Manual

MutaPLEX® CoV-2 MUT 2

Real-Time-RT-PCR-Kit

For the simultaneous detection of the SARS-CoV-2 Spike protein mutations E484K and N501Y

Valid from 2021-05-12

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1 INTENDED USE

The MutaPLEX® CoV-2 MUT 2 Real-Time RT-PCR Kit is an assay for the detection of point mutations in the spike protein of SARS-CoV-2 from biological specimens. The test kit is used with samples that have been prequalified with screening PCRs like MutaPLEX® Coronavirus real time RT-PCR Kit (Immundiagnostik AG, Cat. No. KG192696). The determination of a specific lineage requires another test kit, e.g. MutaPLEX® CoV-2 MUT (Immundiagnostik AG, Cat. No. 193196).

2 PRINCIPLE OF THE TEST

The MutaPLEX® CoV-2 MUT 2 Real-Time RT-PCR Kit contains specific primer and probe systems for the detection of two SARS-CoV-2 spike protein mutations present in most of the Variants of Concern. The two detected mutations are E484K (present in P.1 (Brazilian Variant) and B.1.351 (South African Variant) and N501Y which is present in most of the Variants of Concern, including B.1.1.7 (UK Variant), P.1 and B.1.351. The result of the melting curve does not allow to determine a specific strain or variant of SARS-CoV-2, but it shows the presence of crucial point mutations that are suspected to alter the characteristics of the virus.

3 PACKAGE CONTENTS

The reagents supplied are sufficient for 96 reactions.

Table 1: Components of the MutaPLEX® CoV-2 MUT 2 Real-Time-RT-PCR Kit .

Label	Lid Colour	Content
Reaction Mix	yellow	1 x 1 325 µl
Enzyme	blue	1 x 19.2 µl
10 x Positive Control WT	red	1 x 50 µl
10 x Positive Control Mut	violett	1 x 50 µl
Negative Control	green	1 x 150 µl

4 EQUIPMENT AND REAGENTS TO BE SUPPLIED BY USER

- Sterile microtubes
- Calibrated precision pipets (adjustable volume) and sterile single-use tipps with filter
- Disposable gloves
- Table centrifuge

- PCR grade water *
- Vortexer
- Real-Time PCR instrument
- Optical PCR reaction tubes with lid or optical PCR reaction plate with optical foil
- Optional: Liquid handling system for automation

  * Immundiagnostik AG recommends the use of Ultra Pure Water (Water Type 1; ISO 3696), which is free of undissolved and colloidal ions and organic molecules (free of particles > 0.2 µm) with an electrical conductivity of 0.055 µS/cm at 25 °C (≥ 18.2 MΩ cm).

5 TRANSPORT, STORAGE AND STABILITY

The MutaPLEX® CoV-2 MUT 2 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 to +8 °C for up to 6 months. Protect kit components from direct sunlight during the complete test run.

6 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.

7 SAMPLE MATERIAL

Starting material for MutaPLEX® CoV-2 MUT 2 Real-Time RT-PCR Kit is RNA qualified SARS-CoV-2 positive by Real-Time RT-PCR (e.g. MutaPLEX Coronavirus Real-Time RT-PCR Kit, Imundiagnostik, Cat. No. KG 192696).

Eluates with very low copy numbers resulting in CT values > 32 are not suitable for testing with the MutaPLEX® CoV-2 MUT 2 Real-Time RT-PCR.

8 REAL-TIME-RT-PCR

8.1 Important points before starting

- Please pay attention to chapter 6 "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 WT, one Positive Control Mut 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.

8.2 Procedure

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)

8.3 Preparation of the Positive Controls

The Positive Control WT and the Positive Control Mut are stored in an extra storage buffer which may alter the peak of the melting curves. For a better comparison with the samples, both Positive Controls need to be **freshly** diluted 1:10 in PCR grade water.

Table 3: Preparation of the Positive Control

Component	Volume
Positive Control WT or Mut	2 µl
PCR grade water	18 µl

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, the two Positive Controls 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

8.4 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	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
Melting Curve	see the tables below for individual cycler settings			

LightCycler 480II

Programm Step	Melting Curve			Cooling
Parameter				
Analysis Mode	Melting Curves			None
Cycles	1			1
Target [°C]	95	40	75	40
Hold [hh:mm:ss]	00:00:30	00:02:00	-	00:00:30
Ramp Rate [°C/s]	4.4	1.5	0.29	1.5
Acquisition Mode	None	None	Continuous	None
Acquisitions [per °C]	-	-	1	-

Bio-Rad CFX96

Programm Step	Melt Curve
Parameter	
Melt from	52.0 °C to 72.0 °C
Increment	0.5 °C for 0:05 + Plate Read

Mic qPCR Cycler

Programm Step	Melt
Parameter	
Melt from	52.0 °C to 72.0 °C at 0.1 °C/s
Acquire on	Green

Programm Step	Melt
Parameter	
Melt from	52.0 °C to 72.0 °C at 0.1 °C/s
Acquire on	Red

NEOS-96 qPCR / NEOS-48 qPCR

Programm Step	Continuous Melt	
Parameter		
Cycle	1	
Step	1	2
Temperature	52.0 °C	72.0 °C
Time	00:01	-
Fluorescence	None	5 Readings/°C

QuantStudio 5

Programm Step	Melt Curve Stage	
Parameter		
Step	1	2 (Dissociation)
Temperature	52.0 °C	72.0 °C
Time	00:01	00:01
Ramp Rate	1.6 °C/s	0.1 °C/s

Dependent on the Real-Time instrument used, further instrument settings have to be adjusted according to table 6.

Table 6: Overview of the instrument settings required for the MutaPLEX® CoV-2 MUT 2 Real-Time-RT-PCR.

Real-Time-PCR-Instrument	Parameter Reaction Mix	Detection channel	Notes
LightCycler 480II	E484K N501Y	465 – 510 618 – 660	Colour Compensation not required Melt factor: 1 / Quant factor: 10 / Max integration time (s): 1 Melt factor: 1 / Quant factor: 10 / Max integration time (s): 3
QuantStudio 5 Bio-Rad CFX96	E484K N501Y	FAM Cy5	Reference Dye: None
NEOS-48 qPCR NEOS-96 qPCR	E484K N501Y	FAM Cy5	Reference Dye: None
Mic qPCR Cycler	E484K N501Y	Green Red	Gain 8 Gain 10

9 DATA ANALYSIS

9.1 Interpretation of the PCR Signals

SARS-CoV-2 positive samples should show curves in the FAM and Cy5 channel. The presence of the curves in the amplification process is no criteria for the data analysis. SARS-CoV-2 negative sample must show no amplification curve.

9.2 Interpretation of the melting curve

Figure 1 and Figure 2, show examples for Real-Time RT-PCR melting curve results of the mutations and the wildtype (WT) of SARS-CoV-2 spike proteins.

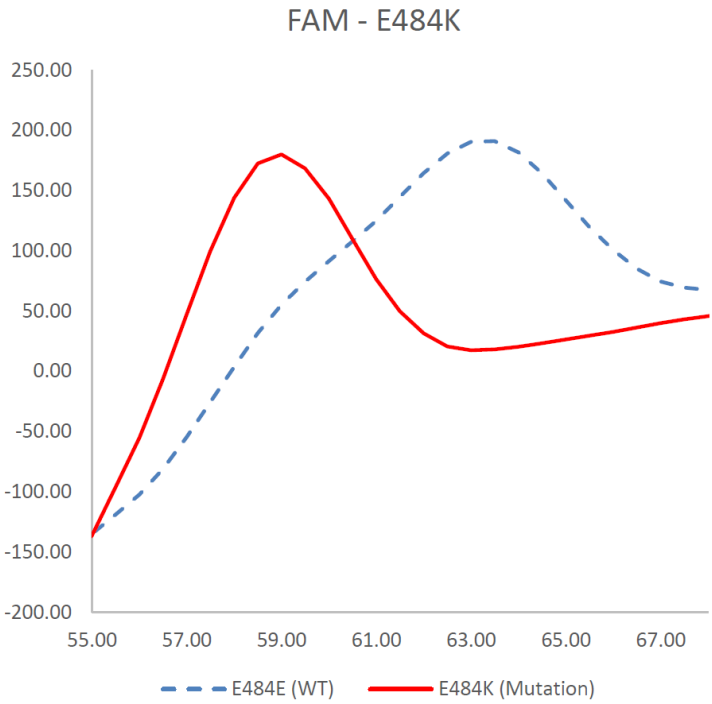

Fig 1: The melting curve of a sample positive for the E484K mutation (red line, Peak at 59 °C) in comparison to the melting curve of a wildtype sample (dashed blue line, Peak at 63.5 °C).

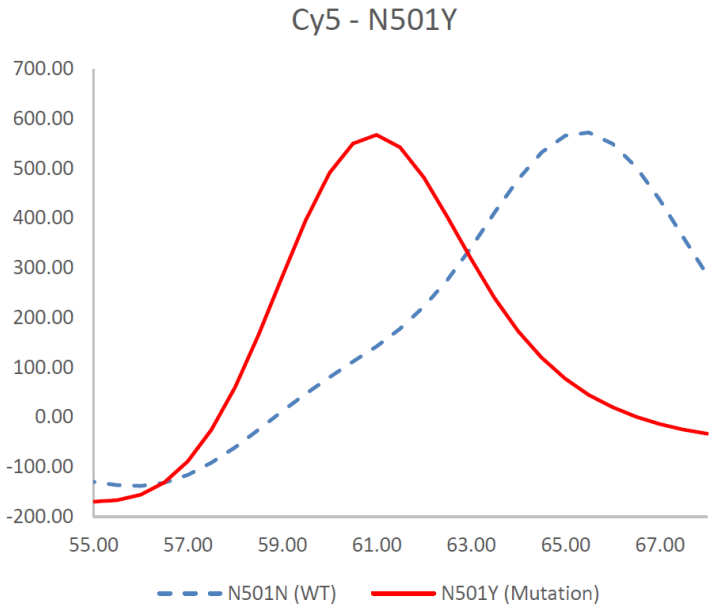

Fig 2: The melting curve of a sample positive for the N501Y mutation (red line, Peak at 61 °C) in comparison to the melting curve of a wildtype sample (dashed blue line, Peak at 65 °C).

9.3 Interpretation of the results

The melting point of the Positive Control WT should be around 4 degrees higher than the melting point of the Positive Control Mut. It must be possible to clearly assign the peaks of the samples to one of the peaks of the Positive Controls. Their melting points may only deviate by ± 1.0 degrees from that of the corresponding Positive Control.

Furthermore, it is essential to look at the melting curves for the associated values given by the cycler. Melting peaks can occur which are not evaluated by the cycler but can be clearly evaluated optically.

Table 7: Interpretation of the results for MutaPLEX® CoV-2 MUT 2

Channel	Melting Peak	Interpretation
FAM	Melting peak of the sample aligned with melting peak of Positive Control Mut	**E484K mutation** is detected
	Melting peak of the sample aligned with melting peak of Positive Control WT	**Wildtype** is detected
	Melting peak of the sample not aligned with melting peak of one of the Positive Controls	another mutation is possible
	No melting peak	not enough sample material or SARS-CoV-2 negative
Cy5	Melting peak of the sample aligned with melting peak of Positive Control Mut	**N501Y mutation** is detected
	Melting peak of the sample aligned with melting peak of Positive Control WT	**Wildtype** is detected
	Melting peak of the sample not aligned with melting peak of one of the Positive Controls	another mutation is possible
	No melting peak	not enough sample material or SARS-CoV-2 negative

10 ASSAY VALIDATION

Negative Control

The Negative Control must show no peak in the melting curve in the FAM and the Cy5 channel.

Positive control WT

The Positive Control WT should show a peak in the melting curve in the FAM and Cy5 channel.

Positive control Mut

The Positive Control Mut must show a peak in the melting curve in the FAM and Cy5 channel which is around 4 degrees lower than the peak of the Positive Control WT.

11 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.
- As with any diagnostic test, results of the MutaPLEX® CoV-2 MUT Real-Time-RT-PCR Kit need to be interpreted in consideration of all clinical and laboratory findings.

12 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 melting curve peaks in the FAM and/or Cy5 channel of the Positive Controls

The selected channel for analysis does not comply with the protocol

Select the detection channels according to table 6.

Incorrect preparation of the Master Mix

Make sure the enzyme is added to the master mix (chapter 8).

Incorrect configuration of the Real-Time-RT-PCR

Check your work steps and compare with chapter 8.

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 chapter Transport, storage and stability.

No melting curve peaks in the FAM and/or Cy5 channel in a sample

The sample does not contain enough RNA to guarantee a proper result

Review the prequalification of the screening PCR. If the Ct value for SARS-CoV-2 detection is > 35, the eluate is not suitable. If the Ct value is < 35 repeat the PCR with this sample.

Detection of a melting curve peak in the FAM and/or Cy5 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.

The peaks of the melting curve do not align with the 'Data Analysis'

Atypical peaks appear at the beginning or ending of the melting curve

Peaks close to the beginning or the ending of the melting curves should not be considered in the data analysis.

The Positive Controls were not diluted for the PCR reaction

Peaks of the Positive Controls are lowered by 2 degrees.

Sensitivity, fluorescence intensity and melt peak may differ on individual real time PCR Cyclers

The samples should be aligned with the two provided Positive Controls.

13 KIT PERFORMANCE

Detailed information based on the latest state of knowledge is available at Immundiagnostik AG. Please address your inquiry to info@immundiagnostik.com.

14 ABBREVIATIONS AND SYMBOLS

RT-PCR	Reverse transcription-PCR	REF	Catalog number
RNA	Ribonucleid acid	IVD	*In vitro* diagnostic medical device
REACTION MIX	Reaction mix		Contains sufficient for <n> test
ENZYME	Enzyme		Upper limit of temperature
CONTROL WT +	Positive control WT		Manufacturer
CONTROL MUT +	Positive control Mut		Use by YYYY-MM-DD
CONTROL –	Negativ control		Consult instructions for use
→REF	use with	LOT	Batch code

15 LITERATURE

1. www.who.int/health-topics/coronavirus
2. Rambaut et al. Preliminary genomic characterisation of an emergent SARS-CoV-2 lineage in the UK defined by a novel set of spike mutations. nCoV-2019 Genomic Epidemiology
3. Garry. Mutations arising in SARS-CoV-2 spike on sustained human-to-human transmission and human-to-animal passage. nCoV-2019 Genomic
4. Faria et al. Genomic characterisation of an emergent SARS-CoV-2 lineage in Manaus: preliminary findings. nCoV-2019 Genomic Epidemiology

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