Time to Unbox a New Assay for Mpox

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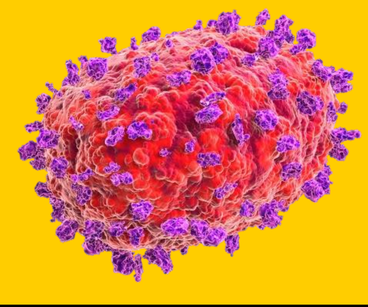


BACKGROUND

- Mpox is a double-stranded DNA virus belonging to the *Orthopoxvirus* genus. There are two pathogenic clades of the virus, denoted as clade I and II.
- In early May 2022, clade II Mpox was reported outside of Central/Western Africa for the first time since 2003, causing a global outbreak in which the United States led in cases as well as deaths.[1,2,3]
- CDC recommended the Non-variola *Orthopoxvirus* Generic Real-Time PCR Test.
  - Limitations: low-volume, no clade specificity, and laborious.
  - Solution: optimize and validate a clade II-specific PCR laboratory-developed assay (LDA) on the Hologic Panther Fusion® using the Open Access™ System.[4]
- Clade II Mpox continues to circulate in the United States but at low levels.[5]
- Clade I Mpox has recently caused outbreaks throughout Central/Eastern Africa beginning in early 2024, and there have been several travel-associated cases from these outbreaks reported in various countries. The first travel-associated case in the United States was detected in November 2024.[6]
  - Previous Fusion® Assay Limitations: only clade II-specific.
  - Current Challenges: clade I Mpox is a select agent, and *confirmed* specimens cannot be handled outside of a BSL-3 registered space. Entities possessing, using, or transferring such agents must comply with the HHS Select Agent and Toxin Regulations.[7] Inactivation of all specimens in a BSL-3 registered space prior to running on the Panther Fusion® poses a significant barrier to high-volume testing in the event of an outbreak.
  - Solution: optimize and validate an updated PCR LDA on the Hologic Panther Fusion® targeting both clade II Mpox as well as Non-variola *Orthopoxvirus* (NVO). The NVO target serves as a "catch-all" for Mpox clade I, clade II, or novel clade, but is not considered confirmatory for clade I.

METHODS

Primer-Probe Mix (PPM) Optimization

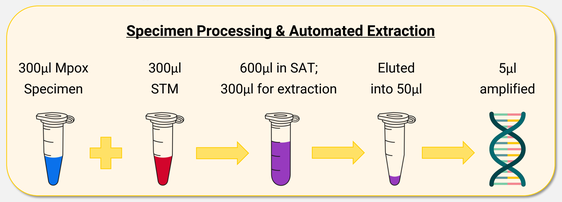
- Various concentrations of reagents (clade II Mpox primers/probe, NVO primers/probe, Hologic's® IC-X primers/probe, KCl, $MgCl_2$) were assessed using a gBlock from IDT designed for both clade II Mpox and NVO target sequences. PPM also contained Tris Buffer, nuclease-free water, and an oil overlay according to Hologic's® recommendations.

Analyte Name	Sequence	Gene Target
Clade II Mpox F Primer	CAC ACC GTC TCT TCC ACA GAT A	Clade II Mpox TNF Receptor Gene
Clade II Mpox R Primer	GAT ACA GGT TAA TTT CCA CAT CGA	
Clade II Mpox Probe	/56-FAM/AAC CCG TCG TAA CCA GCA ATA CAT TT/3BHQ_1/	
NVO F Primer	TCA ACT GAA AAG GCC ATC TAT GA	DNA Polymerase Gene
NVO R Primer	GAG TAT AGA GCA CTA TTT CTA AAT CCC A	
NVO Probe	/56-ROXN/CCA TGC AAT ATA CGT ACA AGA TAG TAG CCA AC/3IAbRQSp/	
Hologic® IC-X Primers	N/A	N/A
Hologic® IC-X Probe	N/A	

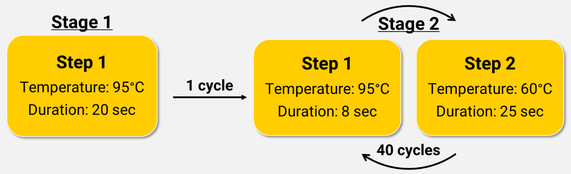
Table 1. Primer and probe sequences.[8,9]

Panther Fusion® – Extraction, PCR, & Open Access™

- Extraction Reagent-X: Low – Viral/Bacterial



- PCR Cycling Parameters – Thermocycler Runtime: 45 m 28 s



- Open Access™ Parameters
  - Clade II Mpox Analyte: FAM Channel – 1000 CT Threshold
  - NVO Analyte: ROX Channel – 1000 CT Threshold
  - IC-X Analyte: Quasar 705 Channel – 1000 CT Threshold

RESULTS

Final Multiplexed PPM

- The PPM was optimized to achieve the most robust, reliable, and accurate assay.

Component	Stock Concentration	Units	Final Concentration	Concentration for 1.25X PPM	Volume (µl) for PPM
Water					818.75
KCl	1000	mM	60	75	75
$MgCl_2$	1000	mM	3	3.75	3.75
Tris Buffer	1000	mM	10	12.5	12.5
CLII Mpox F Primer	100	µM	0.6	0.75	7.5
CLII Mpox R Primer	100	µM	0.6	0.75	7.5
CLII Mpox Probe	100	µM	0.4	0.5	5
NVO F Primer	100	µM	0.6	0.75	7.5
NVO R Primer	100	µM	0.6	0.75	7.5
NVO Probe	100	µM	0.4	0.5	5
IC-X Primers	37.5	µM	0.75	0.9375	25
IC-X Probe	25	µM	0.5	0.625	25
Total					1000

Table 2. Final optimized PPM for 30 tests of the clade II Mpox/NVO/IC-X multiplexed LDA on the Panther Fusion®. PPM also includes a 400 µl oil overlay. Calculation format provided for use by Hologic® Open Access™ training.

Validation

- Inactivated clade I Mpox, clade II Mpox, and Vaccinia virus material used in this validation was provided by the CDC Poxvirus Branch. HSV1, HSV2, and VZV material used was acquired from in-house testing.
- **Limit of Detection (LOD) & Sensitivity**
  - Plan: Accept LOD if 19/20 (95%) of replicates are positive.
  - Result: LOD = 13 copies/µl
  - PCR Efficiency: Clade II Mpox – 99.26% / NVO – 83.05%
- **Accuracy**
  - Plan: ≥90% specimens must agree w/ Poxvirus Branch.
  - Result: 100% accuracy between instruments and days.
- **Precision**
  - Plan: Positive specimens must be within +/- 3 CTs.
  - Result: 100% precision between instruments and days.
- **Specificity**
  - Plan: HSV1, HSV2, and VZV must be negative.
  - Result: 100% specificity between instruments and days.

CONCLUSION

- There are several benefits of this updated clade II Mpox/NVO multiplexed PCR LDA compared to previous testing methods:
  - Automated extraction and PCR saves labor, costs, and time and increases both capacity and reproducibility of results.
  - Capability for bi-directional interfacing with LIMS.
  - Detection of clade II Mpox as well as NVO, serving as a "catch-all" for all Mpox.
  - No initial processing and inactivation required in a BSL-3 registered space, removing the largest barrier for high-volume testing in the event of an outbreak.
  - Mpox testing in Iowa no longer requires epidemiologists' prior approval, improving testing accessibility and outbreak response.
- The methods outlined here can be applied to the development of numerous PCR LDAs on the Panther Fusion® for other pathogens of public health significance.

Limitations

- Specimens resulting positive for NVO but negative for clade II Mpox, i.e., presumptive clade I Mpox or novel clade, will need to be sent to CDC for confirmatory testing.

Next Steps

- The State Hygienic Laboratory at the University of Iowa plans to develop additional PCR LDAs on the Panther Fusion®, which will improve workflow efficiencies during future public health emergencies in which rapid, high-volume testing may be necessary.

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