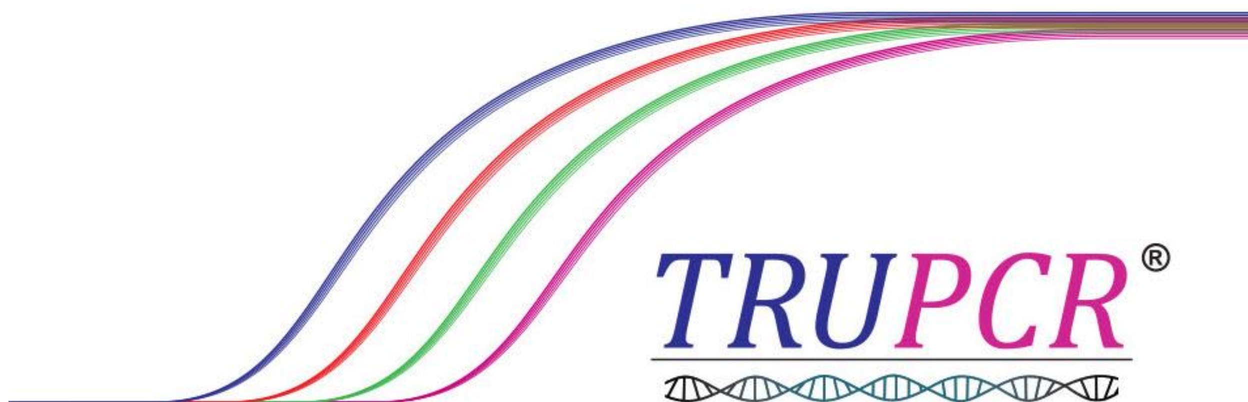


TRUPCR® SARS-CoV-2 RT qPCR KIT

Real-Time PCR based detection of SARS-CoV-2 (COVID-19) virus

Version 2.0

3B BlackBio Biotech India Ltd

7-C Industrial Area, Govindpura Bhopal-462023 (M.P.) India

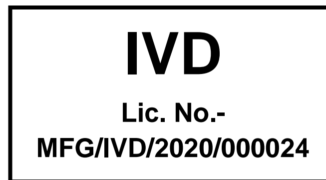
Phone: +91-755-4076518; 4077847 Fax: +91-755-2580438

Website: www.3bblackbio.com E-mail: info@3bblackbio.com

TRUPCR® SARS-CoV-2 RT qPCR KIT

Real-Time PCR based detection of SARS-CoV-2 (COVID-19) virus

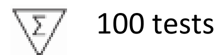
Version 2.0



FOR IN VITRO DIGNOSTIC USE

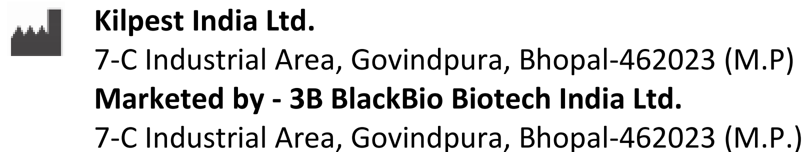
RECOMMENDED BY
Indian Council of Medical Research (ICMR) and
APPROVED BY
National Institute of Virology (NIV), PUNE, India
https://icmr.nic.in/sites/default/files/upload_documents/Guidance_on_PCRKits_COVID19_0204020.pdf

Product No.: 3B304


100 tests

Temperature Limitation

April 2020


Kilpest India Ltd.
7-C Industrial Area, Govindpura, Bhopal-462023 (M.P)
Marketed by - 3B BlackBio Biotech India Ltd.
7-C Industrial Area, Govindpura, Bhopal-462023 (M.P.)

CONTENTS	PAGE NO.

INTENDED USE 3

PRINCIPLE 3

REAGENTS 4

INSTRUCTIONS FOR USE 5

RESULT ANALYSIS 8

SPECIFICATIONS 10

TROUBLESHOOTING REAL TIME PCR 12

STORAGE AND HANDLING 13

MATERIAL AND DEVICES REQUIRED BUT NOT PROVIDED 13

GENERAL PRECAUTIONS 14

REFERENCE 15

INTENDED USE

TRUPCR® SARS-CoV-2 RT qPCR kit is an in vitro nucleic acid amplification test for the qualitative detection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) specific RNA from respiratory specimens (including: **nasopharyngeal or oropharyngeal aspirates or washes, nasopharyngeal or oropharyngeal swabs, bronchoalveolar lavage, tracheal aspirates & sputum**) using Real time PCR.

The human *RNaseP* gene serves as an internal positive control for human nucleic acid, also included in this kit.

The result from TRUPCR® SARS-CoV-2 RT qPCR kit should be interpreted in conjunction with other clinical and laboratory findings.

PRINCIPLE

The TRUPCR® SARS-CoV-2 RT qPCR kit is RT-qPCR assay based on oligonucleotide hydrolysis principle which allows higher specificity and sensitivity. Real-time RT-PCR technology utilizes reverse-transcriptase (RT) reaction to convert RNA into complementary DNA (cDNA), in real-time PCR; the fluorescent signal is generated from the presence of an oligonucleotide probe specific for target DNA sequence. The probe contains a fluorescent dye molecule on its 5’ end and a quencher molecule on its 3’ end. When the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence primarily by Fluorescence resonance energy transfer (FRET). The probe hybridizes with one of the chains of the amplified fragment. During synthesis of a complementary chain, Taq DNA polymerase which possesses 5' - 3' exonuclease activity cleaves the probe. As a result, the fluorescent dye and quencher dye are separated, and the total fluorescence of reaction volume increases in direct proportion to the number of amplicon copies synthesized during PCR. This process occurs in every cycle and does not interfere with the exponential accumulation of product. The fluorescent signal is measured in each cycle of reaction, and the threshold cycle value is determined from the obtained curve. The threshold cycle is proportional to the initial number

of RNA copies in a sample and its value allows qualitative comparisons of analyzed and control samples.

The TRUPCR® SARS-CoV-2 RT qPCR kit composed of 2 tube assays. Tube 1 contains primers and probes specific to E gene (FAM) for the detection of the Sarbecovirus (of Genus B-betacoronavirus (B-βCoV)) and endogenous internal control *RNaseP* (HEX), tube 2 contains primers and probes specific to RdRp gene (FAM) and N gene (FAM) for the detection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) along with endogenous internal control *RNaseP* (HEX) . Internal control gene is included in both the tubes, to verify the extracted RNA quality, amplification procedure and possible presence of inhibitors, which may cause false negative results and this design, makes this kit highly reliable.

REAGENTS

The Kit contains amplification reagents for performance of 100 amplification reactions. Thaw and handle reagents on ice. Do not freeze/thaw Kit vials repeatedly. In case of frequent use, we recommend to aliquot the contents of the vials into 10 reactions each.

Reagent	Description	Volume in 100 reactions pack size
Master Mix	• Hot-start DNA polymerase • Reaction Buffer • dNTPs (dATP, dCTP, dGTP, dTTP) • MgCl2 and stabilizers	1000 µL X 2
Enzyme Mix	• Enzyme Mix for RT	35 µL X 2
Primer Probe Mix-1	• Primer Probe mix for E gene and human *RNaseP* gene detection	233 µL X 2
Primer Probe Mix-2	• Primer Probe mix for RdRp gene and N gene and human *RNaseP* gene detection	233 µL X 2
RNase free water	• Sterilized water	500 µL X 2
Negative Control	• Sterilized water	500 µL X 2
Positive Control	• Positive control	100 µL X 2

INSTRUCTIONS FOR USE

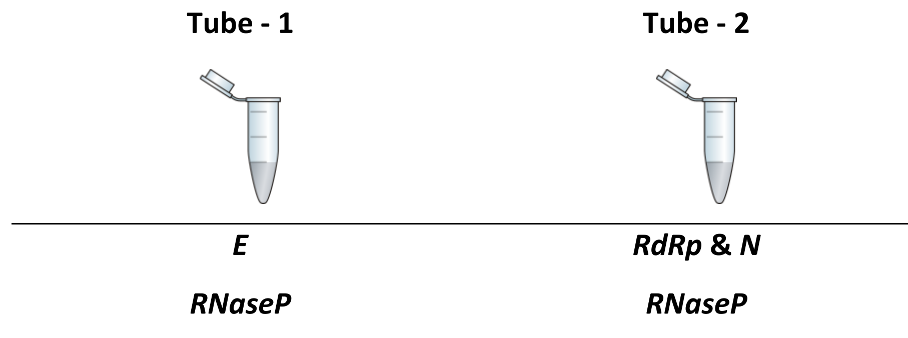
RNA Extraction Step

Performance of RT-PCR amplification-based assays depends on the amount and quality of sample template RNA. RNA extraction procedures should be qualified and validated for recovery and purity before testing specimens. Commercially available extraction procedures including TRUPCR® Viral RNA Extraction Kit, QIAamp® Viral RNA Mini Kit (52904), PureLink™ RNA Mini Kit (Invitrogen), GenElute™ Total RNA Purification Kit, ReliaPrep™ RNA Miniprep System, RNASure® Virus Kit (250) have been shown to generate highly purified RNA when following manufacturer's recommended procedures for sample extraction.

Real Time PCR Protocol

1. **Reaction Preparation for Samples**

   NOTE: Two separate tubes must be run for every single sample



Prepare the PCR Mix as follows:

Name of the Reagent	Tube-1	Tube-2
Master Mix	10 µl	10 µl
Enzyme Mix	0.35 µl	0.35 µl
Primer Probe Mix-1	4.65 µl	-
Primer Probe Mix-2	-	4.65 µl
RNase free water	5 µl	5 µl
Total reaction volume	**20 µl**	**20 µl**

a) Transfer 20 µl of the above prepared Reaction mix in 0.2 ml PCR tubes and close the tubes.

b) For 20 µl of above reaction mix, add 5 µl of RNA samples or positive control or negative control and make up the final volume 25 µl.

2. Program set up

Define the following setting for Temperature Profile and Dye Acquisition

Step	Temperature, °C	Time	Dye Acquisition	Cycles
1	50	15 min	-	1
2	95	05 min	-	1
3	95	05 sec	-	40
4	60	40 sec	Yes	
5	72	15 sec	-	

Passive Reference Dye – ROX

3. Channel Selection

Define the following setting for channel selection

Detection	Detector Name	Reporter	Quencher	Gain Setup
Tube 1	*E gene*	FAM	None	Auto
	RNaseP	HEX	None	Auto
Tube 2	*RdRp*	FAM	None	Auto
	N	FAM	None	Auto
	RNaseP	HEX	None	Auto

Threshold Value for some popular cyclers

For the TRUPCR® SARS-CoV-2 RT qPCR kit**,** threshold values are different in different real time PCR instruments as below:

S. No.	Real Time Instrumental	Threshold value range*	
		FAM	HEX
1	Applied Biosystems® with ROX	0.35 - 0.65	0.2 - 0.4
2	Applied Biosystems without ROX	20000-40000	5000-20000
3	Rotor-Gene Q	0.03 -0.06	0.03 -0.06

*Range of threshold: An absolute value of threshold varies in between the range mentioned in the above table. Absolute value varies from instrument to instrument depending upon instrument's age, model and calibration. Please get in touch with our tech-support team for any query.

RESULT ANALYSIS

1. The negative control reactions for probe/primer sets should not exhibit fluorescence growth curves that cross the threshold line. If a false positive occurs with one or more of the primers and probe non template control (NTC) reactions, sample contamination may have occurred.

2. The positive control reactions for each probe/primer reactions should give following Ct values:

Serial Number	Positive Control	Expected Ct Values
1	Tube 1	22±5
2	Tube 2	22±5

3. All clinical samples should exhibit *RNaseP* reaction curves that cross the threshold line at or before 35 cycles, thus indicating the presence of sufficient RNA from human *RNaseP* gene indicating the specimen is of acceptable quality. However, it is possible that some samples may fail to give positive reactions due to low cell numbers in the original clinical sample. Also, samples taken from animal/avian species or cell culture typically exhibit either no *RNaseP* reaction, or a weak *RNaseP* reaction. Failure to detect *RNaseP* in any of the clinical samples may indicate:

(a) Improper extraction of nucleic acid from clinical materials resulting in loss of RNA or carry-over of RT-PCR inhibitors from clinical specimens

(b) Absence of enough human cellular material in sample to enable detection

(c) Improper assay set up and execution

(d) Reagent or equipment malfunction

4. Cutoff- This assay runs for 40 cycles however for any interpretation, threshold cutoff cycle Ct is 35. (In cases where Ct values are in between 34 and 35 Ct, sample shall be repeated with double the input volume of RNA i.e instead of 5 µl input RNA, use 10 µl of input RNA by avoiding adding RNase-free water. If upon repetition, Ct value appears before Ct 35, the sample shall be considered presumptive positive.)

5. When all controls meet stated requirements, a specimen is considered with following interpretations

Case	Amplification Signals in *RNaseP* (HEX)	Amplification Signals		Results	Interpretation
		Tube 1 (FAM)	Tube 2 (FAM)		
1	+/-	+	+	SARS-CoV-2 Positive	Sarbecovirus (E gene) and SARS-CoV-2 (N or/and RdRp) specific RNA detected
2	+/-	-	+	SARS-CoV-2 Presumptive Positive	Repeat test with more RNA from the sample, up to 10 µL, instead of adding RNase-free water. If positive again, Test sample is Presumptive Positive for SARS-CoV-2
3	+/-	+	-	Sarbecovirus Positive	Repeat test with more nucleic acids from the sample, up to 10 µL, instead of adding RNase-free water. If positive again, Test sample is Presumptive Positive for Sarbecovirus. For samples with the same result on a repeated test, additional confirmatory testing may be conducted, if it is necessary to differentiate between SARS-CoV-2 and SARS-CoV-1 or other Sarbecovirus currently unknown to infect humans, for epidemiological purposes or clinical management.
4	+	-	-	Negative	Test Sample is negative for SARS-CoV-2.
5	-	-	-	Invalid	Results are invalid. Repeat test. If the result is still invalid, a new specimen should be obtained.

NOTE:

1. If needed, kindly manually inspect amplification curves for all the samples. Assign a Ct value to verify the positive amplification.

2. Detection of the Internal Control in the HEX detection channel is not required for positive results in the FAM™ detection channel. A high Sarbecovirus (target E gene) and/or SARS-CoV-2 (target N/RdRp gene) RNA load in the sample can lead to reduced or absent Internal Control signals.

3. In cases where Ct values are in between 34 and 35 Ct, sample shall be repeated with double the input volume of RNA i.e instead of 5 µl input RNA, use 10 µl of input RNA by avoiding adding RNase-free water. If upon repetition, Ct value appears before Ct 35, the sample shall be considered presumptive positive.

SPECIFICATIONS

A. Sensitivity:

The **TRUPCR® SARS-CoV-2 RT qPCR Kit** demonstrated the ability to reproducibly detect the presence of **5.2 RNA copies/reaction** (https://www.who.int/docs/default-source/coronaviruse/protocol-v2-1.pdf? sfvrsn=a9ef618c_2).

B. Specificity

The main advantages of TRUPCR® SARS-CoV-2 RT qPCR kit are its higher sensitivity and specificity compared with other diagnostic methods, as well as its rapidity and possibility of automation. Specificity of this kit was assured by selection of WHO recommended primers and probes as well as the selection of stringent reaction conditions (https://www.who.int/docs/default-source/coronaviruse/protocol-v2-.pdf?sfvrsn=a9ef618c_2).

C. Cross-Reactivity Studies with Clinical Specimens

The specificity of the assay was evaluated by testing on samples that were positive for at least one of the following virus markers listed in below table. All tests returned negative results.

Organism	Number of samples tested	Result
HCoV-HKU1	2	Negative
HCoV-OC43	2	Negative
HCoV-NL63	2	Negative
HCoV-229E	2	Negative
MERS-CoV	2	Negative
Influenza A (H1N1/09)	10	Negative
Influenza A (H3N2)	6	Negative
Influenza B	5	Negative
Rhinovirus	4	Negative
Respiratory Syncytial Virus	4	Negative
Parainfluenza 1 virus	6	Negative
Parainfluenza 2 virus	5	Negative
Parainfluenza 3 virus	2	Negative
Parainfluenza 4 virus	2	Negative
Metapneumovirus	2	Negative
Enterovirus	2	Negative
Adenovirus	2	Negative
Legionella spp.	2	Negative
Mycoplasma spp.	10	Negative
Rhinovirus	2	Negative
S. pneumoniae	10	Negative
S. aureus	10	Negative
S. agalactiae	10	Negative
K. pneumoniae	10	Negative
E. coli	10	Negative
E. cloacae	10	Negative
H. influenzae	10	Negative
K. aerogenes	10	Negative
P. aeruginosa	10	Negative

D. Reproducibility

Precision data of the TRUPCR® SARS-CoV-2 RT qPCR kit was determined as intra-assay variability and inter-assay variability. Variability data are expressed in terms of standard

deviation and coefficient of variation. The study was performed on positive controls of *E, N, RdRp* and *RNaseP* gene. For intra-assay variability assay 20 replicates per control were analyzed. Inter-assay experiment was performed on 4 alternate days with 5 replicates per control per run were analyzed. An estimate of assay precision based on the standard deviation of the daily means and corresponding coefficients of variation all controls are shown in Table below:

Inter Run data: -

Gene	RUN 1 Avg. Ct	RUN 2 Avg. Ct	RUN 3 Avg. Ct	RUN 4 Avg. Ct	Avg. Ct	SD	CV%
N	22.29	22.76	22.74	22.48	22.5675	0.195	0.862
E	19.77	19.22	19.62	19.52	19.5325	0.201	1.030
RdRp	22.29	22.06	22.18	22.63	22.29	0.212	0.953
RNaseP	21.46	21.52	21.79	21.93	21.675	0.193	0.889

Intra run data: -

Gene	Avg. Ct	SD	CV%
N	22.81	0.19	0.833
E	19.35	0.136	0.703
RdRp	22.47	0.164	0.730
RNaseP	21.22	0.122	0.575

TROUBLESHOOTING REAL TIME PCR

No.	Observation	Probable causes	Comments
1	Amplification signal in negative control	Cross contamination during handling	Check for contamination of kit's component
2	No amplification signal with positive controls	Incorrect PCR mixture	Check whether all components are added.
		Missing control sample during RNA mixing	Be careful when pipetting
		Changing RNA during RNA mixing	Write down sample number on the 1.5 ml micro centrifuge tube and the PCR tube

		Leaving reagents at room temperature for a long time or incorrect storage condition	Please check the storage condition and the expiration date (see the kit label) of the reagents and use a new kit, if necessary
		The PCR conditions do not comply with the protocol	Repeat the PCR with corrected settings
3	Weak or no signal of the *RNaseP* gene (Internal control)	Reagent has been thawed and frozen too often or exposed to inappropriate storage conditions	Please mind the storage conditions given in manual
		The PCR was inhibited	RNA of Poor quality may interfere with the PCR reaction, use a recommended isolation method

STORAGE AND HANDLING

All the components of TRUPCR® SARS-CoV-2 RT qPCR kit should be stored at -20˚C and stable until the date of expiry stated. The reagents can be aliquoted and stored at -20˚C in-order to maintain the stability and sensitivity.

MATERIAL AND DEVICES REQUIRED BUT NOT PROVIDED

- Adjustable pipettes with sterile filter or positive displacement tips
- Disposable powder-free gloves
- Sterile bidistilled water
- Sterile 1.5 ml and 2 ml microcentrifuge tubes
- 50 ml conical tubes
- Vortex mixer
- Real time PCR
- Laminar airflow cabinet
- PCR vials (0.2 ml, thin-walled)
- 96 – 100% ethanol

- Personal protection equipment (lab coat, gloves, goggles)
- RNA isolation kit

GENERAL PRECAUTIONS

The user should always pay attention to the following:

- After the thawing of reagents make sure to keep back within 15-20 minutes or as soon as possible to -20 freezers.
- Use sterile pipette tips with aerosol barriers and use new tip for every procedure.
- Thaw all components thoroughly at room temperature before starting detection.
- When thawed, mix the components and centrifuge briefly.
- Use disposable gloves, laboratory coats, and protect eyes while samples and reagents handling. Thoroughly wash hands afterwards.
- Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas.
- Do not use a kit after its expiration date.
- Dispose of all samples and unused reagents in compliance with local authorities' requirements.
- Samples should be considered potentially infectious and handled in a biological cabinet in accordance with appropriate biosafety practices. Infected material and disposable plasticware that was in contact with infected material must be treated with chlorine-containing solutions.
- Clean and disinfect all sample or reagent spills using a disinfectant, such as 0.5 % sodium hypochlorite or other suitable disinfectant.
- Avoid contact with the skin, eyes and mucosa. If skin, eyes and mucosa contact, immediately flush with water, seek medical attention.
- Material Safety Data Sheets (MSDS) are available on request.
- Use of this product should be limited to personnel trained in the techniques of DNA amplification.

- The laboratory process must be uni-directional; it should begin in the Extraction Area and then move to the Amplification and Detection Areas. Do not return samples, equipment and reagents to the area in which the previous step was performed.

REFERENCES

1. Diagnostic detection of Wuhan coronavirus 2019 by real-time RT-PCR – **Charité, Berlin Germany (17 January 2020)** https://www.who.int/docs/default-source/coronaviruse/protocol-v2-1.pdf?sfvrsn=a9ef618c_2
2. Detection of 2019 novel coronavirus (2019-nCoV) in suspected human cases by RT-PCR – **Hong Kong University (23 January 2020)** https://www.who.int/docs/default-source/coronaviruse/peiris-protocol-16-10.pdf?sfvrsn=af1aac73_4
3. Corman VM, Eckerle I, Bleicker T, Zaki A, Landt O, Eschbach-Bludau M, et al. Detection of a novel human coronavirus by real-time reverse-transcription polymerase chain reaction. Euro Surveill. 2012;17(39).
4. Drosten C, Gunther S, Preiser W, van der Werf S, Brodt HR, Becker S, et al. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. N Engl J Med. 2003;348(20):1967-76.
5. Drexler JF, Gloza-Rausch F, Glende J, Corman VM, Muth D, Goettsche M, et al. Genomic characterization of severe acute respiratory syndrome-related coronavirus in European bats and classification of coronaviruses based on partial RNA-dependent RNA polymerase gene sequences. J Virol. 2010;84(21):11336-49.
6. Muth D, Corman VM, Roth H, Binger T, Dijkman R, Gottula LT, et al. Attenuation of replication by a 29-nucleotide deletion in SARS-coronavirus acquired during the early stages of human-to-human transmission. Sci Rep. 2018;8(1):15177.