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Original article

Rapid and accurate monkeypox detection with xylenol orange-based colorimetric assay towards point-of-care: insights from clinical samples in Thailand

Kantaphat Kranjanapaisit[1,2], Yada Ongchanchai[2], Ravee Nitiyanontakij[3], Pawita Suwanwatthana[3], Ratchana Ponang[3], Wansadaj Jaroenram[2,*]

[1]Saint Gabriel's College, 565 Samsen Rd., Wachiraphayaban, Bangkok 10300, Thailand
[2]National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), 111 Thailand Science Park, Pathum Thani 12120, Thailand
[3]Bamrasnaradura Infectious Diseases Institute, 126 Tivanon Rd., Talat Khwan, Mueang, Nonthaburi 11000, Thailand

Abstract

Background: Monkeypox has been declared a public health emergency, highlighting the need for rapid and accessible diagnostic tools. While quantitative polymerase chain reaction (qPCR) is the gold standard due to its high sensitivity and specificity, it requires specialized equipment and trained personnel, making it unsuitable for point-of-care testing. Antigen test kits (ATKs) are simpler and more affordable but lack sufficient sensitivity for early outbreak detection.

Objective: This study aims to develop an accurate, rapid, and cost-effective alternative for monkeypox detection based on clinical samples in Thailand.

Methods: A novel colorimetric loop-mediated isothermal amplification (LAMP) assay was developed using xylenol orange (XO) dye for direct, naked-eye detection. This approach enhances visual clarity and simplifies result interpretation while eliminating the need for thermal cyclers.

Results: The assay is completed within 75 minutes, demonstrating 100% accuracy and a detection limit of 10 viral copies per reaction—ten times more sensitive than PCR. It also offers affordability at approximately $3 per test, making it suitable for widespread use.

Conclusion: This is the first study to develop and validate a colorimetric LAMP assay using clinical samples from Thai patients. The assay presents a reliable and accessible alternative to qPCR, with potential applications in broader pathogen diagnostics.

Keyworks: Monkeypox virus, MPOX, MPXV colorimetric LAMP, xylenol orange.

***Correspondence to:** Wansadaj Jaroenram, National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), 111 Thailand Science Park, Pathum Thani 12120, Thailand.
E-mail: wansadaj.jar@biotec.or.th

Currently, the world is facing significant health challenges, including outbreaks of infectious diseases. Two prominent examples are COVID-19 and Monkeypox [1,2], which have affected millions of people worldwide. Focusing on Monkeypox, it is caused by the monkeypox virus (MPXV), a member of the Orthopoxvirus genus. The disease is mainly transmitted through close contact with infected individuals or contaminated materials. The global monkeypox outbreak in 2022 highlighted the urgent need for effective diagnostics. In August 2024, the World Health Organization declared it a public health emergency due to rising cases and its spread to new regions. [3] The virus has two distinct clades, Clade I and Clade II, with Clade IIb responsible for the global outbreak. [4] To date, efforts to control the outbreak include increased surveillance, vaccination campaigns, public health interventions and broad access to testing. [5] Focusing on the testing, current diagnostic methods for monkeypox, including PCR-based assays and antigen test kits, have limitations. To illustrate, PCR-based techniques, although highly sensitive and specific, require advanced laboratory infrastructure and skilled personnel, making them less feasible in resource-limited settings. Antigen test kits provide quicker results but often lack the sensitivity and specificity of PCR-based methods. These limitations highlight the necessity for alternative diagnostic approaches that are both accurate and accessible, particularly in low-resource environments. [6]

Loop-mediated isothermal amplification (LAMP) presents a promising alternative for nucleic acid amplification. Unlike traditional PCR methods, LAMP operates at a constant temperature, eliminating the need for complex thermal cycling equipment. This makes LAMP faster and more cost-effective, and it can be conducted with minimal laboratory infrastructure, making it ideal for point-of-care testing. [7] LAMP can be combined with colorimetric or visual readouts by the inclusion of pH-sensitive dyes into the reactions, so-called colorimetric LAMP (cLAMP), allowing results to be observed with the naked eye. [8] Mechanistically, the cLAMP reaction involves the strand displacement activity of *Bst* polymerase, which generates pyrophosphates and protons (H+). This causes a significant drop in pH in a weakly buffered environment, resulting in a spontaneous alteration in the optical properties of the dye. Xylenol orange (XO) has been successfully used as a colorimetric indicator in LAMP assays for various pathogens including *E. coli*, [9] COVID-19, [10] scale drop disease virus, [11] and *Vibrio parahaemolyticus* [12,13] and for the authentication of cephalopod species. [14] The colorimetric LAMP method using XO leverages the acidification during the LAMP reaction, causing a color change from purple (negative reaction) to yellow (positive reaction), providing a visible and easily interpretable result without the need for specialized equipment. [15] Despite the demonstrated efficacy of XO-based LAMP for other pathogens, its application in monkeypox detection remains unexplored. This study aims to evaluate the feasibility of using xylenol orange-based cLAMP for rapid and accurate detection of monkeypox, potentially offering a valuable tool for early diagnosis and effective outbreak management.

Materials and Methods

The use of leftover DNA clinical samples in this study was approved by the Institutional Review Board of Bamrasnaradura Infectious Diseases Institute (S04 1h/66_ExPD).

Source of samples and total DNA template preparation

Neither live nor dead virus was used in this study. Only leftover total DNA samples derived from patient blood/lesion swab/throat swab provided by the Bamrasnaradura Infectious Diseases Institute were used. The DNA samples were prepared using the automated extraction machine (magLEAD® 12gC) with the MagDEADx SV kit (Precision System Science Co., Ltd., Chiba, Japan), in accordance with the manufacturer's instructions. The quantity of the obtained DNA was measured by spectrophotometric analysis at 260 and 280 nm and then adjusted to 50 ng/µl using DNase-free water. Two microliters of the template were used in further experiments and optimized LAMP reactions unless otherwise stated.

LAMP primer design

LAMP primers were designed from the F3L gene (F3 protein encoding sequence) of the MPXV genome (GenBank No. AF380138) by using NEB LAMP Primer Desing Tool Version 1.4.2. They consist of outer primers (F3, B3), inner primers (FIP, BIP) and loop primers (LF, LB). The primers were synthesized by Macrogen, South Korea, and their details will be shown in a separate manuscript which is in preparation and will support this proceeding.

Colorimetric LAMP assay optimization

To determine the optimal temperature for cLAMP, reactions were performed on a SimpliAmp Thermal Cycler (Thermo Fisher, USA) at 60, 63, and 65 °C for 1 hour. Each reaction contained 1.6 μM each of inner primers (FIP and BIP) and loop primers (LF and LB), 0.16 μM each of outer primers (F3 and B3), 0.4 M Betaine (Sigma-Aldrich, MO, USA), 0.05 mM XO (Sigma-Aldrich, MO, USA), 1.4 mM of dNTP mix, 6 mM MgSO4, 8 U *Bst* 2.0 DNA polymerase, 1× low-buffer 10 mM (NH4)2SO4, 50 mM KCl, 2 mM MgSO4, and 0.1% v/v Tween-20, and the specified amount of template DNA in a final volume of 25 μl. DNA-free LAMP reactions were included as negative controls. After incubation was completed, the products were analyzed by: 1) naked eye observation of color changes in reactions (purple = negative reaction, yellow/orange = positive reaction), and 2) 1.5% agarose gel electrophoresis (AGE). Results from both methods were compared and the optimal LAMP temperature was selected. For reaction time optimization, cLAMP reactions were performed at the optimized temperature for 45, 60, 75, and 90 minutes. The products were analyzed as mentioned above and the results were compared.

Comparative molecular sensitivity of cLAMP, LAMP-AGE, and PCR

Various amounts of MPXV-infected patient-derived total DNA templates (100 pg, 10 pg, and 1 pg/reaction) were subjected to cLAMP assays and LAMP-AGE. Both protocols were carried out at the optimal temperature for 45, 60, 75, and 90 minutes. The same set of templates was tested by an in-house PCR protocol using the F3 and B3 primers (see Section ***LAMP primer design***), and the results were compared to those from the LAMP assays. The number of positive reactions based on colorimetric results was used to calculate the positive rate of detection for each dilution. The last dilution, whose positive rate was still at 100%, was regarded as the detection limit (DL) of the method.

Specificity of cLAMP

The specificity of the cLAMP assay was examined using a panel of human respiratory viruses and other disease agents. These included (1) SARS-CoV-2, (2) Influenza B virus, (3) *Klebsiella pneumoniae* strain ATCC 700603, (4) *Acinetobacter baumannii* strain ATCC19606, (5) *Pseudomonas aeruginosa* strain ATCC 27853, (6) *Staphylococcus aureus*, (7) *Streptococcus pneumonia*, (8) *Listeria monocytogenes* strain ATCC 19115, (9) *S. epidermidis*, (10) Respiratory syncytial virus (RSV), (11) Influenza A virus subtype (PR8), (12) *Mycobacterium tuberculosis*, and (13) MPXV.

Clinical validation of cLAMP assay

The cLAMP was evaluated for its reliability to detect 60 DNA samples extracted from the blood of patients with known MPXV status as determined by two reference assays: the Bioperfectus monkeypox virus real-time PCR kit (BioPerfectus Technologies, Taizhou, China) and the Multiplex Realtime PCR kit for monkeypox virus and central/west African clade typing (Unimedica, Shenzhen, China). The samples were anonymized and provided by the Bamrasnaradura Infectious Diseases Institute, Thailand (IRB: S04 1h/66_ExPD). All test samples were blinded prior to analysis to prevent diagnostic bias regarding result determination. The diagnostic performance of our assay is summarized in **Table 1**.

Table 1. Clinical validation of the cLAMP assay against two qPCR reference assays.

Colorimetric LAMP	**MPXV status by reference assays**	
	Positive	Negative
Positive	30 (TP)[a]	0 (FN)
Negative	0 (FP)	30 (TN)
% Concordance with respect to qPCR results	100 (sensitivity) 100 (accuracy)	100 (specificity)

Sensitivity = [TP/(TP+FN)]×100, Specificity = [TN/(TN+FP)]×100

Accuracy = [(TP+TN)/(TP+TN+FN+FP)]×100

[a]TP, true positive; FP, false positive; FN, false negative; TN, true negative

Results

Overview of cLAMP assay pipeline

The process of colorimetric LAMP assay for MPXV detection is summarized in **Figure 1**. It consists of three main steps from sampling to readouts. 1) Sample preparation, which can be done using any DNA extraction reagent of interest. The sample matrix can be blood or swabs of lesion surfaces or exudate. 2) DNA addition and LAMP incubation under optimal conditions (63 °C for 1 hour). This step can be carried out in a heating block, thermal cycler, or incubator. 3) Result analysis by naked eye observation. During the incubation at 63 °C, in the presence of target DNA (F3L gene), the buildup of excess protons (H^+) in the reactions will result in a pH drop, changing the hues of XO from purple to yellow. In contrast, a lack of detection targets will preserve the original purple hue of the reaction. For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.

Determination of initial cLAMP conditions

Colorimetric LAMP reactions at 60, 63, and 65 °C with templates of 100 pg to 1 pg per reaction showed no difference in purple-to-yellow color density (**Figure 2A**). This result was consistent with that obtained by AGE, showing a similar pattern and yield of LAMP amplicons (**Figure 2B**). Therefore, 63 °C was arbitrarily selected as the optimal temperature.

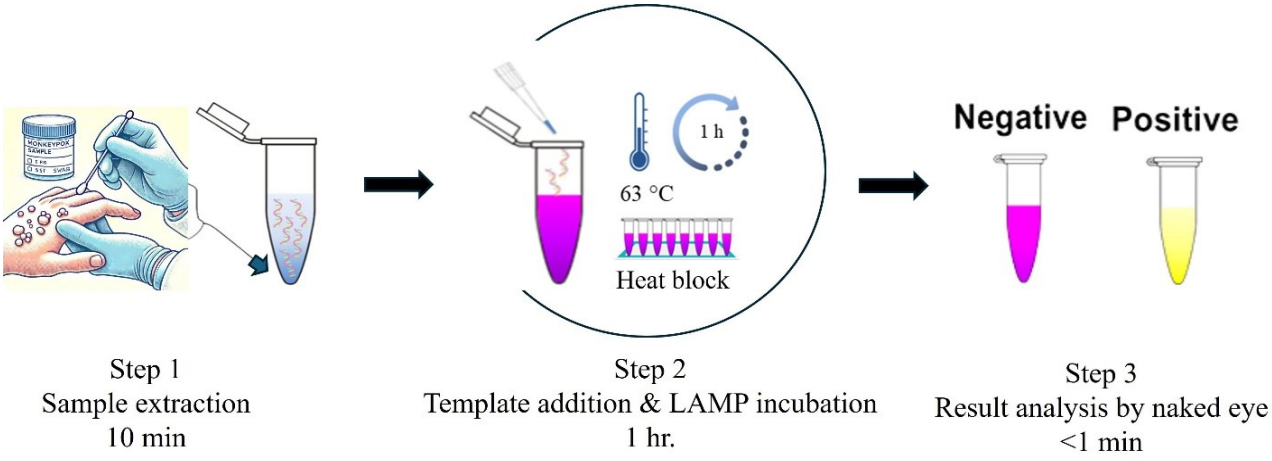

Figure 1. Process of colorimetric LAMP assay for MPXV detection

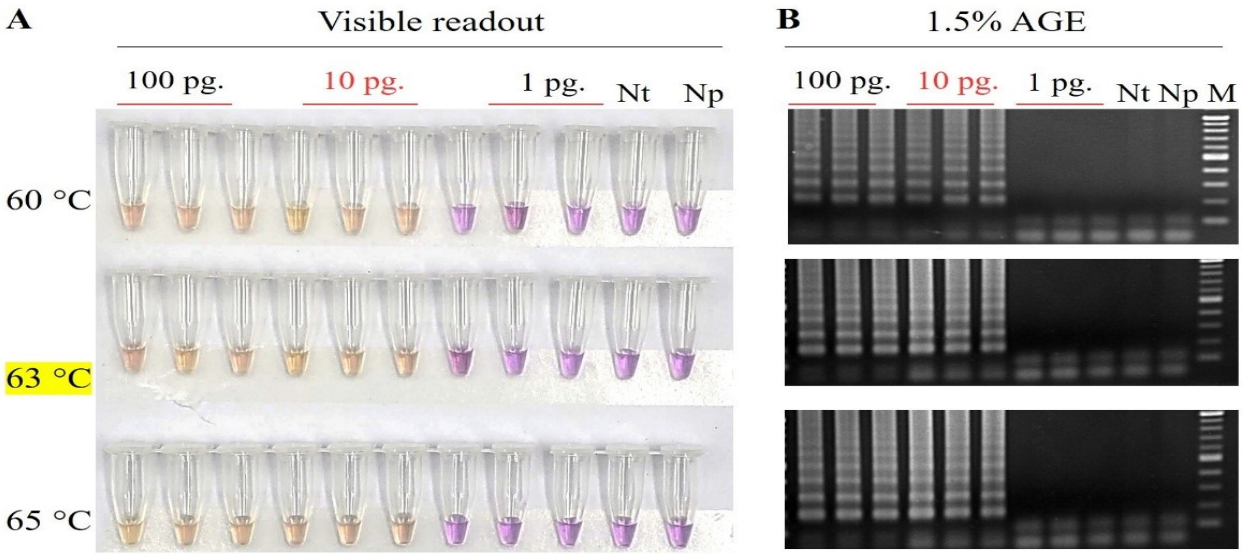

Figure 2. Optimization of cLAMP reaction temperature using various amounts of MPXV-DNA extract as template. The reactions were incubated at the given temperatures for 1 hour, followed by result analysis through naked eye observation **(A)** and AGE **(B)**. Lanes M, Nt, Np: molecular marker, premix-room negative control (DNase-free water added in the premix room), and template-room negative control (DNase-free water added in the template addition room), respectively.

Comparative sensitivity of cLAMP, LAMP-AGE, and PCR-AGE

From cLAMP reactions, they were incubated for various times (45–90 min) using different amounts of DNA template (**Figure 3A**), the change in reaction color from purple (negative result) to yellowish/orange (positive result) started to be detectable at 10 pg/reaction after 45 min. Clearly, the yellow shade was fully developed after 60 min of incubation onwards. All reactions with templates lower than 10 pg remained purple even after 90 min of incubation. The results were consistent with those obtained by AGE (**Figure 3B**). However, to allow maximum purple-to-yellow color development while avoiding non-specific amplification that may occur from excessive incubation, 60 min was selected as the standard assay time. The naked-eye DL of our assay was thus 10 pg/reaction which was 10 times more sensitive than the referred PCR (**Figure 3C**).

Specificity of cLAMP assay

The cLAMP assay challenged by DNA of various pathogens gave a yellow shade (positive result) only for MPXV (lane 13). For all other pathogens (lanes 1-12), reactions remained purple (**Figure 4A**). This result was supported by AGE results (**Figure 4B**), indicating that our cLAMP assay was highly specific for MPXV diagnosis.

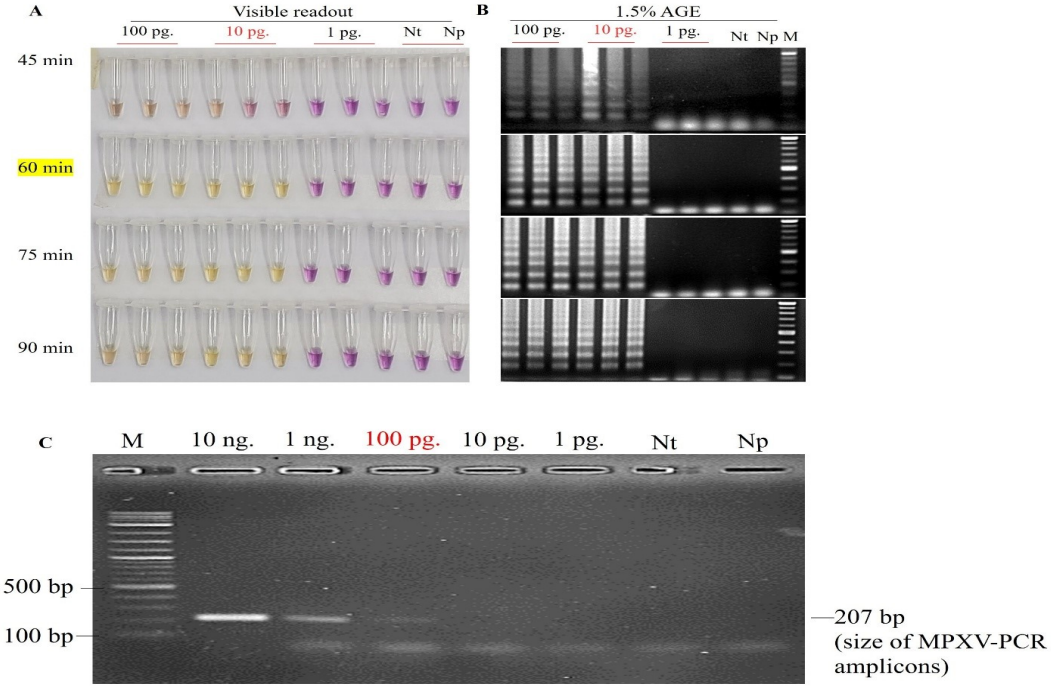

Figure 3. Sensitivity of the cLAMP, LAMP-AGE, and PCR protocols. **(A)** Colorimetric results read by the naked eye at various time points after amplification. The LAMP reactions were performed using 10-fold serially diluted MPXV-DNA extracts. **(B)** and **(C)** Results by LAMP-AGE and PCR, respectively, using the same templates as in (**A**). Lanes M, Nt, Np: molecular marker, premix-room negative control (DNase-free water added in the premix room), and template-room negative control (DNase-free water added in the template addition room), respectively.

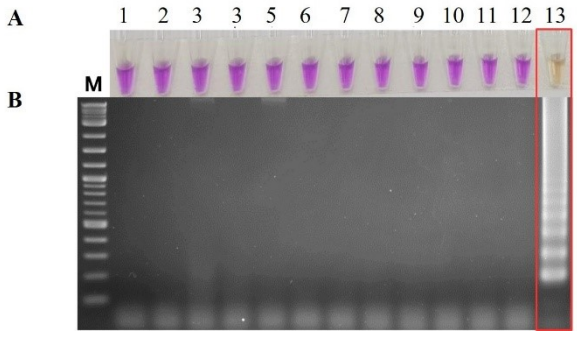

Figure 4. (A) The specificity of cLAMP and LAMP-AGE for MPXV detection as challenged by (1) SARS-CoV-2, (2) Influenza B virus, (3) *Klebsiella pneumoniae* strain ATCC 700603, (4) *Acinetobacter baumannii* strain ATCC19606, (5) *Pseudomonas aeruginosa* strain ATCC 27853, (6) *Staphylococcus aureus*, (7) *Streptococcus pneumonia*, (8) *Listeria monocytogenes* strain ATCC 19115, (9) *S. epidermidis*, (10) Respiratory syncytial virus (RSV), (11) Influenza A virus subtype (PR8), (12) *Mycobacterium tuberculosis*, and (13) MPXV. **(B)** AGE results corresponding to the samples shown in (A). M: molecular marker.

Clinical validation of cLAMP assay

We evaluated the diagnostic performance of our cLAMP assay with 60 blinded samples. As shown in Table 1, 30 samples were identified as positive for MPXV, while the remainder were determined to be negative. The results were identical to those obtained by the reference methods, giving 100% statistical sensitivity, 100% specificity, and 100% accuracy.

Discussion

This study seeks to innovate the current diagnostic landscape by applying XO-based cLAMP for the first time in monkeypox detection, striving to achieve a diagnostic method that is both simple and precise (**Figure 1**). The assay targets the F3L gene, which encodes the F3 protein, believed to play a significant role in the virus's interaction with the host's immune system. [16,17]

For cLAMP assay optimization, a reaction performed at 63 °C for 1 hour was defined as optimal as it maximized DNA amplification-dependent purple-to-yellow color development in the least assay time required (**Figure 2**). Next, we determined the detection sensitivity at different time points. A detection limit (DL) by visual inspection is shown in **Figure 2A**, where a range of MPXV-DNA amounts (100 pg to 1 pg) was detected. At 60 minutes onwards, all template concentrations indicated a positive reaction (yellow), except those below 10 pg/reaction, which remained negative (purple) (**Figure 2A**). Further increasing the incubation time until 90 minutes did not improve the sensitivity, indicating that the colorimetric reactions reached their endpoint with a DL of 10 pg, namely 10 times more sensitive than PCR (**Figure 2C**). We also used recombinant plasmid DNA harboring F3L gene as template and found that our assay detected the plasmid down to 10 copies/reaction (data not shown). Moreover, the assay reacted with MPXV only (**Figure 4**). This high degree of specificity was attributed to the use of well-designed primers to target MPXV-DNA specifically and the experimentally defined optimal assay conditions.

Compared to other existing techniques, our method is superior to other recently reported MPXV colorimetric platforms that leveraged lateral flow biosensing LAMP with respect to the ease of use as our technique abrogates the need for post-amplification workflow associated with hybridization and readout development. [18-21] It is also better than qPCR methods recommended by CDC in terms of simplicity and decentralized applicability. [22-24] Regarding the detection limit of LAMP-XO for various pathogens, studies have shown that LAMP-XO can identify bacteria such as *E. coli* and *V. parahaemolyticus* at concentrations as low as 1 CFU and 100 CFU, respectively. [9,12] Furthermore, it has demonstrated the capability to detect viruses, including SARS-CoV-2 and scale drop disease virus (SDDV), at the respective threshold of 50 copies and 100 copies per reaction. [10,11] This highlights the robustness and broad applicability of LAMP-XO across various sample matrices and applications.

As for limitations, we recognize that our assay still utilizes a standard DNA extraction method to prepare test samples. However, rapid nucleic acid preparation techniques have been demonstrated to be compatible with colorimetric isothermal amplification reactions. [11,25,26,27] Thus, future studies should focus on investigating the feasibility of using direct input samples (i.e. swab) or minimally processed ones with our colorimetric LAMP assay.

Conclusion

We developed the first rapid colorimetric LAMP assay for monkeypox screening using clinical samples collected in Thailand. The test was validated with 60 patient samples, demonstrating 100% accuracy, and its simple analysis makes large-scale testing feasible in low-resource settings. While we acknowledge the current limitations regarding sample size and acquisition methods, the success demonstrated in this study lays the groundwork for future validation with a larger cohort and a broader range of sample types. With a cost of approximately $3 per test and a turnaround time of less than 75 minutes, this test enables frequent and widespread monkeypox screening, improving its effectiveness in the population. We envision that the platform established here will serve as a valuable tool to accelerate the development of diagnostics for emerging pathogens.

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Conflict of interest statement

The authors declare that we do not have any conflict of interest for this manuscript.

Data sharing statement

All data generated or analyzed during the present study are included in this published article. Further details are available for non-commercial purposes from the corresponding author on reasonable request.

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