

EZ-SARS-CoV-2 Real-Time RT-PCR Performance Characteristics

Catalog Number TC-5048-192

For *In Vitro* Diagnostic Use
Rx Only

Validation of this test has not been reviewed by FDA.
Review under the EUA program is pending.

Distributed in accordance with the guidance on
*Policy for Coronavirus Disease-2019 Tests During the Public Health
Emergency, Section IV.C.2.*

Limit of Detection (LoD) - Analytical Sensitivity

The LoD study established the lowest concentration of SARS-CoV-2 (Genomic Copy Equivalents or GCE) that can be detected by the EZ-SARS-CoV-2 Real-Time RT-PCR at least 95% of the time. Negative nasal swab specimens collected in 1mL sterile saline were pooled and spiked with SARS-Related Coronavirus 2, Isolate USA-WA1/2020 (BEI Resources NR-52281) at several concentrations. Pooled specimen material was screened negative using the EZ-SARS-CoV-2 Real-Time RT-PCR prior to spiking. Each viral dilution was added to swabs (50 µL), collected in 1mL sterile saline, and then processed through the EZ-SARS-CoV-2 Real-Time RT-PCR workflow, including nucleic acid extraction using the Qiagen QIAamp® Viral RNA Mini Kit following the manufacturer's recommended procedure. Real-time PCR for the EZ-SARS-CoV-2 Real-Time RT-PCR was carried out on the Applied Biosystems 7500 Fast Real-time PCR System and the T-COR 8™ Real-time PCR Thermocycler. The preliminary LoD was determined to be 10 GCE per reaction for both RT-PCR instruments (see Tables 1 and 2).

Table 1. Preliminary LoD study on the ABI 7500 Fast.

Effective Concentration	Replicate	SARS-CoV-2 Ct Value	IC Ct Value	RNase P Ct Value	Detection Rate
1000 GCE/reaction	1	29.1	27.2	32.3	100%
	2	29.5	27.2	32.0	
	3	31.5	27.7	31.5	
300 GCE/reaction	1	31.1	27.0	32.8	100%
	2	29.8	27.2	31.3	
	3	30.5	27.0	31.5	
100 GCE/reaction	1	32.7	27.1	31.8	100%
	2	31.4	27.4	31.7	
	3	31.8	27.3	31.2	
30 GCE/reaction	1	34.5	27.4	32.2	100%
	2	33.1	27.0	31.7	
	3	33.3	27.1	31.7	
10 GCE/reaction	1	35.0	27.1	31.7	100%
	2	34.6	27.2	31.8	
	3	35.0	27.3	31.5	
3 GCE/reaction	1	36.7	27.1	32.6	33.3%
	2	-	27.1	31.7	
	3	-	27.1	31.6	
1 GCE/reaction	1	-	28.2	33.1	0%
	2	-	27.8	31.8	
	3	-	27.1	31.9	

Table 2. Preliminary LoD study on the T-COR 8™.

Effective Concentration	Replicate	SARS-CoV-2 Ct Value	IC Ct Value	RNase P Ct Value	Detection Rate
1000 GCE/reaction	1	31.6	29.1	33.0	100%
	2	31.5	29.0	33.5	
	3	34.9	30.6	32.7	
300 GCE/reaction	1	32.9	29.1	33.3	100%
	2	32.1	29.3	32.5	
	3	32.2	28.9	33.2	
100 GCE/reaction	1	34.8	29.0	33.7	100%
	2	33.1	29.2	32.6	
	3	33.2	29.1	32.8	
30 GCE/reaction	1	36.1	29.4	33.5	100%
	2	34.6	29.0	32.9	
	3	34.8	29.2	32.4	
10 GCE/reaction	1	36.9	29.0	33.3	100%
	2	36.6	28.9	33.1	
	3	37.3	29.2	32.8	
3 GCE/reaction	1	-	28.8	33.4	66.7%
	2	37.5	29.3	33.1	
	3	37.3	29.2	32.5	
1 GCE/reaction	1	-	31.7	33.7	0%
	2	-	30.2	33.1	
	3	-	29.1	34.5	

The confirmatory LoD study was performed in the same manner as the preliminary LoD study described above. Twenty replicates were tested at the preliminary LoD of 10 GCE per reaction on the ABI 7500 Fast and T-COR 8™ RT-PCR instruments, followed by 20 replicates at 30 GCE per reaction on the T-COR 8™ (see Tables 3 and 4). The LoD was determined to be 10 GCE per reaction on the Applied Biosystems 7500 Fast Real-time PCR System and 30 GCE per reaction on the T-COR 8™ Real-time PCR Thermocycler.

Table 3. Confirmatory LoD study on the ABI 7500 Fast.

Effective Concentration	Replicate	SARS-CoV-2 Ct Value	IC Ct Value	RNase P Ct Value	Detection Rate
10 GCE/reaction	1	36.3	27.5	31.9	95%
	2	35.5	27.7	31.1	
	3	34.7	27.6	30.6	
	4	36.0	27.1	31.6	
	5	36.3	27.7	31.2	
	6	34.0	27.5	30.7	
	7	35.9	27.4	30.6	
	8	36.1	27.4	31.4	
	9	38.3	27.5	32.0	
	10	35.8	28.2	31.2	
	11	36.4	28.8	30.7	
	12	37.3	28.6	31.1	
	13	34.9	27.5	30.6	
	14	-	29.0	31.5	
	15	35.0	27.4	30.8	
	16	36.8	27.5	31.0	
	17	37.8	28.9	31.7	
	18	34.7	27.5	30.6	
	19	36.5	27.8	31.7	
	20	35.6	28.5	31.4	

Table 4. Confirmatory LoD study on the T-COR 8™.

Effective Concentration	Replicate	SARS-CoV-2 Ct Value	IC Ct Value	RNase P Ct Value	Detection Rate
30 GCE/reaction	1	35.2	29.3	32.2	100%
	2	35.2	28.9	32.7	
	3	34.8	29.8	32.1	
	4	34.9	29.4	32.5	
	5	35.6	29.7	32.2	
	6	35.0	29.6	31.9	
	7	35.6	29.9	32.3	
	8	35.5	29.7	32.4	
	9	35.6	29.1	33.1	
	10	36.1	30.0	32.9	
	11	34.3	30.0	31.9	
	12	36.0	30.3	32.4	
	13	35.6	29.6	32.9	
	14	36.0	30.2	32.5	
	15	35.1	29.8	32.6	
	16	34.7	29.6	32.0	
	17	35.3	29.1	31.8	
	18	36.5	29.9	32.4	
	19	35.6	29.4	31.6	
	20	34.9	29.3	31.9	
10 GCE/reaction	1	37.0	30.0	32.9	80%
	2	37.0	30.9	32.1	
	3	37.5	30.6	32.1	
	4	36.9	30.1	33.0	
	5	36.1	30.4	32.5	
	6	36.9	30.7	32.2	
	7	37.0	30.6	31.8	
	8	36.3	30.4	32.4	
	9	35.5	29.7	32.4	
	10	37.2	30.4	33.0	
	11	-	32.0	32.2	
	12	37.1	31.2	31.8	
	13	36.7	30.2	32.1	
	14	37.2	31.7	32.6	
	15	36.7	30.1	31.5	
	16	-	29.9	32.4	
	17	-	32.3	34.3	

	18	36.7	30.3	32.2	
	19	36.8	30.8	32.8	
	20	-	31.8	32.9	

Inclusivity (*In Silico* Analysis)

The nucleotide mismatch frequency of the EZ-SARS-CoV-2 Real-Time RT-PCR N gene primer and probe sequences was evaluated using GISAID sequences (<https://www.gisaid.org>) downloaded on August 4, 2020. The dataset includes 76,047 SARS-CoV-2 sequences collected from December 24, 2019 through July 2020. The combined assay (including all N gene targets) matches >99.5% of all sequences. One mismatch in one of the N gene assay probe sequences is a single point of divergence from 99.5% inclusivity. After filtering for completeness, 2.24% of the sequences (1,649) showed the mismatch. Because the EZ-SARS-CoV-2 Real-Time RT-PCR utilizes a single reporter dye (FAM) for detection of all N gene targets, mismatches would need to be present in all N gene assay regions to produce a false negative result. Twenty four of the SARS-CoV-2 sequences contain a mismatch in each of the EZ-SARS-CoV-2 Real-Time RT-PCR N gene assay regions. None of these 24 sequences were found to have more than one mismatch in any single N gene assay region. Because the EZ-SARS-CoV-2 Real-Time RT-PCR is expected to tolerate a single mismatch greater than 5 bases from the 3’ end of a primer or a single mismatch in a probe, none of the SARS-CoV-2 sequences are anticipated to produce a false negative result.

Cross-reactivity (Analytical Specificity)

In Silico Analysis

BLASTn analysis queries of the EZ-SARS-CoV-2 Real-Time RT-PCR assay primers and probes were performed against public domain nucleotide sequences. The database search parameters were as follows: 1) The nucleotide collection consists of GenBank NT and RefSeq sequences, but excludes EST, STS, GSS, WGS, TSA, patent; 2) The database is non-redundant. Identical sequences have been merged into one entry, while preserving the accession, GI, title and taxonomy information for each entry; 3) Database was updated on 02/11/2020; 4) The search parameters automatically adjust for short input sequences and the expect threshold is 1000; 5) The match and mismatch scores are 1 and -3, respectively. Additionally, Needleman–Wunsch alignments were performed against a defined set of data containing all the sequences in Table 5.

Each primer and probe was aligned to the sequences listed in Table 5. The alignment used the Needleman Wunsch global alignment implemented by seq-align. No gaps were allowed in the alignment and a match matrix was used. The matrix scored the alignment with a 1 for match and a 0 for anything else. The alignment score was the number of matches between the primer or probe and the pathogen. The frequency of the alignment is the number of matches divided by the length of the primer or probe.

The probe sequence of one of the EZ-SARS-CoV-2 Real-Time RT-PCR N gene assays showed 92% sequence identity with SARS-CoV-1. However, forward and reverse primers showed no sequence homology (70% and 88%, respectively) with SARS-CoV-1 genome. Combining primers and probe, there are no significant homologies with human genome, other coronaviruses or human microflora that would predict potential false positive rRT-PCR results. The forward primer sequence of another EZ-SARS-CoV-2 Real-Time RT-PCR N gene assay showed 100% sequence identity to SARS-coronavirus (AY345986.1). The reverse primer and probe sequences showed less significant homology (89% and 78%, respectively) with human genome, other coronaviruses or human microflora. Combining primers and probe, there are no predictions of potential false positive rRT-PCR results.

In summary, the EZ-SARS-CoV-2 Real-Time RT-PCR N gene assays, designed for the specific detection of SARS-CoV-2, showed no significant combined homologies with human genome, other coronaviruses, or human microflora that would predict potential false positive rRT-PCR results.

Table 5. EZ-SARS-CoV-2 Real-Time RT-PCR cross-reactivity (*in silico* analysis).

Pathogen	Strain	GenBank Accession No.
Adenovirus	Human adenovirus type 1, complete genome	AC_000017.1
Bordetella pertussis	Bordetella pertussis strain B3921, complete genome	CP011448.1
Candida albicans	Candida albicans strain L757 mitochondrion, complete genome	NC_018046.1
Chlamydia pneumoniae	Chlamydia pneumoniae genome assembly PB2, chromosome: 1	NZ_LN847241.1
Enterovirus	Human enterovirus 68 isolate EV68_NL_201013421 VP1 protein gene, partial cds	JF896312.1
Haemophilus influenzae	Haemophilus influenzae PittGG, complete genome	CP000672.1
Human coronavirus 229E	Human coronavirus 229E strain 229E/human/USA/932-72/1993, complete genome	KF514432.1
Human coronavirus 229E	Human coronavirus 229E strain 229E/human/USA/933-40/1993, complete genome	KF514433.1
Human coronavirus HKU1	Human coronavirus HKU1 isolate SI17244, complete genome	MH940245.1
Human coronavirus HKU1	Human coronavirus HKU1 strain HKU1/human/USA/HKU1-18/2010, complete genome	KF430201.1
Human coronavirus NL63	Human coronavirus NL63 strain NL63/human/USA/891-4/1989, complete genome	KF530114.1
Human coronavirus NL63	Human coronavirus NL63 strain NL63/human/USA/905-25/1990, complete genome	KF530113.1
Human coronavirus OC43	Human coronavirus OC43 isolate LRTI_238, complete genome	KX344031.1
Human coronavirus OC43	Human coronavirus OC43 strain OC43/human/USA/971-5/1997, complete genome	KF530099.1
Human Metapneumovirus (hMPV)	Human metapneumovirus strain HMPV/Homo sapiens/PER/FPP00726/2011/A, complete genome	KJ627437.1
Influenza A	Influenza A virus (A/New York/PV305/2017(H1N1)) segment 2 polymerase PB1 (PB1) gene, complete cds and functional PB1-F2 protein (PB1-F2) gene, complete sequence	MH798556.1
Influenza B	Influenza B virus (B/Nicaragua/8689_13/2017) segment 2 polymerase PB2 (PB2) gene, complete cds	MK969560.1
Legionella pneumophila	Legionella pneumophila strain Philadelphia_1_CDC, complete genome	CP015928.1
MERS-Coronavirus	Middle East respiratory syndrome-related coronavirus strain HCoV-EMC, complete genome	MH013216.1
Mycobacterium tuberculosis	Mycobacterium tuberculosis DNA, complete genome, strain: HN-506	AP018036.1
Mycoplasma pneumoniae	Mycoplasma pneumoniae strain 14-637 chromosome, complete genome	CP039772.1
Parainfluenza 1	Human parainfluenza virus 1 isolate NM001, complete genome	KX639498.1
Parainfluenza 2	Human parainfluenza virus 2 isolate VIROAF10, complete genome	KM190939.1
Parainfluenza 3	Human parainfluenza virus 3 strain HPIV3/AUS/3/2007, complete genome	KF530243.1

Parainfluenza 4	Human parainfluenza virus 4a isolate HPIV4_DK (459), complete genome	KF483663.1
Pneumocystis jirovecii	Pneumocystis jirovecii isolate SW7_full mitochondrion, complete genome	MH010446.1
Pseudomonas aeruginosa	Pseudomonas aeruginosa UCBPP-PA14, complete genome	CP000438.1
Respiratory syncytial virus	Respiratory syncytial virus strain B/WI/629-Q0190/10, complete genome	JN032120.1
Rhinovirus	Human rhinovirus 14, complete genome	NC_001490.1
SARS-coronavirus	SARS coronavirus A022, complete genome	AY686863.1
SARS-coronavirus	SARS coronavirus CUHK-AG01, complete genome	AY345986.1
SARS-CoV-2	Wuhan seafood market pneumonia virus isolate Wuhan-Hu-1, complete genome	NC_045512.2
Staphylococcus epidermidis	Staphylococcus epidermidis strain SP3 16S ribosomal RNA gene, partial sequence	KY750253.1
Streptococcus pneumoniae	Streptococcus pneumoniae strain D39V chromosome, complete genome	CP027540.1
Streptococcus pyogenes	Streptococcus pyogenes MGAS8232, complete genome	AE009949.1
Streptococcus salivarius	Streptococcus salivarius strain LAB813 chromosome, complete genome	CP040804.1

Wet Testing

To confirm the cross-reactivity of the EZ-SARS-CoV-2 Real-Time RT-PCR in the wet test condition, 40 non-target organisms were prepared by extracting each standard organism (concentration of $> 10^6$ CFU/mL or $> 10^4$ TCID₅₀/mL, when available from the vendor) using the Qiagen QIAamp® Viral RNA Mini Kit. Real-time PCR for the EZ-SARS-CoV-2 Real-Time RT-PCR was carried out on the Applied Biosystems 7500 Fast Real-time PCR System and the T-COR 8™ Real-time PCR Thermocycler. Testing was performed in triplicate on the ABI 7500 Fast, and a single replicate was tested on the T-COR 8™. As a result, all 40 non-target samples were not detected (see Table 6).

Table 6. EZ-SARS-CoV-2 Real-Time RT-PCR cross-reactivity (wet testing).

Organism	Source	Isolate No.	Replicates Detected/Total	
			7500 Fast	T-COR 8™
Human coronavirus 229E	Zeptomatrix	0810229CF	0/3	0/1
Human coronavirus OC43	Zeptomatrix	0810024CF	0/3	0/1
Human coronavirus NL63	BEI Resources	NR-470	0/3	0/1
SARS coronavirus	BEI Resources	NR-9547	0/3	0/1
MERS coronavirus	BEI Resources	NR-45843*	0/3	0/1
Adenovirus Type 7A	Zeptomatrix	0810021CF	0/3	0/1
Adenovirus Type 1	Zeptomatrix	0810050CF	0/3	0/1
Adenovirus Type 4	Zeptomatrix	0810070CF	0/3	0/1
Human metapneumovirus (hMPV) 16 Type A1	Zeptomatrix	0810161CF	0/3	0/1
Parainfluenza virus 1	BEI Resources	NR-48680	0/3	0/1
Parainfluenza virus 2	BEI Resources	NR-3229	0/3	0/1
Parainfluenza virus 3	BEI Resources	NR-3233	0/3	0/1
Parainfluenza virus 4A	BEI Resources	NR-3237	0/3	0/1

Parainfluenza virus 4B	BEI Resources	NR-3238	0/3	0/1
Influenza A H1N1	BEI Resources	NR-13663	0/3	0/1
Influenza A H3N2	BEI Resources	NR-41803	0/3	0/1
Influenza B	BEI Resources	NR-42006	0/3	0/1
Enterovirus Type 68	Zeptomatrix	0810237CF	0/3	0/1
Enterovirus 71	BEI Resources	NR-471	0/3	0/1
Enterovirus D68	BEI Resources	NR-49131	0/3	0/1
Respiratory syncytial virus A1998/3-2	BEI Resources	NR-28529	0/3	0/1
Respiratory syncytial virus B1	BEI Resources	NR-4052	0/3	0/1
Respiratory syncytial virus A1998/12-21	BEI Resources	NR-28528	0/3	0/1
Rhinovirus 20, 15-CV19	BEI Resources	NR-51439	0/3	0/1
Rhinovirus 60, 2268-CV37	BEI Resources	NR-51447	0/3	0/1
Rhinovirus 34, 137-3	BEI Resources	NR-51451	0/3	0/1
<i>Chlamydia pneumoniae</i>	ATCC	53592	0/3	0/1
<i>Haemophilus influenzae</i>	ATCC	33391	0/3	0/1
<i>Legionella pneumophila</i>	Zeptomatrix	0801645	0/3	0/1
<i>Mycobacterium tuberculosis</i>	Zeptomatrix	0801660	0/3	0/1
<i>Streptococcus pneumoniae</i>	ATCC	49619	0/3	0/1
<i>Streptococcus pyogenes</i>	ATCC	10782	0/3	0/1
<i>Bordetella pertussis</i>	BEI Resources	NR-42460	0/3	0/1
<i>Mycoplasma pneumoniae</i>	Zeptomatrix	0801579	0/3	0/1
<i>Pneumocystis jirovecii</i> (PJP)	ATCC	PRA-159	0/3	0/1
Pooled human nasal wash	In-House		0/3	0/1
<i>Candida albicans</i>	ATCC	18804	0/3	0/1
<i>Pseudomonas aeruginosa</i>	ATCC	27853	0/3	0/1
<i>Staphylococcus epidermis</i>	ATCC	14990	0/3	0/1
<i>Streptococcus salivarius</i>	ATCC	13419	0/3	0/1

*Isolate NR-45843 was received as nucleic acid extracted from a preparation of MERS-CoV, EMC/2012 (BEI Resources NR-44260) using the QIAamp® Viral RNA Mini Kit (Qiagen 52906), and was therefore not further extracted.

Clinical Evaluation

A blinded panel of 60 clinical specimens was obtained from a CLIA high-complexity laboratory that characterized the samples for SARS-CoV-2 by the use of an EUA authorized SARS-CoV-2 assay. The specimens were upper respiratory samples (nasopharyngeal, oropharyngeal, and nasal swab specimens in 1mL of sterile saline) collected from patients by qualified personnel. Thirty of the clinical specimens had SARS-CoV-2 positive test results, and 30 had SARS-CoV-2 negative test results.

The blinded specimen panel was processed through the EZ-SARS-CoV-2 Real-Time RT-PCR workflow and tested on the Applied Biosystems 7500 Fast Real-time PCR System and the T-COR 8™ Real-time PCR Thermocycler. All 30 specimens found to be positive for SARS-CoV-2 by the EUA authorized assay also gave positive results when tested with the EZ-SARS-CoV-2 Real-Time RT-PCR, and all 30 specimens found to be negative for SARS-CoV-2 by the EUA authorized assay also gave negative results when tested with the EZ-SARS-CoV-2 Real-Time RT-PCR on both the ABI 7500 Fast and T-COR 8™ RT-PCR instruments (see Tables 7 and 8). Therefore, the positive percent agreement and the negative percent agreement between the EZ-SARS-CoV-2 Real-Time RT-PCR and the EUA authorized assay were both 100%.

Table 7. Clinical performance of the EZ-SARS-CoV-2 Real-Time RT-PCR on the ABI 7500 Fast.

EZ-SARS-CoV-2 Real-Time RT-PCR	EUA Authorized RT-PCR Assay		Total
	Positive	Negative	
Positive	30	0	30
Negative	0	30	30
Total	30	30	60

Table 8. Clinical performance of the EZ-SARS-CoV-2 Real-Time RT-PCR on the T-COR 8™.

EZ-SARS-CoV-2 Real-Time RT-PCR	EUA Authorized RT-PCR Assay		Total
	Positive	Negative	
Positive	30	0	30
Negative	0	30	30
Total	30	30	60