Digital PCR

Wastewater testing using the QuantStudio Absolute Q Digital PCR System

Keywords

poliovirus, mpox virus, influenza A virus (FluA), influenza B virus (Flu B), respiratory syncytial virus (RSV), *Campylobacter jejuni, Campylobacter coli*, *Campylobacter upsaliensis*, *Salmonella* spp., *Shigella* spp., enteroinvasive *E. coli* (EIEC), environmental surveillance, pathogen detection, digital PCR, multiplex absolute quantification, inhibitor tolerance

Introduction

Wastewater-based epidemiology (WBE) is the monitoring of chemicals and microbes in sewage via periodic sampling and chemical analysis. Used for poliovirus surveillance at least as far back as 1939 [1] and proposed in 2001 as a method to monitor illicit drug use in communities [2], this epidemiology tool is increasingly being used for monitoring and trending of infectious disease outbreaks and epidemics [3]. Additionally, WBE can serve as an early warning system for emerging pathogens as well as for the reintroduction or resurgence of previously eradicated infectious diseases [4].

WBE was widely implemented during the global SARS-CoV-2 crisis, in part because of its ability to detect increased case counts or new viral variants in advance of clinical data [5]. The success of WBE programs for SARS-CoV-2 has led to their extension to more pathogens, which are now routinely monitored by wastewater testing in municipalities throughout the world. Recently, the National Wastewater Surveillance System (NWSS) in the United States began considering additional targets, including endemic respiratory and gastrointestinal pathogens (such as influenza viruses, noroviruses, and pathogenic *Escherichia coli* strains), emerging pathogens (such as mpox virus), and antibiotic resistance markers (such as for vancomycin resistance and extended-spectrum β-lactamases). Similarly, the Wastewater Integrated Surveillance for Public Health in Europe (EU-WISH) project is aiming to increase wastewater screening capacity across Europe and build upon existing programs that screen for SARS-CoV-2, poliovirus, respiratory viruses, emerging pathogens, antibiotic resistance, and other pathogens and substances. Wastewater surveillance for pathogens beyond SARS-CoV-2 is also either under consideration or already implemented in countries around the world, including Japan and the Republic of Korea. Furthermore, current SARS-CoV-2 WBE programs in countries such as India and South Africa could potentially be expanded to additional targets.

Detection of microbes in wastewater is most often achieved using variants of the polymerase chain reaction (PCR) technique. Quantitative real-time PCR (qPCR) is a popular choice for wastewater testing because of its specificity and throughput. However, digital PCR (dPCR) is also recommended by the NWSS when quantification of pathogen loads is required, such as for trending and monitoring [6]. dPCR offers multiple benefits for wastewater testing compared to qPCR. First, dPCR provides absolute quantification and does not require a standard curve or reference standards for quantification. Second, dPCR is more tolerant of PCR inhibitors than qPCR because of its use of endpoint detection rather than relying on the efficiency of PCR amplification. Inhibitor tolerance is of particular importance to wastewater testing given the high degree of concentration a sample undergoes prior to analysis and the abundance of inhibitors present in the matrix, such as heavy metals, bile salts and urate, polyphenols, lipids, polysaccharides, industrial waste, and detergents [7].

The Applied Biosystems™ QuantStudio™ Absolute Q™ Digital PCR System is a robust and reliable dPCR system that is compatible with a wide range of nucleic acid extraction techniques. The QuantStudio Absolute Q dPCR System permits multiplexing of up to four dyes, enabling detection of multiple microbial targets, human fecal controls, and matrix recovery controls, all in a single reaction. The QuantStudio Absolute Q dPCR System features a simple and fast workflow, with plate setup in as little as 5 minutes and run times of approximately 90 minutes, and does not have separate process modules that would require manual transfer of the plates during the dPCR workflow (Figure 1).

In the present study, we demonstrate the capability of the QuantStudio Absolute Q dPCR System for detecting various targets of potential interest for WBE. We highlight a full end-to-end workflow with wastewater samples processed using the Applied Biosystems™ MagMAX™ Wastewater Ultra Nucleic Acid Isolation Kit with Virus Enrichment on the Thermo Scientific™ KingFisher™ Flex Purification System and subsequent target quantification on the QuantStudio Absolute Q dPCR System to reliably monitor SARS-CoV-2 concentrations. We also demonstrate the ease of adapting existing qPCR assays to dPCR on the QuantStudio Absolute Q dPCR System, including predesigned Applied Biosystems™ TaqMan™ Assays and multiplex qPCR panels from Thermo Fisher Scientific. Furthermore, we show that published assays designed for qPCR, such as the mpox (formerly known as monkeypox) virus assay from the U.S. Centers for Disease Control and Prevention (CDC) [8], can be successfully used for dPCR. Finally, we showcase the superior performance of dPCR compared to qPCR for confidently obtaining accurate target quantification when working with samples containing PCR inhibitors.

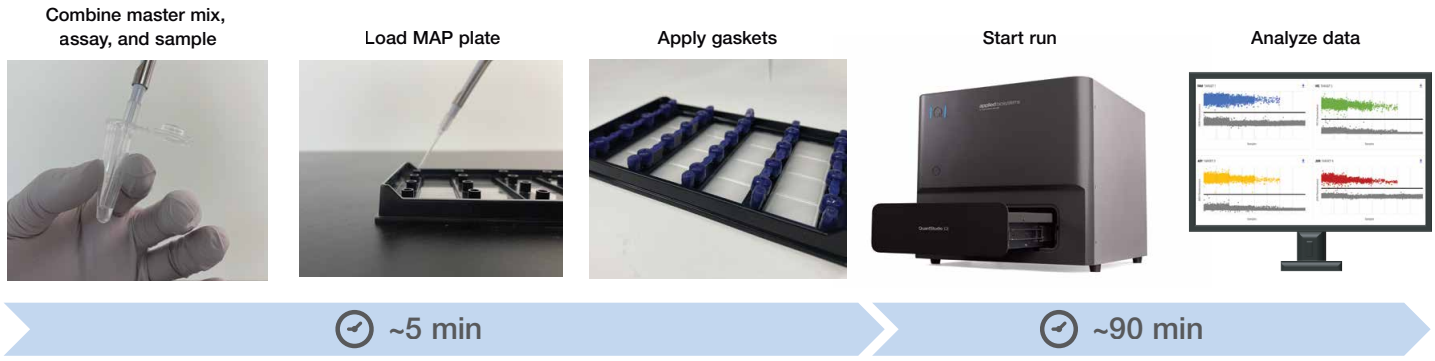

Figure 1. QuantStudio Absolute Q dPCR system workflow. Sample eluates are combined with master mix and assay(s), then loaded into the Applied Biosystems™ QuantStudio™ Absolute Q™ MAP16 dPCR plate, followed by addition of QuantStudio™ Absolute Q™ Isolation Buffer. After gaskets are applied, the plate is inserted into the instrument and the run is started. Compartmentalization, amplification, and detection all occur on the QuantStudio Absolute Q dPCR system without requiring transfer of plates between modules.

Results

SARS-CoV-2 quantification in wastewater samples

To demonstrate the performance of the QuantStudio Absolute Q dPCR System for detecting microbes in wastewater, eluates from raw wastewater samples were spiked with SARS-CoV-2 and tested with the Applied Biosystems™ Absolute Q™ dPCR SARS-CoV-2 Wastewater Surveillance Kit on the QuantStudio Absolute Q dPCR System (Figure 2). Raw wastewater influent was collected at wastewater facilities in Stillwater, Oklahoma (USA). Grab samples were collected at influent sites where permitted, then heat-inactivated for 1 hour at 65°C and stored at 4°C until use. The samples were clarified by centrifugation for 10 minutes at 10,000 x *g*, then spiked with differing amounts of inactivated SARS-CoV-2 virus. Samples were first concentrated using Applied Biosystems™ Dynabeads™ Wastewater Virus Enrichment beads, then nucleic acid was extracted using the MagMAX Wastewater Ultra Nucleic Acid Isolation Kit on the KingFisher Flex System. Finally, extraction eluates were tested using the Absolute Q dPCR SARS-CoV-2 Wastewater Surveillance Kit, a triplex assay targeting two separate regions of the SARS-CoV-2 N gene as well as pepper mild mottle virus (PMMoV).

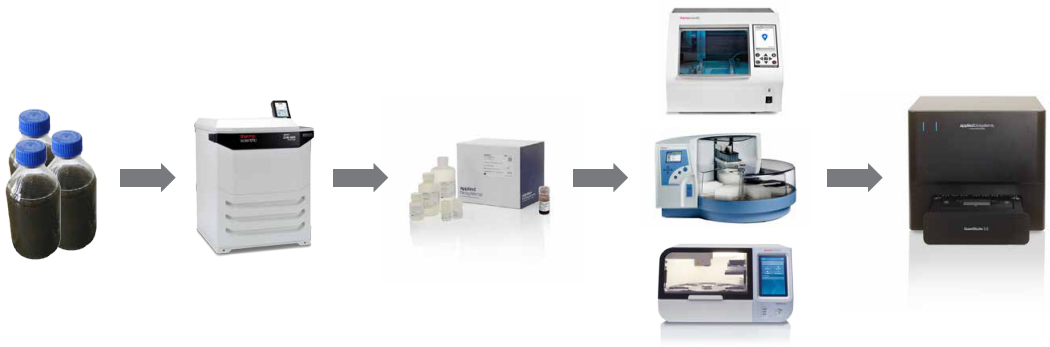

Figure 2. End-to-end workflow of wastewater sample testing with the QuantStudio Absolute Q dPCR System. Wastewater samples are clarified by centrifugation, followed by an optional magnetic bead–based virus enrichment step. Extraction reagents and samples are then loaded onto one of three KingFisher systems (shown are the Thermo Scientific™ KingFisher™ DuoPrime, Flex, and Apex Purification Systems). Eluates are combined with master mix and assay(s), loaded into the QuantStudio Absolute Q MAP16 dPCR plate, and run on the QuantStudio Absolute Q dPCR system.

PMMoV is one of several fecal controls recommended for normalizing wastewater samples to fecal content [9]. As shown in Figure 3, the QuantStudio Absolute Q dPCR System can reliably detect changes in SARS-CoV-2 concentrations down to low levels. Furthermore, multiplexing permits accurate within-reaction normalization to the PMMoV fecal control.

Given the expansion of wastewater testing beyond SARS-CoV-2, we next sought to demonstrate that assays and panels designed for qPCR testing can be easily transferred to the QuantStudio Absolute Q dPCR system. A selection of qPCR assays from the TaqMan Assays library and predesigned panels as well as a qPCR assay developed by the CDC were tested on the QuantStudio Absolute Q dPCR System. Importantly, all assays were tested at the same primer and probe concentrations used for qPCR with the default thermal cycling protocol, without the need for re-optimization of assay or run conditions.

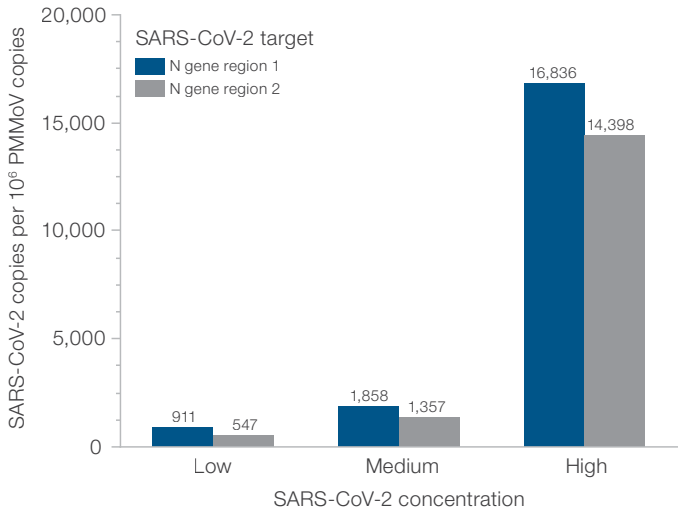

Figure 3. Accurate target detection in wastewater samples. Nucleic acid from wastewater samples spiked with varying levels of SARS-CoV-2 was extracted using the MagMAX Wastewater Ultra Nucleic Acid Isolation Kit with Virus Enrichment on the KingFisher Flex system and tested using the Absolute Q dPCR SARS-CoV-2 Wastewater Surveillance Kit. The concentration of each region of the SARS-CoV-2 N gene is expressed relative to the concentration of PMMoV in the same sample. Concentrations shown are the means of four technical replicates.

Poliovirus detection using a catalog TaqMan Assay

Poliovirus is a target of interest for wastewater testing, particularly in regions where it has not been eradicated or where the oral polio vaccine continues to be administered. Indeed, the World Health Organization (WHO) recommends environmental surveillance of poliovirus, especially where case identification is inconsistent, the virus remains endemic, or periodic reintroduction is known or suspected [10]. To this end we assessed the performance of an existing predesigned TaqMan Assay that targets poliovirus, on the QuantStudio Absolute Q dPCR System. As shown in Figure 4, synthetic nucleic acids representing three poliovirus serotypes were detected, with no re-optimization of assay concentration or thermal cycling conditions required. A range of predesigned assays targeting viruses, bacteria, fungi, and antibiotic resistance markers relevant to WBE can be found in the TaqMan Assay catalog (see ordering information).

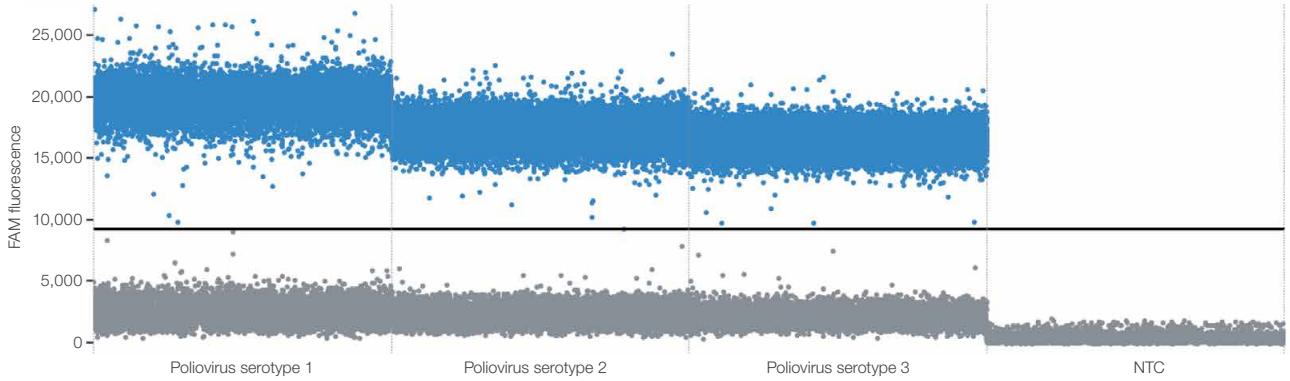

Figure 4. Easy implementation of a predesigned TaqMan Assay on a dPCR platform. A TaqMan Assay for human poliovirus (Cat. No. Vi07922890_po) was tested with synthetic targets representing three poliovirus serotypes and a no-template control (NTC) on the QuantStudio Absolute Q dPCR System using the Applied Biosystems™ Absolute Q™ 1-Step RT-dPCR Master Mix. Blue dots represent chambers called positive for amplification and gray dots represent chambers called negative for amplification, based on the threshold cutoff value indicated by the horizontal black line.

Mpox virus detection using a previously published qPCR assay

Like SARS-CoV-2, mpox has recently emerged as a human pathogen after initially appearing sporadically as a zoonosis. Declared a public health emergency of international concern (PHEIC) by the WHO in 2024, mpox is another pathogen that lends itself well to wastewater surveillance because it is shed in human feces.

To demonstrate that an assay originally designed for qPCR can be successfully transferred to the QuantStudio Absolute Q dPCR System, the mpox generic assay from the CDC's *Monkeypox virus* Generic Real-Time PCR Test [8] was tested with mpox genomic DNA on the QuantStudio Absolute Q dPCR System. The mpox probe sequence was synthesized with a 5′ FAM™ dye label and either an internal Black Hole Quencher™ (BHQ™) dye with other requisite modifications, per the CDC test protocol, or an Applied Biosystems™ TaqMan™ QSY quencher.

As shown in Figure 5, the CDC mpox assay accurately and sensitively quantifies mpox viral gDNA by dPCR on the QuantStudio Absolute Q dPCR System over a range of concentrations, yielding concentration measurements consistent with the assigned values for the test material. Furthermore, no difference was observed in dPCR performance between the TaqMan QSY probe and the probe with an internal BHQ dye.

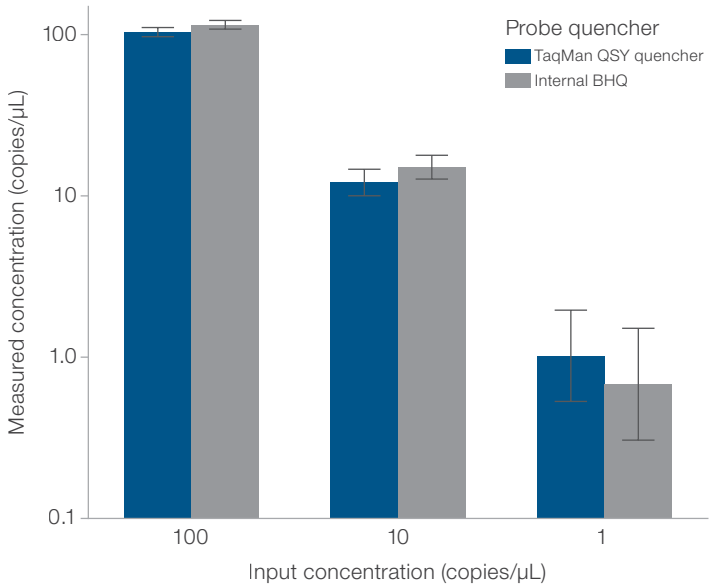

Figure 5. Transfer of the CDC *Monkeypox virus* generic assay to the QuantStudio Absolute Q dPCR System. Genomic DNA from mpox strain hMPX/USA/MA001/2022 was quantified using the Applied Biosystems™ Absolute Q™ Universal DNA Digital PCR Master Mix on the QuantStudio Absolute Q dPCR System across three 10-fold dilutions. The assay probe was synthesized with either a 3′ TaqMan QSY quencher (blue) or an internal BHQ quencher (and other modifications) as specified in the CDC test protocol (gray). Error bars represent Poisson 95% confidence intervals of the calculated concentrations. Input concentrations of mpox gDNA were per the Certificate of Analysis accompanying the product.

Detection of SARS-CoV-2, Flu A, Flu B, and RSV in multiplex format

Respiratory viruses, such as influenza A virus (Flu A) and respiratory syncytial virus (RSV), can be tracked by wastewater to supplement other forms of disease surveillance. The NWSS currently publishes wastewater data for SARS-CoV-2, Flu A, and RSV, and local municipalities often include additional respiratory pathogens such as influenza B virus (Flu B) and human metapneumovirus. Whereas Flu B is restricted to humans, Flu A in wastewater can come from humans, birds (avian flu), livestock, and other sources. When testing of multiple targets per sample is desired, combining targets into the same PCR reaction can provide a more efficient and streamlined testing workflow. To demonstrate the utility of the QuantStudio Absolute Q dPCR System for detecting multiple pathogens in a single well, a multiplex assay targeting SARS-CoV-2, Flu A, Flu B, and RSV was tested with a positive sample containing all four targets. One-dimensional scatter plots for all four dye channels of the multiplex reaction are shown in Figure 6. Compared with the NTC, all four targets are detected with specificity. The assay mix was formulated with standard primer and probe concentrations, and no optimization of thermal cycling conditions was performed.

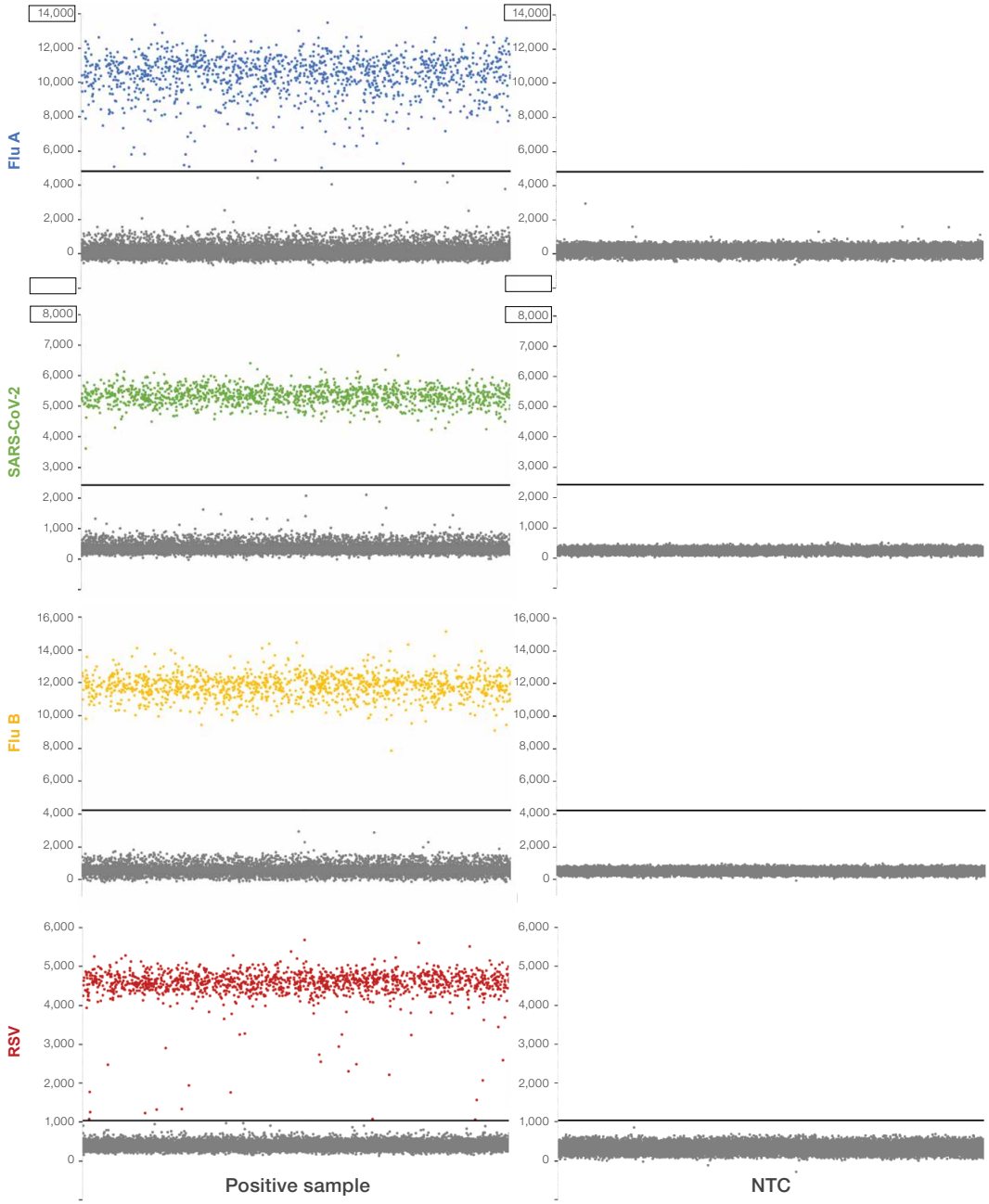

Figure 6. Multiplex detection of Flu A, SARS-CoV-2, Flu B, and RSV. A positive sample containing all four targets and an NTC were tested on the QuantStudio Absolute Q dPCR System using the Absolute Q 1-Step RT-dPCR Master Mix. Flu A, SARS-CoV-2, Flu B, and RSV were detected by the channels for FAM™, VIC™, ABY™, and Cy®5 dyes, respectively.

Enteric pathogen detection using a predesigned TrueMark panel

Several bacterial pathogens are among the targets proposed for expanded wastewater surveillance. Shiga toxin–producing *E. coli* (STEC), also a zoonosis, is typically transmitted to humans through contaminated food, some of which becomes contaminated through contact with contaminated water. Symptoms of STEC infection range from diarrhea and dysentery to hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP). Since its identification in 1984, STEC has caused more than 1,300 outbreaks worldwide, with 22% of infected individuals developing HUS in one of the largest outbreaks [11]. To demonstrate that predesigned multiplex qPCR panels can easily be transferred to the QuantStudio Absolute Q dPCR System, the Applied Biosystems™ TrueMark™ Enteric Bacterial Select Panel I was tested with a control sample containing all four targets. This panel detects STEC as well as other targets of interest for WBE: *Campylobacter jejuni, C. coli*, and *C. upsaliensis*; *Salmonella* spp.; and *Shigella* spp. and enteroinvasive *E. coli* (EIEC). As shown in Figure 7, the TrueMark Enteric Bacterial Select Panel I successfully detected all four targets on the QuantStudio Absolute Q dPCR System over a range of sample concentrations when tested with the Absolute Q Universal DNA Digital PCR Master Mix.

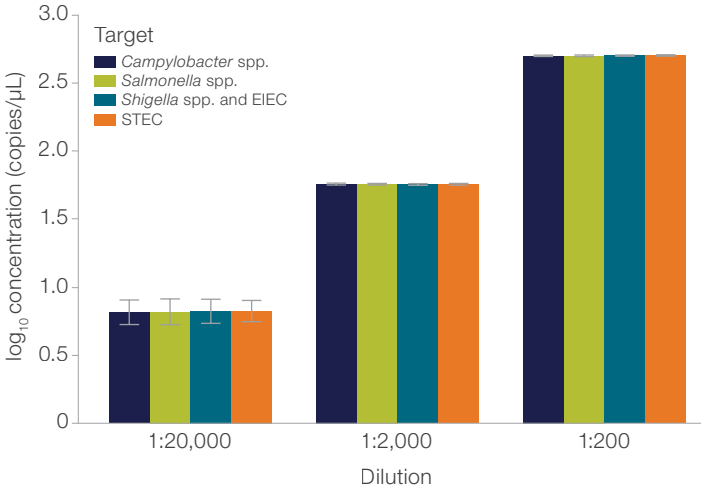

Figure 7. Transfer of the TrueMark Enteric Bacterial Select Panel I to the QuantStudio Absolute Q dPCR System. A control sample containing all four targets was tested using the Absolute Q Universal DNA Digital PCR Master Mix and the TrueMark Enteric Bacterial Select Panel I on the QuantStudio Absolute Q dPCR System with the default system cycling parameters. Values shown are means of two technical replicates, with error bars representing standard deviations. *Campylobacter* spp. are detected in the FAM dye channel, *Salmonella* spp. are detected in the VIC dye channel, *Shigella* spp./EIEC are detected in the ABY dye channel, and STEC is detected in the JUN™/Cy®5 dye channel.

dPCR is more tolerant of PCR inhibitors than qPCR

We sought to demonstrate that dPCR on the QuantStudio Absolute Q dPCR System is more tolerant of PCR inhibitors than qPCR. To mimic inhibitor carryover from sample extraction, purified samples were spiked with different concentrations of bile, a component of wastewater samples, then tested by qPCR and on the QuantStudio Absolute Q dPCR System. At all tested bile concentrations (0.1–2 mg/mL), dPCR shows no inhibitory impact on results (Figure 8A, C) and produces the same target concentrations with high accuracy across the range of PCR inhibitors tested (Figure 8C). In contrast, qPCR begins to show compromised quantification (i.e., less accuracy) due to inhibition (resulting in higher C_q values) even at the lowest level of bile tested (0.1 mg/mL), with no quantification values obtained at 1 mg/mL bile and above (Figure 8B, D). qPCR shows increasing variability between technical replicates as inhibitor concentrations increase, whereas dPCR maintains similar precision across all concentrations tested. Note that the levels of bile used in this experiment are not necessarily expected to carry over from sample extraction, but inhibitor carryover will depend on the chemical characteristics of the inhibitor as well as the methods of sample concentration and nucleic acid extraction used.

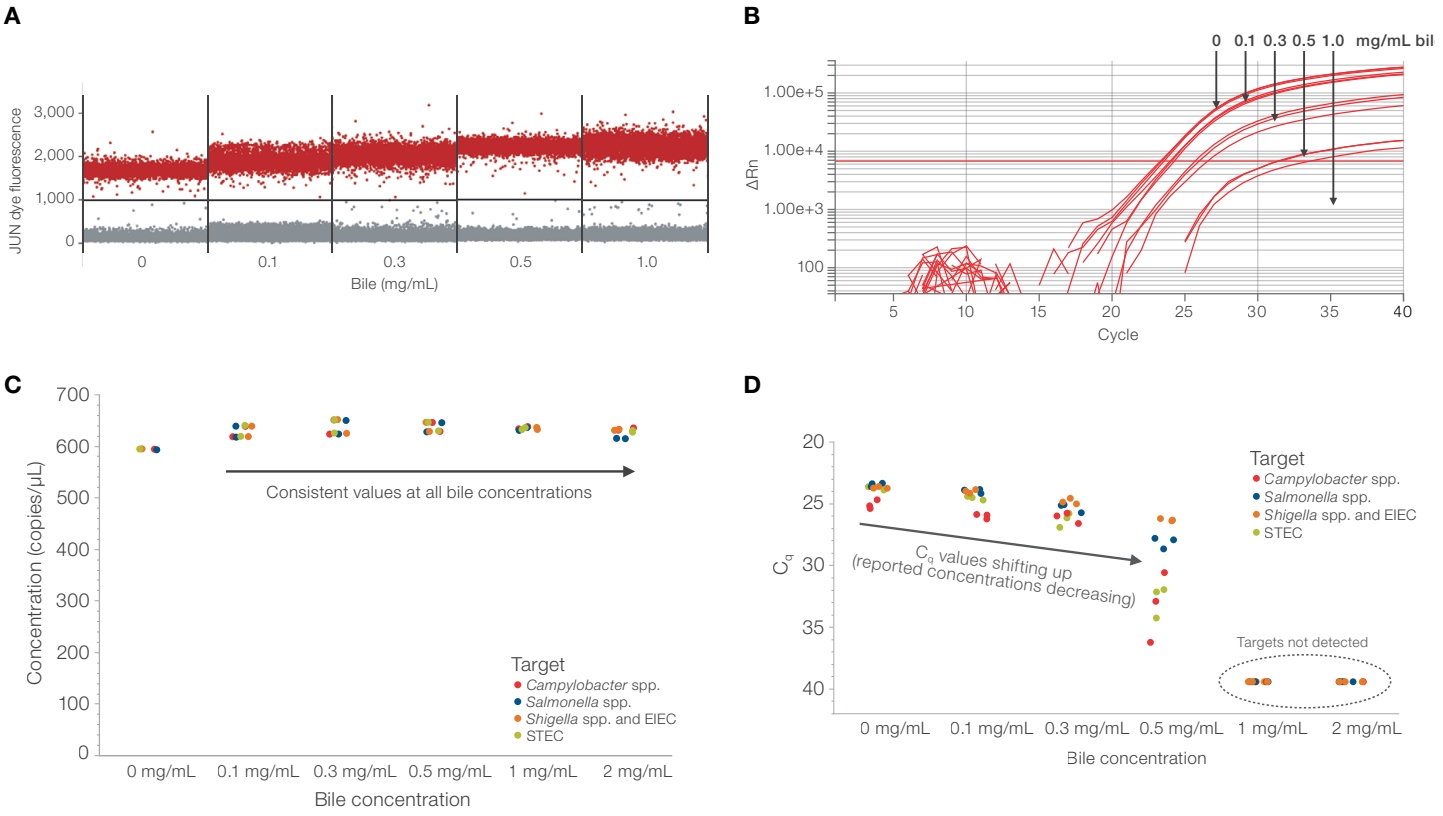

Figure 8. Digital PCR is more tolerant of PCR inhibitors compared to qPCR. A sample positive for all four targets was spiked with the indicated concentrations of unfractionated bovine bile, then tested without further purification on the QuantStudio Absolute Q dPCR System and by qPCR on the Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System, side by side. The same primer and probe concentrations were used for both dPCR and qPCR. **(A)** dPCR results shown as one-dimensional scatter plots from a representative target of the multiplex assay (STEC) in the presence of the indicated bile concentrations. Red dots indicate target-positive chambers and gray dots indicate target-negative chambers. **(B)** Amplification curves for STEC by qPCR in the presence of the indicated bile concentrations. **(C)** dPCR reported concentrations for all four targets in the presence of the indicated bile concentrations. Each dot represents one technical replicate. **(D)** qPCR C_q values for all four targets in the presence of the indicated bile concentrations. Each dot represents one technical replicate.

Conclusions

Here we have shown that the QuantStudio Absolute Q dPCR System provides several advantages for pathogen detection in challenging samples. These include the ability to easily create multiplex assays, high accuracy and precision, and tolerance to PCR inhibitors. Individual TaqMan Assays and Applied Biosystems assay panels designed for qPCR, which contain compatible dyes, can be utilized on the QuantStudio Absolute Q dPCR System with little to no re-optimization required. Paired with the MagMAX Wastewater Ultra Nucleic Acid Isolation Kit, the QuantStudio Absolute Q Digital PCR System can be used for multiplex detection of wastewater-relevant targets, enabling the monitoring of existing and emerging infectious diseases.

References

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Ordering information

Description	Cat. No.
Absolute Q Universal DNA Digital PCR Master Mix (5X)	A72710
Absolute Q 1-Step RT-dPCR Master Mix (4X)	A55146
QuantStudio Absolute Q MAP16 Plate Kit	A52865
MagMAX Wastewater Ultra Nucleic Acid Isolation Kit with Virus Enrichment	A52610
Absolute Q dPCR SARS-CoV-2 Wastewater Surveillance Kit	A55241
TrueMark Enteric Bacterial Select Panel I Combo Kit (Note that the combo kit contains a qPCR master mix, but the assay mix must be paired with the Universal DNA Digital PCR Master Mix for the QuantStudio Absolute Q dPCR System)	A58035
QuantStudio Absolute Q Digital PCR System	A52864, A53267
KingFisher purification systems	Visit website
Predesigned TaqMan Assays	Visit website
Custom respiratory multiplex: Flu A, Flu B, RSV, SARS-CoV-2	Please inquire
Custom mpox assay	Please inquire
TaqMan Gene Expression Assay – Poliovirus	Vi07922890_po

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