DB-1253

DBdirect™ Respiratory panel 2:

SARS-CoV-2/Flu/RSV

Instructions for Use

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CE IVD

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Contents

1 Preface

1.1 Intended purpose and kit usage

DBdirect™ Respiratory panel 2: SARS-CoV-2/Flu/RSV is intended for the simultaneous detection and differentiation of nucleic acids of SARS-CoV-2, Influenza A (IAV), Influenza B (IBV) and Respiratory syncytial virus (RSV) from a wide range of respiratory samples, such as nasal, nasopharyngeal, oropharyngeal or buccal swabs in various transport media, saliva, sputum, nasopharyngeal fluid (aspirate) or nasopharyngeal lavage, oral cavity lavage and pharyngeal gargles – so-called gargling liquid using one-step RT-PCR protocol without the need for prior RNA isolation. The kit is intended for use as an aid in the differential diagnosis of SARS-CoV-2, IAV, IBV and RSV in humans using a real-time PCR device

1.2 Kit description and summary

Viral RNA (SARS-CoV-2, Influenza A, Influenza B and RSV) are detectable in the upper respiratory tract during the infection. A positive result of the test proves presence of viral RNA. However, positive result does not rule out bacterial infection, or other co-infection with other viruses including the viruses tested in this kit. Negative result for any virus does not completely rule out infection by this virus and should not be considered as a sole determinant for patient treatment. The negative result must be combined with clinical description, medical history, and epidemiological information.

DBdirect™ Respiratory panel 2: SARS-CoV-2/Flu/RSV should be used only in a clinical laboratory by trained, qualified personnel specially trained in *in vitro* RT-PCR diagnostics.

1.3 Working Principal of the Kit

The kit consists of primers and probes for real time RT-PCR detection of RNA viruses SARS-CoV-2, Influenza A/B and/or RSV which work on the principal of TaqMan™ hydrolysis probes. **SARS-CoV-2 virus is detected in the fluorescent channel FAM** using amplification of two segments of genomic RNA in the regions of *EndoRNAse* and conserved part of *Spike*. Both of these sequences are used for detection in kits DB-1211 and DB-1219 and have been verified as appropriate targets in millions of tests. Primers used for Influenza A and B are designed according to CDC recommendations with adaptation to match the currently circulating strains. These primers amplify Influenza's A *Matrix protein 1* (M1) at segment 7 and Influenza's B *Nonstructural protein 1* (NS-1) segment 8. Influenza A primers are designed to detect the most common circulating subtypes: H1N1, H1N1-pdm, H3N2, H5N1 and H7N9, but also H2N2, H1N2, H5N6 and H9N2. **Influenza A is detected in the fluorescent channel HEX.** Influenza B primers are designed to detect subtypes Victoria and Yamagata. **Influenza B is detected also in the fluorescent channel HEX.** The kit does not differentiate between Influenza A and Influenza B. If you want to differentiate between these viruses, use the DB-1251 DBdirect™ Respiratory panel 1: SARS-CoV-2/Flu/RSV which uses the same primers and samples, but can differentiate between Influenza A and B by using one additional channel. RSV primers are designed according to WHO

recommendation with adaptation to detect the currently circulating strains and detect both viruses RSV A and RSV B in the *RdRP* region. **RSV is detected in the fluorescent channel Texas Red (TEX)**.

This kit is designed to detect viral RNA directly in respiratory samples without prior RNA isolation. Therefore, the kit includes an external RNA control and primers with a probe for its detection. **This external RNA control must be added to each RT-PCR reaction after sample addition.** This procedure verifies the efficiency of the RT-PCR reaction in each tested sample (detecting any inhibition of reverse transcriptase or polymerase by the sample or unsuitable media, as well as any degradation of RNA by the sample; this will always result in later amplification of this control). **To verify the correct functioning of the kit, at least one negative and at least one positive control (supplied in this kit) must be added to each run, and we also recommend one known clinical positive sample** of the same type as that analyzed in the test: for example, a saliva sample, or a sample in the swab transport medium.

If you want to detect viral RNA in isolated RNA, then use the DB-1252 kit, which is of the same design as this kit (detection of the same viruses in the same channels using the same primers and probes) but is designed for detection in isolated RNA and includes an isolation control. This can be added to the sample prior to RNA isolation and, in addition to inhibiting reverse transcriptase and polymerase or RNA degradation, it can also reveal any losses during the RNA isolation process if inhibited.

1.4 Suitable samples and compatible sampling kits

This kit is suitable for detecting viral RNA directly in a respiratory sample without the need for prior RNA isolation, thanks to the RT-PCR mix formulation that can release RNA from the virion and protect the RNA from degradation by the sample. The kit has been validated for two basic sample types: nasopharyngeal swabs in transport medium and saliva. For saliva collection, we recommend using the DB-1225, DB-1230 or DB-1249 collection kits.

The following samples and media have been validated for use with this kit:

- **Saliva** after heat inactivation: the inactivation will improve sample pipetting and increase the sensitivity of the assay.
- **Nasopharyngeal swabs** in various transport media: common non-inactivating viral transport media are suitable; phenol red does not interfere. Examples of media validated for use with this kit include:
    - Copan eSwab Universal transport medium for viruses (UTM), LMS Corotest VTM, HCUTM medium, VTM DULAB, Qanto VTM and others. For use with other media, the kit must be individually validated.
    - PBS media are also suitable (e.g., test composition 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4; always test for specific composition; strong buffers are inappropriate).
    - Inactivating media, e.g., containing large amounts of guanidium isothiocyanate, are unsuitable.
    - Concentrated media with high salt concentrations may also be unsuitable.
- Swab samples in medium may be heat inactivated, but this is not required:

- Inactivation may slightly increase sensitivity of the assay, but too high a temperature and/or too long an inactivation time may lead to decreased sensitivity (due to precipitation).
- The exact inactivation procedure with a specific medium must be validated by the user.

The kit has been clinically validated for diagnostic use only for nasopharyngeal swabs in non-inactivating viral transport media and saliva but can also be used for other types of respiratory matrices, such as other types of swabs, aspirates, lavages and/or gargling samples. In such cases, the medium is typically PBS or similar buffers that are compatible with this kit. However, the use of a different sample type, saliva collection method, or other medium used must be validated by comparing results for at least 10 positive samples after RNA isolation vs. direct detection with this kit.

1.5 Compatible automation and PCR instruments

This kit can be used manually, but also automated on automatic laboratory pipetting robots. The manufacturer supplies a plastic and a pre-prepared protocol for automation on the Agilent Bravo liquid handling station. The DB-1224 Bravo Installation Package for DBdirect™ contains the necessary protocols for preparation of RT-PCR reactions from both saliva and swabs. The DB-1222 DBdirect™ Bravo Extension Kit for Swab contains the necessary plastic for preparing RT-PCR reactions from swabs, the individual parts can also be used for other laboratory purposes. The DB-1225 Saliva Collection Set 1.4IM, DB-1230 Saliva Collection Set 1.4IF and DB-1249 Saliva Collection Kit contain saliva collection kits suitable for automation. The DB-1226 DBdirect™ Bravo Extension Kit for Saliva 1.4IM contains plastics to automate the preparation of RT-PCR reactions from saliva using these sampling kits, and the individual parts can be used for other laboratory purposes. The DB-1228 Sample Rack 1.4IM and DB-1240 Sample Rack 1.4IF kits contain racks to prepare tubes into a format suitable for automated processing.

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 offer identical technical parameters according to the manufacturer and can be used interchangeably with this kit. All protocols and settings described in these instructions for use apply to and have been validated for these two PCR instruments. The kit can be used on other PCR instruments; however, the exact setup and validation of the protocols is the responsibility of the user. The kit uses detection in FAM, HEX, TEX and Cy5 channels, these channels are on almost all commonly used instruments. The PCR protocol can be validated either by measuring a dilution series of a sample of known concentration or by measuring a set of at least 10 clinical samples of known concentration, several of which should be weakly positive with $C_t > 30$.

2 Kit characteristic

2.1 Analytical reactivity (inclusivity)

A total of 27 different strains (3× Influenza A H1N1, 5× Influenza A H1N1 pandemic, 7× Influenza A H3N2, 8× Influenza B, 2× RSV A and 2× RSV B) were tested to validate the kit's ability to detect

different strains of influenza A and B, and RSV A and B. These were commercially purchased in viral culture form, and because they were in different media, some of which were inhibitory, they were not tested by the direct method without RNA isolation protocol, but the RNA was first isolated with the DB-1206 Automated RNA Isolation Kit and then tested with the DB-1252 RT-PCR Respiratory panel 2: SARS-CoV-2/Flu/RSV kit. This kit is designed for isolated RNA and uses the same primers and probes as this kit, so the data reported here are from measurements with the DB-1252 kit. Isolated RNA from individual cultures (virus strains) were tested at three different dilutions (500×, 5,000× and 50,000×; the lowest concentration tested varied between virus strains but was always in the range of 0.002 to 0.4 $TCID_{50}$ "copies" per reaction) and the results of the measurements were evaluated according to the DB-1250 instructions for use. Viral culture concentrations were reported by the supplier in $TCID_{50}$/mL values, and therefore the resulting LOD analyses were related to these units.

The DB-1252 kit was able to detect all tested influenza A and B and RSV A and B strains in the correct fluorescence channel at less than 1 $TCID_{50}$ per reaction. Table 1 shows the LOD values per reaction and per milliliter for each strain tested (virus copies are given in $TCID_{50}$ units) as well as the approximate C_t value if 1 $TCID_{50}$ virus is present in the reaction. Since $TCID_{50}$ units give (if we simplify it) the number of infectious particles, different LOD values for given strains do not necessarily imply different detection sensitivity, but rather will reflect the different infectivity of the cultures. Determination of the LOD for each strain was made by comparing the C_t values obtained from the analysis of a given culture (three points with different $TCID_{50}$ amounts measured each time) and a dilution series of a standard (one used for each Influenza A or B virus or RSV) with a known amount of RNA copies. The comparison was used to determine how many copies of viral RNA correspond to a given $TCID_{50}$ dilution, and the LOD value (according to the LOD measured for quantitative standards, see Limit of Detection section) in $TCID_{50}$ units was then calculated from this value.

Table 1: **Results of detection of different strains of influenza viruses and RSV**

Virus	Strain	LOD for each strain:		C_t pro 1 $TCID_{50}$/reaction
		$TCID_{50}$/reaction	$TCID_{50}$/mL	
Influenza A H1N1	A/Singapore/63/04	0.04	9	33.2
Influenza A H1N1	A/Brisbane/59/07	0.005	1	30.1
Influenza A H1N1	A/Taiwan/42/06	0.1	26	34.7
Influenza A H1N1pdm	A/Michigan/45/15	0.0007	0.1	27.1
Influenza A H1N1pdm	A/California/07/09	0.0007	0.1	27.2
Influenza A H1N1pdm	A/New York/18/09	0.002	0.4	28.5
Influenza A H1N1pdm	A/NY/03/09	0.07	13	33.8
Influenza A H1N1pdm	A/Mexico/4108/09	0.05	9	33.2
Influenza A H3N2	A/Perth/16/09	0.05	9	32.3
Influenza A H3N2	A/Texas/50/12	0.006	1	29.3
Influenza A H3N2	A/Hong Kong/4801/14	0.003	0.7	28.6
Influenza A H3N2	A/Wisconsin/67/05	0.01	2	30.4
Influenza A H3N2	A/Kansas/14/17	0.01	2	30.1
Influenza A H3N2	A/Brisbane/10/07	0.07	14	33.0

Influenza A H3N2	Singapore/INFIMH-16-0019/16	0.03	5	31.5
Influenza B	B/Brisbane/33/08	0.00009	0.02	22.5
Influenza B	B/Florida/04/06	0.0006	0.1	25.1
Influenza B	B/Brisbane/60/08	0.001	0.2	25.9
Influenza B	B/Texas/2/13	0.0004	0.08	24.6
Influenza B	B/Victoria/504/00	0.004	0.7	27.7
Influenza B	B/Florida/02/06	0.0005	0.1	25.0
Influenza B	B/Colorado/06/17	0.0006	0.1	25.2
Influenza B	B/Malaysia/2506/04	0.002	0.4	27.0
RSV-A	12/2014 Isolate #2	0.0002	0.04	24.5
RSV-A	3/2015 isolate #3	0.0002	0.05	25.0
RSV-B	CH93-18(18)	0.000005	0.0009	19.4
RSV-B	3/2015 Isolate #1	0.00003	0.007	22.2

The table summarizes the strain names (column 2) of each virus (column 1) and the $TCID_{50}$ values per reaction and per milliliter (columns 3 and 4) in RNA isolated from each culture. The last column then shows the expected C_t value for 1 $TCID_{50}$ copy in the reaction. A more detailed description and explanation is provided in the main text.

For detection of SARS-CoV-2, the kit uses the same primers as the DB-1211 and DB-1219 kits. These kits have been used since the beginning of the COVID-19 pandemic and have been proven to detect all major variants of this virus, i.e., wild-type, alpha, beta, gamma, delta, and omicron variants, without any change in sensitivity.

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. Vircell quantitative standards were used to determine the LOD. For each concentration tested, 24-plicates were measured and the number of positive wells was used to determine the LOD. **Table 2** summarizes the approximate $LOD_{95\%}$. For RSV, the real values are probably better because for the LOD determination we used old RSV strains that differ from today's strains and carry substitutions in the targeted sequences. Our primers are optimized for the current RSV strains. The LOD is given in number of copies per well (second column), but also as concentration in mL of sample (third column) depending on if 2 or 4 µL of sample is tested.

Table 2: $LOD_{95\%}$ for selected targeted viruses

DB-1253	$LOD_{95\%}$ in well	$LOD_{95\%}$ mL^{-1} (4 µL sample)	$LOD_{95\%}$ mL^{-1} (2 µL sample)
SARS-CoV-2	2	500	1000
IAV H1N1-pdm	5	1250	2500
IAV H3N2	10	2500	5000
IBV Victoria	5	1250	2500
RSV-A	5	1250	2500
RSV-B	10	2500	5000

2.3 Intraassay a interassay variability

Intra- and interassay variability was tested for selected viral targets (SARS-CoV-2, Influenza A H3N2, Influenza B Victoria, RSV A and RSV B). From each target, three concentrations (1000, 100, or 25 copies per well) were tested in eight replicates (1000 copies in only four replicates) on three different plates, in three different PCR machines, all prepared by three different operators. Commercial quantified RNA standards from Vircell were used as the source of viral RNA. The experiment was performed using a BioRad CFX96™ instrument.

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 a single virus concentration, the expected C_t was calculated as the average of all obtained C_t values. In **Tables 3, 4, and 5** all these three parameters are listed for each of the tested viruses. The number always indicates the mean value of the variable (or, for standard deviations, the square root of the mean of the variance in the C_t values) and then the range defined by the minimum and maximum values for the variable is given in parentheses. The last row in **Table 3** shows the average across all detected RNAs within a single measurement (intraassay), **Table 4** shows the average across all detected RNAs between two measurements (interassay variability), and **Table 5** shows the average across all detected RNAs excluding the control (EC).

Table 3: Intraassay variability values

			Intraassay Variability		
Kit	**RNA**	**Channel**	**1000 copies**	**100 copies**	**25 copies**
DB-1253	SARS-CoV-2	FAM	0.08 (0.05-0.10)	0.09 (0.08-0.10)	0.18 (0.12-0.21)
DB-1253	IAV H3N2	HEX	0.11 (0.06-0.14)	0.20 (0.19-0.21)	0.50 (0.45-0.58)
DB-1253	IBV	HEX	0.09 (0.06-0.11)	0.11 (0.08-0.13)	0.31 (0.31-0.32)
DB-1253	RSV-A	TEX	0.11 (0.08-0.14)	0.12 (0.08-0.16)	0.28 (0.23-0.36)
DB-1253	RSV-B	TEX	0.14 (0.03-0.19)	0.30 (0.23-0.36)	0.61 (0.51-0.76)
DB-1253	EC	Cy5	0.10 (0.05-0.13)	0.09 (0.05-0.14)	0.11 (0.08-0.14)
DB-1253	All	All	0.11 (0.03-0.19)	0.18 (0.08-0.36)	0.41 (0.12-0.76)

Table 4: Interassay variability values

			Intraassay Variability		
Kit	**RNA**	**Channel**	**1000 copies**	**100 copies**	**25 copies**
DB-1253	SARS-CoV-2	FAM	0.41 (0.18-0.57)	0.39 (0.24-0.55)	0.43 (0.28-0.59)
DB-1253	IAV H3N2	HEX	0.31 (0.11-0.41)	0.36 (0.23-0.47)	0.70 (0.51-0.91)
DB-1253	IBV	HEX	0.35 (0.18-0.48)	0.36 (0.17-0.47)	0.43 (0.25-0.56)
DB-1253	RSV-A	TEX	0.50 (0.11-0.66)	0.54 (0.20-0.73)	0.63 (0.37-0.81)
DB-1253	RSV-B	TEX	0.53 (0.22-0.72)	0.71 (0.47-0.96)	0.95 (0.41-1.20)
DB-1253	EC	Cy5	0.30 (0.12-0.44)	0.30 (0.11-0.42)	0.33 (0.10-0.48)
DB-1253	All	All	0.43 (0.11-0.72)	0.49 (0.17-0.96)	0.66 (0.25-1.20)

Table 5: **Expected C_t values**

			Expected C_t values		
Kit	**RNA**	**Channel**	**1000 copies**	**100 copies**	**25 copies**
DB-1253	SARS-CoV-2	FAM	26.66 (26.31-27.11)	29.86 (29.50-30.26)	31.72 (31.29-32.12)
DB-1253	IAV H3N2	HEX	29.55 (29.21-29.78)	32.89 (32.60-33.23)	35.03 (34.47-35.47)
DB-1253	IBV	HEX	28.27 (27.89-28.57)	31.53 (31.16-31.81)	33.51 (33.13-33.77)
DB-1253	RSV-A	TEX	29.63 (29.27-30.20)	32.61 (32.19-33.21)	34.46 (33.97-35.11)
DB-1253	RSV-B	TEX	30.11 (29.69-30.69)	33.29 (32.69-33.99)	35.05 (34.51-36.00)
DB-1253	EC	Cy5	27.20 (26.75-27.52)	27.35 (26.97-27.63)	27.39 (26.97-27.64)
DB-1253	All	All	28.84 (26.31-30.69)	32.04 (29.50-33.99)	33.95 (31.29-36.00)

2.4 Analytical sensitivity in clinical samples (reproducibility)

To determine the analytical sensitivity in clinical samples (reproducibility), 93 swab samples and 79 saliva samples (all samples were negative for all tested viruses when tested with DB-1251, DB-1253 and DB-1255 kits) were sequentially "spiked" with low RNA concentrations of various viruses (SARS-CoV-2, Influenza A H3N2, Influenza B Victoria, RSV A and RSV B). **Table 6** summarizes the results of RNA detection in swab samples, while **Table 7** summarizes the results of RNA detection in saliva samples. Any sample with a C_t in a given channel below cycle 40 was considered as a positive sample.

Table 6: Determination of analytical sensitivity (reproducibility) in nasopharyngeal swabs from 93 individuals

DB-1253	**% Positivity**			**Median C_t**		
Swab (2 µL in well)	**Native**	**Native**	**Inactiv.**	**Native**	**Native**	**Inactiv.**
RNA copies in well	**25**	**100**	**25**	**25**	**100**	**25**
SARS-CoV-2	100 %	100 %	100 %	32.0	30.0	32.0
Influenza A (H3N2)	98 %	98 %	99 %	34.7	33.7	34.9
Influenza B	100 %	100 %	100 %	33.7	31.7	33.7
RSV A	100 %	100 %	100 %	35.2	33.0	34.7
RSV B	99 %	100 %	97 %	34.3	33.3	34.7

Both native nasopharyngeal swab samples and samples after heat inactivation were spiked. 25 and 100 copies of RNA per well were added to the native swabs, and only 25 copies per well were added to the inactivated swabs. The table shows the percentage of samples that were positive, as well as the median C_t for each RNA detected for each measurement (when thresholds were set to recommended values). In the native samples, about 10 % of the samples showed a decrease in the maximum fluorescence of the curves, in some of them to such an extent that the fluorescence threshold had to be lowered from the recommended values. Even so, RNA was not detected in some wells, but these were always wells where the fluorescence in the control Cy5 channel was reduced and/or the C_t in the Cy5 channel was shifted a few cycles higher, or the sample was autofluorescent, which is also detectable at first glance. In contrast, for the heat-inactivated samples, all samples showed virtually the same target fluorescence in all channels and thus the assay after inactivation proved to be more robust. However, low RNA concentration was not detected in inactivated swabs in four samples: SARS-CoV-2 was not detected in one sample, inhibition in Cy5 (more than 3 cycles higher Ct) was present in this well and the sample would have been detected. RSV was not detected in three samples, autofluorescence was detected once and amplification with too low fluorescence would have been evaluated twice.

Table 7: Determination of analytical sensitivity (reproducibility) in saliva from 79 individuals

DB-1253	% Positivity				Median C_t			
Volume in well (µL)	**2**	**2**	**4**	**4**	**2**	**2**	**4**	**4**
RNA copies in well	**25**	**100**	**25**	**100**	**25**	**100**	**25**	**100**
SARS-CoV-2	100 %	100 %	100 %	100 %	31.5	30.4	31.8	29.7
Influenza A (H3N2)	100 %	100 %	99 %	100 %	35.8	33.7	34.6	33.6
Influenza B	100 %	100 %	99 %	100 %	33.1	32.1	33.5	31.3
RSV A	100 %	100 %	100 %	100 %	34.1	33.2	34.7	32.3
RSV B	100 %	100 %	100 %	100 %	34.8	32.6	34.2	33.1

Saliva samples were spiked after heat inactivation (native saliva samples was not tested) in volumes of 2 and 4 µL in the well. 25 and 100 copies of RNA per well were added. The table shows the percentage of samples that were evaluated as positive as well as the median C_t for each detected RNA for each measurement (thresholds were set to recommended values). In wells with 4 µL of saliva, up to 20 % of the samples showed a decrease in maximum fluorescence in one of the channels, but in the vast majority the maximum fluorescence was still several times higher than the thresholds and therefore, the thresholds did not need to be adjusted except for two samples. In wells with 2 µL of saliva, a similar decrease was rare, and the threshold did not need to be adjusted for any of the samples. RNA was not detected in only two cases, at 25 copies of RNA and 4 µL saliva: once for Influenza A and once for Influenza B. In both cases, the sample was the same, where Cy5 was inhibited by 5 and 12 cycles, respectively, and in both measurements the sample would be identified as inhibitory. Thus, measurements in volumes of both 2 and 4 µL of saliva proved to be possible, and at 2 µL, RNA was detected in every channel in every well.

2.5 Competitive interference testing (co-infection detection)

This kit can detect co-infections with two or more viruses at the same time, but if one of the channels is strongly positive, the sensitivity of detection of other viruses in other channels may be impaired. To assess the sensitivity of co-infection detection, the detection of RNA from different viruses was tested at low concentrations when present in a mixture with other viral RNA at high concentrations. Due to the high infectivity of SARS-CoV-2, co-infection with SARS-CoV-2 virus in combination with influenza or RSV is most likely and therefore we tested these combinations.

In the first set of experiments, we tested the detection of low concentrations of Influenza A, Influenza B, RSV A and RSV B RNA (concentrations of 3× LOD, 10× LOD and 50× LOD) in presence of high concentrations of SARS-CoV-2 viral RNA (with a C_t of approximately 20 cycles, corresponding to more than 10,000× LOD) and in a second set of experiments we tested the detection of low concentrations of SARS-CoV-2 RNA (3× LOD, 10× LOD and 50× LOD) in the presence of high concentrations of Influenza A, Influenza B, RSV A and RSV B viral RNA (with a C_t of approximately 25 cycles, corresponding to approximately 1,000× LOD). **Table 8** summarizes the measured results for coinfections.

Table 8: **Ability to detect weak co-infection (competitive interference)**

Virus 1 (C_t~20)	Virus 2	Virus 2 detection	Virus 1 (C_t~25)	Virus 2	Virus 2 detection
SARS-CoV-2	IAV H3N2	50x LOD	IAV H3N2	SARS-CoV-2	3x LOD
SARS-CoV-2	IBV	50x LOD	IBV	SARS-CoV-2	3x LOD
SARS-CoV-2	RSV-A	50x LOD	RSV-A	SARS-CoV-2	3x LOD
SARS-CoV-2	RSV-B	50x LOD	RSV-B	SARS-CoV-2	3x LOD

If high concentrations of SARS-CoV-2 RNA were present in the sample, then significantly weaker amplification was observed for low concentrations of the other viruses, and all were detectable from 50× LOD and even then only with low fluorescence. On the other hand, if there was a large concentration of Influenza A, Influenza B, RSV A, or RSV B in the well and a small concentration of SARS-CoV-2, the latter was reliably detected from the lowest concentration tested, i.e., 3× LOD, and its detection was thus not affected, whereas the presence of a large concentration of Influenza B only led to lower fluorescence when detecting the lowest concentrations of SARS-CoV-2.

Detection of SARS-CoV-2 in the presence of high concentrations of RNA from other viruses is not impaired and is reliable even at low concentrations (3× LOD). In contrast, the detection of other viruses is impaired in the presence of high concentrations of SARS-CoV-2 and all viral RNAs were detected only at 50× LOD concentration and with lower fluorescence. Therefore, negativity in any channel must not be used as the only clue to exclude infection with a given virus.

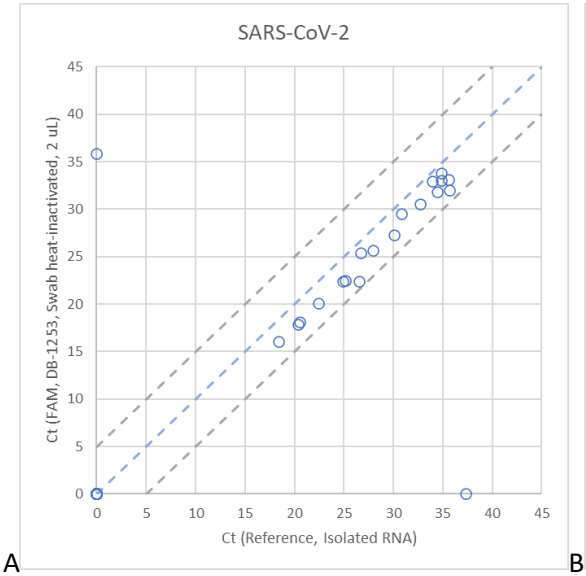
2.6 Clinical Performance – results summary

A total of 186 nasopharyngeal swab samples and 386 saliva samples collected from individuals in the European population for indicated and preventive testing for COVID-19 and other respiratory diseases were tested with at least two reference kits for each virus and the results were compared with the detection of these viruses by this kit. RNA was isolated from the samples using the DB-1206 Automated RNA Isolation Kit from DIANA Biotechnologies. Subsequently, individual viruses were detected in the isolated RNA using reference kits according to the instructions of the manufacturer of the respective RT-PCR kit, either in multiplex or individually (depending on the design of the reference kit). However, viruses were detected with this kit using a protocol without RNA isolation, always in both 2 and 4 µL of saliva and swabs. Two nasopharyngeal swab samples were also in inhibitory media and inhibited the RT-PCR reaction and were therefore excluded from the analysis, another 10 were excluded at the 4 µL measurement because they had already inhibited in larger volume. The positive swab and saliva samples for SARS-CoV-2 are from 2022 and are the omicron variant. RSV-positive swab samples are from 2021 and RSV-positive saliva samples are from 2022. Influenza A-positive swab samples are from 2019, Influenza B-positive swab samples are from 2017 and 2018, and both Influenza A- and B-positive saliva samples are from 2022. The samples came from several different laboratories and the swabs were in different transport media.

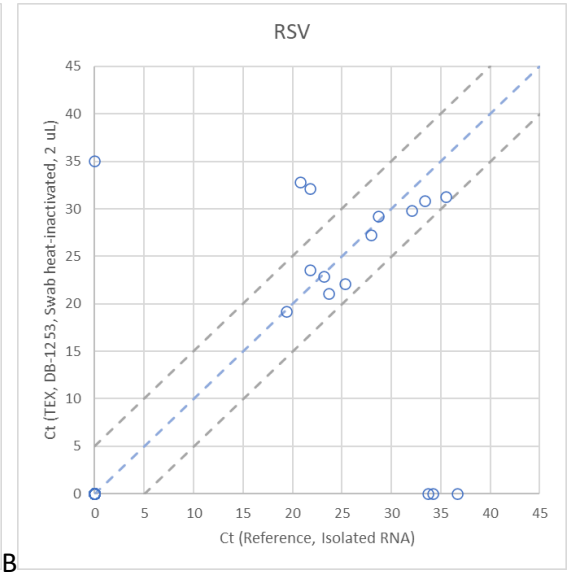
The comparison results for 2 µL of nasopharyngeal swabs are shown in **Figure 1**, and for 2 µL of saliva in **Figure 2**, where the graphs compare the measured C_t values of this kit and the reference kits (each virus in one graph). The numbers of TP (“true positives”), FN (“false negatives”), TN (“true negatives”) and FP (“false positives”) are shown in **Table 9**. The PPA ("positive percent agreement") and NPA ("negative percent agreement") values are also calculated in the table. In

the case of several FN samples, these were samples in which coinfection occurred, with one of the viruses (SARS-CoV-2) at a high load and the other virus at a low concentration (C_t around cycle 35). SARS-CoV-2 was detected in these samples, but a second virus was not detected at low concentrations. Since we also compared the results with kits that detected the viruses individually (and SARS-CoV-2 was not in the multiplex), the PPA* value is calculated in the table, in which these FN results were not taken into account (or were considered as TN).

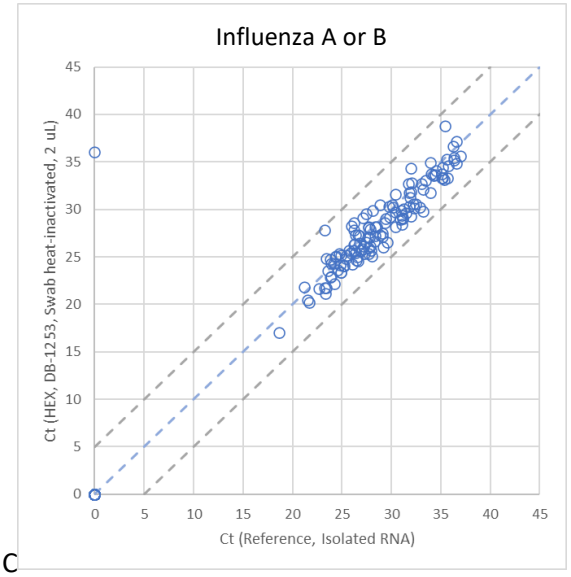
The "positive percent agreement" (PPA) of the device was 99.2% for SARS-CoV-2, 100.0% for Influenza A and B and 95,1% for RSV in saliva and swab samples, while "negative percent agreement" (NPA) for the given viruses reached 97.6%, 98.6% and 97.8% respectively. The PPA and NPA values demonstrate that the product is suitable and effective for the detection of RSV, Influenza A and B and SARS-CoV-2 viruses.



A



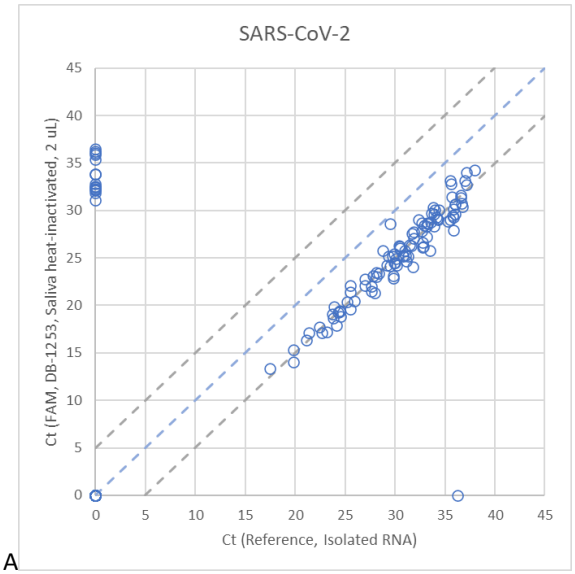
B



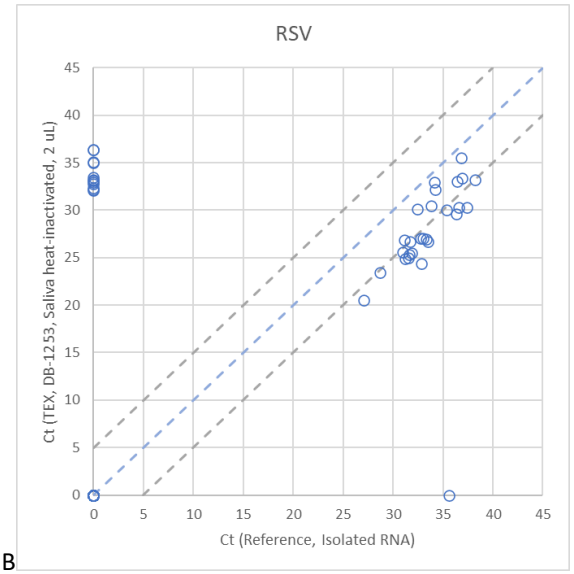
C

Figure 1: **Comparation of measured C_t for 2 µL of nasopharyngeal swab samples with reference measurements**

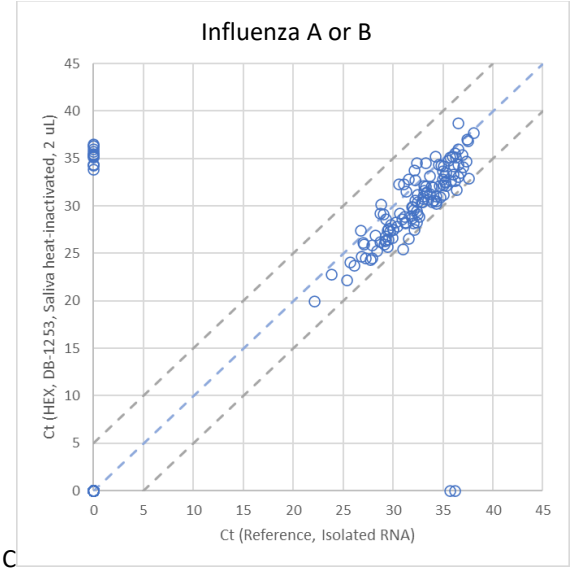
The detection of each virus is plotted in one graph, the **y-axis** of the graph plots the corresponding channel of the DB-1253 testing kit. Specifically, the C_t from the measurements by this testing kit (**y-axis**) is plotted against the C_t measured by the reference testing kits (**x-axis**). If the signal was measured in only one reference kit (C_t < 37), then this C_t is plotted. If the C_t in both sets was lower than 40 (and in at least one set lower than 37), then the average of both C_t's is plotted. Values lying on the x-axis indicate that the sample was detected only in the reference kits, while values on the y-axis indicate that the sample was detected only in the DB-1253 kit. The effect of media inhibition was observed in two RSV samples, which had significantly higher C_t compared to the reference kits that worked with isolated RNA. When remeasured after RNA isolation using the DB-1252 kit with the same set of primers, both samples were detected with the usual C_t, confirming that the C_t is worsened due to medium inhibition and not due to primers. Simultaneously, the average C_t values detected using the DB-1253 kit were lower than the C_t values detected in the reference kits (for all detected viruses), indicating potentially more sensitive detection by the DB-1253 kit.



A



B



C

Figure 2: **Comparation of measured C_t for 2 µL of saliva samples with reference measurements**

The detection of each virus is plotted in one graph, with the corresponding channel of the DB-1253 testing kit plotted on the **y-axis** of the graph. Specifically, the C_t from the measurements done using this testing kit (**y-axis**) is plotted against the C_t measured using the reference testing kits (**x-axis**). If the signal was measured in only one reference kit (C_t < 37), then this C_t is plotted. If the Ct in both sets was lower than 40 (and in at least one set lower than 37), then the average of both C_t's is plotted. Values lying on the x-axis indicate that the sample was detected only in the reference kits, while values on the y-axis indicate that the sample was detected only in the DB-1253 kit. For SARS-CoV-2, Influenza A/B and RSV, many samples were detected only in the DB-1253 kit and not in the reference kit (see points on the y-axis). A closer inspection of the data (**Table 9**) shows that a large proportion of these samples had borderline positive result in some of the reference analyses and the rest of the samples were probably detected thanks to the higher detection sensitivity of DB-1253 testing kit. As all these samples are with C_t > 30 and at the same time the C_t values in the DB-1253 are lower than the reference kits by an average of five cycles for SARS-CoV-2 and RSV and four cycles for Influenza A/B, there is a good chance that these are weak samples that were not detected in the reference measurement.

Table 9: **Results for NPA a PPA determination of nasopharyngeal swabs and saliva samples (2 µL)**

Sample	Interpretation	TP	FN	TN	FP	FP[2]	FP[1]	PPA	PPA*	NPA
Nasopharyngeal swabs	SARS-CoV-2	18	1	164	1	0	0	94.7%	94.7%	99.4%
	Influenza A/B	128	0	55	1	1	0	100.0%	100.0%	100.0%
	RSV	12	3	168	1	0	1	81.3%	86.7%	100.0%
Saliva	SARS-CoV-2	100	1	250	17	6	4	99.1%	99.1%	97.3%
	Influenza A/B	120	2	231	15	8	5	98.5%	100.0%	99.1%
	RSV	26	1	329	12	0	0	96.3%	100.0%	96.5%
Nasopharyngeal swabs + saliva	SARS-CoV-2	118	2	414	18	6	4	98.5%	98.5%	98.1%
	Influenza A/B	248	2	286	16	9	5	99.2%	100.0%	99.3%
	RSV	38	4	497	13	0	1	90.7%	95.1%	97.6%

All samples were first measured with two reference kits and all samples with a C_t in at least one of the kits below 37 cycles were considered as positive. Subsequently, the samples were measured with the DB-1253 kit and samples that had a C_t below 37 and/or below 40 and were also positive in the reference were

considered positive. If the positivity matched with the reference, then they were considered **TP**, if they were positive only in DB-1253 then they were **FP**. In the case of FP, it was further examined whether these samples were borderline positive in one reference set (one sample had a C_t between 37 and 40 and the other was negative, column **FP[1]**) or both reference sets (both had a sample C_t between 37 and 40, column **FP[2]**). These positive samples were treated 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 were considered as **FN**, while samples negative in both DB-1253 and the reference were considered as **TN**. **PPA** was calculated as the number of TP divided by the sum of TP and FN, while **NPA** was calculated as TN decided by the sum of TN and FP. **PPA*** was calculated in the same way as PPA, but FN samples were considered TN if it was true that no virus was detected with C_t >30 that was present in the sample at the same time as another virus with C_t <25. That is, unless a virus was detected that was in the sample at the same time as another virus with a much higher load, see text for details. There were four samples in total.

Table 10: Results for NPA a PPA determination of nasopharyngeal swabs and saliva samples (4 µL)

Sample	Interpretation	TP	FN	TN	FP	FP[2]	FP[1]	PPA	PPA*	NPA
Nasopharyngeal swabs	SARS-CoV-2	19	0	155	0	0	0	100.0%	100.0%	100.0%
	Influenza A/B	118	0	55	1	0	1	100.0%	100.0%	100.0%
	RSV	12	3	159	0	0	0	80.0%	85.7%	100.0%
Saliva	SARS-CoV-2	100	1	246	22	6	6	99.1%	99.1%	96.1%
	Influenza A/B	120	2	233	15	8	3	98.5%	100.0%	98.3%
	RSV	27	0	330	11	0	0	100.0%	100.0%	96.8%
Nasopharyngeal swabs + saliva	SARS-CoV-2	119	1	401	22	6	6	99.2%	99.2%	97.6%
	Influenza A/B	238	2	288	16	8	4	99.2%	100.0%	98.6%
	RSV	39	3	489	11	0	0	92.9%	95.1%	97.8%

All samples were first measured with two reference kits and all samples with a C_t in at least one of the kits below 37 cycles were considered as positive. Subsequently, the samples were measured with the DB-1253 kit and samples that had a C_t below 37 and/or below 40 and were also positive in the reference were considered positive. If the positivity matched with the reference, then they were considered **TP**, if they were positive only in DB-1253 then they were **FP**. In the case of FP, it was further examined whether these samples were borderline positive in one reference set (one sample had a C_t between 37 and 40 and the other was negative, column **FP[1]**) or both reference sets (both had a sample C_t between 37 and 40, column **FP[2]**). These positive samples were treated 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 were considered as **FN**, while samples negative in both DB-1253 and the reference were considered as **TN**. **PPA** was calculated as the number of TP divided by the sum of TP and FN, while **NPA** was calculated as TN divided by the sum of TN and FP. **PPA*** was calculated in the same way as PPA, but FN samples were considered TN if it was true that no virus was detected with C_t >30 that was present in the sample at the same time as another virus with C_t <25. That is, unless a virus was detected that was in the sample at the same time as another virus with a much higher load, see text for details. There were three samples in total.

3 Safety notice

The kit is designed for professional use only. Follow the general principles of chemical safety. Wear protective equipment (gloves, goggles) and avoid contact with chemicals and refrain from eating or drinking in laboratory areas.

Kit components contain 0.02% sodium azide, which is toxic and upon contact with acids produces toxic gas. The material safety data sheet (MSDS) will be provided on request.

If you work 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 areas. Work with infectious samples only in BSL2+ or BSL3 laboratories. Dispose the potentially infectious waste in accordance with applicable legislation.

Continuously check that the work area is free of spilled solutions, chemicals, and / 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, Texas Red and Cy5 channels – **follow the operator's manual for your cycler**
- Calibrated handheld pipettes / multichannel pipettes
- Gloves and other protective equipment

4.2 Recommended laboratory equipment

- Benchtop vortex and centrifuge
- For testing of saliva samples: Incubator with forced air circulation preheated to 90 °C (can be used for swab inactivation as well)
- For use of automated protocol: pipetting robot (e.g., liquid handling station Agilent Bravo)

4.3 Required material which is not included in the kit

- Disposable pipette tips (filter tips recommended)
- Disposable tubes for mixing the components
- PCR plate and adhesive optical foil to seal the plate
- Protocol and plastic needed for automation (e.g., Extension kits DB-1222, DB-1226 compatible with Agilent Bravo liquid handling station)

4.4 Kit components

Table 11: DB-1253 kit components

Kit [7]	Kit component [7]	REF code [6]	Volume (µL) [5]	Storage conditions	Cap number and color
DB-1253-100rxns	Enhancer mix (4x)	RF02930	500	-20 °C [2,3]	1
	Primer mix (4x)	RF02074	500	-20 °C [1,2,3]	2
	Enzyme mix (4x)	RF01000	500	**-80 °C** [2,3]	3
	Positive control A	RF02991	150	-20 °C [2,3]	4A
	Positive control B	RF02557	150	-20 °C [2,3]	4B
	External control	RF02505	500	-20 °C [2,3]	5
	Negative control	RF05353	150	-20 °C [2]	6
DB-1253-1000rxns	Enhancer mix (4x)	RF02930	5000	-20 °C [2,3]	1 [4]
	Primer mix (4x)	RF02074	5000	-20 °C [1,2,3]	2 [4]
	Enzyme mix (4x)	RF01000	5000	**-80 °C** [2,3]	3 [4]
	Positive control A	RF02991	2x 750	-20 °C [2,3]	4A
	Positive control B	RF02557	2x 750	-20 °C [2,3]	4B
	External control	RF02505	5000	-20 °C [2,3]	5 [4]
	Negative control	RF05353	2x 750	-20 °C [2]	6

[1] Store at dark compartment (contains fluorophores, which are photosensitive). **[2] Store the kit frozen at -80 °C**; components except for Enzyme mix (4x) can be stored also at -20 °C. Do not use the kit if any of the components is not frozen upon arrival. **[3] Minimize number of freeze/thaw cycles**, aliquot controls after first thawing. **[4]** Components #1-3 have transparent caps and external control (#5) has violet cap in 1000rxns kit size. **[5]** Actual volume of all reagents is approximately 5-10% higher compared with volumes stated in table 11. **[6]** REF codes and LOT numbers of the kit and each component are indicated on the kit packaging. Keep the packaging for reference until the entire kit is used. Do not mix kit components from different LOTs of the kit. **[7]** Kit packaging and components of the kit contain barcodes with general information.

Enhancer Mix (4x)

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

Primer Mix (4x)

Contains primer pairs and fluorescently labelled probes for detection of SARS-CoV-2 (FAM), Influenza A and Influenza B (HEX), RSV (TEX) and external control (Cy5) Provided as 4x concentrate.

Enzyme Mix (4x)

Contains thermostable Reverse Transcriptase, hot-start Taq DNA Polymerase, nucleotides, buffer, salts, detergents, RNAse inhibitors and other RNA protecting components, and other proprietary additives for extracting RNA from the virus. Provided as 4x concentrate.

Positive control A and Positive control B

Positive control 4A contains mix of genomic RNAs of SARS-CoV-2, Influenza A H3N2 and RSV B. Positive control 4B contains mix of genomic RNAs of SARS-CoV-2, Influenza B Victoria, and RSV A. Opening these vials may cause contamination of the workspace, thus always **spin down vials before opening**!

External control and Negative control

External control contains artificial RNA of length over 2000 bases which is added to each RT-PCR reaction after the sample has been added and thus reveals possible inhibition of Reverse Transcriptase or Polymerase as well as RNA degradation by the sample. Negative control contains PCR grade water.

4.5 Stability of kit components and master mix

The DB-1253 DBdirect™ Respiratory panel 2: SARS-CoV-2/Flu/RSV kit is an innovative formulation of an RT-PCR mix that can extract viral RNA without prior RNA isolation. In addition, the composition allows for RT-PCR analysis to be performed in the presence of a sample without inhibiting or degrading RNA. For this reason, some components contain reagents not commonly used in RT-PCR and have special storage requirements. Based upon thorough stability tests of the kit it is necessary for the time being to store some of the components at **a low temperature of -80 °C** so that the functionality of all components is maintained and the detection in biological matrices is robust. For the same reason, the number of freeze/thaw cycles of some components is limited. The shelf life of the whole kit is indicated on the box.

Component 1 (Enhancer Mix) and Component 2 (Primer Mix) should be kept at -20 °C or -80 °C for long-term storage. Avoid repeated freezing/thawing, never exceed four freeze/thaw cycles. If you intend to use the components more than once, aliquot them after the first thawing.

Component 3 (Enzyme Mix) should be kept at -80 °C for long-term storage. Avoid repeated freezing/thawing, prepare disposable aliquots after the first thawing. However, when thawing at approx. 25 °C and freezing at -80 °C, this component can be thawed/frozen up to four times.

Components 1, 2 and 3 are stable for at least 4 hours at 25 °C until mixed together. However, we recommend using the components as soon as possible after thawing. Keep the components of the kit out of direct sun light, exposure to normal daylight for at least 8 hours does not affect their function. However, the primer mix should be kept in a dark place for long-term storage.

RT-PCR Master Mix (mixture of components 1, 2 and 3; see section 5.6) is stable for up to 2 hours at 25 °C but we recommend using the RT-PCR master mix (i.e., mixing it with the sample) within 30 minutes of its preparation. Keep the master mix out of direct sun light, exposure to normal daylight for at least 8 hours does 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).

Components 4 (positive control) and 5 (external control) contain RNA, thaw them for the necessary time only and store on ice; however, they can be stored cumulatively for at least 24 hours at room temperature. The maximum number of freeze/thaw cycles is nine for the external control and four for the positive control. The optimum temperature for long-term storage of both controls is -80 °C, but they can also be stored at -20 °C.

Negative control (PCR water) can be kept for long-term storage at -20 °C or below and can be repeatedly frozen and thawed. For faster thawing, we recommend preparing smaller aliquots.

5 Instructions for Use

5.1 General precautions

- Do not use kit components, which are not frozen, or which are damaged upon receipt. Keep the kit components for a possible claim and contact the manufacturer.
- Inappropriate handling of kit components and deviations from these instructions for use may adversely affect results.
- Use the corresponding version of these instructions for use (see page header) to that referred to on the package.
- Do not use kit components after kit expiry date indicated on the package.
- Do not mix kit components from different lots of the kit (indicated on the vial).

5.2 Avoiding contamination (false positives)

Good laboratory practice to avoid sample cross-contamination should be followed, including the use of disposable filtered pipetting tips, and using a clean tip for each step.

Handling of clinical samples, positive controls (template RNA), or amplified PCR products (template DNA) should be spatially separated from handling of the stock kit components 1, 2 and 3 to avoid their contamination. The best practice is to prepare the RT-PCR master mix from components 1, 2, and 3 and to transfer this mix into the PCR plate at designated location (e.g., PCR box), in which RNA or DNA templates are never handled. This space should also have assigned equipment (e.g., pipettes, tips, and vials) not used for other purposes (i.e., never used for handling of RNA/DNA templates). PCR plates with master mix are then transferred to other locations (e.g., other PCR box), where the samples or positive controls are added.

Some other general guidelines to avoid accidental contamination:

- **Never open the vial/plate with amplified PCR products.**
- Never open or otherwise handle samples, positive controls, or amplified PCR products in locations where the RT-PCR master mix is prepared.
- Before handling template RNA/DNA close other vials with reagents, and always spin down the vial with positive control before opening.
- 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 control for false-positive and false-negative results, positive and negative controls need to be added to each RT-PCR reaction mixture. Use the provided negative control (PCR grade water, vial #6) or blank sample medium as the negative control. Ideally use both positive controls that are provided (Positive control A, and Positive control B; vials 4A and 4B), or at least one of them.

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 the fridge).
- Spin down each vial to collect all liquid at the vial bottom before opening.
- Mix reagents in vials using vortex or pipetting before use. The pipette should be set to at least ½ the volume of the solution to be mixed, several pipetting up and down is required for proper mixing. Adequate mixing is particularly important before dividing into aliquots. If you vortex the vial, always spin it down briefly before opening.

5.5 Storage and preparation of samples

Saliva samples

Use DB-1225, DB-1230, or DB-1249 collection sets for saliva testing. Saliva samples can be stored in these collection sets for at least 72 hours at 4 °C to 25 °C. Samples can also be frozen at least once (at -80 °C). If you use other collection kits, consult their selection with the manufacturer's representative, never use kits that contain inactivation solutions for the stabilization of RNA or DNA.

In case of saliva, the sample needs to be first thermally inactivated, which will both reduce the infectivity of the sample and increase the sensitivity of detection. If saliva is in the DB-1225, DB-1230, or DB-1249 kit collection tubes, place the closed tubes in a 96-well rack (DB-1228 or DB-1240) so that they fill every second row (thus each rack fits 48 samples). Then, place the rack in a forced air circulation incubator preheated to 90 °C for 30 minutes (never exceed 60 minutes). For a forced air circulation incubator with an internal volume of 60 L, proceed as follows: first, place two 1 liter (or four 0.5 liter) bottles filled with water in the incubator and allow them to warm up for 4 hours, and only then use the incubator to inactivate samples (this will maintain the same conditions for all samples regardless of their number). Place a maximum of 8 half-filled racks, i.e., 384 samples, in the incubator at a time. If you use other collection kits or an incubator with a different internal volume, then calibration/validation needs to be done. Saliva needs to be inactivated by heating the sample to 80 °C for at least 10 minutes. You can achieve this, for example, by transferring 50 µL of the sample to a PCR plate or other micro-tubes and incubating for at least 10 minutes at 80 °C in a thermal cycler (other tubes inserted in a thermo-block can also be used; do not exceed 30 minutes at 80 °C). It is the user's responsibility to properly validate the thermal inactivation procedure for other sampling sets or incubators with different internal volumes. Cool the samples to room temperature by either keeping them at room temperature for another 30 minutes, or at 4 °C for 10 minutes. It would be very difficult to pipette the samples without cooling. It is not necessary to test saliva immediately after inactivation, the viral RNA in it is stable for another 72 hours at 25 °C.

Before pipetting, spin down the saliva at 100 to 200 g for 1 to 2 minutes, this should be enough for spinning down the saliva from the cap and the cap thread, and facilitate better pipetting by spinning down the largest precipitate or other debris in the saliva. Prolonged spinning or spinning at higher speeds may result in the loss of detection sensitivity.

Nasopharyngeal swab samples

Use collection kits containing common transport media (e.g., Copan Universal transport medium for viruses (UTM), or IMPROVIRAL™ Viral Preservative Medium (VPM)) for swabs; inactivation media are not suitable. We recommend storing swabs in the transport medium according to the instructions of the manufacturer of these media. In VPM medium, swabs can be stored for at least 48 hours at 4 °C, and they can be frozen at least once (at -80 °C).

The kit has been validated for more than 10 media (see Section 1.4 for more information or contact the manufacturer's representative for the current datasheet). It is the user's responsibility to validate the use of the kit for any additional media that has not been validated by the manufacturer. To use the kit with a specific media type, it is always necessary to validate by comparing the results of detection of viral RNA in positive samples in that media by RT-PCR after RNA isolation and by direct detection in samples using the kit. At least 10 positive samples should be tested and at least a few of them should be weakly positive with a $C_t > 30$.

Thermal inactivation of the swab samples is not necessary; however, it can be performed to increase safety. Moreover, in some samples it can lead to sensitivity increase. Validation of the precise inactivation procedure for any medium is the responsibility of the user: it is necessary to measure at least 10 positive samples (of which few are weakly positive) using direct detection with this kit without and after thermal inactivation. The inactivation protocol is needed to validate for each used media, since their properties can with inactivation vary considerably. We recommend as initial thermal protocol 15 to 30 minutes at 65 °C. Precipitation of media occurs during longer inactivation times or higher temperatures (typically above 80 °C) which leads to decrease of sensitivity in determination of sample.

If you have heated the samples, let them reach room temperature before pipetting, otherwise they may be difficult to pipet (required if you use a pipetting robot to add samples). If necessary, spin the swabs for 1 to 2 minutes at 100 to 200 g before pipetting. Longer or higher-speed spin may result in loss of detection sensitivity.

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 an RT-PCR master mix for multiple reactions, multiply the volumes by the number of reactions (and add the pipetting reserve) - see also **Table 12**.

1. Thaw and mix all components of the RT-PCR master mix (see **Table 12** and Section 4.4).
2. Pipette **5 µL of Enhancer mix (4x)** into a clean RNase/DNase free vial, **vial #1 (green or clear cap**) in the kit.
3. Add **5 µL of Primer Mix (4x)** to the same vial, **vial #2 (blue or clear cap)** in the kit and mix by repeated pipetting.

4. Add **5 µL of Enzyme Mix (4x)**, in kit **vial #3 (black ● or clear cap)**, to the same vial and mix by repeated pipetting until the mixture is homogeneous (you can also use vortex and spin down briefly at the end).
5. Transfer **15 µL of the mixture (RT-PCR master mix)** into a 96-well plate or 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 the RT-PCR mix).

A dead volume is required for automated plate preparation with RT-PCR mix and sample addition. The total reaction volume and sample volume can therefore be reduced by 10 % compared to the values given in Chapters 5.6 and 5.7 without affecting the detection sensitivity.

Table 12: RT-PCR master mix preparation

Kit components	# and cap color*	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
Total volume of RT-PCR Master Mix		15	1500

* The numbering of the vials corresponds to the order in which the ingredients are added, it is important to keep this order.

Aliquoting solutions for RT-PCR master mix preparation

The numbers and volumes of aliquots of each component of this kit for 1000 tests (1000rxns) are summarized in **Table 11**. If you do not want to use the whole kit at once, it is advisable to prepare single aliquots after the first thawing. To prepare single aliquots for analysis of 96 samples (e.g., using an Agilent Bravo pipetting station and DB-1222 or DB-1226 kits), proceed as follows:

Prepare **10 aliquots of 510 µL** each of Enhancer mix (4x), Primer mix (4x) and Enzyme mix (4x) and **freeze as soon as possible at -80 °C**. To prepare the RT-PCR master mix for analysis of 96 samples, then mix one aliquot of each component and use the entire RT-PCR mix. Details on stability and storage are given in Section 4.5.

Alternatively, you can prepare a ready-made RT-PCR master mix by mixing these three components and after mixing, prepare **10 aliquots of 1530 µL, freeze as soon as possible at -80 °C and use within a month**. Do not refreeze this mix, always use the entire aliquot.

5.7 Adding sample and external control to the RT-PCR reaction

Saliva samples

Add 2 µL of the heat-inactivated and spun saliva sample to wells/microtubes containing 15 µL of RT-PCR master mix and rinse the tip by repeatedly aspirating and dispensing the liquid in the well/microtube while adding the sample. If any precipitate has formed in the saliva during inactivation, try to collect the liquid above the precipitate. Visually check that the saliva has first been aspirated into the tip and then dispensed. Further mixing of the reaction mixture is not necessary.

Next, add **3 µL of RNA from the External control vial (vial #5, purple cap 🟣)** and rinse the tip by repeatedly aspirating and dispensing the liquid in the well/microtube. **Always add the external control after the sample**. Further mixing of the reaction mixture is not necessary.

These volumes are suitable for manual and automated pipetting, but it is possible to add a larger sample volume and a smaller control volume to increase detection sensitivity: **4 µL of sample and 1 µL external control.** If you are adding samples and controls automatically, set the pipetting program so that the sample and control are mixed with the RT-PCR mix immediately after addition.

Nasopharyngeal swab samples

Add 2 µL of the swab sample to the wells/microtubes, containing 15 µL of RT-PCR master mix, and rinse the tip by repeatedly aspirating and dispensing the liquid in the well/microtube during sample addition. Visually check that the sample has first been drawn into the tip and then dispensed. No further mixing of the reaction mixture is necessary.

Next, add **3 µL of RNA from the External control vial (vial #5, purple cap 🟣)** and rinse the tip by repeatedly aspirating and dispensing the liquid in the well/microtube. **Always add the external control after the sample**. Further mixing of the reaction mixture is not necessary.

These volumes are suitable for manual and automated pipetting. If you are adding samples and controls automatically, set the pipetting program so that the sample and control are mixed with the RT-PCR mix immediately after addition.

As with saliva samples, a larger volume of sample and a smaller volume of control (4 µL of sample and 1 µL of external control) can be added to the Master mix to increase sensitivity. However, some media may partially inhibit the RT-PCR assay at this volume and if you wish to use this increased volume, contact the manufacturer's representative to consult whether the media you are using is suitable and validate the procedure on positive samples for each media.

General procedures and use of controls

⚠ An external control must always be added to each well with each sample because if the RT-PCR reaction is inhibited by the sample, this control will detect the inhibition. For proper function, **the external control must always be added after the sample is added**, not the other way around.

After adding the samples and the external control to the 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 run must contain at least one positive and one negative control.** However, the kit includes two positive controls (Positive control A and Positive control B, vials 4A and 4B) and it is recommended to use both controls for each plate. In case of a positive control, add **2 or 4 µL Positive control from vial 4A or 4B (red cap 🔴) instead of the sample.** In case of a negative control, **add 2 or 4 µL Negative control from vial #6 (PCR grade water, white cap ⚪)** instead of the sample. Then **add 1 or 3 µL External control from vial #5 (purple cap 🟣).** Choose the same

volumes for the positive, negative and external controls as you chose for the samples. The total reaction volume after adding the sample and controls is 20 µL.

We also recommend using an additional control – a known positive clinical sample in the same medium as the other samples, or the same biological sample type (e.g., saliva) on each plate. We recommend to aliquoted this control and use repeatedly (control not included in the kit).

A dead volume is required for automated plate preparation with RT-PCR mix and sample addition. The total reaction volume and sample volume can therefore be reduced by 10 % compared to the values given in Chapters 5.6 and 5.7 without affecting the 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 that are capable of simultaneous detection in FAM, HEX, Texas Red (TEX) and Cy5 channels, but it is up to the user to set up the instrument correctly and verify the performance of the kit using appropriate controls. For instructions on setting up the RT-PCR protocol and detection in the relevant channels, please refer to the user manual of the respective instrument.

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

For detection in four channels, use the default instrument settings and filter settings in **Table 13**.

Table 13: Filter settings for RT-PCR detection on BioRad instruments.

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

* The abbreviation of the fluorophore as given here in the text is given in parentheses and the name of the fluorophore as described in the CFX Maestro software is given before the parentheses.

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 14**.

Set the "sample volume" to 20 µL.

Table 14: 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	40
Hold time (hh:mm:ss)	00:10:00	00:02:00	00:00:05	00:01:00	00:00:30
Plate read	NO	NO	NO	YES	NO

The approximate length of this protocol on BioRad instruments is 1 hour and 40 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. We recommend using logarithmic scale to display fluorescence, as well as using color compensation between the FAM and HEX channels, if available on your device. Below are the recommended threshold values for selected instruments, however, 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 auto-fluorescence of the samples (especially saliva), and thus due to a higher background, or due to the differences between instruments (although supplied by the same manufacturer).

For BioRad CFX96™ and BioRad CFX Opus 96, we recommend using the default settings for FAM, HEX, Texas Red (TEX) and Cy5 channels. To determine C_t values use the default Single Threshold mode* method with **manually thresholds values set: 200 RFU for FAM and HEX; 100 RFU for TEX and Cy5.** Because biological samples can be autofluorescent in PCR, manually selected thresholds may need to be increased from the above values. **All C_t values reported in sections 5.9.2 and 5.9.3 correspond to the evaluation with the above-mentioned thresholds.**

In any case, the measured data must be visually checked because the use of threshold fluorescence for the calculation of C_t may result in incorrect evaluation of the curves. For example, the Single Threshold mode method may evaluate a negative sample as a positive sample due to an unusual shape of the curve - e.g., a spike in fluorescence due to a bubble in the reaction mixture, etc. All curves with a steep and steady increase in fluorescence must be evaluated as positive (see Chapter 5.9.4 for the standard curve shape), while other curves must be evaluated as negative (see Chapter 5.9.3 under Table 15 for the appearance of problematic curves). In BioRad instruments we observe about 2% fluorescence bleed-through from FAM to HEX channel. In case of high fluorescence in FAM (FAM fluorescence can reach RFU values over 10 000) it can be wrongly evaluated as a positive HEX channel, even if it is only a bleed-through. We therefore recommend that for wells with FAM fluorescence greater than 5,000 RFU, a HEX threshold of at least 300 RFU (instead of 200 RFU) should be used.

The C_t values obtained will vary depending on the RT-PCR instrument used, the method of evaluation and the threshold settings. Thus, C_t values cannot be used to compare samples if they were analyzed in a different run. To illustrate, the background on BioRad instruments is typically well below the above recommended fluorescence thresholds, and if you set this threshold close to the background value, then you can get C_t values 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-through from another

channel. On the other hand, if you set the fluorescence threshold too high, you can obtain C_t values 3 to 5 cycles higher with minimal risk of false positive interpretation, but you may miss weakly positive samples that do not reach this fluorescence threshold (maximum fluorescence of samples typically ranges between 1,000 and 10,000 RFU units in individual channels). Therefore, we recommend the above values as fluorescence thresholds. However, C_t values are not exactly comparable even between different measurements on the same type of instrument and with the same chosen thresholds: in our experience, fluorescence measurements vary by up to a factor of two between different runs or different BioRad instruments.

All of the expected C_t values listed below for the control evaluations and in **Table 15** 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). If another setting or method is used, the measured C_t values must be shifted by the difference between the C_t value of the positive control and the reference value C_t = 29 (for FAM, HEX and TEX channels)** for accurate interpretation according to **Table 15**. For example, if your determined C_t value for a positive control is 25, either add 4 cycles to the clinical sample C_t values or subtract 4 cycles from the threshold C_t values in **Table 15** to use **Table 15**.

** The method is 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.*

*** Applicable if 2 µL of positive control are added to the reaction; the reference cycles will be smaller by one in case 4 µL of positive control are added.*

5.9.2 Evaluation of controls

For a **positive control**, amplification must occur in three channels: viral genes in FAM, HEX and TEX (depending on the plastic, cycler, and sample volume used, expected C_t values for these channels range from 26-33 cycles). If amplification does not occur in any of these channels (i.e. C_t >35 in any of these channels), the PCR reaction has not been performed correctly and the results of such analysis are not valid and must be repeated. To determine the C_t values, use the Single Threshold mode threshold settings described above. Amplification of the external control in Cy5 channel should result in a C_t < 33 cycles, but this is not necessary for a correct assessment of a positive control.

Negative control must show amplification of the external control in Cy5 channel (<33 cycles), while there must be no amplification in FAM, HEX, TEX or Cy5 channels. Measurable amplification in any of these channels shows contamination of the reagents by template sequence which can cause false positive results. In this case, we recommend testing a larger number of negative controls.

External control must be evaluated for each sample: compare the average C_t value in Cy5 channel for the positive and the negative controls with the C_t of each sample. You do not need to evaluate the control for positive samples because the amplification of the external control may be negatively affected by the amplification of viral genes. If you only evaluate the test qualitatively, **positive samples are considered positive even if the external control fails**. For samples that are negative in FAM, HEX and TEX channels or with C_t > 37, it is necessary to compare the C_t values in Cy5 of the sample and controls. If the C_t value in Cy5 is lower or equal in the sample compared to controls, then there is no inhibition of RT-PCR and the sensitivity of viral RNA detection is not adversely affected by the sample. If the C_t in Cy5 is higher in the sample compared to the controls,

the extent of RT-PCR inhibition can be quantified in proportion to the difference (the external control is significantly more susceptible to inhibition or degradation than the viral RNA, so the shift in C_t for viral channels will always be smaller or at most the same); e.g., if the C_t in Cy5 is one cycle higher in the sample, then the inhibition is up to 50%, i.e., the analytical sensitivity of viral RNA detection is reduced up to twofold. If the C_t value in Cy5 is higher in a sample by about 3.3 cycles, then the analytical sensitivity in such a sample may be up to ten times lower (the lowest detected amount of virus may be up to ten times higher, at this and higher inhibition, a false negative result may occur in this sample if the C_t for viral channels in the uninhibited reaction is higher than about 35). Thus, for samples that have a C_t in the Cy5 channel higher by about 6.6 cycles, the analytical sensitivity of the assay may be up to 100 times lower.

We recommend excluding the results of samples with C_t values in Cy5 more than 4 cycles higher than the control and measure these samples either in a smaller volume (e.g., instead of 4 µL only 2 µL) or in an alternative way with RNA isolation before RT-PCR. The frequency of inhibited samples should be no more than 0.5% for saliva samples and virtually zero for swabs samples. In the case of inhibition in saliva samples pipetted automatically, a re-measurement in which the sample is added manually with a visual check that no inactivation precipitate has been added will usually help.

5.9.3 Results interpretation

Set the detection threshold and read out C_t values in each channel and interpret the results according to **Table 15** 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 limits listed in the previous section (i.e., the C_t for FAM, HEX and TEX channels are between cycle 26 and 33), you can interpret the measured C_t according to this table without further C_t recalculations. Otherwise, the determined C_t must be recalculated before interpreting according to **Table 15**, as described above in Section 5.9.1.

Table 15: Data interpretation

The symbol "-" in FAM, HEX and TEX channels indicates C_t > 40 cycles or an undetectable signal; **the symbol "-"** in the Cy5 channel for the external control indicates a C_t value in the sample 4 or more cycles higher than in the negative control or an undetectable signal; **the symbol "+"** in the Cy5 channel indicates a C_t in the sample that is at most 4 cycles higher than in the negative control. SARS-CoV-2 is detected in the FAM channel, Influenza A/B in HEX, RSV in TEX and External Control in Cy5. The C_t values given in this table assume analysis on BioRad instruments with evaluation according to the procedure and threshold settings described above (see sections 5.8.1 and 5.9.1).

FAM [7,8]	**HEX** [6,7,8]	**TEX** [7,8]	**Cy5**	**Interpretation**
C_t < 37	-	-	+ [1] / - [2]	**SARS-CoV-2 positive** [5]
-	C_t < 37	-	+ [1] / - [2]	**Influenza A/B positive** [5]
-	-	C_t < 37	+ [1] / - [2]	**RSV positive** [5]
C_t 37–40 [3]	-	-	+ [1]	**Weak positive for SARS-CoV-2,** repeat for confirmation [5]
-	C_t 37–40 [3]	-	+ [1]	**Weak positive for Influenza A/B,** repeat for confirmation [5]

-	-	C_t 37–40 [3]	+ [1]	**Weak positive for RSV,** repeat for confirmation [5]
C_t 37–40 [3]	-	-	-	**Weak positive for SARS-CoV-2,** RT-PCR inhibition; repeat for confirmation [4,5]
-	C_t 37–40 [3]	-	-	**Weak positive for Influenza A/B,** RT-PCR inhibition; repeat for confirmation [4,5]
-	-	C_t 37–40 [3]	-	**Weak positive for RSV,** RT-PCR inhibition; repeat for confirmation [4,5]
$C_t < 40$	$C_t < 40$	-	+ [1] / - [2]	**Example of coinfection with two viruses (SARS-CoV-2 and Influenza A/B)** [5,6,9]
$C_t < 40$	$C_t < 40$	$C_t < 40$	+ [1] / - [2]	**Example of coinfection with more than two viruses (SARS-CoV-2, Influenza A/B and RSV)** [5,6,9]
-	-	-	+ [1]	**Undetectable (negative) for all (SARS-CoV-2, Influenza A and B and RSV)** [5]
-	-	-	-	**Unreliable result:** inhibition of RT-PCR, **repeat** or **perform RNA isolation.**

[1] When 1 µL of external control is added to the reaction, the C_t value in the Cy5 channel is around cycle 29 to 30, and when 3 µL of external control is added, the C_t value in the Cy5 channel is around cycle 28.

[2] High concentrations of viral RNA detected in either channel can cause impaired amplification of the external control, resulting in reduced or absent signal in the Cy5 channel (see **Figure 3** for details). The absence of signal in Cy5 does not alter the interpretation of positive signals in FAM, HEX or TEX.

[3] Any amplification in FAM, HEX or TEX with a C_t < cycle 40 can be considered a positive result, but if the C_t value is higher than cycle 37, then the test must be repeated for confirmation (with the same sample to exclude accidental contamination or with a new sample to confirm clinical relevance). A repeated positive result in a given channel is considered as positivity for the virus.

[4] If the C_t value in the FAM, HEX or TEX channels is higher than cycle 37 and inhibition in the Cy5 channel is observed for more than 4 cycles, then the test must be repeated because RT-PCR inhibition has occurred. Alternatively, a standard assay can be performed after RNA isolation using another kit (e.g., DB-1252). A repeated positive result in a given channel is considered as positivity for the virus.

[5] Negativity in any channel must not be used as the sole clue to rule out infection with a given virus. In particular, if any of the other channels is positive, the sensitivity of detection in the other channels may be compromised. Although this kit can detect co-infections with two or more viruses simultaneously, when the signal in FAM was strong (~20th cycle), viral RNAs in the other channels were detected at concentrations up to 50x LOD and even then only with low fluorescence. In contrast, when the signal in HEX or TEX channels was strong (~25th cycle), SARS-CoV-2 detection was not affected even at 3x LOD, and thus weak SARS-CoV-2 coinfection should be reliably detected.

[6] For the interpretation of Influenza A/B and SARS-CoV-2 co-infection, it is necessary to exclude bleed-through from FAM to HEX (exclude curves with maximum RFU in HEX up to 300 if there is more than 5000 RFU fluorescence in FAM).

[7] In any channel, there may be a gradual increase in signal even in negative samples, however, typically with C_t > 30 and with a peak fluorescence of no more than 100-200 RFU. It may be necessary to increase the threshold of the channel in question to eliminate these waveforms. These "non-amplification" curves can be reliably detected by visual inspection of their shape: they have a completely different shape compared to standard amplification curves, where they lack the gradual exponential increase from the low background and end plateau.

[8] In any channel, autofluorescence of the sample can occur, manifested by high fluorescence from early cycles of the PCR protocol, typically with C_t < 10 and end fluorescence in the order of hundreds of RFU.

In addition, for saliva, some samples may have increased background fluorescence and require threshold adjustments for both the viral channels (FAM, HEX and TEX) and the external control in Cy5.

[9] If the C_t value in any of the FAM, HEX or TEX channels is between cycle 37 and cycle 40, then the test must be repeated to confirm positivity in that channel (if the test is also positive in another channel with C_t < 37, then positivity in that channel does not need to be confirmed by repeating; repeat the test with the same sample to exclude accidental contamination or with a new sample to confirm clinical relevance). A repeated positive result (Ct ≤ 40) in a given channel is considered to be a positive result for that virus.

5.9.4 Typical results

Figure 3 shows illustrative data measured with the viral RNA dilution series with the DB-1253 kit.

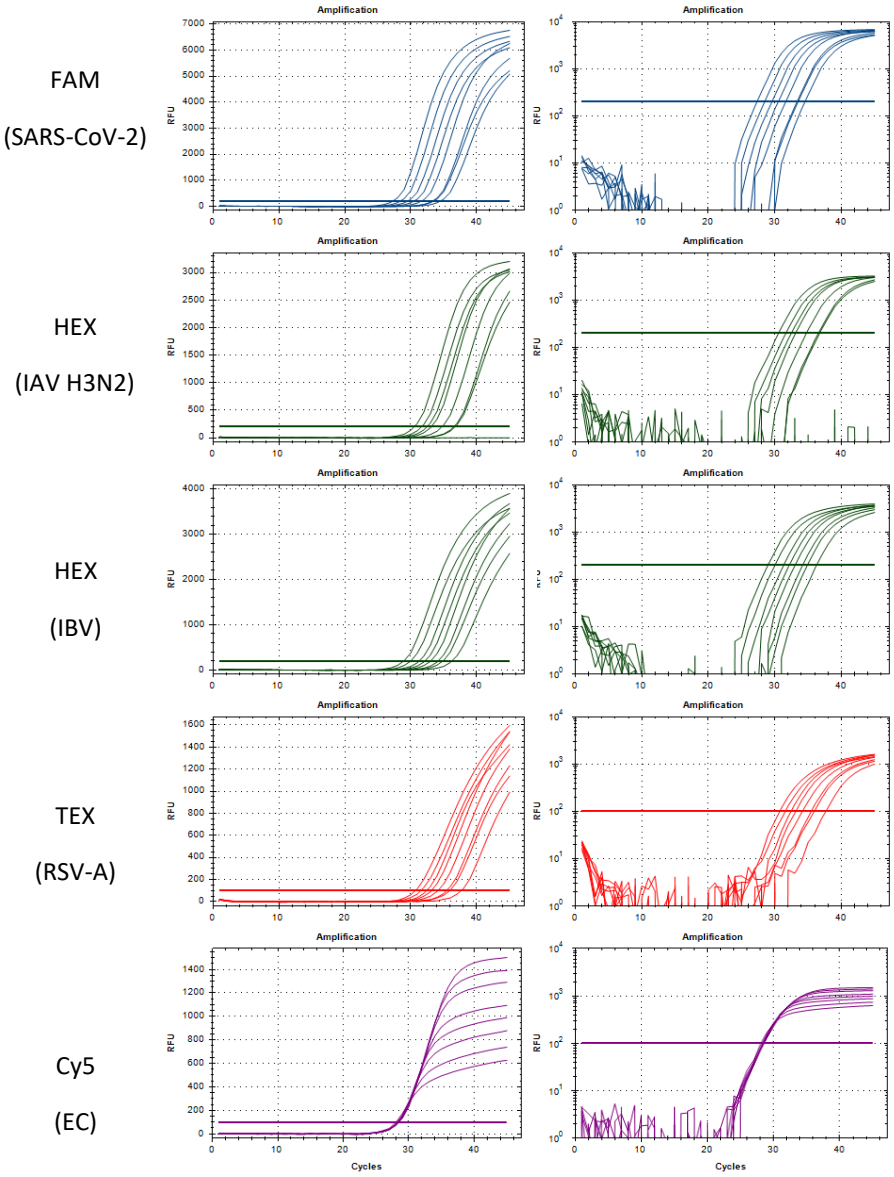


Figure 3: Detection of a dilution series of viral RNAs from 1,000 to 5 copies per well on the BioRad CFX96™ instrument.

The first pair of graphs shows signal amplification in the FAM channel (SARS-CoV-2), the second and third in HEX (Influenza A H3N2 and Influenza B), the fourth in TEX (RSV-A) and the fifth in Cy5 (external control). The graphs on the left show fluorescence in linear scale, while the graphs on the right show fluorescence in logarithmic scale. The end 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). **Material used**: FrameStar 96 Well Semi-Skirted PCR plate (4ti-0951, white wells) a LightCycler 480 Sealing Foil (04729757001).

6 Legal Notice

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7 List of compatible devices

REF DB-1224 Bravo Installation Package for DBdirect™

REF DB-1222 DBdirect™ Bravo Extension Kit for Swab

REF DB-1226 DBdirect™ Bravo Extension Kit for Saliva 1.4IM

REF DB-1225 Saliva Collection Set 1.4IM

REF DB-1230 Saliva Collection Set 1.4IF

REF DB-1249 Saliva Collection kit

REF DB-1228 Sample rack 1.4IM

REF DB-1240 Sample rack 1.4IF

8 One-page summary protocol

8.1 Kit components

Kit components	Volume (µL)		Storage Temperature	Vial Label
	100rxns	1000rxns		
Enhancer mix (4x)	500	5000	-20 °C	1
Primer mix (4x)	500	5000	-20 °C	2
Enzyme mix (4x)	500	5000	**-80 °C**	3
Positive control A	150	2x 750	-20 °C	4A
Positive control B	150	2x 750	-20 °C	4B
External control	500	5000	-20 °C	5
Negative control	150	2x 750	-20 °C	6

8.2 RT-PCR Master Mix preparation

- After thawing, mix all ingredients, spin each vial before opening.
- 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 ingredient using pipet.
- Transfer 15 µL of this RT-PCR master mix into a 96-well plate, add 2 µL or 4 µL of saliva (after heat inactivation) or 2 µL of swab (prior validation required for 4 µL swab), then add up to 20 µL of external control (vial #5): 3 µL and 1 µL, respectively, then seal the plate with optical film and run the RT-PCR reaction as soon as possible.
- For positive and negative controls, add an equal volume of positive (vial 4A or 4B) or negative control (PCR water; vial #6) in place of the sample and make up to 20 µL with external control.

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
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	40
Hold time (hh:mm:ss)	00:10:00	00:02:00	00:00:05	00:01:00	00:00:30
Plate read	NO	NO	NO	YES	NO

Scanning must be set for simultaneous detection of FAM, HEX, TEX and Cy5 channels. The setting for detection can be found in chapter 5.8 and in the manual of the respective instrument.

9 Graphic symbol used

	Manufacturer
	Caution
	Lot Number
	Operator´s manual, operating instructions
	Catalogue Number
	Component volume
	Package contains
	Positive control
	Negative control
	Upper limit of temperature* (*where X stands for particular temperature)
	Do not use if package is damaged
	Use by date
	Do not reuse
	Amount (No. of reactions)** (**for n tests according to the kit variant)
	CE marking
	Diagnostic medical device *in vitro*