## 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.

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 ≤-20°C)
7.3 Microcentrifuge
7.4 Pipetteman, including a p2, p20, and p200 as needed.
7.5 Refrigerator (2–8°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

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 ± 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/µL to 1 fg/µL 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 µM and probes to 10 µM in TE buffer or molecular grade water.

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 ≤-20°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 A human DNA control should be included each time nucleic acid is extracted and run with both the RNase P and assay primers and probes.

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) x 3.5 µL

**ii.** Forward primer = (N+3) x 0.5 µL

**iii.** Reverse primer = (N+3) x 0.5 µL

**iv.** Probe = (N+3) x 0.5 µL

**v.** 2x master mix = (N+3) x 10 µL

**b.** Human DNA reaction mixture (RNaseP reaction from 10.2.2.a listed as example).

**i.** Molecular grade water = (N+3) x 4.25 µL
**ii.** Forward primer = (N+3) x 0.25 µL
**iii.** Reverse primer = (N+3) x 0.25 µL
**iv.** Probe = (N+3) x 0.25 µL
**v.** 2x master mix = (N+3) x 10 µL

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 µL master mix reaction into each assigned well.

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

12.6.1 Pipette 5 µL of molecular grade water into all NTC-labeled wells.
12.6.2 Vortex and centrifuge each DNA specimen tube.
12.6.3 Pipette 5 µL specimen DNA into each specimen-labeled well (both for Orthopoxvirus 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 (see Appendix Table 1).

12.8 **Data retrieval and review**

12.8.1 After run completion, analyze results. Baseline should be determined on your laboratory's SOP that was previously validated for the assay.

## 13. Method Performance Specifications

13.1 Performance specifications need to be established by the laboratory before testing samples.
13.2 If inhibitors are present during DNA extraction, a false negative result may be produced**.**

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

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

## 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 Extraction controls for the RNase P probe and primer set should exhibit a growth curve that crosses a threshold.

16.3.1 If the control(s) does not exhibit a growth curve that cross the threshold line or crosses outside of their expected range, invalidate the plate and repeat the PCR assay.

16.3.2 If the extraction control fails to produce a positive RNaseP result in any replicate the assay must be repeated.

16.4 Extraction controls for the monkeypox virus assay probe and primer set should NOT exhibit a growth curve that crosses the threshold line.

16.4.1 If extraction control with monkeypox virus primers and probes exhibit growth curves that cross the threshold line, specimen contamination may have occurred at the testing, processing or extraction step. Invalidate the plate and repeat the PCR with the same extracted DNA.

16.4.2 If the extraction control still has amplification, re-extract the affected specimens with a new extraction control and repeat the PCR.

16.4.3 If the extraction control still exhibits a growth curve after the repeated DNA extraction and repeated PCR, it should be interpreted that contamination occurred at or before the processing step. The results must be rejected for these specimens. Pause testing and investigate potential cause(s) of contamination which may be associated with the instruments, reagents, or workspaces. Fully document investigations and findings with appropriate NCE forms. If the root cause of contamination has been determined and resolved, re-extract the affected specimens and resume testing.

16.5 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:
- 16.5.1 Inadequate specimen collection.
- 16.5.2 Presence of PCR inhibitor(s) in specimen or collection material.
- 16.5.3 Improper DNA extraction, assay set up and/or execution.
- 16.5.4 Reagent or equipment malfunction.

16.6 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**.**

16.7 Average CT values are calculated when replicates are used, and this average determines the qualitative result (see Table 1 below).

16.8 Interpretation of positive results are as follows:
- 16.8.1 Positive for Non-variola *Orthopoxvirus* Generic: DNA detected from Non-variola *Orthopoxvirus*

16.9 It is recommended any results are communicated to the state or local department of health.

**Table 1: Real-Time PCR Result Interpretation**

| Assay | CT Values | | | |
|---|---|---|---|---|
| | **Positive** | **Inconclusive** | **Negative** | **Valid Extraction/Internal positive control (e.g. RNaseP)** |
| *Orthopoxvirus* | | | | |
| Non-variola *Orthopoxvirus* 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 *Orthopoxirus* 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 |
|---|---|---|---|---|---|
| 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' | | | |

## 22. Revision History

| Rev # | DCR # | Changes Made to Document | Date |
|---|---|---|---|
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