EZ-SARS-CoV-2 Real-Time RT-PCR
Instructions for Use

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

Catalog Number TC-5048-192

For *In Vitro* Diagnostic Use
Rx Only

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

IVD

Tetracore has filed for Emergency Use Authorization with the US FDA. The test has been validated by Tetracore, but the US FDA's independent review of this validation is pending.

Revision: 12 May 2023
Part number: PLM-0618-2

Table of Contents

Intended Use

EZ-SARS-CoV-2 Real-Time RT-PCR is a real-time RT-PCR test intended for the qualitative detection of RNA from the SARS-CoV-2 in nasal swab specimens from individuals suspected of COVID-19 by their healthcare provider. Testing is limited to laboratories certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. §263a, to perform high complexity tests, or by similarly qualified non-U.S. laboratories.

Results are for the identification of SARS-CoV-2 RNA. The SARS-CoV-2 RNA is generally detectable in upper respiratory samples (e.g., nasopharyngeal, oropharyngeal, and nasal 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 definite 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 epidemiological information.

The EZ-SARS-CoV-2 Real-Time RT-PCR is intended for use by qualified and trained clinical laboratory personnel specifically instructed and trained in the techniques of real-time PCR and in vitro diagnostic procedures. The EZ-SARS-CoV-2 Real-Time RT-PCR is distributed in accordance with the guidance on *Policy for Coronavirus Disease-2019 Tests During the Public Health Emergency*, Section IV.C.2.

Summary and Explanation

An outbreak of pneumonia of unknown etiology in Wuhan City, Hubei Province, China was initially reported to WHO on December 31, 2019. Chinese authorities identified a novel coronavirus (2019-nCoV), which has resulted in thousands of confirmed human infections in multiple provinces throughout China and many countries including the United States. Cases of asymptomatic infection, mild illness, severe illness, and deaths have been reported.

The Tetracore, Inc. EZ-SARS-CoV-2 Real-Time RT-PCR is a molecular *in vitro* test that aids in the detection and diagnosis of SARS-CoV-2 and is based on widely used nucleic acid amplification technology. The product contains oligonucleotide primers and dual-labeled hydrolysis probes (TaqMan®) and control material used in RT-PCR for the *in vitro* qualitative detection of SARS-CoV-2 RNA in upper respiratory specimens.

Principles of the Procedure

The oligonucleotide primers and probes for detection of SARS-CoV-2 were selected from regions of the virus nucleocapsid protein (N) gene. The test is designed for specific detection of SARS-CoV-2 viral RNA. An additional primer/probe set to detect the endogenous human RNase P gene in clinical specimens is also included in the test, and a separate primer/probe set is included to detect a full process Inhibition Control (IC) added during sample extraction. Nucleic acids are isolated and purified from nasal swab specimens using the QIAamp® Viral RNA Mini Kit following the manufacturer's recommended procedure. Using either the Applied Biosystems™ (ABI) 7500 Fast Real-time PCR System or the Tetracore, Inc. T-COR 8™ Real-time PCR Thermocycler, the purified nucleic acid is reverse transcribed into cDNA which is then subsequently amplified. In the process, the probe anneals to a specific target sequence located between the forward and reverse primers.

During the extension phase of the PCR cycle, the 5' nuclease activity of Taq polymerase degrades the probe, causing the reporter dye to separate from the quencher dye, generating a fluorescent signal. With each cycle, additional reporter dye molecules are cleaved from their respective probes, increasing the fluorescence intensity. Fluorescence intensity is monitored at each PCR cycle by either the ABI 7500 Fast or the T-COR 8™.

Workflow Summary

Product Description

EZ-SARS-CoV-2 Real-Time RT-PCR
Catalog Number: TC-5048-192

Materials Provided:

Component	Contents
Mastermix	EZ-SARS-CoV-2 Real-Time RT-PCR Mastermix; 4 vials/kit (48 reactions each)
Enzyme	Enzyme Blend; 2 vials/kit
Inhibition Control	Inhibition Control *In Vitro* Transcript (IVT); 2 vials/kit
Positive Control	SARS-CoV-2 Synthetic RNA; 1 vial/kit

Materials and Equipment Required, but Not Provided:

- QIAamp® Viral RNA Mini Kit (Qiagen Cat. No. 52906)
- Ethanol (96–100%)
- 1.5 mL microcentrifuge tubes (DNase/RNase free)
- Applied Biosystems™ 7500 Fast Real-time PCR System with SDS Software v1.4 or v1.5, or Tetracore, Inc. T-COR 8™ Real-time PCR Thermocycler
- MicroAmp™ Fast Optical 96-Well Reaction Plates (Applied Biosystems Cat. No. 4346907) and MicroAmp™ Optical Adhesive Film (Applied Biosystems Cat. No. 311971 or equivalent) or T-COR8™ reaction tubes (Tetracore, Inc. Cat. No. TC-3006-5003)
- T-COR 8™ tube rack (Tetracore, Inc. Cat. No. TC-3014-002)
- Micropipettes and sterile pipette tips with aerosol barriers
- Molecular biology grade water or 1X Tris-EDTA (pH 7.4)
- Microcentrifuge and centrifuge with a rotor that accommodates standard microplates
- Vortex mixer
- Laboratory freezers (-20°C and -80°C)

Storage and Stability

The EZ-SARS-CoV-2 Real-Time RT-PCR Mastermix and Enzyme should be stored at -20°C (-15°C to -25°C), and are stable until the expiration date stated on the label. Avoid repeated (>2x) thawing and freezing of the Mastermix, and return any residual Mastermix to the freezer immediately after use. Protect the fluorogenic probes in the Mastermix from light. The Inhibition Control (IC) and Positive Control should be stored at -80°C (-60°C to -90°C). To avoid repeated freeze-thaws and subsequent degradation of the IC and Positive Control:

- Once thawed, make small working aliquots (≥ 30 µL per tube). Use 0.5 mL sterile microcentrifuge tubes with O-rings to avoid evaporation. Store the aliquots at -80°C (-60°C to -90°C). Limit each aliquot to no more than two freeze-thaws.
- Or make one-time use aliquots (≥ 30 µL per tube). Store the aliquots at -80°C (-60°C to -90°C).
- For short-term storage, the IC and Positive Control can be stored between 2°C and 8°C for no more than 12 hours.

Warnings and Precautions

- For *in vitro* diagnostic use only.
- The EZ-SARS-CoV-2 Real-Time RT-PCR is distributed in accordance with the guidance on *Policy for Coronavirus Disease-2019 Tests During the Public Health Emergency*, Section IV.C.2.
- The EZ-SARS-CoV-2 Real-Time RT-PCR is intended for use by qualified and trained clinical laboratory personnel specifically instructed and trained in the techniques of real-time PCR and *in vitro* diagnostic procedures.
- Handle all specimens as if infectious using safe laboratory procedures. Refer to *Interim Laboratory Biosafety Guidelines for Handling and Processing Specimens Associated with Coronavirus Disease 2019 (COVID-19)* (https://www.cdc.gov/coronavirus/2019-nCoV/lab-biosafety-guidelines.html).
- Specimen processing should be performed in accordance with national biological safety regulations.
- If infection with SARS-CoV-2 is suspected based on current clinical and epidemiological screening criteria recommended by public health authorities, specimens should be collected with appropriate infection control precautions.
- Perform all manipulations of live virus samples within a Class II (or higher) biological safety cabinet (BSC).
- Use personal protective equipment such as (but not limited to) gloves, eye protection, and lab coats when handling kit reagents while performing this assay and handling materials including samples, reagents, pipettes, and other equipment and reagents.
- Amplification technologies such as PCR are sensitive to accidental introduction of PCR product from previous amplifications reactions. Incorrect results could occur if either the clinical specimen or the real-time reagents used in the amplification step become contaminated by accidental introduction of amplification product (amplicon). Workflow in the laboratory should proceed in a unidirectional manner.
- Maintain separate areas for assay setup and handling of nucleic acids.
- Always check the expiration date prior to use. Do not use expired reagent. Do not substitute or mix reagent from different kit lots or from other manufacturers.
- Change aerosol barrier pipette tips between all manual liquid transfers.
- During preparation of samples, compliance with good laboratory techniques is essential to minimize the risk of cross-contamination between samples, and the inadvertent introduction of nucleases into samples during and after the extraction procedure. Proper aseptic technique should always be used when working with nucleic acids.
- Maintain separate, dedicated equipment (e.g., pipettes, microcentrifuges) and supplies (e.g., microcentrifuge tubes, pipette tips) for assay setup and handling of extracted nucleic acids.

- Wear a clean lab coat and powder-free disposable gloves (not previously worn) when setting up assays.
- Change gloves between samples and whenever contamination is suspected.
- Keep reagent and reaction tubes capped or covered as much as possible.
- During use, EZ-SARS-CoV-2 Real-Time RT-PCR Mastermix and Enzyme should be maintained on a cold block at all times during preparation and use.
- Work surfaces, pipettes, and centrifuges should be cleaned and decontaminated with cleaning products such as 10% bleach, DNAZap™ or RNase AWAY® to minimize risk of nucleic acid contamination. Residual bleach should be removed using 70% ethanol.
- RNA should be maintained on cold block or on ice during preparation and use to ensure stability.
- Dispose of unused kit reagents and human specimens according to local, state, and federal regulations.

Specimen Collection and Storage

Nasal swab specimens should be collected, transported, stored, and processed according to CLSI MM13-A. Specimens should be stored at 2°C to 8°C until tested. If specimens cannot be tested within 72 hours of collection, they should be frozen at -70°C or colder until tested. Only swabs with a synthetic tip, such as nylon or Dacron®, and an aluminum or plastic shaft should be used to collect specimens. Calcium alginate swabs are unacceptable and cotton swabs with wooden shafts are not recommended. Place swabs immediately into sterile tubes containing sterile saline.

Note: Sample collection devices are not provided with the kit.

Nucleic Acid Extraction

RNA should be extracted from nasal swab specimens using the QIAamp® Viral RNA Mini Kit (Qiagen Cat. No. 52906) following the manufacturer's recommended procedure. The use of other extraction protocols or commercial products should be validated by the user.

Test Controls

Inhibition Control:
A full process Inhibition Control is provided with the kit, and is added to the lysis buffer during the extraction process. A positive signal for the Inhibition Control indicates that all processing steps were successful.

Positive Control:
A Positive Control is provided with the kit and must be included with each test run (tested on every RT-PCR plate on the ABI 7500 Fast or tested with each prepared mastermix on the T-COR 8™). The Positive Control is provided at a high concentration, and must be diluted before use with molecular biology grade water or 1X Tris-EDTA (pH 7.4) (not provided). See Step 3c of the Real-Time RT-PCR Test Procedure for Positive Control dilution instructions for the ABI 7500 Fast and for the T-COR 8™. The Positive Control is a non-infectious synthetic RNA template that covers the SARS-CoV-2 target regions of the EZ-SARS-CoV-2 Real-Time RT-PCR.

Negative Control:
A Negative Control must be taken through all steps of the analysis process, including extraction. Molecular biology grade water or 1X Tris-EDTA (pH 7.4) (not provided) is recommended for use as a Negative Control. A Negative Control must be included with each extraction run. The Negative Control from each extraction run must be tested on every RT-PCR plate on the ABI 7500 Fast. For example, if samples from four extraction runs are being combined on one 96-well RT-PCR plate, then the four associated Negative Controls must be run on that RT-PCR plate. When testing samples on the T-COR 8™, the associated Negative Control from the

extraction run(s) must be included for each prepared mastermix (see Step 1 of the Real-Time RT-PCR Test Procedure). For example, if an extraction run consisting of 23 samples and one Negative Control is tested with two prepared mastermixes, that Negative Control must be tested with both prepared mastermixes.

Real-Time RT-PCR Test Procedure

1. Preparing the Mastermix

- Determine the total number of reactions needed (the number of extracted samples to be tested plus the required Positive and Negative Controls). Include additional reactions to account for loss during pipetting.
- Each vial of EZ-SARS-CoV-2 Real-Time RT-PCR Mastermix is guaranteed to contain enough reagents for 48 reactions.
- The IC provided with the kit is a proprietary IVT that is utilized as a full process control added to the lysis buffer as detailed below.

a. Incorporate the IC into the RNA extraction process by adding **6.0 µL** per sample of the IC to the extraction kit's lysis buffer (extraction kit is not provided). The eluate at the end of the extraction process should contain the extracted IC.

b. Use the following table to calculate the volume of Mastermix and Enzyme needed per reaction.

1 Reaction

EZ-SARS-CoV-2 Mastermix	17.25 µL
Enzyme	0.75 µL
Total Mastermix Volume	18.00 µL

c. Remove Mastermix vial(s) from the freezer. Thaw at room temperature. Once thawed, keep the Mastermix cold at all times. Gently vortex and briefly centrifuge the vial(s) before use.

d. Remove Enzyme vial(s) from the freezer and briefly centrifuge (do not vortex). Return Enzyme vial(s) to the freezer immediately after use.

Important: Once the Enzyme is added to the Mastermix, the prepared Mastermix must be kept on ice and used within 2 hours.

2. Aliquot 18.0 µL of the prepared Mastermix into each T-COR 8™ tube or well of a 96-well plate.

3. Prepare the Negative Control, Positive Control, and extracted sample reactions.

Note: Always prepare the Negative Control first before handling either an extracted sample or the Positive Control.

a. Negative Control(s):
Add 7.0 µL of the extracted Negative Control to a T-COR 8™ tube or well of a 96-well plate that contains 18.0 µL of the prepared Mastermix.

b. Extracted Sample(s):
Add 7.0 µL of the extracted sample to a T-COR 8™ tube or well of a 96-well plate that contains 18.0 µL of the prepared Mastermix.

c. Positive Control:
Before use, dilute the provided Positive Control as follows:

i. For the ABI 7500 Fast, combine 7.0 µL of the provided Positive Control with 93.0 µL of

molecular biology grade water or 1X Tris-EDTA (pH 7.4) (not provided).

ii. For the T-COR™, combine 21.0 µL of the provided Positive Control with 79.0 µL of molecular biology grade water or 1X Tris-EDTA (pH 7.4) (not provided).

Add 7.0 µL of the diluted Positive Control to a T-COR 8™ tube or well of a 96-well plate that contains 18.0 µL of the prepared Mastermix.

Note: The diluted Positive Control should be used directly after preparation, and should not be stored for subsequent reuse.

4. **Cap the T-COR 8™ tubes, or seal the 96-well plate with MicroAmp™ Optical Adhesive Film and briefly centrifuge.**

   Note: Ensure that all of the liquid is at the bottom of each tube or well, and that no bubbles are present. Centrifuge the plate again or gently tap the T-COR 8™ tubes if needed.

5. **Load the T-COR 8™ tubes into the T-COR 8™ Real-time PCR Thermocycler or the 96-well plate into the Applied Biosystems™ 7500 Fast Real-time PCR System, and follow the thermal cycling protocol below.**

Cycling Conditions and Instrument Settings

The EZ-SARS-CoV-2 Real-Time RT-PCR assay utilizes different reporter dyes to distinguish between targets (see table below).

Target	Reporter Dye Channel
SARS-CoV-2	FAM
IC	TAMRA/DFO
RNase P	Cy5

ABI 7500 Fast Thermal Cycling Protocol and Settings:

Stage 1: 48ºC for 15 minutes
Stage 2: 95ºC for 2 minutes
Stage 3: 45 cycles (2-step PCR):
Step 1: 95ºC for 10 seconds
Step 2: 60ºC for 40 seconds (data collection step)

- Use 7500 System SDS Software Version 1.4 or 1.5
- Select Run Mode: Standard 7500
- Do NOT select ROX as a Passive Reference dye. The Mastermix does NOT contain ROX
- Assay: Standard Curve (Absolute Quantitation)
- SARS-CoV-2: Select detector for FAM as reporter dye and None as quencher
- IC: Select detector for TAMRA as reporter dye and None as quencher
- RNase P: Select detector for Cy5 as reporter dye and None as quencher
- Sample Volume: 25 µL

T-COR 8™ Thermal Cycling Protocol and Settings:

Repeat x1: Go to temperature 48°C, Hold for 300 seconds
Go to temperature 95°C, Hold for 30 seconds
Repeat x5: Go to temperature 95°C, Hold for 10 seconds
Go to temperature 60°C, Hold for 40 seconds
Optics on

Repeat x40: Go to temperature 95°C, Hold for 5 seconds
Go to temperature 60°C, Hold for 20 seconds
Optics on

- Settings: FAM: 160 DFO: 160 TxR: 160 Cy5: 160
  Active cooling
- Analysis: Per-Well SmartCT™

Analysis Settings

ABI 7500 Fast

1. When the run is complete, select the Amplification Plot tab to view the amplification curves. From the Tools menu, click on Graph Settings. Change Post Run Settings to Linear Y-axis and select Auto Scale.
2. Adjust the baseline manually as necessary for each dye channel.
3. For the FAM dye channel, set the Threshold at 3% of the final normalized fluorescence of the Positive Control amplification curve. For the TAMRA dye channel, set the Threshold at 3% of the final normalized fluorescence of the Negative Control amplification curve.

   Example: In the FAM channel, the final Positive Control fluorescence at cycle 45 is approximately 2.0e+006 and 3% of 2.0e+006 is 6.0e+004. Therefore, set the threshold for the FAM channel at 6.0e+004.
4. View each well individually to verify true amplification.

T-COR 8™

1. The user can monitor temperature and fluorescence curves in real time, as well as select graphs for different dye channels in the [View] menu.
2. When the run is complete, the T-COR 8™ software will automatically display a table or graph of the final results.
3. The T-COR 8™ Smart CT™ algorithm calculates the Ct value based on the shape of the curve instead of a threshold value. Select the [View] menu and then select the [SmartCT™ Values] option to view the Smart CT™ Values table.
4. Select graphs for each dye channel in the [View] menu to verify true amplification for each well.

Interpretation of Test Results

All test controls should be examined prior to interpretation of patient results. If the controls are not valid, the patient results cannot be interpreted. Amplification plots for all test controls and patient specimens should be reviewed to discern true amplification from baseline drift.

EZ-SARS-CoV-2 Real-Time RT-PCR Test Controls:
The expected results for the EZ-SARS-CoV-2 Real-Time RT-PCR test controls are detailed in the tables below. Failure of either the Positive Control or the Negative Control invalidates the RT-PCR run and results should not be reported. If an invalid control result is produced, the RT-PCR run should be repeated. The human RNase P gene target serves as an endogenous internal control, and is used for informational purposes only. Detection of the human RNase P gene target (i.e., a Ct value < 40) in either the Positive Control or the Negative Control does not invalidate the RT-PCR run.

Expected Performance of EZ-SARS-CoV-2 Real-Time RT-PCR Test Controls on the ABI 7500 Fast

Control Name	Expected Ct Values		
	SARS-CoV-2	IC	RNase P
Positive Control	< 40	Not Evaluated	-[†*]
Negative Control	-[†]	≤ 30	-[†*]

[†]-, Ct value ≥ 40
[*]The human RNase P gene target is used for informational purposes only.

Expected Performance of EZ-SARS-CoV-2 Real-Time RT-PCR Test Controls on the T-COR 8™

Control Name	Expected Ct Values		
	SARS-CoV-2	IC	RNase P
Positive Control	< 40	Not Evaluated	-[†*]
Negative Control	-[†]	≤ 34	-[†*]

[†]-, Ct value ≥ 40
[*]The human RNase P gene target is used for informational purposes only.

Examination and Interpretation of Patient Specimen Results:
Assessment of clinical specimen test 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. Interpretation of the results from patient specimens is detailed in the table below. Any patient specimen that produces an invalid result must be retested by re-extracting the original sample and repeating the RT-PCR.

Interpretation of Results from Patient Specimens

Results[*]			
SARS-CoV-2	IC	RNase P[†]	Interpretation
+	+	+	SARS-CoV-2 Detected
+	+	-	SARS-CoV-2 Detected
+	-	+	SARS-CoV-2 Detected
+	-	-	SARS-CoV-2 Detected
-	+	+	SARS-CoV-2 Not Detected
-	+	-	SARS-CoV-2 Not Detected
-	-	+	Invalid
-	-	-	Invalid

[*]+, Ct value < 40; -, Ct value ≥ 40
[†]The human RNase P gene target is used for informational purposes only.

Limitations

- The EZ-SARS-CoV-2 Real-Time RT-PCR is designed for use only with extracted nasal swab specimens. Testing of other sample types may result in inaccurate results.
- Negative results in the EZ-SARS-CoV-2 Real-Time RT-PCR do not preclude SARS-CoV-2 infection, and should not be used as the sole basis for treatment or other patient management decisions.
- A false negative result may occur if a specimen is improperly collected, transported or handled. False

negative results may also occur if amplification inhibitors are present in the specimen or if inadequate numbers of organisms are present in the specimen.

- If the virus mutates in the regions targeted by the RT-PCR assay, SARS-CoV-2 may not be detected or may be detected less predictably.
- 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.
- A false positive result may occur if there is cross-contamination by target organisms, their nucleic acids or amplified product.
- Detection of viral RNA may not indicate the presence of infectious virus or that SARS-CoV-2 is the causative agent for clinical symptoms.
- This test cannot rule out diseases caused by other bacterial or viral pathogens.

Performance Characteristics

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 original *in silico* analysis utilized sequences available early in the pandemic. 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.

An updated *in silico* analysis was performed evaluating the mismatch frequency of the N gene primer and probe sequences using GISAID sequences downloaded on September 21, 2021, again on September 29, 2021, and again on May 10, 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.

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 96% sequence identity with SARS-CoV-1. However, forward and reverse primers showed no sequence homology 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 with human genome, other coronaviruses or human microflora. Combining primers and probes, 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 > 10^5 $TCID_{50}$/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	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	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	0/1
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
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 epidermis	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

A blinded panel of 69 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 and nasal swab specimens in sterile saline) collected from patients by qualified personnel. Thirty four of the clinical specimens had SARS-CoV-2 positive test results, and 35 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 (see Tables 7 and 8). All 34 specimens found to be positive for SARS-CoV-2 by the EUA authorized 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 35 specimens found to be negative for SARS-CoV-2 by the EUA authorized comparator assay also gave negative results when tested with the EZ-SARS-CoV-2 Real-Time RT-PCR on the ABI 7500 Fast. Thirty four of the 35 specimens found to be negative for SARS-CoV-2 by the EUA authorized comparator assay also gave negative results when tested with the EZ-SARS-CoV-2 Real-Time RT-PCR on the T-COR 8™.

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		
	Positive	Negative	Total
Positive	34	0	34
Negative	0	35	35
Total	**34**	**35**	**69**

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		
	Positive	Negative	Total
Positive	34	1	35
Negative	0	34	34
Total	**34**	**35**	**69**

Symbols

Symbol	Meaning	Symbol	Meaning
	Catalog Number		Lot Number
	Expiration Date		Manufacturer
	Temperature Limit		*In Vitro* Diagnostic Medical Device
	Consult Instructions for Use		

Revision History

Revision	Date	Description of Change
00	**18 October 2021**	Initial Release
01	**12 May 2023**	Added ISO symbols and temperature ranges; Updated inclusivity *in silico* analysis

How to Obtain More Information

Tetracore, Inc.
77 Upper Rock Circle
Suite 600
Rockville, MD 20850
United States of America

Phone: 240.268.5400
www.tetracore.com

Tel: 240.268.5400
Fax: 240.268.1107
Email: sales@tetracore.com
www.tetracore.com