Rapid detection of monkeypox virus using a CRISPR-Cas12a mediated assay: a laboratory validation and evaluation study

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Summary

Background The 2022 outbreak of mpox (formerly known as monkeypox) led to the spread of monkeypox virus (MPXV) in over 110 countries, demanding effective disease management and surveillance. As current diagnostics rely largely on centralised laboratory testing, our objective was to develop a simple rapid point-of-care assay to detect MPXV in clinical samples using isothermal amplification coupled with CRISPR and CRISPR-associated protein (Cas) technology.

Methods In this proof-of-concept study, we developed a portable isothermal amplification CRISPR-Cas12a-based assay for the detection of MPXV. We designed a panel of 22 primer–guide RNA sets using pangenome and gene-agnostic approaches, and subsequently shortlisted the three sets producing the strongest signals for evaluation of analytical sensitivity and specificity using a fluorescence-based readout. The set displaying 100% specificity and the lowest limit of detection (LOD) was selected for further assay validation using both a fluorescence-based and lateral-flow readout. Assay specificity was confirmed using a panel of viral and bacterial pathogens. Finally, we did a blind concordance study on genomic DNA extracted from 185 clinical samples, comparing assay results with a gold-standard quantitative PCR (qPCR) assay. We identified the optimal time to detection and analysed the performance of the assay relative to qPCR using receiver operating characteristic (ROC) curves. We also assessed the compatibility with lateral-flow strips, both visually and computationally, where strips were interpreted blinded to the fluorescence results on the basis of the presence or absence of test bands.

Findings With an optimal run duration of approximately 45 min from isothermal amplification to CRISPR-assay readout, the MPXV recombinase polymerase amplification CRISPR-Cas12a-based assay with the selected primer–guide set had an LOD of 1 copy per µL and 100% specificity against tested viral pathogens. Blinded concordance testing of 185 clinical samples resulted in 100% sensitivity (95% CI 89·3–100) and 99·3% specificity (95% CI 95·7–100) using the fluorescence readout. For optimal time to detection by fluorescence readout, we estimated the areas under the ROC curve to be 0·98 at 2 min and 0·99 at 4 min. Lateral-flow strips had 100% sensitivity (89·3–100) and 98·6% specificity (94·7–100) with both visual and computational assessment. Overall, lateral-flow results were highly concordant with fluorescence-based readouts (179 of 185 tests, 96·8% concordant), with discrepancies associated with low viral load samples.

Interpretation Our assay for the diagnosis of mpox displayed good performance characteristics compared with qPCR. Although optimisation of the assay will be required before deployment, its usability and versatility present a potential solution to MPXV detection in low-resource and remote settings, as well as a means of community-based, on-site testing.

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Introduction

Mpox (formerly known as monkeypox) is a zoonotic disease caused by the monkeypox virus (MPXV), a large double-stranded DNA virus (~197 kb) belonging to the *Orthopoxvirus* genus, which also encompasses smallpox virus (variola). Since early May, 2022, over 85 000 cases[1] of mpox have been described in multiple countries not previously regarded as endemic for mpox.[2–6] On July 23, 2022, mpox was declared a public health emergency of international concern by WHO, and this was subsequently lifted on May 11, 2023. Mpox cases have primarily been reported in men who have sex with men, with cases also described in women and infants.[7,8] Genomic sequencing has identified two major clades of MPXV (clade I, formerly Congo Basin/Central African, and clade II, formerly West African), with the current

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Research in context

Evidence before this study

We did a search of primary research articles published in PubMed up until Nov 21, 2022, using the search terms "monkeypox" or "monkeypox virus" and "CRISPR". Seven studies were identified, two of which described CRISPR-Cas12a-based assays for the detection of monkeypox virus. However, these studies were limited in terms of primer and guide RNA evaluation, minimal diversity of viral pathogens used for specificity testing, and the low number of clinical samples tested (<20).

Added value of this study

In this proof-of-concept study, we developed and evaluated an assay for monkeypox virus detection using recombinase polymerase amplification coupled with CRISPR-Cas12a. Using genomic DNA from monkeypox virus and a large panel of viral and bacterial pathogens, the test demonstrated high analytical sensitivity (1 copy per µL) and specificity (100%). Importantly, using a large, blinded panel of 185 clinical samples obtained from a range of anatomical sites, the test displayed 100% clinical sensitivity and 99·3% specificity. We also tested the performance of the assay using a lateral-flow readout, illustrating its versatility.

Implications of all the available evidence

In line with current diagnostic innovations and meeting the requirements of WHO ASSURED diagnostics (ie, tests that are accurate, accessible, and affordable), we demonstrated the feasibility of using a CRISPR-Cas-based assay for detection of monkeypox virus. Our platform has broad applicability to other infectious diseases, and further highlights the utility of combining isothermal amplification methods with CRISPR-Cas technology.

outbreak forming a distinct lineage within clade II (clade IIb).[9,10] At present, viral transmission continues at a low level in most countries; however, the explosive emergence of mpox and limited information regarding efficacy of currently used vaccines highlight the need for ongoing vigilance and surveillance to prevent further large outbreaks.[11,12]

In addition to vaccination, rapid detection and isolation of cases is crucial to public health control of mpox. However, the vast majority of testing for MPXV to date has occurred in centralised laboratory settings, often in specialised public health reference laboratories, meaning that test results might not be available for up to several days after sample collection, depending on geographical and logistical considerations.[4,13] This situation can be compounded in low-income and middle-income countries, where there are further resource and access barriers to diagnostic testing.[14]

The COVID-19 pandemic has exemplified the need for fast, affordable, and accurate diagnostics that can be deployed at the point of care. The urgent need for rapid and scalable diagnostics for SARS-CoV-2 catalysed the development of a range of point-of-care tests (PoCTs), including those employing CRISPR and CRISPR-associated protein (Cas) technology.[15] The coupling of isothermal nucleic acid amplification with CRISPR-Cas-mediated target recognition enables high assay sensitivity and specificity and provides an opportunity to implement low-complexity PoCT for infectious diseases, including mpox.

Accordingly, in this study, we describe the design and evaluation of a fluorescence-based CRISPR-Cas12a assay for the detection of MPXV, which we term MPXV-CRISPR. We validated the assay using genomic DNA (gDNA) extracted from a large, blinded panel of clinical samples collected from individuals who had undergone PCR testing for mpox, covering a range of specimen types. Further, we describe implementation of our assay into a lateral-flow device that could be deployed in decentralised settings.

See Online for appendix 1

See Online for appendix 2

Methods

Setting and study design

We performed a single-centre laboratory study in which we designed, optimised, and evaluated a novel MPXV diagnostic assay (appendix 1 p 9). Evaluation and validation of the assay involved the assessment of: (1) analytical sensitivity and specificity, (2) assay precision, and (3) clinical sensitivity and specificity, assessed on gDNA from stored clinical samples. This study was approved by the Royal Melbourne Hospital Research Ethics Committee (QA2022085).

Clinical samples

Anonymised clinical samples sent for MPXV PCR testing between May 1 and Sept 30, 2022, were obtained from the Victorian Infectious Diseases Reference Laboratory (VIDRL) at The Peter Doherty Institute for Infection and Immunity (Melbourne, VIC, Australia). VIDRL is the public health virology laboratory for the state of Victoria, serving a population of about 6·24 million people. Clinical samples were obtained from various testing sites in Victoria and stored in Universal Transport Medium (Copan, Brescia, Italy) at –80°C. In this pilot study, gDNA extracted from 185 clinical samples including oral, anal, and skin lesion specimens was provided blind for processing (appendix 2 p 3).

MPXV quantitative PCR assay

An in-house quantitative PCR (qPCR) assay for MPXV detection, as described by Hammerschlag and colleagues,[16] was used for analysis of clinical samples and as the comparator for the MPXV-CRISPR assay. In brief, the qPCR assay included pan-orthopox primers and an

MPXV-specific probe targeting the orthopox hemagglutinin (B2R) gene. Testing was performed in duplicate on a QuantStudio5 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA), and genome copies per µL were calculated using standard curves, established using the AMPLIRUN Monkeypox Virus DNA Control (Vircell SL, Granada, Spain; appendix 1 pp 3–4, 11). Two cycle threshold (Ct) values were assessed, whereby Ct values of 35 or less or of 30 or less after 45 cycles of amplification were considered positive.

MPXV-CRISPR assay design

Recombinase polymerase amplification (RPA) primers and CRISPR guide RNAs (gRNAs) were designed using a database of 523 MPXV genomes downloaded from the National Center for Biotechnology Information (NCBI; appendix 2 p 4). The design included a pangenome analysis and a gene-agnostic approach that allowed for both coding and non-coding regions of the genome to be targeted. In the pangenome approach, highly conserved genes in MPXV (present in ≥90% of genomes) were identified, individually aligned, and used as targets for primer and gRNA design (appendix 1 pp 4–5). In the gene-agnostic approach, a *k*-mer based method was applied to identify 22-mers to 24-mers unique to MPXV and adjacent to a TTTV protospacer adjacent motif site to serve as a gRNA binding site. Primers were subsequently designed flanking that region. In total, 22 primer–gRNA sets, including a previously published primer set,[17] were shortlisted for further assessment on the basis of in-silico estimates of a sensitivity of 90% or higher and a specificity of 100% (appendix 2 p 5).

MPXV targets were amplified using gDNA extracted from MPXV grown in cell culture (appendix 1 p 3) using RPA with the TwistAmp Basic kit (TwistDx, Maidenhead, UK) at 39°C for 20 min in a thermal cycler (T100 Thermal Cycler, Bio-Rad, Hercules, CA, USA; appendix 1 p 5). 2 µL of RPA product was added to the MPXV-CRISPR reaction, and amplicon detection was performed by CRISPR-Cas12a-mediated trans-cleavage of a dual-labelled single-stranded DNA (ssDNA) reporter, labelled at one end with a quenched 6-FAM (6-carboxyfluorescein) fluorophore and the other with biotin, in the presence of a gRNA at 37°C for 58 min in a ClarioStar Plus microplate reader (BMG Labtech, Ortenberg, Germany; appendix 1 pp 5–6, 9).

Assay readout using fluorescence-based and lateral-flow detection

To assess the endpoint versatility of the MPXV-CRISPR assay, assay readout was measured using two signals, enabled by the dual-labelled reporter molecule design: (1) fluorescence-based readout using the CLARIOstar Plus microplate reader (BMG LABTECH, Ortenberg, Germany) every minute for 58 min, and (2) lateral-flow detection after completion of fluorescence-based readout.

MPXV-CRISPR fluorescence assays included three types of control samples: no-template control, no-guide control, and at least one non-MPXV viral gDNA control (appendix 2 p 6). For the fluorescence-based readout, background fluorescence values of the no-guide control were subtracted from clinical samples. A sample was defined as MPXV-positive if the background-subtracted fluorescence exceeded the threshold of 10 SDs above the mean fluorescence across 58 min of six no-template control samples.

For lateral-flow detection following the fluorescence-based readout, 25 µL of MPXV-CRISPR assay was added to 75 µL of HybriDetect assay buffer (Milenia Biotec, Giessen, Germany) at room temperature. A lateral-flow strip (Milenia GenLine HybriDetect strip, Milenia Biotec, Giessen, Germany) was immersed in the total volume before readout at 5 min (appendix 1 pp 6–7). Visualisation on the lateral-flow strips was achieved through the degradation of the FAM-biotin reporter molecule. Gold nanoparticles with anti-FITC (anti-fluorescein isothiocyanate) antibodies were immobilised at the sample pad of the strip, biotin ligands were present at the control line, and anti-rabbit antibodies at the test line (appendix 1 p 20). The FAM portion of the reporter binds to the anti-FITC antibody as it passes through the sample pad. In the absence of target DNA (negative test), intact reporters are captured at the control line by the biotin ligand, generating a visible control band, with no band in the test line. In the presence of target DNA (positive test), the biotin portion of the cleaved reporter binds to the biotin ligand, generating a control band, and the FAM portion of cleaved reporters bound to the anti-FITC antibody gold nanoparticle is then captured by the anti-rabbit antibody at the test line (appendix 1 p 20). Lateral-flow strips were interpreted blinded to the fluorescence results, visually by eye, and computationally using ImageJ (ImageJ, US National Institutes of Health, Bethesda, MD, USA). Lateral-flow strip band intensities were converted to a hue, saturation, brightness stack in ImageJ, with pixel values ranging from 0 (black; presence of a band) to 255 (white; absence of a band). The minimum brightness of the control and test lines were measured, and the threshold for defining a sample as positive was set at less than 5 SDs from the mean brightness in the no-template control test line (appendix 1 pp 6–7).

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Assessment of analytical sensitivity and specificity

Analytical sensitivity, or limit of detection (LOD), of the MPXV-CRISPR assay was assessed using ten-fold serial dilutions of gDNA extracted from MPXV grown in cell culture (appendix 1 p 3).[16] Serial dilutions were quantified in genome copies per µL by interpolating from standard curves established using the AMPLIRUN Monkeypox Virus DNA Control (Vircell SL, Granada, Spain) with fluorescence assays (appendix 1 p 10). Analytical

specificity was assessed on a range of viral pathogens including those commonly found in skin lesions: vaccinia virus, herpes simplex virus type 1, herpes simplex virus type 2, molluscum contagiosum virus, enterovirus (coxsackie B2), orf virus, and varicella zoster virus. These viruses were grown in cell culture, with gDNA extracted from supernatants (appendix 1 p 3; appendix 2 p 1). Viral loads were quantified and found to be within the range observed in clinical samples (appendix 2 p 1). Given the use of oropharyngeal swabs for detection of MPXV,[18] we also assessed specificity against pathogens commonly found in the oropharynx using a commercial gDNA pooled respiratory control panel (ZeptoMetrix RP2.1, Natrol, Buffalo, New York, NY, USA; appendix 2 p 2). All testing for sensitivity and specificity was performed in triplicate.

Assay precision

We estimated intra-assay and inter-assay precision using serial dilutions of gDNA extracted from MPXV, varicella zoster virus, herpes simplex virus type 1, and no-template control. Three assays were conducted on five technical replicates of each sample, and the experiment was performed three times. Intra-assay and inter-assay precision was estimated using the average fluorescence values obtained at 40 min.

Clinical sensitivity and specificity

Clinical sensitivity and specificity were analysed using the 185 clinical samples obtained from the VIDRL (appendix 2 p 3). We performed blinded concordance testing on gDNA from these samples by comparing MPXV-CRISPR with our in-house qPCR assay targeting the hemagglutinin gene.[16] All testing on clinical samples was performed in duplicate.

Statistical analysis

To achieve an 80% power with 99% sensitivity at a 5% significance level, at least 42 MPXV-positive and 42 MPXV-negative samples were required. Our clinical sample collection comprised 40 MPXV-positive and 145 MPXV-negative samples because of the small number of mpox cases in Australia. Hierarchical clustering analysis was performed to identify which primer–gRNA sets achieved the greatest fluorescence over time. We used unpaired one-sided Wilcoxon rank tests to compare background-subtracted fluorescence of MPXV samples with the no-template control for determining analytical sensitivity, where differences were considered significant if fluorescence signals were higher in MPXV samples (α=0·05). To estimate assay precision, we calculated the average intra-assay and inter-assay coefficients of variation from three assays and three experimental repeats. For clinical sensitivity and specificity calculations, samples were defined as positive if the background-subtracted fluorescence exceeded the mean plus 10 SDs of the no-template control fluorescence values, assuming a normal distribution. We calculated the 95% CIs for clinical sensitivity and specificity using the Agresti-Coull method. The Youden index (sensitivity+specificity−1) was used for determining the optimal fluorescence assay cutoff time. We used the fluorescence values at the determined optimal assay cutoff times for performing the receiver operating characteristic (ROC) curve analysis, with the 95% CIs calculated using the Wilson/Brown method. All statistical analyses and data visualisation were performed using either GraphPad Prism (version 9.4.1) or R (version 4.2.2). Appendix 1 (pp 7–8) shows detailed information on statistical analyses.

Role of the funding source

The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results

We developed an assay encompassing RPA in combination with CRISPR-Cas12a-mediated DNA target recognition and ssDNA cleavage (referred to as MPXV-CRISPR), coupled with high throughput fluorescence-based and lateral-flow strip readouts (appendix 1 p 9). We used a database of 523 MPXV genomes to design a panel of RPA primers and gRNAs for experimental screening (appendix 2 p 4). In total, 21 sets of RPA primers flanking seven gRNAs were obtained (appendix 2 p 5). We also tested a set of previously published MPXV-specific RPA primers targeting a region within the tumour necrosis factor receptor (TNFR)-like protein gene, from which we extracted a gRNA sequence.[17] Collectively, these 22 sets of primers and seven gRNAs targeted three genes and two intergenic regions (appendix 1 p 11), with multiple primer sets for four of the gRNAs (appendix 1 p 13). An in-silico PCR evaluation including 304 non-MPXV genomes (appendix 2 p 4) showed that all primers amplified 96·5% or more of the MPXV genomes tested, encompassing the two currently circulating major clades and exhibiting no in-silico cross-reactivity to other *Orthopoxvirus* species, or to varicella zoster virus, herpes simplex virus type 1, and herpes simplex virus type 2 (appendix 2 p 5).

We next evaluated the performance of all 22 sets in the MPXV-CRISPR assay with a fluorescence readout using 10 000 copies per µL of input MPXV gDNA. All sets were also tested against comparable amounts of herpes simplex virus type 1 and varicella zoster virus, and no-template control. Inspection of fluorescence over 40 min and across three experimental repeats revealed clear differences in sensitivity, specificity, and reproducibility between the sets (appendix 1 pp 11, 14–15). Three main clusters were identified by hierarchical clustering analysis, representing low, intermediate, and high fluorescence (appendix 1 p 11). The high-fluorescence

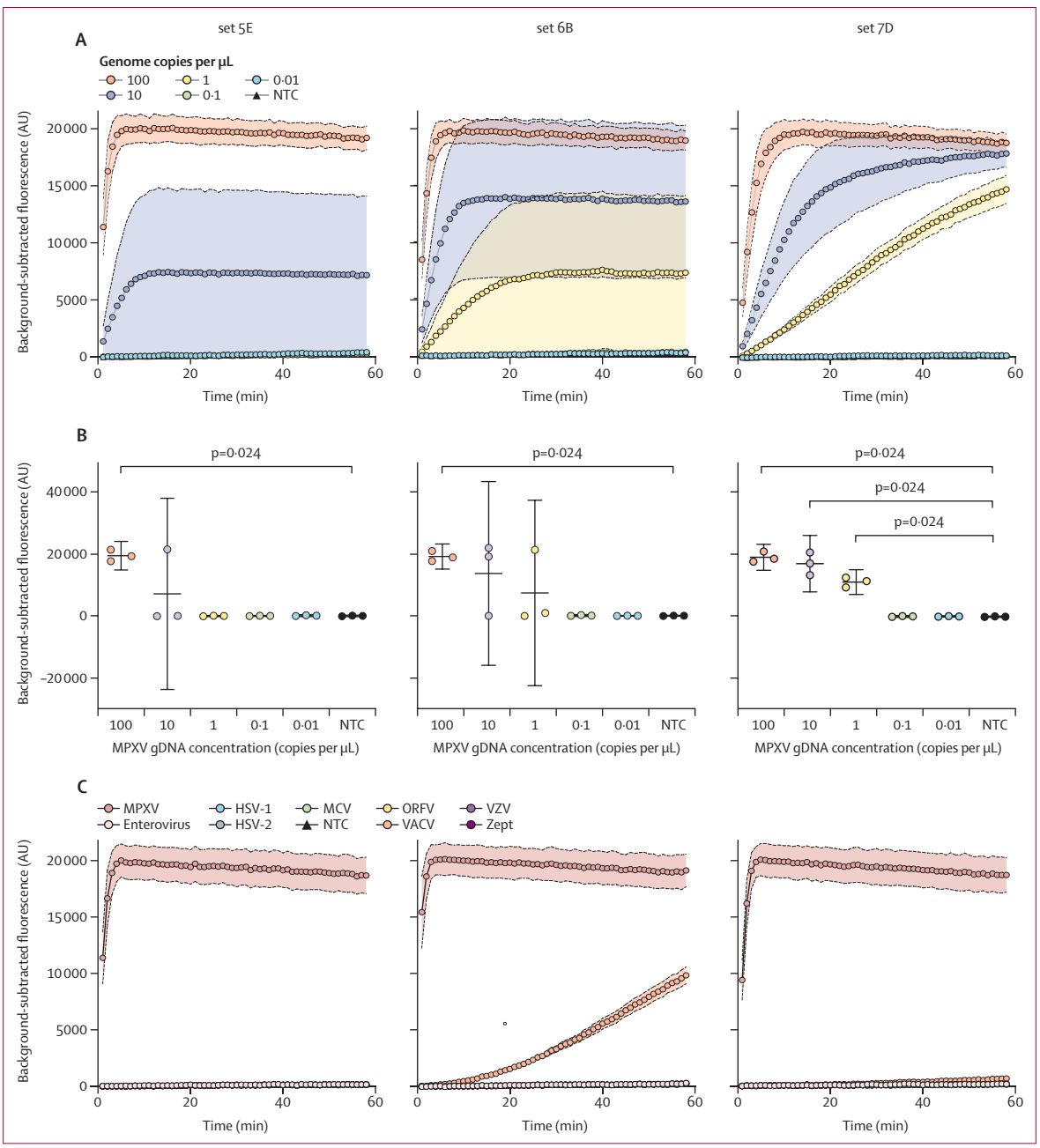
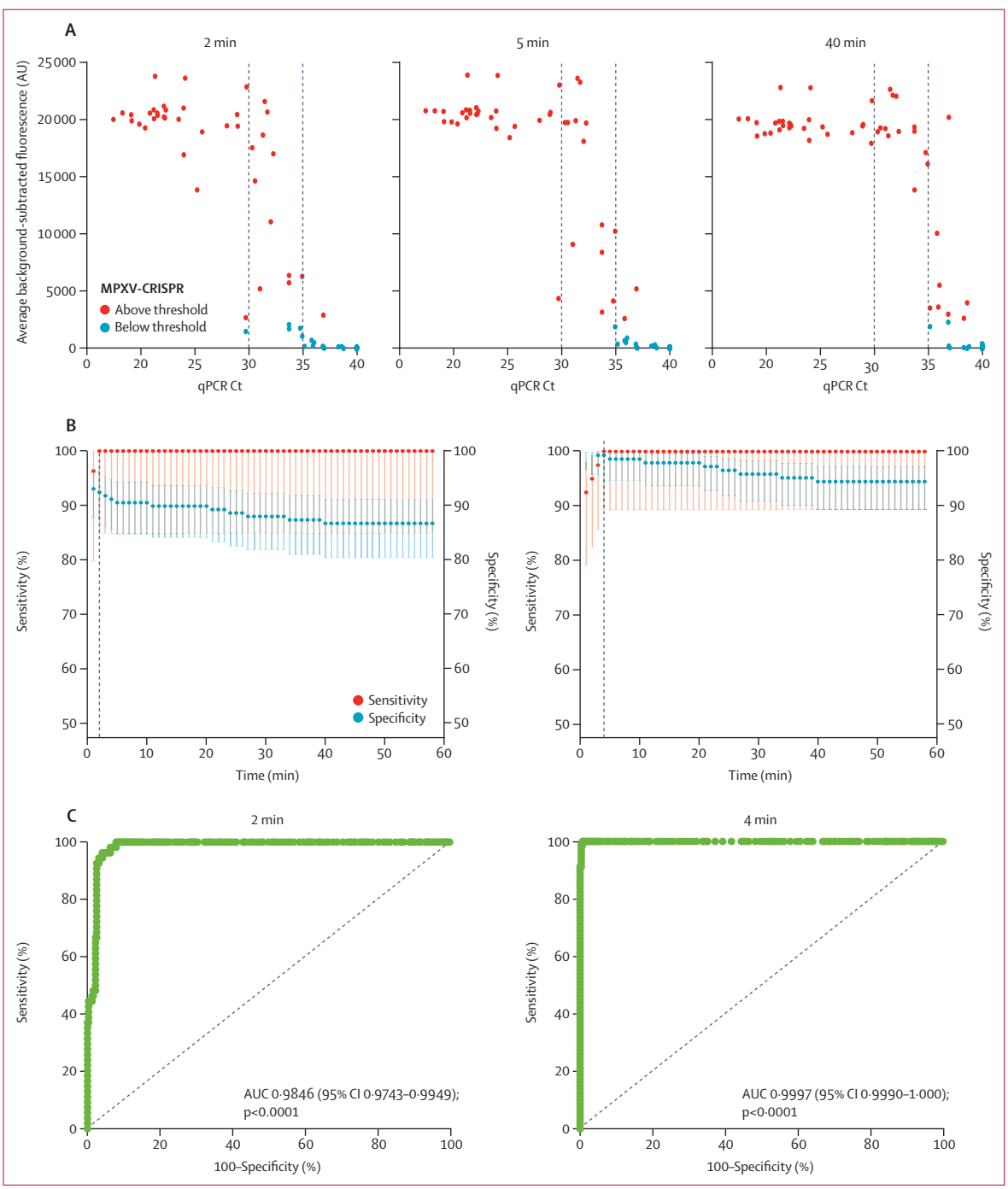


Figure 1: **Analytical sensitivity and specificity of MPXV-CRISPR assay using three shortlisted primer–gRNA sets**
(A) Sensitivity of three shortlisted primer–gRNA sets was determined using 0·01–100 copies per µL (by 10-fold serial dilutions) of MPXV gDNA in the MPXV-CRISPR assay, and an NTC. Background correction of fluorescence was performed by subtracting fluorescence values of the NGC from each sample at each timepoint. Data represent the mean of three experimental repeats over 60 min, and the SEM is depicted by area fill. (B) Statistical limit of detection was calculated at 40 min from three experimental repeats, with pairwise comparisons of background-corrected fluorescence of MPXV gDNA at each dilution with the NTC performed using an unpaired one-sided Wilcoxon rank test. One-tailed p values of 0·05 or lower were considered significant. Lines represent means (95% CIs). (C) Specificity of each primer–gRNA set was determined using a panel of viral pathogen gDNA ranging from ~100 to 9600 copies per µL (appendix 2 p 1): enterovirus (coxsackie B2), HSV-1, HSV-2, MCV, ORFV, VACV, VZV, and Zept. Controls included 10 000 copies per µL of MPXV gDNA and NTC. Background correction of fluorescence was performed by subtracting fluorescence values of the NGC from each sample at each timepoint. Data represent the mean of three experimental repeats over 60 min, and the SEM is depicted by area fill. AU=arbitrary unit. CRISPR=clustered regularly interspaced short palindromic repeats. gDNA=genomic DNA. gRNA=guide RNA. HSV-1=herpes simplex virus type 1. HSV-2=herpes simplex virus type 2. MCV=molluscum contagiosum virus. MPXV=monkeypox virus. NGC=no-guide control. NTC=no-template control. ORFV=orf virus. SEM=standard error of mean. VACV=vaccinia virus. VZV=varicella zoster virus. Zept=ZeptoMetrix NATtrol Respiratory control.

cluster included previously published RPA primers targeting the TNFR-like protein gene,[17] set 2A. However, set 2A, as well as sets 1A and 3A, displayed false positive MPXV detection in the presence of viral controls or no-template controls and were omitted from subsequent experiments (appendix 1 p 11). Of the remaining sets within this cluster, we selected one primer set per gRNA on the basis of earliest fluorescence signal levels. The selected sets were 5E, 6B, and 7D.

We assessed the analytical sensitivity of the three primer–gRNA combinations using serial dilutions of MPXV gDNA purified from cell culture (figure 1A). To determine the LOD of MPXV-CRISPR, we compared background-corrected fluorescence of the MPXV dilution series with no-template control at 40 min and found that set 7D, which targeted a non-genic region present in two copies (appendix 1 p 11), displayed the highest sensitivity (LOD of 1 genome copy per µL),



while sets 5E and 6B had a LOD of 100 genome copies per µL (figure 1B, appendix 1 p 16). The LOD of set 7D was also confirmed using the AMPLIRUN Monkeypox Virus DNA Control (appendix 1 p 10). Specificity testing resulted in false positive signals from vaccinia virus with set 6B, while sets 5E and 7D displayed 100% specificity for MPXV with no cross-reactivity for related orthopoxvirus strains and other viral and bacterial pathogens tested (figure 1C). Taken together, these results demonstrated that set 7D had the highest sensitivity and specificity and was thus selected for subsequent testing using clinical samples. On the basis of in-silico analyses of 523 genomes in the MPXV database, this primer–gRNA set was predicted to amplify all genomes in clade I (49 of 49), 96% (27 of 28) of genomes in clade IIa, and 98% (437 of 446) in clade IIb (appendix 1 p 17).

Assay precision testing was performed using set 7D and dilution series of MPXV gDNA, control gDNA (herpes simplex virus type 1 and varicella zoster virus), and no-template control (appendix 1 pp 6, 18). Intra-assay and inter-assay precision was estimated using the average fluorescence values obtained at 40 min (appendix 1 pp 7–8). The average overall coefficient of variation was 5·2% (SD 3·5) for the intra-assay analysis, and 20·8% (SD 11·3) for the inter-assay analysis (appendix 1 pp 7–8).

To further validate our assay, we performed blinded concordance testing on gDNA from 185 clinical samples (85 from skin lesions, 55 from the anus, 44 from the oral cavity, and one from the nasopharynx; appendix 2 p 3), comparing MPXV-CRISPR with an in-house qPCR assay targeting the hemagglutinin gene.[16] Of the 185 samples, 40 were MPXV-positive and 145 were MPXV-negative. The average Ct values of 51 clinical samples ranged from 17·4 to 38·8, and the remaining 134 samples were below the qPCR detection limit. Given that Ct values for determining MPXV-positive samples might vary between diagnostic laboratories, two Ct values were applied to assess MPXV-positive samples for concordance analysis. At a Ct cutoff of 30 or less, 27 samples were MPXV-positive and 158 were MPXV-negative by qPCR. At a Ct cutoff of 35 or less, 40 samples were MPXV-positive and 145 were MPXV-negative by qPCR. The MPXV-CRISPR assay correctly identified all positive samples, with fluorescence signal above no-template control background detectable within the first 5 min of the assay (figure 2A; appendix 2 p 3). Further, 26 (96·3%) of 27 positive samples (Ct values ≤30) and 33 (82·5%) of 40 positive samples (Ct values ≤35) were detected within 1 min (appendix 1 p 19). Results were consistent across two assay repeats, with 181 (97·8%) of 185 samples producing concordant results. We determined the optimal cutoff time threshold by plotting sensitivity and specificity against time, defined as the timepoint at which the Youden index was highest. For Ct values of 30 or less (ie, with higher viral load), the optimal assay cutoff time was 2 min (100% sensitivity; 92·4% specificity), and for Ct values of 35 or less, the optimal cutoff time was 4 min (100% sensitivity; 99·3% specificity; figure 2B; table 1). ROC curves were used to assess the performance of the assay in distinguishing MPXV-positive and MPXV-negative clinical samples at the different assay cutoff times, with areas under the curve of 0·98 at 2 min and 0·99 at 4 min (figure 2C).

Figure 2: **Clinical sensitivity and specificity of the MPXV-CRISPR assay**
(A) Scatterplot of average background-corrected fluorescence of the MPXV-CRISPR assay using primer–gRNA set 7D and qPCR cycle threshold on a blind cohort of clinical samples (n=185, two experimental repeats) at 2, 5, and 40 min. Each point represents a clinical sample, coloured according to the MPXV-CRISPR fluorescence (red for positive or above threshold, blue for negative or below threshold) at their respective timepoints. Samples with undetermined Ct values by qPCR are assigned a Ct of 40. The threshold used for calling a sample positive was at least 10 SDs above the mean fluorescence across 58 min of six NTC samples. Background correction of fluorescence was performed by subtraction of the NGC fluorescence for each sample at each timepoint, and the average value obtained. The black dotted lines represent the qPCR cutoffs at Cts 30 and 35. (B) Sensitivity and specificity of MPXV-CRISPR was assessed against gold-standard MPXV qPCR at two positive-call cutoffs: Ct ≤30 (5000 copies per µL) and Ct ≤35 (100 copies per µL). Sensitivity (red) and specificity (blue) of MPXV-CRISPR assay at different timepoints (min), with error bars representing the 95% CIs estimated using the Agresti-Coull method. Black dotted lines indicate the assay cutoff time where sensitivity and specificity were optimal based on Youden's index. (C) ROC curve analysis performed using the fluorescence values of the MPXV-CRISPR assay at 2 min (qPCR positive-call cutoff Ct≤30) and 4 min (qPCR positive-call cutoff Ct≤35). The 95% CIs were calculated using the Wilson/Brown method. The diagonal dotted line corresponds to the null hypothesis with area under the curve of 0·5, in which the accuracy of a diagnostic test is no different from random chance. AU=arbitrary unit. AUC=area under the ROC curve. CRISPR=clustered regularly interspaced short palindromic repeats. Ct=cycle threshold. gRNA=guide RNA. MPXV=monkeypox virus. NGC=no-guide control. NTC=no-template control. qPCR=quantitative PCR. ROC=receiver operating characteristic.

	Concordance for a qPCR cutoff of Ct=30	Concordance for a qPCR cutoff of Ct=35
Samples positive by qPCR (Ct≤cutoff)		
True positive	27	40
False negative	0	0
Sensitivity	100% (85–100)	100% (89–100)
Positive predictive value	69·2% (53–81)	97·6% (86–100)
Samples negative by qPCR (Ct>cutoff)		
True negative	146	144
False positive	12	1
Specificity	92·4% (87–96)	99·3% (96–100)
Negative predictive value	100% (97–100)	100% (97–100)

Data are n or % (95% CI). MPXV-CRISPR assay with fluorescence readout was performed with two experimental repeats and assessed against gold-standard MPXV qPCR at two positive-call cutoffs: Ct ≤30 and Ct ≤35. Sensitivity and specificity were calculated at the optimal cutoff time: 2 min for the Ct ≤30 and 4 min for the Ct ≤35 comparisons. A sample is defined as positive by MPXV-CRISPR if the fluorescence of at least one repeat exceeds the threshold of 10 SDs above the mean background fluorescence. CRISPR=clustered regularly interspaced short palindromic repeats. Ct=cycle threshold. MPXV=monkeypox virus. qPCR=quantitative PCR.

Table 1: **Clinical sensitivity and specificity of MPXV-CRISPR with fluorescence readout**

To assess the compatibility of the MPXV-CRISPR assay with a lateral-flow detection method, we repeated analytical and clinical sensitivity and specificity testing immediately after the fluorescence-based assay on lateral-flow strips, made possible by the dual-labelled reporter (figure 3; appendix 1 pp 20–21). Consistent with the fluorescence-based assay, and using either manual inspection or computational image analysis, lateral-flow readouts with set 7D achieved an LOD of 1 copy per µL and 100% specificity for MPXV, with no cross-reactivity against a specificity panel of other viruses, including other poxviruses (figure 3B, C; appendix 1 pp 22–23). Using the computational results of the analytical sensitivity and specificity testing as a training dataset, samples were defined as positive when the background-corrected minimum brightness (measured in pixel intensity [PI]) was at least 5 SDs below the mean PI of the no-template control, corresponding to a PI of 177·6 (figure 3D). This positivity threshold was applied to lateral-flow testing using the 185 clinical samples described above (figure 3E, appendix 1 pp 24–26; appendix 2 p 3). Visual inspection and computational interpretation of the lateral-flow strips resulted in 100% sensitivity (95% CI 84·9–100) and 90·5% specificity (95% CI 84·8–94·2) for samples with Ct values of 30 or less, and 100% sensitivity (89·3–100) and 98·6% specificity (94·7–100) for samples with Ct values of 35 or less (table 2). The presence of weak test bands in negative samples were attributed to the design features of the strip rather than readout errors (appendix 1 p 20). Overall, lateral-flow results were highly concordant with fluorescence-based readouts (179 of 185 tests, 96·8% concordant), with discrepancies associated with samples with low viral load (appendix 2 p 9).

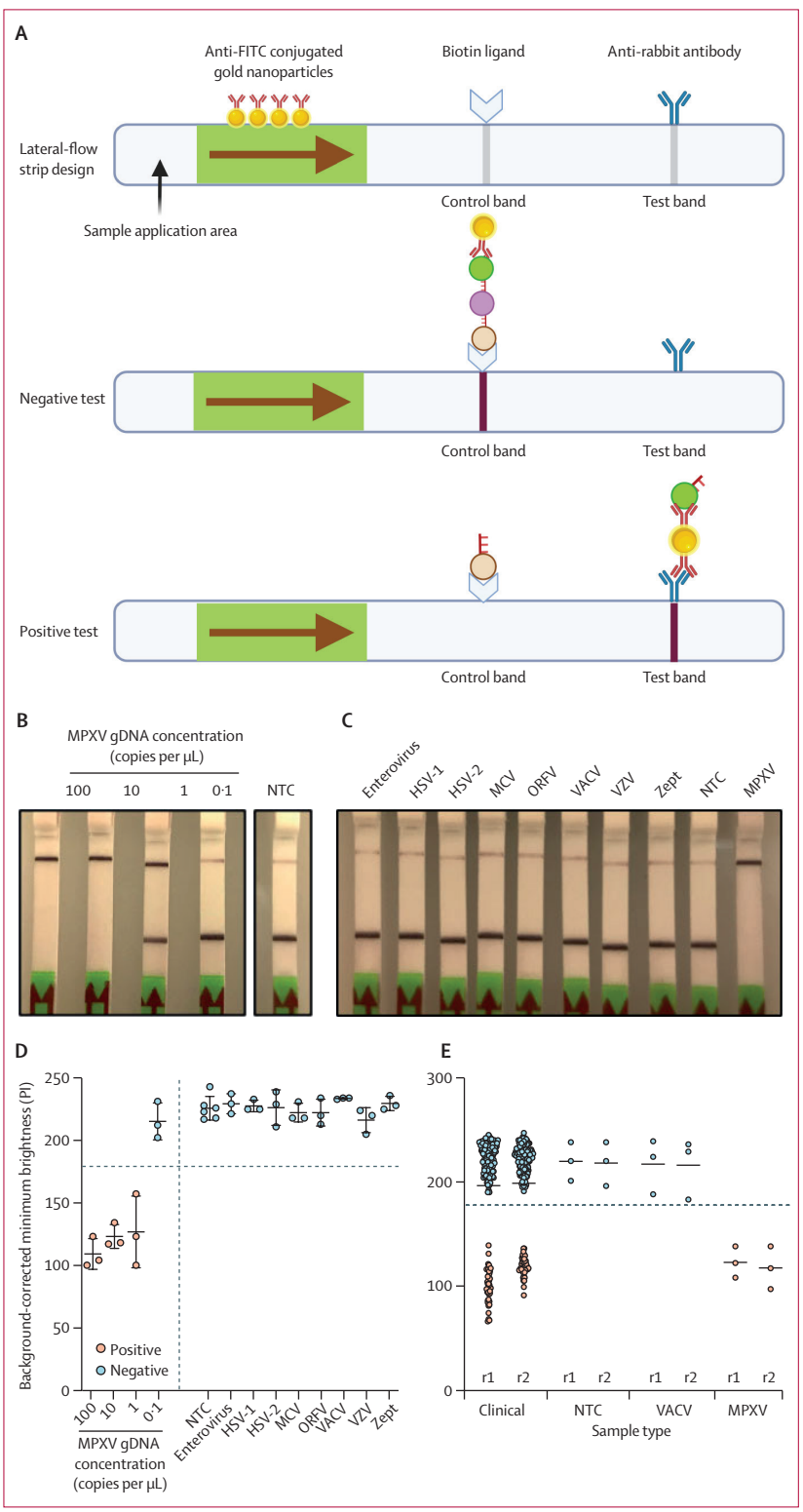


Figure 3: **Assessment of MPXV-CRISPR with lateral-flow device**
(A) Composition of the Milenia GenLine Hybridetect strips and readout interpretation. (B) Limit of detection of MPXV-CRISPR assay with primer–gRNA set 7D using four 10-fold serial dilutions of MPXV gDNA (100–0·1 copies per µL), with NTC included. (C) Specificity of the MPXV-CRISPR assay using primer–gRNA set 7D evaluated using gDNA from a panel of viral and bacterial pathogens: enterovirus (coxsackie B2), HSV-1, HSV-2, MCV, ORFV, VACV, VZV, and Zept. Controls included 10 000 copies per µL of MPXV gDNA and NTC. Lateral-flow testing was performed on three experimental repeats, with representative images presented. (D) Background-corrected minimum brightness of lateral-flow strip test lines (in PI), represented as mean (SD) of at least three experimental repeats. The horizontal dotted line represents the brightness threshold, below which samples were defined as MPXV-positive. The vertical line separates MPXV samples from non-MPXV and negative control samples. (E) Distribution of lateral-flow strip band intensities for a blind cohort of clinical samples (n=185; two experimental repeats, r1 and r2), as quantified by ImageJ. MPXV-positive samples were defined using the threshold determined from D (black dotted line). Positive (MPXV) and negative (NTC, VACV) controls were included. CRISPR=clustered regularly interspaced short palindromic repeats. FITC=fluorescein isothiocyanate. gDNA=genomic DNA. gRNA=guide RNA. HSV-1=herpes simplex virus type 1. HSV-2=herpes simplex virus type 2. MCV=molluscum contagiosum virus. MPXV=monkeypox virus. NTC=no-template control. ORFV=orf virus. PI=pixel intensity. VACV=vaccinia virus. VZV=varicella zoster virus. Zept=ZeptoMetrix NATtrol Respiratory control.

Discussion

In this study, we aimed to develop a rapid, sensitive, and specific PoCT for MPXV in keeping with the

overarching principles of the WHO ASSURED criteria of tests that are accurate, accessible, and affordable.[17] Using the primer–gRNA set determined to produce the best signal, our assay was applied to a large set of clinical samples and compared with a gold-standard MPXV qPCR assay. Overall, the MPXV-CRISPR assay with a fluorescence readout demonstrated 99·5% concordance with qPCR, with the only discrepancy being a sample with a very low viral load. Further, our assay showed no cross-reactivity against other viral pathogens tested, including those with similar clinical presentations such as varicella zoster virus, probably due to our comprehensive in-silico primer and gRNA design, which was subsequently optimised in vitro for increased specificity.

Several in-house and commercial tests have been developed for the diagnosis of mpox; however, these are predominantly PCR-based, designed for use in a centralised laboratory setting.[19] Although there are several PoCTs in development, including both molecular and antigen tests, there are limited data available on the performance of these tests.[20] For SARS-CoV-2, another pathogen to have had an outbreak within the last 5 years, a sensitivity of 90% or higher and specificity higher than 99% were recommended by WHO as desirable performance characteristics for diagnostic assays.[21] Compared with the gold-standard qPCR, our assay with fluorescence readout had a sensitivity of 100% and specificity of 99·3% at 4 min, with an analytical sensitivity at 40 min of 1 genome copy per µL.

The use of a lateral-flow colorimetric readout had no effect on clinical and analytical sensitivity and demonstrated less than 1% reduction in specificity (98·6%) compared with the fluorescence readout. Moreover, the lateral-flow readout was reliably interpreted both manually by independent observers and computationally in ImageJ, further supporting its potential utility as a PoCT. For point-of-care use, further design considerations are required to eliminate potential ambiguity in visual interpretation. For example, the presence of a band in the test line of the lateral-flow strip without a corresponding control line was interpreted as a positive test, unlike conventional lateral-flow tests where this would be considered an invalid result. In addition, a faint band was present at the test line for all negative results due to excess gold nanoparticles; this faint band might be observed when using commercially available strips[22] and can be addressed by optimising the ratio of reporter molecule concentration to available gold nanoparticles or custom manufacturing of lateral-flow strips. Encouragingly, for computational interpretation of strips the presence of a faint test band in a negative test can be accounted for by using predefined thresholds, as demonstrated in our study. In practice, a lateral-flow PoCT for mpox could be used as a first-step rapid test to guide initial decisions regarding isolation and management, with high-specificity confirmatory testing subsequently provided by a reference laboratory, similar to the initial implementation of SARS-CoV-2 lateral-flow devices in some settings.[23–27]

	Concordance for a qPCR cutoff of Ct=30		Concordance for a qPCR cutoff of Ct=35	
	Visual	Computational	Visual	Computational
Samples positive by qPCR (Ct≤cutoff)				
True positive	27	27	40	40
False negative	0	0	0	0
Sensitivity	100% (85–100)	100% (85–100)	100% (89–100)	100% (89–100)
Positive predictive value	64·3% (49–77)	64·3% (49–77)	95·2% (83–99)	95·2% (83–99)
Samples positive by qPCR (Ct>cutoff)				
True negative	143	143	143	143
False positive	15	15	2	2
Specificity	90·5% (85–94)	90·5% (85–94)	98·6% (95–100)	98·6% (95–100)
Negative predictive value	100% (97–100)	100% (97–100)	100% (97–100)	100% (97–100)

Data are n or % (95% CI). MPXV-CRISPR assay with lateral-flow readout was performed with two experimental repeats and assessed against gold-standard MPXV qPCR at two positive-call cutoffs: Ct ≤30 and Ct ≤35. Visual interpretation of the strips from both experimental repeats was performed by three independent observers, and a sample defined as positive by each individual if at least one of the repeats was interpreted as positive. The overall verdict was based on the majority consensus of the three individuals. Computational interpretation of the strips was performed using ImageJ as described in figure 3C, with a sample defined as positive if at least one of the repeats had a minimum test line brightness of ≤177·6. CRISPR=clustered regularly interspaced short palindromic repeats. Ct=cycle threshold. MPXV=monkeypox virus. qPCR=quantitative PCR.

Table 2: **Clinical sensitivity and specificity of MPXV-CRISPR with lateral-flow readout**

Importantly, our assay was able to detect MPXV across a range of clinical specimen types (skin lesions, anal swabs, and oropharyngeal samples). This finding is relevant given that many individuals might present with lesions in the oropharynx or anus.[6,28–30] Of note, a recent study using qPCR suggested that viral loads in lesions from patients with mpox generally have Ct values of 30 or less.[5] Another study found a high correlation between viral DNA concentration and virus infectivity in clinical specimens, suggesting a qPCR threshold of Ct 35 or less (equivalent to ≤4300 copies per mL) for predicting a poorly infectious or non-infectious specimen.[18] In our study, applying a qPCR threshold of Ct 35 or less or of Ct 30 or less did not result in a change in sensitivity, suggesting that our MPXV-CRISPR assay is capable of detecting MPXV in individuals during their infectious period.

A limitation of our study was that, because of the relatively low number of mpox cases in Australia, our clinical validation set included 40 MPXV-positive samples, two samples fewer than the predetermined sample size. All samples were obtained during the 2022 mpox outbreak and were likely to be limited to Clade IIb. However, the primer and gRNA used in this study target a non-coding region and were designed from a comprehensive database of MPXV sequences covering all MPXV clades, spanning over two decades. Future clinical testing should include a broader

geographical range of circulating MPXV lineages. Studies in 2022 have described the hypermutation rate of MPXV[10] and reported deletions in the TNFR-like protein gene,[31] highlighting the potential for diagnostic escape in single-target diagnostic assays. Accordingly, future assay development will include multiplexed primer–gRNA sets, which are not affected by the deletions in the TNFR-like gene.

An additional limitation was that gDNA extraction, amplification, and detection were modularised, although this modularisation enabled us to systematically optimise and troubleshoot each component of the assay at this developmental stage. Future assay development should consider potential differences between laboratory and field setting performance and include exploring simplified DNA extraction methods and a so-called one-tube RPA-CRISPR reaction, which would also reduce opportunities for assay contamination.

Contributors

DAW, SP, SJL, and MTO'N designed the study. GP, TT, JMT, CF, EPFC, and MYC provided clinical samples. SJL, MTO'N, WJK, MK, IS, and LWW performed technical analysis. SJL, MTO'N, WJK, and SP prepared the figures. All authors had full access to all the data in the study and had final responsibility for the decision to submit for publication. LW, JPC, LC, CKL, JP, CSB, and MP verified the underlying data. DAW, SP, SJL, MTO'N, and WJK prepared the Article, which was reviewed by all authors.

Declaration of interests

We declare no competing interests.

Data sharing

Data supporting the findings of this study are available within the text and in supplementary files. Source data are provided with this paper.

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