Validation of the VIR*Seek* 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: laurableichner@eurofins.com

Abstract

Background: The Eurofins GeneScan Technologies' VIR*Seek* 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 (VIR*Seek* PATHO*Swab* kit), RNA extraction (VIR*Seek* RNA*Extractor*), RT-PCR (VIR*Seek* 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 VIR*Seek* RNA*Extractor* kit (3).

Principle of the Method

Eurofins GeneScan Technologies' VIR*Seek* 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 VIR*Seek* 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^{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 VIR*Seek* 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 VIR*Seek* SARS-CoV-2 Mplex kit is validated for use with the Agilent AriaMx^{TM}, Bio-Rad CFX96 Touch^{TM}, CFX96 Touch^{TM} Deep Well and the Applied Biosystems™ 7500 PCR Standard platforms. Each kit contains reagents for 96 reactions. Cycler running time is rapid at approx. 1 h for the Agilent AriaMx^{TM} and the two Bio-Rad cylers. For the Applied Biosystems™ 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; POD_R (reference method POD), POD_C (confirmed candidate method POD), POD_{CP} (candidate method presumptive result POD) and POD_{CC} (candidate method confirmation result POD).

(**b**) *Difference of probabilities of detection (dPOD).*—Difference of probabilities of detection is the difference between any two POD values. If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level (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*.—VIR*Seek* 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 VIR*Seek* SARS-CoV-2 negative control.

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

(**e**) *Extraction control*.—2 vials with 1000 µL VIR*Seek* 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 VIR*Seek* SARS-CoV-2 kit contains glycerol and propane-1,2-diol which may cause mild skin irritation. For more information, please refer to the VIR*Seek* SARS-CoV-2 kit safety information.

General Preparation

(a) Ensure the kit components are kept at −20 ± 2°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 ± 2°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°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°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*[SM] (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 VIR*Seek* 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 VIR*Seek* SARS-CoV-2 Mplex assay, ΔG 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 ΔG=-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 VIR*Seek* SARS-CoV-2 Mplex assay, ΔG 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 ΔG=-4.80 kcal/mol and ΔG=-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 x 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 $\times 10^9$ GC/mL.

The presence of infectious SARS-CoV-2 in the WRCEVA virus stock was verified using standard cell culture techniques. Briefly, 3×10^6 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-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) 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" × 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 VIR*Seek* method were sampled using the VIR*Seek* PATHO*Swab* 50 Surface Sampling Kit. Briefly, the swab was moistened with 2-3 drops of VIR*Seek* 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 VIR*Seek* 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 VIR*Seek* 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™), 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 VIR*Seek* 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 VIR*Seek* 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 VIR*Seek* 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 VIR*Seek* SARS-CoV-2 kit on stainless steel surfaces sampled with the VIR*Seek* PATHO*Swab*, extracted with the VIR*Seek* RNA*Extractor* 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 VIR*Seek* 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.

References

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Tables

Table 1. VIR*Seek* 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 VIR*Seek* 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 VIR*Seek* 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 VIR*Seek* 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 sample[1]. 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.

[1] If 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 [1]	111337	%Ident	65.0%	51.9%	59.3%
		Acc. No	KT253264.1	KT253271.1	KT253270.1
Human coronavirus OC43 [1]	31631	%Ident	N/A	63.0%	N/A
		Acc. No	N/A	AY903460.1	N/A
Human coronavirus NL631 [1]	277944	%Ident	N/A	N/A	66.7%
		Acc. No	N/A	N/A	MK334045.1
Human coronavirus HKU1 [1]	290028	%Ident	N/A	51.9%	59.3%
		Acc. No	N/A	DQ339101.1	AY884001.1
SARS-coronavirus [1]	694009	%Ident	100%	59.3%	89.9%
		Acc. No	AY297028.1	KJ473811.1	KY352407.1
MERS-coronavirus [1]	1335626	%Ident	75.0%	N/A	59.3%
		Acc. No	KJ473821.1	N/A	MG923469.1
Porcine delta coronavirus [2]	CH/JXJGS01/2016	%Ident	45.00%	44.45%	44.45%
		Acc. No	KY293677.1	KY293677.1	KY293677.1

[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 8. Exclusivity N2 system

Organism	Taxon ID/Strain		N2 F1	N2 P1	N2 R1
Human coronavirus 229 E [1]	111337	%Ident	N/A	50.0%	56.5%
		Acc. No	N/A	KT253271.1	KT253272.1
Human coronavirus OC43 [1]	31631	%Ident	N/A	N/A	N/A
		Acc. No	N/A	N/A	N/A
Human coronavirus NL63 [1]	277944	%Ident	N/A	N/A	N/A
		Acc. No	N/A	N/A	N/A
Human coronavirus HKU1 [1]	290028	%Ident	N/A	N/A	N/A
		Acc. No	N/A	N/A	N/A
SARS-coronavirus [1]	694009	%Ident	91.7%	N/A	95.7%
		Acc. No	AY297028.q	N/A	AY297028.1
MERS-coronavirus [1]	1335626	%Ident	N/A	N/A	N/A
		Acc. No	N/A	N/A	N/A
Porcine delta coronavirus [2]	CH/JXJGS01/2016	%Ident	37.53%	45.50%	47.85%
		Acc. No	KY293677.1	KY293677.1	KY293677.1

[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 9. Background viruses, bacteria and Eukaryotes N1 system

Organism		Taxon ID/Strain		N1 F1	N1 P1	N1 R1
Influenza A H1N1 [2]		114727	%Ident	60.0%	44.7%	59.3%
			Acc. No	KF8404 67.1	CY2383 12.1	CY0853 33.2
Influenza A H3N2 [2]		119210	%Ident	55.0%	44.5%	48.2%
			Acc. No	MG784 576.1	KT8391 18.1	MF872 826.1
Influenza A H5N1 [2]		102793	%Ident	55.0%	48.2%	48.2%
			Acc. No	MG664 611.1	JX5345 75.1	KP7429 11.1
Influenza A H7N9 [2]		H7N9 333278	%Ident	55.0%	37.0%	40.7%
			Acc. No	CY1860 06.1	MF629 948.1	KY2381 28.1
Influenza B [1]		11520	%Ident	65.0%	59.3%	59.3%
			Acc. No	NC_002 206.1	NC_002 211.1	NC_002 205.1
Human adenovirus, type 1 [2]		10533 (Ad71 not found)	%Ident	50.0%	48.2%	33.3%
			Acc. No	MH183 293.1	MH183 293.1	MH132 93.1
Human metapneumovirus [1]		162145	%Ident	65.0%	55.6%	59.3%
			Acc. No	KJ62739 7.1	AY5258 43.1	KJ6273 83.1
Respiratory syncytial virus Long A [2]		208893 (Long A not found)	%Ident	50.0%	40.7%	40.7%
			Acc. No	MT4222 73.1	MH181 903.1	MK306 327.1
Rhinovirus [2]		12059	%Ident	65.0%	48.2%	51.9%
			Acc. No	MK993 376.1	MK593 172.1	MF160 542.1
Parainfluenza 1 C 35 [2]		31605	%Ident	N.A	N.A	N.A
			Acc. No	N.A	N.A	N.A
Parainfluenza 2 Greer [2]		11213	%Ident	N.A	N.A	N.A

Organism		Taxon ID/Strain		N1 F1	N1 P1	N1 R1
			Acc. No	N.A	N.A	N.A
Parainfluenza 3 [1]		C-43 not found 11216 used instead (complete parainfluenza 3)	%Ident	60.0%	N/A	66.7%
			Acc. No	KM190 938.1	N/A	KY9735 56.1
Parainfluenza 4 [1]		M-25 not found 1979161 used instead (complete parainfluenza 4)	%Ident	60.0%	44.4%	66.7%
			Acc. No	NC_021 928.1	MH892 407.1	KY4605 15.1
Enterovirus EV68 [2]		EV-D68 found 42789	%Ident	55.0%	40.7%	51.9%
			Acc. No	MN240 500.1	MN245 446.1	MK419 043.1
Human bocavirus [2]		329641	%Ident	45.0%	40.7%	37.0%
			Acc. No	MT4092 20.1	GQ8910 87.1	MH574 945.1
Varicella-zoster virus [2]		10335	%Ident	50.0%	40.7%	40.7%
			Acc. No	MH709 377.1	MH709 377.1	MH709 377.1
Norovirus [2]		142786	%Ident	70.0%	51.9%	48.2%
			Acc. No	MN922 718.1	KC2121 56.1	MT372 476.1
Herpes virus [2]		Herpesviridae 10292	%Ident	N/A	59.3%	59.3%
			Acc. No	DQ1980 83.1	KR2972 53.1	LC0648 08.1
Avian Influenza [2]		H1N1 subtype found 114727	%Ident	See above		
			Acc. No			
Avian Influenza [2]		H4N1 subtype found 282148	%Ident	50.0%	37.0%	40.7%
			Acc. No	KT3383 67.1	JX8786 77.1	LC3493 01.2
Avian Influenza [2]		H6N1 subtype found 119212	%Ident	55.0%	37.0%	40.7%
			Acc. No	CY1808 81.1	CY1786 35.1	MG063 432.1

Organism		Taxon ID/Strain		N1 F1	N1 P1	N1 R1
Avian Influenza [2]		H9N1 subtype found 147762	%Ident	55.0%	37.0%	40.7%
			Acc. No	CY2066 07.1	CY1958 17.1	CY1855 27.1
Avian infectious bronchitis virus [2]		11120	%Ident	60.0%	40.7%	51.9%
			Acc. No	HM486 956.1	MN548 289.1	NC_048 213.1
Bovine coronavirus [2]		11128	%Ident	45.0%	44.5%	40.7%
			Acc. No	LC4941 78.1	LC4941 78.1	LC4941 78.1
Mouse hepatitis [2]		11138	%Ident	55.0%	48.2%	40.7%
			Acc. No	AF2079 02.1	MF416 379.1	FJ64722 3.1
Porcine transmissible gastroenteritis virus [2]		11149	%Ident	45.0%	37.0%	48.2%
			Acc. No	MK272 773.1	KX9004 11.1	KX9004 11.1
Acinetobacter Spp [2]		469	%Ident	75.0%	51.9%	74.1%
			Acc. No	CP0319 76.1	CP0331 33.1	CP0151 10.1
Bacillus thuringiensis [2]		1428	%Ident	70.0%	48.2%	66.7%
			Acc. No	CP0161 94.1	CP0207 54.1	CP0539 34.1
Bacillus cereus [2]		1396 (not only spores)	%Ident	70.0%	51.9%	66.7%
			Acc. No	CP0265 23.1	CP0265 23.1	CP0539 31.1
Bordetella pertussis [1]		520	%Ident	N/A	N/A	N/A
			Acc. No	N/A	N/A	N/A
Candida ablicans [1]		5476	%Ident	60.00%	N/A	59.3%
			Acc. No	NC_002 653.1	N/A	NC_002 653.1
Chlamydia pneumoniae [1]		83558	%Ident	N/A	N/A	51.9%
			Acc. No	N/A	N/A	CP0017 13.1

Organism		Taxon ID/Strain		N1 F1	N1 P1	N1 R1
Clostridium difficile [2]		1496	%Ident	65.0%	48.2%	48.2%
			Acc. No	LK9330 95.1	CP0295 66.1	CP0354 99.1
Escherichia coli [2]		83333	%Ident	70.0%	63.0%	48.2%
			Acc. No	CP0471 27.1	CP0471 27.1	CP0471 27.1
Enterococcus [2]		1350	%Ident	75.0%	51.9%	59.3%
			Acc. No	LR6073 77.1	CP0461 23.1	CP0552 32.1
Haemophilus influenza [1]		727	%Ident	N/A	N/A	74.1%
			Acc. No	N/A	N/A	CP0316 89.1
Klebsiella pneumonia [2]		573	%Ident	75.0%	48.2%	55.6%
			Acc. No	CP0300 70.1	MN543 585.1	CP0183 06.1
Legionella pneumophila[2]		446	%Ident	85.0%	48.2%	70.4%
			Acc. No	CP0459 74.1	CP0459 74.1	LR1343 80.1
Listeria monocytogenes [2]		1639	%Ident	65.0%	48.2%	59.3%
			Acc. No	CP0548 46.1	CP0142 52.2	CP0536 31.1
Mycobacterium tuberculosis [1]		1773	%Ident	N/A	N/A	N/A
			Acc. No	N/A	N/A	N/A
Mycoplasma pneumonia [1]		2104	%Ident	N/A	N/A	N/A
			Acc. No	N/A	N/A	N/A
Pneumocystis jirovecii (PJP) [1]		42068	%Ident	60.0%	N/A	59.3%
			Acc. No	NC_002 653.1	N/A	NC_002 653.1
Pseudomonas aeruginosa [1]		287	%Ident	80.0%	66.7%	N/A

Organism	Taxon ID/Strain		N1 F1	N1 P1	N1 R1
		Acc. No	NZ_CP040684.1	NZ_CP027174.1	N/A
Staphylococcus aureus [2]	1280	%Ident	60.0%	51.9%	55.6%
		Acc. No	AP023034.1	AP020315.1	CP053101.1
Staphylococcus epidermidis [1]	1282	%Ident	N/A	N/A	N/A
		Acc. No	N/A	N/A	N/A
Streptococcus pyogenes [1]	1314	%Ident	N/A	N/A	N/A
		Acc. No	N/A	N/A	N/A
Streptococcus pneumonia [1]	1313	%Ident	80.0%	N/A	N/A
		Acc. No	CP007593.1	N/A	N/A
Streptococcus salivarius [1]	1304	%Ident	70.0%	62.9%	N/A
		Acc. No	NZ_CP040804.1	NZ_CP018187.1	N/A
Torulopsis glabrata [2]	5478	%Ident	75.0%	48.2%	59.3%
		Acc. No	CP048128.1	CP048126.1	CP048120.1
Homo sapiens [2]	9606 (was used instead of HeLa cells)	%Ident	N/A	63.0%	63.0%
		Acc. No	N/A	AC091134.7	AC244112.3
Aedes aegypti (mosquito) [2]	7159	%Ident	65.0%	48.2%	55.6%
		Acc. No	XM_021853659.1	AY431941.1	XM_021854917.1
Aedes albopictus (mosquito) [2]	7160	%Ident	70.0%	51.9%	66.7%
		Acc. No	XM_029853282.1	XM_029852498.1	XM_029856320.1
Dermatophagoides	6956	%Ident	70.0%	51.9%	63.0%

Organism	Taxon ID/Strain		N1 F1	N1 P1	N1 R1
pteronyssinus (dust mite) [2]		Acc. No	XM_02 735053 8.1	XM_02 735021 0.1	XM_02 734229 8.1
Xenopsylla cheopis (flea) [2]	163159	%Ident	45.0%	40.7%	37.0%
		Acc. No	KJ63856 6.1	KU8806 67.1	EF1794 45.1
Drosophila (fruit fly) [2]	7215	%Ident	90.00%	59.3%	66.7%
		Acc. No	XM_01 707178 7.1	CP0233 36.1	CP0233 38.1
Musca domestica (house fly) [2]	7370	%Ident	75.0%	51.9%	70.4%
		Acc. No	NM_00 130906 1.2	XM_02 003627 0.1	XM_00 518253 3.3
Cercopithecus [2]	9533 (was used instead of *C. aethiops* kidney cells)	%Ident	75.0%	40.7%	44.5%
		Acc. No	KJ53175 4.1	MF616 350.1	KJ5318 14.1

[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 10. Background viruses, bacteria and Eukaryotes N2 system

Organism	Taxon ID		N2 F1	N2 P1	N2 R1
Influenza A H1N1 [2]	114727	%Ident	58.4%	50.1%	56.6%
		Acc. No	CY147371.1	KP637780.1	MF098856.1
Influenza A H3N2 [2]	119210	%Ident	58.4%	50.1%	52.2%
		Acc. No	KT888183.1	CY246653.1	CY259529.1
Influenza A H5N1 [2]	102793	%Ident	62.6%	50.1%	69.6%
		Acc. No	GU182155.1	KY635788.1	JQ520362.1
Influenza A H7N9 [2]	H7N9 333278	%Ident	41.7%	45.5%	56.6%
		Acc. No	KY238129.1	MG575606.1	MG575584.1
Influenza B [1]	11520	%Ident	58.3%	59.1%	60.9%
		Acc. No	NC_002207.1	NC_002205.1	NC_002211.1
Human adenovirus, type 1 [2]	10533 (Ad71 not found)	%Ident	37.5%	45.5%	47.9%
		Acc. No	MH183293.1	MH183293.1	MH183293.1
Human metapneumovirus [1]	162145	%Ident	N/A	N/A	56.5%
		Acc. No	N/A	N/A	AF371337.2
Respiratory syncytial virus Long A [2]	208893 (Long A not found)	%Ident	58.4%	41.0%	52.2%
		Acc. No	MT422273.1	MT422273.1	MH181965.1
Rhinovirus [2]	12059	%Ident	58.4%	59.2%	82.7%
		Acc. No	MN812200.1	JX560527.1	AB647319.1
Parainfluenza 1 C 35 [2]	31605	%Ident	N/A	N/A	N/A
		Acc. No	N/A	N/A	N/A
Parainfluenza 2 Greer [2]	11213	%Ident	N/A	N/A	N/A
		Acc. No	N/A	N/A	N/A
Parainfluenza 3 *C-43* [1]	*C-43* not found 11216 used instead (complete parainfluenza 3)	%Ident	N/A	N/A	69.6%
		Acc. No	N/A	N/A	MH678682.1
Parainfluenza 4 M-25 [1]	M-25 not found 1979161 used instead (complete parainfluenza 4)	%Ident	N/A	N/A	47.8%
		Acc. No	N/A	N/A	KF483663.1

Organism	Taxon ID		N2 F1	N2 P1	N2 R1
Enterovirus EV68 [2]	EV-D68 found 42789	%Ident	45.9%	63.7%	47.9%
		Acc. No	KM851231.1	MN240516.1	MN245448.1
Human bocavirus [2]	329641	%Ident	41.7%	41.0%	47.9%
		Acc. No	MK034749.1	KJ492919.1	MN245448.1
Varicella-zoster virus [2]	10335	%Ident	45.9%	54.6%	43.5%
		Acc. No	MH709377.1	MH709377.1	MH709377.1
Norovirus [2]	142786	%Ident	62.6%	59.2%	52.2%
		Acc. No	MT416724.1	NC_044932.1	NC_044046.1
Herpes virus [2]	Herpesviridae 10292	%Ident	66.7%	59.2%	82.7%
		Acc. No	KM924294.1	MK973062.1	HQ698924.1
Avian Influenza [2]	H1N1 subtype found 114727	%Ident			
		Acc. No	See above		
Avian Influenza [2]	H4N1 subtype found 282148	%Ident	41.7%	50.1%	56.6%
		Acc. No	LC349302.2	KT338365.1	CY146544.1
Avian Influenza [2]	H6N1 subtype found 119212	%Ident	54.2%	45.5%	52.2%
		Acc. No	DQ376832.1	KT266926.1	AY862613.1
Avian Influenza [2]	H9N1 subtype found 147762	%Ident	41.7%	45.5%	43.5%
		Acc. No	CY206608.1	CY102721.1	CY206608.1
Avian infectious bronchitis virus [2]	11120	%Ident	50.0%	50.1%	65.3%
		Acc. No	MN548289.1	KP790143.1	KR608272.1
Bovine coronavirus [2]	11128	%Ident	41.7%	45.5%	43.5%
		Acc. No	LC494178.1	FJ938067.1	LC494178.1
Mouse hepatitis [2]	11138	%Ident	41.7%	54.6%	43.5%
		Acc. No	MF416379.1	M55148.1	MF618253.1
Porcine transmissible gastroenteritis virus [2]	11149	%Ident	45.9%	41.0%	56.6%
		Acc. No	KX527917.1	KX900411.1	AF104420.1
Acinetobacter spp [2]	469	%Ident	62.6%	72.8%	69.6%
		Acc. No	CP054822.1	CP041970.1	CP031976.1
Bacillus	1428	%Ident	58.4%	63.7%	60.9%

Organism	Taxon ID		N2 F1	N2 P1	N2 R1
thuringiensis [2]		Acc. No	CP053971.1	CP053969.1	CP054568.1
Bacillus cereus [2]	1396 (not only spores)	%Ident	58.4%	68.3%	60.9%
		Acc. No	CP029454.1	KF240854.1	CP053997.1
Bordetella pertussis [1]	520	%Ident	N/A	N/A	N/A
		Acc. No	N/A	N/A	N/A
Candida ablicans [1]	5476	%Ident	N/A	N/A	65.2%
		Acc. No	N/A	N/A	NC_002653.1
Chlamydia pneumonia [1]	83558	%Ident	70.8%	68.2%	N/A
		Acc. No	AE009440.1	AE009440.1	N/A
Clostridium difficile [2]	1496	%Ident	58.4%	54.6%	56.6%
		Acc. No	CP035499.1	CP035499.1	CP029566.1
Escherichia coli [2]	83333	%Ident	50.0%	68.3%	56.6%
		Acc. No	CP047127.1	CP042184.1	CP047127.1
Enterococcus [2]	1350	%Ident	62.6%	68.3%	69.6%
		Acc. No	AP018545.1	CP055232.1	CP014068.2
Haemophilus influenza [1]	727	%Ident	N/A	86.4%	N/A
		Acc. No	N/A	NC_000907.1	N/A
Klebsiella pneumonia [2]	573	%Ident	58.4%	91.0%	56.6%
		Acc. No	CP052431.1	CP054780.1	AP023148.1
Legionella pneumophila [2]	446	%Ident	54.2%	63.7%	56.6%
		Acc. No	CP048618.1	LR133933.1	CP021266.1
Listeria monocytogenes [2]	1639	%Ident	58.4%	72.8%	60.9%
		Acc. No	CP054846.1	CP038642.1	CP007583.1
Mycobacterium tuberculosis [1]	1773	%Ident	N/A	81.8%	N/A
		Acc. No	N/A	CP000717.1	N/A
Mycoplasma pneumonia [1]	2104	%Ident	N/A	N/A	N/A
		Acc. No	N/A	N/A	N/A
Pneumocystis jirovecii (PJP) [1]	42068	%Ident	54.2%	N/A	78.3%
		Acc. No	AY130996.1	N/A	JX499143.1
Pseudomonas aeruginosa [1]	287	%Ident	N/A	81.9%	N/A
		Acc. No	N/A	NZ_CP007	N/A

Organism	Taxon ID		N2 F1	N2 P1	N2 R1
				147.1	
Staphylococcus aureus [2]	1280	%Ident	54.2%	72.8%	65.3%
		Acc. No	AP023034.1	CP047857.1	LR134088.1
Staphylococcus epidermidis [1]	1282	%Ident	66.7%	N/A	N/A
		Acc. No	NZ_CP018842.1	N/A	N/A
Streptococcus pyogenes [1]	1314	%Ident	N/A	81.8%	N/A
		Acc. No	N/A	AE009949.1	N/A
Streptococcus pneumonia [1]	1313	%Ident	N/A	72.7%	N/A
		Acc. No	N/A	CP001845.1	N/A
Streptococcus salivarius [1]	1304	%Ident	62.5%	77.3%	82.6%
		Acc. No	NZ_CP018189.1	NZ_CP020451.2	NZ_CP020451.2
Torulopsis glabrata [2]	5478	%Ident	62.6%	81.9%	65.3%
		Acc. No	CP048118.1	CP048123.1	CP048124.1
Homo sapiens [2]	9606 (was used instead of HeLa cells	%Ident	75.1%	77.4%	73.95%
		Acc. No	AC116347.2	NG_001273.4	NG_013034.2
Aedes aegypti (mosquito) [2]	7159	%Ident	75.1%	69.6%	65.3%
		Acc. No	XR_002501031.1	XM_021840546.1	XR_002502251.1
Aedes albopictus (mosquito) [2]	7160	%Ident	62.6%	77.4%	65.3%
		Acc. No	XM_019673469.2	XM_019704309.2	XM_029851862.1
Dermatophagoides pteronyssinus (dust mite) [2]	6956	%Ident	54.2%	63.7%	56.6%
		Acc. No	XM_027348820.1	XM_027345041.1	XM_027343841.1
Xenopsylla cheopis (flea) [2]	163159	%Ident	41.7%	45.5%	52.2%
		Acc. No	EF179429.1	EF179446.1	MG668630.1
Drosophila (fruit fly) [2]	7215	%Ident	66.7%	77.4%	78.3%
		Acc. No	XM_017295276.2	XM_034798131.1	XM_032729333.1
		%Ident	54.2%	72.8%	60.9%
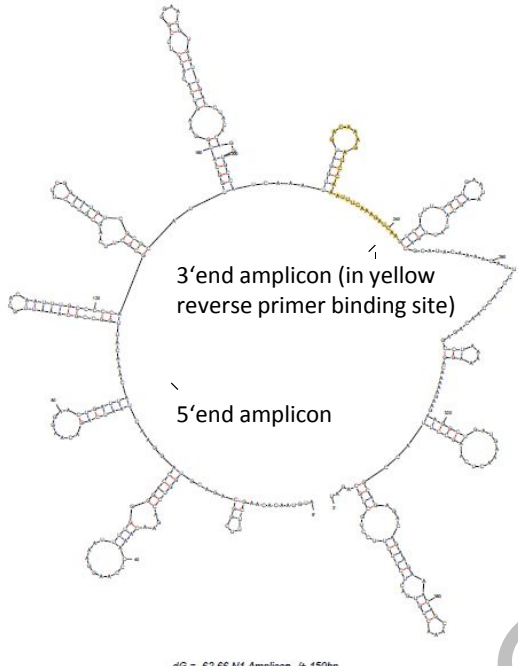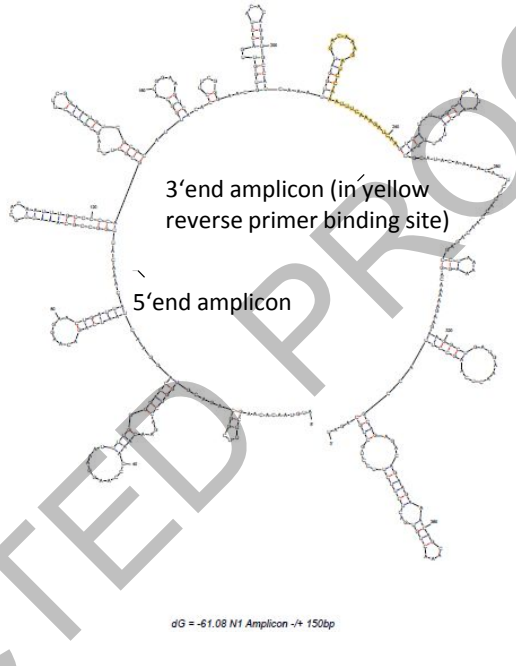
Organism	Taxon ID		N2 F1	N2 P1	N2 R1
Musca domestica (house fly) [2]	7370	Acc. No	XR_00219 4421.1	XM_0200 35518.1	XM_00 517838 3.3
Cercopithecus [2]	9533 (was used instead of *C. aethiops* kidney cells)	%Ident	54.2%	59.2%	47.9%
		Acc. No	JQ256983. 1	AY205096. 1	KJ5317 82.1

[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

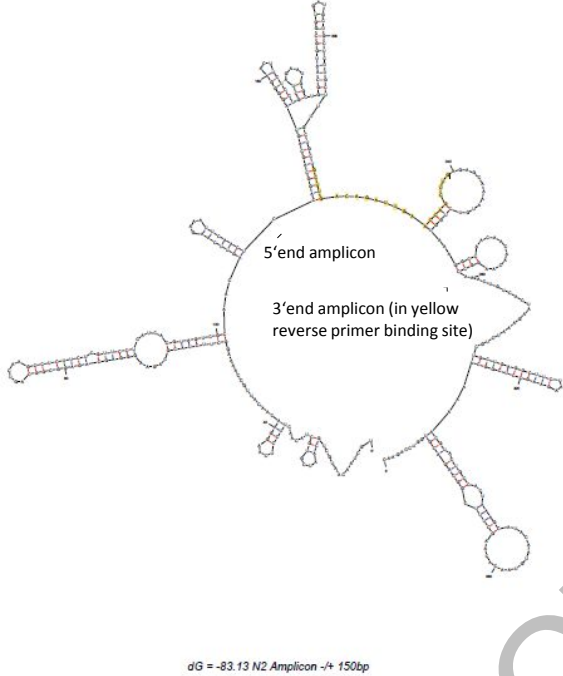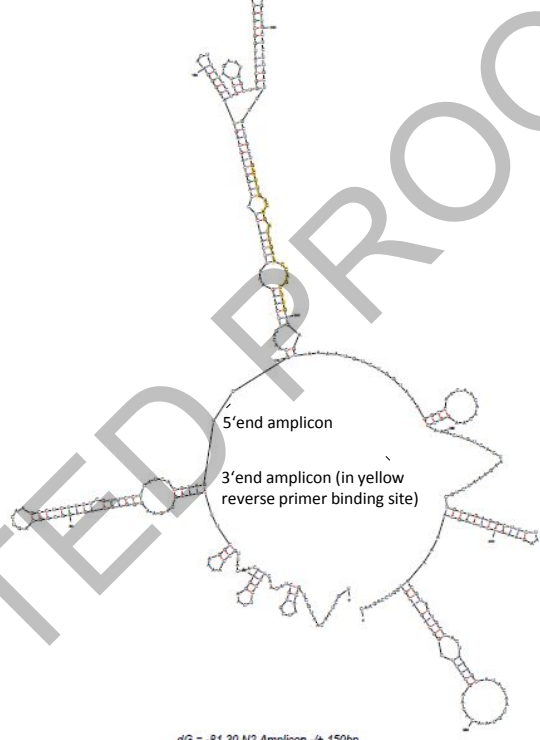**Table 11. Unimolecular folding N1 reverse primer target RNA strand**

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	**Structure 2: ΔG = -61.08 kcal/mol**

3'end amplicon (in yellow reverse primer binding site)

5'end amplicon

dG = -62.66 N1 Amplicon -/+ 150bp

3'end amplicon (in yellow reverse primer binding site)

5'end amplicon

dG = -61.08 N1 Amplicon -/+ 150bp

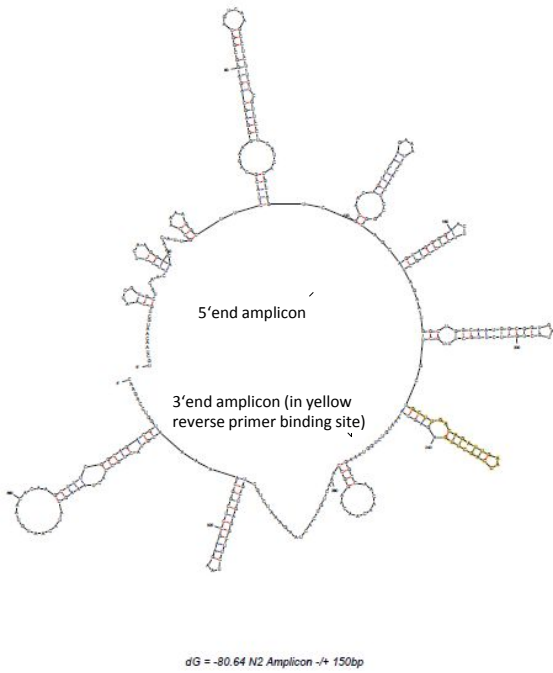*Salt conditions are fixed and can't be changed for RNA secondary structure analysis

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

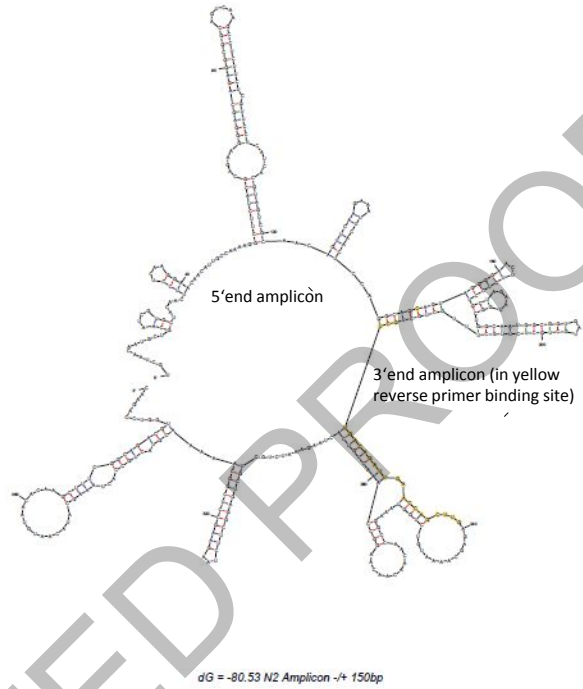| Annealing temperature	60°C
salt conditions	monovalent: 0.08 M; Mg^{2+}: 0.002 M
ΔG total [kcal/mol] structure 1	**ΔG=-1.42**

Table 13. Unimolecular folding N2 reverse primer target RNA strand

reverse transcription temperature	50°C
salt conditions*	[Na+] = 1 M and [Mg++] = 0 M
ΔG total [kcal/mol]	see below for details
Structure 1: ΔG = -83.13 kcal/mol	**Structure 2: ΔG = -81.30 kcal/mol**

5'end amplicon

3'end amplicon (in yellow reverse primer binding site)

dG = -83.13 N2 Amplicon -/+ 150bp

5'end amplicon

3'end amplicon (in yellow reverse primer binding site)
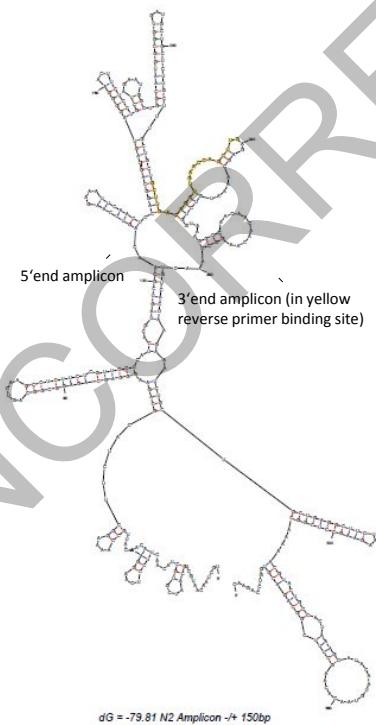
dG = -81.30 N2 Amplicon -/+ 150bp

Structure 3: ΔG = -80.64 kcal/mol

5'end amplicon

3'end amplicon (in yellow reverse primer binding site)

dG = -80.64 N2 Amplicon -/+ 150bp

Structure 4: ΔG = -80.53 kcal/mol

5'end amplicon

3'end amplicon (in yellow reverse primer binding site)

dG = -80.53 N2 Amplicon -/+ 150bp

Structure 5: ΔG = -79.81 kcal/mol

5'end amplicon

3'end amplicon (in yellow reverse primer binding site)

dG = -79.81 N2 Amplicon -/+ 150bp

Structure 6: ΔG = -79.55 kcal/mol

5'end amplicon

3'end amplicon (in yellow reverse primer binding site)

dG = -79.55 N2 Amplicon -/+ 150bp

Structure 7: ΔG = -79.09 kcal/mol

5'end amplicon

3'end amplicon (in yellow reverse primer binding site)

dG = -79.09 N2 Amplicon -/+ 150bp
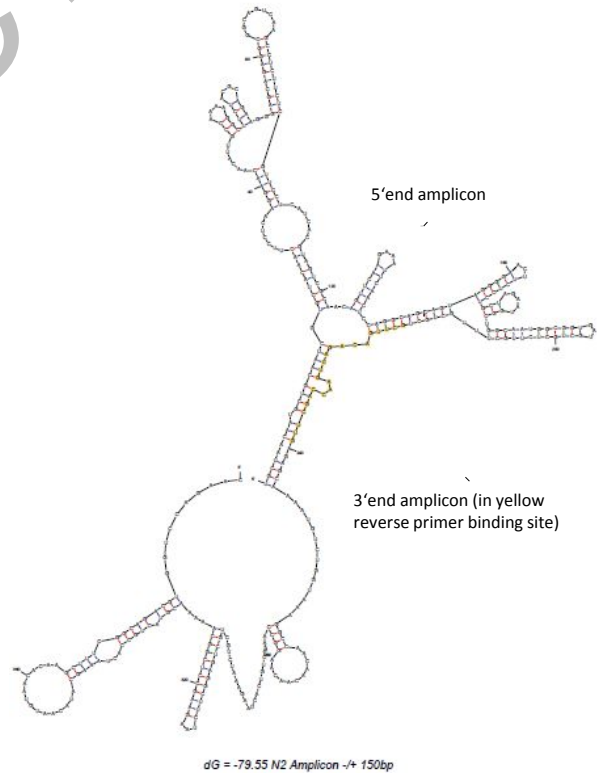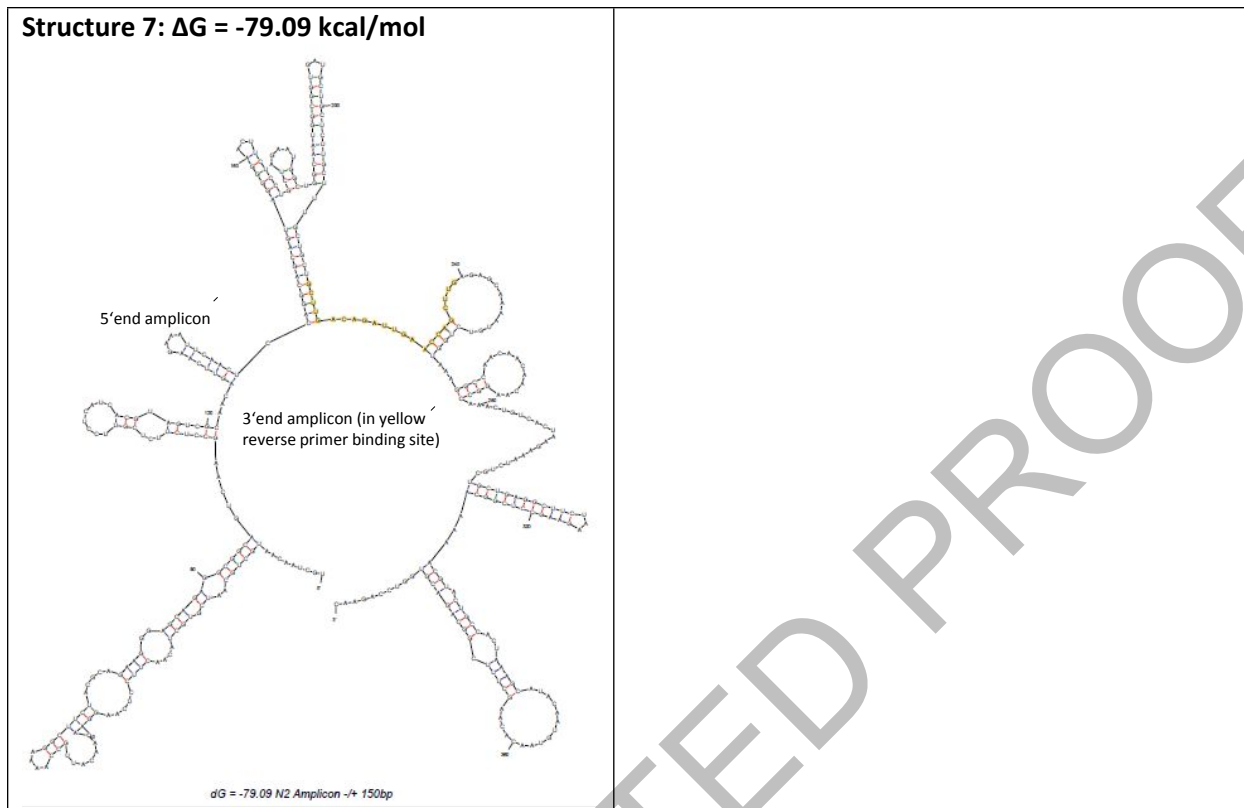
*Salt conditions are fixed and can't be changed for RNA secondary structure analysis

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
ΔG total [kcal/mol] structure 1	ΔG=-4.80
ΔG total [kcal/mol] structure 2	ΔG=-4.02

Table 15. Primer/Probe unimolecular folding N1 system

N1F1		
Predicted structure	**ΔG [kcal/mol]**	**Tm [C°]**
1	1.17	40.4
2	1.87	17.8
3	2.17	34.4

N1P1		
Predicted structure	**ΔG [kcal/mol]**	**Tm [C°]**
1	0.03	59.6

N1R1		
Predicted structure	**ΔG [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

N2F1		
Predicted structure	ΔG [kcal/mol]	Tm [C°]
1	0.17	58.2
2	0.30	59.9
3	1.17	45.8
N2P1		
Predicted structure	ΔG [kcal/mol]	Tm [C°]
1	0.79	48.8
2	1.30	39.6
3	1.77	24.8
N2R1		
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	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

Table 18. Candidate and reference method results

Matrix	Inoculation Strain	GC^a per Test area	n^b	Candidate Method		Reference Method		
				N1 or N2 Positive	Invalid	N1 Positive	N2 Positive	Final Result
Stainless	SARS-CoV-2	0	5	0	0	0	0	0
Steel	USA-	2×10^3	20	8	3	11	19	11
(2" x 2")	WA1/2020	2×10^4	5	5	0	5	5	5

^{a}GC = Genomic copies determined by RT-qPCR.

^{b}n = Number of 2" x 2" test areas.

Table 19. Statistical comparison of VIR*Seek* and CDC method results

Matrix	Inoculation Strain	GC[a] per Test Area	VIR*Seek*				CDC				dPOD_C[f]	95% CI[g]
			n[b]	x[c]	POD_C[d]	95% CI	n	x	POD_R[e]	95% CI		
Stainless Steel (2" x 2")	SARS-CoV-2 USA-WA1/2020	0	5	0[h]	0.00	0.00, 0.43	5	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
		2 x 10³	17[i]	8	0.47	0.26, 0.69	20	11	0.55	0.34, 0.74	-0.08	-0.36, 0.22
		2 x 10⁴	5	5	1.00	0.57, 1.00	5	5	1.00	0.57, 1.00	0.00	-0.43, 0.43

[a]GC = Genomic copies determined by qRT-PCR.

[b]n = Number of test areas.

[c]x = Number of positive test areas.

[d]POD_C = Candidate method confirmed positive outcomes divided by the total number of trials.

[e]POD_R = Reference method confirmed positive outcomes divided by the total number of trials.

[f]dPOD_C= Difference in POD values between the candidate method and reference method results.

[g]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.

[h]One swab initially tested positive, but upon repeat tested negative.

[i]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** ≙ 0.37%

Table 21: Experimental testing for cross reactivity

Pathogen	Source	Concentration	SARS-CoV-2 rRT-PCR C_T	Internal Control C_T
Coronavirus 229E [1]	Zeptometrix	$1x10^{4.10}$ $TCID_{50}$/mL	-	29.47
Coronavirus NL63 [1]	Zeptometrix	$1x10^{3.75}$ $TCID_{50}$/mL	-	30.39
Coronavirus OC43 [1]	Zeptometrix	$1x10^{4.10}$ $TCID_{50}$/mL	-	28.83
SARS NC_004718 [1]	IDT	$5x10^4$ copies/mL	-	N/A
Haemophilus influenza [1]	Zeptometrix	$5x10^4$ CFU/mL	-	29.61
Legionella pneumophila [1]	Zeptometrix	$5x104$ CFU/mL	-	29.71
Mycobacterium tuberculosis [1]	ATCC	$5x10^4$ GEq/mL	-	N/A
Streptococcus pneumonia [1]	Zeptometrix	$5x10^4$ CFU/mL	-	32.70
Streptococcus pyogenes [1]	Zeptometrix	$5x10^4$ CFU/mL	-	29.82
Pseudomonas aeruginosa [2]	Genomic DNA	$2.6x10^4$ copies/mL	-	N/A
Streptococcus salivarius [1]	Zeptometrix	$5x10^4$ CFU/mL	-	29.77
Klebsiella pneumonia [2]	Genomic DNA	$6x10^4$ copies/mL	-	N/A

[1] Analysis Viracor Eurofins

[2] 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$_{50}$ - Tissue Culture Infection Dose 50

GEq - Genome equivalent