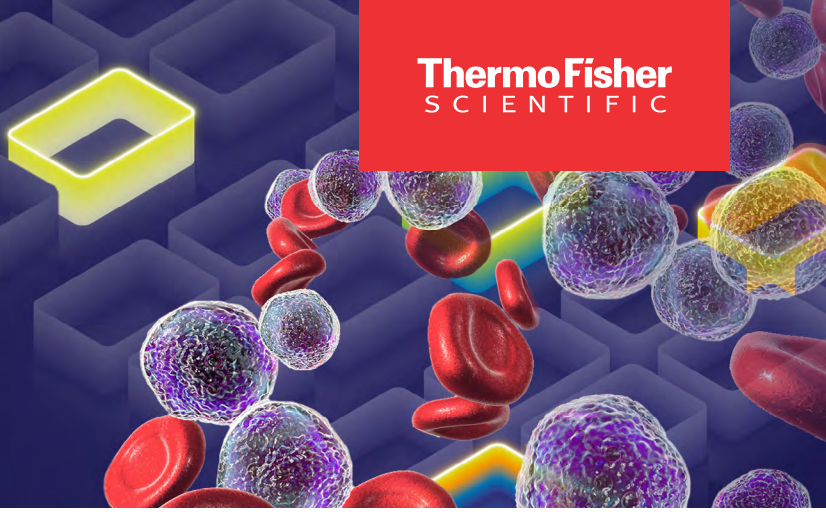

Cancer research

Rare-target quantification on the QuantStudio Absolute Q dPCR System

Leveraging Absolute Q Liquid Biopsy dPCR Assays

Highlights

- Detection of mutation allele frequencies (MAFs) of 0.1% for 30 relevant cancer mutations commonly used in liquid biopsy applications
- Complete, one-step walk-away workflow for digital PCR (dPCR)
- Use of microfluidic array plate (MAP) consumable for emulsion-free dPCR
  - Low sample waste, high microchamber yield
  - Single consumable, single instrument

Introduction

Mutation screening is becoming a standard technique for evaluating treatment options of patients diagnosed with cancer. Notably, certain hotspot mutations can give valuable insight into efficacy of response to various treatments. For example, mutations such as *KRAS* p.G12D [1], *KRAS* p.G12V [2] and *KRAS* p.G13D [3] indicate potential reduced responsiveness to tyrosine kinase inhibitors (TKIs), whereas *PIK3CA* mutations such as p.H1047R indicate positive response to PI3K/AKT/mTOR signaling pathway inhibitors [4]. Liquid biopsies, in which blood is taken and assayed for traces of cancer in circulating cell-free DNA (cfDNA), provide a much less invasive way to examine the genetic composition of a patient's tumors and can even help with diagnosis. However, detecting circulating tumor DNA (ctDNA) for liquid biopsy applications is challenging because the molecules bearing the target of interest is only a small fraction of the total circulating cfDNA collected in the sample.

Liquid biopsy assays must be able to accurately quantify rare, single-nucleotide polymorphisms (SNPs), among high levels of wild type background DNA, with outstanding analytical sensitivity and precision. Real-time or quantitative PCR is a tried-and-true method for detecting mutations with oncological significance, but it can struggle with precise quantitation of rare mutations. With its exceptional precision and analytical sensitivity, dPCR is ideally suited for liquid biopsy applications in which small amounts of relevant mutations exist in the sample.

In this research study, the Applied Biosystems™ QuantStudio™ Absolute Q™ Digital PCR System was used with the Applied Biosystems™ Absolute Q™ Liquid Biopsy dPCR Assays to perform rare-target detection for 30 hotspot cancer mutations in the *KRAS*, *EGFR*, and *PIK3CA* genes. MAFs down to 0.1% were detected among a high-background wild type sample concentration.

Workflow and methods

Titration series preparation

A total of 30 hotspot cancer mutations were selected for this study (Table 1). For each of the mutations selected, DNA mixtures were prepared by combining mutation-bearing plasmids with wild type gDNA. Each DNA mixture contained 50 ng of gDNA, and mutation-encoding plasmid targeted a final MAF of 0.1%. Two replicates of the DNA mixture were tested for each assay alongside 1–2 reactions of a 100% wild type (or reference) control.

Table 1. Assay names and mutations used in the study.

Assay name	Mutation
BRAF 473	*BRAF p.V600K*
BRAF 476	*BRAF p.V600E*
EGFR 12382	*EGFR p.L747 A750>P*
EGFR 12384	*EGFR p.E746_S752>V*
EGFR 12387	*EGFR p.L747_P753delinsQ*
EGFR 12728	*EGFR p.E746_T751delELREAT*
EGFR 6213	*EGFR p.L861Q*
EGFR 6223	*EGFR p.E746_A750delELREA*
EGFR 6224	*EGFR p.L858R*
EGFR 6225	*EGFR p.E746_A750delELREA*
EGFR 6252	*EGFR p.G719S*
EGFR 6253	*EGFR p.G719C*
EGFR 6255	*EGFR p.L747_S752del*
IDH1 28747	*IDH1 p.R132C*
JAK2 12600	*JAK2 p.V617F*
KIT 1314	*KIT p.D816V*
KRAS 19900	*KRAS p.A146V*
KRAS 516	*KRAS p.G12C*
KRAS 517	*KRAS p.G12S*
KRAS 520	*KRAS p.G12V*
KRAS 521	*KRAS p.G12D*
KRAS 527	*KRAS p.G13C*
NPM1 17559	*NPM1 p.W288fs*12*
NRAS 564	*NRAS p.G12D*
PIK3CA 775	*PIK3CA p.H1047R*
PIK3CA 776	*PIK3CA p.H1047L*
TP53 10660	*TP53 p.R273H*
TP53 10662	*TP53 p.R248Q*
NRAS 580	*NRAS 580 p.Q61K*
TP53 10779	*TP53 p.R273L*

QuantStudio Absolute Q dPCR System workflow

Using the simple workflow for the QuantStudio Absolute Q dPCR System (Figure 1), PCR mix was prepared using the Applied Biosystems™ Absolute Q™ DNA dPCR Master Mix (5X), and 9 µL of the sample mix was loaded into the Applied Biosystems™ QuantStudio™ MAP16 Digital PCR Plate. Subsequently, 15 µL of Applied Biosystems™ QuantStudio™ Absolute Q™ Isolation Buffer was overlaid into each sample well, and gaskets were applied across all units of the plate. Finally, the plate was loaded onto the QuantStudio Absolute Q dPCR System where digitization, thermal cycling, and data collection were completed by the instrument in approximately 90 minutes.

The thermal cycling parameters for use of the Absolute Q Liquid Biopsy dPCR Assays on the QuantStudio Absolute Q dPCR System are listed in Table 2. For each assay, a replicate of wild type control gDNA was run in parallel to verify the final placement of the Applied Biosystems™ FAM™ dye channel threshold line. Figure 2 illustrates the resulting dPCR data from both 0.1% MAF DNA and wild type DNA control conditions, with thin black lines lines denoting the threshold placement of the Applied Biosystems™ VIC™ dye and FAM dye channels. If FAM dye–positive microchambers were identified in the wild type DNA controls, these concentrations (cp/µL) were used as a baseline and subtracted from the final reported concentration (cp/µL) of the mutation-bearing DNA mixture conditions.

Table 2. Thermal cycling parameters on the QuantStudio Absolute Q dPCR System.

Temperature	Duration (MM:SS)	Cycles
96°C	10:00	1
96°C	0:05	40
60°C	0:15	

Figure 1. Workflow of the QuantStudio Absolute Q Digital PCR System.

Results

Detection of rare mutation alleles

For this study, 30 cancer-relevant Absolute Q Liquid Biopsy dPCR Assays were selected to demonstrate the high precision and analytical sensitivity of the QuantStudio Absolute Q dPCR System (See "Workflow and methods"). For each assay, the total number of mutation molecule copies and observed MAFs were calculated for an 0.1% MAF condition. Figure 3 illustrates the total copies per reaction detected for the mutant (grey) and reference (blue) alleles for each of the thirty Absolute Q Liquid Biopsy dPCR Assays. As expected, each DNA mixture reported approximately 0.1% mutation copies per reaction.

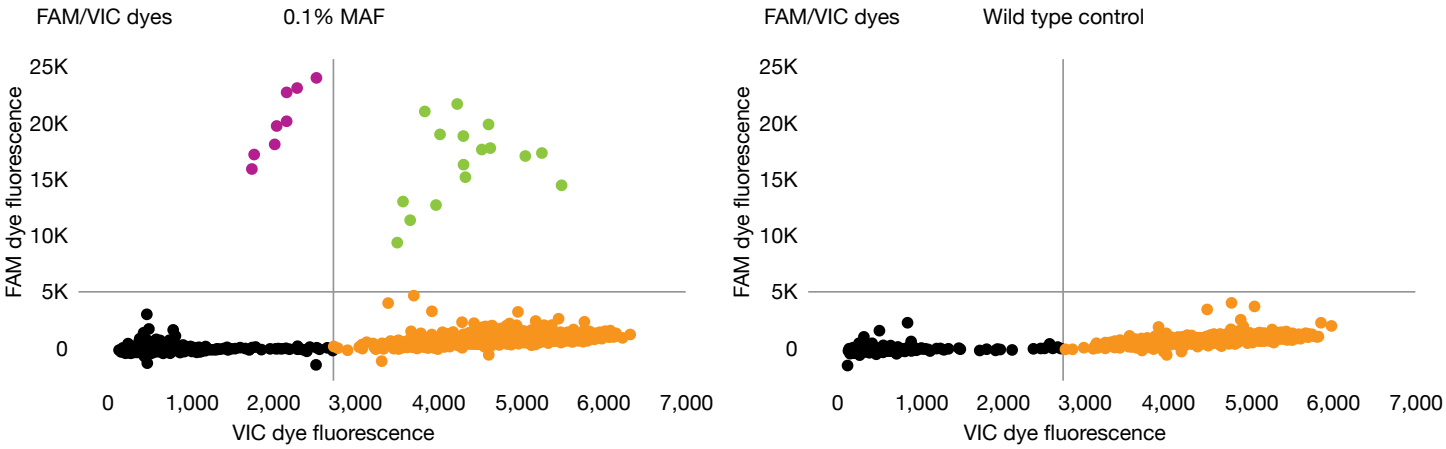

Figure 2. A demonstration of the ability of the QuantStudio Absolute Q dPCR System to detect mutant *KRAS p.G12V* alleles (purple dots) against a background of 99.9% wild type *KRAS* alleles (green dots) using the *KRAS 520* assay. Orange dots represent dPCR microchambers positive for both alleles.

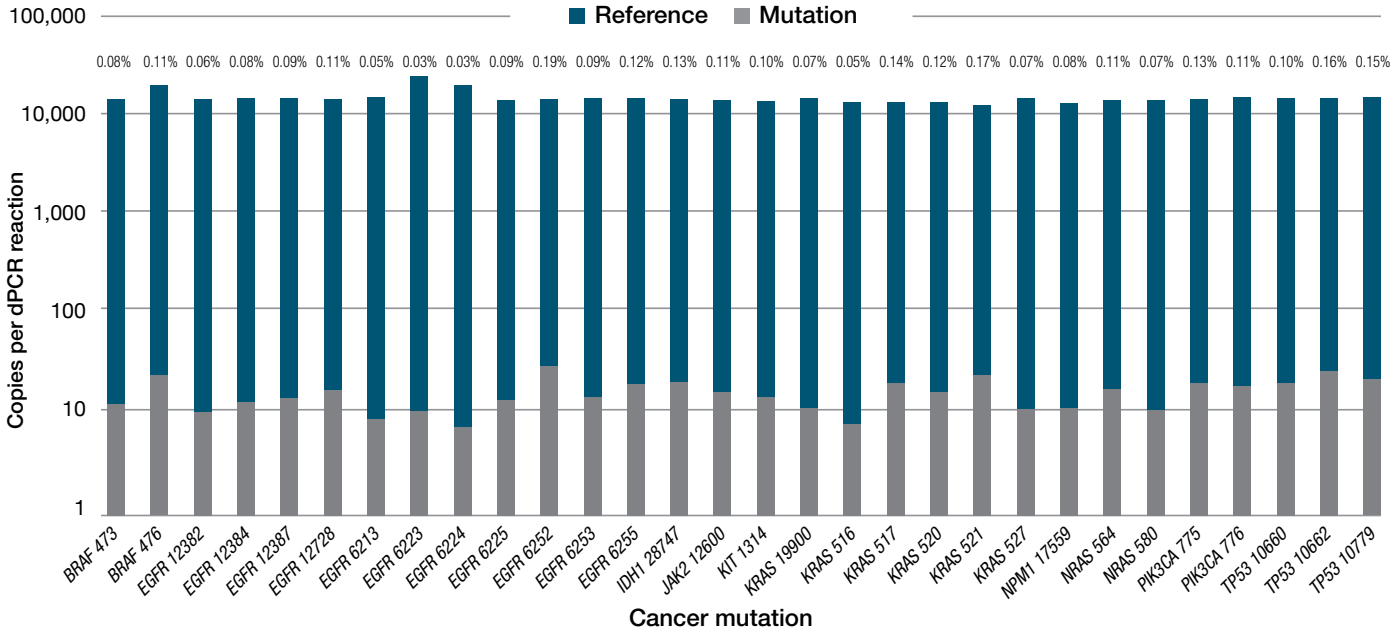

Figure 3. Quantification of cancer mutations (total copies per reaction) using QuantStudio Absolute Q Liquid Biopsy dPCR Assays on the QuantStudio Absolute Q dPCR System for each of the thirty Absolute Q Liquid Biopsy Assays tested.

Absolute Q DNA dPCR Master Mix for low-concentration samples

A major challenge for many liquid biopsy applications is detection of ultrarare variants that exist at extremely low concentrations. Sample input volume alongside dead volume can inhibit the detection of these rare variants by limiting the total number of accessible molecules possible for input. To address this, the Absolute Q DNA dPCR Master Mix was designed at a 5X concentration, which is particularly useful for liquid biopsy assay applications. Use of this formulation enables users to add up to 66% more sample volume to each dPCR reaction than when using a 2X formulation.

Efficient reagent digitization with the QuantStudio MAP16 Digital PCR Plate

The QuantStudio Absolute Q dPCR system utilizes a novel method of reagent distribution on fixed microfluidic array plates (MAPs). Without a reliance on emulsion-based reagent digitization, over 95% of the loaded sample is analyzed in each unit, improving confidence in rare-molecule detection. The QuantStudio MAP16 Digital PCR Plate consumable consistently and uniformly fills approximately 20,000 microchambers with no user interaction or manipulation. Out of a total of 119 dPCR reactions run for this experiment, the average number of microchambers accepted per reaction was 20,477 ± 20 (standard deviation) per dPCR array.

Summary

Digital PCR enables rare-target detection even among a high amount of nontarget presence. The QuantStudio Absolute Q dPCR System consists of a familiar microtiter-plate consumable and a fully integrated instrument. The platform offers a simplified one-step workflow identical to traditional quantitative PCR (qPCR). The QuantStudio Absolute Q dPCR System was used with the Absolute Q Liquid Biopsy dPCR Assays to perform rare-target detection for 30 hotspot cancer mutations. A sensitivity of 0.1% MAF was demonstrated for each assay, showcasing the robustness of Absolute Q assays for relevant rare-target detection.

References

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