Arbeitsanleitung/Manual

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MutaPLEX®
RespiraScreen 2 DIFF

real-time-RT-PCR Kit

For the simultaneous in vitro detection and differentiation of RNA of SARSCoV-2 (E gene, RdRP gene and S gene), Influenza Virus A (Flu A) and Influenza Virus B (Flu B), extracted from biological specimens, with LightCycler® 480 II exclusivly.

Valid from 2021-02-08

REF **KG192896**
KG1928-384
KG1928-768

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1 INTENDED USE

The MutaPLEX® RespiraScreen 2 DIFF real time RT-PCR Kit is a screening assay for the simultaneous detection of three groups of different respiratory viruses. The design allows the differentiation of Influenza Virus A (Flu A), Influenza Virus B (Flu B) from the pandemic coronavirus SARS-CoV-2 (E gene, RdRP gene and S gene), extracted from biological specimens.

2 PATHOGEN INFORMATION

Influenza Viruses belong to the family of Orthomyxoviridae. They are the causative agent of 'the flu'. Influenza A and B viruses have a single stranded RNA genome, consisting of 8 RNA segments. 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 then type A.

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). 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.

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.

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.

3 PRINCIPLE OF THE TEST

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

Furthermore, MutaPLEX® RespiraScreen 2 DIFF 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.

4 PACKAGE CONTENTS

The reagents supplied are sufficient for 96, 384 or 768 reactions, respectively.

Table 1: Components of the MutaPLEX® RespiraScreen 2 DIFF real time RT-PCR Kit

Label	Lid Colour	Content		
		96	384	768
Reaction Mix	yellow	1 x 1 325 µl	4 x 1 325 µl	8 x 1 325 µl
Enzyme	blue	1 x 19.2 µl	1 x 76.8 µl	2 x 76.8 µl
Positive Control	red	1 x 150 µl	1 x 300 µl	1 x 300 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 tipps with filter
- Disposable gloves
- Table centrifuge
- Vortexer
- Roche LC 480II instrument
- Colour Compensation kit (MutaCLEAN CC-1 - KG19-5-CC)
- Optical PCR reaction plate with optical foil
- Optional: Liquid handling system for automation

6 TRANSPORT, STORAGE AND STABILITY

The MutaPLEX® RespiraScreen 2 DIFF 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.

8 SAMPLE MATERIAL

Starting material for MutaPLEX® RespiraScreen 2 DIFF 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 and one Negative Control should be included.
- 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 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)

Real time RT-PCR set-up

- Take an optical PCR reaction plate.
- Pipet 14 µl of the Master Mix into the respective well of the reaction plate.
- Add 6 µl of the eluates from the RNA isolation (including the eluate of the water control), the Positive Control and the Negative Control to the corresponding well of the reaction plate (Table 3).
- Close the reaction plate with optical foil immediately after filling in order to reduce the risk of contamination.

Table 3: Preparation of the real-time-RT-PCR

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

11.3 Instrument Settings

For the real time RT-PCR use the thermal profile shown in Table 4.

Table 4: real-time-RT-PCR thermal profile

Description	Time	Temperature	Number of Cycles	Aquisitions
Reverse Transcription	10 min	45 °C	1	no
Initial Denaturation	5 min	95 °C	1	no
Denaturation	10 sec	95 °C	45	no
Annealing and Extension	40 sec	60 °C		end of step

Further instrument settings have to be adjusted according to the table below.

Table 5: Overview of the instrument settings required for the MutaPLEX® RespiraScreen 2 DIFF real time RT-PCR

real-time-PCR-Instrument	Parameter Reaction Mix	Detection channel	Notes		
LightCycler 480II			Colour Compensation Kit MutaPlex® CC-1 (KG19-5-CC) is required		
			Melt factor	Quant Factor	Max Integration Time (s)
	Flu A	440–488	1	5	1
	SARS-CoV-2 (S gene)	465–510	1	10	1
	Control RNA (IPC)	533–580	1	10	2
	SARS-CoV-2 (E gene, RdRP gene)	533–610	1	10	2
	Flu B	618–660	1	10	3

12 DATA ANALYSIS

12.1 Following results can occur:

Table 6: Interpretation of the results for MutaPLEX® CoV-2 MUT

Signal/ C_T-Values					Interpretation
465–510 SARS-CoV-2	**533–610 SARS-CoV-2**	**440–488 Flu A**	**618–660 Flu B**	**533–580 Control RNA (IPC)**	
positive[1]	**positive**[1]	negative	negative	positive or negative[2]	**Positive result. The sample contains RNA of SARS-CoV-2.**
negative	negative	**positive**	negative	positive or negative[2]	**Positive result. The sample contains RNA of Flu A.**
negative	negative	negative	**positive**	positive or negative[2]	**Positive result. The sample contains RNA of Flu B.**
negative	negative	negative	negative	**≤ 34**	**Negative result. The sample contains no RNA of Flu A, Flu B, or SARS-CoV-2.**
negative	negative	negative	negative	negative or **> 34**[3]	**Caution! The real time RT-PCR is either inhibited or errors occurred while RNA/DNA extraction.**

[1] If the amount of SARS-CoV-2 RNA is below the limit of detection, the results from the 465–510 and the 533–610 channel may differ. In this case, a single positive result in one of the two channels is sufficient for a positive result.

[2] A strong positive signal in the 465–510, 533–610, 440–488 or 618–660 channel can inhibit the IPC. In such cases the result for the Control RNA can be neglected.

[3] In case of high C_T values, the IPC should be compared to the water extraction control as described in the chapter 'Assay validation'.

Figure 1, Figure 2, Figure 3, Figure 4, Figure 5 show examples for positive and negative real time RT-PCR results.

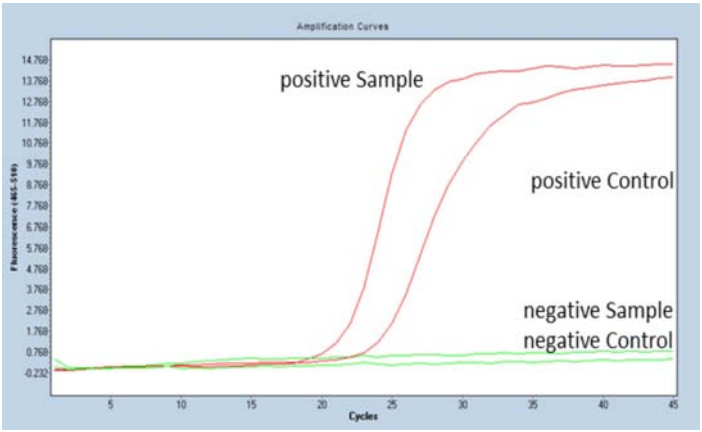

Figure 1: The positive sample shows pathogen specific amplification in the 465–510 channel (positive SARS-CoV-2 (S gene) sample and Positive Control), whereas no fluorescence signal is detected in the negative sample and the Negative Control (LightCycler 480 II).

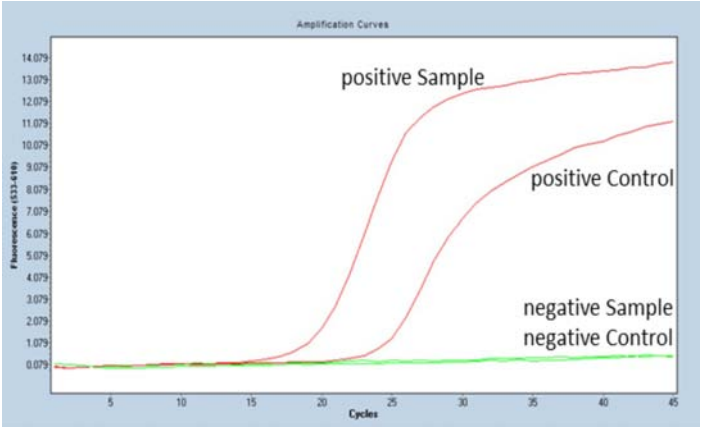

Figure 2: The positive sample shows pathogen specific amplification in the 533–610 channel (positive SARS-CoV-2 (E gene, RdRP gene) sample and Positive Control), whereas no fluorescence signal is detected in the negative sample and the Negative Control (LightCycler 480 II).

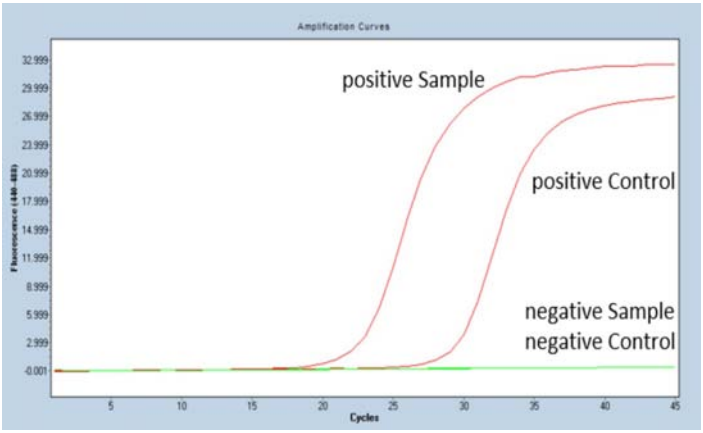

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Figure 3: The positive sample shows pathogen specific amplification in the 440–488 channel (positive Flu A sample and Positive Control), whereas no fluorescence signal is detected in the negative sample or the Negative Control (LightCycler 480 II).

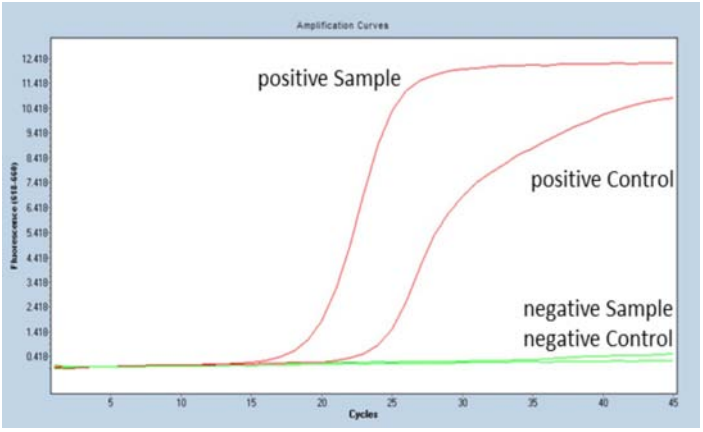

Figure 4: The positive sample shows pathogen specific amplification in the 618–660 channel (positive Flu B sample and Positive Control), whereas no fluorescence signal is detected in the negative sample and the Negative Control (LightCycler 480 II).

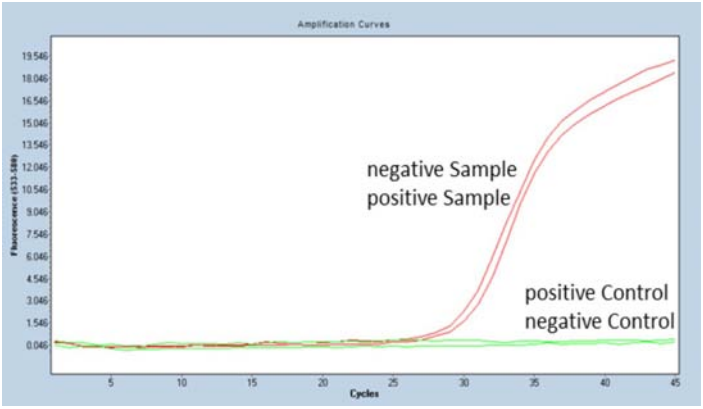

Figure 5: The positive sample and the negative sample show an amplification curve, whereas the Positive Control and the Negative Control don't show a signal in the Control RNA specific 533–580 channel (LightCycler 480 II). The amplification signal of the Control RNA in the negative eluate shows that the missing signal in the specific channels is not due to PCR inhibition or failure of RNA isolation, but that the eluate is a true negative.

13 ASSAY VALIDATION

Negative Control

The Negative Control must show no CT in the channels 440–488, 465–510, 533–580, 533–610 and 618–660.

Positive controls

All parameters in the Positive Control must show a positive (i.e. exponential) amplification curve in the different channels 440–488, 465–510, 533–610 and 618–660. The Positive Controls must fall below a C_T of 30.

Internal Controls

The following values for the amplification of the internal controls are valid using Immundiagnostik nuclic acid extraction kit MutaCLEAN® Mag RNA/DNA. The Control RNA (IPC) must show a positive (i.e. exponential) amplification curve and 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.

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.

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 Flu A, Flu 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 2 DIFF 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 for the Positive Control in the channel 440–488 and/or 465–510 and/or 533–610 and/or 618–660.

The selected channel for analysis does not comply with the protocol

Select the 465–510 channel for analysis of the SARS-CoV-2 (S gene) specific amplification, the 533–610 channel for analysis of the SARS-CoV-2 (RdRP gene and E gene) specific amplification, the 618–660 channel for analysis of the Flu B specific amplification, the 440–488 channel for analysis of the Flu A specific amplification and the 533–580 channel for the amplification of the Control RNA.

Incorrect preparation of the Master Mix

Make sure the enzyme is added to the master mix (chapter 11).

Incorrect configuration of the real-time-RT-PCR

Check your work steps and compare with 'Procedure' (chapter 11.2).

The programming of the thermal profile is incorrect

Compare the thermal profile with the protocol 'Instrument Settings' in Table 5 and Table 6.

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 (533–580) and simultaneous absence of a signal in the channel 440–488 and/or 465–510 and/or 533–610 and/or 618–660.

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 533–580 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 for the Negative Control signal in the channel 440–488 and/or 465–510 and/or 533–580 and/or 533–610 and/or 618–660.

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 Control 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.

16 KIT PERFORMANCE

16.1 Analytical Sensitivity

The limit of detection (LoD) of MutaPLEX® RespiraScreen 2 DIFF real time RT-PCR Kit was determined testing serial dilutions of synthetic RNA-fragments containing the specific gene target sequence on a LightCycler480II real time PCR instrument. The estimated LoD of MutaPLEX® RespiraScreen 2 DIFF real time RT-PCR Kit is ≤ 10 genome copies per reaction for each parameter (SARS-CoV-2, Flu A, Flu B).

16.2 Analytical Specificity

The specificity of the MutaPLEX® RespiraScreen 2 DIFF real time RT-PCR Kit was evaluated with different other relevant viruses and bacteria found in clinical samples and basing on in silico analyses.

The results for the sample analysis are shown in table 6, the results for the in silico analysis are shown in Table 7.

Table 6: Eluted RNA from bacterial and viral pathogens tested for the determination of the analytical specificity of MutaPLEX® RespiraScreen 2 DIFF real time RT-PCR Kit.

Eluates with known status	Result	Result	Result	Result
	SARS-CoV-2 (S gene)	SARS-CoV-2 (E gene, RdRP gene)	Flu A	Flu B
Parainfluenzavirus 1	negative	negative	negative	negative
Parainfluenzavirus 2	negative	negative	negative	negative
Parainfluenzavirus 3	negative	negative	negative	negative
Parainfluenzavirus 4	negative	negative	negative	negative
Metapneumovirus	negative	negative	negative	negative
Adenovirus	negative	negative	negative	negative
Enterovirus	negative	negative	negative	negative
Legionella pneumophila	negative	negative	negative	negative
Mycoplasma pneumophila	negative	negative	negative	negative
Mycobacterium tuberculosis complex	negative	negative	negative	negative
Bordetella pertussis	negative	negative	negative	negative
Bordetella parapertussis	negative	negative	negative	negative
Staphylococcus aureus	negative	negative	negative	negative
MRSA	negative	negative	negative	negative
Streptococcus ssp.	negative	negative	negative	negative
MERS-CoV	negative	negative	negative	negative
HCoV-229E	negative	negative	negative	negative
HCoV-OC43	negative	negative	negative	negative
SARS-CoV-2	positive	positive	negative	negative
Influenza A H1N1	negative	negative	positive	negative
Influenza A H3N2	negative	negative	positive	negative
Influenza A H5N1	negative	negative	positive	negative
Influenzavirus B	negative	negative	negative	positive
Respiratory Syncytial Virus A	negative	negative	negative	negative
Respiratory Syncytial Virus B	negative	negative	negative	negative

Table 7: Inclusivity of the MutaPLEX® RespiraScreen 2 DIFF real time RT-PCR Kit Primers and Probes (in silico analysis).

1000 - 5000 whole genome sequences		Homology	Comment
Flu B	Forward Primer	1 000 sequences: 100 %	no mismatch
	Reverse Primer	1 000 sequences: 100 %	no mismatch
	Probe	998 sequences: 100 %	2 sequences: 96% (1 mismatch)
Flu A	Forward Primer	5 000 sequences: 100 %	no mismatch
	Reverse Primer	5 000 sequences: 100 %	no mismatch
	Probe	5 000 sequences: 100 %	no mismatch
RdRP gene	Forward Primer	2 313 sequences: 100 %	7 sequences: 95% (1 mismatch)
	Reverse Primer	2 320 sequences: 100 %	no mismatch
	Probe	2 318 sequences: 100 %	2 sequences: 95% (1 mismatch)
S gene	Forward Primer	2 315 sequences: 100 %	5 sequences: 96% (1 mismatch)
	Reverse Primer	2 312 sequences: 100 %	8 sequences: 96% (1 mismatch)
	Probe	2 309 sequences: 100 %	11 sequences: 95% (1 mismatch)
E gene	Forward Primer	2 319 sequences: 100 %	1 sequence: 96% (1 mismatch)
	Reverse Primer	2 318 sequences: 100 %	2 sequences: 95% (1 mismatch)
	Probe	2 317 sequences: 100 %	3 sequences: 96% (1 mismatch))

16.3 Clinical Samples

Positive (106) and negative (171) confirmed samples (oral and nasal swabs) from the pandemic COVID-19 outbreak 2020 and 2021 in Europe were tested.

The RNA was extracted by using the MutaCLEAN® Mag RNA/DNA, Immundiagnostik Cat. No. KG1023 or KG1024 on a KingFisher™ Flex 96 Purification System.

The testing of the confirmed samples with MutaPLEX® RespiraScreen 2 DIFF real time RT-PCR Kit showed a sensitivity of 100 % and a specificity of 100 %.

	SARS-CoV-2 positive samples	SARS-CoV-2 negative samples
MutaPLEX® RespiraScreen 2 DIFF positive SARS-CoV-2	106	0
MutaPLEX® RespiraScreen 2 DIFF negative SARS-CoV-2	0	171
	Sensitivity (%)	**Specificity (%)**
	100	100

16.4 Linear Range

The linear range of the MutaPLEX® RespiraScreen 2 DIFF real time RT-PCR Kit was evaluated by analysing logarithmic dilution series of in vitro transcripts of the target sequences.

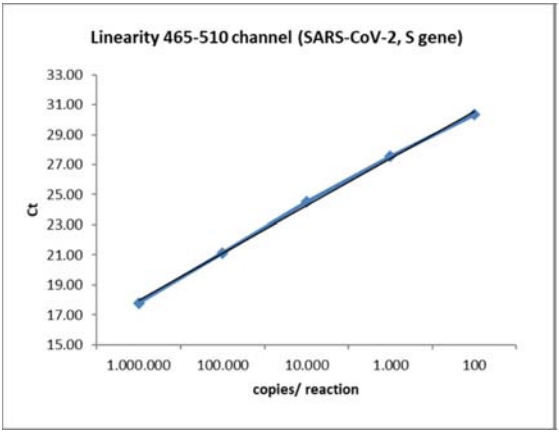

Figure 6: Determination of the linear range of MutaPLEX® RespiraScreen 2 DIFF real time RT-PCR Kit for SARS-CoV-2 (S gene) in the 465–510 channel.

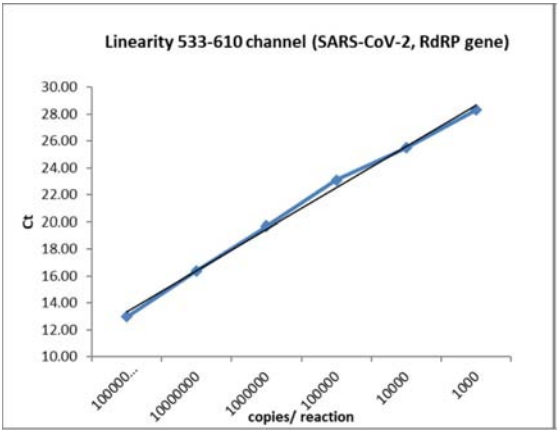

Figure 7: Determination of the linear range of MutaPLEX® RespiraScreen 2 DIFF real time RT-PCR Kit for SARS-CoV-2 (RdRP gene) in the 533–610 channel.

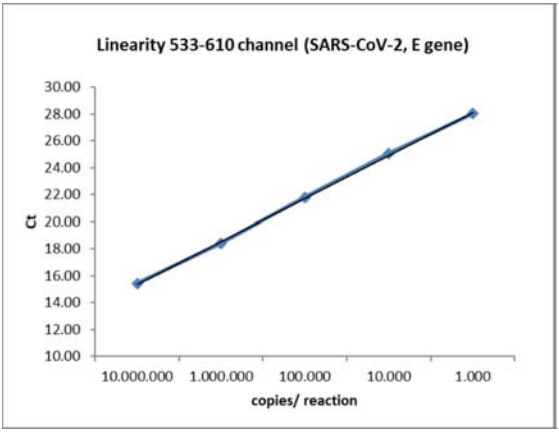

Figure 8: Determination of the linear range of MutaPLEX® RespiraScreen 2 DIFF real time RT-PCR Kit for SARS-CoV-2 (E gene) in the 533–610 channel.

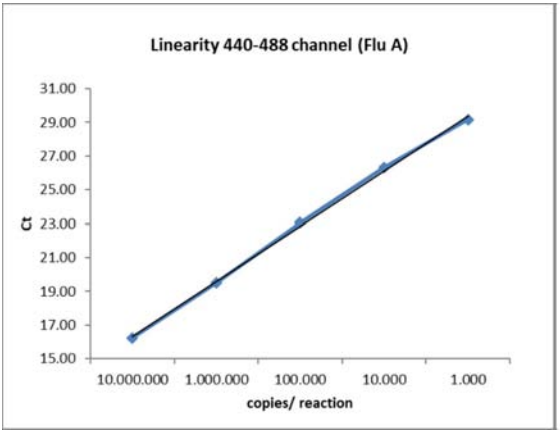

Figure 9: Determination of the linear range of MutaPLEX® RespiraScreen 2 DIFF real time RT-PCR Kit for Flu A in the 440–488 channel.

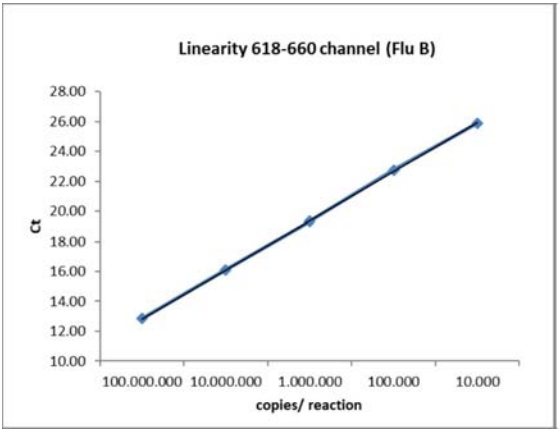

Figure 10: Determination of the linear range of MutaPLEX® RespiraScreen 2 DIFF real time RT-PCR Kit Flu B in the 618–660 channel.

16.5 Precision

The precision of the MutaPLEX® RespiraScreen 2 DIFF 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 Flu A in vitro

transcripts, Flu B in vitro transcripts, SARS-CoV-2 (E gene, RdRP gene and S gene) in vitro transcripts and on the threshold cycle of the Control RNA (IPC). The results are shown in Table 8.

Table 8: Precision of the MutaPLEX® RespiraScreen 2 DIFF real time RT-PCR Kit.

SARS-CoV-2, S gene (465–510)	**copies/ reaction**	**Standard Deviation**	**Coefficient of Variation [%]**
Intra-Assay Variability	100	0.23	0.68
Inter-Assay-Variability	100	0.50	1.63
Inter-Lot-Variability	100	0.00	0.02
SASR-CoV-2, RdRP gene and E gene (533–610)	**copies/ reaction**	**Standard Deviation**	**Coefficient of Variation [%]**
Intra-Assay Variability	500	0.23	0.75
Inter-Assay-Variability	500	0.39	1.3
Inter-Lot-Variability	500	0.40	1.3
Flu A (440–448)	**copies/ reaction**	**Standard Deviation**	**Coefficient of Variation [%]**
Intra-Assay Variability	1 000	0.55	1.81
Inter-Assay-Variability	1 000	0.61	2.0
Inter-Lot-Variability	1 000	0.16	0.52
Flu B (618–660)	**copies/ reaction**	**Standard Deviation**	**Coefficient of Variation [%]**
Intra-Assay Variability	500	0.15	0.48
Inter-Assay-Variability	500	0.28	0.92
Inter-Lot-Variability	500	0.21	0.70
IPC (533–580)	**copies/ reaction**	**Standard Deviation**	**Coefficient of Variation [%]**
Intra-Assay Variability	1 000	0.10	0.36
Inter-Assay-Variability	1 000	0.79	2.81
Inter-Lot-Variability	1 000	0.40	1.43

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.

AccuPlex™ SARS-CoV-2, Flu A/B and RSV Verification Panel member 1 was spiked in 20 samples of pooled confirmed negative pharyngeal swab samples in UTM® with a final concentration of 1 000 copies/ml and 500 copies/ml. Nucleic acids were extracted with theMutaCLEAN® Mag RNA/DNA on the KingFisher™ Flex 96 Purification System as described above. The results of the experiment on the LightCycler 480 II can be found in Table 9 and Table 10.

Table 9: Confirmation of the LoD on the LightCycler 480 II

Concentration 1 000 virus/mL				
replicate	SARS-CoV-2		Flu A	Flu B
	S gene	RdRP gene / E gene		
1	33.17	30.97	33.60	32.85
2	33.62	31.14	33.77	32.88
3	33.32	31.21	34.15	32.92
4	33.63	31.09	33.79	33.14
5	33.21	31.04	34.03	32.88
6	33.24	31.12	34.12	33.04
7	33.17	31.03	33.73	32.52
8	33.05	31.01	34.11	32.84
9	33.30	31.48	34.02	33.16
10	33.18	31.17	33.87	32.73
11	33.39	30.99	33.99	32.58
12	33.04	30.90	---	32.80
13	33.37	30.92	33.88	32.86
14	33.23	31.13	33.97	33.02
15	33.40	31.22	33.90	32.84
16	33.40	31.23	34.16	32.83
17	33.58	31.12	34.02	32.93
18	33.06	31.07	33.86	32.87
19	33.60	31.34	34.07	33.44
20	33.52	31.31	33.97	33.11
Mean CT	33.32	31.12	33.95	32.91
SD	0.19	0.15	0.15	0.20
CoV	0.58	0.47	0.45	0.62
Result	20/20	20/20	19/20	20/20

Table 9: Confirmation of the LoD on the LightCycler 480 II

Concentration 500 virus/mL				
replicate	SARS-CoV-2		Flu A	Flu B
	S gene	RdRP gene / E gene		
1	33.55	31.60	34.07	33.22
2	34.26	31.55	34.10	33.13
3	33.58	31.05	33.87	33.06
4	34.29	31.57	33.96	33.11
5	33.71	---	---	32.97
6	34.10	31.26	---	32.99
7	33.99	31.71	34.28	33.49
8	34.09	31.30	34.31	33.15
9	33.76	31.32	33.87	32.98
10	33.63	31.33	34.27	33.36
11	34.27	31.34	34.02	33.53
12	33.99	31.15	34.03	33.60
13	33.99	31.40	34.00	33.00
14	34.27	31.72	---	33.54
15	34.50	31.50	---	33.47
16	34.30	31.11	33.91	33.07
17	33.55	30.95	33.91	33.04
18	33.68	31.27	34.02	33.05
19	35.00	31.32	34.16	33.34
20	34.60	31.38	34.14	33.36
Mean CT	34.06	31.36	34.06	33.22
SD	0.39	0.21	0.14	0.22
CoV	1.15	0.68	0.42	0.65
Result	20/20	19/20	16/20	20/20

The MutaPLEX® RespiraScreen 2 DIFF real time RT-PCR Kit in combination with the NukEx Mag RNA/DNA nucleic acid extraction kit on a KingFisher™ Flex 96 Purification System and the LightCycler 480 II system detected 20/20 replicates at a

concentration of 500 copies/ml for SARS-CoV-2 and Flu B and 19/20 replicates at a concentration of 1 000 copies/ml for Flu A.

Consequently, the confirmed LoDs are:

- 500 copies/ml for SARS-CoV-2
- 500 copies/ml for Flu B
- 1 000 copies/ml for Flu A

17 LITERATURE

[1] www.who.int/health-topics/coronavirus

[2] Corman et al. Detection of 2019 novel coronavirus (2019-nCoV) by real time RT-PCR. Eurosurveillance, Volume 25, Issue 3, 23/Jan/2020.

[3] www.nature.com/articles/s41564-020-0695-z, 02/March/2020

[4] https://www.ncbi.nlm.nih.gov/research/coronavirus

[5] https://www.nhs.uk/conditions/sars

18 ABBREVIATIONS AND SYMBOLS

RNA	Ribonucleic Acid
RT-PCR	Reverse Transkription Polymerase Chain Reaction
IVD	In Vitro Diagnostic Medical Device
	Manufacturer
LOT	Lot number
CONTROL +	Positive Control
CONTROL -	Negative Control
CONTROL RNA IPC	Control RNA (IPC)
Reaction Mix	Reaction Mix
ENZYME	Enzyme
REF	Catalogue Number
→REF	To be used with
Σ	Contains sufficient for <n> tests
	Use by YYYY-MM-DD
	Consult instructions for use
20°C	Upper limit of Temperature
CE	European Conformity