Non-Invasive Absolute Quantification of EGFR Activating Mutation L858R and Gatekeeper Mutation T790M in NSCLC Plasma Samples Using Droplet Digital PCR

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Introduction

A high proportion of non-small cell lung cancer (NSCLC) patients treated with erlotinib or gefitinib develop resistance via emergence of the gatekeeper epidermal growth factor receptor (EGFR) T790M mutation.[1] The level and amount of the EGFR T790M resistance mutant in relation to EGFR L858R activating mutation, as opposed to simply the presence or absence of the mutations, has been shown to be critical for successful therapy selection in a mouse EGFR mutant lung cancer model.[2] Effective treatment of these patients will likely require both high-sensitivity and quantitative detection of EGFR activating and resistance mutations in liquid biopsies, not obtainable with current platforms. We have developed and optimized a droplet digital qPCR assay (ddPCR) that enables quantification of both EGFR activating L858R and resistance T790M mutations in circulating nucleic acids isolated from plasma.

Figure 1: Monitoring Resistance via Liquid Biopsy

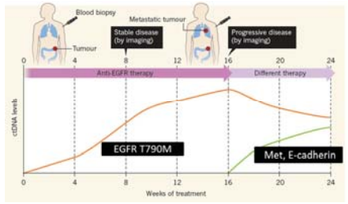

Figure 1. One major barrier to testing any hypothesis about the nature of acquired resistance to EGFR tyrosine kinase inhibitors is limited access to post-treatment tumor tissue. Even when post-treatment tumor tissue is available, sampling bias confounds interpretation because only a small portion of one tumor is usually biopsied, precluding assessment of genetic heterogeneity within or among lesions. Circulating, cell-free DNA (CNA) -- a form of 'liquid biopsy' -- provides a solution to circumvent tissue access problems. Analysis of CNA is informative because not only it can identify a specific mutant genotype, but it can also provide a measurement of the total tumor burden. Specifically, plasma isolated from blood potentially can be used to detect emerging resistance to first-generation EGFR tyrosine kinase inhibitors before detection by CT scanning. Attractive features of using CNA are low invasiveness, ease of obtaining samples at different time points and lack of spatial sampling bias.[3]

Methods

The ddPCR assay was developed using the Bio-Rad QX100 platform. Primers and probes were designed to optimize quantification of EGFR L858R and T790M mutations. The Qiagen EGFR Rotor-Gene (RGQ) assay was used according to manufacturer's protocol. Genomic DNA from the H1975 cell line expressing both EGFR L858R and T790M mutation was diluted into non-amplifiable nucleic acid to determine the assay's limit of detection (LOD). Patient samples enriched for the likelihood of EGFR T790M mutation were analyzed as well.

Figure 2: Droplet Digital PCR - An Enabling Technology Platform to Detect Rare Mutant DNA in Plasma

Figure 2. Digital PCR provides an ideal platform allowing detection of rare mutants in background DNA (Biorad product material). Our assay utilizes the Bio-Rad QX100 platform to detect rare EGFR T790M and L858R mutations in the background of wild type plasma circulating nucleic acids.

- Samples are partitioned into 20,000 droplets (right panels, in blue) using the QX100 cartridge, with target and background DNA evenly distributed among the droplets. Partitioning is completed with the use of a Bio-Rad proprietary surfactant and oil combined with a total 20uL PCR mix containing the target template of interest. Droplets are generated eight (8) reactions at a time and once generated are transferred to a thermal cycling plate and heat sealed with an aluminum non-adhesive seal.
- After PCR amplification, each droplet provides a fluorescent positive or negative signal indicating the target DNA was present or not present post-droplet generation. Following heat activation of the enzyme each well is thermal cycled a total 40 times.
- Each droplet provides an independent digital measurement. Droplets are analyzed with the QX100 Droplet Reader (bottom left) as an endpoint analysis.

Results

The ddPCR assay was designed to detect total EGFR in addition to EGFR mutations. Near the critical low level detection range of less than 1000 copies expected in plasma samples, the assay displayed a range from 770 copies to an LOD of 16 copies for EGFR T790M and L858R. In comparison, the RGQ T790M and L858R assays displayed a range of 770 copies to an LOD of 76 copies. In NSCLC patient plasma samples enriched for the likelihood of EGFR mutations, samples identified as positive for T790M by ddPCR were confirmed by the RGQ assay. In addition to quantifying mutant copies, the ddPCR assay determines the ratio of EGFR mutant to total EGFR template within a sample. In the current set of patient plasma samples analyzed, we were able to accurately quantify the ratio of T790M mutant compared to total EGFR from as low as 2ng DNA without pre-amplification. We compared the sensitivity of ddPCR versus RGQ analysis on healthy donor plasma samples spiked with H1975 cell line measuring both T790M and L858R. The assay additionally detected the Q787Q SNP in patient samples; a worse prognosis is predicted for patients with the Q787Q SNP.[4]

Figure 3: Optimized Conditions for Accurate Detection of EGFR L858R and T790M Mutations

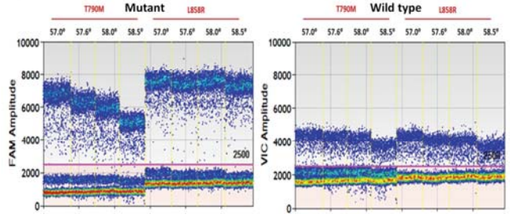

Figure 3. To complete analysis of multiple assays on a single sample, a temperature gradient was completed to visualize the best point of droplet distribution. Both probes for the T790M and the L858R assays are compared on a temperature gradient for the annealing-extension step of 57.0°-58.5° (x-axes, left to right). Though differences in droplet cluster amplitude are not noticeable for either wild type probe in the VIC wavelength, a large variability is seen with the FAM wavelength for T790M probe. These data suggest the best temperature to complete analysis of both assays within a single plate is 57.0 degrees.

Figure 4 : Allelic Frequency Detection of Both L858R and T790M by ddPCR

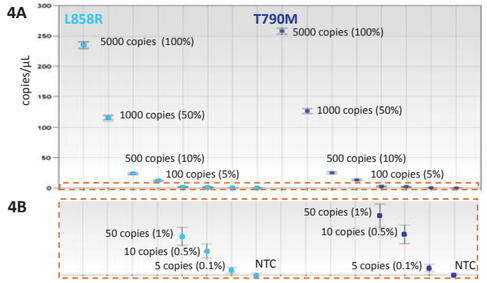

Figure 4A. Amplification products from L858 and T790 SNP PCR reactions were cloned as mutant and wild type templates into TOPO vector backbone. These plasmids were diluted, separately, mutant into wild type template for target percent amounts of 100, 50, 10, 5, 1.0, 0.5, 0.1% (identified in parenthesis); 5 - 5000 copies is a rough estimate (T790M at 0.1%=5.8 copies, L858R at 0.1%=3.62copies). Our ddPCR assay results demonstrate the limit of detection for both T790M and L858R is ~5 copies in a perfect sample.
Figure 4B. This figure contains a magnified view as indicated by the orange dashed line also seen in Figure 4A. Due to separate dilution series for each plasmid, the slight variation in copy number for each assay does not indicate differential efficiencies of the assays.

Table 1: ddPCR Assay Quantitation Confirmed with Copies/uL Analysis

% Mutant	Predicted Horizon (Copies/uL)	MMD-EGFR T790M (Copies/uL)	MMD-EGFR L858R (Copies/uL)
50	189.00	185.00	188.00
20	75.60	74.80	74.90
10	37.80	36.40	38.20
5	18.90	19.00	20.20
1	3.78	4.79	3.99
NTC	0	0	0

Table 1. Dilution series were made from gDNA derived from genetically engineered cell lines. Cell lines were purchased from Horizon Diagnostics at 50% target mutant. The dilution series were made following the suggested experimental outline on the Horizon website. The first column provides the expected % mutation followed by the predicted number of copies/uL at each Horizon dilution point. Copies/uL are calculated assuming 1 cell = 2 copies = 6.6pg. The ddPCR assay results in the two columns on the right specify the experimentally-determined copies/ul for each sample. Both assays correctly identified the copies/uL in association with the predicted Horizon diagnostics copies/uL.

Table 2: Comparison of Sensitivity - ddPCR vs. RGQ on H1975 Spiked Plasma Samples

Dilution Point	% T790M Mutant	ddPCR (Copies/uL)		RGQ (Ct) (ΔCt Cut off = 6.78)			Dilution Point	% L858R Mutant	ddPCR (Copies/uL)		RGQ (Ct) (ΔCt Cut off = 8.58)		
		T790M	T790 WT	Control Assay	ΔCt	T790M Assay			L858R	L858 WT	Control Assay	ΔCt	L858R Assay
A	62.006	38.73	23.73	27.45	2.41	29.86	A	61.948	40.7	25	27.45	3.06	30.51
B	20.075	7.89	31.40	28.05	4.1	32.15	B	20.859	8.83	33.5	28.05	4.76	32.81
C	10.576	3.82	32.30	28.38	4.93	33.31	C	9.509	3.51	33.4	28.38	5.64	34.02
D	2.531	0.81	31.00	28.30	7.51	35.81	D	2.330	0.80	33.7	28.30	8.78	37.08
E	0.728	0.25	34.50	28.09	11.76	39.85	E	0.737	0.27	36.6	28.09	10.2	38.29
F	0.422	0.18	41.37	27.86	-	NA	F	0.061	0.02	39.6	27.86	-	NA
G	0.075	0.03	34.90	28.07	-	NA	G	0.132	0.05	35.6	28.07	-	NA
H	0.068	0.02	36.50	28.25	-	NA	H	0	0	34.5	28.25	-	NA
Plasma ONLY	-	0.02	9.23	30.70	-	NA	Plasma ONLY	-	0	9.5	30.70	-	NA
NTC	-	0.00	0.00	0.00	-	NA	NTC	-	0	0	0.00	-	NA

Table 2. Dilutions of mutant DNA (T790M left columns; L858R right columns) spiked into healthy donor plasma were analyzed by both the ddPCR and RGQ assays for detection of T790M and L858R. Each sample, A – NTC, was analyzed in triplicate. 'Plasma ONLY' is analysis of background CNA purified from the healthy donor. Samples that were below the LOD for a given assay are indicated in red. For both the T790M and L858R ddPCR assays, the lowest copies/uL call for each assay was 0.81 and 0.80 copies/uL for T790M and L858R respectively, or ~2.5%. For the RGQ T790M and L858R assays, both assays failed with samples D-H as defined by the kit's Ct range in relation to the control assay for the same sample; therefore the mutations were detectable down to ~10% with the RGQ assays.

Figure 5. Graphical representation of the data from Table 2 (ddPCR only) at the lower concentration dilutions of both T790M and L858R. The line represents the LOD for both ddPCR assays which is ~10 copies.

Figure 5: H1975 Spiked Plasma ddPCR Results

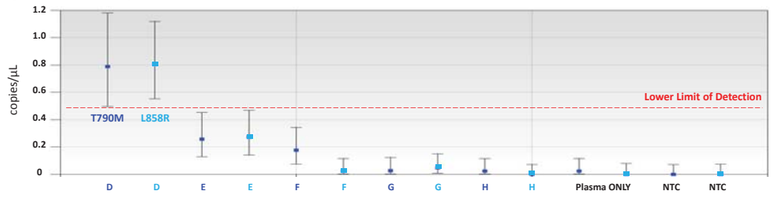

Table 3: ddPCR Detection of EGFR T790M in Blinded Patient Plasma Samples – Assay Reproducibility

MolecularMD ID	T790M (Copies/uL)			
	Day 1		Day 2	
	Replicate 1	Replicate 2	Replicate 1	Replicate 2
MMD-2	0.00	0.00	0.00	0.07
MMD-4	0.00	0.00	0.00	0.00
MMD-5	0.00	0.00	0.14	0.00
MMD-11	0.07	0.07	0.07	0.21
MMD-12	0.17	0.17	0.29	0.17
MMD-13	0.26	0.07	0.15	0.16
MMD-14	**74.80**	**80.60**	**70.60**	**65.20**
MMD-15	0.22	0.22	0.21	0.00
MMD-16	35.20	34.90	36.70	32.60
MMD-17	0.07	0.00	0.15	0.14
MMD-18	0.08	0.21	0.14	0.08
MMD-19	0.00	0.00	0.00	0.08
MMD-20	0.21	0.07	0.08	0.22

Table 3. Blinded NSCLC patient plasma samples were acquired from an outside vendor plasma bank. Patients MMD-14 and MMD-16 were identified via our ddPCR assay as positive for the EGFR T790M mutation. The sample numbering is discontinuous because some of the purifications did not have enough remnant sample to repeat analysis with the minimum two replicates.

Figure 6. Quadrant views for Table 3 data; repeat ddPCR analyses of patient MMD-14 samples on Day 1 (left panel) and Day 2 (right panel).

Figure 6: ddPCR Results Detail – Assay Reproducibility

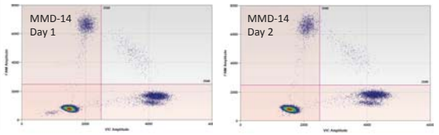

Figure 7: Identification of Q787Q SNP in Plasma Sample

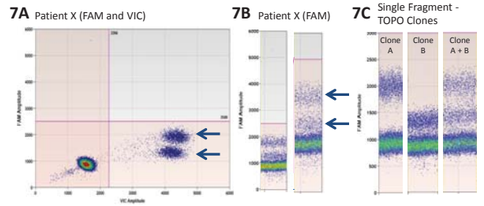

Figure 7. ddPCR EGFR T790M results for Patient X plasma sample indicate a wild type T790 signature with distinct fluorescence clusters (Figure 7A: four quadrant view, lower right corner; Figure 7B: one-dimensional FAM amplitude view, magnified at right). To differentiate the fragments, ddPCR amplification product was cloned into the Invitrogen TOPO Vector and sequenced; the only difference was a single base mutation at amino acid 787 (data not shown). Individual clones analyzed by ddPCR either alone or combined (Figure 7C) created the same fragment signature seen in 7A and 7B, confirming the identity of the two distinct clusters in patient X, and indicating the presence of Q787Q SNP.

Figure 8: ddPCR vs. RGQ EGFR-T790M Identification in Patient Plasma

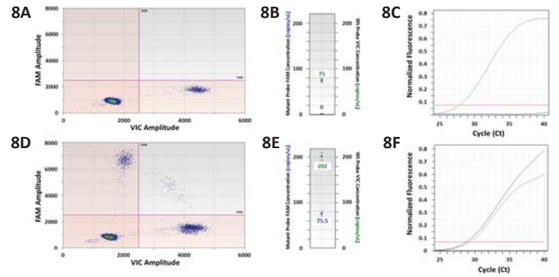

Figure 8. Samples from a patient wild type for EGFR (Panels A-C) and a patient harboring the EGFR T790M mutation (Panels D-F) were analyzed using the ddPCR assay (panels A, B, D, E) or the RGQ assay (Panels C, F). Both systems identified the patient harboring the EGFR T790M mutation as positive for the EGFR T790M mutation. Note that only the ddPCR assay enabled quantification that the mutation represented 27.2% of the total EGFR templates in the T790M-positive patient specimen ((75.5/(202+75.5))x100).

Conclusions

- Droplet digital PCR provides sensitive quantification of low abundance EGFR T790M resistance mutation and L858R activating mutation in plasma without the need for pre-amplification.
- This ddPCR assay provides a useful method for selecting treatment, monitoring disease progression, and providing early detection of treatment failure associated with EGFR acquired resistance

References

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