Supplementary Materials

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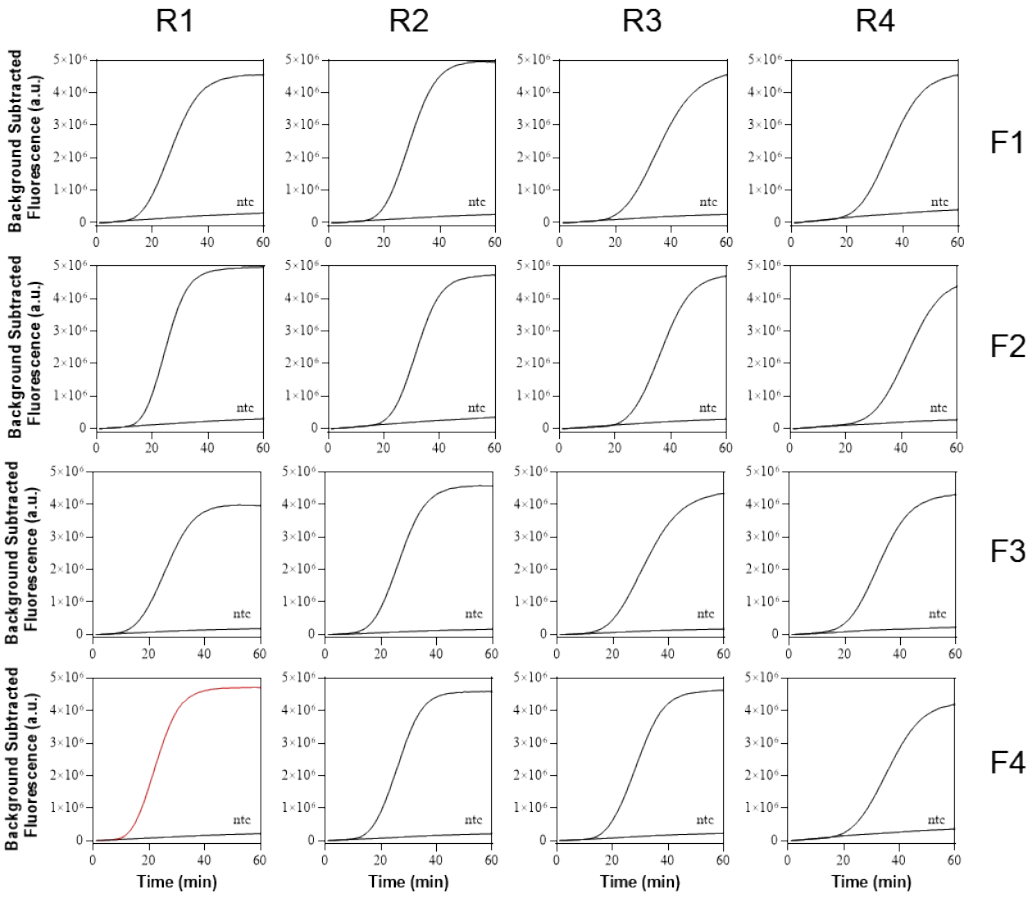

Fig. S1. Real-time fluorescence curves were generated using various primer combinations. Fluorescence was measured every min at 37 °C, and the number of target was 1000 copies.

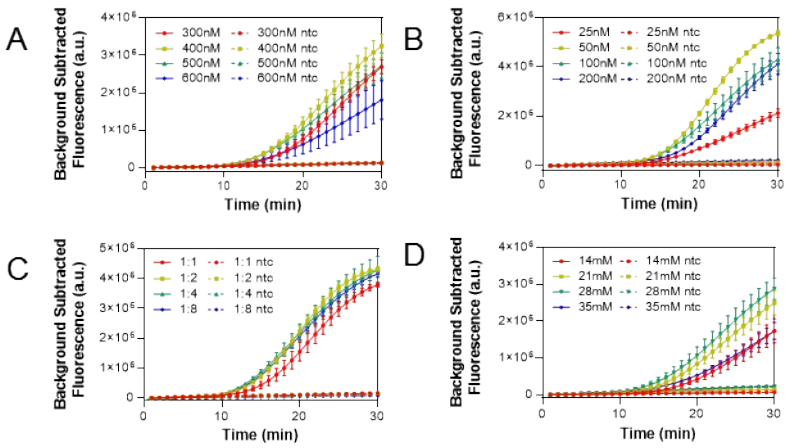

Fig. S2. Optimization of the RPA-CRISPR One-Pot Reaction. The fluorescence profiles were recorded in real time at different concentration of primer (A), LbCas12a (B), ratio of LbCas12a to crRNA (C), and MgOAc (D).

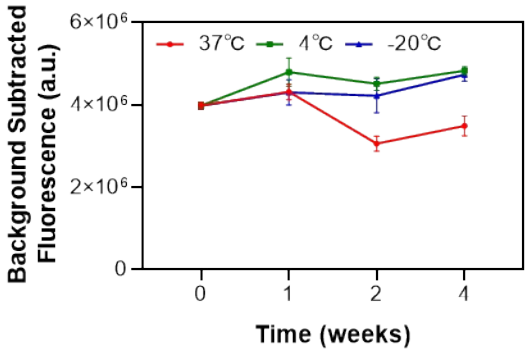

Fig. S3. The end-point fluorescence intensity at 30 min of one-step RPA-CRISPR/Cas12a using the lyophilized reagents stored at 37 °C, 4 °C, and -20 °C for 0, 1, 2, and 4 weeks. Target: 100 copies/reaction.

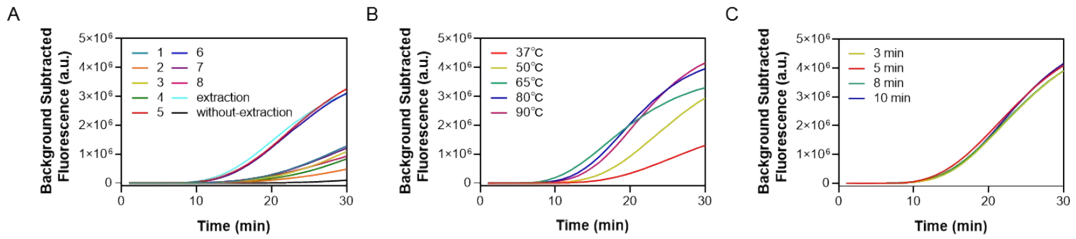

Fig. S4. Optimization of viral lysis. (A) The single-step RPA-CRISPR/Cas12 reaction was tested with 20 copies/μL simulated samples which was lysed by eight kinds of lysis buffers, extraction assay and no treatments at 95 °C for 10 min. (B) Fluorescence profiles with lysis buffer 5 at different lysis temperatures. (C) Fluorescence curves for different lysis times at 80 °C.

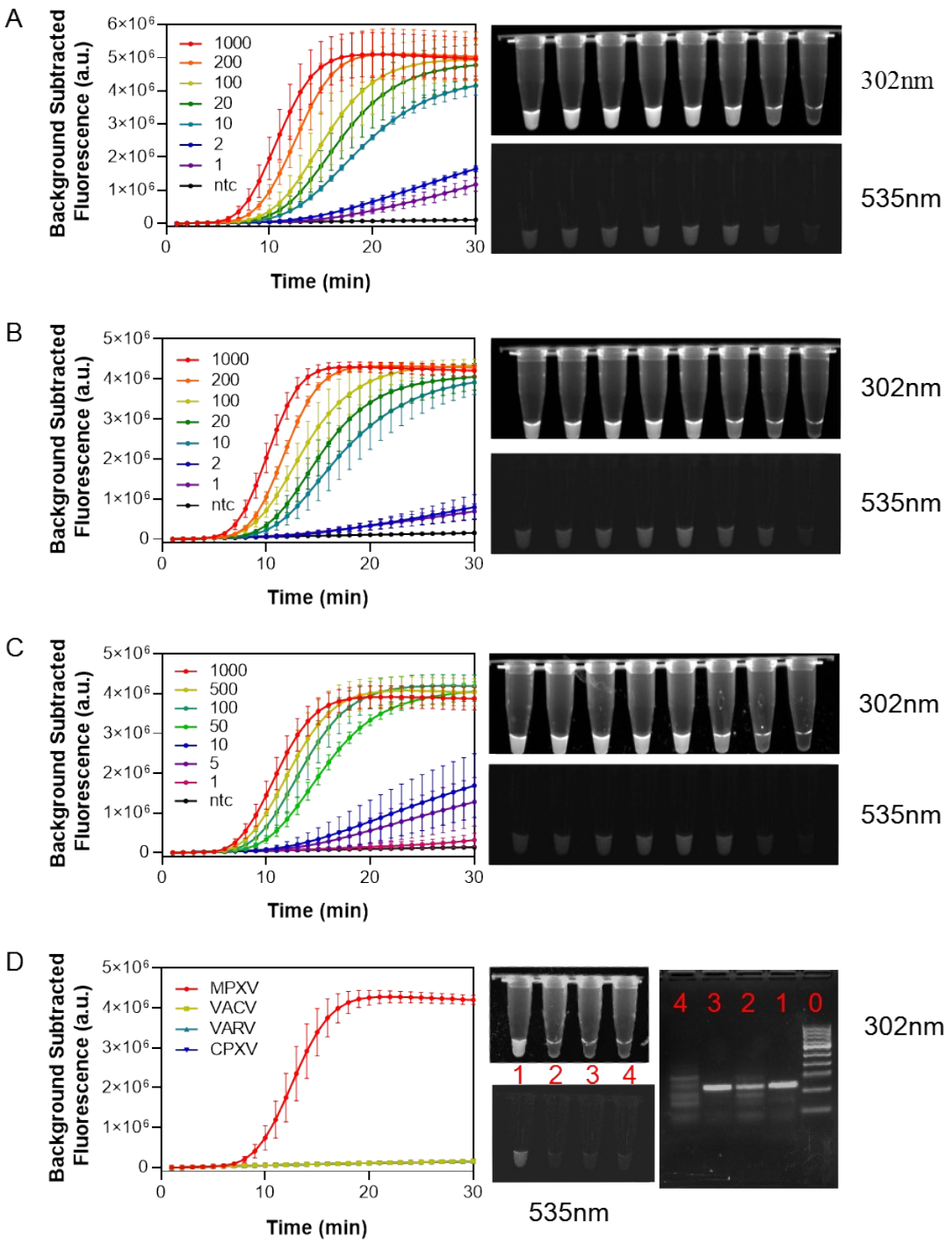

Fig. S5. A–D. Figure on the left shows the real-time fluorescence curve. Figure on the right is an image taken with a gel imager. 302 nm: a light source with a wavelength of 302 nm was used; 535 nm: a blue light source was used, and the wavelength of the filter was 535 nm. (A) Detection of different concentrations of extracted DNA with freshly configured reagents. (B) Detection of different concentrations of extracted DNA using lyophilized reagents. (C) Detection of different concentrations of pseudoviruses in saliva, with lyophilized reagent and lysate. (D) Fluorescence images taken at the endpoint of detection of four orthopoxviruses and electropherograms using a 3 % agarose gel. 0: 50–500 bp marker; 1: MPXV F3L gene; 2: VACV E3L gene; 3: VARV E3L gene; 4: CPXV F3L gene.

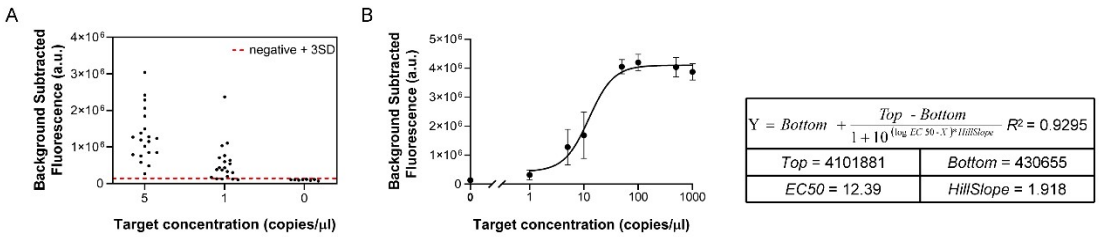

Fig. S6. (A) A scatter plot showing endpoint fluorescence values with background fluorescence removed for 20 replicates of low copy number pseudovirus samples in saliva. (B) A four-parameter fit curve was generated for the fluorescence values in relation to the sample concentration of the pseudovirus simulation.

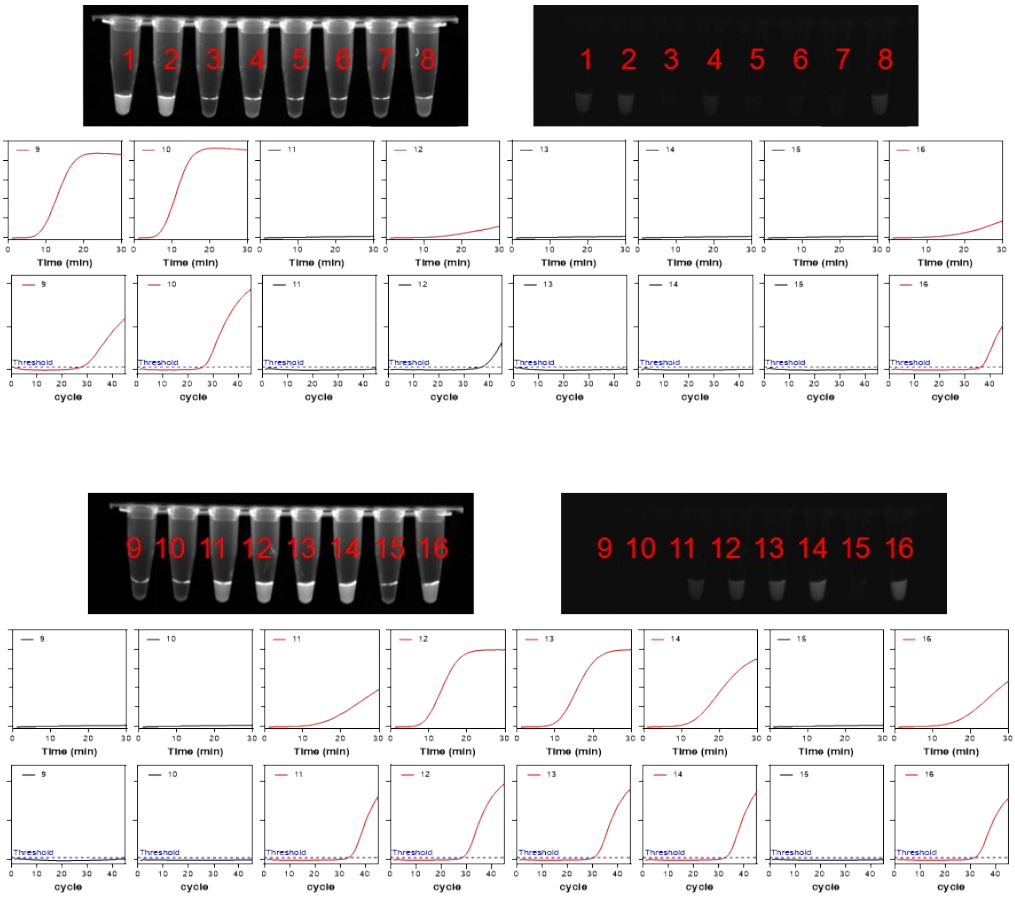

Fig. S7. The detection results of 16 clinical samples are shown, in order of top to bottom, as endpoint (30 min) fluorescence pictures and real-time fluorescence curves for the RPA-CRISPR/Cas12a method and real-time fluorescence curves for the real-time PCR method.

Table S1. The reagents used in this study.

Reagent	Reaction	Manufacturer	Cat.no.	Stock Concentration
LbCas12a	Single-step RPA-CRISPR/Cas12a	Tolo Biotech Co.,Ltd	32108-03	10 μM
RPA lyophilized pellets	Single-step RPA-CRISPR/Cas12a	TwistDx	TABAS03KIT	/
Rehydration buffer	Single-step RPA-CRISPR/Cas12a	TwistDx	TABAS03KIT	/
Magnesium acetate tetrahydrate	Single-step RPA-CRISPR/Cas12a	Sangon Biotech (Shanghai) Co., Ltd.	A501341-0500	/
DNase/RNase-free water	/	Solarbio Life Science	R1600	/
DNA Dilution Buffer (for Real Time PCR)	Gradient dilution of DNA	Sangon Biotech (Shanghai) Co., Ltd.	B639270-0010	/
HiScribe T7 High Yield RNA Synthesis Kit	IVT	New England Biolabs Inc.	E2040	/
Standard Taq buffer	IVT	New England Biolabs Inc.	B9014	10×
RNA Clean & Concentrator-5 Kit	IVT	Zymo Research	R1016	/
DNase I	IVT	Zymo Research	R1016	/
Qubit RNA Broad Range Assay Kits	IVT	Thermo Fisher Scientfic	Q10211	/
Qubit 1× dsDNA HS Assay Kits	Plasmid quantitation	Thermo Fisher Scientfic	Q33230	/
General Agarose	agarose gel electrophoresis	Accurate Biotechnology (Hunan) Co., Ltd.	AG11901	/
GelStain	Agarose gel electrophoresis	Beijing TransGen Biotech Co., Ltd.	GS101-01	10000×
TBE Buffer	Agarose gel electrophoresis	BeiJing Applygen Technologies Inc.	B1111	10×
DNA Marker L (50–500 bp)	Agarose gel electrophoresis	Sangon Biotech (Shanghai) Co., Ltd.	B600305-0050	/

Tris (pH8.0)	Lysis	Thermo Fisher Scientfic	15568025	1 M
EDTA	Lysis	Thermo Fisher Scientfic	AM9260G	0.5 M
NaOH	Lysis	Sinopharm Chemical Reagent Co., Ltd	10019718	/
Triton X-100	Lysis	Thermo Fisher Scientfic	85111	/
Glycine	Lyophilization	Amresco	0167	/
Mannitol	Lyophilization	Solarbio Life Science	M8140	/
Trehalose	Lyophilization	Sinopharm Chemical Reagent	63012666	/
TIANamp Virus DNA/RNA kit	Viral extraction	Tiangen Biotech	DP315	/
MPXV-F3L pseudovirus reference standard	Mock samples	National Institute of Metrology, China	NIM-RM4060	（2.74±0.46）×10^3 copies/μL
MPXV-F3L gene pseudovirus	DNA target	Fubio (Su Zhou) Biomedical Technology Co., Ltd	FNDV4731	/
CPXV-F3L gene pseudovirus	Specificity	Fubio (Su Zhou) Biomedical Technology Co., Ltd	Customised	/
VACV-E3L gene plasmids	Specificity	Sangon Biotech (Shanghai) Co., Ltd.	Customised	/
VARV-E3L gene plasmids	Specificity	Sangon Biotech (Shanghai) Co., Ltd.	Customised	/
AK Taq Master PCR Mix（25×）	qPCR	Fapon Biotech Inc.	MD099M	25×
AK Taq Buffer (with Mg^{2+})	qPCR	Fapon Biotech Inc.	MD099M	5×

Table S2. The sequence list of primers, template for crRNA in vitro transcription.

Item	Sequence (5'–3')
forward primer F1	ATTTTTAGCATCTCGTTTAGATTTTCCATC
forward primer F2	TCTCGTTTAGATTTTCCATCTGCCTTATCG
forward primer F3	CCATCTGCCTTATCGAATACTCTTCCGTCA
forward primer F4	TCAATACGAAAAGACCAATCTCTCCTAGTT
reverse primer R1	AAGTCTTTTGATGATGTTATTCCGGTTAAA
reverse primer R2	ATAAGTCTTTTGATGATGTTATTCCGGTTA
reverse primer R3	TGGTCTACGACAATGGATGCTGATACACGG
reverse primer R4	GTTCCGACGATACTCCTCCTCGTTGGTCTA
T7-3G IVT primer	TAATACGACTCACTATAGGG
qPCR forward primer	TACATCATCTATTATAGCATCAGC
qPCR reverse primer	CTCCAACGATACTCCTCCTC
qPCR probe	FAM-AATCTGTAGGCCGT-MGB
crRNA1	TTATTGGAAAGGTGTTAACC*atctacacttagtagaaattaccctatagtgagtcgtatta*
crRNA2	TTTATTGGAAAGGTGTTAAC*atctacacttagtagaaattaccctatagtgagtcgtatta*
crRNA3	AATGGATGCTGATACACGGC*atctacacttagtagaaattaccctatagtgagtcgtatta*
crRNA4	CAATGGATGCTGATACACGG*atctacacttagtagaaattaccctatagtgagtcgtatta*

Table S3. The list of the lysis buffers used in this study.

Name	Component
Lysis buffer 1	25 mM NaOH, 1 % Triton X-100 in TE buffer [10 mM Tris, 2 mM EDTA (pH 8.0)]
Lysis buffer 2	10 mM NaOH, 1 % Triton X-100 in TE buffer [10 mM Tris, 2 mM EDTA (pH 8.0)]
Lysis buffer 3	40 mM NaOH, 1 % Triton X-100 in TE buffer [10 mM Tris, 2 mM EDTA (pH 8.0)]
Lysis buffer 4	65 mM NaOH, 1 % Triton X-100 in TE buffer [10 mM Tris, 2 mM EDTA (pH 8.0)]
Lysis buffer 5	25 mM NaOH, 0.1 % Triton X-100 in TE buffer [10 mM Tris, 2 mM EDTA (pH 8.0)]
Lysis buffer 6	25 mM NaOH, 0.5 % Triton X-100 in TE buffer [10 mM Tris, 2 mM EDTA (pH 8.0)]
Lysis buffer 7	25 mM NaOH, 2 % Triton X-100 in TE buffer [10 mM Tris, 2 mM EDTA (pH 8.0)]
Lysis buffer 8	25 mM NaOH, 4 % Triton X-100 in TE buffer [10 mM Tris, 2 mM EDTA (pH 8.0)]

Table S4. The synthetic sequences of the MPXV F3L, CPXV F3L, VARV E3L, and VACV E3L.

Name	Sequence (5'−3')
MPXV F3L	TCAGAATCTAATGATGACATAACTAAGAAGTTTATCTACAGCCAATT TAGCTGCATTATTTTTAGCATCTCGTTTAGATTTTCCATCTGCCTTATC GAATACTCTTCCGTCAATGTCTACACAGGCATAAAATGTAGGAGAGT TACTAGGCCCCACTGATTCAATACGAAAAGACCAATCTCTCCTAGTT ATTTGACAGTACTCATTAATAACGGTGACAGGGTTAACACCTTTCCA ATAAATAATTTTTTTAACCGGAATAACATCATCAAAAGACTTATGAT CCTCTCTCATTGATTTTTCGCGGGATACATCATCTATTATAGCATCAG CATCAGAATCTGTAGGCCGTGTATCAGCATCCATTGTCGTAGACCAA CGAGGAGGAGTATCGTTGGAGCTGTAAACCATAGCACTACGTTGAA GATCATACAGAGCTTTATTAACTTCTCGCTTCTCCAT
CPXV F3L	TCAGAATCTAATGATGACGTACCCAAGAAGTTTATCTACAGCCAATT TAGCTGCATTATTTTTAGCATCTCGTTTAGATTTTCCATCGGCCTTAT CGAATACTCTTCCATCGATGTCTACACAGGCATAAAATGTAGGAGAG TTACTAGGTCCCACTGATTCAATACGAAAAGACCAATCTCTCTTAGT TATTTGGCAGTACTCATTAATAATGGTGACAGGGTTAGCATCTTTCC AATCAATAATTTTTTTGGCAGGAATAACATCATCAAAAGACTTATGA TCCTCTCTCATTGATTTTTCGCGGGATACATCATCTATTATGACGTCA GCCATAACATCAGCATCCGTCTTATCCGCCTCCGTTGTCATAAACCA ACGAGGAGGAATATCGTCGGAGCTGTACACCATATCACTACGTTGAA GATCGTACAGAGCTTTATTAACTTCTCGCTTCTCCATATTAAGTTGTC TAGTTAGTTGTGCAGCAGTAGCTCCTTCGATTCCAATGGTTTTAATAG CCTCACACACAATCTCTGCGTCAGAACGTTCGTCGATATAGATTTTA GACAT
VARV E3L	TCAGAATCTAATGATGACGTAACCAAGAAGTTTATCTACAGCCAATT TAGCTGCATTATTTTTAGCATCTCGTTTAGATTTTCCATCGGCCTTAT CGAATACTCTCCCGTCGATGTCTACACAGGCATAAAATGTAGGAGAG TTACTAGGCCCCACTGATTCAATACGAAAAGACCAATCTCTCTTAGT TATTTGGCAGTACTCATTAATAATGGTGACAGGGTTAGCATTTTTCCA ATCAATAATTTTTTTAGCCGGAATAACATCATCAAAAGACTTATGAT CCTCTCTCATTGATTTTTCGCGGGATACATCATCTATTATGACGTCAG CCATAGTCATAGCATCCGGCTTATCCGCCTCAGTTGTCATAAACCAA CGAGGAGGAATATCGTCGGAGCTGTACACCATAGCACTACGTTGAA GATCGTACAGAGCTTTATTAACTTCTCGCTTCTCCATATTAAGTTGTC TAGTTAGTTGTACAGCAGTAACTCCTTCAAGTCCAATGTTTTTAATAG CCTCACATACAATCTCTGCGTCAGAACGCTCGTCAATATAGATCTTA GACAT
VACV E3L	TCAGAATCTAATGATGACGTAACCAAGAAGTTTATCTACTGCCAATT TAGCTGCATTATTTTTAGCATCTCGTTTAGATTTTCCATCTGCCTTATC GAATACTCTTCCGTCGATGTCTACACAGGCATAAAATGTAGGAGAGT TACTAGGCCCCACTGATTCAATACGAAAAGACCAATCTCTCCTAGTA ATTTGGCAGTACTCATTAATAACGGTGACAGGGTTAGCACCTTTCCA

	ATCAATAATTTTTTTAGCCGGAATAACATCATCAAAAGACTTATGAT CCTCTCTCATTGATTTTTCGCGGGATACATCATCTATTATGACGTCAG CCATAGCATCAGCATCCGGCTTATCCGCCTCCGTTGTCATAAACCAA CGAGGAGGAATATCGTCGGAGCTGTACACCATAGCACTACGTTGAA GATCGTACAGAGCTTTATTAACTTCTCGCTTCTCCATATTAAGTTGTC TAGTTAGTTGTGCAGCAGTAGCTCCTTCGATTCCAATGGTTTTAATAG CCTCACACACAATCTCTGCGTTAGAACGCTCGTCGATATAGATTTTA GACAT

Table S5. Detection of MPXV based on isothermal amplification technique.

Technique	Target gene	Limit of detection	One-step	Reference
RPA	G2R	16 DNA molecules/μL	√	[29]
LAMP	ATI D14L	10^2 copies/μL $10^{2.4}$ copies/μL	√	[30]
LAMP	A27L F3L	20 copies/reaction 20 copies/reaction	√	[31]
LAMP	ATI	1 copy/μL	√	[32]
RPA-CRISPR/Cas12a	G2R	1 copy/reaction	×	[33]
RPA-CRISPR/Cas12a	B7R	13.5 copies/μL	×	[19]
RPA-CRISPR/Cas12a	B6R F3L	10.6 viral particles/μL 11 viral particles/μL	×	[27]
RPA-CRISPR/Cas12a	E9L	1 copy/μL	×	[34]
RPA-CRISPR/Cas12a	F3L B6R	1 copy/μL 1 copy/μL	×	[25]
RPA-CRISPR/Cas12a	N4R	1 copy/μL	×	[35]
RPA-CRISPR/Cas12a	F3L	10 copies/μl	×	[36]
HCR-CRISPR/Cas12a	F3L	1 copy	×	[37]
RPA-CRISPR/Cas12a	NA	1 copy/μL	×	[38]
RPA-CRISPR/Cas12a	F3L	5 viral particles/μL	√	This study