



**Test Procedure: *Monkeypox virus* Generic Real-Time PCR Test**

**1. Disclaimers**

The Poxvirus and Rabies Branch (PRB) developed this document as a test procedure for the *Monkeypox virus* generic real-time PCR assay. This is not a controlled document. The performance characteristics as generated at Centers for Disease Control and Prevention (CDC) are specific to the version as written. These documents are provided by PRB laboratory solely as an example for how this test performed within the PRB laboratory. **The recipient testing laboratory is responsible for generating validation or verification data as applicable to establish performance characteristics as required by the testing laboratory’s policies, applicable regulations, and quality system standards.** These data are only for the sample and specimen types and conditions described in this procedure. Tests or protocols may include hazardous reagents or biological agents. No indemnification for any loss, claim, damage, or liability is provided for the party receiving an assay or protocol. Use of trade names and commercial sources are for identification only and do not constitute endorsement by the Public Health Service, the United States Department of Health and Human Services, or the Centers for Disease Control and Prevention.

**2. Purpose / Principle**

The purpose of this protocol is to describe the procedure used for the detection of *Monkeypox virus* DNA in clinical specimens by real-time PCR. This assay detects DNA at varying concentrations, providing a qualitative result of either positive, negative, or inconclusive in the identification of *Monkeypox virus* infections.

**3. Scope**

Applies to all personnel who perform *Monkeypox virus* diagnostic testing by real-time PCR.

**4. Definitions / Keywords**

Term	Definition
CT	Cycle threshold
DNA	Deoxyribonucleic acid
NTC	No template control
PPE	Personal protective equipment
RNase P	Ribonuclease P
RT-PCR	Real-time polymerase chain reaction
SOP	Standard operating procedure
TE	Tris-EDTA

**5. Specimen Information / Processing**

**5.1 Acceptable specimens**

5.1.1 Specimen types need to be validated in the laboratory before testing begins.



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- 5.1.2 DNA extracted from lesion material. DNA can be extracted from acceptable specimen types including, but not limited to, lesion fluid on a dry swab, lesion fluid swab in viral transport media, lesion fluid on a slide, crust, or lesion roof.
- 5.1.3 DNA extract solely from whole blood alone is not a suitable specimen for *Monkeypox virus* diagnostic testing as the viremic phase may have already passed at the time of rash onset. As such, a negative result from only a whole blood specimen cannot rule out a *Monkeypox virus* infection.

**5.2 Rejection criteria**

- 5.2.1 Visible contamination.
- 5.2.2 Incomplete labeling so as contents cannot be identified.
- 5.2.3 Incomplete documentation.
- 5.2.4 Specimen received outside of established storage and shipping acceptance criteria.

**5.3 Specimen shipment conditions and regulations**

- 5.3.1 Specimen shipment conditions and regulations are set forth by the International Air Transport Association (IATA) Dangerous Goods Regulations and government regulations.

**6. Hazards / Safety Precautions**

- 6.1 Follow safety procedures as outlined in the site-specific biological safety plan/laboratory biosafety manual and Biosafety in Microbiological and Medical Laboratories (BMBL), most recent edition ([Link to BMBL](#)). Additional information for laboratory personnel can be found here: <https://www.cdc.gov/poxvirus/monkeypox/lab-personnel/lab-procedures.html> <https://www.cdc.gov/poxvirus/monkeypox/lab-personnel/lab-procedures.html - Manipulating%20Specimens%20Suspected%20to%20Contain%20Monkeypox%20Virus>

**7. Equipment**

- 7.1 Real-time PCR Instrument
- 7.2 Freezer (preferably  $\leq -20^{\circ}\text{C}$ )
- 7.3 Microcentrifuge
- 7.4 Pipettman, including a p2, p20, and p200 as needed.
- 7.5 Refrigerator ( $2-8^{\circ}\text{C}$ )
- 7.6 Vortexer
- 7.7 PCR workstations or BSC class II (preferably separate workstations; a workstation for master mix or reagent preparation and workstation for adding specimen DNA)

**8. Supplies / Materials**

- 8.1 Disposable gloves
- 8.2 Disposable laboratory coat
- 8.3 Ice and ice bucket
- 8.4 Microcentrifuge rack
- 8.5 Microcentrifuge tubes (sterile and nuclease free)
- 8.6 Optical 96-well reaction plates
- 8.7 Optical adhesive covers and applicator or strip caps and capping tool
- 8.8 Pipette tips (aerosol-resistant filter)

**9. Reagents / Media**

- 9.1 **Reagents (Light Sensitive)**



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- 9.1.1 Real-time PCR master mix
- 9.1.2 Positive controls (stored according to manufacturer instructions or laboratory validation)
  - a. *Monkeypox virus* controls
  - b. Human DNA
  - c. Extraction control (such as human cell lines)
- 9.1.3 Primer and probe sets (stored refrigerated protected from light; for long-term storage follow manufacturer instructions)
  - a. *Monkeypox virus* primers and probe (Appendix)
  - b. Human DNA primers and probe (e.g., RNase P,  $\beta$ -actin, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH))
- 9.2 **Reagents (Non-light Sensitive)**
  - 9.2.1 TE buffer (10mM, pH 8.1  $\pm$  0.2)
  - 9.2.2 Water (molecular grade)
- 9.3 **Acceptable surface decontaminants**
  - 9.3.1 Ethanol (70%)
  - 9.3.2 RNase AWAY

## 10. Reagent / Media Preparation

**Note:** Frozen reagents may be used until the manufacturer expiration date as long as they pass QC.

### 10.1 Assay controls

- 10.1.1 *Monkeypox virus* positive control: Potential sources for positive controls for this assay can be DNA from *Monkeypox virus*, or a plasmid containing a cloned PCR target.
- 10.1.2 Human DNA positive control: Any source of human DNA that results in a positive reaction with the assay being used to confirm presence of human DNA is acceptable for use as a positive control. Human genomic DNA is commercially available.
- 10.1.3 Extraction control: Any human cell line or cells can be used with the human DNA primers and probes that results in a positive reaction to confirm the extraction was acceptable.
- 10.1.4 To prepare positive controls and extraction control, dilute DNA or make aliquots of human cells (extraction control) with a known cut-off to create a positive control for use when performing real-time PCR.
  - a. In a 40 cycle PCR, a positive control should have a CT cut-off value between 22–28 and (if used) a second low positive control should have a cut-off value between 30–36.
- 10.1.5 A standard curve can also be used and can be prepared using DNA with a known concentration. For example, logarithmic dilutions from 0.1 ng/ $\mu$ L to 1 fg/ $\mu$ L are created from the known positive control and run-in triplicate with the specimens to be tested.
- 10.1.6 Negative PCR control (e.g., no template control (NTC): control specimen containing all reagents except the target template; molecular grade water is an example of an appropriate NTC. A NTC is a mechanism to detect cross-contamination of specimens.

### 10.2 Primers and probes

- 10.2.1 Dilute assay primers from stock to 20  $\mu$ M and probes to 10  $\mu$ M in TE buffer or molecular grade water.



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- 10.2.2 Dilute Human DNA control primer to concentrations established by the laboratory's validation data.
  - a. Example, RNaseP primers can be diluted to 12.5 nmol and probe diluted to 2.5 nmol.
- 10.2.3 Store in the dark at 2–8°C and use within 6 months. Alternatively, single use aliquots can be prepared and stored frozen up to 24 months (manufacturer instructions should be followed for storage conditions, most frequently  $\leq -20^{\circ}\text{C}$ ). Do not freeze-thaw aliquots.

## 11. Quality Control

- 11.1 No template control (molecular grade water) and positive controls serve as assay quality indicators and must be run during each assay.
- 11.2 Replicates can be used to determine precision.
  - 11.2.1 Replicates, if used, should have a range of less than three CT values. If the range exceeds this value, invalidate the run, and repeat the assay.
- 11.3 An extraction control, human DNA, should be included each time nucleic acid is extracted. If the extraction control fails to produce a positive result with the human DNA primers and probe, the test is inconclusive and must be repeated.
- 11.4 Inconclusive results or deviations must be documented on a real-time PCR worksheet and brought to the attention and reviewed by the laboratory supervisor.
- 11.5 Equipment maintenance and function checks are performed before testing according to manufacturer package insert and/or operating manual.
- 11.6 See the laboratory quality manual for laboratory in which assay is being run, for additional quality control measures.

## 12. Procedure

### 12.1 DNA isolation

- 12.1.1 DNA isolation is performed according to laboratory's DNA extraction SOP.

### 12.2 Plate layout

- 12.2.1 Use a real-time PCR worksheet to establish the plate layout.
- 12.2.2 Each specimen and control may be tested in duplicate or triplicate.
- 12.2.3 Record reagent lot numbers on worksheet.

### 12.3 Workspace preparation for real-time PCR

- 12.3.1 Non disposable laboratory coat and gloves.
- 12.3.2 Decontaminate PCR workstation, e.g., RNase AWAY followed by ethanol (70%).

### 12.4 Master mix cocktail calculations

- 12.4.1 Determine the number of reactions (N) for each assay. Account for specimens being tested in duplicate or triplicate, if applicable. Excess reaction cocktail should be prepared to compensate for volume lost during pipetting (i.e., add 3 extra reactions).
- 12.4.2 The mixture below is assuming a 2x master mix. Adjust volumes as necessary based on master mix instructions and validation of assay
  - a. Assay reaction mixture
    - i. Molecular grade water =  $(N+3) \times 3.5 \mu\text{L}$
    - ii. Forward primer =  $(N+3) \times 0.5 \mu\text{L}$
    - iii. Reverse primer =  $(N+3) \times 0.5 \mu\text{L}$
    - iv. Probe =  $(N+3) \times 0.5 \mu\text{L}$
    - v. 2x master mix =  $(N+3) \times 10 \mu\text{L}$



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- b. Human DNA reaction mixture (RNase P reaction from 10.2.2.a listed as example).
  - i. Molecular grade water =  $(N+3) \times 4.25 \mu\text{L}$
  - ii. Forward primer =  $(N+3) \times 0.25 \mu\text{L}$
  - iii. Reverse primer =  $(N+3) \times 0.25 \mu\text{L}$
  - iv. Probe =  $(N+3) \times 0.25 \mu\text{L}$
  - v. 2x master mix =  $(N+3) \times 10 \mu\text{L}$

**12.5 Reaction mixture preparation and plating best practices (master mix workstation)**

- 12.5.1 Label one microcentrifuge tube per reaction mixture.
- 12.5.2 Thaw frozen reagents on ice and gather remaining reagents from refrigerator.
- 12.5.3 Briefly vortex and centrifuge reagents (5 seconds).
- 12.5.4 Add reagents, with volumes as calculated above, to the appropriately labeled microcentrifuge tube.
- 12.5.5 Briefly vortex and centrifuge reaction mixture tubes (5 seconds).
- 12.5.6 Dispense 15  $\mu\text{L}$  master mix reaction into each assigned well.

**12.6 Specimen and control plating (specimen workstation)**

- 12.6.1 Pipette 5  $\mu\text{L}$  of molecular grade water into all NTC-labeled wells.
- 12.6.2 Vortex and centrifuge each DNA specimen tube.
- 12.6.3 Pipette 5  $\mu\text{L}$  specimen DNA into each specimen-labeled well (both for *Monkeypox virus* assay and for Human DNA assay).
- 12.6.4 Repeat the previous step for both positive controls.

**12.7 Best practices for performing real-time PCR**

- 12.7.1 Peel off the protective covering of the optical adhesive cover and place it over the wells, making sure all wells are covered. Or apply caps if not using optical adhesive.
- 12.7.2 Using the plastic optical adhesive cover applicator, firmly run the edge of the applicator over the cover, ensuring a tight seal. Detach the edges of the adhesive strip along the perforated sections by placing the edge of the applicator against the perforation and gently tearing it off. Alternatively, carefully cap tubes, ensuring they are sealed with cap sealing tool.
- 12.7.3 Inspect wells for bubbles and liquid drops on the sides of the wells above the reaction mixtures. If either bubbles or drops are seen, gently tap or flick the tray repeatedly until the bubbles are dispersed and drops have fallen back into the main reaction mixture. Small drops that do not move after repeated tapping of the plate will not affect assay performance. If possible, use a tabletop centrifuge with plate adapter and centrifuge the plate for 1 minute at 500 x *g* and room temperature. Use a balance plate if necessary.
- 12.7.4 Place the reaction plate into the specimen block of the real-time thermocycler. Make certain that plate orientation is correct, and that the tray is fully inserted into the specimen block.
- 12.7.5 Program the real-time PCR instrument (refer to system user's manual) for the appropriate cycling conditions and volume of the assay run.

**12.8 Data retrieval and review**

- 12.8.1 After run completion, analyze results. Threshold should be set above background as determined by your laboratory's SOP from previous validation data.

**13. Method Performance Specifications**

- 13.1 Performance specifications need to be established by the laboratory before testing specimens.



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- 13.2 If inhibitors are present that are not removed during DNA extraction, a false negative result may be produced.
- 13.3 If RNase P inhibition is suspected, extracted DNA should be tested at 2 or more dilutions (e.g., 1:10 to 1:100) to verify the result.
- 13.4 If any controls fail, the assay run should be invalidated and repeated.

#### 14. Calculations

- 14.1 Adjust calculations if not using triplicates

$$\text{Average CT value} = (\text{Replicate 1} + \text{Replicate 2} + \text{Replicate 3}) \div 3$$

#### 15. Reference / Alert values

- 15.1 The general population is expected to be negative for *Monkeypox virus* DNA.
- 15.2 All positive results are to be considered an alert value for clinical testing.
- 15.3 After PCR results are available, follow laboratory post-analytical SOPs for approval and reporting. For diagnostic testing, it is recommended to report results to the state or local department of health.

#### 16. Result Interpretation

- 16.1 Determine the cut off values for each extraction method and specimen type being used within the PCR assay.
  - 16.1.1 Cut off values must be determined by each laboratory during the validation phase.
  - 16.1.2 Cut off values can vary based on extraction methods, specimen type, PCR instrument, and final cycling conditions.
- 16.2 NTC reactions for all probe and primer sets should NOT exhibit amplification curves that cross the threshold line. If NTCs exhibit amplification curves that cross the threshold line, contamination may be indicated. Invalidate the run and repeat the assay.
- 16.3 The positive control(s) for each assay should exhibit an amplification curve that crosses the threshold line within its intended range (see section 10.1). If the control(s) does not exhibit an amplification curve that crosses the threshold line or crosses outside of their expected range, invalidate the run and repeat the test.
- 16.4 Negative results can only be determined if a specimen is positive for human DNA positive control, otherwise results are inconclusive. Negative or inconclusive RNaseP results may indicate:
  - 16.4.1 Inadequate specimen collection.
  - 16.4.2 Presence of PCR inhibitor(s) in specimen or collection material.
  - 16.4.3 Improper DNA extraction, assay set up and/or execution.
  - 16.4.4 Reagent or equipment malfunction.
- 16.5 Replicates, if used, should have a range of less than three CT values. If the range exceeds this value, invalidate the run, and repeat the assay.
- 16.6 Average CT values are calculated when replicates are used, and this average determines the qualitative result.
- 16.7 Interpretation of positive results are as follows:
  - 16.7.1 Positive for *Monkeypoxvirus* Generic: DNA detected from *Monkeypox virus*
- 16.8 It is recommended any results are communicated to the state or local department of health.

#### 17. Result Reporting

- 17.1 Results are reviewed by the laboratory’s post-analytic review and approval SOPs.
- 17.2 Final reports are distributed according to the laboratory’s specimen test reporting procedures.

**18. Specimen Retention / Storage**

- 18.1 DNA is stored refrigerated prior to testing and frozen (preferably ≤-20°C) for long term storage.
- 18.2 Specimens should be retained and stored according to the laboratory’s specimen and specimen management SOP. At PRB, specimens are frozen (preferably ≤-20°C) for long term storage. Temporary storage (<3 weeks) refrigerated (2-8°C) is permissible.

**19. Scientific publications detailing *Monkeypox virus* real-time PCR assays designed by the CDC and shared with the public**

- 19.1 Li, et al. “Detection of monkeypox virus with real time PCR assays”. Journal of Clinical Virology 36 (2006) 194-203.

**20. References**

- 20.1 [Laboratory Procedures | Monkeypox | Poxvirus | CDC Information For Laboratory Personnel | Monkeypox | Poxvirus | CDC](#)
- 20.2 Laboratory Biological Safety Plan
- 20.3 Biosafety in Microbiological and Biomedical Laboratories, 6th edition
- 20.4 Laboratory quality manual
- 20.5 [Regulations and Interpretative guidelines for laboratories and laboratory services](#)
- 20.6 Laboratory Biosafety Manual

**21. Appendix: Real-time PCR assay sequences and thermal cycling conditions**

Assay	Primer / Probe Sequences		Temp (°C)	Time (sec)	Cycles
<i>Monkeypox virus</i> Generic	Forward	5'-GGA AAA TGT AAA GAC AAC GAA TAC AG-3'	95	20	1
	Reverse	5'-GCT ATC ACA TAA TCT GGA AGC GTA-3'	95	3	
	Probe	5'-FAM-AAG CCG TAA TCT A<BHQ-1dT>GT TGT CTA TCG TGT CC-Spacer C6-3'	60	30	40
Human DNA (e.g., RNase P)	Forward	5'-AGA TTT GGA CCT GCG AGC G-3'	95	20	1
	Reverse	5'-GAG CGG CTG TCT CCA CAA GT-3'	95	3	
	Probe	5'-FAM-TTC TGA CCT GAA GGC TCT GCG CG-BHQ1-3'	60	30	40