Supplementary Information for

Sensitive and Specific CRISPR-Cas12a Assisted Nanopore With RPA For Monkeypox Detection

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1 Materials and methods

1.1 Materials and Chemicals

The study utilized synthetic DNA of MPXV, purchased from ATCC bio (Quantitative Synthetic Monkeypox virus DNA VR-3270SD™, 100 µL, 2.8×10^5 copies/µL, storage -80°C). We also purchased the Cowpox virus strain Brighton VR-302D™ genomic DNA from the same source (100 µL, 3.2×10^3 copies/µL). PCR probe, Nuclease Free Water, DNaseAlert™ Substrate, Alt-R™ L.b. Cas12a crRNA (Cpf1), and crRNA obtained from Integrated DNA Technologies (IDT). Qiagen (#28104) was used for DNA purification. Essential materials, including 1 kb DNA Ladder(#N3232S), Gel Loading Dye, Purple (6X) (#B7025), M13mp18 ssDNA (#N4040S, storage -20°C), and NEBuffer™ r3.1 (#B6003S)were sourced from NEW ENGLAND Biolabs Inc. (NEB). Key reagents such as EDTA (0.5 M) (#AM9260G), HEPES (1 M) (#15630080), $MgCl_2$ (1 M, #AM9530G), NaCl (5 M, #AM9760G), SYBR™ Safe DNA Gel Stain, Ultra Low Range D.N.A. Ladder (#10597012), TaqMan™ Fast Virus 1-Step Multiplex Master Mix (#5555532) and Tris-EDTA-buffer solution were purchased from Thermo Fisher and Sigma-Aldrich. (TwistDx Ltd, Cambridge, UK) provided the RPA reaction basic kits and enzymes (Freeze-dried pellet tubes; # TABAS03KIT). We also purchased 200 µL Greiner 96 Flat Bottom Transparent Polystyrene (#655101). For experimental procedures, Quartz capillaries (inner and outer diameter of 0.5 and 1 mm respectively, Sutter Instrument, USA), Silver Wire (008 Inches, 25 Feet), and accessories like Pipette holder (#663040), Seal (#647110), and Pin Connector (#647110) were sourced from A-M Systems. Furthermore, a microinjector with 34 gauge was purchased from World Precision Instruments.

1.2 MPXV RPA assay

Design. For our synthetic DNA sequences, we employed the F3L genes of the monkeypox virus, which span from the 46,337th to the 46,453rd nucleotide (ID: MPXV_USA_2022_OR0002, source ID: ON563414.3). Our primer designs were specifically aligned to the target genes, as confirmed by a BLAST search (refer to https://blast.ncbi.nlm.nih.gov/Blast.cgi). These primers were developed using PrimedRPA62 and assessed using the PrimerQuest Tool from IDT to ensure no formation of primer dimers or hairpin structures. The primers, summarized in Supplementary

data **Table S1**, underwent commercial synthesis and purification.

Assay. The RPA reaction, with a total volume of 50 µL, incorporates 2.4 µL of each forward and reverse oligos (10 µM), 29.5 µL of rehydration buffer, 12.2 µL of nuclease-free water, 1 µL of DNA sample, and lastly 2.5 µL of MgAcO (280 mM) to prevent premature reaction initiation (refer Supplementary data **Table S2**). This master mix is initially incubated at 39°C for 4 min, then vortexed and centrifuged, and finally left to react at 39°C for 20 min.

1.3 MPXV PCR

The PCR reaction is carried out in a total volume of 20 µL, including 1.2 µL of each of the forward and reverse oligos at a concentration of 10 µM, along with 0.50 µL of a 10 µM probe. We added 5 µL of 4X TaqMan Fast Virus 1-Step Master Mix, 11.10 µL of nuclease-free water, and 1 µL of the DNA sample. The same primers used in the RPA are employed for the PCR reaction. The sequences for the PCR probe can be found in Supplementary Data **Table S1**. The Bio-Rad CFX96 system is used to conduct the PCR reaction and measure the fluorescence signal, following a thermal profile that includes an initial denaturation step at 95°C for 2 min. This is followed by 45 cycles, each consisting of a 30-second denaturation at 95°C, a 45-second primer annealing phase at 55°C, and a 1-min extension phase at 72°C (Chen et al., 2023).

1.4 MPXV CRISPR Assay

RNP formation. The crRNA was reconstituted in nuclease-free water to 100 µM and stored at -80 ºC. 67 µM (1.95 µL) LbCas12a and 100 µM (1.52 µL) crRNA were mixed in a custom-made 1X buffer (0.1 M NaCl, 5 mM $MgCl_2$, 20 mM HEPES, 0.1 mM EDTA, pH 6.5@25°C) to form the unactivated RNP complex. The RNP was left at room temperature for 20 min and stored at -80ºC. It was further diluted by adding 1X buffer, achieving final concentrations of 317.8 nM and 369.80 nM, as detailed in Supplementary Data **Table S6**.

Real-time CRISPR Assay. The CRISPR reaction is executed in a cumulative volume of 100 µL, incorporating 10 µL of the inactive RNP complex, 10 µL of the RPA-amplicons, 50 µL of nuclease-free water, 20 µL of a 5X buffer, and 10 µL (2 µM)) DNaseAlert probe (refer to Supplementary data **Table S7** for more details). Initially, 90 µL master mix excluding DNaseAlert is incubated for 10 min to trigger the RNP complex, followed by adding 10 µL DNaseAlert into the reaction mixture. The master mix is then incubated for another 30-45 min. Fluorescence signals

were measured using the Infinite 200 PRO microplate reader by Tecan, utilizing a 96-well PCR plate. The measurements were set in kinetic mode with a 1-min interval for 45 cycles. Excitation and emission wavelengths were set at 520 nm and 560 nm, respectively. The instrument operated in Fluorescence Top Reading mode with an integration time of 20 μs and a settle time of 5 μs. A manual gain of 75 was selected. Data acquisition involved 10 flashes and was collected using Tecan i-control software (version 2.0.10.0).

CRISPR assay for nanopore detection. The CRISPR is performed in a 20 μL mixture, including 2 μL each of the dormant RNP and RPA-amplicons, 10 μL of nuclease-free water, 4 μL of 5X buffer, and 2 μL of M13mp18 reporter. After a 10-min incubation, 2 μL of M13mp18 reporter is added to the master mix and incubated for another 25 min. The CRISPR reaction is terminated using 180 μL of M KCl.

1.5 Gel analysis of RPA and CRISPR products

Gel analysis of RPA products: Post-RPA, the amplicons are extracted from the 5% agarose gel electrophoresis performed at 10 V/cm using SYBR™ Safe DNA Gel Stain. The required bands are then analyzed using the BIO-RAD GelDoc Go Imaging System with a 20-second exposure.

Gel analysis of CRISPR products: 20 μL of the CRISPR reaction is mixed with 4 μL of DNA loading dye (6X) to halt the reaction. This 24 μL mixture is added to a 1% agarose gel, stained with SYBR™ Safe DNA Gel Stain, and applied at 5 V/cm voltage using the BIO-RAD GelDoc Go Imaging System with 30-second exposure.

1.6 Nanopore fabrication

The quartz capillaries underwent a cleaning process in Piranha solution, lasting for half an hour at 85ºC, followed by a rinse with DI water. They were dried at 120 ºC for 20 min in a vacuum oven. This procedure was executed to remove any organic residues on the quartz capillaries. The capillaries underwent oxygen plasma cleaning for 5 minutes to increase capillaries hydrophilicity (Dong et al., 2022). The capillaries were pulled using a laser pipette puller (P-2000, Sutter Instruments, USA), following a single-stage procedure: The heat was set at 650, the filament at 4, the velocity at 60, the delay at 145, and the pull was adjusted between 160 and 170 (Lastra et al., 2022). The nanopores were analyzed by conducting a current-voltage (I-V) experiment, varying the voltage between -400 and 400 mV (Nouri et al., 2021). A standard nanopore utilized in the

research was approximately 9.5-10 nm in diameter (Lastra et al., 2022).

1.7 Nanopore data acquisition and analysis

Data acquisition. A stable voltage was applied across the nanopore using a 6363 DAQ card (from National Instruments, USA). An amplifier Axopatch 200B (from Molecular Device, USA) was used to amplify the signal. The amplifier's analog output was sampled with the same 6363 DAQ card using the data collection software LabVIEW. The sampling was done at a rate of 100 kHz, with a low-pass filter applied at 10 kHz. The entire measurement setup was built inside a Faraday cage.

Data analysis. The noise performance of the ionic current measurement was analyzed (**Fig. S6b**), with the typical RMS noise in the experiments less than 3.57 pA, and the threshold current dip was set to 15 pA. The current time trace was analyzed, and translocation details (such as dwell time, current drop, and ECD) were extracted using specialized Python 3.8.3 and MATLAB R2020b software. OriginPro 2021 and Python were used for plotting.

2 Supplementary Figures

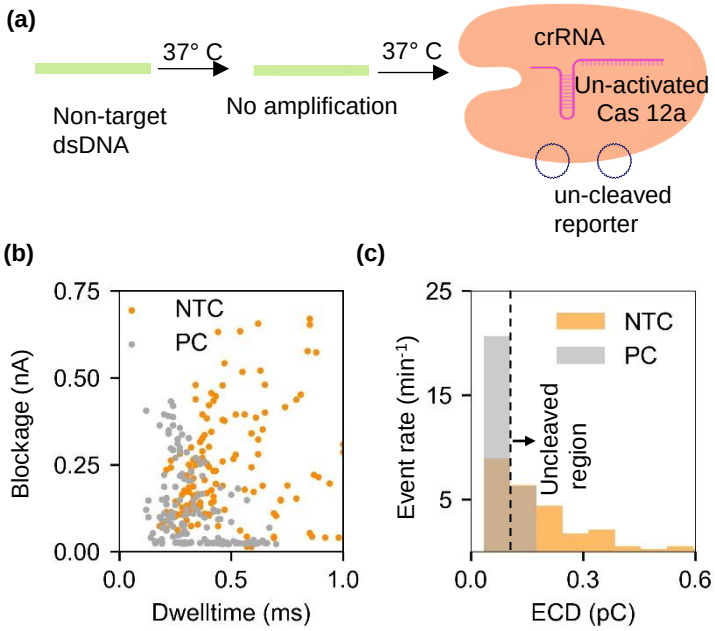


Figure S1. a) Shows non-reactivity of unamplified RPA products without MPXV dsDNAs. b) Illustrates the dwell time and blockage of translocation occurrences in positive and negative cases. The positive sample shows the shorter current blockage and dwell time. and c) Event rate-ECD distribution over time due to CRISPR reaction. Similarly, the positive sample shows higher event rates. Each bar depicts identical ECD lines ranging from .07 pC to 0.6 pC, divided into bins of 75 pC each. The region to the right of the dashed line denotes the area where cleavage has not occurred.

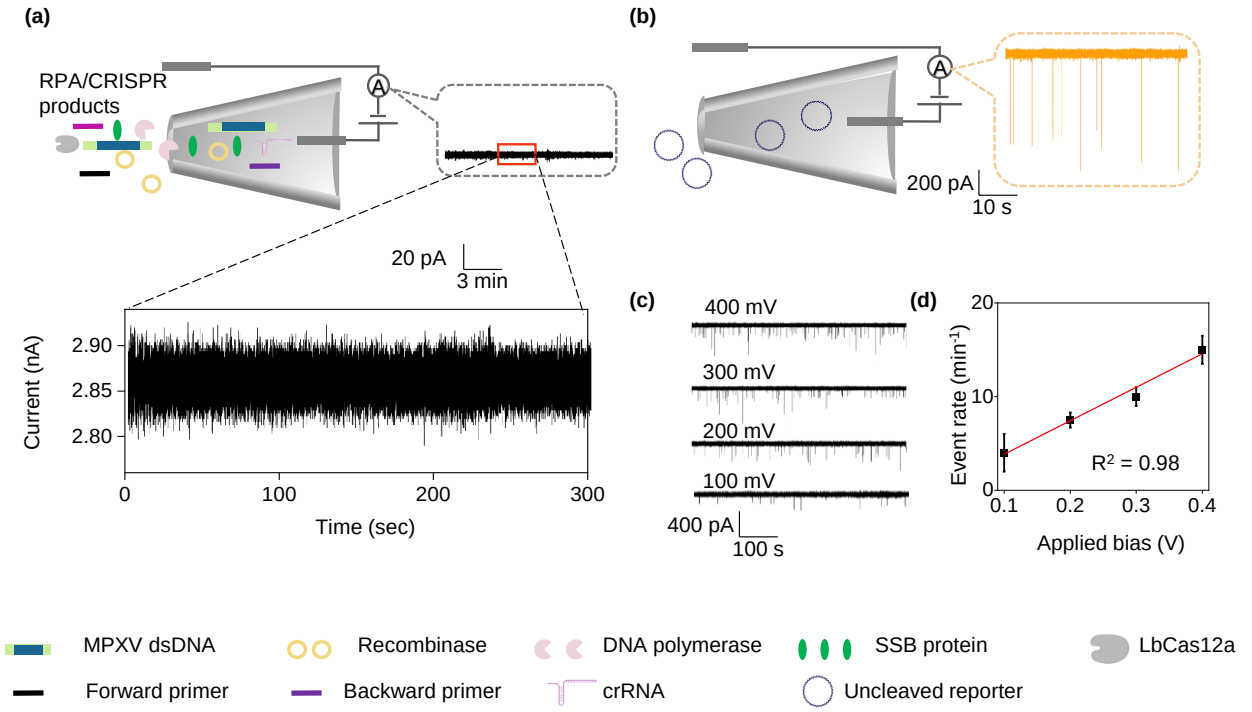


Figure S2. Noise and Background Investigation Experiments. a) Nanopore Analysis of R.P.A. reagents, Pure R.N.P., and M.P.X.V. Targets Without ssDNA Reporter. This illustration indicates the absence of recorded events during the measurement period. b) Expands view of current trace over time. c) Reporter's Current Trace at 0.3 V applied bias d) Event rate at different biases displays a correlation analysis demonstrating the assay's linearity. An R^2 value of 0.98 indicates a strong correlation.

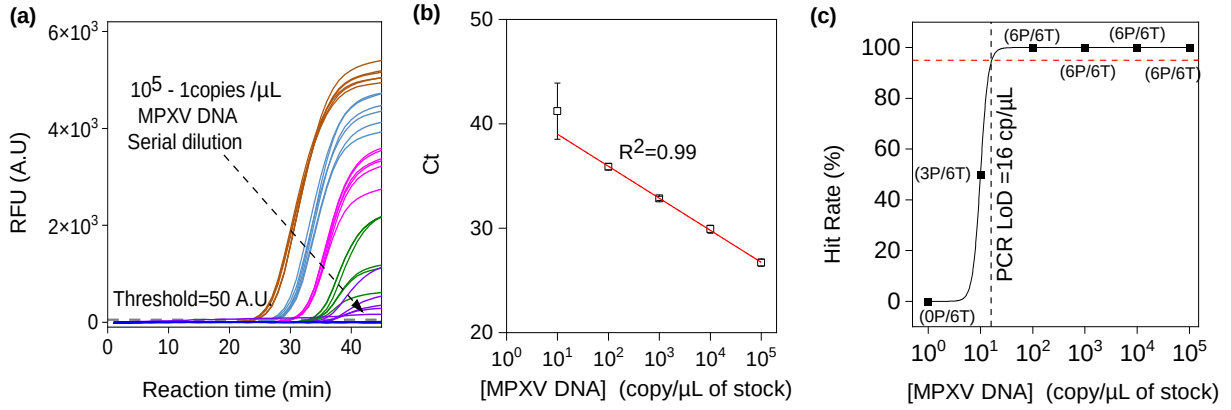


Figure S3. Real-Time PCR Validation for MPXV Assay a) This figure demonstrates real-time PCR validation using the Monkeypox Virus (MPXV). The identical primer set was employed, but a PCR probe was utilized for real-time analysis. b) The linearity of the assay. An R^2 value of 0.99 suggests a high level of correlation. Detailed cycle quantification (Cq) values are provided in Table Sx, and the probe sequence can be found in Table S4. c) LOD the PCR reaction at a confidence level of 95%.

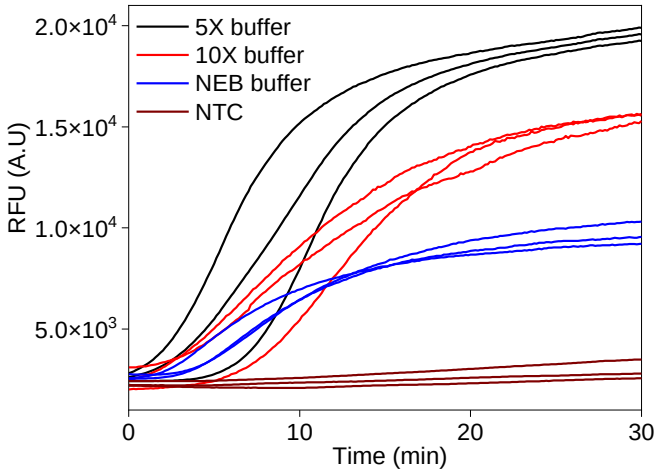


Figure S4. The figure represents the process of optimizing the buffer for the CRISPR assay. The RPA-generated amplicons were utilized for this CRISPR assay, and the stock concentration of these amplicons from the RPA assay was noted as 10^4 copies/µL.

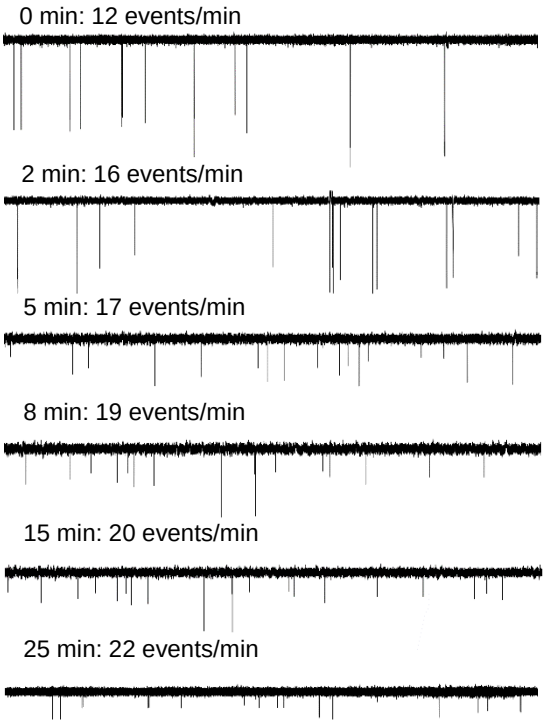


Figure S5. Time-dependent Current Trace Analysis at Various Reaction Termination Intervals from 0 to 25 Minutes.

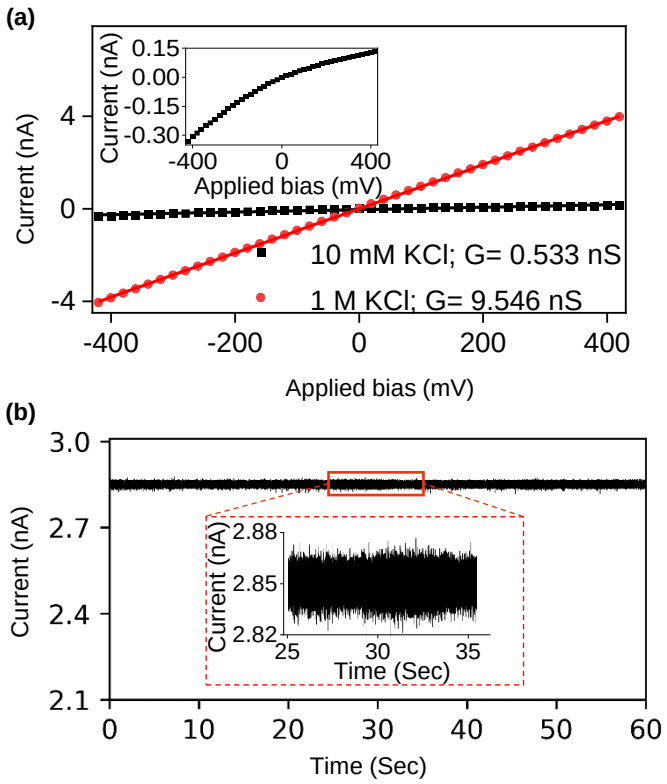


Figure S6. Characterization and Performance of Glass Nanopore. a) The I-V characteristics of the typical glass nanopore are illustrated. As per the findings, the nanopore's average conductance is approximately 9.2 nS, with an estimated pore diameter of 9.5-10 nm. b) This illustrates the baseline for 1M KCl with an applied bias of 0.3V.

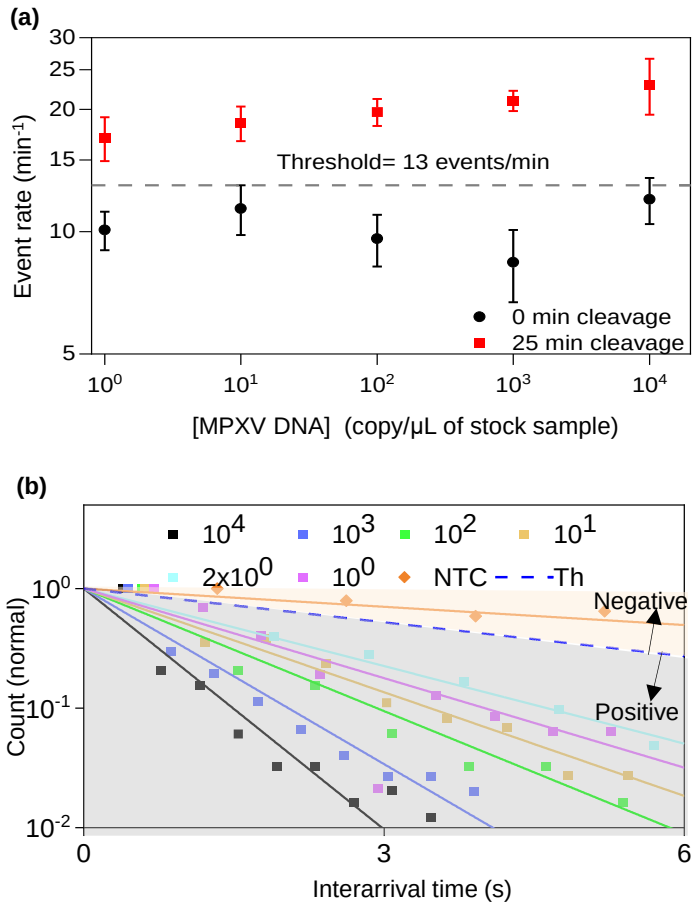


Figure S7. a) Simplified representation of the event rate for a single positive sample across varying concentrations and negative samples. b) Comparative evaluation of positive and negative calls based on normalized count interarrival time, with a threshold slope derived using the Poisson distribution formula $P(t) = \lambda e^{-\lambda t}$, where λ represents the anticipated single-molecule event rate.

3 Supplementary Tables

Table S1. Sequences of Primers, oligos, and crRNAs were employed in this research.

Components	Sequence
F	TTTCGCGGGATACATCATCTATTATAGCAT
R	TGCTATGGTTTACAGCTCCAACGATACTCC
crRNA	TTTCGCGGGATACATCATCTATTATAGC
PCR probe	/5HEX/TGTAGGCCG/ZEN/TGTATCAGCATCCATT/3IABkFQ/
F3 gene fragment	TTTCGCGGGATACATCATCTATTATAGCATCAGCATCAGAATCTGTAGGCCGTGTATCAGCATCCATTGTCGTAGACCAACGAGGAGGAGTATCGTTGGAGCTGTAAACCATAGCA

Table S2. Details of RPA reaction components.

RPA Components	Volume (μL)/test	Stock Concentration
Rehydration buffer, storage -20°C	29.5	1.7X
Forward Primer, storage -20°C	2.4	10 μM
Reverse Primer, , storage -20°C	2.4	10 μM
DNA sample, , storage -80°C	1	1 cp/μL/rxn
$Mg(C_2H_3O_2)_2$ (last step) , storage -20°C	2.5	280 mM
UltraPure Water	12.2	
Total Volume:	50	

Table S3. Reaction table for RPA assays with concentrations ranging from 10^5 to 1 copies/μL.

	Positive/ negative							
DNA cp/μL	Run1	Run2	Run3	Run4	Run5	Run6	Run7	Run8
10^5	+	+	+	+	+	+	+	+
10^4	+	+	+	+	+	+	+	+
10^3	+	+	+	+	+	+	+	+
10^2	+	+	+	+	+	+	+	+
10^1	+	+	+	+	-	+	+	+
$2x10^0$	-	+	+	-	+	-	+	+
10^0	-	-	-	+	-	+	-	+

Table S4. Ct values of PCR assays with concentrations ranging from 10^5 down to 1 copies/µL.

	Ct values							
M.P.X.V. dsDNA cp/µL	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Avg	Standard Deviation
10^5	27.02	27.04	27.02	26.26	26.49	26.35	26.69	0.33
10^4	30.34	29.25	30.07	29.58	29.98	30.21	29.91	0.37
10^3	33.06	32.62	33.09	32.65	33.02	32.67	32.85	0.2
10^2	36.32	36.85	35.64	37.98	35.7	36.91	36.57	0.8
10^1	38.13	None	None	44.69	40.81	None	42.21	2.8
10^0	None	None	None	None	None	None	None	None

Table S5. 5X and 1X buffer components.

5X reaction buffer (TRIS-EDTA)				
Buffer Components	Storage Temp	Stock Conc	Mixing volume (µL)	Final Conc (5X)
NaCl, storage 25°C	25°C	5 M	200	0.5 M
$MgCl_2$, storage 25°C	25°C	1 M	50	25 mM
HEPES, storage 25°C	25°C	1 M	200	100 mM
Water	25°C		550	mM
TRIS-EDTA, pH 8, storage 25°C	25°C	1 mM	1000	0.5 mM
Total volume:			2000	

Table S6. RNP formation method.

RNP formation and dilution	Volume (µL)/test	Stock Concentration
cpf1 (cas protein) , storage -80°C	1.95	67 µM
crRNA, storage -80°C	1.52	100 µM
1X reaction buffer, storage 25°C	1.4	1X
Total Volume:	4.87	
Room temp @ 20 min		
Add 1X reaction buffer	406.158	
Store it -80°C		

Table S7. CRISPR reaction components.

CRISPR Components	Volume (µL)/test	Stock Concentration
diluted cpf1 (cas protein), storage -80°C	1	317.86 nM
diluted crRNA, storage -80°C	1	369.80 nM
5X buffer, storage 25°C	4	5X
Water	10	
Amplified DNA sample, storage -80°C	2	
incubate at 37°C temp @ 10 min		
DNaseAlert, storage -20°C or		2 µM or
M13mp18, storage -20°C	2	111.6 nM
incubate at 37°C temp @ 20 min		
Total Volume	20	

Table S8: Positive/Negative for SCAN measurement with concentrations ranging from 10^5 to 1 copies/µL.

	Positive/ negative							
D.N.A. cp/µL	Run1	Run2	Run3	Run4	Run5	Run6	Run7	Run8
10^5	+	+	+	+	+	+	+	+
10^4	+	+	+	+	+	+	+	+
10^3	+	+	+	+	+	+	+	+
10^2	+	+	+	+	+	+	+	+
10^1	+	+	+	+	-	+	+	+
$2x10^0$	+	+	+	-	+	-	+	+
10^0	+	-	-	+	-	+	-	+

Reference

Chen, Q., Gul, I., Liu, C., Lei, Z., Li, X., Raheem, MA, He, Q., Haihui, Z., Leeansyah, E., Zhang, C.Y., Pandey, V., Du, K., Qin, P., 2023. J. Med. Virol. 95.

Dong, M., Tang, Z.F., Hicks, S., Guan, W.H., 2022. Anal Chem 94, 3865–3871.

Lastra, L.S., Bandara, YMNDY, Nguyen, M., Farajpour, N., Freedman, K.J., 2022. Nat. Commun. 13, 2186.

Nouri, R., Jiang, Y.Q., Tang, Z.F., Lian, X.L., Guan, W.H., 2021. Nano Lett 21, 8393–8400.