Supplementary Information

Direct Repeat Region 3' End Modifications Regulate Cas12a Activity and Expand Its Applications

Wei Zhang,[1]† Yinyin Zhong,[1]† Jiaqi Wang,[1]† Guangrong Zou,[2] Qiaozhen Chen,[3] and Chaoxing Liu[1]*

[1] Guangdong Provincial Key Laboratory of Digestive Cancer Research, Digestive Diseases Center, Scientific Research Center, The Seventh Affiliated Hospital of Sun Yat-sen University, Shenzhen, Guangdong, 518107, P. R. China.

[2] Medical Laboratory Department, The Seventh Affiliated Hospital of Sun Yat-sen University, Shenzhen, Guangdong, 518107, P. R. China.

[3] School of Chemistry and Biological Engineering, University of Science and Technology Beijing, Beijing, 100083, P. R. China.

† These authors contributed equally.

* Corresponding Author: Chaoxing Liu, Email: liuchx69@mail.sysu.edu.cn, Phone: +1-951-888-1287

S1. Chemicals and materials

All chemicals were procured from Macklin (Shanghai, China), unless otherwise specified. Oligodeoxynucleotides (ODNs) were synthesized and purified by Generay Biotechnology (Shanghai, China), unless indicated otherwise. Chemically modified RNA was synthesized and purified by IGE Biotechnology Ltd. (Guangzhou, China). The LbCas12a protein (Cat No. CAS12-010B, 10 μM) was expressed and purified from E. coli by Hylegen Bioscience (Shanghai, China). Super GelRed™ (Cat No. S2002), WonderBlue® High Sensitivity dsDNA Quantitation Kit (Cat No. W2011) were acquired from US EVERBRIGHT (Suzhou, China). T7 RNA Polymerase (Cat No. E00066) was obtained from GenScript Biotech Corporation (Nanjing, China). The Nucleoprotein Extraction Kit (Cat No. C500009-0050) was purchased from Sangon Biotech (Shanghai, China). ALP-labeled Goat Mouse IgG (Cat No. A7028), Recombinant Protein G (Cat No. P5035-10mg), Uracil-DNA Glycosylase (E. coli) (Cat No. D7360M), Anti-Mouse IgG (H+L) (Cat No. A0258), and Albumin/IgG Depletion Kit (Cat No. P2295M) were sourced from Beyotime Biotechnology (Shanghai, China). Fetal bovine serum (Cat No. 209111) was purchased from NEST Biotechnology Co., Ltd (Wuxi, China). Hieff Canace® Plus High Fidelity DNA Polymerase (Cat No. 10153ES76), Alkaline Phosphatase-conjugated Streptavidin (Cat No. 35106ES60), Hieff® qPCR SYBR Green Master Mix (No Rox) (Cat No. 11201ES08), MolPure® PCR Purification Kit (Cat No. 19106ES70), Proteinase K (Cat No. 10401ES60), and T4 Polynucleotide Kinase (Cat No. 14501ES03) were obtained from Yeasen Biotechnology Co., Ltd. (Shanghai, China). Ready-to-use 2XTaq Master Mix (Cat No. GK8005) was purchased from Generay Biotechnology (Shanghai, China). ApexHF HS DNA Polymerase FS (Cat No. AG12201) was purchased from Accurate Biotechnology (Hunan) Co., Ltd. (Changsha, China). Exonuclease I (Cat No. M0293V), Exonuclease III (Cat No. M0206S), Thermostable OGG (Cat No. M0464S), and APE 1 (Cat No. M0282S) were sourced from New England Biolabs. Calf Intestinal Alkaline Phosphatase (Cat No. 18009027) was obtained from Thermo Fisher Scientific Inc. The Human AFP (Alpha-Fetoprotein) ELISA Kit (Cat No. ELK1301) was purchased from ELK Biotechnology Co., Ltd. (Wuhan, China). RT-RPA 2.0 Mix (Cat No. 901221021) and Lateral Flow Detection Paper Strips (Cat No. CAS-cmCSA01) were acquired from KEER LIFE Co., Ltd. (Suzhou, China). 4-Nitrophenyl Phosphate Disodium Salt Hexahydrate (PNPP) (Cat No. P118471), PNPP Substrate Buffer (Cat No. P615628-100ml), and PNPP Stop Solution (Cat No. P616352-100ml) were obtained from Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). Trizol (Cat No. abs60154) was purchased from Absin Biotechnology Co., Ltd. (Shanghai, China). ErCas12a (Cat No. Z03762-100) from GenScript Biotech Corporation (Nanjing, China), FnCas12a (Cat No. D0510S) from Beyotime (Shanghai, China), and ArCas12a (Cat No. 14702ES65) from Yeasen Biotechnology Co., Ltd. (Shanghai, China).

S2. ODN sequences used in this study

Name	Sequence (5'-3')
EBV crRNA	AAU UUC UAC UAA GUG UAG AUC AAA CUC AUA UAU UUG CUG A
DR	AAU UUC UAC UCU UGU AGA U
EBV Spacer	CAA ACU CAU AUA UUU GCU GA
MPXV Spacer	GUA UAU AAG UUG UAC GGC UAA UUC
HCV Spacer	GGC GUG CCC CCG CAA GAC UG
DR-3'PO_4	AAU UUC UAC UAA GUG UAG AU/3Phos/
DR-5'PO_4	/5Phos/AAU UUC UAC UAA GUG UAG AU
DR-DNA	AAU UUC UAC UCU UGU AGA UdGdC dAdTdG dC
DR-PC-DNA	AAU UUC UAC UCU UGU AGA U/PC Linker/UdG dCdAdT dGdC
DR-dU-RNA	AAU UUC UAC UAA GUG UAG AU/dC dTdA(ideoxyU) dTdGdA/ CCG CAU CUA GAU CCU AGA UGC GG
DR-8OG-RNA	AAU UUC UAC UAA GUG UAG AU/dC dTdA(8OdG) dTdGdA/ CCG CAU CUA GAU CCU AGA UGC GG
DR-THF-RNA	AAU UUC UAC UAA GUG UAG AU/dC dTdA(THF) dTdGdA/ CCG CAU CUA GAU CCU AGA UGC GG
EB-RPA-F	GAC CCG GCC CAC AAC CTG GCC CAC TAA GGG
EB-RPA-R	ACT CCA TCG TCA AAG CTG CAC ACA GTC ACC
EB-PCR-F	ATG TCG TAT TAC ACC ATT GAG TCG T
EB-PCR-R	GCG TCT CCT AAC AAG TTA CAT CAC
F-Q	/56-FAM/-CCC CCC CC-/BHQ1/
FAM-Biotin	/56-FAM/-CCC CCC CC-/Biotin/
EBV Target	ATGTCGTATTACACCATTGAGTCGTCTCCCCTTTGGAATGGCCCCTGGA CCCGGCCCACAACCTGGCCCACTAAGGGAGTCCATTGTCTGTTATTTCA TGGTCTTTTTACAAACTCATATATTTGCTGAGGTTTTGAAGGATGCGAT TAAGGACCTTGTTATGACAAAGCCCGCTCCTACCTGCAATATCAAGGT GACTGTGTGCAGCTTTGACGATGGAGTAGATTTGCCTCCCTGGTTTCCA CCTATGGTGGAAGGGGCTGCCGCGGAGGGTGATGACGGAGATGACGG AGATGAAGGAGGTGATGGAGATGAGGGTGAGGAAGGGCAGGAGTGAT GTAACTTGTTAGGAGACGC
MPXV Target	GTAAAACGACGGCCAGTGAATTCGAGCTCGGTACCTCGCGAATGCATC TAGAATGAAAACGATTTCCGTTGTTACGTTGTTATGCGTACTACCTGCT GTTGTTTATTCAACATGTACTGTACCCACTATGAATAACGCTAAATTAA CGTCTACCGAAACATCGTTTAATGATAAACAGAAAGTTACGTTTACAT GTGATTCAGGATATCATTCTTTGGATCCAAATGCTGTCTGTGAAACAGA TAAATGGAAATACGAAAATCCATGCAAGAAAATGTGCACAGTTTCTGA TTATGTCTCTGAACTATATGATAAGCCATTATACGAAGTGAATTCCACC ATGACACTAAGTTGCAACGGTGAAACAAAATATTTTCGTTGTGAAGAA AAAAATGGAAATACTTCTTGGAATGATACTGTCACGTGTCCTAATGCG GAATGTCAACCTCTTCAATTAGAACACGGATCGTGTCAACCAGTTAAA GAAAAATACTCATTTGGGGAATATATGACTATCAACTGTGATGTTGGA TATGAGGTTATTGGTGTTTCGTATATAAGTTGTACGGCTAATTCTTGGA ATGTTATTCCATCATGTCAACAAAAATGTGATATACCGTCCCTATCTAA TGGATTAATTTCCGGATCTACATTTTCTATCGGTGGCGTTATACATCTT AGTTGTAAAAGTGGTTTTACACTAACGGGGTCTCCATCATCCACATGTA TCGACGGTAAATGGAATCCCATACTCCCAACATGTGTACGATCTAACG AAGAATTTGATCCAGTGGATGATGGTCCCGACGATGAGACAGATCTGA GCAAACTCTCGAAAGACGTTGTACAATATGAACAAGAAATAGAATCGT

	TAGAAGCAACTTATCATATAATCATAATGGCGTTGACAATTATGGGTG TCATATTTCTAATCTCCATTATAGTATTAGTTTGTTCCTGTGACAAAAA TAATGACCAATATAAGTTCCATAAATTGCTACCGTGACCCGGGCCCGT CGACTGCAGAGGCCTGCATGCAAGCTTGGCGTAATCATGGTCATAGCT GTTTCCTG
HCV Target	GTAAAACGACGGCCAGTGAATTCGAGCTCGGTACCTCGCGAATGCATC TAGATAATACGACTCACTATAGGGAACTACTGTCTTCACGCAGAAAGC GTCTAGCCATGGCGTTAGTATGAGTGTCGTGCAGCCTCCAGGACCCCC CCTCCCGGGAGAGCCATAGTGGTCTGCGGAACCGGTGAGTACACCGGA ATTGCCAGGACGACCGGGTCCTTTCTTGGATAAACCCGCTCAATGCCT GGAGATTTGGGCGTGCCCCCGCAAGACTGCTAGCCGAGTAGTGTTGGG TCGCGAAAGGCCTTGTGGTACTGCCTGATAGGGTGCTTGCGAGTGCCC CGGGAGGTCTCGTAGACCGTGCACCATGAGCACGAATCCTAAACCTCA AAGACCCGGGCCCGTCGACTGCAGAGGCCTGCATGCAAGCTTGGCGTA ATCATGGTCATAGCTGTTTCCTG
miR-92a	UAUUGCACUUGUCCCGGCCUGU
miR-429	UAAUACUGUCUGGUAAAACCGU
miR-31	AGGCAAGAUGCUGGCAUAGCU
miR-17	CAAAGUGCUUACAGUGCAGGUAG
miR92a TF	GTTGTAAAACGACGGCCAGTTTTGTATTGCACTTGTCCCGGCCTG TCGG
miR92a TR	CCGACAGGCCGGGACAAGTGCAATACAAAACTGGCCGTCGTTTT ACAAC
mR429-TF	GTTGTAAAACGACGGCCAGTTTTGTAATACTGTCTGGTAAAACC GTCGG
mR429-TR	CCGACGGTTTTACCAGACAGTATTACAAAACTGGCCGTCGTTTT ACAAC
miR31 TF	GTTGTAAAACGACGGCCAGTTTTGAGGCAAGATGCTGGCATAGC TCGG
miR31 TR	CCGAGCTATGCCAGCATCTTGCCTCAAAACTGGCCGTCGTTTTAC AAC
miR17 TF	GTTGTAAAACGACGGCCAGTTTTGCAAAGTGCTTACAGTGCAGG TAGCGG
miR17 TR	CCGCTACCTGCACTGTAAGCACTTTGCAAAACTGGCCGTCGTTTT ACAAC

Abbreviations and modifications:

/ideoxyU/: deoxyUridine modification

/PC Linker/: It links two nucleotide sequences via a short, UV-photocleavable C3 spacer arm that can be attached at any position in the sequence.

/3Phos/: 3' Phosphorylation modification

/THF/: dSpacer (1,2'-Dideoxyribose) modification

/56-FAM/: 5' 6-FAM (Fluorescein) modification

/BHQ1/: Black Hole Quencher-1, which is used to quench green and yellow dyes, such as FAM, TET, and HEX.

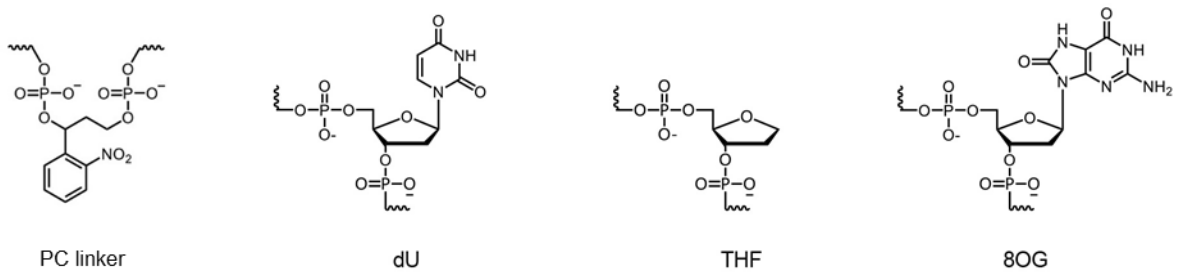
/8OdG/:8-hydroxy-2′-deoxyguanosine

For EBV, MPXV, and HCV targets, which are double-stranded DNA, this table shows only their single-stranded information.

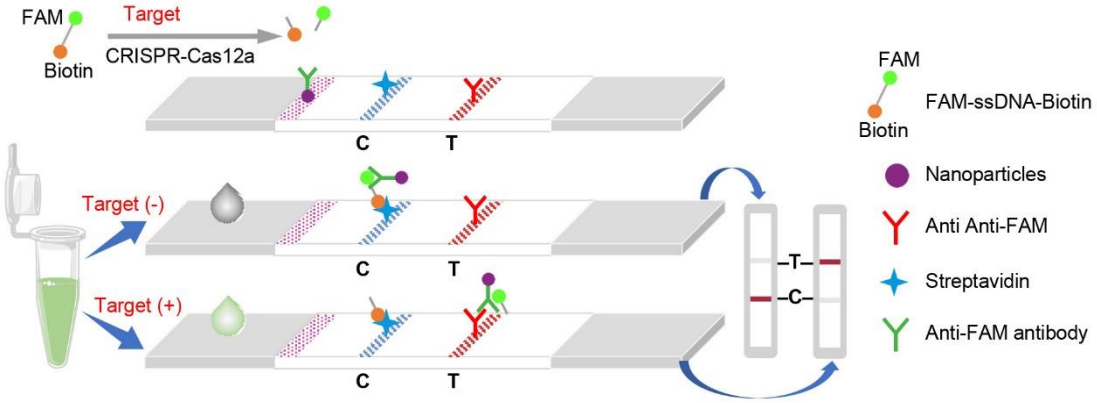
Table S1. Sequences of DNA/RNA oligonucleotides used in this work.

DR	Direct Repeat Region
PC linker	photo-cleavable linker
ALP	alkaline phosphatase
IgG	immunoglobulin G
AFP	alpha-fetoprotein
EBV	Epstein-Barr Virus
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
crRNA	CRISPR RNA
Cas proteins	CRISPR-associated proteins
HCV	Hepatitis C virus
MPXV	Monkeypox virus
UV	ultraviolet radiation
ADP	adenosine diphosphate
PNPP	*p*-nitrophenylphosphate

Table S2. Abbreviation parallel table in this work.



Scheme S1. The chemical structures of PC linker, dU, THF, and 8OG are displayed from left to right, respectively.



Scheme S2. Schematic representation of the paper strip-based visual detection. The paper strips are based on an immunochromatographic assay system and employ a FAM-biotin DNA reporter (a DNA reporter labeled with biotin on one end and FAM on the other) for target detection. The illustration shows the chromatographic migration process, where an anti-FAM antibody conjugated with colloidal gold binds either intact or cleaved FAM-biotin reporters. This complex subsequently interacts with a solid phase comprising streptavidin (control line, C) and anti-mouse IgG antibody (anti-anti-FAM, test line, T).

S3. Extended Data

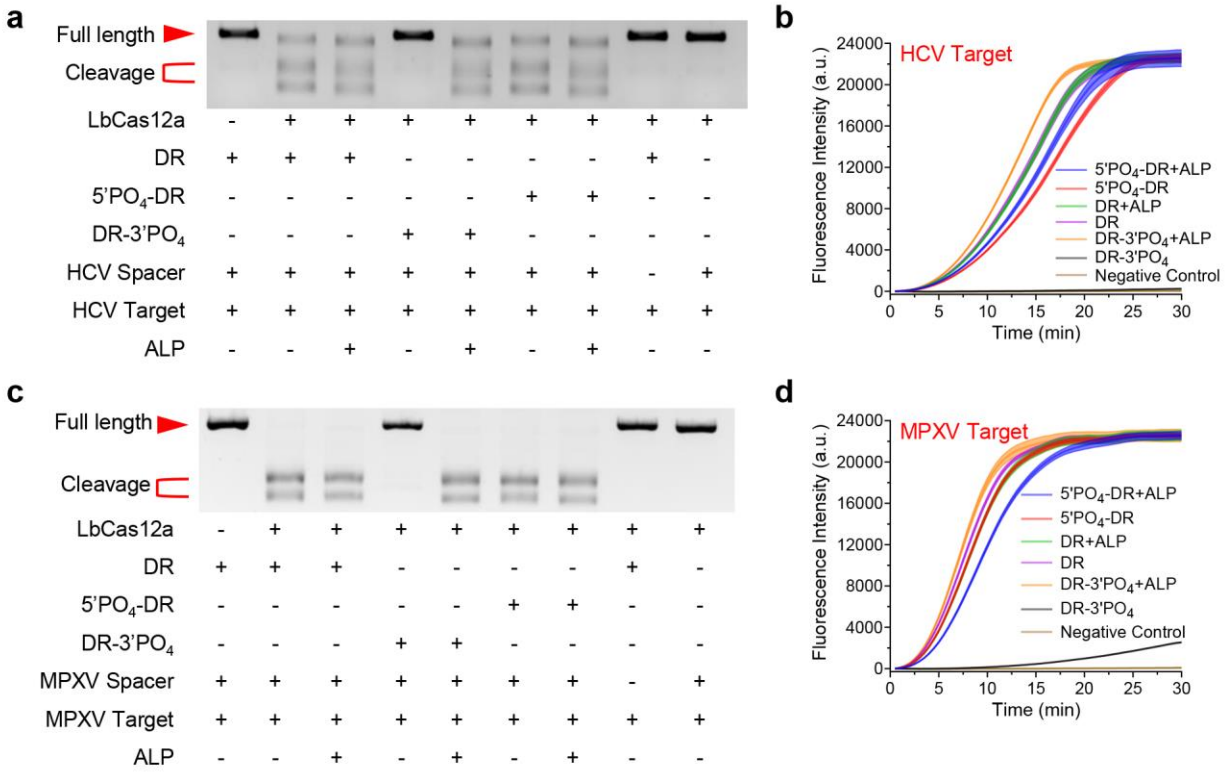


Figure S1. (a) *Cis*-cleavage activity of LbCas12a against HCV targets using classical DR, 5' or 3' phosphorylated DR, with and without ALP, matched with an HCV Spacer. (b) Real-time fluorescence detection curves illustrating *trans*-cleavage activity of LbCas12a for HCV targets, under the above conditions. (c) *Cis*-cleavage activity of LbCas12a against MPXV targets using classical DR, 5' or 3' phosphorylated DR, with and without ALP, matched with an MPXV Spacer. (d) Real-time fluorescence detection curves depicting *trans*-cleavage activity for MPXV targets, under the above conditions.

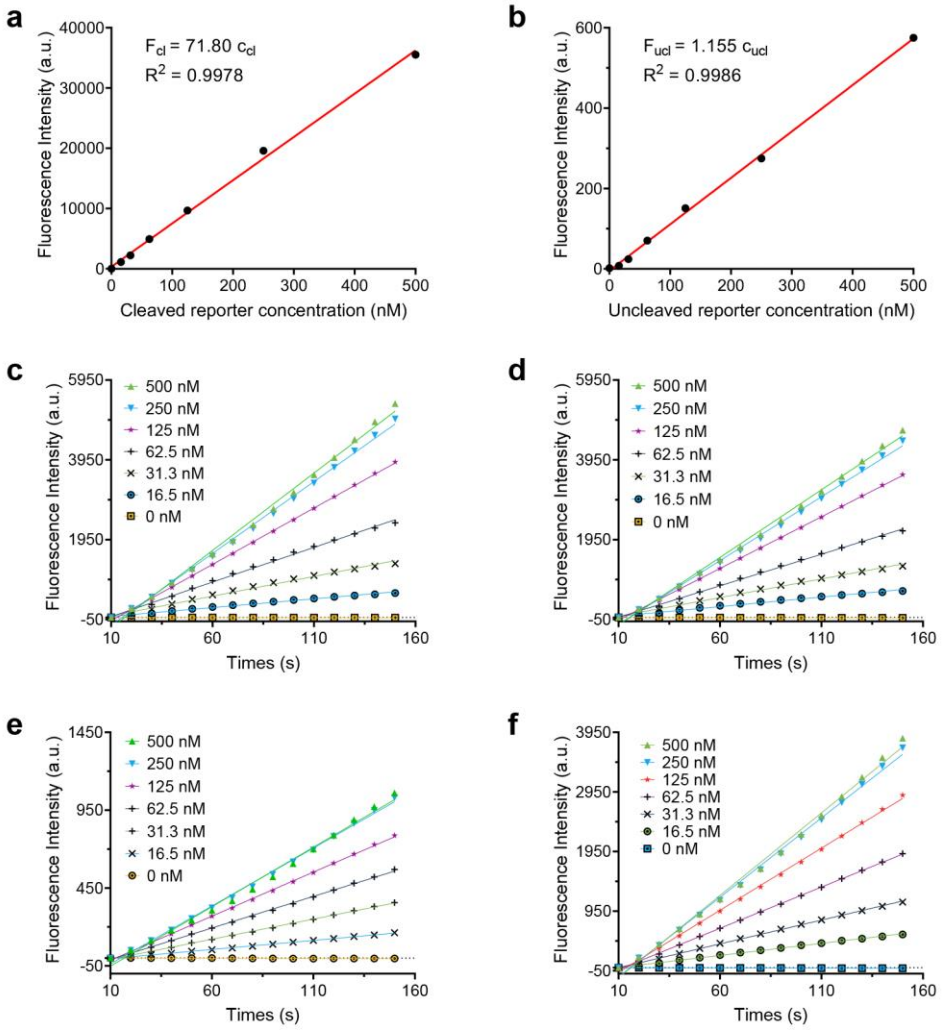


Figure S2. (a) Subtracted fluorescence (F_{cl}) plotted against the concentration of cleaved reporters (c_{cl}). (b) Subtracted fluorescence (F_{ucl}) plotted against the concentration of uncleaved reporters (c_{ucl}). (c) Subtracted fluorescence plotted against time for varying concentrations of reporters, ranging from 16.5 nM to 500 nM, with 50 nM of LbCas12a activated by DR. (d) Subtracted fluorescence plotted against time for varying concentrations of reporters, ranging from 16.5 nM to 500 nM, with 50 nM of LbCas12a activated by DR+ALP. (e) Subtracted fluorescence plotted against time for varying concentrations of reporters, ranging from 16.5 nM to 500 nM, with 100 nM of LbCas12a activated by DR-3'PO_4. (f) Subtracted fluorescence plotted against time for varying concentrations of reporters, ranging from 16.5 nM to 500 nM, with 50 nM of LbCas12a activated by DR-3'PO_4+ALP.

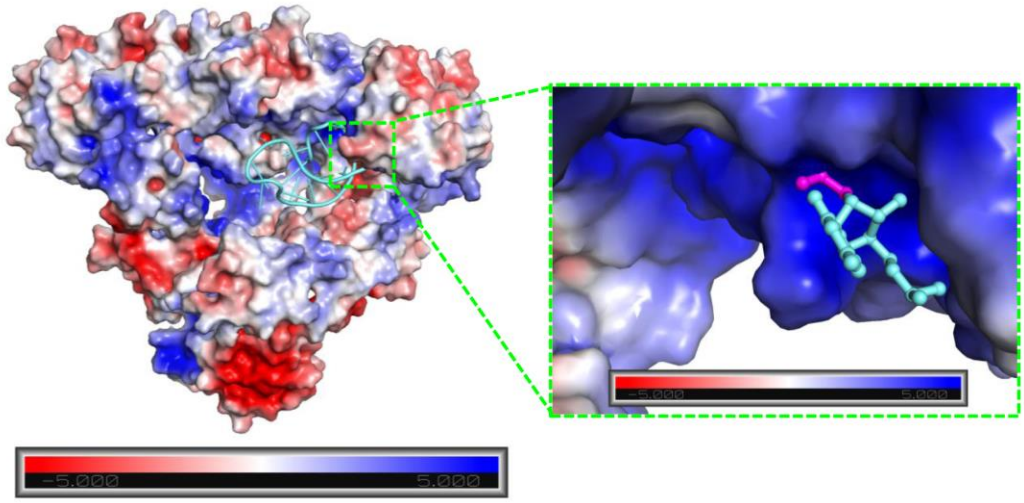


Figure S3. Charge distribution model of LbCas12a protein, highlighting the local positive charge cluster where the PO_4 group of DR-3'PO_4 resides. Red indicates stronger negative charge, while blue indicates stronger positive charge. In the magnified view, cyan represents the terminal nucleic acid of DR, and pink denotes the phosphate group.

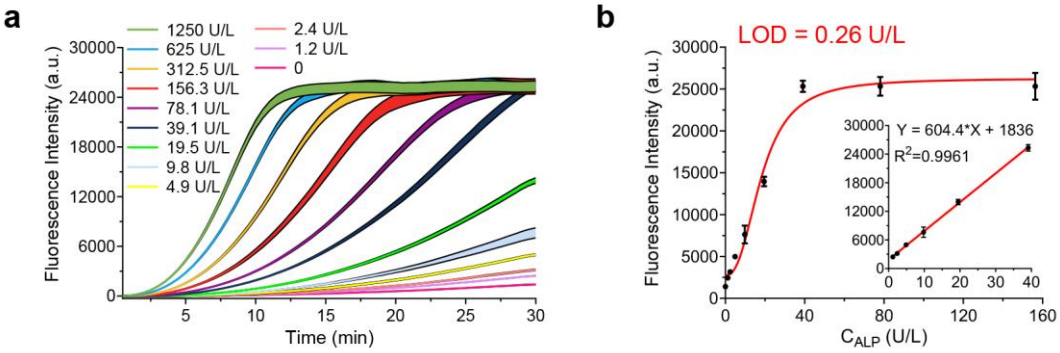


Figure S4. (a) Real-time fluorescence detection of commercial calf alkaline phosphatase at various concentrations using the DR-3'PO_4-assisted CRISPR-Cas12a system. (b) Quantitative relationship, linear fitting, and detection limit of fluorescence values vs. calf alkaline phosphatase concentration.

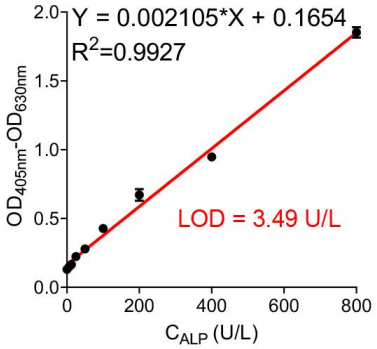


Figure S5. Quantitative relationship, linear fitting, and detection limit of ALP concentration in human serum, measured by the clinical gold standard PNPP method at OD405nm-OD630nm.

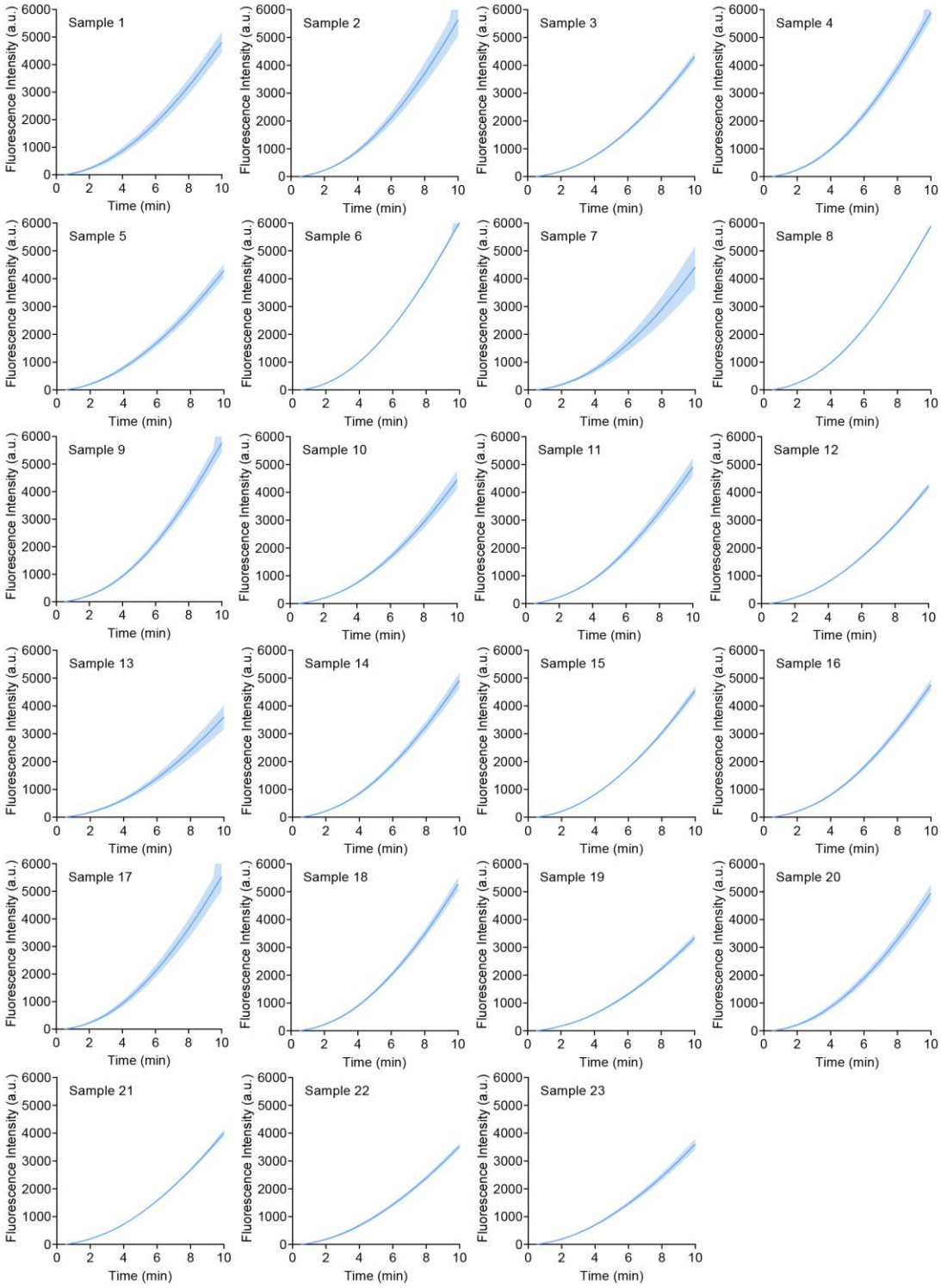


Figure S6. Real-time fluorescence detection of ALP in 23 human serum samples using the DR-3'PO_4-assisted CRISPR-Cas12a system.

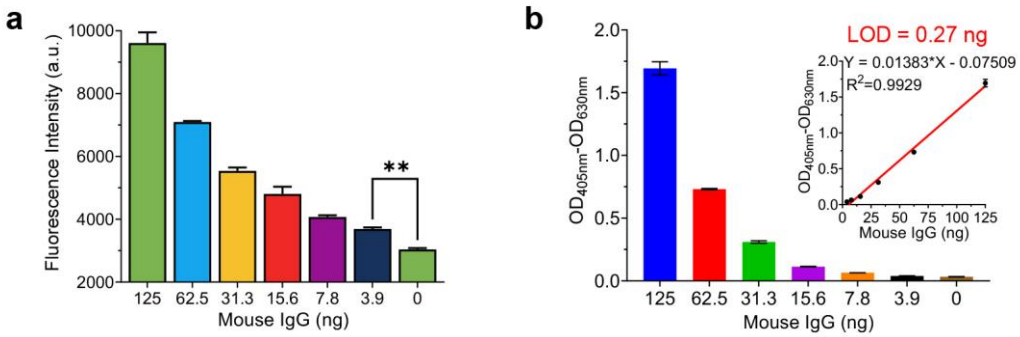


Figure S7. (a) Bar chart analysis of fluorescence values vs. concentration for various amounts of mouse IgG using the DR-3'PO_4-assisted CRISPR-Cas12a system. (b) Quantitative relationship, linear fitting, and detection limit using the classical PNPP method for IgG at OD405nm-OD630nm.

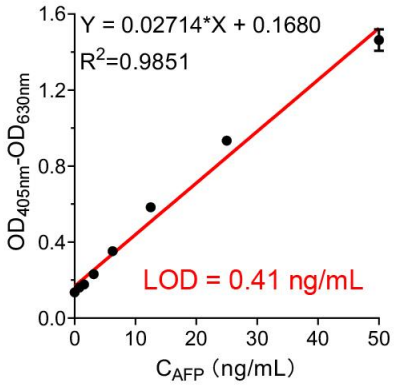


Figure S8. Quantitative relationship, linear fitting, and detection limit of AFP concentrations measured by a commercial Human AFP ELISA KIT at OD405nm-OD630nm.

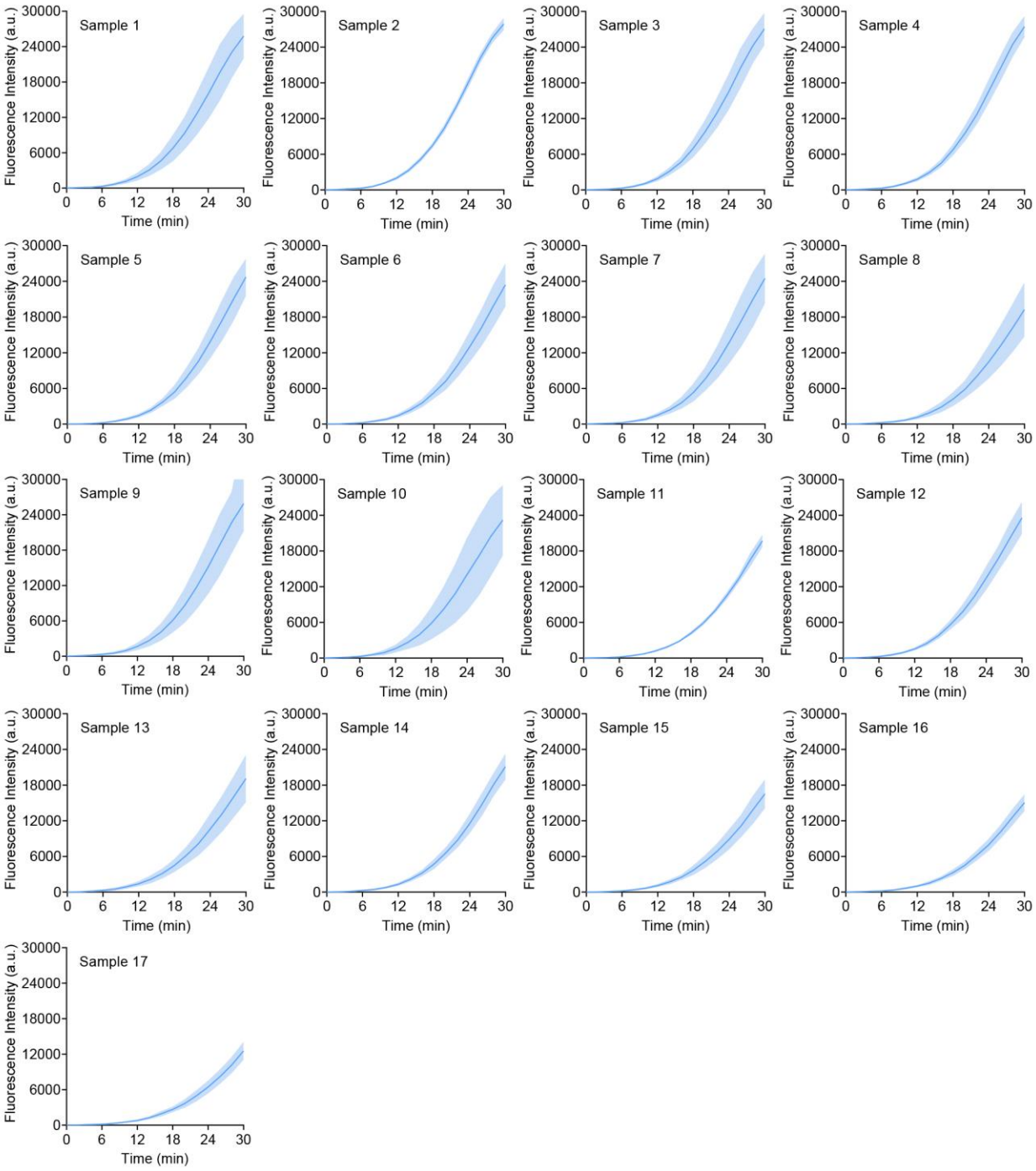


Figure S9. Real-time fluorescence detection of AFP in 17 human serum samples using the DR-3'PO4-assisted CRISPR-Cas12a system.

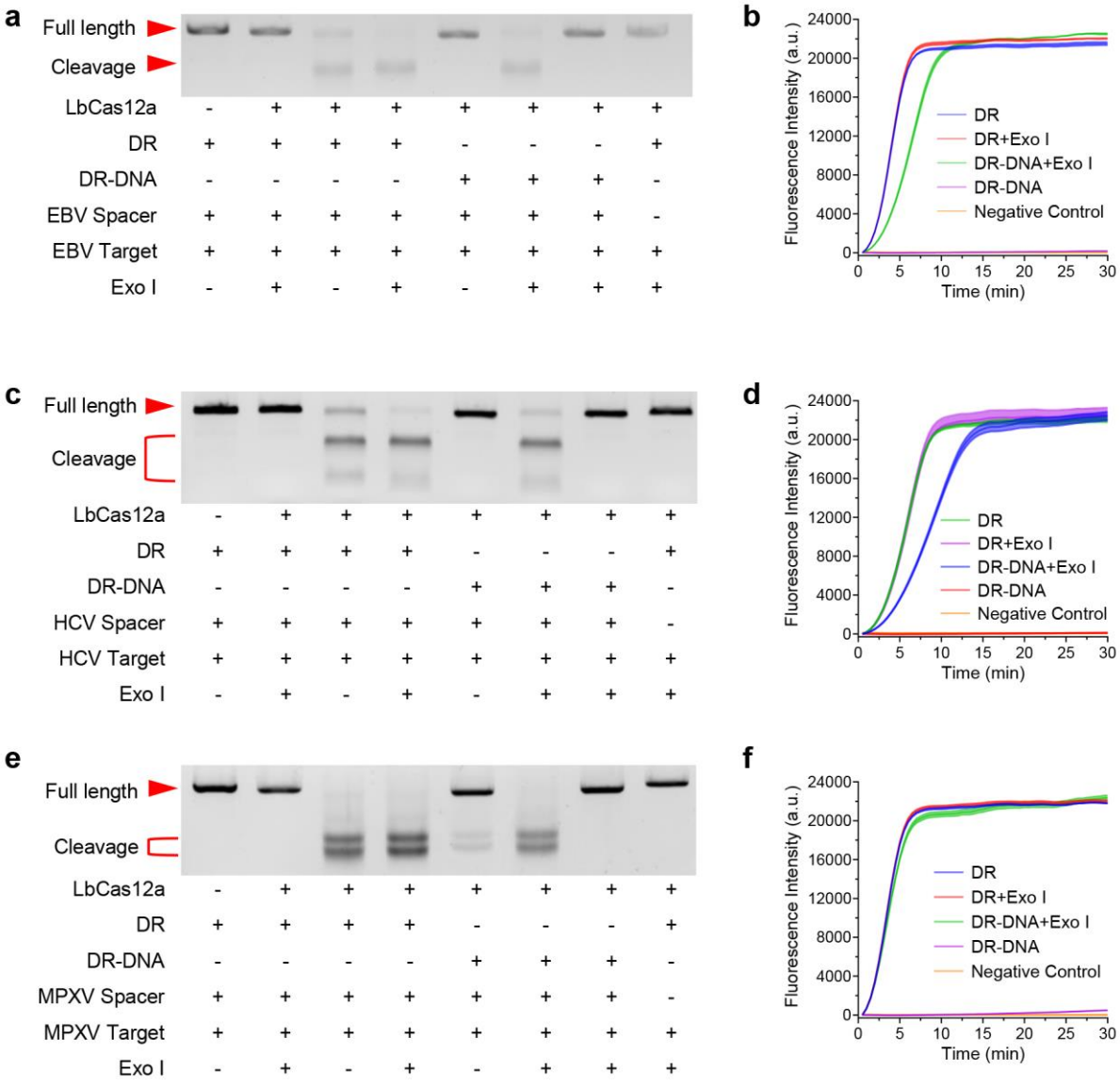


Figure S10. The *cis*-cleavage activity of classical DR or DR-DNA assisted LbCas12a against its corresponding targets with and without the action of exonuclease 1 by matching the EBV Spacer (a), HCV Spacer (c) and MPXV Spacer (e). The real-time fluorescence detection curves for *trans*-cleavage activity of classical DR or DR-DNA assisted LbCas12a against its corresponding targets with and without the action of Exonuclease I (Exo I) by matching the EBV Spacer (b), HCV Spacer (d) and MPXV Spacer (f).

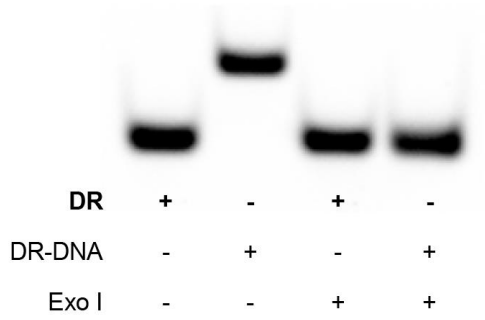


Figure S11.12% TBE-Urea denature PAGE analysis illustrating electrophoresis band results for DR and DR-DNA before and after Exo I treatment. Reaction conditions: 1× RPA-CRISPR Reaction Buffer, 100 ng DR-PC-DNA/DR, exposed to UV light (λ = 365 nm, 35 W) for 15 s (control reactions were not exposed to UV), adjusted to a final volume of 10 μL with deionized water. Reactions were terminated by adding an equal volume of deionized formamide, followed by electrophoresis (12% denaturing gel, 150 V, 35 min).

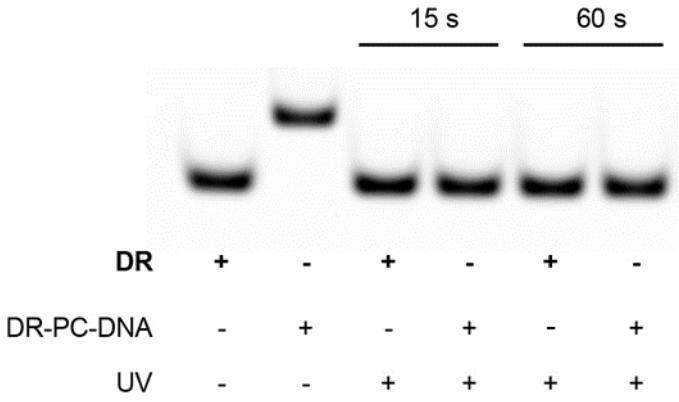


Figure S12.12% TBE-Urea denature PAGE analysis showing electrophoresis band results for DR and DR-PC-DNA under UV exposure for 0, 15 and 60 seconds. Reaction conditions: 1× RPA-CRISPR Reaction Buffer, 100 ng DR-PC-DNA/DR, exposed to UV light (λ = 365 nm, 35 W) for 15 s (control reactions were not exposed to UV), adjusted to a final volume of 10 μL with deionized water. Reactions were terminated by adding an equal volume of deionized formamide, followed by electrophoresis (12% denaturing gel, 150 V, 35 min).

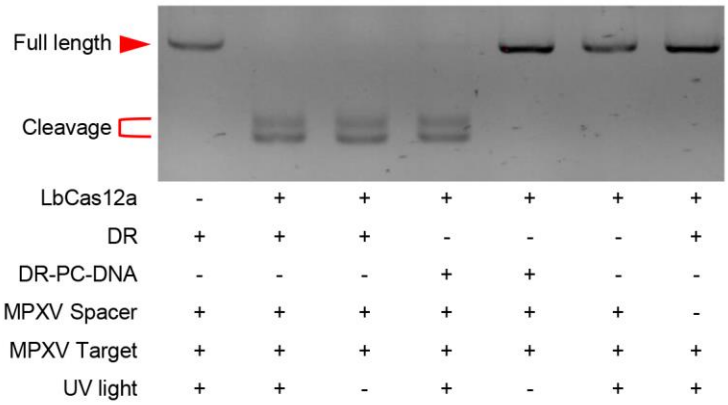


Figure S13.*Cis*-cleavage activity of LbCas12a against MPXV targets using classical DR and DR-PC-DNA, with and without UV light, matched with an MPXV Spacer.

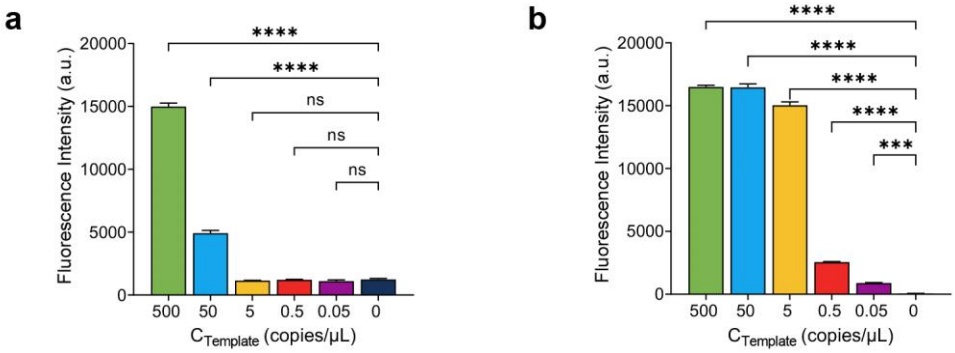


Figure S14. (a) Bar chart quantitative analysis of concentration values detectable by the classical RPA-CRISPR-Cas12a one-pot method. (b) Quantitative analysis for the RPA-DR-PC-DNA CRISPR-Cas12a one-pot trace nucleic acid detection method. Notes: ns, $P > 0.05$, ***, $P < 0.001$, ****, $P < 0.0001$, Error bars are mean ± S.D. (n = 3).

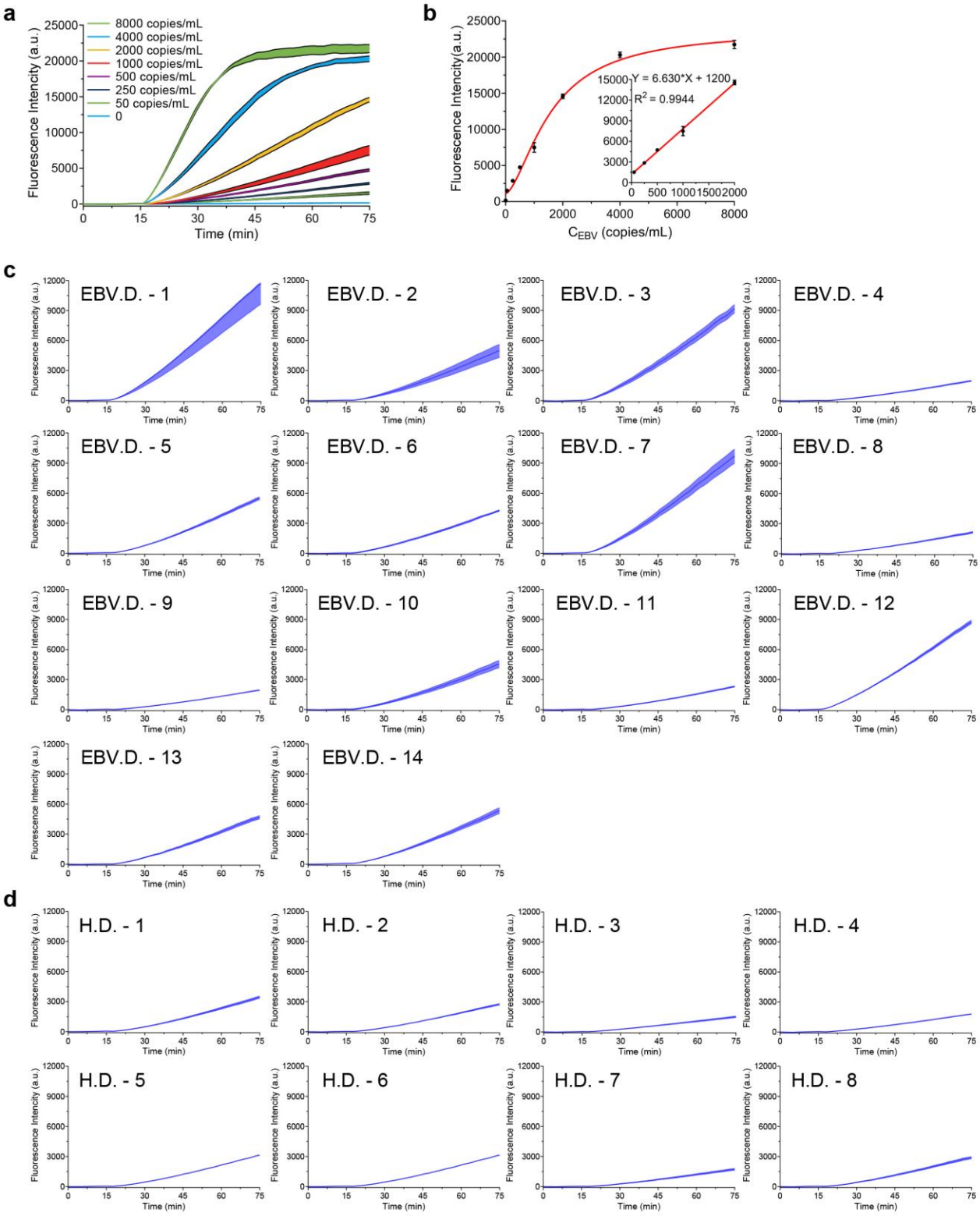

a
Fluorescence Intencity (a.u.)
Time (min)
8000 copies/mL
4000 copies/mL
2000 copies/mL
1000 copies/mL
500 copies/mL
250 copies/mL
50 copies/mL
0
b
Fluorescence Intencity(a.u.)
C_EBV (copies/mL)
Y = 6.630*X + 1200
R² = 0.9944
c
EBV.D. - 1
EBV.D. - 2
EBV.D. - 3
EBV.D. - 4
EBV.D. - 5
EBV.D. - 6
EBV.D. - 7
EBV.D. - 8
EBV.D. - 9
EBV.D. - 10
EBV.D. - 11
EBV.D. - 12
EBV.D. - 13
EBV.D. - 14
d
H.D. - 1
H.D. - 2
H.D. - 3
H.D. - 4
H.D. - 5
H.D. - 6
H.D. - 7
H.D. - 8

Figure S15. (a) Real-time fluorescence curves for various EBV standard concentrations using the RPA-DR-PC-DNA CRISPR-Cas12a one-pot method, with RPA for 15min, followed by 15-second UV exposure and continued incubation at 37°C. (b) Quantitative relationship and linear fitting of fluorescence values vs. EBV concentration. (c) Real-time fluorescence detection of EBV concentrations in the blood of 14 EBV-infected patients (EBV.D.) using the same method. (d) Detection in blood from 8 healthy donors (H.D.).

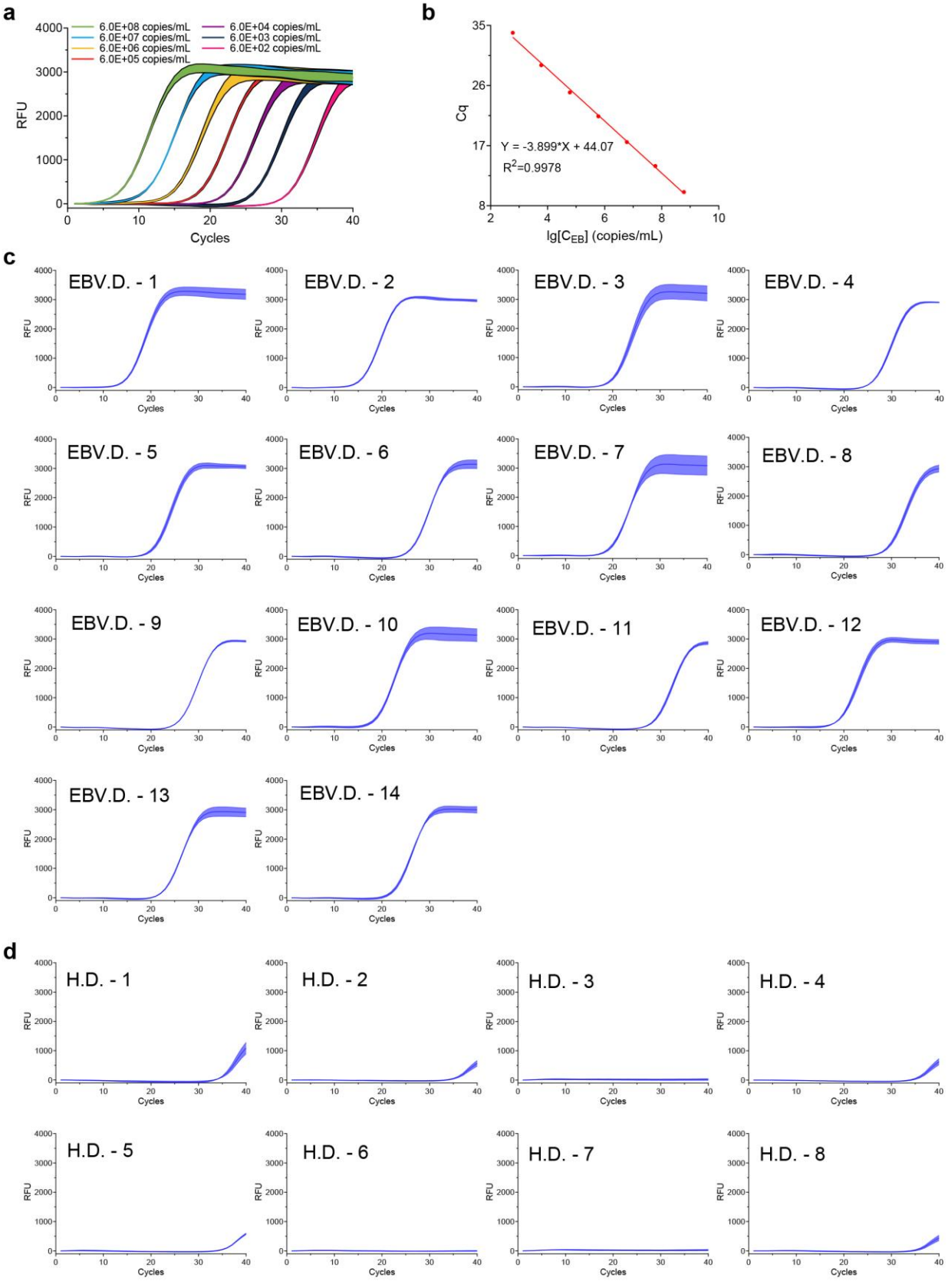

a
6.0E+08 copies/mL
6.0E+07 copies/mL
6.0E+06 copies/mL
6.0E+05 copies/mL
6.0E+04 copies/mL
6.0E+03 copies/mL
6.0E+02 copies/mL
RFU
Cycles
b
Cq
Y = -3.899*X + 44.07
R2=0.9978
lg[CEB] (copies/mL)
c
EBV.D. - 1
EBV.D. - 2
EBV.D. - 3
EBV.D. - 4
EBV.D. - 5
EBV.D. - 6
EBV.D. - 7
EBV.D. - 8
EBV.D. - 9
EBV.D. - 10
EBV.D. - 11
EBV.D. - 12
EBV.D. - 13
EBV.D. - 14
d
H.D. - 1
H.D. - 2
H.D. - 3
H.D. - 4
H.D. - 5
H.D. - 6
H.D. - 7
H.D. - 8

Figure S16. (a) Real-time fluorescence curves for various EBV standard concentrations using the classical qPCR method. (b) Quantitative relationship and linear fitting for cq values vs. EBV concentration. (c, d) Real-time fluorescence detection of EBV concentrations in blood from 14 infected patients and 8 healthy donors.

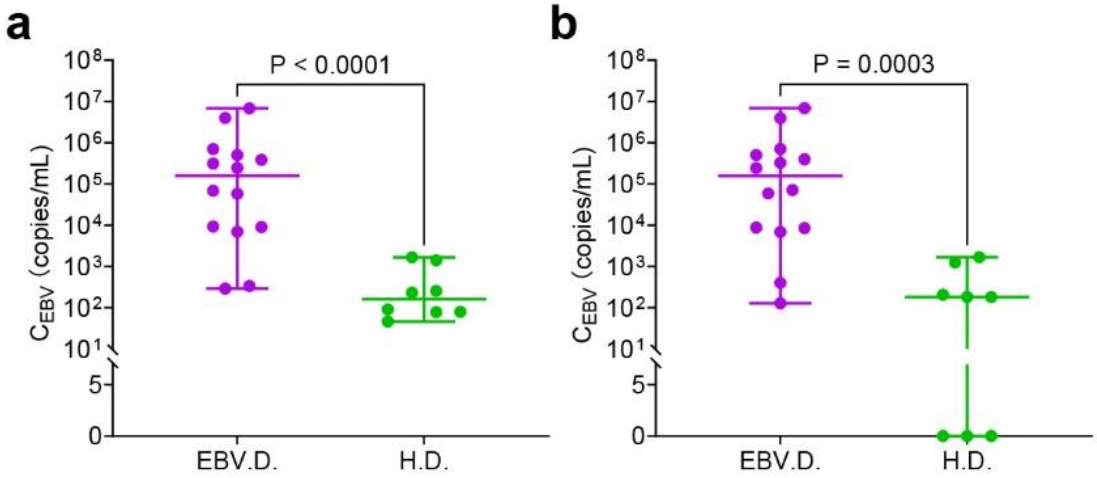


Figure S17.Comparative bar chart of EBV virus copy numbers in blood from 14 infected patients and 8 healthy donors, analyzed using the RPA-DR-PC-DNA CRISPR-Cas12a one-pot method (a) vs. the classical qPCR method (b). Notes: In the qPCR assay, EBV was undetectable in three out of eight healthy individuals (on the right of S17b), resulting in a reported nucleic acid concentration of 0 copies/mL.

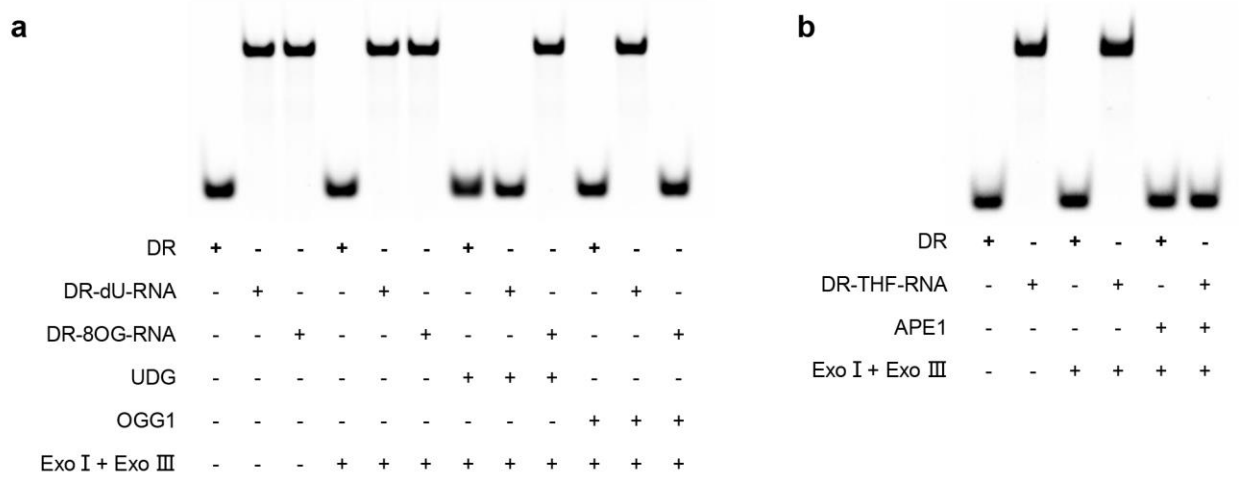


Figure S18. (a) 12% TBE-Urea denature PAGE results for DR, DR-dU-RNA, and DR-8OG-RNA with and without UDG or OGG1, followed by Exonuclease I & III treatment. Only DR-dU-RNA post-UDG

treatment and DR-8OG-RNA post-OGG1 treatment yield band lengths similar to DR. (b) PAGE results for DR and DR-THF-RNA under similar conditions, where DR-THF-RNA only resembles DR in band length after APE1 and Exonuclease I & III treatment. Reaction conditions: 1× LbCas12a-UDG Reaction Buffer (for DR-dU- RNA) or 1× LbCas12a Reaction Buffer (for DR-8OG-RNA/DR-THF-RNA), 100 ng DR-dU-DNA/DR-8OG-RNA/DR-THF-RNA/DR, 1 U UDG/OGG1/APE1, 4 U Exonuclease I and Exonuclease III, adjusted to a final volume of 10 μL with deionized water, incubated at 37°C for 1 h. Reactions were terminated by adding an equal volume of deionized formamide, followed by electrophoresis (12% denaturing gel, 150 V, 35 min).

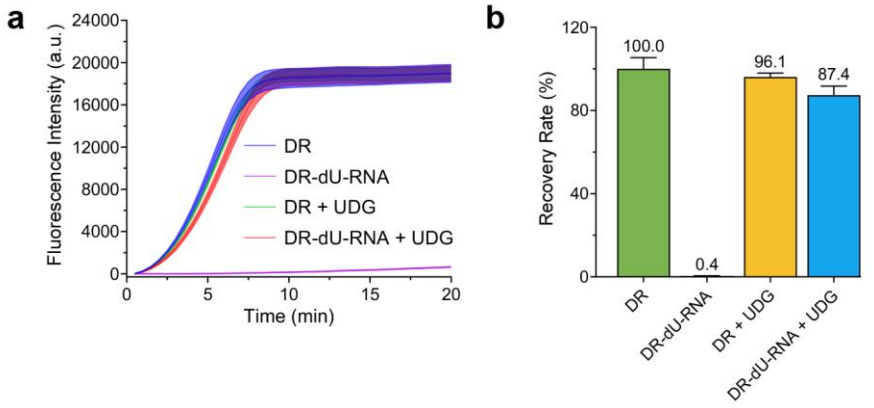


Figure S19. (a) Real-time fluorescence detection curves of LbCas12a *trans*-cleavage activity using DR or DR-dU-RNA with and without UDG, followed by Exonuclease I & III treatment. (b) The currently developed system's UDG treatment recovery rate.

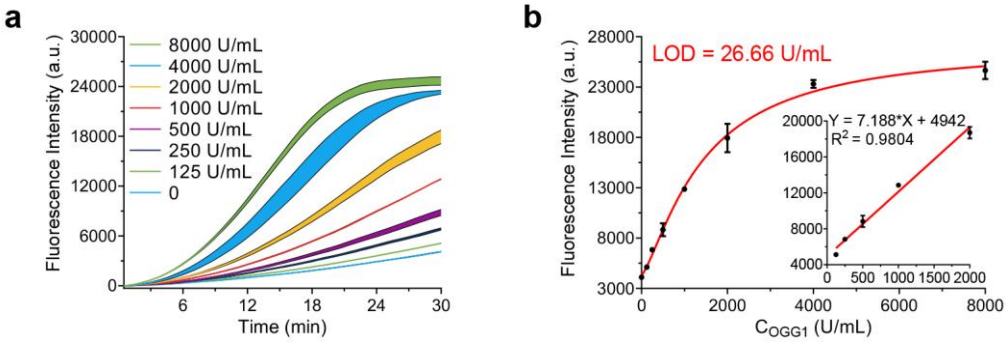


Figure S20. (a) Real-time quantitative fluorescence detection of various OGG1 concentrations using the DR-8OG-RNA-assisted CRISPR-Cas12a system. (b) Quantitative relationship, linear fitting, and detection limit of fluorescence values vs. OGG1 concentration.

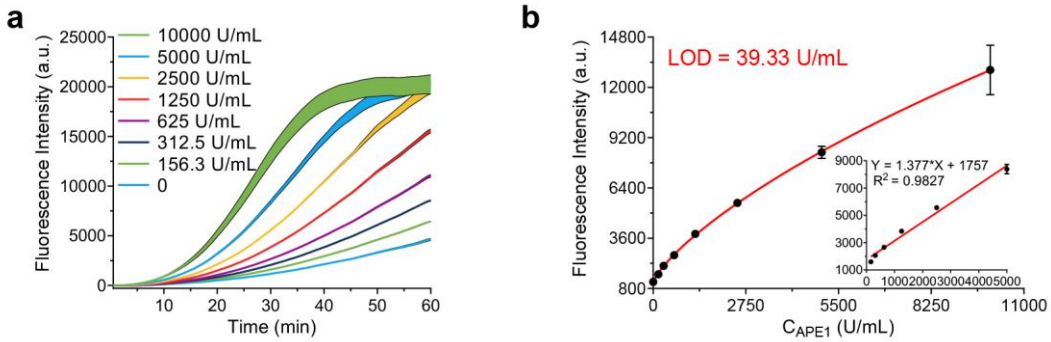


Figure S21. (a) Real-time quantitative fluorescence detection of different APE1 concentrations using the DR-THF-RNA-assisted CRISPR-Cas12a system. (b) Quantitative relationship, linear fitting, and detection limit of fluorescence values vs. APE1 concentration.

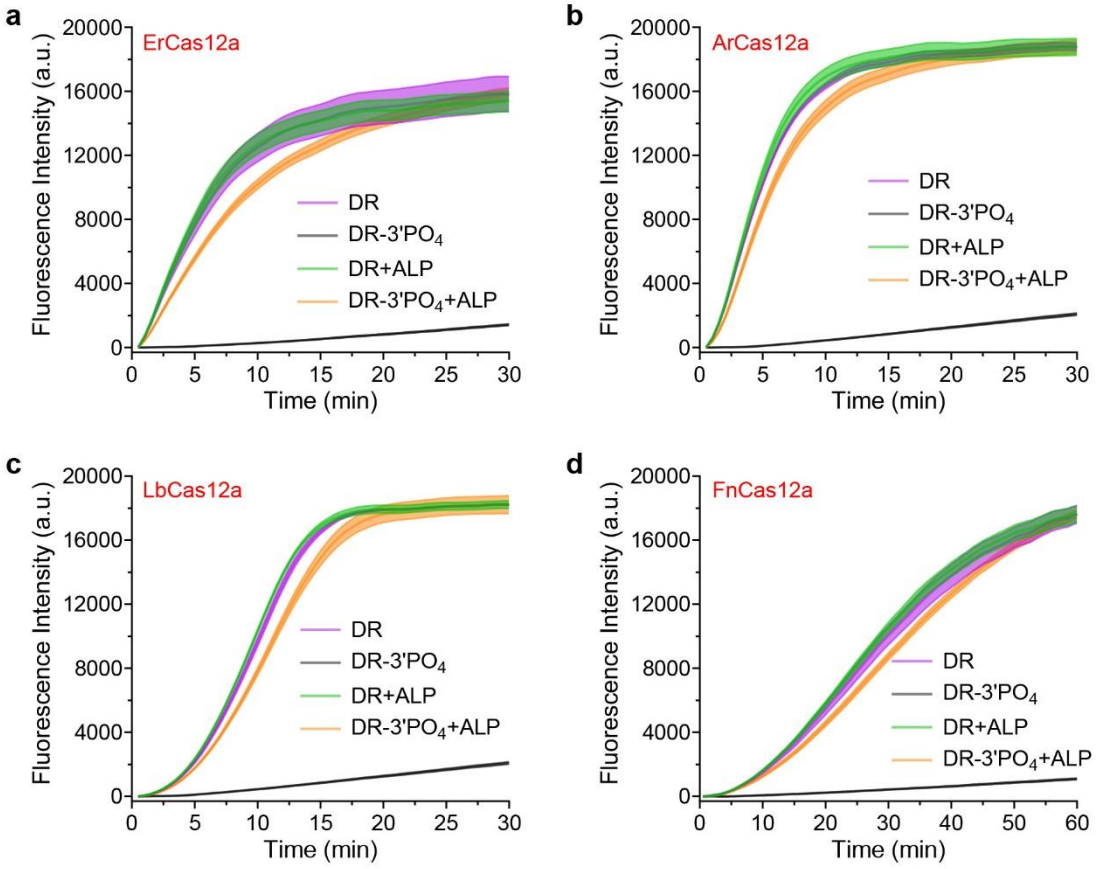


Figure S22. Modulation of *trans*-cleavage activity of different Cas12a variants by DR-3'PO_4: (a) ErCas12a; (b) ArCas12a; (c) LbCas12a; (d) FnCas12a. The reaction was conducted in 1× LbCas12a Reaction Buffer containing either 30 nM DR-3'PO_4 or 30 nM DR, with or without ALP (1 U/μL), and nuclease-free water to achieve a final volume of 10 μL. The mixture was incubated at 37°C for 30 min. Following this, 1×

LbCas12a Reaction Buffer, 100 nM LbCas12a, 250 nM F-Q reporter, 30 nM EBV Spacer, and 2 nM EBV target were added, and the total volume was adjusted to 20 μL with nuclease-free water. After thorough mixing, the reaction was promptly transferred to a qPCR instrument. Fluorescence was monitored at 37°C, with readings taken every 30 s. The detection duration was 30 min for panels (a)-(c) and 60 min for panel (d).

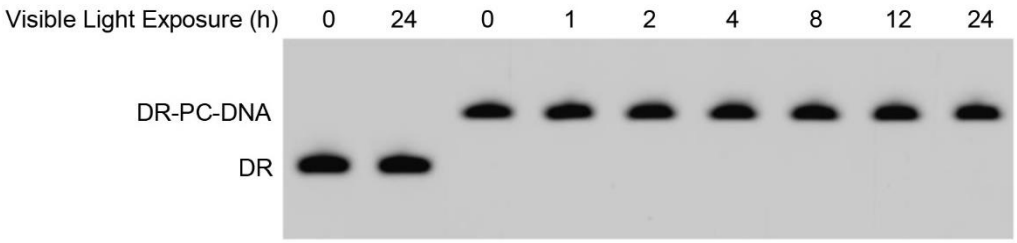


Figure S23. Stability assessment of DR-PC-DNA under laboratory ambient light conditions at different exposure times using 12% denaturing PAGE. The gel image demonstrates that the modified RNA-DNA hybrid remains stable under routine laboratory lighting conditions, indicating that complete light protection is not required during standard experimental procedures. The data suggest that the modification is sufficiently robust to withstand typical visible light exposure encountered in laboratory settings.

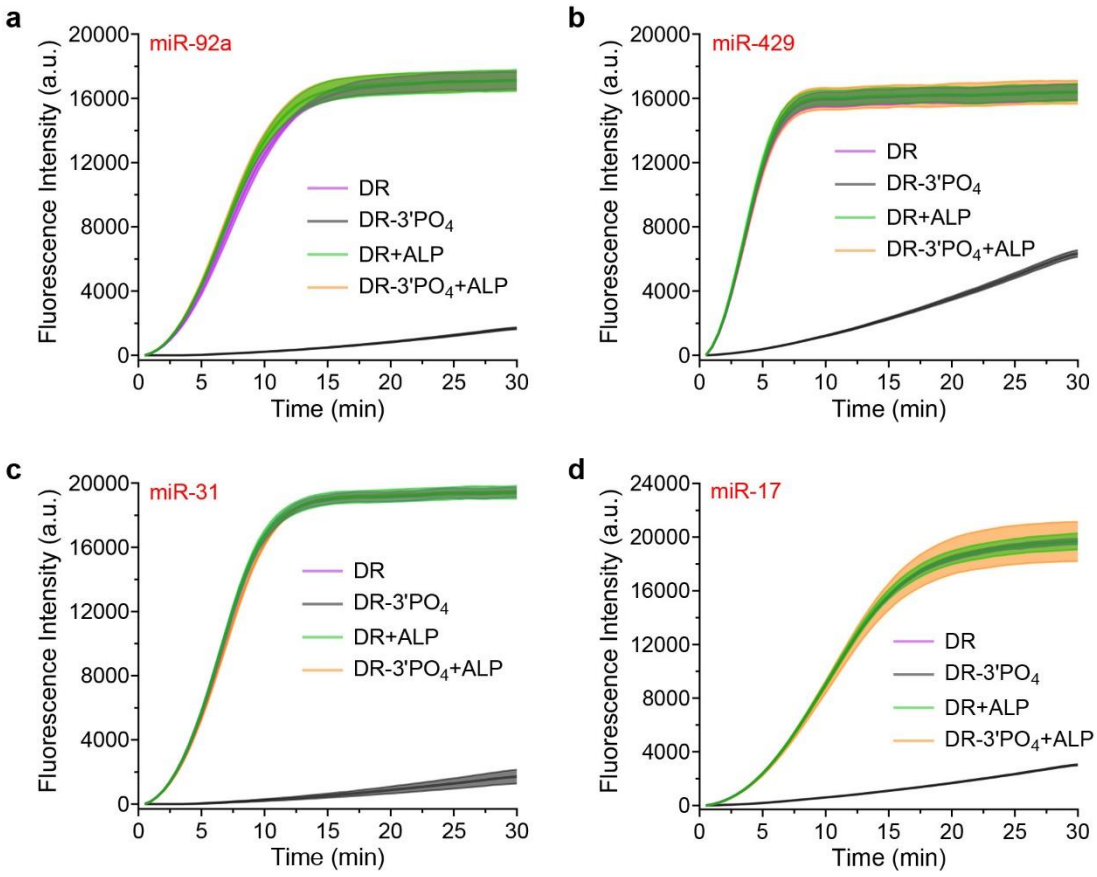


Figure S24. Regulation of LbCas12a *trans*-cleavage activity by DR-3'PO4 and ALP under the guidance of different microRNAs: (a) miR-92a; (b) miR-429; (c) miR-31; (d) miR-17. The reaction was performed in 1× LbCas12a Reaction Buffer with 30 nM DR-3'PO4 or 30 nM DR, with or without ALP (1 U/μL), and adjusted to 10 μL with nuclease-free water. The mixture was incubated at 37 °C for 30 min. Subsequently, 1× LbCas12a Reaction Buffer, 100 nM LbCas12a, 250 nM F-Q reporter, 30 nM miRNA, and 10 nM corresponding miRNA activator were added, and the total volume was adjusted to 20 μL with nuclease-free water. After thorough mixing, the reaction was immediately transferred to a qPCR instrument for fluorescence measurement at 37 °C, with data collected every 30 s for 30 min. The results demonstrate that different miRNAs can guide LbCas12a *trans*-cleavage activity in the presence of their corresponding DNA targets when paired with DR. However, phosphorylation at the 3' end of DR abolishes the guiding function of miRNAs. Upon removal of the 3' phosphate group by ALP, the miRNA-mediated activation of LbCas12a is restored.