Mutation Detection Multiplexing Using the QX600 Droplet Digital PCR System

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Abstract

Precise and rapid mutation detection is critical to the advancement of cancer research, as patient outcomes depend on the early and accurate identification of these mutations. Compared to other methods of mutation detection, Droplet Digital™ PCR (ddPCR™) allows researchers to detect rare mutations at very low fractional abundance, with faster turnaround times, and at lower costs. In this study, we demonstrate how the QX600™ ddPCR System can detect mutations commonly associated with non–small-cell lung cancer (NSCLC) in a single-well, 6-plex reaction. While multiplexing cancer mutations is often challenging due to the need to discriminate multiple sets of highly similar sequences in a single well, here we show the capability of the QX600 ddPCR System to detect mutations with simple analysis.

Introduction

Mutation detection is vital in translational and clinical oncology research, as cancers arise from germline and somatic genetic mutations. Molecular profiling utilizing mutation detection can help to inform early detection and diagnosis, treatment selection and response, and monitoring of minimal residual disease (MRD), ultimately resulting in improved survival rates and patient outcomes. Mutation detection entails the detection of a variant sequence present at a minute frequency in a wild-type (WT) genetic background. The challenge, therefore, is the precise discrimination between two highly similar sequences, one of which is significantly more abundant than the other. The ability to detect and quantify very rare events is particularly important in noninvasive sample types like cell-free DNA (cfDNA) from plasma.

Droplet Digital PCR enables mutation detection with sensitivity and precision levels beyond the capabilities of other methods. In identifying mutations, ddPCR Systems enable researchers to find "a needle in the haystack" by partitioning a sample into nanoliter-sized droplets. Compared to next-generation sequencing (NGS), ddPCR Systems have higher sensitivity and are capable of detecting mutations at a minor allele frequency (MAF) rate as low as 0.001%. Comparatively, most validated clinical NGS platforms have a lower limit of detection between 2–15%, depending on the workflow and mutation being detected (Singh 2020). Most NGS workflows are not ideally suited for specialized applications requiring detection of low-level mutations, such as monitoring therapy response and minimal residual disease by liquid biopsy. Other advantages of ddPCR Systems over NGS include faster turnaround time, less hands-on-time, and lower cost. These combined benefits allow ddPCR technology to advance discoveries and improve outcomes across the patient journey. It is currently utilized in targeted mutational screening and monitoring, therapy response tracking, and molecular relapse monitoring, and appears in over 2,000 peer-reviewed publications related to mutation detection and liquid biopsy (bio-rad.com/ddPCR/publications).

Bio-Rad's QX600 Droplet Digital PCR System has six channels to enable advanced multiplexing, which researchers and investigators can utilize to obtain more answers from precious samples. Here, we demonstrate an elegant 6-plex mutation detection example that leverages the sensitivity and accuracy of the QX600 ddPCR System.

Materials and Methods

The objective of the experiment was to create a multiplex assay for the quantification of multiple mutations and a WT control in a single well on the Bio-Rad QX600 ddPCR System. Mutation targets that were used for the assay design are shown in Table 1, and were combined with the ddPCR *EGFR* Exon 19 Deletions Screening Kit (Bio-Rad Laboratories, Inc., catalog #12002392) to detect the *EGFR/KRAS/BRAF* mutant targets and *EGFR* WT. Assays were combined to create a 1x solution and have been made available as a ddPCR Expert Design Assay (catalog #12008212; assay ID: dEXD95349967). Analytical materials used throughout the experiment were synthetic DNA sequences (gBlocks Gene Fragments, Integrated DNA Technologies, Inc. [IDT]) and Human

Table 1. Mutation detection assays and corresponding detection channels.

Assay	Detection Channel
EGFR Exon 19 Deletions	FAM
EGFR WT	HEX
KRAS G12C	Cy5
EGFR L858R	Cy5.5
BRAF V600E	ROX
EGFR T790M	ATTO 590

WT, wild type.

Table 2. ddPCR reaction setup.

Component	Volume per Reaction, µl	Final Concentration
ddPCR Multiplex Supermix	5	1x
20x 4-Plex Assay (*EGFR L858R*, *KRAS G12C*, *BRAF V600E*, *EGFR T790M*; (ddPCR Expert Design Assay, catalog #12008212; assay ID: dEXD95349967)	1	900 nM primers/ 250 nM probe
20x *EGFR* Exon 19 Deletions Screening Assay (#12002392)	1	1x
Target DNA sample and/or DNase-free water	Variable	
Total volume	**20***	

* For the Automated Droplet Generator, prepare 22 µl per well.

Table 3. Recommended thermal cycling conditions for the Bio-Rad C1000 Touch Thermal Cycler.*

Cycling Step	Temperature, °C	Time	Ramp Rate, °C/sec	Number of Cycles
Enzyme activation	95	10 min	2	1
Denaturation	94	30 sec	2	40
Annealing/ extension	55	1 min	2	40
Enzyme deactivation	98	10 min	2	1
Hold (optional)	4	Infinite	2	1

* The same protocol can be used for the Bio-Rad PTC Tempo Deepwell Thermal Cycler (#12015392; see bulletin 3530).

Genomic DNA (Female) from Promega Corporation (catalog #G1521) for the WT quantification. No template controls (NTCs) were performed using nuclease-free water in place of gBlocks Gene Fragments. Full assay concentrations for the components of a 1x assay were 900 nM of each primer and 250 nM of each probe. ddPCR Multiplex Supermix (Bio-Rad, #12005909) was used. Table 2 shows the reaction components, including the master mix and all other reagents.

Droplet generation was performed on the Automated Droplet Generator (Bio-Rad, #1864101) using DG32 Automated Droplet Generator Cartridges (Bio-Rad, #1864108) and Automated Droplet Generation Oil for Probes (Bio-Rad, #1864110). Thermal cycling was performed on the C1000 Touch Thermal Cycler with 96–Deep Well Reaction Module (Bio-Rad, #1851197) with the thermal cycling protocol listed in Table 3. Detection was performed on the QX600 Droplet Reader (Bio-Rad, #12013328) using ddPCR Droplet Reader Oil (Bio-Rad, #1863004).

Results

These data show that the QX600 System and Bio-Rad multiplex assay enable multiplex mutation detection with simple analysis. The NTC samples are clean, with no positive clusters detected in the NTC control wells, as shown in Figure 1. In Figure 2, the two-dimensional fluorescence amplitude plots that were generated after performing the multiplex ddPCR reaction demonstrate that the distinct targets form unique clusters in a single well. The FAM (mutant)/HEX (WT) channels are derived from the ddPCR *EGFR* Exon 19 Deletions Screening Kit, a nondiscriminatory assay for 15 different *EGFR* mutations on exon 19 of the *EGFR* gene. The remaining four assays belong to different cancer mutation assays: *KRAS G12C*, *EGFR T790M* and *L858R*, and *BRAF V600E*. These mutations are commonly associated with NSCLC samples.

Beyond the mutations shown here, the QX600 System can be used for many types of cancer assays. The Bio-Rad assay catalog and design engine are available to support numerous additional mutation detection assays. An expert design service is also available for advanced design needs and other applications.

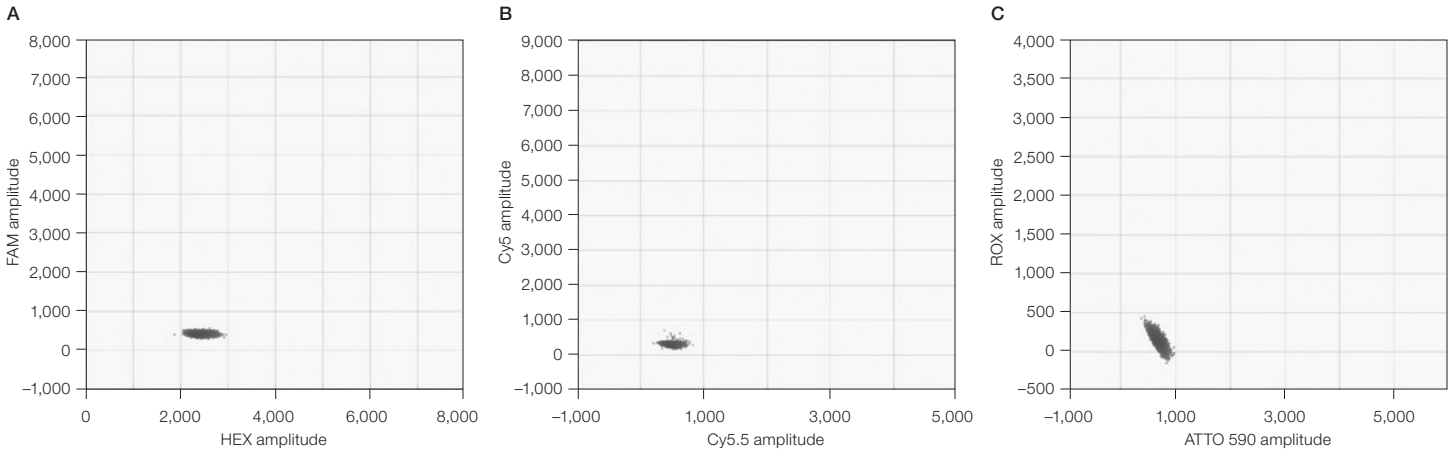

Fig. 1. Multiplex ddPCR analysis of no template control (NTC) samples with a 6-plex mutation detection assay. The two-dimensional plots show no amplification in any of the NTC wells. The assay mix shows a clean assay and environment with no false positives, nonspecific binding, or primer interactions. **A**, data for *EGFR* exon 19 deletions (FAM) and wild-type *EGFR* (HEX). **B**, data for *KRAS G12C* (Cy5) and *EGFR L858R* (Cy5.5). **C**, data for *BRAF V600E* (ROX) and *EGFR T790M* (ATTO 590).

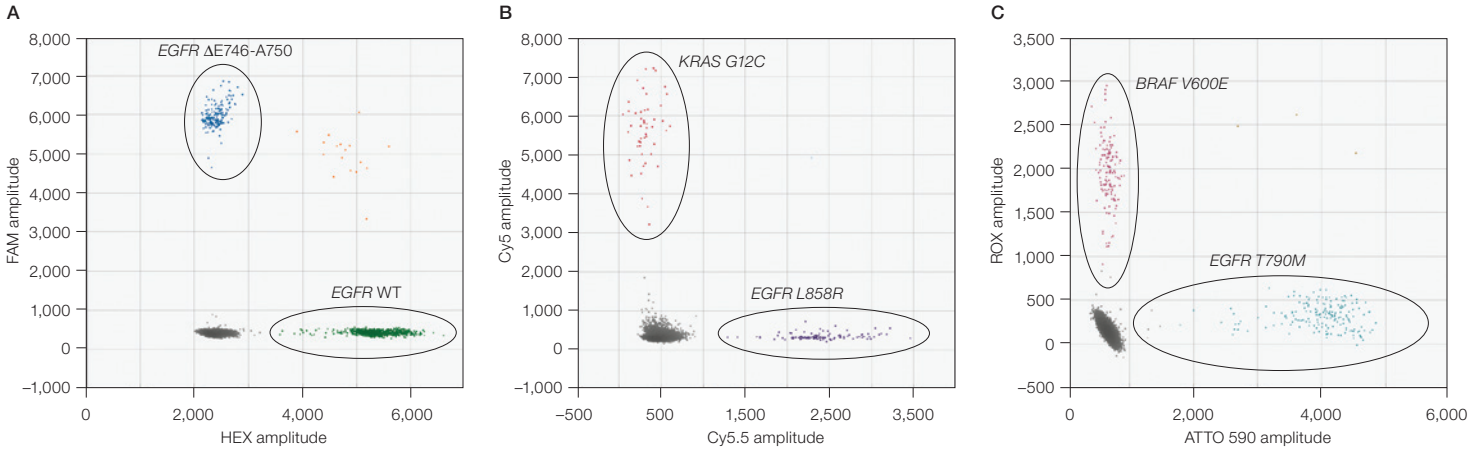

Fig. 2. The two-dimensional fluorescence amplitude plots show a single well of five different mutant targets and a single wild-type (WT) *EGFR* target. A, data for the FAM and HEX channels, showing *EGFR* exon 19 deletion-positive mutants (blue) and *EGFR* WT positive droplets (green). The black cluster represents droplets negative for both targets. **B**, The Cy5/Cy5.5 channels, with *KRAS G12C* positive droplets (light red) and *EGFR L858R* positive droplets (purple). The black cluster represents negative droplets. **C**, The ROX/ATTO 590 channels, with *BRAF V600E* positive droplets (dark red) and *EGFR T790M* positive droplets (cyan). The black cluster represents negative droplets.

Conclusions

Multiplexing allows for the detection of multiple targets from a small sample, saving costs and increasing sample throughput. However, multiplexing in mutation detection is challenging due to the need to discriminate multiple sets of highly similar sequences in a single well. This study demonstrates that the QX600 Droplet Digital PCR System can detect a 6-plex mutation detection assay with simple analysis, which is currently unique among similar systems on the market. The advanced 6-color detection capabilities of the QX600 ddPCR System set a new standard in user-friendliness when developing highly multiplexed assays.

References

Singh RR (2020). Next-generation sequencing in high-sensitive detection of mutations in tumors: Challenges, advances, and applications. J Mol Diagn 22, 994–1007.

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