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EliGene® Monkeypox RT

REF 90095-RT **(for 50 samples)**

Kit components:

5 x 150 µl **MPXV Mix**
5 x 200 µl **IAC**
2 x 50 µl **PC MPXV**
1 x **Instruction for Use**

Storage and shelf life after first opening:

All components of the kit must be transported and stored at -20 °C. Kit and remaining MasterMixes must be stored at -20 °C in a dark.

Intended use

EliGene® Monkeypox RT Kit is intended for detection of Orthopoxvirus causing monkeypox disease (MPXV) from DNA isolated from clinical material.

Principle of the method

This diagnostic kit is based on Real Time PCR (qPCR). For the detection of Monkeypox virus DNA and internal control, primers and fluorescent probes (FAM and HEX) are used.

Primers target the region of the TNF receptor gene located in the inverted terminal repeats (ITR) region. To ensure increased specificity of the kit, additional set of primers targeting the conservative region of the DNA polymerase is present.

Introduction

Monkey pox is a zoonotic disease caused by the Orthopoxvirus Monkeypox virus, closely related to the smallpox virus, which causes smallpox disease. Monkeypox virus is a brick-shaped, enveloped DNA virus 200-250 nm in length that replicates in the cytoplasm, not the nucleus. Human cases were first described in 1970 and outbreaks were reported in Africa in the following decades. Mortality is 1-10%, although the mortality rate is lower for infections caused by the West African strain than for the Central African strain. The number of cases and the geographical prevalence of monkeypox have increased in recent years, probably due to weakening immunity to smallpox. DNA isolated from any clinical samples - swabs from lesions, blisters, nasopharynx and throat or affected tissues is suitable for DNA diagnostics.

Primary sample collection, handling and storage

Clinical material:

Swabs from lesions, blisters, nasopharyngeal and throat swabs

Recommended DNA isolation procedure:

Manual: EliGene® Urine Isolation Kit (ELISABETH PHARMACON)
EliGene® Viral DNA/RNA FAST Isolation Kit
EliGene® Viral RNA/DNA FAST 96 Vacuum Isolation Kit
EliGene® Blood DNA Isolation Kit

Automatic: Prepito NA Body Fluid Kit (PerkinElmer)

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Tissue	Manual: EliGene® Tissue DNA Isolation Kit
	Chemagic DNA Tissue10 Kit (PerkinElmer)
	Automatic: Prepito DNA Tissue10 Kit (PerkinElmer)

Manual isolation:

Add 20 µl of internal control to the sample and then proceed according to the standard isolation protocol. Use isolated DNA immediately for detection or store for several hours to one week at 4 °C. Freeze at -20 °C for storage for more than one week.

Automatic isolation:

Add 20 µl of internal control and isolate DNA from the sample according to the protocol.

Swabs:

Collect these samples according to standard protocol in tubes with virus transport medium or in microtubes with lysis solution. Do not use dry swabs.

Additional required equipment

- Sterile automatic pipettes 5-20 µl and sterile tips with filter DNA-, RNA- free, DNase-, RNase- free (we recommended plastic with CE certificate for diagnostic purposes).
- Sterile stand DNA-, RNA- free, DNase-, RNase- free.
- Equipment for qPCR – the kit is designed for LightCycler® 480 (Roche), QuantStudio 3 and 5 (ThermoFisher Scientific), CFX96 Touch Real-Time PCR Detection System (Bio-Rad) and MIC qPCR Cycler (Bio Molecular Systems).
- Sterile plastic (strips, plates, tubes) DNase-, RNase- free compatible with given qPCR system.
- Lab safety gloves

Configuration of qPCR instrument

- For detection of MPXV, the probe labeled with FAM is used (exc. 494 nm – em. 518 nm).
- For detection of Internal control, the probe labeled with HEX is used (exc. 520 nm – em. 548 nm)

LightCycler® 480 (Roche):

Please, use white plates only intended for LightCycler® 480. The usage of transparent plates can lead to decreased sensitivity of the kit. Do not reuse plates; the contamination of your laboratory could occur during the manipulation with plates.

In option Detection format choose “2 Color Hydrolysis probe”.

Set up the following temperature profile:

Step 1 - Analysis mode “None”, 1 Cycle

95 °C	3 min	Ramp rate (4.4°C/s)	Acquisition mode “None”

Step 2 - Analysis mode “Quantification”, 45 Cycles

95 °C	5 s	Ramp rate (4.4°C/s)	Acquisition mode “None”
60 °C	20 s	Ramp rate (2.2°C/s)	Acquisition mode “Single”

Step 3 - Analysis mode “None”, 1 Cycle

40°C	1 min	Ramp rate (2.2°C/s)	Acquisition mode “None”

The complete temperature profile can be up-loaded from Run Template "EliGene_MPXV_LC480_v00.ixo ". The Run Template can be imported to the software in menu "Navigator" by clicking to icon "Import" from the CD included in the kit.

QuantStudio 3 and 5 (ThermoFisher Scientific):

Use the Experiment type, "Standard curve", Chemistry "TaqMan Probes" and Run Mode "Fast". As reporter dye select FAM (MPXV) and VIC/HEX (IAC) and as the passive reference dye select ROX.

Set up the following temperature profile:

Holding stage

95°C 3 min

Cycling stage – 45 cycles

95°C 5 s

60°C 20 s Data collection ON

Post-Read Stage

40°C 1 min

The complete temperature profile can be up-loaded from Run Template "EliGene_MPXV_QS3_v00.edt" or "EliGene_MPXV_QS5_v00.edt". The Run Template can be copied from the CD included in the kit.

CFX96 Touch (Bio-Rad):

In Startup Wizard Create a new Experiment for CFX96 instrument and Create New Protocol.

Set up the following temperature profile:

Step 1	95°C	3 min
Step 2	95°C	5 s
Step 3	60°C	20 s + Plate Read
Step 4	*GOTO Step 2*	*44x*
Step 5	40°C	60 s

Enter the Sample Volume 20 µl.

For filter settings use the "Scan Mode" All Channels but in Plate Manager select for the samples only fluorophores FAM and HEX. The complete temperature profile can be up-loaded from Run Template "EliGene MPXV_CFX96_v00.pcrl" The Run Template can be copied from the CD included in the kit.

MIC qPCR (Bio Molecular Systems Pty Ltd):

In Run Setup Create a new Run Profile for the MIC instrument. Enter the Sample Volume to 20 µl and Temperature Control "Fast TAQ (v3)"

Set up the following temperature profile:

Holding stage

95°C 3 min

Cycling stage – 45 cycles

95°C 5 s

60°C 20 s Acquiring in channels "Green" and "Yellow"

Holding stage

40°C 60 s

The complete temperature profile can be up-loaded from Run Template "EliGene_MPXV_MIC_v00.mictemplate". The Run Template can be copied from the CD included in the kit.

Reagent preparation

- To avoid the contamination, keep all tubes closed and follow the instructions.
- Before the usage, all reagents must be completely thawed, briefly mixed on vortex and shortly spun.
- In the isolation protocol step, where Proteinase K or Lysis Buffer is added to the sample, also add 20 µl of internal control (IAC). Under no circumstances should the internal control be added to the isolated DNA just prior to analysis.
- If you do not use up the entire volume of the MasterMix, store the tube in the dark at 4 ° C for a maximum of 1 day. Use a freezer (-20 ° C, dark) for long-term storage. MasterMix should not go through more than five freeze-thaw cycles.

WARNING: The contamination in laboratory space is also possible. Use separate pipette for Master Mixes, separate pipette for positive controls and separate pipette for samples! Follow all recommendations for laboratories providing DNA analyses.

Preparation of Master Mix

1. Detection: After complete thawing, pipette 15 µl of MPXV Mix into each amplification tube or well and add 5 µl of isolated DNA. Be careful when pipetting the sample to avoid cross-contamination of the samples.
2. Positive Control: After complete thawing, pipette 15 µl MPXV Mix into an amplification tube or well and add 5 µl PC DNA MPXV. Be careful when pipetting the positive control to avoid contamination of other samples. Use a pipette other than the sample pipette to pipette the positive control; a positive control pipette can be used.

Insert the tubes or plate into the qPCR instrument and run the program according to the chapter qPCR instrument configuration above.

Result reading

LightCycler® 480 (Roche):

In "Sample Editor" menu choose "Abs Quant" workflow.

In menu "Analysis" choose "Abs Quant/2nd Derivative Max" option.

Analyze the results by clicking on the "Calculate" icon.

Positive result for MPXV: The positive result is characterized by the growth of fluorescence signal in FAM channel (465-510) in MPXV Mix. In a case of negative results, the amplification will not occur.

The Internal Control must be amplified in each sample. The Internal Control amplification is characterized by a growth of signal in HEX channel (533-580).

QuantStudio 3 and 5 (ThermoFisher Scientific):

In "Analyse Settings" choose "Automatic Treshold" and "Automatic Baseline" option and analyze results.

Positive result for MPXV: The positive result is characterized by the growth of fluorescence signal in FAM channel. In a case of negative results, the amplification will not occur.

The Internal Control must be amplified in each sample. The Internal Control amplification is characterized by

the growth of fluorescence signal in VIC (HEX) channel.

CFX96 Touch (Bio-Rad):

In Data Analysis window choose "Quantification". In "Settings" menu choose option "Baseline Threshold" and select "Baseline Cycles" option as "Auto Calculated" and "Single Threshold" option as "Auto calculated".

In Data Analysis window select a single fluorophore (FAM or HEX) by the clicking the box next to the fluorophore name located under the amplification chart and read the results for individual samples.

Positive result for MPXV: The positive result is characterized by the growth of fluorescence signal in FAM channel (em. 518 nm). In a case of negative results, the amplification will not occur.

The Internal Control must be amplified in each sample. The Internal Control amplification is characterized by the growth of fluorescence signal in HEX channel (em. 548 nm).

MIC qPCR (Bio Molecular Systems):

To view and analyze acquired data, click the small cross next to Cycling under Analysis module and then select the target – Non-Assay Green (FAM) and Non-Assay Yellow (HEX). In the down right corner increase "Fluorescence Cut-off Level" to 20 %. A quantification cycle (Cq) value is displayed for each positive well. This is the cycle number at which the sample fluorescence rises above the threshold (the red horizontal line near the bottom of the amplification curve chart). In the Samples panel on the right, click sample names to hide or display the associated amplification curves and result data.

Positive result for MPXV: The positive result is characterized by the growth of fluorescence signal in „Green channel". In a case of negative results, the amplification will not occur.

The Internal Control must be amplified in each sample. The Internal Control amplification is characterized by the growth of fluorescence signal in „Yellow channel".

Interpretation of results

Negative result:

If the increase of amplification signal in FAM channel does not appear before cycle number 40, the result of test should be interpreted as probably negative for the presence of MPXV DNA or the concentration is below the detection limit of this kit (10 genomic DNA/reaction). The signal for Internal Control must be positive. This result does not exclude the occurrence of MPXV, because results of this test are dependent on proper sample collection and processing. Results also depend on adequate quantity of analyzed MPXV DNA.

Positive result:

Amplification signal in FAM channel appears before cycle number 40. MPXV DNA was detected in the sample. The sample is MPXV DNA positive.

Inhibited sample:

In case that increase of the amplification signal specific for MPXV DNA in FAM channel and increase of the amplification signal specific for internal control in HEX channel is not observed at the same time, the analysis should be repeated preferably with newly isolated DNA samples.

Control procedure

EliGene® Monkeypox RT kit involves Internal Control. Internal Control follows the quality of DNA isolation and detects the occurrence of possible mistakes during DNA isolation process. It detects also possible inhibition of

amplification process. In the case that the sample is MPXV DNA negative, cycle number (Cq) of Internal Control must be lower than 32.

The internal control must be added directly to the sample with Lysis Buffer before viral DNA isolation begins.

Additionally, **cycle number values of all the samples undergoing the same procedure of DNA isolation must have cycle number value of IAC in the HEX channel within the range of 3 cycles.** The higher fluctuation of the cycle number values in a single qPCR run indicates non-standard conditions in the DNA isolation. However, this condition does not apply to highly positive samples (cycle number in FAM channel < 20).

Use a negative control for each run. Use the molecular biology water used in your laboratory as a negative control. Use a DNA sample pipette for the negative control.

Reference material:

To monitor all examination processes covering DNA isolation and RealTime PCR detection is possible to use reference viral material. Positive material can be ordered in specialized collections focused on orthopoxviruses or the WHO Collaborating Center for Smallpox and Other Poxvirus Infections.

Troubleshooting:

1. If there is no amplification of Internal Control (IAC), there is some problem in the isolation of DNA, or the kit is after the expiration date or there is qPCR instrument malfunction.
2. If the cycle number range of the Internal Amplification Control is higher than 3 cycles, there is probably a non-homogeneity in the DNA isolation, which caused the partial inhibition of the qPCR. Repetition of the DNA isolation should be considered in this case. Another possibility is to dilute the isolated DNA twice, alternatively more times.
3. If there is no amplification of Positive Control, the kit is after the expiration date or there is qPCR instrument malfunction. It may also be a failure to follow the recommended procedure for sample preparation and analysis.

Performance characteristics

Analytical performance characteristics:

Analytical sensitivity of EliGene® Monkeypox RT kit is 10 genomic DNA added in Master Mix.

The sensitivity of method was verified as follows. A cloned insert with a specific DNA concentration was prepared, which was diluted to obtain the desired concentrations of the target sequence.

Analytical sensitivity is 10 copies of MPXV DNA in reaction mix.

Analytical specificity of the methodology is 100% and was verified both by using DNA sequence databases and by adding human DNA to the MasterMix. 50 different human DNA samples did not cause a false positive result. The addition of the following DNA did not cause a false positive result either - *M. tuberculosis*, *M. cansasii*, *M. xenopii*, *M. avium* and *M. marinum*, *C. trachomatis*, *E. coli*, *A. niger, C. albicans*, ADV, HBV, EBV, CMV, VZV, HCV.

Clinical specificity was tested on clinical swab samples from nasopharynx, oropharynx and cutaneous inflammatory pustules.

Diagnostic performance characteristics:

A total of 30 clinical specimens were analyzed as part of the EliGene® Monkeypox RT kit functional testing. The monkey pox virus target DNA sequence was added to 20 clinical samples. All these samples were tested by the reference method recommended by the WHO: Li Y, Zhao H, Wilkins K, Hughes C, Damon IK. Real-time PCR assays for the specific detection of monkeypox virus West African and Congo Basin strain DNA. Journal of virological methods. 2010; 169 (1): 223-7. The EliGene® Monkeypox RT kit evaluated all 30 samples correctly.

Sensitivity: 100%

Specificity: 100%

Measuring interval

The kit enables the detection of 10^1–10^8 MPXV DNA molecules in Reaction Mix.

Internal control of quality

As an internal control of quality, the Internal Control for checking the process of DNA isolation and amplification is used. Positive Control for functional control of Master Mix and as a reference sample is used.

Limitation of the examination procedure

The sensitivity of kit depends on handling with sample (isolation of DNA). It is strictly recommended to use isolation kits and procedures recommended in this manual.

Biological reference intervals

Not applicable information for this kit.

Warning

The unused contents of the MasterMix tube are stable for 2 weeks at -20 ° C. Do not freeze MasterMix tubes repeatedly! Do not mix kit components from different lots.

Warnings and general precautions

This kit is intended for *in vitro* use only.

- Treat all biological samples as potentially infectious material. Avoid direct contact with biological samples. Avoid spilling samples and aerosols. Any material that has come into contact with the biological samples must be autoclaved at 121 ° C for at least 60 minutes before being disposed of.
- You work with all reagents and materials used in the knowledge that they may transmit infectious agents. Avoid direct contact with reagents. Waste must be disposed of in accordance with adequate safety regulations. Consumables must be incinerated. Liquid waste containing acids or bases must be neutralized before disposal.
- Wear appropriate protective clothing, gloves and eye / face protection.
- Never pipette solutions by mouth.
- Do not eat, drink and do not smoke or apply cosmetics in laboratory areas.
- Wash your hands thoroughly after handling samples and reagents.
- Dispose of remaining reagents and waste in accordance with adequate safety regulations.
- Read all instructions in this manual carefully before starting work.
- Follow the operating instructions exactly when working.
- Do not use the kit after the expiration date stated on the package.
- Use only reagents provided in the kit and reagents recommended by the manufacturer.
- Do not mix reagents from different lots!
- Do not use reagents from other manufacturers kits!

Warnings and precautions for molecular biology

- Molecular biology procedures, such as extraction, reverse transcription, amplification and detection of nucleic acids, require qualified staff to prevent the risk of erroneous results, especially due to degradation of the nucleic acids contained in the samples or due to sample contamination by amplification products.
- It is necessary to have separate areas for the extraction/preparation of amplification reactions and for the amplification/detection of amplification products. Never introduce an amplification product in the area designed for extraction/preparation of amplification reactions.
- It is necessary to have lab coats, gloves and tools which are exclusively employed in the extraction/preparation of amplification reactions and for the amplification/detection of amplification products. Never transfer lab coats, gloves or tools from the area designed for the amplification/detection of amplification products to the area designed for the extraction/preparation of the amplification reactions.
- The samples must be exclusively employed for this type of analysis. Samples must be handled under a laminar flow hood. Tubes containing different samples must never be opened at the same time. Pipettes used to handle samples must be exclusively employed for this specific purpose. The pipettes must be of the positive displacement type or be used with aerosol filter tips. The tips employed must be sterile, free from DNases and RNases, free from DNA and RNA.
- Reagents must be handled in PCR box. The reagents required for amplification must be prepared in such a way that they can be used in a single session. The pipettes employed to handle the reagents must be used exclusively for this purpose. The pipettes must be of the positive displacement type or be used with aerosol filter tips. The tips employed must be sterile, free from DNases and RNases, free from DNA and RNA.
- Amplification products must be handled in such a way as to reduce dispersion into the environment as much as possible, in order to avoid the possibility of contamination.

Warnings and precautions specific to components of the kit

- Microtubes containing mixes are for single use only and must only be used to prepare the reaction mixture.
- The microtube containing IAC DNA is for single use only and must only be used to prepare the reaction mixture.
- These Mixes carry the following safety warnings (P):

P280 Wear protective gloves/protective clothing/eye protection/face protection.
P281 Use personal protective equipment as required.

Literature

Li Y, Zhao H, Wilkins K, Hughes C, Damon IK. Real-time PCR assays for the specific detection of monkeypox virus West African and Congo Basin strain DNA. J Virol Methods. 2010;169(1):223-227.

Symbols

	Catalog number
	Upper limit of temperature
	Batch code
	Use by (last day of month)
	*in vitro* diagnostic medical device
	Fulfilling the requirements of European Directive 98\79\EC for *in vitro* diagnostic medical device.
	Contains sufficient for "N" tests
	Attention, consult instructions for use
	Manufacturer

Manufacturer

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