Validation of the VIRSeek SARS-CoV-2 Mplex assay for Detection of SARS-CoV-2 on Stainless Steel surfaces: AOAC Performance Tested Method SM 122006

Laura Bleichner, Vera Bleicher, and Nadine Göhring
Eurofins GeneScan Technologies GmbH, Engesserstraße 4, D-79108 Freiburg im Breisgau, GERMANY

Steven B. Kleiboeker
Viracor Eurofins, 1001 NW Technology Drive, Lee’s Summit, MO 64086, USA

Corresponding author’s email: laurileichner@eurofins.com

Abstract

Background: The Eurofins GeneScan Technologies’ VIRSeek SARS-CoV-2 Mplex kit is a RT-qPCR assay for the detection of two targets on the N-gene (nucleocapsid) of SARS-CoV-2. An extraction control, that allows monitoring of the extraction procedure and PCR inhibition, is included. Objective: In-silico analysis and wet testing showed inclusivity and exclusivity of the assay. The complete workflow starting from surface swabbing (VIRSeek PATHOswab kit), RNA extraction (VIRSeek RNAExtractor), RT-PCR (VIRSeek SARS-CoV-2 Mplex) and evaluation with FastFinder was validated in comparison to the CDC method for detection of SARS-CoV-2 on stainless steel. Methods: In-silico analysis was performed by using the MFOLD online program. The matrix study was performed for stainless steel inoculated with SARS-CoV-2 isolated from the first documented US case of a traveler from Wuhan, China. Results: For inclusivity, 15,764 sequences were analyzed and all mismatches (0.37% of the sequences had single mismatches) were considered non-critical. Cross reactivity for closely related viruses and background organisms was performed, resulting in correct exclusion of all. No significant differences were observed for the POD study when comparing to the CDC method. Conclusions: Results of the inclusivity and exclusivity study show that the assay is specific for detection SARS-CoV-2. The POD study showed no statistically significant difference compared to the CDC reference method, results were identical for the uninoculated and the high level. For the fractional recovery level, the candidate method detected 9/17 samples leading to a POD of 0.47, the reference method detected 11/20 samples leading to a POD of 0.55.

General Information

Due to the SARS-CoV-2 pandemic of coronavirus disease 2019 (COVID-19) many customers have the need for environmental and food surface monitoring. Knowledge of the presence of SARS-CoV-2
genomic RNA would enable businesses, individuals and state agencies to take adequate decisions in regards to cleaning and decontamination as well as assessing the effectiveness of these procedures. Human-to-human transmission of SARS-CoV-2 has been confirmed during the 2019–20 coronavirus pandemic., with transmission occurring primarily via respiratory droplets (from coughs and sneezes). However, indirect contact via contaminated surfaces is another possible cause of infection.

The virus concentration and extraction of viral RNA isolated from surfaces (food as well as environmental surfaces) are performed as described in the protocol for food surfaces recommended by the ISO 15216 [1]. The swab surface area for environmental swabbing is 25 cm$^2$ as described in the protocol recommended by World Health Organization [2]. The RNA extraction is performed according to the protocol described in the VIRSeek RNAExtractor kit [3].

**Principle of the Method**

Eurofins GeneScan Technologies’ VIRSeek SARS-CoV-2 Mplex assay is a test kit for the qualitative detection of SARS-CoV-2 genomic RNA using a multiplex assay format by real-time RT-qPCR for environmental surface samples. The VIRSeek SARS-CoV-2 Mplex kit is based on reverse transcription and specific detection of two targets within the N-gene (nucleocapsid), N1 and N2 by PCR, which are both detected in the FAM$^\text{TM}$ channel.

The primer/probe combination of this PCR system is highly specific for SARS-CoV-2 and does not cross-react with SARS-CoV, MERS-CoV, or the seasonal human coronaviruses HKU1, OC43, NL63, 229E. Furthermore, cross-reactivity was experimentally excluded for various animal corona viruses, food-related viruses, typical background flora and several food matrices.

The VIRSeek SARS-CoV-2 Mplex kit provides all reagents for the rapid detection of the SARS-CoV-2 in environmental and food surfaces via real-time RT-qPCR. Furthermore, an optional extraction control in order to verify the extraction as well as potential PCR inhibition, is included.

The VIRSeek SARS-CoV-2 Mplex kit is validated for use with the Agilent AriaMx$^\text{TM}$, Bio-Rad CFX96 Touch$^\text{TM}$, CFX96 Touch$^\text{TM}$ Deep Well and the Applied Biosystems$^\text{TM}$ 7500 PCR Standard platforms. Each kit contains reagents for 96 reactions. Cycler running time is rapid at approx. 1 h for the Agilent AriaMx$^\text{TM}$ and the two Bio-Rad cylers. For the Applied Biosystems$^\text{TM}$ 7500 cycler the thermal protocol is approx. 1 h 45 min long [4].
Scope of Method

(a) Analyte(s).—SARS-CoV-2.

(b) Matrix.—Stainless steel (2” × 2”).

(c) Performance claim.—Performance comparable to the Center for Disease Control and Prevention (CDC) 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel (5) 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; PODₐ (reference method POD), PODₐₑ (confirmed candidate method POD), PODₐₑₚ (candidate method presumptive result POD) and PODₐₑₙ (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 (1).

(c) RT-PCR.—Reverse transcription polymerase chain reaction.

(d) Multiplex.—Use of polymerase chain reaction to amplify several different DNA sequences simultaneously (as performing many separate PCR reactions all together in one reaction).

(e) N1/N2.—Gene regions coding for the nucleocapsid protein.

Materials and Methods

Test Kit Information

(a) Kit name.—VIRSeek SARS-CoV-2 Mplex.

(b) Cat. No.—5728201101.

(c) Ordering information.—Eurofins GeneScan Technologies GmbH Engesserstr. 4, 79108 Freiburg, Germany, Phone: +49-(0)761-5038-200, Fax: +49-(0)761-5038-211, kits@eurofins.de, Kits can also be ordered over the web shop: https://www.eurofins-technologies.com/

Test Kit Components

(a) OligoMix.—2 vials with 159 µL primer / probe mix, each for 48 reactions.

For information regarding the primers and probes see Table 1.
(b) **BasicMix.**—2 vials with 265 µL enzyme mix, each for 48 reactions.

(c) **Negative control.**—1 vial with 500 µL VIRSeek SARS-CoV-2 negative control.

(d) **Positive control.**—1 vial with 250 µL VIRSeek SARS-CoV-2 positive control (containing both N1 and N2 target sequences).

(e) **Extraction control.**—2 vials with 1000 µL VIRSeek SARS-CoV-2 extraction control.

**Additional Supplies and Reagents**

(a) **VIRSeek RNAExtractor.**—For manual handling, Cat. No. 5524400101.

(b) **Disposable sampler.**—e.g., Nest Biotechnology, Cat. No. 202005.

(c) **PBS buffer.**—e.g., VWR, Cat. No. K812.

(d) **Ethanol absolute (≥99.7%).**—DNase-, RNase-free, e.g., VWR, Cat. No. SERA39556.02.

**Apparatus**

(a) **Agilent AriaMx™ (up to Software v. 1.5).**—Cat No. G8830A.

(b) **Bio-Rad CFX96 Touch™ Deep Well.**—CFX Manager™ Software v. 3.1/CFX Maestro™ Software v. 1.1.

(c) **Applied Biosystems™ 7500 Standard.**—7500 Software v2.3.

(d) **Bio-Rad CFX96 Touch™ Deep Well.**—Used for the validation.

**Safety Precautions**

Use the product only in accordance with the regional/national biosafety regulations. Use only RNase-free equipment, consumables, and chemicals. All samples should be handled with caution, ideally in a biosafety cabinet class II, as they are potentially infectious. Viruses should not be handled by pregnant women, children, elderly, and immuno-compromised individuals due to the high infection risk and potentially fatal health consequences for these groups, in particular for the unborn child in case of pregnant women.

The VIRSeek SARS-CoV-2 kit contains glycerol and propane-1,2-diol which may cause mild skin irritation. For more information, please refer to the VIRSeek SARS-CoV-2 kit safety information.

**General Preparation**
(a) Ensure the kit components are kept at \(-20 \pm 2^\circ C\) until usage. Each kit contains enough volume for 96 reactions. Remove only the volume of reagents required and re-freeze the remaining reagents.

(b) Ensure that the cooling block for pipetting the real-time RT-qPCR is kept at \(-20 \pm 2^\circ C\) until usage.

**Sample Preparation**

For information regarding the sample preparation please refer to the kit inserts for the VIRSeek RNAExtractor (3) for a manual extraction and VIRSeek RNAExtractor AE1 (6) for an automated extraction.

For the PCR setup, prepare the final reaction mix fresh each time and immediately before starting the RT-qPCR run. The required volume for the number of reactions are calculated, and the single components are pipetted together.

**PCR Setup**

(a) Place the PCR plate or strips into the 96-well cooling block which has been cooled to \(-20^\circ C\).

(b) Add 8 µL of final reaction mix to each test well.

(c) Add 12 µL Positive Control SARS-CoV-2, Negative Control, negative extraction control sample, negative sampling control and negative sampling device control to the corresponding wells.

(d) Add 12 µL of each sample to the corresponding reaction well of the PCR plate.

(e) Use optical caps or foil to seal the PCR plate/strips.

(f) Spin down the plate/strips in a centrifuge.

(g) Transfer the cooled PCR plate/strips to the RT-qPCR instrument and start the run according to the thermocycler’s instructions.

(h) Store samples at \(-20^\circ C\) or below in case PCR needs to be repeated.

**Agilent AriaMx™**

(a) **Verifying run parameters.**

1. Select “Plate Setup” on the right side of the interface under the header “Wells”.

2. Under “Add Dyes”, verify FAM™ and Cy5™ are selected for all 96 wells.

(b) **Verifying run thermal profile.**
(1) Select “Thermal Profile” under “Set Up” on the left side of the screen.

(2) Verify that the cycler profile reflects Table 2.

(3) Select “Run” on the “Thermal Profile” page near the top right of the screen.

(4) In the instrument explorer, select the correct cycler and click on “Send Config”. Save the experiment under the desired name and location. The run information has been sent to the instrument.

(5) To open the run, select the computer icon on the bottom right of the cycler screen.

(6) Click “Open Primed Experiment” on the pop-up and the run will open. Select “Sync Plate” if the message is displayed.

(7) Select “Thermal Profile” on the left side of the screen to initiate the run.

Bio-Rad CFX96 Touch™ Standard/Deep Well

(a) Verifying run thermal profile. — Verify that the sample volume is 20 µL and the thermal profile in the “Protocol” tab reflects Table 3.

(b) Verifying run parameters. —

(1) In the “Plate Editor”, verify that the plate type is “BR White”, “Scan Mode” is “All Channels” and FAM™ and Cy5™ are selected for all 96 wells.

(2) All samples should be defined as “Unknown”.

(3) Click “OK” to close the “Plate Editor” and select “Next” in the “Run Setup” tab.

(4) Select the proper cycler and click “Open Lid”. Load the plate into the cyclers and press “Close Lid” followed by “Start Run”.

(5) Choose the location the run will be saved to and select “Save”.

Applied Biosystems™ 7500 Standard

(a) Verifying run parameters. —
(1) Select “Create New Document” in the starter window.

(2) Under “Select detectors”, verify FAM™ and Cy5™ are selected for all 96 wells.

(3) Make sure that “ROX” is selected for the “passive reference” above the “Detectors” window.

(b) Verifying run thermal profile. —

(1) Select “Instrument”

(2) Verify that the cycler profile reflects Table 4.

(3) Select “Start”

(4) Save the experiment under the desired name and location. The run information has been sent to the instrument.

Analysis
(a) Open the FastFinder software and login using your username and password.

(b) Under the “Analysis” tab, select “Browse” and choose the location in which the file that will be analyzed is saved. On the “Start” page, under “To be Processed”, choose the file that will be analyzed.

(c) Verify the plate layout to make sure the proper name tags are corresponding with the wells. (”C+” for the positive kit control, “NTC” for the negative kit control, “S” for sample and “EC” for positive extraction control). Once they are correctly assigned, click “Next Step”.

(d) Select the assay lot for the assays assigned to the plate and then continue by clicking “Analyze”.

Result Interpretation
For information on how to interpret the results see Table 5.

Validation Study
This Emergency Response Validation study was conducted under the AOAC Research Institute Performance Tested Method™ (PTM) program and the AOAC INTERNATIONAL Methods Committee Guidelines for Validation of Microbiological Methods for Food and Environmental Surfaces (7). The in silico analysis was conducted by Eurofins GeneScan Technologies and Viracor Eurofins. The matrix study...
was conducted by the independent laboratory, MRI Global, with candidate method analyses on blind coded swabs performed by Eurofins EMLab P&K (Marlton, NJ). The data is submitted to fulfill the requirements for the Emergency Response Validation PTM certification. Robustness, product consistency, and product stability data will be submitted for full PTM status by March 31, 2021.

**Method Developer Studies**

**Inclusivity.**—Inclusivity was evaluated by *in silico* analysis using the provided SARS-CoV-2 sequences by AOAC “COVID accession for AOAC”. In total, 15,764 sequences were analyzed. Table 6 shows the number of sequences with mismatches in the N1 and N2 system (N1 and N2 system were considered separately).

**Exclusivity and background strains.**—Exclusivity and background organisms were evaluated by *in silico* analysis with BlastN using the organisms provided by AOAC – “exclusivity and background lists”. For each organism the taxon or strain ID and the accession number with the highest percent identity for the primer/probes of the N1 and N2 system are listed. The results of the in silico analysis of exclusivity organisms (closely related viruses) are shown in Table 7 (N1 system) and Table 8 (N2 system).

The results of the in silico analysis of background organisms (other viruses, bacteria, fungi and eukaryotes) are shown in Table 9 (N1 system) and Table 10 (N2 system).

**Detailed in silico analysis for unimolecular folding and hybridization.**—To perform the *in silico* analysis for unimolecular folding of the amplicon region of the N1 and N2 system (±150 bp) and the single primers and probes as well as for the 2 state bimolecular hybridization, the sequence from genome with the NCBI Accession MN908947.3 was used. All *in silico* analyses were performed with the free MFOLD online program (8).

In the VIRSeek SARS-CoV-2 Mplex assay a proprietary enzyme mix is used, therefore no information in regards to the exact salt concentration is available. For all predictions of DNA strands (unimolecular folding and hybridization), the salt conditions stated by AOAC (monovalent: 0.08 M, Mg$^{2+}$: 0.002 M) were used. For all predictions of RNA strands it was not possible to change the salt conditions and default settings were used.
**Unimolecular folding N1.—Prediction of the secondary structure of N1 reverse primer target RNA sequence.**—For the unimolecular folding of the N1 target RNA sequence the amplicon region ±150 bp was chosen for analysis (see Table 11). The reverse transcription temperature was set to 50°C (as used in the kit) and salt conditions were not adjustable and therefore presets with 1M Na+ and 0M Mg++ were used.

**Prediction of the secondary structure of N1 cDNA target strand (binding region forward primer) and N1 probe-binding DNA target strand (binding region probe).**—The probe and the forward primer bind to the antisense strand. As the annealing temperature and the extension temperature are the same in the VIRSeek SARS-CoV-2 Mplex assay, ∆G does not differ for the cDNA target strand and the Probe-binding DNA target strand.

For the unimolecular folding of N1 cDNA target strand and the N1 probe-binding DNA target strand the amplicon region ±150 bp was chosen for analysis (see Table 12). The annealing temperature was set to 60°C (as used in the kit) and salt conditions were set to 0.08M for monovalent ions and 0.002M for Mg++. For the unimolecular folding of N1 cDNA target strand and N1 probe-binding DNA target strand one structure was predicted with ∆G=-1.42 kcal/mol.

**Unimolecular folding N2.—Prediction of the secondary structure of N2 reverse primer target RNA sequence.**—For the unimolecular folding of the N2 target RNA sequence the amplicon region ±150 bp was chosen for analysis (see Table 13). The reverse transcription temperature was set (as in the kit) to 50°C and salt conditions were not adjustable and therefore presets with 1M Na+ and 0M Mg++ were used.

**Prediction of the secondary structure of N2 cDNA target strand (binding region forward primer) and probe-binding DNA target strand (binding region probe).**—The probe and the forward primer bind to the antisense strand. As the annealing temperature and the extension temperature are the same in the VIRSeek SARS-CoV-2 Mplex assay, ∆G does not differ for the cDNA target strand and the Probe-binding DNA target strand.

For the unimolecular folding of N2 cDNA target strand and the N2 probe-binding DNA target strand the amplicon region ±150 bp was chosen for analysis (see Table 14). The annealing temperature was set to 60°C (as used in the kit) and salt conditions were set to 0.08M for monovalent ions and 0.002M for
Mg++. For the unimolecular folding of N2 cDNA target strand and N2 probe-binding DNA target strand two structures were predicted with $\Delta G=-4.80$ kcal/mol and $\Delta G=-4.02$ kcal/mol, respectively.

**Primer and probe unimolecular folding.**—For each primer and probe of the N1 system (Table 15) and N2 system (Table 16) the secondary structure was predicted. The annealing temperature was set to 60°C and the salt conditions were set to 0.08 M for monovalent ions (Na$^+$) and to 0.002 M for Mg$^{2+}$. For all other parameters the default settings of the free MFOLD online program were used. Some of the Tm values are extremely high or low. Nevertheless, the complete analysis is reported here.

**Two-state bimolecular hybridization.**—The 2-state bimolecular hybridization for each primer/probe to the respective target strand was predicted using online DINAMelt software (9). The strand concentration for prediction of Tm was set to 0.01 mM (default settings). The salt conditions for DNA target strains were set to 0.08 M for monovalent ions to and to 0.002 M for Mg$^{2+}$. For the analysis of RNA target strands it was not possible to set the salt concentrations and therefore default settings were used. The temperature used for the analysis is listed in Table 17.

**Independent Laboratory Studies**

**Matrix study.**—**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 (6) and was sourced from the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA, Lot TVP23155). The virus stock was received from WRCEVA as a lyophilizate of a 1 mL aliquot containing $3.6 \times 10^6$ plaque-forming units. Upon receipt, the lyophilizate was suspended in 2 mL of Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12 medium) and single-use 50 µL aliquots were frozen at –70°C.

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 VR-3276SD). The qPCR procedure used the N1 primer and probe sequences published by the CDC. Primers and probes were purchased from Integrated DNA Technologies (IDT 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) RT-qPCR Diagnostic Panel Instructions for Use (5).

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/µL. 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. The master mix was prepared from 5.0 µL TaqPath™ 1-step RT-qPCR Master Mix, CG, 1.5 µL primer/probe mix, and 8.5 µL nuclease-free water per reaction. 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 analyzed 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-treated 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 cells.

**Test Plate Inoculation**

SARS-CoV-2 virus frozen stock was thawed and diluted in viral transport medium (VTM, prepared according to CDC instructions [https://www.cdc.gov/coronavirus/2019-ncov/downloads/Viral-Transport-Medium.pdf](https://www.cdc.gov/coronavirus/2019-ncov/downloads/Viral-Transport-Medium.pdf)) starting from two pooled virus stock aliquots to produce a low level concentration expected to yield fractional positive results by the CDC method and a high level at 10-fold the concentration of the low level.

Square 14” × 14” stainless steel plates (grade 304) were used for the studies to mimic food preparation surfaces. All test plates were cleaned, disinfected, 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, 150 µL of the low level, high level or negative control (VTM only) suspension were pipetted onto the
appropriate test area and spread evenly with a sterile 10 µL inoculating loop. Inoculated plates were left until visibly dry (up to 1 hour) in a biosafety cabinet, then transferred to a sealed plastic container and stored overnight at room temperature (20 hours). Temperature and humidity ranged from 21.7–25.2°C and 28–34% relative humidity during the plate inoculation and drying process.

**CDC Method Plate Sampling**

After drying overnight, the CDC method test areas were sampled as follows: A swab (Puritan 25-1607 1PFSC) was moistened by dipping into a 15 mL conical tube containing 2.0 mL of VTM. The 2” x 2” test area was swiped back and forth in two different directions 10x each while applying pressure to the surface and rotating the swab head. The swab was snapped at the break point and placed back into the VTM tube. A random sample ID was assigned to each swab. Swabs were placed in a refrigerator (2–8 °C) within 15 min of test area swiping and stored overnight (22 h) before nucleic acid extraction.

**VIRSeek Method Plate Sampling**

Test areas for the VIRSeek method were sampled using the VIRSeek PATHOSwab 50 Surface Sampling Kit. Briefly, the swab was moistened with 2-3 drops of VIRSeek Sample Acquisition Solution. The moistened swab was then used to swipe the 2” x 2” test area with a back-and-forth motion in at least two different directions while applying light pressure to the surface and rotating the swab head. After sampling the test area, the swab tip was cut off with sterile scissors and the tip was placed into a dry sample collection tube. Each sample tube was assigned a random ID number. Swab samples were shipped overnight to Eurofins EMLab P&K (Marlton, NJ) with a cool pack and temperature data logger on the day of sampling.

**CDC Method RT-PCR Testing**

Blind coded swabs were transferred to an operator unaware of the blinded sample identities for testing with the CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel. RNA was extracted from 140 µL of VTM sample using the Qiagen QIAamp Viral RNA Mini Kit per the manufacturer’s instructions. Extracted RNA was analyzed by the CDC Panel on an Applied Biosystems 7500 Fast Dx Real-Time PCR instrument following published instructions (5). CDC method test results were sent to AOAC for comparison with the VIRSeek Method.
Results.—While there is no true reference method for detection of SARS-CoV-2 on surfaces, the CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel for clinical diagnosis was modified for use with environmental swabs recommended by CDC based on internal data (personal communication to AOAC). This modified CDC method was used as the “reference” method for the purposes of this validation study.

Individual swab results are presented in Table 18. The candidate and reference methods yielded no positives at the non-inoculated control level, fractional positives at the low level, and all positives at the high level. There were 3 candidate method swabs at the low level that yielded invalid results even after dilution and retesting according to the package insert instructions. It was not possible to collect new swab samples for extraction and analysis and only valid data were used for the statistical analysis. After unblinding of the data, one result from the non-inoculated control level was positive. That extract was reanalyzed in replicates and found to be negative, indicating a potential cross-contamination occurred in the first analysis. A summary of results and statistical analysis is presented in Table 19. The probability of detection (POD) was calculated as the number of positive outcomes divided by the number of trials (10). POD and the 95% confidence interval were calculated for the candidate method, POD\(_C\), and the reference method, POD\(_R\). Bias was determined as the difference between the candidate and reference method POD values, dPOD\(_C\), with a 95% confidence interval based on an unpaired study design. The 95% confidence intervals on dPOD\(_C\) included zero, indicating that no statistically significant difference was detected between methods.

Discussion

Inclusivity

Both target regions - N1 and N2 - of the VIRSeek SARS-CoV-2 RT-qPCR assay are located in the N gene, which encodes for the nucleocapsid, of the SARS-CoV-2 genome. As both probes of the N1 and N2 system are labelled with the same fluorophore (FAM\(^\text{TM}\)), the amplification signals are detected in the same channel. For this reason, only the sequences with mismatches in both, the N1 and the N2 system, are considered for evaluation of inclusivity. An overview of the sequences with mismatches in both systems is given in Table 20.

Seven sequences were found with mismatches in the binding region of the N1 forward primer, which also had mismatches in the N2 system (all in the binding region of the N2 forward primer, none in the binding region of the N2 probe, or N2 reverse primer). 35 sequences were found with mismatches in
the binding region of the N1 probe, which had also mismatches in the N2 system (33 in the binding region of the N2 forward primer, one in the binding region of the N2 probe and one in the binding region of the N2 reverse primer). 16 sequences were found with mismatches in the binding region of the N1 reverse primer, which had also mismatches in the N2 system (all in the binding region of the N2 forward primer, none in the binding region of N2 probe or N2 reverse primer).

Taken together, 0.37% of the sequences had mismatches in both, the N1 and the N2 system. All sequences with mismatches in the N2 system had only single mismatches in the N1 system with one exception, where 2 mismatches were found in the binding region of the N1 reverse primer. This finding is considered uncritical, since the mismatches are not located at the 3’-end.

None of the other sequences with mismatches in the N2 system had mismatches at the first nucleotide of the 3’ binding region of the N1 forward/reverse primer or probe.

Thus, all mismatches are considered non-critical and 100% inclusivity is expected.

Exclusivity

Each primer and probe was checked for cross reactivity (Table 7–10) of closely related viruses and background organisms (viruses, bacteria, fungi, eukaryotes). Some sequences of background organisms showed more than 80% identity for individual primers or probes, but there was no potential cross reactivity identified in full primer/probe sets.

Cross reactivity was tested experimentally for all sequences with more than 80% identity in a PCR-assay with no detectable amplification signals for background organisms (Table 18). Two organisms, Drosophila and *Torulopsis glabrata*, with more than 80% identity for individual primers and probes were not tested in a PCR. As for Drosophila the N1 reverse primer and N1 probe have only 59.3 and 66.7% identity and for *Torulopsis glabrata* the N2 forward and reverse primer have only 62.6 and 65.3% identity, no amplification is expected for both of them.

In addition, three common respiratory coronaviruses (strains 229E, NL63, and OC43) and DNA templates corresponding to N gene sequences of SARS (29034 – 29233 and 28669 – 28868 of NC_004718.3) were tested experimentally with no detectable amplification signal (Table 21).

Matrix Study
The VIRSeek SARS-CoV-2 Mplex kit successfully detected 100% of the high-level inoculated surfaces and correctly showed all non-inoculated surfaces to be negative. One of five samples in the non-inoculated level was found to be positive after unblinding the data. The reanalysis, which was performed in replicates, was found to be negative, indicating that a cross-contamination potentially occurred in the first test. In the fractional recovery level, 8 samples were tested positive with the VIRSeek SARS-CoV-2 method and three samples remained invalid, even after dilution. The outcome of those three samples could be either positive or negative. According to the kit insert if inhibition remains, the sampling of the surface should be repeated. As this was not possible within this study, the results were not considered during statistical evaluation. The reference method detected 11 samples positive for N1 and 19 samples positive for N2, final results are only rated positive if both targets are positive. Therefore, the final result of the reference method in the fractional positive level is 11, so three more positives compared to the alternative method, which is not statistically significant. Potentially the three invalid results are exactly making the difference here, but as discussed, this cannot be finally proven. However, it is somehow remarkably that the difference between N1 and N2 positive samples in the reference method is that big, especially since all surfaces were thoroughly cleaned prior to inoculation with the SARS-CoV-2 isolate. Since only results which are positive in both targets are considered to be positive this has no influence on the final results.

Conclusions

Results from the in silico analysis for inclusivity, exclusivity and background organisms demonstrated that the VIRSeek SARS-CoV-2 test kit can accurately detect the 15,764 inclusivity sequences and correctly exclude all non-target organisms.

The data from the matrix study, within their statistical uncertainty, support the product claims of the VIRSeek SARS-CoV-2 kit on stainless steel surfaces sampled with the VIRSeek PATHOswab, extracted with the VIRSeek RNAExtractor and evaluated with FastFinder. The results obtained by the POD analysis of the method comparison study demonstrated that there were no statistically significant differences between the number of positive samples detected by the VIRSeek SARS-CoV-2 kit compared to the CDC reference method. However, it should be mentioned that there is no direct comparison between the candidate and reference method possible, since they are using different logic for final result reporting. The reference method requires two targets to be positive individually, the final result is positive only
when both individual target results are positive. The candidate method also detects two targets, but there is no discrimination of them possible since they are both detected in the same channel.

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Submitting Company: Eurofins GeneScan Technologies GmbH, Engesserstraße 4, D-79108, Freiburg im Breisgau, Baden-Württemberg, Germany

Supporting in-silico analysis: Steven B. Kleiboeker, Viracor Eurofins, 1001 NW Technology Drive, Lee’s Summit, MO 64086, USA

Independent Laboratory: MRI Global, 425 Volker Boulevard, Kansas City, MO, 64110

Contract Laboratory: Eurofins EMLab P&K, 3000 Lincoln Drive E, Suite A, Marlton, NJ 08053

Reviewers: William Burkhardt, FDA CFSAN; John SantaLucia, Wayne State University; Chengzhu Liang, Qingdao Customs District

AOAC Technical Consultant: Sharon Brunelle, Corvallis, OR

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(4) Kit insert VIRSeek SARS-CoV-2 Mplex, Version 1

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### Tables

#### Table 1. VIRSeek SARS-CoV-2 Mplex N1 and N2 primers and probes

| Primer/Probe         | Name                                           |
|----------------------|------------------------------------------------|
| N1 Forward Primer    | SARS_COV-2_N1_F1_EF (N1F1)                     |
| N1 Reverse Primer    | SARS_COV-2_N1_R1_EF (N1R1)                     |
| N1 Probe             | SARS_COV-2_N1_P1P_EF:FAM:BHQ1 (N1P1)           |
| N2 Forward Primer    | SARS_COV-2_N2_F1_EF (N2F1)                     |
| N2 Reverse Primer    | SARS_COV-2_N2_R1_EF (N2R1)                     |
| N2 Probe             | SARS_COV-2_N2_P1P_EF:FAM:BHQ1 (N2P1)           |

#### Table 2. Thermal Profile for the VIRSeek SARS-CoV-2 kit on the Agilent AriaMx™

| Step | Stage                                           | T (°C) | Time (min:sec) | Data collection | Cycles |
|------|-------------------------------------------------|--------|----------------|-----------------|--------|
| 1    | Reverse Transcription                           | 50     | 10:00          | no              | 1x     |
| 2    | Initial Enzyme Activation/ Reverse Transcriptase Inactivation | 95     | 3:00           | no              | 1x     |
| 3    | Denaturation                                    | 95     | 00:10          | no              | 40x    |
| 4    | Annealing & Extension                           | 60     | 00:30          | yes             |        |

#### Table 3. Thermal Profile for the VIRSeek SARS-CoV-2 kit on the Bio-Rad CFX96 Touch™ Standard / Deep Well

| Step | Stage                                           | T (°C) | Time (min:sec) | Data collection | Cycles |
|------|-------------------------------------------------|--------|----------------|-----------------|--------|
| 1    | Reverse Transcription                           | 50     | 10:00          | no              | 1x     |
| 2    | Initial Enzyme Activation/ Reverse Transcriptase Inactivation | 95     | 3:00           | no              | 1x     |
| 3    | Denaturation                                    | 95     | 00:10          | no              | 39x    |
| 4    | Annealing & Extension                           | 60     | 00:30          | yes             |        |
Table 4. Thermal Profile for the VIRSeek SARS-CoV-2 kit on the Applied Biosystems™ 7500 Standard

| Step | Stage                                                                 | T (°C) | Time (min:sec) | Data collection | Cycles |
|------|------------------------------------------------------------------------|--------|----------------|----------------|--------|
| 1    | Reverse Transcription                                                  | 50     | 20:00          | no             | 1x     |
| 2    | Initial Enzyme Activation/ Reverse Transcriptase Inactivation         | 95     | 10:00          | no             | 1x     |
| 3    | Denaturation                                                          | 95     | 00:10          | no             | 40x    |
| 4    | Annealing & Extension                                                 | 60     | 01:00          | yes            |        |
Table 5. Final result interpretation for qualitative screening assay RT-qPCR system (including Extraction control MS2 phage)

| Target Result       | Extraction control | Final results                | Result interpretation                                                                 | Next Steps                                                                 |
|---------------------|--------------------|------------------------------|--------------------------------------------------------------------------------------|---------------------------------------------------------------------------|
| Positive for N1/N2  | Not inhibited/inhibited | Positive for N1/N2 gene     | Specific SARS-CoV-2 N1/N2 gene target RNA detected.                                  | n/a                                                                       |
| Negative for N1/N2  | Not inhibited      | Negative for N1/N2 gene      | No detection of SARS-CoV-2 N1/N2 gene target RNA. Sample does not contain detectable amounts of specific N1/N2 target RNA. | n/a                                                                       |
| Negative for N1/N2  | Inhibited          | Inhibited real-time RT-PCR / Extraction efficiency of MS2 too low | No evaluation                                                                      | Test 1:10 dilution of RNA extract of undiluted sample. As option: test also 1:5 dilution of RNA extract of undiluted sample¹. If 1:10 dilution is still inhibited, repeat RNA extraction of the sample. For using the option: If both dilutions (1:10 and 1:5) are still inhibited, repeat RNA extraction of the sample. | ¹If only 1:10 dilution is still inhibited, use the result of 1:5 dilution. |
Table 6. *In silico* analysis of inclusivity for primer and probes of the N1 and N2 system

| N1-SYSTEM          | Number of sequences with mismatches (analyzed sequences: 15,764) |
|--------------------|---------------------------------------------------------------|
| N1 F1              | 30 ± 0.19%                                                    |
| N1 R1              | 64 ± 0.41%                                                    |
| N1 P1              | 74 ± 0.47%                                                    |

| N2-SYSTEM          | Number of sequences with mismatches (analyzed sequences: 15,764) |
|--------------------|---------------------------------------------------------------|
| N2 F1              | 3814 ± 24.19%                                                |
| N2 R1              | 32 ± 0.20%                                                    |
| N2 P1              | 41 ± 0.26%                                                    |

Table 7. Exclusivity N1 system

| Organism                        | Taxon ID/Strain | N1 F1 | N1 P1 | N1 R1 |
|---------------------------------|-----------------|-------|-------|-------|
| Human coronavirus 229 E         | 111337          | 65.0% | 51.9% | 59.3% |
|                                 | %Ident          | Acc. No |       |       |
|                                 | %Ident          | KT253264.1 | KT253271.1 | KT253270.1 |
|                                 | Acc. No         | N/A |       |       |
|                                 | Acc. No         | N/A |       |       |
|                                 | %Ident          | N/A |       |       |
|                                 | Acc. No         | N/A |       |       |
|                                 | %Ident          | N/A |       |       |
|                                 | Acc. No         | N/A |       |       |
| Human coronavirus OC43          | 31631           | 63.0% | N/A | N/A |
|                                 | %Ident          | Acc. No |       |       |
|                                 | %Ident          | N/A |       |       |
|                                 | Acc. No         | N/A |       |       |
|                                 | %Ident          | N/A |       |       |
|                                 | Acc. No         | N/A |       |       |
| Human coronavirus NL631         | 277944          | 66.7% | N/A | N/A |
|                                 | %Ident          | Acc. No |       |       |
|                                 | %Ident          | N/A |       |       |
|                                 | Acc. No         | N/A |       |       |
|                                 | %Ident          | N/A |       |       |
|                                 | Acc. No         | N/A |       |       |
| Human coronavirus HKU1          | 290028          | 51.9% | 59.3% | N/A |
|                                 | %Ident          | Acc. No |       |       |
|                                 | %Ident          | N/A |       |       |
|                                 | Acc. No         | N/A |       |       |
|                                 | %Ident          | N/A |       |       |
|                                 | Acc. No         | N/A |       |       |
| SARS-coronavirus                | 694009          | 100%  | 59.3% | 89.9% |
|                                 | %Ident          | Acc. No |       |       |
|                                 | %Ident          | Ay297028.1 | Kj473811.1 | Ky352407.1 |
|                                 | Acc. No         | N/A |       |       |
|                                 | Acc. No         | N/A |       |       |
|                                 | %Ident          | N/A |       |       |
|                                 | Acc. No         | N/A |       |       |
| MERS-coronavirus                | 1335626         | 75.0% | N/A | 59.3% |
|                                 | %Ident          | Acc. No |       |       |
|                                 | %Ident          | Kj473821.1 | N/A |       |
|                                 | Acc. No         | N/A |       |       |
|                                 | %Ident          | N/A |       |       |
|                                 | Acc. No         | N/A |       |       |
| Porcine delta coronavirus       | CH/JXJGSO1/2016 | 45.00% | 44.45% | 44.45% |
|                                 | %Ident          | Acc. No |       |       |
|                                 | %Ident          | Ky293677.1 | Ky293677.1 | Ky293677.1 |
|                                 | Acc. No         | N/A |       |       |
|                                 | Acc. No         | N/A |       |       |
|                                 | %Ident          | N/A |       |       |
|                                 | Acc. No         | N/A |       |       |

1 Analysis Viracor Eurofins
2 Analysis Eurofins GeneScan Technologies GmbH

%Ident - Percent Identity of primer/probe identity, Acc. No - Accession number NCBI, N/A - Not Applicable

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Table 8. Exclusivity N2 system

| Organism                          | Taxon ID/Strain | N2 F1 | N2 P1  | N2 R1  |
|-----------------------------------|-----------------|-------|--------|--------|
| Human coronavirus 229 E           | 111337          | N/A   | 50.0%  | 56.5%  |
|                                   |                 |       | KT253271.1 | KT253272.1 |
| Human coronavirus OC43            | 31631           | N/A   | N/A    | N/A    |
|                                   |                 |       |         |        |
| Human coronavirus NL63            | 277944          | N/A   | N/A    | N/A    |
|                                   |                 |       |         |        |
| Human coronavirus HKU1            | 290028          | N/A   | N/A    | N/A    |
|                                   |                 |       |         |        |
| SARS-coronavirus                 | 694009          | 91.7% | N/A    | 95.7%  |
|                                   |                 |       | AY297028.q | AY297028.1 |
| MERS-coronavirus                 | 1335626         | N/A   | N/A    | N/A    |
| Porcine delta coronavirus         | CH/JXJGS01/2016 | 37.53%| 45.50% | 47.85% |

1 Analysis Viracor Eurofins
2 Analysis Eurofins GeneScan Technologies GmbH
%Ident - Percent Identity of primer/probe identity, Acc. No - Accession number NCBI, N/A - Not Applicable
Table 9. Background viruses, bacteria and Eukaryotes N1 system

| Organism                     | Taxon ID/Strain | N1 F1  | N1 P1  | N1 R1  |
|------------------------------|-----------------|--------|--------|--------|
| Influenza A H1N1<sup>2</sup> | 114727          | 60.0%  | 44.7%  | 59.3%  |
| Acc. No                      |                 | KF8404 | CY2383 | CY0853 |
|                             |                 | 67.1   | 12.1   | 33.2   |
| Influenza A H3N2<sup>2</sup> | 119210          | 55.0%  | 44.5%  | 48.2%  |
| Acc. No                      |                 | MG784  | KT8391 | MF872  |
|                             |                 | 576.1  | 18.1   | 826.1  |
| Influenza A H5N1<sup>2</sup> | 102793          | 55.0%  | 48.2%  | 48.2%  |
| Acc. No                      |                 | MG664  | JX5345 | KP7429 |
|                             |                 | 611.1  | 75.1   | 11.1   |
| Influenza A H7N9<sup>2</sup> | H7N9 333278     | 55.0%  | 37.0%  | 40.7%  |
| Acc. No                      |                 | CY1860 | MF629  | KY2381 |
|                             |                 | 06.1   | 948.1  | 28.1   |
| Influenza B<sup>1</sup>      | 11520           | 65.0%  | 59.3%  | 59.3%  |
| Acc. No                      |                 | NC_002 | NC_002 | NC_002 |
|                             |                 | 206.1  | 211.1  | 205.1  |
| Human adenovirus, type 1<sup>2</sup> | 10533 (Ad71 not found) | 50.0%  | 48.2%  | 33.3%  |
| Acc. No                      |                 | MH183  | MH183  | MH132  |
|                             |                 | 293.1  | 293.1  | 93.1   |
| Human metapneumovirus<sup>1</sup> | 162145          | 65.0%  | 55.6%  | 59.3%  |
| Acc. No                      |                 | KJ62739| AY5258 | KJ62739|
|                             |                 | 7.1    | 43.1   | 83.1   |
| Respiratory syncytial       | 208893 (Long A not found) | 50.0%  | 40.7%  | 40.7%  |
| virus Long A<sup>2</sup>     |                 | MT4222 | MH181  | MK306  |
|                             |                 | 73.1   | 903.1  | 327.1  |
| Rhinovirus<sup>2</sup>       | 12059           | 65.0%  | 48.2%  | 51.9%  |
| Acc. No                      |                 | MK993  | MK593  | MF160  |
|                             |                 | 376.1  | 172.1  | 542.1  |
| Parainfluenza 1 C35<sup>2</sup> | 31605           | N.A    | N.A    | N.A    |
| Acc. No                      |                 | N.A    | N.A    | N.A    |
| Parainfluenza 2 Greer<sup>2</sup> | 11213           | N.A    | N.A    | N.A    |
| Organism               | Taxon ID/Strain                                                                 | Acc. No | N1 F1 | N1 P1 | N1 R1 |
|-----------------------|---------------------------------------------------------------------------------|---------|-------|-------|-------|
| **Parainfluenza 3** 1 | C-43 not found 11216 used instead (complete parainfluenza 3)                   |         | 60.0% | N/A   | 66.7% |
|                       |                                                                                  | KM190 938.1 | N/A   |       | KY9735 56.1 |
| **Parainfluenza 4** 1 | M-25 not found 1979161 used instead (complete parainfluenza 4)                 |         | 60.0% | 44.4% | 66.7% |
|                       |                                                                                  | NC_021 928.1 | MH892 407.1 |       | KY4605 15.1 |
| **Enterovirus EV68** 2| EV-D68 found 42789                                                               |         | 55.0% | 40.7% | 51.9% |
|                       |                                                                                  | MN240 500.1 | MN245 446.1 |       | MK419 043.1 |
| **Human bocavirus** 2 | 329641                                                                          |         | 45.0% | 40.7% | 37.0% |
|                       |                                                                                  | MT4092 20.1 | GQ8910 87.1 |       | MH574 945.1 |
| **Varicella-zoster virus** 2 | 10335                                                                           |         | 50.0% | 40.7% | 40.7% |
|                       |                                                                                  | MH709 377.1 | MH709 377.1 |       | MH709 377.1 |
| **Norovirus** 2       | 142786                                                                          |         | 70.0% | 51.9% | 48.2% |
|                       |                                                                                  | MN922 718.1 | KC2121 56.1 |       | MT372 476.1 |
| **Herpes virus** 2    | Herpesviridae 10292                                                             |         | N/A   | 59.3% | 59.3% |
|                       |                                                                                  | DQ1980 83.1 | KR2972 53.1 |       | LC0648 08.1 |
| **Avian Influenza** 2 | H1N1 subtype found 114727                                                         | See above |       |       |       |
|                       |                                                                                  |         |       |       |       |
| **Avian Influenza** 2 | H4N1 subtype found 282148                                                          |         | 50.0% | 37.0% | 40.7% |
|                       |                                                                                  | KT3383 67.1 | JX8786 77.1 |       | LC3493 01.2 |
| **Avian Influenza** 2 | H6N1 subtype found 119212                                                          |         | 55.0% | 37.0% | 40.7% |
|                       |                                                                                  | CY1808 81.1 | CY1786 35.1 |       | MG063 432.1 |
| Organism                                           | Taxon ID/Strain                      | N1 F1  | N1 P1  | N1 R1  |
|----------------------------------------------------|-------------------------------------|--------|--------|--------|
| Avian Influenza                                   | H9N1 subtype found 147762           | 55.0%  | 37.0%  | 40.7%  |
| Avian infectious bronchitis virus                  | 11120                               | 60.0%  | 40.7%  | 51.9%  |
| Bovine coronavirus                                | 11128                               | 45.0%  | 44.5%  | 40.7%  |
| Mouse hepatitis                                   | 11138                               | 55.0%  | 48.2%  | 40.7%  |
| Porcine transmissible gastroenteritis virus       | 11149                               | 45.0%  | 37.0%  | 48.2%  |
| Acinetobacter Spp                                | 469                                 | 75.0%  | 51.9%  | 74.1%  |
| Bacillus thuringiensis                            | 1428                                | 70.0%  | 48.2%  | 66.7%  |
| Bacillus cereus                                   | 1396 (not only spores)              | 70.0%  | 51.9%  | 66.7%  |
| Bordetella pertussis                              | 520                                 | N/A    | N/A    | N/A    |
| Candida albicans                                  | 5476                                | 60.0%  | N/A    | 59.3%  |
| Chlamydia pneumonia                               | 83558                               | N/A    | N/A    | 51.9%  |
| Organism                        | Taxon ID/Strain | N1 F1 %Ident | N1 P1 %Ident | N1 R1 %Ident |
|--------------------------------|----------------|--------------|--------------|--------------|
| **Clostridium difficile**      | 1496           | 65.0%        | 48.2%        | 48.2%        |
|                                |                | LK9330 95.1  | CP0295 66.1  | CP0354 99.1  |
| **Escherichia coli**           | 83333          | 70.0%        | 63.0%        | 48.2%        |
|                                |                | CP0471 27.1  | CP0471 27.1  | CP0471 27.1  |
| **Enterococcus**               | 1350           | 75.0%        | 51.9%        | 59.3%        |
|                                |                | LR6073 77.1  | CP0461 23.1  | CP0552 32.1  |
| **Haemophilus influenza**      | 727            | N/A          | N/A          | 74.1%        |
|                                |                | N/A          | N/A          | CP0316 89.1  |
| **Klebsiella pneumonia**       | 573            | 75.0%        | 48.2%        | 55.6%        |
|                                |                | CP0300 70.1  | MN543 585.1  | CP0183 06.1  |
| **Legionella pneumophila**     | 446            | 85.0%        | 48.2%        | 70.4%        |
|                                |                | CP0459 74.1  | CP0459 74.1  | LR1343 80.1  |
| **Listeria monocytogenes**     | 1639           | 65.0%        | 48.2%        | 59.3%        |
|                                |                | CP0548 46.1  | CP0142 52.2  | CP0536 31.1  |
| **Mycobacterium tuberculosis** | 1773           | N/A          | N/A          | N/A          |
|                                |                | N/A          | N/A          | N/A          |
| **Mycoplasma pneumonia**       | 2104           | N/A          | N/A          | N/A          |
|                                |                | N/A          | N/A          | N/A          |
| **Pneumocystis jirovecii (PJP)**| 42068         | 60.0%        | N/A          | 59.3%        |
|                                |                | NC_002 653.1 | N/A          | NC_002 653.1 |
| **Pseudomonas aeruginosa**     | 287            | 80.0%        | 66.7%        | N/A          |
| Organism                        | Taxon ID/Strain | N1 F1     | N1 P1     | N1 R1     |
|--------------------------------|----------------|-----------|-----------|-----------|
| Staphylococcus aureus          | 1280           | NZ_CP0 40684.1 | NZ_CP0 27174.1 | N/A       |
| Staphylococcus epidermidis     | 1282           | N/A       | N/A       | N/A       |
| Streptococcus pyogenes         | 1314           | N/A       | N/A       | N/A       |
| Streptococcus pneumonia        | 1313           | 80.0%     | N/A       | N/A       |
| Streptococcus salivarius       | 1304           | 70.0%     | 62.9%     | N/A       |
| Torulopsis glabrata            | 5478           | 75.0%     | 48.2%     | 59.3%     |
| Homo sapiens                   | 9606           | N/A       | 63.0%     | 63.0%     |
| Aedes aegypti (mosquito)       | 7159           | 65.0%     | 48.2%     | 55.6%     |
| Aedes albopictus (mosquito)    | 7160           | 70.0%     | 51.9%     | 66.7%     |
| Dermatophagoides               | 6956           | 70.0%     | 51.9%     | 63.0%     |
| Organism                      | Taxon ID/Strain | N1 F1  | N1 P1  | N1 R1  |
|------------------------------|----------------|--------|--------|--------|
| *ptero*nyssinus* (dust mite) 2* | Acc. No        | XM_02 735053 8.1 | XM_02 735021 0.1 | XM_02 734229 8.1 |
| *Xenopsylla cheopis* (flea) 2 | %Ident 45.0%   | 163159 | 40.7%  | 37.0%  |
| *Drosophila* (fruit fly) 2    | %Ident 90.00%  | 7215   | 59.3%  | 66.7%  |
| *Musca domestica* (house fly) 2 | %Ident 75.0%   | 7370   | 51.9%  | 70.4%  |
| *Cercopithecus* 2             | %Ident 75.0%   | 9533   | 40.7%  | 44.5%  |

1Analysis Viracor Eurofins
2Analysis Eurofins GeneScan Technologies GmbH
%Ident - Percent Identity of primer/probe identity, Acc. No - Accession number NCBI, N/A - Not Applicable
Table 10. Background viruses, bacteria and Eukaryotes N2 system

| Organism                        | Taxon ID | N2 F1   | N2 P1   | N2 R1   |
|---------------------------------|----------|---------|---------|---------|
| Influenza A H1N1               | 114727   | 58.4%   | 50.1%   | 56.6%   |
|                                 |          | CY147371.1 | KF637780.1 | MF098856.1 |
| Influenza A H3N2               | 119210   | 58.4%   | 50.1%   | 52.2%   |
|                                 |          | KF888183.1 | CY246653.1 | CY259529.1 |
| Influenza A H5N1               | 102793   | 62.6%   | 50.1%   | 69.6%   |
|                                 |          | GU182155.1 | KY635788.1 | JQ520362.1 |
| Influenza A H7N9               | 333278   | 41.7%   | 45.5%   | 56.6%   |
|                                 |          | KY238129.1 | MG57560.6 | MG575584.1 |
| Influenza B                    | 11520    | 58.3%   | 59.1%   | 60.9%   |
|                                 |          | NC_00220.7 | NC_00220.5 | NC_002211.1 |
| Human adenovirus, type 1       | 10533    | 37.5%   | 45.5%   | 47.9%   |
| (Ad71 not found)               |          | MH18329.3 | MH18329.3 | MH183293.1 |
| Human metapneumovirus          | 162145   | N/A     | N/A     | 56.5%   |
|                                 |          | N/A     | N/A     | AF371337.2 |
| Respiratory syncytial virus    | 208893   | 58.4%   | 41.0%   | 52.2%   |
| Long A                         |          | MT42227.3 | MT42227.3 | MH181965.1 |
| Rhinovirus                     | 12059    | 58.4%   | 59.2%   | 82.7%   |
|                                 |          | MN81220.0 | JX560527.1 | AB647319.1 |
| Parainfluenza 1 C35            | 31605    | N/A     | N/A     | N/A     |
|                                 |          | N/A     | N/A     | N/A     |
| Parainfluenza 2 Greer          | 11213    | N/A     | N/A     | N/A     |
|                                 |          | N/A     | N/A     | N/A     |
| Parainfluenza 3 C-43           | C-43 not found | N/A | N/A | 69.6% |
|                                 |          | N/A | N/A | MH678682.1 |
| Parainfluenza 4 M-25           | M-25 not found | N/A | N/A | 47.8% |
|                                 |          | N/A | N/A | KF483663.1 |
| Organism                      | Taxon ID                  | N2 F1  | N2 P1  | N2 R1  |
|------------------------------|---------------------------|--------|--------|--------|
| Enterovirus EV68^1           | EV-D68 found 42789        | 45.9%  | 63.7%  | 47.9%  |
| Human bocavirus^2            | 329641                    | 41.7%  | 41.0%  | 47.9%  |
| Varicella-zoster virus^2      | 10335                     | 45.9%  | 54.6%  | 43.5%  |
| Norovirus^2                  | 142786                    | 62.6%  | 59.2%  | 52.2%  |
| Herpes virus^2               | Herpesviridae 10292       | 66.7%  | 59.2%  | 82.7%  |
| Avian influenza^2            | H1N1 subtype found 114727 | See above |        |        |
| Avian influenza^2            | H4N1 subtype found 282148 | 41.7%  | 50.1%  | 56.6%  |
| Avian influenza^2            | H6N1 subtype found 119212 | 54.2%  | 45.5%  | 52.2%  |
| Avian influenza^2            | H9N1 subtype found 147762 | 41.7%  | 45.5%  | 43.5%  |
| Avian infectious bronchitis virus^2 | 11120                     | 50.0%  | 50.1%  | 65.3%  |
| Bovine coronavirus^2         | 11128                     | 41.7%  | 45.5%  | 43.5%  |
| Mouse hepatitis^2            | 11138                     | 41.7%  | 54.6%  | 43.5%  |
| Porcine transmissible gastroenteritis virus^2 | 11149                     | 45.9%  | 41.0%  | 56.6%  |
| Acinetobacter spp^2          | 469                       | 62.6%  | 72.8%  | 69.6%  |
| Bacillus                     | 1428                      | 58.4%  | 63.7%  | 60.9%  |
| Organism                          | Taxon ID | N2 F1             | N2 P1             | N2 R1             |
|----------------------------------|----------|-------------------|-------------------|-------------------|
| *thuringiensis*                  |          | CP053971.1        | CP053969.1        | CP054568.1        |
| *Bacillus cereus*                | 1396     | N/A               | N/A               | N/A               |
| (not only spores)                |          | CP029454.1        | KF240854.1        | CP053997.1        |
| *Bordetella pertussis*           | 520      | N/A               | N/A               | N/A               |
|                                  |          | CP054568.1        | CP053997.1        | CP053968.1        |
| *Candida albicans*               | 5476     | N/A               | N/A               | N/A               |
|                                  |          | CP054568.1        | CP053997.1        | CP053968.1        |
| *Chlamydia pneumonia*            | 83558    | N/A               | N/A               | N/A               |
|                                  |          | CP054568.1        | CP053997.1        | CP053968.1        |
| *Clostridium difficile*          | 1496     | N/A               | N/A               | N/A               |
|                                  |          | CP054568.1        | CP053997.1        | CP053968.1        |
| *Escherichia coli*               | 83333    | N/A               | N/A               | N/A               |
|                                  |          | CP054568.1        | CP053997.1        | CP053968.1        |
| *Enterococcus*                   | 1350     | N/A               | N/A               | N/A               |
|                                  |          | CP054568.1        | CP053997.1        | CP053968.1        |
| *Haemophilus influenza*          | 727      | N/A               | N/A               | N/A               |
|                                  |          | CP054568.1        | CP053997.1        | CP053968.1        |
| *Klebsiella pneumonia*           | 573      | N/A               | N/A               | N/A               |
|                                  |          | CP054568.1        | CP053997.1        | CP053968.1        |
| *Legionella pneumophila*         | 446      | N/A               | N/A               | N/A               |
|                                  |          | CP054568.1        | CP053997.1        | CP053968.1        |
| *Listeria monocytogenes*         | 1639     | N/A               | N/A               | N/A               |
|                                  |          | CP054568.1        | CP053997.1        | CP053968.1        |
| *Mycobacterium tuberculosis*     | 1773     | N/A               | N/A               | N/A               |
|                                  |          | CP054568.1        | CP053997.1        | CP053968.1        |
| *Mycoplasma pneumoniae*          | 2104     | N/A               | N/A               | N/A               |
|                                  |          | CP054568.1        | CP053997.1        | CP053968.1        |
| *Pneumocystis jirovecii (PJP)*   | 42068    | N/A               | N/A               | N/A               |
|                                  |          | CP054568.1        | CP053997.1        | CP053968.1        |
| *Pseudomonas aeruginosa*         | 287      | N/A               | N/A               | N/A               |
|                                  |          | CP054568.1        | CP053997.1        | CP053968.1        |
| Organism                  | Taxon ID | N2 F1 %Ident | N2 P1 %Ident | N2 R1 %Ident |
|---------------------------|----------|--------------|--------------|--------------|
| Staphylococcus aureus     | 1280     | 54.2%        | 72.8%        | 65.3%        |
|                           |          | AP023034.1   | CP047857.1   | LR134088.1   |
| Staphylococcus epidermidis| 1282     | 66.7%        | N/A          | N/A          |
|                           |          | NZ_CP018842.1| N/A          | N/A          |
| Streptococcus pyogenes    | 1314     | N/A          | 81.8%        | N/A          |
|                           |          | N/A          | AE009949.1   | N/A          |
| Streptococcus pneumonia   | 1313     | N/A          | 72.7%        | N/A          |
|                           |          | N/A          | CP001845.1   | N/A          |
| Streptococcus salivarius  | 1304     | 62.6%        | 77.3%        | 82.6%        |
|                           |          | NZ_CP018189.1| NZ_CP020451.2| NZ_CP020451.2|
| Torulopsis glabrata       | 5478     | 62.6%        | 81.9%        | 65.3%        |
|                           |          | CP048118.1   | CP048123.1   | CP048124.1   |
| Homo sapiens              | 9606     | 75.1%        | 77.4%        | 73.95%       |
|                           |          | AC116347.2   | NG_001273.4  | NG_013034.2  |
| Aedes aegypti (mosquito)  | 7159     | 75.1%        | 69.6%        | 65.3%        |
|                           |          | XR_002501031.1| XM_021840546.1| XR_002502251.1|
| Aedes albopictus (mosquito)| 7160   | 62.6%        | 77.4%        | 65.3%        |
|                           |          | XM_019673469.2| XM_019704309.2| XM_029851862.1|
| Dermatophagoides pteronyssinus (dust mite) | 6956 | 54.2% | 63.7% | 56.6% |
|                           |          | XM_027348820.1| XM_027345041.1| XM_027345384.11.1|
| Xenopsylla cheopis (flea) | 163159  | 41.7%        | 45.5%        | 52.2%        |
|                           |          | EF179429.1   | EF179446.1   | MG6668630.1  |
| Drosophila (fruit fly)    | 7215     | 66.7%        | 77.4%        | 78.3%        |
|                           |          | XM_017295276.2| XM_034798131.1| XM_032729333.1|

| %Ident | Acc. No     | Acc. No     | Acc. No     |
|--------|-------------|-------------|-------------|
|        | LR134088   | LR134088   | LR134088   |
|        | N/A         | N/A         | N/A         |
|        | AE009949.1  | AE009949.1  | AE009949.1  |
|        | N/A         | CP001845.1  | N/A         |
|        | N/A         | CP001845.1  | N/A         |
|        | N/A         | N/A         | N/A         |
|        | CP048124.1  | CP048124.1  | CP048124.1  |
|        | N/A         | N/A         | N/A         |
|        | AC116347.2  | NG_001273.4 | NG_013034.2 |
|        | XM_021840546.1| XM_021840546.1| XM_021840546.1|
|        | XM_019673469.2| XM_019673469.2| XM_019673469.2|
|        | XM_027348820.1| XM_027348820.1| XM_027348820.1|
|        | EF179429.1  | EF179446.1  | MG6668630.1 |
|        | XM_017295276.2| XM_034798131.1| XM_032729333.1|

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| Organism                  | Taxon ID | N2 F1            | N2 P1            | N2 R1            |
|--------------------------|----------|------------------|------------------|------------------|
| Musca domestica (house fly) | 7370     | XR_00219         | XM_0200          | XM_00517838      |
|                          |          | 4421.1 Acc. No   | 35518.1          | 3.3              |
| Cercopithecus            | 9533     | 54.2% %Ident     | 59.2%            | 47.9%            |
| (was used instead of C. aethiops kidney cells) |          | JQ256983.1 Acc. No | AY205096.1   | KJ5317 82.1     |

1 Analysis Viracor Eurofins
2 Analysis Eurofins GeneScan Technologies GmbH

%Ident - Percent Identity of primer/probe identity, Acc. No - Accession number NCBI, N/A - Not Applicable
Table 11. Unimolecular folding N1 reverse primer target RNA strand

| Reverse transcription temperature | 50°C |
|----------------------------------|------|
| salt conditions*                | [Na+] = 1 M and [Mg++] = 0 M |
| ΔG total [kcal/mol]             | see below for details |

Structure 1: ΔG = -62.66 kcal/mol  
3’end amplicon (in yellow reverse primer binding site)  
5’end amplicon  

Structure 2: ΔG = -61.08 kcal/mol  
3’end amplicon (in yellow reverse primer binding site)  
5’end amplicon  

*Salt conditions are fixed and can’t be changed for RNA secondary structure analysis

Table 12. Unimolecular folding N1 forward primer-binding cDNA target strand and N1 probe-binding DNA target strand

| Annealing temperature | 60°C |
|-----------------------|------|
| salt conditions       | monovalent: 0.08 M; Mg²⁺: 0.002 M |
| ΔG total [kcal/mol] structure 1 | ΔG=-1.42 |
Table 13. Unimolecular folding N2 reverse primer target RNA strand

| reverse transcription temperature | 50°C |
|----------------------------------|------|
| salt conditions*                | [Na+] = 1 M and [Mg++] = 0 M |
| $\Delta G$ total [kcal/mol]     | see below for details |

**Structure 1: $\Delta G = -83.13$ kcal/mol**

**Structure 2: $\Delta G = -81.30$ kcal/mol**
Structure 3: $\Delta G = -80.64$ kcal/mol

Structure 4: $\Delta G = -80.53$ kcal/mol

Structure 5: $\Delta G = -79.81$ kcal/mol

Structure 6: $\Delta G = -79.55$ kcal/mol
Structure 7: $\Delta G = -79.09$ kcal/mol

*Salt conditions are fixed and can’t be changed for RNA secondary structure analysis

Table 14. Unimolecular folding N2 forward primer-binding cDNA target strand and N2 probe-binding DNA target strand

| Annealing temperature | 60°C |
|-----------------------|------|
| salt conditions       | monovalent: 0.08 M; Mg$^{2+}$: 0.002 M |
| $\Delta G$ total [kcal/mol] structure 1 | $\Delta G$=-4.80 |
| $\Delta G$ total [kcal/mol] structure 2 | $\Delta G$=-4.02 |
Table 15. Primer/Probe unimolecular folding N1 system

| Predicted structure | ∆G [kcal/mol] | Tm [°C] |
|---------------------|---------------|---------|
| 1                   | 1.17          | 40.4    |
| 2                   | 1.87          | 17.8    |
| 3                   | 2.17          | 34.4    |

| Predicted structure | ∆G [kcal/mol] | Tm [°C] |
|---------------------|---------------|---------|
| 1                   | 0.03          | 59.6    |

| Predicted structure | ∆G [kcal/mol] | Tm [°C] |
|---------------------|---------------|---------|
| 1                   | 2.14          | -5.8    |
| 2                   | 2.32          | 25.7    |
| 3                   | 2.47          | 40.4    |
| 4                   | 2.57          | -27.6   |
| 5                   | 2.58          | 16.3    |
| 6                   | 2.61          | 1320.2  |
| 7                   | 2.61          | 1320.2  |
| 8                   | 2.61          | 1320.2  |
| 9                   | 2.78          | -44.3   |
| 10                  | 2.78          | 2.8     |
| 11                  | 2.92          | -262.1  |
| 12                  | 2.94          | -146.6  |
| 13                  | 2.94          | -146.6  |
| 14                  | 2.94          | -146.6  |
| 15                  | 2.94          | -11.8   |
| 16                  | 2.95          | -165.9  |
| 17                  | 2.96          | -15.3   |
| 18                  | 3.01          | -25.6   |
| 19                  | 3.01          | -33.6   |
| 20                  | 3.07          | -137.8  |
Table 16. Primer/Probe unimolecular folding N2 system

| Predicted structure | ΔG [kcal/mol] | Tm [°C] |
|---------------------|----------------|---------|
| 1                   | 0.17           | 58.2    |
| 2                   | 0.30           | 59.9    |
| 3                   | 1.17           | 45.8    |

| Predicted structure | ΔG [kcal/mol] | Tm [°C] |
|---------------------|----------------|---------|
| 1                   | 0.79           | 48.8    |
| 2                   | 1.30           | 39.6    |
| 3                   | 1.77           | 24.8    |

| Predicted structure | ΔG [kcal/mol] | Tm [°C] |
|---------------------|----------------|---------|
| 1                   | 1.94           | -12.3   |
| 2                   | 2.06           | 32.8    |
| 3                   | 2.26           | 13.4    |
| 4                   | 2.58           | -5.7    |
| 5                   | 2.66           | 9.0     |
| 6                   | 2.71           | -14.6   |
| 7                   | 2.8            | -23.3   |
| 8                   | 2.8            | inf °C  |
| 9                   | 2.81           | -12.0   |
| 10                  | 2.9            | -115.7  |
| 11                  | 2.9            | -10.0   |

Table 17. Two-state bimolecular hybridization for the N1 and N2 system

| Primer/Probe to        | Temperature | ΔG annealing temperature [kcal/mol] | Tm       |
|------------------------|-------------|-------------------------------------|----------|
| N1F1 to cDNA           | 60°C        | -12.3                               | 68.2°C   |
| N1P1 to DNA            | 60°C        | -18.3                               | 75.8°C   |
| N1R1 to RNA            | 50°C        | -28.3                               | 79.3°C   |
| N1R1 to DNA            | 60°C        | -11.7                               | 65.6°C   |
| N2F1 to cDNA           | 60°C        | -14.1                               | 70.1°C   |
| N2P1 to DNA            | 60°C        | -16.9                               | 76.5°C   |
| N2R1 to RNA            | 50°C        | -30.3                               | 85.4°C   |
| N2R1 to DNA            | 60°C        | -14.0                               | 70.0°C   |
| Matrix         | Inoculation Strain | GC\(^a\) per Test area | Candidate Method | Reference Method |
|---------------|--------------------|-------------------------|------------------|-----------------|
|               |                    |                         | N1 or N2 Positive | N1 Positive     |
|               |                    |                         | Invalid          | N2 Positive     |
|               |                    |                         |                  | Final Result    |
| Stainless     | SARS-CoV-2         | 0                       | 5                | 0               |
| Stainless     | USA-               | 2 x 10\(^3\)            | 20               | 11              |
| Stainless     | WA1/2020           | 2 x 10\(^4\)            | 5                | 5               |

\(^a\)GC = Genomic copies determined by RT-qPCR.

\(^b\)n = Number of 2” x 2” test areas.
Table 19. Statistical comparison of VIRSeek and CDC method results

| Matrix            | Inoculation Strain     | GC<sup>a</sup> per Test Area | VIRSeek | CDC |
|-------------------|------------------------|-------------------------------|---------|-----|
|                   |                        |                               | n<sup>b</sup> | x<sup>c</sup> | POD<sub>C</sub><sup>d</sup> | 95% CI | n | x | POD<sub>R</sub><sup>e</sup> | 95% CI | dPOD<sub>f</sub> | 95% CI<sup>g</sup> |
| Stainless Steel   | SARS-CoV-2 USA-WA1/2020| 2 x 10<sup>3</sup>             | 5       | 0<sup>h</sup> | 0.00 | 0.00, 0.43 | 5       | 0 | 0.00 | 0.00, 0.43 | 0.00 | -0.43, 0.43 |
|                   |                        | 2 x 10<sup>4</sup>             | 17<sup>i</sup> | 8 | 0.47 | 0.26, 0.69 | 20 | 11 | 0.55 | 0.34, 0.74 | -0.08 | -0.36, 0.22 |
|                   |                        |                               | 5       | 5 | 1.00 | 0.57, 1.00 | 5       | 5 | 1.00 | 0.57, 1.00 | 0.00 | -0.43, 0.43 |

<sup>a</sup>GC = Genomic copies determined by qRT-PCR.
<sup>b</sup>n = Number of test areas.
<sup>c</sup>x = Number of positive test areas.
<sup>d</sup>POD<sub>C</sub> = Candidate method confirmed positive outcomes divided by the total number of trials.
<sup>e</sup>POD<sub>R</sub> = Reference method confirmed positive outcomes divided by the total number of trials.
<sup>f</sup>dPOD<sub>C</sub> = Difference in POD values between the candidate method and reference method results.
<sup>g</sup>95% CI = 95% confidence interval. If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.
<sup>h</sup>One swab initially tested positive, but upon repeat tested negative.
<sup>i</sup>Three invalid results were retested at 1/10 dilution and were still invalid.
Table 20: Overview Mismatches in N1- and N2-system

| N1 System | Mismatches in N1 | N2 System | Mismatches in N2 |
|-----------|-----------------|-----------|-----------------|
| N1 F1     | Single mismatch | N2 F1     | 7               |
|           |                 | N2 P1     | 0               |
|           |                 | N2 R1     | 0               |
| N1 P1     | Single mismatch | N2 F1     | 33              |
|           |                 | N2 P1     | 1               |
|           |                 | N2 R1     | 1               |
| N1 R1     | Single mismatch (one sequence with 2 mismatches) | N2 F1 | 16 |
|           |                 | N2 P1     | 0               |
|           |                 | N2 R1     | 0               |

Total number of sequences with mismatches in the N1 and N2 system: \(58 \pm 0.37\%\)

Table 21: Experimental testing for cross reactivity

| Pathogen                  | Source          | Concentration | SARS-CoV-2 rRT-PCR C<sub>T</sub> | Internal Control C<sub>T</sub> |
|---------------------------|-----------------|---------------|----------------------------------|-------------------------------|
| Coronavirus 229E<sup>1</sup> | Zeptometrix     | 1x10<sup>4</sup> TCID<sub>50</sub>/mL | -                               | 29.47                         |
| Coronavirus NL63<sup>1</sup> | Zeptometrix     | 1x10<sup>3.75</sup> TCID<sub>50</sub>/mL | -                               | 30.39                         |
| Coronavirus OC43<sup>1</sup> | Zeptometrix     | 1x10<sup>4.10</sup> TCID<sub>50</sub>/mL | -                               | 28.83                         |
| SARS NC_004718<sup>1</sup> | IDT             | 5x10<sup>4</sup> copies/mL            | -                               | N/A                           |
| Haemophilus influenza<sup>1</sup> | Zeptometrix     | 5x10<sup>4</sup> CFU/mL              | -                               | 29.61                         |
| Legionella pneumophila<sup>2</sup> | Zeptometrix     | 5x10<sup>4</sup> CFU/mL              | -                               | 29.71                         |
| Mycobacterium tuberculosis<sup>1</sup> | ATCC           | 5x10<sup>4</sup> GEq/mL             | -                               | N/A                           |
| Streptococcus pneumonia<sup>1</sup> | Zeptometrix     | 5x10<sup>4</sup> CFU/mL              | -                               | 32.70                         |
| Streptococcus pyogenes<sup>1</sup> | Zeptometrix     | 5x10<sup>4</sup> CFU/mL              | -                               | 29.82                         |
| Pseudomonas aeruginosa<sup>2</sup> | Genomic DNA     | 2.6x10<sup>4</sup> copies/mL         | -                               | N/A                           |
| Streptococcus salivarius<sup>1</sup> | Zeptometrix     | 5x10<sup>4</sup> CFU/mL              | -                               | 29.77                         |
| Klebsiella pneumonia<sup>2</sup> | Genomic DNA     | 6x10<sup>4</sup> copies/mL           | -                               | N/A                           |

<sup>1</sup> Analysis Viracor Eurofins
<sup>2</sup> Analysis Eurofins GeneScan Technologies GmbH

not detected
N/A - Not Applicable (no extraction control was added since the test was performed with extracted DNA)

TCID<sub>50</sub> - Tissue Culture Infection Dose 50

GEq - Genome equivalent