QuantStudio 3D Digital PCR System

Application considerations

Purpose

This Experiment Design Guide provides helpful information and additional guidance for application-specific experiments performed on the Applied Biosystems™ QuantStudio™ 3D Digital PCR System. The information is organized by general application.

Application index

Section 1: Digital PCR basics

To fully exploit the capabilities of digital PCR (dPCR), it is important to have a basic understanding of the principles of the QuantStudio 3D digital PCR platform (Figure 1). Three criteria must be met to successfully complete a dPCR experiment:

1. A DNA molecule of interest (containing a specified sequence) must be present at limiting concentration in a standard PCR reaction mix.
2. The PCR reaction mix must be partitioned into a large number of independent reaction wells such that not all reaction wells receive a copy of the molecule of interest. (Note: The Applied Biosystems™ QuantStudio™ 3D Digital PCR Chip contains 20,000 independent reaction wells.)
3. Amplification, or lack thereof, in each reaction well must be easily distinguishable, and false amplification detection must be minimized.
   (Note: Standard Applied Biosystems™ TaqMan™ Assays are recommended for use on the QuantStudio 3D Digital PCR System.)

The goal is to detect the presence or absence of the DNA molecules of interest in each independent reaction chamber through determination of whether amplification has occurred or not. Based on this digital outcome, a highly precise and sensitive calculation of absolute target concentration can be made.

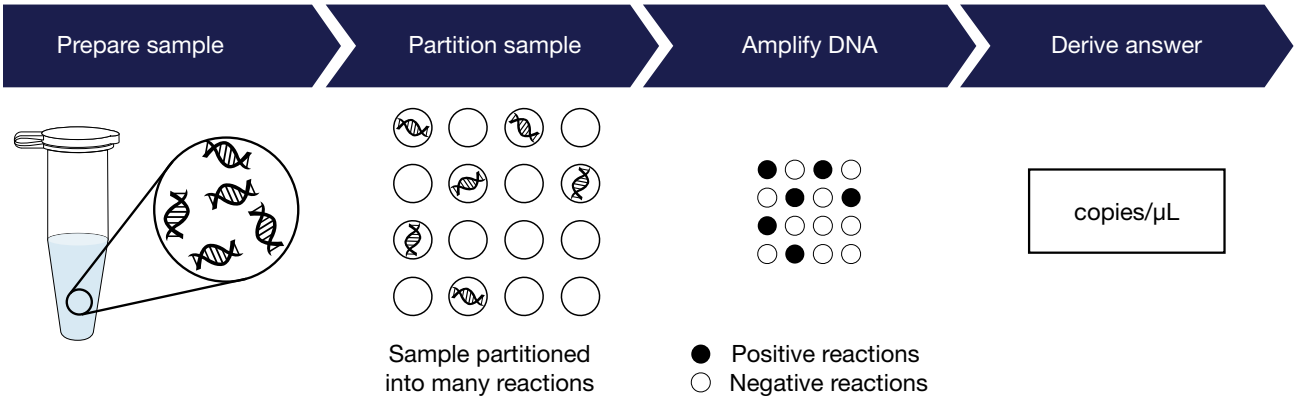

Figure 1. dPCR enables absolute quantification of target sequences. To perform dPCR, a nucleic acid mixture is partitioned into many reaction wells, such that some wells receive a target molecule and some do not. Reactions are subjected to standard PCR to identify wells that have not received any target molecules. A standard statistical correction model accounts for wells that may have received more than a single target molecule, and a final concentration value is produced. Given that dPCR does not rely on C_t values to quantify copy number, comparison to a known standard is not required for absolute quantification.

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Section 2: Copy number variation analysis

The purpose of copy number variation (CNV) analysis is to confirm whether the copy number of a sequence of interest deviates from wild type and by how much. Traditional real-time PCR platforms offer sufficient resolution to resolve low copy numbers (e.g., copy numbers between 0 and 5); however, higher copies require more precise measurements to determine an exact copy number. dPCR is well-suited for analysis of higher copy numbers, as measurement precision exceeding ±10% can be achieved. When analyzing copy number changes on the QuantStudio 3D Digital PCR System, the following points should be taken into consideration:

- Genomic DNA (gDNA) may require predigestion. Since dPCR only considers whether or not amplification has occurred in each partition, each sequence must be able to segregate to a partition independently from all other target sequences. As a result, tightly linked duplications may not be distinguishable since single fragments of DNA may contain more than one copy of the target. This limitation can be overcome by fragmenting the DNA prior to analysis (Figure 2). Since fragmentation must separate tandem duplications into discrete fragments, the most controllable approach is to use a carefully selected restriction enzyme that cuts between the duplicated sequences but leaves the PCR target sequence intact. Mechanical shearing may be used, but results are generally not as clean, as shearing to completion is likely to also shear within the target sequences.
- Use an invariant control locus to normalize for genome number. While the general goal of any CNV analysis is to determine the number of copies per genome of a sequence being analyzed, the answer provided in a dPCR experiment is concentration in copies/µL. To convert to copies/genome, the number of genome equivalents analyzed in the dPCR experiment must be estimated. Utilizing the second color channel on the QuantStudio 3D Digital PCR System, copies/µL for an invariant control locus (sequence known to be at wild type levels) can be measured in the same dPCR experiment. Dividing the determined copies/µL of the target of interest by copies/µL for the invariant control in the same experiment normalizes for genome number, the result of which will be the copy number of the gene of interest per haploid genome.

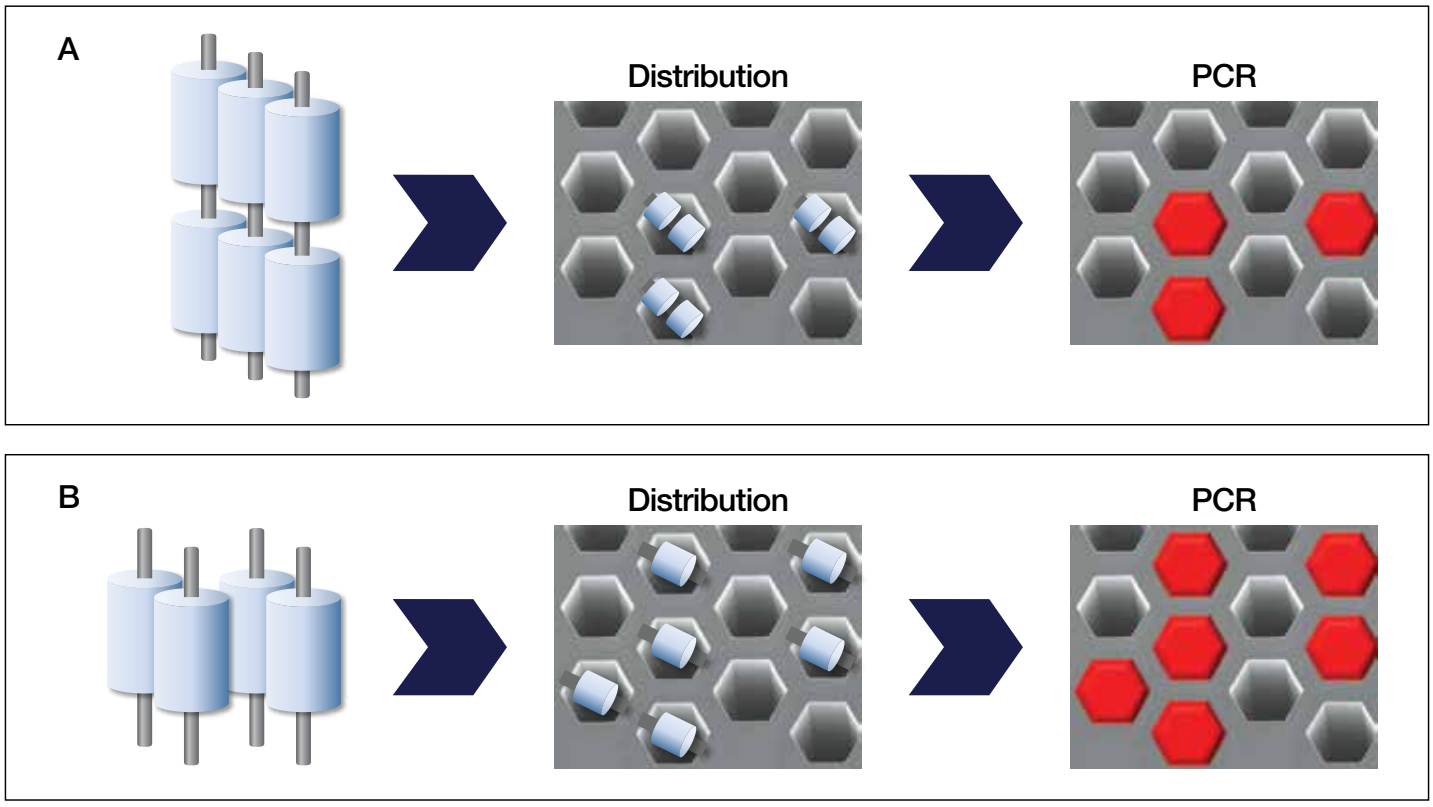

Figure 2. Genomic DNA may require predigestion for copy number variation analysis. (A) A CNV experiment is performed without the use of a restriction enzyme. In this case, the tandem copies will both occupy the same reaction well, resulting in inaccurate quantification. **(B)** A CNV experiment is performed with predigestion with a restriction enzyme to segregate the tandem repeats into individual reactions, resulting in accurate quantification.

Section 3: Low target copy detection

A number of applications, such as pathogen detection, viral reservoir monitoring, and GMO screening, require the detection of very low numbers of specific target nucleic acid sequences. These experiments differ from rare allele detection in one important way: competition by closely related sequence is not a consideration. Two critical confounding factors when performing such low target copy detection experiments are signal-to-noise ratio (with false positive rates ultimately determining the lower limit of detection) and the total sample interrogated (with the sensitivity inherently limited by the total amount of material present in the experiment). For instance if nucleic acid from 10 µL of material is isolated and interrogated with dPCR, the theoretically best possible sensitivity is 1 measured amplification event per 10 µL sample. With this in mind, the following points should be considered when running a low target copy detection experiment:

- Characterize assay signal-to-noise ratio in the absence of sample. Not all assays are equal; therefore, it is important to confirm the noise for any assay used for low target copy detection. Running no-template controls (omitting any sample from the PCR reaction supermix) and no-target controls (replacing the experimental sample with a sample composed of similar DNA sequence composition and complexity but excluding the target sequence of interest) can be critical when considering samples that are expected to contain tens or fewer copies of the molecule of interest. Should the determined lower limit of detection not be sufficient for the application, redesigning the assay is recommended.
- Interrogating a larger amount of sample increases sensitivity. For molecule numbers approaching the lower limit of detection of the digital system, increasing the total sample interrogated increases the absolute number of molecules available for detection. This can be achieved on the QuantStudio 3D Digital PCR System by increasing the concentration of the sample or running a larger volume of the same sample on multiple chips and then allowing the Applied Biosystems™ QuantStudio™ 3D AnalysisSuite™ Cloud Software to pool the data into one larger “virtual” chip.

Section 4: Rare allele detection

The accumulation of mutations in crucial regulatory genes (e.g., oncogenes or tumor suppressor genes) is an important aspect of tumorigenesis. Acquisition of these mutations in a tiny subset of somatic cells could be sufficient for cancer initiation or progression. Detecting this minority population of mutant target loci in the background of loci with wild type sequence is extremely challenging using classical approaches such as Sanger sequencing or real-time PCR, due to classic signal-to-noise problems. Nevertheless, sensitivities less than 1% can be achieved using a dPCR platform. As loci are partitioned into very low numbers per well during the dPCR process, the rare mutant sequences will be absent from most wells; however, in those wells where mutant targets are present, their concentration relative to the wild type background will have been greatly increased. The high enrichment of the mutant sequences increases the signal-to-noise ratio and facilitates their detection using standard Applied Biosystems™ TaqMan™ SNP Genotyping Assays. When detecting rare alleles on the QuantStudio 3D Digital PCR System, the following points should be considered:

- Thermal cycling protocols may require optimization. Since TaqMan SNP Genotyping Assays contain separate but competing probes for mutant and wild type alleles situated within the same amplified target region, the reaction dynamics are significantly more complex compared to single-probe assays. As a result, incorporating adjustments to the standard cycling protocol provided in the quick reference card (QRC) may yield higher-quality results. When taking this approach, starting with small adjustments to the annealing temperature (e.g., 2°C increments) is recommended, as this typically has the largest impact on the performance of an assay.
- Try multiple assay designs. dPCR cannot make up for poor assay performance. Off-the-shelf TaqMan SNP Genotyping Assays are primarily designed for detecting germline differences and not necessarily for the sensitivity needed for rare allele detection, and therefore may not perform satisfactorily in dPCR. Be prepared to try different assay designs if the cycling protocol optimization process does not yield the desired results.

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Section 5: Low-level differential gene expression

Real-time PCR is commonly used to detect gene expression differences; however, this approach is generally limited to detecting differences two-fold or greater. For some studies, detecting expression changes smaller than two-fold may be required. With precision measurements of ±10% or better, dPCR offers the capability to resolve these smaller differences. When performing a gene expression experiment on the QuantStudio 3D Digital PCR System, the following points should be considered:

- Convert RNA to cDNA prior to dPCR analysis. Like other PCR-based platforms, the QuantStudio 3D Digital PCR System is designed to detect DNA. RNA samples must be first converted to cDNA prior to analysis. For efficient RNA conversion, the Applied Biosystems™ High Capacity cDNA Reverse Transcription Kit (Cat. No. 4368814) with no modifications to the described protocol is recommended.
- Use an endogenous control to normalize for cell number. Although dPCR provides an estimate of the absolute number of copies of cDNA in a given sample, differences in copy number between samples may not truly reflect differences in gene expression, due to variability in the many steps (RNA extraction, reverse transcription, and pipetting errors) prior to dPCR analysis. In most cases, to minimize the impact of these sources of error, dPCR quantification of a second gene, an endogenous control gene for which expression is highly stable across the sample set, is advised. By using the endogenous control to normalize for the number of cell equivalents loaded into the dPCR experiment, gene expression differences can be accurately assessed.

Section 6: General commentary

- Achievable precision is a function of target concentration. At the extremes of the digital range the uncertainty of the measurement significantly increases. As such, the greatest precision can be achieved by working in the middle of the digital range (the peak actually lies slightly off the center, at ~20% of the reactions remaining negative). It is, therefore, of value to accurately estimate sample dilution such that the number of copies to be detected falls within this optimal range. For the QuantStudio 3D Digital PCR Chip, 200 to 2,000 copies/µL is recommended.
- Increasing the number of reactions will boost achievable precision. With greater numbers of data points considered in the digital calculations, increasing the total number of reactions used to determine a digital answer decreases the level of uncertainty in the value. This increased level of precision in the measurement can be important in resolving higher copy numbers. This can be achieved on the QuantStudio 3D Digital PCR System by running the same sample on multiple chips and then allowing the AnalysisSuite Cloud Software to pool the data into one larger “virtual” chip.

Consistent with running any PCR experiments, including appropriate controls and replicates in dPCR experimental design is important for identifying workflow errors, reagent issues, instrument malfunctions, and understanding the overall variability of the measurement system. This is not limited to only the dPCR experiment itself, but extends to all sample manipulations from procurement to answer.

For information on ensuring experimental repeatability through robust experimental design, please refer to the following publication: Huggett JF, Foy CA, Benes V et al. (2013) The digital MIQE guidelines: minimum information for publication of quantitative digital PCR experiments. *Clinical Chem* 6:892–902.

Find out more at **thermofisher.com/quantstudio3d**

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