Instructions for use

SARS-CoV-2 PCR Reagent Kit

REF **R2K-288-30**

Research Use Only

Gesellschaft zur Forschung, Entwicklung und Distribution von Diagnostika im Blutspendewesen mbH
Altenhöferallee 3, D-60438 Frankfurt/Main, Germany
Tel: +49 (0) 69 / 400 5513 - 0
Fax: +49 (0) 69 / 400 5513 - 21

Table of contents

1. Notes on the use of the product

The GFE SARS-CoV-2 PCR Reagent Kit can be applied for **research use only** (RUO) purposes. It is neither validated nor approved for use in diagnostic procedures.

2. Intended use

The SARS-CoV-2 PCR Reagent Kit is designed to amplify low copy numbers of templates containing nucleic acid sequences typically found in SARS-CoV-2. Along with all components required for amplification, the reagents include fluorescence probes to measure the formation of PCR products with a suitable real-time PCR instrument.

3. Summary of the procedure

The SARS-CoV-2 PCR Reagent Kit can be used with real-time PCR systems, *e.g.* Roche LightCycler® 480 II or Applied Biosystems QuantStudio 5™, and resembles a dual fluorescent channel multiplex real-time RT-PCR. The detection of the SARS-CoV-2 RNA is covered by simultaneous amplification of two viral targets in the FAM channel. A second amplification system indicates the presence of human genomic DNA in the Cy5 channel. This serves as a control for the sampling process and the PCR reagents in each reaction.

For easier reading, the procedure of amplifying and detecting SARS-CoV-2 sequences is called 'test' in the remainder of the text.

4. Test principle

The SARS-CoV-2 test is based on multiplex PCR. Three amplifications are carried out simultaneously in the presence of SARS-CoV-2 RNA and human genomic DNA.

The detection of SARS-CoV-2 RNA is covered by two amplicons, one specific for Orf1ab and the other specific for the N gene. The presence of human genomic DNA is indicated by amplification of the human RNase P, confirming that sampling and extraction were successful.

Amplification is carried out as real-time RT-PCR comprising in a first step a reverse transcription, which generates complementary DNA (cDNA) copies from virus RNA by a reverse transcriptase (RT) reaction, and in a second step a DNA amplification by the use of the polymerase chain reaction (PCR) for the amplification of specific target sequences.

The target sequences of the amplified DNA are detected by target sequence specific probes. The probes are labelled with fluorescent reporter and quencher dyes. The SARS-CoV-2 specific probes are labelled with FAM and the human RNase P probe is labelled with Cy5 on the 5-prime ends. Each probe has a corresponding quencher on its 3-prime end. The change of the fluorescent signal is mediated by the Taq-Polymerase, which degrades hybridized probe molecules by its 5'-3' exonucleolytic activity. Through probe degradation, the quencher mediated FRET (Förster resonance energy transfer) is eliminated, leading to the increased emitted fluorescence of the reporting fluorophores (FAM and Cy5).

This process occurs as a single tube assay without the need of setting up an additional reaction.

The reaction mix also contains a heat labile Uracil-DNA-Glycosylase (UNG) and dNTPs with a small proportion of dUTP. This leads to the incorporation of a certain amount of Uracil instead of Thymidine into amplified sequences. This suppresses carryover of PCR amplicons to subsequent reactions. The UNG cuts out Uracil from double stranded DNA leading to an AP site (apyrimidinic). DNA with AP sites is chemically degraded by strand breaks while heating up to 95°C at the beginning of the PCR cycling steps. As consequence a contamination with amplicons is diminished.

5. Information about the virus SARS-CoV-2

In December 2019 a novel corona virus was discovered in Wuhan in China where pneumonia of unknown cause in a huge number of cases was reported. After identifying that a corona virus spreading from person to person was the causative agent, the disease was officially named COVID-19 („Corona Virus disease 2019“). As the new coronavirus is closely related to the Severe acute respiratory syndrome coronavirus (SARS-CoV) it was named SARS-CoV-2. Since December 2019 the disease led to a worldwide pandemic [1]. Due to the highly transmissible characteristics of SARS-CoV-2, testing is a mandatory task for all countries [2][3].

The SARS-CoV-2 belongs to the family of corona viruses. The virus contains a single segment of positive single stranded RNA (29’000 – 30’000 bases). Like other human pathogenic corona viruses (Middle East respiratory syndrome Coronavirus – MERS-CoV, Coronavirus 229E, NL63, OC43, HKU1) SARS-CoV-2 is related to severe respiratory diseases. Phylogenetically, SARS-CoV-2 is assigned to the subgenus Sarbecovirus (beta-CoV lineage B) [4]. The mutation frequency is relatively low and genotypes are not assigned [5][6].

The design of the SARS-CoV-2 PCR Reagent Kit was examined by multiple sequence alignments of all available sequences until March 2020 and the primer/probe design was reviewed to provide full coverage of sequences.

6. Reagents provided

Component	Vial	Volume	Reactions/ test	Tests/kit
enzyme mix (EM v1)	5 mL Screw cap tube Cap colour: White	4’040 µL	288	30
oligo mix SARS-CoV-2 (O_CoV2 v1)	5 mL Screw cap tube Cap colour: Blue	610 µL	288	30

Table 1: Kit Components

The overall number of reactions per kit is 8’640 (288 × 30).

7. Storage and handling of reagents

The kit is shipped on dry ice.

Immediately upon arrival the kit should be stored in a freezer at -18°C or below. Storage at other temperatures may influence the performance of the reagents and is not recommended. The individual reagents can be subjected to 3 freeze/thaw cycles. It is recommended to mark the tubes which are already in use.

Do not use reagents past their expiration date.

Thaw the reagents for at least 40 min at ambient temperature without exposure to light before use.

After setting up the master mix the tube can be refrigerated at +2°C to +8°C and kept for 3 hours.

The oligo mix is light sensitive – make sure that the vials are protected against light when thawing or during storage.

8. Required equipment

The execution of the SARS-CoV-2 test requires the following laboratory equipment and disposables:

- Pipettes (0.5 - 20 µL)
- Pipettes (20 - 200 µL)
- Pipettes (200 - 1000 µL)
- Pipettes (500 - 5000 µL)
- Pipette tips with filters as specified by the pipette manufacturer
- Multi-dispense/repetitive pipette (optional/recommended)
- Powder-free disposable gloves
- Vortex Mixer
- Centrifuge with rotor for PCR vessels or plates
- Real-time PCR system, *e.g.* LightCycler® 480 II or QuantStudio 5™
- Consumables for real-time PCR as specified by the real-time PCR system manufacturer

Note: when using real-time PCR systems with PCR plates it is recommended to seal the PCR plates with suitable transparent sealing films and a compatible heat-sealing device. Please check compatibility with the real-time PCR system prior to use to avoid damaging the instrument.

9. Safety instructions

- The test should be carried out by trained laboratory personnel only.
- Follow the general laboratory safety instructions.
- Personal safety equipment (gloves, lab coat, safety glasses) should be worn when working in the laboratory.
- Handle clinical samples as potentially infectious material and take appropriate precautionary measures.
- Take caution regarding cross contamination. Consider a spatial separation of sample extraction processes with PCR setup and the location of the PCR devices.
- The instructions for use of this test described in this handbook must be followed strictly.
- Notice the expiration date of the reagent kit. If expired, discard the reagent kit.
- Ensure that all instruments are maintained and calibrated according to the manufacturers' instructions.
- Do not mix reagents from different kit lots.
- After running the tests, the materials must be disposed of in an appropriate manner. Take cross contamination risks into account when disposing of the consumables.
- Further precautionary, safety and disposal instructions can be taken from the safety data sheets (SDSs). This product does not contain hazardous substances. Safety data sheets are provided on request.

10. Test procedure

The test procedure comprises five stages:

1. Nucleic acids extraction of sample material (not provided with this kit)
2. Preparation of the PCR master mix
3. PCR setup
4. Setup and start of the instrument
5. Data analysis

10.1. Sample extraction and storage

The SARS-CoV-2 test requires extracted virus RNA and human genomic DNA. The nucleic acid quality has a strong impact on the assay performance. The following extraction systems are recommended:

- QIAamp® Viral RNA Mini Kit (QIAGEN)
- QIAamp® MinElute® Virus Spin Kit (QIAGEN)

When using alternative nucleic acid extraction systems and kits, please verify that these are suitable for your purpose and compatible with the SARS-CoV-2 PCR Reagent Kit.

So far the SARS-CoV-2 test compatibility was tested for the following sample types:

- Nasopharyngeal swabs
- Oropharyngeal swabs

Please verify the compatibility of the specific make or brand of swab to be used in combination with the SARS-CoV-2 PCR Reagent Kit, especially when buffer solutions or other liquids are used as transport media.

Other sample types like Sputum or Bronchoalveolar lavage (BAL) resemble difficult materials, which require specialized nucleic acid extraction systems and must be established by the user.

Sample storage must be done according to the recommendations of the sampling system manufacturer.

10.2. Preparation of the PCR master mix

In each SARS-CoV-2 test reaction 15 µL of master mix are used. The master mix is made up of two components:

Component	Volume/rxn
oligo mix SARS-CoV-2 (O_CoV2 v1)	2 µL
enzyme mix (EM v1)	13 µL
Total volume:	15 µL

Table 2: Master mix components and volumes

If multiple reactions are needed, it is recommended to prepare a bulk master mix with enough volume to cover all necessary reactions. In this case, some excess volume has to be considered to compensate for pipetting errors. The exact amount depends on the total number of samples (including controls, see below), methods and equipment in use and needs to be determined by the user.

15 µL of the bulk master mix are dispensed into each reaction vessel or into each well of a PCR plate. Ideally, multi-dispense/repetitive pipettes are employed (for ease of use).

Good PCR practice recommends integrating controls into a PCR run. Therefore at least one positive control (RNA based) and one negative control (NTC, e.g. the buffer used for elution in the nucleic acids extraction kit) should be planned in order to interpret the results.

If PCR positive controls or PCR negative controls are used to evaluate the results (see also section 10.5), the number of controls needs to be added to the number of samples, when calculating the required volume of the bulk master mix.

The easiest way to set up the master mix is to pipette 3'965 µL of enzyme mix (EM) into an unused tube of oligo mix O_CoV2 (OM) containing 610 µL. This results in a master mix volume containing 305 reactions, which is sufficient for 288 samples.

Before you start setting up the master mix:

- Calculate the volume of required master mix and thaw the corresponding number of tubes.
- Thaw the amount of required reagents at least 40 min at ambient temperature and protect them from exposure to light prior to use.
- Separately resuspend the oligo mix and the enzyme mix by gently pipetting the solutions up and down for at least 5 times with a suitable pipette before setting up of the master mix.

After setting up the master mix

- Resuspend the solution by gently pipetting up and down for at least 5 times with a suitable pipette before pipetting the working master mix to the reaction vessels or PCR plates.
- Please note the master mix can be stored at +2°C to +8°C for 3 hours.
- The opened oligo mix and enzyme mix tubes can be stored in a freezer at -18°C or below. It is recommended not to apply more than 3 freeze/thaw cycles, because the reagent performance may decrease. It is recommended to mark the tubes which are in use.

10.3. Reaction setup

Each SARS-CoV-2 test reaction has a final volume of 25 µL. This reaction volume is assembled by combining 15 µL of the master mix with 10 µL of the template (*e.g.* eluates from extractions, positive controls or NTCs).

Component	Volume
Master mix	15 µL
Template	10 µL
Total volume	25 µL

Table 3: Reaction composition

For reaction setup, the following steps have to be carried out:

- Add 10 µL template (eluate, positive controls or NTCs) to the appropriate reaction vessel or well of the PCR plate, which already contains 15 µL of master mix.
- Close or seal the reaction vessels/plate, *e.g.* with heat-sealing film using a heat-sealing device
- Apply a short spin to the reaction vessel or PCR plate with a centrifuge

Note: The reaction vessels or PCR plates can be stored at +2 to +8°C for 90 min prior to starting the thermo cycler.

10.4. Real-time PCR instruments

The GFE SARS-CoV-2 PCR Reagent Kit was tested with the following real-time PCR instruments:

- QuantStudio 5™, Applied Biosystems
- LightCycler® 480 II, Roche

10.4.1. LightCycler® 480 II

Recommended cycling parameters:

Program Name	Cycles	Analysis Mode	Temperature Targets (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/sec)
Reverse Transcription	1	None	55	None	00:15:00	4.4
Activation of Taq-Polymerase	1	None	95	None	00:02:00	4.4
Pre-cycles	5	None	95	None	00:00:10	2.2
			60	None	00:00:30	2.2
Main-cycles	45	Quantification	95	None	00:00:05	4.4
			60	Single	00:00:20	2.2

Table 4: Cycling profile for LightCycler® 480 II with Xenon halogen or LED lamp unit

Note: Set the reaction volume to 50 µL, even though the PCR reaction has a final volume of 25 µL!

Recommended detection format:

Excitation Filter [nm]	Emission Filter [nm]	Name	Melt Factor	Quant Factor	Max Integration Time [sec]
465	510	FAM (465-510)	1	1	2
618	660	Cy5 (618-660)	1	1	2

Table 5: LightCycler® 480 II detection format

Recommended data analysis:

Define a subset for the analysis of the SARS-CoV-2 samples according to the plate layout as described in the LightCycler® 480 Instrument Operator's Manual. In the sample editor, specify the sample numbers and their positions as well as the positions of the controls (*e.g.* "NTC" for PCR negative control, "Pos" for PCR positive control).

Choose “Abs Quant/Fit Points” as analysis method and create an analysis for the parameter SARS-CoV-2. Use the information provided in the LightCycler® 480 Instrument Operator's Manual to determine suitable values for Background and Noiseband (STD Multiplier) and execute the analysis.

10.4.2. QuantStudio 5™

Recommended cycling parameters:

Program Name	Cycles	Temperature Targets	Data collection	Hold (mm:ss)	Ramp Rate
Hold Stage	1	55.0°C 95.0°C	Off Off	15:00 02:00	2.74°C/s 2.74°C/s
PCR Stage	5	95.0°C 60.0°C	Off Off	00:03 00:20	2.7°C/s 2.1°C/s
PCR Stage	45	95.0°C 60.0°C	Off On	00:03 00:20	2.7°C/s 2.1°C/s

Table 6: Cycling profile for QuantStudio 5™

Note: Set the cover temperature to 105.0°C and the reaction volume to 50 µL, even though the PCR reaction has a final volume of 25 µL!

Recommended optical filter settings for PCR Filter

	m1(520±15)	m2(558±11)	m3(586±10)	m4(623±14)	m5(682±14)	m6(711±12)
x1(470±15)	☑	☐	☐	☐	☐	☐
x2(520±10)		☐	☐	☐	☐	☐
x3(550±11)			☐	☐	☐	☐
x4(580±10)				☐	☐	☐
x5(640±10)					☑	☐
x6(662±10)						☐

Table 7: QuantStudio 5™ filter settings

Use the information provided in the QuantStudio™ Design and Analysis Software USER GUIDE to determine suitable values for Baseline and Threshold and execute the analysis.

10.5. Result interpretation

Result interpretation is dependent on several factors and needs to be aligned with overall work-flows and the particular use case. Therefore, the following section is meant to be understood as a guideline on establishing a result interpretation algorithm when processing samples of unknown properties regarding the presence of SARS-CoV-2 RNA.

As mentioned in section 10.2 PCR positive controls and PCR negative controls are often employed to establish a framework for result interpretation. In this case, the PCR controls can be used to assess nucleic acid amplification results for SARS-CoV-2. To assess the results of each individual reaction, the SARS-CoV-2 PCR Reagent Kit also includes an amplification system for human RNase P, which works independently of the SARS-CoV-2 amplification reaction.

When using suitable controls, the validity of the PCR conditions may be evaluated according to the following criteria after each run:

Criterion	Comment
PCR negative control/NTC is negative for SARS-CoV-2 and RNase P	PCR negative controls can be an aid in recognising false reactive results. *Recommended evaluation:* If the negative control is positive for SARS-CoV-2 or RNase P, all SARS-CoV-2 results in the same analysis run should be considered invalid.✋
PCR positive control is positive	PCR positive controls can be used to demonstrate that the reagents in use are capable of performing reverse transcription and PCR amplification. When using PCR positive controls with defined RNA concentrations for SARS-CoV-2 sequences the range of the Ct value can also indicate performance issues. *Recommended evaluation:* If the PCR positive control is negative, all SARS-CoV-2 results in the same analysis run should be considered invalid.✋
RNase P is positive	This result indicates that the PCR amplification reaction was successful in the respective reaction vessel/well of the PCR plate. Positive results for RNase P also indicate that sampling has been carried out with sufficient thoroughness. An unexpectedly high Ct value for RNase P may indicate PCR inhibition or insufficient sampling. *Recommended evaluation:* If the RNase P is negative, the SARS-CoV-2 result of the respective sample should be considered invalid.✋

Table 8: Validity criteria

✋ **Repeat testing using original sample or collect and test a new sample.**

When the validity criteria in table 8 are met, the PCR results may be analyzed according to the following rules:

SARS-CoV-2 Result	RNase P Result	Interpretation
Negative	Positive	SARS-CoV-2 was not detected
Positive	Positive	SARS-CoV-2 was detected

Table 9: Result interpretation

11. Performance characteristics

The performance of the SARS-CoV-2 PCR Reagent Kit was evaluated by testing the assay in different test series and setups. The following sub-chapters summarize the results.

11.1. Analytical sensitivity

The limit of detection (p = 0.95) was determined by testing eluates of serial dilutions of a Zeptometrix Natrol Standard (SARS-Related Coronavirus 2 (SARS-CoV-2) cat no: 0810587CFHI-0.5ml) spiked in Nasopharyngeal Swab material. Extraction was performed by using the QIAamp® MinElute® Virus Spin Kit (QIAGEN) and the QIAcube instrument (QIAGEN). The viral RNA concentrations of the eluates were quantified prior to dilution by using quantified *in-vitro* transcripts of gBlocks (Integrated DNA Technologies) coding for the PCR target regions of both virus genes (orf1ab and N-gene). The serial dilutions ranged from 3 cp/rxn to 30 cp/rxn and the hit-rate was determined with replicate numbers of N > 24. The LoD95 was calculated by Probit Regression Analysis (log_{10}) using IBM SPSS Statistics Version 19.0.0 software.

Cycler	QuantStudio 5	LightCycler 480 II (Xenon light source)
LoD95	19 cp/rxn	13 cp/rxn
Confidence Interval	14 - 31	11 – 20
LoD95 confirmation by hit-rate	95.7% at 19 cp/rxn	94.7% at 13 cp/rxn

Table 10: Analytical sensitivity

11.2. Analytical specificity

The analytical specificity was measured by testing extracted nucleic acids from SARS-CoV-2 negative donors of nasopharyngeal and oropharyngeal samples and by reactions using elution buffer, only. Based on N = 950 samples an analytical specificity of 99.4% was determined.

11.3. Cross reactivity

The GFE SARS-CoV-2 PCR Reagent Kit was tested with the following extracted nucleic acids for the occurrence of false reactive signals, which are indicating a cross reactivity to other non-SARS-CoV-2 related viruses and a selection of bacteria. These tests were all negative and together with the *in-silico* sequence comparison no cross reactivity to other species was observed.

Viruses	Strain
Adenovirus Type 3	N/A
Coronavirus 229E	N/A
Coronavirus HKU-1	N/A
Coronavirus NL63	N/A
Coronavirus OC43	N/A
Influenza A 2009 H1N1pdm	A/NY/02/09**
Influenza A H1N1	A/New Caledonia/20/99
Influenza A H3	A/Brisbane/10/07
Influenza B	B/Florida/02/06
MERS-CoV	Florida/USA-2_Saudi Arabia_2014
Metapneumovirus 8	Peru6-2003
Parainfluenza virus type 1	N/A
Parainfluenza virus type 2	N/A
Parainfluenza virus type 3	N/A
Parainfluenza virus type 4	N/A
Respiratory Syncytial Virus A	N/A
Rhinovirus 1A	N/A
SARS-CoV	2003-00592

Bacteria	Strain /Source
Bacillus cereus	PEI-B-P-57-S-01-02
Bordetella pertussis	A639
Chlamydophila pneumoniae	CWL-029
Escherichia coli	PEI-B-P-19-(TK)-02-01
Klebsiella pneumoniae	PEI-B-P-08-(TK)-02-01
Mycoplasma pneumoniae	M129
Pseudomonas fluorescens	PEI-B-P-77-01-03
Serratia marcescens	PEI-B-P-56-01-02
Staphylococcus aureus	PEI-B-P-63-01-02
Staphylococcus epidermidis	PEI-B-P-06-(TK)-02-01
Streptococcus pyogenes	PEI-B-P-20-(TK)-02-01

Table 11: Tested organisms for cross reactivity

11.4. Limitations of the method

Limitations of the method and therefore the kit are related to

- Insufficient sampling
- Insufficient quality of extracted nucleic acids, due to *e.g.* degradation by nucleases or carry-over of PCR inhibitors
- Cross contamination of reagents by amplicons to a large extend
- Insufficient laboratory practice or training of personnel
- Emergence of SARS-CoV-2 sequences with too many changes at the oligo hybridisation sites

12. Explanation of the symbols

	Symbol for “batch code”
	Symbol for “catalogue number”
	Symbol for “use by date” (year-month)
	Symbol for “sufficient for 8640 reactions”
	Symbol for “upper limit of temperature”
	Symbol for “observe instructions for use”
	Symbol for “research use only”
	Symbol for „keep away from sunlight“
	Symbol for “manufacturer”

13. Abbreviations

cp	copies
DNA	Deoxyribonucleic acid
EM	enzyme mix
NTC	No Template Control
OM	oligo mix
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
RT	Reverse Transcription
rxn	reaction

14. Technical service

For customer support, please contact your local GFE representative.

15. Literature

[1] Guo, Y., Cao, Q., Hong, Z. et al. The origin, transmission and clinical therapies on coronavirus disease 2019 (COVID-19) outbreak – an update on the status. Military Med Res 7, 11 (2020). https://doi.org/10.1186/s40779-020-00240-0

[2] Wang W, Xu Y, Gao R, et al. Detection of SARS-CoV-2 in Different Types of Clinical Specimens. JAMA. Published online March 11, 2020. doi:10.1001/jama.2020.3786

[3] PAN, Yang, et al. Viral load of SARS-CoV-2 in clinical samples. The Lancet Infectious Diseases, 2020, 20. Jg., Nr. 4, S. 411-412. DOI: https://doi.org/10.1016/S1473-3099(20)30113-4

[4] Letko, M., Marzi, A. & Munster, V. Functional assessment of cell entry and receptor usage for SARS-CoV-2 and other lineage B betacoronaviruses. Nat Microbiol 5, 562–569 (2020). https://doi.org/10.1038/s41564-020-0688-y

[5] Xiaolu Tang, Changcheng Wu, Xiang Li, Yuhe Song, Xinmin Yao, Xinkai Wu, Yuange Duan, Hong Zhang, Yirong Wang, Zhaohui Qian, Jie Cui, Jian Lu, On the origin and continuing evolution of SARS-CoV-2, National Science Review, , nwaa036, https://doi.org/10.1093/nsr/nwaa036

[6] Peter Forster, Lucy Forster, Colin Renfrew, Michael Forster, Phylogenetic network analysis of SARS-CoV-2 genomes, PNAS April 28, 2020 117 (17) 9241-9243

16. Trademarks and disclaimers

Trademarks

- The SARS-CoV-2 PCR Reagent Kit contains Invitrogen™ SuperScript® III reverse transcriptase, a trademark of Thermo Fisher Scientific Corporation. The SuperScript® III reverse transcriptase included in the SARS-CoV-2 PCR Reagent Kit is obtained under licence and may only be used for the Intended use specified in these Instructions for use.

- QuantStudio 5™ (Applied Biosystems); FAM™ (Life Technologies); Cy (GE Healthcare); LightCycler® 480 (Roche); QIAamp®, MinElute® (QIAGEN)

- Registered names, trademarks, etc. used in this document are not to be considered unprotected, even if they are not specifically marked as such.

Disclaimer

For research use only (RUO)! Not for use in diagnostic procedures.

17. Version History

Version	Date [JJJJ-MM-TT]	Comment
Version 1	2020-06-17	First release

Notes:

Notes:

Gesellschaft zur Forschung, Entwicklung und Distribution von Diagnostika im Blutspendewesen mbH
Altenhöferallee 3, D-60438 Frankfurt/Main, Germany
Tel: +49 (0) 69 / 400 5513 - 0
Fax: +49 (0) 69 / 400 5513 - 21