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Research Article

MICROBIOLOGICAL METHODS

Detection of SARS-CoV-2 Virus on Stainless-Steel Surfaces: AOAC *Performance Tested Method*^{SM} 022102

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Abstract

Background: The SureFast® SARS-CoV-2 PLUS Test is a reverse transcription qPCR (RT-qPCR) assay for the direct, qualitative detection of novel coronavirus (SARS-CoV-2) RNA from stainless-steel environmental sample swabs.
Objective: To validate the SureFast SARS-CoV-2 PLUS Kit as part of the AOAC Research Institute's Emergency Response Validation *Performance Tested Method(s)*^{SM} program.
Method: The SureFast SARS-CoV-2 PLUS Kit was evaluated for specificity using in silico analysis of 15 764 SARS-CoV-2 sequences and 65 exclusivity organisms (both near neighbors and background organisms) using the ThermoBLAST program. The candidate method was evaluated in an unpaired study design for one environmental surface (stainless steel) and compared to the US Centers for Disease Control and Prevention 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel, Instructions for Use (Revision 4, Effective 6/12/2020).
Results: Results of the in silico analysis demonstrated 99.99% selectivity of the method in being able to detect target sequences of the known CoV-2 genomes and discriminate them from near neighbors. In the matrix study, the candidate method demonstrated statistically significant better recovery of the target analyte than the PCR detection reference method.
Conclusions: The SureFast SARS-CoV-2 PLUS Kit is a rapid and accurate method that can be utilized by food producers to detect the causative agent of COVID-19 on stainless-steel contact surfaces.
Highlights: SureFast SARS-CoV-2 PLUS test method is highly specific for primer/probe binding to the E target genome region for the SARS-CoV-2 virus, 99.99% binding specificity using in silico analysis.

General Information

The novel coronavirus disease 2019 (COVID-19), was initially reported in China on December, 30 2019 (1) and the World Health Organization declared the growing outbreak a pandemic on March 11, 2020 (2). On January 10, 2020 Chinese and Australian scientists released the genome of the novel coronavirus (SARS-CoV-2) (3). The pandemic's growth rate at the end of 2020 sharply increased due to many factors. As of March 18, 2021, there have been 123.3 million COVID-19 cases and 2.7 million deaths worldwide (4). The biotechnology industry's ability to quickly develop test methods for the detection of the novel coronavirus-19 has been unprecedented and has provided tools to aid global public health workers.

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Principle

The SureFast® SARS-CoV-2 PLUS is a real-time reverse transcriptase-polymerase chain reaction (RT-PCR) for the direct, qualitative detection of intact SARS-CoV-2 RNA from stainless-steel swab samples. Each reaction contains an internal control RNA (ICR, consisting of MS2-bacteriophage) as an internal control of sample preparation procedure and to monitor possible PCR-inhibition. The RT-quantitative (q)PCR assay can be performed with commonly used real-time PCR instruments, equipped for detection of two fluorescence emissions at the channels FAM and VIC/HEX simultaneously.

Scope of Method

(a) *Analyte(s)*.—SARS-CoV-2 virus.
(b) *Matrix*.—Stainless-steel surface (2 × 2").
(c) *Performance claim*.—Performance comparable to the US Centers for Disease Control and Prevention (CDC) 2019-Novel Coronavirus Real-Time RT-PCR Diagnostic Panel, Revision 04 (5).

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/level dependent. Two different POD measures can be calculated: POD_R (reference method POD) and POD_C (candidate method POD).
(b) *Difference of probabilities of detection (dPOD)*.—Difference of probabilities of detection is the difference between any two POD values. If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.
(c) *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.
(f) *Selectivity*.—Ability of the method to detect analyte without interference from matrix or other components of similar behavior.

Materials and Methods

Test Kit Information

(a) *Kit names*.
  (1) *Swabbing kit with universal transport medium (UTM), Copan UTM Regular Flocked Swab + 1 mL UTM*.
  (2) *SureFast PREP DNA/RNA Virus*.
  (3) *SureFast SARS-CoV-2 PLUS*.
(b) *Cat. No*.
  (1) *Oropharyngeal Sample Collection Kit*.—359C.
  (2) *SureFast PREP DNA/RNA Virus*.—F1051.
  (3) *SureFast SARS-CoV-2 PLUS*.—F7110.
(c) *Ordering information*.
  (1) Worldwide: R-Biopharm AG, An der neuen Bergstr. 17, 62497 Darmstadt, Germany, info@r-biopharm.de, http://www.r-biopharm.de. Telephone: +49 61518102 0; Fax: +4961418102 -40.
  (2) US: R-Biopharm Inc., 870 Vossbrink Drive, Washington, MO 63090, USA. Telephone: (877) 789-3033; Fax: (866) 922-5856, C (636) 667-4388.
  (3) Swabbing kit: Copan Diagnostics, 26055 Jefferson Ave, Murrieta, CA 92562, USA, www.copanusa.com/. Telephone: (800) 216–4016; distributed in US exclusively by: DHI/Quidel (order no.: 405C); Headquarters/worldwide: Copan Diagnostics COPAN ITALIA spa Via F. Perotti 10, 25125 Brescia—Italy.

Test Kit Components

(a) *Swabbing*.—Tube with 1 mL UTM medium and designated swab.
(b) *SureFast PREP DNA/RNA Virus*.
  (1) *RNA/DNA Prep (F1051)*.—Kit components (per box).
  (2) *Binding buffer*.—One bottle 30 mL.
  (3) *Elution buffer*.—One bottle 5 mL.
  (4) *Pre-wash buffer*.—One bottle 40mL.
  (5) *Wash buffer*.—One bottle 60 mL.
  (6) *Extraction tubes*.—One bag 50×.
  (7) *One bag receiver tubes*.—One bag 2.0 mL, 50×.
  (8) *Receiver tubes*.—One bag 1.5 mL, 50×.
  (9) *One spin filter set*.—50×.
(c) *SureFast SARS-CoV-2 PLUS*.
  (1) *Real TimeRT- qPCR detection kit*.—F7110.
  (2) *Two vials reaction mix*.—2 × 1050 μL.
  (3) *One vial enzyme mix*.—80 μL.
  (4) *Two vials internal control RNA (ICR)*.—2 × 1800 μL.
  (5) *One vial PCR grade water*.—50 μL.
  (6) *One vial positive control*.—250 μL.

Additional Supplies and Reagents

(a) *Optional 5 × 5 cm stencil*.—To achieve reliable swabbing area size, according to relevant sampling SOP of the user.
(b) *Reaction tubes*.—1.5 mL.
(c) *Waterproof pen and tags*.—For labelling the reaction tubes.
(d) *Unpowdered disposable gloves*.
(e) *Pipets with filter tips*.
(f) *Vortex mixer*.
(g) *Microcentrifuge*.
(h) *Heating block (100 ± 1°C)*.
(i) *DNAse-/RNAse-free PCR grade water*.
(j) *Ethanol*.—≥96%.
(k) *Real-time qPCR instrument*.—With two detection channels for 510 and 580 nm, Bio Rad CFX 96 thermocycler.
(l) *Real-time PCR consumables*.—Plates, tubes, capillaries, foils, caps.
(m) *Heating block*.—For 1.5 and 2 mL tubes with shaking function for 65°C.

Apparatus

(a) *Safety cabinet and safety equipment*.—Suitable for working in a BSL2 environment (according to US standards).
(b) *Real-time qPCR instrument*.—As described in supplies and reagents: Bio Rad CFX 96 thermocycler detecting the channels 510 nm (FAM) and 580 nm (VIC, HEX).

Reference Materials Not Provided

(a) *NATtrol™ SARS-Related Coronavirus 2 (SARS-CoV-2) External Run Controls*.—Cat. No. NATSARS(COV2)-ERC. This is not a reference material control material for nucleic acid detection (ZeptoMetrix Corp., 878 Main Street Buffalo, NY 14202, 800-274-5487; www.zeptometrix.com).

(b) *MS2 phage*.—Used as a surrogate marker supplied in the detection kit as ICR. The ICR is detected in the ICR channel (VIC/HEX). MS2 phage is also available as *Escherichia* phage MS2 (*Enterobacteria phage MS2*) from DSMZ (DSM No.: 13767; or ATCC 15597-B1, NCCB 3463, NCIMB 10108).

Safety Precautions

SARS-CoV-2 is a highly infectious human pathogenic virus. Handling such classified human pathogenic material requests high safety precautions and safety practices. Cultures of SARS-CoV-2 strains needs to be handled in Biosafety Level 3 (BSL-3) laboratories, while routine analysis should be performed in at least BSL2+ laboratories. The national laws and guidelines for detection of potential positive SARS-CoV-2 samples should be followed.

Sample Preparation

(a) *RNA preparation with F1051*.—Before starting the preparation, add 20 mL ethanol to the pre-wash buffer and mix thoroughly. Add 48 mL ethanol to the wash buffer and mix thoroughly.

(b) The swab should be pre-moistened with 100 μL UTM directly prior to swabbing the surface. Swab a surface of approximately 25 cm^2 [equivalent to 2 × 2 inches] with the wet swab.

(c) Swab the surface according to the guidelines and put the swab into the vial of UTM. Label the sample and ship the sample to a designated laboratory used for analysis (48 h maximum transport duration at 2–8°C).

(d) *Sample preparation for surface swabbing after the external transport*.—Place the swab with the shaft in an extraction tube and cut the shaft so that the lid of the extraction tube can be closed. Add 400 μL of the transported UTM.

Sample Analysis

(a) Place the extraction tubes into a Thermomixer and incubate under continuously shaking for 15 min at 65°C and for 10 min at 95°C. After lysis, carefully squeeze out the swab on the wall of the tube and discard the swab.

(b) Add 400 μL binding buffer and mix the sample by vortexing.

(c) Transfer the complete sample in a spin filter set. Close the cap and centrifuge for 1 min at 12 000 rpm. Discard the receiver tube with the filtrate and place the spin filter in a new 2.0 mL receiver tube.

(d) Add 500 μL pre-wash buffer to the spin filter and centrifuge at 1 min for 10 000 rpm.

(e) Discard the filtrate and place the spin filter back into the receiver tube.

(f) Add 700 μL wash buffer to the spin filter and centrifuge at 1 min for 10 000 rpm.

(g) Discard the filtrate and place the spin filter back into the receiver tube.

(h) Remove the residual wash buffer by final centrifugation for 4 min at maximum speed.

(i) Place the spin filter into a clear 1.5 mL receiver tube and add 60 μL of the preheated (65°C) elution buffer directly onto the spin filter.

(j) Incubate for 3 min and centrifuge for 1 min at 10 000 rpm.

(k) After centrifugation discard the spin filter.

(l) The eluted nucleic acid is ready to use for PCR or RT-PCR. Store the nucleic acids at –20 or –80°C.

Analysis

(a) The test assay contains an ICR, which can either be used as PCR inhibition control or as positive extraction control for the sample preparation procedure. If the ICR is used only as a PCR inhibition control, 1 μL per reaction of the ICR should be added to the master mix. If the ICR is used as an extraction control for the sample preparation procedure and as PCR inhibition control, 20 μL of the ICR should be added during the extraction procedure.

(b) The ICR should be pipetted to the swab after removal from the transport tube directly prior the addition of the UTM medium in the extraction tube.

(c) Calculate the total number of reactions needed (samples and control reactions) for the specific PCR assay. Recommended control reactions for the specific PCR assay: negative control, negative extraction control (blank control), positive control.

(d) The test assay contains an ICR, which can either be used as PCR inhibition control (1μL/reaction in the master mix) or as positive extraction control (20 μL/reaction in the swab/UTM buffer at the earliest point of the RNA extraction). Three reactions for controls (1 × no-template control, 1 × extraction control, 1 × positive control) and reactions for samples should be calculated for the master mix preparation with 10% additional volume in total in order to compensate for reagent loss.

(e) Allow the reagents to thaw, then mix and centrifuge before opening and use.

(f) Pipet 20 μL of the master mix into all appropriate tubes/wells.

(g) Negative control.—Pipet 5 μl of PCR water into the designed tubes/wells and close them.

(h) Pipet 5 μL of sample RNA into the designated tubes/wells and close them.

(i) Pipet 5 μL of positive control into the designated tubes/wells and close them.

(j) Centrifuge all tubes/plates or capillaries for a short time at low speed.

(k) Place tubes/plates into the real-time PCR instrument and start the run according to the setup.

Calculations, Interpretation, and Test Result Report

(a) The evaluation has to be made according to the analysis program recommended by the real-time PCR instrument manufacturer.

(b) The control reactions have to show the correct results (also summarized in Table 1).

(c) SARS-CoV-2 RNA is detected in the FAM-channel. In the VIC/HEX-channel the internal amplification or positive extraction control (ICR) is detected.

Table 1. SureFast® SARS-CoV-2 PLUS interpretation of results

Result in the respective channel		
FAM channel Sars-CoV-2	VIC/HEX channel ICR	Interpretation
Positive	Positive/negative	SARS-CoV-2 detected
Negative	Positive	SARS-CoV-2 not detectable
Negative	Negative	Invalid

(d) High amplicon concentrations can result in a weak or absent signal of the internal amplification control (IAC). A Cp value for the IAC is not needed to obtain a positive result of the positive control.

(e) A sample is stated **positive** for SARS-CoV-2 if the sample RNA, transcribed during real-time RT-PCR in cDNA, shows amplification in the FAM-channel.

(f) A sample is stated **negative** for SARS-CoV-2 if the sample RNA shows no amplification in the FAM-channel and if the internal amplification or positive extraction control (VIC/HEX-channel) of the sample is **positive** with a shift in Cp-value ≤ 2 compared to the negative control.

(g) If the sample RNA in the VIC/HEX-channel shows **no amplification** or a shift in Cp-value > 2 compared to the negative control, it contains PCR inhibiting substances. A significant decrease in the fluorescence signal can also show the presence of PCR inhibiting substances. Under these circumstances, RNA isolation and purification of the sample need to be improved. Alternatively, the RNA can be diluted (recommendation 1:2 in PCR water) and analyzed again for inhibition. Please note that the dilution factor also affects the detection limit of the specific SARS-CoV-2 PCR assay.

Validation Study

Study Overview

The study was conducted according to the procedures outlined in the AOAC Research Institute (RI) Emergency Response Validation PTM Study Outline: *Validation Outline for Molecular Methods that Detect SARS-CoV-2 on Surfaces (V14, September 2020)*. The in silico analysis was performed by an in silico contractor for R-Biopharm. Stainless-steel test portions for the matrix studies were prepared by the independent laboratory, MRIGlobal. The surfaces were swabbed and shipped blind-coded to Microbac Laboratories, Inc. for analysis.

In Silico Analysis

Inclusivity and exclusivity.— To evaluate the inclusivity and exclusivity of the SureFast SARS-CoV-2 PLUS PCR Kit assay primers and probes, they were tested against a collection of 15 764 SARS-CoV-2 genomes obtained from Global Initiative on Sharing All Influenza Data (GISAID) (6). Nine near neighbors and 53 environmental background organisms were also evaluated. This genome list was provided by the AOAC RI and represents genomes collected between December 2019 and June 26, 2020. The list was filtered to remove duplicates and low-quality sequences defined as >300 Ns. We used ThermoBLAST™ (DNA Software, Inc., https://www.dnasoftware.com) to evaluate the inclusivity of our primer set against this genome data set.

ThermoBLAST analysis was used to search the complete genomes of 15 764 SARS-Cov-2 viruses for the locations of the primers and probes on the genome. ThermoBLAST returned 15 761 full-length forward primer hits with 100% identity (99.7%), 15 720 reverse primer hits (99.7%), and 15 629 full-length probe hits (99.1%). When combined, there are 15 747 genomes (99.9%) having all three primer and probe binding sites with at least partial identity. At least some of the missing binding sites appear to be due to ambiguous sequences (NNNN) in the primer or probe binding sites.

Forward Primer, Reverse Primer, and Probe Analysis

Target amplicons were identified using BLAST (nblast) by searching the forward primer, reverse primer, and probe sequences against the 15 764 SARS-Cov-2 complete genomes, allowing up to 90% homology and an Evalue of 1. The amplicon locations were converted to BED format using a custom script, which kept only the best match for every genome. Then the amplicon sequences were extracted using the scripts faToTwoBit and twoBitToFa from the UCSC Genome browser.

Correct binding sites and folded structures for the primers and probes were obtained using ThermoBLAST using the buffer and primer concentrations, as well as temperatures, set in the above sections, and using the amplicons generated by BLAST as the target sequences. Custom scripts were used to feed sequences to ThermoBLAST, to process the results, and to generate alignment structures.

Binding free energies, melting temperatures, and percentage bound (%Bound) were calculated using OMP DE (DNA Software, Inc.) using the above buffer and primer concentrations, as well as temperatures and sequences set in the above sections. Custom scripts were used to feed sequences to OMP DE and to process the results.

Unimolecular Folding

Amplicon sequences were extracted from the SARS-Cov-2 complete genomes with a pad of 150 bp on either side of the 104 bp amplicon. These were folded using OMP DE. Folding was done under the conditions described above for both the starting RNA and the amplified cDNA. Each primer/amplicon pair was assigned a PairID to make it easier to track individual interactions and their results. The calculated binding free energies and Tms varied from 66.32–74.47°C for the target monomers.

It was shown that the RNA folds have substantially more stability than the DNA folds, but all of the structures should be largely melted out in the 95°C denaturation steps of each PCR cycle. Thus, the primers and probes should not be prevented from binding.

The predicted folded structures for the forward primer, the reverse primer, and the probe sequences at the annealing temperature of 60°C showed that none of these structures are significant or any cause for concern. The T_ms for these structures (39, 22, and 52°C, respectively) are predicted to be well below the annealing temperatures used in the PCR reaction.

Bimolecular Hybridization

Hybridization reactions were carried out using OMP DE (DNA Software, Inc.). The net Tm for the oligo binding to the target

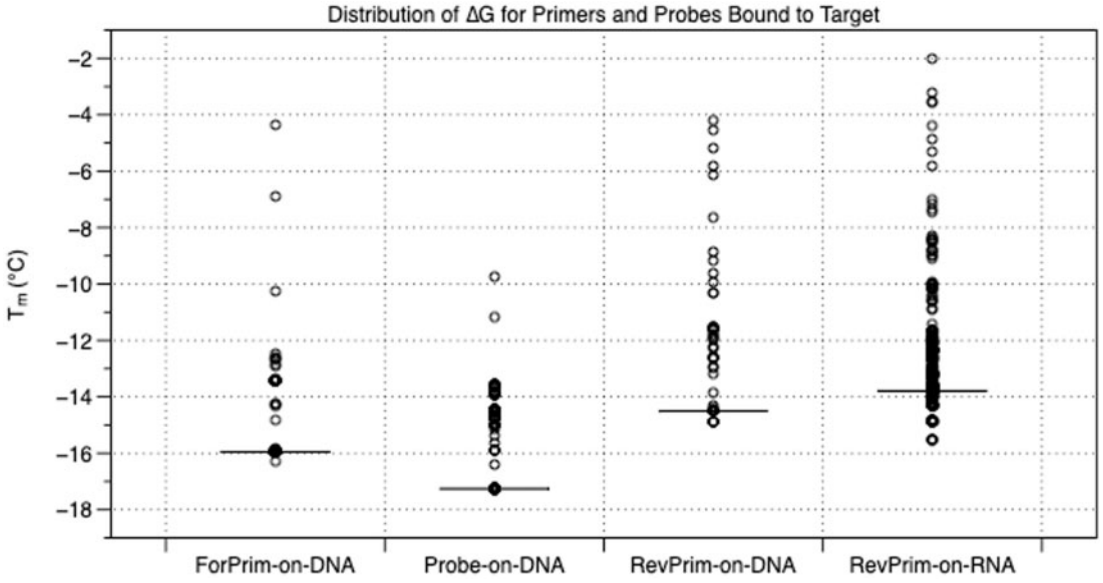

Figure 1. Distribution of binding free energies (ΔG) for oligo-target pairs at the annealing temperature (60°C for DNA; 58°C for RNA).

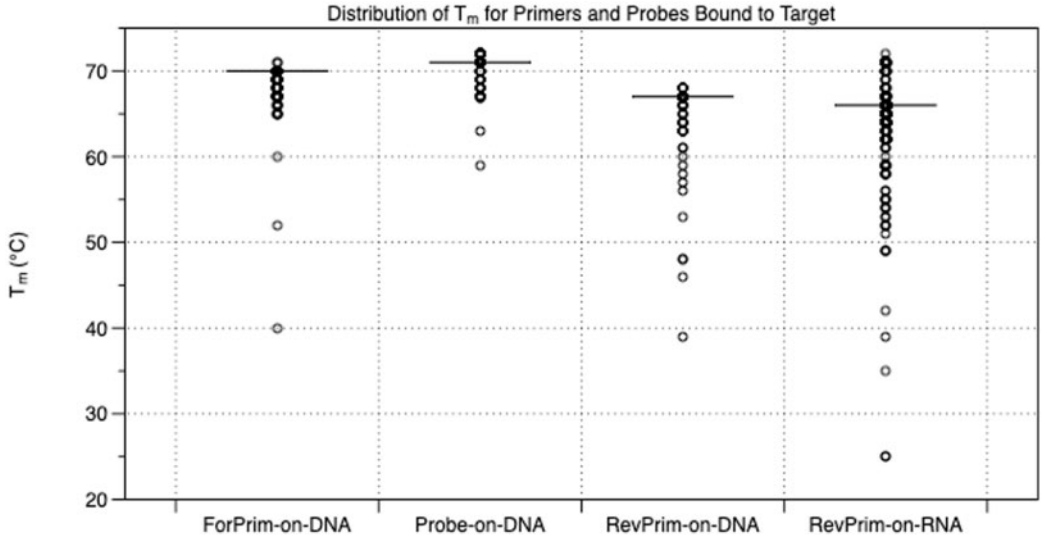

Figure 2. Distribution of melting temperatures (T_m) for oligo-target pairs at the annealing temperature (60°C for DNA; 58°C for RNA).

fragment is 69–70°C and the predicted percentage of target bound is 99.991%.

In Figures 1–3, the entire distributions are shown for binding free energies (Figure 1), T_ms (Figure 2), and predicted %Bound at the annealing temperature (Figure 3) for the oligo-target interactions. In each case, a small number of outliers show weak binding, low T_ms, and poor %Bound. Table 2 shows the number of targets with less than 80% of the oligo bound at annealing temperature or a Tm below 65°C—a safe 5–7°C above the annealing temperature. The reverse primer binding to RNA has the worst predicted %Bound (<80%) and low T_ms. However, even in this worst case, the number of "weak" T_m values is only 1.1% of all the SARS-Cov-2 genomes.

Exclusivity Analysis

The exclusivity of the PCR primer and probe set was evaluated by searching for the primers and probe on a set of nine viral genomes defined by AOAC (Table 3).

Both primers (forward and reverse) and the probe sequence were searched on these genomes using Thermo BLAST, and hits were examined to determine: (1) whether they had sufficient binding free energy to bind under annealing conditions; (2) whether the 3' ends of the bound oligos could be extended by polymerase (i.e., at least three base pairs present at the 3' end); and (3) whether the two primers and the probe would all form a suitable amplicon of 50–200 bp if the primers did indeed get amplified by polymerase. To determine suitable potential off-target binding, a Tm lower limit of 48°C was set. At this temperature, with the annealing temperature set at 60°C, less than 0.1% of the oligo is expected to be bound to the off-target sequence. Four potential off-target sites met the Tm cutoff, of which three met the extensibility criteria. None of those potential off-target interactions could form an effective amplicon, however, because a complete set of forward primer, reverse primer, and probe were lacking to form a complete amplicon. Thus,

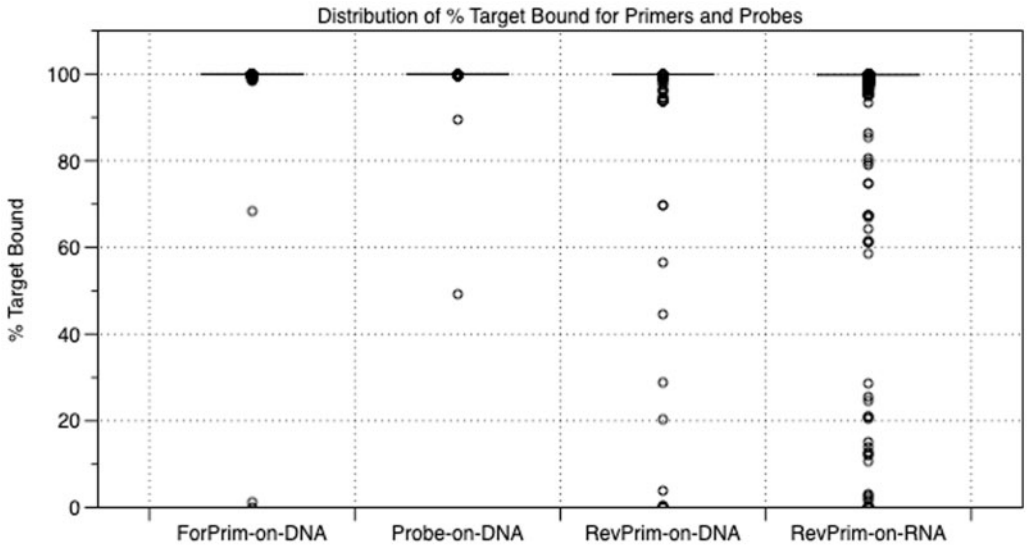

Figure 3. Distribution of predicted %Bound for oligo-target pairs at the annealing temperature (60°C for DNA; 58°C for RNA).

Table 2. Number of oligo-target interactions with %Bound < 80% or Tm < 65°C

Oligo Group	%Bound < 80 %, *n*	Tm < 65°C, *n*
Forward Primer	4	3
Probe	1	2
Reverse Primer on DNA	12	31
Reverse Primer on RNA	37	172

Table 3. List of genomes used for the exclusivity evaluation

Species name	Accession No.
Human coronavirus 229E	NC_002645.1
Human coronavirus OC43	NC_006213.1
Human coronavirus NL63	NC_005831.2
Human coronavirus HKU1	NC_006577.2
SARS-coronavirus	NC_004718.3
	NC_019843.3
MERS-coronavirus	NC_038294.1
	NC_045512.2
Porcine deltacoronavirus	NC_039208.1

there is no risk of amplicon generation from any of the nine genomes evaluated.

Background Analysis

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

A minimum set of background genomes established by AOAC guidelines was used (27 viruses, 23 bacteria and fungi, and eight eukaryotes).

Viruses

The viral analysis included 27 virus species established by the AOAC protocol (Table 4). The same methods and criteria were used as were described in *Exclusivity Analysis*. Only one forward primer had sufficiently high Tm (52°C) but it failed the extensibility criteria. Thus, there is no risk of amplicon generation from any of the 27 genomes evaluated.

Bacteria and fungi

This analysis included 23 species of bacteria and fungi established by the AOAC guidelines (Table 5). ThermoBLAST failed to return any hits whatsoever against this data set, indicating either that the potential duplexes were so poor that they were eliminated from the analysis or that some defect of the data caused ThermoBLAST to fail. To check for the latter possibility, a (regular) BLAST search was performed on the data set allowing as low as 65% sequence identity over the full-length oligos and up to three internal mismatches. The search returned six hits, all to different genomes. Thus, there is no risk of amplicon generation from any of the 23 species evaluated.

Eukaryotes

The eukaryotic analysis included eight eukaryotic species established by the AOAC guidelines (Table 6). The same methods and criteria were used as were described in *Exclusivity Analysis*, except that human genomic DNA and RefSeq transcriptomic RNA was searched separately to ensure complete coverage of the human genome. The search returned 300 hits that showed Tm values in excess of 48°C and for which the primers could potentially be extended by polymerase, however, only one set of four hits contained a forward primer, reverse primer, and probe hitting the same target (human chr6). That set also fails in that the forward and reverse primers would generate an amplicon of 24 000 000 bp in length, with the two probe binding sites outside of the amplicon. Thus, there is no risk of amplicon generation from any of the eight eukaryotic species evaluated.

Table 4. Viral genomes used for background analysis

Species name[a]
Bovine coronavirus
Enterovirus
Enterovirus D68
Human adenovirus 1
Human alphaherpesvirus 3
Human bocavirus
Human metapneumovirus
Human orthorubulavirus 2
Human orthorubulavirus 4
Human respirovirus 1
Human respirovirus 3
Infectious bronchitis virus
Influenza A H7N9 subtype
Influenza A virus
Influenza A virus H1N1
Influenza B virus
Norovirus
Respiratory syncytial virus
Simplexvirus
Transmissible gastroenteritis virus

[a] Accession numbers available upon request.

Table 5. Bacterial and fungal genomes used for background analysis

Species name[a]
[Candida] glabrata
Acinetobacter baumannii
Acinetobacter baylyi
Acinetobacter bereziniae
Acinetobacter calcoaceticus
Acinetobacter chinensis
Acinetobacter cumulans
Acinetobacter defluvii
Acinetobacter dispersus
Acinetobacter equi
Acinetobacter guillouiae
Acinetobacter haemolyticus
Acinetobacter junii
Acinetobacter lactucae
Acinetobacter lanii
Acinetobacter larvae
Acinetobacter nosocomialis
Acinetobacter oleivorans
Acinetobacter phage ZZ1
Acinetobacter pittii
Acinetobacter schindleri
Acinetobacter seifertii
Acinetobacter shaoyimingii
Acinetobacter wanghuae
Bacillus cereus
Bacillus thuringiensis
Bordetella pertussis
Candida albicans
Chlamydia pneumoniae
Clostridioides difficile
Enterococcus casseliflavus
Enterococcus cecorum
Enterococcus faecium
Enterococcus hirae
Enterococcus lactis
Enterococcus mundtii
Enterococcus rotai
Enterococcus saigonensis
Enterococcus thailandicus
Enterococcus wangshanyuanii
Escherichia coli O157: H7 str. Sakai
Escherichia coli str. K-12 substr. MG1655
Haemophilus influenzae
Klebsiella pneumoniae
Legionella pneumophila
Listeria monocytogenes
Acinetobacter seifertii
Acinetobacter shaoyimingii
Acinetobacter wanghuae
Bacillus cereus
Bacillus thuringiensis
Bordetella pertussis
Candida albicans
Chlamydia pneumoniae
Clostridioides difficile
Enterococcus casseliflavus
Enterococcus cecorum
Enterococcus faecium
Enterococcus hirae
Enterococcus lactis
Enterococcus mundtii
Enterococcus rotai
Enterococcus saigonensis
Enterococcus thailandicus
Enterococcus wangshanyuanii
Escherichia coli O157: H7 str. Sakai
Escherichia coli str. K-12 substr. MG1655
Haemophilus influenzaeinfluenza
Klebsiella pneumoniae
Mycobacterium tuberculosis
Mycoplasma pneumoniae
Pneumocystis jirovecii MT seq
Pseudomonas aeruginosa
Staphylococcus aureus
Staphylococcus epidermidis
Streptococcus pneumoniae
Streptococcus pyogenes
Streptococcus salivarius

[a] Accession numbers available upon request.

Independent Laboratory Studies

Coronavirus isolate and genomic copies/mL determination.— 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). SARS-CoV-2 was sourced from the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA). The virus stock was received from WRCEVA as a 1 mL lyophilisate. Upon receipt the lyophilisate (lot TVP23155) was resuspended in 2 mL of DMEM/F12 media and single-use aliquots frozen at –70°C. The plug forming units (PFU) per mL quantitation information was provided by WRCEVA (3.6×10^6 PFU/mL). Genomic copies (GC) per mL were determined by MRIGlobal as described below using one of the frozen viral stock aliquots.

Table 6. List of eukaryote and fungal genomes used for background analysis

Species name	Accession No.
Homo sapiens	GCF_000001405
Aedes aegypti	GCF_002204515
Aedes albopictus	GCF_006496715
Dermatophagoides pteronyssinus	GCF_001901225
Musa domestica	GCF_000371365
Drosophila	GCF_000001215
Chlorocebus sabaeus	GCA_000409795

Table 7. CDC assay thermal cycling parameters

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

Table 8. CDC assay master mix preparation

Reagent	Volume per reaction, μL
Nuclease-free water	8.5
Primer/probe mix	1.5
TaqPath 1-step RT-qPCR Master Mix	5.0
Total master mix	15.0
Sample volume	5.0
Well total	20.0

Viral genomic copies per mL (GC/mL) was determined by quantitative real time RT-PCR using a Bio-Rad CFX96 Real-Time Detection System. The standard curve was prepared using synthetic SARS-CoV-2 RNA (ATCC #VR-3276SD). The qPCR procedure used N1 primer and probe sequences published by the CDC (7). 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-nCoV Real-Time RT-PCR Diagnostic Panel Instructions for Use and are summarized in Table 7 (8).

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}. Master mix was prepared as noted in Table 8.

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

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

The presence of infectious SARS-CoV-2 in the WRCEVA virus stock was verified using standard cell culture techniques. Briefly, 3×10^6 Vero E6 cells were plated into a T75 flask with 15 mL infection media (Dulbecco's Modified Eagle's medium supplemented with 5% fetal bovine serum and nonessential amino acids) and incubated in a humidified incubator with 5% CO_2. The following day the Vero cells were re-fed with infection media and inoculated with virus stock. Cells were incubated for 5 days at which point widespread cytopathic effect (CPE) was apparent by microscopic examination of the Vero cells.

Matrix Study

Test plate inoculation.— Dilutions of SARS-CoV-2 virus stock were prepared in Virus transport medium (VTM) starting from two pooled virus stock aliquots as shown in Table 9. The same concentrations of virus were used for inoculating test areas for both the reference (CDC RT-PCR) and candidate (SureFastSARS-CoV-2 PLUS) methods.

Square 14 × 14" grade 304 stainless-steel plates were used for the studies to mimic food preparation surfaces. All test plates were cleaned, disinfected, and autoclaved prior to use. Test grids of 2 × 2" test areas were created on the test plates using laboratory tape. To inoculation the test plates, the volume specified in Table 9 was pipetted onto the appropriate test area and spread evenly over the entire test area with a sterile 10 μL inoculating loop. Inoculated plates were left until visibly dry (up to 1 h) in a biosafety cabinet (BSC) then transferred to a sealed plastic container and stored overnight at room temperature (21 h). Temperature and humidity ranged from 21.7–25.2°C and 28–34% relative humidity, respectively.

Reference method plate sampling.— After drying overnight, test areas on the reference method test plates were sampled as follows: A swab (Puritan, 25-1607 1PFSC; lot 7168) was pre-moistened by dipping into a 15 mL conical tube containing 2.0 mL of VTM. The pre-moistened swab was used to sample the 2 × 2" test area by rubbing the swab in at least two different directions while applying pressure to the surface and rotating the swab head. After sampling the test area, the swab was snapped at the break point and placed back into the VTM tube. A random sample ID was assigned to each test area sample. Swab samples were placed in a refrigerator (2–8°C) within 15 min of test area sampling and stored overnight (22 h) before nucleic acid extraction.

Candidate method plate sampling.— Test areas on the candidate method test plates were sampled using the provided Copan swab collection kit. Briefly, the swab tip was pre-wetted by dipping the swab into the tube of UTM provided in the Copan kit. The pre-moistened swab was then used to sample the 2 × 2" test area by rubbing the swab back and forth in at least two different directions while applying pressure to the surface and rotating the swab head. After sampling the test area, the swab tip was broken off at the score mark and placed back into the UTM tube that was used to pre-wet the swab. Each sample tube was assigned a unique random ID number (a key correlating test area sample to random ID number was created and sent to AOAC). Swab samples were shipped overnight to

Table 9. SARS-CoV-2 dilutions in VTM

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

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

Table 10. Components used for reference method RT-PCR testing

Component	Vendor/manufacturer	Part No.	Lot No.	Expiration
QIAamp Viral RNA Mini Kit	Qiagen	52906	166023562	3/1/2022
2019-nCoV CDC RUO Kit	IDT	10006713	0000535573	4/8/2022
TaqPath 1-Step RT-qPCR Master Mix, CG	ThermoFisher	A15299	2220404	4/30/2021

Microbac Laboratories with an ice pack on the day of sampling.

Reference method RT-PCR testing.— Samples from the reference method test plate were transferred to an operator, who was not aware of the blinded sample identities, for testing with the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel test kit. RNA was extracted from 140 μL of sample using the Qiagen QIAamp Viral RNA Mini Kit per the manufacturer's instructions. Extracted RNA was tested with the CDC Panel on an Applied Biosystems 7500 Fast Dx Real-Time PCR Instrument following published instructions (9). Components used for the reference method RT-PCR testing are summarized in Table 10. Fractional positive results were seen with the 0.5 POD sample set. Reference method test results were sent to AOAC for comparison with the candidate method.

Testing with the R-Biopharm SureFast SARS-CoV-2 PLUS kit started on November 3, 2020 and was completed on November 4, 2020, with shipment of the swab samples to Microbac Laboratories. Reference method testing started on November 3, 2020 and was completed on November 5, 2020, with nucleic acid extraction and RT-PCR. Reference method test results were sent to AOAC on November 5, 2020. Fractional positive results were seen with the 0.5 POD sample set on the teference method. See the overall POD results for the candidate versus the reference method in Table 11. Results for individual test portions for the candidate and the reference method may be found in Table 12.

Discussion

Results from the POD analysis demonstrate that the the SureFast SARS-CoV-2 RT-PCR is better at detecting low concentrations (2×10^3 GU/2 × 2” test surface or 25 cm^2) of deposited SARS-CoV-2 on a stainless-steel surface compared to the CDC reference method when using the same swabbing sample preparation and swabbing procedure for both the RT-qPCR primers and probes of the candidate method and the reference method.

The in silico analysis of the primers and probes utilized in the SureFast SARS-CoV-2 RT-PCR test method are specific and sensitive enough (99.99% binding of the oligomer and the target binding region) to detect low levels of SARS-CoV-2 without exhibiting false negatives when compared to the CDC reference method. The high level of specificity could be due to the single target assay (E gene) requirement of the SureFast SARS-CoV-2 RT-PCR test method in comparison to the double-target assay (N1 and N2 SARS-CoV-2 gene targets) of the CDC reference method. Competition in amplification efficiency between two targets and/or RNA degradation on surfaces may contribute to a single target assay readily detecting one target over a double-target assay. Another reason that the SureFast SARS-CoV-2 PLUS RT-PCR test method provides better results than the CDC reference method may be due to the different swabs used. The swab used in the SureFast method may have better recovery of the virus from stainless-steel surfaces. Since there is no prescribed swabbing method in the CDC reference method, it is unknown what role the swab material plays in virus recovery.

Conclusions

The in silico analysis indicates that the SureFast SARS-CoV-2 PLUS qPCR primers and probes are well designed to bind to and detect the current known genomes of SARS-CoV-2. The sequences successfully target 99.991% of the known Cov-2 genomes. Self-folding of the primers, probes, and amplicon targets do not appear to be a problem. The oligonucleotide sequences largely bind to their targets with affinities and predicted Tms that should ensure effective amplification under the buffer conditions and cycling protocols described here.

Table 11. Stainless steel candidate versus reference method—POD results

				Candidate SureFast SARS-CoV-2			Reference			$dPOD_C$[f]	95% CI[g]
Matrix	Strain	GU/test area[a]	N[b]	x[c]	POD_C[d]	95% CI	x	POD_R[e]	95% CI		
Stainless Steel (2 x 2")	SARS-CoV-2 BEI NR-52281	0	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	−0.43, 0.43
		2.0×10^3	20	20	1.00	0.84, 1.00	11	0.55	0.34, 0.74	0.55	0.20, 0.66
		2.0×10^4	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	−0.43, 0.43

[a] Results of the GU/test area were determined by plating the inoculum for each matrix in triplicate.
[b] N = Number of test portions.
[c] x = Number of positive test portions.
[d] POD_C = Candidate method confirmed positive outcomes divided by the total number of trials.
[e] POD_R = Reference method confirmed positive outcomes divided by the total number of trials.
[f] $dPOD_C$= Difference between the confirmed candidate method result and reference method confirmed result POD values.
[g] 95% CI = If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.

Table 12. Individual results for the stainless-steel matrix study

Sample No.	SureFast SARS-CoV-2 Plus	CDC N1 target result	CDC N2 target result	CDC final result
1	+	+	+	+
2	+	-	-	-
3	+	-	+	-
4	+	-	+	-
5	+	+	+	+
6	+	+	+	+
7	+	+	+	+
8	+	-	+	-
9	+	+	+	+
10	+	+	+	+
11	+	+	+	+
12	+	-	+	-
13	+	+	+	+
14	+	-	+	-
15	+	-	+	-
16	+	+	+	+
17	+	-	+	-
18	+	-	+	-
19	+	+	+	+
20	+	+	+	+
Total	20/20	11/20	19/20	11/20
	High level			
1	+	+	+	+
2	+	+	+	+
3	+	+	+	+
4	+	+	+	+
5	+	+	+	+
Total	5/5	5/5	5/5	5/5
	Non-inoculated control level			
1	-	-	-	-
2	-	-	-	-
3	-	-	-	-
4	-	-	-	-
5	-	-	-	-
Total	0/5	0/5	0/5	0/5

The matrix data from this study supports the product claim that the SureFast SARS-CoV-2 RT-PCR test method can detect SARS-CoV-2 from stainless-steel surface samples and should be granted Emergency Response Validation for PTM certification.

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Conflict of Interest

The author declares that they have no conflict of interest.

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