Analyze more samples and improve sensitivity for rare targets by pooling digital PCR data

Highlights

- Digital pooling combines data from multiple digital PCR (dPCR) arrays to enable analysis of more sample volume than conventional quantitative PCR.
- The Applied Biosystems™ QuantStudio™ MAP16 Digital PCR Plate contains 16 arrays with a total of 327,680 microchambers that can be digitally pooled.

PCR sample input volume and concentration can affect your ability to detect ultra-rare targets. In this technical note, we show how a low concentration sample containing a target variant with 0.1% mutant allele frequency (MAF) can be detected using multiple dPCR arrays on a QuantStudio MAP16 plate, with the digital pooling feature of Applied Biosystems™ QuantStudio™ Absolute Q™ Analysis Software.

Introduction

A primary application of dPCR is rare-target detection. Unlike threshold cycle (C_t) values, absolute quantitation enables users to determine the original number of target molecules in a sample. Absolute quantitation eliminates the need to interpret amplification curves or rely on a reference to perform quantitative measurements, improving both the accuracy and reproducibility of rare-target concentration measurements. However, the limit of detection for any dPCR reaction depends on several factors. These include the concentration of the target of interest and the volume of sample that can be loaded per reaction (Figure 1). When the target of interest is rare, and the sample has a very low, it may be necessary to run more than one reaction to have enough sample to detect the target.

Low PCR input concentration

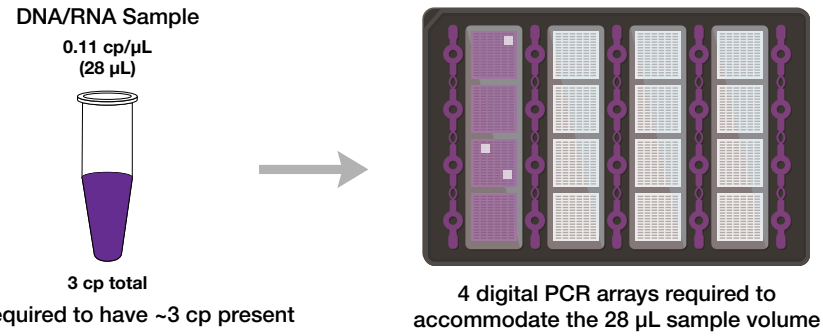

Figure 1. Digital pooling can improve sensitivity for rare targets by increasing the total sample volume analyzed.

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An example of rare-target detection is the measurement of circulating tumor DNA (ctDNA) amidst normal circulating cell-free DNA (ccfDNA) in liquid biopsy samples from individuals diagnosed with cancer. For this application, the total number of ctDNA molecules can vary from person to person and from day to day. The ctDNA concentration is often very low—typically only several molecules per microliter. If the concentration of ctDNA molecules is exceptionally low, the amount of sample tested will directly affect the number of ctDNA molecules that can be detected. Many existing dPCR platforms do not generate a consistent and reproducible number of microreactions due to limitations in the underlying compartmentalization technology, which results in unreliable detection. This inconsistency can cause the total amount of sample analyzed per reaction to vary and generate variability between experiments. Sample wastage can also contribute to subsampling error. Unlike emulsion-based dPCR platforms like droplet dPCR, the microfluidic array plate (MAP) technology designed for the Applied Biosystems™ QuantStudio™ Absolute Q™ Digital PCR System is entirely automated and highly consistent. An industry-leading 95% of loaded sample can be analyzed across >20,000 microchambers every time.

This technical note describes how to apply the digital pooling feature of the QuantStudio Absolute Q Digital PCR System quantifying the cancer mutation *PIK3CA* p.H1047R. A contrived sample with an MAF of 0.1% was created for testing and digitally pooled across 4 dPCR reaction arrays (~81,920 microchambers).

DNA mixture and dPCR reaction preparation

A DNA mixture was prepared that contained human male genomic DNA as a control and plasmid DNA with the *PIK3CA* p.H1047R mutation. The mixture had an MAF of 0.1% and a final genomic DNA concentration of approximately 1.15 ng/µL. The Absolute Q™ Liquid Biopsy dPCR Assay (Catalog No. A52749) for *PIK3CA* p.H1047R detects both the wild-type and mutation-bearing alleles with VIC™ dye– and FAM™ dye–labeled probes, respectively. The dPCR reactions were prepared according to the volumes shown in Table 1.

Plate loading and digital pooling

After preparing the dPCR mix, 9 µL was loaded into each of 4 arrays on a QuantStudio MAP16 plate, followed by a 15 µL overlay of isolation buffer. The prepared plate was then loaded onto the QuantStudio Absolute Q dPCR system. Standard thermal parameters on the QuantStudio Absolute Q system were used. A total of 8 reactions were run using the sample, and 2 replicates were generated by digitally pooling 4 arrays per replicate.

As an orthogonal test, a positive control containing a mixture of 0.1% MAF and DNA in a higher concentration was prepared using the same plasmid and human genomic DNA. This DNA sample was used for 2 independent replicates and loaded at a final concentration of 33 ng per reaction, or approximately 10,000 wild-type and 10 mutation-bearing molecules per reaction. After dPCR was complete, all reactions were analyzed using QuantStudio Absolute Q Analysis Software.

Workflow and methods

Table 1. dPCR reagent setup for the detection of cancer mutation *PIK3CA* p.H1047R on the QuantStudio Absolute Q Digital PCR System.

Reagent	Final concentration
Absolute Q DNA Digital PCR Master Mix (5X)	1X
Liquid Biopsy dPCR Assay (20X)	1X
Wild-type genomic DNA + mutation-bearing plasmid DNA	Maximum volume
Water	Fill to 9 µL

applied
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Results

The dPCR results for the 0.1% MAF samples pooled across 4 arrays and the positive control are shown in Table 2. The concentrations of the mutation-bearing and wild-type *PIK3CA* molecules were close to the expected concentrations. Across approximately 81,815 digitally pooled microchambers, the concentration of the *PIK3CA* mutation–bearing allele was calculated to be 11.5 copies per reaction. This was similar to the positive control concentration of 6.8 copies per reaction. As expected, the average MAF concentration in the pooled samples was 0.1%. The 11.5 copies per reaction are also well above limit of detection. This indicates that digital pooling and its increased sensitivity can also enable variant detection at < 0.1% MAF.

Summary

Digital pooling is an effective way to increase sensitivity by increasing the total volume that can be analyzed for a given sample. This technical note describes the use of 4 arrays (36 µL dPCR mix) on a QuantStudio MAP16 plate to analyze a total of 27 µL of a 0.1% MAF DNA mixture. This method could be used for a multitude of rare-target detection applications in the precision medicine space, such as monitoring treatment response, screening for minimal residual disease, and rapid identification of mutation-linked drug resistance.

Table 2. Results of rare-target detection with 0.1% MAF DNA mixtures and a positive control containing 0.1% MAF and a high genomic DNA concentration. The total number of target molecules detected in 36 µL reactions prepared with the pooled replicates, in 9 µL (1 array) reactions prepared with the positive control, and the total number of microchambers analyzed are shown. Concentrations were calculated using QuantStudio Absolute Q Analysis Software.

0.1% MAF sample (4 pooled arrays per replicate)				Positive control with high DNA concentration (1 array per replicate)		
Replicate	*PIK3CA* p.H1047R copies per reaction (MAF)	Wild-type copies per reaction	Total number of microchambers	*PIK3CA* p.H1047R copies per reaction (MAF)	Wild-type copies per reaction	Total number of microchambers
1	10.44 (0.09%)	11,904.84	81,809	5.31 (0.06%)	9,293.67	20,361
2	12.60 (0.11%)	11,452.32	81,821	8.46 (0.08%)	11,129.31	20,462
Average	11.52 (0.10%)	11,678.58	81,815	6.84 (0.07%)	10,211.49	20,411.5

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