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

For the qualitative detection of SARS-CoV-2 viral RNA
extracted from mid-turbinate nasal swab specimens

Catalog Number TC-5048-192

For *In Vitro* Diagnostic Use
Rx Only
For Use Under Emergency Use Authorization Only

IVD

Revision: 13 March 2024

Performance Characteristics

Limit of Detection (LoD) - Analytical Sensitivity

The LoD study established the lowest detectable concentration of SARS-CoV-2 (genomic copy equivalents or GCE) at which 95% of all (true positive) replicates test positive with the EZ-SARS-CoV-2 Real-Time RT-PCR. Mid-turbinate nasal swab specimens collected in 1 mL sterile saline obtained from individuals who tested negative for SARS-CoV-2 were pooled and spiked with gamma-irradiated SARS-Related Coronavirus 2, Isolate USA-WA1/2020 (BEI Resources NR-52287). The pooled clinical matrix was screened negative using the EZ-SARS-CoV-2 Real-Time RT-PCR prior to spiking. To estimate the LoD, serial dilutions of SARS-CoV-2 were made in the clinical matrix and tested in three replicates. Each viral dilution was added to three swabs (50 µL per swab head). The swabs were each then eluted in 1 mL sterile saline and 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 with the Inhibition Control added to the lysis buffer. 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 lowest concentration at which all three replicates were positive was treated as the preliminary LoD for each real-time PCR instrument. The preliminary LoD was determined to be 3 GCE per reaction for both real-time PCR instruments (see Tables 1 and 2).

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

Effective Concentration	Replicate	SARS-CoV-2 Ct Value	IC Ct Value	RNase P Ct Value	Detection Rate
300 GCE/reaction	1	28.6	26.8	29.2	100%
	2	28.7	26.6	29.2	
	3	28.4	26.9	29.1	
100 GCE/reaction	1	30.2	26.5	29.2	100%
	2	30.3	27.1	29.1	
	3	30.0	27.0	29.2	
30 GCE/reaction	1	32.0	27.2	29.1	100%
	2	31.9	27.0	29.1	
	3	31.8	27.1	29.5	
10 GCE/reaction	1	33.7	27.2	29.1	100%
	2	34.2	26.9	29.4	
	3	33.8	27.0	29.1	
3 GCE/reaction	1	34.9	26.6	29.2	100%
	2	35.5	27.0	29.3	
	3	36.2	26.8	29.3	
1 GCE/reaction	1	-	27.0	29.2	66.7%
	2	36.7	27.2	29.6	
	3	36.7	27.2	29.1	
0.3 GCE/reaction	1	-	26.7	29.5	0%
	2	-	26.8	29.3	
	3	-	26.9	29.3	

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

Effective Concentration	Replicate	SARS-CoV-2 Ct Value	IC Ct Value	RNase P Ct Value	Detection Rate
300 GCE/reaction	1	30.7	28.3	30.3	100%
	2	31.0	28.3	30.2	
	3	30.7	28.9	30.4	
100 GCE/reaction	1	32.9	28.7	30.5	100%
	2	32.3	28.8	30.5	
	3	32.1	28.7	30.4	
30 GCE/reaction	1	34.1	28.9	30.6	100%
	2	33.7	28.8	30.1	
	3	34.2	28.9	30.9	
10 GCE/reaction	1	35.2	28.6	30.2	100%
	2	35.7	28.8	30.6	
	3	35.8	28.8	30.3	
3 GCE/reaction	1	36.9	28.6	30.5	100%
	2	36.8	28.7	30.7	
	3	36.1	28.8	30.9	
1 GCE/reaction	1	-	28.8	30.8	66.7%
	2	37.5	29.0	30.7	
	3	38.0	29.0	30.8	
0.3 GCE/reaction	1	-	28.5	30.9	0%
	2	-	28.7	30.7	
	3	-	28.8	30.6	

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 3 GCE per reaction on the ABI 7500 Fast and T-COR 8™ real-time PCR instruments (see Tables 3 and 4). The LoD was determined to be 3 GCE per reaction for both real-time PCR instruments.

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

Effective Concentration	Replicate	SARS-CoV-2 Ct Value	IC Ct Value	RNase P Ct Value	Detection Rate
3 GCE/reaction	1	35.0	27.0	29.1	100%
	2	35.0	26.6	29.1	
	3	35.2	26.6	29.4	
	4	35.1	26.2	29.0	
	5	34.7	26.6	29.1	
	6	35.6	26.4	29.0	
	7	35.6	26.7	29.1	

	8	34.8	26.6	29.0	
	9	35.0	26.5	29.0	
	10	34.4	26.7	29.0	
	11	34.9	26.6	29.0	
	12	36.1	26.6	29.1	
	13	34.8	26.4	29.2	
	14	36.2	26.3	29.1	
	15	35.3	26.4	29.0	
	16	36.0	26.5	29.1	
	17	34.7	26.6	29.1	
	18	34.2	26.6	29.0	
	19	36.8	26.7	29.3	
	20	34.9	26.4	29.0	

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

Effective Concentration	Replicate	SARS-CoV-2 Ct Value	IC Ct Value	RNase P Ct Value	Detection Rate
3 GCE/reaction	1	36.5	28.7	30.0	100%
	2	37.0	28.8	30.4	
	3	36.1	28.8	30.8	
	4	36.9	28.6	30.6	
	5	37.1	28.9	30.5	
	6	36.3	28.4	30.5	
	7	36.0	28.8	30.5	
	8	37.5	28.9	30.5	
	9	36.5	28.8	30.4	
	10	37.5	28.7	30.4	
	11	36.0	28.3	30.2	
	12	37.5	28.5	30.5	
	13	35.7	28.3	30.5	
	14	36.7	28.3	30.6	
	15	37.4	28.4	30.5	
	16	36.6	28.7	30.5	
	17	36.2	28.6	30.5	
	18	36.5	28.4	30.3	
	19	36.9	28.7	30.6	
	20	36.4	28.5	30.4	

Inclusivity (*In Silico* Analysis)

Updated *in silico* analyses are performed on a regular basis evaluating the mismatch frequency of the N gene primer and probe sequences using SARS-CoV-2 sequences in the GISAID database (https://www.gisaid.org). As of October 11, 2023, the assessment of homology between these SARS CoV-2 sequences shows that the risk of significant loss of signal amplification and/or false negative results is very low due to the absence of a significant numbers of mismatches. 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. Furthermore, 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.

Cross-reactivity (Analytical Specificity)

***In Silico* Analysis**

BLASTn analysis queries of the EZ-SARS-CoV-2 Real-Time RT-PCR assays 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(s) was updated on 08/15/2021; 4) The search parameters automatically adjust for short input sequences and the expected 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 as well as NCBI BLASTn tools. 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 matches to the genome of SARS coronavirus Urbani with an identity of 96%. However, the forward and reverse primers show reduced homology (forward primer 70% and reverse primer 92% with two key mismatches). These primers and probe have no significant homologies with the human genome, other coronaviruses or human microflora that would predict potential for interference or false positive real-time RT-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 Urbani. The reverse primer and probe sequences showed less significant homology with SARS coronavirus Urbani, as well as the human genome, other coronaviruses or human microflora (the BLASTn expectation value was greater than 1.0). Wet testing was performed with SARS coronavirus Urbani as part of the Cross-reactivity wet testing study and was shown to not cross-react with the EZ-SARS-CoV-2 Real-Time RT-PCR assay (see Table 6).

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 the human genome, other coronaviruses, or human microflora that would predict potential false positive real-time RT-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 Urbani, complete genome	AY278741.1
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 for bacteria/fungi or > 10^4 $TCID_{50}$/mL for viruses, 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 minimum of one 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	Zeptometrix	0810229CF	0/3	0/1
Human coronavirus OC43	Zeptometrix	0810024CF	0/3	0/1
Human coronavirus NL63	BEI Resources	NR-470	0/3	0/1
SARS coronavirus Urbani	BEI Resources	NR-9547	0/3	0/1
MERS coronavirus	BEI Resources	NR-45843*	0/3	0/1
Adenovirus Type 7A	Zeptometrix	0810021CF	0/3	0/1
Adenovirus Type 1	Zeptometrix	0810050CF	0/3	0/1
Adenovirus Type 4	Zeptometrix	0810070CF	0/3	0/1
Human metapneumovirus (hMPV) 16 Type A1	Zeptometrix	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	Zeptometrix	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	0/3
Respiratory syncytial virus B1	BEI Resources	NR-4052	0/3	0/3
Respiratory syncytial virus A1998/12-21	BEI Resources	NR-28528	0/3	0/3
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
Chlamydia pneumoniae	ATCC	53592	0/3	0/1
Haemophilus influenzae	ATCC	33391	0/3	0/1
Legionella pneumophila	Zeptometrix	0801645	0/3	0/1
Mycobacterium tuberculosis	Zeptometrix	0801660	0/3	0/1
Streptococcus pneumoniae	ATCC	49619	0/3	0/1
Streptococcus pyogenes	ATCC	10782	0/3	0/1
Bordetella pertussis	BEI Resources	NR-42460	0/3	0/1
Mycoplasma pneumoniae	Zeptometrix	0801579	0/3	0/1
Pneumocystis jirovecii (PJP)	ATCC	PRA-159	0/3	0/1
Pooled human nasal wash	In-House		0/3	0/1
Candida albicans	ATCC	18804	0/3	0/1
Pseudomonas aeruginosa	ATCC	27853	0/3	0/1
Staphylococcus epidermidis	ATCC	14990	0/3	0/1
Streptococcus salivarius	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

Blinded panels totaling 86 clinical specimens were tested at CLIA high-complexity laboratories which characterized the samples for SARS-CoV-2 by the use of an FDA-Emergency Use Authorized high sensitivity RT-PCR comparator SARS-CoV-2 assay that includes solid phase nucleic acid extraction. The specimens were mid-turbinate nasal swabs in sterile saline collected from patients suspected of COVID-19 by their healthcare provider. Fifty-four of the clinical specimens had SARS-CoV-2 positive test results, and 32 had SARS-CoV-2 negative test results by the comparator assay. Among the 54 positive specimens, >50% were considered low positives by the comparator assay. 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. Fifty-three of the 54 specimens found to be positive for SARS-CoV-2 by the comparator assay also gave positive results when tested with the EZ-SARS-CoV-2 Real-Time RT-PCR on both the ABI 7500 Fast and the T-COR 8™. All 32 specimens found to be negative for SARS-CoV-2 by the comparator assay also gave negative results when tested with the EZ-SARS-CoV-2 Real-Time RT-PCR on both the ABI 7500 Fast and the T-COR 8™. The clinical performance of the EZ-SARS-CoV-2 Real-Time RT-PCR when run on the ABI 7500 Fast and T-COR are shown in Table 7 and Table 8, respectively.

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	Comparator RT-PCR Assay		
	Positive	Negative	Total
Positive	53	0	53
Negative	1	32	33
Total	**54**	**32**	**86**
Positive Percent Agreement (PPA)		98.1% (53/54), 95% CI: (90.2%, 99.7%)	
Negative Percent Agreement (NPA)		100% (32/32), 95% CI: (89.3%, 100%)	

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	Comparator RT-PCR Assay		
	Positive	Negative	Total
Positive	53	0	53
Negative	1	32	33
Total	**54**	**32**	**86**
Positive Percent Agreement (PPA)		98.1% (53/54), 95% CI: (90.2%, 99.7%)	
Negative Percent Agreement (NPA)		100% (32/32), 95% CI: (89.3%, 100%)	

Symbols

Symbol	Meaning	Symbol	Meaning
IVD	*In Vitro* Diagnostic Medical Device		Manufacturer

How to Obtain More Information

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