Droplet Digital™ PCR: 3rd Generation PCR Provides Improved Detection, Quantification, Identification, and Characterization of Microbial Targets and Novel Approaches for Molecular Microbiology

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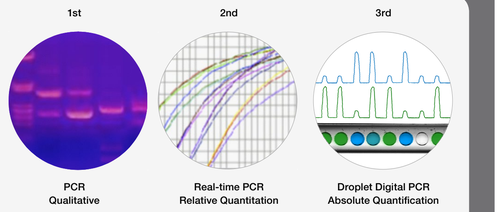
Droplet Digital PCR: The Third Generation of PCR



1 Abstract

Background

Just as PCR and qPCR have revolutionized the field of microbiology over the past few decades, Droplet Digital PCR offers another advance that can provide novel insights into nearly every discipline of molecular microbiology. Digital PCR provides unrivaled precision in qPCR applications. Additionally, partitioning of the sample into thousands of discrete droplets provides many other advantages including improved detection of rare targets through massive reductions in effective background. The flexibility to measure multiple targets independently or, alternatively, to identify linked targets, provides additional improvements for many applications including viral load, microbial quantification and identification, pathogen detection, and microbial ecology.

Methods

We used the commercially available QX100™ Droplet Digital™ PCR system and qPCR assays to investigate the advantages of digital PCR and droplet partitioning. Using both viral and bacterial targets we characterized several advantages of Droplet Digital PCR using model systems in the lab. Additionally, we utilized natural samples to determine applicability to real world data collection.

Results

Using natural samples and controlled model systems, we achieved precise detection, quantification, and identification of microbial targets.

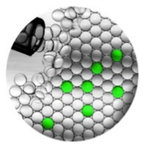
Conclusions

Droplet Digital PCR provides many advantages for molecular microbiology. The application of ddPCR™ to microbiology is just beginning. Droplet Digital PCR is already a powerful technique, and will provide novel insights into many aspects of microbiology. We demonstrate here advantages for several applications including viral load, detection of rare targets (e.g., pathogen detection and food microbiology), and novel methods of microbial identification.

2 How Droplet Digital PCR Works

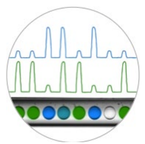
Sample is partitioned into many thousands of nanoliter droplets

Each sample results in thousands of discrete measurements



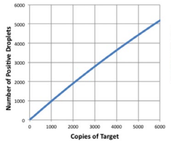
PCR is performed on the droplets

Droplets with 1 or more templates amplify and generate fluorescence



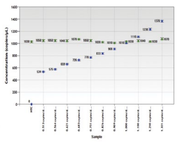
Droplet fluorescence is read using 2 channels (colors)

Droplets are identified as positive or negative for each channel



Percentage of positive droplets is directly related to concentration

Highly precise target concentration measured directly — no ΔΔCq. Up to 5 copies per droplet can be accurately quantified

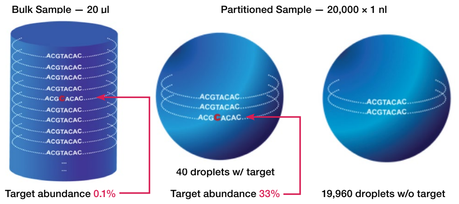


Unparalleled precision with absolute quantitation

Sample differences of 10% can readily be resolved

3 Other Benefits of Partitioning — Droplet Partitioning Increases Rare Target Abundance

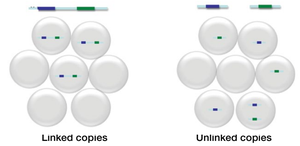
When pathogens or other microbes are distinguished by only small differences (e.g., SNPs) background signal from non-target organisms can compromise the limit of detection by limiting amplification through competition for common reaction components, even when the probe provides good specificity. Droplet partitioning helps avoid this problem by reducing the effective background. Many droplets contain no target molecules, but the droplets that do contain a target have much lower competing background than the bulk solution.



4 Linking Physiology to Phylogeny in Environmental Samples

Simultaneous detection of SAR11 proteorhodopsin and 16S rDNA

Using previously published primer sets targeting 16S rDNA (Suzuki et al. 2001) and proteorhodopsin (Campbell et al. 2008) from the ubiquitous marine microbial clade SAR11, we tested the ability of ddPCR to identify physical linkage between the physiological gene of interest (proteorhodopsin) and the 16S rDNA target. Previous work to link phylogenetic identification and physiological capability typically relied on laborious and expensive techniques such as BAC library construction and sequencing or environmental genome sequencing.



Linked copies co-localize in the same droplet more often than expected by random chance. Unlinked copies segregate randomly.

To investigate linkage by ddPCR, DNA from coastal seawater samples was quantified by assays targeting the SAR11 proteorhodopsin gene (FAM) and SAR 11 specific 16S (HEX). One set of samples was partitioned directly into droplets. Another set was restriction digested to separate the two targets prior to partitioning into droplets.

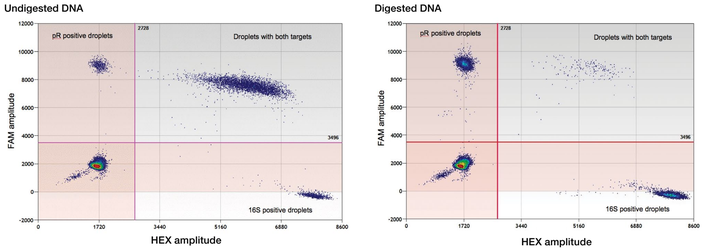


Fig. 1. 2-D fluorescence plots of ddPCR droplets with probes targeting proteorhodopsin (FAM) and 16S (HEX). Shown are composites of 4 replicate wells for each treatment.

- Undigested DNA has overabundance of droplets with both targets in the same droplet (Fig. 1)
- Digested DNA shows fewer dual positive droplets (Fig. 1)
- Concentration estimates of each target are essentially unchanged, indicating digestion separates targets (Fig. 2)
- QuantaSoft™ ddPCR software calculates B-score values to provide a quantitative measure of the degree of association between two loci

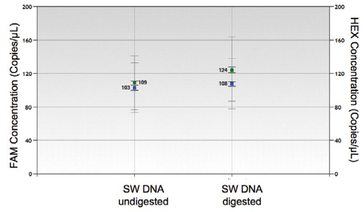


Fig. 2. ddPCR concentration estimates for seawater DNA samples based on composite data from four replicate wells per treatment. Dark error bars represent poisson error for composite data. Lighter error bars represent total error, which includes poisson error and variation among replicates.

5 Pathogen Detection and Quantification

Directly Quantify Targets with High Precision

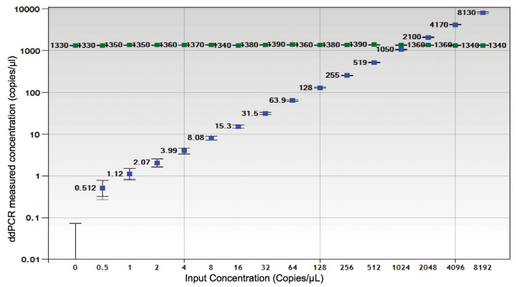
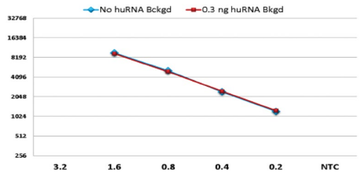


Fig. 3. Twofold dilution series of *Staphylococcus aureus* genomic DNA quantified against a background of human gDNA. Absolute quantification that results from ddPCR yields precise measurements of target concentration directly without the need for reference to a standard curve for quantification.

6 Viral Quantification and Viral Load Measurements for RNA and DNA Viruses

Quantification of HCV RNA

To quantify HCV targets we used the One-Step RT-ddPCR Kit for Probes (Bio-Rad) together with an HCV specific primer/probe set to detect and quantify armored HCV RNA.



Quantification and Viral Load Measurements for HSV1 and HSV2

To quantify HSV1 and HSV2 targets, genomic DNA was extracted from lysates of human cell lines infected with herpesvirus. Viral targets were quantified by primer/probe sets for the respective viral strains. Human cell equivalents were determined using an RPP30 primer/probe set.

Herpesvirus	HSV (copies/well)	RPP30 (copies/well)	Viral load (virus/cell)
HSV 1	48200	49.4	1951
HSV 2	5980	12.72	940

7 Conclusions

Droplet Digital PCR is a new and powerful tool with great potential for improving existing experiments and creating novel approaches to microbial science.

Sample partitioning provides several advantages in addition to improved quantification including:

- reduced effective background for rare target detection
- the ability to identify physically linked targets

Bio-Rad's QX100 ddPCR system – the 3rd generation PCR system that provides unparalleled precision and accuracy in the quantification of DNA and RNA and takes molecular microbiology to the next level

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