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Applying biosensor development concepts to improve preamplification-free CRISPR/Cas12a-Dx†

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Development of CRISPR/Cas-based *in vitro* diagnostic devices, or CRISPR/Cas-Dx, has become an intensely researched area. Among the different classes of CRISPR/Cas-Dx, the class based on the Cas12a enzyme (*i.e.*, CRISPR/Cas12a-Dx or simply Cas12a-Dx), is predominantly employed for detecting DNA targets. Current research in Cas12a-Dx has focused on appending Cas12a-Dx to preamplification techniques or coupling Cas12a-Dx to different detection modalities, which has inevitably overshadowed the detection performance of Cas12a-Dx and overlooked its intrinsic detection capability without preamplification. We recognize that Cas12a-Dx, which relies on DNA-activated Cas12a to cleave single-stranded DNA, shares significant similarity with other nuclease-based DNA biosensors, whose performances can be influenced by parameters ranging from the reaction buffer to the reaction temperature. We are thus inspired to probe the limits of preamplification-free Cas12a-Dx by exploring and systematically evaluating several potential parameters that may impact its detection sensitivity and time. Using a previously reported fluorescence-based Cas12a-Dx as the test bed, we have identified that the Cas12a enzyme, the reaction buffer, the substrate label, the substrate concentration, and the reaction temperature can be optimized to significantly improve the signal-to-background ratio and the reaction rate of Cas12a-Dx. Based on these findings, we have improved the limit of detection (LOD) of the Cas12a-Dx to 100 fM, while reduced the time-to-positive to <46 min, representing the most sensitive LOD without preamplification and the fastest time-to-positive for this LOD to date. More broadly, our work provides a roadmap for further advancing Cas12a-Dx and perhaps other classes of CRISPR/Cas-Dx.

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Introduction

As Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR associated (Cas) (*i.e.*, CRISPR/Cas) systems take the center stage in biotechnology research, development of *in vitro* diagnostic devices enabled by CRISPR/Cas systems, or CRISPR/Cas-Dx, has also become the focus of widespread research efforts.^{1–4} Currently, the most common CRISPR/Cas-Dx are those employing the Cas12a enzyme (*i.e.*, CRISPR/Cas12a-Dx) for DNA detection^{5–10} and those employing the Cas13 enzyme family (*i.e.*, CRISPR/Cas13-Dx) for RNA detection.^{11–13} CRISPR/Cas12a-Dx (or simply Cas12a-Dx) operates *via* DNA-activated collateral cleavage of single-stranded

DNA (ssDNA), in which hybridization between the target DNA and the CRISPR guide RNA (gRNA) in the Cas12a-gRNA complex triggers the complex to cleave not only the target DNA, but also the surrounding ssDNA as “collaterals”.⁵ This mechanism allows Cas12a-Dx to detect, typically, 10–100 pM of target DNA *via* a fluorescence-based read-out that employs ssDNA dually-labeled with a fluorophore–quencher pair as the fluorogenic substrate.⁵ Improvements in the detection sensitivity of Cas12a-Dx are generally achieved by coupling preamplification techniques such as RPA (*i.e.*, DETECTR⁵ and Cas12aVDet¹⁰), PCR (*i.e.*, HOLMES⁶), and LAMP (*i.e.*, ULC⁸). Although preamplification techniques significantly boost the sensitivity of Cas12a-Dx, they inevitably overshadow Cas12a and neglect the intrinsic detection capability of Cas12a. Meanwhile, a handful of researchers have already coupled preamplification-free Cas12a-Dx with different read-out modalities (*e.g.*, electrochemical detection⁷ or platinum nanoreporter reduction-based visual detection⁹), but these novel methods reported similar detection sensitivities as fluorescence-based preamplification-free Cas12a-Dx. Thus, there remains an unfulfilled gap in exploring the DNA detection capability and improving the performance of preamplification-free Cas12a-Dx.

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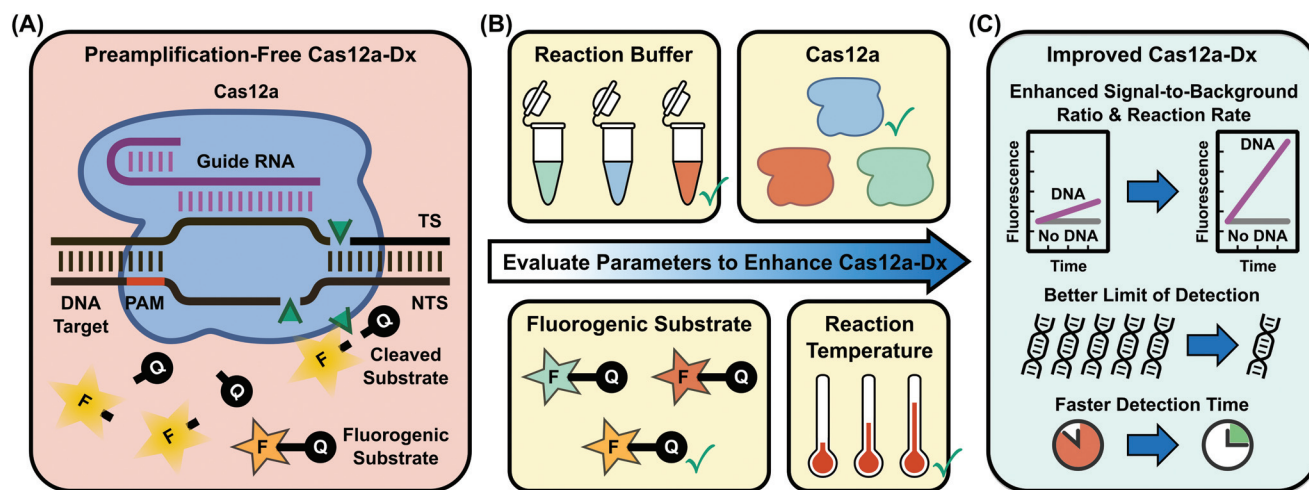


Fig. 1 Applying biosensor development concepts to improve fluorescence-based preamplification-free Cas12a-Dx. (A) Fluorescence-based preamplification-free Cas12a-Dx is composed of the Cas12a enzyme, the guide RNA (gRNA), the reaction buffer, the single-stranded DNA substrate that is dually labeled with a fluorophore (F)-quencher (Q) pair, and the DNA target that contains the protospacer adjacent motif (PAM) sequence on its non-target strand (NTS). Hybridization between the target strand (TS) of the DNA target and gRNA in Cas12a-gRNA complex activates Cas12a, which first cuts both TS and NTS of the DNA target (green arrows) and then collaterally cleaves multiple substrate molecules (green arrow), thus generating fluorescence signals. (B) Conceptually, Cas12a-Dx is highly similar with other nuclease-based DNA biosensors, whose performances can be influenced by parameters such as the reaction buffer, the Cas12a enzyme, the substrate, and the reaction temperature. Systematic evaluation of these parameters and optimization of the condition within each parameter (represented by green checkmarks) therefore offers a path to (C) enhancing the signal-to-background ratio and the reaction rate of Cas12a-Dx, ultimately improving its limit of detection and accelerating its detection time.

As Cas12a-Dx relies on an enzyme that is activated by DNA targets to collaterally cleave ssDNA substrates, its operation and working principles are analogous to DNA biosensors that leverage nuclease-based digestion of DNA. The detection capability of those nuclease-based DNA biosensors is known to be significantly impacted by parameters such as the enzyme, the buffer, and the reaction temperature. We therefore hypothesize that systematic evaluation of potential parameters offers a path to exploring and ultimately improving the DNA detection capability of preamplification-free Cas12a-Dx (Fig. 1). Thus motivated, we employ a previously reported fluorescence-based Cas12a-Dx⁵ as the test bed and investigate several parameters that can potentially improve the signal-to-background ratio and the reaction rate of this Cas12a-Dx, which can lead to improved limit of detection (LOD) and detection time. Upon investigating the Cas12a enzyme, the reaction buffer, the substrate fluorophore label, the substrate concentration, and the reaction temperature, we demonstrate that our improved Cas12a-Dx can detect 100 fM of target DNA in <46 min. Our work not only represents the most sensitive and most rapid preamplification-free Cas12a-Dx to date but also provides a roadmap for advancing Cas12a-Dx and potentially other classes of CRISPR/Cas-Dx.

Methods

Reagents and materials

All CRISPR guide RNA (gRNA) and DNA oligonucleotides including substrates and targets (Table S1†), as well as

AsCas12a V3 and AsCas12a Ultra were purchased from Integrated DNA Technologies (IDT; Coralville, IA). All gRNA sequences were modified with IDT's proprietary 5' AltR1 and 3' AltR2 modifications. Lyophilized gRNA, DNA substrates, and DNA targets were reconstituted in nuclease-free water (Promega, Madison, WI) at 10 μ M, 100 μ M, and 100 μ M, respectively. Reconstituted oligonucleotides and AsCas12a enzymes were stored at -20 $^{\circ}$ C. LbCas12a and NEB 2.1 buffer were purchased from New England Biolabs (Ipswich, MA) and stored at -20 $^{\circ}$ C. Chemical solutions were purchased from various vendors and used without further purification, including 1 M Tris-HCl (pH 7.5) and 1 M MgCl₂ from Quality Biological, Inc. (Gaithersburg, MD), 2 M KCl and 1 M HEPES from ThermoFisher Scientific (Waltham, MA), and 5 M NaCl and sterile glycerol from VWR (Radnor, PA). Chemicals including DTT and heparin sodium salt (from porcine intestinal mucosa) were purchased from MilliporeSigma (Burlington, MA).

Overview of experiments and experimental conditions

We divided this work into the following key categories of experiments that were performed in succession: (A) Cas12a and reaction buffer experiments, (B) Substrate label experiments, (C) Substrate concentration experiments, (D) Reaction temperature experiments, (E) LbCas12a : gRNA concentration ratio experiments, and (F) Limit of detection experiments.

In all experiments, we first hybridized the target strand (TS) and the non-target strand (NTS) to produce the double-stranded DNA (dsDNA) target that was used throughout this work. Briefly, 1 μ M of TS and NTS were mixed in 1 $\times$ NEB2.1

buffer in PCR strips (Bio-Rad, Hercules, CA), and then in a CFX96 Touch Real-time PCR Detection System (Bio-Rad, Hercules, CA), heated at 95 °C for 5 min, and then gradually cooled by 1 °C every 20 s to 4 °C. During dsDNA target formation, the Cas12a-Dx reactions were assembled in a PCR hood (AirClean Systems, Creedmoor, NC) at room temperature. All reactions were assembled in 1.5 mL protein low-binding microcentrifuge tubes (MilliporeSigma, Burlington, MA) before aliquoted in either PCR strips or 96-well plates (Bio-Rad, Hercules, CA) for performing the reactions. Target was added lastly in a separate biosafety cabinet (The Baker Company, Sanford, ME) to prevent any carryover contamination. The final volume for all reactions was 20 µL. All reactions were performed in a Bio-Rad CFX96 Touch Real-time PCR Detection System at specified reaction temperatures for 60 min and the fluorescence signals were measured every 1 min.

For Experiments A through E, 2 µL of dsDNA target was added to the reaction mix at reaction concentrations of 100 pM and 10 pM. Two µL of nuclease-free H₂O was added to the reaction mix as the NTC. We performed technical duplicates (*i.e.*, $n = 2$) for each 100 pM sample, 10 pM sample, and NTC.

(A) Cas12a and reaction buffer experiments. These experiments examined 3 different Cas12a enzymes, each in 3 types of reaction buffers. The Cas12a-Dx reaction mix (18 µL) contained the following: 1× reaction buffer (NEB2.1 buffer (10 mM Tris-HCl, 50 mM NaCl, 10 mM MgCl₂, 100 µg mL⁻¹ BSA, pH 7.9 at 25 °C), “JAD buffer”⁵ (20 mM Tris-HCl, pH 7.5, 100 mM KCl, 5 mM MgCl₂, 1 mM DTT, 5% glycerol, 50 µg mL⁻¹ heparin), or “FZ buffer”¹² (20 mM HEPES, 60 mM NaCl, 6 mM MgCl₂, pH 6.8)), 100 nM Cas12a (NEB LbCas12a, IDT AsCas12a V3, or IDT AsCas12a Ultra), 100 nM gRNA (LbCas12a gRNA-20 for LbCas12a or AsCas12a gRNA for both AsCas12a enzymes), 100 nM Alexa647 substrate, and nuclease-free H₂O. Because NEB2.1 buffer came in 10× stock concentration, we also prepared 10× stocks of both JD buffer and FZ buffer. All reactions were incubated at 37 °C in these experiments.

(B) Substrate label experiments. These experiments examined different fluorophore-quencher pairs of the substrate. The Cas12a-Dx reaction mix (18 µL) contained the following: 1× NEB2.1 buffer, 100 nM LbCas12a, 100 nM gRNA (LbCas12a gRNA-20), 100 nM substrate (Alexa647, FAM or HEX), and nuclease-free H₂O. All reactions were incubated at 37 °C in these experiments.

(C) Substrate concentration experiments. These experiments examined the concentration of the Alexa647 substrate. The Cas12a-Dx reaction mix (18 µL) contained the following: 1× NEB2.1 buffer, 100 nM LbCas12a, 100 nM gRNA (LbCas12a gRNA-20), 300, 100, or 30 nM Alexa647 substrate, and nuclease-free H₂O. All reactions were incubated at 37 °C in these experiments.

(D) Reaction temperature experiments. These experiments examined the reaction temperature. The Cas12a-Dx reaction mix (18 µL) contained the following: 1× NEB2.1 buffer, 100 nM LbCas12a, 100 nM gRNA (LbCas12a gRNA-20), 300 nM

Alexa647 substrate, and nuclease-free H₂O. The reactions were incubated at 37 °C, 42 °C, or 45 °C in these experiments.

(E) LbCas12a : gRNA concentration ratio experiments. These experiments examined the ratio between LbCas12a and gRNA. The Cas12a-Dx reaction mix (18 µL) contained the following: 1× NEB2.1 buffer, 100 or 200 nM LbCas12a, 100 or 200 nM gRNA (LbCas12a gRNA-20), 300 nM Alexa647 substrate, and nuclease-free H₂O. All reactions were incubated at 45 °C in these experiments.

(F) Limit of detection experiments. These experiments examined the limit of detection for our Cas12a-Dx. The Cas12a-Dx reaction mix (18 µL) contained the following: 1× NEB2.1 buffer, 200 nM LbCas12a, 100 nM gRNA (LbCas12a gRNA-20), 300 nM Alexa647 substrate, and nuclease-free H₂O. Two µL of dsDNA target at various stock concentrations was added to the reaction mix at reaction concentrations of 10 pM, 3 pM, 1 pM, 300 fM, or 100 fM (equivalent to $\sim 1.2 \times 10^8$, $\sim 3.6 \times 10^7$, $\sim 1.2 \times 10^7$, $\sim 3.6 \times 10^6$, or $\sim 1.2 \times 10^6$ copies of dsDNA target per 20 µL reaction). Two µL of nuclease-free H₂O was added to the reaction mix as NTC. We performed 4 technical replicates (*i.e.*, $n = 4$) for each sample and NTC. All reactions were incubated 45 °C in these experiments.

Data analysis

In all experiments, real-time fluorescence intensities measured by the Bio-Rad CFX96 Touch Real-time PCR Detection System were exported, without background subtraction, as Microsoft Excel files, and then analyzed *via* Microsoft Excel (Office 365) and Origin 8.5 (OriginLab Corporation, Northampton, MA). All plots in this work were plotted in Origin 8.5.

For Experiments A through E, we used Microsoft Excel to normalize the real-time fluorescence intensities from each sample to the fluorescence intensity of the respective NTC at $t = 1$ min (*i.e.*, $F/F_{\text{NTC}(t=1)}$), which allowed us to compare various versions of Cas12a-Dx that have different fluorescence colors and intensities.

For Experiment F, we first used the built-in “Fit Linear” function in Origin 8.5 to fit the real-time fluorescence intensities of each NTC (*i.e.*, $F_{\text{NTC Fit}}$). Using Microsoft Excel, we then subtracted the linearly fitted real-time fluorescence intensities of the NTC from the real-time fluorescence intensities of each sample (*i.e.*, $F - F_{\text{NTC Fit}}$), from which we then computed the slope of each sample. For 3 pM, 1 pM, and 300 fM samples, because $F - F_{\text{NTC Fit}}$ increased linearly, we computed the slopes by simply using the Origin 8.5 built-in “Fit Linear” function. For 100 fM samples, we noticed that $F - F_{\text{NTC Fit}}$ increased only after an initial lag. The two-phased (*i.e.*, the lag phase and the increase phase) $F - F_{\text{NTC Fit}}$ from 100 fM samples were poorly fitted by linear functions. We therefore employed a piecewise linear function to fit the two segments in $F - F_{\text{NTC Fit}}$ from 100 fM samples and compute the slopes in the increase phase. This piecewise linear function is expressed as:

$$F_{\text{Fit}} = \begin{cases} \frac{F_1(t_3 - t) + F_3(t - t_1)}{t_3 - t_1}, & \text{if } t < t_3 \\ \frac{F_3(t_2 - t) + F_2(t - t_3)}{t_2 - t_3}, & \text{if } t \geq t_3 \end{cases} \quad (1)$$

where t_1 , t_2 , and t_3 represent the initial time point, the final time point, and the transition time point between the lag phase and the increase phase, respectively, and F_1 , F_2 , and F_3 represent the $F - F_{\text{NTC Fit}}$ values at t_1 , t_2 , t_3 , respectively. The slope in the increase phase is then computed by:

$$\text{Slope} = \frac{F_2 - F_3}{t_2 - t_3} \quad (2)$$

Both piecewise linear fitting of $F - F_{\text{NTC Fit}}$ from 100 fM samples and computation of slopes in the increase phase for these 100 fM samples were achieved in Origin 8.5 by a custom-defined fitting function that incorporates eqn (1) and (2).

Time-to-positive (t_{Positive}) of each sample was calculated in Microsoft Excel. In this work, we define t_{Positive} as when the fitted fluorescence intensity of the sample increased by >3-fold of the noise from the NTC. To determine the noise from the real-time fluorescence intensities of each NTC, we calculated the standard deviation of the residuals, or the difference between the measured fluorescence intensities and the fitted fluorescence intensities. To ensure that the fitted fluorescence intensities from the sample had indeed increased beyond 3-fold of the noise of the NTC due to the presence of dsDNA targets, as opposed to well-to-well variations, we subtracted from it the initial fitted fluorescence intensity. For 3 pM, 1 pM, and 300 fM samples, this initial intensity was at $t = 1$ min. For 100 fM samples, because we focused on the slope of the increase phase, the initial intensity was F_3 . The subtracted fitted fluorescence intensity from each sample (*i.e.*, $F_{\text{Fit}} - F_{\text{Fit}(t=1)}$ for 3 pM, 1 pM, and 300 fM samples and $F_{\text{Fit}} - F_3$ for 100 fM samples) at every 1 min was then compared with 3-fold of the noise of the NTC to determine t_{Positive} .

Results and discussion

In this work, we use a test bed Cas12a-Dx, which is slightly modified from a previously published fluorescence-based Cas12a-Dx⁵ that can detect ~10 pM HPV 16 (human papillomavirus type 16) DNA, to explore potential parameters that can enhance its detection performance. This assay is composed of the Cas12a enzyme, the guide RNA (gRNA), the reaction buffer, the substrate, and the DNA target (Fig. 1). The substrate is a 5-nt single-stranded DNA (ssDNA) sequence dually-labeled with a 5' fluorophore (*e.g.*, Alexa647) and a corresponding 3' quencher (*e.g.*, Iowa Black RQ, IABRQSp). The target is a synthesized 40 bp double-stranded DNA (dsDNA) sequence from the HPV 16 genome (Table S1†) and contains the required protospacer adjacent motif (PAM) sequence.⁵ We used a BioRad real-time PCR machine to perform the assay and measure the fluorescence in real time, where increasing fluorescence indicates that the dsDNA target activates the Cas12a-gRNA complex to cleave the ssDNA substrate. We first focused on investigating the impact of potential parameters on our Cas12a-Dx. Specifically, we created versions of Cas12a-Dx by testing different conditions within each parameter and compared their fluorescence signals from detecting technical

duplicates (*i.e.*, $n = 2$) of the no-target control (NTC) and both 10 pM and 100 pM dsDNA targets – target concentrations that we empirically found to result in significant fluorescence to facilitate semi-quantitative comparisons of the versions of Cas12a-Dx. The version of Cas12a-Dx from the best-performing condition of the tested parameter was then included as the baseline condition and compared to the conditions from the subsequent parameters. This process allowed us to progressively improve upon previous versions of Cas12a-Dx and achieve optimization. Finally, we used our improved Cas12a-Dx to detect dsDNA targets below 10 pM while recording the fluorescence in real time, from which we determined the LOD and the detection time.

We first found that the selections of both the Cas12a enzyme and the reaction buffer can significantly enhance pre-amplification-free Cas12a-Dx (Fig. 2). Here, we compared 9 versions of Cas12a-Dx by pairing each of 3 Cas12a enzymes (LbCas12a (purchased from New England BioLabs, NEB), AsCas12a V3, AsCas12a Ultra (both purchased from Integrated DNA Technologies, IDT) coupled to their respective guide RNA sequences, see Table S1†) with each of 3 reaction buffers (NEB2.1, “JAD buffer”,⁵ and “FZ buffer”¹²). These 9 Cas12a-Dx all used 100 nM Cas12a, 100 nM guide RNA, and 100 nM Alexa647-IABRQSp ssDNA substrate (hereafter referred as Alexa647 substrate) and were incubated at 37 °C for 60 min. To facilitate consistent comparisons between different fluorescence signals from various Cas12a-Dx, we normalized real-time fluorescence signals from each sample to the fluorescence signal of the respective NTC at its initial time point, or $t = 1$ min (*i.e.*, $F/F_{\text{NTC}(t=1)}$). Among the 9 Cas12a-Dx, we found that the Cas12a-Dx with NEB LbCas12a and NEB2.1 buffer outperformed other versions, likely because this pairing had already been optimized by NEB. For this Cas12a-Dx, with an input of 100 pM target (Fig. 2, top left panel, red curves), Alexa647 fluorescence rapidly increased, and plateaued after ~30 min at ~3-fold of the fluorescence of the NTC (Fig. 2, top left panel, gray curves), indicating that the 100 nM substrate had been mostly cleaved. With an input of 10 pM target (Fig. 2, top left panel, blue curves), Alexa647 fluorescence steadily increased to ~1.6-fold of the fluorescence of the NTC after 60 min. In contrast, the 2 Cas12a-Dx with either AsCas12a V3 (Fig. 2, top center panel) or AsCas12a Ultra (Fig. 2, top right panel) in NEB2.1 buffer generated considerably lower fluorescence at both target concentrations. These results show that, in NEB2.1 buffer, both AsCas12a appeared to cleave the substrate at slower rates than LbCas12a. The 2 Cas12a-Dx that paired LbCas12a with the 2 published buffers yielded still lower fluorescence at both target concentrations (Fig. 2, middle left panel and bottom left panel), suggesting that LbCas12a was less active in these buffers than the commercially suggested NEB2.1 buffer. Upon comparing the formulation of the buffers (see Methods), we speculate that the elevated concentration of MgCl_2 , the presence of BSA, and the pH of NEB2.1 buffer likely contribute to the improved performance. Finally, the 4 Cas12a-Dx with either AsCas12a V3 or AsCas12a Ultra in either of the 2 published buffers produced

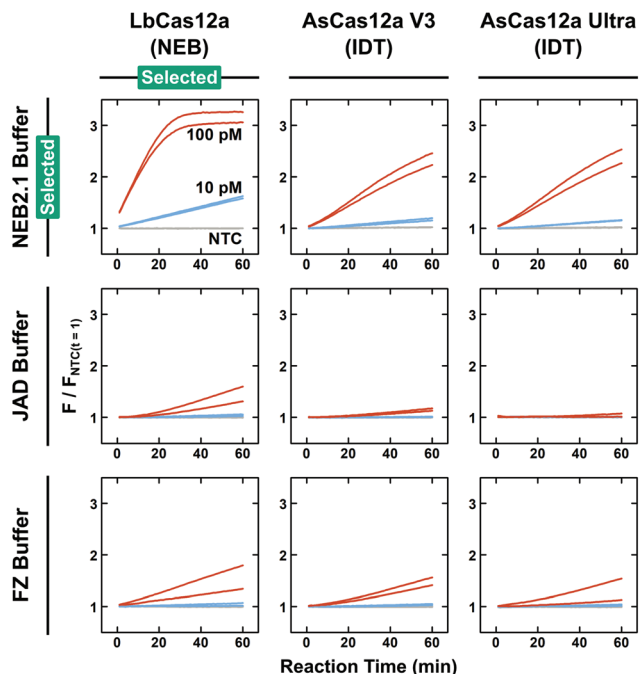


Fig. 2 Significance of Cas12a enzyme and reaction buffer on preamplification-free Cas12a-Dx. Comparisons of 9 versions of Cas12a-Dx, which are created by pairing each of 3 Cas12a enzymes (LbCas12a, AsCas12a V3, or AsCas12a Ultra coupled to their respective guide RNA sequence) with each of 3 reaction buffers (NEB2.1, JAD buffer,⁵ or FZ buffer¹²), reveal that both the Cas12a enzyme and the reaction buffer can significantly improve preamplification-free Cas12a-Dx. The 9 Cas12a-Dx here all use 100 nM Cas12a, 100 nM guide RNA (gRNA), and 100 nM Alexa647-labeled ssDNA substrate and are incubated at 37 °C for 60 min. In this figure and 2 subsequent figures, all Cas12a-Dx are tested against technical duplicates ($n = 2$) of 100 pM dsDNA target (red curves), 10 pM dsDNA target (blue curves), and no-target control (NTC; gray curves) in a 60 min assay, and real-time fluorescence signals from each sample are normalized to the fluorescence signal of the respective NTC at $t = 1$ min (i.e., $F/F_{\text{NTC}(t=1)}$) to facilitate consistent comparisons between different fluorescence signals from various versions of Cas12a-Dx. The Cas12a-Dx with LbCas12a and NEB2.1 buffer (top left panel) outperforms other 8 Cas12a-Dx in detecting 100 pM dsDNA target – indicated by rapidly increasing fluorescence that plateaus after ~30 min at ~3-fold of the fluorescence of the NTC – and 10 pM dsDNA target – indicated by steadily increasing fluorescence to ~1.6-fold of the fluorescence of the NTC. In the presence of NEB2.1 buffer, the 2 Cas12a-Dx with either AsCas12a V3 (top center panel) or AsCas12a Ultra (top right panel) generate considerably lower fluorescence at both target concentrations, suggesting that both AsCas12a enzymes in NEB2.1 buffer cleave the substrate slower than LbCas12a. The 2 Cas12a-Dx that pair LbCas12a with the 2 published buffers also yield lower fluorescence at both target concentrations (middle left panel and bottom left panel), suggesting that LbCas12a cleaves the substrate slower in these buffers. Finally, the 4 Cas12a-Dx with either AsCas12a V3 or AsCas12a Ultra in either of the 2 published buffers produce even lower fluorescence, showing even less optimal detection of 100 pM and 10 pM dsDNA targets. LbCas12a and NEB2.1 buffer are therefore selected for further improving Cas12a-Dx.

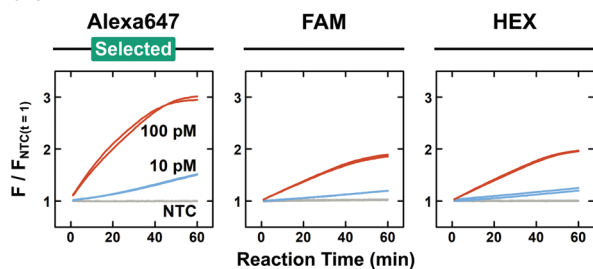
even lower fluorescence, showing a consistent trend along with the other 5 Cas12a-Dx. Based on these results, we selected NEB LbCas12a and NEB2.1 buffer toward improving our Cas12a-Dx. More broadly, our results demonstrate the significant role of the Cas12a enzyme and the reaction buffer in

enhancing the reaction rate (and thus the signal-to-background ratio) of Cas12a-Dx.

We next modified the fluorophore label of the fluorogenic ssDNA substrate and determined that this parameter can affect the signal-to-background ratio of Cas12a-Dx (Fig. 3A). To demonstrate this, we incubated 3 versions of Cas12a-Dx in NEB2.1 buffer with 100 nM LbCas12a, 100 nM gRNA, and 100 nM of 3 distinct substrates at 37 °C for 60 min. Each 5-nt ssDNA substrate is dually-labeled with either Alexa647, FAM, or HEX fluorophore and the corresponding quencher (Table S1†). Here, we hypothesized that the brightness of Alexa647, which has been noted in both research^{14–17} and commercial (e.g., ThermoFisher Scientific) literatures, could enhance the signal-to-background ratio of fluorescence-based Cas12a-Dx when compared to FAM- and HEX-labeled substrates that had been previously reported. For these 3 Cas12a-Dx, at an input of 100 pM dsDNA target, all normalized fluorescence rapidly increased and began plateauing after ~40 min, indicating that LbCas12a cleaved all 3 types of substrates at similar rates. Despite the similar reaction rates, the Cas12a-Dx paired with Alexa647 substrate generated the highest signal-to-background ratio among the 3 substrates. Indeed, this pair yielded fluorescence at ~3-fold of the NTC (Fig. 3A, left panel, red curves), whereas the Cas12a-Dx paired with either FAM substrate (Fig. 3A, center panel, red curves) or HEX substrate (Fig. 3A, right panel, red curves) only yielded fluorescence at ~2-fold of the NTC. The superior signal-to-background ratio for the Cas12a-Dx paired with Alexa647 substrate was also observed from 10 pM dsDNA target: ~1.6-fold of the NTC fluorescence after 60 min from Alexa647 substrate (Fig. 3A, left panel, blue curves) compared to ~1.3-fold of the NTC fluorescence from both FAM substrate (Fig. 3A, center panel, blue curves) and HEX substrate (Fig. 3A, right panel, blue curves). Of note, we also compared Cas12a-Dx reacting with either the HEX substrate and the previously employed DNaseAlert substrate⁵ and observed comparable fluorescence signals (Fig. S1†). These results demonstrate that, at least when performed in our BioRad real-time PCR machine, the bright Alexa647 substrate indeed enhances the signal-to-background ratio of Cas12a-Dx. We therefore selected Alexa647 substrate toward improving our Cas12a-Dx. More broadly, our results also illustrate that the label of the substrate can be a boost for fluorescence-based Cas12a-Dx.

Plateauing fluorescence signals yielded from Cas12a-Dx reacting with 100 nM Alexa647 substrate suggested that the substrate may have become increasingly scarce during the reaction and began limiting the reaction rate and fluorescence generation. We therefore adjusted the Alexa647 substrate concentration to further enhance the signal-to-background ratio of our Cas12a-Dx. Here, we set up our Cas12a-Dx in NEB2.1 buffer with 100 nM LbCas12a and 100 nM gRNA, paired with either 300 nM, 100 nM, or 30 nM Alexa647 substrate, and incubated at 37 °C for 60 min. This experiment revealed that the elevated substrate concentration of 300 nM boosted the signal-to-background ratio of our Cas12a-Dx (Fig. 3B). Specifically, at an input of 100 pM dsDNA target, we measured Alexa647

(A) Substrate Label



(B) Alexa647 Substrate Concentration

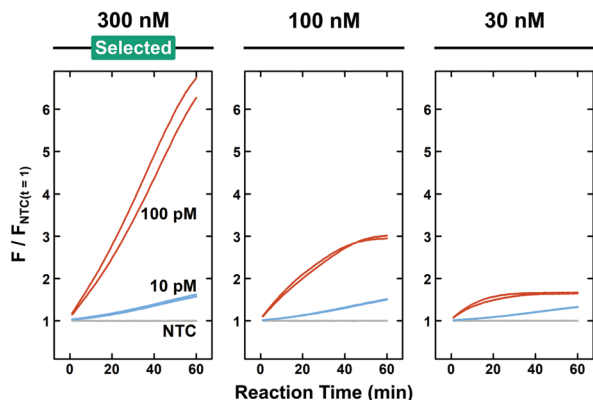


Fig. 3 Significance of substrate label and substrate concentration on preamplification-free Cas12a-Dx. The 6 Cas12a-Dx shown here all use 100 nM LbCas12a and 100 nM gRNA and are incubated in NEB2.1 buffer at 37 °C. (A) The fluorophore label in the ssDNA substrate can dictate the signal-to-background ratio of Cas12a-Dx, which can be seen by comparing 3 Cas12a-Dx reacting with 100 nM of substrate labeled with either Alexa647 (left panel), FAM (center panel), or HEX (right panel). The Cas12a-Dx with bright Alexa647 substrate yields higher normalized fluorescence than its counterparts with FAM and HEX substrates from both 100 pM dsDNA target (red curves) and 10 pM dsDNA target (blue curves). (B) Decreasing the Alexa647 substrate concentration from 300 nM to 100 nM and to 30 nM reduce the signal-to-background ratio of the Cas12a-Dx, which can be clearly observed from both 100 pM dsDNA targets (red curves) and 10 pM dsDNA targets (blue curves). Based on these results, 300 nM Alexa64-labeled substrate is selected for further improving Cas12a-Dx.

fluorescence at ~ 6 -, ~ 3 -, and ~ 1.8 -fold of the NTC for 300 nM, 100 nM, and 30 nM substrate, respectively (Fig. 3B, red curves). Notably, only at 300 nM substrate, Alexa647 fluorescence steadily increased for 60 min without plateauing, suggesting that substrate was not fully cleaved and therefore no longer a limiting factor. In contrast, at both 100 nM and 30 nM substrate, Alexa647 fluorescence plateaued before 60 min, which indicates that these low substrate concentrations had become limiting during the reaction. It is therefore unsurprising that, at an input of 10 pM dsDNA target, Alexa647 fluorescence fold change were ~ 1.8 -, ~ 1.6 -, and ~ 1.3 -fold of the NTC for 300 nM, 100 nM, and 30 nM substrate, respectively (Fig. 3B, blue curves). Consequently, we selected 300 nM as the Alexa647 substrate concentration toward improving our Cas12a-Dx. Our results also illustrate that elevating the substrate concentration such that the substrate no longer limits the reaction offers an effective means to improve the signal-to-ground ratio of Cas12a-Dx.

LbCas12a is active across a range of temperatures according to NEB. We were therefore inspired to tune the reaction temperature of our Cas12a-Dx and found that elevated temperatures can in fact enhance our Cas12a-Dx. For example, when we incubated our Cas12a-Dx in NEB2.1 buffer with 100 nM LbCas12a, 100 nM gRNA, 300 nM Alexa647 substrate for 60 min at 37 °C, 42 °C, or 45 °C, we observed the highest rate of Alexa647 fluorescence generation and the greatest signal-to-background ratio at 45 °C (Fig. 4A). Specifically, in the case of 100 pM dsDNA target, we observed steadily increasing Alexa647 fluorescence without plateauing at 37 °C, steadily increasing Alexa647 fluorescence plateauing after ~ 45 min at 42 °C, and steadily increasing Alexa647 fluorescence plateauing after only ~ 30 min at 45 °C (Fig. 4A, red curves). In the case of 10 pM dsDNA target, Alexa647 fluorescence steadily increased to ~ 1.6 -, ~ 1.7 -, and ~ 1.9 -fold of the NTC at 37 °C, 42 °C, and 45 °C, respectively (Fig. 4A, blue curves). These results therefore prompted us to select 45 °C as the reaction temperature toward improving our Cas12a-Dx. We make two additional remarks on these results. First, plateauing fluorescence signals yielded from the Cas12a-Dx reacting with 100 pM dsDNA target at 45 °C suggest that 300 nM Alexa647 substrate may have again become limiting to the reaction rate and fluorescence generation and that an increase in the substrate concentration may provide another boost to our Cas12a-Dx. Second, the true optimal reaction temperature is in principle governed simultaneously by multiple mechanisms, including the cleavage activity of LbCas12a, the hybridization between dsDNA target and gRNA, and the diffusion of molecules in the reaction. Thorough investigation into the true optimal reaction temperature and into each possible mechanistic underpinning would require finely tuned experiments, which we consider beyond the scope of this work.

We also tweaked the concentrations of LbCas12a and gRNA, though these tweaks only had minor effect on our Cas12a-Dx. We examined 3 versions of Cas12a-Dx with various LbCas12a:gRNA concentration ratios – 100 nM:100 nM, 100 nM:200 nM, and 200 nM:100 nM. All 3 versions of Cas12a-Dx were reacted in NEB2.1 buffer with 300 nM Alexa647 substrate at 45 °C for 60 min. Among them, the Cas12a-Dx with 200 nM LbCas12a and 100 nM gRNA concentrations detected 100 pM dsDNA target with slightly higher rates of fluorescence generation and 10 pM dsDNA target with slightly higher signal-to-background ratio (Fig. 4B). Notably, at 10 pM DNA, this Cas12a-Dx yielded fluorescence at >2 -fold of the NTC after 60 min (Fig. 4B, right panel, blue curves), which represents significant improvements upon the non-optimal Cas12a-Dx that we had tested. In contrast, the Cas12a-Dx with 100 nM LbCas12a and 200 nM gRNA generated fluorescence at a comparatively lower ~ 1.8 -fold of the NTC from 10 pM DNA (Fig. 4B, center panel, blue curves), suggesting that excess gRNA can worsen the performance of Cas12a-Dx. We note that additional versions of Cas12a-Dx with LbCas12a:gRNA concentrations at 600 nM:300 nM and 60 nM:30 nM failed to improve the detection performance (Fig. S2†). Finally, another

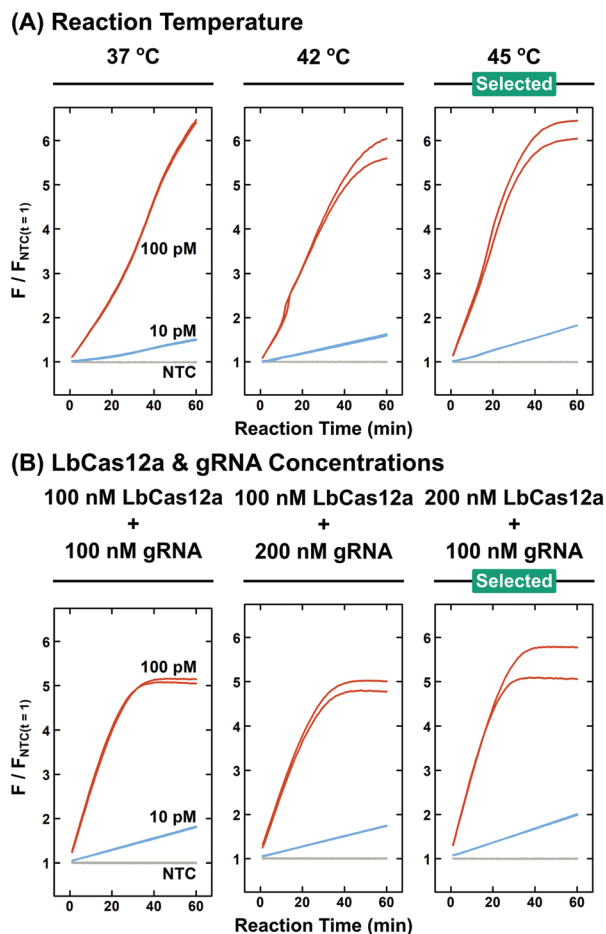


Fig. 4 Effect of reaction temperature and concentrations of LbCas12a and gRNA on preamplification-free Cas12a-Dx. (A) For 3 Cas12a-Dx performed in NEB2.1 buffer with 100 nM LbCas12a, 100 nM gRNA, and 300 nM Alexa647 substrate, increasing the reaction temperature from 37 °C (left panel) to 42 °C (center panel) and to 45 °C (right panel) leads to increased rates of fluorescence generation, which can be clearly observed from 100 pM dsDNA targets (red curves), as well as greater signal-to-background ratio, which can be clearly observed from 10 pM dsDNA targets (blue curves). The reaction temperature is therefore selected at 45 °C for further Cas12a-Dx optimization. (B) A minor effect on Cas12a-Dx from LbCas12a and gRNA concentrations is observed when comparing 3 Cas12a-Dx that vary in LbCas12a:gRNA concentration ratios – 100 nM:100 nM (left panel), 100 nM:200 nM (center panel), and 200 nM:100 nM (right panel). The 3 Cas12a-Dx here are reacted with 300 nM Alexa647 substrate and incubated in NEB2.1 buffer at 45 °C to detect 100 pM dsDNA targets (red curves) and 10 pM dsDNA targets (blue curves). In particular, the Cas12a-Dx with 200 nM LbCas12a and 100 nM gRNA can yield fluorescence at > 2-fold of the NTC even from 10 pM dsDNA targets (right panel, blue curves), which represents a significant improvement from our previous, non-optimal Cas12a-Dx. These conditions are therefore selected for improving Cas12a-Dx.

version of Cas12a-Dx that employed an alternate gRNA with a 24-nt target region also failed to improve the detection performance (Fig. S3†). These results highlight that tuning the concentrations of LbCas12a and gRNA may still enhance Cas12a-Dx, albeit much lesser than other parameters that we have examined.

We lastly measured the LOD for our Cas12a-Dx by detecting 3 pM, 1 pM, 300 fM, and 100 fM dsDNA targets, using our improved condition of NEB2.1 buffer, 200 nM LbCas12a, 100 nM gRNA, 300 nM Alexa647 substrate, and 45 °C reaction temperature. With the improvements we have made, our Cas12a-Dx clearly detected 3 pM, 1 pM, and 300 fM dsDNA, and even yielded a distinctly measurable fluorescence from 100 fM dsDNA above that of the NTC (Fig. 5A). To correct a downward signal drift that we observed from the NTC, which likely stemmed from minor photobleaching of Alexa647 in our PCR machine, we performed a linear fit of the fluorescence of the NTC (*i.e.*, $F_{\text{NTC Fit}}$) and subtracted the fit line from the fluorescence signals of all samples and the NTC (*i.e.*, $F - F_{\text{NTC Fit}}$). This resulted in a near-zero, flat line from the NTC, and the increasing signal originating from 100 fM dsDNA could be more clearly visualized.

To quantitatively validate the LOD of our improved Cas12a-Dx, we performed 4 technical replicates ($n = 4$) for each sample and the NTC and calculated the slopes from the real-time fluorescence measurements. Compared to oft-used end-point fluorescence intensities, which are susceptible to well-to-well and run-to-run variations, slopes are more impervious to such variations and provide direct evidence of functioning Cas12a-Dx. For 3 pM, 1 pM, and 300 fM dsDNA, we computed the slopes by simply fitting each highly linear $F - F_{\text{NTC Fit}}$ to a linear function (Fig. S4†). For 100 fM dsDNA, we determined the slopes by fitting the segmented $F - F_{\text{NTC Fit}}$ with piecewise linear functions instead of linear functions (Fig. S4† and see Methods for details), as piecewise linear functions improved the fits (Fig. S5†). The slopes for 3 pM, 1 pM, 300 fM, and 100 fM dsDNA were 9.46 ± 0.42 , 3.26 ± 0.18 , 0.94 ± 0.08 , and 0.45 ± 0.10 AU min⁻¹, respectively (mean $\pm$ 1 standard deviation; Fig. 5B). These results indicate that, based on the 4 dsDNA concentrations we tested, the measured LOD of our optimal Cas12a-Dx was indeed 100 fM. To the best of our knowledge, our results represent the most sensitive LOD for preamplification-free Cas12a-Dx.

Leveraging real-time fluorescence measurements, we also measured “time-to-positive” (t_{Positive}) for each sample, which offers the potential benefit of shortening the assay time for Cas12a-Dx. In general, t_{Positive} refers to the reaction time at which the signal of the sample becomes definitively detectable from the noise of the NTC. We specifically define t_{Positive} as when the fitted fluorescence signal of the sample increased by >3-fold of the noise from the NTC, where the noise is the standard deviation of the residuals of the fluorescence of the NTC (see Methods for details). The t_{Positive} for 3 pM, 1 pM, 300 fM, and 100 fM dsDNA were 2.3 ± 0.5 , 4.3 ± 0.5 , 10.8 ± 1.7 , and 41.5 ± 4.4 min, respectively (mean $\pm$ 1 standard deviation; Fig. 5C). In other words, our Cas12a-Dx can detect 3 pM and 1 pM dsDNA in <5 min, 300 fM dsDNA in <13 min, and 100 fM dsDNA in <46 min. To our knowledge, these represent the fastest detection times for achieving these LODs to date. In addition, our results exemplify that real-time fluorescence measurements offer added benefit of potentially shortening the assay time for preamplification-free Cas12a-Dx.

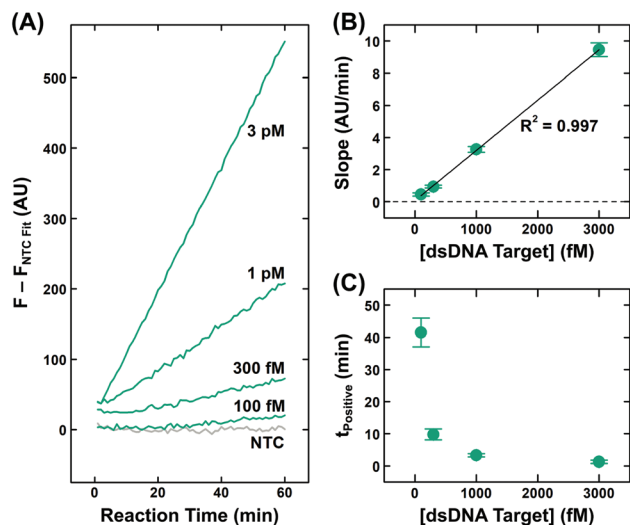


Fig. 5 Sensitive and rapid detection of dsDNA via improved Cas12a-Dx. (A) The Cas12a-Dx with improved conditions of NEB2.1 buffer, 200 nM LbCas12a, 100 nM gRNA, 300 nM Alexa647 substrate, and 45 °C can detect 3 pM, 1 pM, 300 fM, and 100 fM dsDNA, and even yield a measurable fluorescence from 100 fM dsDNA above that of the NTC in 60 min. The fluorescence signals of samples and the NTC shown here are calculated by subtracting a linear fit of the fluorescence signals of the NTC (*i.e.*, $F_{\text{NTC Fit}}$). (B) The LOD of the improved Cas12a-Dx is quantitatively validated with 4 technical replicates (*i.e.*, $n = 4$) for each sample and the NTC and calculated by the slopes from the real-time fluorescence measurements upon subtraction of a linear fit of the fluorescence signals of the NTC (see Methods). The slopes of the NTC-subtracted fluorescence signals from 3 pM, 1 pM, 300 fM, and 100 fM dsDNA are 9.46 ± 0.42 , 3.26 ± 0.18 , 0.94 ± 0.08 , and 0.45 ± 0.10 AU min⁻¹, respectively (mean $\pm$ 1 SD). Based on the 4 tested dsDNA concentrations, the measured LOD of the improved Cas12a-Dx is indeed 100 fM. (C) The time-to-positive (t_{positive}) for each sample – the reaction time at which the signal of the sample becomes definitively detectable from the noise of the NTC – can be determined from real-time fluorescence measurements (see Methods) to potentially shorten the Cas12a-Dx assay time. The t_{positive} for 3 pM, 1 pM, 300 fM, and 100 fM dsDNA are 2.3 ± 0.5 , 4.3 ± 0.5 , 10.8 ± 1.7 , and 41.5 ± 4.4 min, respectively (mean $\pm$ 1 SD, $n = 4$).

Conclusions

Inspired by the process for developing and optimizing biosensors, in this work, we explored and systematically evaluated potential parameters that can enhance preamplification-free Cas12a-Dx. Among the parameters we have investigated, we have determined that the reaction buffer, the Cas12a enzyme, the substrate fluorophore label, the substrate concentration, and the reaction temperature can significantly impact the performance of Cas12a-Dx. Based on our experiments, we enhanced our Cas12a-Dx by using 200 nM LbCas12a and 100 nM gRNA in NEB 2.1 buffer and reacting with 300 nM Alexa647 substrate at 45 °C. Compared to other less optimal Cas12a-Dx that we had tested, our Cas12a-Dx displayed significantly improved signal-to-background ratio and reaction rate, leading to a LOD of 100 fM that can be definitively detected in <46 min. For fluorescence-based Cas12a-Dx without the boost of preamplification methods, these represent the most sensi-

tive LOD and the fastest time-to-positive for the given LOD to date. More broadly, our work demonstrates that applying the process for developing and optimizing biosensors offers an effective means to probe the limits of preamplification-free Cas12a-Dx and ultimately improve its detection performance.

We envision making several improvements upon our promising but admittedly preliminary exploration of preamplification-free Cas12a-Dx in follow-up studies. Among them, we could continue optimizing the buffer composition and substrate concentration of our Cas12a-Dx, evaluate batch-to-batch variabilities of the components in Cas12a-Dx, generalize our observation by testing additional Cas12a-Dx with different gRNA and dsDNA targets, determine the specificity of our Cas12a-Dx by testing the selectivity against noncognate dsDNA sequences, compare fluorescence detection instruments by replicating our Cas12a-Dx in plate readers and under fluorescence microscopes, and perhaps even explore other non-fluorescence detection modalities. On the other hand, the specific findings and the broader conceptual ideas that we present in this work, which are supported by our results, can already be leveraged toward innovating Cas12a-Dx for detecting beyond DNA (*e.g.*, small molecules and exosomes^{18,19}) and perhaps other classes of CRISPR/Cas-Dx^{20–24} – either free of preamplification or coupled to preamplification. Thus, our work has charted a useful roadmap for the burgeoning research area of CRISPR/Cas-Dx.

Conflicts of interest

There are no conflicts of interest to declare.

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