

Diatherix SARS-CoV-2 TEM-PCR™ Assay

PERFORMANCE EVALUATION

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

The analytical sensitivity of the SARS-CoV-2 TEM-PCR™ assay was determined in Limit of Detection (LoD₉₅) studies. LoD₉₅ is defined as the concentration that can accurately be detected 95% of the time (19/20 replicates positive). Since no quantified viral isolates were available at the time of the study, assays were tested from extraction with characterized genomic RNA (Human 2019-nCoV strain 2019-nCoV/Italy-INMI1) and stocks of *in vitro* transcribed (IVT) RNA for a partial sequence of the spike (S) gene. Genomic RNA and IVT RNA of known titers (copies/μL) were spiked into non-reactive, pooled sputum clinical matrix and simulated lung fluid (SLF) to mimic respiratory swab specimens.

To first determine performance of the assay, 10-fold serial dilutions of IVT RNA in Simulated Lung Fluid (SLF) were prepared (ranging from 10¹⁰ copies/μL to 10⁰ copies/μL) and extracted in triplicate. Once assay performance was assessed, broad-range LoD for the assay in clinical matrix was determined by testing triplicate samples of genomic RNA spiked in pooled sputum from 10³ to 10⁰ copies/μL. Confirmation of the LoD was determined using 10-fold serial dilutions of genomic RNA from 10² to 10⁰ copies/μL in sputum with 20 extracted replicates at each concentration. **The LoD₉₅ of the SARS-CoV-2 TEM-PCR™ assay is 1 copy/μL (19/20 positive).**

2) Inclusivity (Analytical Sensitivity):

An alignment was performed with the oligonucleotide primer and detection sequences designed for the Diatherix SARS-CoV-2 TEM-PCR™ panel using all publically available sequences for SARS-CoV-2 found in NCBI Virus Resource and GenBank as of March 15, 2020 to predict the inclusivity of the panel. **All alignments show 100% identity to the primer sequences utilized by the SARS-CoV-2 TEM-PCR™ panel.**

Wet lab testing was performed on genomic RNA (Human 2019-nCoV strain 2019-nCoV/Italy-INMI1 and BetaCoV/Germany/BavPat1/2020 p.1), as well as *in vitro* transcribed RNA (partial S gene). **Both strains of genomic RNA and IVT RNA were detected at or below LoD₉₅ by the SARS-CoV-2 assay.**

3) Cross-reactivity (Analytical Specificity)

A total of 31 viral strains, 34 bacterial strains, and two fungal strains were wet tested for cross-reactivity against the SARS-CoV-2 panel (Table 1). The organisms were selected for their likelihood to be found in the respiratory tract, for genetic similarities, or for likelihood of carriage within the clinical matrix. **No positive signals for SARS-CoV-2 were observed in this cross-reactivity evaluation.**

Table 1. List of Organisms Analyzed by Wet Testing

Strain	Vendor	Catalog #	Concentration Tested
Adenovirus Type 4	Zeptomatrix	0810070CF	10 ³ TCID ₅₀ /mL
Coxsackie Virus Type A16	Zeptomatrix	0810107CF	10 ² TCID ₅₀ /mL
Influenza A H3N2 Texas/50/12	Zeptomatrix	0810238CF	10 ² TCID ₅₀ /mL
Human metapneumovirus Type B2 Strain IA18-2003	Zeptomatrix	0810162CF	10 ³ TCID ₅₀ /mL
Parainfluenza virus Type 4A	Zeptomatrix	0810060CF	10 ² TCID ₅₀ /mL
Parainfluenza virus Type 4B	Zeptomatrix	0810060BCF	10 ² TCID ₅₀ /mL
Human metapneumovirus Type A2 Strain IA27-2004	Zeptomatrix	0810164CF	10 ³ TCID ₅₀ /mL
Adenovirus Type 7A	Zeptomatrix	0810021CF	10 ⁴ TCID ₅₀ /mL
Enterovirus D68 2014 Isolate 1	Zeptomatrix	0810300CF	10 ² TCID ₅₀ /mL
Influenza A H1N1 Michigan/45/15	Zeptomatrix	0810538CF	10 ² TCID ₅₀ /mL
Parainfluenza Virus Type 1	Zeptomatrix	0810014CF	10 ² TCID ₅₀ /mL
Adenovirus Type 1	Zeptomatrix	0810062CF	10 ³ TCID ₅₀ /mL
Echovirus Type 30	Zeptomatrix	0810078CF	10 ³ TCID ₅₀ /mL
Human Coronavirus NL63	Zeptomatrix	0810228CF	10 ² TCID ₅₀ /mL
Influenza A H7N9 Shanghai/2/2013 (BPL Treated)	IRR	FR-1281	10 ² TCID ₅₀ /mL
Influenza B/Phuket/3073/2013 (Yamagata lineage)	IRR	FR-1364	10 ⁶ CEID ₅₀ /mL
MERS-CoV (Heat-inactivated)	BEI	NR-50171	10 ³ TCID ₅₀ /mL
Human Coronavirus OC43	Zeptomatrix	0810024CF	10 ² TCID ₅₀ /mL
Parainfluenza Virus Type 2	Zeptomatrix	0810015CF	10 ³ TCID ₅₀ /mL
Respiratory Syncytial Virus Type B 3/2015 Isolate #2	Zeptomatrix	0810480CF	10 ² TCID ₅₀ /mL
Human Coronavirus 229E	Zeptomatrix	0810229CF	10 ² TCID ₅₀ /mL
Influenza B Brisbane/33/08	Zeptomatrix	0810253CF	10 ³ TCID ₅₀ /mL
Parainfluenza virus Type 3	Zeptomatrix	0810016CF	10 ³ TCID ₅₀ /mL
Rhinovirus A16	Zeptomatrix	0810285CF	10 ⁴ TCID ₅₀ /mL
Respiratory Syncytial Virus Type A 3/2015 Isolate #3	Zeptomatrix	0810482CF	10 ³ TCID ₅₀ /mL
<i>Acinetobacter baumannii</i>	Zeptomatrix	801597	10 ⁶ cfu/mL
<i>Bordetella parapertussis</i>	ATCC	BAA-587	10 ⁶ cfu/mL

Strain	Vendor	Catalog #	Concentration Tested
<i>Bordetella pertussis</i>	ATCC	BAA-1335	10 ⁶ cfu/mL
<i>Candida albicans</i>	ATCC	11006	10 ⁶ cfu/mL
<i>Candida glabrata</i>	ATCC	32554	10 ⁶ cfu/mL
<i>Chlamydia trachomatis</i>	Zeptomatrix	801775	10 ⁶ ifu/mL
<i>Chlamydophila pneumoniae</i>	IRR	FR-319	10 ⁴ TCID ₅₀ /mL
<i>Corynebacterium sp.</i>	ATCC	35515	10 ⁶ cfu/mL
<i>Escherichia coli</i>	ATCC	11775	10 ⁶ cfu/mL
<i>Fusobacterium necrophorum</i>	ATCC	51357	10 ⁶ cfu/mL
<i>Haemophilus influenzae</i>	Zeptomatrix	801680	10 ⁶ cfu/mL
Herpes simplex virus 1	Zeptomatrix	0810005CF	10 ⁴ TCID ₅₀ /mL
Herpes simplex virus 2	Zeptomatrix	0810006CF	10 ⁴ TCID ₅₀ /mL
Herpesvirus 3 (VZV)	Zeptomatrix	0810172CF	10 ³ TCID ₅₀ /mL
<i>Klebsiella pneumoniae</i>	ATCC	35555	10 ⁶ cfu/mL
<i>Lactobacillus acidophilus</i>	Zeptomatrix	801540	10 ⁶ cfu/mL
<i>Lactobacillus plantarum</i>	IRR	FR-324	10 ⁶ cfu/mL
<i>Legionella pneumophila</i>	IRR	FR-317	10 ⁶ cfu/mL
<i>Listeria monocytogenes</i>	Zeptomatrix	801534	10 ⁶ cfu/mL
<i>Moraxella catarrhalis</i>	IRR	FR-327	10 ⁶ cfu/mL
<i>Mycoplasma genitalium</i>	Zeptomatrix	0804094-1	10 ⁵ ccu/mL
<i>Mycoplasma hominis</i>	Zeptomatrix	804011	10 ⁶ ccu/mL
<i>Mycoplasma pneumoniae</i>	IRR	FR-318	10 ⁶ ccu/mL
<i>Neisseria elongata</i>	ATCC	25295	10 ⁶ cfu/mL
<i>Neisseria gonorrhoeae</i>	Zeptomatrix	801482	10 ⁶ cfu/mL
<i>Neisseria meningitidis</i>	Zeptomatrix	801511	10 ⁶ cfu/mL
<i>Neisseria sicca</i>	Zeptomatrix	801754	10 ⁵ cfu/mL
Parechovirus 1	IRR	FR-308	10 ⁴ TCID ₅₀ /mL
Parechovirus 2	Zeptomatrix	0810146CF	10 ⁴ TCID ₅₀ /mL
Parechovirus 3	Zeptomatrix	0810147CF	10 ⁴ TCID ₅₀ /mL
<i>Proteus vulgaris</i>	ATCC	33420	10 ⁶ cfu/mL
<i>Pseudomonas aeruginosa</i>	Zeptomatrix	801519	10 ⁶ cfu/mL
<i>Serratia marcescens</i>	ATCC	43861	10 ⁶ cfu/mL
<i>Staphylococcus aureus</i>	Zeptomatrix	801531	10 ⁶ cfu/mL
<i>Staphylococcus epidermidis</i>	Zeptomatrix	801651	10 ⁶ cfu/mL
<i>Staphylococcus haemolyticus</i>	Zeptomatrix	801591	10 ⁶ cfu/mL
<i>Streptococcus agalactiae</i>	Zeptomatrix	801545	10 ⁶ cfu/mL
<i>Streptococcus dysgalactiae</i>	Zeptomatrix	801516	10 ⁶ cfu/mL
<i>Streptococcus pneumoniae</i>	Zeptomatrix	801439	10 ⁶ cfu/mL
<i>Streptococcus pyogenes</i>	Zeptomatrix	801512	10 ⁶ cfu/mL
<i>Streptococcus salivarius</i>	ATCC	13419	10 ⁶ cfu/mL
<i>Ureaplasma urealyticum</i>	ATCC	33699	10 ⁶ cfu/mL

A total of 183 individual clinical samples from patients exhibiting signs and symptoms of respiratory infection (nasal swabs, nasopharyngeal swabs, throat swabs, bronchial washes, and sputum swabs) were tested against the SARS-CoV-2 assay. **No positive signals for SARS-CoV-2 were observed, indicating that the diverse microflora and genomic material found within the human respiratory tract will not cross-react with the assay.**

For strains not available for wet testing, *in silico* analysis of the SARS-CoV-2 primers and detection sequence was performed. BLAST analysis of the TEM-PCR™ amplicon indicated no significant homology with the human genome, human coronaviruses, human microflora, or other high-priority respiratory organisms that could be circulating within the population. One sequence (MN996532.1 Bat coronavirus RaTG13, complete genome) aligned to the amplicon with 96% homology. **Alignment of the specific SARS-CoV-2 assay to this sequence contained mismatches (>25% sequence mismatch within primers and detection sequence), indicating that amplification and detection would likely not occur.**

4) Clinical Evaluation:

A contrived clinical study was performed to evaluate the performance of the SARS-CoV-2 assay. A total of 219 individual respiratory specimens, comprised of nasal swabs, nasopharyngeal swabs, throat swabs, bronchial washes, and sputum were tested in this study. Positive samples were contrived by spiking genomic RNA or *in vitro* transcribed RNA into clinical specimens assumed to be negative for SARS-CoV-2. Twenty specimens were spiked with genomic RNA at 2X LoD (20 copies/μL). As the genomic RNA material was limited, IVT RNA was used to create the remainder of the contrived specimens (n=16), spanning the assay testing range.

No positive signals for SARS-CoV-2 were observed in the negative samples (100% agreement). Percent agreement for contrived samples was 95% (19/20 detected) at 2X LoD, and 100% for the remainder of the samples.

As an additional quality monitor for clinical evaluation, an orthogonal qRT-PCR assay that amplifies the N-gene of SARS-CoV-2 (2019-nCoV CDC qPCR Probe Assay, Integrated DNA Technologies (IDT), Coralville, Iowa), was developed and experimentally validated. This assay was modified from the published CDC extraction protocol by using the well-established, high-throughput KingFisher Flex instrument for extraction, and the QuantStudio 12K Flex Real-Time PCR System (Thermo Fisher) for 96-well amplification. TaqPath™ 1-Step qPCR Master Mix, CG (Applied Biosystems, Waltham, MA) was used as directed for primer-probe amplification. This qRT-PCR assay was performed independently in the Research and Development (R&D) laboratory at Diatherix.

For experimental validation of the qPCR assay, negative controls were run from RT-PCR as follows: no template control (NTC), MERS-CoV control (IDT) and SARS-CoV control (IDT). Negative clinical sputum samples and Simulated Lung Fluid (Diatherix) were tested as negative sample controls from extraction. The 2019-nCoV_N_Positive Control (IDT) was amplified as per directed as a positive control for amplification. Human extraction positive control material (Hs_RPP30 Positive Control, IDT) was diluted (ranging from 2,000 to 20 copies/μL) and spiked into simulated lung fluid for determination of the RNase P gene (human sample control) assay's lower limit of detection from extraction. For limit of detection determination for contrived clinical specimens, genomic RNA for SARS-CoV-2 (10^3 to 10^0 copies/μL) was spiked into sputum matrix and extracted per Diatherix protocols.

Cycle thresholds for all assays (N1, N2, N3, and RP) were set at <40.0 Ct for positive detection. Experimental validation of the orthogonal assay indicated efficient extraction of human genomic material from Hs_RPP positive control and human clinical material. **The analytical LoD of the orthogonal N-gene assay was between 10 copies/μL and 1 copy/μL in sputum matrix, which is consistent with LoD of the Diatherix SARS-CoV-2 assay.**

After laboratory validation and implementation of the Diatherix TEM-PCR™ SARS-CoV-2 assay, the first twenty-two batched clinical samples were extracted per established Diatherix protocol. Immediately after extraction and template addition into TEM-PCR™ panels, the resultant elution plate containing RNA was kept cold and was transferred to the R&D laboratory for confirmation testing with the orthogonal method. All positive and negative results obtained with the Diatherix SARS-CoV-2 assay were confirmed concordant with the N-gene assay results. Furthermore, all samples exhibited passing RP gene results, indicating successful RNA extraction. Additional runs of confirmation samples were performed, for a total of 86 samples undergoing orthogonal testing. Diatherix utilized this modified version of the CDC qPCR reagents and assay as an in-house QA monitor program for the initial series of testing for COVID-19. As this orthogonal approach is not an EUA-approved assay, the QA monitor program was set up solely as a temporary method and is not part of the normal clinical testing procedures. **The purpose of this approach was to ensure the resultant Diatherix SARS-CoV-2 results were confirmed during the initial phase of clinical testing. All samples (n=86) yielded concordant results between methods (100% PPA/100% NPA).**

As part of the initial assay confirmation testing and as recommended by the FDA for laboratories seeking EUA for SARS-CoV-2 testing Diatherix referred 10 original specimens that were tested in our laboratory for SARS-CoV-2. These specimens represented the first 5 Positive specimens and first 5 negative specimens (SARS-CoV-2). The specimens were blinded and coded with number 1 through 10 and sent to:

Sentinel Laboratory Liaison
ADPH Bureau of Clinical Labs
Montgomery, AL 36124

The original Specimen results and the results reported by ADPH Bureau of Clinical Labs were evaluated and in each case, the results from each lab matched 100% (10/10).

Diatherix Eurofins SARS-CoV-2 virus has not been FDA cleared or approved. This test has been authorized by FDA under an EUA for use by authorized laboratories, and has been authorized only for the detection of nucleic acid from SARS-CoV-2, not for any other viruses or pathogens. The test is only authorized for the duration of the declaration that circumstances exist justifying the authorization of emergency use of in vitro diagnostic tests for detection and/or diagnosis of COVID-19 under Section 564(b)(1) of the Act, 21 U.S.C. § 360bbb-3(b)(1), unless the authorization is terminated or revoked sooner.