White Paper

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Development and Evaluation of Two SARS-CoV-2 RT-PCR Laboratory Developed Tests on the ARIES® Automated, Sample-to-Answer, Real-Time PCR System

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Introduction

In December 2019, pneumonia caused by a novel coronavirus was detected in the Wuhan province of China. In a matter of weeks, the new coronavirus SARS-CoV-2 evolved from its first zoonotic human infection, likely transmitted from a bat and/or a pangolin (scaly anteater) source, to the global health threat now known as coronavirus disease-2019 (COVID-19). By early March 2020, the disease had infected more than 100,000 people in more than 100 countries and caused over 3,500 deaths.[1]

Coronaviruses are a significant source of novel zoonotic respiratory infections, often transmitted from bats.[2] In 2002, an outbreak of severe acute respiratory syndrome (SARS) in China killed nearly 800 people, with more than 8,000 people infected.[3] The Middle East respiratory syndrome (MERS) coronavirus was first identified in 2012; since then, it has routinely infected hundreds of people each year in the Middle East. It also led to an outbreak in South Korea in 2015 that killed dozens.[4]

Thanks to rapid technological advances, scientists can characterize the biology of these emerging coronaviruses faster than ever.[5] Within weeks of the first identified cases of the virus that would become known as SARS-CoV-2, scientists had sequenced the viral genome.[6] By mid-February, more than 80 genome assemblies had been produced in laboratories around the world.[7] The publication of the clinical characteristics of COVID-19 in hospitalized patients also sheds light on the virus and its course of infection in humans.[8,9]

This critical information paved the way for accelerated development of primers and probes for molecular detection of the virus. Primer designs were quickly published by laboratories in Germany, China, and the United States, and were then tested by public health agencies. These developments have made it possible for laboratories to develop and perform assays for SARS-CoV-2.[10-13] By mid-February, international teams made tests available for laboratories around the world to run SARS-CoV-2 assays at scale.[14]

As the number of confirmed infections rises and coincides with the peak of flu season in the northern hemisphere, global demand for testing has expanded well beyond existing reagent production capacity. On February 28, 2020, the American Society for Microbiology (ASM) submitted a letter to the US Food and Drug Administration (FDA) voicing concerns about the impact of Emergency Use Authorization (EUA) regulations on the use of diagnostic tests by clinical laboratories during the COVID-19 outbreak.[15] The FDA responded the next day and took steps to expand testing for SARS-CoV-2, allowing clinical laboratories to begin using their local laboratory developed tests (LDTs) before FDA clearance.[16]

Laboratories performing LDTs would benefit from having a SARS-CoV-2 assay that could be performed on an automated, sample-to-answer instrument system with the flexibility to handle STAT or batch testing, as well as the capability of performing different assays at the same time. The ARIES® System from Luminex Corporation is a sample-to-answer PCR instrument that can run LDTs, as well as Luminex's IVD assays. ARIES® Systems allow users to develop LDTs using the ARIES® Extraction cassettes, an appropriate ARIES® PCR Ready Mix, the proprietary SYNCT™ Software, and the User-Defined Protocol (UDP) application.

Here, we report the development and evaluation of two SARS-CoV-2 automated, real-time reverse transcriptase PCR (RT-PCR) assays using primer and probe sequences published by the China National Institute for Viral Disease Control and Prevention (China CDC) and the US Centers for Disease Control and Prevention (US CDC) on the ARIES® System.[11-13]

Materials and Methods

Participating Laboratories

Four diagnostic laboratories located in North America participated in the study: Site 1, Baylor Scott and White Health, Temple, TX; Site 2, Montefiore Medical Center, Bronx, NY; Site 3, Geisinger, Danville, PA; and Site 4, University of Louisville, Louisville, KY.

Samples

Positive samples were derived from synthetic double-stranded DNA gBlocks corresponding to SARS-CoV-2 gene fragments (Integrated DNA Technologies, Inc. (IDT), Coralville, IA). Each gBlock was 1.5–2 kilobases (kb) in length, with the primer/probe target region residing approximately in the middle of the gBlock.

gBlock gene targets were resuspended in IDTE buffer (IDT) at an approximate concentration of 10 µg/mL, based on molecular weight. DNA concentrations were verified by spectrophotometry on a Nanodrop 1000 according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA).

Positive samples for limit of detection (LoD) experiments were prepared by combining the appropriate gBlock targets at equal concentrations and diluting to 50,000 copies/mL in M10 buffer (1 mM MOPS, pH 7.5, 0.1 mM EDTA, pH 8.0) containing 0.1 ng/mL of a 20-mer poly dA oligonucleotide. The target mixture was further diluted to 1,500 copies/mL of each target in Universal Transport Medium (UTM) viral transport medium (Copan Diagnostics, Murrieta, CA) containing 0.5 µg/mL human genomic DNA (gDNA) (Promega, Madison, WI) and 50 µg/mL poly-A carrier RNA (Qiagen, Germantown, MD). Finally, 200 µL of the target mixture was loaded into the sample chamber of the ARIES® cassette, providing 300 copies of each SARS-CoV-2 gene target, 100 ng gDNA, and 10 µg carrier RNA.

For experiments using contrived human nasopharyngeal swab (NPS) sample matrix, positive samples were prepared by combining the appropriate gBlock targets at equal concentrations and diluting to 1,000,000 copies/mL using M10 buffer. The target mixture was further diluted to 7,500 copies/mL of each target in negative NPS sample matrix containing 0.5 µg/mL gDNA and 50 µg/mL carrier RNA. Finally, 200 µL was loaded into the sample chamber of the ARIES® cassette, providing 1,500 copies of each SARS-CoV-2 gene target, 100 ng gDNA, and 10 µg carrier RNA. For this study, the NPS sample matrix was derived from individual NPS samples and not from pooled samples.

Negative samples consisted of either UTM or negative NPS sample matrix, each containing 0.5 µg/mL gDNA and 50 µg/mL carrier RNA, providing final amounts of 100 ng and 10 µg per cassette, respectively.

Oligonucleotides

Oligonucleotide primers and probes were obtained from IDT. Assay Version 1 was based on the sequences published by the China CDC.[11] The RNase P internal control target was derived from the assay published by the US CDC.[12, 13] Gene targets, nucleotide sequences, and final concentrations are shown in **Table 1**. Assay Version 2 was based on the sequences published by the US CDC.[12, 13] Gene targets, nucleotide sequences, and final concentrations are shown in **Table 2**. See **Appendices A-D**[18-21] for *in silico* inclusivity/cross-reactivity analysis of the primers and probes for each assay.

Table 1. Assay 1 Oligonucleotides[a]

Gene Target	ORF1ab	HKU-N	RNase P
Forward Primer			
Primer ID	China_ ORF_F	China _N_F	RP-F
Sequence (5′->3′)	CCCTGTGGGTTTTACACTTAA	GGGGAACTTCTCCTGCTAGAAT	AGATTTGGACCTGCGAGCG
Final Concentration (µM)	0.2	0.2	0.1
Reverse Primer			
Primer ID	China_ORF_R	China_N_R	RP-R
Sequence (5′->3′)	ACGATTGTGCATCAGCTGA	CAGACATTTTGCTCTCAAGCTG	GAGCGGCTGTCTCCACAAGT
Final Concentration (µM)	0.2	0.2	0.1
Probe			
Primer ID	China_ORF_Probe_FAM	China_Probe_TxRed	RP-P_TYE665
Sequence (5′->3′)	/56-FAM/CCGTCTGCG/ZEN/ GTATGTGGAAAGGTTATGG/3IABkFQ	/5TexRD-XN/ TTGCTGCTGCTTGACAGATT/3IAbRQSp	/5TYE665/ TTCTGACCTGAAGGCTCTGCGCG/ 3IAbRQSp
Final Concentration (µM)	0.3	0.3	0.1

[a]56-FAM = 5′ 6-FAM (Fluorescein), 3IABkFQ = 3′ Iowa Black® FQ, 5TexRD-XN = 5′ Texas Red®-X (NHS Ester), 3IAbRQSp = 3′ Iowa Black® RQ, 5TYE665 = 5′ TYE™ 665

Table 2. Assay 2 Oligonucleotides[a]

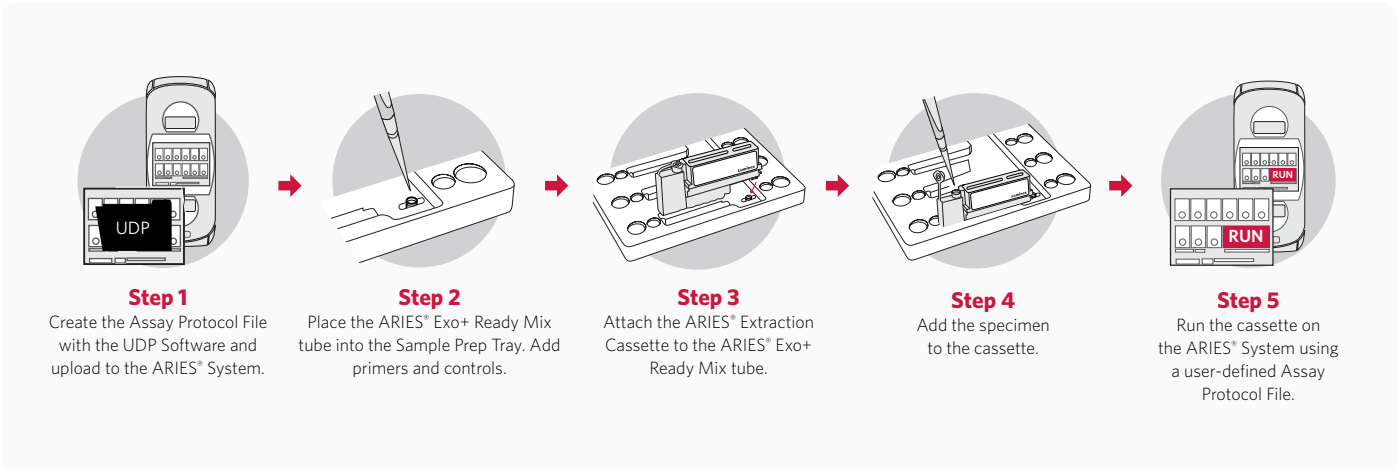
Gene Target	N1	N3	RNase P
Forward Primer			
Primer ID	2019-nCoV_N1-F	2019-nCoV_N3-F	RP-F
Sequence (5′->3′)	GACCCCAAAATCAGCGAAAT	GGGAGCCTTGAATACACCAAAA	AGATTTGGACCTGCGAGCG
Final Concentration (µM)	0.2	0.2	0.1
Reverse Primer			
Primer ID	2019-nCoV_N1-R	2019-nCoV_N3-R	RP-R
Sequence (5′->3′)	TCTGGTTACTGCCAGTTGAATCTG	TGTAGCACGATTGCAGCATTG	GAGCGGCTGTCTCCACAAGT
Final Concentration (µM)	0.2	0.2	0.1
Probe			
Primer ID	2019-nCoV_N1-P	2019-nCoV_N3-P	RP-Pr
Sequence (5′->3′)	/56-FAM/ACCCCGCAT/ZEN/ TACGTTTGGTGGACC/3IABkFQ/	/56-TAMN/AYCACATTGGCACCCGCAATCCTG /3IAbRQSp/	/5TYE665/TTCTGACCTGAAGGCTCTGCG CG/3IAbRQSp/
Final Concentration (µM)	0.3	0.3	0.1

[a]56-FAM = 5′ 6-FAM (Fluorescein), 3IABkFQ = 3′ Iowa Black® FQ, 56-TAMN = 5′ TAMRA (NHS Ester), 3IAbRQSp = 3′ Iowa Black® RQ, 5TYE665 = 5′ TYE™ 665

ARIES® SARS-CoV-2 Assay

ARIES® Extraction Cassettes contain lysis and extraction reagents designed to rupture cells, extract nucleic acid, and remove inhibitors. Released nucleic acids are bound by magnetic nucleic acid capture particles, which are then conveyed via a series of turnstiles where they are washed. 50 µL of purified nucleic acids are eluted into the attached PCR tube containing the ARIES® Ready Mix (PCR master mix) appropriate for the application. For this assay, we used the ARIES® Exo+ Ready Mix. ARIES® Exo+ Ready Mix provides the PCR master mix components needed to run user-defined protocols based on traditional hydrolysis probe chemistry on any ARIES® System. The mix comes in individually sealed PCR tubes to which users add target primers and probes, and then attach the tube to an ARIES® Extraction Cassette. See **Figure 1**.

Figure 1. ARIES® Exo+ Ready Mix Workflow



Step 1
Create the Assay Protocol File with the UDP Software and upload to the ARIES® System.

Step 2
Place the ARIES® Exo+ Ready Mix tube into the Sample Prep Tray. Add primers and controls.

Step 3
Attach the ARIES® Extraction Cassette to the ARIES® Exo+ Ready Mix tube.

Step 4
Add the specimen to the cassette.

Step 5
Run the cassette on the ARIES® System using a user-defined Assay Protocol File.

The UDP App (an application in the SYNCT instrument software) was used to create an assay file which was then installed on the ARIES® Instruments. The assay file controls sample extraction, real-time RT-PCR, and data analysis. The UDP App is used to program the assay-specific thermal profile, RT-PCR cycle threshold (C_t) cutoffs, and to set result language. Initial sample testing data was used to set the C_t cutoffs, which were then used for all subsequent experiments. Finalizing these parameters should be based on data from the validation study, then used for sample testing. Any changes made to the assay parameters would need to be re-validated before subsequent sample testing.

For both Assay 1 and Assay 2, real-time RT-PCR was performed according to the following thermal cycling profile:

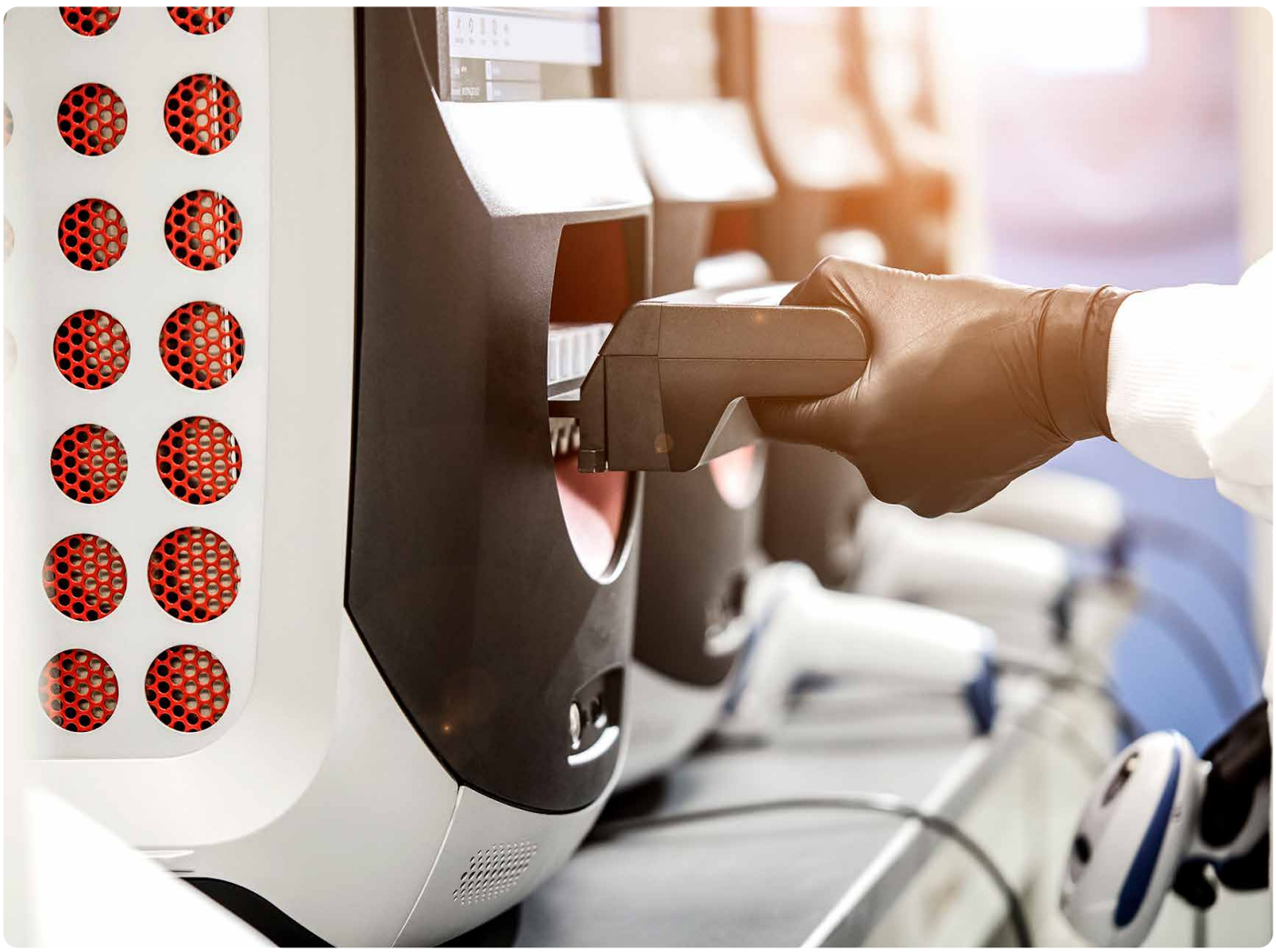
Preheat/RT step – 1 cycle for 7 minutes at 50°C

Activation step – 1 cycle for 2 minutes at 95°C

Cycling – 45 cycles of 95°C for 15 seconds and 60°C for 30 seconds

For each reaction, the appropriate primers and probes (see **Tables 1 and 2**) were diluted such that 5.0 µL of multiplexed primer and probe mixture was added to the ARIES® Exo+ Master Mix tube. The reaction tube was then snapped into place on an ARIES® Extraction Cassette. A 200 µL aliquot of the sample was pipetted into the sample chamber of the cassette, the cap was closed, and the cassette was placed into a magazine for loading on the ARIES® Instrument (see **Figure 2**).

Figure 2. Loading the ARIES® System



Result Interpretation

Results of each assay were interpreted according to the call algorithm shown in **Table 3**.

Table 3. Interpretation of Assay Results

Assay 1 Call Algorithm			
Gene Target			Result Interpretation
ORF1ab	N	RNase P	
+	+	+/-	SARS-CoV-2 detected
-	-	+	SARS-CoV-2 not detected
+	-	+/-	Presumptive SARS-CoV-2 positive[a]; retest
-	+	+/-	Presumptive SARS-CoV-2 positive[a]; retest
-	-	-	Invalid result
Assay 2 Call Algorithm			
Gene Target			Result Interpretation
N1	N3	RNase P	
+	+	+/-	SARS-CoV-2 detected
-	-	+	SARS-CoV-2 not detected
+	-	+/-	Presumptive SARS-CoV-2 positive[a]; retest
-	+	+/-	Presumptive SARS-CoV-2 positive[a]; retest
-	-	-	Invalid result

[a]Based on *in silico* analysis

Results

Each site tested 20 positive samples in UTM (300 copies of each SARS-CoV-2 gene target, 100 ng gDNA, and 10 µg carrier RNA per cassette) to determine the C_t cutoffs for one or both assays (data not shown). C_t cutoffs were established at 6 standard deviations above the mean C_t for each target.

For LoD verification experiments, each site tested 20 positive samples of UTM containing 300 copies per SARS-CoV-2 gene target, 100 ng gDNA, and 10 µg carrier RNA per cassette, and 20 negative samples of UTM containing 100 ng gDNA, and 10 µg carrier RNA per cassette.

Each site then tested 10 contrived samples in human NPS sample matrix containing 1,500 copies of each SARS-CoV-2 gene target, 100 ng gDNA, and 10 µg carrier RNA per cassette, and 10 negative NPS samples containing 100 ng gDNA, and 10 µg carrier RNA per cassette.

The overall results for each assay (all samples, all sites) are shown in **Tables 4 and 5**.

Table 4. Overall Results for Assay 1 on the ARIES® System

ARIES® SARS-CoV-2 Assay 1 Observed Result	Expected Result		
	Positive	**Negative**	**Total**
Positive	93	0	93[a]
Negative	0	89	89[b]
Total	93	89	182
	95% Confidence Interval[c]		
Positive Percent Agreement	100%	96.03%–100%	
Negative Percent Agreement	100%	95.86%–100%	

[a]One positive UTM sample generated an invalid result and was not re-run. This sample was excluded from the calculations. Three positive UTM samples were presumptive positive on initial testing (ORF1ab not detected = 2; N not detected = 1). Two of these (ORF1ab not detected = 1; N not detected = 1) were re-run and were positive. These results were included in the calculations. Two positive NPS samples were presumptive positive on initial testing (ORF1ab not detected). Both were positive on re-run.

[b]One negative UTM sample generated an invalid result and was not re-run. This sample was excluded from the calculations.

[c]95% confidence intervals were determined according to the methods described by Newcombe using the calculator available at http://vassarstats.net/clin1.html.

Table 5. Overall Results for Assay 2 on the ARIES® System

ARIES® SARS-CoV-2 Assay 1 Observed Result	Expected Result		
	Positive	**Negative**	**Total**
Positive	60	0	60[a]
Negative	0	60	60
Total	60	60	120
	95% Confidence Interval[b]		
Positive Percent Agreement	100%	93.98%-100%	
Negative Percent Agreement	100%	93.98%-100%	

[a]One positive UTM sample generated an invalid result and was not re-run. This sample was excluded from the calculations.

[b]95% confidence intervals were determined according to the methods described by Newcombe using the calculator available at http://vassarstats.net/clin1.html.

The results per site are shown in **Tables 6 and 7**.

Table 6. Results Obtained at Each Site for Assay 1

Sample Type[a]	Agreement with Expected Results					
	Site 1		Site 2		Site 3	
UTM Positive	22/22	100%	20/20	100%	19/19	100%
UTM Negative	20/20	100%	20/20	100%	21/21	100%
NPS Positive	10/10	100%	10/10	100%	12/12	100%
NPS Negative	10/10	100%	10/10	100%	8/8	100%

[a]UTM Positive samples contained 300 copies of each SARS-CoV-2 gene target per cassette. NPS Positive samples contained 1,500 copies of each SARS-CoV-2 gene target per cassette. See Materials and Methods.

Table 7. Results Obtained at Each Site for Assay 2

Sample Type[a]	Agreement with Expected Results			
	Site 1		Site 4	
UTM Positive	20/20	100%	19/19	100%
UTM Negative	20/20	100%	19/19	100%
NPS Positive	10/10	100%	11/11	100%
NPS Negative	10/10	100%	11/11	100%

[a]UTM Positive samples contained 300 copies of each SARS-CoV-2 gene target per cassette. NPS Positive samples contained 1,500 copies of each SARS-CoV-2 gene target per cassette. See Materials and Methods.

The mean C_t and standard deviations for each target in each assay are shown in **Tables 8 and 9.**

Table 8. Overall and Per Site Mean C_t Results for Assay 1

	Gene Target					
	ORF1ab		N		RNase P	
	Mean C_t	St Dev[a]	Mean C_t	St Dev	Mean C_t	St Dev
UTM Samples						
Overall	28.22	2.016	32.30	0.856	27.92	0.849
Site 1	30.00	1.293	32.49	0.847	27.89	0.491
Site 2	27.87	1.959	32.46	0.901	28.01	0.803
Site 3	26.54	0.770	31.91	0.745	27.86	1.151
NPS Samples[b]						
Overall	28.86	2.564	33.10	1.619	26.12	2.619
Site 1	31.08	1.177	33.10	1.051	24.49	2.982
Site 2	29.53	1.744	33.39	1.265	26.82	1.463
Site 3	26.46	1.930	32.87	2.245	27.04	2.462

[a]St Dev = standard deviation.

[b]Note that a higher St Dev for RNase P is expected due to variation in gDNA content of NPS sample matrix.

Table 9. Overall and Per Site Mean C_t Results for Assay 2

	Gene Target					
	N1		N3		RNase P	
	Mean C_t	St Dev[a]	Mean C_t	St Dev	Mean C_t	St Dev
UTM Samples						
Overall	31.55	2.114	31.23	1.907	27.86	1.144
Site 1	30.58	2.416	30.44	2.197	27.56	0.738
Site 4	32.56	1.069	32.06	1.081	28.21	1.399
NPS Samples[b]						
Overall	33.00	1.721	32.77	1.687	26.53	3.214
Site 1	33.10	1.549	32.94	1.467	24.97	3.457
Site 4	32.90	1.934	32.62	1.907	27.95	2.221

[a]St Dev = standard deviation.

[b]Note that a higher St Dev for RNase P is expected due to variation in gDNA content of NPS sample matrix.

Inclusivity and cross-reactivity were assessed by *in silico* analysis (**Appendices A-D**[18-21]). For Assay 1 (China CDC), inclusivity analysis included 177 ORF1ab and 188 N gene target sequences with full coverage of all three oligo-binding regions (all sequences available in the GISAID database as of March 5, 2020, and the SARS-CoV-2 reference sequence from GenBank). All genetic sequences were included in the analyses for both the ORF1ab and N oligonucleotide sets. Except for six sequences in the N gene primers and probe, all sequences showed 100% homology with SARS-CoV-2. For cross-reactivity assessment, 263 sequences representing multiple strains of the SARS coronavirus and the bacteria described in FDA's Table 1[17] were assessed by *in silico* analysis. Except for two strains of SARS-coronavirus, no organisms reached homology of >80%. Homology for the two *in silico* cross-reactive strains ranged from 80.0%–90.1%. In addition, 19 sequences representing multiple strains of the bacteria described in FDA's Table 2[17] were analyzed in silico for cross-reactivity. No organisms showed homology >80%. Cross-reactivity analysis included all sequences available from GenBank as of February 24, 2020 (see **Appendices A and B**[18, 19]).

For Assay 2 (US CDC), inclusivity analysis included 188 N gene target sequences with full coverage of all three oligo-binding regions (all sequences available in the GISAID database as of March 5, 2020, and the SARS-CoV-2 reference sequence from GenBank). All genetic sequences were included in the analyses for both N1 and N3 oligonucleotide sets. Except for two sequences in the N1 primers and probe, and six sequences in the N3 primers and probe, all sequences showed 100% homology. For cross-reactivity, 590 sequences representing multiple strains of the SARS coronavirus and the bacteria described in FDA's Table 1[17] were assessed by *in silico* analysis. Except for two strains of SARS-coronavirus, no organism showed homology >80%. Homology for the two SARS strains ranged from 90.5%–95.8%. In addition, 19 sequences representing multiple strains of the bacteria described in FDA's Table 2[17] were analyzed *in silico* for cross-reactivity. No organisms showed homology >80%. Cross-reactivity analysis included all sequences available from GenBank as of February 24, 2020 (see **Appendices C and D**[20, 21]).

Discussion

Here we report the results of a multicenter study to evaluate published SARS-CoV-2 primers and probes in an automated, sample-to-answer, real-time RT-PCR format using the Luminex ARIES® System. We developed two assays based on the primer and probe designs from the China CDC and the US CDC using traditional hydrolysis probe chemistry. ARIES® Systems allow users to develop LDTs, in addition to using ARIES® IVD assays. The SARS-CoV-2 assays were performed on the ARIES® Instrument using ARIES® Extraction Cassettes with the ARIES® Exo+ Ready Mix PCR master mix.

Each site initially tested 20 contrived UTM samples to determine C_t thresholds, which were verified using 20 positive UTM samples (300 copies of each synthetic gene target) and 20 negative UTM samples to verify the threshold settings. Subsequently, each site tested 10 contrived positive samples in negative human NPS matrix (1,500 copies of each synthetic gene target) and 10 negative NPS. Both assays performed extremely well at each site, as shown by the 100% overall positive and 100% overall negative agreement for both assays. Results were consistent across sites with only 1 invalid result of 182 total samples tested on Assay 1. Of a total of 5 presumptive positive samples, 4 were re-run and yielded positive results upon repeat. Of the 120 samples tested on Assay 2, there was 1 invalid result and no presumptive positive results. Based on the contrived nature of the samples and the limited number of tests performed, the mean C_t values and variation are in the expected range. Variation in the lots of materials used in this study may have also impacted the variation in C_t values within and across sites.

There are some limitations to this study as only a low number of samples were tested by each site for the evaluation. In addition, testing was limited to samples contrived with dsDNA templates and no viral RNA has been tested to date. We are continuing to test samples and evaluate these assays, as well as conducting further experiments to determine the performance of these assays in our laboratories.

With the rising number of COVID-19 cases globally, rapid identification and detection of the SARS-CoV-2 is of paramount importance to help prevent spread of the virus and ensure effective infection control measures. With the ability of clinical laboratories to develop and use LDTs for testing, an automated, sample-to-answer instrument platform such as the ARIES® System can provide rapid results with minimal hands-on time. The ARIES® System is capable of running LDTs as well as Luminex IVD Assays, with the flexibility to handle STAT or batch testing. Furthermore, the closed system architecture minimizes the chance of contamination, reducing the risk of false positive results. As we continue to learn about SARS-CoV-2, developing a multiplex assay that can detect other coronavirus strains and other respiratory pathogens in addition to SARS-CoV-2 might be useful in the future.

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21. APPENDIX D_Cross Reactivity Alignments_SARS-CoV-2_Assay 2_US CDC_Feb 24 2020. Access from: **www.luminexcorp.com/APPENDIX-D**.