Supporting Information for

Two-Dimensional Nanozyme-Catalyzed Colorimetric CRISPR Assay for the Microfluidic Detection of Monkeypox Virus

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S1 Experimental section

S1.1. Clinical Samples and Ethical Statement.

Clinical samples for this study were collected from the Beijing Ditan Hospital, and negative samples were collected from healthy volunteers, with informed written consent from all participants. Rash fluid swab and oral swab were collected and stored in disposable virus sampling tube. This study was performed following the guidelines approved by the Ethics Committee of Beijing Ditan Hospital (approval ID: DTEC-YJ2023-001-02).

S1.2. Materials and Chemicals.

Monolayer GO (Catalog #100682, 200–500 nm) was supplied by XFNANO, Inc. (Nanjing, China). Polyethyleneimine branch (PEI, MW 25 kDa), phosphate buffered saline (PBS), bovine serum albumin (BSA), fetal bovine serum, and Tween-20 were provided by Sigma–Aldrich (USA). Nuclepore track-etch membrane filtration with 2 μm pore size was obtained from Whatman (Germany). LwaCas13a (Catalog #32117-01) was supplied by Tolo Biotech Co, Ltd. Lyophilized RPA pellets and rehydration buffer (Catalog #TABAS03KIT) were provided by TwistDx (UK). T7 RNA polymerase (Catalog #M0251), RNase H (Catalog #M0297), and rNTP mix (Catalog N0466) were provided by New England Biolabs Inc (USA). RNase Inhibitor (Catalog #R8061), DNase/RNase-free water (Catalog #R1600), and Chelex-100 (Catalog #C8230) were purchased from Solarbio Life Science (Beijing, China). MPXV were quantified by digital PCR after being diluted 1000 times. The quantification results are presented in **Figure S1**. RNA Clean & Concentrator-5 Kit (Catalog #R1013) was supplied by Zymo Research (USA). RQ1 RNase-Free DNase (Catalog #M6101) was provided by Promega. Qubit™ RNA Broad Range Assay Kits (Catalog #Q10211) were obtained from Thermo Fisher Scientific (USA). Viral Extraction kits (Catalog #DP315-R) were acquired from Tiangen Biotech (Beijing, China).

S1.3. Preparation of Au seeds.

Au seeds of 3 nm diameter are prepared by trisodium citrate (TSC) reduction. In brief, 1 mL of 1% (w/w) $HAuCl_4$ solution was mixed with 98 mL of deionized water, and the mixture was heated. After boiling, 10 mL of 1% (w/w) TSC solution was rapidly added

to form 3 nm Au seeds. The solution was held at 100 °C with magnetic stirring for 15 min. Finally, the Au seed suspensions were naturally cooled and then stored at room temperature until use.

S1.4. Antibody modification of GO@Pt nanozyme.

GO@Pt (200 μL) was centrifuged at 5000 rpm for 5 min, and the supernatant was discarded. Then, 200 μL of 0.01M Tris-HCl (pH=8.8) was added and ultrasonic shock was performed for 10–20 s. The solution was added with 15 μg of FAM antibody, mixed, and oscillated at 800 rpm and room temperature for 2–2.5 h. After adding 100 μL of BSA for sealing for 1–1.5 hours, the mixture was centrifuged at 5000 rpm for 5 min and the supernatant was discard. Then, 500 μL of 0.01M Tris-HCl (pH=8.8) was added, ultrasonic shock was performed for 10 s and centrifugation at 5000 rpm for 5 min, and the supernatant was discarded. Finally, 200 μL of Tris-HCl and 100 μL of storage solution (10 mM Tris-HCl containing 1 mg BSA (w/v), 0.5% sucrose (w/v), 0.02% NaN3 (w/v)) was added.

S1.5. Preparation of crRNA.

The ssDNA template with the reverse complementary sequence of the crRNA was synthesized with a T7 promoter by Sangon. In brief, 1 μL of DNA template (100 μM), 1 μL of Standard Taq buffer (10×), 1 μL of T7-3G-IVT primer (100 μM), and 7 μL of DNase/RNase-free water mixed for annealing reaction. The master mix was denatured at 95 °C for 5 min, followed by gradient annealing with the ramp rate at 0.1 °C/s until cooling to 4 °C using a thermal cycler (Thermo, USA). The annealing products were then transcribed at 37 °C for 8–12 h with HiScribe T7 High Yield RNA Synthesis Kit. The transcribed crRNA was purified by RNA purification reagent RNA Clean & Concentrator-5 Kit. Finally, the crRNA concentration was quantified by the Qubit™ Flex Fluorometer (Thermo, USA) with the Qubit™ RNA Broad Range Assay Kits and stored at –80 °C.

S1.6. MPXV detection by qPCR.

The total reaction system was 25 μL consisting of 6.5 μL of reaction buffer, 1 μL of enzyme, 5 μL of target DNA, and 12.5 μL of DNase/RNase-free water. The details of MPXV-PCR primer and probe are displayed **Table S1**. Real-time fluorescence PCR

was conducted with the following thermocycling program: 50 °C for 2 min, 95 °C for 2.5 min, and 40 cycles of 95 °C for 30 s and 55 °C for 30 s.

S1.7. Characterization.

The TEM and HR-TEM images of fabricated nanomaterials were taken on Hitachi H-7650 TEM and Tecnai G2 F20 microscope, respectively. Elemental mapping images were obtained by energy-dispersive X-ray spectroscopy (EDS) using a Philips Tecnai G2 F20 microscope with a STEM unit. Zeta potential was measured by a Zetasizer Lab (Malvern, UK). The real-time fluorescence curves of the optimization of RPA-CRISPR/Cas13a experiment were determined using a PCR instrument (ABI-7500, Thermofisher, USA). A smartphone (Xiaomi Mix2) is utilized to capture images of the final result, and software image J is employed to analyze the color intensity.

S1. Digital PCR for quantification of monkeypox virus.

Monkeypox viruses were quantified by digital PCR after being diluted 1000 times. 2 μL template was incorporated into the ddPCR reaction system, and the test result was presented as follows.

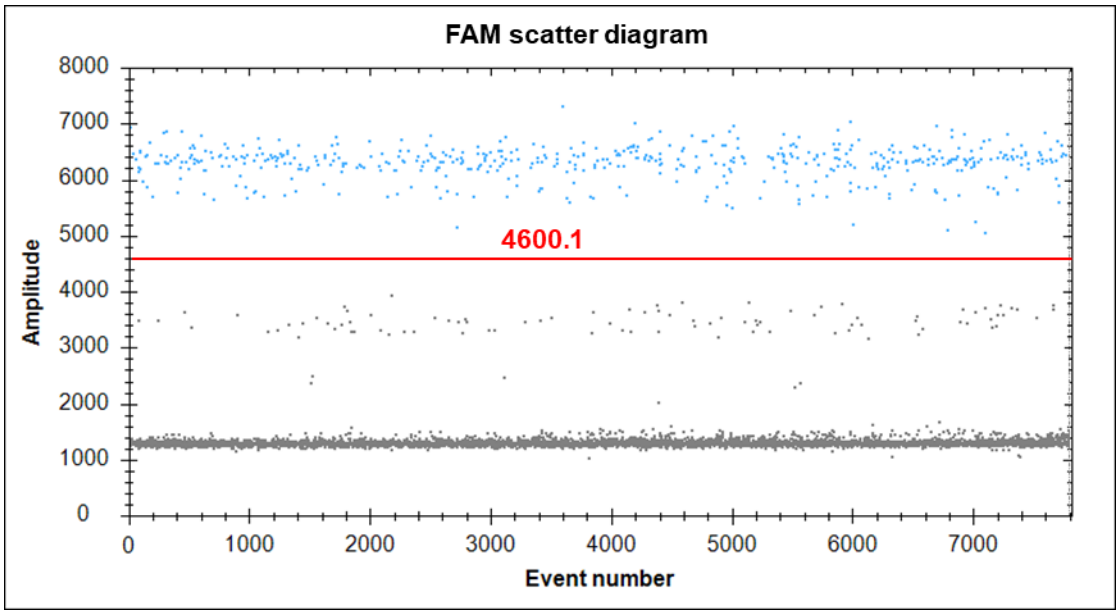


Figure S1 Quantification of monkeypox virus by ddPCR assay.

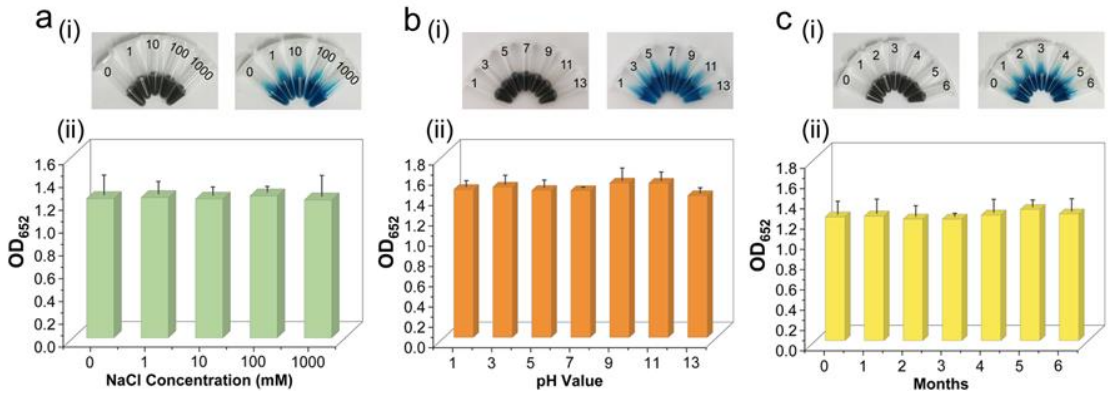


Figure S2. Stability of GO@Pt nanozyme in different circumstance. The GO@Pt nanozyme demonstrates outstanding stability in highly saline solution (a) and aqueous solutions within the different pH (b), and the storage duration (c).

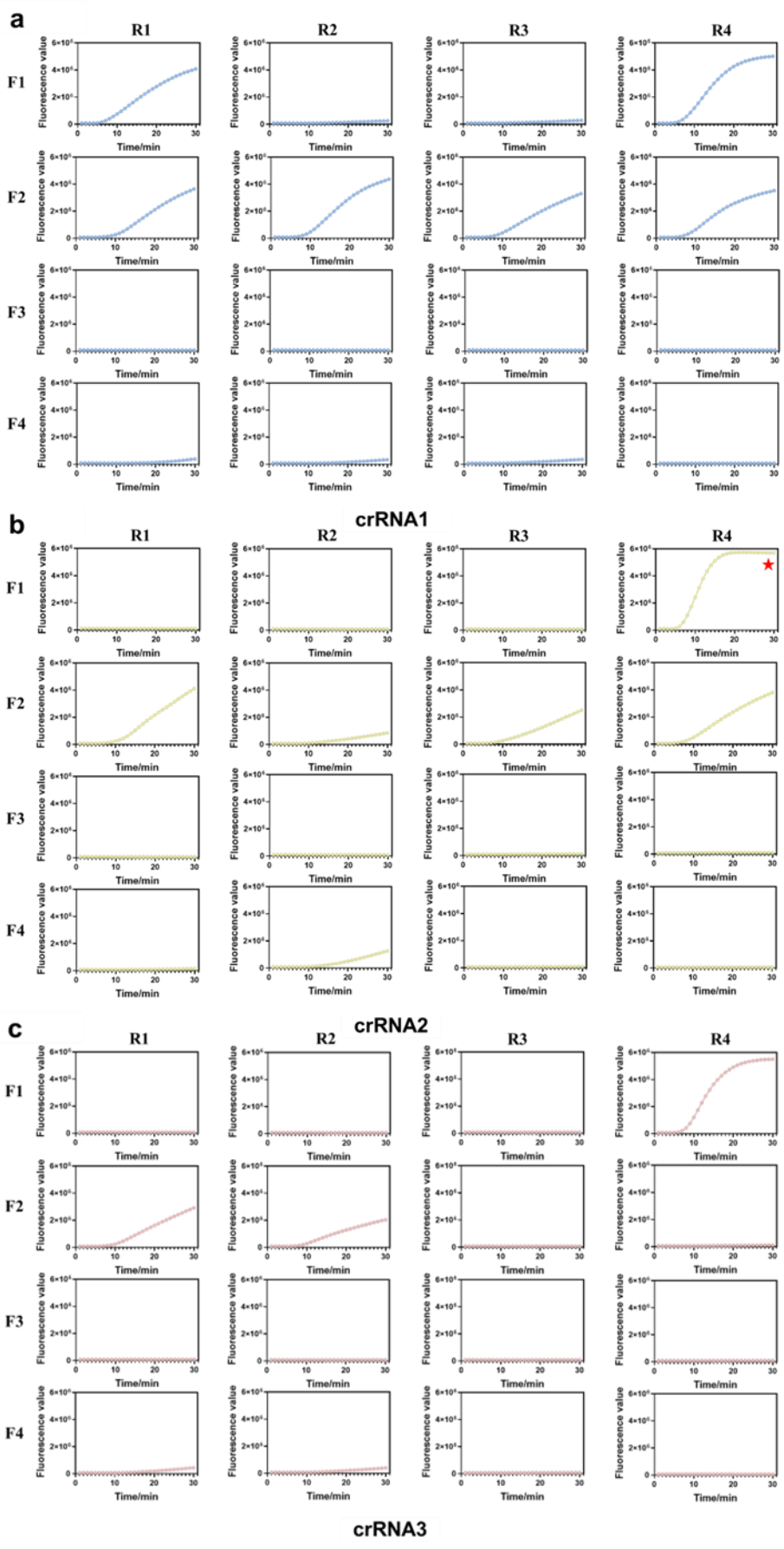

a
R1
R2
R3
R4
F1
F2
F3
F4
Fluorescence value
Time/min
crRNA1
b
crRNA2
c
crRNA3

Figure S3. Primers and crRNAs screening. (a-c) Real-time fluorescence curves of RPA primers and Cas13a crRNA screening were tested by PCR instrument ABI-7500 using the single-step RPA-CRISPR/Cas13a reaction for 30 minutes to detect the MPXV B6R gene. Target: MPXV B6R gene of 10^3 copies/reaction.

S2. Verification of the target-triggered trans-cleavage of CRISPR/Cas13a

MPXV nucleic acid with a concentration of 10^4 copies/μL was selected for RPA at 37 ℃ and T7 transcription, then denatured at 95 ℃ for 10 minutes, and purified by RNA purification kit. The products were divided into two groups: positive group with Cas13a protein added, and negative group without Cas13a protein. The real-time fluorescence curve shown in **Figure S5** verified the target-triggered trans-cleavage of CRISPR/Cas13a.

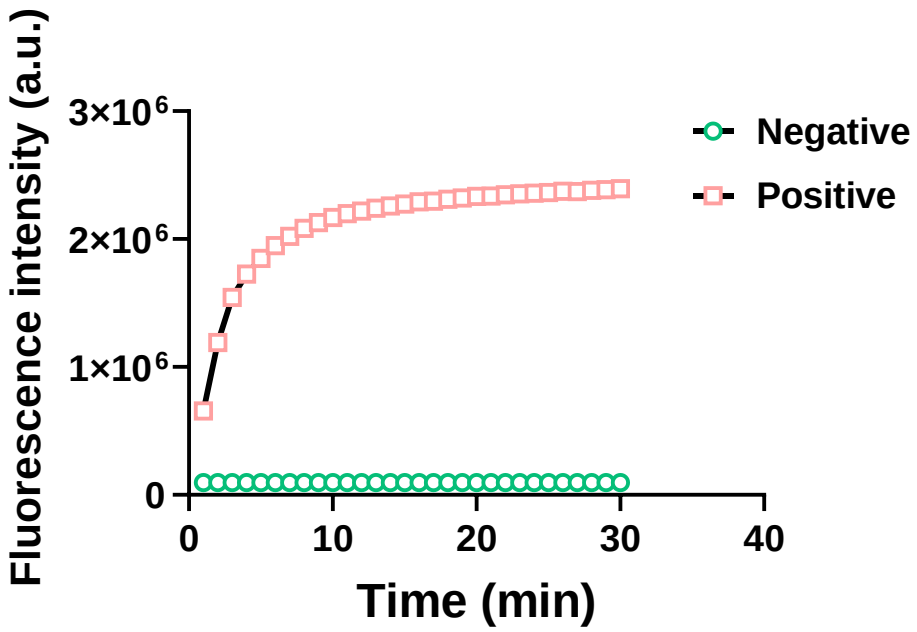


Figure S4. Verification of the target-triggered trans-cleavage of CRISPR/Cas13a.

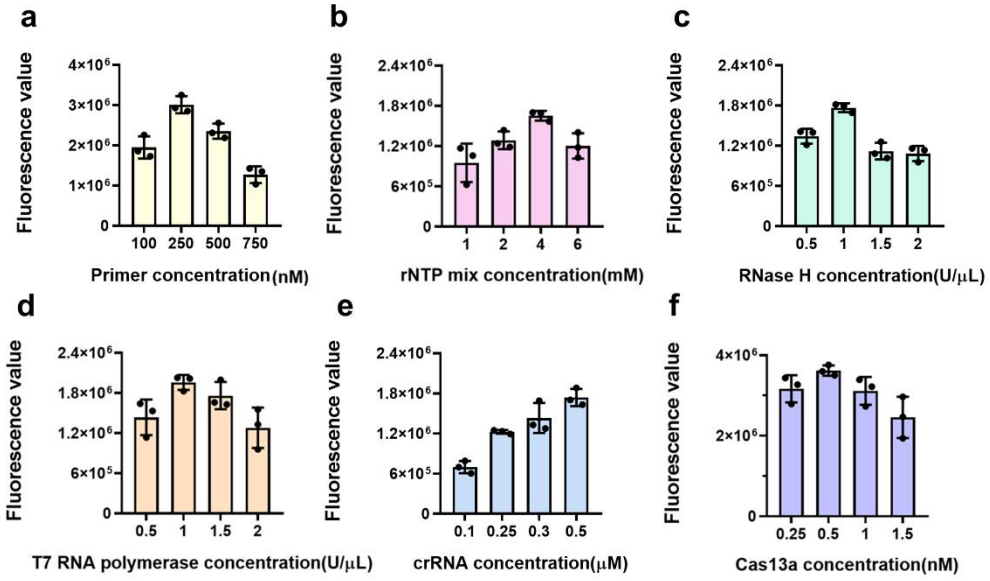


Figure S5. Optimization of CRISPR/Cas13a detection system. (a-f) The end-point fluorescence value of the CRISPR/Cas13a detection for the optimization of six decisive reaction conditions, including primer concentration of 100, 250, 500 and 750 nM; the rNTP concentration of 1, 2, 4, and 6 mM; the RNase H concentration of 0.5, 1, 1.5, and 2 U/μL; the T7 RNA polymerase concentration of 0.5, 1, 1.5, and 2 U/μL; crRNA concentration of 0.1, 0.25, 0.3 and 0.5 μM; Cas13a concentration of 0.25, 0.5, 1, and 1.5 nM. Error bars represent the standard deviation of three repetitive experiments.

S3. Optimization of GO@Pt.

GO/Au seed nanosheets of 200, 400, 800, and 1600 μL were centrifuged at 6000 rpm for 6 minutes and then resuspended in 800 μL, 0.4% PVP solution respectively, and dispersed by ultrasound. In the above solution, 40 μL AA (100 mg/mL) was added to the product for ultrasonic dispersion, and 40 μL of H_2PtCl_4 (100 mM) was added drop by drop, and incubated at 56 ℃ for 35 minutes. After reaction, the products were centrifuged for 5 minutes at 5000 rpm, washed once with deionized water and then resuspended in 200, 400, 800, and 1600 μL anhydrous ethanol respectively.

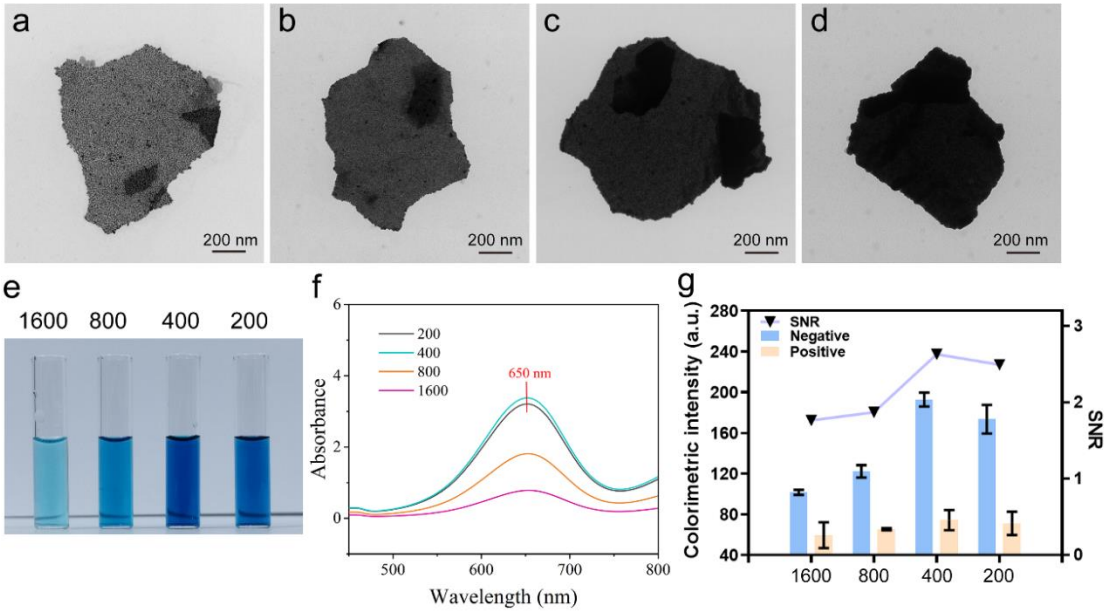


Figure S6. Optimization of GO-Au seeds amount. (a-d) TEM images of GO@Pt nanozymes prepared with 1600, 800, 400, and 200 μL of GO/Au seeds, respectively. **(e)** Photographs and **(f)** absorbance spectra of TMB solutions catalyzed by GO@Pt nanozymes prepared with 1600, 800, 400, and 200 μL of GO/Au seeds. **(g)** Signal-to-noise ratio determined by using GO@Pt nanozymes prepared with 1600, 800, 400, and 200 μL of GO/Au seeds. Error bars represent the standard deviation of three repetitive experiments.

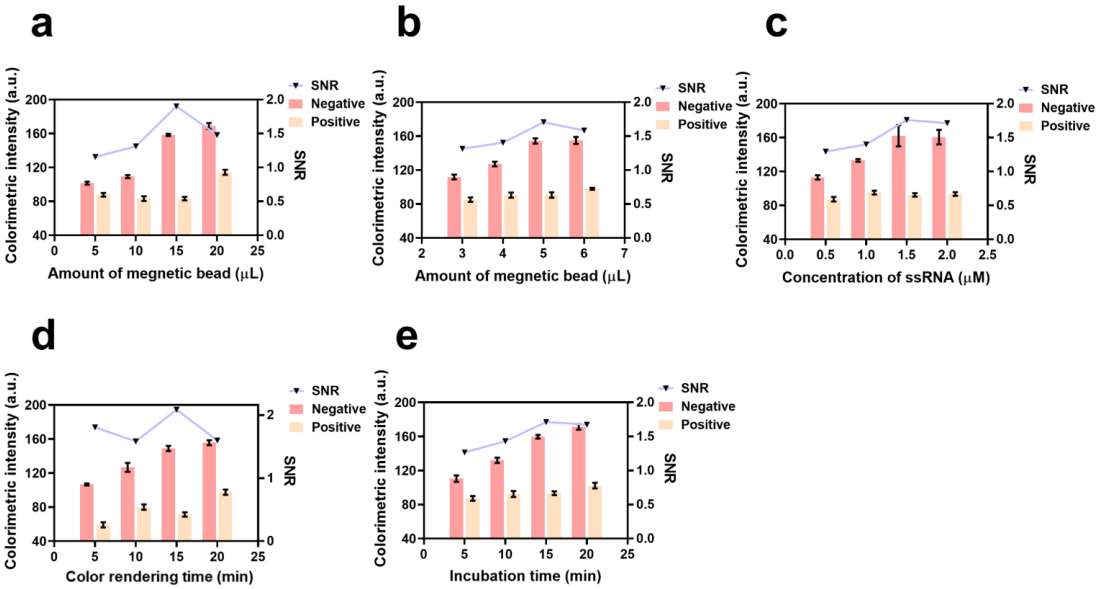


Figure S7. Optimization of experimental conditions related to nanozymes. (a-e) The detection intensity of several vital experimental conditions of CRISPR/Cas13a detection system based on nanozyme, such as the nanozyme amount of 5, 10, 15 and 20 μL; the magnetic beads amount of 3, 4, 5 and 6 μL; ssRNA concentration of 0.5, 1, 1.5 μM; Color rendering time of 5, 10, 15 and 20 minutes; Incubation time of 5, 10, 15, and 20 minutes. Error bars represent the standard deviation of three repetitive experiments.

S4. Synthesis and characterization of Au@Pt nanozymes.

Au seed solution (480 μL)into 520 μL of deionized water. Then, the mixture was added with 40 μL of 10% (w/w) polyvinylpyrrolidone (PVP) solution, and the solution was swirled briefly and incubated for 5 min so that the polymer can evenly coat and stabilize the Au seeds. Subsequently, 40 μL of L-ascorbic acid (L-AA) solution (100 mg/mL) and 40 μL of chloroplatinic acid hydrate (100 mM) were added dropwise into the solution, followed by constant oscillations (800 rpm, 30 min) at 65 ℃. During this process, Pt gradually deposited onto the Au seeds and eventually formed a fluffy-like Pt shell. Finally, the Au@Pt nanoparticles were collected by centrifugation (7600 rpm, 6 min), washed twice with deionized water, and resuspended in 960 μL of deionized water for future use. With the increase of the concentration of the two nanozymes, the color rendering effect is better. However, under the same concentration, the color rendering effect of GO@Pt nanozymes is better than that of Au@Pt nanozymes.

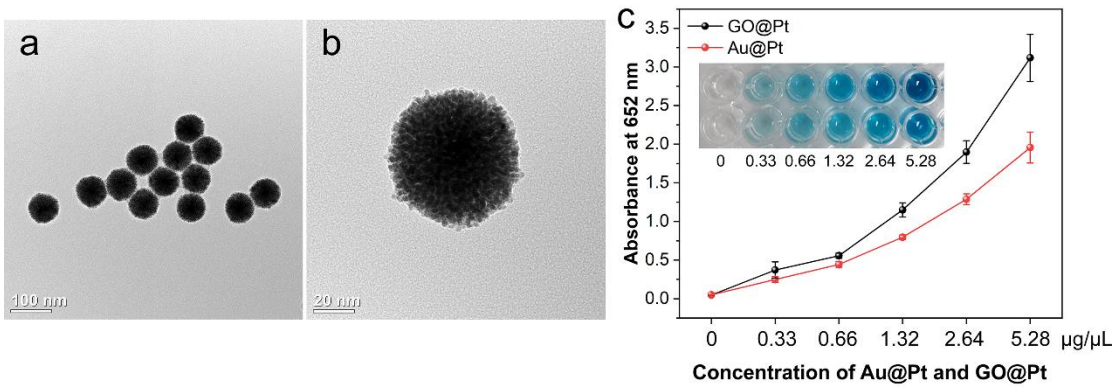


Figure S8. (a-b) TEM images of Au@Pt nanozymes. (c) The catalytic color development of two nanozymes at the same concentration.

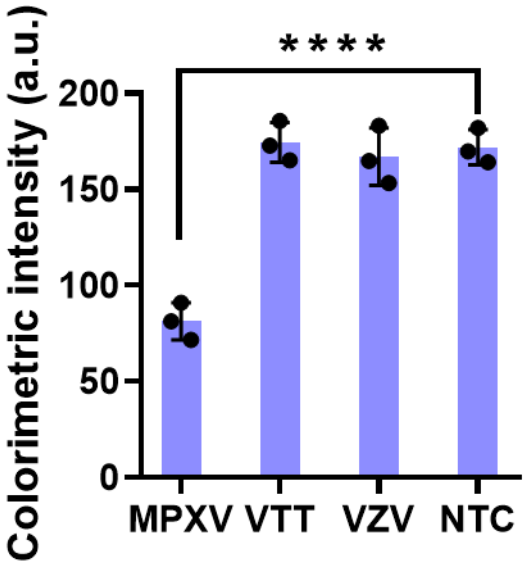


Figure S9. Specificity of this diagnostic system. VTT and VZV are selected to verify assay specificity.

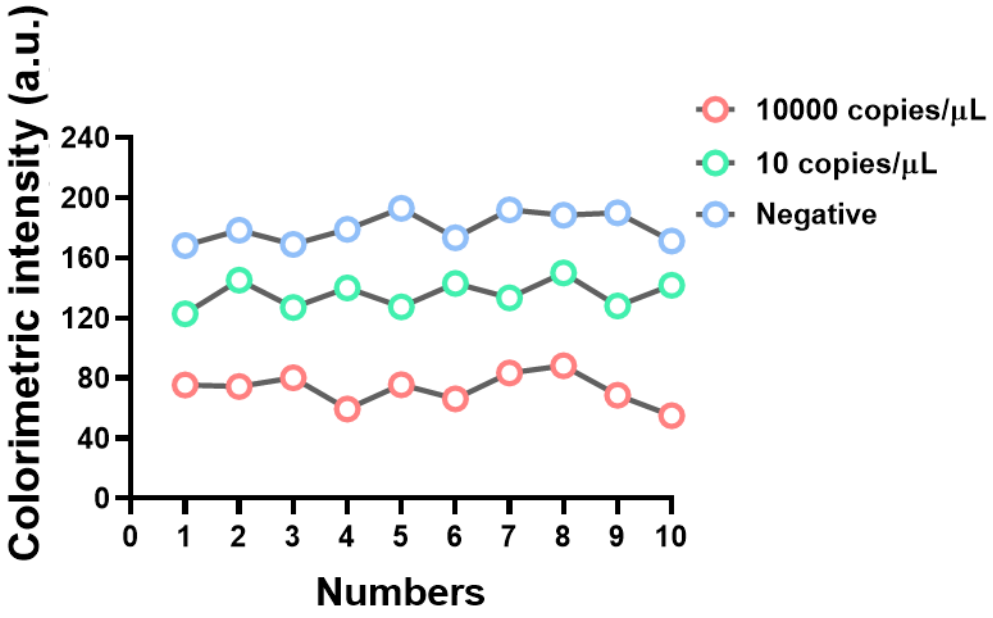


Figure S10. Repeatability of this diagnostic system. The repetitive detection results of Monkeypox virus nucleic acid at the concentrations of 0, 10, 10000 copies/μL.

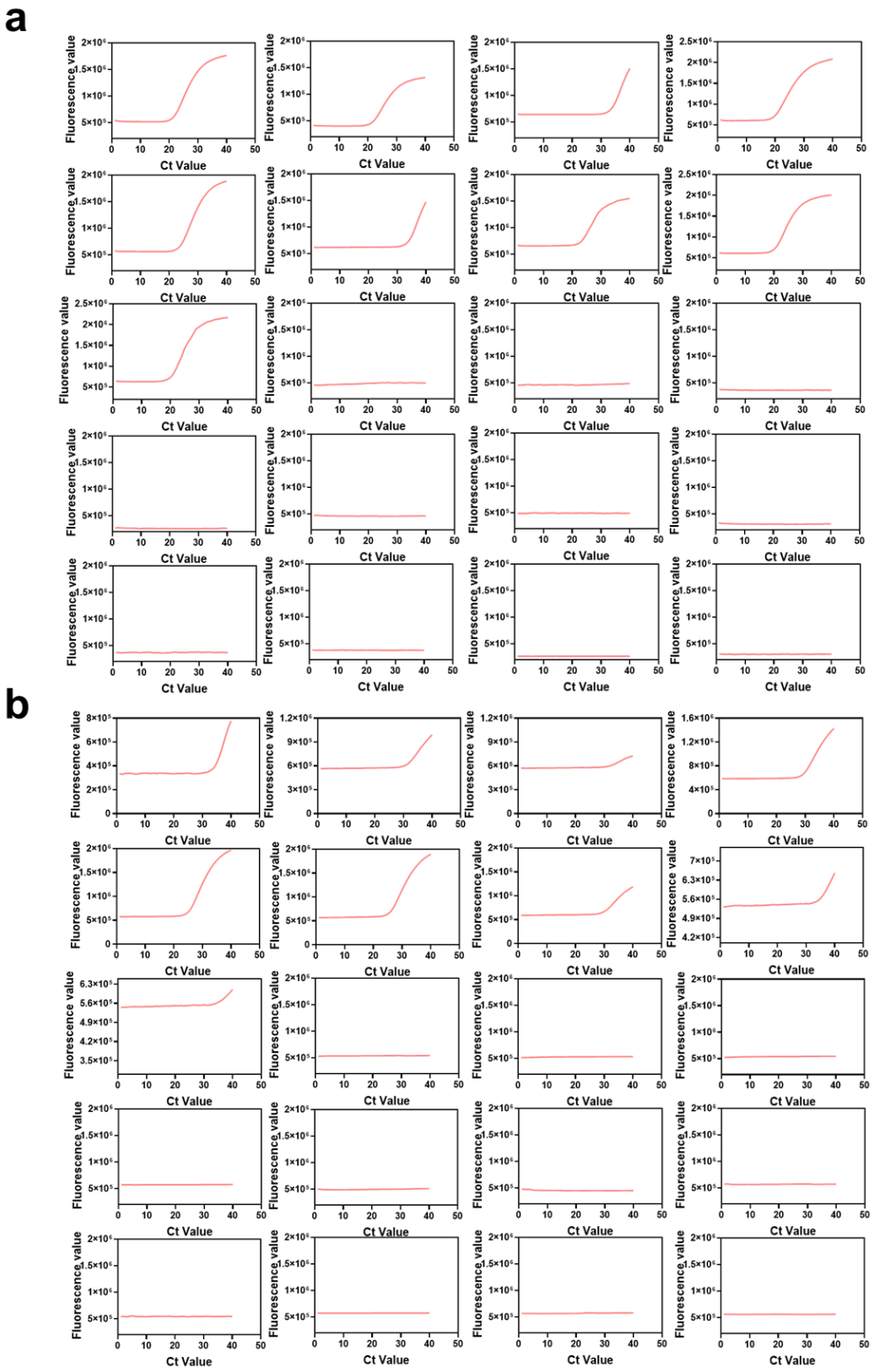


Figure S11. Clinical sample detection via qPCR. (a) The real-time fluorescence curve of rash fluid swab samples (9 positive samples and 11 negative samples) tested by qPCR. **(b)** The real-time fluorescence curve of oral swab samples (9 positive samples and 11 negative samples) tested by qPCR.

Table S1. The sequences of primers and CRISPR crRNA used in this study.

Name	***Sequence (5' - 3')***
MPXV-B6R-F1	*cctctaatacgactcactatagggACTATATGATAAGCCATTATACGAAGT*GAA
MPXV -B6R-F2	*cctctaatacgactcactatagggTCTCTGAACTATATGATAAGCCATTAT*ACG
MPXV -B6R-F3	*cctctaatacgactcactatagggATTATGTCTCTGAACTATATGATAAGC*CA
MPXV -B6R-F4	*cctctaatacgactcactatagggTTTCTGATTATGTCTCTGAACTATATG*AT
MPXV -B6R-R1	GTATTTCCATTTTTTTCTTCACAACGAAAA
MPXV-B6R-R2	GAAGTATTTCCATTTTTTTCTTCACAACGA
MPXV-B6R-R3	GTGACAGTATCATTCCAAGAAGTATTTCCA
MPXV-B6R-R4	GGACACGTGACAGTATCATTCCAAGAAGTA
MPXV-crRNA1	CCACCATGACACTAAGTTGCAACGGTGA*gttttagtccccttcgttttgggg* *gtagtctaaatcccctatagtgagtcgtattaatttc*
MPXV-crRNA2	ACCATGACACTAAGTTGCAACGGTGAAA*gttttagtccccttcgttttgggg* *gtagtctaaatcccctatagtgagtcgtattaatttc*
MPXV-crRNA3	CATGACACTAAGTTGCAACGGTGAAACA*gttttagtccccttcgttttgggg* *gtagtctaaatcccctatagtgagtcgtattaatttc*
MPXV-PCR-F	GCATTATTTTTAGCATCTCGT
MPXV-PCR-R	TTATGCCTGTGTAGACATTGAC
MPXV-PCR-P	FAM-TCCATCTGCCTTATCGAATACTCT-BHQ1
T7-3G-IVT primer	GAAATTAATACGACTCACTATAGGG

Table S2. Comparison of our developed method with the existing CRISPR assays for MPXV detection.

Assay	Target	Sample pretreatment	Detection method	Readout	LoD	Assay time	Reference
Amplification-free CRISPR/Cas12a	Pseudoviruses	Nucleic acid extraction	Surface plasmon resonance	Fiber tip biosensor	59.5 copies/μL	90 min	11
DNAzyme-based chemiluminescence CRISPR/Cas12a	MPXV plasmid	/	Chemiluminescence	Smartphone-assisted imaging cartridge	5.2 copies/μL	>60 min	45
Two-step RPA, Cas12a	Pseudoviruses	Nucleic acid extraction	Fluorescence	Plate reader	5 copies /reaction	60 min	46
Two-step RPA, Cas12a	Virus	Nucleic acid extraction	Fluorescence/ Colorimetric	Plate reader or lateral flow strip	1 copy/μL	45 min/ >60 min	1
One-pot RPA, Cas12a	Synthetic DNA	/	Fluorescence	Visual detection or plate reader	15 copies/μL	35 min	47
Single-step RPA-Cas12a	Pseudoviruses	Viral lysis	Fluorescence	Homemade palm-size pouch	10.6 particles/μL	25 min	48
Single-step RPA-Cas13a	Virus	Viral lysis	Fluorescence	Homemade tiny device	0.5 copies/μL	15 min	49
Nanozyme-catalyzed RPA-Cas13a	Virus	Viral lysis	Colorimetric	Naked eyes	1 copy/μL	~60 min	This work