Multiplex Assay Design for Epidemiological Wastewater Monitoring with Droplet Digital™ PCR (ddPCR™) Technology

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Abstract

Wastewater-based epidemiology (WBE) is a common method to monitor and quantify the incidence of pathogens in wastewater within a population. The rate of incidence of these pathogens can be used to model pathogenic infections in a population. Due to the heterogeneous and complex composition of environmental wastewater samples, sensitive techniques are necessary to accurately identify waterborne pathogens responsible for millions of infections each year (Collier et al. 2021). Furthermore, rapid and high-throughput methods are critical for efficient monitoring in response to high occurrences of waterborne disease and subsequent testing (Collier et al. 2021). Here, we demonstrate that the Bio-Rad Laboratories, Inc. QX600™ Droplet Digital PCR System reliably accomplishes highly sensitive detection of commonly monitored pathogens in a rapid and accurate manner.

Introduction

The presence of enteric viruses and other pathogens in treated and untreated waters poses a significant health risk to the general population and is responsible for a wide range of diseases (Collier et al. 2021). Pathogens frequently enter aquatic environments through urban waste, agricultural spills, and sewage leaks. While water treatment facilities treat wastewater before release into the broader environment, high pathogen levels still pose a significant risk for disease outbreaks. This risk necessitates water and wastewater monitoring for appropriate assessment of the type of pathogens present and their relative prevalence.

In 2020, the Centers for Disease Control and Prevention (CDC) launched the National Wastewater Surveillance System (NWSS) in response to the coronavirus disease 2019 (COVID-19) pandemic (CDC 2023). The NWSS was developed to coordinate and build the nation's capacity to monitor the presence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the virus that causes COVID-19, in wastewater samples across the country. The NWSS now has more than 1,400 sites, performing wastewater sampling and testing in all 50 states, collectively representing more than 40% of the American public (Johnson 2023). Wastewater monitoring can serve as an early warning of an emerging outbreak in a community, with studies showing up to 2-week lead times before peak COVID-19 cases (CDC 2023). The CDC plans to expand the wastewater disease surveillance program pioneered during the pandemic, adding surveillance of gastrointestinal, respiratory, and antimicrobial-resistant pathogens using ddPCR Assays (Johnson 2023).

Detection and identification of different types of pathogens known to contaminate wastewater is difficult, expensive, and time-consuming. Established detection methods typically consist of culturing of pathogens and subsequent identification. Limitations of these methods range from long culturing times, difficulty in isolation, and limited accuracy in identification and quantification (Toze 1999). In recent decades, epidemiological wastewater monitoring has become commonplace for tracking and containing disease outbreaks, with PCR becoming the gold standard for pathogen detection (Toze 1999).

New PCR techniques and technologies have resulted in more efficient and cost-effective methods, including ddPCR technology. ddPCR technology enables accurate, precise, and ultrasensitive nucleic acid detection and absolute quantification. Due to these capabilities, ddPCR technology provides a particular advantage when testing heterogeneous environmental samples. With the advent of the QX600 ddPCR System, users can now expand the number of simultaneously detected targets owing to its six-color detection capability. This expansion of detection channels dramatically augments the information acquired from each sample and allows for a significantly greater degree of multiplexing. Further, the separation of targets across multiple channels streamlines data analysis and simplifies assay optimization. Here, we demonstrate

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Table 1. Pathogens and assigned fluorophores targeted in assays 1 and 2 and the MHV control assay.

	FAM	HEX	FAM/HEX	ROX	ATTO 590	Cy5	Cy5.5
Assay 1	Adenovirus	Norovirus	MHV control assay	MPXV (West African Variant 1)	MPXV (West African Variant 2)	Enterovirus	*Legionella*
Assay 2	Adenovirus	Norovirus	MHV control assay	MPXV (West African Variant 1)	MPXV (West African Variant 2)	Influenza A	Influenza B

MHV, mouse hepatitis virus; MPXV, monkeypox virus.

two 6-plex wastewater assays and one single-target control assay well suited for detecting and monitoring common enteric pathogens.

Materials and Methods

ddPCR Technology and Data Analysis

Droplet Digital PCR Expert Design Assays are specifically designed by Bio-Rad experts for the Droplet Digital PCR platforms. The wastewater sample assays are designed to precisely quantify pathogens in wastewater, with positive droplets representing droplets with amplified and detected target molecules and negative droplets representing droplets with no amplification.

The Bio-Rad Expert Design service was used to create two 6-plex wastewater assays and one single-target control assay for the following pathogens: adenoviruses, noroviruses, enteroviruses, *Legionella*, influenza A, influenza B, two monkeypox virus (MPXV) variants, and mouse hepatitis virus (MHV) as control (Table 1) (Assay 1 ID: dEXD77180931; 6-plex, Aden/Nor, ENT/LEG, MPXV WA1/MPXV WA2) (Assay 2 ID: dEXD26108593; 6-plex, Aden/Nor, InfA/B, MPXV WA1/MPXV WA2) (Control Assay ID: dEXD84432885; single-target, MHV). All primers, probes, and synthetic DNA were synthesized by Integrated DNA Technologies, Inc. (IDT) (custom products). Stock solutions were made by resuspending the primers and probes with DNA Suspension Buffer, pH 8.0 (Alpha Teknova, Inc., catalog #T0223) to 100 µM. Working solutions of a 20x assay (18 µM of each primer and 5 µM of probe) in DNA suspension buffer
was prepared in Eppendorf DNA LoBind Microcentrifuge Tubes (Fisher Scientific, #13-698-791) for a final 1x reaction concentration of 900 nM for each primer and 250 nM for the probe.

Synthetic DNA fragments consisting of a linearized custom gBlocks Gene Fragments (IDT) containing the amplicon of each of the pathogens were added to the ddPCR Supermix for Probes (no dUTP) following the manufacturer's instructions (Bio-Rad, #1863010). Samples were pipet-mixed, thoroughly vortexed, pulsed on a microcentrifuge, and emulsified. Twenty-two microliters of the solution was aliquoted into 96-well PCR plates, of which 20 µl was used to create droplets using the Automated Droplet Generator (Bio-Rad, #1864101). Droplets were transferred to ddPCR 96-Well Plates (Bio-Rad, #17005224) and sealed with pierceable foil PCR Plate Heat Seals (Bio-Rad, #1814040). PCR was performed on a C1000 Touch Thermal Cycler (Bio-Rad, #1851196) using a ramp rate of 2°C per sec with the following parameters: 95°C for 10 min, 40 cycles of 94°C for 30 sec then 55°C for 60 sec over a temperature gradient of 60–50˚ C, 98°C for 10 min, and a final hold at 4°C. The fluorescence signal from the droplets was obtained using a QX600 Droplet Digital PCR System (Bio-Rad, #17007769) according to the manufacturer's instructions.

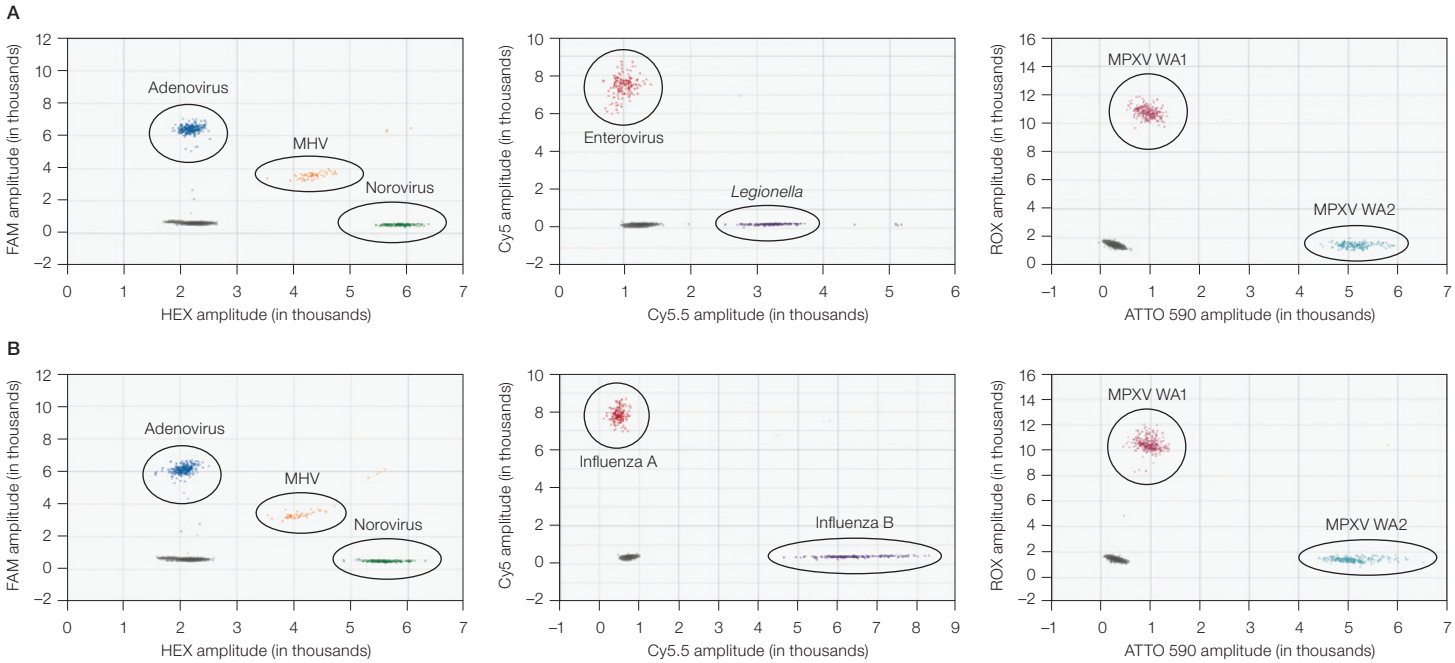

Fig. 1. Two-dimensional fluorescence intensity plots of Assays 1 and 2. A, representative plots for a 7-plex experiment using Assay 1. FAM, adenovirus; HEX, norovirus; FAM/HEX, mouse hepatitis virus (MHV); Cy5, enterovirus; Cy5.5, *Legionella*; ROX, monkeypox virus (MPXV) (West African variant 1); ATTO 590, monkeypox virus (MPXV) (West African variant 2). **B**, representative plots for a 7-plex experiment using Assay 2. FAM, adenovirus; HEX, norovirus; FAM/HEX, MHV; Cy5, influenza A; Cy5.5, influenza B; ROX, MPXV (West African variant 1); ATTO 590, MPXV (West African variant 2).

Results

Representative fluorescence intensity plots for the six pathogens targeted in ddPCR multiplex experiment of Assays 1 and 2 are shown in Figures 1A and 1B, respectively. A titration series was performed on the QX600 ddPCR System to demonstrate its sensitivity on wastewater products. Consistent agreement and linearity across five different concentrations for Assay 1 (Figure 2A, B) and Assay 2 (Figure 2C, D) were observed. Interrogated targets were detected as low as 2.7 copies/µl. Annealing and extension temperature gradient optimization was performed over a range of 50–60°C. Consistent agreement was observed in reported concentrations across all temperatures tested for all targets in Assay 1 (Figure 3A) and Assay 2 (Figure 3B).

Conclusion

The QX600 System enables simultaneous ddPCR evaluation of six or more targets to characterize the identity of numerous pathogens. In addition, the high resolution of ddPCR technology provides absolute quantification of multiple targets per well, showcasing the enhanced multiplexing and the high-throughput capacity of the QX600 System. The ddPCR analysis herein showcases the ability to detect RNA and DNA simultaneously in a representative wastewater sample. The two unique multiplex assays targeting commonly monitored pathogens used here, and many others, can be acquired from the Bio-Rad Expert Design Assay marketplace.

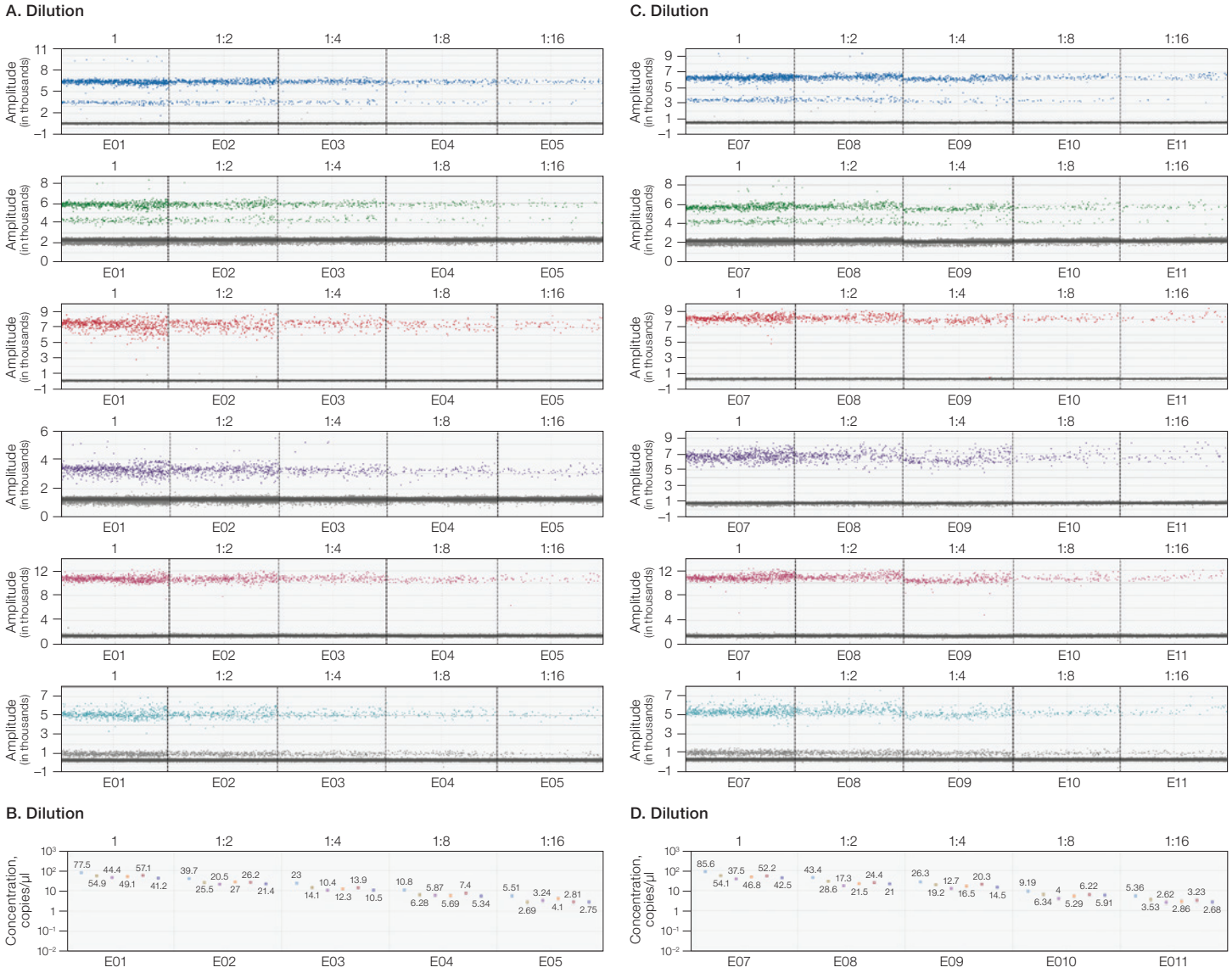

Fig. 2. Titration series demonstrating the sensitivity of the QX600 instrument. Representative titration series one-dimensional and concentration plots of Assays 1 and 2 showing excellent linearity across five dilutions for all interrogated targets. gBlocks Gene Fragments were diluted as designated and thermal cycled at 55°C for Assay 1 (**A**, **B**) and Assay 2 (**C**, **D**). All viral targets could be detected at 5.5 copies/µl or less. The y-axis on concentration plots (**B**, **D**) show concentrations on a logarithmic scale.

Fig. 3. Temperature gradient optimization of Assays 1 and 2. Representative concentration plots of Assay 1 and 2 over a temperature gradient. Undiluted gBlocks Gene Fragments were thermal cycled at a range of 60–50˚C for Assay 1 (**A**) and Assay 2 (**B**). All assays showed a consistency of concentration across a wide range of temperatures. The y-axis shows concentrations on a logarithmic scale.

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