Validation Study for PathogenDx EnviroX-Rv assay for the Detection of SARS-CoV-2 from Stainless Steel Environmental Surface Swabs Performance Tested MethodSM 122003

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Abstract

Background: The PathogenDx EnviroX-Rv uses endpoint PCR + DNA microarray technology to detect SARS-CoV-2, the causative agent of COVID-19, from stainless steel environmental sample swabs. Objective: To validate the PathogenDx EnviroX-Rv assay as part of the Emergency Response Validation Performance Tested MethodSM program. Methods: The PathogenDx EnviroX-Rv assay was evaluated for specificity using in silico analysis of ≥41,000 SARS-CoV-2 sequences and over 50 exclusivity organisms (both near neighbors and background organisms). The candidate method was evaluated in an unpaired study design for one environmental surface (stainless steel) and compared to the U.S. Centers for Disease Control and Prevention 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel, Instructions for Use (Revision 4, Effective 6/12/2020). Results: Results of the In silico analysis demonstrated the high specificity of the method in being able to detect target SARS-CoV-2 sequences and discriminate them from near neighbors and environmental background organisms. In the matrix study, the candidate method demonstrated a statistically significant difference when compared to the results of the CDC method utilized in this study, with the candidate method resulting in more positive replicates as it only requires 1 target to be present for a positive sample. Conclusions: The EnviroX-Rv assay rapidly and accurately detected SARS-CoV-2 RNA on environmental swabs
from stainless steel surfaces at concentration of 2000 genomic copies per 2” × 2” test area.

*Highlights:* The Enviro^X^-Rv assay employs dual PCR and hybridization techniques to provide highly accurate results when detecting SARS-CoV-2 from surfaces.

**General Information**

In late 2019, a novel coronavirus (SARS-CoV-2) was discovered that has resulted in a global pandemic and millions of confirmed human infections globally (1). While easily spread person to person, there remains a high level of uncertainty about its ability to transmit on surfaces including food contact surfaces, which has resulted in the banning of certain imported products from manufacturing facilities where outbreaks have occurred (2). Reducing the risk associated with these surfaces includes both effective cleaning and disinfection and a robust environmental monitoring program, including rapid detection methods (3). Having access to this information can lead to better infection prevention and improved control measures aimed at reducing surface transmission which has resulted in a need for rapid assays that can detect the virus from surfaces.

**Principle of the Method**

The PathogenDx Enviro^X^-Rv assay is a test based on end-point reverse transcription polymerase chain reaction (RT-PCR) coupled to DNA microarray hybridization for the detection of multiple genes within SARS-CoV-1 and SARS-CoV-2 viruses. The Enviro^X^-Rv method requires a few hours longer to obtain results compared to quantitative RT-PCR (qRT-PCR), however, the advantage of end point PCR coupled to DNA microarrays in comparison to the more widely utilized qRT-PCR approach to viral detection is: (1) The ability to increase the assay sensitivity as demonstrated by the lower limit of detection of this assay as compared to the reported qRT-PCR assays approved by the FDA-EUA; (2) The ability to multiplex at a much greater capacity than qRT-PCR. The qRT-PCR method is generally not able to detect more than 5 unique
targets in a single reaction. In qRT-PCR the primer and the probe are in the same reaction limiting the reactions capability. In comparison, microarrays can multiplex hundreds or thousands of unique targets due to the separation of the RT-PCR reaction (primers) from the DNA microarray detection (probes). The DNA microarray contains:

(a) *Enviro*-Rv Kit.—*Enviro*-Rv SARS-CoV-2 Multiplex Assay—contains 5 (five) SARS-CoV-2 primer sets; 4 (four) SARS-CoV-2 probes targeting each N1, N2, and N3 genes; *Enviro*-Rv Control—internal process control for nucleic acid extraction using a primer and probe sets as an internal positive control; *Enviro*-Rv Swabs – WorldBio PUR-Blue™ Swabs in Hi-Cap Broth.

(b) *Enviro*-Rv SARS-CoV-2 Control – RNA control that contains targets specific to the SARS-CoV-2 genomic regions that are targeted by the assay.

Viral and host nucleic acids are isolated and purified from surface swabs using the Zymo Research *Quick*-DNA/RNA™ Viral MagBead (R2140 or R2141) magnetic silica bead extraction kit. Subsequently, five microliters of the purified RNA product are reverse transcribed using RT-PCR System. Following RT-PCR, two microliters of that primary RT-PCR (amplified cDNA) product are then PCR amplified in a second, nested and biased, PCR reaction in which the PCR target is labeled with a Cy3 fluorophore for detection. The resulting PCR product is then ready for hybridization to the DNA microarrays without additional denaturation or purification. The DNA microarray is formatted in a standard 96-well SBS plate format, one microarray per well (i.e., 96 microarrays per plate) each microarray containing up to 144 synthetic ssDNA probes in a 12 × 12 array configuration. The array contains probes to identify genes in SARS-CoV-1, SARS-CoV-2, internal and external positive controls. The labeled PCR product is hybridized to the DNA microarray at room temperature, over the course of 1 hour, to determine if viral RNA is present in the surface sample. Following the hybridization, the arrays are scanned to determine the fluorescence intensity of each spot, using a Sensospot™ (Sensovation Inc.) scanner. The microarray results are uploaded to a secure server, quantified, and interpreted automatically using Augury™ software.
Data from the AOAC ERV PTM study indicated that a concentration of 2000 genomic copies per 2” × 2” test area resulted in 100% detection by the assay.

Scope of method

(a) Analyte(s).—SARS-CoV-2 viral strains.

(b) Matrixes.—Environmental Surface Swabs (2” × 2”): Stainless Steel.

(c) Summary of validated performance claim.—Performance comparable to the Centers for Disease Control and Prevention (CDC) 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel (1) .

Definitions

(a) Probability of detection (POD).—The proportion of positive analytical outcomes for a qualitative method for a given matrix at a given analyte level or concentration. POD is concentration dependent. Several POD measures can be calculated: POD_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.

(c) In silico.—The use of computer simulation to evaluate target and non-target sequences for molecular methods.

(d) Microarray.—A laboratory tool used to detect the expression of thousands of genes at the same time. DNA microarrays are 96-well plates that are printed as a matrix of oligonucleotide probe “Spots” in defined positions, with each spot containing a known DNA sequence.
Materials and Methods

Test Kit Information

(a) Kit name.—PathogenDx EnviroX-Rv Assay.

(b) Cat. No.—EnviroX-Rv.

(c) Ordering information.—www.pathogendx.com - orders@pathogendx.com.

Test Kit Components

(a) Sampling components.—PUR-Blue™ Swabs in Hi-Cap Broth.

(b) RT-PCR components.—

(1) One Step Reverse Transcription Kit.—100 reactions.

(2) Primer Set 1.—1 vial (225 µL).

(3) EnviroX-Rv Positive Control.

(c) Labeling PCR components.—

(1) PCR Master Mix.—Two bottles (4.5 mL).

(2) Primer Set 2.—One vial (225 µL).

(3) Taq polymerase.—One vial (50 µL)

(d) DNA microarray components.—

(1) PathogenDx microarrays.—96 per 96-well plate.

(2) Buffer 1.—One bottle (4 mL).

(3) Buffer 2.—One bottle (1.5 mL).

Additional Supplies and Reagents

(a) Quick DNA/RNA Viral MagBead Extraction Kit.—R2140 – 96 Preps.

(1) ZR-96 MagStands.—P1005.
(2) Collection Plate.—C2002.

(3) 96-Well Block.—P1001.

(4) Elution Plate.—C2003.

(5) Cover Foil.—C2007.

(6) Beta-mercaptoethanol.

(7) Isopropanol, molecular grade.

(8) Ethanol, molecular grade.

(9) DNase/RNase Free Water.

(10) DNase/RNase Free Cleaning Reagent.—Argos Technologies, 04397-24C2007.

(b) 96 well reaction plates.—MicroAmp, N8010560.

(c) Clear adhesive film.—MicroAmp, 4306311.

(d) Microcentrifuge tubes.—1.7 mL, DNase/RNase Free.

(e) 15 mL conical tubes.

(f) Molecular Grade Water.

Apparatus

(a) SensoSpot® Fluorescence Microarray Analyzer.

(b) Micropipettors.—Capable of delivering 1–1000 µL.

(c) Freezer.—Capable of maintaining –20°C.

(d) Heat Block.—Capable of maintaining 37 ± 1°C, 55 ± 1°C, and 95 ± 1°C.

(e) Vortex mixer.—Capable of 3000 rpm.

(f) Centrifuge.—Capable of 50 × g, 1000 × g, and 14 000 × g.

(g) Thermal cycler.—Applied Biosystems 2720 Thermal Cycler or Applied Biosystems MiniAmp Thermal Cycler.
(h) *Slide or Plate Spinner.*

**Reference Materials**

Organisms used in the study were obtained from the Biodefense and Emerging Infections Research Resources Repository (BEI, Manassas, VA).

**Safety Precautions**

Follow standard precautions. All collected samples and positive controls should be considered infectious and/or biohazardous and handled accordingly with safe laboratory procedures. This kit is designed to identify viral RNA from contaminated surfaces. Follow necessary precautions when handling specimens. Use personal protective equipment consistent with current guidelines for the handling of potentially infectious samples. Handle all samples and controls as if they are capable of transmitting infectious agents. Always use pipette tips with aerosol barriers. Tips that are used must be sterile and free from DNases and RNase. Modifications to assay reagents, assay protocol, or instrumentation are not permitted. Primer Set 2 is light sensitive and must be stored away from light. All frozen reagents must be stored at –20 ± 5°C. They must NOT be stored at -80°C as this will cause degradation of reagents. PathogenDx microarrays are light and moisture sensitive and should be stored in the moisture barrier bag with desiccant packet provided with the kit. Buffer 1, and Buffer 2 can cause irritation upon contact, always wear gloves and eye protection when handling this product. Upon contact, rinse with water. See MSDS. In the post-hybridization protocol, centrifuge speed should not exceed 70 × g or slides may break. Refer to the Material Safety Data Sheets on the PathogenDx company website. Kit components from different lot numbers should not be mixed.

**General Preparations**

*PathogenDx Enviro<sup>®</sup> RV Assay*

(a) Change pipette tips in between samples.

(b) Prewarm Thermomixer heat block before initiating extraction
(c) Separate work areas for the following: media preparation, sample preparation, PCR, Post PCR and Hybridization.

(d) Always keep PCR Reagents separate from areas where genomic DNA or the resulting -PCR product is used.

(e) PCR setup should be conducted in an area devoted solely to PCR.

(f) All supplies and instruments to be used for PCR should be kept in the PCR area, and NEVER placed in areas where genomic DNA or amplicons are used

(g) PCR Master Mix and Buffer solution mixes should be made just prior to analysis

(h) When pipetting with the multichannel onto the microarray plate, only dispense to the first stop. DO NOT depress the multichannel to the second stop, or full evacuation of the tips to avoid cross contamination

(i) Do not allow the microarray to air dry.

(j) Always keep the microarray in the hybridization chamber to limit exposure to light.

Sample Preparation

Stainless Steel Environmental Swabs (2” x 2” test area).—Label the outside of the swab container.

Remove wet swab from vial by depressing the swab against the inside of the tube to ring out excess liquid.

Swab the desired surface in an N or S shaped pattern, in 4 directions, if a flat surface, which should take between 10 to 15 s from start to finish (see Figure 1). Place the swab back into the sterile storage container. Close and seal the container. The swab is stable at room temperature until the user is ready for RNA extraction.

Note: Alternatively, if the user has not prepared the samples within 5 days of receipt then the swabs can be stored at –20 or –80°C until use. In that case the swabs should be thawed on ice or at 4°C prior to analysis.
Prior to performing RNA extraction, vortex the samples and remove 1 mL of the swab storage solution and centrifuge at 1000 rpm for 3 min, after centrifugation, remove 400 µL of the precleared sample and proceed to RNA extraction per the Zymo Research Quick-DNA/RNA Viral MagBead Kit.

**Analysis**

**Zymo Kit reagent preparation.**—Note: The Zymo kit comes in 100 sample or 400 sample kit volumes – be sure to follow the manufactures instructions for reagent preparation dependent on the kit size).

(a) Clean the benchtop thoroughly with 10% Bleach followed by 70% Ethanol and finally with RNase/DNase Away.

(b) Add 500 µL of beta-mercaptoethanol to 100 mL of Viral DNA/RNA Buffer

(c) Reconstitute the lyophilized Proteinase K with 1040 µL of Proteinase K Storage Buffer. Mix by vortex. Aliquot 200 µL into sterile tubes and store frozen at –20°C until ready for use.

(d) Add 20 mL of molecular biology grade isopropanol to the MagBead DNA/RNA Wash 1 concentrate

(e) Add 30 mL of molecular biology grade isopropanol to the MagBead DNA/RNA Wash 2 concentrate

(f) Prepare the DNA/RNA Shield (2x concentrate) with an equal volume of Nuclease-free water to obtain a 1x solution.

**Zymo research sample prep (each step is performed at room temperature).**—

(a) Clean the benchtop thoroughly with 10% Bleach followed by 70% Ethanol and finally with RNase/DNase Away.

(b) Vortex the swab. Pipet 1 mL of fluid from the swab solution to a sterile 1.5 mL tube.

(c) Centrifuge the 1 mL solution at 1000 × g for 3 min. Carefully pipet 400 µL of the supernatant to a new sterile 1.5 mL tube or plate without disturbing the pellet.
(1) To prepare the NTC (no template control) pipet 400 μL of 1x DNA/RNA Shield (prepare the 1x solution from the 2x stock) to a new tube. Continue as normal.

(2) To prepare the Extraction Control - Add 5 μL of positive control directly to a swab and follow the Sample Preparation directions above, continue as normal.

(d) Add 4 μL of Proteinase K to the 400 μL sample, vortex the tubes or pipet up and down to mix in plates.

(e) Add 800 μL of Viral DNA/RNA Buffer to the 400 μL sample, vortex the tubes or pipet up and down to mix in plates.

(f) Vortex the MagBinding Beads. (Beads settle quickly, beads must be kept in suspension while dispensing.) Pipet 20 μL of MagBinding Beads to each sample and mix well for 10 min. The sealed tubes or plates may be placed on a shaker at 1000 rpm for 10 min. Carefully remove the seal to avoid any splash over.

(g) Transfer the plate/tube to a magnetic stand and allow to sit until the beads have pelleted. Aspirate and discard the supernatant and retain the pellet.

(h) Add 500 μL MagBead DNA/RNA Wash 1 and vortex the tubes or pipet up and down to mix in plates.

(i) Transfer the plate/tube to a magnetic stand and allow to sit until the beads have pelleted. Aspirate and discard the supernatant and retain the pellet.

(j) Add 500 μL MagBead DNA/RNA Wash 2 and vortex the tubes or pipet up and down to mix in plates.

(k) Transfer the plate/tube to a magnetic stand and allow to sit in the magnetic field until the beads have pelleted. Aspirate and discard the supernatant and retain the pellet.

(l) Add 500 μL ethanol (95–100%) and vortex the tubes or pipet up and down to mix in plates.

(m) Transfer the plate/tube to a magnetic stand and allow to sit until the beads have pelleted.
Aspirate and discard the supernatant and retain the pellet.

(n) Add 500 μL ethanol (95–100%) and vortex the tubes or pipet up and down to mix in plates. Then transfer the entire solution (beads and liquid) to a new tube/plate.

(o) Transfer the plate/tube to a magnetic stand and allow to sit in the magnetic field until the beads have pelleted. Aspirate and discard the supernatant and retain the pellet.

(p) Dry the beads for 10 min or until fully dry. Place the tubes/plates on a heat block at 55°C. Aseptically open the tubes and leave them cracked but not fully open to allow for drying. For the plates, without removing the sticker, place seal over the plate allowing drying to occur. There must be airflow into the tube or plate for drying to occur.

(q) Once the beads are dry, they should turn from a glossy black to a dull brown. Elute the DNA/RNA from the beads by adding 50 μL DNase/RNase-Free water. Vortex the tubes or pipet up and down to mix in plates. Allow the tubes/plates to sit at room temperature for 2 min.

(r) Transfer the plate/tube to a magnetic stand and allow to sit until the beads have pelleted. Aspirate the eluted DNA/RNA and transfer to a new tube/plate.

(s) The DNA/RNA may be used immediately or stored frozen at –20°C. Avoid freeze/thaw cycles once the DNA/RNA is stored frozen. More than 6 freeze/thaw cycles may result in RNA degradation.

PathogenDx Enviro® RV assay.—

(a) Mix and briefly centrifuge each component before use. Combine the following into a master mix, multiply per reaction as shown in Table 1.

(1) Determine the number of samples in the reaction and multiply each reagent to prepare the master mix, leaving out the Purified RNA Template from the master mix and add individually to each designated well.

(2) Mix by pipetting and add 45 μL of the master mix per well.

(3) Add 5 μL of the magnetic bead purified RNA template to each designated well making sure to
change tips between samples.

(b) Cover the plate with clear adhesive film and seal.

(c) Centrifuge the plate in a plate spinner for 30 s.

(d) Place the plate in the thermal cycler and cover with a compression pad before closing the thermal cycler.

(e) Input the Reverse Transcriptase and PCR cycling program as shown in Table 2.

(f) Proceed to Labeling PCR Amplification.

(g) Plates may be stored @4°C for up to 2 weeks in not used immediately.

*Labeling PCR Reaction*

(a) Thaw PCR Master Mix and Primer Set 2 and remove the Taq Polymerase from the freezer just prior to making the master mix.

(b) Mix and briefly centrifuge each component before use.

(c) Mix the indicated reagent volumes (calculated from Table 3) in a microfuge tube to prepare Labeling PCR Master Mix (made fresh each run).

(d) Briefly vortex Labeling PCR master mix and centrifuge at 1000 × g for 3–5 s.

(e) Store all reagents at –20°C after use.

*Note:* Reaction volumes have been scaled to account for a negative control and to account for pipetting losses and volume lost on reservoir/tube walls. If a reservoir is not used for multichannel pipetting, there will be extra volume remaining in the PCR Master Mix tube.

(f) Pipette 48 μL of the Labeling PCR Master Mix into the bottom of PCR tubes or PCR plate.

(g) In the Post PCR area, pipette 2 μL of the RT-PCR Product into the bottom of the corresponding tube or well for a final volume of 50 μL per PCR reaction. Pipet up and down to mix.

(1) Always check pipette tip volumes before and after to ensure accuracy and release.

(2) Warning: Add the Loci Enhancement PCR template into the Labeling PCR reaction outside of the
PCR area to prevent contamination.

(h) The Loci Enhancement PCR plate may be re-sealed and returned to 4°C.

(i) Cap tubes, or seal plates with PCR film ensuring every well is completely sealed.

(j) Centrifuge for 30 s.

(k) Place tubes or plate into the thermal cycler with a pressure pad if necessary, before closing the thermal cycler lid.

(l) Refer to Table 4 to run the Labeling PCR Program.

(m) Labeling PCR product may be stored for 7 days at 4°C protected from light.

**DNA hybridization.**—General guidelines to follow for hybridization: When pipetting with the multichannel onto the microarray plate, only dispense to the first stop. DO NOT depress the multichannel to the second stop, or full evacuation of the tips to avoid cross contamination.

*Caution:* Avoid contact with the array surface of the plate during processing. Use slide edges or barcoded area for handling.

*Caution:* The directions below are for 96 well plates. Please note that the bracketed volumes refer to the volume that should be added to the 96 well plates as opposed to the 12 well slides.

(a) Before starting, thaw Buffer 2 at room temperature.

(b) Cut paper towel to size to fit the bottom of the hybridization chamber provided.

(c) Place the slides to be used in the Hybridization Chamber.

(d) Apply 200 μL of Molecular Biology Grade Water to each well of the 96-well while being careful to avoid contact with the array.

(e) Aspirate and then again, dispense 200 μL of Molecular Biology Grade Water to each well of the 96-well plate and allow to sit covered in the Hybridization Chamber for 5 minutes before aspirating water from the slides.
(f) Prepare the Pre-hybridization Buffer and Hybridization Buffers in clean tubes for the number of microarrays that will be hybridized as per Tables 5 and 6. Vortex briefly to mix.

(g) Aspirate the water wash and add 200 μL of Pre-hybridization Buffer to each well of the 96-well plate without touching the pipette tip to the array surface. Close the Hybridization Chamber box lid.

(h) Allow Pre-hybridization Buffer to stay on the arrays for 5 min; do not remove slides from the Hybridization Chamber.

(i) Briefly centrifuge the PCR tubes or PCR plate containing the Labeling PCR product.

(j) Add 18 μL of Hybridization Buffer to each well of the Labeling PCR product within the 96-well PCR plate or tubes, pipette up and down to mix. It is important that no cross-contamination occurs during this step. The PCR product and the Hybridization Buffer mix constitute the Hybridization Cocktail.

(k) Aspirate Pre-hybridization Buffer from the arrays.

Caution: Do not allow the arrays to air dry. Avoid contact with the array surface.

(l) Immediately add 68 μL (Total Volume of PCR Reaction + Hyb Buffer) of the Hybridization Cocktail to each array of the 96-well being careful not to touch the array surface with the pipette tip. Ensure that the sample ID and location are recorded.

(m) Close the Hybridization Chamber lid.

(n) Allow to hybridize for 30 min at room temperature in the Hybridization Chamber.

Post hybridization PathogenDx slide processing.—

(a) Prepare Wash Buffer according to the number of microarray wells to be used in the 96-well plate. (Table 7). Washing must be performed according to the protocol to ensure detectable signal and adequate washing to prevent elevated background signals.

(b) Aspirate Hybridization Cocktail from the slides.

(c) Add 200 μL of Wash Buffer to each well of the 96-well plate, then aspirate.

(d) Add 200 μL of Wash Buffer to each well of the 96-well plate, allow buffer to remain on the slides.
for 10 min, aspirate

*Note:* Steps (c) and (d) need to occur quickly to ensure no drying occurs.

(a) Perform a final wash by dispensing and aspirating 200 μL of Wash Buffer to each well of the 96-well plate.

(b) Following the last aspiration step, remove the plate from the Hybridization Chamber.

*Note:* Do not allow drying to occur on slide surface.

(c) Load the plate, face down, into the Reusable Laboratory Micro Array Plate Centrifuge. (1 min is adequate to completely dry the plates.)

(d) PathogenDx plates should be placed back into a slide case and moisture barrier bag with desiccant until scanning may be performed to protect the slides from light. Plates should be scanned within two weeks of hybridization.

*Scanning conditions and data acquisition.—*

(a) Please refer to the Augury® User Manual for detailed instructions on plate imaging and how to use the software.

(b) Access the Sensovation scanner desktop, select the application “Array Reader”.

(c) Open the tray, select “Open Tray”.

(d) Place the plate in the tray oriented with the barcode towards the technician and face down.

(e) Close the tray, select “Close Tray”.

(f) Select “Scan”.

(g) Select the number of wells that are being scanned in the 96-well plate (ex. 1, 2, 3, 4)

*Note:* All other information on this screen is preprogrammed – do not alter.

(h) Select the Blue Arrow to begin the scanning process.

(i) While the plate is being scanned, select “Result Overview” to review the images of the wells.

(j) When the plate is finished scanning and the screen displays the digital image of a plate with all
green wells, select the Red X to exit the scanning process.

(k) Open the tray, select “Open Tray”.

(l) Remove the plate and store in the moisture barrier bag with the desiccant packets.

(m) Close the tray, select “Close Tray”.

(n) Exit the Array Reader application, select “Exit”.

(o) On the Sensovation Scanner desktop, select the folder “Scan Results”.

(p) Locate the folder associated with your plate and rename the folder with the slide barcode number by scanning the barcode located either on the outside of the barrier bag or on the plate itself. (ex. rename: ScanJob-191108130334_1 to 7024001001)

(q) Submit the whole barcode labeled folder to the “Image Folder” within Dropbox.

(r) The folder will automatically begin uploading, the PathogenDx Augury© Software will analyze the data and directly deposit the reports into the “Reports” folder within Dropbox.

Quality control and validity of results.—

(a) One Negative Control (No Template Control) and one Positive Process Control are processed with each run.

(b) Validation of results is performed automatically by the Augury Software based on the performance of internal positive and negative controls in each well (Table 8).

Validation Study

Study Overview

The study was conducted 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 in silico analysis was performed by PathogenDx. Matrix studies were performed by the independent laboratory, MRIGlobal.
Additional PTM parameters, robustness and product consistency and stability will be submitted for full PTM certification by March 31, 2021.

In Silico Analysis

Primer and probe sequences.—Methodology.—The oligonucleotide primer and probe sequences of the PathogenDx EnviroX-Rv test were evaluated against >41 000 “High Quality Genomes” resident in the Global Initiative on Sharing All Influenza Data (GISAID, https://www.gisaid.org) database as of July 15, 2020, to demonstrate the predicted inclusivity of the PathogenDx EnviroX-Rv test.

The frequency of mismatches between the primer and probe sequences of the EnviroX-Rv test vs the entire GISAID database are shown in the three figures below (Top, Middle and Bottom Panels). These three figures represent PCR Primer and Hybridization Probe Inclusivity Analysis. Each figure consists of four rows as mentioned below:

Row 1.—Title
Row 2.—PCR primer length (bases)
Row 3.—Average Mismatch Frequency (%) Throughout the entire PCR Primer sequence
Row 4.—Average Mismatch Frequency (%) per Nucleotide within each PCR Primer

The Top Panel is an analysis of the entire sequence of each PCR Primer used, relative to identical Primer mismatch frequency calculations for a published q-RT-PCR based test from the CDC (bold). Data are presented in two formats:

Average Mismatch Frequency (%) Throughout the entire PCR Primer sequence (Figure 2, Row 3).
Average Mismatch Frequency (%) per Nucleotide within each PCR Primer (Figure 2, Row 4).

which is obtained by dividing the data in Row 3 by Primer Length. The method of data analysis in Row 3 (prevalence summed over the entire primer length) is as suggested by GISAID. The analysis in Row 4 is length independent and defines the average mismatch probability per nucleotide in the probe sequence.
With the exception of one PCR Primer (N3, Locus, FP) which displayed an average mismatch frequency of 1.68%, the frequency of mismatches within the Primers was ≤1%, indicating that the prevalence of the mismatches was sporadic among the primers. It should be noted that the relatively higher values obtained for N3, Locus, FP was due to a mutation at the 25th base from the 3’ end of the primer, otherwise it would be equivalent to that observed for the N3 Primer (CDC N3 FP) in the published CDC assay (0.98%).

The Middle Panel is an analysis of the last 5 bases at the 3’ terminus of each of the PCR Primers used, relative to Primer mismatch frequency calculations for a published q-RT-PCR based test from the CDC (bold). The 3’ terminus is known to be the region within a PCR Primer where mismatches will most readily affect PCR efficiency, due to its role as the initiation site for PCR elongation. Consequently, GISAID has suggested that this alternative PCR Primer Inclusivity analysis be used (GISAID, https://www.gisaid.org).

Data are presented in two formats:

- Average Mismatch Frequency (%) in the 5-base-long 3’ PCR primer sequence (Figure 3, Row 3).
- Average Mismatch Frequency (%) per Nucleotide within the 5 base long region (Figure 3, Row 4) which is obtained by dividing the data in Row 3 by (5). With the exception of a single PCR Primer (N1, Locus, RP) which displayed an average mismatch frequency of 0.24% in its 3’ region (identical to CDC’s), the frequency of mismatches within all other PCR Primers was <0.1%, indicating that prevalence of the mismatches within the last 5 bases near the 3’ terminus was sporadic among the Primer terminus region and occurred at a lower incidence than the overall incidence of mismatching throughout the full Primer region.

The Bottom Panel is an analysis of the entire sequence of each Hybridization Probe used, relative to Probe mismatch frequency calculation for a published q-RT-PCR based test from the CDC (bold). Data are presented in two formats:
Average Mismatch Frequency (%) throughout the entire Probe sequence (Figure 4, Row 3). Average Mismatch Frequency (%) per Nucleotide within each Probe (Figure 4, Row 4) which is obtained by dividing the data in Row 3 by Probe Length. The method of data analysis in Row 3 (summed over the entire probe) is as suggested by GISAID. That in Row 4 is length independent and defines the average probability per nucleotide in the probe.

With the exception of 2 Probes (N1 RE.1.2, N1 RE.1.4) which displayed an Average Mismatch Frequency throughout the entire hybridization Probe sequence of 2.2%, the frequency of mismatches within all other Probes was <0.2%, indicating that prevalence of the mismatches were sporadic among the probes. It should be noted that the relatively higher values obtained for N1 RE.1.2 & 1.1 N1 RE.4 were equivalent to that observed for the N1 probe in the published CDC assay (2.4%).

*PCR primer inclusivity.*—At the low incidence observed, the risk that the calculated PCR Primer mismatches would result in a significant loss in reactivity to cause a false negative result is extremely low due to the design of the PCR Primers of this assay, with melting temperatures of 65°C and with annealing temperature at 55°C that can thus tolerate up to 3 mismatches (i.e., 12% mismatching, so long as removed from the 3’terminus.

*Hybridization probe inclusivity.*—Similarly, at the low incidence observed, the risk that the calculated Hybridization Probe mismatches would result in a significant loss in reactivity causing a false negative result is extremely low due to the design of the Hybridization Probes of this assay, with melting temperatures of 60°C and with hybridization temperature at 25°C that can thus tolerate up to 2 mismatches (8%) anywhere in the span of the probe.

Inclusivity Testing

**Results.**—Figure 5 homology analysis to compare the sequence of the PCR primers used to support the 2-step tandem PCR reaction of the Enviro®-Rv test (i.e “Locus” and Labelling”) for each of the 3 SARS-Cov-2
domains analyzed (N1, N2, N3). The Figure also shows homology analysis for each of the microarray probes used in the EnviroX-Rv test to interrogate the PCR product of the tandem PCR assay.

Analysis was performed on all available “High Quality Genomes”, as defined by GISAID, resident in the GISAID database as of July 15, 2020 (@41,000 in total). Details of the analysis are described in more detail in the Appendix. In all cases, as suggested by GISAID in their own analysis of Inclusivity for the various q-RT-PCR assays, the PCR primer and q-RT-PCR probe sequences designed by the CDC have been used as a standard in these calculations.

PCR Primer Inclusivity. As seen in the Figure (Top and Middle Panels) these PCR Primer Inclusivity calculations show that, among the @41,000 GISAID SARS-CoV-2 “High Quality Genome” sequences analyzed here, the incidence of SARS-CoV-2 genome sequences with 1 or more mismatches anywhere in the PCR Primer (Top Panel) or mismatches within 5 bases proximal to the 3’ terminus of the Probes sequence (Middle Panel) for the PCR Primers of this assay (Blue Bars) are equal or lower than that obtained for the CDC PCR Primers (Red Bars).

There is a single example where there is an “apparent” 40% loss of inclusivity relative to the CDC PCR Primer Standard [N3, Locus FP] Top Panel. But that apparent increase is due to the fact that the “N3 Locus FP” Primer is several bases longer than the CDC N3 FP.

Independent analysis of the [N3, Locus FP] PCR primer region among 10,340 total SARS-CoV-2 genomes available (July 22, 2020) on the on the NCBI database (data not shown here) resulted in 10,242 perfect matches 99.05%, 88 single base mismatches at the terminal 25th base from the 3’ end i.e., 0.85% of “nearly” perfect matches and 10 other mismatches distributed elsewhere within the 25 base span of the [N3, Locus FP] PCR primer (0.1%).

That same calculation cannot presently be performed on the GISAID data base, without new code writing. However, such 5’end positioning of genome changes within the [N3, Locus FP] PCR primer can be
seen by evaluation, as per GISAID guidelines, of the last 5 bases near the 3’ terminus of each PCR Primer (Middle Panel).

As GISAID has already noted, the greatest effect on PCR efficiency will be result from mismatches near the 3’ terminus. That secondary GISAID calculation for [N3, Locus FP] and all other PCR primers of the assay (Middle panel) demonstrates that from all primers >95% of the base changes detected are outside the (crucial) 5 base long primer domain at the 3’ terminus: e.g. the incidence of SARS-COV-2 genomes possessing (debilitating) mismatches near the 3’ terminus for [N3, Locus FP] = @0.07% of the 41 000 genomes (Middle Panel) and as expected from the NCBI calculations is approximately 20 fold less than the total incidence of mismatched genomes in [N3, Locus FP] overall, @1.4.% of the 41 000 genomes (Top Panel).

Thus, based on these PCR Primer Inclusivity calculations among all 41 000 “High Quality” SARS-CoV-2 genomes of the GISAID database (July 15, 2020) the PCR primer Inclusivity in the EnviroX-Rv test is calculated to be comparable to PCR Primer Inclusivity seen with the assay (CDC q-RT-PCR) chosen by GISAID as a reference standard.

Probe Inclusivity. As seen in the Figure (Bottom Panel) Microarray Hybridization Probe Inclusivity calculations show that, among the @41 000 “High Quality Genome” GISAID SARS-Cov2 sequences analyzed here, the incidence of SARS-CoV-2 genome sequences with 1 or more mismatches relative to the target sequence for the Probes of this assay (Blue Bars) are in all cases equal to or lower than that obtained for the CDC Probes (Red Bars). For the Microarray Hybridization Probes (Blue) and CDC q-RT-PCR Probes, the highest incidence of homology loss (@2%) is seen for the N1 locus with N2 and N3 showing much lower values (<0.5%).

Thus, based on these Probe Inclusivity calculations among all 41 000 SARS-CoV-2 “High Quality” SARS-CoV-2 genomes of the GISAID database (July 15,2020) Microarray Hybridization Probe Inclusivity of the
Enviro\textsuperscript{X}-Rv test is calculated to be comparable to Probe Inclusivity seen with the assay (CDC q-RT-PCR) chosen by GISAID as a reference standard.

**Exclusivity Testing**

**Results.**—*In silico* analysis of the recommend organisms (Table 9) and the SARS-CoV-2 N1, N2 and N3 PCR primers was performed. No primer homology greater than 80% was detected among any of the microbial target sequences in the NCBI/Genbank non-redundant databases [GISAID databases only contain SARS-CoV-2/Influenza sequences]. The only partial homology detected was in the human genome for the N1-F Locus primer for Enviro\textsuperscript{X}-Rv, which showed 90% complementarity to a single site within the Human genome. However, the homology included a 3’ inhibitory mismatch, which would have been expected to disable N1-F primer function in PCR and there were no human sequences found suitable to serve as a complementary primer to N1-F within 20 kb of the above mentioned 90% homologous region. Further, the lack of cross-reactivity in the human genome has been confirmed among the numerous negative human matrix samples tested with no evidence of cross-reactivity.

In addition, an *in silico* analysis demonstrated that the PCR amplicon derived from each of the 4 RT-PCR reactions (N1, N2, N3, RNaseP) produced an amplicon which would only hybridize to its cognate probes and not measurably to microarray probes specific for other SARS-CoV-2 regions or to any of the “species-specific” probes not engaged directly in SARS-CoV-2 analysis (Table 1). The amplicon sequences for each SARS-CoV-2 target and RNaseP were compared using BLAST against each of the probe sequences in the array. Calculated cross-hybridization for 5 closely related SARS-CoV-2 probes, per GISAID were detected above the 80% cutoff: 86% PANG-1 and -2, 81% BAT2, 80% SARS-rel, 88% hCoV19 homology, none of these probes are utilized in the Enviro\textsuperscript{X}-Rv assay and based on LoD and clinical experiments we did not observe a measurable cross-hybridization signal that impacted the correct SARS-CoV-2 call (Figure 6).

**Wet-testing**

**Results.**—Cross reactivity analysis through wet-testing was also performed to determine the specificity of the Enviro\textsuperscript{X}-Rv assay against closely related and recommended viruses and bacteria. The Exact
Diagnostics RP Positive Run Control (Catalog Number: RPPOS) is a combination of whole, intact virus and bacteria that have been heated or chemically inactivated, and synthetic RNA transcripts, which was combined with the negative Tricor NP samples to create five replicate samples. RNA was extracted from each of the six replicate samples using the Zymo Research Quick DNA/RNA Viral MagBead kit and 5 µL was used as the input template for the exclusivity wet-testing assay. The results are presented in Table 10 with no cross-reactivity detected. The remaining wet-testing samples will be conducted within 90 days of approval. We have been unable to obtain these samples rapidly due to delays with our vendors.

*Unimolecular Folding Protocol*

*Methodology.*—The purpose of performing the thermodynamic folding simulations is to deduce if the PCR primers and probe are able to bind to their targets without substantial unfolding of the target. Primers that require substantial unfolding or the target are often “fragile” and can give false negatives if a mutation occurs at a primer binding site or if the salt concentrations vary slightly (e.g., due to a bad master mix lot or user intentionally diluting reagents). The steps below provide a recipe for determining the quality of the designs. The numbering referred to below is the numbering for the sense strand of the RNA virus. Use a program (e.g., MFOLD, RNAstructure, VisualOMP, etc.) to predict the secondary structure of the RNA and DNA target regions. An expert will evaluate the reported results to deduce if primers and probes are likely to have problems. We understand that providing primer and probe sequences are sensitive information and thus we request only the amplicon positions, though if the user does provide the primer and probe sequences, then we can provide more specific guidance can be given about the potential problems of the user’s design.

*Testing the Reverse Primer (RP) Binding Region*

Target RNA sequence: Parse out the region where the amplicon is made with an extra 150 nts. on either side of the amplicon (these extra regions are called the 5’-tail and the 3’-tail). For example, if the amplicon covers positions 1000 to 1100 (i.e., amplicon length of 101 nts.), then the RNA region to parse
out would be from 850 to 1250 (i.e., 401 nts long). Since SARS-CoV-2 virus is positive single-stranded RNA virus, this is the strand to which the reverse transcription primer (i.e., RP) will bind.

Perform the folding using “RNA” as the strand type and use the reverse transcription temperature for the PCR reaction (e.g., 50°C) and a salt concentration representative of the conditions in the reverse transcription/PCR reaction (a suggested default values are [monovalent] = 0.08 M and [Mg2+] = 0.002 M). This is DG (total).

Folding of the sequences (amplicons ± 150 nucleotides), primers, and probes were carried out with MFOLD program with the default conditions except the conditions specified in Table 2 and 4. In the folding of the target RNA, complementary DNA, and sense DNA sequences, the approximate locations and the start and the end of the amplicons are marked with red and blue arrows, respectively. The lowest energy structure for each molecule was presented in the file.

We analyzed the RNA reverse primer binding region with additional 150 nts on either side of the amplicon, using MFOLD. In Table 11, we display the resulting total DG of the reverse primer binding region at a temperature of 45°C, Mg2+ salt concentration at 1 mM, and default monovalent concentration. In Figures 7–9, we have the MFOLD structures for each sequence.

*Testing of the Forward Primer (FP) binding region*

Make the reverse compliment of the target region and convert all U's to T's. This is the DNA complementary strand to which the Forward primer will bind (which we refer to as the cDNA target strand).

Predict the secondary structure of the cDNA target region. Perform the folding using “DNA” as the strand type and use the annealing temperature for the PCR reaction (e.g., 60°C) and a salt concentration representative of the conditions in the reverse transcription/PCR reaction (suggested default values are [monovalent] = 0.08 M and [Mg2+] = 0.002 M). This is DG(total).
We analyzed the cDNA Forward Primer target region with additional 150 nts on either side of the amplicon, using MFOLD. In Table 12, we display the resulting total DG of the reverse transcription primer binding region at a temperature of 55°C, Mg2+ salt concentration at 1 mM, and default monovalent concentration. In Figures 10–12, we have the MFOLD structures for each sequence.

**Testing of the Probe binding region**

If the probe binds to the antisense strand, then use the cDNA region described in step 3. If the probe binds to the sense strand, then use the region described in step 1, but convert all the U’s to T’s (call this “DNA sense strand”). Whichever is the appropriate strand, we will call this region the “probe-binding DNA target strand”.

Predict the secondary structure of the probe-binding DNA target strand. Perform the folding using “DNA” as the strand type and use the extension temperature for the PCR reaction (e.g., 72°C) and a salt concentration representative of the conditions in the reverse transcription/PCR reaction (suggested default values are [monovalent] = 0.08 M and [Mg2+] = 0.002 M). This is DG (total).

We analyzed the cDNA of the Probe target region with additional 150 nts on either side of the amplicon, using MFOLD. In Table 13, we display the resulting total DG of the PCR extension temperature at 25°C, Mg2+ salt concentration at 1 mM, and 600 mM Na+ concentration. In Figures 13–15, we have the MFOLD structures for each sequence.

**Primer and Probe Unimolecular Folding**

For each primer and probe, predict their secondary structure. Perform the folding using “DNA” as the strand type and use the annealing temperature for the PCR reaction (e.g., 60°C) and a salt concentration representative of the conditions in the reverse transcription/PCR reaction (suggested default values are [monovalent] = 0.08 M and [Mg2+] = 0.002 M). This is DG (total).
We analyzed the DNA of the Primer and Probe target region with additional 150 nts on either side of the amplicon, using MFOLD. In Table 14, we display the resulting total DG of the PCR extension temperature at 25°C, 45°C, or 55°C, Mg2+ salt concentration at 1 mM, and appropriate monovalent concentration (80 mM Primers and 600 mM Probes).

**Hybridization Protocol**

Perform simulation of 2-state bimolecular hybridization for FP binding to the cDNA target (from step 3) under PCR salt conditions and using the annealing temperature. See Table 15.

Perform simulation of 2-state bimolecular hybridization for RP binding to the RNA target (from step 1) under the PCR salt conditions and using the reverse transcription temperature. See Table 16.

Perform simulation of 2-state bimolecular hybridization for RP binding to the DNA target (from step 1 but with U’s converted to T’s) under the PCR salt conditions and using the annealing temperature. See Table 17.

Perform simulation of 2-state bimolecular hybridization for Probe binding to the DNA target (from step 5) under the PCR salt conditions and using the extension temperature. See Table 18.

**Independent Laboratory Study**

**Matrix Studies**

**Methodology.**—The SARS-CoV-2 isolate used for these studies, USA_WA1/2020, was isolated from the first documented US case of a traveler from Wuhan, China (5). This isolate was sourced from the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA). The virus stock was received from WRCEVA as a 1 mL lyophilizate. Upon receipt the lyophilizate was resuspended in 2 mL of PBS and single-use aliquots frozen at −70°C. Table 19 summarizes the characteristics of the SARS-CoV-2 stock used for these studies. The PFU/mL quantitation information was provided by WRCEVA. GC/mL was determined by MRIGlobal as described below using one of the frozen viral stock aliquots.
Viral genomic copies per mL (GC/mL) was determined by quantitative RT-PCR using a Bio-Rad CFX96 Real-Time Detection System. The standard curve was prepared from Synthetic SARS-CoV-2 RNA (ATCC No. VR-3276SD). The qPCR procedure used N1 primer and probe sequences published by the CDC. Primers and probes were purchased from Integrated DNA Technologies (IDT No. 10006713). TaqPath™ 1-step RT-qPCR Master Mix, CG was sourced from ThermoFisher. Thermal cycling conditions followed those published in the CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel Instructions for Use and are summarized in Table 20.

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. Master mix was prepared as noted in Table 21.

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% 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 (CPE) was apparent by microscopic examination of the Vero cells.
Test Plate Inoculation

Dilutions of SARS-CoV-2 virus stock were prepared in VTM from a frozen viral stock aliquot as shown in Table 22. The same concentrations of virus were used for inoculating test areas for both the Reference (CDC RT-PCR) and Candidate (EnviroX-RV) 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, the volume specified in Table 23 (135 µL) 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 16.7–23.5°C and 25–42% RH (relative humidity) during the plate inoculation and drying process. Components used in the test plate inoculation procedure are listed in Table 23.

Reference Method Plate Sampling

After drying overnight, test areas on the Reference Method test plates were sampled as follows: 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. It will take between 10–15 s to complete. After sampling the test area, the swab was snapped at the break point and placed back into the VTM tube. A random sample ID was assigned to each test area sample. Swab samples were placed in a refrigerator (2-8°C) within 15 min of test area sampling and stored overnight (22 h) before nucleic acid extraction. Components used in the Reference Method test plate sampling are listed in Table 24.
Candidate Method Plate Sampling

Test areas on the Candidate Method test plates were sampled using the provided World Bioproducts swab samplers. Each swab sampler contains a swab fitted to a threaded cap inside a tube containing transport media. Briefly, the excess transport media was expressed from the wet swab by pressing on the inside of the collection tube above the level of the liquid. 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 was screwed back into the collection tube containing transport media. 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 PathogenDx with an ice pack the day of sampling.

Components used in the Candidate Method test plate sampling are listed in Table 25.

Reference Method RT-PCR Testing

Samples from the Reference Method test plate were transferred to an operator not aware of the blinded sample identities for testing on the CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel test kit. Swabs were vortexed for 30 s, and a 140 µL aliquot was transferred to the well of a 96 well microplate. RNA was extracted from the 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. Components used for the Reference Method RT-PCR testing are summarized in Table 26. 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.

Results.—As per criteria outlined in Appendix J of the Official Methods of Analysis Manual, fractional positive results were obtained for the CDC reference method. The probability of detection (POD) was calculated as the number of positive outcomes divided by the total number of trials (6). POD was...
calculated for the candidate presumptive results, POD$\text{C}$ and the reference method, POD$\text{R}$ as well as the difference in the candidate and reference methods, dPOD$\text{C}$. The POD analysis between the Enviro$^\text{X}$Rv assay and the reference method indicated that there was a statistically significant difference when compared to the results of the CDC method utilized in this study, with the candidate method resulting in more positive replicates as it only requires 1 target to be present for a positive sample. A summary of POD analyses is presented in Table 27. Individual results are provided in Table 28.

**Discussion**

In the matrix study, the Enviro$^\text{X}$Rv assay successfully detected the target analyte from stainless steel environmental surface samples. The Enviro$^\text{X}$Rv method demonstrated a high level of specificity in detecting the target analyte in over 40,000 accession numbers from the GISAD database and showing low affinity for non-target organisms. The POD statistical analysis in Table 28 (POD$\text{c}$ 0.90 vs POD$\text{r}$ 0.55), indicated that the candidate method performance was statistically different than the reference method (95% CI 0.12, 0.61) with the candidate method detecting more positive samples.

Based on the principle for detection of the candidate and CDC method, it is not surprising that a difference in the number of positive results was obtained. The CDC method result interpretation requires more than one signature being positive (N1 and N2), increasing the probability of a positive result being due to the presence of intact virus. This makes practical sense as the CDC method is designed for clinical use and has been adapted for surface detection with the WHO sampling method in this validation study. The Enviro$^\text{X}$-Rv assay requires only a single target to be present (N1 and/or N2 and/or N3 conserved target regions), making it more likely to call a sample positive due to these RNA fragments than the CDC method which requires both targets to be present to be called positive.

**Conclusion**
The data from this study supports the product claim that the Enviro\textsuperscript{R} assay can detect SARS-CoV-2 from stainless steel environmental surface samples (LOD of 2000 genomic copies per 2” x 2” test area). Data from the \textit{in silico} analysis indicates the method is highly specific and can detect a wide range of target sequences and discriminate them from background organisms and near neighbors. The results obtained by the POD analysis of the method comparison study demonstrated that the candidate methods performance was not statistically different than that of the reference method.

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Figure 1: Sampling diagram for the Enviro-X-Rv Method
### N1, N2 and N3 PCR Primers with CDC PCR Primers as Reference

**Average Mismatch Frequency throughout the Entire PCR Primer Sequence**

And **Average Mismatch Frequency per Nucleotide within Each PCR Primer Sequence**

| PCR Primer Length (bases) | N1 locus- FP | N1 locus- RP | N1 label- FP | N1 label- RP | CDC N1- FP | CDC N1- RP | N2 locus- FP | N2 locus- RP | N2 label- FP | N2 label- RP | CDC N2- FP | CDC N2- RP | N3 locus- FP | N3 locus- RP | N3 label- FP | N3 label- RP | CDC N3- FP | CDC N3- RP |
|--------------------------|--------------|--------------|--------------|--------------|-----------|-----------|--------------|--------------|--------------|--------------|-----------|-----------|--------------|--------------|--------------|--------------|-----------|-----------|
|                          | 25           | 25           | 25           | 25           | 20        | 24        | 25           | 25           | 25           | 20           | 18        | 25        | 25           | 25           | 25           | 25           | 22        | 21        |
| Average Mismatch Frequency (%) Throughout the PCR Primer sequence | 0.10         | 0.12         | 0.18         | 0.41         | 0.25      | 0.41      | 0.62         | 0.32         | 0.25         | 0.08         | 0.20      | 0.12      | 1.68         | 0.36         | 1.08         | 0.23         | 0.98      | 0.23      |
| Average Mismatch Frequency (%) per nucleotide in the PCR Primer | 0            | 0            | 0.01         | 0.02         | **0.01**  | **0.02**  | 0.02         | 0.01         | 0.01         | 0            | **0.01**  | **0.01**  | **0.01**     | **0.01**     | **0.04**     | **0.01**     | **0.04**  | **0.01**  |

**Figure 2: The Top Panel**
N1, N2 and N3 PCR Primers with CDC Primers as Reference
Average Mismatch Frequency throughout the 3’ End of each PCR Primer Sequence and
Average Mismatch Frequency per Nucleotide within that 5 base long 3’ Region

| Length of 3’ Terminal Domain (bases) | N1 locus PP | N1 locus RP | N1 label PP | N1 label RP | CDC N1 PP | CDC N1 RP | N2 locus PP | N2 locus RP | N2 label PP | N2 label RP | CDC N2 PP | CDC N2 RP | N3 locus PP | N3 locus RP | N3 label PP | N3 label RP | CDC N3 PP | CDC N3 RP |
|-------------------------------------|-------------|-------------|-------------|-------------|-----------|-----------|-------------|-------------|-------------|-------------|-----------|-----------|-------------|-------------|-------------|-------------|-----------|-----------|
| 5                                   | 5           | 5           | 5           | 5           | 5         | 5         | 5           | 5           | 5           | 5           | 5         | 5         | 5           | 5           | 5           | 5           | 5         | 5         | 5         |
| Average Mismatch Frequency (%) Throughout the 3’ Primer Domain | 0.00        | 0.01        | 0.08        | 0.24        | 0.01      | 0.24      | 0.01        | 0.04        | 0.08        | 0.00        | 0.08      | 0.05      | 0.02        | 0.02        | 0.00        | 0.03        | 0.03      | 0.03      | 0.03      |
| Average Mismatch Frequency (%) Per Nucleotide in the 3’ Domain | 0           | 0           | 0.02        | 0.05        | 0         | 0.05      | 0           | 0.01        | 0.02        | 0         | 0.02      | 0.01      | 0.02        | 0           | 0           | 0.01        | 0.01      | 0.01      | 0.01      |

Figure 3: The Middle Panel
### N1,N2 and N3 Hybridization Probes with CDC Probes as Reference

**Average Mismatch Frequency Throughout the Entire Probe Sequence and Average Mismatch Frequency per Nucleotide within Each Probe Sequence**

| Probe Length (bases) | N1 RE1.1 | N1 RE1.2 | N1 RE1.4 | N1 pub CDC | N2 RE1.1 | N2 RE1.2 | N2 RE1.3 | N2 RE1.4 | N2 pub CDC | N3 RE1.1 | N3 RE1.2 | N3 RE1.3 | N3 pub CDC |
|----------------------|----------|----------|----------|-------------|----------|----------|----------|----------|-------------|----------|----------|----------|------------|
|                      | 17.00    | 17.00    | 17.00    | **24.00**   | 15.00    | 16.00    | 17.00    | 17.00    | **23.00**   | 17.00    | 18.00    | 17.00    | **24.00**  |
| Average Mismatch Frequency (%) Throughout the probe sequence | 0.04     | 2.20     | 2.21     | **2.42**    | 0.00     | 0.01     | 0.00     | 0.17     | **0.42**    | 0.07     | 0.00     | 0.13     | **0.07**   |
| Average Mismatch Frequency (%) per nucleotide in the probe | 0.00     | 0.13     | 0.13     | **0.10**    | 0.00     | 0.00     | 0.00     | 0.01     | **0.02**    | 0.00     | 0.00     | 0.01     | **0.00**   |

**Figure 4: The Bottom Panel**
Figure 5: PCR Primer and Probe Inclusivity Calculation
### Table: In Silico Analysis of Cross-Hybridization Potential Among Probes

| Probe Position | Probe Specificity | Probe Specificity |
|----------------|-------------------|-------------------|
| COVID-19       | ND                | ND                |
| ND*            | ND                | ND                |

**White boxes = data used for COVID-19 detection**

**Grey boxes = data used for confirmation of specificity**

**Legend:**
- ND* - Less than 25% calculated homology with PCR Primer generated targets
- Homology <80% is not expected to contribute to hybridization signals as discussed by FDA

**SARS specificity control:** Probes with SARS sequence within test loci.

**Species specific controls:** Probes for other respiratory virus & human

**CDC Probe:** Sequences derived from CDC 2019-Novel Coronavirus assay

**COVID-19** = (2019-nCoV)

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**Figure 6. In Silico Analysis of Cross-Hybridization Potential Among Probes**

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Homology was obtained using NCBI BLAST using Accession nos. NC_045512.2 for COVID-19 and NM_006413.5 for RNAse P.

- No - less than 25% calculated homology with PCR Primer generated targets
- Homology <80% is not expected to contribute to hybridization signals as discussed by FDA

**SARS specificity control:** Probes with SARS sequence within test loci.

**Species specific controls:** Probes for other respiratory virus & human

**CDC Probe:** Sequences derived from CDC 2019-Novel Coronavirus assay

**COVID-19** = (2019-nCoV)

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**UNCORRECTED PROOF**
Figure 7. N1 Reverse Primer predicted RNA structure.
Figure 8. N2 Reverse Primer predicted RNA structure.
Figure 9. N3 Reverse Primer predicted RNA structure.
Figure 10. N1 Forward Primer predicted cDNA structure.
Figure 11. N2 Forward Primer predicted cDNA structure.
Figure 12. N3 Forward Primer predicted cDNA structure.
Figure 13. N1 Probe predicted cDNA structure.
Figure 14. N2 Probe predicted cDNA structure.
Figure 15. N3 Probe predicted cDNA structure.
| Component                                      | Vol, µL per reaction | Final concentration |
|------------------------------------------------|----------------------|----------------------|
| AccessQuick master mix, 2X                    | 25                   | 1X                   |
| RT-PCR Primer Set 1                           | 2                    | 1µM                  |
| AMV Reverse Transcriptase (5u/µL)             | 1                    | 0.1u/µL              |
| Internal EnviroX-Rv positive control          | 2                    | N/A                  |
| Purified RNA sample                           | 5                    | N/A                  |
| Nuclease-Free water (final volume 5 µL)       | 17                   | N/A                  |
| Total volume per reaction                     | 50                   | N/A                  |
Table 2. AccessQuick RT-PCR System reactions conditions

| Steps                        | Temp., °C | Time    | Cycles |
|------------------------------|-----------|---------|--------|
| First strand cDNA synthesis  |           |         |        |
| Reverse transcription        | 45        | 45 min  | 1      |
| AMV RT inactivation and RNA/cDNA/primer denaturation | 94 | 2 min  | 1      |
| Denaturation                 | 94        | 30 s    |        |
| PCR amplification            |           |         |        |
| Annealing                    | 55        | 30 s    | 35     |
| Extension                    | 68        | 1 m     |        |
| Final extension              | 68        | 7 m     | 1      |
| Number of reactions | PCR master mix, µL | Primer set 2, µL | Taq polymerase, µL | Final vol., µL |
|---------------------|--------------------|-----------------|-------------------|----------------|
| 1                   | 62                 | 2.6             | 0.6               | 65.2           |
| 6                   | 372                | 16              | 4                 | 392            |
| 12                  | 698                | 30              | 8                 | 735            |
| 24                  | 1256               | 54              | 14                | 1323           |
| 36                  | 1860               | 80              | 20                | 1960           |
| 48                  | 2418               | 104             | 26                | 2548           |
| Steps | Labeling PCR |
|-------|--------------|
|       | Temp., °C   | Time    | Cycles |
| 1     | 95          | 4 min   | 1      |
| 2     | 95          | 20 s    |        |
| 3     | 55          | 20 s    | 30     |
| 4     | 72          | 30 s    |        |
| 5     | 72          | 7 min   | 1      |
| 6     | 15          | Hold ready for next step | 1 |
Table 5. Reagent volumes for preparation of Pre-hybridization buffer

| 96-Well Plate | Volumes needed for the number of wells being pre-hybridized in the plate<sup>a</sup> |
|--------------|---------------------------------|
|              | 32 Wells | 64 Wells | 96 Wells |
| Molecular biology grade water, mL | 5.587    | 11.174   | 16.761   |
| Buffer 1, mL  | 1.658    | 3.317    | 4.976    |
| Buffer 2, mL  | 0.872    | 1.745    | 2.618    |

<sup>a</sup>Calculations include 20% extra volume to account for pipetting errors.
Table 6. Reagent volumes for preparation of Hybridization Buffer

| 96-Well Plate | Volumes needed for the number of wells being hybridized in the plate<sup>a</sup> |
|---------------|--------------------------------------------------------------------------------|
|               | 32 Wells                      | 64 Wells                      | 96 Wells                      |
| Buffer 1, mL  | 0.463                         | 0.926                         | 1.390                         |
| Buffer 2, mL  | 0.233                         | 0.466                         | 0.700                         |

<sup>a</sup>Calculations include 20% extra volume to account for pipetting errors.
Table 7. Reagent volumes for preparation of Wash Buffer

| 96-Well Plate | 32 Wells | 64 Wells | 96 Wells |
|---------------|----------|----------|----------|
| Buffer 1, mL  | 0.333    | 0.667    | 1.0      |
| Molecular Grade Water, mL | 46.0 | 93.0 | 139.0 |

\(^a\)Volumes are measured in mL.

\(^b\)Calculations include 20% extra volume to account for pipetting errors.
| N1 CoV-2 | N2 CoV-2 | N3 CoV1/2 | RNase P | Status | Results | Action |
|----------|----------|-----------|---------|--------|---------|--------|
| POS      | NEG      | NEG       | POS or NEG | Valid  | SARS-CoV-2 Positive | Report results to test requester and respective authorities. |
| NEG      | POS      | NEG       | POS or NEG | Valid  | SARS-CoV-2 Positive | Report results to test requester and respective authorities. |
| POS      | NEG      | POS       | POS or NEG | Valid  | SARS-CoV-2 Positive | Report results to test requester and respective authorities. |
| NEG      | POS      | POS       | POS or NEG | Valid  | SARS-CoV-2 Positive | Report results to test requester and respective authorities. |
| POS      | POS      | POS       | POS or NEG | Valid  | SARS-CoV-2 Positive | Report results to test requester and respective authorities. |
| NEG      | NEG      | POS       | POS or NEG | Valid  | SARS-CoV-1 and/or SARS-CoV-2 Positive | Repeat test to confirm that the patient is not positive for SARS-CoV-2. Report results to test requester and respective authorities. |
| NEG      | NEG      | NEG       | POS       | Valid  | SARS-CoV-2 Negative | Report results to test requester and respective authorities. Consider testing for other viruses. |
| NEG      | NEG      | NEG       | NEG       | Invalid | N/A | Repeat test. If the repeat results remain invalid, consider collecting a new specimen. |

SARS-CoV-1 and/or SARS-CoV-2 Positive: Repeat test to confirm that the patient is not positive for SARS-CoV-2.
### Table 9. Recommended List of organisms to be analyzed in silico

| Organism                                      | TaxID     |
|-----------------------------------------------|-----------|
| Human genome (taxid:9606)                     |           |
| HCoV-SARS (taxid:694009)                      |           |
| Human coronavirus 229E (taxid:11137)          |           |
| Human coronavirus OC43 (taxid:31631)          |           |
| Human coronavirus HKU1 (taxid:290028)         |           |
| Human coronavirus NL63 (taxid:277944)         |           |
| MERS-coronavirus (taxid:1335626)              |           |
| Human Metapneumovirus (taxid:162145)          |           |
| Adenovirus (taxid:1643649)                    |           |
| Parainfluenza virus 1 (taxid:11210)           |           |
| Parainfluenza virus 3 (taxid:11216)           |           |
| Parainfluenza virus 4a (taxid:11224)          |           |
| Influenza A (taxid:11320)                     |           |
| Influenza B (taxid:11520)                     |           |
| Enterovirus (taxid:12059)                     |           |
| Parainfluenza virus 4a (taxid:1124)           |           |
| Parainfluenza virus 4b (taxid:1126)           |           |
| Streptococcus pneumoniae (taxid:1313)         |           |
| Human parainfluenza virus 2 (taxid:11214)     |           |
| Respiratory syncytial virus (taxid:11250)     |           |
| Rhinovirus (taxid:12059)                      |           |
| Parainfluenza virus 4b (taxid:11226)          |           |
| Haemophilus influenza (taxid:157239)          |           |
| Legionella pneumophila (taxid:446)            |           |
| Mycobacterium tuberculosis (taxid:1773)       |           |
| Streptococcus pyogenes (taxid:1314)           |           |
| Bordetella pertussis (taxid:520)              |           |
| Mycoplasma pneumonia (taxid:2104)             |           |
| Pneumocystis jirovecii (taxid:42068)          |           |
| Candida albicans (taxid:5476)                 |           |
| Pseudomonas aeruginosa (taxid:287)            |           |
| Staphylococcus epidermis (taxid:1282)         |           |
| Streptococcus salivarius (taxid:1304)         |           |
| Chlamydia pneumonia (taxid:83558)             |           |
Table 10. Analytes tested for cross-reactivity wet-testing – Results Table from Emergency Use Authorization

| Analyte                              | Manufacturing targets concentration, cp/mL<sup>a</sup> | Results | Final result |
|--------------------------------------|--------------------------------------------------------|---------|--------------|
| Adenovirus                           | 500 000                                                | 0/5     | Negative     |
| *Bordetella parapertussis*           | 5000                                                   | 0/5     | Negative     |
| *Bordetella pertussis*               | 500 000                                                | 0/5     | Negative     |
| *Chlamydia pneumoniae*               | 1000                                                   | 0/5     | Negative     |
| Coronavirus 229E                     | 500 000                                                | 0/5     | Negative     |
| Coronavirus HKU                      | 20 000 000                                             | 0/5     | Negative     |
| Coronavirus NL63                     | 2 000 000 000                                          | 0/5     | Negative     |
| Coronavirus OC43                     | 25 000                                                 | 0/5     | Negative     |
| Human metapneumovirus                | 20 000 000                                             | 0/5     | Negative     |
| Influenza A H1N1                     | 100 000                                                | 0/5     | Negative     |
| Influenza A H1N1-09                  | 500 000 000                                            | 0/5     | Negative     |
| Influenza A H3N2                     | 500,000,000                                            | 0/5     | Negative     |
| Influenza B                          | 500 000                                                | 0/5     | Negative     |
| *Mycoplasma pneumoniae*              | 1000                                                   | 0/5     | Negative     |
| Parainfluenza 1                      | 50 000                                                 | 0/5     | Negative     |
| Parainfluenza 2                      | 5000                                                   | 0/5     | Negative     |
| Parainfluenza 3                      | 5000                                                   | 0/5     | Negative     |
| Parainfluenza 4a                     | 500 000                                                | 0/5     | Negative     |
| Rhinovirus 1A                        | 500 000                                                | 0/5     | Negative     |
| RSV A                                | 10 000                                                  | 0/5     | Negative     |
| RSV B                                | 10 000                                                  | 0/5     | Negative     |
| MERS                                 | 5 000 000                                              | 0/5     | Negative     |
| SARS-CoV                             | 200 000 000                                            | 0/5     | Negative     |

<sup>a</sup>cp/mL = Copies per milliliter.
### Table 11. Normalized energy (DG) of predicted RNA structures

| Sequences | Temp., °C | Monovalent, mM | Mg++, mM | DG, kcal/mol |
|-----------|-----------|----------------|----------|--------------|
| N1_RNA    | 45        | 80             | 1        | -68.94       |
| N2_RNA    | 45        | 80             | 1        | -83.69       |
| N3_RNA    | 45        | 80             | 1        | -82.02       |
| Sequences | Temperature (°C) | Monovalent (mM) | Mg++ (mM) | DG (kcal/mol) |
|-----------|-----------------|-----------------|-----------|---------------|
| N1_cDNA\(^a\) | 55              | 80              | 1         | -2.09         |
| N2_cDNA   | 55              | 80              | 1         | -3.72         |
| N3_cDNA   | 55              | 80              | 1         | -1.97         |

\(^a\)cDNA = Complementary DNA reverse transcribed from viral RNA.
| Sequences | Temp., °C | Monovalent, mM | Mg++, mM | DG, kcal/mol |
|-----------|-----------|----------------|----------|--------------|
| N1_cDNA   | 25        | 600            | 1        | –82.91       |
| N2_cDNA   | 25        | 600            | 1        | –84.56       |
| N3_cDNA   | 25        | 600            | 1        | –75.67       |

*cDNA = Complementary DNA reverse transcribed from viral RNA.*
Table 14. Normalized energy (DG) of predicted cDNA structures

| Sequences   | Temp., °C | Monovalent, mM | Mg++, mM | DG, kcal/mol |
|-------------|-----------|----------------|----------|--------------|
| N1_FP<sup>a</sup> | 55       | 80             | 1        | 2.49         |
| N1_Probe<sup>b</sup> | 25       | 600            | 1        | -2.06        |
| N1_RP<sup>c</sup>_R | 45       | 80             | 1        | -0.46        |
| N1_RP_P     | 55       | 80             | 1        | 0.57         |
| N2_FP       | 55       | 80             | 1        | 1.62         |
| N2_Probe    | 25       | 600            | 1        | -1.03        |
| N2_RP_R     | 45       | 80             | 1        | 0.75         |
| N2_RP_P     | 55       | 80             | 1        | 1.45         |
| N3_FP       | 55       | 80             | 1        | 2.14         |
| N3_Probe    | 25       | 600            | 1        | -1.62        |
| N3_RP_R     | 45       | 80             | 1        | -0.24        |
| N3_RP_P     | 55       | 80             | 1        | 0.66         |

<sup>a</sup>FP = Forward primer.

<sup>b</sup>Probe = Oligonucleotide probes for detection of viral target sequence in complementary DNA strand.

<sup>c</sup>RP = Reverse primer.
| Sequence A      | Sequence B      | Annealing T., °C | Conc, mm | DG, kcal/mol | Tm, °C |
|----------------|----------------|------------------|---------|--------------|--------|
| N1_cDNA\(^a\)  | N1_FP\(^b\)    | 55               | 200     | -13.4        | 60.9   |
| N2_cDNA        | N2_FP          | 55               | 200     | -13.5        | 60.6   |
| N3_cDNA        | N3_FP          | 55               | 200     | -14.1        | 61.1   |

\(^a\)DNA = Complementary DNA reverse transcribed from viral RNA.
\(^b\)FP = Forward primer.
| Sequence A | Sequence B | Annealing T., °C | Concn, mm | DG, kcal/mol | Tm, °C |
|------------|------------|------------------|-----------|--------------|--------|
| N1_RNA a   | N1_RP b    | 45               | 200       | -34.7        | 81.9   |
| N2_RNA     | N2_RP      | 45               | 200       | -28.0        | 78.8   |
| N3_RNA     | N3_RP      | 45               | 200       | -30.5        | 79.6   |

aRNA = Viral sense RNA.  
bRP = Reverse primer.
| Sequence A  | Sequence B | Annealing T., °C | Conc., mm | DG, kcal/mol | Tm, °C |
|-------------|------------|------------------|-----------|--------------|--------|
| N1_sDNAa    | N1_RPb     | 55               | 200       | –15.8        | 63.5   |
| N2_sDNA     | N2_RP      | 55               | 200       | –14.0        | 61.7   |
| N3_sDNA     | N3_RP      | 55               | 200       | –15.0        | 62.9   |

\( ^a \)sDNA = DNA strand in the same sense as viral RNA.

\( ^b \)RP = Reverse primer.
| Sequence A | Sequence B | Annealing T., °C | Concн | DG, kcal/mol | Tm, °C |
|------------|------------|------------------|-------|--------------|--------|
| N1_cDNA    | N1_Probe   | 25               | 200 mm N/A | -38.9 N/A   | 76.7 N/A |
| N2_cDNA    | N2_Probe   | 25               | 200 mm N/A | -37.5 N/A   | 75.8 N/A |
| N3_cDNA    | N3_Probe(T) | 25               | 200 mm N/A | -37.8 N/A   | 74.8 N/A |
| N3_cDNA    | N3_Probe(C) | 25               | 200 mm N/A | -36.9 N/A   | 75.0 N/A |

\(^a\) cDNA = Complementary DNA reverse transcribed from viral RNA.

\(^b\) Probe = Oligonucleotide probes for detection of viral target sequence in complementary DNA strand.

\(^c\) N/A = The probe is tethered to the surface, rather than in solution, and we are unable to accurately predict any secondary structure formation.

\(^d\) N3_Probe(T) = Degenerate nucleotide Y converted to T in the analysis.

\(^e\) N3_Probe(C) = Degenerate nucleotide Y converted to C in the analysis.
### Table 19. Summary of SARS-CoV-2 virus stock used in the studies

| Virus     | Isolate     | Source/No | Lot   | Lyophilization date | PFU/mL<sup>a</sup> | GC/mL<sup>b</sup> |
|-----------|-------------|-----------|-------|---------------------|----------------------|-------------------|
| SARS-CoV-2| USA_WA1/2020| WRCEVA    | TVP23155 | 2/19/2020           | 3.6 × 10<sup>6</sup> | 1.6 × 10<sup>9</sup> |

<sup>a</sup>PFU/mL = Plaque Forming Units/mL (pre-lyophilization).

<sup>b</sup>GC/mL = Genomic Copies/mL.
| Stage | Temp., °C | Time  | Cycles |
|-------|-----------|-------|--------|
| 1     | 25        | 2 min | 1      |
| 2     | 50        | 15 min| 1      |
| 3     | 95        | 2 min | 1      |
| 4     | 95        | 3 min | 45     |
|       | 55        | 30 s  |        |
| Reagent                              | Vol. per reaction, µL |
|-------------------------------------|-----------------------|
| Nuclease-free water                 | 8.5                   |
| Primer/probe mix                    | 1.5                   |
| TaqPath™ 1-step RT-qPCR Master Mix  | 5.0                   |
| Total                               | 15                    |

Table 21. CDC Assay master mix preparation
| Sample                                      | Method     | Test area size | No. of test areas | GC/mL | µL/Test area | GC/test area |
|---------------------------------------------|------------|----------------|-------------------|-------|--------------|--------------|
| High (1 POD<sup>a</sup>/test area)          | Reference  | 2” × 2”        | 5                 | 1.3 × 10<sup>5</sup> | 135          | 2.0 × 10<sup>4</sup> |
| Low (0.5 POD/test area)                     | Reference  | 2” × 2”        | 20                | 1.3 × 10<sup>4</sup> | 135          | 2.0 × 10<sup>3</sup> |
| Negative VTM control (0 POD/test area)      | Reference  | 2” × 2”        | 5                 | 0     | 135          | 0            |
| High (1 POD/Test Area)                      | Candidate  | 2” × 2”        | 5                 | 1.3 × 10<sup>5</sup> | 135          | 2.0 × 10<sup>4</sup> |
| Low (0.5 POD/test area)                     | Candidate  | 2” × 2”        | 20                | 1.3 × 10<sup>4</sup> | 135          | 2.0 × 10<sup>3</sup> |
| Negative VTM control (0 POD/test area)      | Candidate  | 2” × 2”        | 5                 | 0     | 135          | 0            |

<sup>a</sup>POD = Probability of detection. The POD is based on range-finding studies conducted with the Reference Method.
Table 23. Components Used for Test Plate Inoculation

| Component           | Vendor/Manufacture | Lot. No. | Expiration   |
|---------------------|--------------------|----------|--------------|
| Viral transport media | MRIGlobal          | 9/24/2020 | 9/20/2021    |
Table 24. Components Used for Reference Method Test Plate Sampling

| Component          | Vendor/manufacturer | Part No.     | Lot. No. | Expiration    |
|--------------------|---------------------|--------------|----------|---------------|
| Viral transport media | MRIGlobal           | NA<sup>a</sup> | 9/24/2020 | 9/20/2021     |
| Swabs              | Puritan             | 25-1607 1PFSC | 7168     | 3/1/2025      |

<sup>a</sup>NA = Not available.
| Component        | Vendor/manufacturer   | Part No. | Lot. No.       | Expiration |
|------------------|-----------------------|----------|----------------|------------|
| Swab Sampler     | World Bioproducts     | BLU-SHC  | BL-53815-10149 | 5/5/2022   |
| Component                          | Vendor/manufacture | Part No. | Lot. No.  | Expiration |
|-----------------------------------|--------------------|----------|-----------|------------|
| QIAamp Viral RNA Mini Kit         | Qiagen             | 52906    | 166023562 | 3/1/2022   |
| 2019-nCoV CDC EUA Kit             | IDT                | 10006713 | 535573    | 4/8/2022   |
| TaqPath™ 1-Step RT-qPCR Master Mix, CG | ThermoFisher       | A15299   | 2220404   | 4/30/2021  |
| Matrix                  | Strain          | GU\(^a\)/test area | \(N\)\(^b\) | \(x\)\(^c\) | POD\(_C\)\(^d\) | 95% CI  | Candidate | \(x\) | POD\(_R\)\(^e\) | 95% CI  | dPOD\(_C\)\(^f\) | 95% CI  |
|------------------------|-----------------|---------------------|-------------|-------------|----------------|-------|-----------|--------|----------------|-------|----------------|-------|
| Stainless steel (2” × 2”) | SARS-CoV-2      |                     | 0           | 5           | 0.00           | 0.00, 0.43 | 0.00     | 0.00     | 0.00, 0.43 | 0.00     | –0.43, 0.43 |
|                        | BEI NR-52281    | 1.8 × 10\(^3\)     | 20          | 18          | 0.90           | 0.70, 0.97 | 11       | 0.55     | 0.34, 0.74 | 0.40     | 0.12, 0.61 |
|                        |                 | 1.8 × 10\(^4\)     | 5           | 5           | 1.00           | 0.57, 1.00 | 5        | 1.00     | 0.57, 1.00 | 0.00     | –0.43, 0.43 |

\(^a\)GU/Test Area = 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 28: Individual results for the stainless steel matrix study

| Sample number | EnviroX RV N1 result | EnviroX RV N2 result | EnviroX RV N3 result | EnviroX RV Final result | CDC N1 target result | CDC N2 target result | CDC final result | Low Level |
|---------------|----------------------|----------------------|----------------------|-------------------------|----------------------|----------------------|------------------|-----------|
| 1             | +                    | +                    | +                    | +                       | +                    | +                    | +                | 9/20      |
| 2             | -                    | +                    | +                    | +                       | +                    | +                    | +                | 14/20     |
| 3             | -                    | -                    | +                    | +                       | +                    | +                    | +                | 13/20     |
| 4             | -                    | -                    | +                    | +                       | +                    | -                    | -                | 18/20     |
| 5             | +                    | +                    | -                    | +                       | +                    | +                    | +                | 11/20     |
| 6             | +                    | +                    | +                    | +                       | -                    | -                    | -                | 18/20     |
| 7             | -                    | +                    | -                    | +                       | -                    | -                    | -                | -         |
| 8             | +                    | +                    | +                    | +                       | -                    | -                    | -                | -         |
| 9             | +                    | +                    | +                    | -                       | -                    | -                    | -                | -         |
| 10            | -                    | -                    | -                    | -                       | +                    | +                    | +                | -         |
| 11            | -                    | +                    | -                    | -                       | -                    | +                    | +                | -         |
| 12            | +                    | +                    | +                    | +                       | +                    | +                    | +                | -         |
| 13            | -                    | -                    | +                    | +                       | +                    | +                    | +                | -         |
| 14            | +                    | +                    | +                    | +                       | -                    | +                    | +                | -         |
| 15            | -                    | -                    | -                    | -                       | -                    | -                    | -                | -         |
| 16            | -                    | +                    | -                    | -                       | +                    | +                    | +                | -         |
| 17            | -                    | -                    | +*                   | +                       | -                    | -                    | -                | -         |
| 18            | -                    | +                    | +                    | +                       | +                    | +                    | +                | -         |
| 19            | +                    | +                    | +                    | +                       | +                    | +                    | +                | -         |
| 20            | +                    | +                    | -                    | +                       | +                    | +                    | +                | -         |
| Total         | 9/20                 | 14/20                | 13/20                | 18/20                   | 11/20                | 18/20                | 11/20            |           |

| Sample number | EnviroX RV N1 result | EnviroX RV N2 result | EnviroX RV N3 result | EnviroX RV Final result | CDC N1 target result | CDC N2 target result | CDC final result | High Level |
|---------------|----------------------|----------------------|----------------------|-------------------------|----------------------|----------------------|------------------|-----------|
|               |                      |                      |                      |                         |                      |                      |                  |           |
| 1             | +                    | +                    | +                    | +                       | +                    | +                    | +                | 5/5       |
| 2             | +                    | +                    | +                    | +                       | +                    | +                    | +                | 5/5       |
| 3             | +                    | +                    | +                    | +                       | +                    | +                    | +                | 5/5       |
| 4             | +                    | +                    | +                    | +                       | +                    | +                    | +                | 5/5       |
| 5             | +                    | +                    | +                    | +                       | +                    | +                    | +                | 5/5       |
| Total         | 5/5                  | 5/5                  | 5/5                  | 5/5                     | 5/5                  | 5/5                  | 5/5              |           |

| Sample number | EnviroX RV N1 result | EnviroX RV N2 result | EnviroX RV N3 result | EnviroX RV Final result | CDC N1 target result | CDC N2 target result | CDC final result | Non-inoculated Control Level |
|---------------|----------------------|----------------------|----------------------|-------------------------|----------------------|----------------------|------------------|----------------------------|
| 1             | -                    | -                    | -                    | -                       | -                    | -                    | -                | -             |
| 2             | -                    | -                    | -                    | -                       | -                    | -                    | -                | -             |
| 3             | -                    | -                    | -                    | -                       | -                    | -                    | -                | -             |
| 4             | -                    | -                    | -                    | -                       | -                    | -                    | -                | -             |
| 5             | -                    | -                    | -                    | -                       | -                    | -                    | -                | -             |
| Total         | 0/5                  | 0/5                  | 0/5                  | 0/5                     | 0/5                  | 0/5                  | 0/5              | 0/5            |

aN3 only positive targets may be positive for SARS-CoV-1 or SARS-CoV-2. Samples are considered positive. Refer to Table 8 for more details.