Primerdesign™ Ltd

Monkeypox virus

M3L gene

genesig®Advanced Kit

150 tests

GENESIG

Kits by Primerdesign

For general laboratory and research use only

Introduction to Monkeypox virus

What is Monkeypox?
Monkeypox virus is a double-stranded DNA zoonotic virus from the genus Orthopoxvirus. This genus includes cowpox, camelpox, mousepox, variola virus (causative agent of smallpox) and vaccinia virus (used for the smallpox vaccine).

Transmission
Transmission primarily occurs from zoonotic transfer from other animals to humans, but human-to-human transmission can occur from contact with skin lesions, body fluids and respiratory secretions of infected animals/humans. Contact with contaminated clothing and bedding of infected individuals can also lead to transmission of the virus. The virus can be transmitted to the unborn foetus during pregnancy which can lead to stillbirth and other complications. The fatality rate of human monkeypox can range from 1% up to 11%, with younger age groups having the highest rate.

History of Infection
The first cases of monkeypox in humans were discovered in 1970 in the Democratic Republic of the Congo (formerly Zaire). There are two clades of Monkeypox, the Congo Basin clade (associated with higher human-to-human transmission and higher mortality) and the West African clade (associated with milder infection). Most cases are reported from the Democratic Republic of the Congo and Nigeria.

On the 7 May 2022, a single case was confirmed in the UK from an individual who recently travelled from Nigeria. On the 14 May 2022, 2 additional cases were confirmed with no known links to the first case. Further cases have been reported since in Europe, North America, and other parts of the globe:
https://www.cdc.gov/poxvirus/monkeypox/response/2022/world-map.html

Symptoms
Clinical presentations in humans are similar to smallpox with fever, headache, swollen lymph nodes, fatigue, muscle aches along with characteristic pox lesions. The incubation period for onset of symptoms can be between 5-21 days with 6-13 days being the average. Symptoms can last from 2-4 weeks.

Diagnosis
PCR blood tests are not conclusive in diagnosing human monkeypox. PCR tests of skin lesions (surface and/or exudate, roofs or crusts) is the preferred diagnostic testing method for virus detection[1]. WHO also encourage the additional collection of oropharyngeal swabs from the patient, although the accuracy of such samples in detecting the presence of monkeypox is limited[1]. Antigen detection methods are not recommended due to serological cross-reactivity and do not provide monkeypox-specific detection.

1. Laboratory testing for the monkeypox virus: Interim guidance.
https://www.who.int/publications/i/item/WHO-MPX-laboratory-2022.1

Specificity

The Primerdesign genesig Advanced Kit for Monkeypox is designed for the quantitative and qualitative detection of the monkeypox genome from DNA extracted from human serum, lesion exudate, or scab samples from human/animal patients of all ages. The patient may be exhibiting signs and symptoms of monkeypox virus infection at any point during infection or be being screened after identification as a potential close contact.

The Primerdesign genesig Advanced Kit for Monkeypox is configured for use with any real-time PCR instrument equipped to detect amplification in the FAM and VIC/HEX channels.

The kit is designed to have a broad detection profile. Specifically, the primers represent 100% homology with over 95% of the NCBI database reference sequences of all Monkeypox viral clades available at the time of design (May 2022).

The dynamics of genetic variation mean that new sequence information may become available after the initial design. Primerdesign periodically reviews the detection profiles of our kits and when required releases new versions.

If you require further information or have a specific question about the detection profile of this kit then please send an email to enquiry@primerdesign.co.uk and our bioinformatics team will answer your question.

Kit contents

Monkeypox specific primer/probe mix (150 reactions BROWN)
FAM labelled

Monkeypox positive control template (for Standard curve RED)

Internal extraction control primer/probe mix (150 reactions BROWN)
VIC labelled as standard

Internal extraction control DNA (150 reactions BLUE)

Endogenous control primer/probe mix (150 reactions BROWN)
FAM labelled

RNase/DNase-free water (WHITE)
for resuspension of primer/probe mixes

Template preparation buffer (YELLOW)
for resuspension of internal control template, positive control template and standard curve preparation

Reagents and equipment to be supplied by the user

Real-time PCR Instrument

Extraction kit
This kit is recommended for use with genesig Easy DNA/RNA extraction kit. However, it is designed to work well with all processes that yield high-quality DNA with minimal PCR inhibitors.

oasig™ lyophilised or Precision®PLUS 2X qPCR Master Mix
This kit is intended for use with oasig or PrecisionPLUS 2X qPCR Master Mix.

Pipettors and Tips

Vortex and centrifuge

Thin-walled 0.1 ml PCR reaction tubes

Kit storage and stability

This kit is stable at room temperature but should be stored at -20ºC on arrival. Once the lyophilised components have been resuspended, they should not be exposed to temperatures above -20°C for longer than 30 minutes at a time and unnecessary repeated freeze/thawing should be avoided. The kit is stable for six months from the date of resuspension under these circumstances.
If a standard curve dilution series is prepared for quantitative detection, this can be stored frozen for an extended period. If you see any degradation in this serial dilution a fresh standard curve can be prepared from the positive control.
Primerdesign does not recommend using the kit after the expiry date stated on the pack.

Suitable sample material

All kinds of sample material suited for PCR amplification can be used. Please ensure the samples are suitable in terms of purity, concentration, and DNA integrity. Always run at least one negative control with the samples. To prepare a negative control, replace the template DNA sample with RNase/DNase-free water.

Dynamic range of test

Under optimal PCR conditions, genesig detection kits have very high priming efficiencies of >90% and can detect less than 100 copies of the target template.

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Principles of the test

Real-time PCR
A Monkeypox specific primer/probe mix is provided and this can be detected through the FAM channel.

The primer/probe mix provided exploits with the so-called TaqMan® principle. During PCR amplification, forward and reverse primers hybridize to the Monkeypox viral DNA. A fluorogenic probe is included in the same reaction mixture which consists of a DNA probe labelled with a 5`-dye and a 3`-quencher. During PCR amplification, the probe is cleaved, and the reporter dye and quencher are separated. The resulting increase in fluorescence can be detected on a range of qPCR platforms.

Positive control – quantitative versus qualitative analysis
For copy number determination and as a positive control for the PCR set-up, the kit contains a positive control template This can be used to generate a standard curve of the Monkeypox copy number/Cq value. Alternatively, the positive control can be used at a single dilution where full quantitative analysis of the samples is not required, hence making the tests in that run purely qualitative.

Each time the kit is used, at least one positive control reaction must be included in the run. A positive result indicates that the primers/probes for detecting the target Monkeypox genome worked properly in that particular experimental scenario. If a negative result is obtained the test results are invalid and must be repeated. Care should be taken to ensure that the positive control does not contaminate any other kit component which would lead to false-positive results. This can be achieved by handling this component in a Post PCR environment. Care should also be taken to avoid cross-contamination of other samples when adding the positive control to the run. This can be avoided by sealing all other samples and negative controls before pipetting the positive control into the positive control well.

Negative control
To validate any positive findings a negative control reaction should be included every time the kit is used. For this reaction, RNase/DNase-free water should be used instead of the template. A negative result indicates that the reagents have not become contaminated while setting up the run.

Internal DNA extraction control
When performing DNA extraction, it is often advantageous to have an exogenous source of DNA template that is spiked into the lysis buffer. This control DNA is then co-purified with the sample DNA and can be detected as a positive control for the extraction process. Successful co-purification and qPCR for the control DNA also indicate that PCR inhibitors are not present at a high concentration.

A separate primer/probe mix is supplied with this kit to detect the exogenous DNA using qPCR. The primers are present at PCR limiting concentrations which allows multiplexing with the target sequence primers. Amplification of the control DNA does not interfere with the detection of the Monkeypox target DNA even when present at a low copy number. The Internal control is detected through the VIC channel and gives a Cq value of 28+/-3.

Endogenous control
To confirm the extraction of a valid biological template, a primer/probe mix is included to detect an endogenous gene. Detection of the endogenous control is through the FAM channel, and it is NOT therefore possible to perform a multiplex with the Monkeypox primers. A poor endogenous control signal may indicate that the sample did not contain sufficient biological material.

Resuspension Protocol

To minimize the risk of contamination with foreign DNA, we recommend that all pipetting be performed in a PCR clean environment. Ideally, this would be a designated PCR lab or PCR cabinet. Filter tips are recommended for all pipetting steps.

1. **Pulse-spin each tube in a centrifuge before opening.**
   This will ensure that the lyophilised primer/probe mix is in the base of the tube and is not spilt upon opening the tube.

2. **Resuspend the kit components in the RNase/DNase-free water supplied, according to the table below.**
   To ensure complete resuspension, vortex each tube thoroughly

Component - resuspend in water	Volume
Monkeypox primer/probe mix (BROWN)	165 µl
Internal extraction control primer/probe mix (BROWN)	165 µl
Endogenous control primer/probe mix (BROWN)	165 µl

1. **Resuspend the internal control template and positive control template in the template preparation buffer supplied, according to the table below:**
   To ensure complete resuspension, vortex each tube thoroughly.

Component - resuspend in template preparation buffer	Volume
Internal extraction control DNA (BLUE)	600 µl
Monkeypox Positive Control Template (RED) *	500 µl

* This component contains a high copy number template and is a VERY significant contamination risk. It must be opened and handled in a separate laboratory environment, away from the other components.

DNA extraction

The internal extraction control DNA can be added either to the DNA lysis/extraction buffer or to the DNA sample once it has been resuspended in the lysis buffer.

DO NOT add the internal extraction control DNA directly to the unprocessed biological sample as this will lead to degradation and a loss in signal.

1. **Add 4 µl of the Internal extraction control DNA (BLUE) to each sample in DNA lysis/extraction buffer per sample.**

2. **Complete DNA extraction according to the manufacturer's protocols.**

qPCR detection protocol

1. For each DNA sample prepare a reaction mix according to the table below:
Include sufficient reactions for positive and negative controls.

Component	Volume
oasig or PrecisionPLUS 2X qPCR Master Mix	10 µl
Monkeypox primer/probe mix (BROWN)	1 µl
Internal extraction control primer/probe mix (BROWN)	1 µl
RNase/DNase-free water (WHITE)	3 µl
Final Volume	**15 µl**

2. For each DNA sample prepare an endogenous control reaction according to the table below (Optional):
This control reaction will provide useful information regarding the quality of the biological sample.

Component	Volume
oasig or PrecisionPLUS 2X qPCR Master Mix	10 µl
Endogenous control primer/probe mix (BROWN)	1 µl
RNase/DNase-free water (WHITE)	4 µl
Final Volume	**15 µl**

3. Pipette 15 µl of each mix into individual wells according to your qPCR experimental plate set-up.

4. Prepare sample DNA templates for each of your samples.

5. Pipette 5 µl of DNA template into each well, according to your experimental plate set up.
For negative control, wells use 5 µl of RNase/DNase-free water. The final volume in each well is 20 µl.

6. If a standard curve is included for quantitative analysis, prepare a reaction mix according to the table below:

Component	Volume
oasig or PrecisionPLUS 2X qPCR Master Mix	10 µl
Monkeypox primer/probe mix (BROWN)	1 µl
RNase/DNase-free water (WHITE)	4 µl
Final Volume	**15 µl**

7. Preparation of a standard curve dilution series.

a. Pipette 90 µl of template preparation buffer into 5 tubes and label 2-6
b. Pipette 10 µl of Positive Control Template (RED) into tube 2
c. Vortex thoroughly
d. Change pipette tip and pipette 10 µl from tube 2 into tube 3
e. Vortex thoroughly

Repeat steps d and e to complete the dilution series.

Standard Curve	Copy Number
Tube 1 Positive control (RED)	2×10^5 per µl
Tube 2	2×10^4 per µl
Tube 3	2×10^3 per µl
Tube 4	2×10^2 per µl
Tube 5	20 per µl
Tube 6	2 per µl

8. Pipette 5 µl of the standard template into each well for the standard curve according to your experimental plate set-up.

The final volume in each well is 20 µl.

qPCR amplification protocol

Amplification conditions using oasig or PrecisionPLUS 2X qPCR Master Mix.

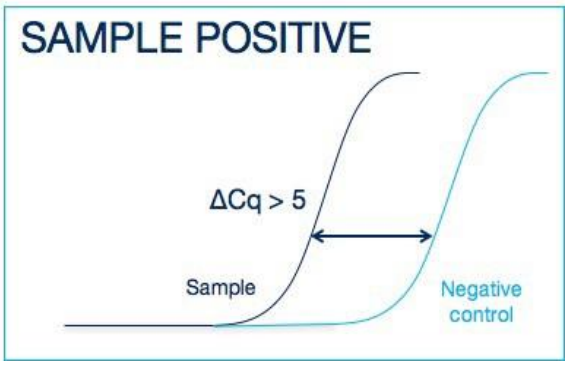
	Step	Time	Temp
Cycling x50	Enzyme activation	2 min	95°C
	Denaturation	10 s	95°C
	DATA COLLECTION *	60 s	60°C

* Fluorogenic data should be collected during this step through the FAM and VIC/HEX channels

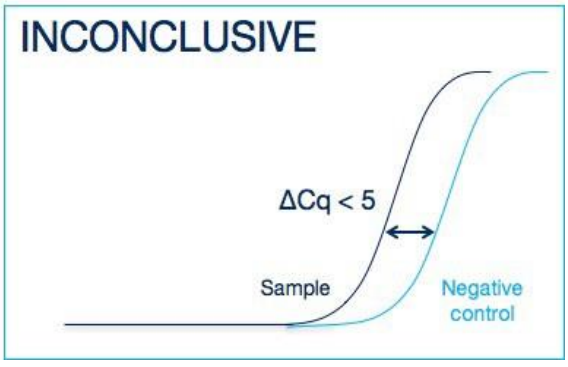
Interpretation of results

Target (FAM)	Internal control (VIC)	Positive control	Negative control	Interpretation
≤ 30	**+ / -**	**+**	**-**	**POSITIVE QUANTITATIVE RESULT** calculate copy number if standard curve used
> 30	**+**	**+**	**-**	**POSITIVE QUANTITATIVE RESULT** calculate copy number if standard curve used
> 30	**-**	**+**	**-**	**POSITIVE QUALITATIVE RESULT** do not report the copy number as this may be due to poor sample extraction
-	**+**	**+**	**-**	**NEGATIVE RESULT**
+ / -	**+ / -**	**+**	**≤ 35**	**EXPERIMENT FAILED** due to test contamination
+ / -	**+ / -**	**+**	**> 35**	*****
-	**-**	**+**	**-**	**SAMPLE PREPARATION FAILED**
+ / -	**+ / -**	**-**	**+ / -**	**EXPERIMENT FAILED**

A positive control template (**RED**) is expected to amplify between Cq 16 and 23. Failure to satisfy this quality control criterion is a strong indication that the experiment has been compromised.
*Where the test sample is positive and the negative control is positive with a Cq > 35, the sample must be reinterpreted based on the relative signal strength of the two results:



If the sample amplifies > 5 Cq earlier than the negative control, then the sample should be reinterpreted (via the table above) with the negative control verified as negative.



If the sample amplifies < 5 Cq earlier than the negative control, then the positive sample result is invalidated, and the result should be determined inconclusive due to test contamination. The test for this sample should be repeated.

Internal PCR control
The Cq value obtained with the internal control will vary significantly depending on the extraction efficiency, the quantity of DNA added to the PCR reaction and the individual machine settings. Cq values of 28±3 are within the normal range. When amplifying a Monkeypox sample with a high genome copy number, the internal extraction control may not produce an amplification plot. This does not invalidate the test and should be interpreted as a positive experimental result.

Endogenous control
The signal obtained from the endogenous control primer/probe set will vary according to the amount of biological material present in a given sample. An early signal indicates the presence of a good yield of biological material. A late signal suggests that little biological material is present in the sample.