Manual

MutaPLEX®
Monkeypox virus
real-time-PCR Kit

For in vitro detection of the DNA of Monkeypox virus extracted from biological specimens

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1 INTENDED PURPOSE

The MutaPLEX® Monkeypox virus real time PCR Kit is designed for the qualitative detection of the nucleic acid of Monkeypox virus in eluates from biological specimens.

For reasearch use only. Not for use in diagnostic procedures.

2 PATHOGEN INFORMATION

Monkeypox virus of the genus Orthopox in the family Poxviridae is a double-stranded DNA virus and is the causative agent of monkeypox. Monkeypox is a viral zoonosis (a virus transmitted to humans from animals) with symptoms similar to those seen in the past in smallpox patients, although it is clinically less severe. Symptoms include skin eruption, fever, intense headache, myalgia and intense asthenia. In rare cases, death has been reported after an infection with Monkeypox virus. The disease however is self limited with the symptoms lasting 2-4 weeks.

With the eradication of smallpox in 1980 and subsequent cessation of smallpox vaccination, monkeypox has emerged as the most important Orthopox virus for public health. Animal hosts include a range of rodents and non-human primates [1]. In May 2022, multiple cases of monkeypox were identified in several non-endemic countries. The outbreak seems to be based on human-to-human transmission.

3 PRINCIPLE OF THE TEST

The MutaPLEX® Monkeypox virus real time PCR Kit contains specific primers and dual-labeled probes for the amplification of the DNA of Monkeypox virus (OPG185 gene) extracted from biological specimens.

The presence of nucleic acid is detected by an increase in fluorescence due to hydrolysis of the probes during amplification. The fluorescence of the Monkeypox virus specific probe is measured in the FAM channel. The real time PCR protocol includes a melting curve to ensure the specificity of the detected signal.

Furthermore, the MutaPLEX® Monkeypox virus real time PCR Kit contains a Control DNA (Internal Process Control, IPC), which is added during DNA extraction and detected in the same reaction by a HEX-labeled probe.

The Control DNA allows the detection of 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 reactions.

Table 1: Components of the MutaPLEX® Monkeypox virus real time PCR Kit

Label	Lid Colour	Content
Reaction Mix	yellow	1 x 1344 µl
Positive Control	red	1 x 50 µl
Negative Control	green	1 x 150 µl
Control DNA	colourless	1 x 480 µl

5 EQUIPMENT AND REAGENTS TO BE SUPPLIED BY USER

- DNA isolation kit (e. g. MutaCLEAN® Mag RNA/DNA, KG1023 or KG1024).
- PCR grade water
- 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® Monkeypox virus real time 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 mix components from different lots.

8 SAMPLE MATERIAL

Starting material for MutaPLEX® Monkeypox virus real time PCR Kit is DNA isolated from biological specimens.

Biological specimen used for the DNA extraction can be respiratory swabs or swabs of blisters or crusts of the human skin.

9 SAMPLE PREPARATION

Commercial kits for DNA 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 DNA in the samples to the amplification of the internal control in the water control will give insights on possible inhibitions of the real time PCR. Furthermore, possible contaminations during DNA extraction will be detectable.

Please note the chapter ‚Control DNA'.

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

10 CONTROL DNA

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

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

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

11 REAL-TIME-PCR

11.1 Important points before starting

- Please pay attention to the chapter 7 ‚Warnings and Precautions'.
- Before setting up the real time 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 PCR set up.
- In every 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 and centrifuged very briefly.

11.2 Preparation of the Positive Control

The Positive Control is stored in an extra storage buffer which may alter the peak of the melting curves. For a better comparison with the samples, the Positive Control must be freshly diluted 1:10 in PCR grade water before each PCR run.

Prepare the Positive Control according to Table 2.

Table 2: Preparation of the Positive Control

Component	Volume
Positive Control	2.0 µl
PCR grade water	18.0 µl

11.3 Procedure

The Master Mix contains all of the components needed for the real time 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 3: Preparation of the master mix

Volume per reaction	Volume master mix
14.0 µl Reaction Mix	14.0 µl x (N+1)

11.4 Real time 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 DNA isolation (including the eluate of the water control), the diluted Positive Control 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-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 PCR use one of the thermal profiles shown in Table 5 and Table 6. For Immundiagnostik AG real time PCR kits used for amplification of DNA, the reverse transcription can be omitted.

Table 5: real-time-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
Denaturation	10 sec	95 °C	45	no
Annealing and Extension	40 sec	60 °C		end of step
Melt Curve	see the table below for individual cycler settings			

Table 6: real-time-PCR thermal profile without reverse transcription

Description	Time	Temperature	Number of Cycles	Aquisition
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
Melt Curve	see the table 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	76	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 / CFX Opus96

Programm Step	Melt Curve
Parameter	
Melt from	52.0 °C to 76.0 °C
Increment	0.5 °C for 0:05 + Plate Read

Mic qPCR Cycler

Programm Step	Melt
Parameter	
Melt from	52.0 °C to 76.0 °C at 0.1 °C/s
Acquire on	Green

NEOS-48 qPCR

Programm Step	Continuous Melt	
Parameter		
Cycle	1	
Step	1	2
Temperature	52.0 °C	76.0 °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	76.0 °C
Time	00:01	00:01
Ramp Rate	1.6 °C/s	0.1 °C/s

MyGo Mini S

Programm Step	Melt
Parameter	
Melt from	52.0 °C to 76.0 °C
Initial Stage Ramp	1.5 °C
Final Stage Ramp	0.1 °C

Dependent on the real time PCR instrument used, further instrument settings have to be adjusted according to Table 7.

Table 7: Overview of the instrument settings required for the MutaPLEX® Monkeypox virus real time PCR

<table>
<tr><th>Real-Time-PCR-Instrument</th><th>Parameter
Reaction Mix</th><th>Detection channel</th><th>Notes</th></tr>
<tr><td>LightCycler 480II</td><td>Monkeypox virus
Control DNA (IPC)</td><td>465 – 510
533 – 580</td><td>Color Compensation Kit needed, e.g. pre-installed universal CC FAM (510) – VIC (580)
<table><tr><th>Melt factor</th><th>Quant factor</th><th>Max integration time (s)</th></tr><tr><td>1</td><td>10</td><td>1</td></tr><tr><td>1</td><td>10</td><td>2</td></tr></table></td></tr>
<tr><td>QuantStudio 5
CFX96
CFX Opus96
NEOS-48 qPCR
MyGo Mini S</td><td>Monkeypox virus

Control DNA (IPC)</td><td>FAM

HEX</td><td>Option Reference Dye ROX: NO</td></tr>
</table>

Real-Time-PCR-Instrument	Parameter Reaction Mix	Detection channel	Notes
Mic qPCR Cycler	Monkeypox virus	Green	Gain 8
	Control DNA (IPC)	Yellow	Gain 10

12 DATA ANALYSIS

Following results can occur:

Signal / C_T Values		Melting Curve / T_m Values [4]	
FAM Channel	**HEX Channel**	**FAM Channel**	**Interpretation**
Monkeypox virus	**IPC**		
positive	positive or negative [1]	**68 - 72 °C [2]**	**Positive result. The sample contains Monkeypox virus.**
positive or negative	positive or negative [1]	**< 68 °C**	**Negative result,** the sample contains no Monkeypox virus DNA, but another Orthopox virus like Vaccinia virus is present.
negative	≤ 34 [3]		**Negative result,** the sample contains no Monkeypox virus DNA.
negative	negative or ≥ 34 [3]		**No statement can be made.** The real time PCR is either inhibited or errors occurred during DNA extraction.

[1] A strong positive signal in the FAM channel can inhibit the IPC. In such cases the result for the Control DNA can be neglected.

[2] The peak of the melting curve depends on the real time Instrument. The peak of a positive sample must match the peak of the diluted Positive Control (± 1 °C).

[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'.

[4] Only the melting curves in the FAM channel are evaluated

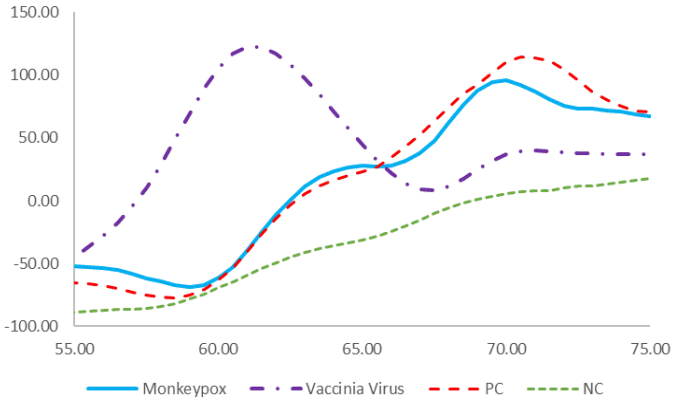

Figure 1: Example for the melting curves of the MutaPLEX® Monkeypox virus real time PCR Kit in the FAM channel.

13 ASSAY VALIDATION

Negative Control

In the Negative Control must show no C_T and no T_m in the FAM and HEX channel.

Positive control

The Positive Control must show a positive (i.e. exponential) amplification curve in the FAM channel. The Positive Control must be below C_T 30. The peak of the melting curve in the FAM channel must be between 68 and 72 °C.

Internal Control

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

The IPC must be below a C_T of 34. If the IPC 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 be 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 DNA (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.
- 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 PCR inhibitors may cause false negative or invalid results.

15 TROUBLESHOOTING

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

No fluorescence signal in the FAM channel of the Positive Control.

The selected channel for analysis does not comply with the protocol

Select the FAM channel for analysis of the Monkeypox virus specific amplification and the HEX channel for the amplification of the Control DNA (IPC).

Incorrect configuration of the real-time-PCR

Check your work steps and compare with chapter *Procedure*.

The programming of the thermal profile is incorrect

Compare the thermal profile with the protocol in chapter *Instrument settings*.

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*.

Weak or no signal of the Control DNA (IPC) and simultaneous absence of a signal in the specific FAM channel.

real time PCR conditions do not comply with the protocol

Check the real time PCR conditions in (chapter *Real time PCR*).

real time 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.

DNA loss during isolation process

In case the Control DNA was added before extraction, the lack of an amplification signal can indicate that the DNA 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*.

Detection of a fluorescence signal in the FAM channel of the Negative Control.

Contamination during preparation of the real-time PCR

Repeat the real time 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 work space and instruments are decontaminated regularly. Use a new kit and repeat the real time PCR.

16 KIT PERFORMANCE

16.1 Analytical Sensitivity

For the FAM channel the limit of detection (LoD) of MutaPLEX® Monkeypox virus real time PCR Kit was determined using serial dilutions of synthetic DNA. The determination of the LoD was done on a CFX Opus 96 (Bio-Rad).

The LoD of MutaPLEX® Monkeypox virus real time PCR Kit is < 2.5 genome copies per µl for the FAM channel.

16.2 Analytical Specificity

The specificity of the MutaPLEX® Monkeypox virus real time PCR Kit was evaluated with different ring trial samples of known status and different other relevant viruses and bacteria found in biological samples and basing on in silico analyses.

The results for the sample analysis are shown in Table 8.

Table 8: Eluted DNA/RNA from bacterial and viral pathogens tested for the determination of the analytical specificity of MutaPLEX® Monkeypox virus real time PCR Kit, FAM channel.

Eluates with known status	**MutaPLEX® Monkeypox virus**
	FAM channel
Adenovirus Typ 1	negative
Herpes Simplex Virus Type 1	negative
Herpes Simplex Virus Type 2	negative
Treponema pallidum	negative
Varizella Zoster Virus Genotyp 3	negative
Streptococcus agalactiae	negative
Enterovirus Coxsackievirus A9	negative
Amplirun® Chlamydia trachomatis DNA Control	negative
Amplirun® Mycoplasma genitalium DNA Control	negative
Amplirun® Neisseria gonorrhoeae DNA Control	negative
Amplirun® Gardnerella vaginalis DNA Control	negative
Amplirun® Trichomonas vaginalis DNA Control	negative
Amplirun® Mycoplasma hominis DNA Control	negative
Amplirun® Ureaplasma parvum DNA Control	negative
Amplirun® Ureaplasma urealyticum DNA Control	negative
Amplirun® Monkeypox Virus DNA Control	**positive**

16.3 Linear Range

The linear range of the MutaPLEX® Monkeypox virus real time PCR Kit was evaluated by analysing logarithmic dilution series of synthetic DNA of the target sequences with both thermal profiles.

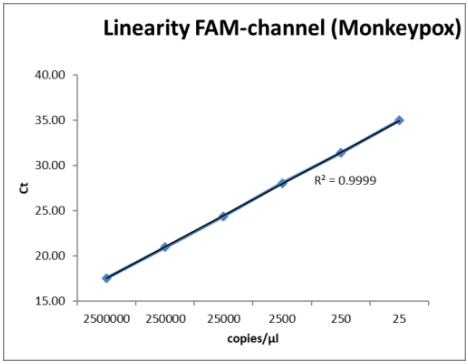

Figure 2: Determination of the linear range of the MutaPLEX® Monkeypox virus real time PCR Kit in the FAM

16.4 Precision

The precision of the MutaPLEX® Monkeypox virus real time 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 Monkeypox virus specific synthetic DNA and on the threshold cycle of the Control DNA (IPC). The results are shown in Table 9.

Table 9: Precision of the MutaPLEX® Monkeypox virus real time PCR Kit.

Monkeypox virus (FAM)	**copies/µl**	**Standard Deviation**	**Coefficient of Variation [%]**
Intra-Assay Variability	250	0.37	1.16
Inter-Assay-Variability	250	0.07	0.23
Inter-Lot-Variability	250	0.21	0.67
IPC (HEX)	**copies/µl**	**Standard Deviation**	**Coefficient of Variation [%]**
Intra-Assay Variability	2500	0.17	0.62
Inter-Assay-Variability	2500	0.09	0.31
Inter-Lot-Variability	2500	0.02	0.07

17 LITERATURE

[1] https://www.who.int/news-room/fact-sheets/detail/monkeypox

18 ABBREVIATIONS AND SYMBOLS

DNA	Deoxyribonucleic acid	REF	Catalog number
PCR	Polymerase chain reaction	→REF	To be used with
REACTION MIX	Reaction mix		Contains sufficient for <n> test
CONTROL +	Positive Control		Upper limit of temperature
CONTROL –	Negative Control		Manufacturer
CONTROL DNA IPC	Control DNA (IPC)		Use by
CONTENT	Content	LOT	Lot number
	Consult instructions for use	RUO	*research use only*

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