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# Monkeypox Virus Inactivation Guidance for Nucleic Acid Testing Validation

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## I. Introduction for Use of This Guidance

To reduce potential biosafety risks and enhance laboratory safety for handling patient specimens that may contain monkeypox virus (MPXV), laboratories may consider implementing one or more methods to inactivate MPXV during nucleic acid amplification testing (NAATs). The Centers for Disease Control and Prevention (CDC) has recommended that laboratories which test lesions from suspected MPXV patients perform viral inactivation before putting the specimen on an automated platform for extraction or testing (1). Given the overlapping clinical presentations and similar specimen collection devices for MPXV and other pathogens (such as herpes simplex virus (HSV), varicella zoster virus (VZV) and others), the addition of viral inactivation steps may be desired for both MPXV NAAT and other similar viral tests. Inactivation methods may be employed and validated as part of new test development by individual laboratories (laboratory developed tests [LDTs]) or by commercial test manufacturers seeking test authorization, approval, or clearance. Alternatively, individual laboratories may wish to modify an existing test to include viral inactivation steps. In this document, we provide recommendations for completing a test performance evaluation (also referred to here as validation) when modifying an existing qualitative NAAT to include viral inactivation steps, to ensure the performance of the test is not negatively impacted by the modification. These validation studies should be completed before implementing the modified method for patient testing. For laboratories that are considering implementing MPXV inactivation methods, we also refer readers to [ASM's Monkeypox Virus Biosafety and Testing Recommendations White Paper, Section 7](#) for a summary of inactivation approaches and reported efficacies. Laboratories may also refer to the UK Health Security Agency (UKHSA) assessments of inactivation methods for various specimens and instrument platforms (2).

## II. Background

Verification and/or validation of a new or modified assay is an integral part of implementing any test in a clinical laboratory. Verification and validation studies should be conducted in consultation with and final approval of each laboratory's Clinical Laboratory Improvement Amendments (CLIA) Director, and individual validation and verification studies may differ somewhat by director discretion. The need for a laboratory to perform either validation or verification depends on the FDA classification of the test being implemented and how the laboratory is using the test (i.e., modified or unmodified). The following definitions and interpretations were applied in the development of this document:

Verification: If the test has FDA 510(k) *in vitro* diagnostic (IVD) clearance, the manufacturer has already validated the test (i.e., demonstrated that the test performance characteristics are acceptable to be used for clinical testing through a robust evaluation of required test elements). Therefore, the laboratory can perform a straightforward verification- a process to confirm that the established performance specifications [accuracy, precision, reportable range (when applicable) and reference interval (when applicable)] are achieved when the test is performed in that laboratory (3). On September 7, 2022, the FDA implemented an Emergency Use Authorization (EUA) pathway for commercial developers of MPXV PCR tests, which remains active as of the time of writing this document. When implementing a commercial assay which has been granted EUA, individual laboratories can verify the assay similarly to the process used for assays with FDA clearance or approval. ASM has previously published guidance for verifying tests with EUA (4, 5).

However, verification only applies when there are no deviations or modifications from the manufacturer's instructions for use of the test. If there is a modification, such as addition of an inactivation step not included in the instructions for use by the manufacturer, then a validation is required (see below).

Validation: Test validation is the process that must be performed when a laboratory modifies a clinical test that has been granted FDA clearance or EUA and for tests that are developed and implemented by an individual laboratory as LDTs. If a manufactured test is granted EUA from the FDA, the need for individual laboratories to perform verification or validation, if modified, is similar to that of tests with FDA 510(k) clearance.

- If the test is a laboratory-developed test (LDT), a more in-depth validation is required. In addition to accuracy and precision studies, LDT validations also include analytical sensitivity/limit of detection (LOD), specificity/cross-reactivity, interference, and carry-over testing, depending on the test platform being used (3).
- If the test has 510(k) IVD clearance/approval or EUA but an inactivation step is added or a different reagent is used/added to an existing extraction protocol, the procedure has been changed, thus making the test an LDT (e.g., modified FDA-cleared test) and additional validation is required.

At the time of the writing of this document, a public health emergency declaration and the justification for the authorization of emergency use of IVD assays by the FDA are active policies for MPXV. While EUA is an available pathway for the authorization of tests developed by both commercial and non-commercial entities, individual laboratory development and implementation of a MPXV NAAT is currently also permitted as a laboratory developed test (without EUA), when both the laboratory and test meet specific requirements dictated by the FDA (6). Laboratories are required to notify the FDA of having validated such an assay for MPXV. Additionally, at this time, test modifications by high-complexity CLIA-certified laboratories (*such as implementing an additional viral inactivation step to an already authorized or cleared assay*) are permitted without additional authorizations if the modification 1) has been validated by the laboratory and shown not to adversely impact the test performance, 2) does not alter the indication for use of the test, and 3) does not change the analyte specific reagents. Laboratories that modify existing MPXV assays with inactivation steps must also notify the FDA within 5 days of offering the modified test (6). Note that laboratories do not need to notify the FDA upon validation of modifications (including viral inactivation steps) for assays that do not target MPXV (such as those for HSV or VZV) but should ensure all processes comply with CLIA '88 regulations.

Given the evolving nature of policies during a public health emergency, during any period in which the FDA utilizes EUA we recommend that each laboratory should communicate with the FDA to determine the required steps *before* implementing a MPXV LDT or laboratory modified assay.



## III. Materials Required

- Appropriate biosafety and protective equipment (1).
- Quantified MPXV material (or other target analyte) to perform LOD studies.
- Positive and negative patient specimens tested on a comparator assay. Or, contrived samples (negative specimens spiked with either positive sample or positive control) if needed due to insufficient number of positive patient specimens.
- Standard reagents for performing the original, unmodified test.
- Inactivation procedure-specific reagents, as applicable.
- Positive and negative controls provided by manufacturer or purchased separately.
- Ancillary supplies (tubes, pipette tips, etc.) necessary to perform the test.

## IV. Instruments Required

Instrument(s) for performing the NAAT test, as approved by the manufacturer for 510k IVD, EUA tests, or employed in an existing LDT.

## V. Validation Procedure

### 1. Analytical Sensitivity/Limit of Detection (LOD)

If a laboratory chooses to add a MPXV inactivation step to a test, an LOD study must be performed to determine if the modification has altered the analytic sensitivity of the test. Importantly, if an inactivation step negatively impacts test performance by significantly decreasing the analytic sensitivity (i.e., increasing the LOD), false negative results may occur. Therefore, it is critical to demonstrate that the LOD is similar with and without the inactivation modification.

Prior to performing LOD studies, acceptance criteria for allowable difference in LOD with and without an inactivation step should be determined. It is recommended that the LOD of the test with the added inactivation step be equivalent if not better than the LOD without the inactivation step.

- a. Purchase quantified material for the pathogen(s) that is/are being detected by the test
  - i. Whole, quantified inactivated pathogen is preferred.
  - ii. If whole pathogen is not available, laboratories may consider testing quantified preparations of pseudovirus containing the target analyte nucleic acid sequence or purified nucleic acid (genomic or otherwise), the latter of which is least preferred.
- b. Perform serial dilutions of the control in negative matrix.
  - i. For 510k IVD/EUA tests, compare to the claimed LOD in the manufacturer's instructions for use (IFU).
  - ii. For established LDTs, compare to the previously established LOD in the initial validation.
- c. Using the modified protocol that includes MPXV inactivation, test each dilution 3 times to estimate the range of dilutions (2 or 3 dilutions) where the LOD may be located.

NOTE: Laboratories may consider initially focusing on concentrations near the claimed LOD of the test to streamline or reduce the number of dilutions needed. However, if there is significant impact of inactivation on the LOD, additional dilutions may be necessary.



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- d. For each dilution near the estimated LOD, test 20 replicates to determine with 95% confidence which dilution is the LOD (3).
- e. Compare the LOD obtained from the above testing to the manufacturer's reported or laboratory's originally established LOD without the inactivation step.

NOTE: Due to the lack of a universal standard for quantifying MPXV material, the method by which the reagent manufacturer quantifies MPXV materials, and the type of material quantified (whole live virus, inactivated whole virus, genomic nucleic acid), there may be variability between previously reported LODs and the validation LOD. If discrepancies are observed and suspected to be due to the difference in materials used for LOD studies, side-by-side comparison of the unmodified assay LOD and modified assay LOD using the same quantified reagent may be appropriate to determine if addition of an inactivation procedure alters test performance. We recommend consulting with the FDA to determine if a side-by-side LOD comparison validation approach will be acceptable, particularly if this approach yields a numeric LOD result that is significantly higher than the originally reported LOD.

## 2. Accuracy

Specimen selection: Patient specimens with known comparator results are preferred. Residual specimens may include fresh aliquots taken during processing or frozen aliquots from previously tested specimens. If residual specimen testing is not possible (e.g., a single dry swab is used entirely for the patient test), additional specimens obtained side-by-side may be employed, after appropriate patient consent and institutional approval is obtained. Alternatively, a laboratory may use contrived samples whereby negative specimens are spiked with either known positive samples or positive control material, the latter of which is the least preferred method. When possible, include specimens spanning the detection range of the assay (e.g., relatively high, medium, and low amounts of analyte for qualitative tests).

Number of specimens: Accuracy validation for molecular assays to detect MPXV or other pathogens must include both known positive and known negative specimens. CLSI MM19A recommends testing 50 positive specimens and 50 negative specimens. However, given the emergence of MPXV and regional differences in prevalence, 50 positive specimens may be challenging for some laboratories to acquire. At the discretion of the medical director, less than 50 positive specimens may be used for validation purposes, especially if MPXV is considered to be rare in the region that the laboratory serves. While 50 positive specimens are ideal, in such situations described above, as few as 20 specimens may be sufficient for accuracy studies. While a public health emergency for MPXV is declared, if a laboratory has access to fewer than 30 positive and 30 negative specimens (7), we recommend consulting the FDA before initiating the modified assay validation.

Specimen type: Laboratories must validate every specimen type that will be approved for testing. For MPXV testing, lesion material is the preferred specimen type. If the laboratory plans to test additional specimen types, including multiple collection devices (swabs and/or transport media) that are not equivalent to one another, those specimen types must also be included in the validation. The accuracy approaches detailed above should be taken for each unique specimen type. If multiple specimen collection containers are used, validation should include a sampling of various collection devices.



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Data analysis: Data should be analyzed and performance assessed by calculating positive and negative agreement between the validation result (with inactivation step) and the comparator result (testing without inactivation or expected results from known contrived specimens). At least 95% agreement is generally considered acceptable test performance. Discrepant results between the comparator method and the test being verified should be fully investigated. Specimens should be re-tested and, if significant discrepancy continues, additional troubleshooting should occur. The new assay or modified protocol (i.e., inactivation steps) should not be implemented for patient testing until discrepancies are resolved or scientifically justified and/or the pre-determined acceptance criteria are met.

### 3. Precision

Precision testing must occur with any test type (510(k) cleared, EUA, or LDT). Depending on test method and platform design, laboratories may need to perform both intra (within run) and inter (between days) precision studies. Each replicate specimen should be tested individually throughout the entire test procedure (e.g., inactivation, extraction, and amplification).

Specimen selection: At least one positive and one negative specimen should be included for qualitative test validation. Select specimens with sufficient volume for all precision replicates. To obtain specimens with sufficient volume, laboratories may employ one of the following approaches:

- a. Select high volume residual patient specimens.
- b. Selected a single high titer patient specimen and dilute in negative patient matrix (positive precision only).
- c. Pool multiple patient specimens with the same qualitative result.
- d. Dilute commercially available virus in patient matrix.
- e. Use quality control materials.

Consideration should be given to the concentration tested. Testing low positivity specimens is recommended for qualitative assays (e.g., target concentration to be within 10-fold of the assay LOD). However, testing positive specimens that are too close, or below, the test's LOD for precision studies, may inaccurately reflect the test's true precision and may fail to meet acceptability criteria.

Number and timing of replicates: For qualitative molecular LDTs, CLSI MM19A recommends testing a positive and a negative specimen 20 times over 20 different days. This may be challenging for many labs, especially if validating modifications to commercially manufactured test devices which can be costly. At the discretion of the medical director, the number of replicates and days may be modified or reduced. If reduced precision studies are needed, it is recommended to perform 3 replicates per day (intra-precision) of a positive and a negative sample over 3-4 days (inter-precision).

Data analysis: Results for the positive and negative specimens should be consistent across runs and days, with at least 95% agreement.

### 4. Reportable Range

Verification of reportable range is not applicable for the available, qualitative PCR tests for MPXV, HSV, and VZV.



## 5. Specificity/Cross-reactivity

Interfering substances data can be taken from the manufacturer's IFU or previous studies performed for LDT initial validation. Since an inactivation procedure may introduce possible interfering substances, if interference is observed with the test, this will likely be revealed in LOD and accuracy studies and does not warrant additional studies unless potential cross-reactivity or interference is observed during accuracy studies. If possible cross-reactivity is encountered, further evaluation with an *in silico* analysis for exclusivity of primers/probes may be performed, in addition to wet testing.

## 6. Carry-over Testing

Any change to the specimen composition or viscosity which may occur due to inactivation methods could impact instrument performance including potential for increased carry-over. Throughout validation, positive and negative specimens should be interspersed on the testing runs to assess potential carryover on instrumentation where carryover contamination is a risk based on instrument layout, design, or test set-up. If false positive results are noted, additional carry-over studies are recommended.

## 7. Interference Testing

If additional reagents are used for the viral inactivation protocol, assay interference is possible. Similarly, heating steps may change the composition of the specimen, potentially liberating compounds within the specimen in formats that were not previously present. Interference may cause false positive results due to cross-reactivity (see section 6 above) or false negative results due to assay inhibition. Assay inhibition should be assessed throughout the Accuracy and Limit of Detection studies, described above. Inhibition may be identified by analyzing internal control measurements, when included in the assay, and positive specimen detection. If false negative results are noted, additional interference studies are recommended.

## VI. Quality Control Testing

Daily quality control (QC), using a positive and negative control, is recommended when validation testing is performed. If QC fails, the results from that day's testing are invalid and additional testing should pause for investigation and resolution. Testing can resume and results used when QC passes. After validation, external positive and negative QC must be run every day of patient testing or no less than the manufacturer's instructions (8). In general, external QC should be run at least each day of patient testing. More extensively, external QC may be performed every shift, every 24 hours or every batch, as appropriate. Acceptability of QC criteria should be established for quality assurance. Commercial QC products provided by the test manufacturer are preferred over residual patient specimens or third-party vendors, unless recommended by the manufacturer as an alternative QC option for the test.

## VII. Biosafety Requirements

Prior to onboarding MPXV molecular testing, the laboratory should perform a site-specific biosafety risk assessment to identify and mitigate risk. See [ASM's Monkeypox Virus Biosafety and Testing Recommendations White Paper](#) for a summary of biosafety and risk assessment considerations and approaches to viral inactivation.

## VIII. Limitations

The recommendations detailed in this document provide one approach to validating test performance after making modifications (i.e., inactivation steps) to the original test protocol. However, this validation does not ensure effective inactivation of MPXV or other infectious agents in the specimens.

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