Multiplex real-time PCR detection of monkeypox virus using Luna® qPCR Reagents

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INTRODUCTION

Monkeypox virus (MPXV) is a double-stranded DNA poxvirus that causes mpox (formerly known as monkeypox), which historically has been a rare disease that results in similar symptoms to smallpox (1). Prior to the 2022 outbreak, mpox was mainly found in several countries in Central and West Africa. There are two clades of mpox virus, Clade I and Clade II, and infections from the current outbreak stem from Clade II (2). As of October 3, 2022, monkeypox virus has spread to 106 countries with 68,874 cases, resulting in outbreaks in 100 non-endemic countries (3). The WHO Director-General declared the ongoing mpox outbreak a Public Health Emergency of International Concern on July 23, 2022. U.S. CDC testing guidance recommends hydrolysis probe-based (e.g., TaqMan®) qPCR for detection of viral targets in DNA purified from patient samples, owing to the high accuracy and sensitivity of qPCR (4,5).

To facilitate molecular diagnostic development efforts, here we demonstrate the detection of synthetic monkeypox viral DNA using Luna qPCR reagents. We show high specificity and sensitivity in the detection of monkeypox DNA using the Luna Universal Probe qPCR Master Mix (NEB #M3004). The limit of detection (LOD) on synthetic viral DNA is five copies per reaction. The exceptional performance in multiplex amplification allows users to detect control human DNA in the same reaction. Similar results were achieved with the Luna Probe One-Step RT-qPCR 4X Mix with UDG (NEB #M3019), which was evaluated due to the concurrent SARS-CoV-2 pandemic and allows users to rely on a single reagent for detection of both viruses, if desired.

RESULTS

Detecting Synthetic MPXV Using the Luna Universal Probe qPCR Master Mix

Both the non-variola orthopoxvirus generic and monkeypox virus generic single-plex test procedures outlined by the CDC were evaluated using the Luna Universal Probe qPCR Master Mix (NEB #M3004) on three different real-time PCR instruments: Bio-Rad® CFX Opus, Applied Biosystems® (ABI) 7500 and Thermo Fisher Scientific® QuantStudio® 6. Each CDC test procedure requires a minimum of two individual reactions per patient sample: one for non-variola orthopoxvirus or monkeypox virus and a second reaction for human DNA as a control (e.g., RNase P). The non-variola orthopoxvirus single-plex reaction (CDC-OP) includes a pair of primers and a FAM labeled probe that targets a region of the monkeypox virus F8L gene (4). The CDC-OP primer/probe set also detects other orthopoxviruses (e.g., cowpox) except for smallpox, which is caused by variola virus. Although not specific for monkeypox virus, positive detection using the CDC-OP assay and clinical presentation is sufficient for treatment. The monkeypox virus generic test (MP-Generic) targets the tumor necrosis factor (TNF) receptor gene (J2L) (5,6). However, three recent cases in California have shown a significant deletion in this gene that may lead to false negative results, suggesting caution must be used when using this assay. In our evaluation of these two target designs, the quenchers of both probes were modified according to recommendations provided by the oligo vendor. Additionally, the forward primer for the MP-Generic test was truncated by a single base. Data was collected using our standard concentration recommendations for primer (0.4 µM) and probe (0.2 µM) with slightly modified cycling condition for NEB #M3004 (Table 1). For ABI instruments, the passive reference dye was set to ROX to enable data normalization.

The Luna Universal Probe qPCR Master Mix detected the F8L or J2L gene target from 53,000 copies down to 5.3 copies of synthetic viral DNA template (Synthetic Monkeypox Virus DNA from ATCC #VR-3270SD) on all three instruments with exceptional sensitivity, reproducibility, and qPCR performance using either the non-variola Orthopoxvirus primers/probes or the monkeypox virus generic prim-

MATERIALS

Reagents

- Luna® Universal Probe qPCR Master Mix (NEB #M3004)
  or
  Luna Probe One-Step RT-qPCR 4X Mix with UDG (NEB #M3019)

Primers and probes

- See Table 1

Template

- ATCC® Quantitative Synthetic Monkeypox virus DNA (#VR-3270SD)

TABLE 1: **Primers and Probes**

Cycling conditions (NEB recommendation): Single-plex or 2-plex: 95°C for 1 min., 45 cycles of 95°C for 10 sec., 60°C for 30 sec.
PCR instruments: Bio-Rad CFX Opus (Default mode), Applied Biosystems (ABI) 7500, Thermo Fisher Scientific QuantStudio 6

ASSAY	PRIMER/PROBE		REF.	NOTE	CONC. (µM)
	NAME	SEQUENCE			
CDC-Non-variola Orthopoxvirus	CDC-OP-F	5´-TCAACTGAAAAGGCCATCTATGA-3´	CDC	Probe quencher modified	Primer: 0.4 Probe: 0.2
	CDC-OP-R	5´-GAGTATAGAGCACTATTTCTAAATCCCA-3´			
	CDC-OP-FAM	5´-FAM-CCATGCAAT/ZEN/ATACGTACAAGATAGTAGC-CAAC-3´IABkFQ			
RP-DNA	RP-DNA-F	5´-AGATTTGGACCTGCGAGCG-3´	CDC	Probe fluorescent dye and quencher modified	Primer: 0.4 Probe: 0.2
	RP-DNA-R	5´-GAGCGGCTGTCTCCACAAGT-3´			
	RP-DNA-Cy5	5´-Cy5-TTCTGACCT/TAO/GAAGGCTCTGCGCG-3´ IABRQ			
MP-Generic	MP-G-F	5´-GGAAAATGTAAAGACAACGAATACA-3´	CDC	F primer and probe quencher modified	Primer: 0.4 Probe: 0.2
	MP-G-R	5´-GCTATCACATAATCTGGAAGCGTA-3´			
	MP-G-FAM	5´-FAM-AAGCCGTAA/ZEN/TCTATGTTGTCTATCGT-GTCC-3´IABkFQ			

ers/probes (Figure 1). Similar performance was observed using the Luna Probe One-Step RT-qPCR 4X Mix with UDG (NEB #M3019, data not shown). Although this Luna one-step mix is typically intended for RNA detection, the reagent allows higher sample input volumes given its 4X concentration and includes thermolabile UDG for carryover prevention. Given the ongoing SARS-CoV-2 pandemic, this single mix can be used to detect either virus. Furthermore, the CDC's recommendations for primer/probe concentrations and cycling conditions can also be used with either Luna reagent with no observable impact to MPXV detection.

Sensitive Detection of Synthetic MPXV using the Luna Universal Probe qPCR Master Mix in 2-plex assay

Multiplex assays offer a more efficient testing option in diagnostic settings, allowing a single sample to be interrogated for various targets simultaneously. Each MPXV CDC test design described above utilizes detection of RNase P in a second independent reaction as a control to confirm the presence of human DNA. We investigated the use of a 2-plex assay that allows for detection of human RNase P and the monkeypox virus F8L gene (CDC-OP) in a single reaction.

The FAM fluorophore of the human RNase P probe was changed to Cy5™ to accommodate the multiplex design. This allows detection of the internal control in the Cy5 channel while non-variola Orthopoxvirus detection remains in the FAM channel.

The Luna Universal Probe qPCR Master Mix detected the monkeypox F8L target from 53,000 copies down to 5.3 copies of synthetic viral DNA template (ATCC #VR-3270SD) in the presence of 5 ng Jurkat DNA on all 3 instruments (Figure 2), consistent with the performance in the single-plex assay. To determine the LOD (95%

FIGURE 1: **The Luna Universal Probe qPCR Master Mix offers exceptional sensitivity, reproducibility and qPCR performance**

Detection of the monkeypox F8L gene using the CDC-OP primers/probe (A) and the J2L gene using the CDC MP-G primers/probe (B) was performed using the Luna Universal Probe qPCR Master Mix over a 5-log range of input template (53,000 cp – 5.3 cp synthetic monkeypox virus DNA, ATCC #VR-3270SD) with 2 replicates at each dilution. Instruments: CFX Opus (Bio-Rad®), ABI 7500 and QuantStudio 6 (Thermo Fisher Scientific®).

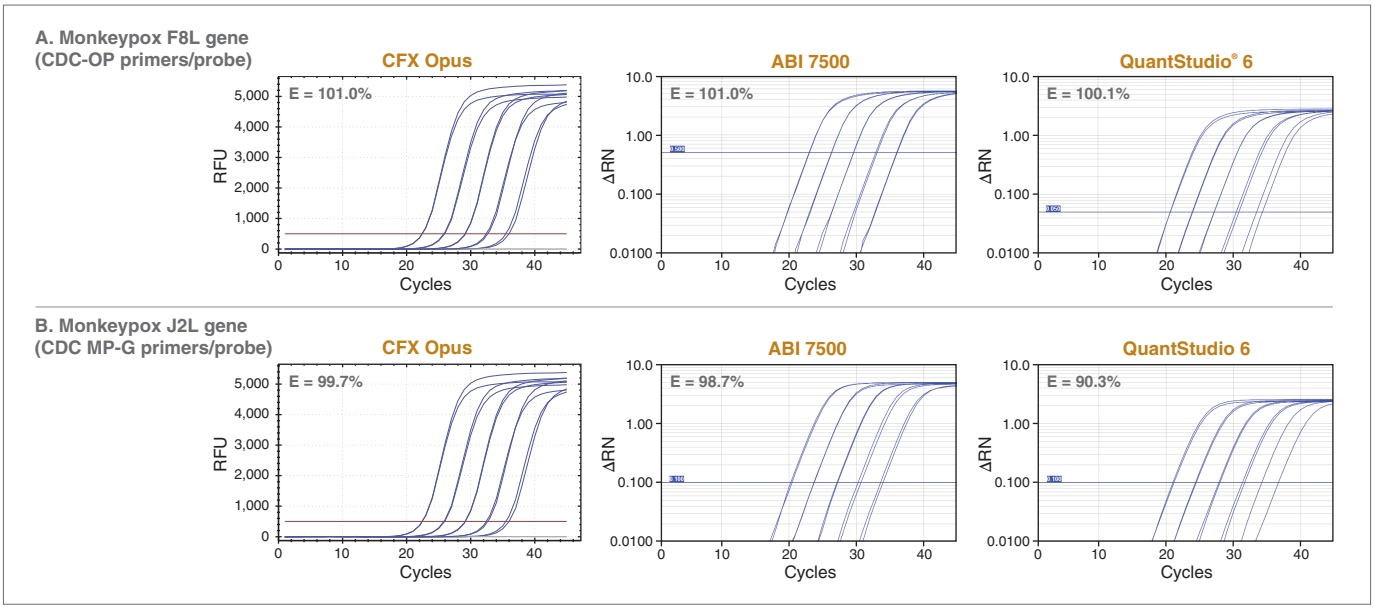
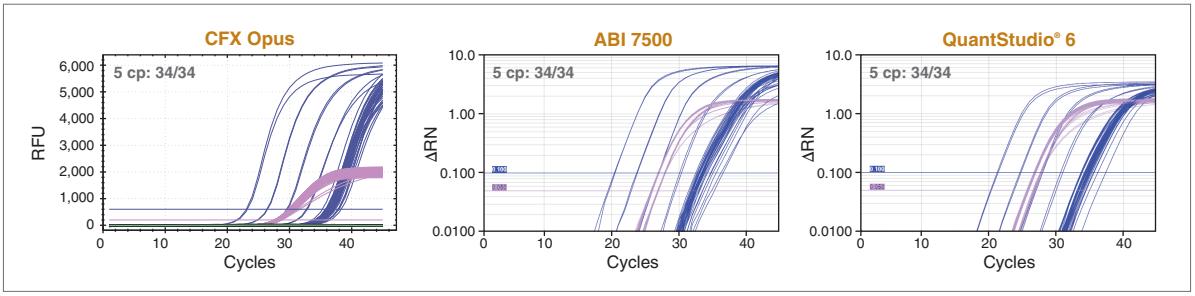


FIGURE 2: **The Luna Universal Probe qPCR Master Mix offers robust performance in 2-plex detection of synthetic monkeypox and human RNase P control DNA**

2-plex qPCR assays targeting the monkeypox F8L gene (CDC-OP, blue) and human RNase P DNA (RP-DNA, light purple) were performed using the Luna Universal Probe qPCR Master Mix over a 5-log range of input template (53,000 cp – 5.3 cp synthetic monkeypox virus DNA, ATCC #VR-3270SD, diluted in 5 ng/µl Jurkat genomic DNA) with 2 replicates at each dilution. To verify the limit of detection (LOD) of the Luna qPCR reagent, an additional 34 replicates of 5 cp input was tested in the 2-plex assay. Across all three instruments, 34 out of 34 replicates were detected. Instruments: CFX Opus (Bio-Rad), ABI 7500 and QuantStudio 6 (Thermo Fisher Scientific).



confidence) of the monkeypox 2-plex assay, we evaluated the Luna reagent on all three real-time PCR instruments. The LOD for each instrument was established by testing 34 replicates of 5-copy synthetic viral DNA input and 2 non-template controls. The Luna Universal Probe qPCR Master Mix detected all 34 replicates on all three qPCR instruments, while the non-template control reactions lacked amplification. RNase P was also 100% detected simultaneously on Cy5 channel. Similar results were also observed using the Luna Probe One-Step RT-qPCR 4X Mix with UDG.

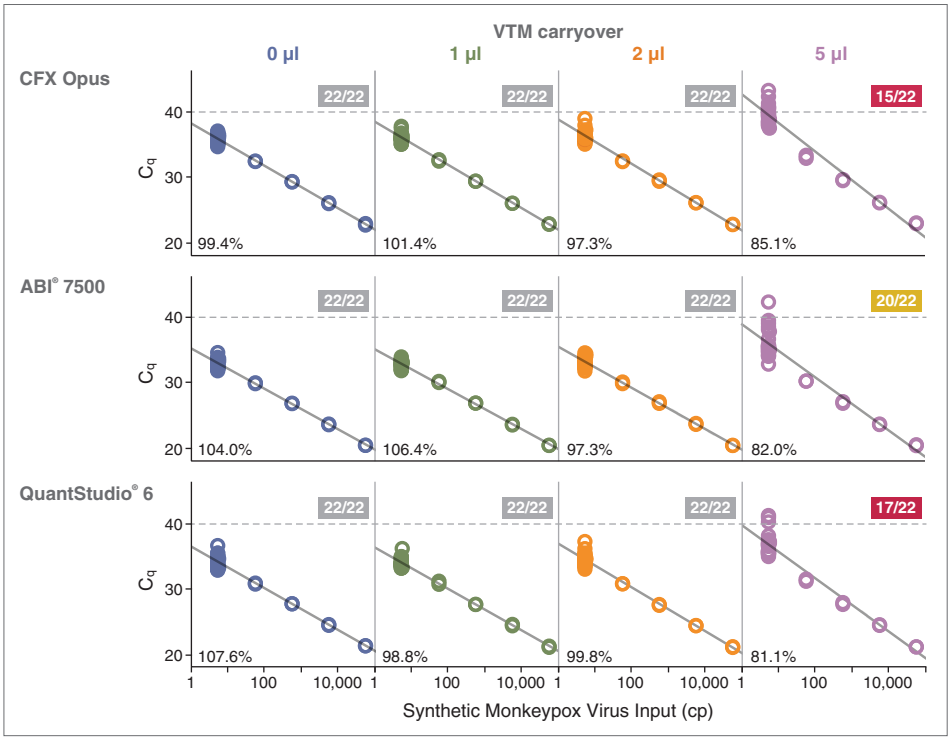
Reagent Tolerance to Viral Transport Medium (VTM)

Currently, both the WHO and the CDC recommend specimen collection from skin lesion swabs and DNA extraction for diagnostic testing. However, extraction-free workflows may be possible. Given that swabs can be stored dry or in Viral Transport Medium (VTM), VTM tolerance is critical for development of direct detection workflows of monkeypox virus DNA in patient samples. We therefore tested the effects of VTM on the Luna Universal Probe qPCR Master Mix.

The Luna reagent was highly tolerant of VTM, with no detectible effect on quantitation and LOD detection with 2 µl VTM presence per 20 µl reaction (10% v/v) (Figure 3). Detection of low input (5 copies/reaction) was impacted by 5 µl VTM per 20 µl reaction (25% v/v), but negligible effects were observed for high inputs.

FIGURE 3: **Luna Universal Probe qPCR Master Mix tolerates up to 10% Viral Transport Medium**

2-plex qPCR assays targeting the monkeypox F8L gene (CDC-OP) and human RNase P (RP-DNA) were performed using the Luna Universal Probe qPCR Master Mix over a 5-log range of input template amount (53,000 cp – 5.3 cp synthetic monkeypox virus DNA, ATCC #VR-3270SD in 10 ng of Jurkat RNA) with 2 replicates at each dilution. To test any impact to LOD, an additional 22 replicates of 5 cp input was tested in the 2-plex assay. The assays were performed in the absence or presence of a gradient of Viral Transport Medium (VTM) up to 5 µl in a 20 µl reaction. Instruments: CFX Opus (Bio-Rad), ABI 7500 and QuantStudio 6 (Thermo Fisher Scientific).



CONCLUSION

Molecular diagnostics continue to play a critical role in the detection and diagnosis of infectious diseases. The recent rise in mpox cases around the globe has sparked concern and many labs are turning to nucleic acid amplification tests to help prevent the spread of the disease. The details outlined herein showcase how the Luna reagents can be used in previously developed single-plex real-time PCR assays by the CDC for detection of monkeypox virus while achieving an LOD of 5 copies of synthetic DNA per reaction. Modification of the fluorophores and use of the Luna reagents allows these real-time PCR assays to be converted into multiplex tests. We hope the high specificity and sensitivity data and recommendations presented will help labs develop mpox assays that meet their specific needs.

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