



Test Procedure: Non-variola Orthopoxvirus Generic Real-Time PCR Test

Rev. No. 02

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1. Disclaimers

The Poxvirus and Rabies Branch (PRB) developed this document as a test procedure for the non-variola *Orthopoxvirus* 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 non-variola virus *Orthopoxvirus* 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 *Orthopoxvirus* infections, other than smallpox (caused by variola virus).

3. Scope

Applies to all personnel who perform *Orthopoxvirus* 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
RNaseP	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 *Orthopoxvirus* 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 an *Orthopoxvirus* 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>

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 (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
- 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)**
 - 9.1.1 Real-time PCR master mix



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- 9.1.2 Positive controls (stored according to manufacturer instructions or laboratory validation)
 - a. *Orthopoxvirus* 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. *Orthopoxvirus* primers and probe (Table 1)
 - b. Human DNA primers and probe (e.g., RNaseP, β -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 *Orthopoxvirus* positive control: Potential sources for positive controls for this assay can be virus or DNA from appropriate orthopoxviruses, or a plasmid containing 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/ μ L to 1 fg/ μ L are created from the known positive and run-in triplicate with the specimens to be tested.
- 10.1.6 Negative PCR control (e.g., no template control (NTC): control sample containing all reagents except the target template; molecular grade water is an example of an appropriate NTC. An 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 μ M and probes to 10 μ 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, 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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- 13.3 If RNaseP 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 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 *Orthopoxvirus* 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 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.
- 16.2 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.
- 16.3 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:
 - 16.3.1 Inadequate specimen collection.
 - 16.3.2 Presence of PCR inhibitor(s) in specimen or collection material.
 - 16.3.3 Improper DNA extraction, assay set up and/or execution.
 - 16.3.4 Reagent or equipment malfunction.
- 16.4 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.
- 16.5 Average CT values are calculated when replicates are used, and this average determines the qualitative result (see Table 1 below).
- 16.6 Interpretation of positive results are as follows:
 - 16.6.1 Positive for Non-variola *Orthopoxvirus* Generic: DNA detected from Non-variola *Orthopoxvirus*
- 16.7 It is recommended any results are communicated to the state or local department of health.



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Table 1: Real-Time PCR Result Interpretation

Assay	CT Values			Valid Extraction/Internal positive control (e.g. RNaseP)
	Positive	Inconclusive	Negative	
<i>Orthopoxvirus</i>				
Non-variola <i>Orthopoxvirus</i> Generic	<37	37 ≤ CT < 40	≥40	<37

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 non-Variola *Orthopoxvirus* 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



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21. Appendix

Real-time PCR assay sequences and thermal cycling conditions

Assay	Primer / Probe Sequences		Temp (°C)	Time (sec)	Cycles
Non-variola Orthopoxvirus Generic	Forward	5'-TCA ACT GAA AAG GCC ATC TAT GA-3'	95	20	1
	Reverse	5'-GAG TAT AGA GCA CTA TTT CTA AAT CCC A-3'	95	3	40
	Probe	5'-FAM-CCA TGC AAT A (T-BHQ1) A CGT ACA AGA TAG TAG CCA AC-Phos-3'	63	30	
Human DNA (e.g., Rnase P)	Forward	5'-AGA TTT GGA CCT GCG AGC G-3'			
	Reverse	5'-GAG CGG CTG TCT CCA CAA GT-3'			
	Probe	5'-FAM-TTC TGA CCT GAA GGC TCT GCG CG-BHQ1-3'			