

Tolerance of Droplet-Digital PCR vs Real-Time Quantitative PCR to Inhibitory Substances

To the Editor:

Real-time quantitative PCR (qPCR)¹ is a rapid and sensitive method that forms the foundation for many clinical diagnostic tests. Droplet digital PCR (ddPCR) shares these qualities with qPCR, but owing to reaction partitioning, ddPCR is proposed to exhibit increased tolerance to interfering substances, making it an attractive alternative to qPCR for diagnostic applications (1, 2). The data to support this phenomenon and its mechanism, however, are currently lacking in the literature (3).

Herein, we describe a series of experiments to compare the inhibition tolerance of laboratory-developed CMV qPCR and ddPCR (Bio-Rad Laboratories, QX-100) assays by introducing a panel of clinically relevant inhibitors (SDS, EDTA, and heparin) directly into the PCR reactions (4). Differences in the resulting inhibition curves and the half-maximal inhibitory concentrations (IC₅₀) were then assessed. The laboratory-developed CMV qPCR is a double primer/probe Taqman assay that amplifies and detects the genes *UL123* (IE)² (enhances activation by IE2; interacts with basal transcriptional machinery and cellular transcription factor; disrupts ND10; involved in gene regulation [Human herpesvirus 5]) and *UL55* (gB) (type 1 membrane protein; possible mem-

brane fusogen; binds cell surface heparan sulphate; involved in cell entry; involved in cell-to-cell spread [Human herpesvirus 5]) with primers and probes previously described (5). The ddPCR assay uses the same primers and probes, with the dyes HEX (hexachlorofluorescein) replacing FAM (6-carboxyfluorescein) on the gB probe and BHQ1 (black hole quencher 1) replacing TAM (*N,N,N',N'*-tetramethyl-6-carboxy-rhodamine) on both probes (Sigma Aldrich).

SDS, EDTA, and heparin were serially diluted and added directly to CMV (AD169 whole virus; Advanced Biotechnologies), qPCR, and ddPCR reactions. On average, 14 143 (439) droplets were analyzed per well of any given ddPCR reaction. The mean concentration of the CMV template in uninhibited samples of the ddPCR was 9.27 copies/ μ L (in a 20- μ L reaction) over 3 biological replicates. Droplets in partially inhibited samples show fluorescent units ranging between those of positive and negative droplets and can be easily visualized in 1-dimensional amplitude plots (Fig. 1, wells 4–7). In a typical uninhibited sample, a tight threshold (Fig. 1, orange line) is placed near the cluster of positive droplets and is constant for a specific target. However, in the case of partial inhibition, a broad threshold is applied to the ddPCR QX100 analysis near the negative droplets to appropriately incorporate all partially inhibited droplets (Fig. 1, blue line).

Using this strategy, we calculated log IC₅₀ values from the resulting inhibition curves. Greater than a half log increase in IC₅₀ was observed for both the IE and gB targets of ddPCR over qPCR for both SDS (absolute log difference in IC₅₀ qPCR vs ddPCR IE, 0.554, and vs ddPCR gB, 0.628) and heparin (absolute log difference in IC₅₀ qPCR vs ddPCR IE, 0.655, and vs ddPCR gB, 0.855).

The probability of difference between the data sets for ddPCR and qPCR was >99.99% for both inhibitors and both ddPCR targets, indicating that ddPCR tolerated the presence of these inhibitors better than qPCR. However, this difference was not noted when we compared ddPCR and qPCR in the presence of EDTA for both ddPCR targets (log difference in IC₅₀ qPCR vs ddPCR IE, 0.116, and vs ddPCR gB, 0.0198), possibly owing to different inhibition mechanisms. EDTA is a calcium chelator, whereas SDS and heparin both act on DNA polymerase.

The ddPCR CMV assay is more tolerant to SDS and heparin than the qPCR assay, indicating that reaction partitioning through digitization may reduce susceptibility to these traditional PCR inhibitors. The data suggest that individual microreactions mitigate the impact of inhibitors on PCR amplification by retaining discernible positive signals even when moderate PCR inhibition is occurring in a droplet. Since PCR reactions are not partitioned in qPCR, amplification is dependent on the concentration of inhibitor in the entire reaction, leading to an increased number of amplification cycles required to reach a signal above a given threshold. In turn, this will result in inaccurate quantification of template in the original sample. In ddPCR, on the other hand, quantification is dictated by the Poisson distribution. Theoretically, each droplet ideally contains 1 or 0 copies of template. This distribution can also be applied to the presence of inhibitory substances. Amplification will depend on the presence or absence of template and the presence or absence of inhibitory concentrations of inhibitor in each droplet. Delayed amplification or reduced amplification efficiency per cycle due to partial

¹ Nonstandard abbreviations: qPCR, real-time quantitative PCR; ddPCR, droplet digital PCR; CMV, cytomegalovirus; IC₅₀, half-maximal inhibitory concentration.

² Genes: IE (*UL123*), enhances activation by IE2; interacts with basal transcriptional machinery and cellular transcription factor; disrupts ND10; involved in gene regulation [Human herpesvirus 5]; gB, (*UL55*), (type 1 membrane protein; possible membrane fusogen; binds cell surface heparan sulphate; involved in cell entry; involved in cell-to-cell spread [Human herpesvirus 5]).

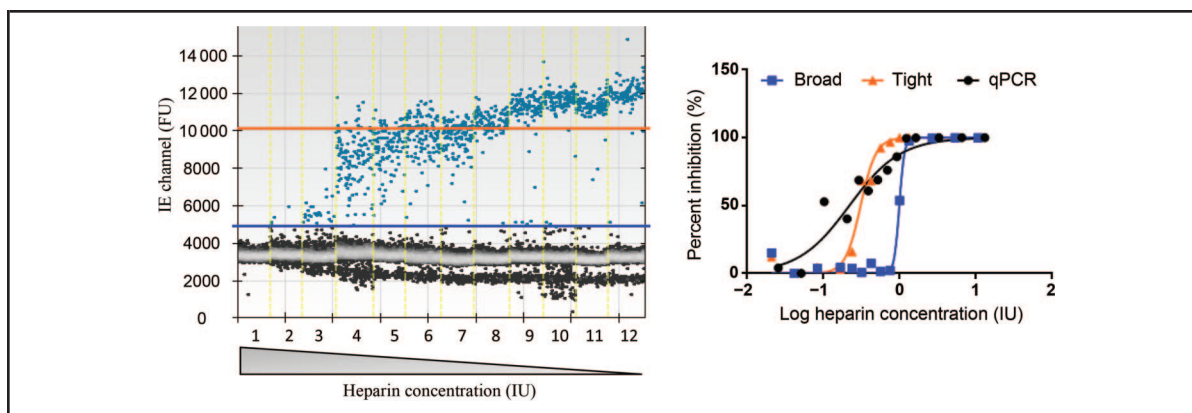


Fig. 1. Amplitude plots.

(Left panel), Droplet plot of the CMV IE ddPCR assay for 12 wells containing a dilution series of heparin. Each well is demarcated by a yellow dashed line. The heparin concentration decreases from well 1 to well 12. Gray droplets are negative for CMV. Blue droplets are positive for CMV. The orange line represents the typical thresholding level for noninhibited samples (wells 10–12). The blue line represents the broad threshold applied to inhibited samples for accurate quantification in the presence of inhibitory concentrations of heparin. (Right panel), Inhibition curves corresponding to the threshold applied to the data analysis of the ddPCR are shown. The inhibition curve for the CMV qPCR is also indicated.

inhibition can be visualized by using amplitude plots. This mechanism allows optimal placement of the analysis threshold to include positive droplets that exhibit amplitude shifts due to the effects of inhibitors (Fig. 1). The results from the inhibitor-spiked PCR reactions provide proof-of-concept that ddPCR may offer an advantage over qPCR when dealing with inhibition-prone samples. Other clinical specimen types, such as stool, sputum, and tissue, are known to be more recalcitrant to removal of inhibitors through typical extraction methods, so ddPCR may prove especially useful for such specimens.

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