**Department of Virology**
**Public Health Laboratories Division (PHLD)**
**National Institutes of Health (NIH)**
**Ministry of National Health Services,**
**Regulations & Coordination**
Phone: (+92-51) 9255082-9255232



17 June, 2022 (Version:01)


This document is based on the World Health Organization and PAN American Health Organization [1-2] interim guidance on "Laboratory testing for the monkeypox virus" (23 May 2022) and is intended to provide guidance to Provincial Public Health Reference Laboratories on laboratory diagnosis of monkeypox.

## Introduction

Monkeypox is a viral disease caused by the monkeypox virus (MPXV), a member of the *orthopoxvirus* genus within the *Poxviridae* family. MPXV is a double-stranded DNA virus around 190 kb in size having two phylogenetically distinct clades: Central African (Congo Basin) clade and the West African clade.

The typical presentation of monkeypox initiates with a short febrile prodromal period followed by progressive development of a classic rash with indurated and umbilicated (centrally depressed) lesions, starting on the head or face and progressing to the limbs and trunk. Lesions progress all at the same stage from macules, to papules, to vesicles, to pustules and eventually to crusts which dry up and fall off after two to four weeks. There are often enanthems (sores or ulcers) in the mouth and lesions can affect the eyes and/or genital area. Swollen lymph nodes are typical of monkeypox.


**Figure 1:** Images of individual monkeypox lesions (Photo credit: UK Health Security Agency) [3].

The first human case of MPXV was detected in 1970 in the Democratic Republic of the Congo (DRC), and since then the infection has been reported in a number of central and western African countries. During the ongoing multi-country outbreak, 1,472 laboratory confirmed cases have been reported from 33 countries from the Americas, Eastern Mediterranean Region, Europe and Western Pacific region (as of June 08, 2022).

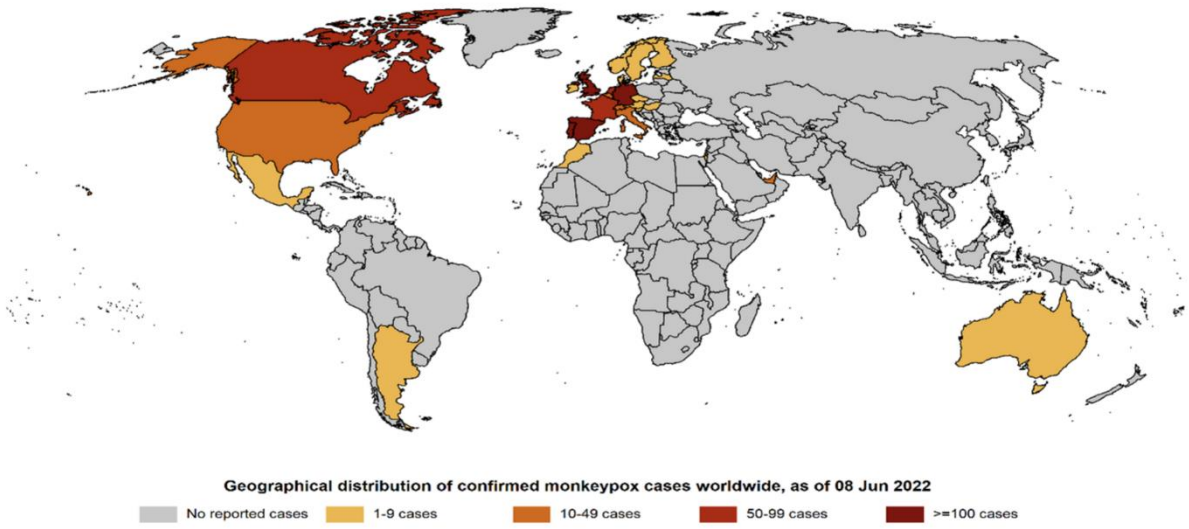


**Figure 2:** Geographical distribution of confirmed cases of monkeypox worldwide, as of June 08, 2022 [4].

Because of range of conditions that cause skin rashes and because clinical presentation may more often be atypical in this outbreak, it can be challenging to differentiate monkeypox solely based on the clinical presentation. Therefore, decision to test should be based on both clinical and epidemiological factors, linked to an assessment of the likelihood of infection. Given the current multiple detection of MXPV world-wide, any individual meeting the definition for a suspected case should be offered testing. In this sense, the National Institutes of Health (NIH) recommends to provincial health departments to ensure the timely identification of suspect cases, the timely collection of samples and the implementation of molecular detection protocols at the provincial public health reference laboratories according to the existing capacity. Where necessary, shipping of samples to NIH may be considered.

**Address:** Department of Virology, Public Health Laboratories Division, National Institutes of Health, Islamabad. +92 (51) 9255082; +92 (51) 9255238.

## Case Definitions

| Suspected case | Probable case | Confirmed case |
|---|---|---|
| A person of any age presenting with an unexplained acute rash<br><br>**AND**<br><br>One or more of the following signs or symptoms, since 15 March 2022:<br><br>• Headache<br>• Acute onset of fever (>38.5 ºC)<br>• Lymphadenopathy<br>• Backpain<br>• Myalgia<br>• Asthenia<br><br>**AND**<br><br>For which the following common causes of acute rash do not explain the clinical picture: varicella zoster, herpes zoster, measles, herpes simplex, dengue, bacterial skin infections, and any other relevant common causes of papular or vesicular rash.<br><br>**Note:** It is not necessary to obtain negative laboratory results for listed common causes of rash illness in order to classify a case as suspected. | A person meeting the case definition for a suspected case<br><br>**AND**<br><br>One or more of the following:<br><br>• Has an epidemiological link to a probable or confirmed case of monkeypox in the 21 days before symptom onset<br>• Reported travel history to a monkeypox endemic country in the 21 days before symptom onset<br>• Is hospitalized due to the illness | A case meeting the definition of either a suspected or probable case<br><br>**AND**<br><br>Is laboratory confirmed for monkeypox virus by detection of unique sequences of viral DNA either by real-time polymerase chain reaction (PCR) and/or sequencing |

## Sample Collection and Management

## Safety Procedures

Use of adequate standard operating procedures (SOPs) must be ensured, and laboratory personnel must be trained for appropriate use of personal protective equipment (PPE) including disposable antifluid gown, latex gloves, goggles or full-face cover, face mask, head cover, shoe covers, and for the disposal of used PPE. Additionally, staff should be appropriately trained for specimen collection, storage, packaging, and transport.

## Biological Risk Management

Measures should be taken to minimize the risk of laboratory transmission based on a risk assessment at institutional level when testing routine clinical specimens from confirmed or suspected monkeypox patients. These may include limiting the number of staff testing specimens only to those with proven competency, wearing appropriate PPE, using rigorously applied standard precautions, using effective disinfectants (which include quaternary ammonium compounds and 0.5% (or 200ppm) bleach (0.5%), and avoiding any procedures that could generate aerosols. Rigorous adherence to infection prevention and control guidelines must be ensured during specimen collection and handling.

It is recommended that all manipulations of specimens originating from suspected, probable or confirmed cases of monkeypox in the laboratory be conducted according to a risk-based approach. Each laboratory should conduct an Institutional risk assessment and when manipulating biological specimens, core biosafety requirements (biosafety level 2) must be met and heightened control measures should be applied based on risk assessment.

MPXV may be contracted during the specimen processing stage from contaminated material or faulty processes. Therefore, heightened biosafety measures are recommended in addition to the core requirements, including the following for the purpose of clinical testing without virus propagation:

- Specimens from patients with suspected MPXV infection must be handled in a functioning and certified Class II biosafety cabinet, prior to sample inactivation. Properly inactivated specimens do not require a biosafety cabinet.
- Laboratory personnel should wear appropriate PPE, especially for handling specimens before inactivation.
- Where use of a centrifuge is required for a procedure, safety cups or sealed rotors should be used.

Additional control measures should be considered for specific procedures, including aerosol-forming procedures, according to the local risk assessment. For more information on core biosafety requirements and heightened control measures, please see the fourth edition of the WHO Biosafety Manual [5].

## Specimen to be collected

The recommended specimen type for laboratory confirmation of monkeypox is skin lesion material, including:

- Swabs of lesion surface and/or exudate,
- Roofs from more than one lesion, or
- Lesion crusts

Lesions swabs, crusts and vesicular fluids should not be mixed in the same tube.

Swab the lesion vigorously using Dacron or polyester flocked swabs, to ensure adequate viral DNA is collected. Both dry swabs and swabs placed in viral transport media (VTM) can be used. Two lesions of the same type should be collected in one single tube, preferably from different locations on the body and which differ in appearance. In addition to a lesion specimen, the collection of an oropharyngeal swab is encouraged. However, data on the accuracy of this

specimen type for diagnosis is limited for monkeypox, therefore a negative throat swab specimen should be interpreted with caution.

## Specimen Collection and Storage

| Specimen Type | Collection Materials | Storage Temperature | Collection purpose |
|---|---|---|---|
| Skin lesion material, including:<br>• Swabs of lesion exudate<br>• Lesion roofs<br>• Lesion crusts | Dacron or polyester flocked swabs with VTM or dry swab | Refrigerate (2-8 °C) or freeze (- 20°C or lower) within 1 hour of collection; -20°C or lower after 7 days * | Recommended for diagnosis |
| Oropharyngeal swab | Dacron or polyester flocked swabs with VTM or dry swab | Refrigerate (2-8 °C) or freeze (- 20°C or lower) within 1 hour of collection; -20°C or lower after 7 days * | Recommended for diagnosis if feasible, in addition to skin lesion material |

*Long term specimen storage (>60 days from collection) is recommended at -70°C. Repeated freeze-thaw cycles should be avoided because they can reduce the quality of specimens.

## Sample Transportation

Specimens should be stored refrigerated or frozen within an hour of collection and transported to the laboratory as soon as possible after collection. All specimens being transported should have;

- Appropriate triple packaging
- Proper labelling
- Patient history form (Annex-I)
- Coolers and cold packs or dry ice

## Laboratory Testing

Testing for the presence of MPXV should be performed in appropriately equipped laboratories (BSL-2) by staff trained in the relevant technical and safety procedures. Measures should be taken to minimize the risk of laboratory transmission based on risk assessment when testing routine clinical specimens from confirmed or suspected monkeypox patients. Laboratories with no molecular diagnostic protocol implemented for MPXV detection should send suspected clinical samples (strictly fitting case definition) to National Institutes of Health.

## Molecular Methods

Confirmation of MPXV infection is based on nucleic acid amplification testing (NAAT), using real-time or conventional polymerase chain reaction (PCR), for detection of unique sequences of viral DNA. PCR can be used alone, or in combination with sequencing. Few groups have developed validated PCR protocols for the detection of MPXV, some of which include distinction of Congo Basin and West African clades. PCR kits detecting MPXV are under development, and available commercial validated PCR kits can be used to detect the virus.

## DNA Extraction

DNA can be extracted from samples mentioned above using any standard extraction protocols or kits. In general, the sample lysis step in DNA extraction inactivates any live virus. Thus, it is recommended that the sample lysis step is performed under a Class II biosafety cabinet. For crust samples, DNA extraction kit for tissue samples should be used to insure appropriate sample lysis.

## Molecular Detection

On the basis of the protocol suggested for the detection of MPXV by Li et al., [6], a working protocol has been designed and mentioned as Annex-II. The protocol is based on the initial detection of MPXV through a real-time PCR/sequencing that detects all MPXV strains. If positive, the sample will be subjected to detection of specific clade (Western Africa and Congo Basin) using real-time PCR.

It is important that each NAAT run must include external, internal, positive and negative controls to validate the results. Controls provide the information about (1) sample quality, (2) nucleic acid quality, and (3) process quality. In order to avoid contamination, negative controls on every run should be utilized to ensure contamination has not occurred. Sample integrity controls (e.g., RNase P), extraction, positive and inhibition controls should also be added to distinguish a false negative from a true negative. Controls should be utilized following laboratory SOPs. If any of the assay controls fail, testing should be repeated.

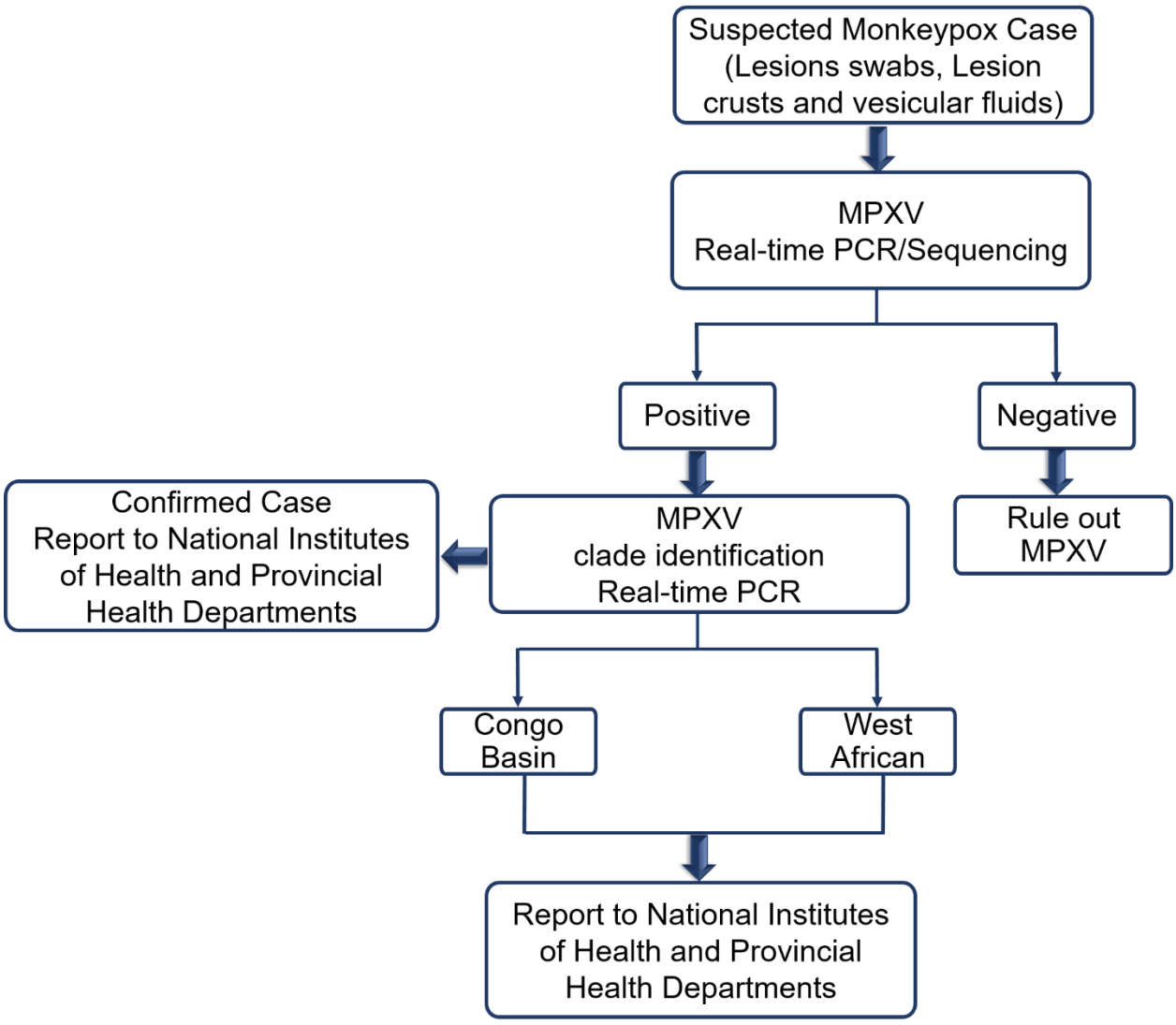


**Figure 3:** Algorithm for the molecular diagnosis of monkeypox virus.

## Interpretation of Results

Confirmation of MPXV infection should consider clinical and epidemiological information. Positive detection by confirmation of MPXV via PCR and/or sequencing in suspected cases confirm MPXV infection. When the clinical presentation and epidemiology suggest an infection with MPXV despite negative PCR results, serological testing may be useful to further investigate prior infection for epidemiological purposes. A number of factors could contribute to false-negative results, such as poor quality of specimen, wrong handling or shipping, or technical reasons inherent to the test, DNA extraction failure.

Moreover, the genetic sequencing data, generated either by sanger or next-generation sequencing (NGS) methods also provide additional information regarding virus characteristics, its origin and epidemiology. It also provides additional information regarding introductions or community transmission.

## Differential Diagnosis

It is important to consider other potential causes of discrete skin lesions or a disseminated rash and other etiologies for similar-appearing skin lesions at the different stages of development including herpes simplex virus, varicella zoster virus, enterovirus, measles, chikungunya, dengue, Treponema pallidum (syphilis), bacterial skin infections, parapoxviruses and among others.

## Data Reporting

Because of the public health risks associated with a single case of monkeypox, clinicians should report suspected cases immediately to provincial and federal health authorities, regardless of whether they are also exploring other potential diagnosis. Cases should be reported immediately, according to the case definitions (mentioned above). All the confirmed cases should be notified immediately to National Institutes of Health and Provincial Health Departments.

Laboratories that perform sequencing (sanger or next-generation sequencing) are encouraged to submit MPXV genome to GISAID or NCBI.

## References

1. World Health Organization. Laboratory testing for the monkeypox virus. Geneva: WHO; 2022. Available from: https://www.who.int/publications/i/item/WHO-MPX-laboratory-2022.1
2. Pan American Health Organization Laboratory Guidelines for the Detection and Diagnosis of Monkeypox Virus Infection 2022. Available from: https://www.paho.org/en/documents/laboratory-guidelines-detection-and-diagnosis-monkeypox-virus-infection
3. https://www.gov.uk/government/collections/monkeypox-guidance
4. https://www.ecdc.europa.eu/en/news-events/epidemiological-update-monkeypox-multi-country-outbreak-8-june
5. https://www.who.int/publications/i/item/9789240011311
6. 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 Oct;169(1):223-7. doi: 10.1016/j.jviromet.2010.07.012

## Annex-I

NIH Pakistan request form

# Monkeypox Testing Request Form

**Department of Virology**
National Institute of Health
Islamabad, Pakistan

Phone +92 (051) 9255082 (8am - 4pm)
Website: www.nih.org.pk

### LAB DETAILS

Lab name and address

Phone/Cell #

### PATIENT DETAILS

Full Name

Gender ☐ Male ☐ Female

Date of birth | D D | M M | Y Y Y Y | Age

### SAMPLE DETAILS

**Sample type**

- ☐ Lesion swabs/dry crusts in VTM
- ☐ EDTA whole blood
- ☐ Plasma
- ☐ Serum
- ☐ Other *(please specify)*

**LAB ID** _____

Date of collection | D D | M M | Y Y | Time

### CLINICAL/EPIDEMIOLOGICAL DETAILS

**Foreign Travel** within previous 21days?

YES/NO

Date of travel | D D | M M | Y Y

Date returned | D D | M M | Y Y

**Onset date** | D D | M M | Y Y

**Countries visited**

- ☐ Acute onset of fever (>38.5 ºC)
- ☐ Lymphadenopathy
- ☐ Intense Headache
- ☐ Backpain
- ☐ Myalgia
- ☐ Asthenia
- ☐ Chills and/or sweats
- ☐ Malaise/listlessness
- ☐ Coryza
- ☐ Sore throat and/or cough
- ☐ Prostration/distress
- ☐ Rash

**Other clinical details**

**Travel Vaccination History**

**Suspected Diagnosis?**

### REFERRED BY

Name Signature Date | D D | M M | Y Y

Version effective from June-2022 V1.0

## Annex-II

## Monkeypox virus (MPVX): Real-time PCR protocol

Assays for the generic detection of MPXV (species: *Monkeypox virus*, genus: *Orthopoxvirus*) and the

detection of its two clades:*1*

• Assay with G2R_G primers and probe: detects all MPXV strains

• Assay with G2R_WA primers and probe: detects Western African clade viruses

• Assay with C3L primers and probe: detects Congo Basin clade viruses

• Primers and probes sequences at the end of the document.

• All probes are hydrolysis (“TaqMan”) probes labelled with the FAM dye and the BHQ-1 quencher.

## Master Mix

| **Reagent** | Volume per reaction **EXPRESS qPCR Supermix Universal**[2] | Volume per reaction **TaqMan® Universal PCR Master Mix**[3] |
|---|---|---|
| Reaction buffer (2x) | 10 µl | 10 µl |
| Forward primer (10 µM) | 0.8 µl | 0.8 µl |
| Reverse primer (10 µM) | 0.8 µl | 0.8 µl |
| Probe (10 µM) | 0.4 µl | 0.4 µl |
| Nuclease Free Water | 3.0 µl | 3.0 µl |
| **Total Volume** | 15 µl | |

## Template

Pipette **5 µl** of sample DNA in 15 µl master mix (total reaction volume: 20 µl). Finally add **5 µl** negative and positive control to assess the validity of the run.

## PCR Amplification Condition for G2R_G assay

| EXPRESS qPCR Supermix Universal | | TaqMan® Universal PCR Master Mix | |
|---|---|---|---|
| | **Condition** | | **Condition** |
| UNG Incubation | 50°C – 2min | UNG Incubation | 50°C – 2min |
| Polymerase Activation | 95°C - 06min | Polymerase Activation | 95°C - 10min |
| PCR Amplification (45 Cycle) | 95°C - 15sec | PCR Amplification (45 Cycle) | 95°C - 15sec |
| Fluorescence Acquisition (FAM) | 60°C – 30sec | Fluorescence Acquisition (FAM) | 60°C – 30sec |

## PCR Amplification Condition for G2R-WA Assay

| EXPRESS qPCR Supermix Universal | | TaqMan® Universal PCR Master Mix | |
|---|---|---|---|
| | **Condition** | | **Condition** |
| UNG Incubation | 50°C – 2min | UNG Incubation | 50°C – 2min |
| Polymerase Activation | 95°C - 06min | Polymerase Activation | 95°C - 10min |
| PCR Amplification (45 Cycle) | 95°C - 15sec | PCR Amplification (45 Cycle) | 95°C - 15sec |
| Fluorescence Acquisition (FAM) | 62°C – 30sec | Fluorescence Acquisition (FAM) | 62°C – 30sec |

## PCR Amplification Condition for C3L Assay

| **EXPRESS qPCR Supermix Universal** | | **TaqMan® Universal PCR Master Mix** | |
|---|---|---|---|
| | **Condition** | | **Condition** |
| UNG Incubation | 50°C – 2min | UNG Incubation | 50°C – 2min |
| Polymerase Activation | 95°C - 06min | Polymerase Activation | 95°C - 10min |
| PCR Amplification (45 Cycle) | 95°C - 15sec | PCR Amplification (45 Cycle) | 95°C - 15sec |
| Fluorescence Acquisition (FAM) | 60°C – 30sec | Fluorescence Acquisition (FAM) | 60°C – 30sec |

## Primers and Probes

| **Assay for Generic Detection of Monkeypox Virus (G2R_G assay)** [1] | | |
|---|---|---|
| **Primers/Probe** | **Sequence (5' > 3')** | **Length** |
| G2R_G Forward Primer | GGAAAATGTAAAGACAACGAATACAG | 26 |
| G2R_G Reverse Primer | GCTATCACATAATCTGGAAGCGTA | 24 |
| G2R_G Probe | FAM-AAGCCGTAATCTATGTTGTCTATCGTGTCC-BHQ1 | 30 |
| **Assay for Detection of Western African Clade Virus (G2R-WA Assay)** [1] | | |
| G2R_WA Forward Primer | CACACCGTCTCTTCCACAGA | 20 |
| G2R_WA Reverse Primer | GATACAGGTTAATTTCCACATCG | 23 |

| | | |
|---|---|---|
| G2R_WA Probe | FAM-AACCCGTCGTAACCAGCAATACATTT-BHQ1 | 26 |
| **Assay for Detection of Congo Basin Clade Viruses (C3L Assay) [1]** | | |
| C3L Forward Primer | TGTCTACCTGGATACAGAAAGCAA | 26 |
| C3L Reverse Primer | GGCATCTCCGTTTAATACATTGAT | 24 |
| C3L Probe | FAM-CCCATATATGCTAAATGTACCGGTACCGGA-BHQ1 | 30 |

1. Li et al., Journal of Virological Methods 169, 223–7 (2010).
2. Invitrogen, cat. no.: 11785-200, 11785-01K, 11795-200 or 11795-01K.
3. Applied Biosystems, cat. no.: 4304437, 4364338, 4364340, 4305719, 4318157 or 4326708.

**Disclaimer: The mention of specific companies or of certain manufacturers' products does not imply that they are endorsed or recommended by the National Institute of Health in preference to others of a similar nature that are not mentioned.**