COVID-19 Wastewater Project: Validating SARS-CoV-2 Detection Techniques

David Kim[1], David Vang[2] , David Ojcius[2], Der Thor[2]
[1]Doctor of Dental Surgery Program (DDS 2022), [2]Department of Biomedical Sciences
University of the Pacific, Arthur A. Dugoni School of Dentistry, San Francisco, CA

Introduction

- COVID-19 is caused by SARS-CoV-2 virus that are largely enveloped and contains single-stranded RNA.
- Out of the four structural proteins that SARS-CoV-2 encodes, Nucleocapsid (N) protein is one of the most crucial structural components in identifying the virus.[1]
- Therefore, N1 and N2 genes are used as 2019-nCoV markers, and the RNase P is used as extraction control for human RNase P gene.
- RT-qPCR has been used widely in identifying other viral diseases, such as Ebola virus and Zika virus. However, it has less amount of studies demonstrating the application to SARS-CoV-2.

Objectives

- To validate and test the current viral RNA detection techniques
- To determine sensitivity and optimal temperature of RT-qPCR technique

Methods

Primer/Probe Validation
- N and RP gene containing plasmids were prepared with corresponding to primer/probe labeled N1, N2, and RP. One-step qPCR method was used to amplify the gene in interest.
- Sequences for primer/probe sets

Name	Description	Sequence	Label	Final Conc.
2019-nCoV_N1-F	2019-nCoV_N1 Forward Primer	GAC CCC AAA ATC AGC GAA AT	None	500nM
2019-nCoV_N1-R	2019-nCoV_N1 Reverse Primer	TCT GGT TAC TGC CAG TTG AAT CTG	None	500nM
2019-nCoV_N1-P	2019-nCoV_N1 Probe	FAM-ACC CCG CAT TAC GTT TGG TGG ACC-BHQ1	FAM, BHQ-1	125nM
2019-nCoV_N2-F	2019-nCoV_N2 Forward Primer	TTA CAA ACA TTG GCC GCA AA	None	500nM
2019-nCoV_N2-R	2019-nCoV_N2 Reverse Primer	GCG CGA CAT TCC GAA GAA	None	500nM
2019-nCoV_N2-P	2019-nCoV_N2 Probe	FAM-ACA ATT TGC CCC CAG CGC TTC AG-BHQ1	FAM, BHQ-1	125nM
RP-F	RNAse P Forward Primer	AGA TTT GGA CCT GCG AGC G	None	500nM
RP-R	RNAse P Reverse Primer	GAG CGG CTG TCT CCA CAA GT	None	500nM
RP-P	RNAse P Probe	FAM – TTC TGA CCT GAA GGC TCT GCG CG – BHQ-1	FAM, BHQ-1	125nM

Agarose Gel Electrophoresis
- 5uL of DNA products from each set of primer/probe validation experiment were combined with 1uL of DNA loading dye. DNA marker, each DNA mixture, and control were ran in 2% agarose gel with TAE buffer at 90 volts.

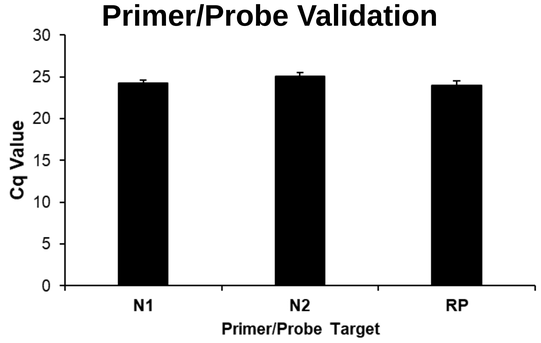
PCR Temperature Gradient
- Eight sets of the same set-up for primer/probe validation experiment were prepared. qPCR was ran with amplification temperature altered for each set ranging between 55 and 63 °C.

DNA Plasmid Serial Dilution
- Stock DNA plasmids (N and RP) with 200,000 copies/uL were prepared in a test tube. 10 µL of stock plasmid was taken and added into another tube with 90 µL of cold sterile water. 10 µL of diluted stock plasmid was taken and mixed with 90 µL of cold sterile water in another tube. Dilution was repeated until the final tube contained 2 copies/µL of DNA plasmid.

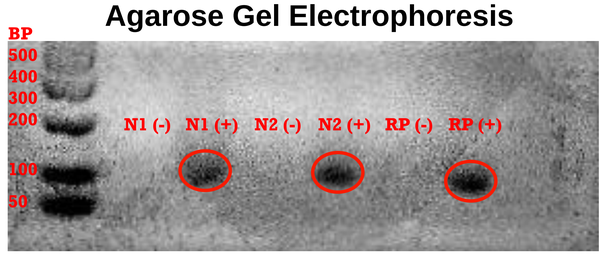
Viral RNA Detection with Dilution
- Concentrate of 10 cVRNA with 250 µL of wastewater is added into a concentrate of 1250 µL of wastewater (250 µL each from five sources) without viral RNA. The combined mixture is then concentrated for RNA extraction and followed by one-step RT-qPCR.

Results

Primer/Probe Validation

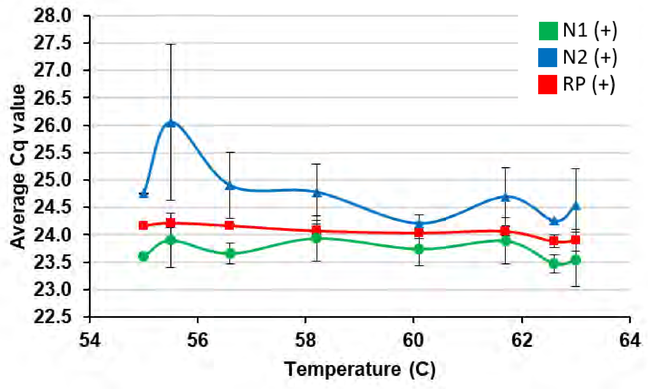


Agarose Gel Electrophoresis

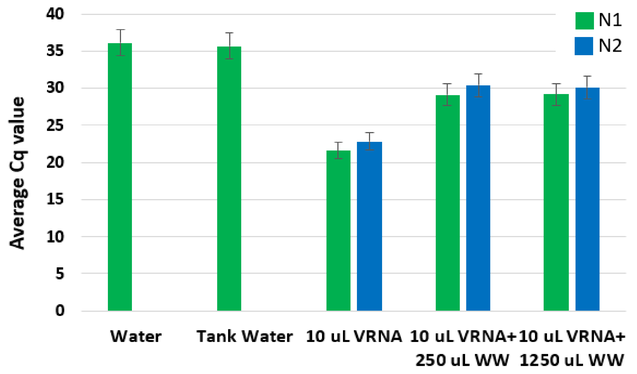


Expected bp size for N1, N2: 117bp and RP: 101bp

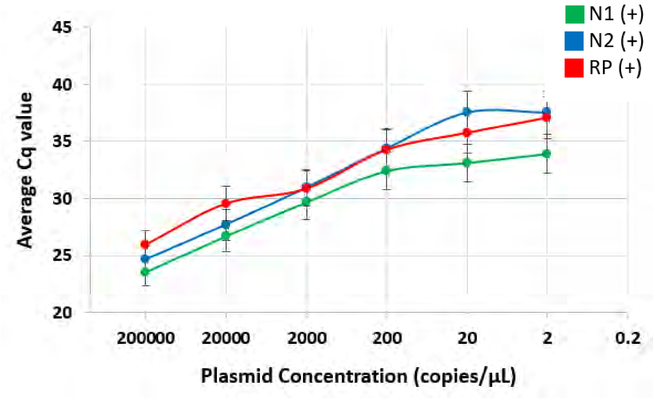
PCR Temperature Gradient



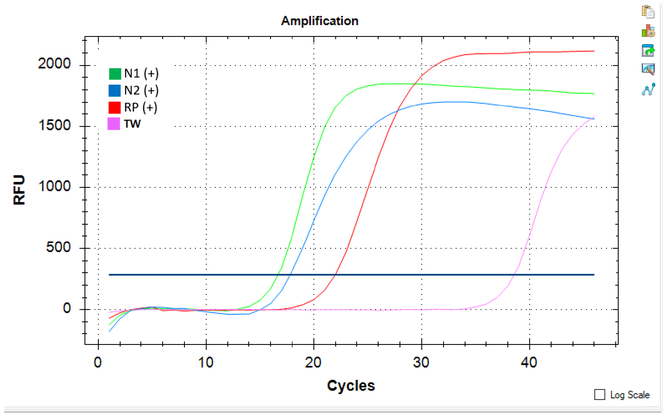
Viral RNA detection with Dilution



DNA Plasmid Serial Dilution



Sample PCR Run For N1, N2, RP With/Without Viral load in Wastewater



Results

Primer/Probe Validation
- Results showed Cq values that range within 24-25.1 for the detection of N1, N2, and RP gene.

Agarose Gel Electrophoresis
- Approximately 100BP was shown for each amplicons of N1, N2, and RP.

PCR Temperature Gradient
- 62.6 °C had lowest average Cq for all three primer and probe sets. 60 °C had the next lowest average Cq.

DNA Plasmid Serial Dilution
- Plasmid concentration and average Cq value had an inverse correlation.

Viral RNA Detection with Dilution
- 10 µL VRNA sample showed lowest Cq value, and both of the diluted 10 µL VRNA+250 µL wastewater and 10 µL VRNA + 1250 µL wastewater showed similar Cq values.

Conclusions

- Each primer and probe sets showed effective detection among different plasmids.
- Agarose gel electrophoresis of DNA products showed base pair size of 100 BP which correlates to the expected range of N and RP gene base pairs.[2]
- Optimal annealing temperatures are 62.6 °C and 60 °C. Based on published data, 60 °C is proposed to be the ideal temperature for primer annealing.[3] Greater number of samples should be included in the future for greater reproducibility and reliability.
- Inverse correlation of plasmid concentration to Cq value in a linear trend indicates the sensitivity of qPCR to be highly sensitive.
- Viral RNA detection was possible in a diluted solution with multiple pool of wastewater. However, there were no difference between the Cq value of highly diluted (+1250 µL WW) sample and less diluted (+250 µL WW) sample.
- Further study with greater number of samples would bring more insight into determining optimal lab set-up for RT-qPCR techniques.

References

1. Bai, Zhihua et al. “The SARS-CoV-2 Nucleocapsid Protein and Its Role in Viral Structure, Biological Functions, and a Potential Target for Drug or Vaccine Mitigation.” *Viruses* vol. 13,6 1115. 10 Jun. 2021, doi:10.3390/v13061115

2. Park, M., Won, J., Choi, B.Y. *et al.* Optimization of primer sets and detection protocols for SARS-CoV-2 of coronavirus disease 2019 (COVID-19) using PCR and real-time PCR. *Exp Mol Med* **52,** 963–977 (2020).

3. Kidd, K. K., and G. Ruano. "PCR 2: A practical approach." (1995): 1-22.

Acknowledgements:
This research was supported by intramural funding from the University of the Pacific

OKU Sutro Excellence Day Project Cover Sheet

(ONE Cover Sheet per project)

Project Title: COVID-19 Wastewater Project: Validating SARS-CoV-2 Detection Techniques

Award Category: DDS & IDS - Research

List names of all contributors to this project:

1. Student Name: David Kim #989 338940
   Program: DDS Class Year 2022
2. Student Name: _____ #989_____
   Program: Please select... Class Year_____
3. Student Name: _____ #989_____
   Program: Please select... Class Year_____
4. Student Name: _____ #989_____
   Program: Please select... Class Year_____
5. Student Name: _____ #989_____
   Program: Please select... Class Year_____
6. Student Name: _____ #989_____
   Program: Please select... Class Year_____
7. Student Name: _____ #989_____
   Program: Please select... Class Year_____

Last field on next page...

8. Enter your abstract text here (300 word max) :

Objectives

This research was done to validate and test the current viral RNA detection techniques and to determine sensitivity and optimal temperature of RT-qPCR technique.

Methods

In Primer/probe validation, N and RP gene containing plasmids were prepared with corresponding to primer/probe labeled N1, N2, and RP. qPCR method was used to amplify the gene in interest. In gel electrophoresis, 5uL of DNA products from each set of primer/probe validation experiment were ran in 2% agarose gel with TAE buffer at 90 volts. In PCR temperature gradient, qPCR was ran with amplification temperature altered for each set ranging between 55 and 63C. In Plasmid Serial Dilution, stock DNA plasmids with 200,000 copies/uL were prepared in a test tube. Dilution was repeated with 0.1x decrease until the final tube contained 2 copies/uL. In Viral RNA Detection with Dilution, concentrate of 10 cVRNA with 250 uL of wastewater is added into a concentrate of 1250 uL of wastewater (250 uL each from five sources) without viral RNA. The combined mixture is then concentrated for RNA extraction and followed by one-step RT-qPCR.

Results

Cq values ranged 24-25.1 for all genes. Approximately 100BP was shown for all amplicons. 62.6C had lowest average Cq and subsequently 60C. Plasmid concentration and average Cq value had an inverse correlation. 10 uL VRNA sample showed lowest Cq value, and both of the diluted 10 uL VRNA+250 uL wastewater and 10 uL VRNA + 1250 uL wastewater showed similar Cq values.

Conclusions

Each primer and probe sets showed effective detection among different plasmids. Agarose gel electrophoresis of DNA products correlated to the expected range of base pairs of ~100BP. Based on published data, 60C is proposed to be the ideal temperature for primer annealing. Greater number of samples should be included in the future for greater reproducibility and reliability. Inverse correlation of plasmid concentration to Cq value in a linear trend indicates the sensitivity of qPCR to be highly sensitive. Viral

Thank you for filling out the OKU Sutro Excellence Day Project Cover Sheet!Please merge this Cover Sheet with your Final Project Materials (ie, research poster, clinical case, paper, or other creative production) before uploading to the Final Project Submission Form.