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Probing CRISPR-Cas12a Nuclease Activity Using Double-Stranded DNA-Templated Fluorescent Substrates

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Abstract

The CRISPR-Cas12a nuclease shreds short single-stranded DNA (ssDNA) substrates indiscriminately through transcleavage upon activation with a specific target DNA. This shredding activity offered the potential for development of ssDNA-templated probes with fluorescent dye (F) and quencher (Q) labels. However, the formulations of double-stranded DNA (dsDNA)-templated fluorescent probes have not been reported possibly due to unknown (or limited) activity of Cas12a against short dsDNAs. The ssDNA probes have been shown to be powerful for diagnostic applications; however, limiting the probe selections to short ssDNAs could be restrictive from an application and probe diversification standpoint. Here, we report a dsDNA substrate (*probe-full*) for probing Cas12a trans-cleavage activity upon target detection. A diverse set of Cas12a substrates with alternating dsDNA character were designed and studied using fluorescence

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Supporting Information

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Results of additional experiments and probe designs (PDF)

The authors declare no competing financial interest.

spectroscopy. We have observed that *probe-full* without any nick displayed trans-cleavage performance that was better than that of the form that contains a nick. Different experimental conditions of salt concentration, target concentration, and mismatch tolerance were examined to evaluate the probe performance. The activity of Cas12a was programmed for a dsDNA frame copied from a tobacco curly shoot virus (TCSV) or hepatitis B virus (HepBV) genome by using crRNA against TCSV or HepBV, respectively. While on-target activity offered detection of as little as 10 pM dsDNA target, off-target activity was not observed even at 1 nM control DNAs. This study demonstrates that trans-cleavage of Cas12a is not limited to ssDNA substrates, and Cas12a-based diagnostics can be extended to dsDNA substrates.

Graphical Abstract

Advances in clustered regularly interspaced short palindromic repeat (CRISPR) systems have attracted substantial attention due to implications for precision gene editing. CRISPR systems are single-stranded guide RNA (crRNA)-dependent enzymes, named Cas proteins, that act on foreign genetic materials.^{1,2} Upon activation, Cas proteins serve as RNA-guided nucleases, both binding and cleaving nucleic acids, playing an adaptive part in the bacterial immune system.¹⁻³ The most popular and exciting discovery of these proteins is Cas9, which has been on the forefront of RNA-guided DNA editing studies.⁴⁻⁸ Since the discovery of Cas9, other CRISPR-Cas nucleases have been revealed such as Cas12a (Cpf1), Cas13a (C2c2), and Cas14a that display different nuclease behaviors.^{1,2,9} Specifically, the Cas12a nuclease functions by utilizing a single-component CRISPR RNA (crRNA) that can recognize an exact DNA complement.¹⁰⁻¹³ DNA recognition is due to a T-rich protospacer adjacent motif (PAM) within a target double-stranded DNA (dsDNA) sequence (Scheme 1a), generating a 5' staggered break within the DNA.¹⁰⁻¹³ Upon recognition, Cas12a undergoes an induced conformational change in its RuvC active site allowing for trans-cleavage activity (shredding) indiscriminately on single-stranded DNA (ssDNA) in the vicinity.¹⁴⁻¹⁶

The trans-cleavage activity of Cas12a has been adapted for detection of sequence-specific DNA for disease diagnostics.^{17,18} The most popular of these diagnostic applications have been DNA endonuclease-targeted CRISPR trans reporter (DETECTR)¹ and specific high-sensitivity enzymatic reporter unlocking (SHERLOCK),¹⁹ which both take advantage of the trans-cleavage activity of a target-specific Cas12a. The primary premise of these applications uses a signal amplification step via recombinase polymerase amplification of target DNA and uses a fluorescent (F)- and quencher (Q)-labeled ssDNA molecule that is cleaved upon Cas12a trans-activity generating a fluorescence readout.^{1,2} Because the DNA is a substrate for Cas12a trans-activity, the probes assembled using DNA molecules have

promise in the development of CRISPR-based diagnostic assays. Since the development of ssDNA reporter techniques, many more techniques have been developed using the Cas12a trans-cleavage activity.²⁰⁻²⁵ However, there has been little observation of Cas12a activity on a dsDNA reporter probe.²⁶

Here, we have explored the behavior of *Lachnospiraceae* bacterium Cas12a (Lba Cas12a) on ssDNA/dsDNA hybrid substrates. A number of dsDNA substrates with and without ssDNA spacing were designed and complexed to examine the Cas12a trans-cleavage activity upon target recognition. The substrates were labeled with F and Q to monitor the target detection using fluorescence spectroscopy. We observed that Cas12a indiscriminately cleaves the ssDNA/dsDNA hybrid complex generating a recovery in quenched fluorescence. Upon observing this, we examined Cas12a trans-cleavage activity on a nicked dsDNA, fully intact dsDNA, and several dsDNA substrates with different ssDNA spacings under various experimental conditions (Scheme 1).

MATERIALS AND METHODS

All oligonucleotides were purchased from Integrated DNA Technologies (IDT, Coralville, IA) with the following sequence information.

crRNA Sequences.

Underlined regions are the crRNA guiding scaffold: TCSV-crRNA, 5'-UAAUUUCUACUAAGUGUAGAUUUAAUUUGUUACGGAAUCAUA-3'; HepBV-crRNA, 5'-UAAUUUCUACUAAGUGUAGAUUGGGCAUGGACAUUGAUCCUU-3'.

Target Sequences.

PAM is highlighted in bold, and mutations are underlined: TCSV-tDNA, 5'-GATCTATTTCTATGATTCCGTAACAAATTAATAAATATTGAATTT-3'; TCSV-cDNA, 5'-AAATTCAATATTTATTAATTTGTTACGGAATCATAGAAATAGATC-3'; TCSV(mutPAM)-tDNA, 5'-GATCTATTTCTATGATTCCGTAACAAATTAAGGATATTGAATTT-3'; TCSV(mutPAM)-cDNA, 5'-AAATTCAATATCCTTTAATTTGTTACGGAATCATAGAAATAGATC-3'; TCSV(mut12)-tDNA, 5'-GATCTATTTCTATGATTCCGTAACAAATTGGTAAATATTGAATTT-3'; TCSV(mut12)-cDNA, 5'-AAATTCAATATTTACCAATTTGTTACGGAATCATAGAAATAGATC-3'; TCSV(mut78)-tDNA, 5'-GATCTATTTCTATGATTCCGTAAAGAAATTAATAAATATTGAATTT-3'; TCSV(mut78)-cDNA, 5'-AAATTCAATATTTATTAATTCCTTTACGGAATCATAGAAATAGATC-3'; HepBV-tDNA, 5'-CTTTATAAGGATCAATGTCCATGCCCCAAAGCCACCCAAGGCACAGCTTG-3'; HepBV-cDNA, 5'-CAAGCTGTGCCTTGGGTGGCTTTGGGGCATGGACATTGATCCTTATAAAG-3'.

Probe Sequences.

probe-full is formed by hybridization of strands a and b: (a) 5'-/56-FAM/AGAACCGAATTTGTGTAGCTTATCAGACTG-3' and (b) 5'-CAGTCTGATAAGCTACACAAATTCGGTTCT/3IABkFQ/-3'.

probe-0-nt is formed by hybridization of strands c–e: (c) F-labeled strand, 5'-/56-FAM/CACAAATTCGGTTCT-3'; (d) Q-labeled strand, 5'-CAGTCTGATAAGCTA/3IABkFQ/-3'; and (e) zero-nucleotide linker DNA, 5'-AGAACCGAATTTGTGTAGCTTATCAGACTG-3'.

probe-3-nt is formed by hybridization of strands c, d, and f: (f) three-nucleotide linker DNA, 5'-AGAACCGAATTTGTGTTATTAGCTTATCAGACTG-3'. The underlined region is the ssDNA spacing in the final probe assembly.

probe-5-nt is formed by hybridization of strands c, d, and g: (g) five-nucleotide linker DNA, 5'-AGAACCGAATTTGTGTTATTTAGCTTATCAGACTG-3'.

probe-9-nt is formed by hybridization of strands c, d, and h: (h) nine-nucleotide linker DNA, 5'-AGAACCGAATTTGTGTTATTATTATTAGCTTATCAGACTG-3'.

probe-12-nt is formed by hybridization of strands c, d, and i in a 1:1:1 ratio: (i) 12-nucleotide linker DNA, 5'-AGAACCGAATTTGTGTTATATTATTATTAGCTTATCAGACTG-3'.

EnGen LbaCas12a (catalog no. M0653), M13mp18 single-stranded DNA (ssM13) (catalog no. N4040S), M13mp18 RF I DNA (dsM13) (catalog no. N4018S), and gel loading dye purple (6×) (catalog no. B7024S) were all purchased from New England Biolabs (Ipswich, MA). Bovine serum albumin (BSA) (catalog no. 9048-46-8) was purchased from Amresco (Solon, OH). GelRed Nucleic Acid Stain (10000×) was purchased from Biotum (Fremont, CA, catalog no. 41003-T). Target DNAs were prepared in stock solutions by being diluted to a final concentration of 10 µM and hybridized in 10 mM Tris-HCl (pH 7.9), 50 mM NaCl, 10 mM MgCl₂, and 100 µg/mL BSA as suggested by the supplier (NEB). The probe DNAs (100 nM) were hybridized with the probe contents in a 1:1 ratio in working buffer. Autoclaved and filtered doubly distilled water was used to prepare buffers and stock oligonucleotide solutions.

dsDNA Target and crRNA Designs.

DNA target strands were designed from regions of tobacco curly shoot virus (TCSV) (GenBank accession number [AF240675.1](#)) and hepatitis B virus (HepBV) (GenBank accession number [MK355500.1](#)) that contained a PAM sequence of TTTN (N is any DNA oligonucleotide) within the complementary DNA region. The crRNA scaffold sequence was based on the supplier's recommendation and literature reports.¹ The recognition site in the crRNA was made to be complementary to tDNA strands of target dsDNAs.

Monitoring Lba Cas12a Trans-Cleavage Activity Using DNA Probes.

To activate Cas12a with crRNA (Cas12a-[crRNA] complex), Lba Cas12a and TCSV-crRNA were mixed together in a 1:1.2 ratio to a final working buffer concentration of 1 μ M and incubated at 37 °C for 30 min. Target TCSV-dsDNA (10 μ M) was assembled by mixing TCSV-tDNA with TCSV-cDNA in a 1:1 ratio in working buffer; 100 nM substrates (probe designs) were assembled using the aforementioned strands in working buffer.

Then, 100 μ L of probe and 1 nM dsDNA target were added to the wells of a 96-well microplate. Initial fluorescence readings were recorded with 1 min intervals with 5 s linear shaking between each read. After the 5 min initial readings, 6 μ L of the Cas12a-[crRNA] complex (60 nM) was added to each well. Immediate fluorescence recovery was observed in experimental wells. Fluorescence intensity was monitored for 3 h. Negative control experiments were performed in the absence of crRNA, Cas12a, or TCSV-dsDNA target. Fluorescence measurements were performed using a Synergy BioTek H1 Microplate Reader (excitation at 485 nm, emission at 520 nm, gain of 90). Fluorescence recovery (F%) was calculated using the expression $(F_{\text{obs}} - F_{\text{q}}/F_{\text{max}} - F_{\text{q}}) \times 100\%$, where F_{obs} is the observed fluorescence at each time point, F_{max} is the maximum fluorescence of 100 nM FAM-labeled strand, and F_{q} is the quenched fluorescence of the assembled probe. F_{q} is determined during the initial readings in the absence of activated Cas12a.

Lba Cas12a Cleavage at Various NaCl Concentrations.

Three different working buffers [10 mM Tris-HCl (pH 7.9), 10 mM MgCl₂, and 100 μ g/mL BSA] were prepared at NaCl concentrations of 50, 100, and 150 mM. The detection studies were performed in each buffer according to the aforementioned protocol using 100 μ L of *probe-full* and 1 nM TCSV-dsDNA target.

Concentration-Dependent Cleavage Activity.

The target concentration dependence of Lba Cas12a activity was determined according to the aforementioned protocol using 5, 1, 0.5, 0.1, 0.01, 0.001, and 5×10^{-4} nM TCSV-dsDNA target. Fluorescence readings were recorded for 3 h at 37 °C.

Specificity toward Different Targets.

The specificity of Cas12a toward different dsDNA targets was evaluated using TCSV-crRNA, HepBV-crRNA, and dsDNA targets for TCSV and HepBV. Cas12a was mixed with TCSV-crRNA or HepBV-crRNA to form 1 μ M Cas12a-[crRNA] complex in working buffer. One target sequence at 1 nM was added to a 100 nM *probe-full* mixture according to the aforementioned protocol. *probe-full* and target mixtures were added to the wells of a 96-well plate; 60 nM Cas12a-[crRNA] complex with either TCSV-crRNA or HepBV-crRNA was added to the probe/target mixture. Fluorescence readings were recorded according to the aforementioned parameters.

Tolerance of Lba Cas12a to Target Mismatch.

Mutations in the target strands were placed either in the PAM sequence (mut_{PAM}), one to two nucleotides from the PAM sequence (mut₁₂), or seven to eight nucleotides from the

PAM sequence (mut₇₈). Mutated targets were hybridized using target (t) and complementary (c) strands in working buffer; 100 nM *probe-full* was mixed with each mutated target at 1 nM. Fluorescence readings were recorded after incubation for 1 h using the aforementioned parameters.

Cleavage of dsM13 and ssM13 with Lba Cas12a.

Electrophoresis studies were performed using the activated Cas12a-[crRNA] complex formed by incubating 1 μ M stock Lba Cas12a and TCSV-crRNA in a 1:1.2 ratio at 37 °C in working buffer. Both single-stranded M13 (ssM13, 7249 bp) and double-stranded circular M13 (nicked dsM13, 7420 bp) were incubated separately with 50 nM TCSV-DNA and 60 nM Lba Cas12a-crRNA complex at 37 °C for 30 min. The reaction products were measured using gel electrophoresis (at 70 V for 100 min) with 1 $\times$ gel loading dye and GelRed, 0.8% agarose gel, and 0.5 $\times$ TBE buffer.

Statistical Analysis.

Data are expressed as means $\pm$ the standard deviation. Statistical differences were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons and a Student's *t* test using GraphPad Prism (San Diego, CA). A *p* value of <0.05 was taken to be statistically significant. Experiments were performed in triplicate.

RESULTS AND DISCUSSION

The activity of the Cas12a enzyme against a variety of substrate formulations was studied. The goal was to understand the substrate preference of Cas12a for the development of alternative fluorescent probes. Previous studies have demonstrated that Cas12a cleaves ssDNA substrates, leading to the synthesis of ssDNA-templated fluorescent probes.¹ However, dsDNA-based fluorescent probes have not yet been reported possibly due to the unknown (or limited) activity of Cas12a against short dsDNA substrates.²⁶ This study demonstrates that Cas12a cleavage is not limited to short ssDNA and can be extended to dsDNA probe formulations. These findings offer broader opportunities for designing DNA-based fluorescent probes for Cas12a-based diagnostics.

First, we designed a series of substrates using a nicked dsDNA with and without a ssDNA cleavage frame (Scheme 1b and Table S1). The spacing between the nicked region is systematically increased from 0 to 12 nucleotides to understand whether the extension of the ssDNA frame favors substrate cleavage. Fully complementary dsDNAs with and without a nick (*probe-0-nt* and *probe-full*, respectively) were designed for the purpose of comparison. The substrates are labeled with F and quencher Q, which are placed in the proximity on opposite strands. Upon activation of the Cas12a-[crRNA] complex with the corresponding target, the substrates would be cleaved resulting in a recovery of quenched fluorescence (Scheme 1). Fluorescein and Iowa Black Dark Quencher were chosen as the F and Q, respectively, which are standard FRET pairs for DNA labeling studies performed under physiological conditions. The DNA molecules in these studies have a phosphodiester backbone and are stable under experimental conditions in this study.

First, we tested whether the Lba Cas12a system could recognize a dsDNA target and cleave a select substrate. As a proof of principle, we used the substrate with a 3-nucleotide spacing (*probe-3-nt*) because it has an ssDNA cleavage site (Figure 1a) with the lowest fluorescence background (Figure 2c) due to the proximity of F and Q. We have demonstrated that Cas12a could cleave *probe-3-nt* only when the target dsDNA and the guide RNA are present, simultaneously (Figure 1). Both are known to be essential for trans/cis activation of Cas12a (Figure 1a).¹ In the absence of either crRNA or target DNA, the substrate cleavage and fluorescence recovery were not observed (Figure 1b,c). The studies were also performed in the presence of crRNA and target DNA without Cas12a. As expected, no substrate cleavage was observed (Figure 1 b,c). The maximum rate of cleavage was determined to be 1.45 min⁻¹ (percent fluorescence recovery per minute) and was significantly different ($p < 0.0001$; $n = 3$) than the control studies according to an ANOVA test (Figure 1b, inset).

After demonstrating the activity of the Cas12a system using *probe-3-nt*, we tested each of our probes (*probe-0-nt*, *-3-nt*, *-5-nt*, *-9-nt*, and *-12-nt*) against target DNA with the activated Cas12a. Our hypothesis was that as the level of ssDNA character (ssDNA spacing between F and Q) increases within the substrate both the cleavage rate and yield would increase due to the accessibility of Cas12a to the cleavage site. We have observed that the rate of the recovery with *probe-12-nt* (largest ssDNA spacing) is greater than the rest while the rate with *probe-0-nt* was the lowest (1.97, 1.77, and 1.09 min⁻¹) (Figure 2a and Figure S1). This was predicted as Cas12a is known to preferably cleave ssDNA. A significant difference in rate ($p < 0.0001$; $n = 3$) was observed between *probe-12-nt* and *probe-0-nt*. However, we observed that the overall recovery with all probes including the nicked dsDNA substrate (*probe-0-nt*) was not significantly different after incubation for 3 h (Figure 2b). The comparable recovery with the *probe-0-nt* substrate was surprising because even though it was nicked there was not any available ssDNA region between F and Q for Cas12a cleavage (Table S1).

Later, we evaluated whether the nick in the *probe-0-nt* substrate was the reason for the unexpected cleavage activity. Thus, we have designed a dsDNA substrate (*probe-full*) of the same length and sequence but without a nick. The F and Q were placed on opposite strands, and the fluorescence was quenched upon dsDNA substrate formation (Scheme 1b). *probe-full* was tested against 1 nM target with activated Cas12a and was compared to the *probe-0-nt* substrate. Surprisingly, both substrates were cleaved very efficiently (Figure 2e). *probe-full* displayed a hyperbolic rate of recovery, whereas *probe-0-nt* displayed a sigmoidal recovery (Figure 2d). The maximum rates of recovery for *probe-full* and *probe-0-nt* were determined to be 1.21 and 1.04 min⁻¹, respectively, and the difference was found not to be statistically significant ($p = 0.1661$; $n = 3$; Student's *t* test) (Figure 2d, inset). Though it was unexpected, it is worth noting the observation of cleavage of the dsDNA substrate from a detection standpoint as this offers a greater number of probe choices for Cas12a-based diagnostic.

Because *probe-full* was shown to be a better substrate due to its lower fluorescence background (Figure 2c), earlier recovery (Figure 2d), and comparable degree of fluorescence recovery (Figure 2e), we turned our attention to study it more in detail. It is interesting to observe the cleavage of a dsDNA when only ssDNA-templated probes are reported. Our gel

electrophoresis observations suggest that a large dsDNA (ds-M13) is much more resistant to Cas12a cleavage than large ssDNA (ss-M13) (Figure S2), consistent with the literature.²⁶ Thus, we hypothesized that the cleavage of *probe-full* could be due to the instability of the short dsDNA substrate in a low-saline environment that could be enriched with an increasing NaCl concentration. Thus, we explored the Cas12a cleavage of *probe-full* with various NaCl concentrations (50, 100, and 150 mM). The maximum rates of recovery were calculated to be 1.53, 0.95, and 0.24 min⁻¹, respectively, and found to be significantly different according to an ANOVA test ($p < 0.0001$; $n = 3$) (Figure 3a,c,e). The studies demonstrate that the rate of cleavage of *probe-full* drastically decreased with an increase in NaCl concentration.

The salt-dependent cleavage studies were repeated with *probe-3-nt* to evaluate whether the observed decrease in activity with *probe-full* is largely due to the increased stability of the dsDNA character but not the decrease in Cas12a enzyme activity. *probe-3-nt* has available ssDNA spacing between F and Q regardless of the increase in NaCl concentration; thus, it is hypothesized that increase in NaCl concentration would have a weaker effect on *probe-3-nt* cleavage. As predicted, the effects of NaCl on the recovery rate and overall cleavage were less pronounced with *probe-3-nt* (Figure 3b,d,e). The maximum rates of recovery were calculated to be 1.25, 0.99, and 0.96 min⁻¹, respectively, and the difference was found not to be statistically significant ($p = 0.0144$; $n = 3$; ANOVA test) (Figure 3d). The results suggest that the stability of the dsDNA character in the *probe-full* body is inversely related to its cleavage, which could be tuned by NaCl concentration. This finding could be instrumental for fine-tuning the detection window of fully intact short dsDNA probes using NaCl for various diagnostic scenarios.

Regardless of the salt effect, *probe-full* is an ideal Cas12a substrate for biodetection studies due to its lower background and greater fluorescence recovery rate with 50 mM NaCl (Figure 2c-e). The fluorescence recovery with various concentrations of target dsDNA was studied using *probe-full* (Figure 4). As expected, the rate of recovery increased with an increase in target concentration (Figure 4a). The maximum rates of recovery were calculated to be 3.38, 1.54, 0.88, 0.17, and 0.02 min⁻¹ for 5 nM, 1 nM, 0.5 nM, 100 pM, and 10 pM, respectively (Figure 4b, inset). The changes in higher concentrations (from 5 to 0.1 nM) were significantly different according to an ANOVA test, whereas differences at concentrations of <100 pM were found not to be statistically significant.

The entire probe consumption was complete in 30 min with 5 nM target demonstrating the efficiency of the cleavage. An obvious fluorescence recovery was observed with as little as 1 fmol (10 pM) of target (Figure 4a). The fluorescence recoveries at the end of a 3 h incubation were compared, and the linear detection range was observed to be between ~10 pM and 1 nM (Figure 4b,c). However, the limit of detection can be improved dramatically using various isothermal amplification methods.^{1,2,27-29} The goal of this study is to demonstrate the utility of *probe-full* for Cas12a-based detection methods. The studies were repeated with *probe-3-nt* (ssDNA spacing) and *probe-0-nt* (no ssDNA spacing, but nick), and comparable results were obtained in all three probe types (Figure S3).

To evaluate the programmability of Cas12a using *probe-full*, we have tested it against two different dsDNA targets (Figure 5a). First, the Cas12a was programmed for the detection of

45-nucleotide dsDNA (1 nM) copied from the conserved genomic region of tobacco curly shoot virus (TCSV) using crRNA against TCSV. As expected upon detection of TCSV, there was an immediate fluorescence recovery with a maximum rate of 1.33 min^{-1} (Figure 5b), whereas no fluorescence recovery was observed with the control sequence copied from hepatitis B virus (HepBV). Later, Cas12a was programmed against 50-nucleotide dsDNA from HepBV using crDNA against HepBV. Fluorescence recovery was observed only with HepBV with a maximum rate of 1.42 min^{-1} but not with TCSV (Figure 5c). The rate of recovery with the control samples was insignificant, and the difference between experimental samples was found to be statistically significant ($p < 0.0001$ using a Student's t test; $n = 3$).

Finally, effects of mutations were studied using the TCSV with three different mutation sites: mut_{PAM} , mut_{12} , and mut_{78} (Figure 5d). The three-nucleotide mutations in PAM (mut_{PAM}) and the two-nucleotide mutations close to PAM (mut_{12}) negatively affected the overall recovery. On the other hand, PAM-distant two-nucleotide mutations (mut_{78}) had a weaker effect on overall recovery, consistent with a previous report.¹ The results confirm that the mutations in the dsDNA target influence the activity of Cas12a depending on the mutation sites.¹

CONCLUSION

We have discovered that dsDNA-templated fluorescent substrates can be used to probe Cas12a activity. This offers a larger number of probe choices for Cas12a-based diagnostics because the current fluorescent probes are limited to ssDNA-templated substrates.¹ We have demonstrated that the dsDNA-templated probe cleavage happens in a concentration-dependent manner and is highly specific to guided target DNA. Even though hybrid dsDNA substrates with or without an ssDNA cleavage frame are shredded by Cas12a upon target recognition, fully intact dsDNA substrates serve as better probes due to the lower background and faster fluorescence recovery rate. The expansion of the DNA substrate library for probing Cas12a activity could be important from a diagnostic standpoint. The F and Q can be placed on the 5' and 3' ends of opposite strands, and the sequence and length of the dsDNA probe can be changed indefinitely without altering the F and Q spacing and background fluorescence (Scheme S1). In addition, the stability of the probe formulation can be regulated with the temperature, salt concentration, and length of the sequence that could be used to fine-tune the detection window and rate of target recognition.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

(a) All four components (I, Cas12a; II, crRNA; III, target DNA; IV, *probe-3-nt*) are required for target detection, probe cleavage, and fluorescence recovery. (b) Rate of fluorescence recovery with or without Cas12a, crRNA, or 1 nM target. (c) Change in fluorescence at the end of the 3 h kinetic study. Experiments were performed in triplicate (mean $\pm$ standard deviation).

Figure 2.

(a) Rate of fluorescence recovery with *probe-12-nt*, *-3-nt*, and *-0-nt*. (b) Change in fluorescence with *probe-12-nt*, *-9-nt*, *-5-nt*, *-3-nt*, and *-0-nt* after enzyme reaction for 3 h. (c) The fluorescence of all substrates demonstrates that *probe-full* and *-0-nt* have lower background fluorescence due to the proximity of F and Q. (d) Rate and (e) degree of fluorescence recovery with *probe-full* and *-0-nt*. The difference in panel e is considered not to be statistically significant. Experiments are performed in triplicate (mean $\pm$ the standard deviation).

Figure 3.

Rate of fluorescence recovery with (a and c) *probe-full* and (b and d) *probe-3-nt* in the presence of 50, 100, and 150 mM NaCl. (e) The increase in the NaCl concentration influences the fluorescence recovery in *probe-full* more dramatically than in *probe-3-nt*. Experiments were performed in triplicate (mean $\pm$ the standard deviation).

Figure 4. Concentration-dependent detection of target dsDNA using *probe-full*. (a) A 3 h kinetic study and (b) end-point readings demonstrate that as little as 10 pM target DNA can be detected with the cleavage of *probe-full* with Cas12a. The inset shows the rate of recovery. (c) The linear detection window is determined to be between ~ 10 pM and 1 nM under these conditions. Experiments were performed in triplicate (mean $\pm$ the standard deviation).

Figure 5.

(a) Schematic representation of binding of crRNA to the TCSV-dsDNA target. Cas12a is programmed for the detection of dsDNA copied from (b) tobacco curly shoot virus (TCSV) genome or (c) hepatitis B virus (HepBV) by using crRNA against TCSV and HepBV, respectively. (d) The site of mutations placed in the TCSV target has a differential effect on fluorescence recovery. Mutations around PAM decrease the fluorescence recovery more dramatically under these experimental conditions upon incubation for 1 h. Experiments were performed in triplicate (mean $\pm$ the standard deviation).

Scheme 1. Schematic Illustration of the Process Used in This Work ^a

^a(a) The Cas12a-[crRNA] complex binds to dsDNA target, which triggers its trans-nuclease shredding activity. (b) A number of fluorescently silent ssDNA/dsDNA hybrid substrates are used to monitor the Cas12a-[crRNA] activity. (c) Upon shredding, the quenched fluorescence of the substrates recovers, enabling enzyme activity analysis and target detection.