Wastewater-Based Epidemiology Surveillance For Early Detection SARS-COV-2: Method Development

BY

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THESIS

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DEDICATION

I dedicate this thesis to my mother Shelia, grandmother Dorothy, great-grandmother Ophelia. I am their wildest dreams come to life. I dedicate this thesis to my husband, James. His love and support helped me accomplish my goals. Most importantly, I dedicate this thesis to my Lord and Savior, who set before me a path of purpose and passion and the one in which I walk in by accomplishing this calling in my life.

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LIST OF ABBREVIATIONS

BCoV	Bovine coronavirus
CV	Coefficient of variation
COVID-19	Coronavirus disease 2019
R^2	Correlation of coefficients
CCJ	Cook County Department of Corrections Jail
GC/RXN	Gene copies per reaction
LLOQ	Lower limit of quantification
MeB	Method blank
NTC	No template control
PMMoV	Pepper mild mottle virus
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SD	Standard deviation
Ct	Cycle threshold
WW	Wastewater (WW)
WBE	Wastewater-based epidemiology
WWTP	Wastewater treatment plant

SUMMARY

Evaluation of RT-qPCR assay for the detection of SARS-CoV-2 quantitatively measured in standard calibration curves using gene targets that yield precise, sensitive, specific, and efficient results. Two gene targets were used to detect two regions in the SARS-COV-2 nucleocapsid N1 and N2 loci of the nucleocapsid (N) gene, specific to SARS-CoV-2, bovine coronavirus (BCoV) to determine virus recovery, and Pepper mild mottle virus (PPMoV), a marker of human stool. Evaluation required standard calibration curves for each of the two targets using primers and probes. The purpose of these standard calibration curves was to evaluate whether the RT-qPCR assay and instruments give consistent data with low to no variability from run to run. Thus, the curves can be used to estimate the RNA viral concentrations in wastewater samples. Six standard calibration curves for two gene markers were performed. Data analysis included examining the linearity of the calibration curves, residual analysis to determine the consistency of slope with repeated calibrations and determining the lower limit of quantification (LLOQ) for the four targets of interest.

Indicate that the calibration curves of log-transformed concentration versus cycle threshold (Ct) are linear over 5 orders of magnitude for N1 and N2, 6 orders of magnitude for BCoV, and seven orders of magnitude for PMMoV. Average slopes (standard deviation) are -3.19 (0.06), -3.38 (0.12), -3.55 (0.09), and -3.32 (0.05) for N1, N2, BCoV, and PMMoV, respectively. Residual analysis of the slopes for each experiment showed average residuals equal to zero for each of the four gene targets with a small upward but low correlation trend

for N1 and small downward trends, with low correlations for N2, BCoV, and PMMoV. The LLOQ values are 0.018 , 0.018, 0.017, 0.017 for N1, N2, BCoV, and PMMoV, respectively.

This analysis reveals the methodology and instruments are giving consistent data with low variability over time allowing for confidence in the detection of SARS-CoV-2. The consistency gives confidence that the four targets (N1, N2, BCoV, PMMoV) can be used to detect and quantify SARS-CoV-2 in wastewater. Our laboratory will use this method to test for SARS-CoV-2 in environmental wastewater samples.

I. INTRODUCTION

A. Wastewater-Based Epidemiology Background

Coronavirus disease 2019 (COVID-19) is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) officially named COVID-19 by the World Health Organization (WHO). It was first reported as an idiopathic pneumonia in Hubei Province, Wuhan, China, in December 2019. It is difficult to distinguish SARS-CoV-2 from other common respiratory viruses such as influenza viruses, because of their highly similar symptoms. A genetic assay with high specificity is necessary to detect SARS-CoV-2. (Chung et al., 2021).

Individuals infected with SARS-CoV-2, the virus that causes COVID-19, may shed the virus in stool before, during, and after developing symptoms, as well as being asymptomatic suggesting that measurements of SARS-CoV-2 concentrations in wastewater could be a "leading indicator" of COVID-19 prevalence at the community population level (Olesen et al., 2021). Wastewater-based epidemiology (WBE),is defined as the use of measurements from wastewater for public health surveillance, is being used in the COVID-19 pandemic as a complement to more traditional monitoring methods like diagnostic testing (Olesen et al., 2021).

Wastewater surveillance is being utilized in many countries to monitor possible outbreaks of COVID-19 by using SARS-CoV-2 viral RNA presence and levels in community wastewater (Ahmed et al., 2022). A multipart solution using WBE and reverse transcription quantitative polymerase chain reaction (RT-qPCR) is a public health strategy that may help to reduce or mitigate the spread of SARS-CoV-2 by giving public health professionals,

stakeholders, and policymakers real time evidence-based data about outbreaks within their communities. Allowing resources to be directed to outbreak locations. Utilizing WBE is an approach that can estimate COVID-19 illness caused by SARS-COV-2 prevalence in the population by detecting SARS-CoV-2 RNA fragments in community wastewater.

Combining centralized and decentralized WBE approaches**.** Centralized WBE is an approach where samples are collected at wastewater treatment plants (WWTPs) and is a common method. A more recent concept is a decentralized approach for WBE where samples are collected at different points of a sewer system. A decentralized approach can be used to focus on parts of the population where an outbreak is likely to happen (i.e., congregate living settings like college dorms, retirement homes, hospitals, and correctional facilities (jails, prisons, detention centers). "Targeted-WBE" as defined by Goncalves et al., (2022) is a decentralized approach that can be used to focus the value of diagnostic tests.

Using decentralized or targeted-WBE approaches to monitor SARS-CoV-2 outbreaks in jails. Incarcerated populations have experienced disproportionately higher rates of COVID-19-related illness and death. Correctional and detention facilities are not closed systems. Highly communicable make COVID-19 easily transmissible to and from the surrounding community through staff member and visitor movements and entry, transfer, and release of incarcerated persons (Hagan et al., 2020). Testing in these settings has often been limited to symptomatic persons. However, it is well known that infected persons can be asymptomatic. Asymptomatic COVID-19 infection has been reported to range from 18% to 31%, which are unaccounted for by health surveillance except for targeted studies of COVID-19 prevalence. "A recent study in clinical testing data to determine the abundance of asymptomatic versus symptomatic patients

revealed that 79.2% of the COVID-19-positive individuals were asymptomatic. However, many symptomatic and asymptomatic people shed the virus in stool, that can be detected in wastewater. WBE embraces both symptomatic and asymptomatic groups." (Shrestha et al., 2021).

According to the World Health Organization, "Infectious diseases are an important problem in prisons, interacting dynamically with other problems of incarcerated persons such as mental illness, addiction, or homelessness. Contextual factors such as overcrowding, limited access to water, or delays in diagnosis contribute to higher transmission rates [of infectious diseases]. Every prison health care service should have a comprehensive vaccination program for prisoners and prison staff"(World Health Organization, 2014).

According to a report from the Justice Collaborative, "It is not a matter of if but when the coronavirus will enter prisons and jails, according to health experts (Justice Collaborative, 2020). The consequences of that eventuality could be devastating. Outbreaks of SARS-CoV-2 in prisons and jails will spread "like wildfire" due to close quarters, unsanitary conditions, a population more vulnerable to SARS-CoV-2, and the large number of people that cycle through the criminal legal system. The risk extends far beyond those who are incarcerated. Outbreaks in jails and prisons threaten the larger public as hundreds of thousands of individuals churn through jails daily. Other staff interact with the incarcerated population and circulate back into the local communities. With 2.3 million people in the United States in prison or jail on any given day, an outbreak in these facilities threatens the entire country."

Testing for SARS-CoV-2 in a sizeable congregate population is costly, time-consuming, and not viable for long term monitoring. The number of tests needed, healthcare professionals required to perform/analyze, and cost of shipping and testing COVID kits daily to local clinical laboratories make this option impractical. Symptoms of COVID-19 are varied and often non-specific, including, fever, cough, diarrhea, loss of smell, flu-like symptoms, and unexplained rash. This can mean that conventional public health measures such as isolation or quarantine are ineffective when symptoms are not easily recognizable and do not have the space to quarantine individuals in a large congregate carceral setting.

The Bureau of Justice Statistics states, “An estimated 2.1 million U.S. adults are housed within approximately 5,000 correctional and detention facilities on any given day (Justice Collaborative, 2020).” The challenges of combating infectious disease in prisons are overcrowding where incarcerated persons are housed, shared toilets/showers, inadequate medical access, poor isolation/quarantine resource spaces, and the daily entry and exit of staff members or visitors. The repeated introduction of newly incarcerated or incarcerated persons and the transportation of persons in multi-person vehicles for court appearances, medical treatment, or security reasons.

The Morbidity and Mortality Weekly Report from May 15, 2020, states, “During April 22–28, 2020, aggregate data on SARS-CoV-2 cases were reported to CDC by 37 of 54 state and territorial health department jurisdictions (MMWR. CDC. Weekly / September 24, 2020). Thirty-two (86%) jurisdictions reported at least one laboratory-confirmed case from 420 correctional and detention facilities. Among these facilities, SARS-CoV-2 was diagnosed in 4,893 of

incarcerated persons and 2,778 facility staff members, resulting in 88 deaths among incarcerated persons and 15 deaths among staff members."

On September 28, 2020, the Wisconsin Department of Health Services (DHS) contacted CDC to report a SARS-CoV-2 outbreak in state prison (MMWR. CDC. Weekly April 2, 2021). During October 6–20, a CDC team investigated the outbreak, which began with 12 cases detected from specimens collected during August 17–24 from incarcerated persons housed within the same unit, 10 of whom were transferred together on August 13 and under quarantine following prison intake procedures (intake quarantine). After early detection of SARS-CoV-2 in six newly transferred persons during intake quarantine in a Wisconsin prison, 79.4% of incarcerated persons and 22.6% of staff members contracted SARS-CoV-2 from August 14–October 22, 2020. Whole-genome sequencing from 172 incarcerated persons with SARS-CoV-2 determined that all specimens clustered in the same lineage.

As of April 16, 2021, U.S. correctional and detention facilities reported 399,631 cases of SARS-CoV-2 in incarcerated persons, resulting in 2,574 deaths; during July 14–November 30, 2020, SARS-CoV-2 was diagnosed in 382 persons incarcerated in Idaho prisons with work-release programs(MMWR. CDC. Weekly / April 23, 2021).

The Prison Policy Initiative conducted an in-depth study of the SARS-CoV-2 caseloads outbreaks in non-metro mass incarceration jails/prisons. The data showed the more densely populated a jail/prison, the more caseloads of SARS-CoV-2 among the incarcerated population and the greater risk of spread to surrounding communities, counties, etc.

In the Polo (2020) study, WBE is an approach that relies on the assumption that any substance excreted by humans and is stable in wastewater can be used to back-calculate the original concentration excreted by the serviced population (Hooks and Sawyer, 2020). This same concept can be translated to virus surveillance. “Therefore, human viruses in wastewater can represent the concentrations excreted by the corresponding human population as long as they persist long enough (2–4 days) to be detected. Therefore, monitoring temporal changes in viral concentrations and diversity in community wastewater samples can be used not only to determine the true extent of the infection in the population, but also the emergence of new viral strains and the early detection of new viral outbreaks.”

In the Randazzo (2020) study, wastewater from metropolitan Valencia, Spain was analyzed for SARS-CoV-2 epidemiological surveillance was deployed to help a significant metropolitan area monitor for confirmed cases of SARS-CoV-2. Recent studies indicate that SARS-CoV-2 can also be excreted in feces and urine in asymptomatic carriers and recently recovered patients (W. Randazzo et al., 2020). Specifically, viral RNA was detected in feces up to 10 days after viral clearance from the respiratory tract, regardless of disease severity. The studies suggest that WBE is a possible solution and more realistic than individual testing of people. Data that confirms SARS-CoV-2 can be reproducibly detected by RT-qPCR “in longitudinal samples from sewage treatment plants that receive wastewaters from over one million inhabitants in the metropolitan area of Valencia, Spain. The study analyzed 15 samples taken at three wastewater treatment plants between February 12 and April 14, 2020. The sewage treatment plants under study collected wastewaters from approximately 1,200,000 inhabitants in 22 townships of the Valencian, Spain metropolitan area.”

The Hart (2020) study used WBE and RT-qPCR in Tempe, Arizona. Tempe has a population of “185,038 according to the 2017 U.S. Census and a density of 1779 people per square kilometer (O. Hart and R. Halden, 2020).” Land use is predominantly residential, with some industrial and commercial activity. According to Hart, the “practical limit” of detection of SARS-CoV-2 in community wastewater is well within the useful range and potentially superior to the alternative approach of randomly testing 100 to 2 million people to establish the presence or absence of symptomatic or asymptomatic cases in a population of interest.

The presence of SARS-CoV-2 in feces and wastewater constitutes a paradigm shift in surveillance. The utility and potential of a wastewater surveillance system have been previously demonstrated. “During the global polio eradication program, it was utilized as a tool to assess polio circulation within populations and the evaluation of immunization efficacy against poliovirus (Hovi et al., 2012).

Use of WBE has potential as a surveillance and predictive tool during the SARS-CoV-2 pandemic. Certain limitations could be solved by adding it to existing surveillance systems that have been highlighted during the pandemic. Specifically, the sensitivity and specificity of the syndromic surveillance approach greatly depend on the reporting and severity of clinical symptoms and how much these signs overlap with existing diseases within the population (Polo, 2020). In the case of SARS-CoV-2, a significant proportion of patients are either “asymptomatic, presymptomatic, or experience mild, non-specific symptoms and therefore go unreported, resulting in considerable underestimation of infection.”

Useful as an unbiased surveillance system WBE reflects the community's health. Human viruses in wastewater are genetic markers of their transmission in populations due to their RNA. Viral detection in influents of WWTP indicates human sources and hence indicates what diseases are circulating within a population in virtual real-time. Additionally, WBE is cost-effective even in low-resourced community settings by providing rapid results besides screening for various emergent viral infections. In addition, the Cook County Jail has a centralized wastewater collection system (where SARS-CoV-2 is most prevalent); thus, the viral load from infected individuals can be captured in a single sample, facilitating the analysis of the whole incarcerated population. The ethical risks of data collection with WBE are low as it cannot capture data on individuals only populations.

The World Health Organization created guidelines for the first comprehensive international ethics guidelines on public health surveillance, “highlighting those ethical guidelines should be appropriately adapted to different social, economic, and epidemiological circumstances. In this sense, care must be taken in disease reporting and to reduce media misinterpreting the publication's findings (WHO, 2014).”

B. Validation and Verification of Assay Performance and Method Development

It is important to know how well the standard calibration curve assay performs because the validation/verification of the assay must be reliable and reproducible for accuracy of test results and laboratory quality control/quality assurance. If the results are not accurate it may lead to false-positives or false-negatives. If the negative controls used to establish a baseline for the absence of SARS-CoV-2 viral RNA were frequently positive, then this could mean

contamination is present and adversely affect the reliability of experimental methods or results. If the recovery of the SARS-CoV-2 viral RNA is highly variable, then viral RNA may not be adequately captured during the experimental methods. Validation/verification of assays may address these issues. A search of studies produced by other researchers engaged in validation/verification assay performance for the detection of SARS-CoV-2 viral RNA was conducted (Table X, Appendix).

C. Aims

The aims of this study are A) Evaluate a method using RT-qPCR assay and quantification of the SARS-CoV-2 viral RNA and B) Validation and verification of standard calibration curves using precision, sensitivity, efficiency, and recovery parameters.

II. METHODS

A. RT-qPCR for SARS-CoV-2 Detection

Quantification was performed using reverse-transcription quantitative polymerase chain reaction (RT-qPCR). Quantification of SARS-CoV-2 occurred at the N1 and N2 loci of the nucleocapsid (N) gene using primers and probes published by the United States Centers for Disease Control and Prevention (CDC, 2020). BCoV was quantified using the transmembrane (M) gene with primers and probes from Decaro et al., (2008). As a human fecal load indicator, pepper mild mottle virus (PMMoV) was quantified using a forward primer and probe from Zhang et al., (2006), and reverse primer from Haramoto et al., (2013). Primer and probe sequences and thermal cycling conditions for each assay are shown (Table VIII, Appendix).

B. Standard Calibration Curves

The calibration curves were run in three replicate reactions for each sample, no-template control (NTC), method blanks (MeB), and recovery control (BCoV) samples for all assays. The negative controls were method blank (Phosphate-Buffered Saline (PBS) 25mL spiked with BCoV 60µL as a part of the quality assurance/quality control (QA/QC)) and no template control (NTC) (RT-qPCR reactions where sample SARS-CoV-2 RNA was replaced with nuclease-free water). Six standard curves were performed using four targets (N1, N2, BCoV, PMMoV) on November 11, 2020; December 8, 2020; January 7, 2021; February 11, 2021; March 10, 2021; April 8, 2021 (Table IV). Standard calibration curves for N1 and N2 were generated using the 2019-nCoV_N_Positive Control (Integrated DNA Technologies, Inc.), a plasmid containing the entire N gene, with concentrations ranging from 5 to 5 x 10^5 copies/reaction. For BCoV,

standard calibration curves were generated using gBlocks Gene Fragments with concentrations from 8.42 to 8.42 x 10^5 copies/reaction. For PMMoV, standard calibration curves were generated using Ultramer RNA Oligos with concentrations ranging from 1 x 10^2 to 1 x 10^8 copies/reaction. Assays for RT-qPCR were performed using QuantStudio 3 thermal cyclers (Applied Biosystems). Targets were run in triplicates for five concentration ranges from 10^1 to 10^5 (5.69, 4.69,3.69, 2.69, and 1.69).

Our evaluation is based on the use of calibration curves which is using them to reverse calculate the concentration value of RNA gene targets of interest. The equation of the calibration curve is:

$$Ct = m*x + b \rightarrow Ct = slope*\log sequence + y\ intercept \qquad (Eq\ 1)$$

where Ct is the threshold cycle value, m is the slope of the line, x is the log sequence, and b is the y intercept. Calculation of the concentration of an environmental sample can then be determined by solving the equation for the log sequence:

$$\log sequence = Ct - y\ intercept / slope \qquad (Eq\ 2)$$

Once the log sequence is determined the equation for the inverse is:

$$\log sequence^{-1} = sample\ concentration\ (GC/RXN) \qquad (Eq\ 3)$$

will provide the sample concentration.

C. Data Analysis

The performance characteristics of RT-qPCR assay. Precision was assessed by regression analysis and the coefficient of determination (R^2), used to explain how much variability of one factor can be caused by its relationship to another factor.

Analytical sensitivity for the RT-qPCR assay was assessed by determining the lower limit of quantification (LLOQ) for each gene target.

Normalizing community concentration. Used as a surrogate and/or domestic wastewater marker in various situations PMMoV is useful as an enteric virus for population monitoring. As an index virus PMMoV excels for enteric viruses in environmental waters exposed to untreated domestic wastewater because it was detected more frequently and in higher concentrations than other human viruses in groundwater (72.2%) and surface waters (freshwater, 94.5% and coastal, 72.2%), with pathogen co-detection rates as high as 72.3%. (Symonds et al., 2018). In this study PMMoV is used as a fecal load indicator or "fecal associated virus surrogate" in the RT-qPCR assay. The detection of PMMoV is useful to determine concentration level when comparing communities. For example, one community may have 1,000 residents and another community may have 10,000 residents. This would mean that the larger community will have higher concentrations of SARS-CoV-2 and PMMoV when compared to the smaller community. To effectively calculate and report those concentration levels PMMoV acts to normalize the data so that results are not skewed. Linear regression analysis may indicate a proportional correlation. Suggesting that high concentrations of fecal load

indicator will be found in conjunction with high concentrations of SARS-CoV-2 RNA in wastewater.

The PCR efficiency can be defined as the increase in amplicon per cycle. During the exponential phase of the PCR reaction this efficiency is constant (Ruijter, 2009). The slope of standard curve can be translated into a PCR efficiency value which indicates how well the RT-qPCR assay preforms. The efficiency value is measured by the following equation, Efficiency (E) = $10^{(-1/\text{slope})} - 1$. A slope of (-3.32) is considered 100 percent efficient (Thermo Fisher Scientific, 2016). It is important to measure this parameter according to (Bustin, 2009), "Calibration curves provide a simple, rapid, and reproducible indication of the mean PCR efficiency, the analytical sensitivity, and the robustness of the assay.

The purpose of recovery in the RT-qPCR assay was to evaluate the ability of the process method to capture and quantify the gene target of interest. BCoV recovery was assessed using the mean Ct value, standard deviation (SD), and coefficient of variation (CV).

III. RESULTS

A. Statistical Analysis

Datasets were generated in Microsoft Excel 2016 and data analysis preformed using ANCOVA. Reliability of each calibration assay was determined from the F values (test statistic)and *P* values (statistically significant). The P values greater than 0.05 for all targets indicates statistically significant results. The F values are slightly above, equal to, or less than 5.00 in all targets which indicates statistically significant results. The coefficient of variation (CV), a measure of the ratio of the standard deviation to the mean, is used more often when a comparison of the variation between two different datasets is needed (Table I). The CV values are less than five percent which indicates good method performance (Zady, 2019). “The CV provides a general "feeling" about the performance of a method. CVs of five percent or less generally give a feeling of good method performance, whereas CVs of ten percent and higher sound bad” (Zady, 2019).

Table I. ACCURACY AND PRECISION OF RT-QPCR AMPLIFICATION

Assay	F value	P value	CV (%)
N1	5.095	0.0005	2.79
N2	4.885	0.0007	4.10
BCoV	5.776	0.0001	3.98
PMMoV	4.716	0.0006	3.50

Linear regression analysis revealed mean correlation coefficient R^2 range from 0.997 to 0.999. The data adhere nearly perfect to the line of fit (Figures 1-4). Standard deviations of 0.938, 1.391, 1.483, 1.255 for N1, N2, BCoV, and PMMoV respectively. The spread of the data is low and close to the mean value. The mean slope values of -3.19, -3.39, -3.55, and -3.-3 for N1, N2, BCoV, and PMMoV respectively (Table III; Figures. 1-4). The slope measures the efficiency of the PCR reaction. A slope of -3.32 is considered 100% efficient (Thermo Fisher Scientific, 2016). The slopes of each target are nearly at or exceed -3.32. "The efficiency of the PCR should be between 90–100% (−3.6 ≥ slope ≥ −3.3). If the efficiency is 100%, the Ct values of the 10-fold dilution will be 3.3 cycles apart (there is a 2-fold change for each change in Ct). If the slope is below –3.6, then the PCR has poor efficiency" (Thermo Fisher Scientific, 2016).

Analytical sensitivity of the RT-qPCR assay was assessed by determining the lower limit of quantification (LLOQ). The mean Ct value and the corresponding gene copies per reaction for the LLOQ for all the targets was calculated. Individual LLOQ are not calculated for each standard curve run. "They were pooled using the data from six individual runs to generate a master standard curve (Abhilasha Shrestha Ph.D., personal unpublished communication)." The LLOQ for RT-qPCR assays were 38.78, 40.68, 36.26, and 43.04 Ct values for N1, N2, BCoV, and PMMoV respectively.

The PMMoV had a mean value of 38.64 Ct and a SD of 1.38. The SD indicates low spread of data points from the mean value. Indicating the RT-qPCR assay is detecting the appropriate target rather than other or nonspecific targets also present in the sample (Bustin, 2009). Conversely, high spread of data from the mean value could indicate non-specificity or detection of nonspecific targets.

The RT-qPCR assay efficiencies are 105%, 97%, 91%, and 99% for N1, N2, BCoV, and PMMoV respectively. All values were nearly 100% or exceed 100% which indicates high levels of efficiency suggesting, the RT-qPCR assay performed well.

The mean MeB Recovery was 24.35%, 23.93 mean Ct value,1.34 SD, and 5.59% CV. The SD and CV indicate low spread of data from the mean value however, the recovery percent was low. This may indicate a need to adjust for recovery in the assay because it is underestimating the actual concentration in the standard calibration curve. There is no simplified way to say that an adjusted recovery value is ten times an actual value thus recovery is needed to calculate actual value or true concentration after considering the loss of most of the gene target. Assay adjustments may lead to improvements in the assay method and greater outcomes of BCoV recovery.

A correlation analysis between N1 and N2 gene targets were performed. The data show a negative correlation between the two gene targets in Table II.

Table II. CORRELATION ANALYSIS N1 VS N2

	Log Sequences (X)	**Ct (Y)**
Log Sequences (X)	1	
Ct (Y)	-0.965159172	1

B. Quality Control

All method blanks were negative for N2. With the exception of one all method blanks for N1 were negative; two of three RT-qPCR replicates were negative, and the third had contamination which resulted in a Ct value of 36.97. All NTC were negative for N1, N2, and BCoV. Tables IV-VII show the performance characteristics for these four gene targets.

Table III. RT-QPCR PERFORMANCE CHARACTERISTICS

Assay	E (%)	R^2	SD	Slope	Intercept	LLOQ (GC/RXN)
N1	105	0.999	0.938	-3.19	42	0.0186
N2	97	0.997	1.391	-3.39	43	0.0187
BCoV	91	0.997	1.483	-3.55	39	0.0179
PMMoV	99	0.998	1.255	-3.32	46	0.0179

Table IV. RT-QPCR PERFORMANCE CHARACTERISTICS FOR N1 GENE TARGET

Date	Assay	R^2	Slope	Intercept
11/11/2020	N1	0.997	-3.288	41.649
12/8/2020	N1	0.991	-3.377	42.844
1/7/2021	N1	0.996	-3.170	41.083
2/11/2021	N1	0.999	-3.236	41.790
3/10/2021	N1	0.998	-3.165	42.647
4/8/2021	N1	0.994	-2.986	41.250

Table V. RT-QPCR PERFORMANCE CHARACTERISTICS FOR N2 GENE TARGET

Date	Assay	R^2	Slope	Intercept
11/11/2020	N2	0.997	-3.408	43.546
12/8/2020	N2	0.997	-3.383	42.295
1/7/2021	N2	0.998	-3.167	41.257
2/11/2021	N2	0.989	-3.242	41.710
3/10/2021	N2	0.993	-3.302	45.009
4/8/2021	N2	0.994	-3.627	46.141

Table VI. RT-QPCR PERFORMANCE CHARACTERISTICS FOR BCoV GENE TARGET

Date	Assay	R^2	Slope	Intercept
11/11/2020	BCoV	0.997	-3.648	38.787
12/8/2020	BCoV	0.997	-3.444	37.614
1/7/2021	BCoV	0.997	-3.541	38.765
2/11/2021	BCoV	0.992	-3.370	38.789
3/10/2021	BCoV	0.994	-3.598	40.521
4/8/2021	BCoV	0.997	-3.814	42.787

Table VII. RT-QPCR PERFORMANCE CHARACTERISTICS FOR PMMoV GENE TARGET

Date	Assay	R^2	Slope	Intercept
11/11/2020	PPMoV	0.992	-3.383	44.882
12/8/2020	PPMoV	0.990	-3.135	45.278
1/7/2021	PPMoV	0.984	-3.107	45.690
2/11/2021	PPMoV	0.988	-3.214	44.941
3/10/2021	PPMoV	0.996	-3.432	47.332
4/8/2021	PPMoV	0.998	-3.497	47.260

IV. DISCUSSION

This study evaluates the verification and validation of a RT-qPCR assay for SARS-CoV-2 viral RNA. This study also displays the utility of using molecular methods which are more rapid, accurate, and sensitive for virus detection than culture methods (Chung, 2021).

A. Summary of Method Parameters

In this study we evaluated the precision, sensitivity, efficiency, and recovery of RT-qPCR assay for detection of SARS-CoV-2 standard calibration curves. The data provides confidence in the validity of the assay as it is linear, repeatable, and reproducible by several orders of magnitude. The efficiency is nearly 100% for each gene targets. The data has significantly small number of outliers; the removal of such decreased the standard error. The LLOQ is detectable up to 45 quantifiable cycles. The data has significantly low residuals showing no obvious trend or discernable pattern in the data points (Figures 5 to 8, Appendix). The calibration standard curve assay and the data generated provide reliable, repeatable, and reproducible data. The calibration standard curves data are consistent over time with significantly small to no residual effects. The reliability, repeatability, and reproducibility give the method development confidence to analytically derive correct concentration values for environmental samples.

The calibration curve assay and the data generated provide reliable, repeatable, and reproducible results. The calibration curves data are consistent over time with significantly small to no residual. The reliability, repeatability, and reproducibility give the method development confidence to analytically derive correct concentration values for viral RNA of interest.

The (2022) study from Ahmed et al. and their performance characteristics of Efficiency, R^2, and slope were comparable to our study findings. Ahmed et al. used RT-qPCR assay for N1, and N2 gene targets of interest. This study found values of 97.7%, 0.99, and -3.37 for N1 Efficiency, R^2, and slope respectively. For the N2 gene target the values were 95.9%, 0.98, and -3.42 for Efficiency, R^2, and slope respectively. The Ahmed study evaluated an assay limit of detection with GC/reaction. Their study found 9.50 and 26.7 GC/reaction for N1 and N2 respectively. The study conducted by Navarro et al., 2021 performed RT-qPCR using N1, N3, and S gene targets. This study found values of 102%, 0.998 for N1 Efficiency and R^2; values of 95.9% and 0.997 for N3 Efficiency and R^2.

In the data, a difference in sensitivity between the N1 and N2 RT-qPCR assays was observed. Detected in a greater number of samples was the N1 gene target. Most of the samples had greater detected concentrations for N1 compared to N2. Other studies such as Ahmed, (2020) found the N1 assay to be more sensitive than N2. The reason for this difference is uncertain. It may be that changes to the sequences of primer binding sites have occurred in the SARS-CoV-2 population since the primers and probes were initially developed at the beginning of the pandemic. Those changes may have amassed faster in the N2 region thus, making the N2 region detectability less sensitive during amplification. Despite differences in average sensitivity, Ct values for N1 and N2 were still moderately correlated ($R^2 = 0.58$) and followed comparable dynamics over time.

Frequency of sample contamination. All method blanks were negative for N2. With the exception of one all method blanks for N1 were negative; two of three RT-qPCR replicates were negative, and the third had contamination which resulted in a Ct value of 36.97. All NTC were

negative for N1, N2, and BCoV. These data suggest that contamination of samples with SARS-CoV-2 did not occur in the laboratory.

High linearity with correlation of coefficients (R^2) at or nearly 1 suggests Low SD and slopes at or above the -3.3 100% efficient mark. These measured parameters strongly suggest the method is precise (Table III).

The assay was able to detect SARS-CoV-2 utilizing an LLOQ for the gene targets. The RT-qPCR assay was able to detect between 36 and 43 Ct values. The assay utilizes 45 Ct which strongly suggests the method is noticeably sensitive. The SD for sensitivity was low with minimal spread of the data which suggests consistency over time and with repeated standard calibration curve measures (Table III).

Robust and precise qPCR assays are usually correlated with high PCR Efficiency. All PCR efficiencies were near or greater than 99% which indicates the method is efficient. The method utilized in this study generated data that strongly suggests it is 1) “repeatable (short-term precision or intraassay variance) refers to the precision and robustness of the assay with the same samples repeatedly analyzed in the same assay and 2) reproducible (long-term precision or interassay variance) refers to the variation in results between runs or between different laboratories” (Bustin, 2009).

The recovery was calculated using the ratio of BCoV gene copies per reaction and reference spike BCoV per reaction (40uL). The BCoV recovery ranged from 0.003% to 19.93%. The data has a 25^{th} percentile of 0.51, a 75^{th} percentile of 2.12 and the interquartile range (IQR)

of 1.6. The distribution of the recovery values shows there is significant variability in measures of recovery over time.

The Dimitrakoppulos, (2022) study calculated the recovery using copies of spiked control samples minus endogenous copies of each sample divided by theoretical copies spiked in each replicate times 100. That study found using spiked samples, recoveries were estimated 2.1–37.6% using different extraction kits and 0.1–2.1% using different concentration kits. While our study utilized only one viral RNA extraction method with six concentrations and three replicates each.

Although BCoV behaves similarly to SARS-CoV-2 virus, on average samples lost approximately 75%. This loss may be due to the complexity of wastewater matrix which contains many PCR inhibitors, including some components of human waste (i.e., bile salts, complex polysaccharides, lipids, and urea), humic acids, detergents, metal ions, calcium ions, and polyphenols (Owen, 2022). Additionally, the process of wastewater filtration during the experimental methods may be another source of BCoV loss. The low recovery may suggest that adjustments to the BCoV assay and or methods be examined.

B. Limitations

Evaluate the utility of using RT-qPCR assay to quantify SARS-CoV-2 viral RNA. Some limitations of this study's evaluation method performance are standard calibration curve sample size, variable quantity recovery of spiked BCoV MeB, and low RT-qPCR replicates of gene targets of interest. This study only preformed n=6 calibration curves. A larger number of calibration curves like n≥30 would create a more robust dataset and generally speaking is more

statistically significant. This study only preformed replicates of three for the calibration curves. In the future more replicate reactions (i.e., six or twelve) from multiple SARS-CoV-2 gene targets (N1, N2, N3, S spike protein, and M membrane) may enhance evaluation of precision, sensitivity, and efficiency.

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APPENDIX

Figure 1. N1 log sequences vs Ct line fit

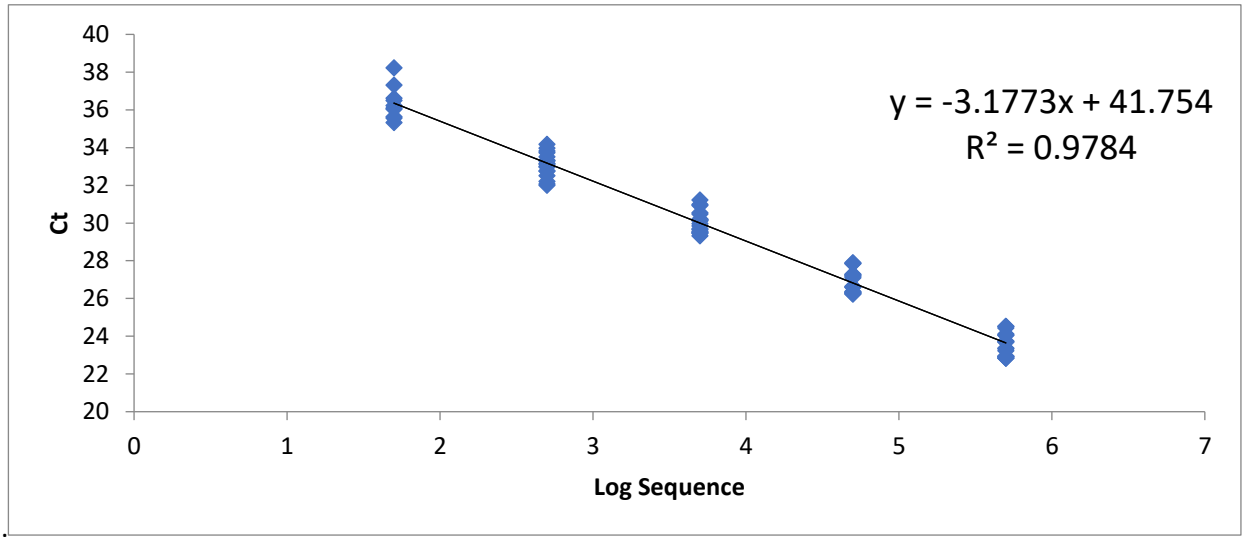

Figure 2. N2 log sequences vs Ct line fit

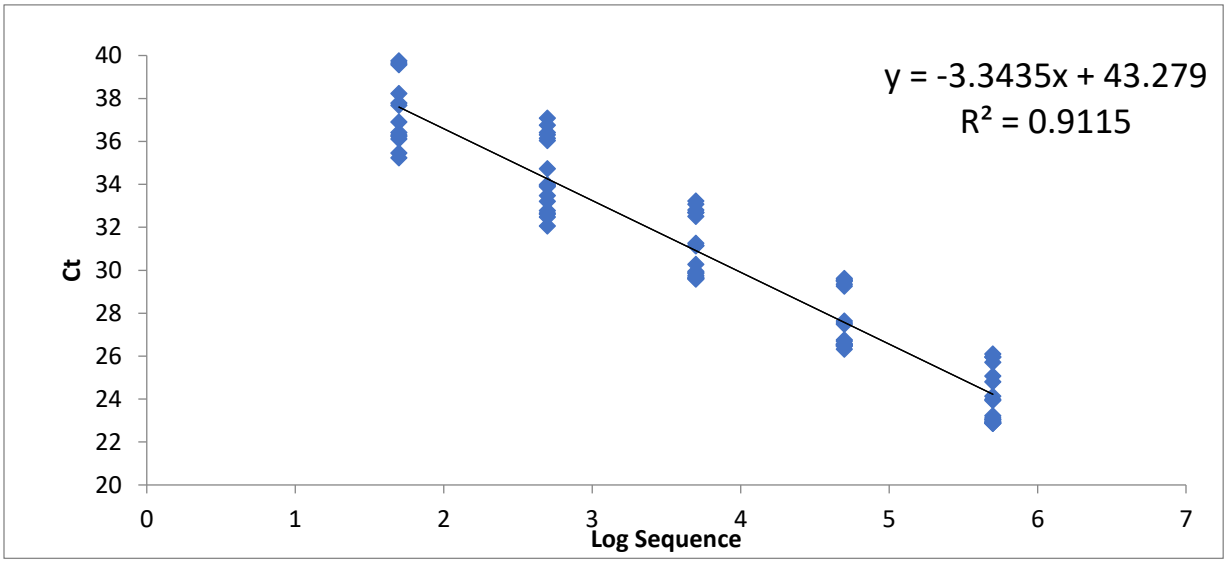

Figure 3. BCoV log sequence vs Ct line fit

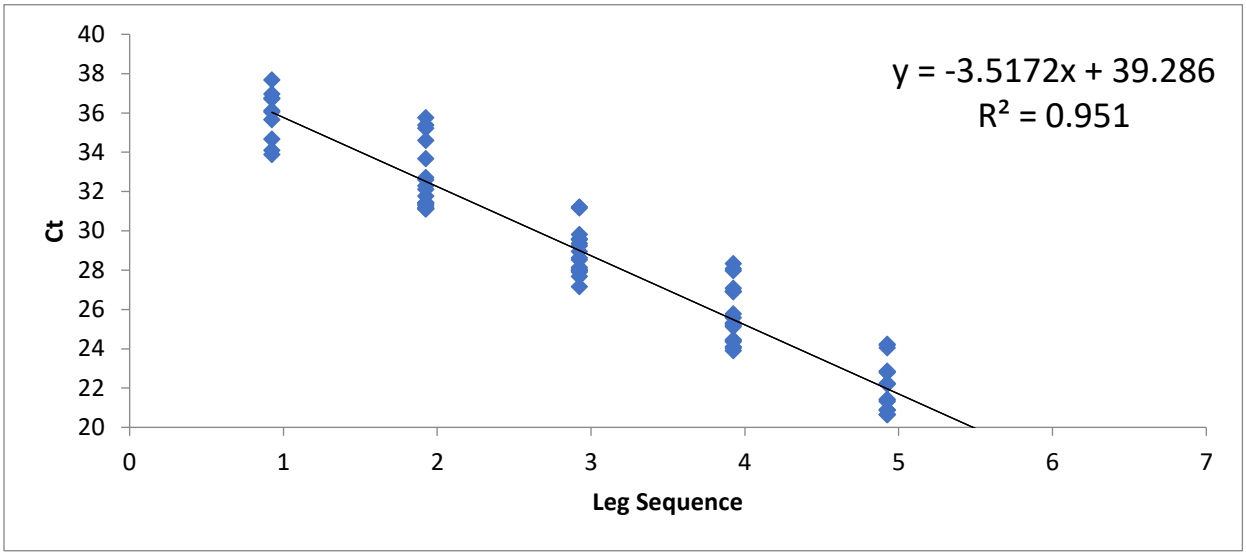

Figure 4. PMMoV log sequence vs Ct line fit

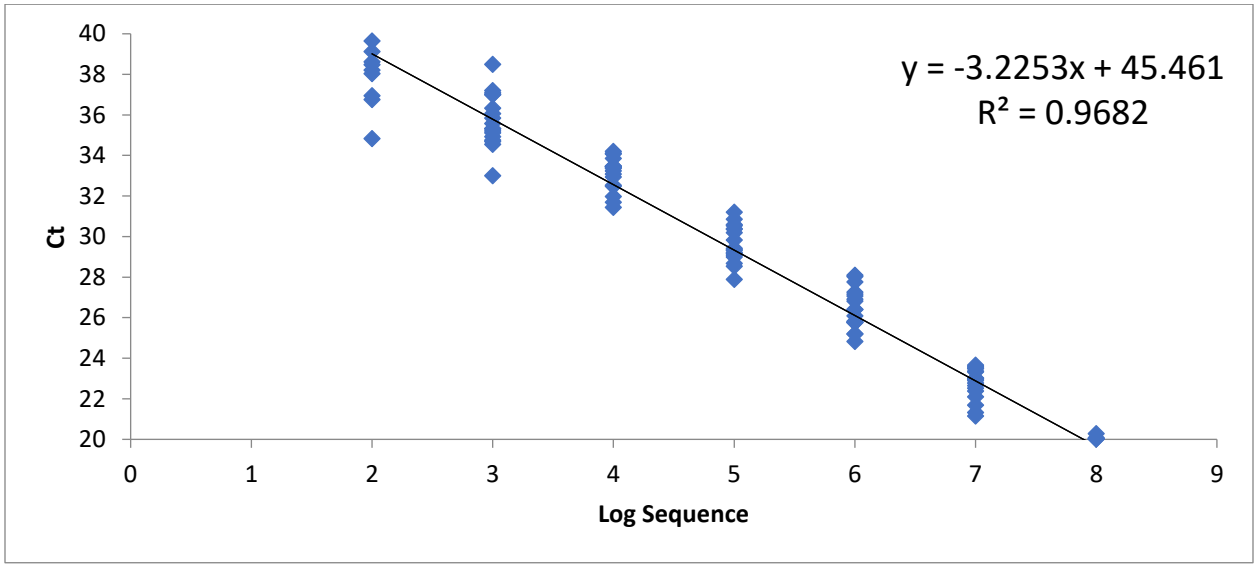

Figure 5. N1 log sequences vs residual

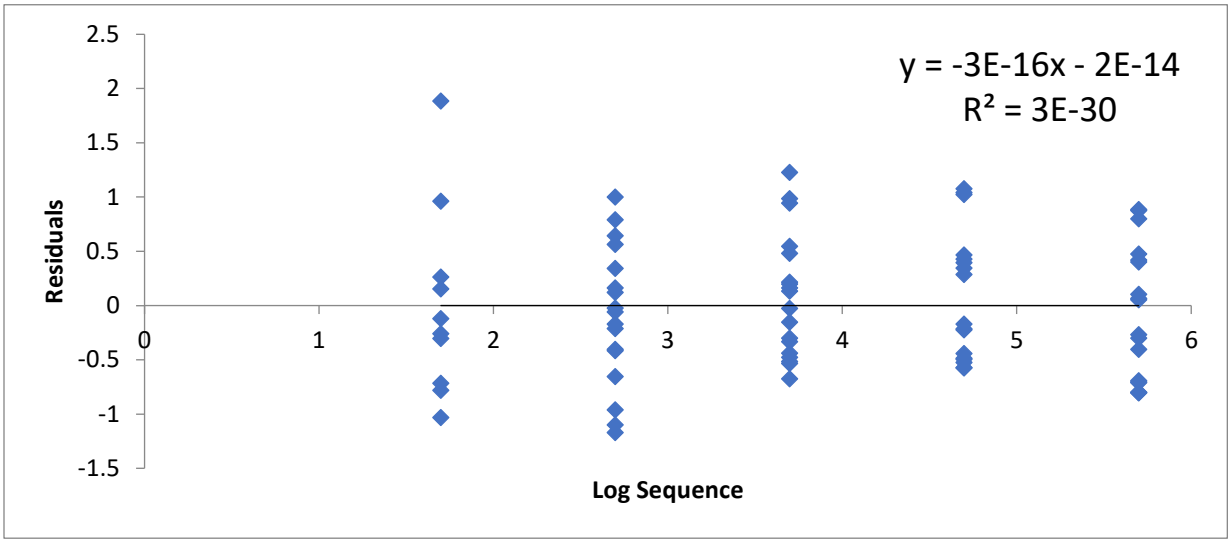

Figure 6. N2 log sequences vs residual

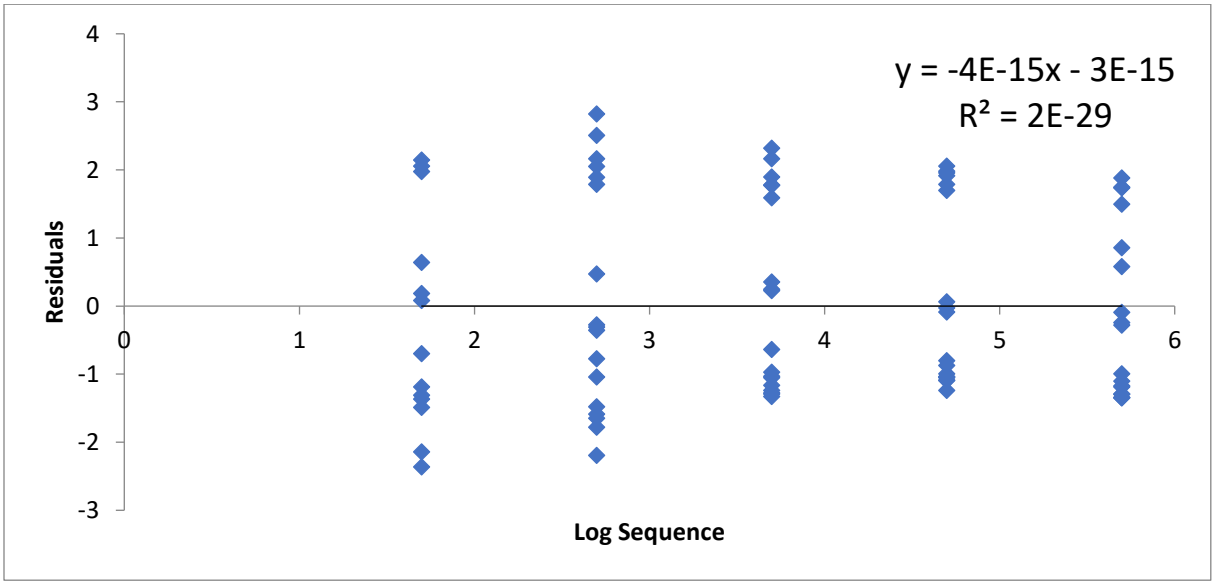

Figure 7. BCoV log sequences vs residual

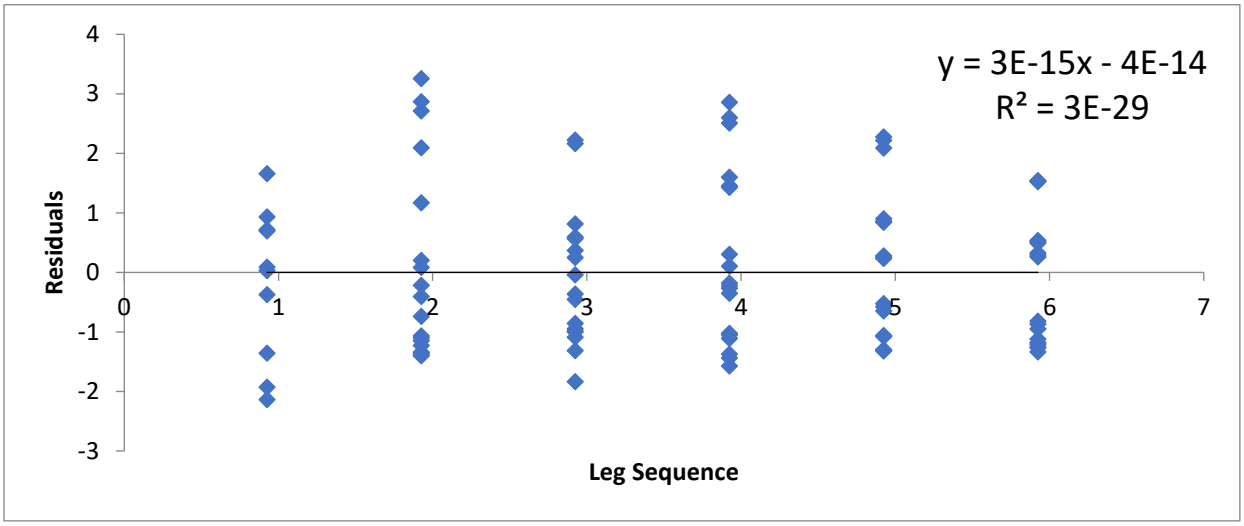

Figure 2. PMMoV log sequences vs residual

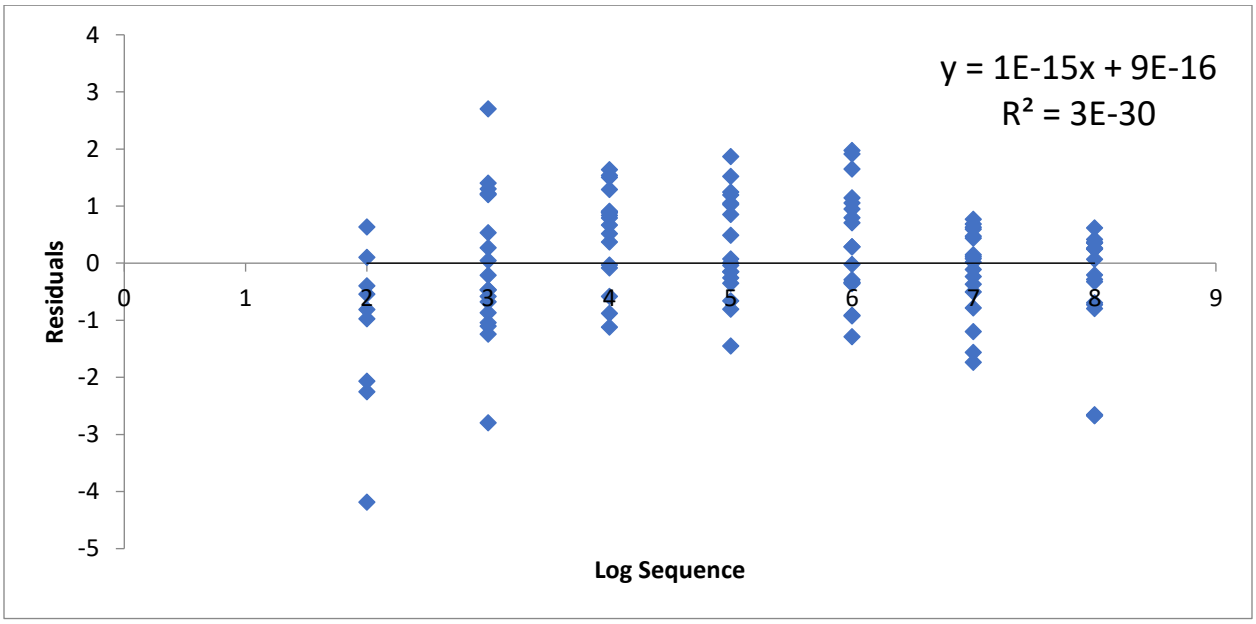

Table VIII. TARGETS, PRIMERS, PROBES, AND RT-QPCR THERMAL CYCLING

Assay	Forward primer	Reverse primer	Probe	Thermal cycling protocol
2019-nCoV_N1. United States Centers for Disease Control (CDC, 2020).	GAC CCC AAA ATC AGC GAA AT	TCT GGT TAC TGC CAG TTG AAT CTG	FAM-ACC CCG CAT TAC GTT TGG ACC-BHQ1	25 °C(2:00) + 50 °C(15:00) + 95 °C(2:00) + [95 °C(0:30) + 55 °C(0:30)] x 45
2019-nCoV_N2. United States Centers for Disease Control (CDC, 2020).	TTA CAA ACA TTG GCC GCA AA	GCG CGA CAT TCC GAA	FAM-ACA ATT TGC CCC CAG CGC TTC AG-BHQ1	25 °C(2:00) + 50 °C(15:00) + 95 °C(2:00) + [95 °C(0:30) + 55 °C(0:30)] x 45
Bovine Coronavirus (BCOV) (Decaro et al., 2008).	CTG GAA GTT GGT GGA GTT	ATT ATC GGC CTA ACA TAC ATC	FAM-CTT TCA TAT CTA TAC ACA TCA AGT TGT T-ZEN	25 °C(2:00) + 50 °C(15:00) + 95 °C(2:00) + 95 °C(10:00) + [95 °C(0:15) + 60 °C(0:30) + 60 °C(1:00) x 45
Pepper Mild Mottle Virus (PPMOV). (Zhang et al., 2006). (Haramoto et al., 2013).	GAG TGG TTT GAC CTT AAC GTT TGA	TTH TCG GTT GCA ATG CAA GT	HEX-CCT ACC GAA GCA AAT G-ZEN	25 °C(2:00) + 50 °C(15:00) + 95 °C(2:00) + [95 °C(0:30) + 55 °C(0:30)] x 45

Table IX. GENE TARGETS FOR RNA QUANTIFICATION

Gene Target	RNA Quantification	Concentration
2019-nCoV_N1 and N2	Loci of the nucleocapsid (N) gene. 2019-nCoV_N_Positive Control Integrated DNA Technologies, Inc. A plasmid containing the entire N gene. (CDC)	5 to 5 x 105 copies/reaction
Bovine Coronavirus (BCoV)	Transmembrane (M) gene with primers and probes from Decaro et al., 2008. (gBlocks Gene Fragments)	8.42 to 8.42 x 105 copies/reaction
Pepper Mild Mottle Virus (PPMoV)	Forward primer and probe from Zhang et al., (2006), and reverse primer from Haramoto et al., 2013 (Ultramer RNA Oligos)	1 x 102 to 1 x 108 copies/reaction

Table X. RT-QPCR SARS-COV-2 ASSAY EVALUATION OF OTHER STUDIES

Author, Year	Sample	RNA Extraction	RT-qPCR Assay	Thermocycler Instrument	Findings
Wright-Foulkes, 2022. Validated the precision, sensitivity, specificity, and efficiency of the RT-qPCR assay for SARS-CoV-2 detection.	2019-nCoV_N_Positive Control (Integrated DNA Technologies, Inc.)	QIAamp Viral RNA Mini kit (QIAGEN, Hilden, Germany).	TaqPath™ 1-Step RT-qPCR Master Mix, CG Catalog number: A15300 (Applied Biosystems™).	QuantStudio™ 3 Real-Time PCR System, 96-well, 0.2 mL, laptop Catalog number: A28567 (Applied Biosystems™).	The RT-qPCR assay data strongly suggests significant precision, sensitivity, specificity, and efficiency of the RT-qPCR assay for SARS-CoV-2 detection.
Chung, 2021. Validated the sensitivity, specificity, precision, linearity, accuracy, and robustness of the RT-qPCR assay for SARS-CoV-2 detection.	Cultured medium of SARS-COV-2 cells from Korean CDC.	QIAamp Viral RNA Mini kit (QIAGEN, Hilden, Germany).	AgPath-ID one-step RT-PCR reagents (Applied Biosystems, Foster City, CA, USA)	7500 Fast Real-Time PCR System. (Applied Biosystems, Foster City, CA, USA)	The RT-qPCR assay showed excellent specificity and sensitivity, including a low LOD. The assay exhibited high specificity and sensitivity for SARS-CoV-2 and good analytical performance using cloned SARS-CoV-2 genes and/or virus isolated.
Tombuloglul, 2021. Developed a multiplex rRT-PCR diagnostic method, which targets two viral genes (RdRP and E) and one human gene (RP) simultaneously.	Viral RNA was extracted from nasopharyngeal swabs in virus transport medium (VTM)	QIAamp Viral RNA Mini kit (Qiagen, Hilden, Germany)	1) 2 µL of 10x Buffer, 0.25 µL of dNTPs (10 mM each), 0.2 µL of uracil-DNA glycosylase (UDG) (1 U/µL), 0.4 µL of VitaTaq1 HS polymerase (2 U/µL), 0.05 µL VitaScript1Enzyme mix including M-MLV (Procomcure, Austria), 0.05 µL of Triton™ X-100 (molecular biology grade, Merck). 2) Xpert Xpress SARS-CoV-2 kit (n = 14) (Cepheid, Sunnyvale, CA, USA)	1) 7500 Fast Real-Time PCR System (Applied Biosystems™). 2) GeneXpert Dx instrument (Cepheid, Sunnyvale, CA, USA).	

Table X. RT-QPCR SARS-COV-2 ASSAY EVALUATION OF OTHER STUDIES (Continued)

Author, Year	Sample	RNA Extraction	RT-qPCR Assay	Thermocycler Instrument	Findings
Fenaux, 2022. Specificity and sensitivity.	Serial tenfold dilutions of SARS-CoV-2 culture supernatants were obtained from the French National Reference Centre for Respiratory Viruses (NRC)	QIAprep&amp Viral RNA UM Kit (Qiagen, Hilden, Germany) allows preparation and detection of viral RNA targets from viral transport media, dispensing the nucleic acids extraction step.	Alinity m SARS-CoV-2 AMP Kit (Abbott Diagnostics, Chicago, Illinois, USA),	ViiA7 (ThermoFisher).	the QIAprep reliably detected samples with Ct<34, a surrogate marker of infectivity. The QIAprep assay was proven a reliable method with excellent specificity and acceptable sensitivity for SARS-CoV-2 detection from nasopharyngeal swabs.
Pierri, 2022. Sensitivity, specificity and accuracy. nsp14 (genomic region, a target previously developed for SARS-CoV-2 monitoring in wastewaters), rt RT-qPCR vs dd RT-qPCR.	258 nasopharyngeal swabs (NP).	an automated platform (GeneQuality X120, AB Analitica, Italy) with a magnetic bead-based protocol, using the GeneQuality X120 Pathogen kit (AB Analitica)	Assay based on nsp14. RNA UltraSense One-Step Quantitative RT-PCR System (Life Technologies),	Quant Studio 12 K Flex instrument (Thermo Scientific).	rt RT-qPCR nsp14 assay revealed better performances than the dd RT-PCR in terms of sensitivity (100.0 % vs 92.4 %) and accuracy (99.6 % vs 94.7 %).
Brotons, 2021 Validate and implement an optimized screening method for SARS-CoV-2 RNA combining use of self-collected raw saliva samples, RNA extraction, and direct RT-qPCR.	Saliva samples from the Biobank of SJDH a research biorepository at the Spanish Biobank Network of Instituto de Salud Carlos III.	Direct RT-qPCR no RNA extraction step.	RNA amplification was performed using two RT-qPCR kits (GeneFinder COVID-19 Plus RealAmp kit, Elitech, France; TaqPath COVID-19 RT-PCR kit, Thermo Fisher, USA)	Two thermal cycler platforms (Applied Biosystems QuantStudio 7 and Applied Biosystems Prism 7500, Thermo Fisher, USA).	The sensitivity (95.7%) and specificity values (100.0%) validated in a diverse cohort of teenagers and young and older adults without symptoms were comparable to those of standard RT-qPCR protocols that use NP samples for clinical diagnosis.

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	Solar Powered Microplasma-Generated Ozone: Assessment of a Novel Point-of-Use Drinking Water Treatment Method. International Journal of Environmental Research & Public Health. 13 March 2020. DOI: https://doi.org/10.3390/ijerph17061858
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