Validation of the COVID-19 Indoor Test™ by Phylagen for Detection of SARS-CoV-2 Virus on Stainless-Steel Surfaces: AOAC Performance Tested Method℠ 122004

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Abstract

Background: The COVID-19 Indoor Test™ by Phylagen uses a real-time PCR Assay to detect nucleic acid from SARS-CoV-2, the causative agent of COVID-19, which is extracted from swabs sampled from environmental surfaces. This information can be used to detect the presence of the virus in indoor environments.

Objective: To validate the COVID-19 Indoor Test™ by Phylagen as part of the AOAC Research Institute’s Emergency Response Validation Performance Tested Method(s)℠ program.

Method: The COVID-19 Indoor Test by Phylagen assay was evaluated for specificity using in silico analysis of 15,764 SARS-CoV-2 sequences and 65 exclusivity organisms. The candidate method was also evaluated in an unpaired matrix 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 (Revision 4, Effective 6/12/2020).

Results: Results of the in silico analysis demonstrated the specificity of the method in being able to detect SARS-CoV-2 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 (2^(-Ct) test surface).

Conclusions: The COVID-19 Indoor Test by Phylagen is a rapid and accurate method that can be utilized to monitor the presence of SARS-CoV-2, the causative agent of COVID-19, on stainless-steel surfaces in built environments.

Highlights: The COVID-19 Indoor Test by Phylagen assay performed significantly better than the reference method when used to detect SARS-CoV-2 from environmental surfaces.

General Information

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is responsible for the ongoing coronavirus disease (COVID-19) pandemic. The virus primarily spreads from person to person through respiratory droplets. These droplets can land on surfaces, and people could get sick by touching those surfaces and subsequently their mouth, nose, or eyes. The detection of SARS-CoV-2 on these surfaces can signal the need for more effective cleaning and sanitizing measures.

The presence of the virus’s genetic material on environmental surfaces is most easily, quickly, and reliably detected using (quantitative reverse transcription PCR RT-qPCR), which
identifies the novel coronavirus and distinguishes it from other viruses through its unique RNA sequence. This is the same technology used to diagnose patients with COVID-19 based on samples from nasal swabs or saliva.

**Principle of the Method**

The COVID-19 Indoor Test™ by Phylagen is intended to detect SARS-CoV-2 RNA on common surfaces in our everyday environment. Environmental surface swabs are collected by the user and shipped back to Phylagen, Inc. for sample processing and report generation. SARS-CoV-2 RNA is extracted and concentrated from environmental samples and detected through the use of RT-qPCR. Sample data are analyzed and results are reported through our tracking portal.

**Scope of method**

(a) **Analyte(s).**—SARS-CoV-2 virus.
(b) **Matrixes.**—Example: stainless steel-surface.
(c) **Summary of validated performance claims.**—No statistical difference was detected compared to the U.S. Centers for Disease Control and Prevention 2019-Novel Coronavirus Real-Time RT-PCR Diagnostic Panel, Revision 04 (1).

**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$_H$ (reference method POD), POD$_C$ (confirmed candidate method POD), POD$_{CPR}$ (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) **GU.**—Genetic units
(d) **Quantitative reverse transcription polymerase chain reaction (RT-qPCR).**—A laboratory method used for the detection and quantification of RNA.
(e) **N1/N2.**—Gene regions coding for the nucleocapsid protein.
(f) **Selectivity.**—Ability of the method to detect analyte without interference from matrix or other components of similar behavior.

**Materials and Methods**

**Test Kit Information**

(a) **Kit name.**—COVID-19 Indoor Test™ by Phylagen.
(b) **Catalog number.**—COVID-19 Indoor Test™ by Phylagen.
(c) **Ordering information.**—Phylagen.com, Phylagen, Inc., 164 Welsh Street, San Francisco, CA 94107, USA.

**Test Kit Components**

(a) **Wetting solution.**—Also negative control.
(b) **Pre-labeled sample tubes.**—Containing shipping/lysis buffer shown to inactivate viruses (10 or 25 reactions).
(c) **Swabs.**

(d) **Gloves.**
(e) **Box seal.**
(f) **Return shipping label.**
(g) **Instructions.**

**Additional Supplies and Reagents**

(a) **Smart phone, tablet, or computer—**With Phylagen application portal.
(b) **Internet connection.**
(c) **Refuse receptacle.**

**Safety Precautions**

All samples should be viewed as potentially containing SARS-CoV-2 virus. Wear appropriate personal protective equipment.

**General Preparation**

The test kit should be kept at room temperature. Confirm that the user has access to internet signal in order to access the Phylagen portal. This is integral to being able to enter necessary metadata for each sample collected. Before sample collection, the user is advised to wash their hands.

**Sample Collection**

Prior to collecting samples, the user should register their contact information using the secure Phylagen web portal. The Phylagen portal will act as the primary source of metadata collection during the sampling process. As each sample is collected, important information about the location and surface type of the sample are recorded. This information will follow the sample through collection, laboratory processing, and report generation to the customer through the use of the barcode on the side of the tube. When ready to collect samples, sign into the Phylagen portal, review the test plan (if one has been uploaded), and ensure a trash receptacle is nearby for easy disposal of refuse. Open the test kit box, put on the gloves provided in the box. The use of a mask and eye protection is recommended. Open one wetting solution tube and a single sample tube and place them in the designated locations in the workstation. Open a swab and dip it into the wetting solution. Swab up to 1 x 1 square foot surface, moving top to bottom and left to right. Swab surface for approximately 10 s. Limit each swab up to one 1 x 1 square foot surface area.

Holding the sample tube in one hand, insert the swab into the open tube and press the stem at the back of the swab to release the swab head. Using the prompts in the portal, follow the directions to record your sample information, along with the barcode. This process will be repeated for each sample. After recording the metadata, the operator will close the tube, making sure that the lid snaps securely. Make sure the tube is closed tightly and return it to its original position in the box.

After collecting samples, the user must complete the control. Open a swab and dip it in the wetting solution. Eject the swab end directly into the control tube, without swabbing any surface. Place the control tube back in its demarcated spot in the box. The control tube is used for internal process quality check, and its result will only be reported to the tester if it returns a positive call. This may indicate that any environmental sample with a positive SARS-CoV-2 result may be called into question due to sampling contamination.
Confirm that all tubes are closed tightly, close the box, and seal it with the sticker provided. Place the overnight mailing label on the outside of the box and send it back to Phylagen. Samples should be shipped within 24 h after collecting samples. Once received at Phylagen, results are available after 24 h.

Targets
COVID-19 Indoor Test by Phylagen uses two assays run independently. SARS-CoV-2 is detected with the N1 gene target from the CDC (Centers for Disease Control) 2019-nCoV TaqMan primer and probe set. In addition to the SARS-CoV-2 target assay, an internal process control (IPC) is used to control for sample preparation failures or PCR failures.

Sample Intake and Accessioning at Phylagen
Upon package receipt at Phylagen, all samples are transported into a biosafety cabinet (BSC) following all BSL-2 safety protocols as prescribed by the CDC’s guidelines for Biosafety in Microbiological and Biomedical Laboratories (BMBL). Sample barcodes are scanned into Phylagen’s Laboratory Information Management System (LIMS). These barcodes align with the metadata entered into the Phylagen portal by the customer following swabbing/sampling surfaces. In addition, all process documentation followed through the sample extraction, RT-qPCR, and analysis for each sample, resides in the LIMS.

Sample Extraction and Preparation at Phylagen
Once samples have been accessioned, two negative extraction controls (NEC) are prepared for each batch of kits to be processed (one control negative for both the IPC and N1, and one control negative for N1 but positive for the IPC). A batch can contain up to three complete 25-swab kits, seven complete 10-swab kits, or 15 5-swab kits. A static concentration of process control MS2 bacteriophage is added directly to the customer sample tubes containing sample swabs and added to the IPC positive NEC. Samples are lysed and nucleic acids are recovered using proprietary methods developed by Phylagen. Recovered nucleic acids are resuspended in tris-ethylenediaminetetraacetic acid (Tris-EDTA) buffer and are ready for analysis using the RT-qPCR assays.

RT-qPCR
RT-qPCR mastermix is performed in a 384-well PCR format. The 1-Step RT-qPCR format is performed. Positive template controls (PTCs) serve as reference dilution series for both assays. IPC and SARS-CoV-2 RNA are diluted independently in 5-fold from 100 000 copies to 6.4 copies. The PTCs dilutions are run in triplicate, for a total of 21 PCR controls per assay. In addition to the PTCs, no template controls (NTCs) are included in triplicate, for each assay.

The plate is sealed with an optical seal and loaded onto an Applied Biosystems QuantStudio thermal cycler. The LIMS integrates with QuantStudio by setting a run plate layout, which assigns sample or control identifiers to be used in run analysis.

Analysis and Calculations
Following qPCR, qualitative inspection of PTCs, NECs, NTCs, and all N1 positives, is performed to assess the validity of the test batch. Observation of any NEC or NTC replicates crossing the N1 assay threshold results in undetermined calls for all samples on the plate. In this case, these samples will be reprocessed. N1 positives are manually inspected to ensure results fall within accepted quality control metrics established in the product development phase of Phylagen’s testing. The QuantStudio instrument software assigns a CT (threshold cycle) to a sample if the amplification for that sample crosses the set threshold value of 0.05, which was determined through product development. If a sample does not cross the threshold, it is assigned a non-amplification designation. The qPCR output file is uploaded into the LIMS, where the sample results are linked with the sample data previously entered by the kit user, and a report is generated.

Interpretation and Test Result Report
There are three interpretations of the data, which result in the final calls reported to a customer. The assignments are linked to the sample barcodes through LIMS, and a report is accessible on the Phylagen results portal (my.phylagen.com). Table 1 describes the logic used, and the resulting output call.

Validation Study
This validation study was conducted under the AOAC Research Institute (RI) Performance Tested MethodSM program and the AOAC INTERNATIONAL Methods Committee Guidelines for Validation of Microbiological Methods for Food and Environmental Surfaces (2). Method developer studies were conducted in the laboratories of Phylagen, Inc., and included the in silico analysis for inclusivity/exclusivity. The independent laboratory study was conducted by MRI Global, and included a matrix study for SARS-CoV-2 virus on a stainless-steel surface.

Method Developer Studies
Inclusivity
The inclusivity of the COVID-19 Indoor Test by Phylagen primer set (CDC design N1) was evaluated by in silico analysis against a collection of 15 766 SARS-CoV-2 genomes obtained from Global Initiative on Sharing All Influenza Data (GISAID; 3). This genome collection was provided by the AOAC RI and represents genomes collected between December 2019 to June 26th, 2020. The list was filtered to remove duplicates and low-quality sequences defined as >300 Ns. We used thermonucleotideBLAST (4) to evaluate the inclusivity of our primer set against this genome dataset.

Forward Primer
A total of 15 717 genomes (99.7%) had a perfect match with the forward primer. For the remaining 47 genomes, we detected various mismatches against the forward primer: two genomes

Table 1. Interpretation

| N1 assay | IPC assay |
|----------|----------|
| CT < 40 | SARS-CoV-2 positive |
| CT = 40 | SARS-CoV-2 negative |

CT = threshold cycle.
with a mismatch of seven nucleotides, eight genomes with a mismatch of four nucleotides, six genomes with a mismatch of three nucleotides, nine genomes with a mismatch of two nucleotides, and 24 genomes with a mismatch of one nucleotide.

Reverse Primer
A total of 15 678 genomes (99.4%) had a perfect match with the reverse primer. The remaining 86 genomes had different numbers of mismatches against the reverse primer: five genomes with a mismatch of three nucleotides, one genome with a mismatch of two nucleotides, and 80 genomes with a mismatch of one nucleotide.

Probe
A total of 15 720 genomes (99.7%) had a perfect match when evaluating the N1 probe used in the COVID-19 Indoor Test by Phylagen assay. The remaining 46 genomes (0.3%) had a mismatch of one nucleotide when aligned to the probe sequence (24 nucleotides) against the reference genomes.

Exclusivity Analysis
The exclusivity of the genomes was evaluated by searching for the primers on a set of seven viral genomes defined by AOAC (Table 2). Both primers (forward and reverse) were searched on these genomes using thermonucleotideBLAST (4). There were no matches between the primers and any of the seven genomes evaluated.

Possible background amplification of the primers was evaluated against a dataset of different genomes, including multiple viral, bacterial, fungal, and other eukaryotic genomes. The procedure was similar as before, where thermonucleotideBLAST was used to evaluate the match of the primer set against these genomes.

Background Analysis
We used the minimum set of background genomes established by AOAC guidelines (29 viruses, 21 bacteria, two fungi, and eight eukaryotes), and expanded it to also include all avian influenza genomes available in the National Center for Biotechnology Information (NCBI) database.

Viruses
The viral analysis included 29 viruses established by the AOAC guidelines (Table 3). In addition, we included all available complete genomes (as of July 31, 2020) for avian influenza available at NCBI. This dataset included: 802 genome sets for avian influenza H1; 1694 for H4; 1400 for H6; and 1426 for H9. None of the primers showed any amplification against this genome dataset.

Bacteria
To establish the background bacterial signal, we used 31 190 reference genomes available from the Genome Taxonomy Database (GTDB; 5), which included the minimum set of background genomes established by AOAC. Only four bacterial species (Flavobacterium fontis DSM25660, Micavibrio aeruginosavorus ARL-13, Synechocystis sp PCC6803, Hymenobacter psychrophilus CGMCC 1.8975) had hits with the primers, but with a high number of mismatches (Table 4).

None of these bacteria are expected to be present in surfaces samples tested for SARS-CoV-2. F. fontis and Synechocystis sp. PCC6803 are commonly found in freshwater, M. aeruginosavorus ARL-13 was isolated from wastewater samples, while H. psychrophilus CGMCC 1.897 was isolated from oil-contaminated soil samples.

Fungi and Eukaryotes
We did not detect any matches against the background fungal or eukaryotic genomes analyzed (Table 5).

### Table 2. List of genomes used for the exclusivity evaluation

| Species name                      | Accession number |
|-----------------------------------|------------------|
| Human coronavirus 229E            | NC_002645        |
| Human coronavirus OC43            | NC_006213        |
| Human coronavirus NL63            | NC_005831        |
| Human coronavirus HKU1            | NC_006577        |
| SARS-coronavirus                  | NC_004718        |
| MERS-coronavirus                  | NC_038294        |
| Porcine deltacoronavirus          | KY293677         |

### Table 3. Viral genomes used for background analysis

| Species name                        | Accession number |
|-------------------------------------|------------------|
| Influenza A H1N1                    | GCF_00134785.1   |
| Influenza A H3N2                    | GCF_00065085.1   |
| Influenza A H5N1                    | GCA_000864105.1  |
| Influenza A H7N9                    | GCF_000928555.1  |
| Influenza B                         | GCA_000820495.2  |
| Human adenovirus, type 1. Ad71      | KF268207         |
| Human metapneumovirus               | NC_039199        |
| Respiratory syncytial virus LongA   | AY911262         |
| Rhinovirus A                        | NC_001617        |
| Rhinovirus B                        | NC_038312        |
| Rhinovirus C                        | NC_038878        |
| Parainfluenza 1 C35                 | JQ901971         |
| Parainfluenza 2 Greer               | AF530102         |
| Parainfluenza 3 C-43                | NC_0017296       |
| Parainfluenza 4 M-25                | NC_021928        |
| Enterovirus EV78                    | NC_038308        |
| Human bocavirus                     | MG953829         |
| Varicella-zoster virus              | NC_001948        |
| Norovirus                           | NC_039897        |
| Herpes virus 1                      | NC_001806        |
| Herpes virus 2                      | NC_001798        |
| Avian infectious bronchitis virus   | NC_001451        |
| Bovine coronavirus                  | NC_003045        |
| Mouse hepatitis                     | NC_048217        |
| Porcine transmissible gastroenteritis virus | HM776941       |
Reverse Primer (RP) Binding Region

The RNA sequence was selected based on the positions where the amplicon matched on the reference sequence (28136–28359), and an additional 150 nucleotides were added on each side of the amplicon. The resulting RNA sequence was:

UCCUGUUUACCUUUUACAAUUAAUUGCCAGGAACCUAAAUUGGGUAGUCUUGUAGUGCGUUGUUCGUUCUAUGAAGACUUUUUAGAGUAUCAUGACGUUCGUGUUGUUUUAGAUUUCAUCUAAACGAACAAACUAAAAUGUCUGAUAAUGGACCCCAAAAUCACGGAAAAUUGCAGCCCCGCAUAUGCUGUUUGGUGGACCCUGAGAUAUGCAACUUGGAGUAACCCGAAGGGACACCAACCAACACACACUUGGUUAAACCAACCUUGCGUCUIUGGUUCACCCUGUCUCACCAUAAACAAUGGACGAAGGCUUCCAAUAACCAACCAUAGCA

The prediction was done using the reverse transcription temperature at 55°C, using the previously mentioned salt concentrations. One secondary structure was predicted (Figure 1), with a ΔG value of –40.09 kcal/mol.

Forward Primer (FP) Binding Region

The FP region was obtained by making the reverse complement of the target region (from the previous step) and converting all Us to Ts. The generated sequence was:

TGCTATTGGTGTTAATTGGAACGCCTTGTCCTCGAGGGAATTTAAGGTCTTCCCTGCAGTGTGAGTAGAGGCCGAGGTGTTTGTATCGGCACCCACTGCCTTCCATTCTGTACTGTCACTGCCTGAATTGAGTGAATCAGGGTCCAAACAGTAACGCGGTTGACATTGTTGGATTTTCTTTTTGTCCTTTCATTTAAAAGACAAACGACGAGCTCTGACTGATCTCATTAAAAAGCTTCTCTTATAAGCA

Prediction of the secondary structure was done using the annealing temperature at 60°C, with the resulting structure having a ΔG of –1.49 kcal/mol (Figure 3).

Probe Binding Region

Given that the assay probe binds to the sense DNA strand, we selected this strand for the prediction of the secondary structure of the probe binding region. For this, the target region RNA (step 1) was used, with all Us replaced by Ts. The prediction was performed using the extension temperature (60°C), with the resulting structure having a ΔG of –2.8 kcal/mol (Figure 4).

Primer and Probe Unimolecular Folding

The secondary structure of the forward and reverse primers, and the probe, were predicted using VisualOMP (version 7.8.42.0). For all predictions, the annealing temperature was used (60°C), and using the PCR salt concentrations (monovalent ions, 0.08 M; Mg²⁺, 0.002 M).

The predicted structure for the forward primer (Figure 4), had a ΔG of 2.8 kcal/mol. The structure for the reverse primer (Figure 5) had a ΔG of 0.56 kcal/mol and the predicted structure of the probe had a ΔG of 1.15 kcal/mol (Figure 6).

Hybridization Protocol

Using VisualOMP (Version 7.8.42.0), we performed two-state bimolecular hybridization simulations for different molecule combinations. All simulations were performed using the appropriate temperature parameter, assuming salt concentrations of 0.08 M for monovalent salts and 0.002 M for Mg²⁺, and
with a strand concentration of 100 nM, primer concentrations of 300 nM, and probe of 150 nM. The results are shown in Table 6.

Independent Laboratory Study

**Coronavirus Isolate and Genomic Copies/mL Determination**

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 (7). 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 (Phosphate Buffered Saline) and single-use aliquots (50 μL) frozen at −70°C. Table 7 summarizes the characteristics of the SARS-CoV-2 stock used for these studies. The plaque forming units per mL (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 qPCR 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-qPCR 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 8.

The synthetic RNA standard curve consisted of the following concentrations: $1 \times 10^1$, $1 \times 10^2$, $1 \times 10^3$, $1 \times 10^4$, and $1 \times 10^5$ GC/L. SARS-CoV-2 virus stock was diluted in nuclease-free water for testing at the following dilutions: $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$, and $10^{-5}$. Master mix was prepared as noted in Table 9.
For the RT-PCR reaction, 15 µL prepared master mix was added to each well followed by 5 µL 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 $1.6 \times 10^9$ GC/mL.

The presence of infectious SARS-CoV-2 in the WRCEVA virus stock was verified using standard cell culture techniques. Briefly, $3 \times 10^6$ 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% CO$_2$. 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 viral transport medium (VTM) from a frozen viral stock aliquot as shown in Table 10. The same concentrations of virus were used for inoculating test areas for both the CDC RT-qPCR reference and the COVID-19 Indoor Test by Phylagen methods.

Square $14 \times 14$" 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 $2 \times 2$" 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. The temperature and humidity ranges were 18.1–21.0°C and 33–46% relative humidity (RH) during the plate inoculation and drying process. Components used in the test plate inoculation procedure are listed in Table 11.

Reference method plate sampling. — After drying overnight, test areas on the reference method test plates were sampled according to the WHO (World Health Organization) procedure as follows: A swab was pre-moistened by dipping into a 15 mL conical tube containing 2.0 mL VTM. The pre-moistened swab was used to sample the $2 \times 2$" test area by rubbing the swab in

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**Table 6.** Predicted results for two-state bimolecular hybridizations

| Hybridization type             | Temperature used, °C | Delta G, kcal/mol | Calculated Tm, °C | Bound, % |
|--------------------------------|----------------------|-------------------|-------------------|---------|
| Forward primer—cDNA target    | 60 (annealing temperature) | $-16.45$          | 64.7              | 99.25   |
| Reverse primer—RNA target     | 55 (reverse transcription temperature) | $-49.98$          | 73.2              | 21.56   |
| Reverse primer—DNA target     | 60 (extension temperature) | $-19.88$          | 68.5              | 9.03    |
| Probe—DNA target              | 60 (extension temperature) | $-22.78$          | 74.3              | 90.97   |

**Table 7.** Summary of SARS-CoV-2 virus stock used in the studies

| Virus   | Isolate     | Source/No. | Lot     | Lyophilization date | PFU/mL$^a$ | GC/mL  |
|---------|-------------|------------|---------|---------------------|------------|--------|
| SARS-CoV-2 | USA_WA1/2020 | WRCEVA     | TVP23155 | 2/19/20            | $3.6 \times 10^6$ | $1.6 \times 10^9$ |

$^a$Pre-lyophilization.
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 reference method test plate sampling are listed in Table 12.

**Table 12. Components used for WHO reference method test plate sampling**

| Component | Vendor/manufacturer | Part No. | Lot No. | Expiration |
|-----------|---------------------|----------|---------|------------|
| VTM       | MRI Global          | N/A      | 24Sep2020 | 9/24/2021  |
| Swabs     | Puritan             | 25–1607 1PFSC | 7168 | 3/1/2025  |

* N/A = Not available.

Candidate method plate sampling. — Test areas on the candidate method test plates were sampled per the instructions provided in the COVID-19 Indoor Test by Phylagen. Briefly, a swab was pre-moistened by dipping it in a tube of wetting solution. The pre-moistened swab was used to sample the 2 × 2" test area by rubbing the swab in at least two different directions (as indicated in the COVID-19 Indoor Test by Phylagen instructions) 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 stored in a randomly selected pre-numbered tube of sample buffer (a key correlating test area sample to random ID number was created and sent to AOAC). A negative control sample (swab and wetting solution only) was processed per the kit instructions. Swab samples were placed in the shipping container and shipped overnight to Phylagen at ambient conditions immediately after sampling. Components used in the candidate method test plate sampling are listed in Table 13.

Reference method RT-qPCR testing. — Samples to be analyzed by the CDC reference method were transferred to an operator unaware 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 sample 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 14. Fractional positive results were seen with the 0.5 POD

**Table 8. CDC assay thermal cycling parameters**

| Stage | Temperature, °C | Time     | Cycles |
|-------|-----------------|----------|--------|
| 1     | 25              | 2 min    | 1      |
| 2     | 50              | 15 min   | 1      |
| 3     | 95              | 2 min    | 1      |
| 4     | 95              | 3 s      | 45     |
| 5     | 55              | 30 s     |        |

**Table 9. 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 10. 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/test area)* | Reference | 2 × 2" | 5 | 1.3 × 10⁵ | 150 | 2.0 × 10⁴ |
| Low (0.5 POD/test area) | Reference | 2 × 2" | 20 | 1.3 × 10⁴ | 150 | 2.0 × 10³ |
| 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 × 10⁵ | 150 | 2.0 × 10³ |
| Low (0.5 POD/test area) | Candidate | 2 × 2" | 20 | 1.3 × 10⁴ | 150 | 2.0 × 10³ |
| Negative VTM control (0 POD/test area) | Candidate | 2 × 2" | 5 | 0 | 150 | 0 |

*The POD is based on range-finding studies conducted with the reference method.

**Table 11. Components used for test plate inoculation**

| Component | Vendor/manufacturer | Part No. | Lot No. | Expiration |
|-----------|---------------------|----------|---------|------------|
| VTM       | MRI Global          | N/A      | 24Sep2020 | 9/24/2021  |

*N/A = Not available.

**Table 13. Components used for candidate method test plate sampling**

| Component         | Vendor/manufacturer | Part No.    | Lot No.     | Expiration |
|-------------------|---------------------|-------------|-------------|------------|
| VTM               | MRI Global          | N/A*        | 24Sep2020   | 9/24/2021  |
| Swabs             | Puritan             | 25–1607 1PFSC | 7168 | 3/1/2025  |

*N/A = Not available.
Results. — As per criteria outlined in Appendix J of the Official Methods of Analysis Manual (2), fractional positive results were obtained for the CDC reference method. The POD was calculated as the number of positive outcomes divided by the total number of trials. POD was calculated for the candidate presumptive results, PODC, and the reference method, PODR, as well as the difference in the candidate and reference methods, dPODC. The POD analysis between the Phylagen COVID-19 Indoor Test 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 as described in AOAC Appendix J policies. A summary of POD analyses is presented in Table 15. Individual results are provided in Table 16.

Discussion

Results from the POD analysis demonstrate that the COVID-19 Indoor Test by Phylagen is statistically better at detecting low concentrations \((2 \times 10^3 \text{ GU/}2 \times 2\text{ test surface})\) of deposited SARS-CoV-2 on a stainless-steel surface compared to the CDC reference method. In addition, all high concentration samples were positive and no false negatives were detected with the COVID-19 Indoor Test by Phylagen, at either concentration. These data support high analytical sensitivity for SARS-CoV-2. The high sensitivity may be attributed, in part, to viral degradation. Viruses deposited onto surfaces undergo some degradation due to environmental factors, such as heat and humidity. Lysis of viral particles due to degradation could make it more likely that a single target assay would have a higher probability of detecting one target than an assay that requires the detection of both targets to be interpreted as a positive result. The Phylagen COVID-19 Indoor Test employs a single detection of the N1 target; whereas the CDC reference method requires the detection of both N1 and N2 SARS-CoV-2 gene targets to be interpreted as a positive result, this difference resulted in more positive results for the COVID-19 Indoor Test than the CDC reference method.

Assay sensitivity and specificity was further explored and confirmed through in silico analysis for inclusivity and exclusivity against background organisms. It was demonstrated that the COVID-19 Indoor Test by Phylagen can accurately detect 15,745 genomes of the 15,766 that are part of the inclusivity dataset (99.88%) with up to two nucleotide mismatches on the forward and/or reverse primer. While mismatches against these genome sequences could be due to natural variation on the strains, they may be attributed to de novo sequence errors, given the low number of genomes with some mismatches to the primer sequences. Furthermore, none of the exclusivity viral targets were amplified by the primer set, and from the background set only four microbial genomes (Table 4) showed any possible amplification in silico. These are uncommon microorganisms not expected to be found on indoor surfaces tested for SARS-CoV-2. Additionally, in silico amplification required a large number of mismatches on both primers to proceed. This data suggests
Conclusions

The data from this study supports the product claim that the COVID-19 Indoor Test by Phylagen can detect SARS-CoV-2 from stainless-steel surface samples. Monitoring high risk surfaces for SARS-CoV-2 may signal the presence of an infected person in the facility before they would be identified through other means. Research suggests that many individuals with COVID-19 are asymptomatic or presymptomatic (8). Even if infected individuals ultimately develop symptoms, they may not realize they are sick for many days. The virus they shed will nonetheless accumulate on surfaces. The COVID-19 Indoor Test by Phylagen is superior at detecting SARS-CoV-2 from stainless-steel surfaces. Therefore, surface monitoring with COVID-19 Indoor Test by Phylagen can serve as an early sentinel of asymptomatic and presymptomatic transmission risk in the built environment: offices, schools, stores, restaurants, hotels, warehouses, factories, etc.

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Table 16. Individual results for the stainless-steel matrix study

| Sample No. | Phylagen COVID-19 indoor result | 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                           | 13/20                | 13/20                | 9/20             |

|            | High level                      |                      |                      |                  |
| 1          | +                               | +                    | +                    | +                |
| 2          | +                               | +                    | +                    | +                |
| 3          | +                               | +                    | +                    | +                |
| 4          | +                               | +                    | +                    | +                |
| 5          | +                               | +                    | +                    | +                |
| Total      | 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              |
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