The detection efficiency of digital PCR for the virulence genes of waterborne pathogenic bacteria

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ABSTRACT

Waterborne pathogens are the primary concern for the safe reuse of wastewater. Although digital PCR (dPCR) is considered promising for absolutely quantitating genes, the detection efficiency of dPCR is affected by many factors. This study tested eight virulence genes of pathogenic bacteria on a control plasmid and reclaimed water samples with reported primer–probe sets and designed ones on quantitative PCR (qPCR) and dPCR. Probe efficiency, data analysis, and PCR inhibition were found to affect the detection efficiency of dPCR. Firstly, poor probe quality, which is determined by probe quenching and activation efficiencies, was the main cause of PCR failure. Secondly, even if the PCR was successful, the probe quality and signal intensity could still affect the quantitative process. Manual analysis of dPCR data on the weak signal intensity would significantly reduce errors. And lastly, the sensitivity of PCR inhibition was lower in dPCR than qPCR, but inhibition still existed. The dPCR produced various detection efficiencies for different targets in one sample indicating inconstant inhibitory effects. Dilution was still the proper approach to overcome inhibition, but decreased the detection limit. More studies are required to ensure accurate waterborne pathogen quantitation by dPCR.

Key words | digital PCR, pathogenic bacteria, PCR inhibition, probe efficiency, reclaimed water

HIGHLIGHTS

- Successful primers and probes are PCR reagent, protocol, and equipment dependent.
- Probe quality is determined by quenching and activation efficiencies.
- Peak separation of dPCR is critical and needed manual adjustment sometimes.
- dPCR is much more inhibition-tolerant than qPCR.
- PCR inhibitory effect is different for different PCR reactions.
INTRODUCTION

The intense pressure on water resources globally has led to efforts in applying reclaimed water for many purposes. Reclaimed water is perceived as risky due to the occurrence of waterborne pathogens. Waterborne pathogens can be classified as bacteria, molds, viruses, and parasites. They can cause cellular damage and lead to clinical outcomes of morbidity or mortality. According to the statistics of the United States Centers for Disease Control and Prevention, public health officials of 46 states reported approximately 495 outbreaks associated with recreational water, and among 363 outbreaks, 57 (16%) caused by Legionella spp., and 47 (13%) by Pseudomonas spp. during the years 2000–2014. Moreover, six out of eight reported deaths were caused by Legionella spp. (Hlavsa et al. 2018). It seems that among the pathogens, bacteria are the common etiological agents for illness.

To address the public health concerns relating to pathogens, detection methods have been extensively investigated. For reclaimed water, traditional culture-dependent plate-count methods of indicator organisms have been used over past decades (Asano et al. 2007). But most of the indicator organisms are nonpathogenic. The enumerations do not correlate with the actual pathogens or opportunistic pathogens such as Aeromonas spp., Campylobacter spp., Escherichia coli 157:H7, Salmonella spp., Legionella spp., Mycobacterium spp., Pseudomonas spp. (Brandi et al. 1999; Chhipi-Shrestha et al. 2017; Garner et al. 2018). Consequently, molecular tools for detecting pathogens have been developed. Rapid detection methods can be categorized into three general technologies: (1) biosensor technology involving optical biosensors and electrochemical biosensors; (2) immunology-based assays like lateral flow immunoassay and enzyme-linked immunosorbent assay (ELISA); (3) nucleic-acid-based assays such as polymerase chain reaction (PCR), multiplex PCR, qPCR, ring-mediated isothermal amplification, dPCR, and DNA microarray (Law et al. 2015).

A biosensor’s basic structure contains a substrate of silicon or polymers with specific biological recognition elements such as aptamers, or antibodies of known pathogens. The binding effect will generate corresponding biological responses and be converted into readable signals ready to be analyzed (Leonard et al. 2016). Despite the advantages of portability and miniaturizations of the techniques, the applications of a biosensor still face the challenges of low sensitivity due to the environmental influences of dirt, pH and temperatures (Bridle & Desmulliez 2014).

Immunology-based and nucleic-acid-based assays are still considered the most robust technologies nowadays (Hameed et al. 2018). Immunology-based assays involved the specific interactions between antibodies and antigens on the surface of the target bacteria. They are rapid and specific, but lack sensitivity and have high values of cost,
while for nucleic acid-based detection technologies, they depend on the detection of specific DNA or RNA sequences. In this case, the virulence genes of pathogens are widely adopted. Virulence is defined as the ability of the microorganisms to overcome the host defense. A typical nucleic-acid-based detection strategy used in the field is the qPCR technique. The amplification process of the target DNA is simultaneously monitored by the fluorescence produced during each cycle of a PCR reaction. One of the advantages of qPCR is the use of fluorescent dyes or probes of different wavelengths, which can be used in an automated system for multiple detections, although qPCR will result in larger error, when the target sequence is at very low abundance or the differences between samples are less than two-fold. Consequently, it leads to the emergence of dPCR.

The key characteristic of dPCR is that a sample is first partitioned into thousands of individual reactions. After thermal cycling, the target gene copy can be calculated by the positive and negative numbers of partitions and the Poisson distribution law. The main advantage is that it allows absolute quantification of the target sequence rather than the relative quantification of qPCR. The dPCR technique is best suited to applications requiring precision, high sensitivity and reproducibility, such as low abundance target detection. Because of the target concentration effect of partitioning, dPCR is also less susceptible to contaminants that affect PCR detection efficiency (Monteiro & Santos 2017). The disadvantages of dPCR are that it is costly and time-consuming. The cost of one dPCR reaction is about 6–10 times higher than that of qPCR, and the entire procedure of dPCR takes two or three times as long in operation. Recently, dPCR has been applied in detecting microbial agents associated with waterborne diseases. Ricchi et al. (2017) suggested that dPCR approaches were considered valid based in the testing of waterborne pathogens such as Listeria monocytogenes, Francisella tularensis, and Mycobacterium avium subsp. paratuberculosis. Jikumaru et al. (2020) were able to detect the Shiga-toxin gene (STX2) in Escherichia coli from a river sample at a concentration of 32 copies/100 mL. And Li et al. (2018) proved the success of dPCR amplification on Staphylococcus, Salmonella, Listeria and other infectious microorganisms.

There are several versions of dPCR from different companies, like ddPCR from Bio-Rad and dPCR from Stilla Technologies. Although these digital PCR technologies have distinct ways to generate droplets and count positive ones, their core principles are the same, and they are all capable of performing absolute quantitation. Nonetheless, the quality of primers and probes, the way of analyzing data, and the interference of impurity from DNA samples could still lower the detection efficiency of dPCR. The detection efficiency means the ratio of experimental quantification result to the real gene copy (the specificity of testing different sub-species is not discussed in this study).

This study investigated the influence of PCR reaction conditions, analytical methods, and PCR inhibitions on the detection efficiency of absolute quantification of waterborne pathogenic bacteria by detecting the eight virulence genes in dPCR, and provided corresponding solutions.

**MATERIALS AND METHODS**

**Synthesis of the control plasmid**

In this study, eight virulence genes of eight common pathogenic bacteria were chosen (Table 1). A plasmid containing eight virulence gene fragments, invA, cadF, ipaH2, regA, eae, hsp, mip, yst, and a human GAPDH gene fragment was synthesized as the external control (Figure S1). The entire sequence is available in the Supplementary Materials. The plasmid was linearized to mimic the form of genomic DNA extracted from water. The quantity of plasmid was determined by UV260 absorbance.

**Extraction of bacterial genomic DNA**

The DNA purification method was adapted from a recent paper (Shi et al. 2020). The extraction method was proven to have higher DNA recovery efficiency for the samples on filter membranes than commercial kits. Briefly, water samples from a river, a lake, and an artificial pond (water quality data is available in Table S1) were filtered with 0.45 μM MCE membranes (BOJIN, China), which were stored at –80 °C before DNA extraction. The procedure started with adding lysozyme lysis buffer (1 mg/mL in Tris-HCl, pH 8.0) to break the cell
wall of gram-positive cells (37 °C, 30 min) followed by degradation of proteins by proteinase K (0.4 mg/mL, 60 °C, 2 h). Bath sonication (100 W, 5 min) and syringe shearing were used to assist cell lysis. Then phenol/chloroform/isoamyl alcohol (25:24:1) was added to remove proteinase K. The upper phase was finally precipitated by isopropanol.

qPCR and dPCR amplification

To test the PCR performance of different probes, two sets of primers and probes were synthesized for eight bacterial virulence genes, namely BHQ and MGB. Each set contained forward and reverse primers and a probe. The primers and probes of BHQ sets were all reported from the literature (Table S2). The probes used the traditional TaqMan Fluorescein amidites (FAM) dye label with black hole quencher (BHQ). The MGB sets were designed by ThermoFisher Scientific with Primer Express 3.0. And all used minor groove binder (MGB), epoch dark quencher and non-fluorescent quencher. The qPCR was performed as a pre-test for dPCR with recombinant plasmid as the template. Six MGB sets were successful, but only four BHQ sets gave significant signals (Figure S2).

The qPCR amplifications were performed with Applied Biosystems™ QuantStudio™ 5 or 1 (ThermoFisher Scientific, USA). The reaction mixture (20 μL) of qPCR contained 2,340 copies of control plasmid, 200 nM of each primer, 200 nM of TaqMan BHQ probe (or 2 μL of 1:40,000 diluted SYBR Green from Solarbio, China, when testing the primers), and 10 μL of QuantStudio™ 3D Digital PCR Master Mix v2. The amplification protocol was as follows: 96 °C for 10 min, 60 °C for 2 min and 98 °C for 30 s, 40 cycles. The amplification protocol for the primer test in qPCR was as follows: 50 °C for 2 min, 95 °C for 2 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min, followed by the melt curve setting of one cycle of 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s.

The dPCR amplifications were performed with the QuantStudio™ 3D Digital PCR system (ThermoFisher Scientific, USA). The reaction mixture (15 μL) of dPCR contained 2,340 copies of control plasmid, 200 nM of each primer, 250 nM MGB probe, and 7.5 μL QuantStudio™ 3D Digital PCR Master Mix v2. The amplification protocol of dPCR was as follows: 96 °C for 10 min, 60 °C for 2 min and 98 °C for 30 s, 40 cycles.

Assessment of PCR detection efficiency

In all experiments, 2,340 copies of control plasmids were added for each reaction. Inhibition of environmental samples was assessed by comparing their absolute copy numbers

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Table 1 | Functions of virulence genes in bacterial pathogens

| Pathogens  | Virulence genes & GenBank records | Functions                              | References               |
|-----------|-----------------------------------|----------------------------------------|--------------------------|
| Salmonella enterica | invA U43271.1 | the invasion of epithelial cells | Ike et al. (2018)        |
| Campylobacter jejuni | cadF FJ940601.1 | bonding pathogens to intestinal epithelial cells | Konkel et al. (1999)     |
| Shigella flexneri | ipaH2 EU743831.1 | invasion of epithelial cell | Clark et al. (2011)      |
| Pseudomonas aeruginosa | mgA X12566.1 | regulator of exotoxin A expression | Wick et al. (1990)       |
| Escherichia coli O157:H7 | eae NC_002695.1:c4599262-4596458 | intimate attachment onto intestinal epithelial cells | Loukiadis et al. (2006) |
| Mycobacterium tuberculosis | hsp CP003900.2 | immunodominant antigen | Bajaj & Batra (2012)     |
| Legionella pneumophila | mip AF095230.1 | lung macrophage infection | Engleberg et al. (1989)  |
| Yersinia enterocolitica | yst X65999.1 | heat-stable enterotoxin | Delor & Cornelis (1992)  |
(dPCR) to the deionized water control. The detection efficiency of dPCR was calculated by the following equation:

\[ E = \frac{D_{pe} - D_{e}}{D_{p}} \times 100\% \]  

(1)

where

- \( E \) represents the PCR detection efficiency;
- \( D_{pe} \) represents the detected target gene copies in the environmental sample together with the plasmid control;
- \( D_{e} \) represents the detected target gene copies in the environmental sample;
- \( D_{p} \) represents the detected target gene copies from the plasmid control only.

**Data analysis and statistics**

For calculating the standard deviation of dPCR detection efficiency, the positive counts and the upper and lower confidence limits of \( D_{pe}, D_{e}, \) and \( D_{p} \) from the analysis software QuantStudio 3D AnalysisSuite™ were used. The premise is that the counts of these three variables conform to normal distributions. Hence, the standard deviation is calculated by taking the absolute value of the difference between the two 95% confidence limits and the count values, and dividing the larger value by 2. Random numbers of normal distributions were generated from the rand function in Microsoft Excel and the simulations of the Monte Carlo model. They were used to obtain the distribution of the PCR detection efficiency based on Equation (1), as well as its 95% confidence limits and standard deviations.

**RESULTS**

**The influence of primer/probe design on dPCR detection efficiency**

All MGB sets and a commercial GAPDH control were tested on a dPCR instrument, since MGB sets had stronger signal and smaller Ct values. From the scatter plots, six of them were successful including the GAPDH control (Figure 1, the negative controls were normal as shown in Figure S3). Among them, \( invA, regA, eae, \) and \( mip \) got clear separations between the negative and positive dot groups (Figure 1(a), 1(e), 1(f) and 1(h)). In dPCR software, a separation line is drawn between the negative and positive dots to determine the exact positive dot numbers. It is the key step of absolute quantitation (this will be further discussed below). However, the negative and positive groups seemed to merge in the cases of GAPDH and \( hsp \) (Figure 1(c) and 1(g)). The overlap makes it difficult to determine the separation line, which significantly increases the error and affects the detection efficiency of dPCR.

Poor primer and probe qualities will seriously affect dPCR detection efficiency. If all the reactions were as successful as that of \( invA \)-MGB (Figure 1(a) and Figure S4(a)), the data analysis would be easy. Nevertheless, there is no guarantee for working primers and probes, and it is often costly to screen them. The eight sets of BHQ primers and probes have all been successfully amplified and published in the literature (Table S2), but only 50% of them worked in this study. This suggests that the successful design of a PCR primer–probe set is also related to the PCR reagents, protocols, and even instruments. All MGB sets were designed by Primer Express 3.0, which was developed by the same company as that of the dPCR reagent and instrument. It is fairly acceptable that six out of eight primer-probe sets were successful. But whether the failure of PCR reaction is due to inferior primers or probes is still not clear. In addition, all the primers were examined by SYBR Green dye independent of probes. All the primers from MGB sets had positive results and the primers of two genes, \( eae \) and \( yst \), from BHQ sets did not have significant signal (Figure S5). The results indicate that probes were the main cause of PCR reaction failure.

Another cause of poor amplification signal could be PCR conditions. The PCR mix (QuantStudio™ 3D Digital PCR Master Mix v2) provided by ThermoFisher has a very special recommended PCR protocol. The protocol has the annealing and extension combined and sets the temperature at 60 °C with a duration time of 2 min. It has been optimized specifically for the QuantStudio™ 5D Digital PCR system. Under such pre-set conditions, all the primers and probes were designed to reach the annealing temperature of 60 °C. And the long extension time is due to the low extension activity of Taq polymerase at 60 °C.
Consequently, it reduced the options for further optimization of PCR conditions. Optimization of reaction temperature or primer/probe ratio for the cadF gene showed that lower temperature could slightly improve the signal (Figure S6), but the signal was still much lower compared with other genes. Increasing probe to primer concentration ratio did not affect the results (Figure S7). The reactions of the two previous unsuccessful genes remained negative regardless of the changing conditions (data not shown). These data also support that the quality of the probe is most important.

**The influence of data analysis of dPCR detection efficiency**

Aside from the well-defined negative–positive separation on the scatter plots (Figure 1), determinations of positive dot cutoff for dPCR analysis is critical since this is one of the...
primary sources of error for absolute quantification. Good separation means small error, and vice versa. The analysis software, QuantStudio 3D AnalysisSuite™, applies a bimodal distribution fitting and calculates a value in a histogram (Figure 2). The negative dots usually show a good normal distribution; whereas the distribution of positive dots is unpredictable. Sometimes the software does not generate the best estimate. Figure 2(a) shows the result for GAPDH from Figure 1(c). The red separation line given by the software is not as good as the manually placed one (the black line in Figure 2(a)), which is at the lowest point between the two peaks based on observations (the red line in Figure 2(b)). Another option might be to model these two distributions. But the positive dots have a skewed distribution, and it does not have a general model and might not be more accurate than the simple separation especially when there are not many positive dots.

It is noted that the cadF-MGB had significant amplification on qPCR, but with a large Ct value of 31 (Figure S2(b)), while all the other five successful ones had much smaller Ct within 19–22 for the same template copies (Figure S2). The result showed that the final signal of the cadF-MGB reaction on qPCR was one order of magnitude lower. And it could be the cause for its poor performance on dPCR. The fluorescence of the amplification curve showed weak positive signals but still rose ten-fold higher than the baseline. In contrast, the dPCR amplification seemed to be a negative result. It appeared that the fluorescence intensity on both qPCR and dPCR instruments is a relative value. Thus, adjusting the scale might show much more detailed information. Unfortunately, the software does not support manual adjustment of the abscissa. The data was exported and redrawn as Figure 2(c). Although the positive and negative peaks are very close, they separate well. Hence, it is important to manually check the histogram for peak separation.

**The influence of PCR inhibition on dPCR detection efficiency**

The effective amplifications of MGB primer–probe sets for the control plasmid do not guarantee successful

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**Figure 2** | The separation of negative and positive wells of GAPDH by (a) the software, QuantStudio 3D AnalysisSuite™, (b) hypothesized model in histogram, and (c) the enlarged histogram of cadF-MGB from Figure 1(b) and Figure S4(b). The signals are drawn in distribution forms. The red line is the separation line in both (a) and (b). The black line in (b) denotes the partial data line at the overlapping site, while the yellow and blue lines denote the theoretical negative and positive peaks. The negative peak is cut off at 200 counts for better viewing of the positive peak in Figure 2(a) and 2(b). The negative peak is cut off at 1,000 counts to view the positive peak in Figure 2(c). Please refer to the online version of this paper to see this figure in colour. [http://dx.doi.org/10.2166/ws.2021.056](http://dx.doi.org/10.2166/ws.2021.056).
amplifications on the environmental samples. When the plasmid was mixed with an environmental DNA sample, which was extracted from the water of a reclaimed-water-replenished river (Table S1), none of the four positive sets above had significant amplification in the qPCR instrument (Figure 3(a)). This result indicates that the PCR reaction was completely inhibited by the environmental sample. This sample had a noticeably greenish color indicating impurity, but fairly good A260/A230 and A260/A280 values (1.37 and 1.83 respectively). It has been described that there are many PCR inhibitors. They might be coextracted with DNA, such as bile salt, humic compounds, complex polysaccharides in feces, proteinase in milk, urea in urine, heavy metals and excessive DNA (Pillai et al. 1991; Lantz et al. 1997; Monteiro et al. 1997; Alaeddini 2012). Many of them might have been present in the sample, since the river was mainly supplied by reclaimed water. The most studied inhibitory substance in the environment is the humic acids (Young et al. 1993; Crecchio & Stotzky 1998; Schrader et al. 2012). The inhibition mechanism of humic acids is that inhibitors bind with DNA polymerase enzyme and form an enzyme–substrate complex making the reaction inactive and also shift the DNA melting point to a higher temperature (Sutlovic et al. 2008).

Nonetheless, the reactions were all successful in dPCR without observable inhibitory effect (Figure 3(b)). This suggests that dPCR is not as sensitive to PCR inhibitors as qPCR. Furthermore, when three more environmental samples were tested, it showed that the inhibition also happened with dPCR (Figure 4). Sample 1 was taken from a reclaimed-water-filled river in spring, and did not exhibit apparent inhibitory effect for six genes. Sample 2 was from a mock reclaimed-water-filled pond in summer, and showed significant inhibition. Sample 3 was from a reclaimed-water-filled lake in summer, and had the strongest inhibitory effect on PCR amplification. It seemed that the inhibitory potentials were higher with the samples having smaller A260/A230 or A260/A280 values (Table S1). But the data was too limited to draw significant conclusions, and the changes of these two values were small. It is also challenging to identify the possible inhibitor due to its mixture form and there is not even a feasible method to measure humic acids in the DNA samples.

Although the dPCR data sometimes showed no inhibition with the environmental samples, the inhibitory effect could still be seen from the histogram of dPCR (Figure 5). It is clear that the positive peak moved towards the negative peak from (b) to (c) with the addition of environmental sample 1. This weak inhibition did not affect the dPCR quantification very much as the peak separation was still good enough (Figure 5(c)). Nevertheless, if this reaction was performed on qPCR, a larger Ct value would be expected since the fluorescence signal would be weakened and the quantity of the target gene would be underestimated. The reaction of sample 2 had merged peaks with moderate inhibition (Figure 5(d)). This result
severely affects the quantification detection efficiency by losing the positive counts to the negative peak and increasing the error in data analysis. Sample 3 completely inhibited the reaction (Figure 5(e)).

DISCUSSION

From the results, it is clear that poor probe design, inadequate data analysis, and PCR inhibitors could significantly affect the detection efficiency of dPCR. Possible solutions for improving the performance of dPCR are discussed.

The reason why some probes do not produce strong signals is not well understood to date. On the two ends of a probe, a reporter emits fluorescence and a quencher inhibits the fluorescence from the reporter. The fluorescence of the reporter should be quenched before the reaction and activated during the reaction. However, the quenching and activation efficiencies usually are not 100%, which results in a narrowed signal interval (Figure S8). From Figure S7, it is shown that the background fluorescence signal increased dramatically with the increasing probe concentration, which was even higher than the signal increase during the amplification. This result demonstrated that the quenching efficiency of this probe is significantly low. The quenching efficiency mostly depends on the distance between the reporter and quencher on the probe. Probes containing a hairpin structure have been designed to maximize the quenching efficiency like molecular beacons (Navarro et al. 2015). In this study, the activation efficiency could not be maximized, since reporter and quencher were separated due to the break of the hairpin structure during the hybridization step of the PCR reaction, and they were still on the probe. Thus, the fluorescence could not be completely activated.

According to this defect, the hydrolysis probe was invented to optimize activation efficiency, like the popular TaqMan probe (Navarro et al. 2015). By contrast, the quenching efficiency is compromised since the distance between reporter and quencher is much longer than for the hybridization probe. Both types of probes would have a relatively long sequence to ensure a higher annealing temperature ($T_m$) and faster hybridization than primers. The primer will start elongation immediately after hybridization and completely block the binding probe and inhibit signals entirely. The longer hydrolysis probe will then have an even lower quenching efficiency. As a result, many companies have developed short probes with comparative high $T_m$, like the MGB probe with a length of 16 nt, and the LNA probe as short as 8 nt (Navarro et al. 2015). The AllGlo probe not only applies modified nucleic acid to enhance the $T_m$, but also creates novel signal molecules. These molecules simultaneously emit their own fluorescence and quench fluorescence from other signal molecules through interactions with each other. And the molecules can be synthesized at any location on the probe, which further enhances the quenching efficiency by shortening the distance between signal molecules and increases activation efficiency.

Another way of improving probes other than by modification of nucleic acid or signal molecules is to combine the idea of molecular beacon and TaqMan probe. Jiang et al. (2018) kept the 5’ end of the TaqMan probe unchanged to gain the property of hydrolysis and expanded the 3’ end to form a hairpin or probe dimer to get high quenching efficiency. Despite all these efforts to ameliorate probes, more investigation and understanding of probe designs are required for successful and optimized PCR amplification.

There has been no accurate model for the data analysis to now, because the distribution of the peaks is unpredictable. Sometimes the positive droplets will be too few to be considered significant. Approaches using a quantitated

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**Figure 4** | Detection efficiencies for dPCR in the environmental samples with control plasmids. Samples 1, 2, and 3 represent a reclaimed-water-filled river, pond, and lake, respectively (water quality indices are shown in Table S1 and virulence gene copies in environmental samples are shown in Table S3). For each assay, 2,340 copies of the synthesized control plasmids were spiked. Errors were calculated based on the method section ‘Data analysis and statistics’.
positive control and others to prove the detection efficiency should be considered. The ideal way to prove the detection efficiency of dPCR is to test with known copy numbers of templates, whereas the dPCR itself might be the most accurate quantification test. One indirect approach to verify the detection efficiency of the absolute quantification of dPCR is to compare all the reactions within one plasmid template. When 2,340 copies of plasmid were added for the three independent tests with MGB primer–probe sets, the coefficients of variation were relatively larger at 10.4%, 7.9%, 17.4%, respectively (Figure 6(a)–6(c)), while the average values had a satisfactory CV of 4.4% (Figure 6(d)). No
single reaction had consistently higher or lower levels. This result indicates that random errors happened during the experiments that were possibly caused by the operation and could be alleviated by replicates. And the comparable result of absolute quantification for six genes suggests that the peak separation method is successful and the absolute quantification seems reliable, but whether possible systemic errors exist is unknown. These results also suggest that dPCR is relatively insensitive to the performance of the probes, if they are good enough to show positive signals. It can partially overcome the problem of out-of-linear quantification range caused by the high Ct value in qPCR.

PCR inhibition is a serious problem for the quantitation of specific targets in environmental samples. If the inhibition effect is similar when amplifying different genes, only one control test is needed to determine the PCR detection efficiency. Samples can be directly applied for the quantification of multiple targets. Unfortunately, the inhibitory effects were clearly distinct for each gene (Figure 5). It suggests that the PCR detection efficiency of each gene should be examined for absolute quantification. This might be due to the varied sensitivities of different reactions to the PCR inhibition. The reaction with a perfect peak separation, the *invA* gene, was supposed to have a larger anti-interference space, but in practice it did not, for unknown reasons. Therefore, it would be better to have all the reaction free of inhibition. Dilution is the most common approach (McKee *et al*. 2015). An environmental sample, which had a strong inhibitory effect for some genes, was tested for the dilution effect (Figure 7). Five-fold dilution significantly improved the PCR detection efficiencies, and ten-fold dilution completely eliminated inhibition effects. Although dilution can alleviate the inhibition problem, it significantly lowers the detection limit. It is not recommended for

![Figure 6](image-url)
particular pathogens at low abundance. The other strategy is to enhance the DNA purity either by adding more purification steps or using different commercial kits. Nevertheless, there is no guarantee of clearance of inhibitors.

**CONCLUSIONS**

This study reported that probe design, data analysis, and PCR inhibition are the three critical factors affecting dPCR detection efficiency when detecting waterborne pathogenic bacteria. Probe quenching and activation efficiencies are important considerations of design. Successful primer-probe sets are PCR reagent, protocol, and instrument dependent. Peak separation of dPCR needs manual adjustment when the negative and positive data peaks merge together. The PCR inhibitory effect varies for different PCR reactions. Thus, each reaction efficiency needs to be determined for quantitation or other measures need to be taken to remove the inhibition like dilution. Nonetheless, dPCR is much more inhibition-tolerant than qPCR.

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**DATA AVAILABILITY STATEMENT**

All relevant data are included in the paper or its Supplementary Information.

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