DB-1254

COVID-19 Multiplex RT-PCR Kit 2

Instructions for Use

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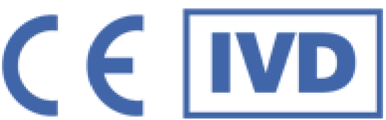

REF DB-1254-100rxns contains reagents for 100 reactions

REF DB-1254-1000rxns contains reagents for 1000 reactions

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1 Preface

1.1 Intended purpose and Kit Usage

COVID-19 Multiplex RT-PCR Kit 2 is intended for the detection of the SARS-CoV-2 virus (the cause of the disease COVID-19) using a one-step RT-PCR protocol from RNA isolated from various biological samples, such as nasal, nasopharyngeal, oropharyngeal or buccal swabs, nasopharyngeal fluid (aspirate), nasopharyngeal lavage, saliva, sputum, oral cavity lavage and pharyngeal gargles – so-called gargling liquid, stool, urine, tissue biopsies, FFPE tissue samples or water. The kit is intended for use as an aid in the diagnosis of SARS-CoV-2 in humans using a real-time PCR device.

1.2 Kit description and summary

RNA from the SARS-CoV-2 virus is detectable in human upper respiratory tract samples during infection. A positive result of the test indicates the presence of the SARS-CoV-2 RNA genome but does not rule out bacterial infection or co-infection with other viruses. A negative result of this test does not rule out SARS-CoV-2 infection and should not be used as the sole basis for treatment decisions. A negative result must be combined with clinical observations, patient history and epidemiological information.

The COVID-19 Multiplex RT-PCR Kit 2 is intended for use exclusively in clinical laboratory by qualified personnel specially trained in real-time PCR *in vitro* diagnostic methods.

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1.3 Principle of the test

The kit contains primers and probes for real-time RT-PCR detection of SARS-CoV-2 RNA, where fluorescence is detected using TaqMan™ probe hydrolysis technique. The **SARS-CoV-2** virus is detected by **amplifying three** non-overlapping **segments of genomic RNA**: two segments are located in the RNA-dependent RNA polymerase gene *(RdRP*; FAM and HEX channels) and the third one in the Envelope gene *(E-gene*; ROX channel). The design is therefore similar to "Charité Berlin" and "Institut Pasteur" designs because it targets similar parts of the genome, but the targeted sequences here are different and more conserved compared to the sequences in the other two published primers and probes.

This kit is designed to detect viral RNA from RNA isolated from various biological samples. The kit includes RNA isolation control along with primers and probes for its detection. **Adding a control to each sample prior to RNA isolation is recommended** in order to verify the isolation yield. Add the control to the lysis/binding buffer from the RNA isolation kit, which will subsequently be mixed with the sample from which the RNA is isolated. This procedure verifies both the efficiency of the RT-PCR reaction itself (detecting possible inhibition) and a sufficient yield of RNA isolation, which is a key prerequisite for correct diagnosis. A less preferred option is to add the RNA control directly to the RT-PCR master mix, but this only checks the efficiency of the RT-PCR reaction. **To verify that the kit is working correctly, a negative and a positive control, supplied in this kit, must be added to each analysis.**

If you want to detect viral RNA directly in respiratory samples without prior RNA isolation, use the DB-1255 kit, designed the same way (detection of the same targets in the same channels using the same primers and probes), but for direct detection with no need for RNA isolation.

1.4 Suitable samples and compatible sampling kits

This kit is suitable for the detection of viral RNA isolated from a various of biological samples, e.g. nasal, nasopharyngeal, oropharyngeal or buccal swabs, nasopharyngeal fluid (aspirate), nasopharyngeal lavage, saliva, sputum, oral and pharyngeal gargle lavage – the so-called "gargling liquid", stool, urine, tissue biopsies, FFPE of tissue samples and/or water using the dedicated kits. Both column isolation kits and magnetic particle-based kits can be used for RNA isolation. The isolated RNA must be eluted in water or in a buffer that does not inhibit the RT-PCR reaction. The DIANA Biotechnologies Automated RNA Isolation Kit (Cat. No. DB-1206) is recommended for optimal results in detecting SARS-CoV-2 from saliva or nasopharyngeal swabs in UTM or PBS.

Saliva and swab samples can be inactivated prior to RNA isolation when the DB-1206 Automated RNA Isolation Kit is used. If the saliva is in DIANA Biotechnologies collection kits, such as the DB-1225 Saliva Collection Set 1.4IM, DB-1230 Saliva Collection Set 1.4IF or DB-1249 Saliva Collection Kit, the saliva can be inactivated in an incubator. Sample inactivation procedures are described in the manual for the DB-1206 Automated RNA Isolation Kit and are identical to those for the DB-1255 DBdirect™ COVID-19 Multiplex RT-PCR Kit 2 isolation-free kit. The procedure for saliva inactivation has been validated by the manufacturer, while the procedure for media inactivation is only a recommendation and is user's responsibility to validate it for each type of media (to verify on approximately 10 positive samples, some of which must be weak with $C_t > 30$ so inactivation does not lead to reduced RNA yields).

1.5 Compatible automation and PCR instruments

This kit can be used both manually and with automatic laboratory pipetting machines allowing automation. The necessary protocols and plastics for automation on the Agilent Bravo liquid handling station are supplied by the manufacturer. The DB-1206 Automated RNA Isolation Kit contains plastics and reagents for automated RNA isolation from various biological samples on the Agilent Bravo liquid handling station and for subsequent automated RT-PCR plate preparation. The DB-1214 Agilent Bravo Installation Package for Automated RNA Isolation Kit contains protocols for automated RNA isolation on the Agilent Bravo liquid handling station.

This kit has been validated on the BioRad CFX96™ Real-Time PCR Detection System (BioRad CFX96™) and the BioRad CFX Opus 96 Real-Time PCR System (BioRad CFX Opus 96), which the manufacturer claims offer identical capabilities and can be used interchangeably with this kit. All protocols and settings described in this manual apply to these two PCR machines and have been validated for them. However, the kit can also be used with other PCR machines (e.g. Roche LC96), although users will need to set up the protocols exactly and validate them. The kit uses detection in FAM, HEX, ROX and Cy5 channels and these channels are on almost all commonly used machines. The PCR protocol can be validated by measuring either a dilution series of a sample of known concentration or a set of at least 10 clinical samples of known concentration, at least a few of which should be weakly positive with $C_t > 30$.

2 Kit characteristics

2.1 Analytical reactivity (inclusivity)

Amplification by this kit was tested on internally quantified standards of isolated RNA from SARS-CoV-2 "wild-type", alpha, beta, gamma, delta and omicron variants and these variants were determinably detected with equal efficiency.

2.2 Limit of Detection (LOD)

The limit of detection was determined as the approximate number of copies in the reaction at which 95% of the wells would be positive and is summarized in **Table 1**. The LOD was determined using the quantitative SARS-CoV-2 delta standard from Vircell. For each concentration tested, the 24 replicates were measured and the LOD was determined by the number of positive wells. The LOD is given as a number of copies per well (second column), but also as concentration per mL of sample (third column), assuming that 5 µL of isolated RNA is used for the assay and no sample concentration is re-concentrated during isolation (when isolation is re-concentrated five times, the LOD per mL will be 5 times lower).

Table 1: $LOD_{95\%}$ for one or two or three positive channels out of three monitored

DB-1254	$LOD_{95\%}$ in the well	$LOD_{95\%}$ mL^{-1} (5 µL sample)
at least 1 channel out of 3 positive	1	200
at least 2 channels out of 3 positive	2	400
3 channels out of 3 positive	5	1000

2.3 Intraassay and interassay variability

Intra- and interassay variability was tested for the detection of SARS-CoV-2 (RNA-dependent RNA polymerase gene and Envelope gene in FAM, HEX and ROX channels). Three concentrations (1000, 100, or 25 copies per well) were tested in eight replicates on three different plates, in three different PCR machines and all prepared by three different operators. A commercial quantified RNA standard SARS-CoV-2 manufactured by Vircell was used as the source of viral RNA. The experiment was performed on a BioRad CFX96™ machine.

Standard deviations for intraassay variability were calculated from the well-to-well variance within a single plate, and standard deviations for interassay variability were calculated from the well-to-well variance between plates. From all measurements of one concentration, one gene (channel), the expected C_t was calculated as the average of the obtained C_t values. **Tables 2, 3 and 4** give all these three parameters for each of the tested genes (channels). The number always indicates the average value of the given variable (or, in the case of standard deviations, the square root of the average of the variances in the number of cycles) and the range defined by the minimum and maximum value for the variable is given in parentheses. The last row in **Table 2** shows the average over all detected RNAs within a single measurement (intraassay), in **Table 3,** the average over all detected RNAs between two measurements (interassay variability), and in **Table 4,** the average over all detected RNAs excluding the control (IC).

Table 2: Intraassay variability values

Kit	RNA	Channel	Intraassay Variability		
			1000 copies	100 copies	25 copies
DB-1254	RdRP1	FAM	0.08 (0.04-0.10)	0.26 (0.19-0.33)	0.37 (0.32-0.42)
DB-1254	RdRP2	HEX	0.07 (0.05-0.09)	0.21 (0.16-0.26)	0.38 (0.34-0.44)
DB-1254	Envelope	ROX	0.09 (0.07-0.11)	0.17 (0.15-0.19)	0.30 (0.20-0.41)
DB-1254	IC	Cy5	0.11 (0.08-0.15)	0.15 (0.11-0.17)	0.13 (0.09-0.16)
DB-1254	All	All	0.08 (0.04-0.11)	0.21 (0.15-0.33)	0.35 (0.20-0.44)

Table 3: Interassay variability values

Kit	RNA	Channel	Interassay Variability		
			1000 copies	100 copies	25 copies
DB-1254	RdRP1	FAM	0.09 (0.07-0.16)	0.22 (0.15-0.26)	0.23 (0.19-0.25)
DB-1254	RdRP2	HEX	0.15 (0.09-0.13)	0.23 (0.17-0.31)	0.39 (0.34-0.43)
DB-1254	Envelope	ROX	0.24 (0.16-0.21)	0.27 (0.16-0.36)	0.46 (0.41-0.55)
DB-1254	IC	Cy5	0.16 (0.15-0.33)	0.15 (0.07-0.19)	0.21 (0.13-0.28)
DB-1254	All	All	0.17 (0.07-0.33)	0.24 (0.15-0.36)	0.37 (0.19-0.55)

Table 4: Expected C_t values

Kit	RNA	Channel	Expected C_t values		
			1000 copies	100 copies	25 copies
DB-1254	RdRP1	FAM	29.46 (29.39-29.52)	32.55 (32.49-32.60)	34.48 (34.41-34.53)
DB-1254	RdRP2	HEX	30.02 (29.86-30.16)	33.12 (32.97-33.29)	35.15 (34.89-35.35)
DB-1254	Envelope	ROX	28.12 (27.90-28.35)	31.30 (31.06-31.51)	33.15 (32.83-33.55)
DB-1254	IC	Cy5	27.43 (27.34-27.56)	27.42 (27.32-27.57)	27.34 (27.14-27.52)
DB-1254	All	All	29.20 (27.90-30.16)	32.32 (31.06-33.29)	34.26 (32.83-35.35)

2.4 Clinical performance and summary results

A total of 93 nasopharyngeal swab samples and 182 saliva samples, collected from individuals in the European population for indicated and preventive testing of COVID-19 and other respiratory diseases, were tested for SARS-CoV-2 with two reference kits and the results were compared with the SARS-CoV-2 detection results of this kit. RNA was isolated from the samples using the DB-1206 Automated RNA Isolation Kit manufactured by DIANA Biotechnologies. Subsequently, SARS-CoV-2 was detected in the isolated RNA as instructed by the manufacturer of the RT-PCR kit used to detect it. The swab and saliva samples tested positive for SARS-CoV-2 had been taken in 2022 and they are Omicron variants. The samples came from several different laboratories and the swabs were in different transport media.

Results from the comparison for nasopharyngeal swabs are shown in **Figure 1** and for saliva in **Figure 2**, where the graphs compare the C_t values measured by this kit with the reference kits. The numbers of TP ('true positives'), FN ('false negatives'), TN ('true negatives') and FP ('false positives') are shown in **Table 5**. The table also gives the calculated PPA ("positive percent agreement") and NPA ("negative percent agreement") values.

The "positive percent agreement" (PPA) of the device reached 97.5% in the saliva and swab samples for SARS-CoV-2, while the "negative percent agreement" (NPA) reached 100.0%. Both the PPA and NPA values demonstrate that the product is suitable and effective for the detection of SARS-CoV-2.

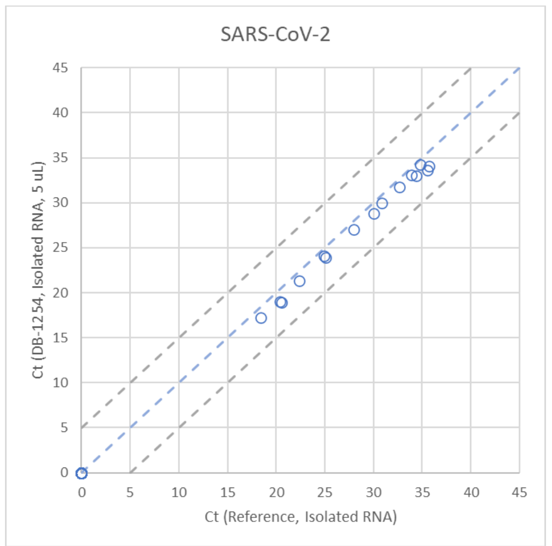

Figure 1: Comparison of measured C_t values for nasopharyngeal swab samples with reference measurements

The **y-axis** of the graph plots the minimum value of C_t from two or three channels of the DB-1254 kit and this is compared with the C_t measured by the reference kits **(x-axis**). If the signal was measured in only one reference kit ($C_t < 37$), then this C_t is plotted here, if the C_t in both sets was below 40 (and at least in one kit below 37), then the average of both C_t values is plotted. Values lying on the x-axis indicate detection of the sample only in the reference kits, while values lying on the y-axis indicate detection only in the DB-1254 kit. On average, C_t values were 1 cycle lower in the DB-1254 kit than in the reference kits, indicating a potentially more sensitive detection of SARS-CoV-2 in the DB-1254 kit.

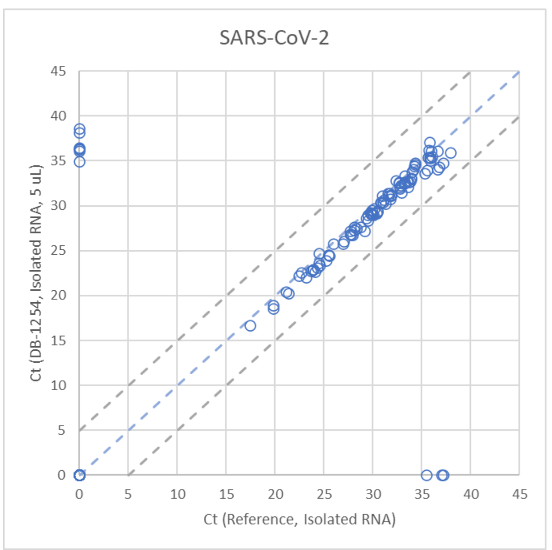

Figure 2: Comparison of measured C_t values for saliva samples with reference measurements

The **y-axis** of the graph plots the corresponding channel of the DB-1254 kit. Specifically, the minimum C_t from 2 or 3 channels **(y-axis**) is plotted against the C_t measured by the reference kits **(x-axis**). If the signal was measured in only one reference kit ($C_t < 37$), then this C_t is plotted here, if the C_t in both sets was below 40 (and at least in one kit below 37), then the average of both C_t values is plotted. Values lying on the x-axis indicate detection of the sample only in the reference kits, while values on the y-axis indicate it only in the

DB-1254 kit. Several SARS-CoV-2 samples were detected only in the DB-1254 kit and not in the reference kit (see points on the y-axis). A closer analysis **(Table 5**) shows a large proportion of them borderline positive in some of the reference measurements and the rest of the samples. This is probably due to the higher sensitivity of the DB-1254 kit detection. All these samples are with C_t >30, while the C_t values in the DB-1254 kit are on average one cycle lower than in the reference kit, so there is a strong probability of these being weak samples not detected in the reference measurement.

Table 5: Results of NPA and PPA determination for RNA isolated from nasopharyngeal swabs and saliva samples

Sample	Interpretation	TP	FN	TN	FP	FP(2)	FP(1)	PPA	NPA
Nasopharyngeal swabs	SARS-CoV-2*	15	0	78	0	0	0	100.0%	100.0%
	SARS-CoV-2 (FAM)	15	0	78	0	0	0	100.0%	100.0%
	SARS-CoV-2 (HEX)	15	0	78	0	0	0	100.0%	100.0%
	SARS-CoV-2 (ROX)	15	0	78	0	0	0	100.0%	100.0%
Saliva	SARS-CoV-2*	95	3	77	7	6	1	97.1%	100.0%
	SARS-CoV-2 (FAM)	95	3	79	5	4	1	97.1%	100.0%
	SARS-CoV-2 (HEX)	94	4	78	6	3	1	96.1%	97.5%
	SARS-CoV-2 (ROX)	96	2	71	13	6	3	98.1%	94.7%
Nasopharyngeal swabs + saliva	SARS-CoV-2*	110	3	155	7	6	1	97.5%	100.0%
	SARS-CoV-2 (FAM)	110	3	157	5	4	1	97.5%	100.0%
	SARS-CoV-2 (HEX)	109	4	156	6	3	1	96.6%	98.7%
	SARS-CoV-2 (ROX)	111	2	149	13	6	3	98.4%	97.4%

All samples were first measured with two reference kits and all the samples with a C_t below 37 cycles in at least one of the kits were considered positive. Subsequently, the DB-1254 kit was used to measure these samples and those with a C_t below 40 and positive in at least two of the three channels were considered positive. Sample positivity was evaluated both in terms of overall sample positivity for the condition of "positivity in at least two of the three channels" (*) and positivity in individual FAM, HEX and ROX channels. If the positivity matched the reference kit, they were considered TP; if they were positive only in DB-1254, they were considered FP. In the case of FP, there was a further examination of whether these samples were borderline positive in one reference kit (in one kit, a sample had a C_t between 37 and 40 while the other was negative - column FP1) or in both reference kits (both kits had samples with a C_t between 37 and 40 - column FP2). These positive samples were considered as TP for the purpose of calculating PPA and NPA (their sum was added to TP and subtracted from FP). Samples positive only in the reference kit were considered FN, while samples negative in both DB-1254 and the reference kit were considered TN. PPA was calculated as the number of TPs divided by the sum of TPs and FNs, while NPA was calculated as TN divided by the sum of TNs and FPs. The PPA and NPA values of the sample's overall positivity score for fulfilling the condition of "positivity in at least 2/3 of the channels" (*) as well as within the individual FAM, HEX and ROX channels was 100% for samples of RNA isolated from nasopharyngeal swabs. The lower PPA value for saliva isolated RNA samples was due to 3 weak FN samples with C_t >35.

3 Safety notice

This kit is intended for professional use only. Follow general principles of chemical safety. Wear protective equipment (gloves, goggles), avoid direct contact with the chemicals and avoid eating or drinking in laboratories.

Kit components contain 0.02% sodium azide, which is toxic and upon contact with acids produces toxic gas. The relevant Material Safety Data Sheets (MSDS) will be provided on request.

When working with biological samples, pay attention to the safety rules for working with infectious biological material, use appropriate protective equipment (e.g., shield, respirator), and work with samples only in designated biohazard boxes or designated work areas. Work with infectious samples only in BSL2+ or BSL3 laboratories. Dispose of the potentially infectious waste in accordance with applicable legislation.

Continually check the work area for spilled chemicals or biological samples. In the event of a spill, decontaminate the work area immediately. In case of skin or eye contact with reagents, rinse the affected area under running water immediately.

4 List of materials

4.1 Required laboratory equipment

- Real-time PCR cycler with software capable of multiplex detection in FAM, HEX, ROX and Cy5 channels – **follow the manual provided by the instrument's manufacturer**
- Calibrated handheld single-/multichannel pipettes
- Gloves and other protective equipment

4.2 Recommended laboratory equipment

- Benchtop vortex and centrifuge
- If using an automated protocol: pipetting robot (e.g. Agilent Bravo liquid handling station is recommended)

4.3 Required material not included in the kit

- Disposable pipette tips (filter tips recommended)
- Disposable tubes for mixing individual components
- PCR plate and adhesive optical foil for sealing the PCR plate
- Kit or reagents for RNA isolation (e.g. DB-1206)
- Material to be used as a negative control (see Section 5.3)
- If using an automated protocol: protocols and plastics for automation (e.g. DB-1214 installation package and DB-1206 kit for Agilent Bravo liquid handling station)

4.4 Kit components

Table 6: Components of DB-1254 kit

Kit [8]	Kit components [8]	REF code [7]	Volume (µL) [6]	Storage conditions	Description and color of the cap
DB-1254-100rxns	Enhancer mix (4x)	RF00506	500	-20 °C [2,3]	1
	Primer mix (4x)	RF06288	500	-20 °C [1,2,3]	2
	Enzyme mix (4x)	RF07929	500	**-20 °C** [2,3]	3
	Positive control	RF09960	150	-20 °C [2,3]	4
	Isolation control [4]	RF05323	150	-20 °C [2,3]	5
DB-1254-1000rxns	Enhancer mix (4x)	RF00506	5000	-20 °C [2,3]	1 [5]
	Primer mix (4x)	RF06288	5000	-20 °C [1,2,3]	2 [5]
	Enzyme mix (4x)	RF07929	5000	**-20 °C** [2,3]	3 [5]
	Positive control	RF09960	2x 750	-20 °C [2,3]	4
	Isolation control [4]	RF05323	2x 750	-20 °C [2,3]	5

[1] Store in a dark place (contains fluorophores, which are photosensitive). **[2] Store the kit at -20 °C or low**; you can also store it at -40 °C or -80 °C. The product must be transported on dry ice and the distributor should guarantee sufficient quantities of dry ice available for the duration of transport. Do not use the kit if the components are thawed upon delivery and check their condition upon handover for whether there was little or no dry ice in the shipping box. **[3] Minimize the number of freeze/thaw cycles,** and aliquot the solutions after first thaw. **[4]** Add to the lysis/binding buffer either before extracting the RNA or directly to the RT-PCR master mix. **[5]** In kits for 1000 reactions, components 1-3 have transparent caps. **[6]** The tubes are filled with a volume of 2-10% higher than indicated in the table. **[7]** The packaging indicates the REF codes and lot numbers (LOT) for the kit and individual components, so keep it for reference until you have used the entire kit. Do not mix components from different LOTs of kits. **[8]** Barcodes with basic information are provided in the packaging of the kit and its individual components.

Enhancer Mix (4x)

Contains proprietary additives which improve efficiency of RT-PCR. Supplied as 4x concentrate.

Primer Mix (4x)

Contains primers and hydrolysis probes for detection of SARS-CoV-2 (two regions of the RdRP gene in the FAM and HEX channels; the Envelope gene in the ROX channel) and an isolation control (Cy5). Supplied as 4x concentrate.

Enzyme Mix (4x)

Contains thermostable Reverse Transcriptase, “hot-start” Taq DNA polymerase, nucleotides, buffer, salts, detergents, RNase inhibitors and other additives. Supplied as 4x concentrate.

Positive control

Contains genomic SARS-CoV-2 RNA at a concentration of approximately 400 copies per microliter. Opening this vial may cause work area contamination, thus **always spin down** this vial before opening!

Isolation control

Contains artificial sequences of RNA of more than 2000 bases in length. This RNA is added to each sample prior to RNA isolation to check the efficiency of the isolation and to detect possible inhibition of the RT-PCR reaction.

4.5 Stability of kit components and master mix

For long-term storage, keep **Components 1, 2 and 3 (Enhancer, Primer and Enzyme Mix) at -20°C or below** (shelf life indicated on the packaging). Avoid repeated freezing/thawing, never exceed four freeze/thaw cycles. If you intend to use these components more than once, aliquot them after they have first been thawed.

Components **1, 2 and 3** are stable for at least 4 hours at 25 °C unless they are mixed together, and the components should be used as soon as they have been thawed. Keep the components of the kit out of direct sunlight. Exposure to normal daylight for at least 8 hours does not affect their function. However, for long-term storage, the primer mix should be kept in a dark place.

The **RT-PCR Master Mix** (a mixture of components 1, 2 and 3; see Section 5.6) is stable for up to 2 hours at 25 °C, but we recommend to use the RT-PCR Master Mix (i.e. mixing it with samples) within 30 minutes of its preparation. Keep the Master Mix out of direct sunlight. Exposure to normal daylight for at least 8 hours will not affect its function.

The **RT-PCR Master Mix** can be frozen once. It is therefore possible to prepare a master mix for several PCR plates in advance or to prepare ready-to-use PCR plates directly, however, everything needs to be aliquoted and frozen as soon as possible after the master mix has been prepared. Aliquots need to be frozen at -80 °C, where they can be stored for up to 1 month (see Section 5.6).

Because **components 4 and 5 (positive and isolation controls)** contain RNA, thaw them only for the time necessary and keep them on ice. Nonetheless, they can be stored cumulatively for at least 24 hours at room temperature. Avoid repeated freezing/thawing of positive and isolation controls. Do not exceed four freeze/thaw cycles. The optimum temperature for long-term storage is -80 °C, but they can also be stored at -20 °C.

5 Instructions for Use

5.1 General precautions

- Do not use kit components that are damaged or thawed upon receipt. On handover, check whether there is no or little dry ice in the shipping box. Keep the kit components for possible claims and contact the manufacturer.
- Improper handling of kit components and failure to follow the procedures in these instructions for use may adversely affect the results.
- Use the same version and current revision of this instructions for use (see page header), which is referenced on the packaging.
- Do not use the kit after the expiry date indicated on the packaging.
- Do not mix components from different lots of the kit (indicated on the vial).

5.2 Avoiding contamination (false positives)

Good laboratory practice should be followed to avoid sample cross-contamination. Use disposable filtered pipetting tips and use clean tips for each step of the protocol.

Handling of clinical samples, positive controls (template RNA) and amplified PCR products (template DNA) should be spatially separated from the handling of stock kit components 1, 2 and 3 to minimize the risk of accidental contamination. Best practice is preparing an RT-PCR master mix from components 1, 2 and 3 and pipette this mixture into a PCR plate in a space where the RNA/DNA templates are never handled (e.g. a PCR box). This area should also have assigned equipment (e.g. pipettes, laboratory plastics) not used for other purposes and that never comes into contact with an RNA/DNA template. The PCR plates with the master mix should then be transferred to another location (e.g. another PCR box) for adding samples and positive controls.

Other general guidelines on how to prevent accidental contamination - not exhaustive:

- **Never open a vial/plate with amplified PCR products.**
- Never open or otherwise handle samples, positive controls, or amplified PCR products in areas where the RT-PCR master mix is being prepared.
- Before handling the template RNA/DNA, close other vials with reagents and always spin down the vial with the positive control properly before opening it.
- Keep reagent containers open only for the time necessary.
- Use ultra-pure or PCR-grade water (or buffers prepared from it) to dilute the sample.

5.3 Required controls in each analysis

To detect possible false positive and false negative results, add a positive and a negative control to each RT-PCR reaction mixture. There are two ways for creating a negative control. The best way is to perform RNA isolation with a known negative sample or pure medium and add the same amount of eluate to the RT-PCR master mix as if it were a real sample. Such a negative control will detect contamination at any step of the process. The other way of setting up a negative control is less robust and involves directly adding the pure elution solution or ultra-pure/PCR grade water to the RT-PCR master mix as if it were a sample. However, this procedure only detects contamination of the elution buffer or RT-PCR master mix. As a positive control, use the SARS-CoV-2 genomic RNA included in the kit (Positive control, vial #4).

5.4 Before you start

The kit components are supplied and stored frozen, therefore before each use:

- Thaw the components at room temperature (do not thaw on ice or in a refrigerator).
- Spin down each vial before opening to collect all the liquid at the bottom.
- Mix the reagents in the vials using vortex or pipetting them before use. The pipette should be set to at least ½ of the volume of the solution to be mixed, multiple pipetting up and down is required for proper mixing. Sufficient mixing is particularly important before making aliquots. When vortexing, always spin the vial briefly before opening.

5.5 Adding RNA Isolation control

An RNA Isolation control should be added to the lysis/binding buffer prior to adding a sample and subsequent isolation of the RNA. Add **1 µL of Isolation Control (vial #5, purple cap)** to the lysis/binding buffer for each isolated sample (e.g., per 10 isolated samples; add 10 µL of isolation control to volume of lysis/binding buffer). This procedure is strongly recommended as it reveals both possible inhibition of the RT-PCR reaction and possible reduced efficiency of RNA isolation in each sample.

If it is not possible to add the RNA isolation control prior to RNA isolation, add **0.1 µL of Isolation Control (vial #5, purple cap) for each reaction** directly to the RT-PCR master mix (e.g., add 1 µL to the master mix for 10 reactions, see Step 5 in Section 5.6).

5.6 RT-PCR master mix preparation

The preparation of the RT-PCR master mix for one reaction is described below. If you are preparing it for multiple reactions, multiply the volumes by the number of reactions (and add the pipetting reserve) – see also **Table 7**.

1. Thaw and mix all components of the RT-PCR master mix (see **Table 7** and Section 4.4).
2. Into a clean RNase/DNase free vial, pipette **5 µL of Enhancer mix (4x) vial #1 in the kit (green or transparent cap)**.
3. Add **5 µL of Primer Mix (4x)** vial #2 in the kit (blue **or transparent cap)** to the same vial and mix by repeated pipetting.
4. Add **5 µL of Enzyme Mix (4x)** vial #3 in the kit (black **or transparent cap)**, to the same vial and mix by pipetting until the mixture is homogeneous (you can also briefly vortex and spin down).
5. ***Optional***: *if you are not adding RNA isolation control to the sample prior to RNA isolation,* ***add 0.1 µL of the Isolation Control – vial #5 in the kit (violet cap),*** *and either repeatedly pipette to mix it (the pipette should be set at least to ½ volume of the solution to be mixed) or vortex it.*
6. Transfer **15 µL of the mixture (RT-PCR master mix)** into a 96-well plate or into micro-tubes (depending on the type of PCR cycler used). If you cannot proceed immediately with sample addition and subsequent RT-PCR, cover the plates/microtubes with a lid (see Section 4.5 for details on the stability of the individual kit components and RT-PCR master mix).

A dead volume is required for automated plate preparation when adding the RT-PCR master mix and sample. Therefore, the total reaction volume and sample volume can be reduced by 10%, compared to the values given in Sections 5.5, 5.6 and 5.7, without affecting detection sensitivity.

Table 7: Preparation of RT-PCR master mix

Kit components	# and cap color	µL per 1 reaction	µL per 100 reactions
Enhancer mix (4x)	1 [1]	5	500
Primer mix (4x)	2 [1]	5	500
Enzyme mix (4x)	3 [1]	5	500
Isolation control (*optional*) [2]	5	0.1	10
Total volume of RT-PCR master mix		15	1500

[1] The vials are numbered by the sequence for adding individual components and it is crucial for this order to be maintained. **[2]** The Isolation control volume is neglected; it is only added to the RT-PCR master mix if it was not added during RNA isolation (see Section 5.5 for details).

Aliquoting solutions for RT-PCR master mix preparation

Table 6 summarizes the numbers and volumes of aliquots for each component of this kit to cover 1000 tests (1000rxns). Unless you desire to use the whole kit at once, it is advisable to prepare single aliquots only after first thawing them. There are two possible ways of preparing single aliquots for the analysis of 96 samples (e.g., using the Agilent Bravo liquid handling station and DB-1206 kit):

1. **If the aliquots can be stored at -80 °C,** then mix all of Enhancer mix (4x), Primer mix (4x) and Enzyme mix (4x) contents. Follow this sequence and always mix before adding the Enzyme mix. Then mix thoroughly again and divide into 10 aliquots of 1.53 mL. This will result in 10 vials with RT-PCR master mix. The RT-PCR master mix prepared this way must be frozen as soon as possible and stored at -80 °C. Once thawed, use the RT-PCR master mix as soon as possible, ideally within 30 minutes, although it will remain stable at room temperature for up to 2 hours.
2. **If the aliquots cannot be stored at -80 °C**, then prepare 10 aliquots of 510 µL of each solution of the Enhancer mix (4x), Primer mix (4x) and Enzyme mix (4x). Store them at -20 °C. To prepare the RT-PCR master mix for the analysis of 96 isolations, mix one aliquot of each component and use the entire mix. The individual components of the RT-PCR master mix will remain stable at room temperature for 4 hours after they have been thawed and before they are mixed.

Aliquoting positive control

Prepare 5 single use aliquots of the positive control, each with a volume of 150 µL. These aliquots can be stored at either -80 °C or -20 °C as required.

Aliquoting isolation control

Prepare 5 single use aliquots of the isolation control, each with a volume of 150 µL. These aliquots can be stored at either -80 °C or -20 °C as required.

Section 4.5 provides details on the stability and storage of individual solutions.

5.7 Adding a sample to the RT-PCR reaction

Add 5 µL of the sample into each well/PCR-microtube, containing 15 µL of RT-PCR master mix. After adding the samples to a 96-well plate, seal it with an adhesive optical foil and run the RT-PCR reaction (within 60 minutes of adding the samples) as described in Section 5.8.

Each analysis must contain at least one positive and one negative control. For positive control, add **5 µL of positive control from Vial #4 (red cap ●)** instead of the sample. For negative control, add either **5 µL of RNA isolated from a known negative sample**, **5 µL of elution buffer** from the RNA isolation kit, or **5 µL of ultra-pure/PCR grade water** (see Section 5.3) instead of the sample. The total volume of the reaction after adding the sample or controls is 20 µL.

A dead volume is required for automated plate preparation when adding the RT-PCR master mix and sample. Therefore, the total reaction volume and sample volume can be reduced by 10 %, compared to the values given in Sections 5.5, 5.6 and 5.7, without affecting detection sensitivity.

5.8 RT-PCR protocol

The RT-PCR protocol described here for this kit has been validated on the **BioRad CFX96™and BioRad CFX Opus 96**, which have identical setups. It can also be used with other instruments capable of simultaneous detection in FAM, HEX, ROX and Cy5 channels, although users should use appropriate controls to set up the instrument correctly and verify the kit's performance. For instructions on setting up the RT-PCR protocol and detection in the relevant channels, please refer to the instrument's user manual.

5.8.1 Setting up the BioRad CFX96™ and BioRad CFX Opus 96

For detection in four channels, use the default instrument settings and filter settings shown in **Table 8**.

Table 8: Filter settings for RT-PCR detection on BioRad instruments.

Fluorophores	Excitation (nm)	Detection (nm)
FAM*	450-490	515-530
HEX*	515-535	560-580
ROX*	560-590	610-650
Cy5*	620-650	675-690

*The names of the fluorophores are the same as they are described in the CFX Maestro Software.

The program consists of 4 steps:

1. Reverse transcription of viral RNA (RT step)
2. Taq polymerase activation (Denature)
3. PCR amplification (45 cycles; Cycling)
4. Plate cooling (Cooling)

The target temperature setting, and timing of each step is shown in **Table 9.**

Set the "sample volume" to 20 µL.

Table 9: Protocol for RT-PCR detection on BioRad instruments.

Variable	RT step	Denature	Cycling			Cooling
Cycles	1	1	45			1
Temperature (°C)	50	95	95	60	72	40
Hold time (hh:mm:ss)	00:10:00	00:02:00	00:00:05	00:00:15	00:00:15	00:00:30
Plate read	NO	NO	NO	YES	NO	NO

The approximate duration of this protocol on BioRad instruments is approximately 1 hour and 16 minutes.

5.9 Data analysis

5.9.1 Determination of cycle threshold value (C_t)

Perform data analysis as described in the operator's manual for your real-time PCR system. A logarithmic scale is recommended be used to display both fluorescence and color compensation between the FAM and HEX channels if available on your device. Below are the recommended thresholds for selected instruments, if necessary, adjust their value so that the curves intersect in their linear section (in logarithmic representation, this does not apply to linearly displayed curves); and at the same time the threshold always needs to be above the background for all negative samples.. Adjustment of thresholds may be required either due to higher autofluorescence of

samples (especially saliva) because of a higher background, or due to differences between instruments (although supplied by the same manufacturer).

Use the default settings for FAM, HEX, ROX and Cy5 channels for BioRad CFX96™ and BioRad CFX Opus 96. To determine C_t values, use the default Single Threshold mode* method with manually set thresholds: **200 RFU for FAM and HEX and 100 RFU for ROX and Cy5. All C_t values in Sections 5.9.2 and 5.9.3 correspond to the evaluation with the thresholds set up in this way.** As biological samples can be autofluorescent in PCR, you should activate the "Apply Fluorescence Drift Correction" setting on BioRad machines to minimize the occurrence of autofluorescence curves. If necessary, you can manually increase the selected thresholds, compared to the above values.

In any case, the measured data must be visually checked, as the use of threshold fluorescence to calculate of C_t may result in incorrect evaluation of the curves. For example, the Single Threshold mode method may evaluate a negative sample as positive because of the unusual curve shape – e.g. a spike in fluorescence due to a bubble in the reaction mixture, etc. All the curves with a steep and sustained increase in fluorescence must be evaluated as positive (see Section 5.9.4 for the standard curve shape), while the other curves must be evaluated as negative (see Section 5.9.3 under Table 10 for the appearance of problematic curves. As in BioRad instruments, an approximately 2% fluorescence bleed-trough from FAM to HEX is observed, in case of high fluorescence in FAM (around 10 000 RFU), using a higher threshold for HEX of 300 RFU (instead of 200 RFU) is recommended.

The C_t values obtained will vary depending on the RT-PCR instrument used, the method of evaluation and the threshold settings. Therefore, the C_t values cannot be used to compare samples if they were analyzed in a different run. To illustrate, the background on BioRad machines is typically well below the above recommended fluorescence thresholds, and if you set this threshold close to the background value, then you could obtain C_t values of as much as 3 to 5 cycles lower than with the recommended thresholds. The disadvantage of such low thresholds is the susceptibility to false positive interpretation of wells with autofluorescent samples or bleed-trough from another channel. Conversely, if you set the threshold fluorescence high, you can obtain C_t values of 3 to 5 cycles higher with a minimum risk of false positive interpretation, but you may overlook the weakly positive samples that do not reach this fluorescence threshold (the maximum fluorescence of samples typically ranges between 1,000 and 10,000 RFU units in individual channels). Therefore, the above values are recommended as fluorescence thresholds. However, the C_t values are not exactly comparable even between different measurements on the same type of instrument and with the same chosen threshold. Experience has shown the measured fluorescence to vary by up to a factor of two between different runs or different BioRad instruments.

All the expected C_t values listed below in the evaluation of controls and in **Table 10** assume compliance with the instructions (e.g., added component volumes) and the BioRad instrument setup described above (Single Threshold mode method with manually set thresholds). For accurate interpretation according to **Table 10**, if another setting or method is used, the measured C_t values will must be shifted by the difference between the C_t value of the positive control and the reference C_t value (cycle 28 for FAM and HEX channels and cycle 27 for the ROX channel). For example, if the defined FAM channel C_t value for a positive control is 24, for the values in **Table 10** either add 4 cycles to the clinical sample C_t values or subtract 4 cycles from the threshold C_t values in **Table 10**.

** Also called Threshold Crossing, Cycle Threshold or Fit Points, where the C_t value corresponds to the cycle where the fluorescence rises above the background level and exceeds a predetermined threshold.*

5.9.2 Evaluation of controls

For a **positive control**, amplification must occur in the three channels: viral genes in the FAM, HEX and ROX (depending on the plastic, cycler and sample volume used, expected C_t values for these channels range from 26-33 cycles). If there is no amplification in any of these channels (i.e. C_t>35 in any of these channels), the PCR reaction has not been performed correctly, the results of such analysis are not valid and the analysis must be repeated. To determine the C_t values, use the Single Threshold mode method described above. Amplification of the isolation control in Cy5 should result in C_t <35 cycles, but this is not necessary for a correct evaluation of the positive control (the signal of the control in Cy5 will only be measurable if the positive control has been isolated. If you add 5 µL of the positive control directly to the RT-PCR reaction and do not add the isolation control separately, then Cy5 will be negative).

Negative control must show amplification of the isolation control in the Cy5 channel (<40 cycles), while there must be no amplification in all other channels. A measurable amplification in FAM, HEX or ROX channels indicates possible contamination of reagents with the template, which may cause false positive results. In this case, it will be necessary to test a larger number of negative controls (the signal of the control in Cy5 would only be measurable had the negative control been isolated; if you add 5 µL of the negative control directly to the RT-PCR reaction and do not add the isolation control separately, then Cy5 will be negative).

The **isolation control** must be evaluated for each sample. However, in positive samples, the amplification of the isolation control may be negatively affected by viral gene amplification, and the C_t values in the Cy5 channel may be significantly higher than in the negative control or even below the threshold. If you are only evaluating the test qualitatively, **positive samples are considered positive even if the isolation control fails**. For samples that are negative in the FAM, HEX and ROX channels or with C_t > 37, check the amplification of the control in Cy5. If the C_t in Cy5 of such a sample is greater than cycle 40 or the signal is undetectable, low RNA isolation efficiency or inhibition of the RT-PCR reaction can be assumed and the RNA isolation from the sample must be repeated.

5.9.3 Results interpretation

Set the detection cycle threshold (C_t) values in each channel and interpret the results according to both **Table 10** and the results of the positive control evaluation. If you are using the recommended procedure for determining C_t and the C_t values you obtained for the positive control match the thresholds in the previous section (i.e., the C_t values for FAM, HEX, and ROX channels are between cycle 26 and 33), you can interpret the measured C_t according to this table without any further C_t recalculations. Otherwise, the determined C_t must be recalculated before interpreting according to **Table 10**, as described above in Section 5.9.1.

Table 10: Data interpretation

The symbol „-" indicates Ct > 40 cycles or undetectable signal; the symbol „+" indicates Ct ≤ 40 cycles. SARS-CoV-2 genes are detected in FAM, HEX and ROX channels and an isolation control in Cy5. The C_t values given in the table assume the analysis has been performed on the BioRad machines according to the evaluation procedure and threshold settings described above (see Sections 5.8.1 and 5.9.1).

FAM [8,9]	HEX [7,8,9]	ROX [8,9]	Cy5	Interpretation
C_t < 40	C_t < 40	C_t < 40	+ [1] / - [2]	**SARS-CoV-2 positive** [3]
C_t < 40	C_t < 40	-	+ [1] / - [2]	**SARS-CoV-2 positive** (any two of the three viral channels are positive) [3]
C_t < 40	-	C_t < 40	+ [1] / - [2]	
-	C_t < 40	C_t < 40	+ [1] / - [2]	
C_t 30-40	-	-	+ [1]	**Weak SARS-CoV-2 positive**, repeat for confirmation [4]
-	C_t 30-40	-	+ [1]	
-	-	C_t 30-40	+ [1]	
C_t 30-40	-	-	-	**Weak SARS-CoV-2 positive**, RT-PCR inhibition, repeat for confirmation [5]
-	C_t 30-40	-	-	
-	-	C_t 30-40	-	
C_t < 30	-	-	+ [1] / - [2]	**Unreliable result:** may indicate contamination by the amplification product or mutations in the detected genes. It is recommended **to repeat with an RT-PCR kit that detects different genes than this kit**. [6]
-	C_t < 30	-	+ [1] / - [2]	
-	-	C_t < 30	+ [1] / - [2]	
-	-	-	+ [1]	**Undetectable (negative) for SARS-CoV-2** [10]
-	-	-	-	**Unreliable result:** inhibition of RT-PCR, **repeat** or **perform an RNA isolation.**

[1] If an RNA isolation control was added to the sample, in the amount as directed, prior to RNA isolation, and the standard isolation protocol was followed (i.e., approximately 1/10 of the elution used for RT-PCR, e.g., 5 µL of 50 µL), the C_t value for Cy5 should be around cycle 35 or lower.

[2] High concentrations of viral RNA detected in any channel can cause impaired amplification of the isolation control, which is reflected by a reduction or complete absence of a signal in the Cy5 channel (see

Figure 3 for details). Nevertheless, the absence of a signal in Cy5 does not change the interpretation of positive signals in FAM, HEX or ROX.

[3] A positive signal in at least two of the three viral channels indicates a positive result, and typically, amplification in one channel may be absent in weak samples with a C_t around cycle 35 or higher. If the signal of positive viral channels was high ($C_t < 30$) and one channel was still negative, this may be an indication of a mutation occurring in the relevant gene for which amplification is missing. Even so, the result indicates that the sample is positive for SARS-CoV-2 virus. But if this were to occur in such samples, please contact the manufacturer's application specialists.

[4] If only one of the viral channels positive with a C_t between cycle 30 and 40 and the Isolation control is also positive, the result can be considered positive, but the test must be repeated for confirmation (either with the same sample to exclude accidental contamination or with a new sample to confirm clinical relevance). A repeatedly positive result in any channel is considered a positive result for the virus.

[5] If the C_t in the FAM, HEX or ROX channels is between cycle 30 and 40, and the signal in the Cy5 channel is higher than cycle 40 or undetectable, then the assay must be repeated because the RT-PCR is likely to be inhibited or the efficiency of RNA isolation has been reduced. A repeatedly positive result in any channel is considered a positive result for the virus.

[6] The result is unreliable if the C_t in any of the FAM, HEX or ROX channels is lower than cycle 30 and the other two channels are negative. This may indicate contamination from the product of amplification or mutations in two target sequences that came out negative. Therefore, it is recommended to repeat the test with an RT-PCR kit that detects different target genes than this kit. Were such samples to occur, please contact the manufacturer's application specialists.

[7] Because of the signal overlap from the FAM to the HEX channel, it is necessary to exclude curves whose maximum RFU in HEX is up to 300 when the FAM fluorescence is around 10 000 RFU.

[8] Even in negative samples, there can be a gradual increase of the signal in any channel, although typically with a $C_t > 30$ and a final fluorescence of at most 100-200 RFU. It may be necessary to increase the threshold of the relevant channel to eliminate these curves. Visually inspecting their shape can reliably detect these "non-amplification" curves. Their shape is completely different than standard amplification curves, which lack the gradual exponential increase from a low background and a final plateau.

[9] Autofluorescence of the sample can occur in any channel due to high fluorescence from early cycles of the PCR protocol, typically with a $C_t < 10$ and a final fluorescence in the order of hundreds of RFU. Autofluorescence is most common in the ROX channel.

[10] A negative result does exclude infection with this virus and should not be used as the sole basis for decisions about patient treatment.

5.9.4 Typical results

Figure 3 shows illustrative data measured with the viral RNA dilution series by the DB-1255 kit, which uses identical primers and probes as the DB-1254.

FAM (RdRP)

HEX (RdRP)

ROX (Envelope)

Cy5 (EC)

Figure 3: Detection of a dilution series of viral RNA from 10,000 to 5 copies per well on BioRad CFX96™.

The first pair of graphs shows the signal amplification in the FAM channel (gene for RdRP of the SARS-CoV-2 virus), the second in the HEX channel (gene for RdRP of SARS-CoV-2 virus), the third in ROX (gene encoding Envelope of the SARS-CoV-2 virus) and the fourth in Cy5 (external control used instead of the isolation control in the DB-1255 kit). The left-hand graphs show fluorescence on a linear scale, while the right-hand graphs show it on a logarithmic scale. The final fluorescence (RFU) and curve shapes may vary between channels/runs. Amplification in the Cy5 channel is reduced at high viral RNA titers (curves with decreasing maximum fluorescence correspond to wells with increasing amounts of viral RNA). **Materials used**: FrameStar 96 Well Semi-Skirted PCR plate (4ti-0951, white wells) and LightCycler 480 Sealing Foil (04729757001).

6 Legal notice

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7 List of compatible kits

REF DB-1206 Automated RNA Isolation Kit

REF DB-1214 Agilent Bravo Installation Package for Automated RNA Isolation Kit

8 One-page summary protocol

8.1 Kit components

Kit components	Volume (µL)		Storage temperature	Cap label and color
	100rxns	1000rxns		
Enhancer mix (4x)	500	5000	-20 °C	1
Primer mix (4x)	500	5000	-20 °C	2
Enzyme mix (4x)	500	5000	**-20 °C**	3
Positive control	150	2x 750	-20 °C	4
Isolation control	150	2x 750	-20 °C	5

8.2 RT-PCR Master Mix preparation

- After the vials have been thawed, spin each vial before opening it and mix all of the components
- Mix in the following order: 5 µL of Enhancer mix (vial #1), 5 µL of Primer mix (vial #2) and 5 µL of Enzyme mix (vial #3). Mix after adding each component.
- Transfer 15 µL of this RT-PCR master mix into a 96-well plate, add 5 µL of the sample (isolated RNA), seal the plate with optical foil and run the RT-PCR reaction as soon as possible.
- For positive and negative controls, add 5 µL of positive (v ial #4) or negative control instead of the sample.

Table summarizing the volumes of individual RT-PCR master mix components required for 1 and 100 reactions:

Kit components	Label	µL per 1 reaction	µL per 100 reactions
Enhancer mix (4x)	1	5	500
Primer mix (4x)	2	5	500
Enzyme mix (4x)	3	5	500
Isolation control (optional)	5	0.1	10
Total volume of RT-PCR master mix		15	1500

8.3 RT-PCR protocol

Table summarizing RT-PCR cycler settings:

Variable	RT step	Denature	Cycling			Cooling
Cycles	1	1	45			1
Temperature (°C)	50	95	95	60	72	40
Hold time (hh:mm:ss)	00:10:00	00:02:00	00:00:05	00:00:15	00:00:15	00:00:30
Plate read	NO	NO	NO	YES	NO	NO

Scanning must be set for simultaneous detection in FAM, HEX, ROX and Cy5 channels. The procedure for setting the detection can be found in Section 5.8 and in the instructions for use of the instrument to be used.

9 Graphical symbols used

Symbol	Meaning
	Manufacturer
	Caution
LOT	Lot Number
	Operator´s instructions for use, operating instructions
REF	Catalogue Number
VOL	Component volume
CONT	Package contains
CONTROL +	Positive control
x °C	Upper temperature limit* (*for X corresponding to a specific temperature)
	Do not use if package is damaged
	Use by date
	Do not reuse
Σ n	Amount (number of reactions)** (**for <n> tests depending on kit variant)
CE	CE marking
IVD	*In vitro* diagnostic medical device