Supporting Information

Single molecule characterization of CRISPR-Cas12a for amplification-free genetic testing

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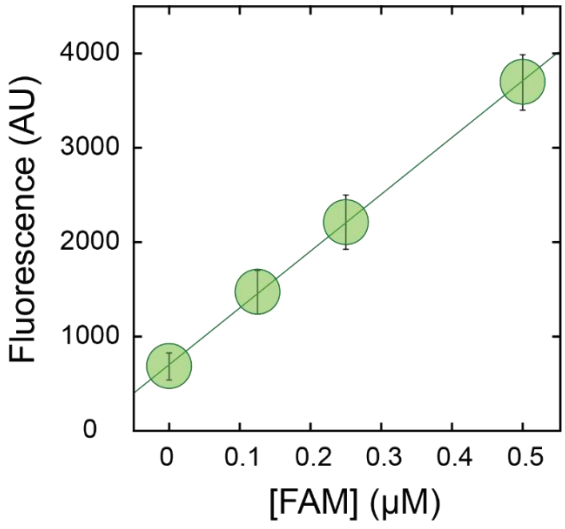


Figure S1. Calibration curve of FAM-conjugated nucleotide. 5' FAM-conjugated dTdTdTdTdT oligonucleotides was sealed in CD-based microchamber devices, and the fluorescence images were recorded. The solid line represents the linear regression, and the data are mean ± SD (n = 3 technical replicates).

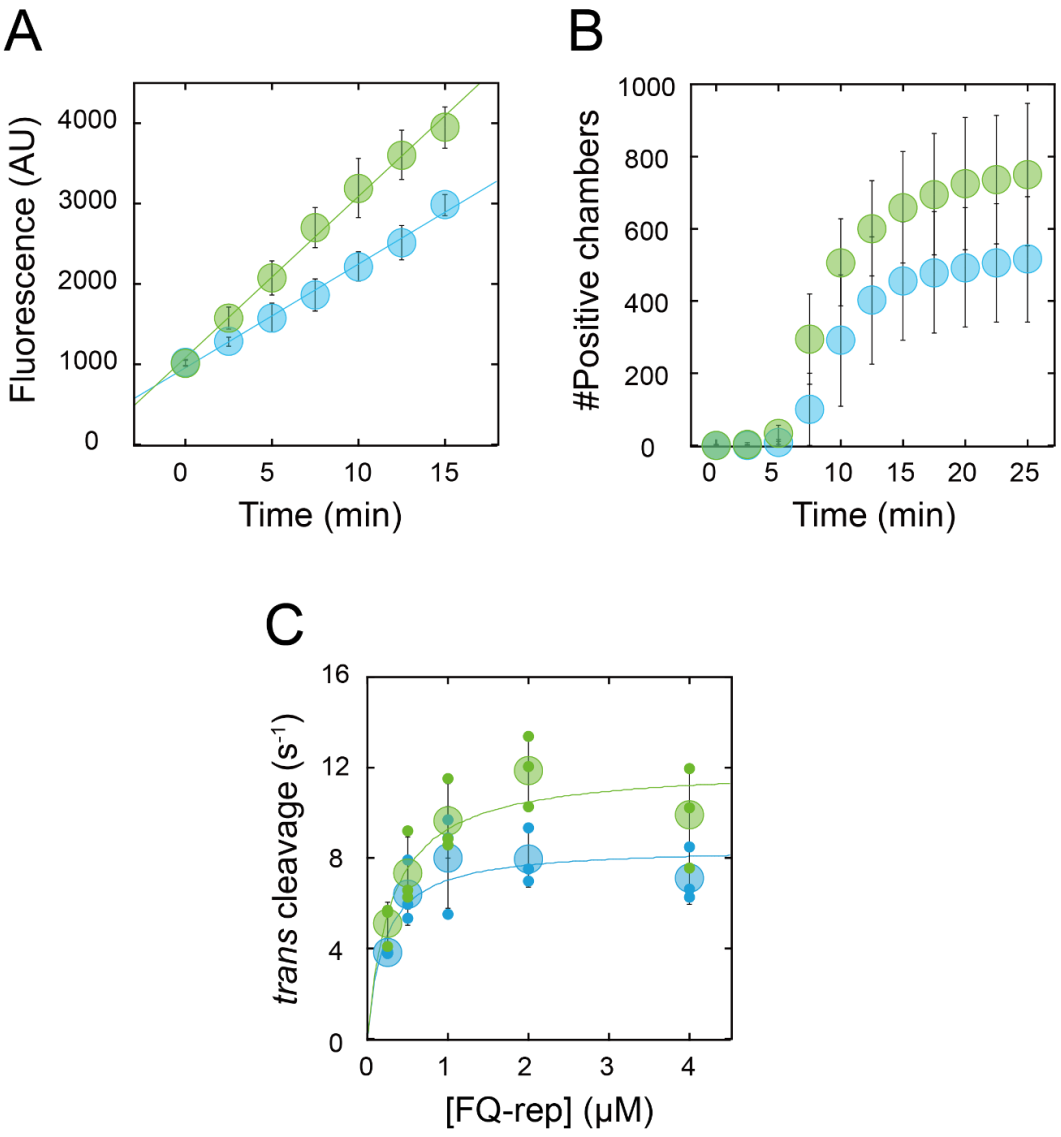


Figure S2. Single-molecule analysis of en*Lb*Cas12a. (A, B) Time course of fluorescence intensity increase (A) and number of positive chambers (B) at 45 ℃ using crRNA with (green) or without (blue) a 7-base DNA appending at the 3' terminus. Data was obtained in the presence of 100 fM MPXV J2R gene fragment. (C) *Trans*-cleavage rate measured at various concentrations of FQ reporters. The data were fitted to the Michaelis–Menten equation. V_{max} and K_m values were 8.5 s^{-1} and 0.21 μM for crRNA, and 12.0 s^{-1} and 0.30 μM for the 7-nt DNA-appended crRNA.

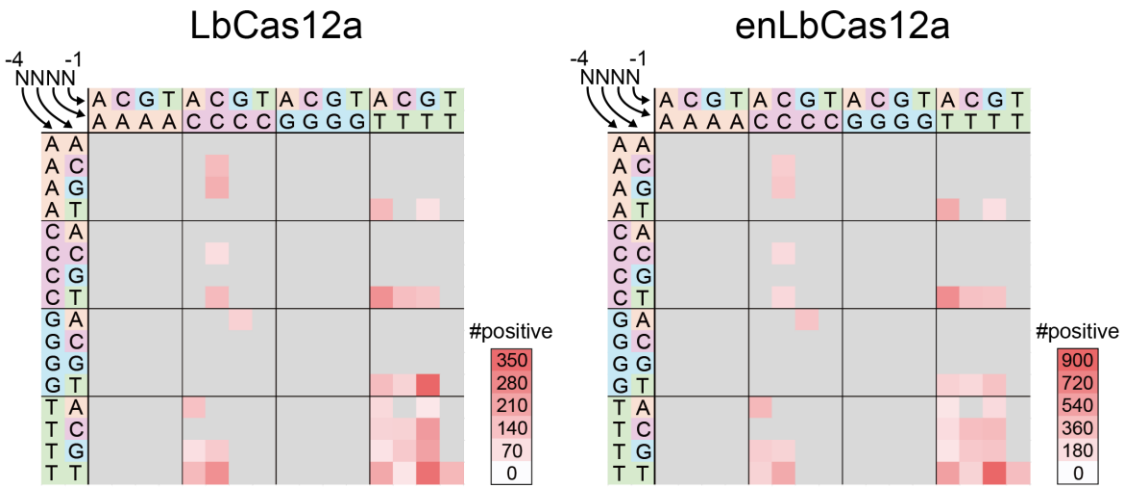


Figure S3. A classification analysis of PAM activity patterns for *Lb*Cas12a and en*Lb*Cas12a. The number of positive chambers obtained by single-molecule analysis with 1 pM tgDNA bearing different PAM sequences are shown as a heat map (n = 3 technical replicates).

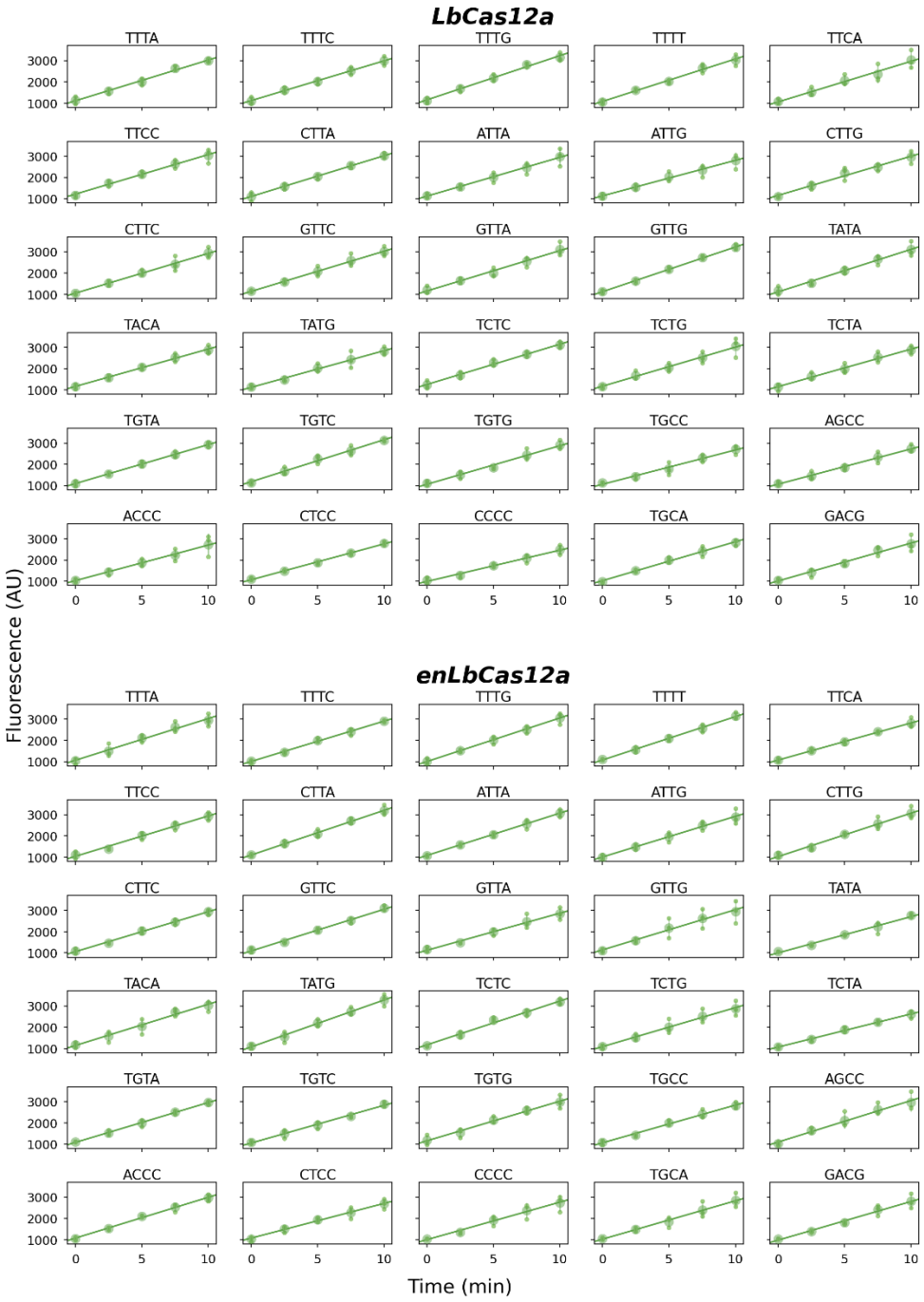


Figure S4. Time course of fluorescence increase from *trans*-cleavage activity of *Lb*Cas12a and en*Lb*Cas12a on tgDNAs bearing different PAM sequences. Single-molecule analyses were performed with 1 pM tgDNA at 45 °C. Solid lines represent linear regressions fitted to the data between 0 and 10 min. Data are presented as mean ± SD (n = 3 technical replicates).

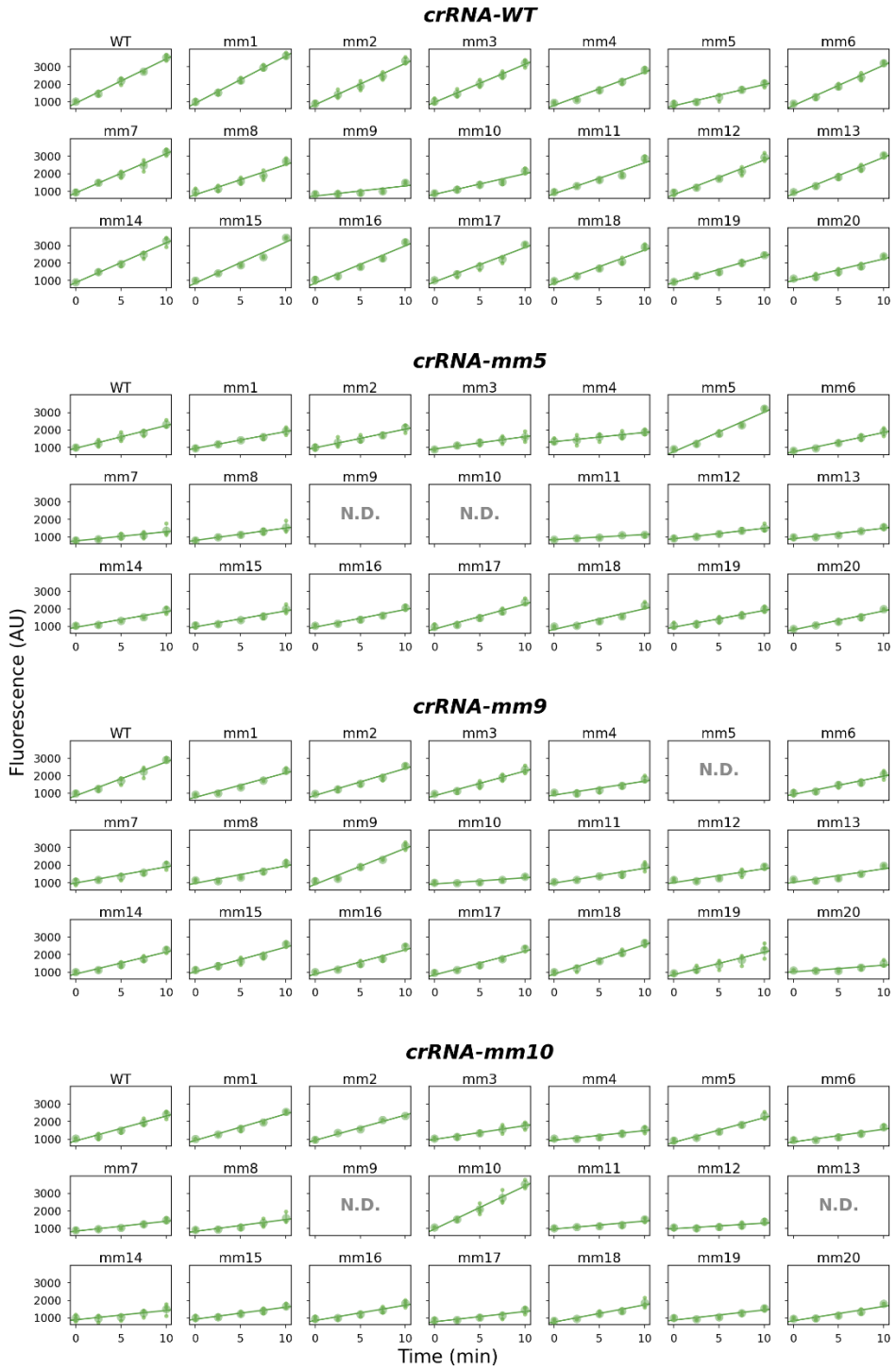


Figure S5. Time course of fluorescence increase from *trans*-cleavage activity of *Lb*Cas12a and en*Lb*Cas12a on mismatch-containing tgDNAs. Single-molecule analyses were performed with 1 pM tgDNA at 45 °C. The sequences of crRNA and tgDNA are shown in Figure 3A. Solid lines represent linear regressions fitted to the data between 0 and 10 min. Data are presented as mean ± SD (n = 3 technical replicates).

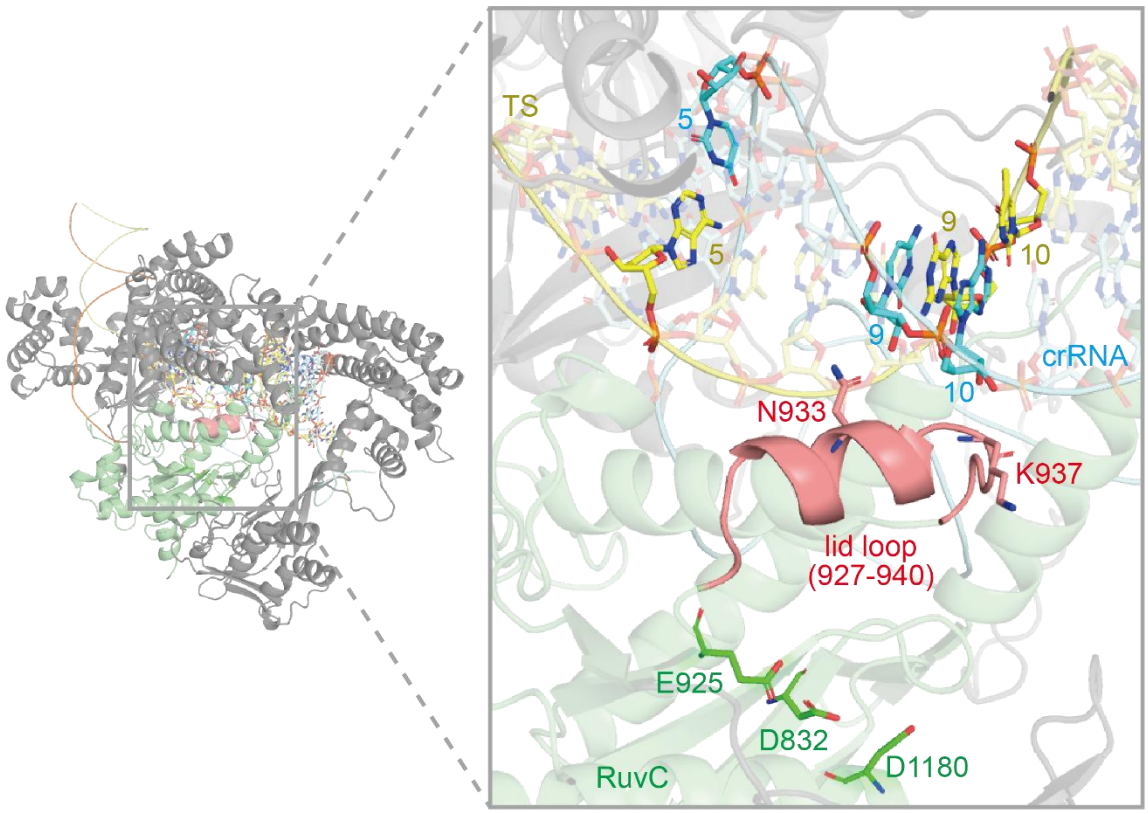


Figure S6. AlphaFold 3 model of en*Lb*Cas12a complex with crRNA and dsDNA target. Structure was predicted by AlphaFold 3(1). A close-up view of the α-helical lid region is shown on the right. The protein is shown in grey, with the lid loop in pale red, the crRNA in cyan, the target strand (TS) in yellow, and the non-target strand (NTS) in orange. Hybrid positions 9 and 10 are placed adjacent to the RuvC-occluding "lid" loop. The DNA sequences used in the model were: TS, 5′-attgtttctctcatgtataataaTAAAcggaagagatatagcaccacatgcaccatccaatggaaagtgtaaag-3′; NTS, 5′-ctttacactttccattggatggtgcatgtggtgctatatctcttccgTTTAttattat-3′. The PAM and PAM-complement are shown in uppercase. The crRNA carried a 7-nt DNA extension at its 3′ end (sequence crRNA-a in Table S1).

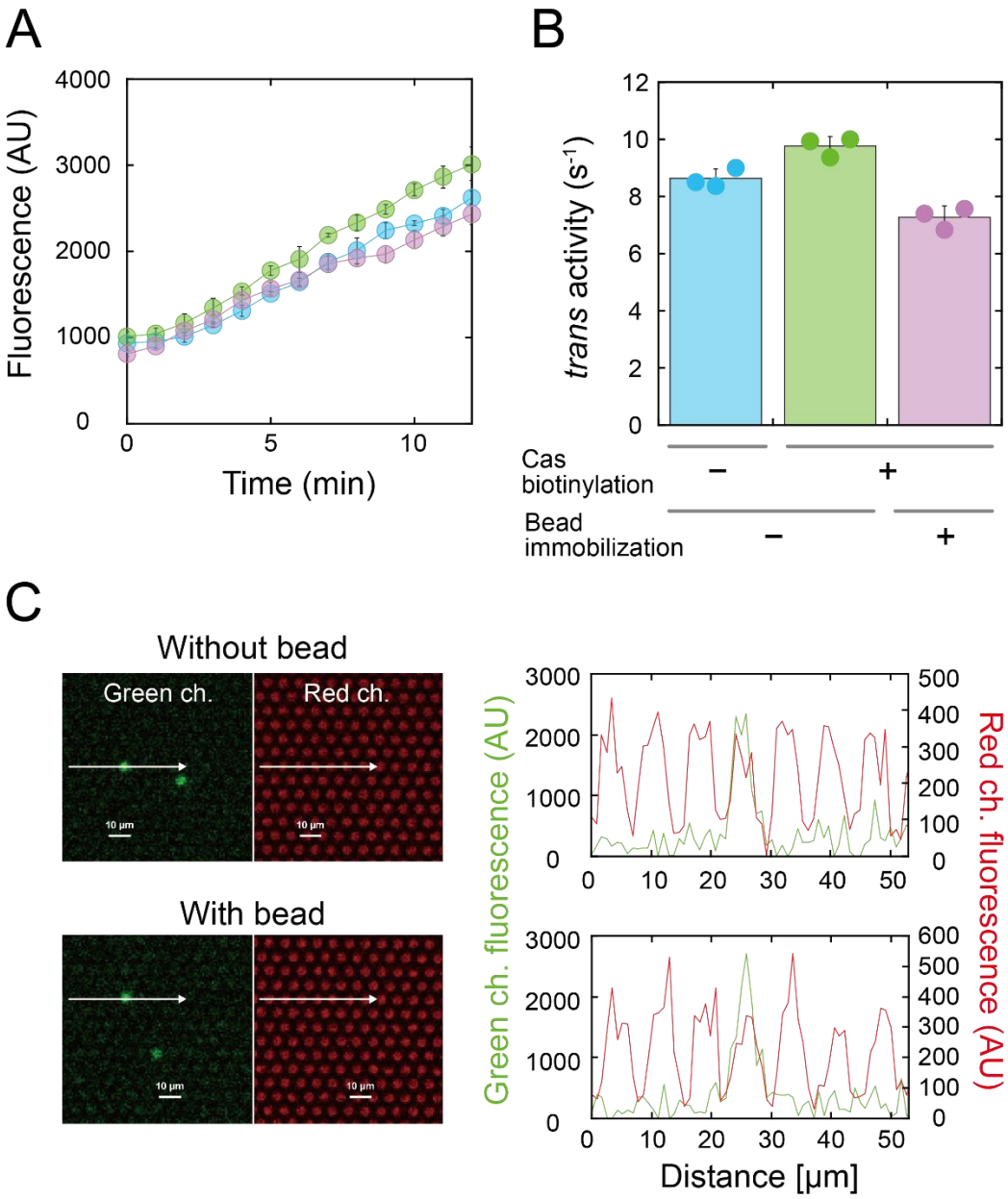


Figure S7. Effects of biotinylation and bead immobilization of en*Lb*Cas12a. (A, B) Time course of fluorescence increase (A) and the corresponding *trans*-cleavage rates (B) for non-biotinylated en*Lb*Cas12a (blue), biotinylated en*Lb*Cas12a (green) in solution, and biotinylated en*Lb*Cas12a with magnetic bead immobilization (purple). Single-molecule measurements of *trans*-cleavage activity were performed with 100 fM tgDNA for the non-biotinylated and biotinylated conditions (blue and green) in solution, and with 3 fM tgDNA for the magnetic-bead immobilized condition (purple). Data are presented as mean ± SD (n = 3 technical replicates). (C) Representative fluorescence images obtained with and without the magnetic bead immobilization (left). Line profiles of fluorescence intensity along the indicated lines are shown for the green and red channels (right), corresponding to FAM and Alexa Fluor 647 fluorescence, respectively.

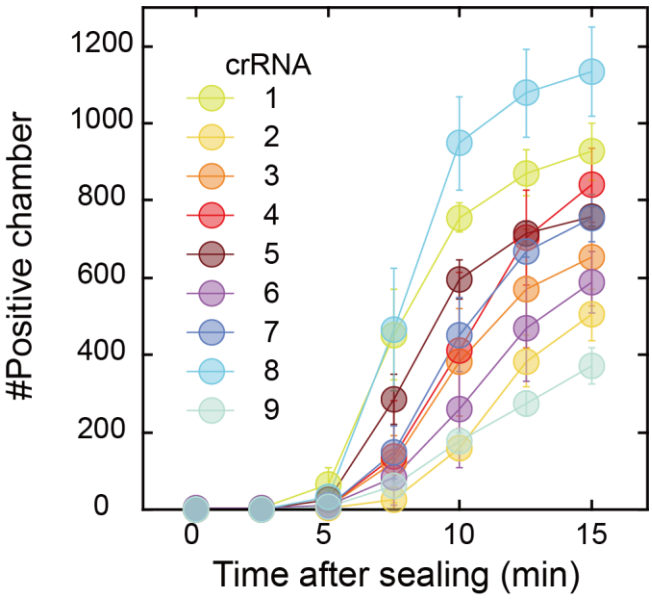


Figure S8. Screening of optimal crRNA for MPXV dsDNA detection. Amplification-free DNA detection assay using en*Lb*Cas12a was conducted without magnetic bead–enrichment process. The number of positive chambers was measured at the indicated time points after oil sealing with 100 fM MPXV J2R gene. crRNA-8 (Lb12-cr-MPXV-DNA-8) was identified as the optimal crRNA for MPXV dsDNA detection.

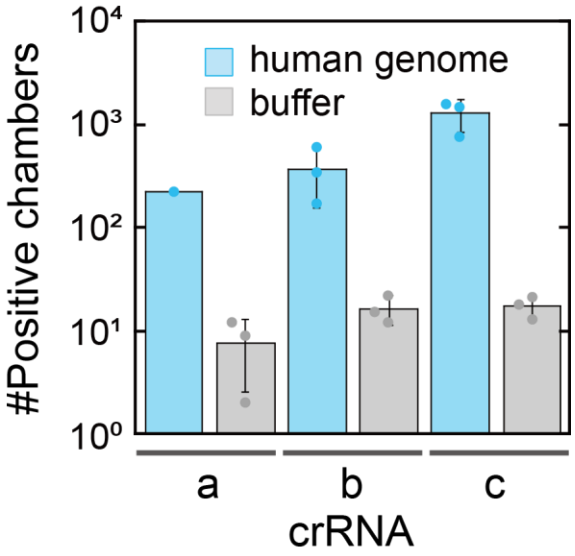


Figure S9. Cross-reactivity of *Lb*Cas12a-K538R. The number of positive chambers was measured in the presence of 100 ng/mL genomic DNA derived from human using three different crRNAs targeting distinct sequences in MPXV gene.

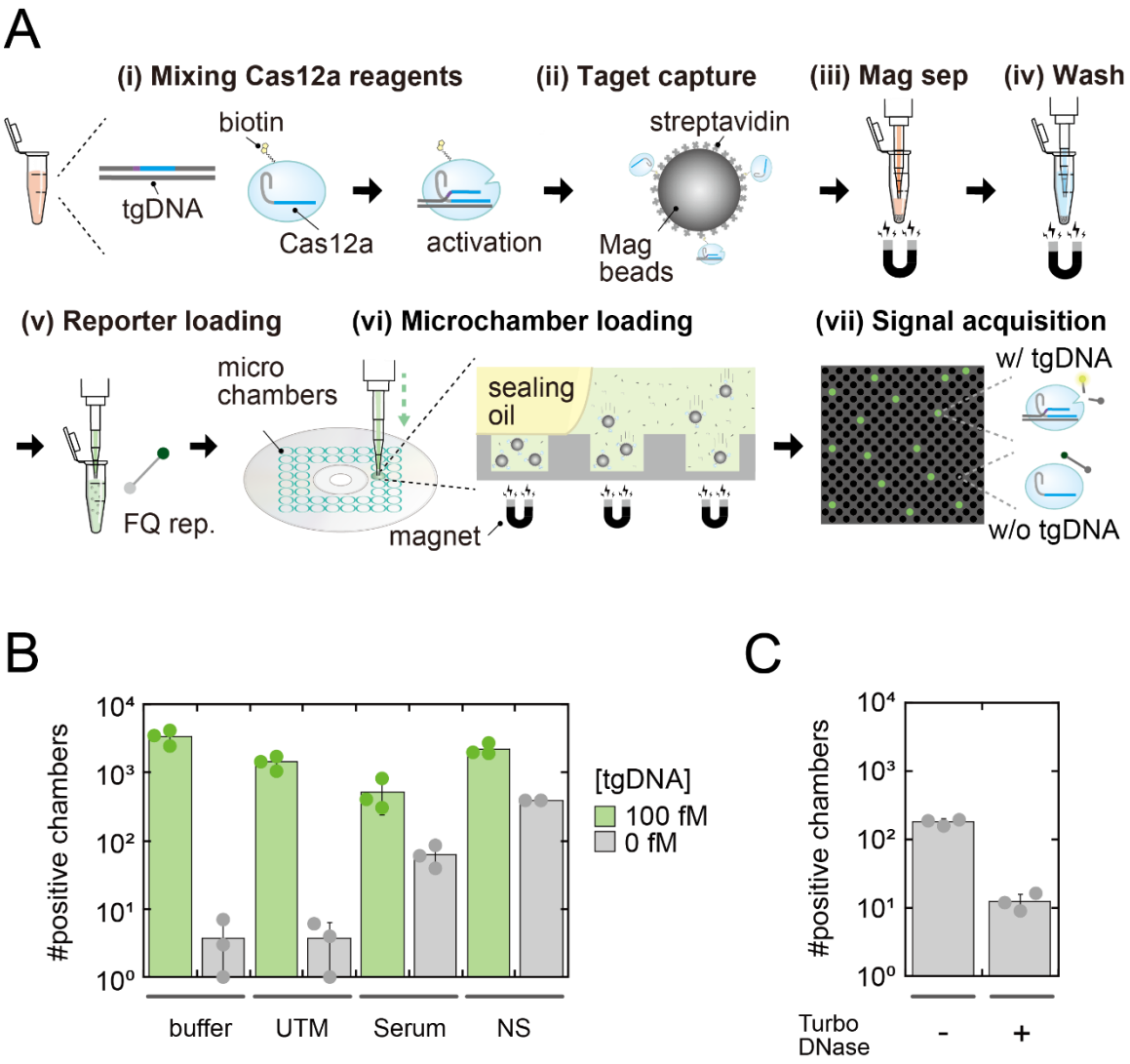


Figure S10. Direct detection of dsDNA targets from biological samples. (A) Schematic of direct single-molecule DNA detection from biological samples. (B) Direct detection of dsDNA targets spiked into buffer, universal transport medium (UTM), healthy-donor serum, or nasopharyngeal swab (NS) samples. The number of positive chambers was measured for each condition. Substantial background signals were observed in non-spiked serum and NS samples, suggesting that en*Lb*Cas12a may be nonspecifically activated by endogenous DNA present in these samples. (C) Effect of DNase treatment on swab samples. 10-fold diluted swab samples were treated with or without 0.2 U/μL Turbo DNase (Thermo Fisher Scientific, AM2238) in the reaction buffer supplied by the manufacturer for 1 h at 37 °C, followed by heat inactivation at 90 °C for 5 min, and then subjected to the direct SATORI assay. Data are presented as mean ± SD (n = 3 technical replicates).

Table S1. crRNA sequences.

Name	sequences	Fig.
Lb12-cr-MPXV-1	GGAAUUUCUACUAAGUGUAGAU UUAUUAUACAUGAGAGAAAC	Fig. 1, S2
Lb12-cr-MPXV-DNA-1 (crRNA-a)	GGAAUUUCUACUAAGUGUAGAU UUAUUAUACAUGAGAGAAAC dTdAdTdTdAdTdT	Fig. 1-3,4E, S2-6, S8-9
Lb12-cr-MPXV-DNA-1-mm5	GGAAUUUCUACUAAGUGUAGAU UUAUAAUACAUGAGAGAAAC dTdAdTdTdAdTdT	Fig. 3, S5
Lb12-cr-MPXV-DNA-1-mm9	GGAAUUUCUACUAAGUGUAGAU UUAUUAUAGAUGAGAGAAAC dTdAdTdTdAdTdT	Fig. 3, S5
Lb12-cr-MPXV-DNA-1-mm10	GGAAUUUCUACUAAGUGUAGAU UUAUUAUACUUGAGAGAAAC dTdAdTdTdAdTdT	Fig. 3, S5
Lb12-cr-MPXV-DNA-2	GGAAUUUCUACUAAGUGUAGAU CAUCUCACAAUAAUCAUUUA dTdAdTdTdAdTdT	Fig. S8
Lb12-cr-MPXV-DNA-3	GGAAUUUCUACUAAGUGUAGAU CAGGCUUGUCUAAGUUGUAA dTdAdTdTdAdTdT	Fig. S8
Lb12-cr-MPXV-DNA-4	GGAAUUUCUACUAAGUGUAGAU GAAAUACAUGUUCUACACCC dTdAdTdTdAdTdT	Fig. S8
Lb12-cr-MPXV-DNA-5	GGAAUUUCUACUAAGUGUAGAU UCUGUGGAAGAGACGGUGUG dTdAdTdTdAdTdT	Fig. S8
Lb12-cr-MPXV-DNA-6	GGAAUUUCUACUAAGUGUAGAU ACUAUAUCGAUGUGGAAAUU dTdAdTdTdAdTdT	Fig. S8
Lb12-cr-MPXV-DNA-7 (crRNA-b)	GGAAUUUCUACUAAGUGUAGAU UGAUUCAUGGUAAUAGUUAG dTdAdTdTdAdTdT	Fig. 4E, S8-9
Lb12-cr-MPXV-DNA-8 (crRNA-c)	GGAAUUUCUACUAAGUGUAGAU CAGGAGAAAAUAGAUAUCAG dTdAdTdTdAdTdT	Fig. 4B-E, S7-10
Lb12-cr-MPXV-DNA-9	GGAAUUUCUACUAAGUGUAGAU AUCUCGAAAUUCAGAGUACA dTdAdTdTdAdTdT	Fig. S8

Table S2. dsDNA target sequences.

Name	sequences	Fig.
tgDNA_TTTA-PAM	ctttacactttccattggatggtgcatgtggtgctatatctcttcc gTTTAttattatacatgagagaaacaatatatacgagtataatacg gacctcat	Fig. 2, S3-4
tgDNA_TTTC-PAM	ctttacactttccattggatggtgcatgtggtgctatatctcttcc gTTTCttattatacatgagagaaacaatatatacgagtataatacg gacctcat	Fig. 2, S3-4
tgDNA_TTTG-PAM	ctttacactttccattggatggtgcatgtggtgctatatctcttcc gTTTGttattatacatgagagaaacaatatatacgagtataatacg gacctcat	Fig. 2, S3-4
tgDNA_TTTT-PAM	ctttacactttccattggatggtgcatgtggtgctatatctcttcc gTTTTttattatacatgagagaaacaatatatacgagtataatacg gacctcat	Fig. 2, S3-4
tgDNA_TTCA-PAM	ctttacactttccattggatggtgcatgtggtgctatatctcttcc gTTCAttattatacatgagagaaacaatatatacgagtataatacg gacctcat	Fig. 2, S3-4
tgDNA_TTCC-PAM	ctttacactttccattggatggtgcatgtggtgctatatctcttcc gTTCCttattatacatgagagaaacaatatatacgagtataatacg gacctcat	Fig. 2, S3-4
tgDNA_CTTA-PAM	ctttacactttccattggatggtgcatgtggtgctatatctcttcc gCTTAttattatacatgagagaaacaatatatacgagtataatacg gacctcat	Fig. 2, S3-4
tgDNA_ATTA-PAM	ctttacactttccattggatggtgcatgtggtgctatatctcttcc gATTAttattatacatgagagaaacaatatatacgagtataatacg gacctcat	Fig. 2, S3-4

tgDNA_ATTG-PAM	ctttacactttccattggatggtgcatgtggtgctatatctcttcc gATTGttattatacatgagagaaacaatatatacgagtataatacg gacctcat	Fig. 2, S3-4
tgDNA_CTTG-PAM	ctttacactttccattggatggtgcatgtggtgctatatctcttcc gCTTGttattatacatgagagaaacaatatatacgagtataatacg gacctcat	Fig. 2, S3-4
tgDNA_CTTC-PAM	ctttacactttccattggatggtgcatgtggtgctatatctcttcc gCTTCttattatacatgagagaaacaatatatacgagtataatacg gacctcat	Fig. 2, S3-4
tgDNA_GTTC-PAM	ctttacactttccattggatggtgcatgtggtgctatatctcttcc gGTTCttattatacatgagagaaacaatatatacgagtataatacg gacctcat	Fig. 2, S3-4
tgDNA_GTTA-PAM	ctttacactttccattggatggtgcatgtggtgctatatctcttcc gGTTAttattatacatgagagaaacaatatatacgagtataatacg gacctcat	Fig. 2, S3-4
tgDNA_GTTG-PAM	ctttacactttccattggatggtgcatgtggtgctatatctcttcc gGTTGttattatacatgagagaaacaatatatacgagtataatacg gacctcat	Fig. 2, S3-4
tgDNA_TATA-PAM	ctttacactttccattggatggtgcatgtggtgctatatctcttcc gTATAttattatacatgagagaaacaatatatacgagtataatacg gacctcat	Fig. 2, S3-4
tgDNA_TACA-PAM	ctttacactttccattggatggtgcatgtggtgctatatctcttcc gTACAttattatacatgagagaaacaatatatacgagtataatacg gacctcat	Fig. 2, S3-4
tgDNA_TATG-PAM	ctttacactttccattggatggtgcatgtggtgctatatctcttcc gTATGttattatacatgagagaaacaatatatacgagtataatacg gacctcat	Fig. 2, S3-4
tgDNA_TCTC-PAM	ctttacactttccattggatggtgcatgtggtgctatatctcttcc gTCTCttattatacatgagagaaacaatatatacgagtataatacg gacctcat	Fig. 2, S3-4
tgDNA_TCTG-PAM	ctttacactttccattggatggtgcatgtggtgctatatctcttcc gTCTGttattatacatgagagaaacaatatatacgagtataatacg gacctcat	Fig. 2, S3-4
tgDNA_TCTA-PAM	ctttacactttccattggatggtgcatgtggtgctatatctcttcc gTCTAttattatacatgagagaaacaatatatacgagtataatacg gacctcat	Fig. 2, S3-4
tgDNA_TGTA-PAM	ctttacactttccattggatggtgcatgtggtgctatatctcttcc gTGTAttattatacatgagagaaacaatatatacgagtataatacg gacctcat	Fig. 2, S3-4
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tgDNA_CTCC-PAM	ctttacactttccattggatggtgcatgtggtgctatatctcttcc gCTCCttattatacatgagagaaacaatatatacgagtataatacg gacctcat	Fig. 2, S3-4
tgDNA_CCCC-PAM	ctttacactttccattggatggtgcatgtggtgctatatctcttcc gCCCCttattatacatgagagaaacaatatatacgagtataatacg gacctcat	Fig. 2, S3-4
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tgDNA_GACG-PAM	ctttacactttccattggatggtgcatgtggtgctatatctcttcc gGACGttattatacatgagagaaacaatatatacgagtataatacg gacctcat	Fig. 2, S3-4
tgDNA_MPXV_WT	ctttacactttccattggatggtgcatgtggtgctatatctcttcc gTTTAttattatacatgagagaaacaatatatacgagtataatacg gacctcat	Fig. 3, S5

tgDNA_MPXV_MM1	ctttacactttccattggatggtgcatgtggtgctatatctcttcc gTTTAAtattatacatgagagaaacaatatatacgagtataatacg gacctcat	Fig. 3, S5
tgDNA_MPXV_MM2	ctttacactttccattggatggtgcatgtggtgctatatctcttcc gTTTAtAattatacatgagagaaacaatatatacgagtataatacg gacctcat	Fig. 3, S5
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tgDNA_MPXV_MM20	ctttacactttccattggatggtgcatgtggtgctatatctcttcc gTTTAttattatacatgagagaaaGaatatatacgagtataatacg gacctcat	Fig. 3, S5
MPXV-J2R	atgaggtccgtattatactcgtatatattgtttctctcatgtataa taataaacggaagagatatagcaccacatgcaccatccaatggaaa gtgtaaagacaacgaatacagaagccgtaatctatgttgtctatcg tgtcctccgggaacttacgctttcagattatgtgatagcaagacta atacacaatgtacaccgtgtggttcggataccttacatctcacaa taatcatttacaggcttgtctaagttgtaacggaagatgtgatagt aatcaggtagagacgcgatcgtgtaacacgactcacaatagaatct gtgaatgctctccaggatattattgtcttctcaaaggagcatcagg gtgtagaacatgtatttctaaaacaaagtgtggaataggatacgga gtatccggatacacgtctaccggagacgtcatctgttctccgtgtg	Fig. 1, 4A-B, S2, S7-8, S10

	gtcccggaacatattctcacaccgtctcttccacagataaatgcga acccgtcgtaaccagcaatacatttaactatatcgatgtggaaatt aacctgtatccagtcaacgacacatcgtgtactcggacgaccacta ccggtctcagcgaatccatctcaacgtcggaactaactattaccat gaatcataaagattgtgatccagtctttcgtgcagaatacttctct gtccttaataatgtagcaacttcaggattctttacaggagaaaata gatatcagaatacttcaaagatatgtactctgaatttcgagattaa atgtaacaacaaagattcatcttccaaacagttaacgaaaacaaag aatgatactatcatgccgcattcagagacggtaactctagtgggcg actgtctatctagcgtcgacatctacatactatatagtaataccaa tactcaagactacgaaaatgatacaatctcttatcatatgggtaat gttctcgatgtcaatagccatatgcccgctagttgcgatatacata aactgatcactaattcccagaatcccacccacttatag	

Table S3. DNA used for target DNA preparation.

Name	Sequence
Fw_tgDNA	ctttacactttccattggatggtgcatgtggtgctatatctcttccg
Rv_tgDNA_TTTA-PAM	atgaggtccgtattatactcgtatatattgtttctctcatgtataataa TAAA cggaagagatatagcaccac
Rv_tgDNA_TTTC-PAM	atgaggtccgtattatactcgtatatattgtttctctcatgtataataa GAAA cggaagagatatagcaccac
Rv_tgDNA_TTTG-PAM	atgaggtccgtattatactcgtatatattgtttctctcatgtataataa CAAA cggaagagatatagcaccac
Rv_tgDNA_TTTT-PAM	atgaggtccgtattatactcgtatatattgtttctctcatgtataataa AAAA cggaagagatatagcaccac
Rv_tgDNA_TTCA-PAM	atgaggtccgtattatactcgtatatattgtttctctcatgtataataa TGAA cggaagagatatagcaccac
Rv_tgDNA_TTCC-PAM	atgaggtccgtattatactcgtatatattgtttctctcatgtataataa GGAA cggaagagatatagcaccac
Rv_tgDNA_CTTA-PAM	atgaggtccgtattatactcgtatatattgtttctctcatgtataataa TAAG cggaagagatatagcaccac
Rv_tgDNA_ATTA-PAM	atgaggtccgtattatactcgtatatattgtttctctcatgtataataa TAAT cggaagagatatagcaccac
Rv_tgDNA_ATTG-PAM	atgaggtccgtattatactcgtatatattgtttctctcatgtataataa CAAT cggaagagatatagcaccac
Rv_tgDNA_CTTG-PAM	atgaggtccgtattatactcgtatatattgtttctctcatgtataataa CAAG cggaagagatatagcaccac
Rv_tgDNA_CTTC-PAM	atgaggtccgtattatactcgtatatattgtttctctcatgtataataa GAAG cggaagagatatagcaccac
Rv_tgDNA_GTTC-PAM	atgaggtccgtattatactcgtatatattgtttctctcatgtataataa GAAC cggaagagatatagcaccac
Rv_tgDNA_GTTA-PAM	atgaggtccgtattatactcgtatatattgtttctctcatgtataataa TAAC cggaagagatatagcaccac
Rv_tgDNA_GTTG-PAM	atgaggtccgtattatactcgtatatattgtttctctcatgtataataa CAAC cggaagagatatagcaccac
Rv_tgDNA_TATA-PAM	atgaggtccgtattatactcgtatatattgtttctctcatgtataataa TATA cggaagagatatagcaccac
Rv_tgDNA_TACA-PAM	atgaggtccgtattatactcgtatatattgtttctctcatgtataataa TGTA cggaagagatatagcaccac
Rv_tgDNA_TATG-PAM	atgaggtccgtattatactcgtatatattgtttctctcatgtataataa CATA cggaagagatatagcaccac
Rv_tgDNA_TCTC-PAM	atgaggtccgtattatactcgtatatattgtttctctcatgtataataa GAGA cggaagagatatagcaccac
Rv_tgDNA_TCTG-PAM	atgaggtccgtattatactcgtatatattgtttctctcatgtataataa CAGA cggaagagatatagcaccac
Rv_tgDNA_TCTA-PAM	atgaggtccgtattatactcgtatatattgtttctctcatgtataataa TAGA cggaagagatatagcaccac
Rv_tgDNA_TGTA-PAM	atgaggtccgtattatactcgtatatattgtttctctcatgtataataa TACA cggaagagatatagcaccac
Rv_tgDNA_TGTC-PAM	atgaggtccgtattatactcgtatatattgtttctctcatgtataataa GACA cggaagagatatagcaccac
Rv_tgDNA_TGTG-PAM	atgaggtccgtattatactcgtatatattgtttctctcatgtataataa CACA cggaagagatatagcaccac
Rv_tgDNA_TGCC-PAM	atgaggtccgtattatactcgtatatattgtttctctcatgtataataa GGCA cggaagagatatagcaccac

Rv_tgDNA_AGCC-PAM	atgaggtccgtattatactcgtatatattgtttctctcatgtataataa GGCT cggaagagatatagcaccac
Rv_tgDNA_ACCC-PAM	atgaggtccgtattatactcgtatatattgtttctctcatgtataataa GGGT cggaagagatatagcaccac
Rv_tgDNA_CTCC-PAM	atgaggtccgtattatactcgtatatattgtttctctcatgtataataa GGAG cggaagagatatagcaccac
Rv_tgDNA_CCCC-PAM	atgaggtccgtattatactcgtatatattgtttctctcatgtataataa GGGG cggaagagatatagcaccac
Rv_tgDNA_TGCA-PAM	atgaggtccgtattatactcgtatatattgtttctctcatgtataataa TGCA cggaagagatatagcaccac
Rv_tgDNA_GACG-PAM	atgaggtccgtattatactcgtatatattgtttctctcatgtataataa CGTC cggaagagatatagcaccac
Fw_tgDNA_MPXV	ctttacactttccattggatggtgcatgtggtgctatatctcttccg
Rv_tgDNA_MPXV_MM0	atgaggtccgtattatactcgtatatatt gtttctctcatgtataataa TAAA cggaagagatatagcaccac
Rv_tgDNA_MPXV_MM1	atgaggtccgtattatactcgtatatatt gtttctctcatgtataataT TAAA cggaagagatatagcaccac
Rv_tgDNA_MPXV_MM2	atgaggtccgtattatactcgtatatatt gtttctctcatgtataatTa TAAA cggaagagatatagcaccac
Rv_tgDNA_MPXV_MM3	atgaggtccgtattatactcgtatatatt gtttctctcatgtataaAaa TAAA cggaagagatatagcaccac
Rv_tgDNA_MPXV_MM4	atgaggtccgtattatactcgtatatatt gtttctctcatgtataTtaa TAAA cggaagagatatagcaccac
Rv_tgDNA_MPXV_MM5	atgaggtccgtattatactcgtatatatt gtttctctcatgtatTataa TAAA cggaagagatatagcaccac
Rv_tgDNA_MPXV_MM6	atgaggtccgtattatactcgtatatatt gtttctctcatgtaAaataa TAAA cggaagagatatagcaccac
Rv_tgDNA_MPXV_MM7	atgaggtccgtattatactcgtatatatt gtttctctcatgtTtaataa TAAA cggaagagatatagcaccac
Rv_tgDNA_MPXV_MM8	atgaggtccgtattatactcgtatatatt gtttctctcatgAataataa TAAA cggaagagatatagcaccac
Rv_tgDNA_MPXV_MM9	atgaggtccgtattatactcgtatatatt gtttctctcatCtataataa TAAA cggaagagatatagcaccac
Rv_tgDNA_MPXV_MM10	atgaggtccgtattatactcgtatatatt gtttctctcaAgtataataa TAAA cggaagagatatagcaccac
Rv_tgDNA_MPXV_MM11	atgaggtccgtattatactcgtatatatt gtttctctcTtgtataataa TAAA cggaagagatatagcaccac
Rv_tgDNA_MPXV_MM12	atgaggtccgtattatactcgtatatatt gtttctctGatgtataataa TAAA cggaagagatatagcaccac
Rv_tgDNA_MPXV_MM13	atgaggtccgtattatactcgtatatatt gtttctcAcatgtataataa TAAA cggaagagatatagcaccac
Rv_tgDNA_MPXV_MM14	atgaggtccgtattatactcgtatatatt gtttctGtcatgtataataa TAAA cggaagagatatagcaccac
Rv_tgDNA_MPXV_MM15	atgaggtccgtattatactcgtatatatt gtttcActcatgtataataa TAAA cggaagagatatagcaccac
Rv_tgDNA_MPXV_MM16	atgaggtccgtattatactcgtatatatt gtttGtctcatgtataataa TAAA cggaagagatatagcaccac
Rv_tgDNA_MPXV_MM17	atgaggtccgtattatactcgtatatatt gttActctcatgtataataa TAAA cggaagagatatagcaccac
Rv_tgDNA_MPXV_MM18	atgaggtccgtattatactcgtatatatt gtAtctctcatgtataataa TAAA cggaagagatatagcaccac
Rv_tgDNA_MPXV_MM19	atgaggtccgtattatactcgtatatatt gAttctctcatgtataataa TAAA cggaagagatatagcaccac
Rv_tgDNA_MPXV_MM20	atgaggtccgtattatactcgtatatatt Ctttctctcatgtataataa TAAA cggaagagatatagcaccac
MPXV-J2R gene fragment	gtttctctcatgtataataataaacggaagagatatagcaccacatgcacc atccaatggaaagtgtaaagacaacgaatacagaagccgtaatctatgttg tctatcgtgtcctccgggaacttacgctttcagattatgtgatagcaagac taatacacaatgtacaccgtgtggttcggatacctttacatctcacaataa tcatttacaggcttgtctaagttgtaacggaagatgtgatagtaatcaggt agagacgcgatcgtgtaacacgactcacaatagaatctgtgaatgctctcc aggatattattgtcttctcaaaggagcatcagggtgtagaacatgtatttc taaaacaaagtgtggaataggatacggagtatccggatacacgtctaccgg agacgtcatctgttctccgtgtggtcccggaacatattctcacaccgtctc ttccacagataaatgcgaacccgtcgtaaccagcaatacatttaactatat cgatgtggaaattaacctgtatccagtcaacgacacatcgtgtactcggac gaccactaccggtctcagcgaatccatctcaacgtcggaactaactattac catgaatcataaagattgtgatccagtctttcgtgcagaatacttctctgt ccttaataatgtagcaacttcaggattctttacaggagaaaatagatatca

	gaatacttcaaagatatgtactctgaatttcgagattaaatgtaacaacaa agattcatcttccaaacagttaacgaaaacaaagaatgatactatcatgcc gcattcagagacggtaactctagtgggcgactgtctatctagcgtcgacat ctacatactatatagtaataccaatactcaagactacgaaaatgatacaat ctcttatcatatgggtaatgttctcgatgtcaatagccatatgcccgctag ttgcgatatacataaactgatcactaattcc
Fw_MPXV_J2R	atgaggtccgtattatactcgtatatattgtttctctcatgtataataata aacg
Rv_MPXV_J2R	ctataagtgggtgggattctgggaattagtgatcagtttatg

Table S4. qPCR for MPXV full-length genome quantification.

Name	Sequence
MPXV F3L upper	CATCTATTATAGCATCAGCATCAGA
MPXV F3L lower	GATACTCCTCCTCGTTGGTCTAC
MPXV F3L probe	(FAM)-TGTAGGCCGTGTATCAGCATCCATT-(MGBNFQ)
MPXV F3L (79 bp)	GATCCATCTATTATAGCATCAGCATCAGAATCtgtaggccgtgtatcagca tccattGTCGTAGACCAACGAGGAGGAGTATCTCGA
MPXV_F3L_tg_Fw-p	GATCCATCTATTATAGCATCAGCATCAGAATCtgtaggccgtgtatcagca tc
MPXV_F3L_tg_Rv-p	TCGAGATACTCCTCCTCGTTGGTCTACGACaatggatgctgatacacggcc

Table S5. Genome-wide off-target counts for three crRNAs. Number of putative off-target sites identified by Cas-OFFinder v2.4.1 on both strands of human (GRCh38), mouse (GRCm39), and *E. coli* K-12 MG1655 (NC_000913.3) reference genomes, stratified by spacer-region mismatch count (0–4). Two PAM models were evaluated: the canonical *Lb*Cas12a 5′-TTTN-3′ PAM, and an unconstrained 5′-NNNN-3′ PAM. Mismatches in the 4-nt PAM region are not counted.

In case of TTTN PAM

crRNA	Genome	0 mm	1 mm	2 mm	3 mm	4 mm
a	Human	0	0	2	27	328
	Mouse	0	0	1	15	265
	E. coli	0	0	0	0	1
b	Human	0	0	0	3	150
	Mouse	0	0	0	9	88
	E. coli	0	0	0	0	0
c	Human	0	0	3	34	280
	Mouse	0	0	1	17	188
	E. coli	0	0	0	0	1

In case of NNNN PAM

crRNA	Genome	0 mm	1 mm	2 mm	3 mm	4 mm
a	Human	0	2	44	795	8,578
	Mouse	0	3	40	595	7,182
	E. coli	0	0	0	0	4
b	Human	0	1	7	214	2,972
	Mouse	0	0	7	156	2,620
	E. coli	0	0	0	0	2
c	Human	0	2	52	669	7,660
	Mouse	0	2	38	603	6,172
	E. coli	0	0	0	0	6

<**References**>

1. J. Abramson *et al.*, Accurate structure prediction of biomolecular interactions with AlphaFold 3. *Nature* **630** (2024).