Comparing the efficiencies of qPCR and ddPCR using SARS-CoV-2 nucleocapsid primers and probes

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Introduction

Polymerase chain reaction (PCR) provides the ability to amplify specific fragments of DNA and synthesize millions of copies in a rapid qualitative reaction for various areas of biological research and vaccine testing (Kralik & Ricchi, 2017). During the PCR process, small primers bind to the ends of the target sequence after the DNA strands are denatured. These primers serve as templates that extend until they are the same length as the original strand. More current quantitative PCR procedures have been developed to use fluorescence to monitor the amplification by attaching fluorescence to the probe.

One such method is quantitative PCR (qPCR), which utilizes quantification cycles (C_t) to determine when the fluorescence intensity is proportional to the initial number of DNA molecules in the samples. This allows the relative quantity of target DNA to be calculated using a calibration curve and a standard curve during analysis (Kralik & Ricchi, 2017). Another quantitative method is droplet digital PCR (ddPCR), where mixture of template DNA, primers, probes, fluorescence, and other materials is divided into nanoliter-sized droplets that facilitate amplification (Hindson et al., 2011).

Despite these method's differences, both have improved the detection of many pathogens. For example, qPCR and ddPCR have been used to help analyze and diagnose SARS-CoV-2, which is the strain of coronavirus that caused COVID-19. SARS-CoV-2 is made up of four structural proteins, including the spike protein, the envelope protein, the membrane protein, and the nucleocapsid protein (Vindeirinho et al., 2022). These proteins all contain DNA that can be amplified as reference genes during a PCR process,. However, the nucleocapsid protein is used more often due to its low likeliness to mutate compared to the other proteins (Vindeirinho et al., 2022). The purpose of this project is to compare the efficiencies (accuracy and reliability) of qPCR and ddPCR through utilizing SARS-CoV-2 nucleocapsid primers and probes.

Materials and Methods

The reference RNA sequence MT039890 and was chosen as the reference gene from the SARS-CoV-2 nucleocapsid protein. Integrated DNA Technologies (IDT) generated five different designs for the forward primer, reverse primer, and probe based on the reference gene. The five designs generated for the forward primer and information provided for each design are shown in Table 1. The first design was chosen for the primers and probes based on their %GC content and melting temperature. After they were delivered, they were rehydrated and tested against standard nucleocapsid primers and probes. This was done to determine if there were any errors present in the designs that would cause problems during future runs of qPCR and ddPCR.

Materials and Methods (continued)

	Sequence	Length (Bases)	Melting Temperature	%GC
Set 1	TCATCACGTAGTCGCAACAG	20	61.92	50
Set 2	CCCACCAACAGAGCCTAAA	19	61.59	53
Set 3	GTGATGCTGCTCTTGCTTTG	20	61.93	50
Set 4	GTGATGCTGCTCTTGCTTTG	20	61.92	50
Set 5	CAACTGAGGGAGCCTTGAATAC	22	62.53	50

Table 1 (above): The five designs as generated by IDT. A primer should have a %GC content of 50–55% and a melting temperature of 60–64 °C to properly function.

Four dilutions of the SARS-CoV-2 RNA were made and labelled Dilutions 1 though 4, with decreasing concentrations of RNA in each dilution. Copies of RNA per reaction in each dilution are shown in Tables 3 and 4. There was no standard concentration set applied to the qPCR runs and the ddPCR runs due to the differences in methodology. The C100 Touch Thermal Cycler and Thermocycler CFX Touch were used for the qPCR runs. The QX200 Droplet Reader, QX200 Droplet Generator, PX1 PCR Plate Sealer, and C100 Touch Thermal Cycler were used for the ddPCR runs. Four runs were completed for each dilution in each PCR method. Mean values were calculated for analysis.

Results

In order to compare accuracy of qPCR and ddPCR methods, mean observed output of DNA after each run is compared to expected values, measured in DNA copies per reaction. Expected values are known based on set dilutions. Observed values are measured in quantification values. Calculations to determine these values are shown in Table 2. Percent error was used to determine the accuracy of each dilution run in each type of PCR. Expected values, observed quantification values, and percent error for each dilution in qPCR and ddPCR are shown in Table 3 and Table 4, respectively. The coefficient of variation (%CV) was also calculated for each dilution. This was used to measure variability in both methods. A greater %CV indicates greater variability of data and less reliability. This is shown in Table 5.

Methods	Equations
qPCR	$y = 2\left(10^{\left(\frac{C_t - y - intercept}{slope}\right)}\right)$
ddPCR	$c = 4.4\left(-\frac{\ln\left(\frac{Negative\ droplets}{Total\ droplets}\right)}{0.85}\right)$

Table 2 (left): Equations used in conversion of output data to the value of copies/reaction. The qPCR equation was multiplied by two to account for the 2 µL volume of template used in qPCR reactions.

	Expected Value (copies/reaction)	Quantification Value (copies/reaction)	Percent Error (%)
Dilution 1	625	621	−0.64
Dilution 2	125	131	4.8
Dilution 3	25	30	20
Dilution 4	5	4	−20

Table 3 (above): The results of the qPCR runs after the quantification values were calculated and compared to the expected values. The equation used for percent error was

$$E = 100\left(\frac{(Quantification\ Value - Expected\ Value)}{Expected\ Value}\right).$$

Results (continued)

	Expected Value (copies/reaction)	Quantification Value (copies/reaction)	Percent Error (%)
Dilution 1	625	578	−7.5
Dilution 2	125	124	−0.8
Dilution 3	25	26	4.0
Dilution 4	5	4	−20

Table 4 (above): The results of the ddPCR runs after the quantification values were calculated and compared to the expected values. The same equations used in Table 3 were used in Table 4.

Method	Dilution 1 %CV	Dilution 2 %CV	Dilution 3 %CV	Dilution 4 %CV
qPCR	6.16	5.13	16.6	21.1
ddPCR	2.45	2.86	10.9	35.4

Table 5 (above): The %CV values of qPCR and ddPCR for each dilution. The equation, $CV = 100(Standard\ Deviation / Mean)$ was used to calculate each %CV value.

Conclusion

The purpose of this project was to compare the efficiencies of qPCR and ddPCR through utilizing SARS-CoV-2 nucleocapsid primers and probes. The results of the comparison of the quantification values and expected values of each dilution indicated that qPCR was more accurate than ddPCR due to ddPCR having a greater percent error overall. This conclusion does not align with previous studies regarding this comparison. Possible sources of this disparity could include small sample size tested, the need for more precision with input concentrations, and increased complexity of ddPCR setup.

Variation of %CV between dilutions of each PCR method indicate varied precision of each dilution, regardless of method. Greater precision in the creation of dilutions would have likely resulted in lower %CV values and should be considered in the future.

Recommendations for further studies include providing more training in the processing and analysis of both PCR methods to ensure accuracy and collection of more values to improve statistical analysis. Comparative accuracy of qPCR versus ddPCR can also be done on different primer and probe combinations.

References

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