Application Note

A test panel for rapid Monkeypox virus detection using multiplex probe-based qPCR

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Introduction

An outbreak of the viral monkeypox disease was confirmed in May 2022 [1], i.e., at the end of the coronavirus world pandemic. The first case was detected in London on 6 May 2022 [2], in a patient returning from Nigeria. Immediately after, cases were reported in several other countries around the world, with a high incidence in Europe and North America, marking the first spread of monkeypox outside Central and West Africa, the regions where it was originally endemic [1]. On 23 July, the World Health Organization (WHO) declared the outbreak a public health emergency of international concern [3]. As of 2 March, 2023, there have been a total of 86,173 confirmed cases (of which 100 resulted in the patient's death) in over 110 countries [4, 5].

Monkeypox is an infectious disease in humans and other animals, which manifests with fever, swollen lymph nodes, and a rash that forms blisters and crusts over. It may take up to 21 days to show symptoms from initial exposure, and they may last for two to four weeks. There may be mild or no classic symptoms at all, making the disease difficult to diagnose and, therefore, to contain [6, 7].

The disease is caused by the monkeypox virus (MPXV), a zoonotic DNA virus belonging to the genus Orthopoxvirus, the same genus of variola virus (known to cause smallpox). There are 2 types of MPXV affecting humans, of which clade II (formerly known as the West African clade) causes a less severe disease than the Central African / Congo basin (clade I) type [8]. MPXV spreads from infected animals by contact (e.g., with infected meat and via bites or scratches). Human-to-human transmission happens by exposure to infected body fluids or contaminated objects, by droplets, and possibly via the airborne route. People can spread the virus from the onset of symptoms until all the lesions have fallen off (or even later) [8, 9].

Molecular diagnosis could therefore be crucial to limit the spread of the disease and to confirm the diagnosis. Being a pathology known to the scientific community since the 1970s, several papers have already provided validated primers for molecular diagnosis [10]. This application note describes a reliable and rapid (40-70 min) multiplex assay for the detection of monkeypox and the identification of the viral type. We envision the use of such panels to be commonplace even after the current outbreak and have therefore designed this assay to enable streamlined diagnostic testing of this pathogen.

Method

At PCR Biosystems we have established a protocol for simultaneous detection of MPXV (Generic MPXV) and the identification of both clade I (Congo basin type of MPXV, MPXV_CB) and clade II (West African type, MPXV_WA) in a multiplex assay, based on the oligonucleotide sequences already available in literature, i.e., using primer and probe sequences identified and verified for their diagnostic validity in a peer reviewed journal report [10], listed in Table 1. We also used our newly developed Clara™ Probe Mix in this protocol. Test templates were either a purified complete microbial genome (Vircell Amplirun®

Name	Dye-Sequence	Concentration	Target
Generic-F	GGAAAATGTAAAGACAACGAATACAG	400 nM	
Generic-R	GCTATCACATAATCTGGAAGCGTA	400 nM	
Generic-P	**FAM**-AAGCCGTAATCTATGTTGTCTATCGTGTCC-**BHQ1**	200 nM	
MPXV_WA-F	CACACCGTCTCTTCCACAGA	400 nM	
MPXV_WA-R	GATACAGGTTAATTTCCACATCG	400 nM	*TNF receptor gene*[10]
MPXV_WA-P	**Cy5**-AACCCGTCGTAACCAGCAATACATTT-**BHQ2**	200 nM	
MPXV_CB-F	TGTCTACCTGGATACAGAAAGCAA	400 nM	
MPXV_CB-R	GGCATCTCCGTTTAATACATTGAT	400 nM	
MPXV_CB-P	**HEX**-CCCATATATGCTAAATGTACCGGTACCGGA-**BHQ1**	200 nM	

Table 1: Primers, probes and targets used

MBC146-R, West African clade, Spanish clinical isolate extract) or a synthetic DNA viral sequence (containing the TFN Receptor sequences used for the amplification). After assaying different conditions, we present the optimised multiplex qPCR protocol for detection of this pathogen.

Primer and template preparation

For the multiplex reaction, a primer set including the oligonucleotides for the 3 targets was prepared by mixing 400 µL 10 µM primers (F + R) to 200 µL 10 µM probes (which is equivalent to adding 0.8 µL 10 µM primers F + R and 0.4 µL of 10 µM probe to each 20 µL reaction mix).

Clinical extract template was prepared in a 4-point, 10-fold dilution series ranging from 7,000 copies to 7 copies per reaction. Synthetic DNA template was prepared in an 8-point, 10-fold dilution series ranging from 5 x 10^7 to 5 copies per reaction. No template control samples (NTCs) were included for each target. Four technical replicates were run for each sample.

Reaction setup

Reactions were set up using a Qiagen QIAgility robot in a final volume of 20 µL to include: 8.8 µL Milli-Q water, 5 µL 4x Clara™ Probe Mix, 1.2 µL x primers/probes mix (described above) and 5 µL of DNA sample. Table 2 summarises the final concentration of the components added.

Cycling conditions

Thermocycling was carried out on a Bio-Rad CFX96 Touch™ qPCR machine (for regular cycling) or on a BMS MIC qPCR machine (for fast cycling), with the cycling parameters outlined in Table 3. Fluorescence measurements were acquired at the end of each cycle.

Reagent	Volume	Final conc.
4x Clara™ Probe Mix	5 µL	1x
Primer mix (10 µM)	0.8 µL	400 nM
Probe (10 µM)	0.4 µL	200 nM
DNA template	5 µL	Variable
PCR grade dH_2O	8.8 µL	–

Table 2: Reaction setup and composition

Cycles	Temperature	Time (regular)	Time (fast)	Notes
1	95 °C	3 min	30 sec	Polymerase activation and RTase inactivation
50	95 °C 60 °C	15 sec 30 sec	1 sec 5 sec	Denaturation Annealing/Extension

Table 3: Cycling conditions

Results

In the clinical extract, as expected, only the generic and the West African targets were successfully detected before 40 cycles (Figure 1), even at the lowest concentration (7 copies per 20 µL reaction) with no product detected in the NTCs. The clade-specific oligonucleotides for the Congo basin MPXV type did not result in any amplification as desired. Efficiency for the two targets was 94-96%, with an R^2 value above 99% in all cases. The mean ± SD target Ct values for the 2 targets were 26.99 ± 0.03 and 37.21 ± 0.26 (Generic MPXV) and 28.05 ± 0.05 and 38.02 ± 0.44 (MPXV_WA), at their highest (7,000 copies per reaction) and lowest concentration (7 copies per reaction), respectively.

On the other hand, with synthetic DNA target, all 3 targets were successfully detected before 40 cycles (Figure 2), even at the lowest concentration (5 copies per 20 µL reaction), with no product detected in the NTCs. Efficiency for the three targets was 94-95%, with an R^2 value above 99% in all cases. The mean ± SD target Ct values for all targets were 5.40 ±

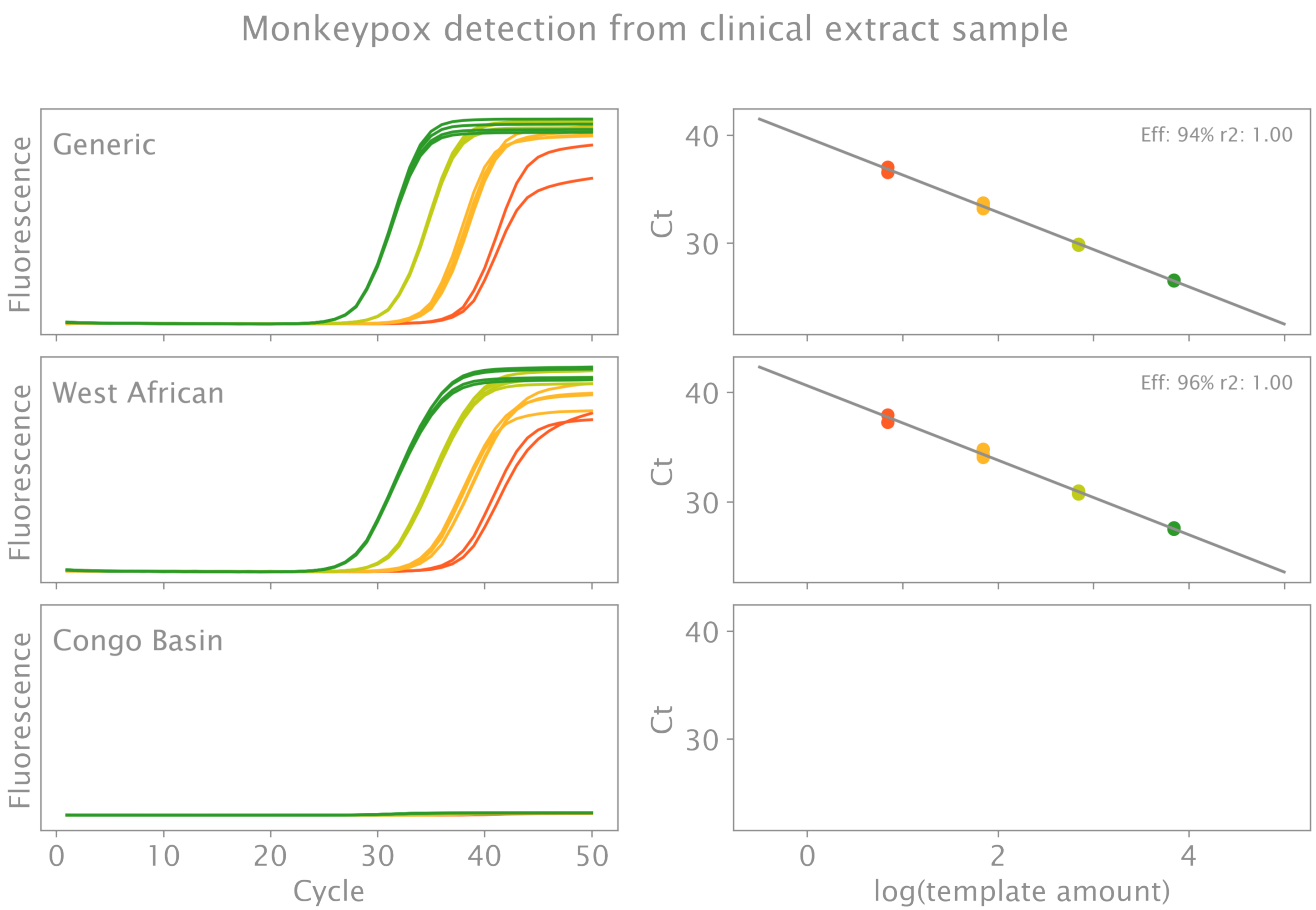

Figure 1: Triplex detection of MPXV targets in a clinical extract sample. See main text for experimental details.

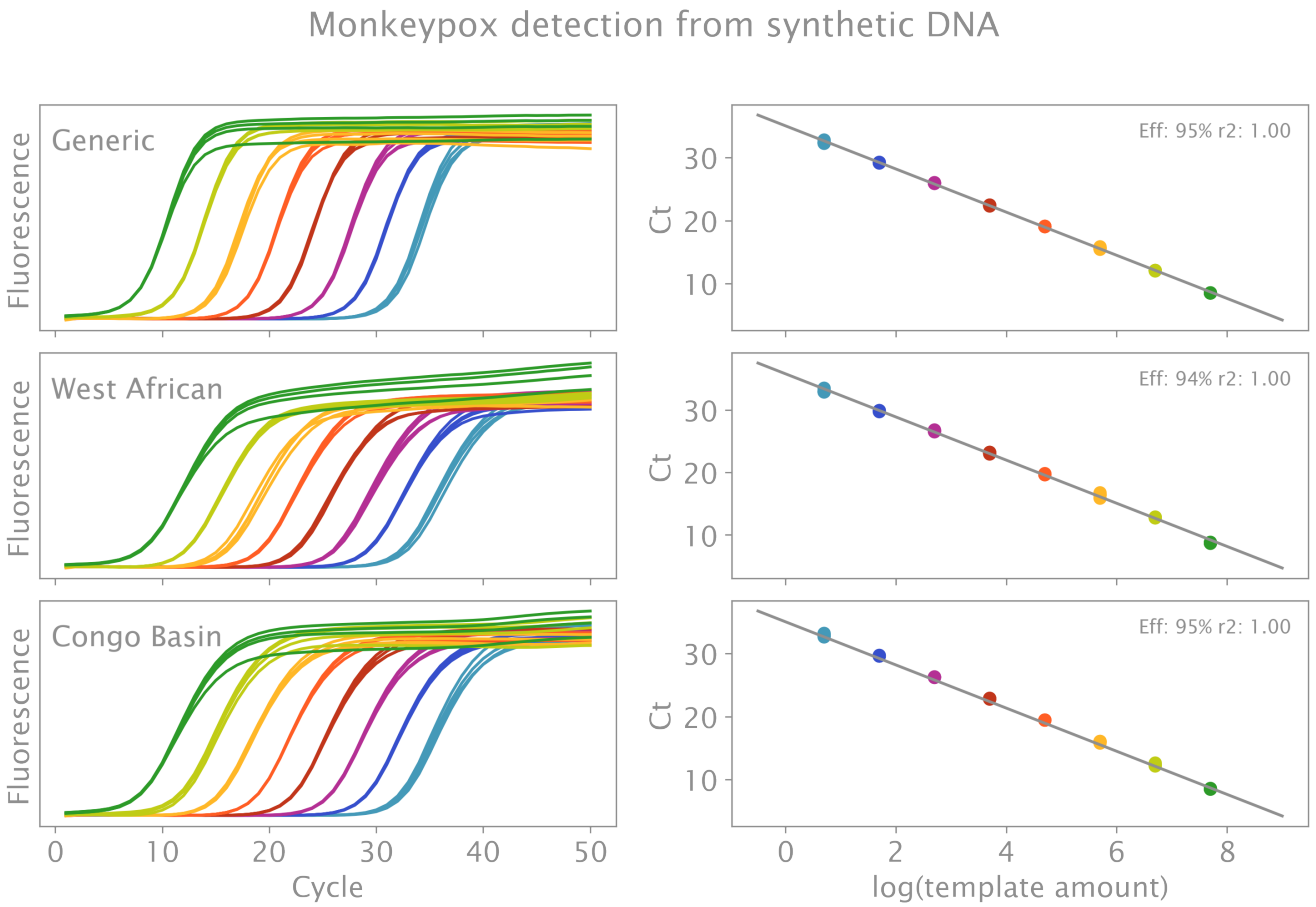

Figure 2: Triplex detection of MPXV targets from synthetic cDNA. See main text for experimental details.

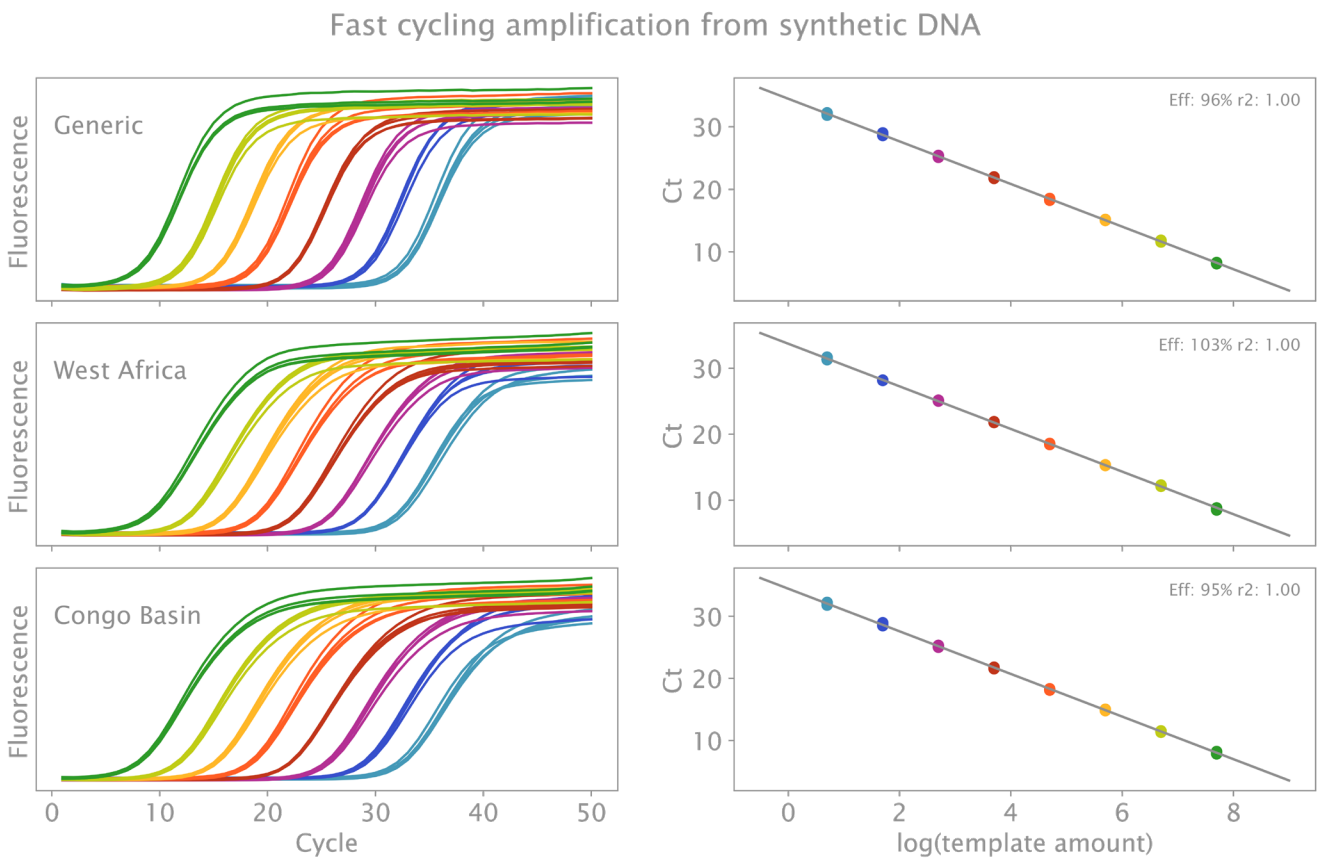

Figure 3: Triplex detection of MPXV targets from synthetic cDNA using fast cycling conditions. See main text for experimental details.

0.03 and 29.97 ± 0.21 (Generic MPXV), 7.19 ± 0.07 and 31.81 ± 0.24 (MPXV_CB), and 9.51 ± 0.06 and 33.78 ± 0.30 (MPXV_WA), at their highest (50,000,000 copies per reaction) and lowest concentration (5 copies per reaction), respectively.

In regular cycling conditions, amplification of MPXV could be achieved in about 70 minutes. We therefore repeated the experiment shown in figure 2 with a fast-cycling program, allowing the entire detection in about half of the time. Also under these conditions, all 3 targets were successfully detected before 40 cycles (Figure 3), even at the lowest concentration (5 copies per 20 µL reaction), with no product detected in the NTCs. Efficiency for the three targets was between 95-105%, with an R2 value above 99% in all cases. The mean ± SD target Ct values for all targets were 8.21 ± 0.09 and 32.07 ± 0.16 (Generic MPXV), 8.07 ± 0.17 and 32.07 ± 0.19 (MPXV_CB), and 8.68 ± 0.14 and 31.40 ± 0.19 (MPXV_WA), at their highest (50,000,000 copies per reaction) and lowest concentration (5 copies per reaction), respectively.

Discussion

We report here a fast and reliable detection protocol for the monkeypox disease. Our approach has proven to be successful in detecting very low amounts of viral targets in a multiplex qPCR reaction (less than 10 copies per 20 µL reaction). Using the BMS MIC qPCR instrument, detection can be achieved in under 40 minutes. This offers a rapid means of detecting MPXV and of identifying the clade in a single test tube, and therefore constitutes a valid tool to limit the spreading of this pathogen. These results also highlight the power and efficiency of multiplex qPCR reactions. In this context, it is crucial to state that additional targets can be included in this panel, to confirm or exclude pathologies with a similar aetiology .

At the time of writing, due to the limitation of available vaccines, the spread of monkeypox is still ongoing. Therefore, a fast-screening program could help in identifying and isolating cases even before symptoms have developed.

Our Clara™ Probe Mix allows the rapid setup of new multiplex assays and is designed to be reliable under a range of template amounts, sample types, and target nucleotide compositions, eliminating the need for laborious multiplex assay optimisation.

Product use

PCR Biosystems products, including Clara™ Probe Mix, alone do not provide diagnostic results and are supplied for research use only. However, all products are

manufactured under an ISO 13485-compliant management system and are suitable for use as components in molecular diagnostic assays, where applicable country laws allow and after clinical validation of an assay itself.

If you would like to discuss which products are best suited to your application, or need further technical advice on how to use Clara™ Probe Mix for MPXV or other DNA pathogen testing, contact our team of experts at technical@pcrbio.com.

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