# Enhancement of CRISPR/Cas12a trans-cleavage Activity Using Hairpin DNA Reporters

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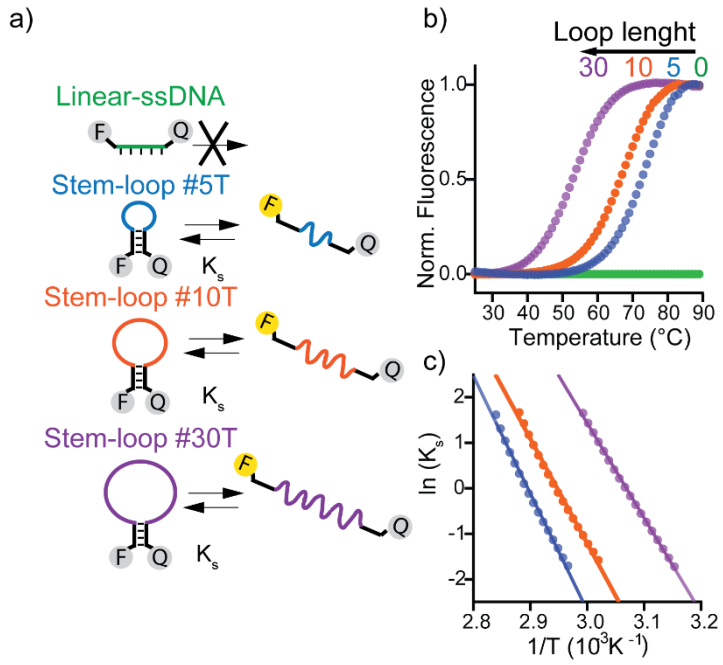
**SUPPLEMENTARY DATA**




| | $T_m$(°C) | $\Delta G$(Kcalmol$^{-1}$) | $K_s$ |
|---|---|---|---|
| Stem-loop #5T | 72.4 ± 0.3 | 4.5 ± 0.2 | (7 ± 2) × $10^{-4}$ |
| Stem-loop #10T | 67 ± 1 | 3.9 ± 0.1 | (1.7 ± 2.2) × $10^{-3}$ |
| Stem-loop #30T | 53.3 ± 0.5 | 2.19 ± 0.015 | (2.9 ± 0.2) × $10^{-2}$ |

**Figure S1.** Thermodynamic characterization of the FRET-based hairpin DNA reporters. a) Schematic representation of the structure-switching mechanism occurring between the closed and opened states of the hairpin structures. The Linear-ssDNA reporter does not show any conformational equilibrium. b) Melting curve and c) van't Hoff analysis of the melting assays carried out to provide the quantitative information reported in table about the switching equilibrium constants ($K_s$) associated to the specific hairpin-forming DNA reporters.

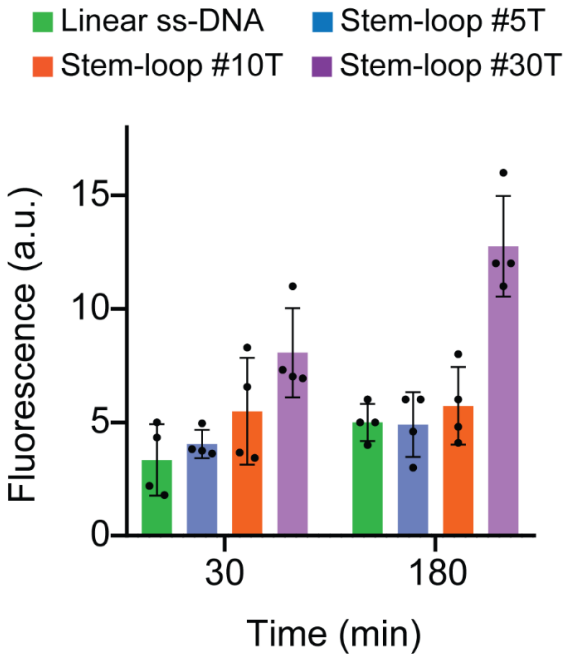



**Figure S2.** Left) Fluorescence background of the DNA reporters (100 nM) at 37°C after 30 and 180 min showing very low background change over time and higher background of fluorescence for Stem-loop #30T according to the fraction of this reporter in the unfolded state at temperature of the assays (T = 37°C).

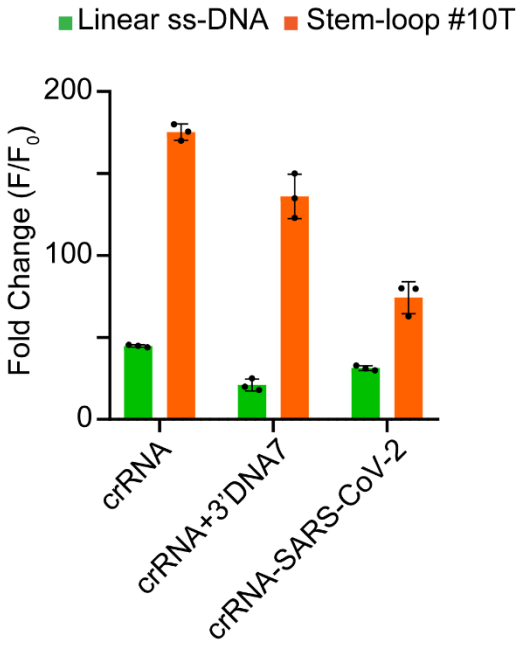



**Figure S3.** Trans-cleavage activity was tested by using different crRNA and dsDNA activators (see Oligonucleotides in Materials and Methods). Fluorescence is plotted as $F/F_0$ (F is the fluorescence signal subtracted of the background $F_0$). $F_0$ is the fluorescence background in the absence of the dsDNA activator collected at time t = 60 min. The bar graph shows the mean and SD as error bars (n = 3) at the assay endpoint (t = 60 min). All the experiments were carried out by adding 4.5 µl of 10x concentrated solution of RNP complex to a solution (final volume 45 µl) containing the FRET-based DNA reporter (final concentrations: Cas12a/crRNA/dsDNA activator 20/20/1 nM, [reporter] = 100 nM).

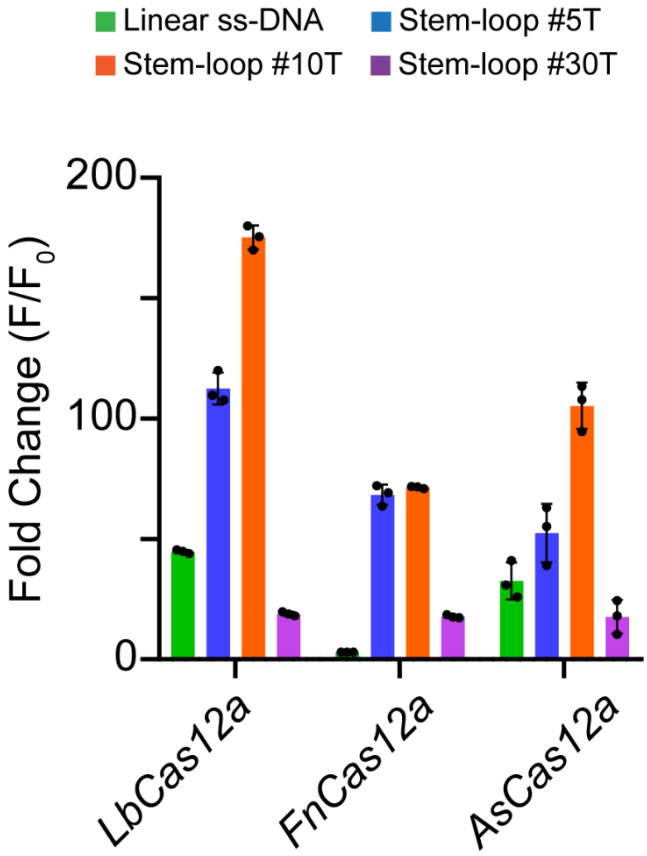



**Figure S4.** *Trans*-cleavage activity was tested using different orthologs of Cas12a enzyme. Fluorescence is plotted as $F/F_0$ at time t =60 min ($F_0$ is fluorescence background measured in the absence of the dsDNA activator). The bar graph shows the mean and SD as error bars (n = 3) at the assay endpoint (t = 60 min). All the experiments were carried out by adding 4.5 µl of 10x concentrated solution of RNP complex to a solution (final volume 45 µl) containing the FRET-based DNA reporter (final concentrations: Cas12a/crRNA/dsDNA activator 20/20/1 nM, [reporter] = 100 nM).

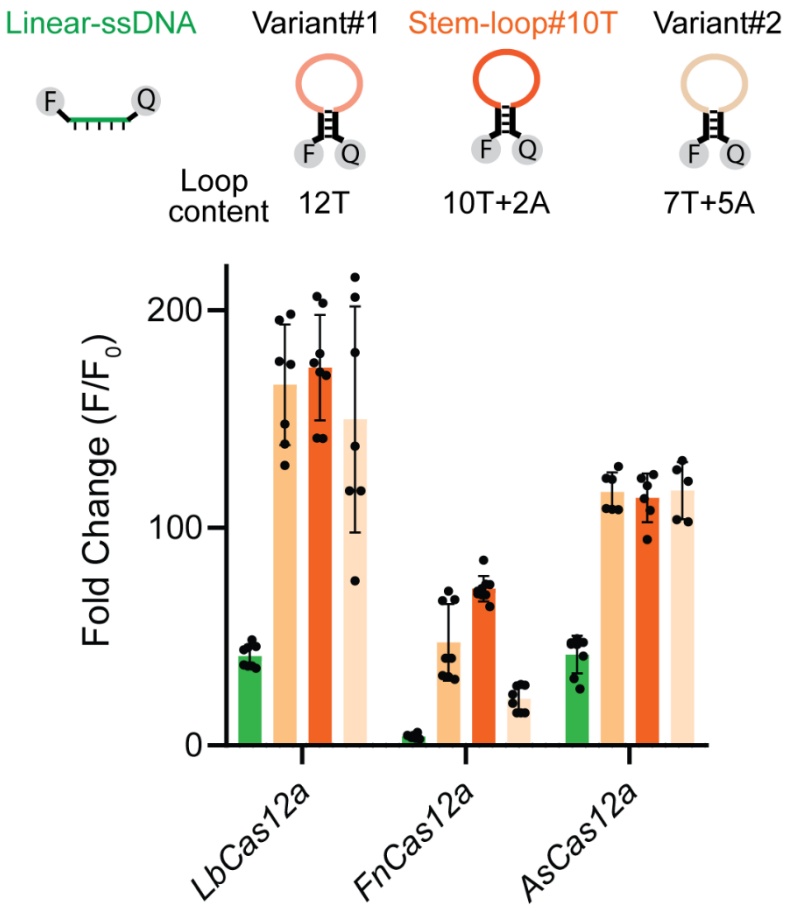



**Figure S5.** *Trans*-cleavage activity of different Cas12a orthologs using variants of Stem-loop #10T reporter. The three hairpin reporters have a different number of thymine in the loop but they show very similar fluorescence fold change. Fluorescence is plotted as $F/F_0$ at time t =60 min ($F_0$ is fluorescence background measured in the absence of the dsDNA activator). The bar graph shows the mean and SD as error bars (n = 7) at the assay endpoint (t = 60 min). All the experiments were carried out by adding 4.5 µl of 10x concentrated solution of RNP complex to a solution (final volume 45 µl) containing the FRET-based DNA reporter (final concentrations: Cas12a/crRNA/dsDNA activator 20/20/1 nM, [reporter] = 100 nM).

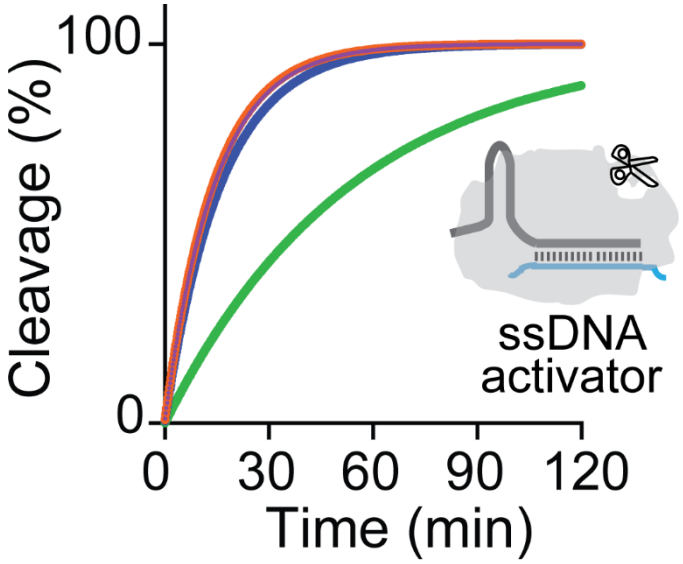



**Figure S6.** Kinetic analysis of *Lb*Cas12a *trans*-cleavage activity. Fraction of DNA reporters cleaved by the *Lb*Cas12a/crRNA complex activated by ssDNA activators *vs* time. The assays were performed at 37°C by adding 4.5 µl of Cas12a/crRNA complex (200 nM) preassembled with the ssDNA activator (10 nM) to a solution containing the DNA reporter (100 nM).

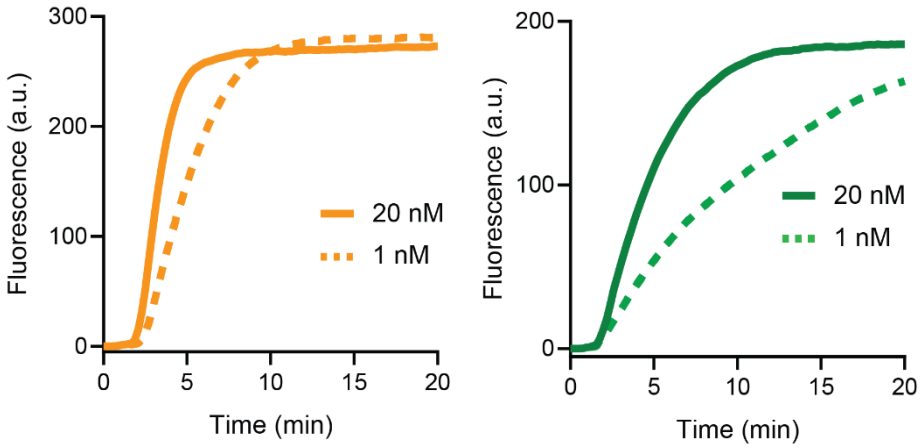



**Figure S7.** Kinetic analysis of *Lb*Cas12a *trans*-cleavage activity on Stem-loop #10T (left) and Linear ss-DNA (right) reporters, tested at high (20 nM) and low concentration (1 nM) of ds-DNA activator. The assays were performed at 37°C by adding a 10X concentrated solution of Cas12a/crRNA complex (200 nM) preassembled with the dsDNA activator (10 nM) to a solution containing the DNA reporter (final concentration 100 nM).

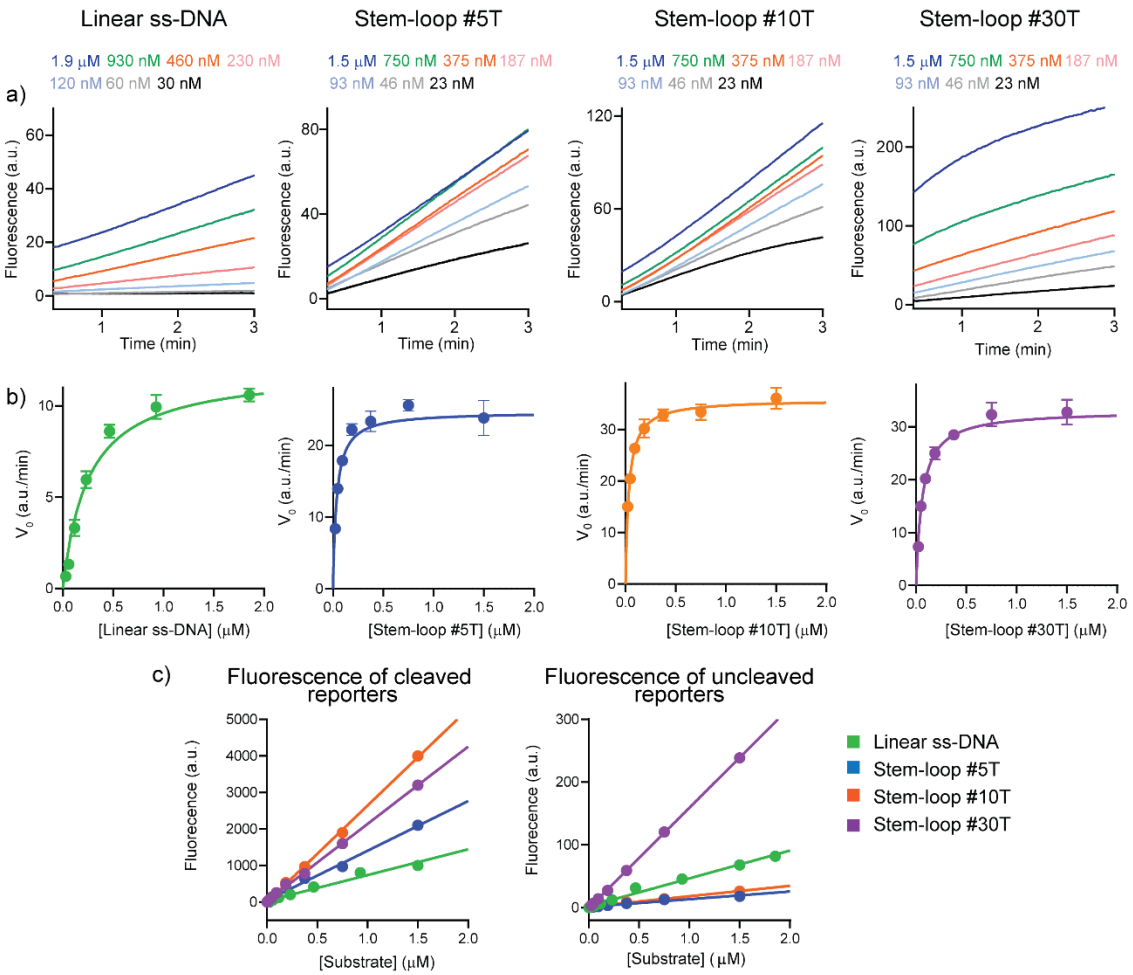



**Figure S8.** Michaelis-Menten analysis. a) Representative plots of initial velocity *versus* time using a fixed concentration of *Lb*Cas12a/crRNA/dsDNA activator ribonucleoprotein complex (1nM) and different reporter substrate concentrations. b) Michaelis-Menten plots for the corresponding data reported in (a). c) Calibration curves of cleaved and uncleaved reporters used to convert reaction velocities from a.u./min to nM/min. All the values reported represent the mean and SD as error bars (n = 3).

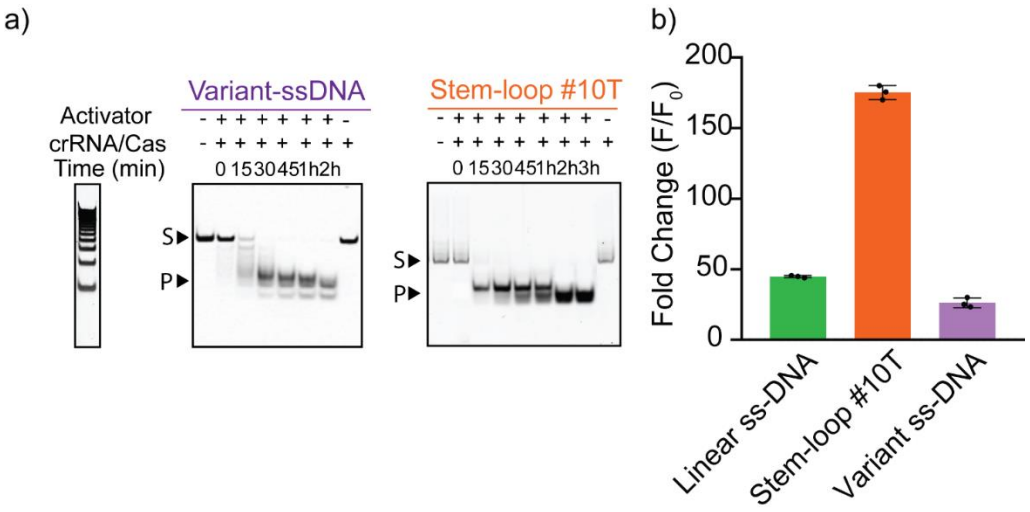



**Figure S9.** *Trans*-cleavage activity of Cas12a on Variant-ssDNA and Stem-loop #10T reporters. (a) PAGE assay showing the time-dependent *trans*-cleavage activity of the activated RNP complex on FRET-labelled DNA reporters (250 nM). S represent the substrate (e.g. DNA reporters, S) and P the degraded products P. The intrinsic fluorescence background of the DNA reporters (lines 1 and 2) and signal change over time is consistent with the increased amount of degraded oligonucleotides over time (lines 3 to 7). (b) Fluorescence fold change after 1h of *trans*-cleavage activity indicating enhanced signal transduction using Stem-loop #10T reporter.

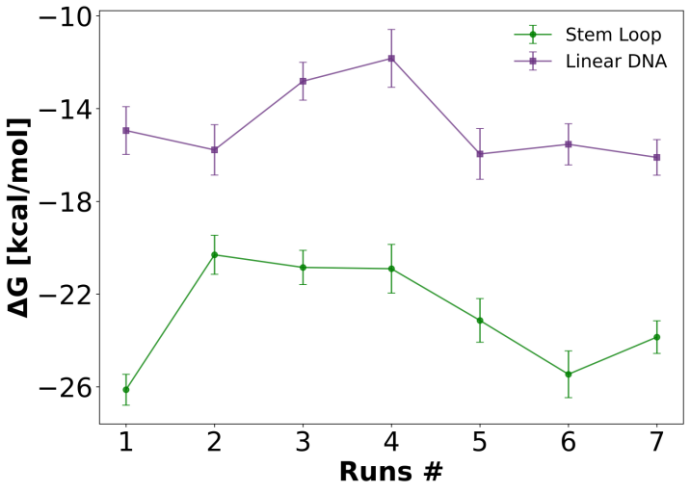



**Figure S10.** The binding free energy for the stem loop (green) and linear ssDNA(purple) binding Cas12a. Data are shown for the individual runs of the MM-GBSA solvation method, showing stronger association of the stem loop with Cas12a than the linear ssDNA. The average free energy of the individual runs is plotted with the standard error of mean.

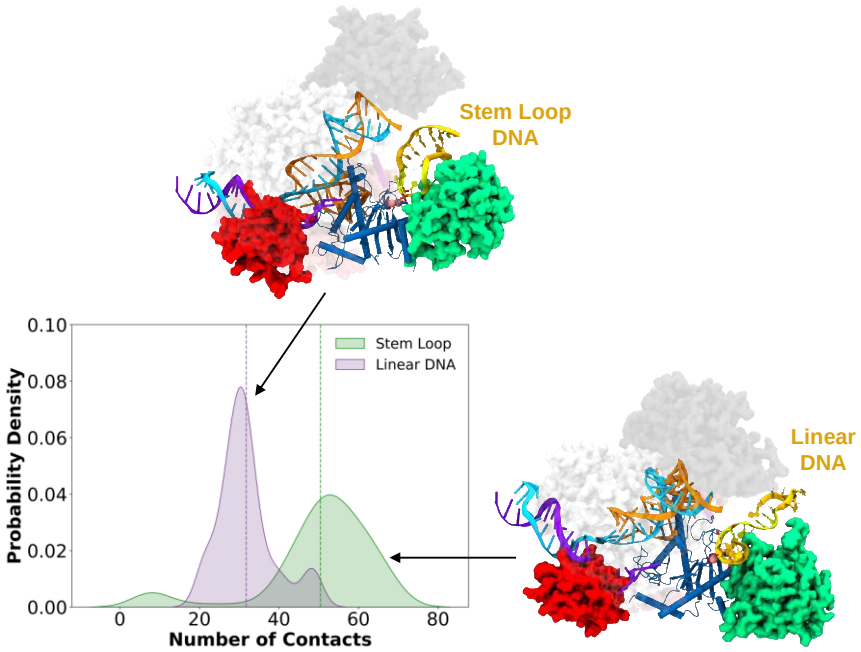



**Figure S11.** Contact analyses of the *trans*-cleavage substrates (i.e., stem loop and linear DNA) with the Nuc domain along the MD simulations. The shift in the probability density plots (dashed lines representing the mean) shows larger extent of interactions between the stem loop DNA and the Nuc domain, compared to the linear DNA. Contacts were considered for interactions within 5 Å, to account for both strong and weak interactions.

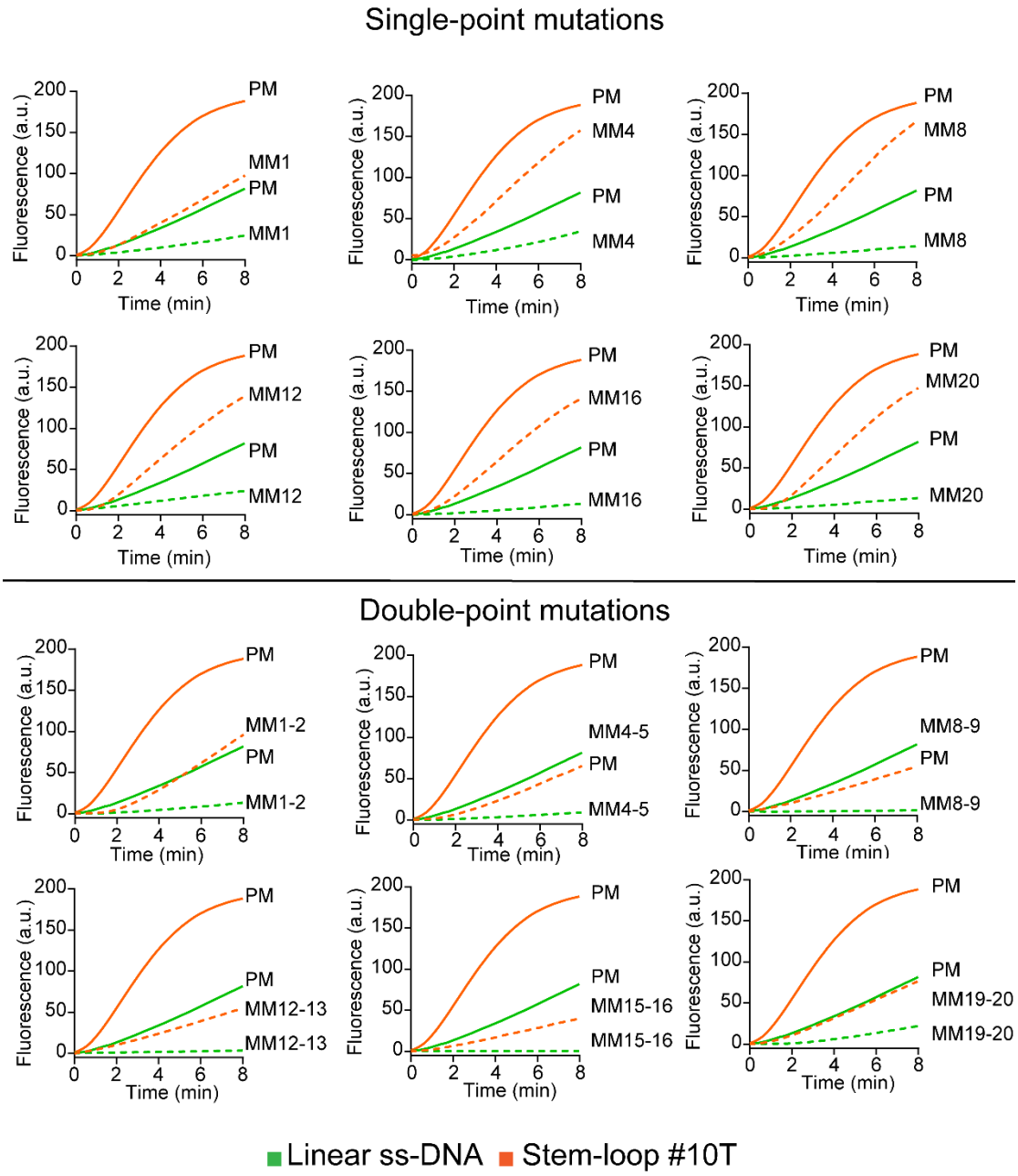



**Figure S12.** *Trans*-cleavage reaction profile over time to evaluate the specificity of *Lb*Cas12a in the presence of wilt type and mutant dsDNA target. All the experiments were carried out by adding 4.5 µl of dsDNA (final concentration 10 nM) to 45 µl of solution containing the DNA reporter (final concentration 100 nM) and 20 nM of *Lb*Cas12a/crRNA complex.

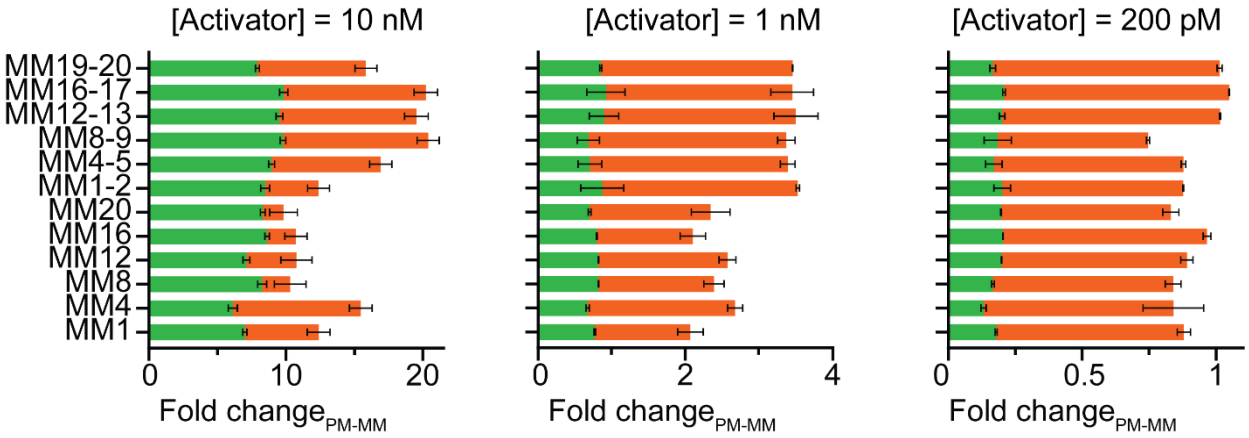



**Figure S13.** Specificity test on single-nucleotide mismatch mutations (from MM1 to MM20) and double-nucleotide mismatch mutations (from MM1-2 to MM19-20) on synthetic SARS-CoV-2 dsDNA activator at different concentrations (10 nM, 1 nM, 200 pM). Superimposed bar graphs showing the difference between the fold change related to the perfect match (PM) and the fold change related to the mismatch (MM) target (right, t=4 min). All the values reported show the mean ± SD, where n =3 replicates.

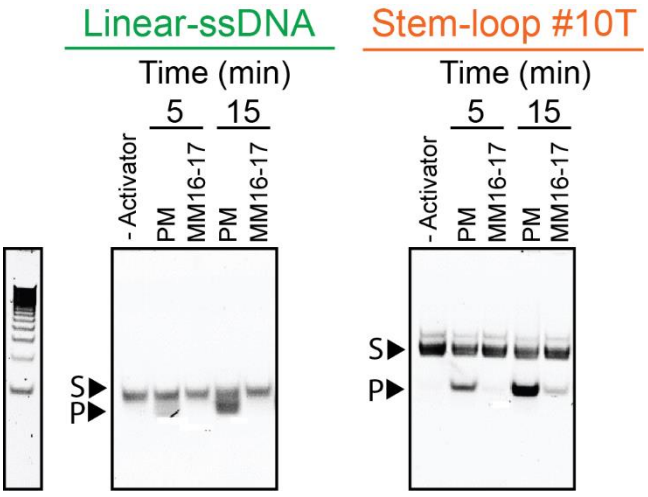



**Figure S14.** Time-dependent *trans*-cleavage activity of *Lb*Cas12a activated by native ds-DNA (PM) and double-point mutations ds-DNA (MM16-17) activators. PAGE assay showing the time-dependent *trans*-cleavage activity of activated *Lb*Cas12a on Linear-ssDNA and Stem-loop #10T reporters (250 nM). S indicate the substrate (e.g. DNA reporters) whereas P indicate the degraded products. The intrinsic fluorescence background of the DNA reporters (lines 1) and signal changes at 5 and 15 min indicating the enhanced specificity obtained using Stem-loop #10T reporter.

| | Linear ss-DNA | Stem-loop #5T | Stem-loop #10T | Stem-loop #30T |
|---|---|---|---|---|
| Time | Cleavage % (PAGE assay) | Cleavage % (PAGE assay) | Cleavage % (PAGE assay) | Cleavage % (PAGE assay) |
| 15 min | 18 | 22 | 33 | 42 |
| 30 min | 34 | 43 | 71 | 55 |
| 45 min | 40 | 61 | 82 | 79 |
| 1 h | 65 | 70 | 87 | 83 |
| 2 h | 100 | 100 | 100 | 100 |

**Table S1.** PAGE analysis of LbCas12a trans-cleavage activity. The fraction of DNA reporters cleaved by the LbCas12a/crRNA complex activated by dsDNA vs time and resolved by Polyacrylamide gel electrophoresis (PAGE) are reported as cleavage %. Samples were prepared by adding a 10X concentrated solution of RNP complex (Cas12a 200 nM/crRNA 200 nM/dsDNA activator 10 nM) to a solution containing the DNA reporter (final concentration 250 nM) at 37°C. The intensity is measured by stopping the trans-cleavage reaction at different times (0, 15, 30, 45, 60 and 120 min), by heating the solutions for 10 min at 75 °C to inactivate Cas12a enzyme.

| | $K_m$ (nM) | $K_{cat}$ ($s^{-1}$) | $K_{cat}/K_m$ ($s^{-1}$ $M^{-1}$) |
|---|---|---|---|
| Linear ss-DNA | 250 ± 50 | 0.30 ± 0.02 | (1.2 ± 0.2) x 10^6 |
| Stem-loop #5T | 42 ± 5 | 0.24 ± 0.02 | (6.1 ± 0.9) x 10^6 |
| Stem-loop #10T | 34 ± 2 | 0.23 ± 0.01 | (6.7 ± 0.7) x 10^6 |
| Stem-loop #30T | 69 ± 5 | 0.28 ± 0.02 | (4.1 ± 0.6) x 10^6 |

**Table S2.** Measured $K_m$, $k_{cat}$, and $k_{cat}/K_m$ values reported as mean ± SD, where n = 3.

| Reporter | $K_m$ (nM) | $K_{cat}$ ($s^{-1}$) | $K_{cat}/K_m$ ($s^{-1}$ $M^{-1}$) | Reference |
|---|---|---|---|---|
| DNaseAlert | 1010 | 17 | 1.71 x 10^7 | Chen et al., 2021, *Science*, 371, No. eabh0317 |
| DNaseAlert | 26 | 1.30 | 5.1 x 10^7 | Nalefski et al., 2021, *iScience*, 24, 102996 |
| /56-FAM/TTATTATT/BHQ-1 | 213<br>274 | 0.09<br>0.07 | 4.2 x 10^5<br>2.5 x 10^5 | Ramachandran et al., 2021, *Anal. Chem*., 93, 7456–7464. |

**Table S3.** Comparison of enzyme kinetic parameters with the most relevant published data reported in literature for LbCas12a and dsDNA activator.