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Development and Clinical Utility of a Blood-Based Test Service for the Rapid Identification of Actionable Mutations in Non–Small Cell Lung Carcinoma

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Nearly 80% of cancer patients do not have genetic mutation results available at initial oncology consultation; up to 25% of patients begin treatment before receiving their results. These factors hinder the ability to pursue optimal treatment strategies. This study validates a blood-based genome-testing service that provides accurate results within 72 hours. We focused on targetable variants in advanced non–small cell lung carcinoma—epidermal growth factor receptor gene (*EGFR*) variant L858R, exon 19 deletion (ΔE746–A750), and T790M; GTPase Kirsten ras gene (*KRAS*) variants G12C/D/V; and echinoderm microtubule associated protein like and 4 anaplastic lymphoma receptor tyrosine kinase fusion (*EML4-ALK*) transcripts 1/2/3. Test development included method and clinical validation using samples from donors with ($n = 219$) or without ($n = 30$) cancer. Clinical sensitivity and specificity for each variant ranged from 78.6% to 100% and 94.2% to 100%, respectively. We also report on 1643 non–small cell lung carcinoma samples processed in our CLIA-certified laboratory. Mutation results were available within 72 hours for 94% of the tests evaluated. We detected 10.5% mutations for EGFR sensitizing ($n = 2801$ samples tested), 13.8% mutations for EGFR resistance ($n = 1055$), 13.2% mutations in *KRAS* ($n = 3477$), and 2% mutations for *EML4-ALK* fusion ($n = 304$). This rapid, highly sensitive, and actionable blood-based assay service expands testing options and supports faster treatment decisions. *(J Mol Diagn 2017, 19: 404–416; http://dx.doi.org/10.1016/j.jmoldx.2016.11.004)*

Somatic variants, including rearrangements, point mutations, and indels, are critical genetic alterations that influence malignant transformation and ultimately may result in disease progression. The clinical significance and importance of aberrations in epidermal growth factor receptor (EGFR), Kirsten ras (KRas), and anaplastic lymphoma receptor tyrosine kinase (ALK) have been previously reported.[1–5] The identification of the driver genomic alterations (oncogenic drivers) and targeting those specific alterations with therapy are critical aspects of today's approach to the management of cancer.[6,7]

The standard approach to the identification of actionable variants in patients with non–small cell lung carcinoma (NSCLC) is analysis of a tissue biopsy sample. The US Food and Drug Administration has approved formalin-fixed, paraffin-embedded–based tests for the detection of *EGFR* mutations [the Cobas (Roche, Basel, Switzerland) and Therascreen (Qiagen, Valencia, CA) kits], *KRAS* mutations (Therascreen), and *ALK* rearrangements (Vysis ALK Break Apart FISH Probe Kit; Abbott Diagnostics, Lake Forest, IL), and for ALK immunohistochemistry testing (clone D5F3; Ventana/Roche, Tucson, AZ). However, there are

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limitations of tissue-based mutation testing. For instance, approximately one fourth of patients with NSCLC are either not candidates for biopsy or have insufficient tissue samples recovered from the initial biopsy.[8,9] This can limit the treating physicians' ability to fully diagnose the cancer genotype. Additionally, tissue biopsies may be accompanied by discomfort in the patient and inherent clinical risks, including bleeding in the lungs, infection, and pneumothorax. A tissue biopsy sample may also not be representative of the total load and spectrum of mutated cells, especially in patients with advanced disease stages and metastases. Genetic changes can occur in the interval between removal of the initial biopsy sample and the start of the specific targeted treatment, especially in patients who are first treated with conventional chemotherapy or radiotherapy; however, surgical complications and economic considerations make multiple or serial tissue biopsies impractical in most cases.[1,10] Importantly as well, results from tissue-based testing can take weeks to obtain and can delay time to treatment.[7,11]

Prior studies have demonstrated that circulating nucleic acids of cellular origin from normal and cancer cells can be isolated from blood.[11–14] Importantly, genomic information from a patient's tumor can be obtained within days of a blood draw. Thus, tracking tumor-associated genetic aberrations in the blood can be used for quickly and noninvasively determining whether targeted therapies are a treatment option; assessing the presence of residual disease, recurrence, or relapse; and detecting the emergence of therapy-resistant cancer cells more quickly than conventional tissue-based methods.[14–16]

The EGFR gene (*EGFR*) is mutated in an estimated 10% to 40% of patients with NSCLC.[17] Approximately 90% of these *EGFR* mutations occur in either exon 19 from E746-A750 or as an amino acid substitution in exon 21 at codon 858 (L858R),[18] both of which confer sensitivity to the EGFR tyrosine kinase inhibitors gefitinib, erlotinib, and afatinib. The *EGFR* T790M mutation is the most commonly recognized mechanism of drug resistance to these first-line EGFR tyrosine kinase inhibitors, accounting for nearly 50% of the acquired resistance.[19,20] T790M, initially considered as relevant only as a resistance marker, is also now actionable with the regulatory approvals of osimertinib for use in *EGFR* T790M mutation–positive NSCLC cases.

The ALK gene (*ALK*) encodes a kinase that promotes cellular proliferation; genomic rearrangements in *ALK* can drive aberrant proliferation and tumorigenesis in a number of cancers. The most prevalent rearrangement involving the *ALK* locus in NSCLC is a chromosome-2 inversion that leads to juxtaposition of the associated echinoderm microtubule associated protein like 4 gene (*EML4*), resulting in the *EML4-ALK* transcript variant 1 (E13:A20), variant 2 (E20:A20), and variant 3 (E6:A20).[21,22] Patients with *ALK* rearrangements have generally mutually exclusive mutations in *EGFR* and *KRAS* and do not respond to EGFR tyrosine kinase inhibitors.[23] In this event though, there are targeted therapies for ALK-positive tumors, including crizotinib, ceritinib, and alectinib.

KRAS variants account for approximately 15% to 25% of the driver mutations found in patients with NSCLC.[17,24] The *KRAS* G12C, G12V, and G12D mutations, for which we developed assays in this study, are the most prevalent and together account for approximately 76% of all *KRAS* mutations.[24] Importantly, *KRAS* encodes a GTPase protein that functions downstream of EGFR; thus, tumors that contain *KRAS* driver mutations are nonresponsive to EGFR tyrosine kinase inhibitors.[6,25] Additionally, the detection of a *KRAS* mutation in a liquid biopsy sample can serve as an indicator that the quantity and quality of collected tumor circulating cell-free (cf) DNA are sufficient for identifying a cancer-specific driver mutation. Since oncogenic drivers are typically mutually exclusive,[26] the detection of a *KRAS* mutation can increase confidence that another mutation has not been missed. For these reasons, *KRAS* mutation testing is commonly performed even in the absence of a currently approved targeted agent directed at specific *KRAS* variants in NSCLC.

We used Droplet Digital (dd) PCR (Bio-Rad, Pleasanton, CA), which was optimized for rare mutation detection from blood. ddPCR is a highly sensitive gene-mutation detection method that is based on the partitioning of DNA into droplets.[27] There are now several ddPCR assays that have been developed for various common and rare cancer mutations; these assays have demonstrated utility in the clinical setting.[11,14,28,29] Our assays were developed using several specimen types and included synthetic DNA oligonucleotides, cell line (tumor)-derived RNA and DNA, cell line materials spiked into normal plasma, as well as circulating nucleic acids isolated from whole-blood samples from donors with or without cancer. In the *EGFR* and *KRAS* rare mutation–detection tests, we measured the presence of DNA somatic variants and the relevant wild-type (WT) sequences in dual-detection assays. In the case of the *EML4-ALK* gene expression test, cDNA copied from the plasma circulating RNA was measured. Here, the fusion transcript assays and reference glucuronidase-ß (control) gene (*GUSB*) assay were multiplexed to perform *EML4-ALK* testing. We report on the results from technical feasibility and verification and validation studies using the aforementioned reference and donor specimens. A schematic outline of these studies is shown in Supplemental Figure S1. We further detail analyses of >1600 clinical cases tested using these assays after the assays were made available through our CLIA-certified laboratory.

We have developed a highly sensitive and robust blood-based assay to identify EGFR-sensitizing mutations (L858R and ΔE746–A750), the EGFR-resistance mutation (T790M), *KRAS* G12C/D/V in circulating DNA, and *EML4-ALK* variants 1/2/3 in circulating RNA. Our results indicate that, in clinical practice, circulating nucleic acids can be used as a rapid and reliable indicator of positive mutation status of a tumor in the absence of tissue.

Table 1 Clinical Validation Sample Sets

Mutation	Total eligible positive samples	Total eligible negative samples
EGFR ΔE746-A750	30	62
EGFR L858R	19	54
EGFR T790M	15	40
KRAS G12C	8	34
KRAS G12V	11	19
KRAS G12D	14	13
EML4-ALK	15	9

Materials and Methods

Donor Specimens

All human-donor samples in these studies were procured under collaborative agreements with partners, prospectively under the healthy-donor program at our laboratory, or as remnant specimens purchased under commercial use licenses. All donor specimens were consented for research use and no donor-specific identifying information was collected or used in the course of this study. Sample sets sizes used for clinical validation studies are shown in Table 1.

Analytical Controls

Synthetic Controls

Synthetic gene fragments were synthesized as gBlocks by Integrated DNA Technologies (Coralville, IA). The sequences for *EGFR* and *KRAS* were designed so that each single nucleotide variant was represented by a unique gBlock along with a wild type of the same length and position (Table 2). Lyophilized gBlocks were suspended in Tris-EDTA buffer to a stock concentration of 10 ng/μL and were further diluted to working concentrations as needed. Synthetic DNA oligos were used for qualifying the instrument (Supplemental Figure S2) and for assessing initial analytical sensitivity and specificity (Supplemental Table S1), at concentrations between 1 attogram and 1 femtogram per well as input into the respective ddPCR reactions.

gBlock DNA-mimicking cDNA products from the *EML4-ALK* variants 1/2/3 (E13:A20, E20:A20, and E6:A20) (Table 2) were used at 0.2 femtogram per well of the ddPCR reaction (Supplemental Figure S3 and Supplemental Table S1). Of note, the *EML4-ALK* multiplexed assay detects variant 2 at a lower amplitude than those of variants 1 and 3 (Supplemental Figure S3). *EML4-ALK* and *GUSB* analytical control RNA molecules were generated using *in vitro* transcription of the gBlocks, with a T7 promoter sequence. The *in vitro* transcription reaction was performed using the TranscriptAid T7 High Yield Transcription Kit (Thermo Fisher Scientific, Kalamazoo, MI) according to the manufacturer's instructions. Synthesized RNA was treated with DNase I and analyzed for appropriate size using a 2% agarose gel. Synthesized RNAs were quantified by Qubit (Life Technologies, Carlsbad, CA) and mixed at equal molar concentrations to generate the final multiplexed analytical control. The multiplexed RNA control was used at concentrations ranging from 0.25 to 2.5 femtograms as input into the reverse-transcription reaction.

Cell Line Controls

Positive cell line controls were purchased from Horizon Discovery (Cambridge, UK) as genomic DNA or cell pellets (for mRNA isolation) derived from engineered and genetically defined human cell lines. The genomic DNA custom mixture contained *EGFR* and *KRAS* mutations. Extracted DNA was added at 2.5 ng per well into the ddPCR reactions. The *EML4-ALK* variant 1 rearrangement–positive cells were previously characterized by Horizon Discovery to be biologically relevant control material used to assess the performance of PCR-based assays aimed at detecting the EML4-ALK fusion transcript. Frozen cell pellets were processed using the RNeasy Plus Mini Kit (Qiagen) to isolate total RNA.

Recovery of cfDNA from Plasma

Retrospectively and prospectively collected samples were evaluated. Whole-blood samples were prospectively collected into either Cell-Free DNA BCT blood-collection tubes (Streck, La Vista, NE) or BD Vacutainer K_2 EDTA tubes (lavender top) (Becton, Dickinson and Company, Franklin Lakes, NJ). Samples collected into BD Vacutainer tubes were immediately processed by the collection sites to plasma and were frozen before being sent to Biodesix, Inc. (Boulder, CO). The methods for processing whole blood to plasma on-site were per the manufacturer's instructions (room temperature at a speed of 1000 to 1300 relative centrifugal force for 10 minutes in a swinging-bucket centrifuge and 15 minutes in a fixed-angle centrifuge). Frozen plasma specimens were received and centrifuged at high speed (16,000 × *g* for 10 minutes at 4°C). For those cases in which whole blood was collected into the Cell-Free DNA BCT tubes, samples were shipped overnight to the Biodesix CLIA-certified laboratory and processed to plasma first by centrifugation at low speed (1900 × *g* for 10 minutes at 4°C), followed by the same high-speed spin.

cfDNA was isolated using the QIAamp Circulating Nucleic Acid Kit (Qiagen) according to the manufacturer's instructions. DNA was quantitated by the Qubit dsDNA HS (High-Sensitivity) Assay Kit (Life Technologies/Thermo Fisher). When available, cfDNA was used at 2.5 ng per reaction in the ddPCR workflow.

Recovery of Circulating RNA from Plasma

Retrospectively procured samples were purchased as frozen plasma (Indivumed GmbH, Hamburg, Germany). Prospectively procured whole-blood samples were collected into Cell-Free RNA BCT tubes (Streck). All samples were

Table 2 Synthetic DNA Generated as an Analytical Control for Variant-Specific Assays

Variant	Synthetic DNA sequence
EGFR ΔE746-A750	5′-AACGTCTTCCTTCTCTCTCTGTCATAGGGACTCTGGATCCCAGAAGGTGAGAAAGTTAAAATTCCCGTCGCTA-TCAAAACATCTCCGAAAGCCAACAAGGAAATCCTCGATGTGAGTTTCTGCTTTGCTGTGTGGGGGTCCATGGCT-CTGAACCTCAGGCCCACCTTTTCTCATGTCTGGCAGCTGCTCTGCTCTAGACCCTGCTCAT-3′
EGFR L858R	5′-CGTCGCTTGGTGCACCGCGACCTGGCAGCCAGGAACGTACTGGTGAAAACACCGCAGCATGTCAAGATCACAG-ATTTTGGGCGGGCCAAACTGCTGGGTGCGGAAGAGAAAGAATACCATGCAGAAGGAGGCAAAGTAAGGAGGTGG-CTTTAGGTCAGCCAGCATTTTCCTGACACCAGGGACCAGGCTGCCTTCCCACTAGCTGTATTGTTTAACACAT-3′
EGFR T790M	5′-TACGTGATGGCCAGCGTGGACAACCCCCACGTGTGCCGCCTGCTGGGCATCTGCCTCACCTCCACCGTGCAGC-TCATCATGCAGCTCATGCCCTTCGGCTGCCTCCTGGACTATGTCCGGGAACACAAAGACAATATTGGCTCCCAG-TACCTGCTCAACTGGTGTGTGCAGATCGCAAAGGTAATCAGGGAAGGGAGATACGGGGAGGGGAGATAAGGAG-3′
KRAS G12C	5′-TGCACCAGTAATATGCATATTAAAACAAGATTTACCTCTATTGTTGGATCATATTCGTCCACAAAATGATTCT-GAATTAGCTGTATCGTCAAGGCACTCTTGCCTACGCCATCAGCTCCAACTACCACAAGTTTATATTCAGTCATT-TTCAGCAGGCCTTATAATAAAAATAATGAAAATGTGACTATATTAGAACATGTCACACATAAGGTTAATACAC-TAT-3′
KRAS G12D	5′-TGCACCAGTAATATGCATATTAAAACAAGATTTACCTCTATTGTTGGATCATATTCGTCCACAAAATGATTCT-GAATTAGCTGTATCGTCAAGGCACTCTTGCCTACGCCAATCGCTCCAACTACCACAAGTTTATATTCAGTCATT-TTCAGCAGGCCTTATAATAAAAATAATGAAAATGTGACTATATTAGAACATGTCACACATAAGGTTAATACAC-TAT-3′
KRAS G12V	5′-TGCACCAGTAATATGCATATTAAAACAAGATTTACCTCTATTGTTGGATCATATTCGTCCACAAAATGATTCT-GAATTAGCTGTATCGTCAAGGCACTCTTGCCTACGCCAACAGCTCCAACTACCACAAGTTTATATTCAGTCATT-TTCAGCAGGCCTTATAATAAAAATAATGAAAATGTGACTATATTAGAACATGTCACACATAAGGTTAATACAC-TAT-3′
EML4-ALK variant 1	**5′-CAGAGATGCATAATACGACTCACTATAGGGAGA**GAAAAGCCAAAATTTGTGCAGTGTTTAGCATTCTTGGGGA-ATGGAGATGTTCTTACTGGAGACTCAGGTGGAGTCATGCTTATATGGAGCAAAACTACTGTAGAGCCCACACCT-GGGAAAGGACCTAAAGTGTACCGCCGGAAGCACCAGGAGCTGCAAGCCATGCAGATGGAGCTGCAGAGCCCTGA-GTACAAGCTGAGCAAGCTCCGCACCTCGACCATCATGACCGACTACAACCCCAACTACTGCTTTGCTGGCAAGA-CCTCCTCCATCAGTGACCTGAAGGA-3′
EML4-ALK variant 2	**5′-CAGAGATGCATAATACGACTCACTATAGGGAGA**TATAGCAGATATGGAAGGTGCACTGGACATTCCAGCTACA-TCACACACCTTGACTGGTCCCCAGACAACAAGTATATAATGTCTAACTCGGGAGACTATGAAATATTGTACTTG-TACCGCCGGAAGCACCAGGAGCTGCAAGCCATGCAGATGGAGCTGCAGAGCCCTGAGTACAGCTGAGCAAGCTC-CGCACCTCGACCATCATGACCGACTACAACCCCAACTACTGCTTTGCTGGCAAGACCTCCTCCATCAGTGACCT-GAGGAGGTGCCGCGGAAAAACATCACCCTCATTCG-3′
EML4-ALK variant 3	**5′-CAGAGATGCATAATACGACTCACTATAGGGAGA**TGTCGAAAATACCTTCAACACCCAAATTAATACCAAAAGT-TACCAAAACTGCAGACAAGCATAAAGATGTCATCATCAACCAAGTGTACCGCCGGAAGCACCAGGAGCTGCAAG-CCATGCAGATGGAGCTGCAGAGCCCTGAGTACAAGCTGAGCAAGCTCCGCACCTCGACCATCATGACCGACTAC-AACCCCAACTACTGCTTTGCTGGCAAGACCTCCTCCATCAGTGACCTGAAGGAGGTGCCGCGGAAAAACATCAC-CCTCATTCG-3′
GUSB control	**5′-CAGAGATGCATAATACGACTCACTATAGGGAGA**TGATCGCTCACACCAAATCCTTGGACCCCTCCCGGCCTGT-GACCTTTGTGAGCAACTCTAACTATGCAGCAGACAAGGGGGCTCCGTATGTGGATGTGATCTGTTTGAACAGCT-ACTACTCTTGGTATCACGACTACGGGCACCTGGAGTTGATTCAGCTGCAGCTGGCCACCCAGTTTGAGAACTGG-TATAAGAAGTATCAGAAGCCCATTATTCAGAGCGAGTATGGAGCAGAAACGATTGCAGGGTTTCACCAGGATCC-ACCTCTGATGTTCACTGAAGAGTACCAGAAAAGTCTGCTAGAGCAGTACCATCTGGGTCTGGATCAAAAACGCA-GAAAATACGTGGTTGGAGAGCTCATTTGGAATTTTGCCGATTTCATGACTGAACAGTCACCGACGAGAGTGCTG-GGGAATAAAAA-3′

Bold text represents T7 promoter used for *in vitro* transcription; *EML4-ALK* and *GUSB* only.

processed to plasma by centrifugation at 900 × *g* for 10 minutes at 4°C. The plasma supernatant was then aspirated without disturbance of the buffy coat layer. Approximately 4 to 5 mL of plasma was obtained from one 10-mL whole blood—collection tube. Circulating RNA was isolated using the Plasma/Serum Circulating and Exosomal RNA Purification Kit, slurry format (Norgen Biotek, Thorold, ON, Canada) according to the manufacturer's guidelines. Samples were eluted into 100 μL of prewarmed nuclease-free water and subsequently concentrated using the RNA Clean-Up and Concentration Kit (Norgen Biotek). RNA levels were generally undetectable using the Qubit measurement method.

Circulating RNA was reverse-transcribed using SuperScript IV Reverse Transcriptase (Thermo Fisher) according to the manufacturer's instructions. cDNA was isolated from remaining reverse transcriptase primers and reaction mix by elution through the DNA Clean and Concentrator-5 column with a lower limit of capture of 50 bp. cDNA eluted from this column was then used for ddPCR. A no—reverse transcriptase control was run with every batch of samples and was processed identically but without reverse transcriptase.

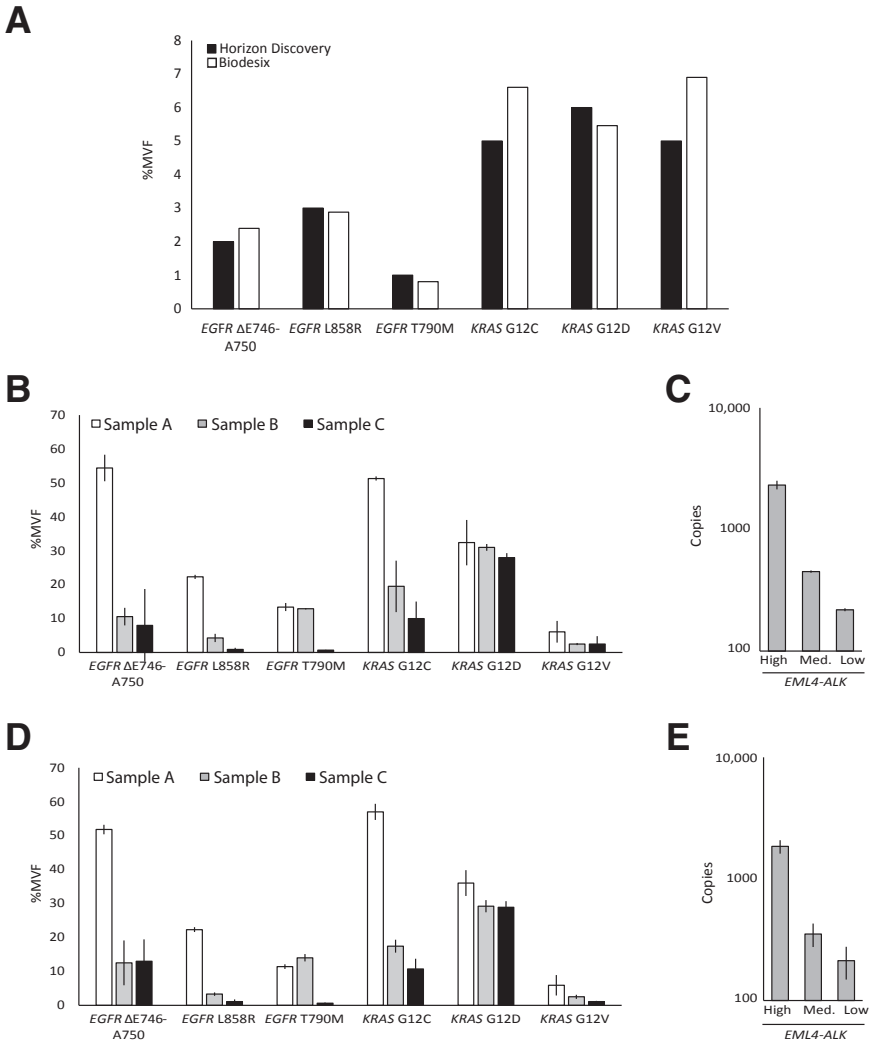


Figure 1 Precision testing. **A:** Assays for genetic variants were evaluated by Droplet Digital (dd) PCR (Bio-Rad, Pleasanton, CA) at Horizon Discovery (Cambridge, UK) with a prequalified standard of known percent minor variant frequencies (%MVF). The same standards were evaluated at Biodesix, Inc. (Boulder, CO) using the QX200 ddPCR system (Bio-Rad). *EML4-ALK* was not evaluated in the study. **B:** Intrarun studies for each *EGFR* and *KRAS* variant were run with three plasma samples from donors with cancer or, in the case of L858R, using analytical cell line standards (Horizon Discovery). **C:** Intrarun studies for the *EML4-ALK* multiplexed assay was run with three concentrations of analytical RNA standard. **D:** Inter-run studies of *EGFR* and *KRAS* were performed as in **B**. **E:** Interday testing of the *EML-ALK* assay was performed as in **C**. Data are expressed as means ± SD. $n = 3$ (**B** and **C**, independent runs; **D** and **E**, consecutive days of testing). Med., medium.

Droplet Digital PCR

ddPCR PrimePCR (Bio-Rad) reactions were set up in duplicate. Each reaction well was mixed to a final volume of 20 μL with the following: 10 μL of 2× ddPCR supermix for probes, no 2′-deoxyuridine 5′-triphosphate; 1 μL of 20× variant target primers/probe set (450 nmol/L primers/250 nmol/L fluorescein amidite OR 6-carboxyfluorescein probe); 1 μL of 20× control target primers/probe set (450 nmol/L primers/250 nmol/L 5′-hexachloro-fluorescein-CE phosphoramidite probe); and up to 7 μL of the test template DNA (either cfDNA or cDNA). Restriction digestion of template DNA was executed within the PCR mix by adding two to five units of enzyme (*Hind*III, *Alu*I, *Mse*I) per nanogram of DNA where applicable. *Hind*III was used for *EGFR* T790M and L858R; *Alu*I, for *EGFR* ΔE746-A750; and *Mse*I, for all *KRAS* assays (New England BioLabs, Ipswich, MA). The *EML4-ALK* assay did not include a restriction enzyme. No-template control reactions were performed with water in place of the DNA template and were run with every assay within a plate. PrimePCR assays used for variant detection are listed in Supplemental Table S2.

Droplet generation was performed with either a manual or automated droplet generation QX200 system (Bio-Rad) according to the manufacturer's guidelines. Once emulsions were generated, plates were placed into a C1000 Touch thermocycler (Bio-Rad). The thermocycling profile was optimized as follows: enzyme activation, one cycle (95°C, 10 minutes), followed by denaturation (94°C, 30 seconds) and annealing/extension (55°C, 1 minute) at a ramp rate of approximately 2°C/second for 40 cycles. To conclude the procedure, enzyme was deactivated at 98°C for 10 minutes, followed by hold at 4°C (ramp rate, approximately 1°C/second). After amplification, the plate was transferred to the droplet reader (Bio-Rad). Samples were read using the Rare Event Detection module on the reader (QuantaSoft version 1.7.4.0917; Bio-Rad).

Data Analysis and Review, and Generation of the Results

Data on negative and positive droplet counts in each sample were analyzed and reviewed using the QuantaSoft

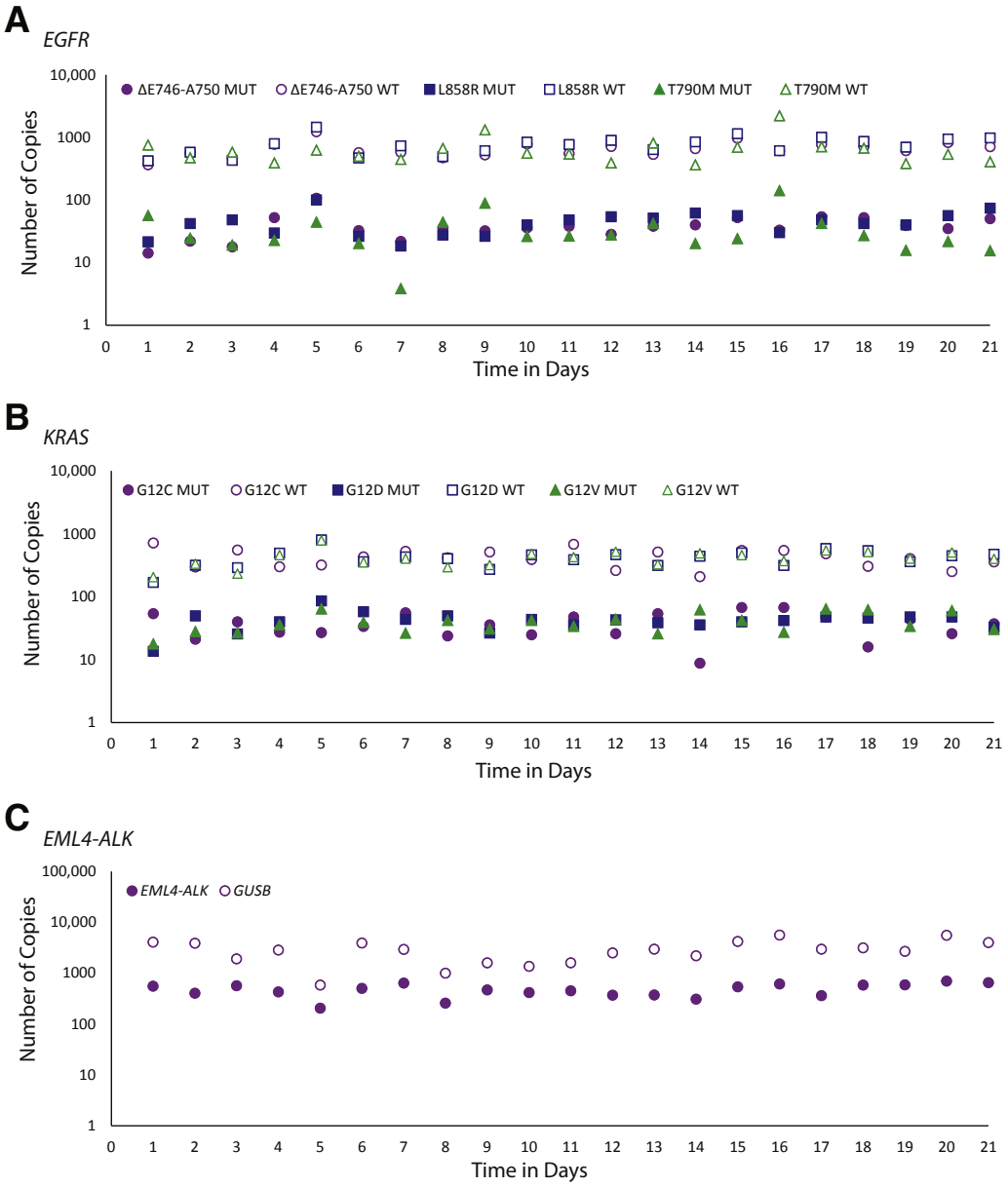


Figure 2 Robustness testing. Analytical positive control was spiked into normal human plasma, extracted, and tested by Droplet Digital PCR (Bio-Rad, Pleasanton, CA) over 21 consecutive business days. Both mutant (MUT) and wild-type (WT) copies are reported. **A:** *EGFR* variants ΔE746-A750, L858R, and T790M. **B:** *KRAS* variants G12C, G12D, and G12V. **C:** *EML4-ALK* fusion copies and control gene copies detected using the *EML4-ALK* multiplex for the detection of variants 1, 2, and 3.

analysis modules for calculating mutant and WT copy numbers. The *GUSB* RNA and the WT DNA variants were used as assay-quality controls to verify the presence of circulating nucleic acids of quality and quantity sufficient for testing. *EML4-ALK* and *GUSB* results were reported as the total number of copies per sample. The clinical cutoff for validation studies was defined as two or more copies of fusion variant. The DNA-variant (*EGFR* and *KRAS*) test results were expressed as either the number of copies or the percent minor variant frequency (%MVF) of the mutation in relation to wild type. The clinical cutoff for calling a positive sample in the validation studies was defined as 0.02% MVF. Variant frequencies were calculated as follows:

$$\%\text{MVF} = \frac{(\text{Mutation copy number})}{(\text{Mutation} + \text{Wild type copy number})} \times 100 \qquad (1)$$

Results

System Limit of Detection and Assay Verification

System qualification of the QX200 was conducted using the ddPCR System Demonstration Kit (Bio-Rad). Serial

Clinical Validation

A

ddPCR	Reference Result: ΔE746-A750 +	Reference Result: ΔE746-A750 -	Total
ΔE746-A750 +	28	0	28
ΔE746-A750 -	2	62	64
Total	30	62	92

B

ddPCR	Reference Result: L858R +	Reference Result: L858R -	Total
L858R +	19	0	19
L858R -	0	54	54
Total	19	54	73

C

ddPCR	Reference Result: T790M +	Reference Result: T790M -	Total
T790M +	13	0	13
T790M -	2	40	42
Total	15	40	55

D

ddPCR	Reference Result: G12C +	Reference Result: G12C -	Total
G12C +	7	0	7
G12C -	1	34	35
Total	8	34	42

E

ddPCR	Reference Result: G12D +	Reference Result: G12D -	Total
G12D +	11	0	11
G12D -	3	13	16
Total	14	13	27

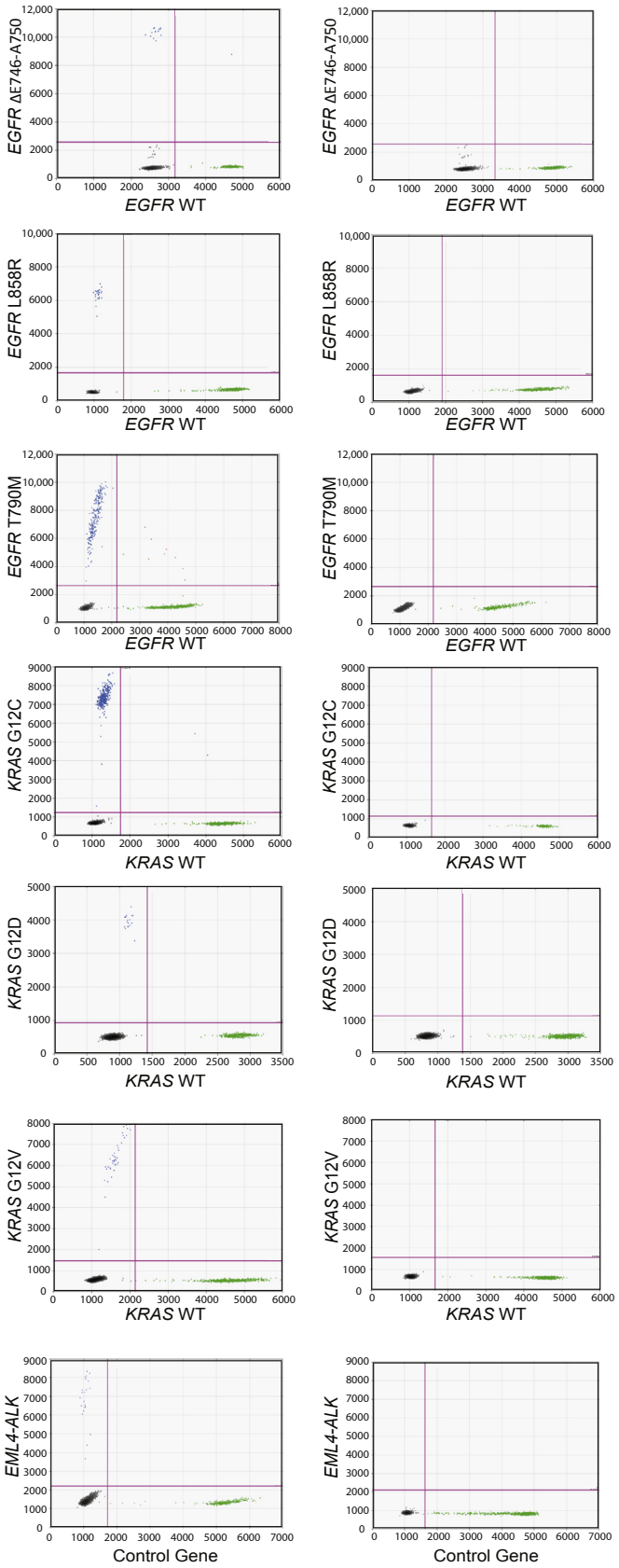
F

ddPCR	Reference Result: G12V +	Reference Result: G12V -	Total
G12V +	11	0	11
G12V -	0	19	19
Total	11	19	30

G

ddPCR	Reference Result*: *EML4-ALK* +	Reference Result*: *EML4-ALK* -	Total
EML4-ALK +	10	0	10
EML4-ALK -	5	9	14
Total	15	9	24

Representative Data



dilutions were performed by diluting mutant-allele samples into WT background at 1:8, 1:40, 1:200, 1:1000, and 1:5000. In the 1:5000 dilution condition, a mean of 8 copies of the mutant allele and 48,400 copies of the WT allele were detected to generate a lower limit of detection of 0.02% MVF (Supplemental Figure S2). This analytical limit of detection demonstrates the sensitivity of the system. In practice, although we have observed a few cases that have reached this lower bound, the majority of samples from cancer patients (when blood is processed according to our protocol) do not have enough circulating WT DNA to permit detection at the 0.02% MVF limit.

Following initial system qualification, an initial verification study was performed to evaluate the sensitivity and specificity of each assay for its intended target and not off-targets. These experiments were performed using synthetic DNA control templates designed to mimic either circulating tumor (ct) DNA fragments for *EGFR* mutations L858R, ΔE746−A750, and T790M, *KRAS* mutations G12C, G12D, G12V, or the cDNA generated from circulating *EML4-ALK* fusion transcript variants 1, 2, and 3 (Supplemental Table S1). For example, the *EGFR* T790M assay was tested using synthetic DNA templates harboring either the ΔE746−A750, L858R, or T790M mutation. As expected, the T790M assay detected only T790M template, but neither of the other two *EGFR* templates. These results indicate that the T790M assay is specific to *EGFR* T790M template but no off-target activity toward the other *EGFR* variants.

Although synthetic templates such as gBlocks are valuable tools, they are not the ideal surrogate for cfDNA because they are homogenous in nature and thus do not reflect the true fragmentation status of cfDNA. Thus, in addition to the gBlock studies, we used the combination of sonication and restriction enzyme digestion to evaluate genomic DNA from cell lines. Each assay was evaluated using either genomic DNA or total RNA extracted from cell lines expressing each variant of interest (Horizon Discovery). Individual assays successfully detected the cell line reported to express such variant but did not detect cell lines that expressed alternative variants (data not shown). Additional feasibility and verification were completed to optimize each step in the workflow. Studies included optimization of extraction volumes, thermocycling conditions, and reverse-transcription enzyme selection (data not shown).

Evaluation of Assay Precision

Technical concordance between the results from the assays developed for this study and reported reference results was assessed using ddPCR methods performed in an external laboratory (Horizon Discovery). The data were generated using genomic DNA from the predefined cell line reference standards described in *Materials and Methods* for *EGFR* and *KRAS*. The %MVF detected in each laboratory (Figure 1A), as well as the mutant copy numbers (Supplemental Table S3), are reported ($r = 0.8117$, $P = 0.061$). Interoperator studies were also performed for all variants. Two individual operators generated results (Supplemental Table S4) with a high level of concordance ($r = 0.9333$, $P = 0.0007$).

Interday and intraday precision testing was performed on three samples for each of the variants. Where available, these studies were executed using plasma samples from donors with cancer that were predefined as having the desired mutation at variable frequencies. For the L858R and *EML4-ALK* assays, we were unable to identify donor samples of a quantity sufficient for use in the precision studies. Thus, L858R precision test data were supplemented using analytical cell line control material (Horizon quantitative standards), and *EML4-ALK* analytical performance data were generated using the *EML4-ALK* multiplexed analytical RNA standard. Intraday testing was completed with three runs of each sample performed in a single day (Figure 1, B and C). Interday testing was completed with one run of each sample on 3 consecutive days (Figure 1, D and E). Using predefined performance criteria based on the detection of copies in all replicates and in all samples, all samples passed evaluation.

Robustness evaluation was conducted for each variant assay by analysis of the performance of the assay over 21 consecutive business days (Figure 2). *EGFR* and *KRAS* robustness studies were executed using a multiplexed cell line standard (custom-blended by Horizon Discovery; same as in Supplemental Table S3), whereas testing for the *EML4-ALK* multiplex assay was performed using multiplexed RNA standards (Table 2). These controls were spiked into healthy-donor plasma each day, extracted, and processed through the entirety of the workflow. Consistent with the criteria used in our evaluation of precision, all samples passed the robustness evaluation.

Evaluation of Assay Accuracy

The accuracy of each assay was evaluated with plasma samples from healthy donors as well as variant-negative and variant-positive samples from donors with cancer. Twenty healthy-donor plasma samples were processed for isolation of cfDNA and evaluated using each of the *EGFR* and *KRAS* variant assays; in none of these cases were

Figure 3 Sensitivity and specificity clinical validation studies. Validation samples with reference result and Droplet Digital (dd) PCR (Bio-Rad, Pleasanton, CA) result, along with associated sensitivity, specificity, and concordance calculations (left) and an example of a two-dimensional plot of results not detected and detected from each validation set (right) are shown for *EGFR* variants ΔE746-A750 (**A**), L858R (**B**), and T790M (**C**); *KRAS* variants G12C (**D**), G12D (**E**), and G12V (**F**); and *EML4-ALK* multiplex for the detection of variants 1, 2, and 3 (**G**). *Prevalence of *EML4-ALK* variants 1, 2, and 3 represents that 78.4% of the total *ALK* mutations were used to calculate sensitivity and concordance. WT, wild type.

mutations detected by ddPCR. Ten healthy-donor samples were processed for isolation of circulating RNA and evaluated using the *EML4-ALK* multiplexed assay; in none of these cases were fusion variants detected by RT-ddPCR.

The sample sets from donors with cancer evaluated for each variant assay are enumerated in Table 1. Results obtained from each individual clinical validation sample, including initial plasma volumes, quantity of recovered cfDNA, and maximum reachable sensitivities (expressed as %MVF for *EGFR* and *KRAS* genetic variants), as well as initial plasma volumes and mutant copies detected for *EML4-ALK* transcript variants, can be found in Supplemental Tables S5–S11. In many cases, a single donor sample was used to generate multiple reference results because of the mutual exclusivity of the variants. For example, an *EGFR* L858R sample is generally considered to be mutually exclusive with the *EGFR* ΔE746-A750 variant. Thus the number of evaluable validation reference samples ($n = 343$) was more than the number of total individual donors with cancer ($n = 172$).

The clinical factors of cancer tissue of origin and cancer staging varied in the samples from donors with cancer. In addition, the reference methodology included Sanger sequencing, pyrosequencing, and PCR [Cobas (*EGFR*), Roche and Therascreen (*EGFR* and *KRAS*), Qiagen], all performed on tissue for the DNA-variant references. The clinical accuracy evaluation for each individual assay is shown in Figure 3, along with representative results of a two-dimensional plot of a variant detected and variant not detected. A summary of the overall sensitivity, specificity, and concordance results for each individual variant, as well as by class, is shown in Table 3. Clinical sensitivities ranged from 78.6% for the G12D variant to 100% for the G12V and L858R variants, whereas clinical specificities were 100% for all assays. Of note, the prevalence of *EML4-ALK* variants in our test represent 78.4% of the total *EML4-ALK* transcripts generated from *ALK* rearrangements. Since the reference method in this data set was variant agnostic (ie, Vysis break-apart FISH and Ventana immunohistochemistry tissue results), we adjusted our specificity and concordance by estimating that our *EML4-ALK* plasma RT-ddPCR assay would maximally capture 78.4% of ALK tissue–positive samples.[30] Thus, the adjusted clinical sensitivity of the *EML4-ALK* assay was 85%. The overall mean sensitivity, specificity, and concordance for the group of variants described were 90.0%, 100%, and 97.0%, respectively.

Table 3 Summary of Sensitivity, Specificity, and Concordance Studies of Individual Variants, Gene Classes, and Overall

Variant (individual and class)	Sensitivity, %	Specificity, %	Concordance, %
EGFR sensitizing	95.9	100	98.8
ΔE746-A750	93.3	100	97.8
L858R	100	100	100
EGFR resistance (T790M)	86.7	100	96.4
KRAS	87.9	100	96.0
G12C	87.5	100	97.6
G12D	78.6	100	88.9
G12V	100	100	100
EML4-ALK	~85	100	~92
All variants	90.9	100	97.0

EGFR, epidermal growth factor receptor.

Test Utilization in the Clinical Setting

We next evaluated metrics associated with physicians' usage of the seven variant-specific assays. The workflow for clinical sample processing is represented in Figure 4A. Data were extracted from the secure Laboratory Information Management System, and all protected health information was excluded from the analysis. Although we deliberately developed these tests to have utility for NSCLC, up to 6% of the tests requested to date have been for patients with cancer from other anatomic sites (data not shown). For the purpose of this study, we evaluated only samples from patients with a diagnosis of lung cancer. Of the 1643 patients within this data set, most were under the care of community physicians, whereas approximately 11% of orders came from physicians with academic affiliations (Figure 4B). Importantly, results were available within 72 hours of sample receipt in 94% of cases. These data highlight the adoption of mutation testing by the lung cancer community and demonstrate the utility of a centralized laboratory for the evaluation of actionable mutations from blood.

For the majority of test orders, physicians requested variants as a set of two or more. However, in this sample set, 21% of T790M orders and 5% of *EML4-ALK* orders were placed as single-variant test requests (Figure 4C). We found this to be of interest in the case of EGFR-resistance mutation testing, where more recent recommendations are to test for T790M in parallel with the original *EGFR* driver mutation.[31] The proposed reason for testing for the sensitizing and resistance mutations simultaneously is that smaller, more indolent tumors may harbor the resistance mutation but not shed enough ctDNA to allow for detection in plasma.[32] Conversely, if the original *EGFR* mutation is found but T790M is not, this could indicate an alternative mechanism of resistance rather than a problem with cfDNA detection. The total numbers of each sample submitted for variant testing, percentage positive or negative, or no test results, as well as the ranges of clinically relevant sensitivities and variant copies observed are presented in Table 4. Significantly, we recovered enough circulating nucleic acid to report on >99% of samples received for DNA testing, and on >90% of samples received for *EML4-ALK* fusion variant testing. Variant copies ranged from a mean of 5.4 (*EML4-ALK*) to 1216 (*EGFR* ΔE746-A750) and %MVFs ranged from the previously established lower bound of 0.02% (*EGFR* L858R) to 88.8% (*EGFR* ΔE746-A750).

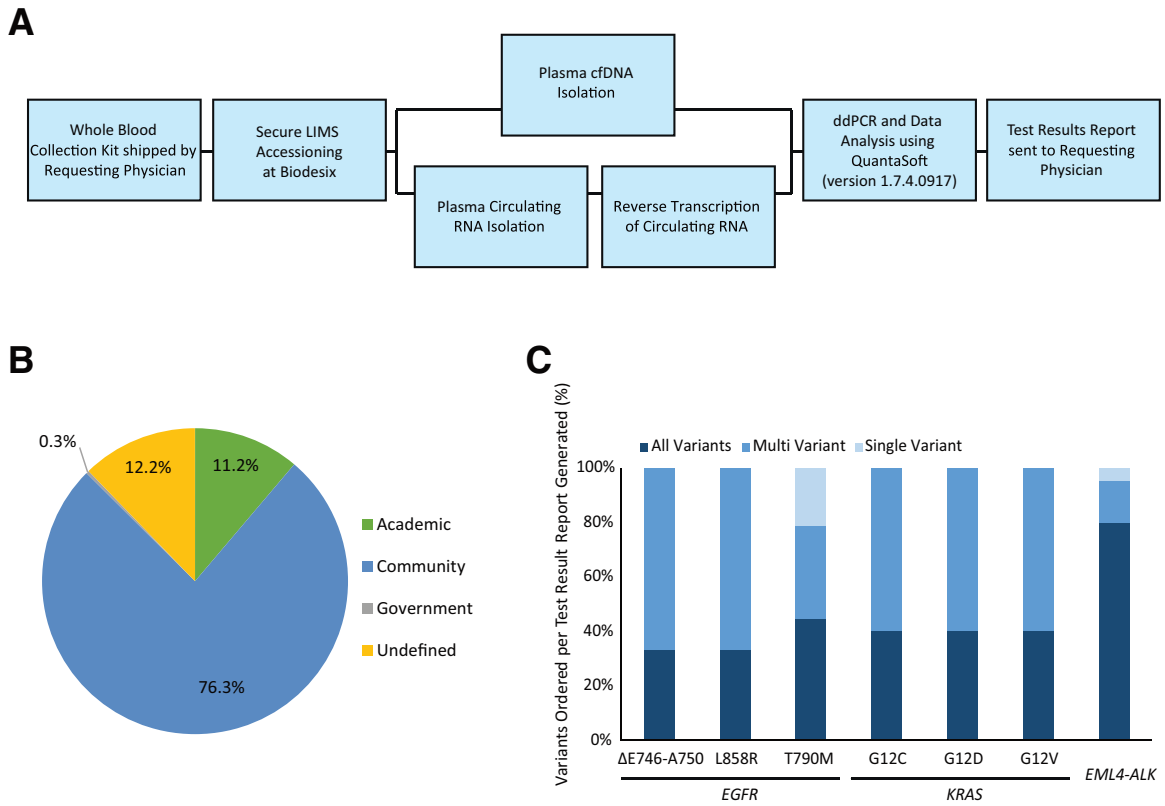


Figure 4 Assay utilization by ordering physicians. **A:** Workflow overview of the CLIA-certified laboratory test (Biodesix, Inc., Boulder, CO). The test process is initiated when whole blood is drawn into two blood-collection tubes (BCT; Streck, La Vista, NE) and the collection kit arrives at the centralized CLIA laboratory. Patient samples are accessioned into the Laboratory Information Management System (LIMS) and processed through parallel workflows to isolate either cDNA or RNA. Following nucleic acid extraction (and cDNA synthesis for RNA), samples are processed using the QX200 Droplet Digital (dd) PCR system (Bio-Rad, Pleasanton, CA). Droplet count evaluation is conducted using QuantaSoft version 1.7.4.0917 (Bio-Rad), and test result reports are generated from the secure LIMS. **B:** The percentages of orders submitted from physicians affiliated with community, academic, and government-run centers. *Undefined* represents those centers for which information was not provided with the sample. **C:** The percentage of test result reports with an individual variant, that variant with at least one other variant, and the variant as one of a full set of seven. cfDNA, circulating free DNA.

EML4-ALK was reported as positive based on copies detected and not %MVF.

Discussion

Measurement of circulating nucleic acids in plasma has shown utility in the detection of metastatic cancer,[12,33] and levels are modulated with tumor burden as disease progresses.[11,34–36] ctDNA can also provide an early measure of treatment response, as has been supported by findings from analyses of ctDNA in other solid cancers.[11,15,16,37,38] However, the accurate detection and quantification of mutated ctDNA in blood has been challenging due to the difficulty in discriminating ctDNA from normal cfDNA.[37] Measurement of circulating RNA, additionally complicated due to a shorter half-life in circulation, has generally been achieved when the RNA is extracted from cells and other vesicles (eg, circulating tumor cells,[39–41] tumor-educated platelets,[42] or exosomes[43,44]) that protect circulating RNA. Notwithstanding these considerations, the potential significance of circulating nucleic acids in diagnosis, prognosis, and monitoring has been clearly established in various recent studies.[10,11,45–47] Additionally, more efficient procedures for the isolation of circulating nucleic acids, coupled with highly sensitive detection procedures,[20,48] have enabled advances in this area of research.[45]

Our goal was to develop rapid, robust, and highly sensitive blood-based genome assays with clinical utility. We thus focused on actionable mutations, including the EGFR-sensitizing (L858R, ΔE746-A750) and EGFR -resistance (T790M) mutations, and *KRAS* mutations G12C, -D, and -V in circulating DNA from donor specimens. Additional studies were conducted to detect the most prevalent *EML4-ALK* variant fusions with treatment implications. We have described in this report a ddPCR testing service for the detection of clinically actionable variants in circulating nucleic acids isolated from whole-blood samples from patients with NSCLC. Variant results are typically reported within 72 hours of sample receipt, consistent with a recent report using a similar ddPCR approach.[11] As

Table 4 Variant Percent and Copy Number Detection in Commercial Processing of NSCLC Patient Cases within the Biodesix CLIA-Certified Laboratory (Boulder, CO) ($N = 1643$)

Variant	Total number of samples	Positive results, %	Negative results, %	No test results, %	Positive samples (variant copies)	Positive samples, %MVF range
EGFR ΔE746-A750	1401	6.2	93.6	0.2	Mean = 1216 Median = 49 SD = 4920	0.08–88.8
EGFR L858R	1400	4.3	95.6	0.1	Mean = 115 Median = 11.4 SD = 234	0.02–85.5
EGFR T790M	1055	13.8	85.9	0.3	Mean = 162 Median = 6.6 SD = 852	0.03–87.0
KRAS G12C	1159	5.1	94.6	0.3	Mean = 30 Median = 8.8 SD = 63	0.07–38.7
KRAS G12D	1159	4.1	95.5	0.3	Mean = 100 Median = 7.9 SD = 318	0.05–67.2
KRAS G12V	1159	4.0	95.8	0.3	Mean = 43 Median = 10.6 SD = 62	0.06–33.6
EML4-ALK	304	2.0	88.5	9.5	Mean = 5.4 Median = 3 SD = 4.8	NA

MVF, minor variant frequency; NA, not applicable; NSCLC, non–small cell lung carcinoma.

described, components of assay development included: i) workflow optimization for nucleic acid recovery from whole blood (Figure 4), ii) test system performance verification/method development studies including precision (Figure 1 and Supplemental Figure S1), iii) robustness (Figure 2), and iv) analytical and clinical sensitivity and specificity (concordance) analyses with reference samples and results (Figure 3 and Table 3). Clinical sensitivity for each variant was individually established, with a range from 78.6% to 100% and a mean of 90.9%. Clinical specificity was established to be 100% for all variants (Table 3). Overall concordance was established, with a range of 88.9% to 100% and a mean of 97.0% for all variants.

In addition, we have described the utility of these tests in clinical practice, with >1600 clinical cases analyzed in this study (Figure 4 and Table 4). Of particular interest is the finding that the tests developed are predominantly ordered by community physicians (76.3%) (Figure 4B). Furthermore, physicians most frequently request the EGFR-sensitizing and -resistance variants and the *KRAS* mutations (Figure 4B). The *EML4-ALK* assay in contrast to the cfDNA tests has been available for a shorter time and thus 304 case results were available for evaluation; however, the utility trends for the RNA variants were similar to those of the DNA test requests. The frequency of detected ALK was consistent with the current reported prevalence in patients with NSCLC in the United States.[49] We observed a higher assay failure rate with the RNA variant assay than with the DNA variants (9.5% versus <1%). This finding likely supports the argument for inherently less stability of RNA in circulation. Additionally, similar to what has been previously reported,[50] the ranges of variant burden, as measured using the validated assay, was highly variable in the patient population (0.02% to 88.8%) (Table 4) and independent of plasma volume (3 mL) or the amount of input cfDNA (2.5 ng per well).

Our data confirm that whole blood is a reliable source for identifying ctDNA mutations and RNA variants and demonstrate the potential for blood-based mutation testing in clinical practice. The assay described herein provides results within 72 hours from sample receipt, with minimal false-positive detection rates, which facilitates more rapid treatment decisions and circumvents the limitations of tissue-based testing.[11] For those patients who do not have an actionable driver mutation identified by ddPCR, the addition of more extensive genome and proteome testing may provide even broader clinical information to support treatment planning.

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Supplemental Data

Supplemental material for this article can be found at *http://dx.doi.org/10.1016/j.jmoldx.2016.11.004*.

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