EMERGENCY USE AUTHORIZATION (EUA) SUMMARY
SARS-CoV-2 Acutis Multiplex Assay
(Acutis Diagnostics)

For *in vitro* Diagnostic Use
Rx Only
For Use Under Emergency Use Authorization (EUA) Only

The SARS-CoV-2 Acutis Multiplex Assay will be performed at Acutis Diagnostics, located at 400 Karin Lane, Hicksville, NY 11801, which is certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. §, and meets requirements to perform high complexity tests, as described in the Laboratory Standard Operating Procedure that was reviewed by the FDA under this EUA.

INTENDED USE

The SARS-CoV-2 Acutis Multiplex Assay is a reverse transcriptase polymerase chain reaction (RT-PCR) test intended for the qualitative detection of nucleic acid from SARS-CoV-2 in nasopharyngeal swab specimens from individuals suspected of COVID-19 by their healthcare provider. Testing is limited to Acutis Diagnostics, located at 400 Karin Lane, Hicksville, NY 11801, which is certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. §263a, and meets requirements to perform high complexity tests.

Results are for the identification of SARS-CoV-2 RNA. The SARS-CoV-2 RNA is generally detectable in nasopharyngeal swab specimens during the acute phase of infection. Positive results are indicative of the presence of SARS-CoV-2 RNA; clinical correlation with patient history and other diagnostic information is necessary to determine patient infection status. Positive results do not rule out bacterial infection or co-infection with other viruses. The agent detected may not be the definitive cause of disease. Laboratories within the United States and its territories are required to report all test results to the appropriate public health authorities.

Negative results do not preclude SARS-CoV-2 infection and should not be used as the sole basis for patient management decisions. Negative results must be combined with clinical observations, patient history, and/or epidemiological information.

The SARS-CoV-2 Acutis Multiplex Assay is intended for use by qualified clinical laboratory personnel specifically instructed and trained in the techniques of real-time RT-PCR and in vitro diagnostic procedures. The SARS-CoV-2 Acutis Multiplex Assay is only for use under the Food and Drug Administration's Emergency Use Authorization.

DEVICE DESCRIPTION AND TEST PRINCIPLE

Device Description

The SARS-CoV-2 Acutis Multiplex Assay is a real-time reverse transcription polymerase chain reaction (RT-PCR) test. The SARS-CoV-2 primer and probe sets is designed to detect RNA from the SARS-CoV-2 virus in nasopharyngeal swabs collected from patients suspected of COVID-19 by their healthcare provider.

The SARS-CoV-2 Acutis Multiplex Assay utilizes the CDC-developed assay (FDA submission number EUA200001) that targets specific regions of the nucleocapsid gene. Two nucleocapsid (N) gene regions, N1 and N2, are used to detect the COVID-19 virus. N1 and N2 dual targets are

multiplexed resulting in complete mitigation of assay failure by having both fluorescent probe in the same channel (FAM dye). Human RNase P target probe is labeled with ATTO 647 fluorescent dye and multiplexed with N gene targets. In the clinical setting, N gene must be detected in order for the sample to be reported as positive. N gene must be negative, and RNase P must be positive for patient samples to be reported as negative.

Description of Test Steps:

1. Specimen Collection

Nasopharyngeal (NP) swabs will be collected from individuals suspected of COVID-19 by their healthcare provider in a healthcare setting. Samples will be placed into viral transport media and can be stored at room temperature (18°C to 30°C) for up to 48 hours or refrigerated (2°C to 8°C) for up to seven (7) days.

2. Specimen Testing

Nucleic acid is extracted from NP swab specimens (400 µL) using the KingFisher Flex extraction platform. This extraction process utilizes the MagMax Viral/Pathogen II (MVP II) Nucleic Acid Isolation Kit following the manufacturer's instructions. Nucleic acid is eluted in 50 µL of elution buffer.

The primer/probe sets for the N (N1 + N2) and RNase P assays were multiplexed into one assay (SARS-CoV-2 Acutis Multiplex Assay). A master mix solution is prepared by using the volumes listed in Table 1: Master Mix Solution.

Table 1: Master Mix Solution

Component	Volume (µL) / Reaction
COVID-19 real-time PCR assay multiplex	1
Nuclease-free water	4
TaqPath 1-Step Multiplex Master Mix	5

The isolated nucleic acid sample is mixed with master mix solution. The 384 well plate is loaded onto the QuantStudio instrument and subject to RT-PCR amplification. The results are analyzed using the instrument software.

Extracted SARS-CoV-2 RNA is subjected to a 1-step RT-PCR test. In 1-step RT-PCR, RNA is initially reverse transcribed to a complementary DNA (cDNA) strand, followed by a PCR reaction carried out in the same reaction vessel. The TaqMan reagents include two pairs of unlabeled primers that target two different regions of the SARS-CoV-2 N protein gene, as well as a pair of unlabeled primers for the internal RNaseP control. The reagents also include specific oligonucleotide probes for each gene target region(s) that are fluorescently labeled (FAM and ATTO 647N for the N gene and RNase P gene, respectively) on the 5' end, ZEN at tenth nucleotide position, and a quencher label (Iowa Black) on the 3' end. TaqMan technology relies on the 5'-exonuclease activity of the Taq polymerase which cleaves the dual-labeled probe after hybridization to the DNA target sequence, and subsequent fluorescent label detection. At the start of a PCR cycle, the temperature is raised to denature the DNA. During this step, the fluorescent label is in close proximity with the quencher label. Therefore, the fluorescent signal is quenched in a phenomenon known as fluorescence resonance energy transfer. In the next step, the reaction temperature is lowered to allow the primers and probe to anneal to their specific target sequence. Taq polymerase synthesizes new DNA strands by extending the unlabeled primers along the DNA template. When the polymerase reaches the probe, its endogenous 5' exonuclease activity cleaves the fluorescent label thus separating the fluorophore from the quencher. With each PCR cycle,

more fluorescent labels are released, resulting in an increase in fluorescence signal intensity proportional to the number of amplicons synthesized.

INSTRUMENTS USED WITH THE TEST

Table 2. Instruments and Software for Use with the SARS-CoV-2 Acutis Multiplex Assay

Instrument	Manufacturer	Software Version
KingFisher Flex	Thermo Fisher Scientific	BindIt 4.1 Software for KingFisher instruments Script – COVID_ONLY.bdz
QuantStudio 12K Flex Real-Time PCR System (384-well Block)	Thermo Fisher Scientific	QuantStudio 12K Flex Software version 1.3
QuantStudio 5 Real-Time PCR Instrument (384-well Block)	Thermo Fisher Scientific	QuantStudio Design & Analysis Software version 1.5.1

REAGENTS AND MATERIALS

Table 3. Reagents and Materials Used for Sample Preparation and to Perform the SARS-CoV-2 Acutis Multiplex Assay

Reagent/Material	Manufacturer/ Supplier	Catalogue/Part Number
MagMAX Viral/Pathogen II Nucleic Acid Isolation Kit	Applied Biosystems	A48383
SARS-CoV-2 Acutis Multiplex Assay	Integrated DNA Technologies	N/A
TaqPath 1-Step Multiplex Master Mix, No ROX	Applied Biosystems	A28522
Water, nuclease-free, Molecular Biology grade	Thermo Scientific	J71786-XCR
Absolute Ethanol (200 proof), Molecular Biology Grade	Fisher Scientific	BP2818-500
Viral Transport Medium	Acutis Diagnostics	N/A
Hs_RPP30 Positive Control	Integrated DNA Technologies	10006626
2019-nCoV_N_Positive Control	Integrated DNA Technologies	10006625
Tris-EDTA buffer solution	Sigma Aldrich	93302-100ML
NATtrol SARS-CoV-2 External Run Control	ZeptoMetrix	NATSARS(COV2)-ERC

CONTROLS

The SARS-CoV-2 Acutis Multiplex Assay controls are included with each PCR plate tested. External Positive Control (EPC), Negative Extraction Control (NEC), No Template Control (NTC), Reverse Transcriptase PCR Control (RT Pos), and a RNase P Internal Control (RP) are added to the RT-PCR plate during the plate preparation. The EPC acts as a positive control for amplification of the N gene target. The NTC acts as a negative control that monitors for contamination of the RT-PCR reagents. The NEC acts as a monitor for cross contamination during the extraction and RT-PCR process. The RT Pos is intended to monitor the reverse transcription reaction, RT-PCR reagent efficiency, and RT-PCR process for every extraction and RT-PCR plate. The RP is intended to monitor the integrity of nucleic acid extraction and RT-PCR for every human respiratory tract specimen and NEC.

Table 4: Assay Controls Used With the SARS-CoV-2 Acutis Multiplex Assay

Control	Description	Purpose	Expected Results	Frequency of Use
External Positive Control (EPC) **250 GCE/mL [20 copies/reaction]**	2019-nCoV_N_Positive Control contains Plasmid with the SARS-CoV-2 N gene near assay LOD and RNase P gene	To monitor the integrity of RT-PCR reagents and process	N and RP genes must all be positive $C_T < 40$	Every RT-PCR plate
Negative Extraction Control (NEC)	Molecular grade nuclease-free water	To monitor cross contamination during extraction, RT-PCR, and reagent contamination	Internal control (RP) should be positive. $C_T < 40$ COVID-19 target (N gene) must be negative. $C_T \geq 40$ or undetermined	Every extraction and RT-PCR plate
No template Control (NTC)	Molecular grade nuclease-free water	To monitor contamination of RT-PCR reagent contamination	all targets (N gene, and RP gene) must be negative $C_T \geq 40$ or undetermined	Every RT-PCR plate
RNase P (RP) Internal Control	The internal control comes from the cells collected within the patient sample	To monitor the integrity of nucleic acid extraction and RT-PCR for every human respiratory tract specimen	Internal control (RP) should be positive. $C_T < 40$ COVID-19 target (N gene) can be either positive or negative.	Every collected human respiratory specimen and NEC.
Reverse Transcriptase PCR Control (RT Pos) **100 GCE/mL [8 copies/reaction]**	Inactivated intact viral particles, SARS-Related Coronavirus 2, Isolate USA-WA1/2020	To monitor the reverse transcription reaction, RT-PCR reagent efficiency, and RT-PCR process	COVID-19 target (N gene) must all be positive $C_T < 40$	Every extraction and RT-PCR plate

INTERPRETATION OF RESULTS

Assessment of SARS-CoV-2 Acutis Multiplex Assay results should be performed after the positive and negative controls have been examined and determined to be valid and acceptable. If the controls are not valid, the patient results cannot be interpreted. There are two levels for the results interpretation process: 1) batch review and 2) patient review.

1) <u>Batch Review: SARS-CoV-2 Acutis Multiplex Assay Controls:</u>

For patient results to be released, the batch must be accepted. After the batch is accepted, the individual patient results are reviewed. The following applies to the batch review process.

a. The negative controls (NTC and NEC) must be negative for the SARS-CoV-2 N (N1 + N2) and RNase P genes with no discernable amplification to accept the batch. If the negative controls are positive for any of the gene targets, then the entire batch will be repeated.
b. The positive external controls must be positive for the SARS-CoV-2 N gene (N1 + N2) and RNase P targets with Ct values < 40 cycles to accept the batch. If the

external positive control is negative for any of the intended targets, then the entire batch must be rejected and repeated.

c. The reverse transcriptase PCR control (RT Pos) must be positive for the SARS-CoV-2 N gene (N1 + N2) target with a Ct value < 40 cycles to accept the batch. If the reverse transcriptase PCR control is negative for the intended target, then the entire batch must be rejected and repeated.

Table 5: Expected Performance of SARS-CoV-2 Acutis Multiplex Assay Controls

Control Type	N gene (FAM)	RP gene (ATTO647N)
Negative Extraction Control	Negative (>40 Ct[1])	Negative (>40 Ct[1])
Positive Control	Positive (<40 Ct[1])	Positive (<40 Ct[1])
No Template Control	Negative (>40 Ct[1])	Negative (>40 Ct[1])
Reverse Transcriptase PCR Control	Positive (<40 Ct[1])	Negative (>40 Ct[1])

[1] Cycle threshold is defined as the number of PCR cycles required for the fluorescent signal to cross the threshold

2) Patient Review: Examination and Interpretation of Patient Sample Results:

During the patient results review process, results can be either accepted or rejected. Patient results that are accepted will be reported as DETECTED or NOT DETECTED. Patient results that are rejected will be retested. In accordance with the Instructions for Use (IFU) for the CDC 2019-nCoV Real-Time RTPCR Diagnostic Panel; SARS-CoV-2 N (N1 + N2) gene must be positive for the sample to be reported as DETECTED. The SARS-CoV-2 N (N1 + N2) gene must be negative, and the RNase P gene must be positive for the sample to be reported as NOT DETECTED.

Table 6: Patient Results Interpretation

SARS-CoV-2 N gene	Internal Control – RNase P	Assay Status	Result Outcome	Action
Negative	Negative	REJECT	N/A	Re-test sample and recollection recommended.
Negative	Positive	ACCEPT	Not Detected	Forward results to LIS[1] and local DOH[1]
Positive	Positive or Negative	ACCEPT	Detected	Forward results to LIS[1] and local DOH[1]

[1]LIS - Laboratory Information System, DOH - Department of Health

PERFORMANCE EVALUATION

1) Limit of Detection (LoD) - Analytical Sensitivity:

The LoD of the SARS-CoV-2 Acutis Multiplex Assay was determined by measuring the lowest concentration of SARS-CoV-2 heat inactivated virus (obtained through ATCC Catalog # VR-1986HK) that can be measured or distinguished from pooled Viral Transport Medium (VTM) collected from real human negative clinical samples.

The preliminary LoD study used sample concentrations of 1 x 10^6, 1 x 10^5, 1 x 10^4, 1 x 10^3, 500, 200, 100, 75 and 50 GCE per mL of heat-inactivated SARS-CoV-2 diluted in pooled VTM. Each sample was processed using the same extraction and analysis process outlined in the "SARS-CoV-2 Acutis Developed Multiplex Assay" Standard Operating Procedure (SOP) on both the QuantStudio 5 and QuantStudio 12K instruments. The samples were analyzed in triplicate for the presence of the SARS-CoV-2 N gene target and the internal control, RNase P. The preliminary LoD for the QuantStudio 5 was determined to be 75 GCE/mL while the QuantStudio 12K was determined to be 50 GCE/mL.

The confirmation LoD study used 20 replicate samples of heat-inactivated SARS-CoV-2

diluted in pooled negative VTM at concentrations of 200, 100, 75, 50 GCE/mL. Each sample was processed using the same extraction and analysis process outlined in the "SARS-CoV-2 Acutis Developed Multiplex Assay" SOP on both the QuantStudio 5 and QuantStudio 12K instruments. The samples were analyzed for the presence of the SARS-CoV-2 N gene target and the internal control, RNase P. The final LoD for the QuantStudio 5 was determined to be 50 GCE/mL while the QuantStudio 12K was determined to be 100 GCE/mL. The overall LoD for the SARS-CoV-2 Acutis Multiplex Assay is therefore determined to be 100 GCE/mL.

Table 7: LoD Study Results

Concentration (GCE/mL)	Number Replicates (n)	QuantStudio 5		QuantStudio 12K	
		# Positive/ Total		# Positive/ Total	
		N Gene	RNase P	N Gene	RNase P
1 x 10^6	3	3/3 (100%)	3/3 (100%)	3/3 (100%)	3/3 (100%)
1 x 10^5	3	3/3 (100%)	3/3 (100%)	3/3 (100%)	3/3 (100%)
1 x 10^4	3	3/3 (100%)	3/3 (100%)	3/3 (100%)	3/3 (100%)
1 x 10^3	3	3/3 (100%)	3/3 (100%)	3/3 (100%)	3/3 (100%)
500	3	3/3 (100%)	3/3 (100%)	3/3 (100%)	3/3 (100%)
200	3	3/3 (100%)	3/3 (100%)	3/3 (100%)	3/3 (100%)
100	3	3/3 (100%)	3/3 (100%)	3/3 (100%)	3/3 (100%)
75	3	3/3 (100%)	3/3 (100%)	3/3 (100%)	3/3 (100%)
50	3	2/3 (66%)	3/3 (100%)	3/3 (100%)	3/3 (100%)
200	20	20/20 (100%)	20/20 (100%)	20/20 (100%)	20/20 (100%)
100	20	20/20 (100%)	20/20 (100%)	20/20 (100%)	20/20 (100%)
70	20	20/20 (100%)	20/20 (100%)	18/20 (90%)	20/20 (100%)
50	20	19/20 (95%)	20/20 (100%)	12/20 (60%)	20/20 (100%)

2) Inclusivity (Analytical Reactivity):

An in-silico analysis was performed to determine the clinical relevance of the SARS-CoV-2 Acutis Multiplex Assay against recent viral mutations and variants of the SARS-CoV-2 virus. The data used in the analysis was collected on 1/31/2023 and included samples with dates ranging from 7/27/2022 to 1/18/2023. A total of 189,859 high-quality genomes were analyzed using the GISAID primer checker program v3.05 (https://www.epicov.org/epi3/frontend#5c1590). Only high-quality genomes (<1% Ns and <0.05% unique non-synonymous mutations), with assigned accession ID #s were evaluated. Omicron variants with high-coverage genome were included in the in-silico analysis. Any mutation present in either N1 or N2 assays in one accession ID was considered acceptable. The N1 and N2 assays are pooled to make one call, hence a signal from either assay is sufficient to detect SARS-CoV-2. Therefore, any mutations present in both N1 and N2 assays, for any given accession ID, were subjected to further investigation. Mutation frequency rates were determined separately for the N1 and N2 sequences. The frequency of mutation was determined by dividing the totaling the number of mutations in the 3'-end of the forward and reverse primers, and any mutations probes by the total number of accession IDs. The percentage specificity inclusivity was calculated using the following formula:

$$\left(1 - \left(\frac{Number\ of\ False\ Negatives}{Total\ number\ of\ representatives\ genomes}\right) x\ 100\right)$$

The mutation frequency was highest for the N1 probe (25%) followed by the N2 probe (0.2%). The mutation rates for the primers ranged from 0.04% to 0.1%. (See Table 8: Mutation Frequency in N1 and N2 Sequences)

Table 8: MUTATION FREQUENCY IN N1 AND N2 SEQUENCES

Gene Target	Oligonucleotides	Number of Accession IDs with Mutation	Frequency of Mutation (GISAID global)
N1	Probe	48,078	25%
N1	Forward Primer	215	0.1%
N1	Reverse Primer	72	0.04%
N2	Probe	361	0.2%
N2	Forward Primer	86	0.05%
N2	Reverse Primer	73	0.04%
Total Number of Accession IDs		189,859	

Of the 136,117 omicron variants evaluated, the 20 sub-variants were further evaluated due to mutations identified in the N1 and N2 sequences. These variants are identified in Table 9: Possible Affected Omicron Variant Lineages. Specifically, the BA.5.2.1 (N = 4,424), BA.5.2 (N = 4,278) and BF.7 (N = 2,156) sub-variants contributed the most mutations in either the N1 and N2 sequences. These sub-variants also contributed most of the accession IDs that have mutations in both the N1 and N2 sequences.

Table 9: POSSIBLE AFFECTED OMICRON VARIANT LINEAGES

INDEX #	OMICRON LINEAGES IDENTIFIED USING IN SILICO ANALYSIS	TOTAL NUMBER WITH AT LEAST ONE MUTATION	NUMBER OF SUB-VARIANTS WITH MUTATIONS ON BOTH N1 AND N2 ASSAYS	PERCENTAGE OF POTENTIALLY AFFECTED LINEAGE MEMBERS
1	BA.5.2	4278	65	0.048
2	BF.10	130	5	0.004
3	BA.5.2.9	203	2	0.001
4	BF.5	834	3	0.002
5	BF.7	2156	19	0.014
6	BA.5.2.1	4424	20	0.015
7	BF.21	61	5	0.004
8	BA.5.2.2	32	1	0.001
9	BA.5.2.19	33	1	0.001
10	BA.5.2.18	64	2	0.001
11	BA.5.2.26	93	2	0.001
12	BA.5.2.42	4	1	0.001
13	BY.1	273	1	0.001
14	BF.7.5	277	2	0.001
15	BA.5.2.13	123	2	0.001
16	BA.5.2.6	312	8	0.006
17	BA.5.2.35	149	3	0.002
18	BA.5.2.3	191	1	0.001
19	BA.5.2.24	37	1	0.001
20	BA.5.2.34	52	1	0.001
	Total	13,726	145	

Based on the analysis completed, the percentage specificity inclusivity was calculated for the SARS-CoV-2 Acutis Multiplex Assay and determined to detect 99.9% of all known SARS-

CoV-2 variants. Monitoring the mutation frequency and potential effect on assay accuracy will be completed on a monthly basis using the methods described above.

3) Cross-Reactivity (Analytical Specificity), Microbial Interference:

The N1 and N2 primers utilized in the SARS-CoV-2 Acutis Multiplex Assay are identical to those of the CDC nCoV Real-Time RT-PCR Diagnostic Panel. The CDC performed both an in silico analysis and wet-testing to evaluate cross-reactivity. The CDC has granted right of reference to use the performance data submitted to the FDA as part of EUA200001.

Additionally, cross reactivity wet testing was completed to determine the potential of the SARS-CoV-2 Acutis Multiplex Assay to provide incorrect results when a non-SARS-CoV-2 respiratory virus is present in a pooled negative clinical VTM sample. Viral, bacterial and fungal pathogens identified in Table 10: Summary of Analytical Specificity - Cross-Reactivity Study were acquired from ATCC and ZeptoMetrix. Viral pathogens were tested at a concentration of 2 x 10^5 copies/mL while bacterial and fungal pathogens were diluted to 1 x 10^6 CFU/mL. Samples were analyzed on both the QuantStudio 5 and QuantStudio 12K for the presence of the SARS-CoV-2 N gene and RNase P gene targets. All samples tested remained negative and showed other respiratory pathogens are not likely to cause a positive result on either instrument.

Table 10: SUMMARY OF ANALYTICAL SPECIFICITY - CROSS-REACTIVITY STUDY

INDEX	ORGANISM NAME	QUANTSTUDIO 5		QUANTSTUDIO 12K	
		OUTPUT	CROSS-REACTS	OUTPUT	CROSS-REACTS
1	Adenovirus (e.g., C1 Ad. 71)	Undetermined	NO	42.42	NO
2	Enterovirus (e.g., EV68)	Undetermined	NO	Undetermined	NO
3	Human Coronavirus 229E	Undetermined	NO	Undetermined	NO
4	Human coronavirus HKU1	Undetermined	NO	Undetermined	NO
5	Human coronavirus NL63	Undetermined	NO	Undetermined	NO
6	Human coronavirus OC43	Undetermined	NO	40.9523	NO
7	Human Metapneumovirus	Undetermined	NO	Undetermined	NO
8	Influenza A	Undetermined	NO	Undetermined	NO
9	Influenza B	Undetermined	NO	Undetermined	NO
10	MERS-coronavirus	Undetermined	NO	Undetermined	NO
11	Parainfluenza virus 1	Undetermined	NO	Undetermined	NO
12	Parainfluenza virus 2	42.68	NO	Undetermined	NO
13	Parainfluenza virus 3	Undetermined	NO	Undetermined	NO
14	Parainfluenza virus 4A	Undetermined	NO	Undetermined	NO
15	Parainfluenza virus 4B	Undetermined	NO	Undetermined	NO
16	Respiratory syncytial virus A	Undetermined	NO	Undetermined	NO
17	Respiratory syncytial virus B	Undetermined	NO	Undetermined	NO
18	Rhinovirus	Undetermined	NO	Undetermined	NO
19	SARS-CoV-1	Undetermined	NO	Undetermined	NO
20	*Bordetella pertussis*	Undetermined	NO	Undetermined	NO
21	*Candida albicans*	Undetermined	NO	Undetermined	NO
22	*Chlamydia pneumoniae*	Undetermined	NO	Undetermined	NO
23	*Haemophilus influenzae*	Undetermined	NO	Undetermined	NO

Table 10: SUMMARY OF ANALYTICAL SPECIFICITY - CROSS-REACTIVITY STUDY					
INDEX	ORGANISM NAME	QUANTSTUDIO 5		QUANTSTUDIO 12K	
		OUTPUT	CROSS-REACTS	OUTPUT	CROSS-REACTS
24	*Legionella pneumophila*	Undetermined	NO	Undetermined	NO
25	*Mycoplasma pneumoniae*	Undetermined	NO	Undetermined	NO
26	*Pseudomonas aeruginosa*	43.85	NO	40.94	NO
27	*Staphylococcus epidermidis*	Undetermined	NO	Undetermined	NO
28	*Streptococcus pneumoniae*	Undetermined	NO	Undetermined	NO
29	*Streptococcus pyogenes*	Undetermined	NO	Undetermined	NO
30	*Streptococcus salivarius*	Undetermined	NO	Undetermined	NO
31	Pooled human nasal wash	Undetermined	NO	Undetermined	NO

4) Interfering Substances

Endogenous/exogenous studies were completed to identify any potentially interfering substance reasonably likely to be encountered by the SARS-CoV-2 Acutis Multiplex Assay and cause false results. Pooled negative VTM from clinical specimens was used to create samples for the interference tests. Heat-inactivated SARS-CoV-2 virus was spiked into the negative VTM samples at concentrations of 200 copies/mL (2x LoD) and 300 copies/mL (3x LoD) to evaluate the potential interfering substances. Each set of samples was then spiked with the interfering substances at the concentrations identified in Table 11: Summary of Interference Study. The samples were analyzed in triplicate for the presence of the SARS-CoV-2 N gene and the internal control, RNase P. Samples were tested on both the QuantStudio 5 and QuantStudio 12K Flex using the procedures identified in the "SARS-CoV-2 Acutis Developed Multiplex Assay" SOP.

All samples spiked with SARS-CoV-2 virus and potential interferents produced positive results with Ct values within ±3 of the neat sample. The negative pooled VTM samples did not produce positive results when samples were spiked with potentially interfering substances. No endogenous or exogenous interfering substances tested demonstrated the potential to interfere with the SARS-CoV-2 Acutis Multiplex Assay.

Table 11: SUMMARY OF INTERFERENCE STUDY							
		POSITIVE SAMPLES				NEGATIVE SAMPLES	
		QUANTSTUDIO 5		QUANTSTUDIO 12K		QUANTSTUDIO 5	QUANTSTUDIO 12K
Interferent	Concentration	2x LOD	3x LOD	2x LOD	3x LOD	Negative VTM	Negative VTM
No Interferent	N/A	3/3	3/3	3/3	3/3	3/3	3/3
Cough Syrup	5% v/v	3/3	3/3	3/3	3/3	3/3	3/3
Nicotine	0.03mg/mL	3/3	3/3	3/3	3/3	3/3	3/3
Nasal Spray (Afrin)	15% v/v	3/3	3/3	3/3	3/3	3/3	3/3
Mucin	2.5 mg/mL	3/3	3/3	3/3	3/3	3/3	3/3
Vicks Lozenge	3 mg/mL	3/3	3/3	3/3	3/3	3/3	3/3
Sore Throat Spray	5% v/v	3/3	3/3	3/3	3/3	3/3	3/3

5) Specimen Stability:

The SARS-CoV-2 Acutis Multiplex Assay is intended to be used with nasopharyngeal swabs (NP) that have been placed in VTM for transport and storage. Samples placed immediately into VTM can be stored at room temperature (18°C to 30°C) for up to 48 hours or refrigerated for up to 6 days prior to testing.

A sample stability study was completed demonstrating the stability of NP swab samples stored in VTM at room temperature (18°C to 30°C) for up to 48 hours and for samples stored in refrigeration (2°C to 8°C) for up to 7 days prior to testing. Pooled VTM collected from negative clinical samples were used to create the samples used for stability testing. For each condition evaluated, a total of 35 samples, 10 samples at 100 copies/mL (1X LoD), 10 samples at 200 copies/mL (2X LoD), 5 samples at 300 copies/mL (3X LoD), 5 samples at 500 copies/mL (5X LoD) and 5 negative sample were tested using the same starting materials. Positive samples were created using heat-inactivated virus acquired from ATCC. Samples were analyzed for the presence of the SARS-CoV-2 N gene and the internal control, RNase P on the QuantStudio 12K Flex using the procedures identified in the "SARS-CoV-2 Acutis Developed Multiplex Assay" SOP.

For the room temperature samples, each sample was analyzed daily for 3 days. Ct values were recorded for all targets (N and RNase P) for each sample. All samples spiked with SARS-CoV-2 remained positive on Days 1 and 2. One failure was observed on Day 3 where the sample's N Gene Ct score was undetermined. All other positive samples tested remained positive when tested on Day 3. All negative samples remained negative for the duration of the testing.

For the refrigerated samples, each sample was analyzed on Day 0, Day 3 and Day 7. Ct values were recorded for all targets (N and RNase P) for each sample. All samples spiked with SARS-CoV-2 remained positive at all three testing points. All negative samples remained negative for the duration of the testing. Results for the Specimen Stability Study are summarized in Tables 12 and 13.

Table 12: Specimen Stability Study Results – Room Temperature

Concentration (GCE/mL)	Number Replicates (n)	Room Temperature (18°C to 30°C) (Day 0)		Room Temperature (Day 1) (18°C to 30°C)		Room Temperature (Day 2) (18°C to 30°C)		Room Temperature (Day 3) (18°C to 30°C)	
		N Gene	RNase P	N Gene	RNase P	N Gene	RNase P	N Gene	RNase P
100 (1x LoD)	10	10/10 (100%)	10/10 (100%)	10/10 (100%)	10/10 (100%)	10/10 (100%)	10/10 (100%)	9/10 (90%)	10/10 (100%)
200 (2x LoD)	10	10/10 (100%)	10/10 (100%)	10/10 (100%)	10/10 (100%)	10/10 (100%)	10/10 (100%)	10/10 (100%)	10/10 (100%)
300 (3x LoD)	5	5/5 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)
500 (5x LoD)	5	5/5 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)
Negative	5	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)

Table 13: Specimen Stability Study Results – Refrigerated

Concentration (GCE/mL)	Number Replicates (n)	Refrigerated (2°C to 8°C) (Day 0)		Refrigerated (2°C to 8°C) (Day 3)		Refrigerated (2°C to 8°C) (Day 7)	
		N Gene	RNase P	N Gene	RNase P	N Gene	RNase P
100 (1x LoD)	10	10/10 (100%)	10/10 (100%)	10/10 (100%)	10/10 (100%)	10/10 (100%)	10/10 (100%)
200 (2x LoD)	10	10/10 (100%)	10/10 (100%)	10/10 (100%)	10/10 (100%)	10/10 (100%)	10/10 (100%)
300 (3x LoD)	5	5/5 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)
500 (5x LoD)	5	5/5 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)
Negative	5	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)

6) Clinical Evaluation:

Clinical performance of the SARS-CoV-2 Acutis Multiplex Assay was evaluated through comparison of archived nasopharyngeal (NP) swab samples stored in viral transport medium, collected from individuals suspected of infection with SARS-CoV-2 with those of an EUA authorized molecular comparator. A total of 314 previously tested specimens, 68 positive and 246 negative NP swabs, were collected at Acutis Diagnostics in Hicksville, NY and held per the "SARS-CoV-2 Acutis Developed Multiplex Assay" SOP until the clinical study was completed. Both the comparator test and the SARS-CoV-2 were tested simultaneously. The comparator assay was performed in accordance with the manufacturer's instructions. The SARS-CoV-2 Acutis Multiplex Assay was executed per the instructions for use both the QuantStudio 5 and QuantStudio 12K Flex instruments.

Results from the QuantStudio 5 instrument indicated 79 positive specimens and 235 negative specimens. All 19 specimens determined to be low positives (i.e., within 3 Ct of the mean Ct at the LoD of the comparator test), were determined to be positive for both the candidate and comparator test. Summary of the results can be found in the Table 14.

Table 14: SUMMARY OF QUANTSTUDIO 5 TO THE COMPARATOR

QUANSTUDIO 5	COMPARATOR ASSAY		
	POSITIVE	NEGATIVE	TOTAL
POSITIVE	68	11	79
NEGATIVE	0	235	235
TOTAL	68	246	314
PPA: 100.0% (95% CI: 94.7% – 100.0%)			
NPA: 95.5% (95% CI: 92.1% – 97.8%)			

Results from the QuantStudio 12K instrument indicated 76 positive specimens and 238 negative specimens. All 19 specimens determined to be low positives (i.e., within 3 Ct of the mean Ct at the LOD of the comparator test), were determined to be positive for both the candidate and comparator test. Summary of the results can be found in Table 15.

Table 15: SUMMARY OF QUANTSTUDIO 12K TO THE COMPARATOR

QUANSTUDIO 12K	COMPARATOR ASSAY		
	POSITIVE	NEGATIVE	TOTAL
POSITIVE	68	8	76
NEGATIVE	0	238	238
TOTAL	68	246	314
PPA: 100.0% (95% CI: 94.7% – 100.0%)			
NPA: 96.8% (95% CI: 93.7% – 98.6%)			

Limitations

- The performance of this test was established based on the evaluation of a limited number of clinical specimens. Clinical performance has not been established with all circulating variants but is anticipated to be reflective of the prevalent variants in circulation at the time and location of the clinical evaluation. Performance at the time of testing may vary depending on the variants circulating, including newly emerging strains of SARS-CoV-2 and their prevalence, which change over time.
- Detection of RNase P indicates that human nucleic acid is present and implies that human biological material was collected and successfully extracted and amplified. It does not necessarily indicate that the specimen is of appropriate quality to enable detection of SARS-CoV-2.

WARNINGS

- For in vitro diagnostic use
- Rx Only
- For use under Emergency Use Authorization (EUA) only
- This product has not been FDA cleared or approved, but has been authorized for emergency use by FDA under an EUA for use by the authorized laboratory;
- This product has been authorized only for the detection of nucleic acid from SARS-CoV-2, not for any other viruses or pathogens; and
- The emergency use of this product is only authorized for the duration of the declaration that circumstances exist justifying the authorization of emergency use of in vitro diagnostics for detection and/or diagnosis of COVID-19 under Section 564(b)(1) of the Federal Food, Drug and Cosmetic Act, 21 U.S.C. § 360bbb-3(b)(1), unless the declaration is terminated or authorization is revoked sooner.