analytical chemistry

pubs.acs.org/ac Article

Dithiothreitol Facilitates LbCas12a with Expanded PAM Preference for Ultrasensitive Nucleic Acid Detection

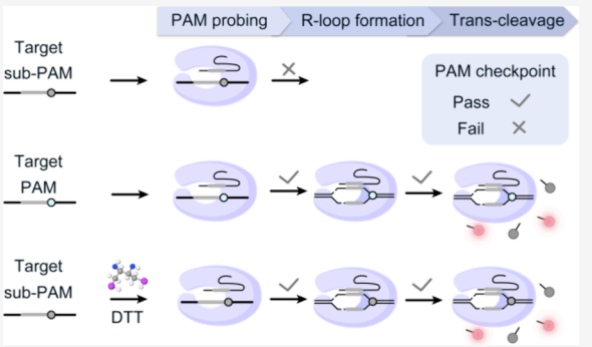
Min Qing,[#] Cheng Huang,[#] Yueyuan Li,[#] Qiubo Yu, Qianfang Hu, Jing Zhou, Ruo Yuan,[*] and Lijuan Bai[*]

Cite This: https://doi.org/10.1021/acs.analchem.5c00280 Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

Downloaded via SHANGHAI NORMAL UNIV on March 18, 2025 at 07:00:49 (UTC).
See https://pubs.acs.org/sharingguidelines for options on how to legitimately share published articles.

ABSTRACT: Clustered regularly interspaced short palindromic repeats-associated (CRISPR/Cas) proteins have been used for a growing class of in vitro molecular diagnostics due to their modularity and high specificity in targeting nucleic acid. However, the requirement of a protospacer adjacent motif (PAM) for Cas protein-catalyzed trans-cleavage poses a challenge for random nucleic acid detection. Here, we demonstrate that dithiothreitol (DTT) enables LbCas12a to adopt a relaxed preference for PAM base pairing, thereby expanding the target sequence space. Accordingly, we propose a DTT-mediated CRISPR/Cas12a toolbox (DTT-deCRISPR) that exhibits relaxed PAM specificity and is readily compatible with nucleic acid amplification techniques including recombinase polymerase amplification (RPA) and polymerase chain reaction (PCR). As a proof of concept, we integrate DTT-deCRISPR with frequently used PCR for sensitively and selectively detecting high-risk human papillomavirus (HPV) 16 and 18. The platform demonstrates the ability to detect synthesized HPV 16 and 18 plasmids down to 1 aM within 60 min. Based on the receiver operating characteristic curve analysis, the clinical sensitivities of the developed method for detecting HPV 16 and 18 are 93.75% and 80.00%, respectively. We further incorporate it into a lateral flow assay (LFA) for point-of-care detection, and the HPV 16 and HPV 18 abundances determined by LFA for clinical samples are consistent with the fluorescence analysis results. Together, this work uncovers an unexpected connection between DTT and PAM preferences of LbCas12a, promoting the universality and flexibility of CRISPR technology in molecular diagnostics.



CRISPR/Cas (clustered regularly interspaced short palindromic repeats−CRISPR-associated proteins) are nucleases paired with a guide RNA (crRNA) to cleave nucleic acid sequences.[1] The RNA-guided property encodes CRISPR/Cas with modularity and high specificity, which has been widely applied in improved existing methods and accelerated the development of point-of-care nucleic acid testing.[2,3] Typically, target sequences are amplified using polymerase chain reaction or isothermal amplification assays, which then activate Cas12 or Cas13 family proteins for trans-cleavage of reporter probes. For example, LbCas12a-based DETECTR (DNA Endonuclease-Targeted CRISPR Trans Reporter), integrated with recombinase polymerase amplification, has been experimentally validated to detect high-risk HPVs with attomolar sensitivity.[4] CRISPR/AapCas12b-assisted loop-mediated isothermal amplification, called STOP (SHERLOCK Testing in One Pot), has been performed in one pot to detect SARS-CoV-2.[5] The in vitro molecular diagnostics are undergoing a seismic shift with the discovery of CRISPR.[6−8] The ever-expanding set of CRISPR-based technologies has come with the growing demand of the improved flexibility and utility of the CRISPR/Cas toolbox.[9−11] However, one major limitation, i.e., the requirement for a protospacer-adjacent motif (PAM), has constrained the target sequence space of CRISPR/Cas.[12]

CrRNA-guided target double-stranded DNA (dsDNA) hybridization is initiated by PAM sequence recognition, which facilitates dsDNA unwinding, strand invasion of the crRNA in the helix, and R-loop formation.[13,14] PAM recognition is a mechanistic requirement for CRISPR/Cas in target dsDNA detection, but this requirement restricts the target sequence space.[15] The low-frequency PAM motif in human genome constrains the universality and flexibility of CRISPR technology.[16] Consequently, recent efforts have focused on relaxing the PAM specificity of Cas proteins to extend the target space.[17,18] Studies have demonstrated that single-stranded DNA (ssDNA) can activate the DNase activity of Cas proteins in a PAM-independent manner.[4,14] Accord-

Received: January 13, 2025
Revised: March 9, 2025
Accepted: March 11, 2025

ACS Publications © XXXX American Chemical Society

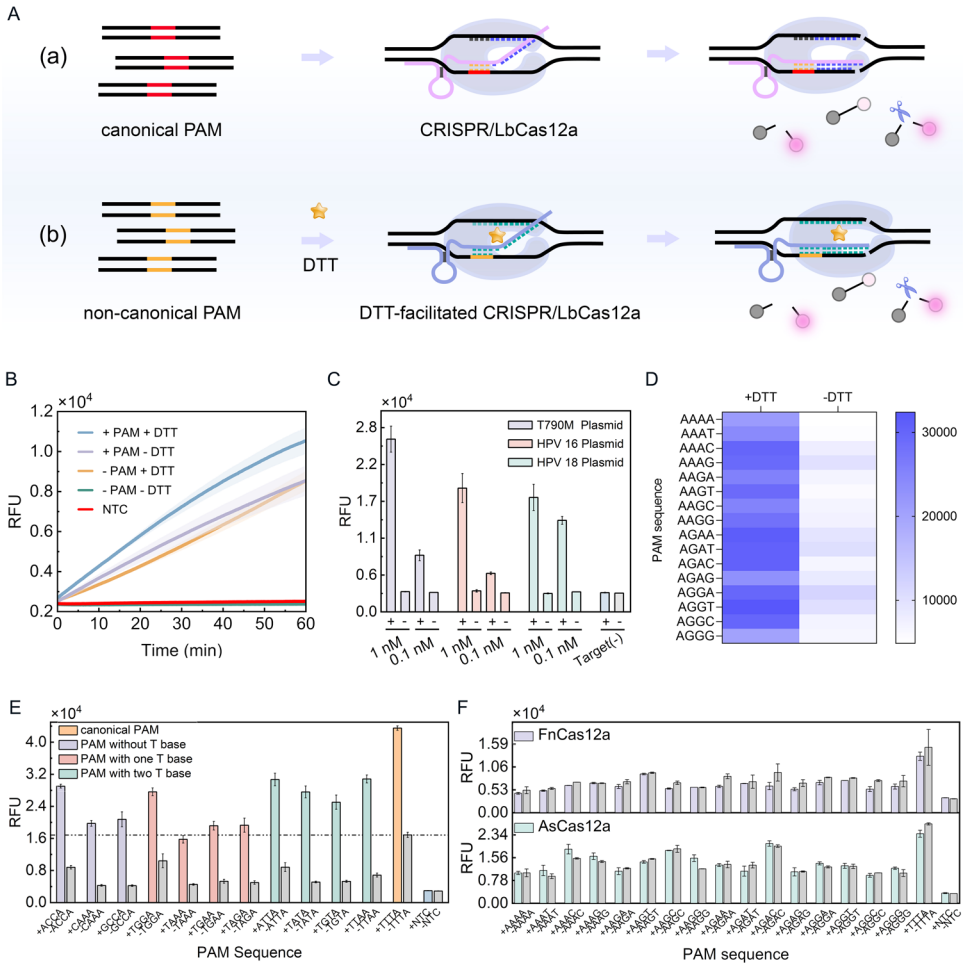


Figure 1. (A) Schematic diagram of (a) CRISPR/Cas12a for detection of canonical PAM dsDNA activators and (b) DTT-deCRISPR/Cas12a for detection of noncanonical PAM dsDNA activators. (B) Evaluation of the effects of DTT and PAM on the trans-cleavage of CRISPR/LbCas12a-crRNA1 in the presence of 200 pM of either canonical PAM or noncanonical PAM activators (plasmids containing the T790M gene). (C) Comparison of the trans-cleavage of DTT-facilitated LbCas12a and LbCas12a in detecting noncanonical PAM activators (plasmids containing T790M, HPV 16, or HPV 18 genes) with various concentrations. (D) Comparison the specificity of DTT-facilitated LbCas12a and LbCas12a across 16 noncanonical PAM combinations. Heat map represents the relative fluorescence intensity (RFU) at 60 min (n = 2). (E) Comparison of the trans-cleavage of DTT-facilitated LbCas12a and LbCas12a in detecting a library of 12 dsDNA activators with different PAMs. The dotted line represents the trans-cleavage activity of LbCas12a in the presence of dsDNA activators with canonical PAMs. (F) Investigation of the effect of DTT on CRISPR/FnCas12a and CRISPR/AsCas12a systems in detecting a library of 17 dsDNA activators with different PAMs. The histogram represents the relative fluorescence intensity at 60 min (n = 2). The symbol "+" indicates that the system performs with DTT, while the symbol "−" indicates that the system performs without DTT. Unless otherwise specified, the histogram presents the relative fluorescence units (RFU) at 60 min, with data expressed as the mean ± standard deviation (n = 3 technical replicates). NTC, no target control.

ingly, enzyme digestion, asymmetric primer extension amplification, and DNA unwinding through high-pH and high-temperature treatment were employed to generate ssDNA activators.[19−22] However, these attempts are complex, require additional steps, and compromise the specificity of CRISPR/Cas. Alternatively, more studies have focused on introducing an additional PAM site upstream of the recognition fragment, such as μReaCH-PAD and RACE.[23,24] While loosening the PAM constraints, these methods require a strict primer design and are susceptible to producing false-positive results. One promising approach is to search for Cas nucleases with relaxed PAM preference via protein engineering.[12,16,25] Nonetheless, these approaches still face some challenges, including technical difficulties, extended target recognition time, and an increased risk of going off-target.

Mechanism studies indicated that the enhanced dsDNA binding with iGeoCas9 dramatically accelerated DNA unwinding and relaxed preference for PAM base pairing.[26] Additionally, a single-molecule fluorescence study showed that dithiothreitol (DTT) can promote >20-fold tighter binding of the LbCas12a−crRNA−DNA complex.[27] Previous studies have shown that DTT facilitates the trans-cleavage of the LbCas12−crRNA−canonical PAM target complex, but its effect on PAM preferences of the Cas12a nucleases remains unclear.[28,29] We speculate that exploring Cas proteins with relaxed PAM specificity will expand the universality and flexibility of CRISPR technology in molecular diagnostics.

Inspired by the above results, we demonstrate that DTT enables LbCas12a to adopt a relaxed preference for PAM base pairing, thereby expanding the target sequence space. Accordingly, we propose a DTT-mediated CRISPR/Cas12a toolbox (DTT-deCRISPR) that exhibits relaxed PAM specificity and is readily compatible with nucleic acid amplification techniques, including RPA and PCR. To

https://doi.org/10.1021/acs.analchem.5c00280
Anal. Chem. XXXX, XXX, XXX−XXX

demonstrate the potential clinical applications of the biosensor, we selected high-risk human papillomaviruses 16 and 18 as the target model, as they are the most prevalent genotype in cervical cancer.[30,31] As a proof of concept, we integrated DTT-deCRISPR with frequently used PCR to develop an enhanced CRISPR biosensor, which can detect synthesized HPV 16 and 18 plasmids down to 1 aM within 60 min. We further engineer DTT-deCRISPR-PCR into lateral flow analysis to meet the criterion of point of care and validate its good clinical performance in detection of clinical samples. Together, this work uncovers an unexpected connection between DTT and LbCas12a, promoting the universality and flexibility of the CRISPR technology in molecular diagnostics.

MATERIALS AND METHODS

DTT-deCRISPR Assay. The DTT-deCRISPR assay was performed in a 20 μL system: 20 nM LbCas12a, noncanonical PAM activators, 20 nM specific crRNA, 12 mM DTT, 250 nM FQ reporter, and 1× deBuffer. Meanwhile, the conventional CRISPR/Cas12a assay was also conducted in a 20 μL system using canonical PAM activators, but without the addition of DTT. Fluorescence intensity was recorded immediately at 37 °C for 60 min with a CFX Connect Real-Time PCR Detection System (Bio-Rad, USA).

To explore the influence of different additives on the trans-cleavage of CRISPR/LbCas12a with noncanonical PAM activators, CRISPR/LbCas12a was performed in a 20 μL system containing 20 nM LbCas12a, 20 nM crRNA 2, 200 pM noncanonical PAM HPV16 plasmids, 250 nM FQ reporter, different additives, and 1× deBuffer. Fluorescence intensity was recorded immediately at 37 °C for 60 min with CFX Connect Real-Time PCR Detection System (Bio-Rad, USA).

PAM Preference of the Cas12a Nucleases. The dsDNA activators with various PAMs were generated by annealing two synthesized oligonucleotides (Table S6), the target strand (TS) and the nontarget strand (NTS), in a molar ratio of 1:5. The annealing protocol included an initial denaturation step at 95 °C for 5 min followed by gradual cooling to 25 °C at a rate of 0.1 °C every 10 s. To investigate the effect of DTT on the PAM preference of various Cas12a nucleases, CRISPR/Cas12a cleavage was performed in a 20 μL system containing 20 nM various Cas12a nucleases, 20 nM specific crRNA, 1 nM noncanonical PAM dsDNA activators, 12 mM DTT (DTT was only used in the reaction system when specified), 250 nM FQ-reporter, and 1× deBuffer.

RESULTS AND DISCUSSION

DTT-Facilitated Cas12a Cleavage Has a Relaxed PAM Requirement. crRNA-guided Cas12a binding to target dsDNA is initiated by the recognition of a canonical PAM (T-rich), which facilitates target DNA duplex unwinding, strand invasion of the crRNA in the target dsDNA, and R-loop formation, thereby activating nuclease activity of Cas12a (Figure 1A-a). However, PAM requirement restricts the ability of CRISPR/Cas12a to target any sequence. According to previous studies,[26,27] we hypothesize that DTT-facilitated Cas12a cleavage has a relaxed PAM requirement. To test this hypothesis, we designed crRNA1 to target a region bearing noncanonical PAMs (CCTT) in a plasmid containing the T790M gene (Figure 1A-b, Table S1). The four bases adjacent to the crRNA-targeted region were mutated from CCTT to TAAA to generate a canonical PAM (Table S2), thereby enabling a comparison of the influence of DTT and PAM on the trans-cleavage activity of CRISPR/LbCas12a. As shown in Figure 1B, the fluorescence intensity of the CRISPR/LbCas12a system increases with DTT or PAM. Moreover, we observed that the CRISPR/LbCas12a system with both DTT and PAM exhibited further signal enhancement, which is consistent with the results of previous studies.[28,29] When plasmids bearing different noncanonical PAMs were used as activators (Tables S1, S3, and S4), we found that DTT-facilitated LbCas12a harbors a much wider tolerance for noncanonical PAMs (CCTT, CCTC, and TACC) compared to conditions without DTT (Figure 1C and Figure S1). Previous research has demonstrated that the catalytic efficiency of CRISPR/Cas12a varies depending on the crRNA sequence.[32,33] We speculate that the differences in the fluorescence signals obtained by the CRISPR/Cas12a system when detecting various activators are related to the specific sequences of the crRNA used. These observations indicate that DTT-facilitated LbCas12a adopts a relaxed preference for PAM base pairing, enabling it to target a broader range of dsDNA activators.

To explore the PAM specificity of LbCas12a, we compared the preferences of DTT-facilitated LbCas12a and LbCas12a across different PAM combinations (Table S6). Previous studies have shown that LbCas12a-crRNA exhibits a preference for thymidine and tolerance for cytosine in central PAM positions.[22,34] The T(1):A(1*) base pair in the PAM duplex does not form base-specific interaction with LbCas12a,[15] and we fixed the first nucleotide to be a constant "A" and designed a library of 16 noncanonical PAMs (Figure 1D and Figure S2). The LbCas12a shows the highest trans-cleavage with canonical PAM (TTTA) and lower but considerable trans-cleavage with AGAT, AGGA, and AGGT. Interestingly, the DTT-facilitated LbCas12a works well with all PAM combinations and shows the highest trans-cleavage with canonical PAM (TTTA), which is consistent with previous studies.[28,29] To further compare the preferences of DTT-facilitated LbCas12a and LbCas12a, we tested their trans-cleavage activities toward 11 dsDNA activators with ACCA, CAAA, GCCA, TGGA, TAAA, TGAA, TAGA, ATTA, TATA, TGTA, or TTAA as the potential PAM (Figure 1E). The 11 dsDNA activators contain potential PAMs with varying numbers of T bases. LbCas12a cleaved the FQ reporter with noncanonical PAMs but less efficiently than the canonical PAM. In contrast, DTT-facilitated LbCas12a revealed an unexpectedly high activity toward all PAM combinations and was more efficient than LbCas12a with canonical PAM. In addition, we investigated the PAM specificity of Cas12a orthologs. The RuvC and Nuc domains of AsCas12a are structurally similar to those of LbCas12a,[15] but DTT-facilitated AsCas12a shows negligible change in trans-cleavage efficiency (Figure 1F). FnCas12a recognizes dsDNA activators with TTN PAM.[35] DTT-facilitated FnCas12a exhibits negligible changes in the trans-cleavage efficiency. These findings are consistent with previous research showing that DTT does not affect DNA binding by AsCas12a or FnCas12a but enhances the binding affinity of LbCas12a by more than 20-fold.[27] These observations indicate that the DTT additive supersedes the role of the PAM in dsDNA target recognition, perhaps by enhancing the binding of the LbCas12a−crRNA−DNA complex and relaxing preference for PAM base pairing.

Profiling the Function of DTT on CRISPR/LbCas12a. To investigate the impact of chemical additives on the trans-cleavage of the CRISPR/LbCas12a system with noncanonical

https://doi.org/10.1021/acs.analchem.5c00280
Anal. Chem. XXXX, XXX, XXX−XXX

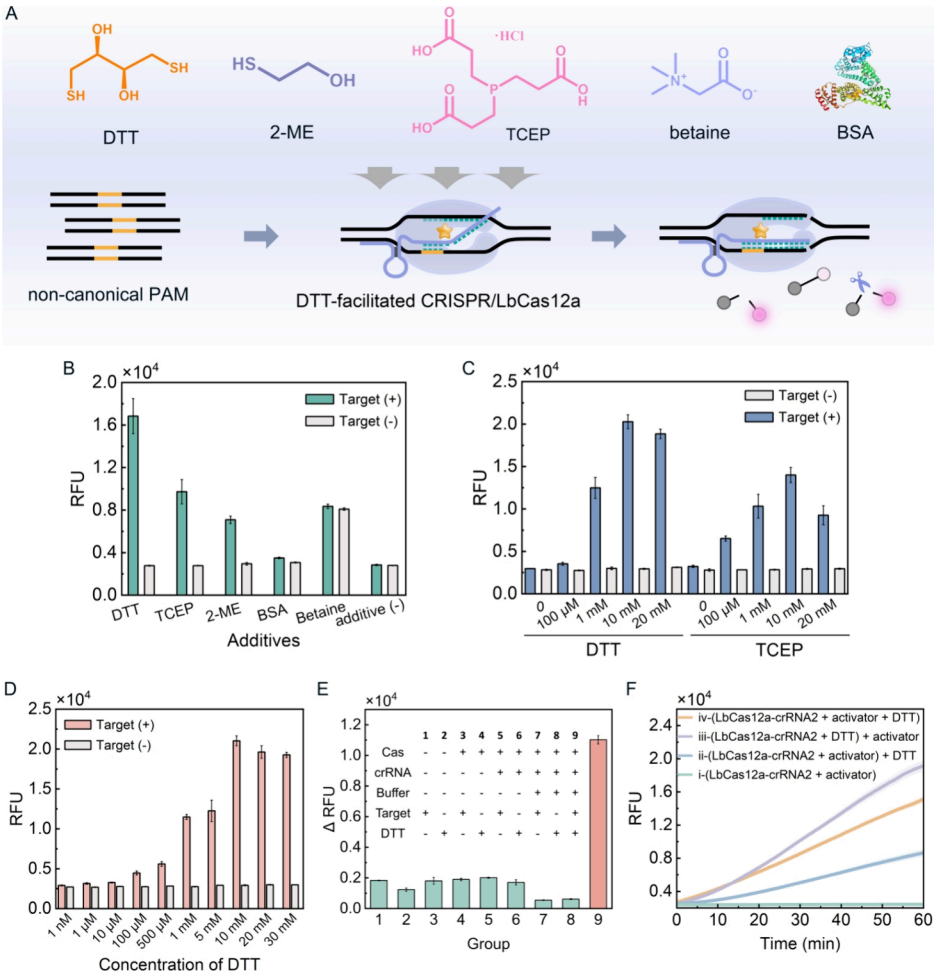


Figure 2. (A) Schematic diagram of chemical additive-mediated CRISPR/LbCas12a system. (B) Investigation of the effects of chemical additives (5 mM DTT, 5 mM TCEP, 5 mM 2-ME, 5 mM betaine, 100 μg/mL BSA) on the trans-cleavage of the CRISPR/LbCas12a system using 200 pM noncanonical PAM activator (plasmid containing the HPV 16 gene). (C) Comparison of the effects of different concentrations of DTT and TCEP on the trans-cleavage of the CRISPR/LbCas12a system using 200 pM noncanonical PAM activator (plasmid containing the HPV 16 gene). (D) Study of the influence of different concentrations of DTT on the trans-cleavage of the CRISPR/Cas12a system using 200 pM noncanonical PAM activator (plasmid containing the HPV 16 gene). (E) Investigation of the key components of LbCas12a-crRNA2 complexes targeting 200 pM noncanonical PAM activator (plasmid containing the HPV 16 gene) through the component deletion experiment. ΔRFU was obtained by subtracting the fluorescence intensity of the experimental groups with only the reporter probe. (F) Investigation of the promotion effect of 12 mM DTT on the recognition process between LbCas12a−crRNA2 complexes and 200 pM noncanonical PAM activator (plasmid containing the HPV 16 gene). Curve i, the LbCas12a−crRNA2 complex and noncanonical PAM activator were incubated at 37 °C for 30 min, followed by the addition of FQ reporter for real-time fluorescence testing; Curve ii, the LbCas12a−crRNA2 complex and noncanonical PAM activator were incubated at 37 °C for 30 min, followed by the addition of DTT and FQ reporter for real-time fluorescence testing; Curve iii, the LbCas12a−crRNA2 complex and DTT were incubated at 37 °C for 30 min, followed by the addition of noncanonical PAM activator and FQ reporter for real-time fluorescence testing; Curve iv, the LbCas12a−crRNA2 complex, noncanonical PAM activator, and DTT were coincubated at 37 °C for 30 min, followed by the addition of FQ reporter for real-time fluorescence testing. The histogram presents the relative fluorescence units (RFU) at 60 min, with data expressed as the mean ± standard deviation (n = 3 technical replicates).

PAM activators, we compared sulfhydryl reductants, including dithiothreitol (DTT), tris(2-carboxyethyl)phosphine (TCEP), and 2-mercaptoethanol (2-ME), along with a common buffer additive (BSA) and a common PCR enhancer (betaine) (Figure 2A). The effects of these additives on the trans-cleavage of the CRISPR/LbCas12a system were assessed under the same testing conditions. The addition of sulfhydryl reductants results in a significantly increased signal-to-noise ratio compared to those of BSA and betaine (Figure 2B). DTT, TCEP, and 2-ME are commonly used reducing agents in laboratory settings, primarily to prevent disulfide bond formation between cysteine residues in proteins. Compared to 2-ME, which has the weakest reducing ability, DTT and TCEP exhibit stronger promoting effects on the trans-cleavage of the CRISPR/LbCas12a system with noncanonical PAM activators. To further evaluate the promoting effect of DTT, we compared the effects of different concentrations of DTT and TCEP on the trans-cleavage of the CRISPR/LbCas12a system. The enhancement effect of DTT was observed to be more pronounced than that of TCEP (Figure 2C), consistent with a previous study.[29] It may be that further degradation of buried disulfide bonds usually results in a loss of the natural conformation of proteins. Specifically, the enhancement effect of DTT on the trans-cleavage of the CRISPR/LbCas12a system with noncanonical PAM activators is concentration-dependent, with significant effects observed at concentrations

https://doi.org/10.1021/acs.analchem.5c00280
Anal. Chem. XXXX, XXX, XXX−XXX

above 10 μM and reaching a peak at approximately 10 mM (Figure 2D). Specifically, a slightly higher concentration of DTT (12 mM) was used to ensure optimal and stable enhancement of Cas12a activity while considering potential variations in reaction conditions. To evaluate the key components of the DTT-facilitated LbCas12a system, we conducted the component deletion assay. The results show that DTT-facilitated LbCas12a can target activators with noncanonical PAMs only in the presence of DTT (Figure 2E). In addition, DTT-facilitated LbCas12a exhibits optimal trans-cleavage efficiency in deBuffer (Figure S3 and Table S8).

We conjectured that the promotion on trans-cleavage of noncanonical PAM activators might be ascribed to how DTT influences the recognition process between the LbCas12a−crRNA complex and the activator. Therefore, we changed the incubation order of the three components (LbCas12a−crRNA2 complex, noncanonical PAM activator, and DTT) and conducted the in vitro cleavage assay to verify this hypothesis (Figure 2F). The FQ reporter served as an indicator for fluorescence signal change. The results indicate that the preincubation of DTT with the LbCas12a−crRNA complex can obviously enhance the trans-cleavage. Especially, preincubation of the LbCas12a−crRNA complex with DTT facilitates a higher fluorescence signal than when the activator is also present. Presumably, DTT assists the LbCas12a−crRNA complex in priming for noncanonical PAM activator recognition, thereby triggering the special DNase activity of LbCas12a.

Furthermore, the cis- and trans-cleavage of DTT-facilitated LbCas12a were demonstrated using 12% polyacrylamide gel electrophoresis (Figure S4). To obtain the linear noncanonical PAM activators, we first designed a pair of primers to amplify the plasmid containing the T790M gene. On incubation of DTT with the LbCas12a−crRNA1 complex, we observed the cis-cleavage of the linear noncanonical PAM activators (Figure S4B, lane 3) and the trans-cleavage of random ssDNA (Figure S4C, lane 2). The DTT-facilitated LbCas12a shows the highest cis-cleavage with canonical PAM (TTTA), which is consistent with previous studies.[28,29] In contrast, the cis-cleavage of linear noncanonical PAM activators (Figure S4B, lane 4) and trans-cleavage of random ssDNA (Figure S4C, lane 3) are not observed in the absence of DTT. The results suggest that DTT facilitates the binding of the LbCas12a−crRNA complex and noncanonical PAM activator, inducing the cis- and trans-cleavage.

Profiling the Compatibility of DTT-deCRISPR with Nucleic Acid Amplification Techniques. Accordingly, we proposed a DTT-mediated CRISPR/Cas12a toolbox (DTT-deCRISPR) for random nucleic acid detection, which enhances the flexibility of nucleic acid testing. To evaluate the flexibility of the DTT-deCRISPR system, we integrated it with nucleic acid amplification techniques, selecting the widely used PCR and the isothermal amplification method, RPA. The noncanonical PAM targets were PCR or RPA amplified, and the amplicons were subsequently added into the LbCas12a/crRNA2-induced in vitro cleavage assay (Figure 3A). The results show that the specific DTT additive could activate the trans-cleavage of CRISPR-LbCas12a (Figure 3B). The non-canonical PAM amplicons originating from PCR cannot activate the trans-cleavage of CRISPR-LbCas12a in the absence of DTT (Figure 3C), while that originating from RPA induces partial trans-cleavage (Figure 3D). The DTT-deCRISPR-RPA reaction carried out a further addition of 12 mM DTT, generating an increased fluorescence signal, which is 1.62-fold higher than that without additional DTT. The RPA buffer contains a certain amount of DTT,[36] which can facilitate the LbCas12a−crRNA complex to prime for noncanonical PAM amplicons. Overall, DTT-deCRISPR adopts a relaxed preference for PAM base pairing and is easily compatible with nucleic acid amplification techniques.

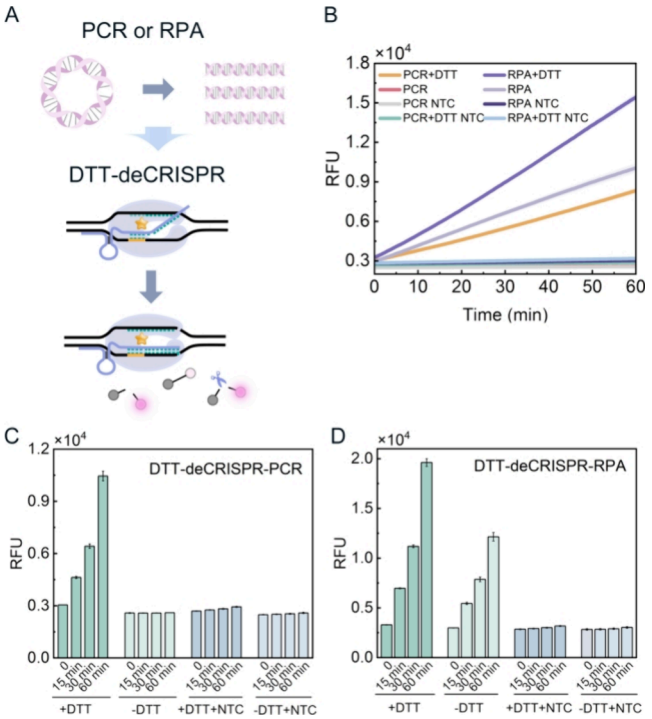


Figure 3. (A) Schematic diagram of the integration of DTT-deCRISPR with PCR and RPA. (B) Time−course results of DTT-deCRISPR-PCR and DTT-deCRISPR-RPA for detecting 40 aM noncanonical PAM activator (plasmid containing the HPV 16 gene). (C) and (D) Comparison of the fluorescence intensities of DTT-deCRISPR-PCR and DTT-deCRISPR-RPA with conventional CRISPR-PCR and CRISPR-RPA for detecting 40 aM noncanonical PAM activator (plasmid containing the HPV 16 gene) at different time points, respectively. The histogram presents the relative fluorescence units (RFU) at 60 min, with data expressed as the mean ± standard deviation (n = 3 technical replicates). NTC, no target control.

Detection of High-Risk HPV Using DTT-deCRISPR-PCR. For sensitive detection, we integrated DTT-deCRISPR with frequently used PCR to develop a real-time CRISPR platform. Cervical cancer remains a major public health concern for women, with an estimated 13,820 new cases and 4360 deaths projected in 2024.[31] High-risk HPV types are detected in over 90% of cervical cancer cases. The WHO recommends HPV nucleic acid testing as the primary method for cervical cancer screening. To demonstrate the potential clinical applications of the proposed DTT-deCRISPR-PCR system, we selected high-risk HPV 16 and 18 as target models, as they are the most prevalent genotypes in cervical cancer. First, we optimized key parameters, including HPV primers and the number of PCR cycles (Figure S5 and Table S7). To further determine the limit of detection (LoD) of the optimized DTT-deCRISPR-PCR system, we used it to detect HPV16 and HPV 18 with gradient concentrations ranging from 0.1 pM to 1 aM. The results indicate that the LoD of the DTT-deCRISPR-PCR system is 1 aM for HPV 16 and HPV

https://doi.org/10.1021/acs.analchem.5c00280
Anal. Chem. XXXX, XXX, XXX−XXX

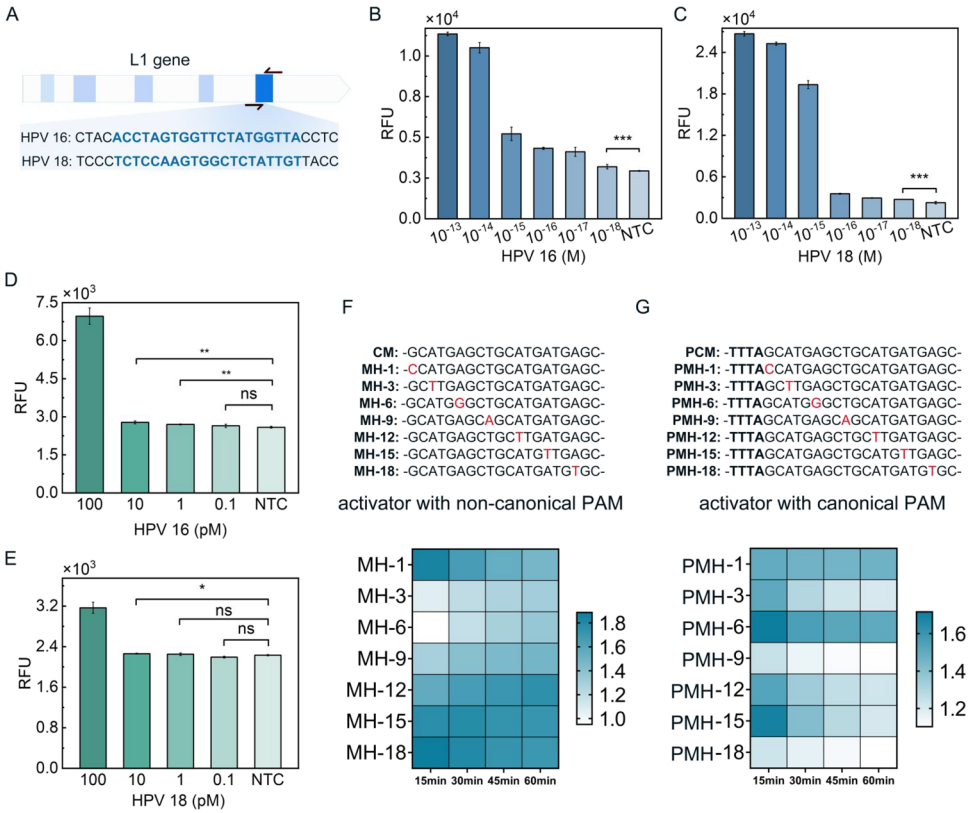


Figure 4. (A) Schematic diagram of the LbCas12a/crRNA-targeted HPV 16 and HPV 18 sequences within hypervariable loop V of the L1-encoding gene. Titration of (B) HPV 16 and (C) HPV 18 were detected by the DTT-deCRISPR-PCR system. Titration of (D) HPV 16 and (E) HPV 18 were detected by the DTT-deCRISPR system. (F) Discrimination factors change in the DTT-facilitated LbCas12a/crRNA1 cleavage assay over time. The top panel shows the complementary sequence (CM) and mismatch sequences (MH1−MH18). (G) Discrimination factor change in the conventional LbCas12a/crRNA1 cleavage assay without DTT over time. The top panel shows the complementary sequence (PCM) and mismatch sequences (PMH1−PMH18). The histogram presents the relative fluorescence units (RFU) at 60 min, with data expressed as the mean ± standard deviation (n = 3 technical replicates). NTC, no target control. ns, not significant; * $p < 0.05$; ** $p < 0.01$; ***, $P < 0.001$.

18 targets (Figure 4B−C). Compared with the analytic performance of the DTT-deCRISPR system (Figure 4D−E), the sensitivity of the DTT-deCRISPR-PCR system is 10^8-fold enhanced. These analytic applications further demonstrate the universality and efficacy of the DTT-deCRISPR-PCR system in gene detection.

In addition, the specificity of the developed DTT-deCRISPR was evaluated through testing single base mismatch differentiation. Previous studies demonstrated that the trans-cleavage rate of CRISPR/Cas12a is affected by mismatches in the dsDNA activator but not by the crRNA-complementary ssDNA activator.[4,14] PAM mutations or mismatches in the PAM-adjacent "seed region" can slow the trans-cleavage rate. Specifically, we designed a hairpin DNA activator featuring a double-stranded stem stabilized by a loop structure (Table S9). The hairpin DNA activators consist of different stems, including sequences (PCM and CM) that are fully complementary to crRNA1, as well as sequences (PMH and MH) with single base mismatches at various locations. First, we compared the mismatch tolerance of the conventional LbCas12a/crRNA1 cleavage assay. The significant differences in fluorescence signal are observed between the completely matched targets and the single base mismatch targets. The single base mismatch differentiation performance is assessed by the discrimination factor (DF) that can be calculated by the formula DF = ([CM] background)/([MH] − background). For the DTT-facilitated LbCas12a/crRNA1 cleavage assay, the DF value of detecting completely matched targets (CM) is generally 1.1−1.69 times higher than that of detecting single base mismatch targets (MH) at the same concentration (Figure 4F). Also, the conventional LbCas12a/crRNA1 cleavage assay shows a DF value of 0.95−1.89 times difference in detecting completely matched targets (PCM) and single base mismatch targets (PMH) (Figure 4G). Especially, the DTT-deCRISPR system shows better discrimination ability for the remote mismatch. In light of the superiority of the DTT-deCRISPR system with relaxed preference for PAM base pairing, we speculate that it is more flexible and practical. In the future, combining the DTT-deCRISPR system with other mutation enrichment methods is expected to meet the needs of clinical diagnosis.

Clinical Sample Analysis. To evaluate the applicability of our method for molecular diagnostics, we performed a clinical study using 26 vaginal or cervical swab samples. The PANTHER detection system is a certified HPVE6/E7 virus mRNA (mRNA) detection system, along with its corresponding detection kits approved by CFDA.[37] By utilizing both the PANTHER detection system and its matching detection kit APTIMA HPV detection system, 26 samples with definitive typing were obtained. The 26 clinical samples with known genotypes consist of 16 HPV 16 positive samples, 5 HPV 18 positive samples, and 5 negative samples (Table S10). To verify the utility of the DTT-deCRISPR assay for analyzing clinical samples, we tested crude DNA extractions from the 26

https://doi.org/10.1021/acs.analchem.5c00280
Anal. Chem. XXXX, XXX, XXX−XXX

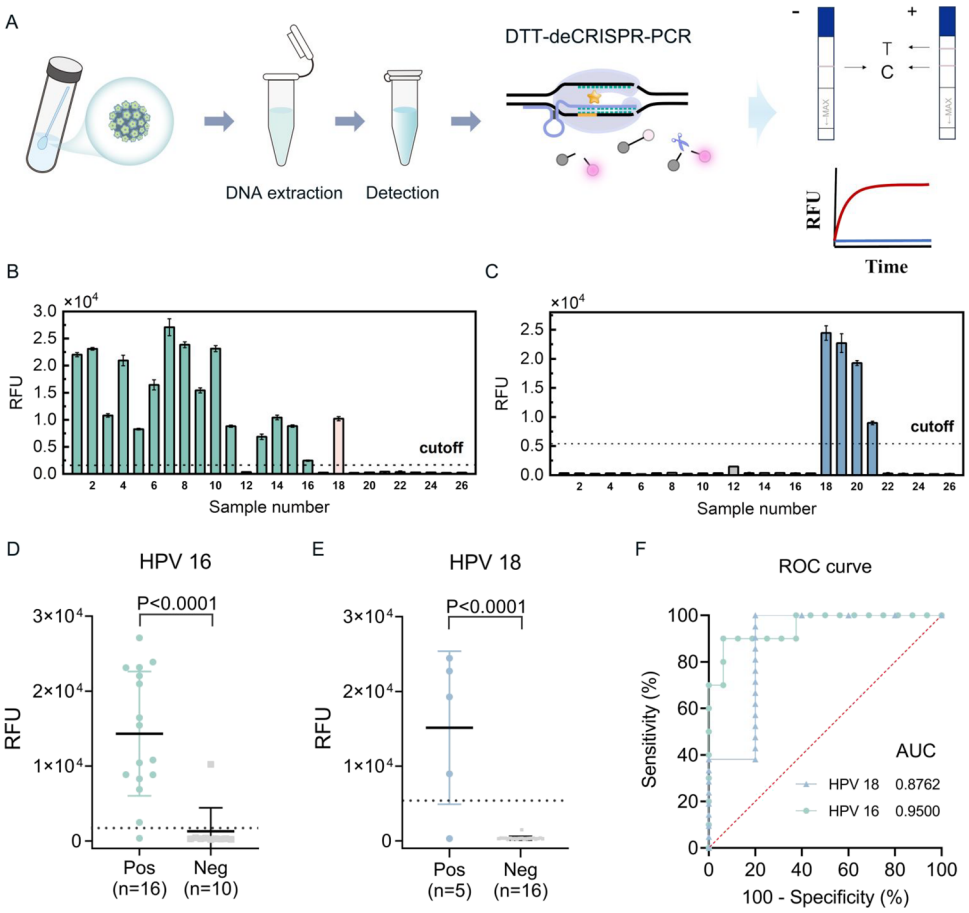


Figure 5. (A) Schematic of clinical diagnosis of HPV 16 and HPV 18 by the DTT-deCRISPR-PCR. DTT-deCRISPR-PCR assay results for (B) HPV 16 and (C) HPV 18. (D) Scatter plots for discriminating HPV 16 positive samples from HPV 16 negative groups. A cutoff value of 1455 for HPV 16 was calculated from the ROC curve. (E) Scatter plots for discriminating HPV 18 positive samples from HPV 18 negative groups. A cutoff value of 5231 for HPV 18 was calculated from the ROC curve. (F) ROC curves and AUC analysis of this method for high-risk HPV infection diagnosis. The two-tailed Student's *t* test.

human cervical cell samples that were previously analyzed using the PANTHER detection system (Figure 5A). The DTT-deCRISPR assay reveals distinct signals between positive and negative groups for both HPV 16 and HPV 18 (Figure 5B−C). The positive or negative status of the 23 samples determined by our assay for HPV 16 and HPV 18 is consistent with the PANTHER results, except for three samples: false positive for HPV 16 (sample no. 18), false negative for HPV 16 (sample no. 12), and HPV 18 (sample #17). Based on the receiver operating characteristic (ROC) curve analysis, the clinical sensitivity and specificity for detecting HPV 16 were 93.75% and 90.00%, respectively, while those for detecting HPV 18 were 80.00% and 100.0%, respectively (Figure 5D−E). The AUC values for HPV 16 and HPV 18 were 0.9500 and 0.8762, respectively (Figure 5F), demonstrating the satisfactory clinical performance of the DTT-deCRISPR assay. Collectively, these results suggest that the proposed assay is an effective diagnostic tool for the treatment of high-risk HPV infections.

In addition, the proposed assay can be integrated into a lateral flow assay (LFA) for point-of-care detection (Figure S6). The results of the lateral flow visualization analysis demonstrate that the DTT-deCRISPR-LFA system successfully differentiates between positive and negative HPV 16 and 18 clinical samples, consistent with the fluorescence analysis results. These findings underscore the potential of this method for point-of-care applications.

CONCLUSIONS

In this study, we discovered that DTT-facilitated LbCas12a nucleases adopt a relaxed preference for PAM base pairing and proposed a DTT-mediated CRISPR/Cas12a toolbox (DTT-deCRISPR) that relaxes the PAM specificity and is readily compatible with nucleic acid amplification techniques. In addition, we developed DTT-deCRISPR-PCR for sensitive detection of high-risk HPV 16 and 18. The platform can detect synthesized HPV 16 and 18 plasmids down to attomolar sensitivity within 60 min. We also investigated the point-of-care detection potential of the DTT-deCRISPR-LFA system through combining it with an LFA strip. Moreover, the positive and negative results for clinical samples are consistent with the fluorescence analysis results. However, tube opening and liquid transfer in the two-step reaction increase the complexity and the risk of aerosol contamination, potentially leading to false positives. Given the superiority of the DTT-deCRISPR system with a relaxed preference for PAM base pairing, we speculate that this system offers greater flexibility and practicality in developing one-pot detection systems.

https://doi.org/10.1021/acs.analchem.5c00280
Anal. Chem. XXXX, XXX, XXX−XXX

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.5c00280.

Materials and reagents; DTT-deCRISPR-RPA assay; DTT-deCRISPR-PCR assay; Single base mismatch differentiation experiments; Human clinical sample detection; PAGE measurement; Statistical analysis; Plasmid information; Sequences information on crRNA, fluorescent probe, dsDNA activators, hairpin DNA activator and primer; Composition of the buffer; Information on the clinical samples; Comparison of real-time fluorescence curves between DTT-facilitated LbCas12a and DTT-free LbCas12a system for the detection of noncanonical PAM (CCTT, CCTC, and TACC) activators; Comparison the specificity of DTT-facilitated LbCas12a and LbCas12a across 16 non-canonical PAM combinations; Optimization of the composition of buffer; Investigation of the cis- and trans-cleavage by PAGE; Optimization of the DTT-deCRISPR-PCR system; Evaluation of the DTT-deCRISPR system for point-of-care detection (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

Ruo Yuan − *Key Laboratory of Luminescence Analysis and Molecular Sensing (Southwest University), Ministry of Education, College of Chemistry and Chemical Engineering, Southwest University, Chongqing 400715, P.R. China;* orcid.org/0000-0003-3664-6236; Email: yuanruo@swu.edu.cn

Lijuan Bai − *Chongqing Research Center for Pharmaceutical Engineering, College of Pharmacy, Chongqing Medical University, Chongqing 400016, P.R. China; Department of Respiratory and Critical Care Medicine, The First Affiliated Hospital of Chongqing Medical University, Chongqing 400016, P.R. China;* orcid.org/0000-0001-9450-4581; Email: bailj1018@cqmu.edu.cn

Authors

Min Qing − *Chongqing Research Center for Pharmaceutical Engineering, College of Pharmacy, Chongqing Medical University, Chongqing 400016, P.R. China;* orcid.org/0000-0001-9491-0360

Cheng Huang − *Chongqing Research Center for Pharmaceutical Engineering, College of Pharmacy, Chongqing Medical University, Chongqing 400016, P.R. China*

Yueyuan Li − *Chongqing Research Center for Pharmaceutical Engineering, College of Pharmacy, Chongqing Medical University, Chongqing 400016, P.R. China*

Qiubo Yu − *Molecular Medical Laboratory and Department of Pathology, Chongqing Medical University, Chongqing 400016, P.R. China*

Qianfang Hu − *Department of Respiratory and Critical Care Medicine, The First Affiliated Hospital of Chongqing Medical University, Chongqing 400016, P.R. China*

Jing Zhou − *Chongqing Research Center for Pharmaceutical Engineering, College of Pharmacy, Chongqing Medical University, Chongqing 400016, P.R. China;* orcid.org/0000-0003-1237-3296

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.analchem.5c00280

Author Contributions

#Min Qing, Cheng Huang, and Yueyuan Li contributed equally.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work is supported by the National Natural Science Foundation of China (82072378, 81601856), Chongqing Talents: Exceptional Young Talents Project, China (CQYC202005015, cstc2021ycjhbgzxm0328), Discipline Talent Training Program of College of Pharmacy in Chongqing Medical University, China (YXY2021BSH04), and CQMU Program for Youth Innovation in Future Medicine, China (172020320220090 W0072).

■ REFERENCES

(1) Kaminski, M. M.; Abudayyeh, O. O.; Gootenberg, J. S.; Zhang, F.; Collins, J. J. *Nat. Biomed. Eng.* **2021**, *5* (7), 643−656.

(2) Broughton, J. P.; Deng, X.; Yu, G.; Fasching, C. L.; Servellita, V.; Singh, J.; Miao, X.; Streithorst, J. A.; Granados, A.; Sotomayor-Gonzalez, A.; Zorn, K.; Gopez, A.; Hsu, E.; Gu, W.; Miller, S.; Pan, C.-Y.; Guevara, H.; Wadford, D. A.; Chen, J. S.; Chiu, C. Y. *Nat. Biotechnol.* **2020**, *38* (7), 870−874.

(3) Hu, M.; Wang, Y.; Zhou, X. *Interdiscip. Med.* **2024**, *2* (3), No. e20240006.

(4) Chen, J. S.; Ma, E.; Harrington, L. B.; Da Costa, M.; Tian, X.; Palefsky, J. M.; Doudna, J. A. *Science* **2018**, *360* (6387), 436−439.

(5) Joung, J.; Ladha, A.; Saito, M.; Kim, N. G.; Woolley, A. E.; Segel, M.; Barretto, R. P. J.; Ranu, A.; Macrae, R. K.; Faure, G.; Ioannidi, E. I.; Krajeski, R. N.; Bruneau, R.; Huang, M. L. W.; Yu, X. G.; Li, J. Z.; Walker, B. D.; Hung, D. T.; Greninger, A. L.; Jerome, K. R.; Gootenberg, J. S.; Abudayyeh, O. O.; Zhang, F. *N. Engl. J. Med.* **2020**, *383* (15), 1492−1494.

(6) Zhang, J.; Li, Z.; Guo, C.; Guan, X.; Avery, L.; Banach, D.; Liu, C. *Angew. Chem., Int. Ed.* **2024**, *63* (20), No. e202403123.

(7) Wang, H.; Li, Y. L.; Fan, Y. J.; Dong, J. X.; Ren, X.; Ma, H.; Wu, D.; Gao, Z. F.; Wei, Q.; Xia, F. *Anal. Chem.* **2023**, *95* (36), 13659−13667.

(8) Bagheri, N.; Chamorro, A.; Idili, A.; Porchetta, A. *Angew. Chem., Int. Ed.* **2024**, *63* (17), No. e202319677.

(9) Dai, Y.; Wu, Y.; Liu, G.; Gooding, J. J. *Angew. Chem., Int. Ed.* **2020**, *132* (47), 20938−20950.

(10) Pickar-Oliver, A.; Gersbach, C. A. *Nat. Rev. Mol. Cell. Biol.* **2019**, *20* (8), 490−507.

(11) Zhang, L.; Liu, Q.; Guo, Y.; Tian, L.; Chen, K.; Bai, D.; Yu, H.; Han, X.; Luo, W.; Feng, T.; Deng, S.; Xie, G. *J. Nanobiotechnol.* **2024**, *22* (1), 189.

(12) Collias, D.; Beisel, C. L. *Nat. Commun.* **2021**, *12* (1), 555.

(13) Strohkendl, I.; Saifuddin, F. A.; Rybarski, J. R.; Finkelstein, I. J.; Russell, R. *Mol. Cell* **2018**, *71* (5), 816−824.

(14) Swarts, D. C.; Jinek, M. *Mol. Cell* **2019**, *73* (3), 589−600.

(15) Yamano, T.; Zetsche, B.; Ishitani, R.; Zhang, F.; Nishimasu, H.; Nureki, O. *Mol. Cell* **2017**, *67* (4), 633−645.

(16) Toth, E.; Varga, E.; Kulcsar, P. I.; Kocsis-Jutka, V.; Krausz, S. L.; Nyeste, A.; Welker, Z.; Huszar, K.; Ligeti, Z.; Talas, A.; Welker, E. *Nucleic Acids Res.* **2020**, *48* (7), 3722−3733.

(17) Feng, W.; Newbigging, A. M.; Tao, J.; Cao, Y.; Peng, H.; Le, C.; Wu, J.; Pang, B.; Li, J.; Tyrrell, D. L.; Zhang, H.; Le, X. C. *Chem. Sci.* **2021**, *12* (13), 4683−4698.

(18) Qing, M.; Chen, S. L.; Sun, Z.; Fan, Y.; Luo, H. Q.; Li, N. B. *Anal. Chem.* **2021**, *93* (20), 7499−7507.

(19) Cao, G.; Yang, N.; Xiong, Y.; Shi, M.; Wang, L.; Nie, F.; Huo, D.; Hou, C. *ACS Sens.* **2023**, *8* (12), 4655−4663.

(20) Yang, L.; Chen, G.; Wu, J.; Wei, W.; Peng, C.; Ding, L.; Chen, X.; Xu, X.; Wang, X.; Xu, J. *Anal. Chem.* **2024**, *96* (14), 5471−5477.

(21) Zhou, S.; Dong, J.; Deng, L.; Wang, G.; Yang, M.; Wang, Y.; Huo, D.; Hou, C. *ACS Sens.* **2022**, *7* (10), 3032−3040.
(22) Rananaware, S. R.; Meister, K. S.; Shoemaker, G. M.; Vesco, E. K.; Sandoval, L. S. W.; Lewis, J. G.; Bodin, A. P.; Karalkar, V. N.; Lange, I. H.; Pizzano, B. L. M.; Chang, M.; Ahmadimashhadi, M. R.; Flannery, S. J.; Nguyen, L. T.; Wang, G. P.; Jain, P. K. *medRxiv* **2024**, No. 24306194.
(23) Huang, D.; Ni, D.; Fang, M.; Shi, Z.; Xu, Z. *Anal. Chem.* **2021**, *93* (50), 16965−16973.
(24) Wang, R.; Zhao, X.; Chen, X.; Qiu, X.; Qing, G.; Zhang, H.; Zhang, L.; Hu, X.; He, Z.; Zhong, D.; Wang, Y.; Luo, Y. *Anal. Chem.* **2020**, *92* (2), 2176−2185.
(25) Kong, X.; Zhang, H.; Li, G.; Wang, Z.; Kong, X.; Wang, L.; Xue, M.; Zhang, W.; Wang, Y.; Lin, J.; Zhou, J.; Shen, X.; Wei, Y.; Zhong, N.; Bai, W.; Yuan, Y.; Shi, L.; Zhou, Y.; Yang, H. *Nat. Commun.* **2023**, *14* (1), 2046.
(26) Eggers, A. R.; Chen, K.; Soczek, K. M.; Tuck, O. T.; Doherty, E. E.; Xu, B.; Trinidad, M. I.; Thornton, B. W.; Yoon, P. H.; Doudna, J. A. *Cell* **2024**, *187* (13), 3249−3261.
(27) Singh, D.; Mallon, J.; Poddar, A.; Wang, Y.; Tippana, R.; Yang, O.; Bailey, S.; Ha, T. *Proc. Natl. Acad. Sci. U.S.A.* **2018**, *115* (21), 5444−5449.
(28) Deng, F.; Li, Y.; Li, B.; Goldys, E. M. *Sens. Actuators B: Chem.* **2022**, *373*, No. 132767.
(29) Habimana, J. d. D.; Mukama, O.; Chen, G.; Chen, M.; Amissah, O. B.; Wang, L.; Liu, Y.; Sun, Y.; Li, A. L.; Deng, S.; Huang, J.; Yan, X.-x.; Rutaganda, T.; Mutangana, D.; Wu, L.-P.; Huang, R.; Li, Z. *Biosens. Bioelectron.* **2023**, *222*, No. 114939.
(30) Cohen, P. A.; Jhingran, A.; Oaknin, A.; Denny, L. *Lancet* **2019**, *393* (10167), 169−182.
(31) Siegel, R. L.; Giaquinto, A. N.; Jemal, A. *CA Cancer J. Clin.* **2024**, *74* (1), 12−49.
(32) Ramachandran, A.; Santiago, J. G. *Anal. Chem.* **2021**, *93* (20), 7456−7464.
(33) Huyke, D. A.; Ramachandran, A.; Bashkirov, V. I.; Kotseroglou, E. K.; Kotseroglou, T.; Santiago, J. G. *Anal. Chem.* **2022**, *94* (27), 9826−9834.
(34) Nalefski, E. A.; Kooistra, R. M.; Parikh, I.; Hedley, S.; Rajaraman, K.; Madan, D. *Nucleic Acids Res.* **2024**, *52* (8), 4502−4522.
(35) Zetsche, B.; Gootenberg, J. S.; Abudayyeh, O. O.; Slaymaker, I. M.; Makarova, K. S.; Essletzbichler, P.; Volz, S. E.; Joung, J.; van der Oost, J.; Regev, A.; Koonin, E. V.; Zhang, F. *Cell* **2015**, *163* (3), 759−771.
(36) Piepenburg, O.; Williams, C. H.; Stemple, D. L.; Armes, N. A. *PLoS. Biol.* **2006**, *4* (7), No. e204.
(37) Forslund, O.; Miriam Elfström, K.; Lamin, H.; Dillner, J. *Int. J. Cancer* **2019**, *144* (5), 1073−1081.

https://doi.org/10.1021/acs.analchem.5c00280
Anal. Chem. XXXX, XXX, XXX−XXX