QX100 ddPCR Assay Development and Optimization for EGFR T790M Mutation: Identification in CNAs

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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] Liquid biopsies (analysis of circulating, cell-free DNA) offer advantages for monitoring development of resistance mutations in patients for whom tumor biopsies are limited.[2] We have developed and optimized a droplet digital qPCR assay that enables quantification the EGFR resistance mutation, T790M, in circulating nucleic acids (CNAs) isolated from patient plasma samples.

Methods

The ddPCR assay was developed using the Bio-Rad QX100 platform. Primers and probes were designed to target quantification of EGFR T790M mutations. The Qiagen EGFR Rotor-Gene (RGQ) assay was used according to manufacturer's protocol. EGFR T790M positive cell line NCI-H1975 (ATCC) genomic DNA (gDNA) was used for assay development and optimization work. Plasma purification of CNAs was carried out using QIAamp DSP Circulating NA Kit (Qiagen).

Results

The designed ddPCR assay detects total EGFR in addition to EGFR T790M mutations. The concentration (copies/µL) value measured using our EGFR T790M assay demonstrated concordance with reference gDNA (Horizon Diagnostics). Viable methodologies for patient plasma processing and CNA isolation were established. Analysis of clinically relevant patient plasma specimens identified a T790M expressing sample, which was confirmed using the Qiagen RGQ platform.

Analytical Development

Figure 1: Temperature Gradient Optimization

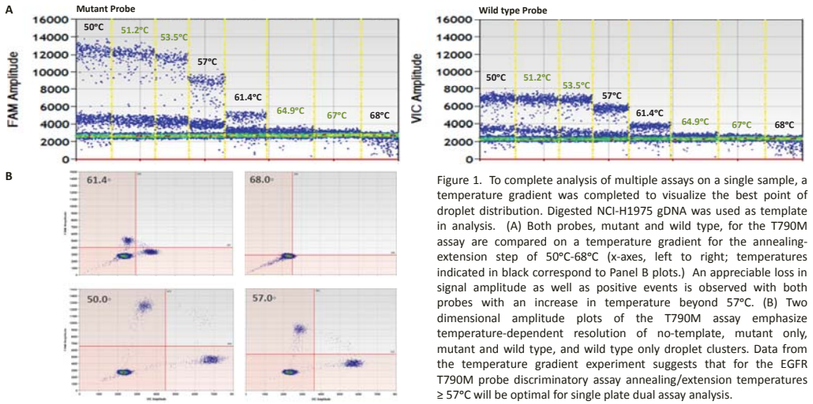

Figure 1. To complete analysis of multiple assays on a single sample, a temperature gradient was completed to visualize the best point of droplet distribution. Digested NCI-H1975 gDNA was used as template in analysis. (A) Both probes, mutant and wild type, for the T790M assay are compared on a temperature gradient for the annealing-extension step of 50°C-68°C (x-axes, left to right; temperatures indicated in black correspond to Panel B plots.) An appreciable loss in signal amplitude as well as positive events is observed with both probes with an increase in temperature beyond 57°C. (B) Two dimensional amplitude plots of the T790M assay emphasize temperature-dependent resolution of no-template, mutant only, mutant and wild type, and wild type only droplet clusters. Data from the temperature gradient experiment suggests that for the EGFR T790M probe discriminatory assay annealing/extension temperatures ≥ 57°C will be optimal for single plate dual assay analysis.

Figure 2: Probe Design Resolution Optimization

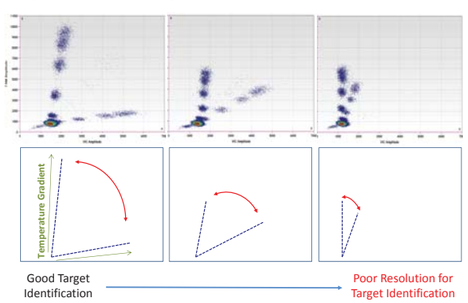

Figure 2. Various EGFR T790M assay probes differing by length and size were analyzed for differences in droplet distribution and amplitude over a temperature gradient identical to Figure 1. Amplitude and resolution of the positive events are shifted based on probe modifications. Slight alterations in SNP location within the probe, probe location on the target template, and probe length affect correct target identification.

Figure 3: Threshold Definition for Lower Limit of Detection

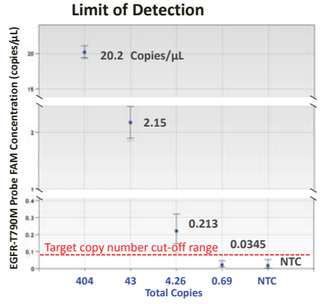

Figure 3. Cell line NCI-H1975 gDNA was restriction enzyme digested and used to create a dilution series to determine the QX100 T790M assay lower limit of detection. The cut-off range is defined as above the no-template control's (NTC) 95% confidence interval. Samples would be disqualified as positive (ie. 0.69) if the respective 95% confidence interval overlaps with that of the no-template control.

Data Interpretation

Figure 4 : Observed Droplet Amplitude Shift

Sample ID	Mutant gDNA input (ng)	Mutant (Copies/µL)	Wild type gDNA input (ng)	Wild type (Copies/µL)
A	2	9.24	0	3.08
B	2	10.30	2	24.6
C	2	9.55	4	43.0
D	2	9.47	6	63.7
E	2	9.81	8	85.2
F	2	9.79	10	104.0
G	2	10.50	12	122.0
H	2	9.07	20	214.0
I	2	10.70	40	419.0
J	2	10.20	60	631.0
K	2	10.00	80	817.0
L	2	10.10	100	1030.0
M	2	11.00	120	1290.0
N	2	11.40	140	1430.0
O	2	11.10	160	1620.0

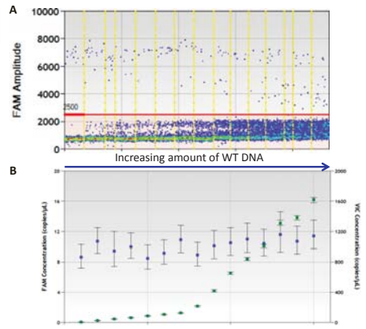

Figure 4: Samples identified A-O each contained 2ng NCI-H1975 gDNA (T790M positive) mixed with wild type gDNA ranging from 0 - 160ng. Copies/µL of EGFR WT (green) and T790M (blue) were determined with the droplet digital PCR assay . With increasing wild type template amounts, droplets positive for the FAM probe exhibit a decrease in fluorescence amplitude (Panel A). Total overall T790M copies/µL identified in each sample exhibits very little fluctuation over the range of concentrations of wild type template (Table, Panel B).

Figure 5: Observation of Abnormal Droplet Distribution Phenomenon

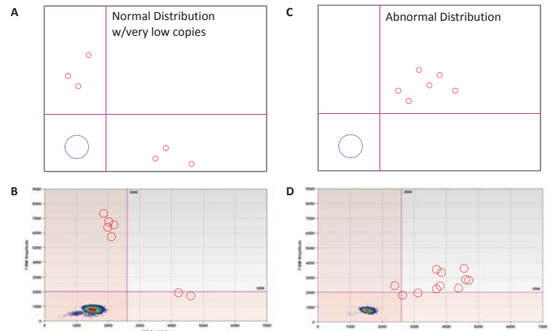

Figure 5. (A) Graphic representation of droplet distribution expected when low copy sample analysis occurs. (B) Two-dimensional plot of a low copy number sample with minimal positive events observed in the FAM (mutant probe) only and VIC (wild type probe) quadrants. (C) Graphic representation of abnormal droplet distribution. Of particular note is the 45° angle droplet distribution of positive events present in FAM(+)-VIC(+) quadrant. (D) Two-dimensional plot of no template control sample showing abnormal distribution of droplets. The probability of double positive droplets present in low copy samples is very unlikely and are most likely the consequence of droplet disruption during transfer between the droplet generation cartridge and thermocycling plate.

Table 1: Concentration Defined by Amplifiable Template

Nanodrop		QX100	Difference
Concentration (ng/µL)	Predicted Total (Copies/µL)	Total (Copies/µL)	Copies/µL vs. Copies/µL (%)
22.74	329.70	282.30	85.62
22.56	327.10	278.00	84.99

Table 1. Identical genomic DNA samples were quantitated for concentration in duplicate using a NanoDrop (Thermo Scientific)(ng/µL) and the QX100 EGFR T790M assay (copies/µL). Concentration values derived from the Nanodrop were converted to copies/µL using the Illumina DNA copy number calculator.[3] Concentrations in copies/µL between the two methods were compared for concordance. The percent difference between methodologies is represented in the far right column. Results indicate differences observed when comparing overall nucleic acid quantification determined by the NanoDrop and amplifiable template by the QX100 EGFR T790M assay. NanoDrop analysis can be skewed by sample contaminants while QX100 assays can be skewed by enzyme/assay efficiency.

Clinical Validation

Table 2: EGFR T790M Assay Accuracy

% Mutant	Horizon (Copies/µL)	MMD-EGFR T790M (Copies/µL)
50	181.25	188.2
20	72.50	75.4
10	36.25	35.1
5	18.10	19.8
1	3.60	3.89
NTC	0	0

Table 2. Dilution series were made from gDNA derived from genetically engineered cell lines. Genomic DNA Reference Standards were purchased from Horizon Diagnostics at 50% target mutant. The dilution series was made following the suggested experimental outline on the Horizon website. The first column provides the expected % mutation followed by the predicted number of copies/µL at each Horizon dilution point. Copies/µL were calculated using the Illumina DNA copy number calculator.[3] Our T790M assay correctly identified the copies/µL in association with the predicted Horizon diagnostics copies/µL.

Figure 6: Inherent Variation in Patient Samples

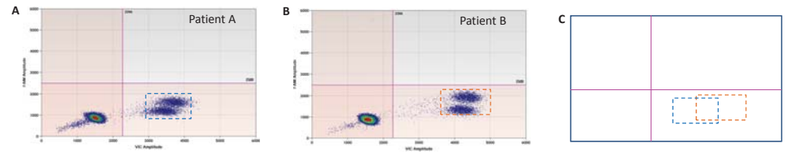

Figure 6. Two-dimensional plots for two unique purified patient plasma specimens (Patient A and Patient B) each containing two distinct clusters in the no template droplet and the wild type template only quadrants. MolecularMD has noted distinct differences in varying patient cluster amplitudes (or cluster locations). (C) Graphic representation of the unique wild type clustering seen with Patient A and Patient B. We attribute cluster differences to variable fragmentation of template input and/or purified DNA quality.

Figure 7: Patient Plasma Concordance Testing

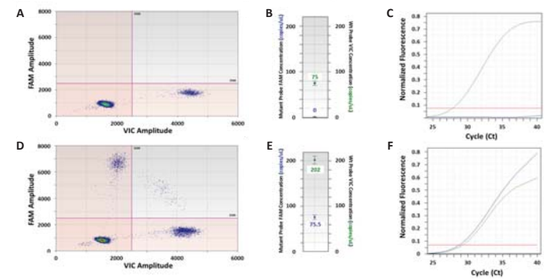

Figure 7. Samples from a patient wild type for EGFR (Panels A-C) and a patient harboring the EGFR T790M mutation (Panels D-F) were analyzed using the ddPCR assay (panels A, B, D, E) or the RGQ assay (Panels C, F). Both systems identified the patient harboring the EGFR T790M mutation as positive for the 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 a sensitive quantification platform for detection of low abundance EGFR T790M resistance mutation in plasma without the need for pre-amplification.
- We have optimized and validated a plasma-based assay for EGFR T790M detection using the QX100 and identified several parameters including threshold determination that need to be taken into account for QX100 assay development.

References

1. Sequist, L.V., Waltman, B.A., *et al., Sci. Transl. Med.* 2011; 3(75)
2. Vilar, E. and Tabernero, J., *Nature* 2012; 486: 482-483
3. Illumina DNA copy number calculator http://www.ecoqpcr.com/support/useful_tools.ilmn

For Further Information

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