Monkeypox Virus Real Time PCR Kit

Kit for the detection of Monkeypox Virus by Real Time PCR

REF **Ref. MAD-003969M**

50 determinations

For in vitro diagnostic use only
Directive 98/79/EC

Rev.: 2022/06/28

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IVD

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1 INTRODUCTION

Monkeypox virus (MPXV) is a zoonotic, double-stranded DNA virus belonging to the genus *Orthopoxvirus* of the family *Poxviridae*. It is one of the orthopoxviruses that infect humans, including smallpox virus (causing smallpox, which has been eradicated), cowpox virus and vaccinia virus (which has been used as an live vaccine and was a key tool in the eradication of smallpox achieved in 1980).

MPXV owes its name to the fact that it was initially detected in monkeys. This virus is mainly found in rodents, although further studies are needed to identify the exact reservoir of the virus and how it maintains circulation in nature.

Clinical signs of monkeypox usually include fever, rash and swollen lymph nodes and can lead to a variety of medical complications. The disease is usually self-limiting, with symptoms lasting two to four weeks, although it can cause severe disease.

MPXV is transmitted to humans by close contact with an infected person or animal, or with material contaminated by the virus. Person-to-person transmission occurs through close contact with lesions, body fluids, respiratory droplets and contaminated materials such as bedding.

Although monkeypox occurs mainly in rainforest areas of central and western Africa, it is sporadically exported to other non-endemic regions. From May 13 to June 10, 2022, the World Health Organization (WHO) received notification of 1,423 confirmed cases of monkeypox in 31 Member States that are not endemic for monkeypox. This outbreak is still ongoing and it is expected that more cases will be identified as surveillance and laboratory capacity is implemented in non-endemic countries.

For laboratory confirmation of samples from a suspect case, the World Health Organization recommends the use of nucleic acid amplification tests, such as real-time or conventional polymerase chain reaction (PCR). These tests can be generic for *Orthopoxvirus* or specific for monkeypox virus, preferably.

2 INTENDED USE

The **Monkeypox Virus Real Time PCR kit** is a kit that allows the qualitative detection of the virus causing monkeypox, covering the variants of the Congo Basin (Central Africa) and West Africa.

The kit functions from DNA extracted from clinical samples (surface swabs and/or skin lesion exudate, as well as nasopharyngeal or oropharyngeal swabs) by real-time PCR using specific fluorescent-labeled primers and probes for the target genes.

The target for detection of monkeypox virus is the F3L gene.

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Specific primers and fluorescent probe are included for the simultaneous detection of the human Beta globin gene as internal quality control of the starting and amplification material. The detection channels of the different targets are:

Target	Fluorophore
Monkeypox virus	FAM
Beta globin	HEX/JOE/VIC

Table 1. Detection channels for the different targets of the Monkeypox Virus Real Time PCR.

Microbiological status: Non-sterile product.

3 COMPONENTS

Monkeypox Virus Real Time PCR kit is commercialized as a ready-to-use Master Mix which includes all the necessary reagents to perform the real-time PCR.
Furthermore, in order to avoid contamination with previous PCR products, the Mix contains the enzyme Uracil-DNA Glycosylase (UDG), which degrades PCR products containing dUTP.
A tube as positive control (PC) and DNase/RNase-free water to include in the negative controls (NTC) are supplied along with the PCR Mix.

REFERENCE (DESCRIPTION)		CONTENT	AMOUNT
MAD-003969M-50 (Monkeypox virus MMIX)	**MAD-003969-MIX (Monkeypox virus MMix)**	Hot Start DNA Polymerase, Uracil DNA glycosylase, primers, fluorescent probes, reaction buffer, dNTPs (dATP, dCTP, dGTP, dTTP, dUTP)	**1 vial with 50 test (660 µL)**
	MAD-DDW-200 (RNAse/DNAse free water)	---	**1 vial (200 µl)**
MAD-MPXV (Monkeypox virus PC)		Synthetic non-infectious DNA containing part of the genome of the monkeypox virus and human DNA	**1 vial (100 µl)**

Table 2. Reagents supplied in the Monkeypox Virus Real Time PCR kit.

4 ADDITIONAL REQUIRED MATERIAL NOT SUPPLIED

4.1 Reagents and materials

- Disposable gloves.
- DNase/RNase-free filtering pipette tips.
- DNA extraction kit.
- Tube strips/plates/optical adhesive films specific for each equipment of Real-Time PCR

4.2 Equipment

- Laminar flow cabinet
- Microcentrifuge for tubes of 1.5ml.

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- Microcentrifuges of PCR tube strips or 96-well plates.
- Vortex.
- Automatic micropipettes: P1000, P200, P20 and P2.
- Real-Time PCR instrument.

5 STORAGE AND STABILITY CONDITIONS

Monkeypox Virus Real Time PCR kit must be transported and stored between -10 °C* and -30 °C*. Nonetheless, besides the recommended transport between -10 °C and -30 °C, it is also possible to transport it at refrigeration temperature (2 °C - 8 °C), as long as the transit period does not exceed a maximum of ten days. In any case, the kit must be stored at a temperature of -10 °C -30 °C upon receipt.

The reaction mix **Monkeypox virus MMix** is sensitive to physical state changes and it has been proven that it supports up to five freeze-thaw cycles. If a run is performed with a low number of samples, it is recommended to aliquot the reagent in advance. The mix contains fluorescent molecules and it must be kept away from direct light.

The positive control is sensitive to physical state changes and it must not undergo more than eight freeze-thaw cycles. It is advisable to handle the positive control vial separately from the clinical samples to avoid potential contamination which might yield false positives.

If stored at recommended temperature, the PCR reagents are stable until the expiration date indicated. The PCR reagents must be stored in areas free of DNA or PCR products contamination.

*A temperature indicator is included in the package to control the conditions during the shipment. In case the cold chain is interrupted, it is recommended to contact the manufacturer before using the reagents.

6 WARNINGS AND PRECAUTIONS

- Read the instructions for use before using this product.
- The kit must be handled by qualified technicians in molecular biology techniques applied to diagnosis.
- Do not use any component of the kit after the expiration date.
- The mix Monkeypox virus MMix must be thawed before use and handled on ice or cold plate and away from light. Mix the solutions by inverting the tubes several times without shaking in vortex, and centrifuge briefly.
- The positive control must be thawed at room temperature, mixed well and centrifuged briefly before use.
- The safety and disposal precautions are described in the Safety Data Sheet of this product. This product is only intended for professional laboratory purposes, and it is not intended for pharmacological, home or any other type of use. The current version of the Safety Data Sheet of this product can be downloaded in the web page www.vitro.bio or requested at regulatory@vitro.bio.
- The Monkeypox Virus Real Time PCR kit uses nucleic acids previously extracted and purified as starting material. It is the client's responsibility to include the necessary controls to verify that the system of extraction of the used genetic material works properly.

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- **General considerations to avoid the contamination with PCR product**

  The most important contamination source is usually the same amplified PCR product. Therefore, it is recommended to carry out the amplification and handling of the amplified products in a different area to the one where the DNA extraction and PCR preparation are performed. It is recommended to work in different pre- and post-PCR areas where the handling of the test DNA and preparation of the PCR tubes (pre-PCR), and the amplification and handling of the amplified products (post-PCR) are performed. These areas must be physically separated and different laboratory material must be used (laboratory coats, pipettes, tips, etc.) to avoid the contamination of the samples with the amplified DNA, which could lead to false positive diagnoses. The workflow must always go in a single direction, from the pre-PCR area to the post-PCR area and never the opposite way. The material and personal flow from the post-PCR area to the pre-PCR area must be avoided. Furthermore, in order to avoid the contamination with previous PCR products, the enzyme *Uracil-DNA Glycosylase (UDG), which* degrades the PCR products containing dUTP, is included in the kit.

  It is recommended to include negative amplification controls replacing the DNA specimen with RNase/DNase-free water, in order to detect and control any possible contamination of the reagents with test samples or amplified products.

- **Waste disposal**

  The handling of wastes generated by the use of the products commercialized by Vitro S.A. must be performed according to the applicable law in the country in which these products are being used. As reference, the following table indicates the classification of waste generated by this kit according to the European Law, specifically according to the European Commission Decision of December 18, 2014 amending decision 2000/532/EC on the list of waste pursuant to Directive 2008/98/EC of the European Parliament and of the Council:

POTENTIAL WASTE GENERATED AFTER USING THIS PRODUCT	ELW CODE*	TYPE OF WASTE ACCORDING TO ELW*
1. Liquid waste disposal	161001	"Aqueous liquid wastes containing dangerous substances" after adding 10% of the total volume of a disinfectant agent. If the disinfection is not carried out, this waste must be considered as "waste whose storage and disposal is subjected to special requirements in order to prevent infection"
2. Perishable material (tubes, tips, etc.) 3. Any element that has been in contact with the starting genetic material	180103	"Waste whose collection and disposal is subject to special requirements in order to prevent infection"
4. Container for reagents used classified as dangerous (according to the Safety Data Sheet)	150110	"Containers containing waste or contaminated by dangerous substances"

Table 3. Classification of wastes generated by this kit according to the European Legislation. *ELW: *European Legislation of Waste.*

*Note: This classification is included as a general guideline of action, being under the final responsibility of the user the accomplishment of all the local, regional and national regulations on the disposal of this type of materials.

7 PREPARATION OF THE CLINICAL SAMPLE FOR ANALYSIS

7.1 Sample collection

The Monkeypox Virus Real Time PCR kit has been validated for use with purified genetic material from various types of clinical specimens, such as surface swabs and/or skin lesion exudate, as well as nasopharyngeal or oropharyngeal swabs.

This kit has been validated with starting genetic material obtained from the following DNA/RNA purification kits and extraction kits* starting with 200 µl of clinical sample and eluting in 100 µl of elution buffer (for purification with Opentrons start with 92 µl of clinical specimen and elute in 60 µl of elution solution):

EXTRACTION KITS	EXTRACTION EQUIPMENT
QIAamp Viral RNA Mini Kit (QIAGEN)	Manual
RNA/DNA pathogen extraction kit (Robot Opentrons OT2) (Vitro, ref. MAD-003955M)	Opentrons OT-2

Table 4. Extraction kits and instruments used for the purification of DNA/RNA from clinical samples.

*Note: The system has not been validated with other DNA/RNA extraction systems. Therefore, if any other purification system is used, this must be verified beforehand.

8 PCR PROTOCOL

8.1 Preparation of the Reaction mix

The PCR is carried out in a final volume of 20 µl. Prepare the Master Mix as indicated below:

1. Thaw and homogenize Monkeypox virus MMix (do not use vortex). Once it is thawed, centrifuge briefly.
2. Mix in each PCR tube the following volumes for each sample:

Reagent	V/test
Monkeypox virus MMix	12 µl
Sample	8 µl

Table 5. Preparation of the mix of the Monkeypox Virus Real Time PCR kit

3. Include a negative control by adding 8 µl of the water included in the kit.
4. Include a positive control by adding 8 µl of the positive ADN control MPXV included in the kit.
5. Centrifuge briefly to make sure there are no air bubbles in the wells.

It is recommended to keep the MMix on cold plate during the preparation of the samples and not thaw the vial more than five times.

8.2 Configuration of the instrument for real-time PCR

Enter the different targets and detection channels for each of them in the instrument's software. Create the samples, the positive control (PC), the PCR targets (NTC) and allocate the positions of the samples in the PCR plate.

Set the real-time PCR instrument following the steps below:

PCR PROGRAM		
25°C	5 min	1 cycle
95°C	5 min	1 cycle
95°C	15 sec	45 cycles
63°C*	30 sec	

Table 6. PCR program of the Monkeypox Virus Real Time PCR kit

*The fluorescence data must be collected during the extension stage (*) by means of the FAM (Monkeypox virus) and HEX, JOE or VIC channels (Internal Control).

This kit has been validated with the platforms:

- QuantStudio™ 5 Real-Time PCR System (Applied Biosystems)
- QuantStudio™ 3 Real-Time PCR System (Applied Biosystems)
- CFX96™ Real-Time PCR Detection System (Bio-Rad)
- Vitrocycler (Vitro S.A.)

For use in other platforms, it is recommended to verify the compatibility of the fluorochromes with the detection channels of each platform, although the fluorochromes included in the kit are compatible with the majority of the most-used real-time instruments available on the market.

In Applied Biosystems QuantStudio™ 3 and 5 Real-Time PCR System thermal cyclers the ROX passive control option must be disabled.

In the thermal cyclers Applied Biosystems QuantStudio™ 3 and 5 Real-Time PCR System, select Ramp Speed Standard in the menu “Select New Experiment/Advanced Setup/Experiment Properties”.

9 INTERPRETATION OF RESULTS

Before interpreting the results of the clinical samples, it is necessary to follow the interpretation guide of the positive and negative controls as in the table below:

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	RESULT	INTERPRETATION
Positive Control MPXV	Signal for the channels FAM and JOE/HEX/VIC*	The control/reaction is correct
	No signal for FAM and/or JOE/HEX/VIC	Problem in the amplification: repeat analysis
Negative control:	Signal for the channels FAM and/or JOE/HEX/VIC	Contamination, repeat analysis
	No signal	The control/reaction is correct

Table 7. Interpretation guide of the controls of the Monkeypox Virus Real Time PCR kit

*The amplification signal must be determined by a rapid and steady increase in the fluorescence values and not by peak phenomena or gradual increase of the background signal (irregular background or increased background noise) (Fig 1).

The run is considered valid when adequate results have been obtained for all the reaction controls.

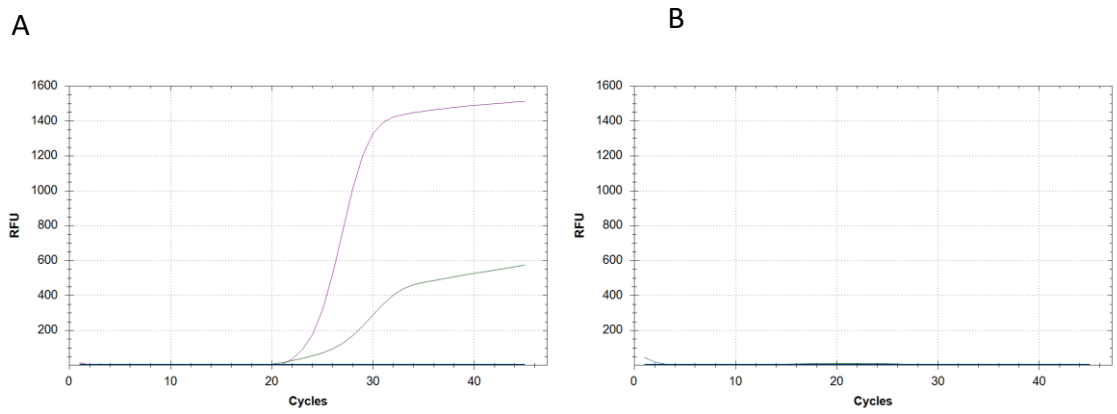

Figure 1: Graphs of amplification of a positive control PC (A) and of a negative control with NTC water (B). Experiment performed on CFX96™ Real-Time PCR Detection System (Bio-Rad). The purple curve corresponds to the amplification of monkeypox virus DNA and the green curve corresponds to the amplification of the internal control (human beta globin).

If the run has been validated, interpret the results of the clinical samples according to the following table:

FAM	JOE/HEX/VIC	Result
+	+	Monkeypox virus
-	+	Negative sample
-	-	Degraded sample

Table 8. Interpretation guide of the results obtained with the Monkeypox Virus Real Time PCR kit

The figures below show the expected amplification curves in each case:

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Monkeypox virus

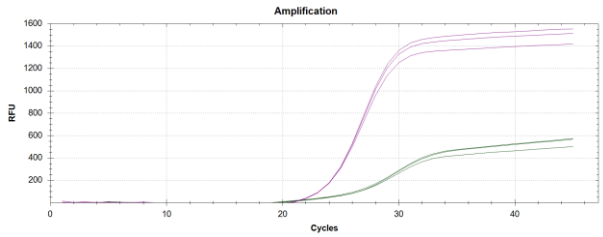

Figure 2: Amplification curves of monkeypox virus

Negative sample

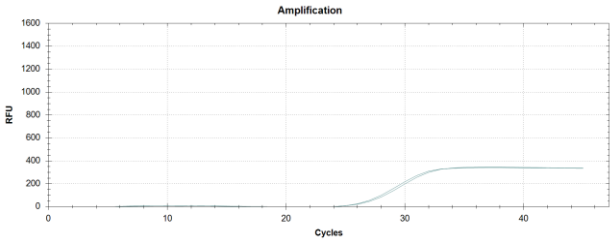

Figure 3: Negative sample

10 PERFORMANCE CHARACTERISTICS

10.1 Analytical sensitivity

The analytical sensitivity of Monkeypox Virus Real Time PCR kit was determined by performing eight serial dilutions of synthetic fragments of the F3L gene at a known concentration.

The amplification efficiency for the target gene F3L was evaluated with eight serial dilutions of a synthetic fragment of the F3L gene from 10^8 copies/rxn up to 10 copies/rxn.

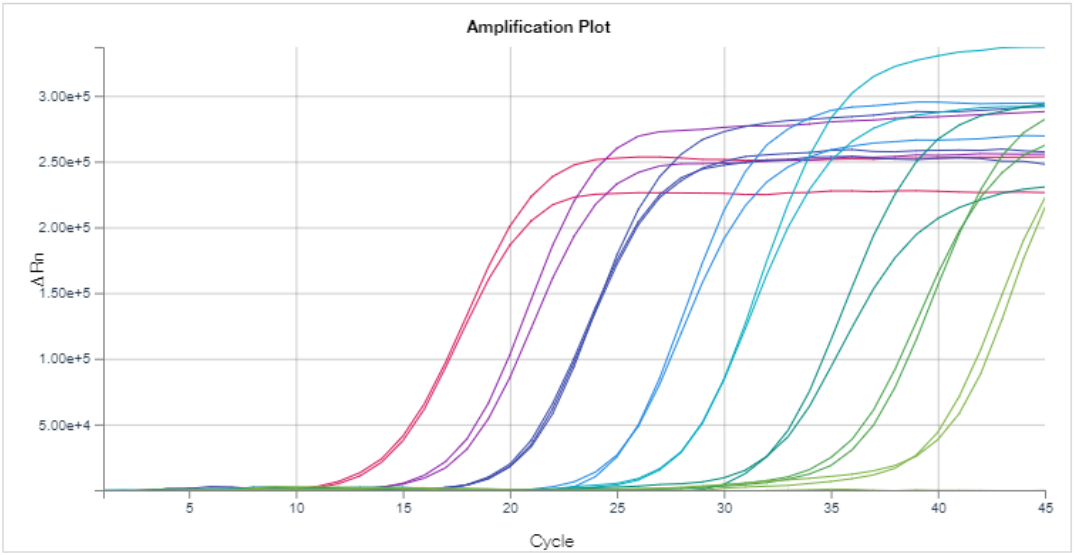

Figure 4: Serial dilutions from 10^8 copies/reaction up to 10 copies/reaction of a synthetic fragment of the F3L gene in the FAM channel. Experiment performed on QuantStudio™5 Real Time PCR System (Applied Biosystems).

By adjusting the Cts data to a line, the amplification efficiency, R^2 and the slope were determined for this gene.

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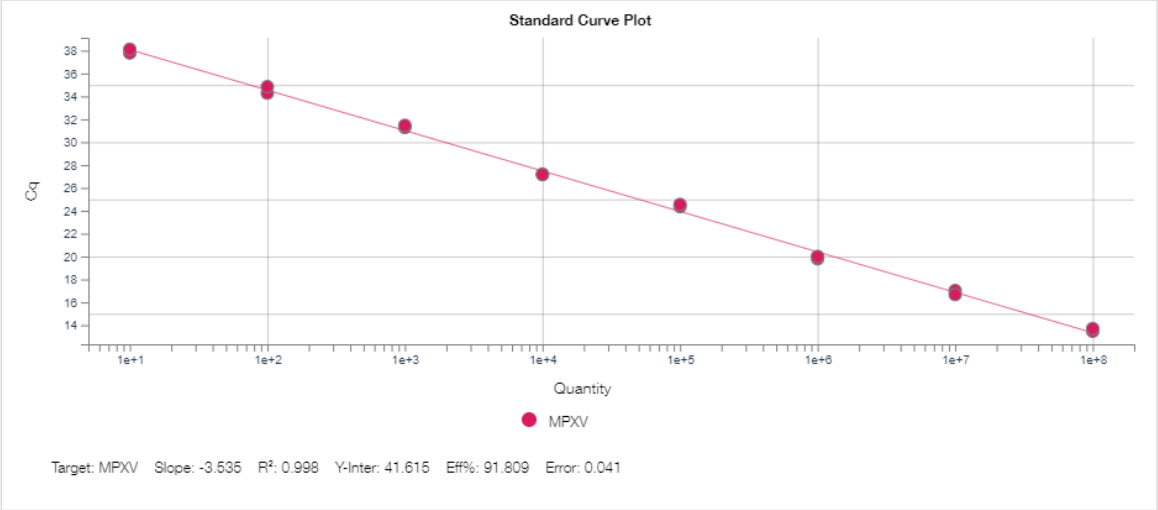

Figure 5: Calibration curve of the F3L gene in the FAM channel

The F3L gene showed an amplification efficiency of 91.81%, an R^2 of 0.998 and a slope of -3.535.

It has been established that the Monkeypox Virus Real Time PCR kit has a limit of detection of 10 copies/reaction for the target gene F3L.

10.2 Analytical specificity

The analytical specificity of the Monkeypox Virus Real Time PCR kit was confirmed *in silico*, using DNA sequences from the databases, and by adding human DNA to the Master Mix. It has also been verified that the addition to the Master Mix of DNA extracted from clinical samples positive for the microorganisms specified in the table below did not yield any false positive results.

The specificity of the Monkeypox Virus Real Time PCR kit is 100%.

Cross-reactivity test	
Microorganism	**Results**
Candida albicans	Negative
Chlamydia trachomatis	Negative
Enterococcus faecalis	Negative
Enterococcus faecium	Negative
Escherichia coli	Negative
HSV-1	Negative
HSV-2	Negative
Klebsiella pneumoniae	Negative
Mycobacterium avium	Negative
Mycobacterium bovis	Negative
Mycobacterium kansasii	Negative
Mycobacterium tuberculosis	Negative
Mycoplasma genitalium	Negative

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Mycoplasma hominis	Negative
Mycoplasma genitalium	Negative
Neisseria gonorrhoeae	Negative
Pseudomonas aeruginosa	Negative
Streptococcus pneumoniae	Negative
Ureaplasma parvum	Negative
Human DNA	Negative

Table 9. Microorganisms tested in the specificity test

10.3 Clinical sensitivity and specificity

The diagnostic capability of Monkeypox Virus Real Time PCR kit was evaluated by studying its diagnostic sensitivity and specificity. These two parameters are defined and calculated as follows:

· The diagnostic specificity is expressed as a percentage (numeric fraction multiplied by 100), calculated as 100 x the number of true negative values (TN) divided into the sum of the number of true negative values (TN) plus the number of false positive values (FP), or 100 x TN/(TN + FP).

· The diagnostic sensitivity is expressed as a percentage (numerical fraction multiplied by 100), calculated as 100 × the number of true positive values (TP) divided by the sum of true positive values (TP) plus the number of false negative values (FN), or 100 × TP/(TP + FN).

The diagnostic performance of the Monkeypox Virus Real Time PCR kit was determined by analyzing a total of 32 clinical samples, 10 of which were negative and 22 positive, of which 2 were from individuals with confirmed monkeypox virus infection. The remaining 20 positive samples were prepared by adding monkeypox virus genomic DNA to 20 clinical samples from different healthy individuals.

DNA from these 32 samples was analyzed in parallel with the Monkeypox Virus Real Time PCR kit and with one of the methods recommended by WHO (Li, Y., Zhao, H., Wilkins, K., Hughes, C. and Damon, I.K. 2010. 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), which was taken as the reference method for the comparative study.

The Monkeypox Virus Real Time PCR kit correctly evaluated all 32 samples, showing a diagnostic sensitivity and specificity of 100%.

Organism	TN	FP	TP	FN	Diagnostic Specificity	95% CI	Diagnostic Sensitivity	95% CI
Monkeypox virus	10	0	22	0	100%	65,54 - 100%	100%	81.5 – 100 %

Table 10. Clinical specificity and sensitivity of Monkeypox virus Real Time PCR kit.

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Organism	TN	FP	TP	FN	VPP	95% CI	VPN	95% CI
Monkeypox virus	10	0	22	0	100%	81.5 – 100 %	100%	65,54 - 100%

Table 11. Positive predictive value and negative predictive value of Monkeypox virus Real Time PCR kit.

Note: the results of the specifications (sensitivity and specificity) declared correspond to the total number of samples tested and the value may vary depending on the type of sample.

10.4 Measurement range

The measurement range of the assay was determined using triplicates of serial dilutions from 10^8 copies/reaction to 10 copies/reaction of a synthetic DNA fragment of the F3L gene, with 5 ng of human genomic DNA.

The Monkeypox Virus Real Time PCR kit allows detection from 10^8 to 10 copies per reaction of monkeypox virus DNA.

11 LIMITATIONS OF THE TEST

1. The results of the test must be evaluated by a healthcare professional in the context of medical history, clinical symptoms, and other diagnostic tests.

2. This test can be used with different types of samples, although it has only been validated with DNA extracted from culture and exudate of skin lesion.

3. The correct functioning of the test depends on the quality of the sample; the nucleic acid must be properly extracted from the clinical samples. Improper collection, storage and/or transport of samples can result in false negatives.

4. A low number of target copies below the detection limit can be detected, but the results may not be reproducible.

5. A positive test for the monkeypox virus does not exclude the possibility that other pathogens are present in the clinical sample.

6. The test works within the genomic regions in which the probes have been designed.

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12 LABEL AND BOX SYMBOLS

IVD	*In vitro* diagnostic medical device		Expiration date
REF	Catalog number		Temperature limit
LOT	Lot code		Manufacturer
	Refer to the instructions for use		Sufficient content for <n> assays
	Safety data sheet		Keep away from sunlight

13 CHANGELOG

Date	Description
2022/06/28	• Creation of the document.

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