

MutaPLEX[®]

RespiraScreen 4 DIFF seq

real-time-RT-PCR Kit

For the simultaneous in vitro detection and differentiation of RNA of Influenza Virus (Flu A and Flu B), Respiratory Syncytial Virus (RSV A and RSV B) and SARS-CoV-2 (E gene and S gene), extracted from biological specimens.

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1 INTENDED PURPOSE

The MutaPLEX® RespiraScreen 4 DIFF seqc real time RT-PCR Kit is designed for the simultaneous qualitative detection of RNA of SARS-CoV-2 corona virus, Influenza Virus A, Influenza Virus B, Respiratory Syncytial Virus A and Respiratory Syncytial Virus B in eluates from biological specimen.

The assay is an in vitro diagnostic medical device and intended to be used by professional users in a laboratory environment. It can be performed manually or using an automated platform. The assay serves as an aid in the diagnosis, screening and monitoring for SARS-CoV-2 corona virus, Influenza Virus A, Influenza Virus B, Respiratory Syncytial Virus A and Respiratory Syncytial Virus B.

2 PATHOGEN INFORMATION

Coronaviruses (CoV) are a large family of viruses that cause illness ranging from the common cold to more severe diseases such as Middle East Respiratory Syndrome (MERS) and Severe Acute Respiratory Syndrome (SARS) [1]. The novel Coronavirus (SARS-CoV-2) is a new strain within the Sarbecoviruses that has been previously identified in humans and causes the pulmonary disease COVID-19 [2].

Coronaviruses are zoonotic, meaning they are transmitted between animals and people. Detailed investigations found that SARS-CoV was transmitted from civet cats to humans and MERS-CoV from dromedary camels to humans. Several known Coronaviruses are circulating in animals that have not yet infected humans [3].

Common signs of infection include respiratory symptoms, fever, cough, shortness of breath and breathing difficulties. In more severe cases, infection can cause pneumonia, severe acute respiratory syndrome, kidney failure and even death.

Standard recommendations to prevent infection spread include regular hand washing, covering mouth and nose when coughing and sneezing, thoroughly cooking meat and eggs. Avoid close contact with anyone showing symptoms of respiratory illness such as coughing and sneezing.

Influenza Viruses belong to the family of Orthomyxoviridae and are the causative agent of 'the flu'. Influenza A and B viruses have a single stranded RNA genome, consisting of 8 RNA segments [4]. The genome of Influenza A Viruses is characterized by a high mutation frequency, the so-called 'antigenic drift'. Numerous subtypes of Influenza A Viruses are known. They can be categorized by their surface antigens H (haemagglutinin) and N (neuraminidase): Influenza A (H1N1) Virus, Influenza A (H5N1) Virus etc. Therefore, yearly in-silico analysis of the sequences of newly emerged subtypes is done, to prevent false negative results caused by primer and/or probe mismatches. Influenza B viruses show a 2 – 3 times slower mutation rate than type

A. Latest results indicate an enhanced infectivity for SARS-CoV-2 when a coinfection with Influenza A is present [5].

Respiratory Syncytial Viruses are enveloped negative-sense, single stranded RNA Viruses of the Pneumoviridae family, genus Orthopneumovirus. RSV are divided into subgroups A and B. RSV is a virus that causes infections of the lungs and respiratory tract. It is so common that most children have been infected with the virus at the age of 2. RSV can also infect adults.

In adults and older, healthy children, the symptoms of RSV infections are mild and typically mimic the common cold [6]. Self-care measures are usually all that is needed to relieve any discomfort. Infection with RSV can be severe in some cases, especially in premature babies and infants with underlying health conditions. RSV can also become serious in older adults, adults with heart and lung diseases, or anyone with a very weak immune system (immunocompromised).

3 PRINCIPLE OF THE TEST

The MutaPLEX® RespiraScreen 4 DIFF seqc real time RT-PCR Kit contains specific primers and dual-labelled probes for the amplification of RNA (cDNA) of Influenza A Virus (M gene, FAM channel), Influenza B Virus (NEP gene, FAM channel), RSV A (G gene, Cy5 channel), RSV B (G gene, Cy5 channel) and SARS-CoV-2 (E gene and S gene, ROX channel) extracted from biological specimen.

Additionally, MutaPLEX® RespiraScreen 4 DIFF seqc real time RT-PCR Kit contains an Internal System Control (ISC). The ISC consists of primers and probes for the detection of a house keeping gene (human succinate dehydrogenase) in the eluate from a biological specimen. The ISC helps preventing false negative results due to insufficient sample drawing or transport. The amplification of the human succinate dehydrogenase target sequence is measured in the Cy5.5 channel.

Furthermore, MutaPLEX® RespiraScreen 4 DIFF seqc real time RT-PCR Kit contains a Control RNA (Internal Process Control, IPC), which is added during RNA extraction and detected in the same reaction by a HEX-labelled probe. The Control RNA allows the detection of RT-PCR inhibition and acts as control that the nucleic acid was isolated from the biological specimen.

In the MutaPLEX® RespiraScreen 4 DIFF seqc real time RT-PCR, both the amplification curves and the melt curves are considered for the evaluation.

4 PACKAGE CONTENTS

The reagents supplied are sufficient for 96 (KG193796), 384 (KG1937-384), or 768 (KG1937-768) reactions, respectively.

Table 1: Components of the MutaPLEX® RespiraScreen 4 DIFF seqc real time RT-PCR Kit

Label	Lid Colour	Content		
		96	384	768
Reaction Mix	yellow	1 x 1325 µl	4 x 1325 µl	8 x 1325 µl
Enzyme	blue	1 x 19.2 µl	1 x 76.8 µl	1 x 153.6 µl
Positive Control 1	red	1 x 50 µl	1 x 100 µl	1 x 100 µl
Positive Control 2	violet	1 x 50 µl	1 x 100 µl	1 x 100 µl
Negative Control	green	1 x 150 µl	1 x 300 µl	1 x 300 µl
Control RNA	colourless	1 x 480 µl	2 x 960 µl	4 x 960 µl

5 EQUIPMENT AND REAGENTS TO BE SUPPLIED BY USER

- RNA isolation kit (e.g. MutaCLEAN® Mag RNA/DNA, KG1023 or KG1024).
- Sterile microtubes
- Calibrated precision pipets (adjustable volume) and sterile single-use tips with filter
- Disposable gloves
- Table centrifuge
- Vortex
- Real time PCR instrument
- Optical PCR reaction tubes with lid or optical PCR reaction plate with optical foil
- Optional: Liquid handling system for automation

6 TRANSPORT, STORAGE AND STABILITY

The MutaPLEX® RespiraScreen 4 DIFF seqc real time RT-PCR Kit is shipped on dry ice or cool packs. All components must be stored at maximum -20°C in the dark immediately after receipt. Do not use reagents after the date of expiry printed on the package. Up to 20 freeze and thaw cycles are possible. For convenience, opened reagents can be stored at +2–8°C for up to 6 months. Protect kit components from direct sunlight during the complete test run.

7 WARNINGS AND PRECAUTIONS

Read the Instruction for Use carefully before using the product.

Before first use check the product and its components for:

- Use of this product is limited to personnel specially instructed and trained in the techniques of Real-Time PCR procedures.
- Specimens should always be treated as infectious and/or biohazardous in accordance with safe laboratory procedures.
- Avoid microbial and nuclease (DNase/RNase) contamination of the eluates and the components of the kit.
- Always use DNase/RNase-free disposable pipette tips with aerosol barriers.
- Always wear protective disposable powder-free gloves when handling kit components.
- Use separated and segregated working areas for (1) sample preparation, (2) reaction setup and (3) amplification/detection activities. The workflow in the laboratory should proceed in unidirectional manner. Always wear disposable gloves in each area and change them before entering a different area.
- Dedicate supplies and equipment to the separate working areas and do not move them from one area to another.
- Store positive and/or potentially positive material separated from all other components of the kit.
- Do not open the reaction tubes/plates post amplification, to avoid contamination with amplicons.
- Additional controls may be tested according to guidelines or requirements of local, state and/or federal regulations or accrediting organisations.
- Do not autoclave reaction tubes after the PCR, since this will not degrade the amplified nucleic acid and will bear the risk to contaminate the laboratory area.
- Discard sample and assay waste according to your local safety regulations.
- Do not combine MutaPLEX® RespiraScreen 4 DIFF seqc real time RT-PCR Kit components of different lot numbers.

8 SAMPLE MATERIAL

Starting material for MutaPLEX® RespiraScreen 4 DIFF seqc real time RT-PCR Kit is RNA isolated from biological specimens (respiratory samples).

9 SAMPLE PREPARATION

Commercial kits for RNA isolation such as the following are recommended:

- MutaCLEAN® Mag RNA/DNA, Immundiagnostik Cat. No. KG1023 or KG1024

Please follow the Instructions for Use of the respective extraction kit.

Important:

In addition to the samples always run a ‚water control‘ in your extraction. Treat this water control analogous to a sample.

Comparing the amplification of the Control RNA in the samples to the amplification of the internal control in the water control will give insights on possible inhibitions of the real time RT-PCR. Furthermore, possible contaminations during RNA extraction will be detectable.

Please note the chapter ‚Control RNA‘.

If the real time RT-PCR is not performed immediately, store extracted RNA according to the instructions given by the manufacturer.

10 CONTROL RNA

A Control RNA is supplied as extraction control. This allows the user to control the RNA isolation procedure and to check for possible real time RT-PCR inhibition.

Add 5 µl Control RNA per extraction (5 µl x (N+1)). Mix well. Perform the RNA isolation according to the manufacturer's instructions.

The Control RNA must be added to the Lysis Buffer of the extraction kit.

11 REAL-TIME-RT-PCR

11.1 Important Points Before Starting

- Please pay attention to the chapter 7 ‚Warnings and Precautions‘.
- Before setting up the real time RT-PCR familiarise yourself with the real time PCR instrument and read the user manual supplied with the instrument.

- The programming of the thermal profile should take place before the RT-PCR set up.
- In every RT-PCR run one Positive Control 1, one Positive Control 2 and one Negative Control should be included. Positive Control 1 and one Positive Control 2 must be freshly diluted 1:10 in PCR grade water before each run.
- Before each use, all reagents should be thawed completely at room temperature, thoroughly mixed (except the Enzyme) and centrifuged very briefly.
- Due to the high viscosity of the Enzyme (blue lid), prewarming at room temperature for 15 min is recommended.

11.2 Procedure

The Master Mix contains all of the components needed for the real time RT-PCR except the sample. Prepare the Master Mix according to Table 2. Prepare a volume of Master Mix for at least one sample more than required, in order to compensate for pipetting inaccuracy.

Table 2: Preparation of the master mix

Volume per reaction	Volume master mix
13.8 µl Reaction Mix	13.8 µl x (N+1)
0.2 µl Enzyme	0.2 µl x (N+1)

11.3 Preparation of the Positive Controls

Positive Control 1 and Positive Control 2 are stored in an extra storage buffer which may alter the peak of the melt curves. For a better comparison with the samples, all Positive Controls need to be freshly diluted 1:10 in PCR grade water before each PCR run. The dilution of the positive controls is mandatory, because the stabilizing buffer of the reagents does affect the melting temperature in the subsequent melting curve analysis. Prepare the Positive Controls according to Table 3.

Table 3: Preparation of the Positive Controls

Component	Volume
Positive Control 1 / 2	2.0 µl
PCR grade water	18.0 µl

11.4 Real time RT-PCR set-up

- Place the number of optical PCR reaction tubes needed into the respective tray of the real time PCR instrument / take an optical PCR reaction plate.

- Pipet **14 µl** of the Master Mix into each optical PCR reaction tube / the optical PCR reaction plate.
- Add **6 µl** of the eluates from the RNA isolation (including the eluate of the water control), the diluted Positive Control 1, the diluted Positive Control 2 and the Negative Control to the corresponding optical PCR reaction tube / the optical PCR reaction plate (Table 4).
- Close the optical PCR reaction tubes / the optical PCR reaction plate immediately after filling in order to reduce the risk of contamination.

Table 4: Preparation of the real-time-RT-PCR

Component	Volume
Master mix	14.0 µl
Sample	6.0 µl
Total volume	20.0 µl

11.5 Instrument Settings

For the real time RT-PCR use the thermal profile shown in Table 5.

Table 5: real-time-RT-PCR thermal profile

Description	Time	Temperature	Number of Cycles	Aquisition
Reverse Transcription	10 min	45 °C	1	no
Initial Denaturation	5 min	95 °C	1	no
Amplification of cDNA				
Denaturation	10 sec	95 °C	40	no
Annealing and Extension	40 sec	60 °C		end of step
pre-Melt Curve Amplification				
Denaturation	10 sec	95 °C	5	no
Annealing and Extension*	40 sec	60 °C		no
Melt Curve	see table below for individual cycler settings			

* No acquisition is done in this step. This will cause an error message before the start of the PCR. The error message can be confirmed with 'OK'.

Bio-Rad CFX Opus 96 / Bio-Rad CFX96 C1000 Touch

Program Step	Melt Curve
Parameter	
Melt from	55.0 °C to 75.0 °C
Increment	0.5 °C for 0:05 + Plate Read

Further instrument settings have to be adjusted according to table 6. If other instruments will be used, please contact our technical support for further information at info@immundiagnostik.com.

Table 6: Overview of the instrument settings required for the MutaPLEX® RespiraScreen 4 DIFF seqc real time RT-PCR

real-time-PCR-Instrument	Parameter Reaction Mix	Detection channel	Notes
Bio-Rad CFX96 C1000 Touch	Flu A / Flu B	FAM	Reference Dye: None
	Control RNA (IPC)	HEX	
	SARS-CoV-2	ROX	
Bio-Rad CFX Opus 96	RSV A / RSV B	Cy5	
	ISC	Cy5.5	

12 DATA ANALYSIS

In the MutaPLEX® RespiraScreen 4 DIFF seqc real time RT-PCR, both the amplification curves and the melt curves are considered for the evaluation. Positive samples have to show both an amplification curve and a melt peak in the specific channel. Influenza Virus is detected in the FAM channel, SARS-CoV-2 in the ROX channel and RSV in the Cy5 channel. The distinction between Influenza Virus A and Influenza Virus B (FAM), SARS-CoV-2 E gene and SARS-CoV-2 S gene (ROX) as well as RSV A and RSV B (Cy5) can be made using the melt curves, where two peaks with different melting temperatures can be observed. The peaks of the samples should be aligned with the melt peaks of the corresponding Positive Control (± 2.0 degrees). In the Cy5.5 and in the HEX channel no differentiation is required. Only the C_T values are evaluated for these two channels. The corresponding peaks and T_m values can therefore be neglected.

Following results can occur:

FAM Channel	ROX Channel	Cy5 Channel	HEX Channel	Cy5.5 Channel	Interpretation
Flu A / Flu B	SARS-CoV-2	RSV A / RSV B	Control RNA (IPC)	ISC	
positive	negative	negative	positive or negative ¹	positive or negative	Positive result. The sample contains RNA of Flu A and/or Flu B. For differentiation between Flu A and Flu B compare the melt curve with the Positive Controls (Figure 2, Figure 4).
negative	positive	negative	positive or negative ¹	positive or negative	Positive result. The sample contains RNA of SARS-CoV-2. Both genes (E gene, S gene) should be shown in the melt curve (Figure 6).
negative	negative	positive	positive or negative ¹	positive or negative	Positive result. The sample contains RNA of RSV A and/or RSV B. For differentiation between RSV A and RSV B compare the melt curve with the Positive Controls (Figure 8, Figure 10).
negative	negative	negative	≤ 34	≤ 34	Negative result. The sample contains no RNA of Flu A, Flu B, RSV A, RSV B or SARS-CoV-2.
negative	negative	negative	≤ 34	> 34	Caution! The sample quality and/or quantity is very low

FAM Channel	ROX Channel	Cy5 Channel	HEX Channel	Cy5.5 Channel	Interpretation
Flu A / Flu B	SARS-CoV-2	RSV A / RSV B	Control RNA (IPC)	ISC	
negative	negative	negative	negative or > 34 ²	positive or negative	Caution! The real time RT-PCR is either inhibited or errors occurred while RNA/DNA extraction.

¹ A strong positive signal in the FAM, ROX or Cy5 channel can inhibit the IPC. In such cases the result for the Control RNA can be neglected.

² In case of high C_t values, the IPC should be compared to the water control as described in the chapter 'Assay validation'.

Figure 1 to Figure 13 show examples for positive and negative real time RT-PCR results.

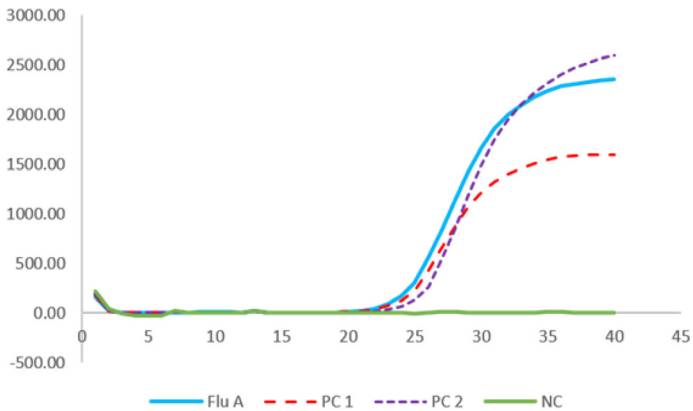


Figure 1: The sample, Positive Control 1 and Positive Control 2 show pathogen specific amplification in the FAM channel, whereas no fluorescence signal is detected in the Negative Control.

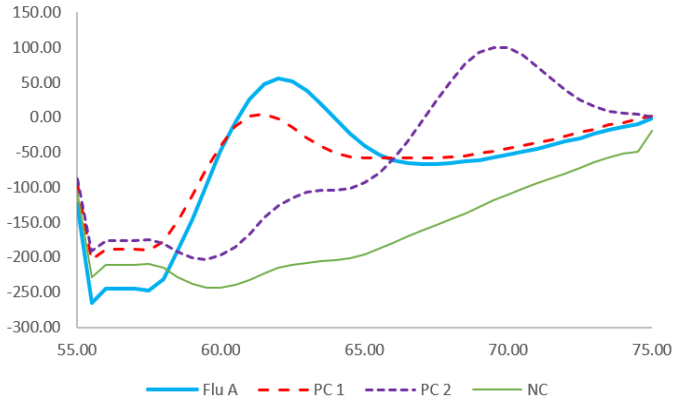


Figure 2: The melt peak of the sample in the FAM channel is aligned with the melt peak of Positive Control 1. The sample is characterized as Flu A positive.

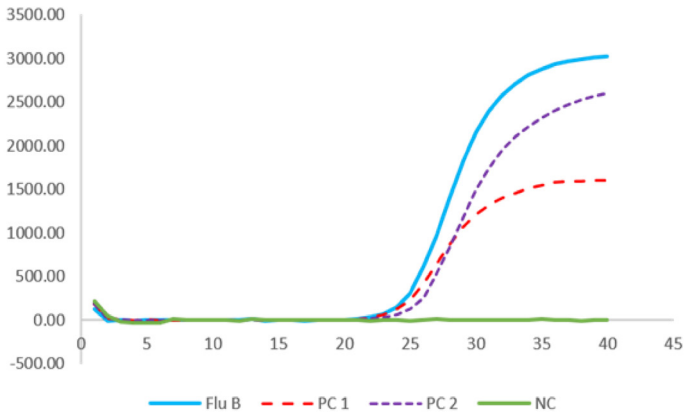


Figure 3: The sample, Positive Control 1 and Positive Control 2 show pathogen specific amplification in the FAM channel, whereas no fluorescence signal is detected in the Negative Control.

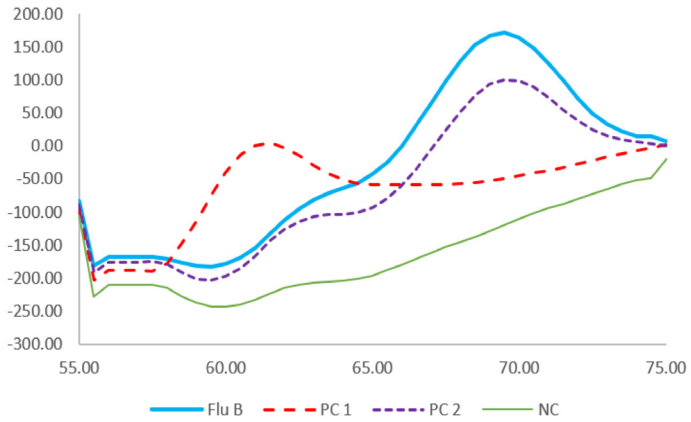


Figure 4: The melt peak of the sample in the FAM channel is aligned with the melt peak of Positive Control 2. The sample is characterized as Flu B positive.

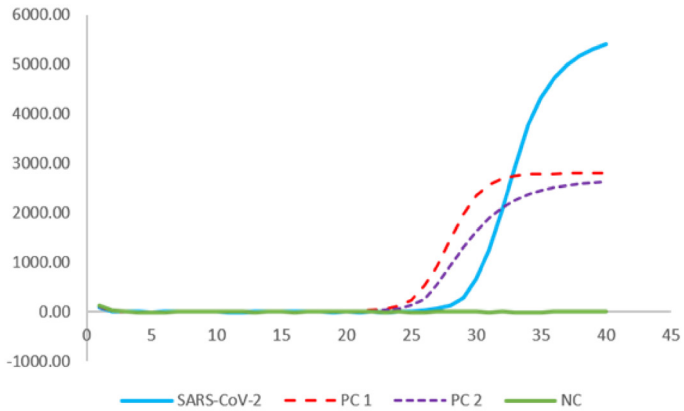


Figure 5: The sample, Positive Control 1 and Positive Control 2 show pathogen specific amplification in the ROX channel, whereas no fluorescence signal is detected in the Negative Control. The sample is characterized as SARS-CoV-2 positive.

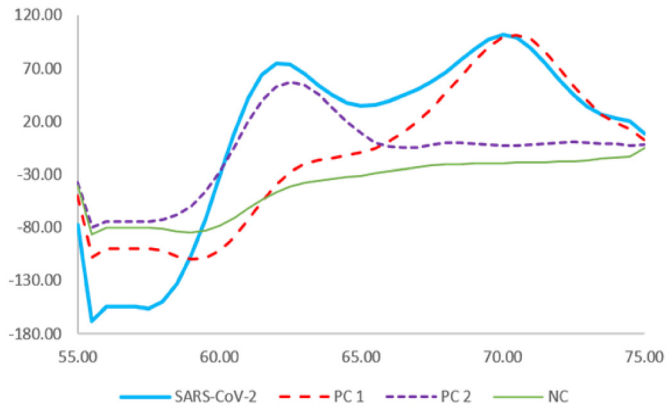


Figure 6: The melt peaks of the sample in the ROX channel are aligned with the melt peak of Positive Control 1 and Positive Control 2. Both genes (E gene, S gene) could be detected.

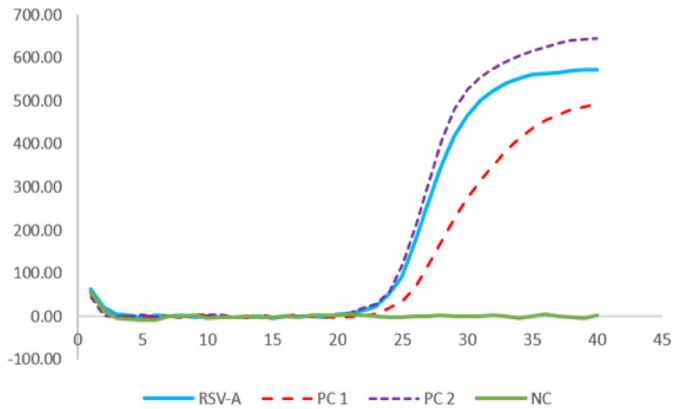


Figure 7: The sample, Positive Control 1 and Positive Control 2 show pathogen specific amplification in the Cy5 channel, whereas no fluorescence signal is detected in the Negative Control.

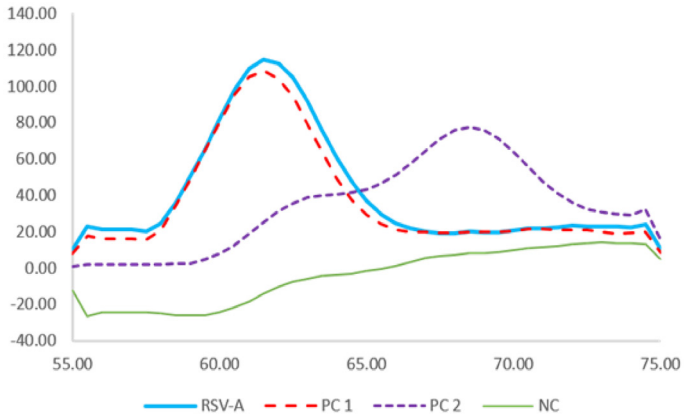


Figure 8: The melt peak of the sample in the Cy5 channel is aligned with the melt peak of Positive Control 1. The sample is characterized as RSV A positive.

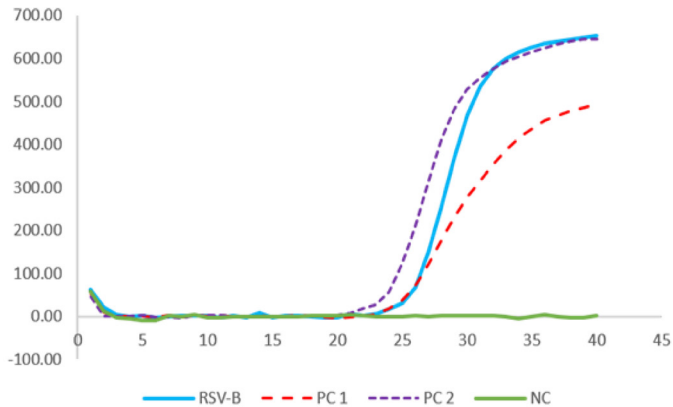


Figure 9: The sample, Positive Control 1 and Positive Control 2 show pathogen specific amplification in the Cy5 channel, whereas no fluorescence signal is detected in the Negative Control.

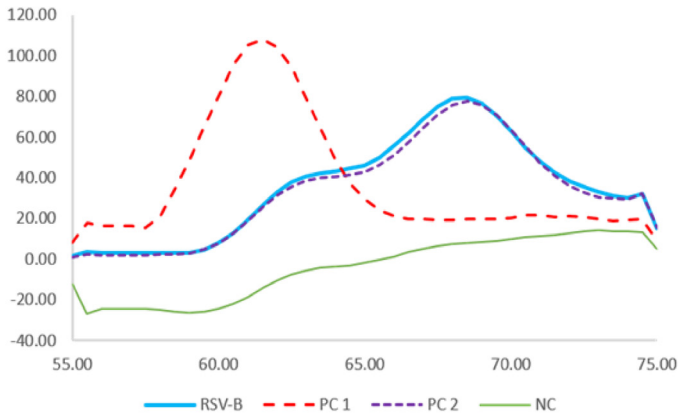


Figure 10: The melt peak of the sample in the Cy5 channel is aligned with the melt peak of Positive Control 2. The sample is characterized as RSV B positive.

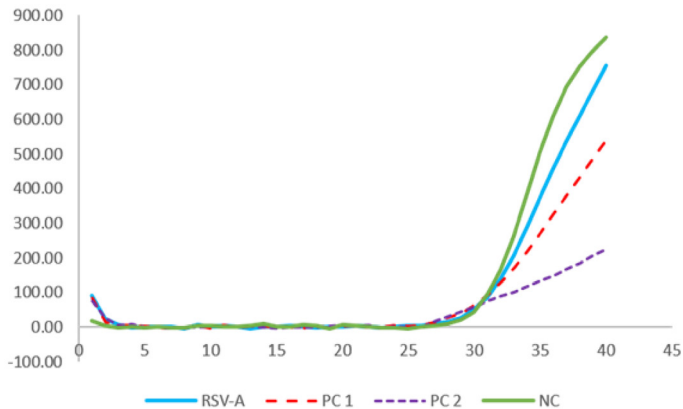


Figure 11: The positive sample, Positive Control 1, Positive Control 2 and the Negative Control show a signal in the Control RNA specific HEX channel (IPC). The amplification signal of the Control RNA in the negative sample shows that the missing signals in the pathogen specific channels FAM, ROX and Cy5 are not due to RT-PCR inhibition or failure of RNA isolation, but that the sample is a true negative sample. The melt curves are not evaluated for the HEX channel.

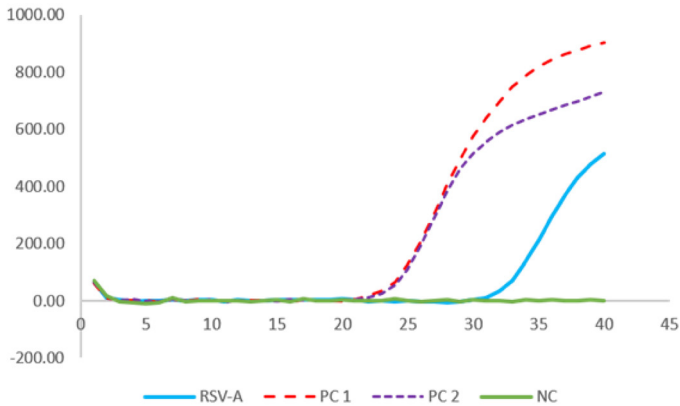


Figure 12: Signals of the amplification of the ISC in the Cy5.5 channel. The figure shows the C_T value of an eluate from a respiratory swab after nucleic acid extraction using MutaPLEX® Mag RNA/DNA nucleic acid extraction kit. The melt curves are not evaluated for the Cy5.5 channel.

Caution: In the case of weakly positive Influenza samples, attention must be paid to the course of the melt curve when differentiating. In the case of Influenza B Virus positive samples with copy numbers < 100 copies/ μ l, the melt peak can be absent. However, the course of the curve differs from negative samples / the Negative Control and can be misinterpreted as Influenza A Virus. Therefore, for a correct differentiation, the beginning of the course of the melt curve must be compared with that of the Positive Control 1 and Positive Control 2 (Figure 13).

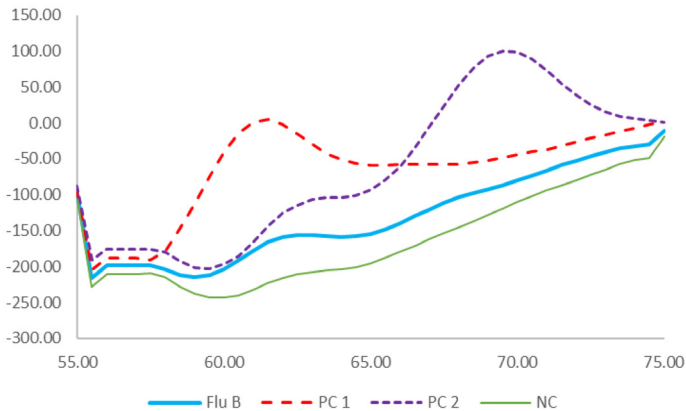


Figure 13: Here the melt curves of a weak Influenza B Virus positive sample, the Positive Control 1, the Positive Control 2 and the Negative Control are shown. The peak for Influenza B Virus is missing in the sample. Here attention must be paid to the beginning of the melt curve of

the sample. This is similar to the beginning of the melt curve of Positive Control 2 (Flu B). The slight wave at the height of the peak of the Positive Control 1 (Flu A) must not be considered as a peak.

13 ASSAY VALIDATION

Negative Control

In the Negative Control, no amplification curves and melt peaks are allowed to appear in the FAM, ROX, Cy5 and Cy5.5 channel. It must show no C_T and no T_m in these channels. There has to be an amplification curve in the HEX channel which must be below a C_T of 34.

Positive controls

All parameters in the Positive Control 1 and the Positive Control 2 must show a positive (i.e. exponential) amplification curve in the different channels FAM, ROX, Cy5 and Cy5.5 and a clearly interpretable melt peak in the FAM, ROX and Cy5 channel. The melt peak in the Cy5.5 channel is not evaluated and can be neglected. The Positive Controls must fall below a C_T of 30 in these channels. The HEX channel of the Positive Controls indicates the Internal Control (IPC) and must be below a C_T of 34.

Positive Control 1 contains target sequences for Influenza Virus A (FAM), SARS-CoV-2 E gene (ROX), RSV A (Cy5), the ISC (Cy5.5) and the IPC (HEX).

Positive Control 2 contains target sequences for Influenza Virus B (FAM), SARS-CoV-2 S gene (ROX), RSV B (Cy5), the ISC (Cy5.5) and the IPC (HEX).

Internal Controls

The following values for the amplification of the Internal Control are valid using gerbion nucleic acid extraction kit MutaCLEAN® Mag RNA/DNA.

All Internal Controls (ISC and IPC, seqc – sample and extraction quality control) must show a positive (i.e. exponential) amplification curve. The Control RNA (IPC) must fall below a C_T of 34. If the Control RNA is above C_T 34 this points to a purification problem or a strong positive sample that can inhibit the IPC. In the latter case, the assay is valid. It is recommended to perform the extraction of a water control in each run. The IPC in the water control must fall below a C_T of 34.

For accurately drawn respiratory swab samples, the ISC shows C_T values from app. 15 to app. 28. A heavily delayed signal of higher than a C_T of 34 indicates a low sample amount. Therefore, false negative results cannot be ruled out. In case of no amplifications in the FAM, ROX or Cy5 channel, there must be an amplification curve in the Cy5.5 channel (ISC) and the HEX channel (IPC).

If other nucleic acid extraction kits are used, the customer must define own cut-offs. In this case the C_T value of the Control RNA (IPC) in an eluate from a sample should

not be delayed for more than 4 C_T in comparison to an eluate from an extracted water control.

The melt peaks in the Internal Control channels (HEX and Cy5.5) are not evaluated and can be neglected.

14 LIMITATIONS OF THE METHOD

- Strict compliance with the instructions for use is required for optimal results.
- Use of this product is limited to personnel specially instructed and trained in the techniques of real-time PCR and *in vitro* diagnostic procedures.
- Good laboratory practice is essential for proper performance of this assay.
- All reagents should be closely monitored for impurity and contamination. Any suspicious reagents should be discarded.
- This assay must not be used on a biological specimen directly. Appropriate nucleic acid extraction methods have to be conducted prior to using this assay.
- The presence of RT-PCR inhibitors may cause false negative or invalid results.
- Potential mutations within the target regions of the Influenza Virus A, Influenza Virus B, RSV A, RSV B and SARS-CoV-2 genomes covered by the primers and/or probes used in the kit may result in failure to detect the respective RNA.
- As with any diagnostic test, results of the MutaPLEX® RespiraScreen 4 DIFF seqc real time RT-PCR Kit need to be interpreted in consideration of all clinical and laboratory findings.

15 TROUBLESHOOTING

The following troubleshooting guide is included to help you with possible problems that may arise when performing a real-time RT-PCR. If you have further questions, please do not hesitate to contact our scientists on info@immundiagnostik.com.

No fluorescence signal in the FAM and/or ROX and/or Cy5 and/or Cy5.5 channel of the Positive Controls.

The selected channel for analysis does not comply with the protocol

Select the FAM channel for analysis of the Influenza Virus specific amplification, the ROX channel for analysis of the SARS-CoV-2 specific amplification, the Cy5 channel for analysis of the RSV specific amplification, the Cy5.5 channel for

analysis of the ISC specific amplification and the HEX channel for analysis of the Control RNA specific amplification.

Incorrect preparation of the Master Mix

Make sure that the Enzyme is added to the Master Mix (chapter 11.2).

Incorrect configuration of the real-time-RT-PCR

Check your work steps and compare with chapter 11.

The programming of the thermal profile is incorrect

Compare the thermal profile with the protocol 'in chapter 11.5.

Incorrect storage conditions for one or more kit components or kit expired

Check the storage conditions and the date of expiry printed on the kit label. If necessary, use a new kit and make sure kit components are stored as described in 'Transport, storage and stability' (chapter 6).

Weak or no signal of the Control RNA and simultaneous absence of a signal in the FAM and/or ROX and/or Cy5 and/or Cy5.5 channel.

real time RT-PCR conditions do not comply with the protocol

Check the real time RT-PCR conditions in Table 5.

real time RT-PCR inhibited

Make sure that you use an appropriate isolation method (see chapter 'Sample Preparation') and follow the manufacturer's instructions. Make sure that the ethanol-containing washing buffers have been completely removed.

sample material not sufficient

Make sure that enough sample material has been applied to the extraction. Use an appropriate isolation method (see chapter 9 - 'Sample Preparation') and follow the manufacturer's instructions.

RNA loss during isolation process

Lack of an amplification signal in the HEX channel can indicate that the RNA isolation was not successful. Make sure that you use an appropriate isolation method (commercial kits are recommended) and stick to the manufacturer's protocol.

Incorrect storage conditions for one or more components or kit expired

Check the storage conditions and the date of expiry printed on the kit label. If necessary, use a new kit and make sure kit components are stored as described in 'Transport, Storage and Stability (chapter 6).

Detection of a fluorescence signal in the FAM and/or ROX and/or Cy5 and/or Cy5.5 channel of the Negative Control.***Contamination during preparation of the real-time RT-PCR***

Repeat the real-time RT-PCR in replicates. If the result is negative in the repetition, the contamination occurred when the samples were pipetted into the optical PCR reaction tubes. Make sure to pipet the Positive Controls last and close the optical PCR reaction tube immediately after adding the sample. If the same result occurs, one or more of the kit components might be contaminated. Make sure that workspace and instruments are decontaminated regularly. Use a new kit and repeat the real-time RT-PCR.

 $C_T > 35$ in the Cy5.5 channel

Since the MutaPLEX® RespiraScreen 4 DIFF seqc real time RT-PCR is very sensitive, airborne contamination with individual human cells can lead to C_T values > 35 in the Cy5.5 channel. The relevant range for the detection of the ISC in clinical samples is between C_T 20 and C_T 32. Therefore, C_T values > 35 in the Cy5.5 channel are not considered positive in this real time RT-PCR. The real time RT-PCR does not need to be repeated.

Problems with the evaluation of the melt curves***Occurrence of an unspecific peak in the ROX channel (between the peaks for E gene and S gene)***

Unspecific peaks between the peaks for the E gene and the S gene are evaluated as negative. A repetition of the sample is recommended.

Absence of the peak in higher RSV positive samples

For RSV positive samples with a C_T value < 20 it is possible that the peak in the melt curve will be missing. If a distinction between RSV A and RSV B is desired, the sample must be diluted to a C_T value > 20 and retested.

16 KIT PERFORMANCE

16.1 Analytical Sensitivity

The limit of detection (LoD) of MutaPLEX® RespiraScreen 4 DIFF seqc real time RT-PCR Kit was determined testing serial dilutions of synthetic RNA-fragments containing the specific gene target sequence on a CFX Opus 96 real time PCR instrument.

For evaluating the Amplification Curves, the LoD of the MutaPLEX® RespiraScreen 4 DIFF seqc real time RT-PCR Kit for Influenza Virus A is ≤ 2.5 genome copies per μl , for Influenza Virus B is ≤ 1 genome copy per μl , for SARS-CoV-2 is ≤ 0.25 genome copies per μl , for RSV A is ≤ 0.1 genome copies per μl and for RSV B is ≤ 2.5 genome copies per μl .

For evaluating the melt curves, the LoD of the MutaPLEX® RespiraScreen 4 DIFF seqc real time RT-PCR Kit for Influenza Virus A is ≤ 2.5 genome copies per μl (corresponds to a C_T value of approximately 36), for Influenza Virus B is ≤ 10 genome copies per μl (corresponds to a C_T value of approximately 31), for SARS-CoV-2 E gene is ≤ 0.25 genome copies per μl (corresponds to a C_T value of approximately 36), for SARS-CoV-2 S gene is ≤ 25 genome copies per μl (corresponds to a C_T value of approximately 30), for RSV A is ≤ 10 genome copies per μl (corresponds to a C_T value of approximately 31) and for RSV B is ≤ 2.5 genome copies per μl (corresponds to a C_T value of approximately 33).

16.2 Analytical Specificity

The specificity of the MutaPLEX® RespiraScreen 4 DIFF seqc real time RT-PCR Kit was evaluated with different ring trial samples of known status, different other relevant viruses and bacteria found in clinical samples and basing on in-silico analyses.

All samples were detected correctly. The results for the sample analysis are shown in Table 7. The results for the in-silico analysis are shown in Table 8.

Table 7: Eluted RNA from bacterial and viral pathogens tested for the determination of the analytical specificity of MutaPLEX® RespiraScreen 4 DIFF seqc real time RT-PCR Kit.

Eluates with known status	Result	Result	Result	Result
	Flu A / Flu B (FAM channel)	SARS-CoV-2 (ROX channel)	RSV A / RSV B (Cy5 channel)	IPC (HEX channel)
Influenza Virus A	positive	negative	negative	positive
Influenza Virus B	positive	negative	negative	positive
SARS-CoV-2	negative	positive	negative	positive
RSV A	negative	negative	positive	positive
RSV B	negative	negative	positive	positive

Eluates with known status	Result	Result	Result	Result
	Flu A / Flu B (FAM channel)	SARS-CoV-2 (ROX channel)	RSV A / RSV B (Cy5 channel)	IPC (HEX channel)
Adenovirus 31 (Spezies A)	negative	negative	negative	positive
Bordetella parapertussis	negative	negative	negative	positive
Bordetella pertussis	negative	negative	negative	positive
cMSSA (S. aureus, oxaS, PVL-pos)	negative	negative	negative	positive
Enterovirus (Echovirus 30)	negative	negative	negative	positive
Human Coronavirus HCoV-229E	negative	negative	negative	positive
Human Coronavirus HCoV-OC43	negative	negative	negative	positive
Human Coronavirus MERS-CoV	negative	negative	negative	positive
Legionella pneumophila SG 1	negative	negative	negative	positive
Metapneumovirus A1	negative	negative	negative	positive
MRSA (S. aureus, PVL-neg)	negative	negative	negative	positive
Mycobacterium tuberculosis complex	negative	negative	negative	positive
Mycoplasma pneumoniae	negative	negative	negative	positive
Parainfluenzavirus Type 1	negative	negative	negative	positive
Parainfluenzavirus Type 2	negative	negative	negative	positive
Parainfluenzavirus Type 3	negative	negative	negative	positive
Parainfluenzavirus Type 4	negative	negative	negative	positive
Pneumocystis jirovecii	negative	negative	negative	positive
Rhinovirus Type 5	negative	negative	negative	positive
Streptococcus agalactiae	negative	negative	negative	positive

Table 8: Inclusivity of the MutaPLEX® RespiraScreen 4 DIFF seqc real time RT-PCR Kit Primers and Probes (in silico analysis).

	120 - 5000 whole genome sequences	Homology	Comment
NCBI / GISAID	Flu B	Forward Primer	1000 sequences: 100% no mismatch
		Reverse Primer	1000 sequences: 100% no mismatch
		Probe	998 sequences: 100% 2 sequences: 96% (1 mismatch)
NCBI / GISAID	Flu A*	Forward Primer	4995 sequences: 100% 5 sequences: 96% (1 mismatch)
		Reverse Primer	4995 sequences: 100% 5 sequences: 96% (1 mismatch)
		Probe	5000 sequences: 100% no mismatch
NCBI / GISAID	SARS-CoV-2** (S gene)	Forward Primer	5000 sequences: 100% no mismatch
		Reverse Primer	5000 sequences: 100% no mismatch
		Probe	5000 sequences: 100% no mismatch
NCBI / GISAID	SARS-CoV-2** (E gene)	Forward Primer	5000 sequences: 100% no mismatch
		Reverse Primer	5000 sequences: 100% no mismatch
		Probe	5000 sequences: 100% no mismatch
NCBI / GISAID	RSV A / RSV B	Forward Primer	120 sequences: 100% no mismatch
		Reverse Primer	120 sequences: 100% no mismatch
		Probe	120 sequences: 100% no mismatch***

* The chosen Flu-A sequences includes H1 – H12 and N1 – N9 strains.

** The chosen SARS-CoV-2 sequences belong to the Delta (AY.x) and Omicron (BA.x) variants.

*** 2 mismatches are used to discriminate RSV-A from RSV-B. Apart from those, no other mismatches were present.

16.3 Clinical Samples

Positive (35) and negative (100) confirmed samples (oral and nasal swabs) for SARS-CoV-2 from the pandemic COVID-19 outbreak 2020 in Europe were tested. In addition, positive (10) confirmed samples (eluates) for SARS-CoV-2 Omicron Variant were tested.

The RNA was extracted by using the MutaCLEAN® Mag RNA/DNA (Immundiagnostik AG, Cat. No. KG1023) extraction kit.

The PCR experiments were performed on a CFX Opus 96 real time PCR instrument. The testing of the confirmed samples with MutaPLEX® RespiScreen 4 DIFF seqc real time RT-PCR Kit showed a sensitivity of 100% and a specificity of 100% when tested in comparison to another commercial kit (MutaPLEX® RespiScreen 1, Immundiagnostik AG). None of the samples were inhibited in the real time RT-PCR

field samples		MutaPLEX® RespiScreen 1 (KG192796)	
		SARS-CoV-2 positive	SARS-CoV-2 negative
MutaPLEX® RespiScreen 4 DIFF seqc	SARS-CoV-2 positive	45	0
	SARS-CoV-2 negative	0	100
		Sensitivity (%)	Specificity (%)
		100	100

field samples		MutaPLEX® RespiScreen 1 (KG192796)	
		Flu positive	Flu negative
MutaPLEX® RespiScreen 4 DIFF seqc	Flu A positive	0	0
	Flu B positive	0	0
	Flu A negative	0	145
	Flu B negative	0	145
		Sensitivity (%)	Specificity (%)
		100	100

field samples		MutaPLEX® RespiraScreen 1 (KG192796)	
		RSV positive	RSV negative
MutaPLEX® RespiraScreen 4 DIFF seqc	RSV A positive	0	0
	RSV B positive	0	0
	RSV A negative	0	145
	RSV B negative	0	145
		Sensitivity (%)	Specificity (%)
	100	100	

16.4 Linear Range

The linear range of the MutaPLEX® RespiraScreen 4 DIFF seqc real time RT-PCR Kit was evaluated by analysing logarithmic dilution series of in vitro transcripts and synthetic DNA fragments.

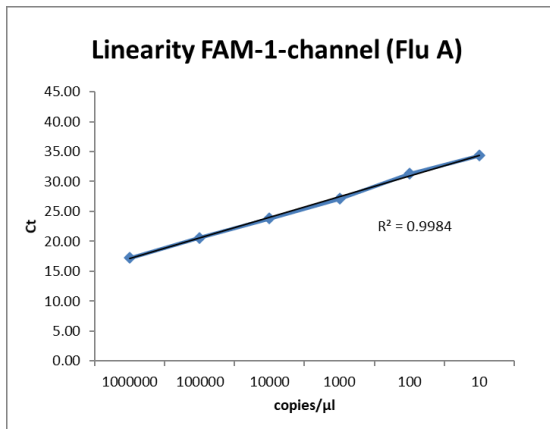


Figure 14: Determination of the linear range of MutaPLEX® RespiraScreen 4 DIFF seqc real time RT-PCR Kit for Influenza A Virus in the FAM channel.

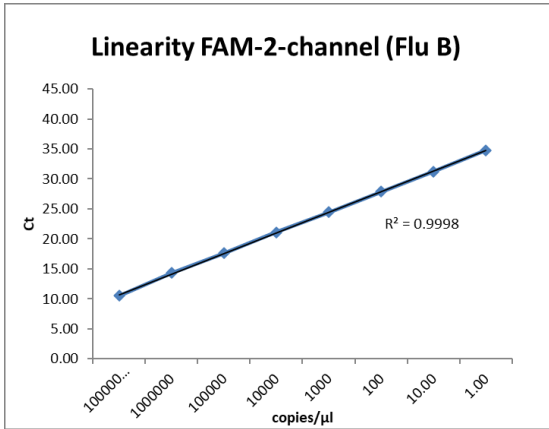


Figure 15: Determination of the linear range of MutaPLEX® RespiraScreen 4 DIFF seqc real time RT-PCR Kit for Influenza B Virus in the FAM channel.

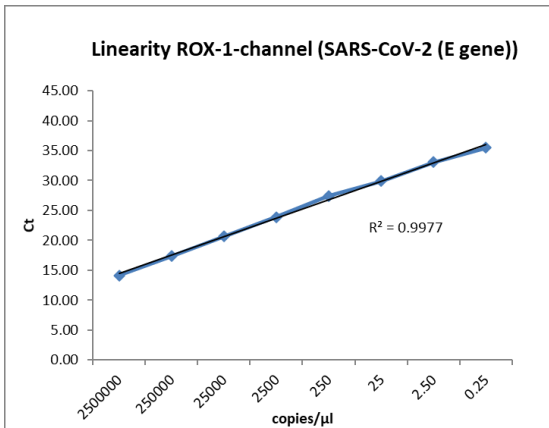


Figure 16: Determination of the linear range of MutaPLEX® RespiraScreen 4 DIFF seqc real time RT-PCR Kit for SARS-CoV-2 (E gene) in the ROX channel

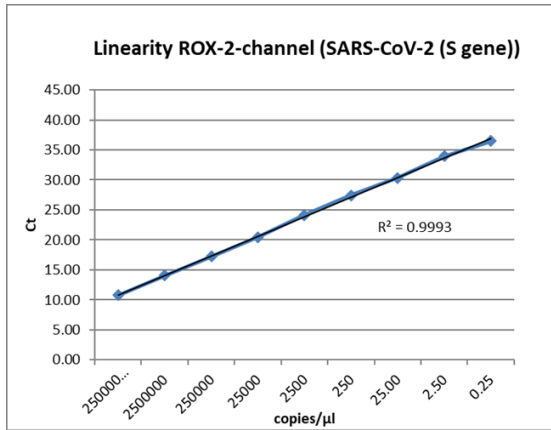


Figure 17: Determination of the linear range of MutaPLEX® RespiraScreen 4 DIFF seqc real time RT-PCR Kit for SARS-CoV-2 (S gene) in the ROX channel.

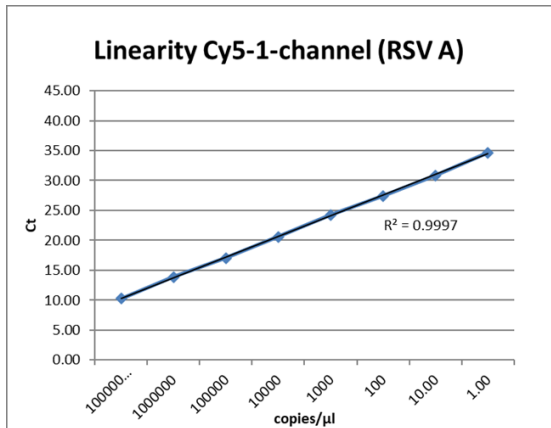


Figure 18: Determination of the linear range of MutaPLEX® RespiraScreen 4 DIFF seqc real time RT-PCR Kit for RSV A in the Cy5 channel.

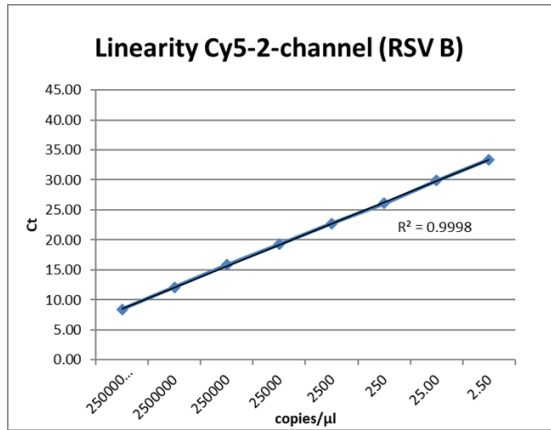


Figure 18: Determination of the linear range of MutaPLEX® RespiraScreen 4 DIFF seqc real time RT-PCR Kit for RSV B in the Cy5 channel.

16.5 Precision

The precision of the MutaPLEX® RespiraScreen 4 DIFF seqc real time RT-PCR Kit was determined as intra-assay variability, inter-assay variability and inter-lot variability.

Variability data are expressed by standard deviation and coefficient of variation. The data are based on quantification analyses of defined concentrations of M gene (Influenza Virus A) in vitro transcripts, NEP gene (Influenza Virus B) in vitro transcripts, SARS-CoV-2 (E gene and S gene) in vitro transcripts, G gene in vitro transcripts (RSV) and on the threshold cycle of the Control RNA (IPC). The results are shown in Table 9.

Table 9: Precision of the MutaPLEX® RespiraScreen 4 DIFF seqc real time RT-PCR Kit.

Flu A (FAM)	copies/μl	Standard Deviation	Coefficient of Variation [%]
Intra-Assay Variability	100	0.12	0.39
Inter-Assay-Variability	100	0.41	1.33
Inter-Lot-Variability	100	0.10	0.34
Flu B (FAM)	copies/μl	Standard Deviation	Coefficient of Variation [%]
Intra-Assay Variability	10	0.20	0.63
Inter-Assay-Variability	10	0.15	0.48
Inter-Lot-Variability	10	0.02	0.07
SARS-CoV-2 E gene (ROX)	copies/μl	Standard Deviation	Coefficient of Variation [%]
Intra-Assay Variability	2.5	0.25	0.77
Inter-Assay-Variability	2.5	0.18	0.55
Inter-Lot-Variability	2.5	0.05	0.15
SARS-CoV-2 S gene (ROX)	copies/μl	Standard Deviation	Coefficient of Variation [%]
Intra-Assay Variability	25	0.12	0.39
Inter-Assay-Variability	25	0.23	0.74
Inter-Lot-Variability	25	0.04	0.14
RSV A (Cy5)	copies/μl	Standard Deviation	Coefficient of Variation [%]
Intra-Assay Variability	10	0.16	0.51
Inter-Assay-Variability	10	0.30	0.97
Inter-Lot-Variability	10	0.03	0.11
RSV B (Cy5)	copies/μl	Standard Deviation	Coefficient of Variation [%]
Intra-Assay Variability	2.5	0.21	0.64
Inter-Assay-Variability	2.5	0.38	1.15
Inter-Lot-Variability	2.5	0.22	0.66

ISC (Cy5.5)	copies/μl	Standard Deviation	Coefficient of Variation [%]
Intra-Assay Variability	2.5	0.28	0.87
Inter-Assay-Variability	2.5	0.18	0.55
Inter-Lot-Variability	2.5	0.20	0.61
IPC (HEX)	copies/μl	Standard Deviation	Coefficient of Variation [%]
Intra-Assay Variability	500	0.17	0.57
Inter-Assay-Variability	500	0.31	1.07
Inter-Lot-Variability	500	0.14	0.47

16.6 Diagnostic Sensitivity

The diagnostic sensitivity of real time (RT-)PCR assays is mainly dependent on the DNA/RNA extraction method used to isolate DNA and RNA from various biological specimens. DNA/RNA extraction reagents are not part of the Immundiagnostik real time (RT-)PCR kits. Immundiagnostik real time (RT-)PCR kits include an extraction control and guidelines for the validation criteria of the extraction control in each reaction. The extraction control indicates inhibition of the real time (RT-)PCR and/or inefficient nucleic acid extraction. It cannot be used as a calibrator.








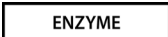







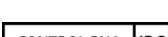


Therefore, Immundiagnostik guarantees the analytical sensitivities and specificities of the real time (RT-)PCR kits, performed with eluted DNA and RNA from reference materials and ring trial samples and with synthetic nucleic acid fragments. Immundiagnostik does not guarantee diagnostic sensitivities. If diagnostic sensitivities are mentioned in manuals of Immundiagnostik real time (RT-)PCR kits, the data are strictly correlated to a specific nucleic acid extraction method that has been used during the validation of the respective kits and cannot be transferred to other extraction methods. It is the responsibility of the user to qualify the extraction methods used for DNA/RNA isolation from biological samples.

17 LITERATURE

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18 ABBREVIATIONS AND SYMBOLS

(c)DNA	(complementary) Deoxyribonucleic acid		Catalog number
RNA	Ribonucleic acid		To be used with
PCR	Polymerase chain reaction		Contains sufficient for <n> test
RT	Reverse transcription		Upper limit of temperature
RT-PCR	Reverse transcription-PCR		Manufacturer
	Reaction mix		Use by
	Enzyme		Lot number
	Positive Control 1		Content
	Positive Control 2		Consult instructions for use
	Negative Control		<i>In vitro</i> diagnostic medical device
	Control RNA (IPC)		European conformity
	Unique Device Identification		

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