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ORIGINAL RESEARCH

Development of a real-time Reverse-Transcription PCR for SARS-CoV-2 on the Luminex ARIES® Platform

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Abstract

The University of Louisville Infectious Diseases Laboratory followed the US Food and Drug Administration (FDA) Emergency Use Authorization (EUA) guidance for developing a molecular diagnostic test for SARS-CoV-2 to help address the novel coronavirus pandemic. As a Clinical Laboratory Improvement Amendment '88 (CLIA) certified, high-complexity clinical laboratory, the Infectious Diseases Laboratory chose to use the Luminex ARIES® platform to evaluate a laboratory developed test. This instrument was already familiar to the Infectious Diseases Laboratory and in use for molecular diagnostic testing for pathogens causing atypical pneumonia and two tick-borne pathogens. The FDA EUA guidance for molecular diagnostic tests recommended limit of detection studies, inclusivity and exclusivity (specificity) analysis, and validation with clinical samples to ensure that the performance of the assay was acceptable for use as a molecular diagnostic tool. Data obtained from these experiments demonstrated acceptable performance per FDA guidance, as well as for CLIA requirements. Thus, the real-time Reverse Transcription PCR assay was implemented for diagnostic use on March 27, 2020, and was of great benefit to the local community in responding to the pandemic.

Introduction

Coronaviruses are a large family of single-stranded RNA viruses. Middle East Respiratory Syndrome virus (MERS) and Severe Acute Respiratory Syndrome virus (SARS) are two recent examples of novel coronaviruses that originated in animals and then spread to humans.[1] The World Health Organization declared that SARS-CoV-2 was a global pandemic on March 11, 2020.[2]

The US Food and Drug Administration's (FDA) Emergency Use Authorization (EUA) guidance for validation of molecular assays for SARS-CoV-2, published originally on February 29, 2020, allowed high-complexity, CLIA-certified laboratories to validate molecular assays and submit their data to the FDA for review. The requirements initially set forth in the EUA included establishing and verifying the limit of detection (LoD) or sensitivity, providing inclusivity and exclusivity analysis (specificity), and performing analysis on contrived clinical samples (30 negative and 30 positive) using the sample type(s) intended to be collected and tested using the assay. As more clinical materials positive for SARS-CoV-2 became available in the course of the pandemic, the FDA updated the EUA guidance (July 2020), requesting that laboratories use true clinical samples rather than contrived samples to supplement the initial data packet.

The University of Louisville Infectious Diseases Laboratory is a CLIA-certified, high-complexity laboratory with previous experience using laboratory-developed tests on the Luminex ARIES® system.[3–6] To meet local testing needs related to the pandemic, the Infectious Diseases Laboratory partnered with Luminex Corporation to validate a SARS-CoV-2 real-time Reverse Transcription PCR assay (RT-PCR) using a slight modification of the CDC primer and probe sets targeting the N1 and N3 nucleocapsid genes, as well as the human RNase P gene, to create a multiplex molecular diagnostic assay. Data was compiled and published, along with that of three other laboratories using the Luminex ARIES® instrument, in a white paper in March 2020.[7]

Table 1. SARS-CoV-2 Primer and Probe Information.[a]

Gene Target	N1	N3	RNase P
Forward Primer			
Primer ID	2019-nCoV_N1-F	2019-nCoV_N3-F	RP-F
Sequence (5μ->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 (5μ->3')	TCTGGTTACTGCCAGTTGAATC TG	TGTAGCACGATTGCAGCATTG	GAGCGGCTGTCTCCACAAGT
Final concentration (μM)	0.2	0.2	0.1
Probe			
Probe ID	2019-nCoV_N1-P	2019-nCoV_N3-P	RP-Pr
Sequence (5μ->3')	/56-FAM/ACCCCGCAT/ZEN/TAC GTTTGGTGGACC/3IABkFQ/	/56-TAMN/AYCACATTGGCACCC GCAATCCTG/3IAbRQSp/	/5TYE665/TTCTGACCTGAAGGC TCTGCGCG/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

This report details our laboratory's experience validating a molecular diagnostic assay to detect SARS-CoV-2, using guidance for EUA from the US FDA, as well as CLIA requirements for high-complexity testing. The aim of this study was to provide data to support a sample-to-answer testing option for laboratories to facilitate the sensitive and specific detection of SARS-CoV-2 RNA in nasopharyngeal swab specimens.

Methods

Reagents

SARS-CoV-2 nucleocapsid specific N1 and N3 gene targets, as well as the internal control human RNase P gene target, primers and probe sets, were obtained from Integrated DNA Technologies (Luminex SARS-CoV-2 Panel 2, Integrated DNA Technologies, Coralville, IA, USA) (**Table 1**). ARIES® TaqMan Exo+ Ready Mix® (Luminex Part number: 3698, Austin, TX) and ARIES® extraction cassettes (Luminex Part number: 50-10026) were purchased, ready to use. Carrier RNA (cRNA) (QIAGEN Part number 1017647, Germantown, MD) prepared as 1 μg/μL solution in AVE elution buffer (QIAGEN Part number 1026956), and stored in the -20°C freezer prior to use.

Instrument used with Test

The SARS-CoV-2 real-time RT-PCR test was performed on the Luminex ARIES® system, a sample-to-answer instrument that can be used with both *In Vitro* Diagnostic PCR assays and Laboratory Developed Tests. The ARIES® instrument required extraction cassettes, ARIES® ReadyMix®, and Luminex ARIES® proprietary SYNCT™ Software, as well as the User-Defined Protocol (UDP) application. No separate extraction instrument was required for this assay. The instrument settings for this protocol are shown in **Table 2**. To be considered positive for N1 detection, the Ct value had to be less than 38; positive for N3, less than 37; and positive for RNase P, less than 45 (**Table 2**). Following completion of the test run, the results were considered acceptable if the assay passed quality control. Results were then interpreted according to **Table 3**.

Luminex ARIES® SARS-CoV-2 real time RT-PCR assay (modified CDC assay)

The original CDC real-time RT-PCR assay authorized by the FDA required a separate extraction and PCR step, as well as three separate wells for amplification of each target (N1, N2, and RNase P). The Luminex ARIES® did not require a separate extraction step, however. The CDC assay used the FAM dye and Black Hole Quencher 1 (BHQ-1) on all three probes. The ARIES® assay combined N1, N3, and RNaseP in one multiplex reaction per cassette.[7] Since the targets were multiplexed in one cassette, the dyes on the probes needed to be modified to facilitate differentiation of the three gene targets. For the N1 target on the ARIES® assay, FAM was used with a double quencher: ZEN™ Internal Quencher positioned between the ninth (9th) and tenth (10th) nucleotide base in the oligonucleotide sequence and Iowa Black® FQ (3IABkFQ) located at the 3'-end. For the N3 target on ARIES®, the TAMRA dye was used

Table 2. ARIES instrument settings for assay protocol channel, dye and cut off values.

Channel	Dye	[a]Ct Lower	Ct Upper	Gene Target
2	6FAM	1	38	N1
3	AP525	1	37	N3
6	AP662	1	45	RNase P

[a]Ct=Cycle threshold.

Table 3. Interpretation of Results for the Luminex ARIES® Laboratory Developed Test.

N1 Result	N3 Result	RNase P Result	Interpretation
Positive	Positive	Positive or Negative	SARS-CoV-2 Detected
Positive	Negative	Positive	Presumptive SARS-CoV-2 Positive; Retest x2
Negative	Positive	Positive	Presumptive SARS-CoV-2 Positive; Retest x2
Negative	Negative	Positive	SARS-CoV-2 Not Detected
Negative	Negative	Negative	Invalid

with 3IAbRQSp and for the RNase P target, the TYE 665 dye was used with 3IAbRQSp. The cycling conditions were modified on ARIES® due to instrument programming limitations (shorter reverse transcription step, no uracil-N-glycosylase/uracil DNA glycosylase step, UNG). The cycling condition for the ARIES® SARS-CoV-2 real-time RT-PCR assay were as follows:

- Reverse Transcription Step, 50°C for 7 minutes
- Enzyme Activation Step, 95°C for 2 minutes
- Amplification Step, including Denaturing and Annealing, 95°C for 15 seconds, 60°C for 22 seconds, 45 cycles

In contrast, the cycle conditions for the CDC SARS-CoV-2 real-time RT-PCR assay were as follows:

- UNG Incubation Step, 25°C for 2 minutes
- RT Incubation Step, 50°C for 15 minutes
- Enzyme Activation Step, 95°C for 2 minutes
- Amplification Step, including Denaturing and Annealing, 95°C for 3 seconds, 55°C for 30 seconds, 45 cycles

Limit of Detection and Analytical Sensitivity

To determine the LoD of this real-time RT-PCR assay, quantitated amounts of heat-inactivated virus were spiked into known negative nasopharyngeal swab samples. Heat- inactivated viral culture fluid (Catalog # 0810587 CFHI-0.5mL, Zeptometrix, Buffalo, NY) at a concentration of 1.50 X10^6 Tissue Culture Infective Dose-50% ($TCID_{50}$)/mL was tested using 10-fold serial dilutions in known negative pooled nasopharyngeal swab samples (concentrations ranging from 1.50x10^6 to 1.50x10^{-2}). These dilutions were tested in triplicate. The lowest concentration that gave positive results 100% of the time was defined as the preliminary LoD. The final LoD concentration was confirmed by testing 24 individual extraction replicates at the preliminary LoD. The FDA defined LoD as the lowest concentration at which all 24 replicates were positive.

Specificity Studies (Inclusivity/Exclusivity)

An *in silico* analysis of published SARS-CoV-2 sequences using the SARS-CoV-2 assay's primers and probes was performed, with 100% being detected, and published previously.[7] In addition, archived respiratory pathogen–positive patient samples, previously tested using the BioFire® Respiratory Pathogen Panel, were tested with this assay. These included 11 different respiratory virus–positive samples.

Stability Study

Two different positive patient nasopharyngeal samples were chosen for the stability study. One fresh positive sample chosen gave Ct values close to the established LoD, while the other fresh sample gave Ct values below the LoD. These two samples were stored in a refrigerator at 4°C for a total of 8 days and were tested daily for 8 days to check the stability of the viral RNA.

Clinical Evaluation:

To assess the clinical performance of the real-time RT-PCR assay, a total of 60 nasopharyngeal swab (clinical) samples collected in saline or Universal Transport Media (UTM) were tested on the ARIES®. Studies

Position	Sample ID	Assay Name	Test Result
B5	Positive sample	UL 04.23.20 VC	SARS-CoV-2 DETECTED N1 RNA DETECTED N3 RNA DETECTED RNase P DNA DETECTED

Channel	Channel Desc.	Ct	Channel Result
1	Not Used	-	-
2	N1	22.5	Detected
3	Not Used	-	-
4	N3	21.3	Detected
5	Not Used	-	-
6	RNase P	21.6	Detected

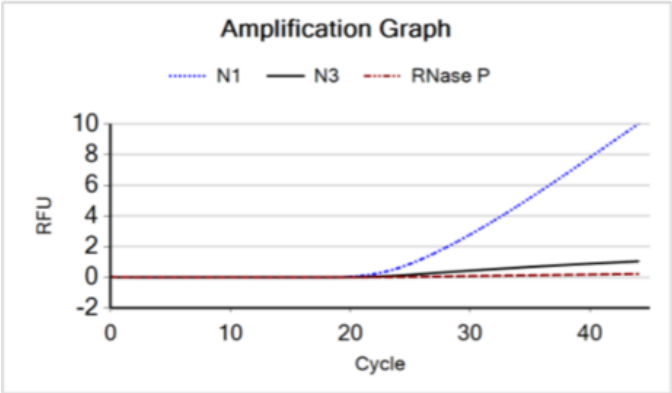

Figure 1. Positive SARS-CoV-2 real-time RT-PCR result. Sample data from ARIES instrument illustrating the amplification curve for the SARS-CoV-2 specific N1 and N3 target and internal control RNase P.

Position	Sample ID	Assay Name	Test Result
B6	Negative sample	UL 04.23.20 VC	SARS-CoV-2 Not Detected N1 RNA not detected N3 RNA not detected RNase P DNA DETECTED

Channel	Channel Desc.	Ct	Channel Result
1	Not Used	-	-
2	N1	ND	Not Detected
3	Not Used	-	-
4	N3	ND	Not Detected
5	Not Used	-	-
6	RNase P	30.0	Detected

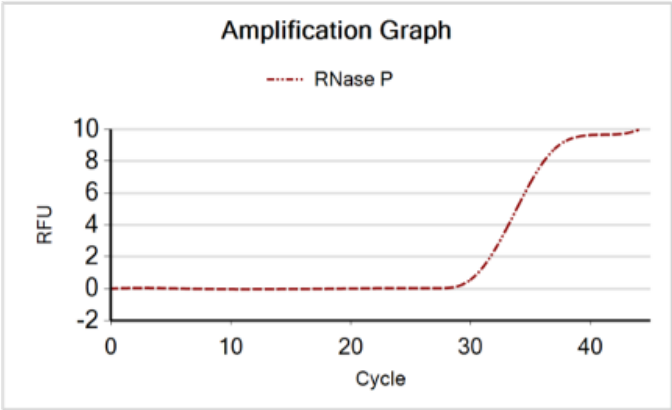

Figure 2. Negative SARS-CoV-2 real-time RT-PCR result. Sample data from ARIES instrument illustrating the amplification curve for the internal control RNase P.

comparing nasopharyngeal swabs collected in saline versus UTM showed no difference in qualitative results for the CDC and ARIES® real-time RT-PCR assays (data not shown). The first five SARS-CoV-2-positive and the first five SARS-CoV-2-negative nasopharyngeal swab patient samples collected in UTM were submitted in early March 2020 to the Kentucky Division of Laboratory Services in Frankfort, KY. In addition, the Louisville Metro Public Health and Wellness Laboratory, Louisville, KY, provided 50 de-identified nasopharyngeal swab (clinical) samples, collected in saline—previously tested by the FDA EUA–approved CDC real-time RT-PCR SARS-CoV-2 assay (gene targets N1, N2, and RNaseP; three individual reactions)—to test on the ARIES® instrument.

Data Analysis

Microsoft Excel was used to calculate averages and standard deviations for Ct values from replicate experiments, while MEDCALC Diagnostic Test Evaluation was used to determine analytic sensitivity and specificity.[8] The User Defined Protocol (UDP) module in the SYNCT® software (proprietary from Luminex) was used to set assay parameters. The SYNCT® software was installed on an external computer for detailed data analysis and editing of Ct cutoff values. After completing data analysis and PCR assay validation, the cutoff values were fixed and locked in the SYNCT® software assay file. Next, the assay file was loaded on the ARIES® instrument for subsequent diagnostic laboratory testing. Example results are shown in **Figures 1 and 2**. For a sample to be interpreted as negative or positive for SARS-CoV-2, the following conditions had to be met as shown in **Tables 2 and 3**:

(1) The internal control (RNaseP) for any NEGATIVE sample must have a valid Ct value to exclude sample inhibition; however, it may be negative if N1 and N3 are positive.

(2) The sample must have Ct values in the established range for each gene target if POSITIVE for N1, N3 or RNaseP.

Table 4. Limit of detection results for the SARS-CoV-2 real-time RT-PCR assay.

Sample Concentration [a]$TCID_{50}$/mL	N1 [b]Ct Average ([c]SD, n=3)	N3 Ct Average (SD, n=3)	RNase P Ct Average (SD, n=3)
$1.50x10^5$	13.63 (0.25)	13.20 (0.10)	[d]ND
$1.50x10^4$	17.63 (0.85)	17.07 (0.91)	NDx2, 18.70 x1
$1.50x10^3$	21.37 (0.84)	20.93 (0.72)	21.50 (0.70)
$1.50x10^2$	23.97 (0.61)	23.57 (0.55)	23.53 (0.35)
$1.50x10^1$	28.67 (0.55)	27.23 (0.61)	25.17 (0.42)
[e]$1.50x10^0$	**33.60 (0.56)**	**32.30 (0.46)**	**25.50 (0.26)**
$1.50x10^{-1}$	NDx2, 37.60x1	ND	27.93 (0.80)
$1.50x10^{-2}$	ND	ND	26.57 (0.31)

[a]TCID=Tissue Culture Infective Dose [b]Ct=Cycle threshold [c]SD=Standard Deviation [d]ND=Not Detected [e]Limit of detection shown in bold

Table 5. Replicate experiments to establish limit of detection.

Sample Concentration (n=24)	N1 Result	N1 [b]Ct Average [c](SD)	N3 Result	N3 Ct Average (SD)	RNase P Result	RNase P Ct Average (SD)	Interpretation
$1.5x10^0$ [a]$TCID_{50}$/mL	N1 Detected	35.07 (0.83)	N3 Detected	34.29 (0.76)	RNase P Detected	25.43 (0.51)	SARS-CoV-2 Detected

[a]TCID=Tissue Culture Infective Dose [b]Ct=Cycle threshold [c]SD= Standard Deviation

Results

Initial data gathered for LoD studies in March 2020 were obtained using genomic blocks of DNA of the novel coronavirus due to lack of viral materials early in the pandemic. Using the Luminex ARIES® platform, we were able to detect 300 DNA copies of the SARS-CoV-2 nucleocapsid gene targets reproducibly, using 20 replicate tests. Contrived nasopharyngeal samples in UTM (created in the laboratory to mimic clinical samples) were spiked with 1500 DNA copies of SARS-CoV-2 synthetic material to further assess performance. As more viral RNA sources became available, the LoD studies were repeated with heat-inactivated culture fluid containing SARS-CoV-2. The LoD established with this viral material was 1.5 $TCID_{50}$/mL, using 24 replicates to confirm this virus concentration (**Tables 4 and 5**), thus serving as precision data as well. Specificity experiments demonstrated no cross-reactivity with 11 other respiratory viruses, including the four endemic coronaviruses (**Table 6**). Testing daily over an 8-day period demonstrated that RNA was detectable the entire time, with little variability in original and final Ct values (**Table 7**).

Per the FDA EUA guidance, a total of 60 clinical samples, 30 positive and 30 negative, were tested using the ARIES® platform. In March 2020, the first five positive clinical samples and the first five negative clinical samples were confirmed by the Kentucky Division of Laboratory Services in Frankfort, KY, the state public health laboratory, using the EUA approved, CDC real-time RT-PCR novel coronavirus assay (data not shown). In July 2020, 25 deidentified positive samples and 25 deidentified negative samples were provided by the Louisville Metro Public Health and Wellness Laboratory, a local, public health, CLIA-certified, high-complexity laboratory, using the same real-time RT-PCR assay as the Kentucky Division of Laboratory Services. All of these results agreed 100% as well, giving 100% analytical sensitivity (95% Confidence Interval 88.43 to 100) and 100% analytical specificity (95% Confidence Interval 88.43 to 100). Remarkably, despite the modifications of the CDC assay and differing real-time RT-PCR platforms, the accuracy was 100% for all 25 sample results. Furthermore, the Ct values for N1 were very similar (less than three Ct units' difference) for both assays on all samples except #13, #21 and #22 (**Table 8**, rows in bold).

Discussion

Our laboratory was able to successfully implement a real-time RT-PCR assay to detect SARS-CoV-2 in nasopharyngeal samples on the Luminex ARIES® platform by March 27, 2020. The assay performance data met both the FDA EUA requirements and CLIA standards for a diagnostic assay by reproducibly detecting the N1 and N3 gene targets at 1.5 $TCID_{50}$/mL. Evaluation of clinical samples achieved 100% accuracy, as well as acceptable analytical sensitivity and specificity for nasopharyngeal swabs. The strengths of this report include following a standardized method validation guidance document from the FDA and supplementing with experiments to meet CLIA requirements for a laboratory-developed test. Results from two independent public health laboratories using the CDC EUA-approved novel coronavirus assay confirmed equivalent performance of the ARIES® multiplex real-time RT-

Table 6. Specificity studies with known respiratory pathogen positive samples.

Sample	Expected Test Result	N1 Result	N1 [a]Ct	N3 Result	N3 Ct	RNase P Result	RNase P Ct
A1	RSV	Negative	[b]ND	Negative	ND	Positive	26
A2	FluA H3	Negative	ND	Negative	ND	Positive	24.4
A3	Para 2	Negative	ND	Negative	ND	Positive	28.6
A4	FluB	Negative	ND	Negative	ND	Positive	26.1
A5	Corona OC43	Negative	ND	Negative	ND	Positive	33.6
A6	Rhino/Enterovirus	Negative	ND	Negative	ND	Positive	29.3
A7	Para 4	Negative	ND	Negative	ND	Positive	29.5
A8	Para 3	Negative	ND	Negative	ND	Positive	28.9
A9	Corona 229	Negative	ND	Negative	ND	Positive	26.1
A10	Corona NL63	Negative	ND	Negative	ND	Positive	25.2
A11	Corona HKU-1	Negative	ND	Negative	ND	Positive	26.2

[a]Ct=Cycle threshold [b]ND=Not Detected

Table 7. Stability assay for SARS-CoV-2 rt RT-PCR assay.

Sample	N1 Result	N1 [a]Ct Average [b](SD)	N3 Result	N3 Ct Average (SD)	RNase P Result	RNase P Ct Average (SD)	Result
1	N1 Detected	21.76 (1.29)	N3 Detected	20.81 (1.37)	RNase P Detected	22.05 (1.69)	SARS-CoV-2 Detected
2	N1 Detected	31.59 (1.70)	N3 Detected	30.51 (1.71)	RNase P Detected	29.31 (1.26)	SARS-CoV-2 Detected

[a]Ct=Cycle threshold [b]SD=Standard Deviation

PCR assay. The advantages of using the ARIES® platform include a combined extraction and detection process and a multiplex PCR assay in a closed system. Furthermore, a true internal control—a human RNaseP primer/probe set—was included to detect sample inhibition, as well as proper extraction of the sample. For diagnostic laboratories, the availability of a sensitive and specific test for SARS-CoV-2 in a closed system, giving results within two hours, greatly improves the testing process and significantly limits potential contamination.

During the pandemic, there has been a clear recognition that laboratory testing is central to helping control and prevent the spread of the disease by identifying infected patients and starting treatment.[9] The ability to have a test available by March 27, 2020, to support the pandemic response in our community was critical since the initial priority population was health care workers from a large health care system in Louisville, KY. As the needs of the community changed, we were able to make testing available to health care systems for inpatients, those scheduled for elective surgery who had been postponed, employees of local large businesses, nursing home staff, and people who traveled via airlines that needed COVID-19 diagnostic results. Having an accurate test with a two-hour turnaround time proved beneficial to these populations and organizations.

Limitations of the study primarily involved supply chain issues, leading to a more limited validation process. For example, early in the pandemic, there was a lack of quantitated RNA control materials derived from the novel coronavirus that could be safely handled in a BSL-2 laboratory. Subsequently, as more laboratories brought on molecular testing, shortages in viral transport media, swabs and primer/probe sets became limiting, affecting our ability to test contrived or true clinical samples. Finally, most laboratories experienced shortages of consumables, especially plastics used for molecular testing, and being placed on allocation for PCR kits. For our laboratory, these shortages limited the validation to only one respiratory sample type, the gold-standard nasopharyngeal swab. For a clinical laboratory, it would have been preferable to validate multiple sample types.

Future studies will include testing the sensitivity of the ARIES® assay when pooling respiratory samples and validating other common upper and lower respiratory tract samples to benefit the local health care community. Another planned study involves comparison of Ct values to viral culture results through a collaboration with a local laboratory with viral culture and BSL-3 capabilities. Finally, we have conducted preliminary studies to determine assay performance for oropharyngeal versus nasopharyngeal samples, as well as self-collected nasal swabs versus provider-collected nasopharyngeal swabs. We also compared the laboratory-developed test for SARS-CoV-2 to the EUA-approved *In Vitro* Diagnostic version for the ARIES® platform offered by Luminex Corporation.

Table 8. Accuracy results for positive clinical samples provided by the Louisville Metro Public Health and Wellness Laboratory, Louisville, KY.

	University of Louisville Infectious Diseases Laboratory			Louisville Metro Public Health and Wellness Laboratory		
Specimen #	**N1 [a]Ct**	**N3 Ct**	**RNase P Ct**	**N1 Ct**	**N2 Ct**	**RNase P Ct**
POS 1	27.1	26.4	26.3	27.62	26.48	31.11
POS 2	26	25.2	24.7	24.04	23.12	31.43
POS 3	26.5	25.5	25.1	25.42	24.26	28.65
POS 4	22.6	22.8	22.9	25.13	23.56	29.58
POS 5	23.2	22.5	22.5	20.46	19.42	28.39
POS 6	34.7	34.1	29.3	27.93	28.14	25.92
POS 7	26.5	25.5	25.7	25.22	24.16	28.37
POS 8	34.1	33.3	29.1	32.14	31.55	28.77
POS 9	17	17.1	[b]ND	17.87	15.97	29.65
POS 10	23.3	22.3	22.5	19.33	18.51	29.97
POS 11	35.9	35.7	29.5	36.21	32.93	28.58
POS 12	33.5	32.5	29.8	34.67	33.03	26.58
[c]POS 13	**22**	**22.1**	**ND**	**15.28**	**15.49**	**26.15**
POS 14	22.5	21.7	22.4	22.4	21.6	28.69
POS 15	22.7	21.5	21.3	22.21	20.26	30.85
POS 16	25.5	24.3	24.3	24.28	ND	30.81
POS 17	22.9	22.2	23.1	21.09	20.31	27.14
POS 18	25	25.5	ND	22.19	22.3	26.95
POS 19	20.9	20.1	ND	21.9	20.6	31.12
POS 20	30.7	29.9	28.2	30.38	29.21	28.31
[c]POS 21	**34.3**	**33.3**	**27.2**	**ND**	**33.67**	**30.73**
[c]POS 22	**32.7**	**31.2**	**29.3**	**25.22**	**24.16**	**28.37**
POS 23	18.6	17.7	ND	18.18	17.97	27.79
POS 24	26.7	25.9	25.6	23.95	23.05	27.68
POS 25	29.9	29.1	27.9	28.33	28.11	30.68

[a]Ct=Cycle threshold [b]ND=Not Detected [c]Significant differences in Ct values shown in bold

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