community study. *J Mol Diagn* 25:143-155, 2023

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Alexander was renowned for his pioneering work in personalized medicine and for developing innovative diagnostic methodologies, including tests for detecting *KRAS*, *BRAF*, and *TP53* mutations. He was the head of the Molecular Pathology Laboratory at the Peter MacCallum Cancer Centre and led the Translational Genomics and Epigenomics Laboratory at the Olivia Newton-John Cancer Research Institute. A passionate scientist, visionary leader, and tireless advocate for advancing public health, Dr Caroline Sigman founded CCS Associates in 1985 with a singular mission: to help others achieve the breakthroughs that could cure some of society's most intractable health challenges, particularly cancer. She was a pioneer in the development of biomarkers and among the first to recognize their transformative potential in identifying disease earlier and intervening more effectively. Her leadership helped shape the future of biomarker science and expanded access to powerful tools for disease prevention and treatment. To those who had the privilege of working with her, Dr Sigman was more than a leader—she was an inspiration.

Coauthor Susan Keating, PhD, died May 8, 2025.

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