DB-1252

RT-PCR Respiratory panel 2:

SARS-CoV-2/Flu/RSV

Instructions for Use

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CE IVD

REF DB-1252-100rxns containing reagents sufficient for 100 reactions

REF DB-1252-1000rxns containing reagents sufficient for 1000 reactions

Contents

1 Preface

1.1 Intended purpose and kit usage

RT-PCR Respiratory panel 2: SARS-CoV-2/Flu/RSV is intended for simultaneous detection and differentiation of nucleic acids of SARS-CoV-2, influenza (Influenza A, IAV and Influenza B, IBV) and Respiratory syncytial virus (RSV) using one-step RT-PCR protocol from RNA isolated from a variety of 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 differential diagnosis of SARS-CoV-2, Influenza A/B 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 samples of the human upper respiratory tract during infection. A positive result of the test proves the presence of SARS-CoV-2, Influenza A/B and/or RSV RNA. However, a positive result for any virus detected does not rule out bacterial infection or co-infection with other viruses, including other viruses tested negative in this kit. A 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.

RT-PCR Respiratory panel 2: SARS-CoV-2/Flu/RSV is intended for use solely by qualified clinical laboratory by qualified clinical laboratory personnel specially trained in *in vitro* real-time PCR diagnostics.

1.3 Principle of operation of the test

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 E*ndoRNAse* 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) at 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 also detected in the fluorescent channel HEX.** The kit does not distinguish Influenza A and Influenza B. If you want to distinguish these viruses, then use the RT-PCR Respiratory panel 1: SARS-CoV-2/Flu/RSV (DB-1250), which uses the same primers and probes, but can distinguish Influenza A and B by using one additional

fluorescent 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 Texas Red channel (TEX)**.

This kit is designed to detect viral RNA from isolated RNA from various biological samples. Therefore, the kit includes an RNA isolation control and primers with a probe for its detection. **Isolation control is recommended to add to the sample prior RNA isolation**, to verify isolation yield. Add the control to the lysis/binding buffer from the RNA isolation kit, which will then 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 the sufficient yield of RNA isolation, which is a key prerequisite for correct diagnosis. A less preferred option is to add control RNA directly to the RT-PCR mix - this only verifies the efficiency of the RT-PCR reaction. **At least one negative control and at least one positive control (supplied in this kit) must be added to each run to verify the correct function of the kit.**

If you want to detect viral RNA directly from primary sample without prior RNA isolation, then use the DB-1253 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 without the need for RNA isolation.

1.4 Suitable samples and compatible sampling kits

This kit is suitable for detecting viral isolated RNA from various biological samples, e.g., nasal, nasopharyngeal, oropharyngeal, buccal swabs, nasopharyngeal liquid (aspirate), nasopharyngeal lavage, saliva, sputum, oral cavity lavage, gargling liquid, stool, urine, tissue biopsies, FFPE samples, and water using dedicated isolation kits for that purpose. Both column isolation kits and magnetic beads-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. For optimal detection results of SARS-CoV-2, Influenza A/B and RSV from saliva or nasopharyngeal swabs in UTM or PBS, we recommend the use of the DIANA Biotechnologies Automated RNA Isolation Kit for Agilent Bravo (Cat# DB-1206).

Saliva and swab samples can be inactivated prior to RNA isolation if the Automated RNA Isolation Kit (DB-1206) is used. Saliva can be inactivated in an incubator, if saliva is collected in DIANA Biotechnologies Sample Collection Sets (e.g., DB-1225 Saliva Collection Set 1.4IM, or the DB-1230 Saliva Collection Set 1.4IF, or the DB-1249 Saliva Collection Kit). Sample inactivation procedures are described in the manual for the DB-1206 Automated RNA Isolation Kit and are identical to those for the isolation-free DB-1253 DBdirect™ Respiratory Panel 2: SARS-CoV-2/Flu/RSV Kit. The procedure for saliva inactivation has been validated by the manufacturer, the procedure for media inactivation is a recommendation only and is the responsibility of the user to validate for each media type (verify on approximately 10 positive samples, some of which must be weak with $C_t > 30$, that inactivation does not result in reduced RNA yields).

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-1206 Automated RNA isolation Kit contains plastic labware and reagents for automated RNA isolation from various samples and consequent RT-PCR plate preparation using the Agilent Bravo liquid handling station. The DB-1214 Agilent Bravo Installation Package for Automated RNA Isolation Kit contains pipetting protocols for automated RNA isolation for 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 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 (e.g., Roche LC96); 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 machines. 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 the RNA was isolated with the DB-1206 Automated RNA Isolation 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 for 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 confirmed in real operation their ability to detect all variants of the disease, i.e., wild-type, alpha, beta, gamma, delta and omicron variants.

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) if 5 µL of isolated RNA is used for the assay and that the sample is not concentrated during isolation (i.e., when isolation is fivefold concentrated, the LOD per mL will be 5× lower).

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

DB-1252	$LOD_{95\%}$ v well	$LOD_{95\%}$ mL^{-1} (5 µL sample)
SARS-CoV-2	2	400
IAV H1N1-pdm	5	1000
IAV H3N2	10	2000
IBV Victoria	5	1000
RSV-A	5	1000
RSV-B	10	2000

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 (IC).

Table 3: Intraassay variability values

			Intraassay Variability		
Kit	**RNA**	**Channel**	**1000 copies**	**100 copies**	**25 copies**
DB-1252	SARS-CoV-2	FAM	0.12 (0.05-0.17)	0.16 (0.11-0.19)	0.24 (0.17-0.29)
DB-1252	IAV H3N2	HEX	0.12 (0.08-0.15)	0.23 (0.15-0.32)	0.63 (0.41-0.78)
DB-1252	IBV	HEX	0.07 (0.03-0.10)	0.22 (0.13-0.27)	0.35 (0.23-0.42)
DB-1252	RSV-A	TEX	0.08 (0.05-0.10)	0.16 (0.10-0.21)	0.29 (0.21-0.34)
DB-1252	RSV-B	TEX	0.13 (0.06-0.18)	0.45 (0.27-0.56)	0.62 (0.56-0.71)
DB-1252	IC	Cy5	0.20 (0.10-0.27)	0.15 (0.11-0.19)	0.13 (0.10-0.20)
DB-1252	All	All	0.11 (0.03-0.18)	0.27 (0.10-0.56)	0.46 (0.17-0.78)

Table 4: Interassay variability values

			Interassay Variability		
Kit	**RNA**	**Channel**	**1000 copies**	**100 copies**	**25 copies**
DB-1252	SARS-CoV-2	FAM	0.21 (0.06-0.27)	0.20 (0.18-0.23)	0.28 (0.17-0.35)
DB-1252	IAV H3N2	HEX	0.29 (0.21-0.40)	0.37 (0.23-0.48)	0.77 (0.59-0.92)
DB-1252	IBV	HEX	0.26 (0.07-0.34)	0.31 (0.19-0.38)	0.46 (0.42-0.48)
DB-1252	RSV-A	TEX	0.30 (0.19-0.42)	0.36 (0.23-0.46)	0.40 (0.31-0.51)
DB-1252	RSV-B	TEX	0.34 (0.22-0.47)	0.54 (0.47-0.59)	0.76 (0.49-0.91)
DB-1252	IC	Cy5	0.42 (0.23-0.63)	0.32 (0.14-0.46)	0.35 (0.15-0.48)
DB-1252	All	All	0.28 (0.06-0.47)	0.37 (0.18-0.59)	0.57 (0.17-0.92)

Table 5: **Expected C_t values**

			Expected C_t values		
Kit	**RNA**	**Channel**	**1000 copies**	**100 copies**	**25 copies**
DB-1252	SARS-CoV-2	FAM	26.54 (26.43-26.73)	29.96 (29.86-30.12)	31.77 (31.61-32.02)
DB-1252	IAV H3N2	HEX	30.06 (29.79-30.34)	33.48 (33.23-33.77)	35.71 (35.25-36.33)
DB-1252	IBV	HEX	28.21 (28.02-28.49)	31.47 (31.34-31.74)	33.59 (33.29-33.86)
DB-1252	RSV-A	TEX	29.97 (29.69-30.28)	33.12 (32.85-33.48)	34.98 (34.65-35.31)
DB-1252	RSV-B	TEX	30.65 (30.30-30.96)	33.82 (33.51-34.13)	35.67 (35.05-36.08)
DB-1252	IC	Cy5	30.30 (29.93-30.86)	30.26 (30.00-30.66)	30.27 (29.98-30.65)
DB-1252	All	All	29.09 (26.43-30.96)	32.37 (29.86-34.13)	34.35 (31.61-36.33)

2.4 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 6** summarizes the measured results for coinfections.

Table 6: 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	3x LOD	IAV H3N2	SARS-CoV-2	3x LOD
SARS-CoV-2	IBV	3x LOD	IBV	SARS-CoV-2	3x LOD
SARS-CoV-2	RSV-A	3x LOD	RSV-A	SARS-CoV-2	3x LOD
SARS-CoV-2	RSV-B	3x LOD	RSV-B	SARS-CoV-2	3x LOD

When a high concentration of SARS-CoV-2 RNA were present in the sample, then significantly weaker amplification was observed for low concentrations of the other viruses, resulting in lower fluorescence, but all were detected from a concentration of 3x LOD. When a high concentration of Influenza A, Influenza B, RSV A or RSV B and a low concentration of SARS-CoV-2 were present in the well, this was also detected from the lowest concentration tested, i.e. 3x LOD, and the SARS-CoV-2 amplification was not affected in any way, nor was the fluorescence decreased.

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 (3x LOD). Detection of other viruses is weaker in the presence of high concentrations of SARS-CoV-2, but all viral RNAs were detected at the lowest concentration tested, i.e. 3x LOD, with only partially reduced fluorescence. Negativity in any channel cannot be used as the only clue to exclude infection by a given virus.

2.5 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). 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 nasopharyngeal swabs are shown in **Figure 1**, and for 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 7**. 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 co-infection 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 percentage agreement" (PPA) of the device was 96.0% for SARS-CoV-2, 97.6% for Influenza A and B and 97.6% for RSV in saliva and swab samples, while the "negative percentage agreement" (NPA) for the given viruses was 99.5 %, 99,7 % and 97.7 % 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.

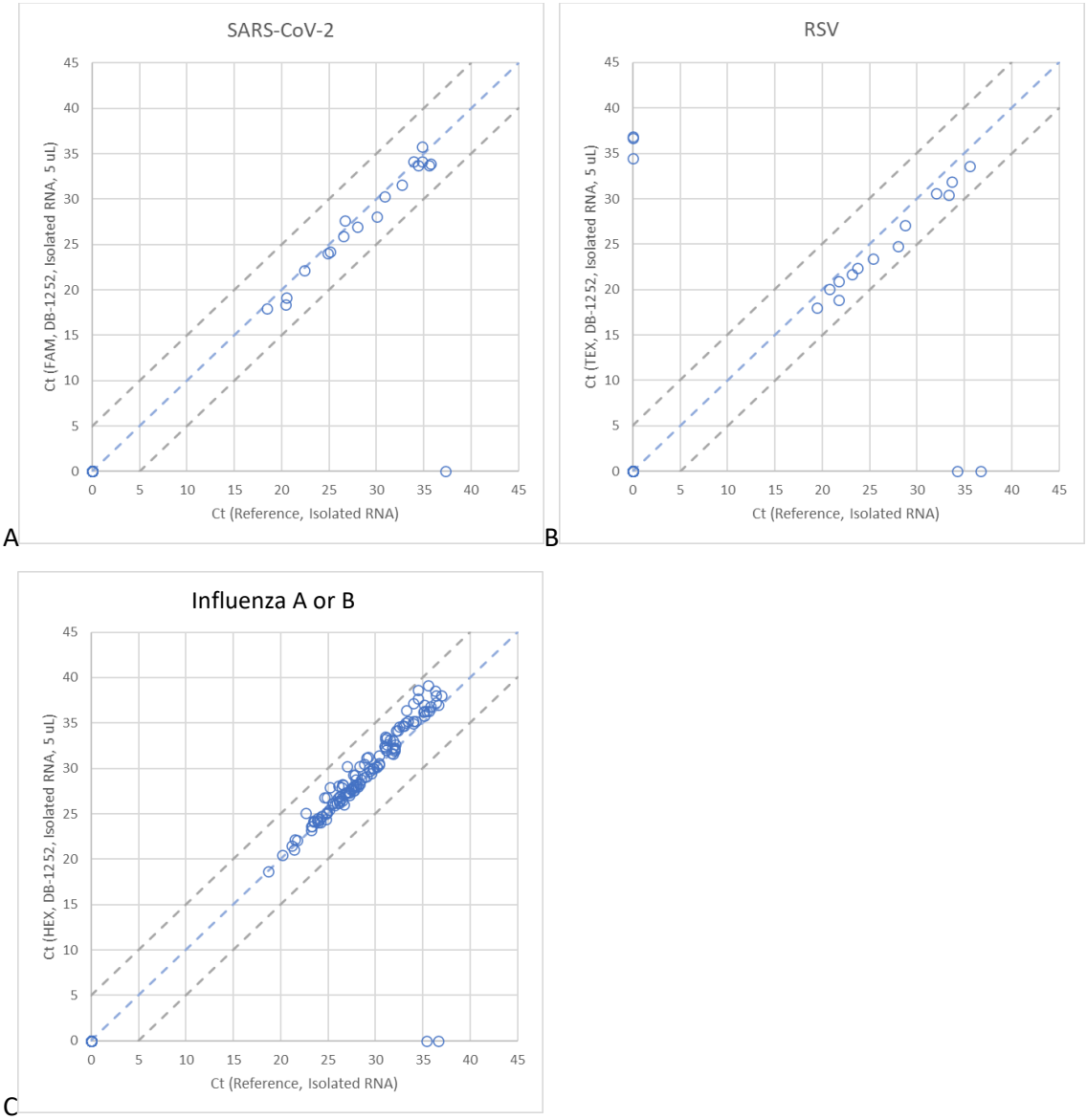

Figure 1: **Comparison of measured C_t for nasopharyngeal swab samples with reference measurements**

The detection of each virus is plotted in one graph, the y-axis of the graph plots the corresponding channel of the DB-1252 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-1252 kit.

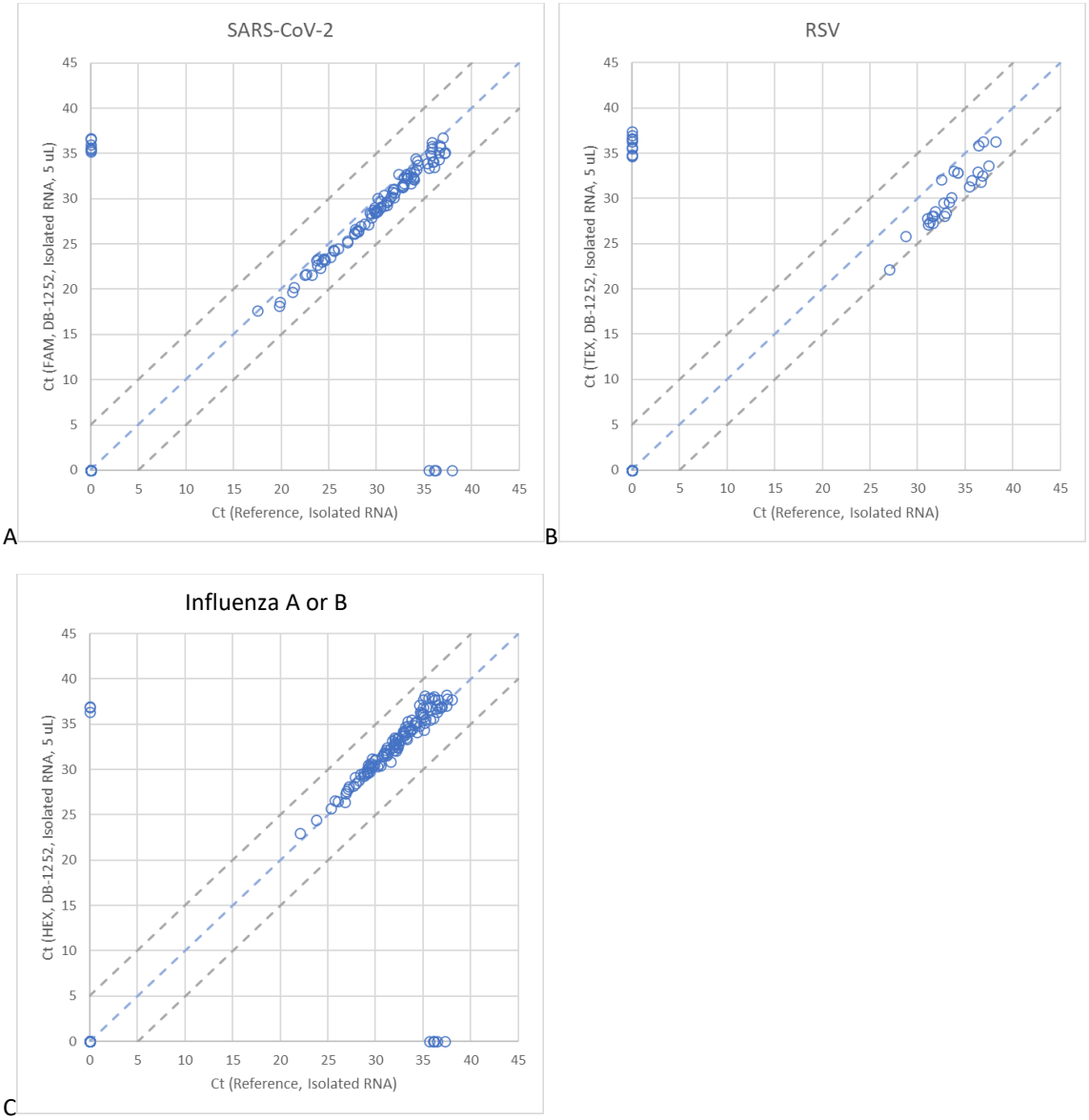

Figure 2: **Comparison of measured C_t for saliva samples with reference measurements**

The detection of each virus is plotted in one graph, with the corresponding channel of the DB-1252 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 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-1252 kit. Many samples were positively detected for SARS-CoV-2, and RSV only in the DB-1250 testing kit without being detected in the reference testing kits (see points on y-axes). A closer inspection of the data (**Table 7**) 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-1252 testing kit. As all these samples are with $C_t > 30$ and at the same time the C_t values in the DB-1252 are lower than the reference kits by an average

of two cycles for SARS-CoV-2 and three cycles for RSV, there is a good chance that these are weak samples that were not detected in the reference measurement.

Table 7: Results for NPA and PPA determination of nasopharyngeal swabs and saliva samples

Sample	Interpretation	TP	FN	TN	FP	FP[2]	FP[1]	PPA	PPA*	NPA
Nasopharyngeal swabs	SARS-CoV-2	18	1	167	0	0	0	94.7%	94.7%	100.0%
	Influenza A/B	128	2	56	0	0	0	98.5%	98.5%	100.0%
	RSV	13	2	168	3	0	1	87.5%	93.3%	98.8%
Saliva	SARS-CoV-2	97	4	259	8	3	3	96.3%	96.3%	99.2%
	Influenza A/B	116	6	243	3	2	0	95.2%	96.7%	99.6%
	RSV	27	0	331	10	0	0	100.0%	100.0%	97.1%
Nasopharyngeal swabs+ saliva	SARS-CoV-2	115	5	426	8	3	3	96.0%	96.0%	99.5%
	Influenza A/B	244	8	299	3	2	0	96.9%	97.6%	99.7%
	RSV	40	2	499	13	0	1	95.3%	97.6%	97.7%

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-1252 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-1252 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-1252 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 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
- Kit or reagents for RNA isolation (e.g., DB-1206)
- Material that will serve as a negative control (see chapter 5.3)
- In case of use of automated protocol: protocols and plastic needed for automation (e.g. installation package DB-1214 and kit DB-1206 for Agilent Bravo pipette station)

4.4 Kit components

Table 8: DB-1252 Kit components

Kit [8]	Kit component [8]	REF code [7]	Volume (µL) [6]	Storage conditions	Cap number and color
DB-1252-100rxns	Enhancer mix (4x)	RF00772	500	-20 °C [2,3]	1
	Primer mix (4x)	RF02074	500	-20 °C [1,2,3]	2
	Enzyme mix (4x)	RF06854	500	**-20 °C** [2,3]	3
	Positive control A	RF02991	150	-20 °C [2,3]	4A
	Positive control B	RF02557	150	-20 °C [2,3]	4B
	Isolation control [4]	RF05323	150	-20 °C [2,3]	5
DB-1252-1000rxns	Enhancer mix (4x)	RF00772	5000	-20 °C [2,3]	1 [5]
	Primer mix (4x)	RF02074	5000	-20 °C [1,2,3]	2 [5]
	Enzyme mix (4x)	RF06854	5000	**-20 °C** [2,3]	3 [5]
	Positive control A	RF02991	2x 750	-20 °C [2,3]	4A
	Positive control B	RF02557	2x 750	-20 °C [2,3]	4B
	Isolation control [4]	RF05323	2x 750	-20 °C [2,3]	5

[1] Store at dark compartment (contains fluorophores, which are photosensitive). **[2] Store the kit frozen at -20 °C or lower**; kit can be stored also at -40 °C, or -80 °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]** Add to lysis/binding buffer prior RNA extraction, or directly to the RT-PCR mix. **[5]** Components 1-3 have transparent caps in 1000rxns kit size. **[6]** Actual volume of all reagents is approximately 5-10% higher compared with volumes stated in table 8. **[7]** 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. **[8]** 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 isolation controls (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 additives. 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**!

Isolation control

Contains artificial RNA of length over 2000 bases. This RNA is added to each sample prior RNA isolation in order to control isolation efficiency and it reveals possible inhibition of RT-PCR reaction.

4.5 Stability of kit components and master mix

Components 1, 2, and 3 (Enhancer, Primer a Enzyme Mix) should be kept at -20 °C or lower for long-term storage (expiry date is indicated on the packaging). 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.

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 and 5 (positive and isolation controls) 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. Avoid repeated freezing and thawing of the positive control and isolation control, do not exceed four freeze/thaw cycles. The optimum temperature for long-term storage of both controls 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, 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 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 minimize the risk of accidental contamination with template RNA/DNA. 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) which is not used for other purposes and which is 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. The negative control can be set up in two ways. The best way is to run the RNA isolation with known negative sample or with a blank sample medium and add the same amount of eluate to the RT-PCR mix as for the actual samples. Such negative control will monitor contaminants at any step of the process. Less robust negative control can be set up by directly adding the clean elution solution, or PCR-grade water to the RT-PCR mix as if it was a sample. This procedure will monitor only the contamination of elution buffer or of RT-PCR mix. 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 Adding isolation control

RNA Isolation control should be added into the lysis/binding buffer prior mixing with the sample and subsequent RNA isolation. Add **1 µL of RNA from Isolation control vial (vial 5, violet cap 🟣) for each isolated sample** (e.g., per 10 isolated samples; add 10 µL of isolation control to volume of lysis/binding buffer). Such control is highly recommended, as it reveals possible reduced efficacy of RNA isolation and possible inhibition of RT-PCR at the same time.

Alternatively, if adding the isolation control to the sample prior RNA isolation is not possible, then add **0.1 µL of Isolation control (vial 5, violet cap 🟣)** per one sample directly to the RT-PCR mix (e.g., add 1 µL of master mix for 10 reactions). See optional step 5 in section 5.6 for more details.

5.6 RT-PCR master mix preparation

Preparation of RT-PCR mix for one reaction is described below. If you are preparing RT-PCR mix for multiple reactions, multiply the volumes by the number of the reaction (and account for some pipetting reserve) – see also **Table 9**.

1. Thaw and mix each component of the RT-PCR master mix (see **Table 9** and section 4.4)
2. Into a clean RNase/DNase free vial, pipette **5 µL of Enhancer mix (4x) from kit vial #1 (green 🟢or transparent cap)**.
3. Add **5 µL of Primer mix (4x) from kit vial #2 (blue 🔵or transparent cap)** to the same vial and mix by pipetting several times up and down.
4. Add **5 µL of Enzyme mix (4x) from kit vial #3 (black ⚫or transparent cap)**, to the same vial and mix by pipetting several times up and down until the mixture is homogeneous (you can also briefly vortex and spin down).
5. ***Optional**: if you do not add the RNA isolation control into the sample prior RNA isolation, **add 0.1 µL of Isolation control kit vial #5 (violet cap 🟣)** and mix by pipetting up and down (use pipette with at least ½ volume of the liquid in the vial) or by vortexing.*
6. 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 Sections 5.5, 5.6, and 5.7 without affecting the detection sensitivity.

Table 9: RT-PCR master mix preparation

Kit Component	# 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 numbering of the vials corresponds to the order in which the ingredients are added, it is important to keep this order. **[2]** The Isolation control volume is omitted; 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

The numbers and volumes of aliquots of each component of this kit for 1000 tests (1000rxns) are summarized in **Table 8**. If you do not want to use the whole kit at once, it is advisable to prepare single aliquots after the first thawing. There are two options how to prepare single aliquots for analysis of 96 samples (e.g., using an Agilent Bravo pipetting station and DB-1206 kit):

1. **Aliquots can be stored at -80°C**: mix all contents of Enhancer mix (4x), Primer mix (4x) and Enzyme mix (4x). Follow this order and always mix before adding the Enzyme mix. Then mix thoroughly again and divide into 10 aliquots of 1.53 mL. The result will be 10 vials of RT-PCR master mix. The RT-PCR master mix thus prepared must be frozen and stored at -80 °C as soon as possible. Once thawed, use the mix as soon as possible, ideally within 30 minutes, but it is stable at room temperature for up to 2 hours.
2. **If storage at -80 °C is not available**: prepare 10 aliquots of 510 µL of each of the Enhancer mix (4x), Primer mix (4x) and Enzyme mix (4x). To prepare the RT-PCR master mix for analysis of 96 isolations, then mix one aliquot of each component and use the entire mix. After thawing and before mixing, the individual components of the RT-PCR master mix are stable at room temperature for 4 hours.

Aliquoting positive control

Prepare 5 single use 150 µL aliquots of positive control. These aliquots can be stored both at -80 °C and at -20 °C.

Aliquoting isolation control

Prepare 5 single use 150 µL aliquots of isolation control. These aliquots can be stored both at -80 °C and at -20 °C.

Details on stability and storage are given in Section 4.5.

5.7 Adding sample to the RT-PCR reaction

Add 5 µL of the sample into each well/PCR-microtube, containing 15 µL of the RT-PCR master mix. After adding the samples to the 96-well plate, seal it with an adhesive optical film and run the RT-PCR protocol (within 60 minutes after 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 **5 µL of the Positive control from vial 4A or 4B (red cap 🔴)** instead of the sample. In case of a negative control, add either **5 µL of the RNA isolated from known negative sample**, or **5 µL of the Elution buffer** from the RNA Isolation Kit, or **5 µL of PCR-grade water** (see Section 5.3). The total reaction volume after adding the sample or controls is 20 µL.

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 5.5, 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™ and BioRad CFX Opus 96

For detection in four channels, use the default instrument settings and filter settings in **Table 10**.

Table 10: 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 11**.

Set the "sample volume" to 20 µL.

Table 11: 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 length of this protocol on BioRad instruments is 1 hour and 16 minutes.

5.9 Data analysis

5.9.1 Determination of cycle threshold value (Ct)

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 selected thresholds: **200 RFU for FAM and HEX and 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 abovementioned 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 12 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 12** 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 = 28 (for FAM, HEX and TEX channels) for accurate interpretation according to **Table 12**. For example, if your determined C_t value for a positive control is 24, either add 4 cycles to the clinical sample C_t values or subtract 4 cycles from the threshold C_t values in **Table 12** to use **Table 12**.

** 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.*

5.9.2 Evaluations of controls

In 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 isolation control in Cy5 should result in a C_t <35 cycles, but this is not necessary to evaluate a positive control (the signal in control Cy5 channel 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 Cy5 channel (<40 cycles), while there must be no amplification in all other channels. Measurable amplification in FAM, HEX or TEX 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 (the signal in control Cy5 channel 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).

Isolation control must be evaluated for each sample, however, for positive samples, the amplification of the isolation control may be negatively affected by viral gene amplification, and C_t values in the Cy5 channel may be significantly higher than in the negative control, or below the threshold. If you only evaluate the test qualitatively, **positive samples are considered positive even if the isolation 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 C_t > 40 or the signal is undetectable, then a low RNA isolation efficiency or inhibition of the RT-PCR reaction can be inferred and RNA isolation from the sample must be repeated.

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 12** 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 12**, as described above in Section 5.9.1.

Table 12: Data interpretation
The symbol „-“ indicates C_t > 40 cycles or undetectable signal; the symbol „+“ indicates C_t ≤ 40 cycles.
SARS-CoV-2 is detected in the FAM channel, Influenza A/Influenza B in HEX, RSV in TEX and Isolation 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/B and RSV)**[5]
-	-	-	-	**Unreliable result: inhibition of RT-PCR, repeat or perform RNA isolation.**

[1] When an RNA isolation control is added to the sample prior to RNA isolation in the amounts as directed and the standard isolation protocol was followed (i.e., approximately $^1/_{10}$ of an elution volume was used for RT-PCR, e.g., 5 µL of 50 µL), the C_t value for Cy5 is around cycle 35 or lower.
[2] High concentrations of viral RNA detected in either channel can cause impaired amplification of the isolation 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 between cycles 37 and 40 and also signal in Cy5 channel is higher than cycle 40 or not detectable, then the test must be repeated because RT-PCR inhibition or a decrease in RNA isolation efficiency is likely to have occurred. 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. 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 of two or more viruses simultaneously, a reduction in amplification efficiency in the other channels was observed when the signal in FAM was strong (~20th cycle), but all were detectable from 3x LOD. In contrast, when the signal in HEX or TEX channels was strong (~25th cycle), SARS-CoV-2 detection was not affected even at 3× LOD, and thus weak SARS-CoV-2 coinfection should be reliably detected.
[6] For the interpretation of Influenza A 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 5,000 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. Autofluorescence is the most common in TEX channel.
[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-1252 kit.

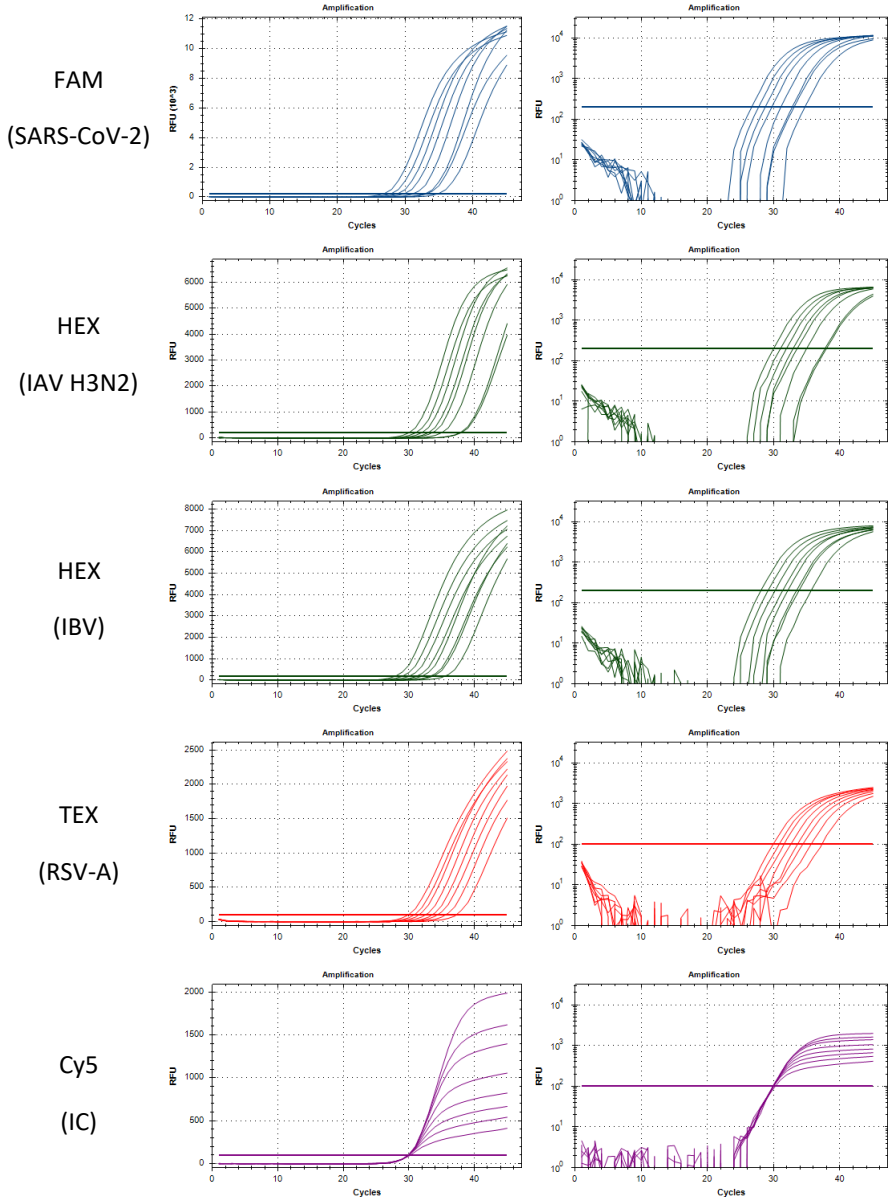

Figure 3: Detection of a dilution series of viral RNAs from 1,000 to 5 copies per well on the BioRad CFX OPUS 96 instrument.

The first pair of graphs shows signal amplification in the FAM channel (SARS-CoV-2), the second and the third in HEX (Influenza A H3N2 and Influenza B), the fourth in TEX (RSV-A) and the fifth in Cy5 (isolation 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

To the fullest extent permitted by the laws of your jurisdiction, DIANA Biotechnologies, s.r.o. shall not be liable for any direct or indirect damages associated with or arising out of this document and its use, or for any direct or indirect damages associated with or arising out of the RT-PCR Respiratory Panel 1: SARS-CoV-2/Flu/RSV Kit and its use.

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 A	150	2x 750	-20 °C	4A
Positive control B	150	2x 750	-20 °C	4B
Isolation control	150	2x 750	-20 °C	5

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 96-well plate, add 5 µL of the sample (isolated RNA), seal the plate with optical film and run the RT-PCR reaction as soon as possible.
- For positive and negative controls, add 5 µL of positive (vial 4A or 4B) or negative control in place 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 rxn	µL per 100 rxn
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 the 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 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 symbols used

	Manufacturer
	Caution
	Lot Number
	Operator´s manual, operating instructions
	Catalogue Number
	Component volume
	Package contains
	Positive 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*