Validation Study for VERIPRO® SARS-CoV-2 Env Assay for the Detection of SARS-CoV-2 from Stainless Steel Environmental Surface Swabs: Emergency Response Validation—AOAC *Performance Tested Method*℠ 122001

Adam Joelsson, Shannon Green, Nicholas Siciliano, Vikrant Dutta, Ron Johnson

bioMérieux, 595 Anglum Road, Hazelwood, MO 63042

Abstract

Background: The VERIPRO SARS-CoV-2 Env assay uses reverse transcriptase PCR (RT-PCR) to detect SARS-CoV-2, the causative agent of COVID-19, from stainless steel environmental sample swabs.

Objective: To validate the VERIPRO SARS-CoV-2 Env assay as part of the AOAC Research Institute's Emergency Response Validation *Performance Tested Method*℠ program.

Methods: The VERIPRO SARS-CoV-2 Env assay was evaluated for specificity using *in silico* analysis of 15 764 SARS-CoV-2 sequences and 65 exclusivity organisms (both near neighbors and background organisms). The candidate method was evaluated in an unpaired study design for one environmental surface (stainless steel) and compared to the U.S. Centers for Disease Control and Prevention 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel, Instructions for Use (Revision 4, Effective 6/12/2020).

Results: Results of the in silico analysis demonstrated the specificity of the method in being able to detect target sequences and discriminate them from near neighbors. In the matrix study, the candidate method demonstrated statistically significant better recovery of the target analyte than the reference method.

Conclusions: The VERIPRO SARS-CoV-2 Env assay is a rapid and accurate method that can be utilized by food producers to detect the causative agent of COVID-19 on food contact surfaces.

Highlights: The VERIPRO SARS-CoV-2 Env assay can be performed without the need for an optional RNA purification step to detect SARS-CoV-2 from environmental surfaces.

General Information

In late 2019, Chinese authorities notified the World Health Organization (WHO) of a novel coronavirus (SARS-CoV-2) that caused an outbreak of pneumonia and has resulted in millions of confirmed human infections globally (1). The detection and spread of the new respiratory viruses has led to uncertainty of its ability to survive on surfaces, including food contact surfaces (2). Previous respiratory outbreaks have resulted in studies indicating the viability of organisms on surfaces for extended periods of time. Reducing the risk associated with these surfaces includes both effective cleaning, disinfection, and a robust environmental monitoring program, including rapid detection methods (3). Having access to this information can lead toward infection prevention and improved control measures aimed at reducing surface-based transmission.

Principle of the Method

The VERIPRO SARS-CoV-2 Env test kit is a reverse transcriptase PCR (RT- PCR) assay for the molecular detection of SARS-CoV-2 from environmental surface swabs. The assay targets the highly conserved N1 and N2 regions of the nucleocapsid gene of SARS-CoV-2. It utilizes a multiplex detection method that detects the targets on the Cy5 channel and an RNA-based internal amplification control (IAC) on the HEX channel. The VERIPRO SARS-CoV-2 Env assay couples the advantages of the real-time format with a streamlined environmental

sampling protocol that provides results in under 2 h following sample collection. Testing is performed on the GENE-UP thermocycler.

The assay is designed to be compatible with purified RNA via approved RNA extraction kits and synthetic environmental sampling swabs. The Limit of Detection (LOD) of the assay is 5-10 genomic copies (1000–2000 genomic copies of sample per mL from either approved collection buffers or extracted samples). Data from the AOAC ERV PTM study indicated that a concentration of 2000 genomic copies per 2” × 2” test area resulted in 100% detection by the assay.

Scope of method

(**a**) *Analyte(s).*—SARS-CoV-2 viral strains.

(**b**) *Matrixes.*—Environmental surface swabs (2” × 2”): stainless steel.

(**c**) *Summary of validated performance claim.*—Performance comparable to the Centers for Disease Control and Prevention (CDC) 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel (1) for detection of SARS-CoV-2 on stainless steel.

Definitions

(a) *Probability of detection (POD).*—The proportion of positive analytical outcomes for a qualitative method for a given matrix at a given analyte level or concentration. POD is concentration dependent. Several POD measures can be calculated; POD_R (reference method POD), POD_C (confirmed candidate method POD), POD_{CP} (candidate method presumptive result POD) and POD_{CC} (candidate method confirmation result POD).

(b) *Difference of probabilities of detection (dPOD).*—Difference of probabilities of detection is the difference between any two POD values. If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.

(c) *In silico.*—The use of computer simulation to evaluate target and non-target sequences for molecular methods.

(d) *Homology.*—Similarity of nucleic acid sequences due to a common evolutionary origin. In this report, a result of no homology would indicate that the two sequences being compared come from different origins.

Materials and Methods

Test Kit Information

(**a**) *Kit name*.—VERIPRO SARS-CoV-2 Env Assay.

(**b**) *Cat. No.*.—IS1093.

(**c**) *Ordering information*.—Contact Invisible Sentinel: 3711 Market Street, Suite 910, Philadelphia PA, 19104 Phone: 215-966-6118. E-mail: orders@invisiblesentinel.com.

Test Kit Components

(a) *VERIPRO SARS-CoV-2 Env PCR Reagent*.—4 packs of 96 tubes at 45 µL.

(b) *VERIPRO SARS-CoV-2 Env RNA Positive Control*.—50 µL; 1000 genomic copies per 5 µL

Additional Supplies and Reagents

(a) *Pipettes and filter tips*.—Capable of 5 µL and 50-1000 µL volume transfers.

(b) *Synthetic sample collection swabs*.—1 mL Genotek OR-100 collection swabs.

(c) *Genotek prepIT*.—2QA AG (PT-QAG) and ST (PT-QST) Reagents (for crude Genotek Swab Sample Prep).

(d) *RNA extraction kit.*—Optional.

Apparatus

(a) *GENE-UP PCR Instruments or comparable instrument capable of detecting HEX & Cy5 fluorophores.*

(b) *Lab Bench-top Vortex*

(c) *Water bath or heat block*.—capable of maintaining 75°C temperatures.

Reference Materials

Organisms used in the study were obtained from:

(a) *Biodefense and Emerging Infections Research Resources Repository (BEI)*.—Manassas, VA

Safety Precautions

Assay users should observe BSL2/3 microbiological practices and use the appropriate PPE and safety precautions when performing this assay. Do not use the VERIPRO® SARS-CoV-2 Env components past indicated expiration date. Deviations from the assay protocol may impact overall test performance. Invisible Sentinel PCR tubes are for single use only. Decontaminate all surfaces, media and reagents and discard in accordance with local, state, and federal regulations.

General Preparations

(a) Use aseptic technique.

(b) Ensure proper use of PPE.

(c) Use a BSL2 Safety Cabinet when opening the swab/buffer tube.

(d) Change pipette tips in between sample analysis.

Sample Preparation

(a) *Stainless Steel Environmental Swabs (2" × 2" test area)*.—Pre-moisten swab in collection buffer and swab area of interest. Securely place swab back into collection tube and mix by inverting 5–10 times.

Note: Prior to sampling, surfaces should be dry and clear of any residual sanitizer as these products may affect final results

Note: For samples that will not be processed in <1 hour from collection, store swab/buffer at 2-6° (for up to 24 h).

Note: Alternatively, the swabs can be stored at –20 or –80°C until use. In that case the swabs should be thawed on ice or at +4°C.

Analysis

(a) After collection swabs are processed via a quick RNA extraction protocol or following purification:

(1) The Invisible Sentinel's quick RNA extraction method (steps **b-i**).

(2) The Qiagen QIAamp Viral RNA mini kit (Cat. No. 52904) following manufacturer's directions. After RNA purification, samples are processed as outlined in step **(g)**.

(b) Transfer 100 µL of the OR-100 buffer from the environmental swab collection tube to a 1.5 mL microcentrifuge tube.

(c) Heat for 75°C for 10 min in water bath or heat block.

(d) Add 10 µL of Reagent AG and 20 µL of Reagent ST and mix thoroughly by vortexing for 2 min at 1300 rpm.

(e) Centrifuge samples for 2500 x *g* for 2 min.

(f) Transfer 10 µL of the upper phase to a new 1.5 mL microcentrifuge tube and add 10 µL of dH_2O. Vortex to mix contents.

(g) Transfer 5 µL from sample to a thawed VERIPRO® SARS-CoV-2 Env PCR tube.

Note: IMPORTANT: Open the VERIPRO® SARS-CoV-2 Env PCR tube only when adding sample and promptly close after addition to avoid cross contamination between tubes.

(h) Load 5 µL of RNA positive control in a separate reaction tube. RNA control is provided at 1000 genomic copies per 5 µL.

(i) Load 5 µL of RNA free water into a separate reaction tube. This will serve as a negative control.

(j) Place PCR Tube into GENE-UP PCR instrument, select "SARS-CoV-2 Env PCR"

program and start program as directed by the GENE-UP PCR instrument User Guide.

Amplification

(a) Place the VERIPRO® SARS-CoV-2 Env PCR tube into the GENE-UP thermal block using the GENE-UP Adapter Plate.

(b) Select “From Template” in GENE-UP Open Software home page window and choose the “SARS-CoV-2 Env PCR” program.

(c) Enter sample ID’s into the Sample Editor module, utilizing the Sample Subset module to indicate wells, if desired.

(d) Save the experiment and select “START RUN” in the Experiment module. Run time is approximately 1.5 h.

(e) Once the run is complete, proceed to the Analysis module. Create a New Analysis by selecting “Qualitative Detection” from the list of analysis options provided. If samples are organized as a subset, select the appropriate sample subset from the dropdown menu.

(f) Select “Calculate” to obtain the Ct/Cp values. Inspect the traces for the characteristic exponential amplification curve shape.

(g) The sample Ct/Cp results table from each channel can be exported as a text file by right clicking on the sample results table. These files can be opened in Microsoft Excel to record, organize, and evaluate the data. Reports can be generated by saving the analysis and navigating to the “Report” module.

(h) Perform Step **(g)-(h)** for each channel by toggling to each individual filter using the “Channel” Button.

Results Interpretation

(a) Amplification curves have a characteristic shape consisting of an initial lag phase, exponential amplification phase and a final plateau phase. The final plateau phase, which represents a decrease in reaction efficiency as reagents are consumed, may not be reached in reactions containing low levels of target RNA. Amplification curves that deviate from the

characteristic shape should be interpreted with caution. For each VERIPRO® reaction, the cycle at which fluorescence signal rises above background fluorescence is determined and is called the “threshold cycle” (Ct) or “crossing point” (Cp), depending on the instrument. The Ct/Cp will occur at an earlier cycle for samples containing high levels of target organisms and will be delayed for reactions containing low levels of target organisms. The software algorithm utilizes a 2nd derivative max option to enhance the sensitivity of the method. The CY5 channel is designed to detect the N1 and N2 regions of the SARS-CoV-2 Nucleocapsid gene (as defined by CDC). The HEX channel serves as an internal amplification control (IAC) to indicate a successful PCR reaction and should be detected at a Ct/Cp value between ~26–30 cycles.

Validation Study

Study Overview

The study was conducted according to the procedures outlined in the AOAC Research Institute (RI) Emergency Response Validation *Performance Tested Methods*^{SM} Study Outline: *Validation Outline for Molecular Methods that Detect SARS-CoV-2 on Surfaces (V14, September 2020).* The in silico analysis was performed by bioMérieux. Test portions for the matrix studies were prepared by the independent laboratory, MRIGlobal, and shipped blind-coded to bioMérieux for analysis. Additional AOAC RI PTM parameters, robustness and product consistency and stability will be submitted for full PTM certification by March 31, 2021 (4).

Robustness.—Data to be collected and submitted for final PTM certification.

Product consistency and stability.—Data to be collected and submitted for final PTM certification.

In Silico Analysis

Inclusivity and Exclusivity.—(1) Methodology.—To evaluate the inclusivity and exclusivity of the VERIPRO SARS-CoV-2 ENV assay primers and probes, 15 764 unique target strains, 8 near neighbors and 57 environmental background organisms were evaluated.

(2) Results.—Of the target genomes analyzed, 15 759 were perfect matches for detection on the Cy5 target channel of our assay (99.97% inclusivity). For the variants that were not perfect matches, it is highly likely that these variants are rare, have ambiguous positions or possible sequence errors. *See* Table 1 for a summary of the inclusivity results.

All exclusivity organisms (near neighbors and background organisms) produced no matching sequences for the VERIPRO SARS-CoV-2 ENV primers and probes. *See* Table 2 for a summary of the results for the closest mismatches for the exclusivity testing.

(3) Quality control.—Figure 2 shows the unimolecular RNA folding of the nucleocapsid protein and Figures 3 and 4 present the N1 and N2 regions, respectively, of the nucleocapsid RNA. While the N2 region shows a stable UUCG hairpin, the N2 probe utilized in the assay does not bind at this site and should not pose an issue to method performance. Table 3 provides a summary of the normalized energy of predicted RNA structures of SARS-CoV-2 nucleocapsid protein. The internal amplification control (IAC) employed by the Invisible Sentinel SARS-CoV-2 ENV Assay is a proprietary, 120 bp synthetic SS RNA sequence derived from spliced segments of non-coding region bacterial DNA. Tables 4 and 5 provide a summary of the delta-G and melting temperature (Tm) of the target primers and probes and internal amplification control (IAC).

Independent Laboratory Study

Coronavirus isolate and genomic copies/mL determination.—(1) Methodology.—The SARS-CoV-2 isolate used for these studies, USA_WA1/2020, was isolated from the first documented US case of a traveler from Wuhan, China (5). SARS-CoV-2 was sourced from the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA). The virus stock was received from WRCEVA as a 1 mL lyophilizate. Upon receipt the lyophilizate was resuspended in 2 mL of PBS and single-use aliquots (50 µL) were frozen at –70°C. Table 1 summarizes the characteristics of the SARS-CoV-2 stock used for these studies. The PFU/mL quantitation

information was provided by WRCEVA. GC/mL was determined by MRIGlobal as described below using one of the frozen viral stock aliquots.

Viral genomic copies per mL (GC/mL) was determined by quantitative RT-PCR using a Bio-Rad CFX96 Real-Time Detection System. The standard curve was prepared from Synthetic SARS-CoV-2 RNA (ATCC No. VR-3276SD). The PCR procedure used N1 primer and probe sequences published by the CDC. Primers and probes were purchased from Integrated DNA Technologies (IDT No. 10006713). TaqPath™ 1-step RT-PCR Master Mix, CG was sourced from ThermoFisher. Thermal cycling conditions followed those published in the CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel Instructions for Use and are summarized in Table 7.

The synthetic RNA standard curve consisted of the following concentrations: 1×10^{1}, 1×10^{2}, 1×10^{3}, 1×10^{4} and 1×10^{5} GC/ L. SARS-CoV-2 virus stock was diluted in nuclease-free water for testing at the following dilutions: 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}. Master mix was prepared as noted in Table 8.

For the RT-PCR reaction, 15 µL of prepared master mix was added to each well followed by 5 µL of standard or sample, for a final total volume of 20 µL per reaction well. Both RNA standards and SARS-CoV-2 sample dilutions were run in triplicate wells.

The GC/mL of the SARS-CoV-2 dilutions was determined using the slope and y-intercept of the synthetic RNA standard curve, as determined by linear regression analysis. The GC/mL of the virus stock was determined based on the average of the triplicate well results for all dilutions within the standard curve range. For the SARS-CoV-2 stock used for these studies, the concentration was calculated to be 1.6×10^{9} GC/mL.

The presence of infectious SARS-CoV-2 in the WRCEVA virus stock was verified using standard cell culture techniques. Briefly, 3×10^{6} Vero E6 cells were plated into a T75 flask with 15 mL infection media (Dulbecco's Modified Eagle's medium supplemented with 5% fetal bovine

serum and nonessential amino acids) and incubated in a humidified incubator with 5% CO2. The following day the Vero cells were re-fed with infection media and inoculated with virus stock. Cells were incubated for 5 days at which point widespread cytopathic effect (CPE) was apparent by microscopic examination of the Vero cells.

Test Plate Inoculation

Dilutions of SARS-CoV-2 virus stock were prepared in VTM from a frozen viral stock aliquot as shown in Table 9. The same concentrations of virus were used for inoculating test areas for both the Reference (CDC RT-PCR) and Candidate (VERIPRO SARS-CoV-2 Env) methods.

Square 14” × 14” grade 304 stainless steel plates were used for the studies to mimic food preparation surfaces. All test plates were cleaned, disinfected, washed with sterile water and autoclaved prior to use. Test grids of 2” × 2” test areas were created on the test plates using laboratory tape. To inoculate the test plates, the volume specified in Table 9 was pipetted onto the appropriate test area and spread evenly over the entire test area with a sterile 10 µL inoculating loop. Inoculated plates were left until visibly dry (up to 1 h) in a biosafety cabinet (BSC) then transferred to a sealed plastic container and stored overnight at room temperature (24 h for the Reference Method plates and 28 h for the Candidate Method plates). This was a deviation from the 16–24 h storage period specified in the protocol for the Candidate Method plates due to an analyst error. Temperature and humidity ranged from 18.1–21.0°C and 33%–46% RH (relative humidity) during the plate inoculation and drying process. Components used in the test plate inoculation procedure are listed in Table 10.

WHO Reference Method Plate Sampling

After drying overnight, test areas on the test plates were sampled according to the WHO procedure as follows: A swab was pre-moistened by dipping into a 15 mL conical tube containing 2.0 mL of VTM. The pre-moistened swab was used to sample the 2” × 2” test area by

rubbing the swab in at least two different directions while applying pressure to the surface and rotating the swab head. After sampling the test area, the swab was snapped at the break point and placed back into the VTM tube. A random sample ID was assigned to each test area sample. Swab samples were placed in a refrigerator (2–8°C) within 15 min of test area sampling and stored overnight (22 h) before nucleic acid extraction. Components used in the WHO Reference Method test plate sampling are listed in Table 11.

Candidate Method Plate Sampling

Test areas on the Candidate Method test plates were sampled using the provided ORAcollect-RNA sample collection kits (ORAcollect kits are intended for collection of oral fluid but were repurposed here for collection of environmental surface samples). Each ORAcollect sample collection kit contains a swab fitted to a threaded cap and a tube containing transport media. Briefly, a swab was pre-moistened by dipping it in the transport media tube. The pre-moistened swab was used to sample the 2” × 2” test area by rubbing the swab in at least two different directions while applying pressure to the surface and rotating the swab head. After sampling the test area, the swab was screwed back into the collection tube and mixed by inverting the tube 15 times. Each sample tube was assigned a unique random ID number (a key correlating test area sample to random ID number was created and sent to AOAC). Swab samples were shipped overnight to Invisible Sentinel at ambient conditions the day of sampling. Components used in the Candidate Method test plate sampling are listed in Table 12.

CDC Reference Method RT-PCR Testing

Samples to be analyzed by the CDC reference method were transferred to an operator not aware of the blinded sample identities for testing on the CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel test kit. RNA was extracted from 140 µL of VTM using the Qiagen QIAamp Viral RNA Mini Kit per the manufacturer’s instructions. Extracted RNA

was tested on the CDC Panel on an Applied Biosystems 7500 Fast Dx Real -Time PCR Instrument following published instructions. Components used for the Reference Method RT-PCR testing are summarized in Table 13. Fractional positive results were seen with the 0.5 POD sample set. Reference Method test results were sent to AOAC for comparison with the Candidate Method.

Candidate Method Testing

Swabs were processed according to the methodologies listed in the *Sample Preparation* section. Each test portion was evaluated using the rapid extraction protocol and using the QIAamp RNA purification assay.

Results.—As per criteria outlined in Appendix J of the *Official Methods of Analysis* Manual, fractional positive results were obtained for the CDC reference method. The probability of detection (POD) was calculated as the number of positive outcomes divided by the total number of trials (6). POD was calculated for the candidate presumptive results, POD_C and the reference method, POD_R as well as the difference in the candidate and reference methods, $dPOD_C$. The POD analysis between the VERIPRO SARS-CoV-2 Env assay and the reference method indicated that there was a significant difference between the two methods, with the candidate method detecting more positive samples than the reference method, which is acceptable according to AOAC Appendix J policies. A summary of POD analyses are presented in Table 14. Individual results are provided in Table 15.

Discussion

The VERIPRO SARS-CoV-2 Env assay successfully detected the target analyte from stainless steel environmental surface samples following two extraction procedures (Invisible Sentinel's quick RNA extraction procedure which requires no RNA purification step or the Qiagen QIAamp viral RNA purification kit).

Using POD analysis, a statistically significant difference was observed between the number of positive samples detected by the candidate method when compared to the reference method, with the candidate method detecting more positive samples. Based on the principle for detection of the candidate and CDC method, it is not surprising that a difference in the number of positive results was obtained. The CDC method result interpretation requires more than one signature being positive (N1 and N2), increasing the probability of a positive result being due to the presence of intact virus. This makes practical sense as the CDC method is designed for clinical use and has been adapted for surface detection with the WHO sampling method. The VERIPRO SARS-CoV-2 Env assay requires only a single target to be present (N1 and/or N2 conserved target regions), making it more likely to call a sample positive due to these RNA fragments than the CDC method which requires both targets to be present to be called positive.

Once the virus begins to lyse on surfaces, RNA will begin to degrade, and some genes may be more labile than others. As the RNA fragments become smaller in size over time, methods that require only a single target may be more likely to detect these fragments. This increases the chances of the candidate method being positive at the limit of detection of the assay as it can likely detect both intact virus and viral RNA fragments from surfaces.

The VERIPRO SARS-CoV-2 Env method allows for the rapid screening of surfaces for the target strain. With no RNA purification protocol requirement, samples can be quickly swabbed and analyzed. Product instructions are well written and simple enough that a technician at any level of training would be able to follow it and achieve accurate results. The assay utilizes the GENE-UP instrument which allows the integration of this test into their routine environmental sampling workflow.

Conclusion

The data from this study supports the product claim that the VERIPRO SARS-CoV-2

Env assay can detect SARS-CoV-2 from stainless steel environmental surface samples (LOD of 2000 genomic copies per 2" × 2" test area). Data from the *in silico* analysis indicates the method is highly specific and can detect a wide range of target sequences and discriminate them from background organisms and near neighbors. The results obtained by the POD analysis of the method comparison study demonstrated that the candidate method's performance was superior to that of the reference method.

Acknowledgments

Reviewer(s)

William Burkhardt

FDA GCSL

1 Iberville Drive

Dauphin Island, AL 36528

John SantaLucia

Wayne State University

42 W Warren Ave

Detroit MI 48202

Sanjiv Shah

EPA ORD CESER HSMMD DCB

1200 Pennsylvania Ave NW

Washington, DC 20004

Submitting Company:

bioMérieux,

595 Anglum Road

Hazelwood, MO 63042

Independent Laboratory:

MRIGlobal

425 Volker Blvd

Kansas City, MO 64110

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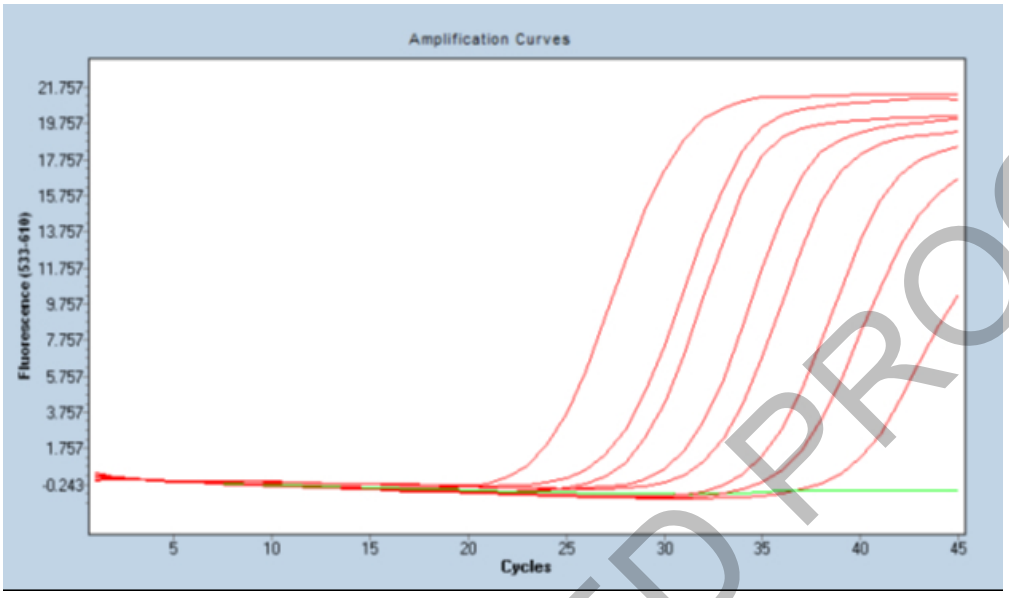

Interpretation of Results

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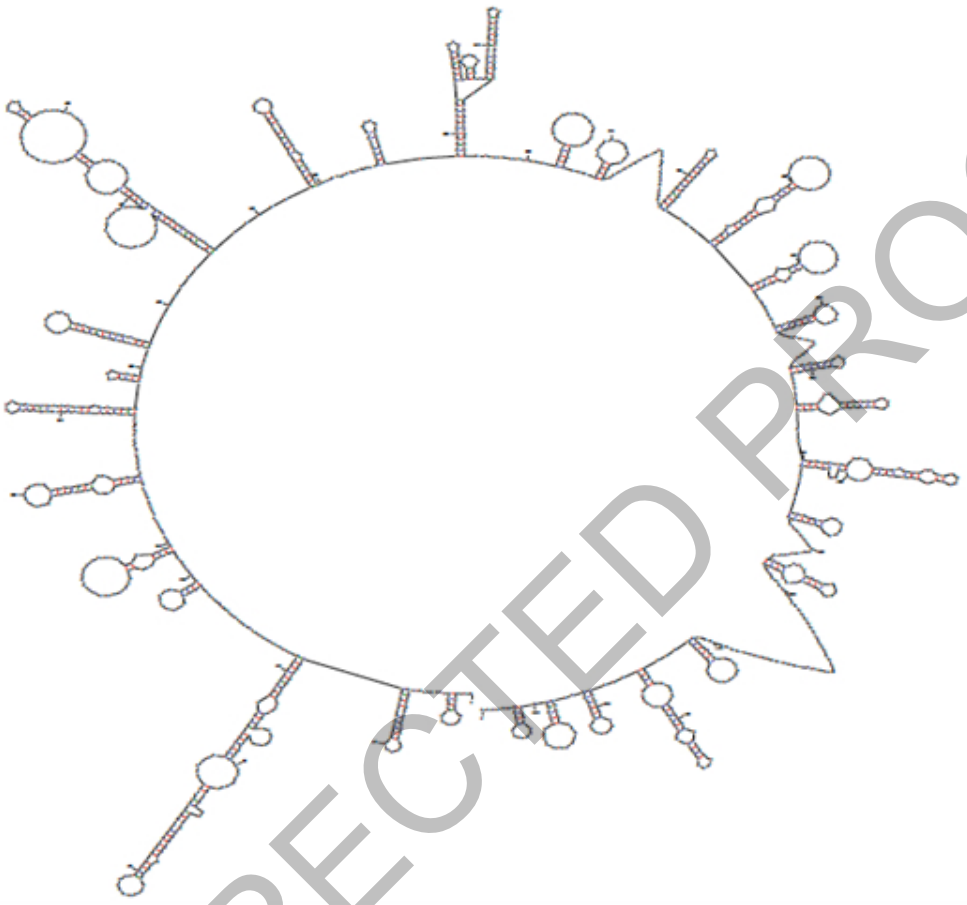
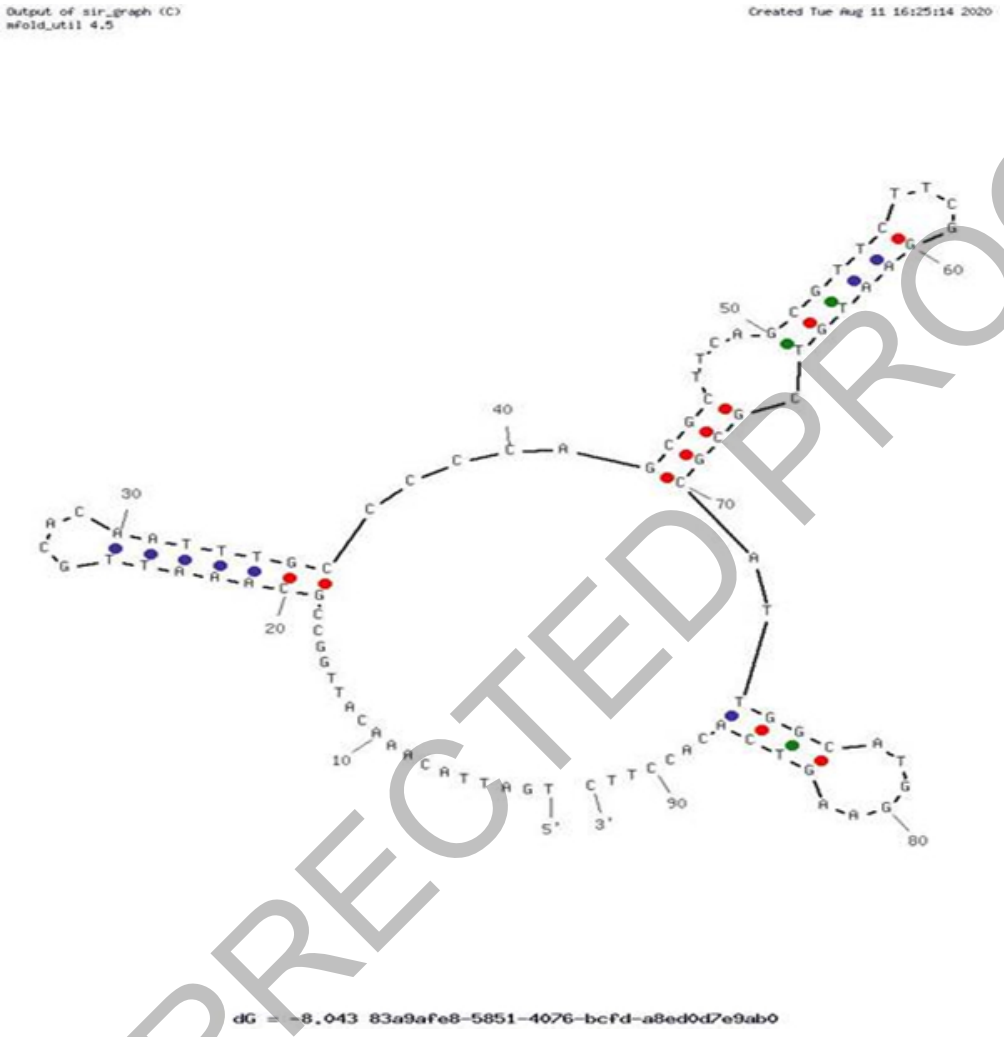


Unimolecular RNA Folding of Nucleocapsid Protein

141x130mm (96 x 96 DPI)

N1 Region of Nucleocapsid RNA

167x165mm (96 x 96 DPI)



N2 Region of Nucleocapsid RNA

158x162mm (96 x 96 DPI)

Table 1. Inclusivity results of the VERIPRO SARS-CoV-2 Env primers and probes

Invisible Sentinel SARS-CoV-2 ENV Primers & Probes			*SARS-CoV-2 Genomes*	
Blast Name	Assay Target Channel	Primers and Probes	# Perfect matches[a] (*n* = 15764)	% Perfect matches[b]
SARS-CoV-2 Genomes	cy5	N1-F	15732	99.82%
	cy5	N1-R	15684	99.49%
	cy5	N1-P	15416	97.79%
	cy5	N2-F	15629	99.14%
	cy5	N2-R	15744	99.87%
	cy5	N2-P	15716	99.70%
	cy5	Aggregate primer/probe specificity[c]	15759	99.97%

[a]# Perfect matches = # of the 15,764 total SARS-CoV-2 genomes that have 100% homology to the oligo queried.

[b]% Perfect matches = % of the 15,764 total SARS-CoV-2 genomes that have 100% homology to the oligo queried. N=5 of SARS-CoV-2 genomes did not yield a blast hit; *Note:* likely due to incomplete reads during genome sequencing.

[c]Aggregate Primer/Probe Specificity = total assay specificity statistic taking single Cy5 channel output with dual targets into account.

Table 2. Exclusivity results of the potential closest mismatches of the VERIPRO SARS-CoV-2 Env Primers and Probes

Coronavirus	Reference	N1 F	N1 R	N1 Probe	N2 F	N2 R	N2 Probe
229E	ATCC VR-740	4BP mismatch - + partial frame shift	No Homology	No Homology	5 BP Mismatch	No Homology	10 BP Mismatch
MERS	GenBank: MK796425.1	No Homology	No Homology	11 BP Mismatch	6 BP Mismatch	6 BP Mismatch	10 BP Mismatch
SARS CoV	GenBank: MG772933.1	4 BP mismatch	6 BP mismatch	1 BP Mismatch	100% Homology	2 BP Mismatch	5 BP Mismatch

Table 3. Summary of normalized energy of predicted RNA structures				
Normalized energy (∆G) of predicted RNA structures of SARS-CoV-2 Nucleocapsid Protein				
Sequences	Temp., °C	Monavalent, mM	Mg2+, mM	∆G, kcal/mol
N1	60	50	3	–5.95
N2	60	50	3	–8.04

Table 4. Delta-G and melting temperature of the VERIPRO SARS-CoV-2 primers and probes[a]

Primers and probes	Delta G @60°C (kcal/mole^{-1})	Tm, °C
N1-F	2.67	61.10
N1-R	0.61	64.50
N1-P	–1.1	70.30
N2-F	–1.03	61.70
N2-R	–0.68	62.60
N2-P	–0.81	69.60

[a]All calculations were performed at Na^+ and Mg^{++} concentration at 50 and 3 mM, respectively.

Table 5: Delta-G and melting temperature of the VERIPRO SARS-CoV-2 internal amplification control [a]

Primers and probes	Delta G @60°C, kcal/mole^{-1}	Tm, °C
IAC F	–0.55	64.20
IAC R	0.98	62.8
IAC Probe	1.94	66.2

[a] All calculations were performed at Na^{+} and Mg^{++} concentration at 50 and 3 mM, respectively.

Table 6. Summary of SARS-CoV-2 virus stock used in the studies

Virus	Isolate	Source/No.	Lot	Lyophilization date	PFU[a]/mL	GC[b]/mL
SARS-CoV-2	USA_WA1/2020	WRCEVA	TVP23155	2/19/20	3.6×10^6	1.6×10^9

[a]PFU/mL = Plaque forming units/mL (pre-lyophilization).
[b]GC/mL = Genomic copies/mL.

Table 7. CDC assay thermal cycling parameters			
Stage	Temp., °C	Time	Cycles
1	25	2 min	1
2	50	15 min	1
3	95	2 min	1
4	95	3 s	45
	55	30 s	

Table 8. CDC assay master mix preparation	
Reagent	Volume per reaction, µL
Nuclease-free water	8.5
Primer/probe mix	1.5
TaqPath™ 1-step RT-qPCR Master Mix	5.0
Total	15

Table 9. SARS-CoV-2 dilutions in VTM

Sample	Method	Test area size	No. of test areas	GC/mL	µL/Test area	GC/test area
High (1 POD[a]/test area)	Reference	2” × 2”	5	1.3×10^5	150	2.0×10^4
Low (0.5 POD/test area)	Reference	2” × 2”	20	1.3×10^4	150	2.0×10^3
Negative VTM control (0 POD/test area)	Reference	2” × 2”	5	0	150	0
High (1 POD/test area)	Candidate	2” × 2”	5	1.3×10^5	150	2.0×10^4
Low (0.5 POD/test area)	Candidate	2” × 2”	20	1.3×10^4	150	2.0×10^3
Negative VTM control (0 POD/test area)	Candidate	2” × 2”	5	0	150	0

[a]POD = Probability of detection. The POD is based on range-finding studies conducted with the reference method.

Table 10. Components used for test plate inoculation				
Component	Vendor/Manufacturer	Part No.	Lot No.	Expiration
Viral Transport Media (VTM)	MRIGlobal	NA[a]	24Sep2020	9/24/2021

[a]NA = Not available.

Table 11. Components used for WHO reference method test plate sampling				
Component	Vendor/Manufacturer	Part No.	Lot No.	Expiration
Viral Transport Media (VTM)	MRIGlobal	NA[a]	24Sep2020	9/24/2021
Swabs	Puritan	25-1607 1PFSC	7168	3/1/2025

[a]NA = Not Available.

Table 12. Components used for candidate method test plate sampling				
Component	Vendor/ Manufacturer	Part No.	Lot No.	Expiration
ORAcollect Collection Kit	DNA Genotek	OR-100	XE16	5/14/2021
			XE19	5/19/2021

Table 13. Components used for CDC reference method RT-PCR testing				
Component	Vendor/Manufacturer	Part No.	Lot No.	Expiration
QIAamp Viral RNA Mini Kit	Qiagen	52906	166023562	3/1/2022
2019-nCoV CDC EUA Kit	IDT	10006713	0000535573	4/8/2022
TaqPath™ 1-Step RT-qPCR Master Mix, CG	ThermoFisher	A15299	2220404	4/30/2021

Table 14. Stainless steel candidate vs. reference method – POD results

Matrix	Strain	GU[a]/ Test Area	N[b]	Candidate[h]			Reference			$dPOD_C$[f]	95% CI[g]
				x[c]	POD_C[d]	95% CI	X	POD_R[e]	95% CI		
Stainless Steel (2” × 2”)	SARS-CoV-2 BEI NR-52281	0	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
		2.0×10^3	20	20	1.00	0.84, 1.00	9	0.45	0.26, 0.66	0.55	0.29, 0.74
		2.0×10^4	5	5	1.00	0.57, 1.00	5	1.00	0.57,1.00	0.00	-0.43, 0.43

[a]GU/Test Area = Results of the GU/Test area were determined by standard cell culture techniques
[b]N = Number of test portions
[c]x = Number of positive test portions
[d]POD_C = Candidate method confirmed positive outcomes divided by the total number of trials
[e]POD_R = Reference method confirmed positive outcomes divided by the total number of trials
[f]$dPOD_C$= Difference between the confirmed candidate method result and reference method confirmed result POD values
[g]95% CI = If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level
[h] Candidate POD results were identical for each extraction method utilized in this study

Table 15. Individual results for the stainless steel matrix study

Sample No.	VERIPRO SARS - CoV-2 Result (Quick RNA extraction)[a]	VERIPRO SARS -CoV-2 Result (QIAamp RNA purification)	CDC N1 target result	CDC N2 target result	CDC final result
Low level					
1	+	+	–	–	–
2	+	+	–	+	–
3	+	+	–	–	–
4	+	+	–	+	–
5	+	+	+	+	+
6	+	+	+	+	+
7	+	+	+	+	+
8	+	+	–	+	–
9	+	+	+	+	+
10	+	+	+	–	–
11	+	+	+	–	–
12	+	+	–	+	–
13	+	+	+	+	+
14	+	+	+	–	–
15	+	+	–	–	–
16	+	+	+	+	+
17	+	+	+	–	–
18	+	+	+	+	+
19	+	+	+	+	+
20	+	+	+	+	+
Total	20/20	20/20	13/20	13/20	9/20
High level					
1	+	+	+	+	+
2	+	+	+	+	+
3	+	+	+	+	+
4	+	+	+	+	+
5	+	+	+	+	+
Total	5/5	5/5	5/5	5/5	5/5
Non–inoculated control level					
1	–	–	–	–	–
2	–	–	–	–	–
3	–	–	–	–	–
4	–	–	–	–	–
5	–	–	–	–	–
Total	0/5	0/5	0/5	0/5	0/5

[a] Positive result indicates either the presence of the N1 gene, the N2 gene or both genes.