Development of a novel qPCR assay to detect SARS-CoV-2 RNA extracted from wastewater

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Abstract

Since the onset of the COVID-19 pandemic, sensitive and accurate diagnostic assays have been essential to detect infected individuals and support efforts to limit further spread of the virus. We initially focused on developing a simple, low-cost assay ($1US) that could be used to directly detect the RNA of SARS-CoV-2 (the virus that causes COVID-19) in unprocessed human saliva, and then applied this assay for detection in wastewater. The assay utilizes PCR primers and a reporter probe designed to target the SARS-CoV-2 nucleocapsid gene, and a novel polymerase called Volcano 2G (V2G) that efficiently reads both RNA and DNA templates. The V2G-qPCR assay is sensitive and specific, simple to execute on standard qPCR instruments, and provides an instantaneous visual readout at PCR end-point through blue light excitation.

Minor modifications to the standard assay allowed for quantitative PCR using a real-time qPCR instrument. Nucleocapsid DNA standards were synthesized by PCR, quantified by droplet digital PCR and used to generate standard curves for determining SARS-CoV-2 RNA quantities of unknowns by qPCR. Based on the standard curves, amplification efficiency is greater than 95%, with a coefficient of determination of 1, or very close to 1. V2G-qPCR assays have also been developed to measure targets to control for sample processing (OC43) and to measure the degree of PCR inhibition (HIV RNA). Assays to measure PMMoV and human beta-2-microglobulin RNA have also been developed for standardization of CoV-2 RNA copy data.

For weekly sampling, wastewater was concentrated on electronegative filters, and total RNA was purified manually using silica-based spin columns. Importantly, eluting the RNA with a minimal volume and increasing the wash buffer volumes was effective in reducing PCR inhibition to negligible levels. SARS-CoV-2 RNA levels, as determined by V2G-qPCR ranged from 10^2 to 10^6 genomic copies per liter raw wastewater and correlated well with quantitation by a more commonly used commercially available RT-qPCR assay. Although standard RT-qPCR assays perform similarly, notably RNA extracted from wastewater is amplified directly by V2G-qPCR, and thus the cDNA synthesis step is bypassed, *thereby reducing both assay time and cost compared to standard RT-qPCR*. Overall, V2G-qPCR is an accurate and cost-effective approach to directly measure target RNA in less than one hour.

Background/ Preliminary Results

Initial work supported by the University of Miami Clinical and Translational Science Institute resulted in a qualitative assay to detect SARS-CoV-2 RNA in unprocessed patient saliva. Primers and fluorogenic reporter probe were designed to amplify a segment of the SARS-CoV-2 nucleocapsid phosphoprotein gene (N). Reagents to detect targets N2 and N3 published by the CDC were evaluated for use, but results indicated a modified N3 set performed best using V2G (Figure 1). Synthetic DNA standards were used to evaluate the efficiency of PCR amplification using the N gene reagents. Standards were subjected to forty cycles of PCR amplification and results were visualized by blue light excitation at PCR end-point (Figure 2) .

References

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Babler, Kristina M. et al., Comparison of Electronegative Filtration to Magnetic Bead-Based Concentration and V2G-qPCR to RT-qPCR for Quantifying Viral SARS-CoV-2 RNA from Wastewater. *ACS ES&T Water* **Article ASAP** DOI: 10.1021/acsestwater.2c00047

Zhan, Qingyu et al., Relationships between SARS-CoV-2 in Wastewater and COVID-19 Clinical Cases and Hospitalizations, with and without Normalization against Indicators of Human Waste. *ACS ES&T Water* **Article ASAP** DOI: 10.1021/acsestwater.2c00045

Results

Figure 1. The CDC N3 target primers and probe were optimized for V2G amplifications to improve sensitivity and specificity.

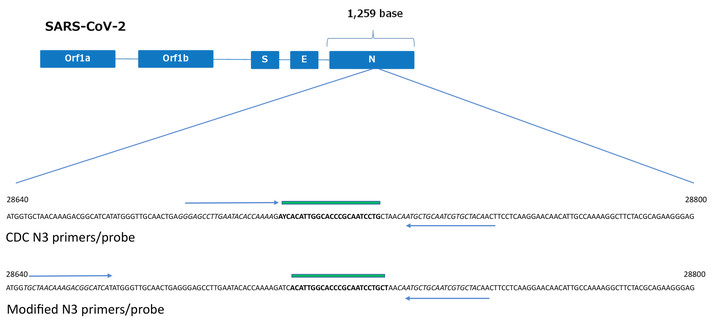


Figure 2. Synthetic DNA standards are amplified efficiently using reagents that target the SARS-CoV-2 nucleocapsid gene.

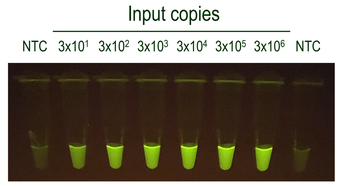


Figure 3. SARS-CoV-2 RNA is efficiently amplified directly from samples of unprocessed patient saliva using V2G (a). Patient saliva diluted with donor saliva from an uninfected individual demonstrates detection sensitivity (b).

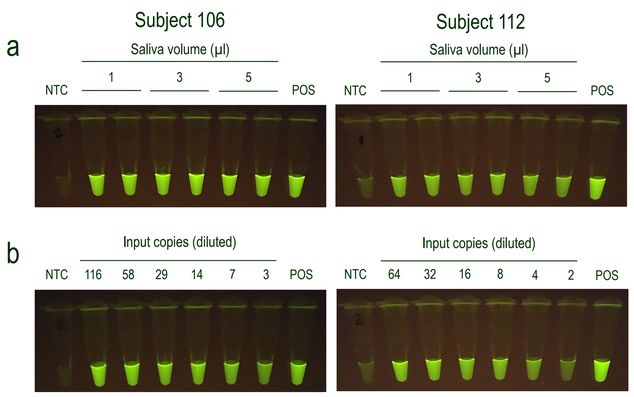


Figure 4. DNA standards were amplified using a BioRad CFX Connect Instrument with an efficiency of 98 percent and an R^2 value of 1.000.

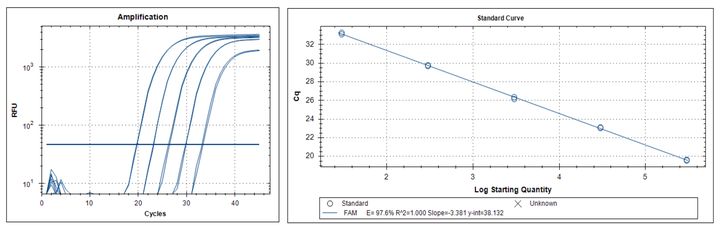


Table 1. Additional RNA targets used as controls. V2G-qPCR assays to measure other targets perform with similar efficiencies.

Target	Function
OC43 betacoronavirus	Processing/ extraction control
HIV-1 RNA spike	Indicator of PCR inhibition
Human beta-2-microglobulin	Normalization of SARS-CoV-2 signal
Pepper Mild Mottle Virus	Normalization of SARS-CoV-2 signal

Results

Figure 5. PCR inhibitors that co-purify with wastewater RNA are effectively eliminated by modifying Zymo's *Quick*-Viral RNA extraction protocol. Eluting RNA with a minimal volume of water and increasing wash solution volumes were most impactful.

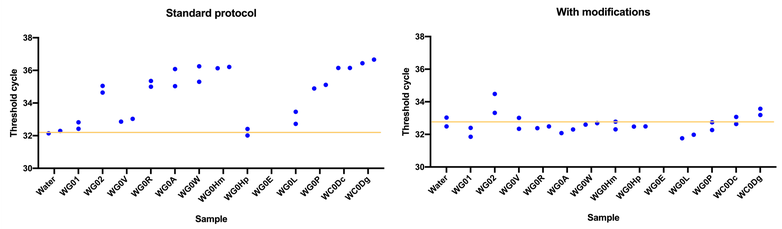
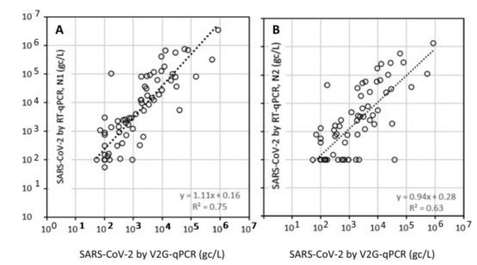


Figure 6. Comparison of SARS-CoV-2 RNA levels by V2G-qPCR and RT-qPCR performed by an independent research group. Good correlations are observed when comparing V2G-qPCR results to RT-qPCR data generated for the CDC N1 target (A) and the CDC N2 target (B).



Conclusions

- CoV-2 RNA detection using unprocessed saliva is highly sensitive
- Positive reactions can be scored using a simple, in-tube visual read-out
- V2G-qPCR simplifies quantification of CoV-2 RNA in wastewater and reduces both assay time and cost
- Simple modification of the RNA extraction protocol profoundly reduces PCR inhibition
- V2G-qPCR data correlates well with more mainstream approaches used to quantitate CoV-2 RNA extracted from wastewater

Future Directions

- Continue weekly monitoring of SARS-CoV-2 RNA levels at the University of Miami, hospitals and wastewater treatment plants
- Analysis of wastewater for SARS-CoV-2 RNA expanded to a set of Miami grade schools
- Utilize sampling and data management systems to monitor alternative organisms or viruses of interest (eg. Candida auris, Influenza A and B)

Acknowledgements

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