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Note

A multiplexed droplet digital PCR assay performs better than qPCR on inhibition prone samples

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ABSTRACT

We demonstrate the development of a multiplex droplet digital PCR assay for human cytomegalovirus (CMV), human adenovirus species F, and an internal plasmid control that may be useful for PCR inhibition–prone clinical samples. This assay performs better on inhibition-prone stool samples than a quantitative PCR assay for CMV and is the first published clinical virology droplet digital PCR assay to incorporate an internal control.

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PCR inhibition remains a problem in some clinical sample types that have high concentrations of PCR-interfering substances, including stool and sputum. Components such as complex polysaccharides, bile salts, lipids, and urate found in stool samples are particularly responsible for PCR inhibition (Schrader et al., 2012). The concentration of these substances varies from sample to sample depending on nutrition, gut microbiota, and environment. PCR inhibition can be overcome by diluting the matrix several fold. However, this approach also dilutes the template DNA, which compromises sensitivity (Alaeddini, 2012).

A previous study utilizing known PCR-inhibitory substances spiked directly into reactions has shown droplet digital PCR (ddPCR) to be more resistant to PCR inhibitors than quantitative PCR (qPCR) (Dingle et al., 2013; Hall Sedlak and Jerome, 2014). Therefore, we evaluated the performance of ddPCR compared to qPCR on clinical stool samples assayed for DNA viruses potentially associated with gastrointestinal disease, human cytomegalovirus (CMV) and human adenovirus species F (AdVs). A multiplexed ddPCR assay that targeted CMV, AdVs, and EXO internal plasmid control (derived from a jellyfish gene (Huang et al., 2008)) was developed and tested on stool samples in comparison to the University of Washington Molecular Virology Laboratory–developed tests (LDTs) for CMV (Boeckh et al., 2004) and AdVs (Huang et al., 2008), each with the same internal plasmid control.

The ddPCR assay was performed on the QX100 droplet generator and reader (Bio-Rad Laboratories, Hercules, CA, USA) as previously described (Sedlak et al., 2014). The primers and probes utilized are as follows for each of the 3 targets: CMV UL55 Forward- TGG GCG AGG ACA ACG AA,

UL55 Reverse- TGA GGC TGG GAA GCT GAC AT, UL55 probe- HEX-TGG GCA ACC ACC GCA CTG AGG – BHQ1; AdVsF Forward- TGTTYG AAGTTTTCGACG TYGT, Reverse- SAGGTAGACGGCCTCGATGA, Probe- FAM- CGCATCCACCAGCCSCACC-BHQ1; Exo internal control Forward- GGC GGA AGA ACA GCT ATT GC, Reverse- GGA ACC TAA GAC AAG TGT GTT TAT GG, Probe-FAM or VIC-AACGCCATCGCACAAT-MGBNFQ. The final concentration of all primers was 900 nmol/L and the concentration of CMV and AdVs probes was 250 nmol/L. For the EXO internal control probe, a 50:50 mixture of FAM- and VIC-labeled probes was used with each at a concentration of 125 nmol/L. The assay was validated using CMV NIST DNA (Haynes et al., 2013), AdVs species F (types 40 and 41) ATCC DNA, and EXO plasmid DNA spiked individually and as mixtures into clinical specimens (Fig. 1). Using a mixture of FAM- and VIC-labeled EXO probes resulted in distinct clusters of positive droplets for each of the 3 targets (Fig. 1). Samples with both single and multiple targets could be differentiated.

Residual clinical stool samples (n = 60) previously tested negative for Rotavirus by qPCR, and non-inhibited buffer controls were evaluated by ddPCR and qPCR assays for CMV and AdVs. A loopful of stool sample was added to 1 mL of Hank's balanced salt solution, 200 μL was extracted on a MagnaPure LC instrument (Roche Diagnostics, Indianapolis, IN, USA) using the Total Nucleic Acid HP Extraction Kit and eluted in 100 μL buffer. Exo internal control was spiked into the lysis buffer of the extraction, resulting in approximately 20 copies Exo template per microliter of DNA extract. The final volumes of the ddPCR (20 μL) and qPCR reactions (CMV: 30 μL and AdVs: 50 μL) differed, but the ratio of extracted template DNA (inhibitor) to reaction volume was kept constant between the ddPCR and qPCR reactions.

Fifteen (25%) of 60 samples tested were inhibited in the CMV qPCR assay, as determined by absence of or very low internal control
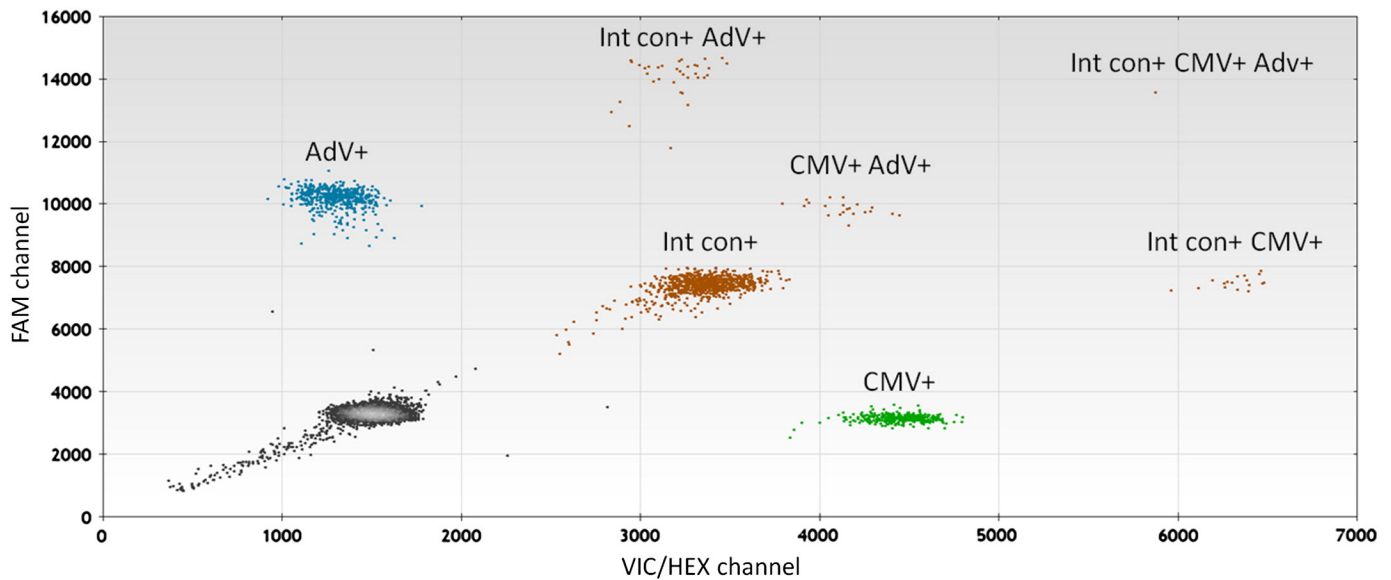
Fig. 1. Multiplex ddPCR assay for AdVs, CMV, and EXO internal control (Int con). Each distinct cluster of dots represents droplets that are positive for a single or multiple DNA targets as labeled. Multiplexed detection beyond 2 targets is achieved in this system, which only has 2 fluorescent detection channels, by introducing a 50:50 mixture of FAM- and VIC-labeled probes for 1 of the targets (Exo internal control).

Table 1

Comparison of PCR inhibition in stool samples with a triplex ddPCR assay for CMV, AdVs, and internal control to qPCR assays for CMV and AdVs with internal controls.

N = 60	ddPCR	CMV qPCR	AdVs qPCR
# inhibited	0	15	0
% inhibited	0	25%	0
# positive	1 (AdVs)	0	2
% positive	1.7%	0	3.3%

amplification (Table 1). The mean cycle threshold (Ct) value for Exo in uninhibited samples in the CMV qPCR assay was 31.3 ± 0.4. Fourteen of the 15 inhibited samples exhibited complete inhibition (Ct = undetermined), while 1 sample had a Ct of 38.7. None of these same samples were inhibited in the ddPCR assay, further validating the inhibition resistance of ddPCR. The mean copies/reaction for Exo in uninhibited samples in the multiplex ddPCR assay was 140.0 ± 28.8. This value closely matched the mean copies/rxn for Exo in samples that exhibited inhibition in the CMV qPCR assay (130.0 ± 34.1). None of the samples were inhibited in the AdVs qPCR assay, reflecting the difference in inhibitor tolerance between different qPCR assays. The 1 sample that was positive for AdVs by qPCR, but not ddPCR had a low viral quantity (200 copies/mL). Since the experiment was designed to normalize the ratio of inhibitory template to reaction volume, more template DNA was added to the qPCR reaction than the ddPCR reaction, accounting for the difference in sensitivity.

Droplet digital PCR performs better than the CMV LDT qPCR when assaying for viral templates in PCR-inhibitory stool samples. The standard procedure for efficient PCR in stool samples is to dilute samples enough that inhibitors are diluted out. For viruses like norovirus, which are typically present at very high viral loads, this strategy is sufficient. However, it will be beneficial to improve sensitivity of PCR stool assays by abrogating the need for dilution. The specimens in this study had a very low positive rate for CMV and AdVs, but these specimens were not selected from a cohort of patients with risk factors for either of these viruses. One study correlating results of mucosal biopsies to CMV stool DNA PCRs found that stool PCR could be a non-endoscopic testing mode for underlying CMV infection in patients with inflammatory bowel disease (Herfarth et al., 2010). A multiplexed ddPCR assay similar to this one, designed specifically for stool samples, may be useful to detect low levels of virus indicative of significant disease. Moreover, this multiplex ddPCR assay is the first published utilizing an internal control, crucial for daily clinical reproducibility, prevention of false negatives, and assurance of accurate quantitation in inhibition prone specimens.

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