Validation of the Thermo Scientific™ SARS-CoV-2 RT-PCR Detection Workflow for the Detection of SARS-CoV-2 from Stainless Steel Environmental Surface Swabs

**AOAC Performance Tested Method**SM 012103

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**Background:** The Thermo Scientific™ SARS-CoV-2 RT-PCR Detection Workflow, packaged with Applied Biosystems™ TaqMan™ 2019-nCoV Assay Kit v1 targets three different SARS-CoV-2 genomic regions in a single RT-PCR reaction.

**Objective:** To validate the Thermo Scientific™ SARS-CoV-2 RT-PCR Workflow, for the detection of SARS-CoV-2 virus on stainless steel surfaces as part of the AOAC Performance Tested MethodSM Emergency Response Validation program.

**Methods:** The Applied Biosystems™ TaqMan™ 2019-nCoV Assay Kit v1, as part of the Thermo Scientific SARS-CoV-2 RT-PCR Workflow was evaluated for specificity using *in silico* analysis of 15,764 SARS-CoV-2 sequences and 65 exclusivity organisms. The Thermo Scientific SARS-CoV-2 RT-PCR Workflow was evaluated in an unpaired study for one environmental surface (stainless steel) and compared to the U.S. Centers for Disease Control and Prevention 2019-Novel Coronavirus RT-PCR Diagnostic Panel,
Results: *In silico* analysis showed that of the 15,756 target SARS-CoV-2 genomes analyzed, 99% of the strains/isolates are perfectly matched to at least two of the three assays, and more than 90% have 100% homology to all three assays (ORF1ab, N-gene, S-gene) in the SARS-CoV-2 Kit. None of the 65 non-target strain genomes analyzed showed matching sequences. In the matrix study, the Thermo Scientific SARS-CoV-2 workflow showed comparable detection to the CDC method.

Conclusions: The Thermo Scientific SARS-CoV-2 RT-PCR Workflow is an effective procedure for detection of RNA from SARS-CoV-2 virus from stainless steel.

Highlights: The workflow provides equivalent performance results with the two tested RNA extraction platforms and the two tested RT-PCR Instruments.

General Information

SARS-CoV-2 is a public health issue, and not a food-borne virus. However, it is obvious that some food production segments have faced contamination in the working environment. Coronaviruses have been shown to survive (remain viable) on different surfaces, for up to twenty-eight days, under various conditions such as temperature, relative humidity, and light (1). SARS-CoV-2 can be transmitted when a healthy person touches contaminated food or environmental contact surfaces (including packaging materials) (2).

Proper cleaning, surveillance, and the prevention of cross-contamination are critical in the control of foodborne illnesses. The application of sound principles of environmental sanitation, personal hygiene and established food safety practices reduces the likelihood that harmful pathogens, including SARS-CoV-2, may threaten the safety of the food supply, regardless of how it is produced. In July 2020, Thermo Fisher Scientific applied to the SARS-CoV-2 Emergency Response Program of the AOAC Research

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Institute’s *Performance Tested Methods*SM Program for the detection of SARS-CoV-2 on environmental surfaces.

**Principle of the Method**

The Applied Biosystems™ TaqMan™ 2019-nCoV Assay Kit v1 contains a set of TaqMan™ RT-PCR assays for the detection and characterization of SARS-CoV-2. The kit includes three assays that target SARS-CoV-2 genes and one positive control assay that targets the human RNase P RPPH1 gene. Features of the TaqMan™ 2019-nCoV Assay Kit include: 2019-nCoV assays targeting three different viral genomic regions that are run together in a single PCR reaction and detected on the same channel, highly specific target sequences generated by robust bioinformatic selection that are unique to SARS-CoV-2 and show no cross-reactivity with one another, and an RNase P internal positive control run in duplex with the 2019-nCoV assays.

The TaqMan™ 2019-nCoV Assay Kit is used with the TaqMan™ 2019-nCoV Control Kit v1 to monitor assay-specific amplification. Additionally, the TaqMan™ 2019-nCoV Control Kit v1 is a synthetic positive control; the TaqMan™ 2019-nCoV Control Kit v1 contains target sequences for each of the assays included in the TaqMan™ 2019-nCoV Assay Kit v1. The control includes synthetic DNA target sequences for three SARS-CoV-2 genes (ORF1ab, S protein, and N protein) and the human RNase P RPPH1 gene. During RT-PCR, 1 μL of the 2019-nCoV Control v1 in 9.75 μL PCR-grade water is used as the sample for the positive-control reaction.

The positive control is intended to demonstrate correct setup of PCR reagents and the correct extraction protocol has been conducted, as well as presence of PCR inhibitors which may negatively impact the other targets. As long as a positive reaction is observed in each well for the positive control,
PCR is proven to be a success. The control can be used in SARS-CoV-2 detection to verify assay performance and to help with troubleshooting.

Both the TaqMan™ 2019-nCoV Assay Kit and the TaqMan™ 2019-nCoV Control Kit v1 use Applied Biosystems™ TaqMan® MGB probes.

Scope of method

(a) Analyte.—SARS-CoV-2 Virus.

(b) Matrices.—Stainless steel environmental surface (e.g. such as those in production or laboratory settings).

(c) Summary of Validated Performance Claims.—The Thermo Scientific SARS-CoV-2 Real Time PCR Workflow demonstrated comparable performance to the CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time Diagnostic Panel Instructions for Use (Revision 4, Effective 6/12/2020;2) for the 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. 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 (4).
In silico.—The use of computer simulation to evaluate target and non-target sequences for molecular methods.

Materials and Methods

Test Kit Information

(a) Kit Names and Catalog numbers.—The Thermo Scientific SARS-CoV-2 Real Time PCR Workflow using: PrepSEQ™ Nucleic Acid Extraction Kit for Food and Environmental Testing with KingFisher™ Flex-96 Deep-Well Magnetic Particle Processor or MagMAX™ Express-96 Deep Well Magnetic Particle Processor, TaqMan™ 2019-nCoV Assay Kit v1, TaqMan™ 2019-nCoV Control Kit v1 (Cat. No. A47533), RNA UltraSense™ One-Step qPCR Master Mix on Applied Biosystems™ QuantStudio™ Design and Analysis Software v1.5.1 or later or Applied Biosystems™ 7500 Software SDS v.1.4.2 or later

(c) Ordering Information.—All materials are available through the Thermo Fisher Microbiology ordering process or thermofisher.com

Test Kit Components

RNA Extraction Kit (one of the following):

(a) PreSEQ™ Nucleic Acid Extraction Kit (Cat. No. 4480466; 100 total NA isolations).

(1) Lysis Buffer.—2 x 50 mL.

(2) Magnetic Particles.—2 x 1.5 mL.

(3) Binding Solution (Isopropanol).—One empty bottle provided.

(4) Wash Buffer Concentrate.—2 x 26 mL.

(5) Elution Buffer.—1 x 25 mL.

(6) Proteinase K Buffer.—1 x 1.25 mL.
(b)  PrepSEQ™ Nucleic Acid Extraction Kit (Cat. No. 4428176; 300 total NA isolations).

1. Lysis Buffer.—6 x 50 mL.
2. Magnetic Particles.—6 x 1.5 mL.
3. Binding Solution (Isopropanol).—Three empty bottles. (user provides?)
4. Wash Buffer Concentrate.—6 x 26 mL.
5. Elution Buffer.—3 x 25 mL.
6. Proteinase K Buffer.—3 x 1.25 mL.

Automated nucleic acid extraction system and materials

Extraction Instrument (one of the following):

(a)  KingFisher™ Flex 96 Deep Well System.

1. KingFisher™ Flex Magnetic Particle Processor with 96 Deep-Well Head (Cat. No. A32681).
2. KingFisher™ Flex Deepwell 96 Plate (Cat. No. 95040450).
3. KingFisher™ 96 tip comb for DW magnets (Cat. No. 97002534).

(b)  MagMAX™ Express-96 Deep well Magnetic Particle Processor.

1. MagMAX™ Express-96 Deep well Particle Processor with 96 Deep-Well Head.
2. KingFisher™ Flex Deepwell 96 Plate.
3. KingFisher™ 96 tip comb for DW magnets.

Assay Kits

(a)  TaqMan™ 2019nCoV Assay Kit v1 (Cat. No. A47532).

1. 2019-nCoV (ORF1ab) Assay, FAM dye, 75 µL, 20X.
2. 2019-nCoV (S protein) Assay, FAM dye, 75 µL, 20X.
(3) 2019-nCoV (N protein) Assay, FAM dye, 75 µL, 20X.

(4) RNase P Assay, VIC dye, 250 µL, 20X.

(b) TagMan™ 2019nCoV Control Kit v1 (Cat. No. A47533).—One tube containing 50 µL (1 x 10^4 copies/µL).

Master Mix

(a) RNA UltraSense™ One-Step Quantitative RT-PCR System (Cat. No. 11732927).

(1) 250 µL RNA UltraSense™ Enzyme Mix.

(2) 1 mL RNA UltraSense™ 5X Reaction Mix.

(3) 300 µL 20X Bovine Serum Albumin (BSA).

(4) 1 mL 50-mM magnesium sulfate (MgSO_4).

(5) 100 µL ROX Reference Dye.

(c) Total RNA Control (Human, Cat. No. 4307281 or equivalent).

Real-Time PCR Instrument (use one of the following)

(a) Applied Biosystems™ QuantStudio™ 5 Food Safety Real-Time PCR System (Cat. No. A36328 OR A36320).—96-well, 0.1 mL Block, Laptop, QuantStudio™ Design Analysis Software v1.5.1 or later (included).

(b) Applied Biosystems™ 7500 Fast Food Safety Real-Time PCR System (Cat. No. A30299 OR A30304).—96-well, 0.1 mL block, laptop, 7500 Software SDS Software v1.4 or later (included).

Apparatus

(a) Laboratory freezers, -30°C to -10°C and ≤-70°C.
(b) **Centrifuge, with a rotor that accommodates deepwell microplates.**

(c) **Microcentrifuge.**

(d) **Laboratory mixer, vortex or equivalent.**

(f) **Single and multichannel adjustable pipettors.**—1.00 µL to 1,000 µL.

(g) **Cold block.**—96 well or 384 well or ice.

(h) **MicroAmp™ Optical 96-Well Reaction Plate, 0.1 mL (Cat. No. 4346907 or equivalent)**

or

**MicroAmp™ Optical 8-Cap Strip (Cat. No. 4323032).**

(i) **MicroAmp™ Optical Adhesive Film (Cat. No. 4313663 or equivalent)**

or

**MicroAmp™ Optical Fast 8-Tube Strip (0.1 mL) (Cat. No. 4358293).**

(l) **Disposable gloves.**

(m) **Micropipette tips, aerosol resistant.**

(n) **Non-cellulose swabs with synthetic tip.**

Reagents

(a) **Thermo Scientific™ Oxoid™ Phosphate Buffered Saline Tablets (Cat. No. BR0014G).**

(c) **Ethanol, 95%.**

(d) **Isoproranol, 100%.**

(e) **Nuclease-free water.**

Safety Precautions

(a) **Resources.**—The Thermo Scientific SARS-CoV-2 Real-Time PCR Workflow should be performed
by qualified and trained staff to avoid the risk of erroneous results. Samples should always be treated as
if infectious and/or biohazardous in accordance with safe laboratory procedures.

Follow necessary precautions when handling specimens. Use personal protective equipment (PPE)
consistent with current guidelines for the handling of potentially infectious samples. Laboratories shall
implement the method and ensure data reporting, in compliance with local regulations.

(b)  Surface sampling and sample transport.—Follow good laboratory practices and all precautions
and guidelines in these user guides to avoid cross-contamination between samples.

Samples must be collected, transported, and stored using appropriate procedures and conditions.
Improper collection, transport, or storage of specimens may hinder the ability of the assay to detect the
target sequences.

Refer to the ISO 15216-1: 2017 standard (5) and the “Surface sampling of coronavirus disease
(COVID-19): A practical “how to” protocol for health care and public health professionals”, published by
the World Health Organization (WHO) (6).

(c)  Real-Time PCR.—This workflow uses purified RNA as a sample for analysis which is processed
through a reverse transcription polymerase chain reaction. The quality of the RNA recovered from
environmental samples is essential for the quality of the results generated with the method. Following
the completion or partial completion of a Real-Time PCR run, it is important that PCR Tubes are not
opened, because amplified DNA (amplicon) in the tubes could easily contaminate the equipment and
surrounding laboratory environment, potentially leading to false-positive results in future analyses. The
reagents should not pose a safety concern when used as directed in the method instructions. Dispose of
waste lysate, eluted RNA and PCR tubes according to local guidelines.

Refer to the Real-Time PCR Instrument Manual for guidelines on cleaning equipment and handling
possible amplicon contamination. Positive and negative test controls must be included to accurately
interpret test results.

(d) **Consumables and reagents.**—For disposal of surface sampling or any of the reagents and materials included in the Thermo Scientific SARS-CoV-2 Real-Time PCR Workflow and associated tests, refer to the manufacturer’s material safety data sheets and apply appropriate local guidelines.

**Sample Collection and Storage**

(a) Pre-moisten swab with PBS or viral transport medium (the workflow utilized PBS in this study).

(b) When sampling, apply pressure with the wet swab onto the surface, move in at least two different directions while rotating the swab stick. Avoid letting the swab dry completely. Swab an area up to 2 x 2 inches, ensuring that the full area is covered in the sampling.

(c) Place the swab back into the transport tube containing 2 mL PBS.

(d) Place swabs at refrigeration temperature (2–8°C) no more than 15 min post sampling and maintain refrigeration until analysis.

(e) Analyze the swabs preferably in the coming 24 h. If the swabs are not likely to be analyzed within 48 h maximum, store the swabs preferably between -70°C and -80°C, and ship on dry ice.

**Sample Preparation and RNA extraction**

The KingFisher™ Flex Purification System with 96 Deep-Well Head or the MagMAX™ Express-96 Deep Well Magnetic Particle Processor can be used to extract RNA with the PrepSEQ™ Nucleic Acid Extraction Kit for Food and Environmental Testing:

(a) Ensure that the KingFisher™ Flex Magnetic Particle Processor with 96 Deep-Well Head is set up with the KingFisher™ Flex 96 Deep-Well Heating Block.
(b) Ensure that the PSNA_Flex_300ul script has been downloaded from the product page and loaded onto the instrument using the BindIt™ software. Use PSNA_MagMAX_300ul if you are using MagMAX™ Express-96 Deep Well Magnetic Particle Processor.

Prepare the PrepSEQ Nucleic Acid Extraction Kit reagents:

(a) Add 74 mL 95% ethanol to the Wash Buffer Concentrate bottle.

(b) Add 35 mL 100% isopropanol to an empty Binding Solution bottle.

(c) Incubate the tube of Magnetic Particles at 37 ± 1°C for approximately 10 min.

Prepare the extraction processing plates:

Prepare and label the side of the processing plates according to Table 1. Cover the plates with an adhesive film, then store at room temperature for up to 1 hour while setting up the sample plate.

Prepare the required amount of Lysis Bead Mix on each day of use:

(a) Vortex the Total Nucleic Acid Magnetic Beads to ensure that the bead mixture is homogeneous.

(b) For the number of required reactions, prepare the Binding Bead Mix according to Table 2.

(c) Invert the Binding Bead Mix five times gently to mix, then add 700 μL to each sample well and the Negative Extraction Control well in the Sample Plate.

Note: Remix the Binding Bead Mix by inversion frequently during pipetting to ensure even distribution of beads to all samples or wells. The Binding Bead Mix is viscous, so pipet slowly to ensure that the correct amount is added. DO NOT reuse pipette tips to add Binding Bead Mix to the samples, as the high viscosity will cause variations in the volumes added.
(d) Add 1 µL Total Human RNA Control to each sample well and the Negative Extraction Control well in the Sample Plate.

(e) Vigorously mix each sample tube containing the swab by vortexing for 30 seconds.

(f) Add 300 µL of sample to each sample well.

(g) Add 300 µL of Nuclease-free Water (not DEPC-Treated) to the Negative Extraction Control well in the Sample Plate.

(h) Select the **PSNA_Flex_300ul** on the KingFisher™ Flex Magnetic Particle Processor with 96 Deep-Well Head. The **PSNA_MagMAX_300ul** script should be used with the MagMAX Express 96 DW Magnetic Particle Processor.

(i) Start the run, then load the prepared plates into position when prompted by the instrument.

(j) After the run is complete (~40 minutes after start), immediately remove the Elution Plate from the instrument, then cover the plate with an adhesive film.

(k) Place the Elution Plate on ice for immediate use in RT-PCR.

**Note:** RNA can be stored at -70°C for long term storage (up to 1 year).

**RT-PCR and Data Analysis**

(a) Use purified, non-degraded total nucleic acid that is free of RNase activity and RT-PCR inhibitors.

(b) Protect the assays and master mix from light.

(c) For each research sample, include the primers and probes for all three 2019-nCoV targets (FAM™ assay) in multiplex with the RNase P target (VIC™ assay).

(d) Before beginning, determine the number of required reactions. In addition to the nucleic acid test samples, include the following reactions:
One negative extraction control per plate

One 2019-nCoV Control reaction per plate

One no-template control (NTC) per plate

(e) Perform PCR using 2019-nCoV assay kit and 2019-nCoV control kit combined with the RNA UltraSense™ Master Mix

**PCR Setup using RNA UltraSense™ Master Mix**

(a) On ice or using a cold block, prepare an RT-PCR Master Mix for the number of reactions required plus 10% overage. See Table 3.

(b) For each reaction combine the following components in PCR reaction tubes or plates as in Table 4.

(c) Cap or seal the reaction vessels, and gently tap on the bench top to make sure that all components are at the bottom of the amplification tube. Centrifuge briefly.

NOTE: It is essential that no bubbles remain in the PCR reaction tubes. If bubbles remain after centrifugation, gently tap the reaction plate/tubes on the bench top and then briefly centrifuge again at a higher speed until all bubbles are removed.

(d) Place reactions in a thermal cycler programmed as described below. The following software should be used with each instrument (Table 5).

(e) Set up and run real-time PCR instrument (Table 6).

**Software and Analysis**

For more information about using a software, see the software user guide or help.

(a) Open the data file (EDS) in the same software used to run PCR.
(b) Perform analysis using the following settings (Table 7).

(c) Analyze the run with no reference dye.

(d) For each plate, confirm that the control reactions perform as expected (Table 8).

(e) Review all of the results for the 2019-nCoV assay to ensure that all positive results are detected. Evaluate the overall shape of the amplification curves. An exponential and continuous increase in signal amplification indicates positive amplification.

(f) Export the results.

**Review Results**

(a) Positive signals:

1. Amplification curves are considered positive if they have a sudden increase in fluorescence into an exponential signal.

2. Shallow or linear amplification curves are acceptable as positive if there is a continuous increase in fluorescence with little or no flattening of the curve after the initial increase.

**Note:** If the amplification signal is weak, the software can be manipulated to allow for easier interpretation of results. This can be achieved with the following steps:

- Set the baseline to remove any anomalous data. Example: 12 - 25 cycles.
- Remove the threshold by setting it to 1.
- Change the graph view to linear.
- Change the y axis range to minimum -100 and maximum 1,000,000.
- Change the x axis range to 12–45 cycles.

(b) Negative Signals:
(1) Amplification curves are considered negative if they have no continuous increase in fluorescence.

(2) If an increase in fluorescence is present, the signal must be minor and reach a plateau rapidly or decrease to result in no overall continuous increase in fluorescence.

(c) Review the amplification plots for the RNase P assay (IPC) and classify according to Table 9.

(d) For each test sample, interpret the results using the table below. It is recommended that each lab perform accuracy testing with appropriate samples to establish guidelines for interpreting results. See Table 10.

(e) See Table 11 for Troubleshooting guidance.

Validation Study

This validation study was conducted under the AOAC Research Institute Performance Tested MethodSM program according to the procedures outlined in the AOAC Research Institute Emergency Response Validation Performance Tested MethodsSM Study Outline: Validation Outline for Molecular Methods that Detect SARS-CoV-2 on Surfaces (V10, July 2020). The inclusivity/exclusivity \textit{in silico} evaluation was conducted by Thermo Fisher Scientific (San Francisco, CA, Vantaa, Finland and Basingstoke, United Kingdom). The matrix study consisted of swabs taken from 2”x 2” stainless steel surface areas. The surfaces were prepared and sampled by the independent laboratory, MRI Global (Kansas City, MO). Swabs were shipped blind-coded to Food Safety Net Services (FSNS; San Antonio, TX) for analysis by the Thermo Scientific SARS-CoV-2 RT-PCR Workflow. MRI Global analyzed the corresponding unpaired swabs by the CDC 2019-nCoV RT-PCR method. Additional PTM parameters, robustness and product consistency and stability will be submitted for full PTM certification by March 31, 2021.
Inclusivity/exclusivity Study

The in silico analysis of the primer and probe sequences was conducted according to the Validation Outline provided by AOAC Research Institute using Appendix 1 with the minimal set of GISAID accession numbers for the inclusivity testing, and Appendix 2 with the minimal set environmental genomes for the exclusivity testing.

The selection and design of the primer and probe sequences were developed according to similar quality measures mentioned in the Appendix 6, i.e. unimolecular folding, bimolecular hybridization.

In silico analysis was performed to determine inclusivity (reactivity) and exclusivity the primer and probe sequences according to the Wuhan in silico Reactivity-Inclusivity Study Protocol. BLASTN 2.6.0+ version was used. Blast was performed using ncbi_blastn -query assays.fas -word_size 7-db <organism genome database>. 15,829 sequences were assessed: 15,764 SARS-CoV-2 genomes from GISAID and GenBank Viral NCBI databases were assessed, 8 near neighbor genomes, 26 environmental virus genomes, 23 environmental bacteria and fungi genomes and 8 eukaryotes environmental genomes. Homology was calculated using: %homology= identity_hsp/len_comp. identity_hsp: Identical nucleotide in the blast hsp (high scoring segment pair). Len_comp: length of the primer or probe. Individual components (forward and reverse primers and probe) of each were realigned against the sequence database. Alignments are reported for which of the three components (forward and reverse primers and probes) resulting in an alignment signal with the blast parameters listed above.

Inclusivity Study Results

A total of 15,756 from 15,764 accessions listed were tested. The remaining eight sequences were withdrawn from the GISAID repository by the submitter.
Of the 15,756 strains/isolates analyzed, more than 90% have 100% homology to all three assays (ORF1ab, N-gene, S-gene) in the SARS-CoV-2 Kit and 99% have 100% homology to at least two of the three assays. The strains/isolates without 100% homology to at least two assays are shown in Table 12. The 15,756 tested genomes are predicted to be detected by at least one of the three 2019-nCoV assays. Continuous monitoring of the inclusivity/homology shall be conducted as new genomes are added to the databases.

Exclusivity Study Results

Analysis was conducted for the assays for potential off-target hybridization against the exclusivity listed in the Appendix 2 of the Validation Outline. Complete genomes were used for all. The background list was evaluated, and complete genomes were used when available. There were no primer/probe combinations where a homology was observed for more than one target sequence. The worst-case scenario is shown in Table 13.

Of the 65 non-target genomes analyzed against the primers and probes of the TaqMan™ 2019-nCoV Assay Kit v1, none showed matching sequences.

Quality Assessment of the unimolecular folding and bimolecular hybridization

The selection and design of the PCR primers and probes were done according to Thermo Fisher Scientific TaqMan™ assay development, good practices and quality management procedures to avoid substantial unfolding of the target. This was done using internal mapping tool for TaqMan™ Assays, that are proprietary to Thermo Fisher Scientific. Additionally, in regard to in silico analysis requirements, all analyses were performed using the RNAStructure program (7). Default salt concentration of the program was used throughout. Reverse primer (RT) analysis was performed as described, with a
temperature of 50°C using the RNA mode of the folding program. Forward primer and probe analysis were performed using the DNA mode and probe orientation was taken into account by choosing the strand that the probe binds to. No folding was reported at annealing/extension temperature of 60°C. The DNA folding was set up at 37°C by default and the energy for folding was indicated much less than reported, i.e. 37°C. Note there is no energy values available in the RNAStructure program for Applied Biosystems™ TaqMan® MGB probes.

The bimolecular folding was performed as instructed. The requested pictures of the primers and probes were presented respectively for 6673_nsp2_fwd primer, 6673_nsp2_probe, 6673_nsp2_reverse primer, 66792_S_fwd primer, 66792_S_probe, 66792_S_reverse primer, 66794_N_fwd primer, 66794_N_probe and 66794_N_reverse primer.

The reverse primer (RP) binding region, forward Primer (FP) binding region, the probe binding region, the primer and probe unimolecular folding and the hybridization protocol have been accurately assessed. No significant predicted off target hybridization was observed.

Independent Laboratory Studies – Matrix Study

Viral stock preparation

The stainless-steel surface samples were prepared for this matrix study by MRI Global under Biosafety Level 3 (BSL-3) conditions. SARS-CoV-2 strain USA_WA1/2020, isolated from the first documented US case of a traveler from Wuhan, China (8) was sourced from the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA) for use in this study. The virus stock contained $3.6 \times 10^6$ Plaque Forming Units/mL (PFU/mL) pre-lyophilization. Upon receipt, the lyophilized virus stock was resuspended by MRI Global in 2 mL of PBS, and single use aliquots of 50 µL were frozen and stored at -70°C.
Viral genomic copies per mL (GC/mL) were determined by quantitative RT-PCR using a Bio-Rad CFX96 Real-Time Detection System. The standard curve was prepared from Synthetic SARS-CoV-2 RNA (ATCC No. VR-3276SD). The qPCR procedure used N1 primer and probe sequences published by the CDC (2). Primers and probes were purchased from Integrated DNA Technologies (IDT No. 10006713). TaqPath™ 1-step RT-qPCR Master Mix, CG was sourced from Thermo Fisher Scientific. Thermal cycling conditions followed those published in the CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel Instructions for Use (3).

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

The presence of infectious SARS-CoV-2 in the WRCEVA virus stock was verified using standard cell culture techniques. Briefly, 3 × 10^6 Vero E6 cells were plated into a T75 flask with 15 mL infection media (Dulbecco’s Modified Eagle’s medium supplemented with 5% fetal bovine serum and nonessential amino acids) and incubated in a humidified incubator with 5% CO2. The following day the Vero cells were re-fed with infection media and inoculated with virus stock. Cells were incubated for 5 days at which point widespread cytopathic effect was apparent by microscopic examination of the Vero cells.

Test Plate Inoculation
Dilutions of SARS-CoV-2 virus stock were prepared in viral transport medium [(VTM), prepared by MRI per CDC instructions (9) from a frozen viral stock aliquot and spread onto test areas as shown in Table 14. The same concentrations of virus were used for inoculating test areas for both the CDC 2019-nCoV RT-PCR and Thermo Scientific SARS-CoV-2 Workflow 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 inoculate the test plates, 135 µL (see Table 16) 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 18.4–21.1°C and 30–35% RH (relative humidity) during the plate inoculation and drying process.

**CDC 2019-nCoV RT-PCR Method Plate Sampling**

After drying overnight, test areas on the CDC 2019-nCoV RT-PCR Method test plates were sampled as follows using Puritan foam tipped swabs (Cat. No. 25-1607 1PFSC): A swab was pre-moistened by dipping into a 15 mL conical tube containing 2.0 mL of VTM. The pre-moistened swab was used to sample the 2” × 2” test area by rubbing the swab in at least two different directions while applying pressure to the surface and rotating the swab head. After sampling the test area, the swab was snapped at the break point and placed back into the VTM tube. A random sample ID was assigned to each test area sample. Swab samples were placed in a refrigerator (2–8°C) within 15 min of test area sampling and stored overnight (22 h) before nucleic acid extraction.
Thermo Scientific SARS-CoV-2 Workflow Method Plate Sampling

Test areas on the Thermo Scientific SARS-CoV-2 Workflow test plates were sampled using the same Puritan foam tipped swabs that were used for the CDC 2019-nCoV RT-PCR Method. A swab was pre-moistened by dipping into a 15 mL conical tube containing 2.0 mL of PBS (provided by Thermo Fisher Scientific). 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 PBS tube. Each tube was capped and then wrapped with Parafilm to avoid moisture loss during shipping. A unique random ID number was assigned to each sample, and a key correlating test area sample to random ID number was created and sent to AOAC. Swab samples were shipped overnight to FSNS with ice packs on the day of sampling.

CDC 2019-nCoV RT-PCR Testing

Samples from the CDC 2019-nCoV RT-PCR method test plate were transferred to an operator not aware of the blinded sample identities for testing on the CDC 2019-nCoV 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 on the CDC Panel on an Applied Biosystems 7500 Fast Dx Real-Time PCR Instrument following published instructions. Fractional positive results were seen with the 0.5 POD sample set. Reference Method test results were sent to AOAC for comparison with the Thermo Scientific SARS-CoV-2 Workflow.

Thermo Scientific SARS-CoV-2 Real-Time PCR Workflow
The Thermo Scientific SARS-CoV-2 Workflow swab samples arrived at FSNS laboratories by 10:00 AM on the day following the sampling. The analyst receiving the samples noted that the ice packs used for shipping had melted, and the temperature of the test samples were 16°C (2–8°C) for shipping was preferred. From each sample tube, four analyses were conducted; 1) KingFisher™ Flex 96 Deep Well System/Applied Biosystems™ 7500 Fast Food Safety Real-Time PCR System, 2) KingFisher™ Flex 96 Deep Well System/Applied Biosystems™ QuantStudio™ 5 Food Safety Real-Time PCR System, 3) MagMAX™ Express-96 Deep well Magnetic Particle Processor/Applied Biosystems™ 7500 Fast Food Safety Real-Time PCR System, and 4) MagMAX™ Express-96 Deep well Magnetic Particle Processor/Applied Biosystems™ QuantStudio™ 5 Food Safety Real-Time PCR System. Results from each analysis were recorded and provided to AOAC for blind sample decoding and POD analysis.

Results

As per criteria outlined in Appendix J of the Official Methods of Analysis Manual (4), fractional positive results were obtained for the CDC 2019-nCoV RT-PCR (reference method for this purpose) at the low level of contamination. POD was calculated for the Thermo Scientific SARS-CoV-2 Workflow (the candidate method) presumptive results, POD_c and the reference method, POD_R as well as the difference in the candidate and reference methods, dPOD_C. The POD calculations for each sample extraction/PCR analysis are presented in Tables 15–19. A summary of results is provided in Table 21. For all analyses, there were no positive responses at the 0/5 contamination level (POD = 0), and there were 5/5 positive responses at the high contamination level (POD = 1). At the low contamination level, there were 11/20 positive responses for the CDC 2019-nCoV RT-PCR (positive for N1 and N2), 14/20 for the KingFisher Flex Purification System with both the 7500 Fast and QuantStudio 5 Real-Time PCR systems, 16/20 for the MagMax Magnetic Processor with the 7500 Fast Real-Time PCR System, and 15/20 for the MagMax
Magnetic Processor with the QuantStudio 5 Real-Time PCR System. There were no statistically significant differences seen between the methods, based on POD analysis, with the exception of the comparison between the MagMax Magnetic Particle Processor with PrepSEQ Nucleic Acid Extraction kit with the 7500 Fast Real-Time PCR analysis (dPOD = 0.25, 95% CI = -0.04, -0.49). If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.

Discussion

Inclusivity

Of the 15,756 target SARS-CoV-2 genomes analyzed, 99% of the strains/isolates are perfectly matched to at least two of the three assays, more than 90% have 100% homology to all three assays (ORF1ab, N-gene, S-gene) in the SARS-CoV-2 Kit.

15,756 out of 15,756 tested genomes are predicted to be detected by at least one of the three SARS-CoV-2 assays: 100% of the strains/isolates matched with at least one of the three assays (ORF1ab, N-gene, S-gene) in the SARS-CoV-2 Kit.

Exclusivity

Of the 65 non-target genomes analyzed against the primers and probes of the TaqMan™ 2019-nCoV Assay Kit v1, none showed matching sequences.

Matrix Study

Data from the matrix study demonstrated that all four options for the Thermo Scientific SARS-CoV-2 Real-Time PCR Workflow, KingFisher Flex Purification System with 96 Deep-Well Head used to extract RNA with the PrepSEQ Nucleic Acid Extraction Kit procedure with the Applied Biosystems 7500 Fast or the Applied Biosystem QuantStudio 5 Real-Time PCR Systems and the MagMAX Express-96 Deep Well
Magnetic Particle Processor used to extract RNA with the PrepSEQ™ Nucleic Acid Extraction Kit
eextraction procedure with the Applied Biosystems 7500 Fast or the Applied Biosystems QuantStudio 5
Real-Time PCR Systems were able to detect low levels of SARS-CoV-2 viral RNA on stainless steel. Each
option gave statistically comparable or slightly better results than the CDC 2019-nCoV RT-PCR method,
based on POD analysis. It should be noted that for the CDC to call a sample positive, both the N1 and N2
targets must give positive responses. For the Thermo Scientific SARS-CoV-2 Real-Time PCR Workflow, at
least one of the targets must give a positive response to call a sample positive. In this study, the N2
target for the CDC method gave significantly more positive responses than the N1 (18 vs 11, dPOD = -
0.35, CIs = -0.57, -0.07). The reason for this difference has not been determined but could depend on
degradation of virus during the application and drying onto the surface for this study. However,
experiments to examine this have not been conducted as part of this evaluation. When comparing the
Thermo Scientific SARS-CoV-2 Real-Time PCR Workflow results to the CDC N2 results only, no statistically
significant differences were seen (data not shown), even with the largest differences between the
methods (14 positive results vs 18 positive results, dPOD = -0.20, CIs = -0.43, 0.05). It should also be
noted that the test samples shipped to FSNS arrived at an elevated temperature (16°C vs 2–8°C), and
although there do not appear to be any adverse effects on the results, there was a potential for the
candidate method samples to be compromised. As presented in this study, the data show that all
evaluated options for the Thermo Scientific SARS-CoV-2 Workflow are a comparable alternative to the
CDC 2019-nCoV Real-Time RT-PCR method for detecting SARS-CoV-2 on stainless steel surfaces.

Conclusion

The Thermo Scientific SARS-CoV-2 Real-Time PCR Workflow is targeting three different SARS-CoV-2
genomic regions in one single RT-PCR reaction. A flexibility feature is offered with the use of multiple
extraction and Real-Time PCR instruments: the RNA extraction can be run either with the KingFisher™ Flex 96 Deep Well System or the MagMAX™ Express-96 Deep well Magnetic Particle Processor; the Real-Time PCR can be conducted either with the Applied Biosystems™ QuantStudio™ 5 Food Safety Real-Time PCR System or the Applied Biosystems™ 7500 Fast Food Safety Real-Time PCR System. Data interpretation supports risk management and decision-making to develop relevant hygiene control measures.

During the Emergency Response Validation AOAC Performance Tested MethodSM program, the Thermo Scientific SARS-CoV-2 Real-Time PCR Workflow delivered outstanding robustness, specificity and sensitivity. All the 15,756 SARS-CoV-2 genomes analyzed in the in silico analysis are predicted to be detected with Thermo Scientific SARS-CoV-2 Real-Time PCR Monitoring Workflow. None of the 65 near neighbor genomes and other environmental microorganisms’ genomes showed matching sequences with the primers and probes of the TaqMan™ 2019-nCoV Assay Kit. The Thermo Scientific SARS-CoV-2 Real-Time PCR workflow showed comparable detection to the CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel for SARS-CoV-2 from stainless steel surface swabs whatever the RNA extraction procedures and RT-PCR instruments.

The Thermo Scientific SARS-CoV-2 Real-Time PCR Workflow is an effective procedure for SARS-CoV-2 detection on stainless steel surfaces (such as those found in a food production environment) in evaluating the efficiency of control measures and develop proper hygiene control measures.

Acknowledgements

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### Table 1. Prepare extraction processing plates

| Plate ID       | Plate type                  | Reagent         | Volume per well (μL) |
|----------------|-----------------------------|-----------------|----------------------|
| Sample         | KingFisher™ Deepwell 96 Plates | Binding Bead Mix | 700 μL               |
| Wash plate 1   |                             | Wash solution   | 300                  |
| Wash plate 2   |                             | Wash solution   | 300                  |
| Elution plate  |                             | Elution buffer  | 100                  |
| Tip comb plate |                             |                 |                      |

*Place a KingFisher™ 96 tip comb for Deep Well magnets in a KingFisher™ 96 microplate*

### Table 2. Prepare Lysis Bead Mix

| Component                                 | Volume per well (μL)* |
|-------------------------------------------|-----------------------|
| Proteinase K Buffer                       | 340                   |
| Binding Solution                          | 325                   |
| Total Nucleic Acid Magnetic Beads         | 25                    |
| Proteinase K                              | 10                    |
| Total Binding Bead Mix per well           | 700                   |

*Include 10% overage when making for multiple reactions*

### Table 3. Prepare RT-PCR master mix

| Component                                                      | Volume per reaction (μL) |
|                                                               |                        |
| RNA UltraSense™ Enzyme Mix                                   | 1.0                    |
| RNA UltraSense™ 5X Reaction Mix                              | 4.0                    |
| 2019-nCoV ORF1ab Assay (20X)                                  | 1.0                    |
| 2019-nCoV N Protein Assay (20X)                               | 1.0                    |
| 2019-nCoV S Protein Assay (20X)                               | 1.0                    |
| RNase P assay (20X)                                          | 1.0                    |
| MgSO₄ (50mM)                                                  | 0.25                   |
| Total volume per reaction                                    | 9.25                   |

### Table 4. RT-PCR Master mix

| Component                                                      | Volume per reaction (μL) |
|                                                               |                        |
| Reaction Mix                                                  | 9.25                   |
| Sample (Elution from RNA Extraction) OR                       |                        |
| 1 μl 2019-nCoV Control v1 + 9.75 μl RT-PCR Grade Water OR    | 10.75                  |
| NTC (RT-PCR Grade Water)                                      |                        |
| Total Reaction Volume                                         | 20.0                   |

### Table 5. Instruments and software

| Instrument                                               | Software                                |
|----------------------------------------------------------|-----------------------------------------|
| 7500 Fast Food Safety Real-Time PCR System               | 7500 Fast SDS v1.4 or later             |
| QuantStudio 5 Food Safety Real-Time PCR System          | QuantStudio Design and Analysis Software v1.5.1 or later |
**Table 6. Thermal cycler program**

| Step                        | Stage          | Temp. | Time |
|-----------------------------|----------------|-------|------|
| Reverse transcription       | Hold           | 50°C  | 15 min |
| Polymerase activation       | Hold           | 95°C  | 2 min |
| Amplification               | 45 cycles      | 95°C  | 3 sec |
|                             |                | 60°C  | 30 sec |

Collect data

**Table 7. Settings to perform analysis**

| Target                                      | Baseline | Threshold |
|---------------------------------------------|----------|-----------|
| 2019-nCoV assay (FAM™ dye)                  | Auto     | Auto      |
| RNAse P assay (IPC) (VIC™ dye)              |          |           |

**Table 8. Confirm control reactions**

| Reaction                       | 2019-nCoV assay (FAM™ dye) | RNAse P assay (IPC) (VIC™ dye) | About expected result                                                                 |
|--------------------------------|-----------------------------|--------------------------------|--------------------------------------------------------------------------------------|
| NTC                            | Undetermined                |                                | If the NTC has an amplification curve that crosses the threshold (false positive), sample contamination may have occurred. Repeat the test with new reagents, following good RT-PCR practices. |
| Negative extraction control    | Undetermined                | C<sub>t</sub> < 40             | If a C<sub>t</sub> value is detected in the 2019-nCoV assay then contamination may have occurred during extraction. Repeat the test with new reagents. |
| 2019-nCoV Control v1           | C<sub>t</sub> ≤ 35          | C<sub>t</sub> < 40             | If the C<sub>t</sub> value for either assay is above the expected result, repeat the test with new reagents. |

**Table 9. Interpretation of results**

| 2019-nCoV assay (FAM™ dye) | RNAse P assay (IPC) (VIC™ dye) | 2019-nCoV assay result |
|-----------------------------|--------------------------------|------------------------|
| Clear positive signal       | Any value<sup>a</sup>          | Positive.              |
| Weak positive signal        | Ct < 40                         | Positive.              |
| No positive signal          | Ct = Undetermined or Ct ≥ 40    | Invalid. Proceed to troubleshooting. |
|                             | Ct < 40                         | Negative               |

<sup>a</sup> An RNase P positive result is expected in most reactions. However, when a strong positive signal is detected in the 2019-nCoV assay the RNase P assay can occasionally give a negative result. These samples should be treated as positive if the amplification curve for the 2019-nCoV assay appears normal.
### Table 10. Guidelines for interpreting results

| 2019-nCoV assay result | Interpretation of results |
|-------------------------|--------------------------|
| Clearly positive.       | High risk positive. Employ full hygiene intervention strategy. |
| Weak positive.          | Lower risk positive. Use risk assessment to employ appropriate hygiene intervention strategy. |
| Negative.               | No need for hygiene intervention strategy. |

### Table 11. Troubleshooting guidance

| Observation | Possible cause | Recommended action |
|-------------|----------------|--------------------|
| Inhibition of downstream PCR, indicated by non-detection of IPC reaction in samples with no or late target-specific signal. | Magnetic Particles were in the Elution Plate. | Avoid disturbing the Magnetic Particles during transfer of eluted RNA to the lyophilized assay. Avoid transfer of Magnetic Particles using one of the following methods (optional): 1. Place the Elution Plate on the 96-Well Magnetic Ring Stand during transfer of eluted RNA sample to the assay. 2. Spin the plate at maximum speed in a plate centrifuge for the equivalent of approximately 4,000 x g for approximately 30 seconds, to pellet the Magnetic Particles to the bottom of the plate. |
| In positive control wells, no target-specific signal is detected. | Elution Plate contains other inhibitory substances. | Dilute the eluted RNA 1:5 with Nuclease-free Water to dilute PCR inhibitors and repeat the assay. If PCR remains inhibited, repeat the sample preparation. |
| In positive control wells or unknown sample wells, no IPC RNA exists in samples, resulting in preferential amplification of the target-specific RNA. | | No action is required. The result is considered positive if the target-specific amplification curve appears normal. |
| In negative extraction control wells, target-specific signal is detected. | Carryover contamination occurred. | 1. Repeat the assay using fresh aliquots of all reagents and clean pipetting equipment. 2. If the negative extraction control continues to show contamination, repeat the assay using a new kit. 3. If the negative extraction control continues to show contamination, contact Technical Support. |
| Amplification curve appear abnormal (differ from an | Auto baseline settings cannot account for large deviations in CT values between samples. | Manually adjust the baseline settings for the sample under investigation. |
Observation | Possible cause | Recommended action
--- | --- | ---
Inhibitory substances carried over from RNA extraction. | Dilute the eluted RNA 1:5 with Nuclease-free Water to dilute PCR inhibitors and repeat the assay. If PCR remains inhibited, repeat the sample preparation. |  
Primers and probes have degraded. | Repeat the PCR assay using fresh reagents. |

**Table 12. Inclusivity results**

| Strain Name | GenBank Accession | Assay | Forward Primer Homology (%) | Reverse Primer Homology (%) | Probe Homology (%) |
|---|---|---|---|---|---|
| hCoV-19/Australia/VIC505/2020| EPI_ISL_426776 | N gene | 96 | 100 | 100 |
| hCoV-19/Australia/VIC644/2020 | EPI_ISL_426940 | ORF1ab | 96 | 100 | 100 |
| hCoV-19/Australia/VIC644/2020 | EPI_ISL_426940 | S gene | 100 | 96 | 100 |
| hCoV-19/Australia/VIC648/2020 | EPI_ISL_426944 | ORF1ab | 88 | 100 | 100 |
| hCoV-19/Australia/VIC951/2020 | EPI_ISL_430646 | ORF1ab | 96 | 100 | 100 |
| hCoV-19/Australia/VIC951/2020 | EPI_ISL_430646 | S gene | 100 | 96 | 100 |
| hCoV-19/Brazil/CV17/2020 | EPI_ISL_429677 | ORF1ab | 88 | 100 | 100 |
| hCoV-19/Brazil/CV41/2020 | EPI_ISL_429694 | ORF1ab | 88 | 100 | 100 |
| hCoV-19/Brazil/CV46/2020 | EPI_ISL_429699 | N gene | 78 | 78 | 100 |
| hCoV-19/Canada/ON_PHL3917/2020 | EPI_ISL_418322 | ORF1ab | 88 | 100 | 100 |
| hCoV-19/Canada/ON_PHL3917/2020 | EPI_ISL_418322 | S gene | 96 | 100 | 100 |
| hCoV-19/Canada/ON_PHL3919/2020 | EPI_ISL_418323 | ORF1ab | 88 | 100 | 100 |
| hCoV-19/Canada/ON_PHL3919/2020 | EPI_ISL_418323 | S gene | 96 | 100 | 100 |
| hCoV-19/England/20104035803/2020 | EPI_ISL_417238 | ORF1ab | 88 | 100 | 100 |
| Strain Name                              | GenBank Accession | Assay      | Forward Primer Homology (%) | Reverse Primer Homology (%) | Probe Homology (%) |
|------------------------------------------|-------------------|------------|-----------------------------|----------------------------|-------------------|
| hCoV-19/England/201361007/2020| EPI_ISL_421784 | ORF1ab     | 100                         | 96                         | 100               |
| hCoV-19/England/201380276/2020| EPI_ISL_421826 | ORF1ab     | 100                         | 96                         | 100               |
| hCoV-19/England/201380277/2020| EPI_ISL_421827 | ORF1ab     | 100                         | 96                         | 100               |
| hCoV-19/England/2013901104/2020| EPI_ISL_421880 | S gene     | 96                          | 100                        | 100               |
| hCoV-19/England/20146017604/2020| EPI_ISL_423580 | ORF1ab     | 100                         | 96                         | 100               |
| hCoV-19/France/OCC-4/2020| EPI_ISL_434619 | ORF1ab     | 100                         | 100                        | 95                |
| hCoV-19/France/OCC-4/2020| EPI_ISL_434619 | S gene     | 96                          | 100                        | 100               |
| hCoV-19/Iceland/143/2020| EPI_ISL_417794 | N gene     | 96                          | 100                        | 100               |
| hCoV-19/Iceland/194/2020| EPI_ISL_417821 | ORF1ab     | 88                          | 100                        | 100               |
| hCoV-19/Iceland/28/2020| EPI_ISL_417767 | N gene     | 96                          | 100                        | 100               |
| hCoV-19/Iceland/545/2020| EPI_ISL_424565 | ORF1ab     | 100                         | 96                         | 100               |
| hCoV-19/Iceland/86/2020| EPI_ISL_417870 | N gene     | 96                          | 100                        | 100               |
| hCoV-19/India/nimh-0834/2020| EPI_ISL_428485 | N gene     | 100                         | 100                        | 0                 |
| hCoV-19/Mexico/Chiapas-InDRE_02/2020| EPI_ISL_424666 | S gene     | 100                         | 68                         | 100               |
| hCoV-19/Netherlands/Gelderland_8/2020| EPI_ISL_422644 | ORF1ab     | 88                          | 100                        | 100               |
| hCoV-19/Netherlands/Gelderland_8/2020| EPI_ISL_422644 | N gene     | 100                         | 100                        | 0                 |
| hCoV-19/USA/CA-CDPH-UC25/2020| EPI_ISL_417329 | N gene     | 100                         | 100                        | 0                 |
| hCoV-19/USA/CA-CZB027/2020| EPI_ISL_429068 | S gene     | 100                         | 76                         | 100               |
| Strain Name                        | GenBank Accession | Assay  | Forward Primer Homology (%) | Reverse Primer Homology (%) | Probe Homology (%) |
|-----------------------------------|-------------------|--------|-----------------------------|----------------------------|-------------------|
| hCoV-19/USA/CA-CZB04/2020| EPI_ISL_429069     | S gene | 100                         | 76                         | 100               |
| hCoV-19/Wales/PHWC-2433A/2020    | EPI_ISL_419398    | N gene | 96                          | 100                        | 100               |
| hCoV-19/Wales/PHWC-243FE/2020    | EPI_ISL_419408    | ORF1ab | 100                         | 96                         | 100               |
| hCoV-19/Wales/PHWC-24CE/2020     | EPI_ISL_419408    | S gene | 96                          | 100                        | 100               |
| hCoV-19/Wales/PHWC-24C11/2020    | EPI_ISL_419500    | S gene | 100                         | 100                        | 0                 |
| hCoV-19/Wales/PHWC-25A4D/2020    | EPI_ISL_420979    | ORF1ab | 100                         | 96                         | 100               |
| hCoV-19/Wales/PHWC-25A4D/2020    | EPI_ISL_420979    | S gene | 72                          | 100                        | 100               |
| hCoV-19/Wales/PHWC-2704F/2020    | EPI_ISL_422376    | S gene | 72                          | 100                        | 100               |
| SARS-CoV-2/human/IRN/HGRC-01-IPI-8206/2020 | MT281530.2 | ORF1ab | 88                          | 100                        | 100               |
| SARS-CoV-2/human/USA/CT-UW-5036/2020 | MT375469.1 | ORF1ab | 92                          | 100                        | 100               |
| SARS-CoV-2/human/USA/CT-UW-1963/2020 | MT326080.1 | ORF1ab | 100                         | 64                         | 100               |
| SARS-CoV-2/human/USA/CT-UW-2225/2020 | MT345837.1 | S gene | 96                          | 100                        | 100               |

Table 13. Exclusivity worst case scenario

| Assay_name | Blast_subject_id | fwd_pcnt_homology | probe_pcnt_homology | rev_pcnt_homology |
|------------|------------------|-------------------|---------------------|-------------------|
| 66792_S    | Aedes albopictus NW_021837454.1 | 0                 | 100%                | 0                 |
Table 14. SARS-CoV-2 Dilutions in VTM

| Target contamination level       | Method     | Test area size | No. of test areas | GC/mL\(^b\)  | µL/test area | GC/test area\(^c\) |
|----------------------------------|------------|----------------|-------------------|--------------|--------------|-------------------|
| High (1 POD)                     | Reference  | 2” x 2”        | 5                 | 1.3 × 10⁵     | 135          | 1.8 × 10⁴         |
| Low (0.5 POD)                    | Reference  | 2” x 2”        | 20                | 1.3 × 10⁴     | 135          | 1.8 × 10³         |
| VTM Control (0 POD)              | Reference  | 2” x 2”        | 5                 | 1.3 × 10⁴     | 135          | 0                 |
| High (1 POD)                     | Candidate  | 2” x 2”        | 5                 | 1.3 × 10⁵     | 135          | 1.8 × 10⁴         |
| Low (0.5 POD)                    | Candidate  | 2” x 2”        | 20                | 1.3 × 10⁴     | 135          | 1.8 × 10³         |
| VTM Control (0 POD)              | Candidate  | 2” x 2”        | 5                 | 0            | 135          | 0                 |

\(^a\)POD = Probability of detection. A POD = 1 targets 5/5 positive responses, POD = 0.5 targets 10/20 positive responses, POD = 0 targets 0/5 responses.

\(^b\)GC/mL = Number of gene copies of SARS-CoV-2 viral RNA per mL viral stock solution.

\(^c\)GC/test area = Number of gene copies of SARS-CoV-2 viral RNA applied per 2” x 2” surface area based on range finding studies using the CDC 2019-nCoV RT-PCR method.
Table 15. Statistical comparison of Thermo Scientific SARS-CoV-2 Real-Time PCR Workflow method results using KingFisher™ Flex 96 Deep Well System with Applied Biosystems™ 7500 Fast Food Safety Real-Time PCR System

| Matrix       | Inoculation Strain     | GC\(^a\) per Test Area | Thermo SARS-CoV-2 KF/7500 Fast\(^c\) | CDC 2019-nCoV RT-PCR |
|--------------|------------------------|-------------------------|-------------------------------------|---------------------|
|              |                        | n\(^b\)                 | X\(^d\) | POD\(_C\)^e | 95% CI | n | x | POD\(_R\)^f | 95% CI | dPOD\(_C\)^g | 95% CI |
| Stainless Steel | SARS-CoV-2              | 0                       | 5           | 0.00        | 0.00, 0.43 | 5 | 0 | 0.00         | 0.00, 0.43 | 0.00 | -0.43, 0.43 |
|              | USA-WA1/2020           | 1.8 x 10\(^3\)          | 20         | 0.70        | 0.48, 0.86 | 20 | 11 | 0.55         | 0.34, 0.74 | 0.150 | -0.14, 0.41 |
|              | Stainless Steel        | 1.8 x 10\(^4\)          | 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.
\(^c\)KF/7500 Fast = Samples extracted using KingFisher™ Flex 96 Deep Well System, RT-PCR by the Applied Biosystems™ 7500 Fast Food Safety Real-Time PCR System.
\(^d\)x = Number of positive test areas.
\(^e\)POD\(_C\) = Candidate method (Thermo SARS-CoV-2 Workflow) positive outcomes divided by the total number of trials.
\(^f\)POD\(_R\) = Reference method (CDC 2019-nCoV RT-PCR) positive outcomes divided by the total number of trials.
\(^g\)dPOD\(_C\) = Difference in POD values between the candidate method confirmed and reference method confirmed results.
\(^h\)95% CI = If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.
Table 16. Statistical comparison of The Thermo Scientific SARS-CoV-2 Real-Time PCR Workflow method results using KingFisher™ Flex 96 Deep Well System with Applied Biosystems™ QuantStudio™ 5 Food Safety Real-Time PCR System

| Matrix | Inoculation | GC\(^a\) per | Test Area | n\(^b\) | Thermo SARS-CoV-2 KF/QS\(^c\) | CDC 2019-nCoV RT-PCR |
|--------|-------------|---------------|-----------|---------|------------------------------|---------------------|
|        | Strain      |               |           |         | X\(^d\) | POD\(_C\)^e | 95% CI | n | x | POD\(_R\)^f | 95% CI | dPOD\(_C\)^g | 95% CI |
| Stainless Steel | SARS-CoV-2 | 0 | 5 | 0 | 0.00 | 0.00, 0.43 | 5 | 0 | 0.00 | 0.00, 0.43 | 0.00 | -0.43, 0.43 |
|          | USA-WA1/2020 | 1.8 x 10\(^4\) | 20 | 14 | 0.70 | 0.48, 0.86 | 20 | 11 | 0.55 | 0.34, 0.74 | 0.15 | -0.14, 0.41 |
|          | Stainless Steel | 1.8 x 10\(^4\) | 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.
\(^c\)KF/QS = Samples extracted using the Thermo Scientific SARS-CoV-2 Real-Time PCR Workflow, KingFisher Flex 96 Deep Well System, RT-PCR by the Applied Biosystems™ QuantStudio™ 5 Food Safety Real-Time PCR System.
\(^d\)x = Number of positive test areas.
\(^e\)POD\(_C\) = Candidate method (Thermo SARS-CoV-2 Workflow) positive outcomes divided by the total number of trials.
\(^f\)POD\(_R\) = Reference method (CDC 2019-nCoV RT-PCR) positive outcomes divided by the total number of trials.
\(^g\)dPOD\(_C\) = Difference in POD values between the candidate method confirmed and reference method confirmed results.
\(^h\)95% CI = If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.
Table 17. Statistical comparison of The Thermo Scientific SARS-CoV-2 Real-Time PCR Workflow method results using MagMAX™ Express-96 Deep well Magnetic Particle Processor and Applied Biosystems™ 7500 Fast Food Safety Real-Time PCR System

| Matrix          | Inoculation Strain | GC* per Test Area | n b | X d | POD c | 95% CI     | n | x | POD R | 95% CI | dPOD C | 95% Cl h |
|-----------------|-------------------|-------------------|-----|-----|-------|------------|---|---|-------|--------|--------|----------|
| Stainless Steel | SARS-CoV-2        | 0                 | 5   | 0   | 0.00  | 0.00, 0.43 | 5 | 0 | 0.00  | 0.00, 0.43 | 0.00   | -0.43, 0.43 |
|                 | USA-WA1/2020      | 1.8 x 10^3        | 20  | 16  | 0.80  | 0.58, 0.92 | 20 | 11 | 0.55  | 0.34, 0.74 | 0.25   | -0.04, -0.49 |
|                 |                   | 1.8 x 10^4        | 5   | 5   | 1.00  | 0.57, 1.00 | 5 | 5 | 1.00  | 0.57, 1.00 | 0.00   | -0.43, 0.43 |

aGC = Genomic copies determined by qRT-PCR.
b n = Number of test areas.
c MM/7500 Fast = Samples extracted using MagMAX™ Express-96 Deep well Magnetic Particle Processor, RT-PCR by the Applied Biosystems™ 7500 Fast Food Safety Real-Time PCR System.
d x = Number of positive test areas.
PODc = Candidate method (Thermo SARS-CoV-2 Workflow) positive outcomes divided by the total number of trials.
POD R = Reference method (CDC 2019-nCoV RT-PCR) confirmed positive outcomes divided by the total number of trials.
dPOD C = Difference in POD values between the candidate method confirmed and reference method confirmed results.
95% CI = If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.
Table 18. Statistical comparison of The Thermo Scientific SARS-CoV-2 Real-Time PCR Workflow method results using MagMAX™ Express-96 Deep well Magnetic Particle Processor and Applied Biosystems™ QuantStudio™ 5 Food Safety Real-Time PCR System

| Matrix          | Inoculation   | GC\(^a\) per Test Area | n\(^b\) | x\(^d\) | Thermo SARS-CoV-2 MM/QS5\(^c\) | POD\(_C\)\(^e\) | 95% CI          | CDC 2019-nCoV RT-PCR | POD\(_R\) \(^f\) | 95% CI          | dPOD\(_C\)\(^g\) | 95% CI\(^h\) |
|-----------------|---------------|-------------------------|---------|---------|-------------------------------|-----------------|-------------------|----------------------|-----------------|-------------------|-----------------|----------------|
| Stainless Steel | USA-          | 1.8 x 10\(^3\)         | 20      | 15      | 0.75                          | 0.53, 0.89      |                   | 20                   | 11              | 0.55              | 0.34, 0.74      | 0.20           | -0.09, 0.45     |
|                 | WA1/2020      | 1.8 x 10\(^4\)         | 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.

\(^c\)MM/QS5 = Samples extracted using MagMAX™ Express-96 Deep well Magnetic Particle Processor, RT-PCR by the Applied Biosystems™ QuantStudio™ 5 Food Safety Real-Time PCR System.

\(^d\)x = Number of positive test areas.

\(^e\)POD\(_C\) = Candidate method (Thermo SARS-CoV-2 Workflow) positive outcomes divided by the total number of trials.

\(^f\)POD\(_R\) = Reference method (CDC 2019-nCoV RT-PCR) confirmed positive outcomes divided by the total number of trials.

\(^g\)dPOD\(_C\) = Difference in POD values between the candidate method confirmed and reference method confirmed results.

\(^h\)95% CI = If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.
Table 19. The Thermo Scientific SARS-CoV-2 Real-Time PCR Workflow and CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel test kit method summary results

| Matrix         | Inoculation Strain | GC\(^{a}\) per Test area | n\(^{b}\) | Thermo SARS-CoV-2 workflow | CDC 2019-nCoV RT-PCR\(^{g}\) |
|----------------|--------------------|---------------------------|----------|----------------------------|-------------------------------|
| Stainless Steel | SARS-CoV-2 USA-WA1/2020 | 1.8 x 10\(^{3}\) | 5        | 0 Fast\(^{c}\) 0 MM/7500 Fast\(^{c}\) 0 MM/QS5\(^{d}\) | 0 Positive 0 Positive 11 Final |
|                |                    | 1.8 x 10\(^{4}\) | 5        | 14 Fast\(^{c}\) 14 MM/7500 Fast\(^{c}\) 16 MM/QS5\(^{d}\) | 5 Positive 5 Positive 5 Final |

\(^{a}\)GC = Genomic copies determined by qRT-PCR.

\(^{b}\)n = Number of test areas.

\(^{c}\)KF/7500 Fast = Samples extracted using KingFisher™ Flex 96 Deep Well System, RT-PCR by the Applied Biosystems™ 7500 Fast Food Safety Real-Time PCR System.

\(^{d}\)KF/QS5 = Samples extracted using KingFisher™ Flex 96 Deep Well System, PCR by the Applied Biosystems™ QuantStudio™ 5 Food Safety Real-Time PCR System.

\(^{e}\)MM/7500 Fast = Samples extracted using MagMAX™ Express-96 Deep well Magnetic Particle Processor, RT-PCR by the Applied Biosystems™ QuantStudio™ 5 Food Safety Real-Time PCR System.

\(^{f}\)MM/QS5 = Samples extracted using MagMAX™ Express-96 Deep well Magnetic Particle Processor, RT-PCR by the Applied Biosystems™ QuantStudio™ 5 Food Safety Real-Time PCR System.

\(^{g}\)CDC 2019-nCoV RT-PCR = Both the N1 and N2 targets must give positive responses to call a sample positive. For the Thermo SARS-CoV-2 workflow, at least one must be detected to call a sample positive.