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Precision Genotyping for EGFR Mutation-Positive Non-Small Cell Lung Cancer (NSCLC) Using Multiplexed Digital PCR on cell-free DNA (cfDNA)

Aaron Marks Horning[1], Rounak Feigelman[1], Wei Wei[1], MinGin Kim[1], Lingxia Jiang[1], Kuo-Jang Kao[2] 1)Thermo Fisher Scientific, 180 Oyster Point Blvd, South San Francisco, CA, 94080 2) Koo Foundation Sun Yat-Sen Cancer Center, Taiwan

INTRODUCTION

Epidermal growth factor receptor (EGFR) mutations can be drivers of cancer and cause resistance to treatments in patients with non-small cell lung cancer (NSCLC), the leading cause of cancer-related death in the US. There are multiple tyrosine kinase inhibitors (TKIs) that can be used to specifically target EGFR-mutated NSCLC for potential treatment. However, cancer cells frequently mutate and acquire new TKI-resistant EGFR mutations that may reduce efficacy. Identifying EGFR mutations in patient cell-free DNA (cfDNA) may enable researchers to detect cancer early, measure therapeutic response, quantify residual tumor burden, and monitor emerging resistance to potential therapies.

Here, we present three multiplex EGFR mutation panels compatible with cfDNA for use on the Applied Biosystems™ QuantStudio™ Absolute Q™ digital PCR (dPCR) system. This platform consists of a single instrument with five optical channels and microfluidic array plate which can compartmentalize up to 16 samples each into 20,480 micro-chambers and acquire multiplexed results in < 2 hours. The panels target known driver, drug resistance, and low frequency mutations, respectively. We demonstrate that the three EGFR mutation panels can detect multiple mutations in the same reaction with high sensitivity (0.01% allele frequency, AF), thus enabling liquid biopsy cancer research for early detection and monitoring, which is critical for understanding how a tumor may respond to potential treatment.

MATERIALS AND METHODS

We developed 3 separate dPCR EGFR multiplex panels targeting various mutant and wild-type alleles on EGFR (Table 1). Each multiplex panel has a single VIC wild-type probe and the rest of the mutant probes in the panel had a combination of FAM, ABY and JUN fluorophores. We tested the panels on mutant EGFR cfDNA reference standards with 5, 1, 0.1 and 0% AF. These standards were purchased from Horizon Discovery (HD, *Cat #*: HD825) and supplemented with custom mutant DNA strings purchased from Thermo Fisher (*Cat #*: 815010DE). For testing false positives, we validated results using CEPH control DNA (*Cat #*: 403062) that were fragmented (fCEPH) with the Covaris (S220). Candidate assays were screened in duplex dPCR with 1 mutant FAM, ABY or JUN probe, and 1 wild-type VIC probe. Selected mutant assays were pooled together and tested against different wild-type assays. All reference standard experiments were performed with dPCR. DNA copy concentrations were determined by first calculating lambda (λ). Next, concentration in copies per microliter of the reaction was calculated by dividing λ by the volume of each microchamber (Equations 1 and 2). To calculate AF, we used equation 3. To determine the extent to which we could distinguish each mutant reference standard to the wild-type sample, we performed a Wilcoxon rank sum test and adjusted the p-values for multiple comparisons using the Holm method. Adjusted p-values are plotted as follows: “ns” ($p \geq 0.05$), “*” ($p \leq 0.05$), “**” ($p \leq 0.01$).

To determine the extent to which we could detect the EGFR mutations in NSCLC donor samples, we generated data on 13 cfDNA banked samples. These samples came from donors who were undergoing treatment and screening at the Koo Foundation Sun Yat-Sen Cancer Center (KFSYSCC) in Taiwan. cfDNA was extracted from 3 ml of plasma and eluted in 40ul of elution buffer using Qiagen’s QIAmp Circulating Nucleic Acid Kit (50 Rxn) (*Cat #: 55114*). Before dPCR, the samples’ mutation burden was measured using quantitative amplification refractory mutation system qPCR (qARMS-PCR) with reference standard plasmids quantified based on the OD260 nm and molecular weights. The results are plasmid copy number equivalence in each mL of plasma assuming 100% recovery of plasmids from extraction. Next, we generated dPCR data on the cfDNA samples using the EGFR multiplex panels. We calculated the copies per ml of plasma as well to compare with the previous results.

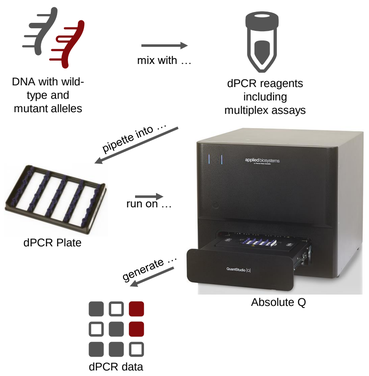
$$\lambda = -ln(1 - \frac{positive\ microchambers}{total}) \quad (1)$$

$$cp/\mu l = \frac{\lambda}{0.000432\ \mu l/microchamber} \quad (2)$$

$$Allele Frequency = \frac{[mut\ cp/\mu l]}{[mut\ cp/\mu l]+[wild\ type\ cp/\mu l]} * 100 \quad (3)$$

RESULTS

Figure 1. Applied Biosystems QuantStudio Absolute Q workflow



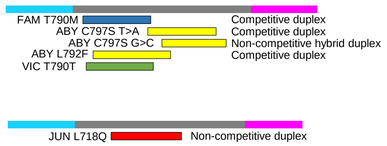
Simplified workflow for performing dPCR steps and identifying mutant and wild-type alleles. Reference standards from HD had predetermined AFs ranging from 5% to 0.1%. Then we tested cfDNA extracted from plasma of 13 donors.

Table 1. EGFR Multiplex Panels

Panel	Mutation	Dye
Driver Mutations	L858R	FAM
	p.E746_A750del	ABY
	L858L	VIC
Drug Resistant Mutations	T790M	FAM
	C797S T>A	ABY
	C797S G>C	
	L792F	
	L718Q	JUN
	T790T	VIC
Low Frequency Mutations	L861Q	FAM
	G719C	ABY
	G719S	
	S768I	JUN
	G719G	VIC

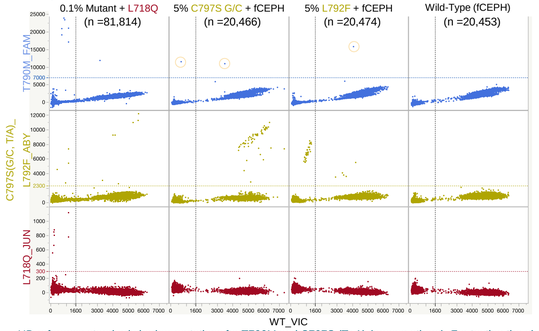
Some mutations’ probes share the same fluorophore because they are associated with identical treatments.

Figure 2. Schematic of the Multiplex Drug Resistant Mutation Panel



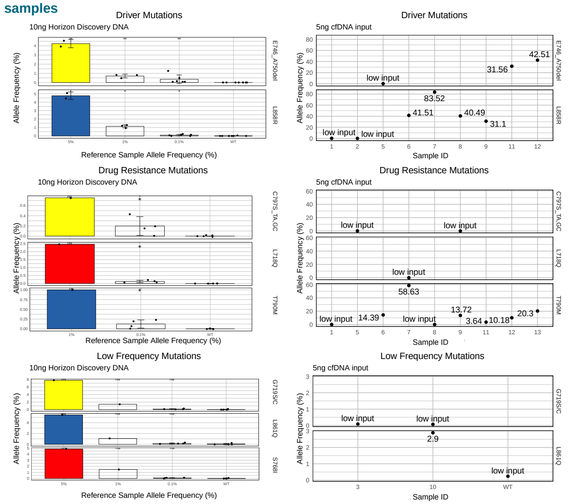
This panel consists of two amplicons (top and bottom) and a mixture of the different types of “duplexes”, or pairs of mutant and wild-type probes. A “duplex” is characterized by whether the mutant probe sequences overlap with the wild-type probe and if it is located on the same amplicon as the wild-type probe. The terms describing “competitive” and “non-competitive (hybrid)” are described by Whale et al. 2016.

Figure 3. Two-dimensional dPCR scatterplots indicating that the drug resistant multiplex panel is sensitive (0.1%) and specific for different C797S mutations



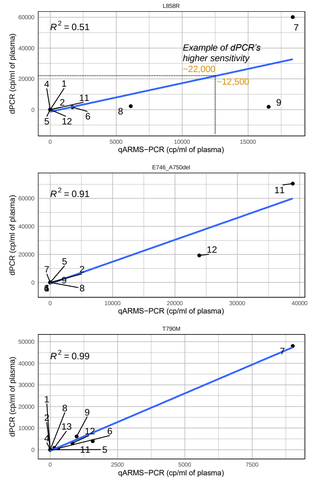
HD reference standards harbor mutations for T790M and C797S (T>A) (among others). For testing the above panel, we supplemented the reference standards with mutant DNA strings for the C797S (G>C), L792F, and L718Q mutations. Though there are false positives detected for T790M, they are very rare (Figure 3, light orange circles) and not dependent on fCEPH or DNA string concentrations (data not shown).

Figure 4. EGFR multiplex panel performance on reference standards and donor samples



Data generated from the dPCR from HD reference standards (left column) and donor samples (right column) for each of the EGFR Mutation Multiplex Panels. The left column shows the measured AF for the reference standards, labelled by their *expected* AF. The right column shows the AF for the donor samples. Different donor samples were selected for panel testing based on previous qARMS-PCR results. The “low input” label indicates that ≤ 3 positive microchambers were detected, suggesting that the result is a false positive.

Figure 5. Comparing Absolute Q Multiplex dPCR to duplex qARMS-PCR



Correlation of EGFR Multiplex Panels results from the Absolute Q dPCR to the duplex results gathered from qARMS-PCR. Each point represents a donor sample for which data was generated with both methods.

RESULTS

We optimized the multiplex dPCR assays to detect 5%-0.1% AF sensitively and specifically for each mutation with 10-40 ng of EGFR mutation-positive reference standard DNA (Figure 3 and 4). Because the expected mutant copies in 0.1% AF reference standards is so low (i.e., 3 mutant copies in 3,000 WT copies in 10ng of cfDNA), we tested 4 wells with the 0.1% AF standards. Although false positives are detected in our T790M results, they are rare and below the range which would be considered a true results (observations with ≤ 3 positive microchambers are unreliable) (Figure 3). Altogether, using the HD reference standards, the multiplex panels can distinguish wild-type DNA from 0.1% AF mutant DNA (Figure 4).

The results of the 13 donor samples show that the EGFR Multiplex panels can detect mutations in cfDNA isolated from donor plasma. Overall, we show good correlation (R^2) between our multiplex dPCR results and the duplex qARMS-PCR results (Figure 5). Importantly however, the dPCR method is frequently more sensitive than qARMS-PCR. An arbitrary point labelled by dotted lines along the blue regression line for the L858R panel indicates that the dPCR method can detect 22,000 copies per ml of plasma where the qARMS-PCR method detects only 12,500.

CONCLUSIONS

We developed a comprehensive multiplex genotyping method with high sensitivity and specificity for detection of EGFR driver, drug resistance and low frequency mutations using a fast and simple workflow. This study demonstrates a powerful multiplex dPCR application for EGFR mutation detection.

REFERENCES

1. Whale, Alexandra S., et al. “Fundamentals of Multiplexing with Digital PCR.” *Biomolecular Detection and Quantification*, Elsevier, 27 May 2016

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