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Review

Viral diagnostics in the era of digital polymerase chain reaction ☆

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ABSTRACT

Unlike quantitative polymerase chain reaction (qPCR), digital PCR (dPCR) achieves sensitive and accurate absolute quantitation of a DNA sample without the need for a standard curve. A single PCR reaction is divided into many separate reactions that each have a positive or negative signal. By applying Poisson statistics, the number of DNA molecules in the original sample is directly calculated from the number of positive and negative reactions. The recent availability of multiple commercial dPCR platforms has led to increased interest in clinical diagnostic applications, such as low viral load detection and low abundance mutant detection, where dPCR could be superior to traditional qPCR. Here we review current literature that demonstrates dPCR's potential utility in viral diagnostics, particularly through absolute quantification of target DNA sequences and rare mutant allele detection.

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Clinical viral diagnostic approaches rely heavily on quantitative polymerase chain reaction (qPCR) as a method to detect and quantify viral load in patient samples. For the past 20 years, fluorescence-based qPCR chemistries have revolutionized nucleic acid diagnostics and become the gold standard for viral load quantification (Mackay et al., 2002) and detection of bacterial pathogens, among myriad other applications. During qPCR, DNA is amplified until it produces a certain level of signal which is supplied through a DNA intercalating dye or sequence-specific fluorescent probe. The cycle threshold, defined as the number of amplification cycles required to reach that signal level, is used to calculate the number of DNA molecules originally present based on a standard curve (Bustin, 2004).

Although qPCR has driven major advances in disease diagnosis, this technology has notable limitations. Quantification is based on a standard curve, which requires careful calibration and consistent source material. Additionally, the choice of signal threshold can be made by the operator, introducing subjectivity into the analysis. Due to differences in standard curve construction and potential subjectivity in analysis, interlaboratory variation can be substantial even when using commercial kits and standardized protocols. Moreover, even within a highly trained laboratory the coefficient of variation for any single assay can be 20–30% or higher at lower template copy number (Cook et al., 2009; Lai et al., 2003). For example, the interassay variability for a cytomegalovirus (CMV) quantitation assay is considered low with a viral load coefficient of variation of 28% (Boeckh et al., 2004).

Digital PCR (dPCR) promises to remedy some of the shortcomings of qPCR by transforming the analog, exponential nature of PCR into a digital, linear signal (Vogelstein and Kinzler, 1999). Here we discuss the theoretical basis for dPCR and the currently available commercial dPCR systems. We also review current literature that demonstrates dPCR's potential utility in viral and microbial diagnostics, particularly through absolute quantification of target DNA sequences and rare mutant allele detection.

1. Digital PCR

First described in the 1990s (Sidransky et al., 1992; Vogelstein and Kinzler, 1999), dPCR uses the same primers and probes as qPCR, but touts increased sensitivity and precision. These improvements are achieved by diluting the sample and partitioning it into individual reactions so that ideally each reaction contains 1 or no copies of the DNA of interest (Fig. 1). The number of positive versus negative reactions is counted to directly calculate the number of DNA molecules in the original sample based on Poisson statistics. If the sample is not dilute, many of the individual reactions will be positive and will have contained 2, 3, or more target molecules. In this case, simply counting the positive reactions would underestimate the true number of molecules. This underestimation can be corrected using the Poisson equation [copies per reaction $= -\ln(1-p)$, where p is the fraction of positive reactions], which calculates the average number of molecules per reaction from the observed proportion of positive reactions (Sykes et al., 1992). Using Poisson statistics, digital PCR provides absolute quantification of nucleic acids, reducing subjectivity in analysis by abrogating the need for signal threshold determination and standard curves.

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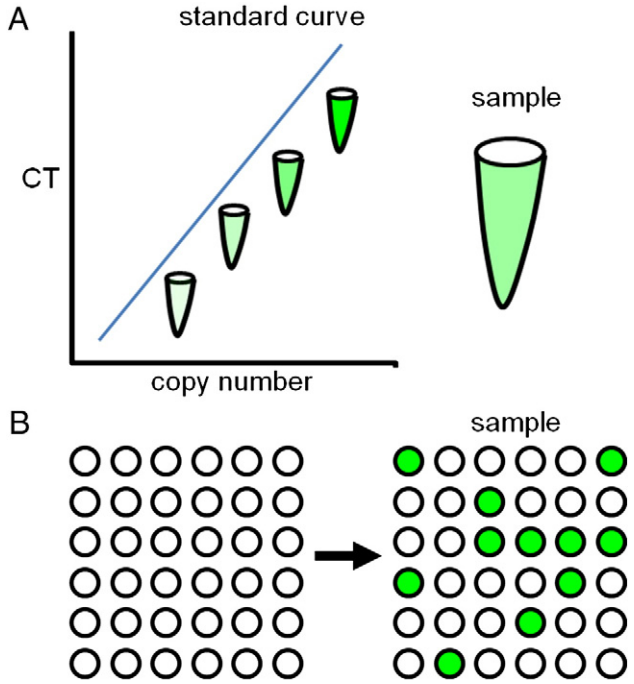

Fig. 1. Comparison of standard quantitative PCR and digital PCR. (A) Quantitative PCR. Quantitation of DNA in a sample is based on comparing the cycle threshold (CT) values for known DNA concentrations to the CT values of the measured sample. (B) Digital PCR. Absolute quantitation of DNA in a sample is achieved by compartmentalizing a sample into hundreds or thousands of separate reactions that are cycled to endpoint, each containing one (green) or no (white) target DNA particles.

Additionally, when amplification is carried out in bulk reactions, it is difficult to quantify poorly represented target sequences in a background of more abundant species. Digital PCR increases sensitivity by isolating rare target species so they are not competing with extraneous DNA targets for primers or other reagents (Fig. 2). Although the concept of dPCR is a powerful one for nucleic acid analysis, the technique has been limited by technical roadblocks associated with the sheer number of reactions required for statistically significant results. The advent of multiple commercially available platforms capable of running reactions on the nano- to picoliter scale has made dPCR a practical tool with great potential in research and clinical settings.

2. Digital PCR platforms

Four different dPCR platforms are currently marketed and differ mainly in their method of individual reaction partitioning (Table 1).

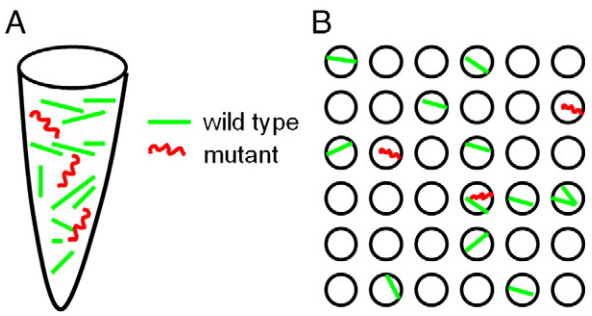

Fig. 2. Detection of rare mutant alleles in a background of wild-type DNA by digital PCR. (A) In conventional qPCR, mutant and wild-type alleles are mixed together in one bulk reaction where rare mutants compete for reagents with more abundant wild-type DNA. (B) Digital PCR increases sensitivity by compartmentalizing wild-type and mutant sequences, giving a less abundant mutant sequence equal access to reagents.

Fluidigm Corporation (San Francisco, CA) and Life Technologies (Carlsbad, CA) offer microfluidics-based systems that partition sample using sophisticated chips designed with microfluidics channels that deliver nanoliter volumes of sample into individual reaction wells. These systems are limited only by the number of reactions that fit onto a single microfluidics chip (hundreds to thousands) and the cost of the consumable chips (in the hundreds of dollars) (Baker, 2012). Bio-Rad Laboratories (Hercules, CA) and RainDance (Lexington, MA) have developed systems that divide diluted sample among many water-in-oil droplets (Hindson et al., 2011; Kiss et al., 2008). Each droplet represents a single reaction allowing simultaneous analysis of thousands (Bio-Rad) to millions (RainDance) of separate reactions (Baker, 2012).

In addition to commercially available dPCR systems, several laboratories are developing simpler dPCR systems with the goal of making this technology practical in resource-limited settings. For example, the SlipChip platform relies simply on the movement or "slipping" of 2 plates to reproducibly and precisely deposit discreet volumes suitable for parallel compartmentalization of nucleic acids (Shen et al., 2010). Any system capable of dividing 1 bulk PCR reaction into many discrete reactions is suitable for dPCR, whose utility derives simply from the ability to identify the amplification of a single nucleic acid template in many separate reactions.

3. Applications of dPCR

Although dPCR promises more sensitive and accurate nucleic acid detection, its use has been mainly limited to research applications. For example, Tadmor et al. (2011) used digital PCR instead of classical phage enrichment to identify virus–bacteria interactions in uncultured bacteria. The group targeted phage-like elements with degenerate primers and targeted bacterial small subunit ribosomal RNA genes with universal "all bacterial" primers in a microfluidics dPCR platform to identify previously unknown, uncultured bacteria in the termite hindgut (Tadmor et al., 2011).

Digital PCR has significantly advanced research capabilities, but its potential for clinical application has been investigated only to a limited degree, partly because devices that are practical, in both cost and dynamic range of detection, are just now becoming commercially available. As commercial systems gain wider use, dPCR could become a standard diagnostic approach for nucleic acid quantitation. Two areas where dPCR has shown potential clinical diagnostic utility are absolute quantification of target DNA sequences and rare mutant allele detection.

3.1. Absolute quantification

Digital PCR provides a sensitive method for the direct measure of viral nucleic acid, providing the absolute number of copies/mL without the need for a standard curve. White et al. (2012) utilized the Fluidigm dPCR system to quantify GB Virus Type C (GBV-C), an occult RNA virus associated with HIV-1 infection. Co-infection of HIV-1 patients with GBV-C has been suggested to lead to a decrease in the temporal progression to AIDS (Bhattarai and Stapleton, 2012; Gretch, 2012). Therefore, tracking the presence of GBV-C early in infection could provide the information needed for a more comprehensive patient prognosis. White et al. compared quantification of GBV-C isolated from transfected cells lines using standard qPCR and dPCR; they found that dPCR had an average coefficient of variation (CV, measure of precision) of 11.7 $\pm$ 2.2% for viral load testing, while standard qPCR had an average CV of 25.8 $\pm$ 4.9%. Using dPCR, they could detect between 3 and 10 DNA molecules/μL, a level that could not be detected by traditional qPCR in parallel experiments.

The second comparison of viral qPCR and dPCR was carried out by Henrich et al. (2012) on HIV-1 quantitation. They found that serial dilutions of HIV-1 or human CCR5 DNA amplicon standards

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