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Intrinsic RNA Targeting Triggers Indiscriminate DNase Activity of CRISPR-Cas12a

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Abstract

The CRISPR-Cas12a system has emerged as a powerful tool for next-generation nucleic acid-based molecular diagnostics. However, it has long been believed to be effective only on DNA targets. Here, we investigate the intrinsic RNA-enabled *trans*-cleavage activity of AsCas12a and LbCas12a and discover that they can be directly activated by full-size RNA targets, although LbCas12a exhibits weaker *trans*-cleavage activity than AsCas12a on both single-stranded DNA and RNA substrates. Remarkably, we find that the RNA-activated Cas12a possesses higher specificity in recognizing mutated target sequences compared to DNA activation. Based on these findings, we develop the "Universal Nuclease for Identification of Virus Empowered by RNA-Sensing" (UNIVERSE) assay for nucleic acid testing. We incorporate a T7 transcription step into this assay, thereby eliminating the requirement for a protospacer adjacent motif (PAM) sequence in the target. Additionally, we successfully detect multiple PAM-less targets in HIV clinical samples that are undetectable by the conventional Cas12a assay based on double-stranded DNA activation, demonstrating unrestricted target selection with the UNIVERSE assay. We further validate the clinical utility of the UNIVERSE assay by testing both HIV RNA and HPV 16 DNA in clinical samples. We envision that the intrinsic RNA targeting capability may bring a paradigm shift in Cas12a-based nucleic acid detection and further enhance the understanding of CRISPR-Cas biochemistry.

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Introduction

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) technology, widely recognized as a groundbreaking genome editing tool[1], has recently attracted considerable attention for its promising potential in nucleic acid-based molecular diagnostics[2]. Among the CRISPR-Cas families, Cas12a is often considered an ideal option for nucleic acid detection due to its distinctive *trans*-cleavage activity. This activity leads to the massive indiscriminate degradation of single-stranded DNA substrates labeled with a fluorophore and quencher pair (ssDNA-FQ) when a DNA sequence complementary to the spacer of CRISPR (crRNA) is recognized[3]. The *trans*-cleavage activity generates visible fluorescence in a rapid, sensitive, and accurate manner. Therefore, extensive studies have been conducted to explore related methodologies in pathogen detection[3-4] and their potential in the development of point-of-care diagnostic devices[5].

While exciting, Cas12a-based detection of double-stranded DNA (dsDNA) targets is inherently limited by a protospacer adjacent motif (PAM) sequence (e.g., TTTV)[3, 6], which facilitates the separation of dsDNA and subsequent crRNA invasion[6a]. In contrast, the PAM sequence is not essential for ssDNA detection[3, 7]. Recently, a series of suboptimal PAMs (e.g., VTTV, TCTV, and TTVV) were discovered to generate an equivalent or even greater *trans*-cleavage fluorescence response compared to the canonical PAM under the conventional CRISPR-Cas12a assay protocol using LbCas12a[8]. However, commonly used Cas12a orthologs including LbCas12a, AsCas12a and FnCas12a still lacks the ability to detect a random sequence within a dsDNA amplicon unless auxiliary attempts are made, such as artificially introducing a PAM sequence through amplification[9], carefully designing a strand displacement reaction pathway[10], utilizing nucleases that degrade dsDNA to ssDNA[11], or implementing target-dependent synthesis of a crRNA matching an effective activator introduced to the reaction[12]. The additional considerations in many of these methods may have significantly limited the selection of target sequences and reduced the detection efficiency in clinical diagnostic applications of Cas12a.

CRISPR-Cas12a has long been believed to be active only toward DNA targets and is widely used to detect nucleic acids by leveraging its DNA-activated *trans*-cleavage activity. However, its RNA-activated *trans*-cleavage property has rarely been investigated, and its ability to detect RNA is not well explored[3, 13]. Recently, our laboratory and others have reported that Cas12a (e.g. LbCas12a, ErCas12a and AsCas12a) can be activated by using a pair of split DNA/RNA targets,

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wherein one half is an RNA fragment (termed "RNA target") targeting the PAM-distal region of the crRNA, and the other half is a DNA fragment (termed "DNA activator") targeting the PAM-proximal seed region[14]. However, these methods still require an artificial DNA activator to boost the *trans*-cleavage activity of Cas12a for RNA detection.

In this work, we investigated the intrinsic *trans*-cleavage activity of AsCas12a and LbCas12a and discovered that both nucleases can be activated by full-size RNA targets. These findings suggest that some Cas12a orthologs share RNA-activating properties with Cas13a but also retains its original DNA-activating capability, enabling universal nucleic acid detection. However, we surprisingly observed that the tested Cas12a orthologs are significantly different from Cas13a in terms of *cis*-cleavage activity, as we observed negligible degradation of RNA targets with the tested Cas12a. Moreover, we found that RNA activation of Cas12a exhibited better specificity on mutated targets compared to DNA activation. Based on these findings, we developed an assay named "Universal Nuclease for Identification of Virus Empowered by RNA-Sensing" (UNIVERSE) for nucleic acid detection by combining recombinase polymerase amplification (RPA) and T7 transcription. We successfully detected multiple PAM-less sequences that were undetectable by the conventional RPA/CRISPR-Cas12a assay, demonstrating that UNIVERSE not only expanded the target selection range but also improved the detection efficiency. Finally, we validated the clinical feasibility of the UNIVERSE assay by detecting HIV RNA in clinical plasma samples and HPV 16 DNA in clinical cervical swab samples. We envision that the discovery of versatile RNA-activated *trans*-cleavage with Cas12a and the associated PAM-free detection scheme will revolutionize our understanding of CRISPR-Cas biochemistry and further expand the toolbox for Cas12a-based molecular diagnostics.

Results and Discussion

RNA-initiated *trans*-cleavage with CRISPR-Cas12a

To investigate the intrinsic RNA-targeting characteristics of Cas12a, we first designed a fully complementary, 20-nt single-stranded RNA (ssRNA) as Cas12a's target. We assessed the RNA-activated *trans*-cleavage activity of four commercially available Cas12a nucleases, including AsCas12a V3, AsCas12a Ultra, LbCas12a Ultra, and EnGen® Lba Cas12a. Surprisingly, we

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observed a significant increase in fluorescence intensity with all four Cas12a nucleases, indicating effective *trans*-cleavage induced by the RNA targets (Figure 1a-d). In particular, AsCas12a (AsCas12a V3 and AsCas12a Ultra) exhibited significantly higher RNA-activated *trans*-cleavage activity compared to LbCas12a (LbCas12a Ultra and EnGen® Lba Cas12a). We further studied the Michaelis–Menten kinetics of AsCas12a and obtained a catalytic efficiency (k_{cat}/K_M) of 2.1 × 10^4 M^{-1} s^{-1} for AsCas12a V3 and 5.4 × 10^4 M^{-1} s^{-1} for AsCas12a Ultra when activated by ssRNA (Figure S1). To compare the *trans*-cleavage efficiency of the ssRNA target with traditional DNA target, we also designed a 20-nt ssDNA target with an identical sequence to the ssRNA and performed the *trans*-cleavage assay (Figure S2). Additionally, we measured the fluorescence intensity of *trans*-cleavage on the RNA substrate by switching the ssDNA reporters to ssRNA reporters in the assay (Figures S3 and S4). When using RNA targets and ssDNA reporters, we observed a weakening of *trans*-cleavage activity for the LbCas12a nucleases and a relatively slighter weakening for the AsCas12a nucleases (Figure 1e). However, it is noteworthy that we did not observe significant differences in sensitivity when detecting both DNA and RNA targets (Figures S5 and S6). With ssRNA reporters being the substrates, the *trans*-cleavage activity of Cas12a sharply declined regardless of RNA or DNA targets, and their sensitivity became correspondingly lower (Figures S7 and S8). Thus, our results indicate that a full-size ssRNA could trigger *trans*-cleavage activity of both AsCas12a and LbCas12a nucleases, albeit with a lower magnitude with LbCas12a, potentially leading to Cas12a-based RNA detection by coupling with the ssDNA reporter.

Next, we were curious whether *cis*-cleavage occurs on the ssRNA target. Strong *cis*-cleavage activity might cut the ssRNA target and potentially reduce the detection sensitivity. A previous study suggested that the *cis*-cleavage site for ssDNA was near the 22nd base counting from the 3' end of the first base pairing with the crRNA[7]. Given that the target used above only contained 20 nt, the *cis*-cleavage site might lie outside the targeting region and within the 5' extension of the target. Considering potential differences in mechanisms between ssDNA-activated and ssRNA-activated *cis*-cleavage, we designed two 5' FAM-labeled ssRNA targets. One was extended at the 5' end by a 20-nt sequence and the other was extended by 20 nt at both the 5' and 3' ends. To avoid any potential interference from non-specific sequence segments, we carefully designed the extension sequences to ensure there is only one valid targeting site for Cas12a throughout the complete sequence. For the *cis*-cleavage analysis, we used the wild-type AsCas12a

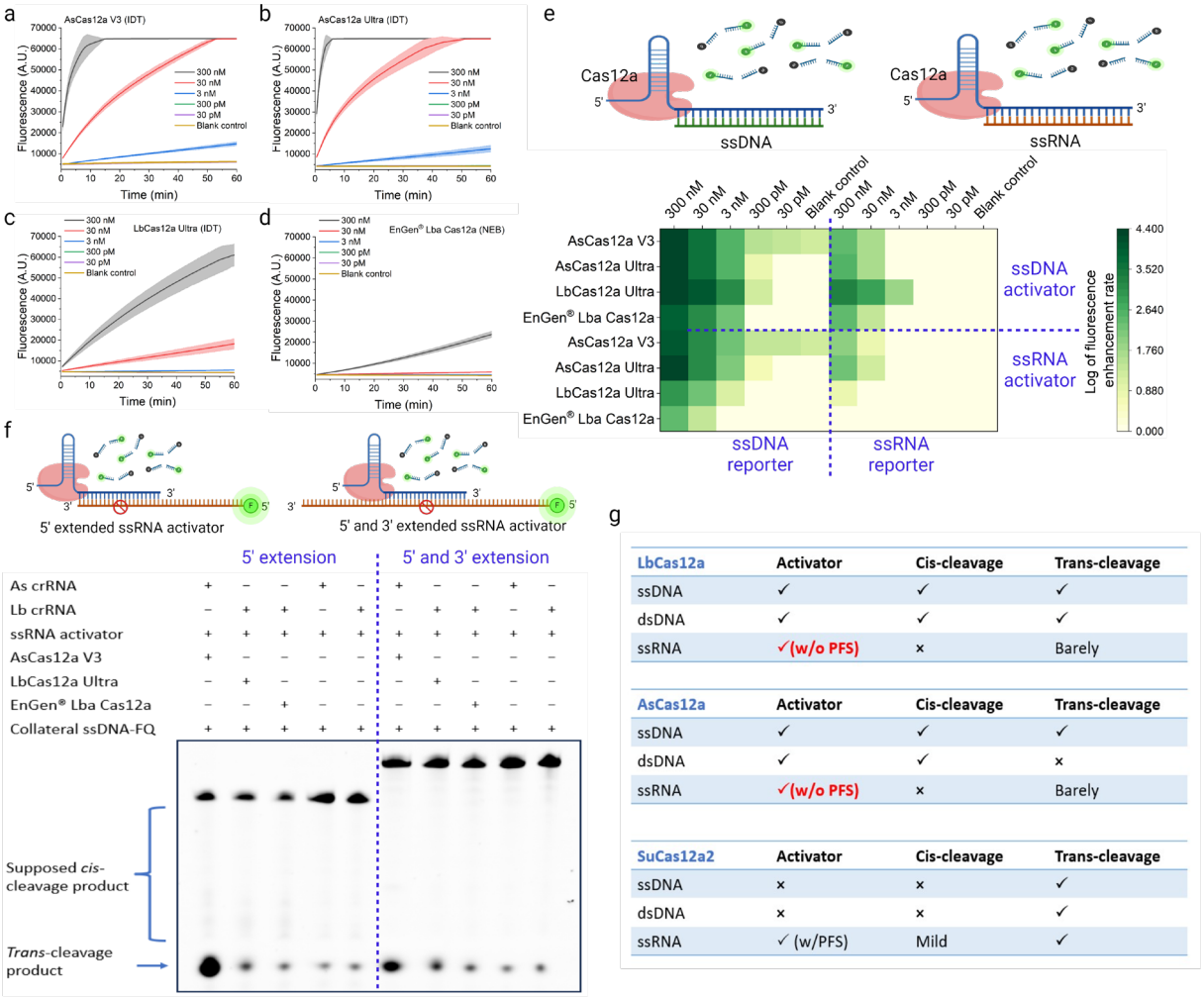
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V3, as its mutant AsCas12a Ultra exhibited minimal differences in the *trans*-cleavage assay with ssRNA targets. After a 60-min incubation at 37°C, none of the Cas12a nucleases, neither AsCas12a nor LbCas12a, exhibited *cis*-cleavage on 5'-extended ssRNA targets or on 5'- and 3'-extended ssRNA targets (Figure 1f), indicating that Cas12a could not cleave the ssRNA targets. To further validate this finding, we designed a 3' FAM-labeled ssRNA target with both 5' and 3' extensions and performed the *cis*-cleavage assay, obtaining consistent results (Figure S9c). In contrast, the *cis*-cleavage was apparent on ssDNA targets with only a 5' extension with all the enzymes tested (Figure S10), consistent with the conclusions from the previous study[7]. Specifically, LbCas12a nucleases could almost completely cleave ssDNA targets within a 60-min incubation period, whereas AsCas12a nucleases cleaved ssDNA targets to a lesser extent, albeit noticeably stronger than the *cis*-cleavage activity observed with ssRNA targets. Thus, both *cis*-cleavage and *trans*-cleavage activities on ssRNA substrates were negligible with LbCas12a and AsCas12a.



g

LbCas12a	Activator	Cis-cleavage	Trans-cleavage
ssDNA	✓	✓	✓
dsDNA	✓	✓	✓
ssRNA	✓(w/o PFS)	×	Barely

AsCas12a	Activator	Cis-cleavage	Trans-cleavage
ssDNA	✓	✓	✓
dsDNA	✓	✓	×
ssRNA	✓(w/o PFS)	×	Barely

SuCas12a2	Activator	Cis-cleavage	Trans-cleavage
ssDNA	×	×	✓
dsDNA	×	×	✓
ssRNA	✓ (w/PFS)	Mild	✓

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Figure 1. RNA-initiated nuclease activity with Cas12a. a-d) Real-time fluorescence of ssRNA-activated *trans*-cleavage with a) AsCas12a V3 (IDT), b) AsCas12a Ultra (IDT), c) LbCas12a Ultra (IDT), and d) EnGen® Lba Cas12a (NEB). For a-d), experiments at each concentration were run in triplicate (n = 3) and graphs represent means (bold line) ± standard deviation (s.d.). e) Comparison of *trans*-cleavage activity among the enzymes tested for different types of targets and reporters. To facilitate the comparison, the intensity of the *trans*-cleavage activity was represented by the logarithm of the average fluorescence enhancement rate from three technical replicates (n = 3) to base 10. For a clearer comparison, the data were corrected to 0 if the average fluorescence enhancement rate or the logarithm value was negative. f) Denaturing PAGE gel electrophoresis for identification of *cis*-cleavage and *trans*-cleavage products using 5' FAM-labeled ssRNA target with a 5' extension and 5' FAM-labeled ssRNA target with both 5' and 3' extensions. For uncropped gel scan see Figure S21 in Supporting Information. g) A summarization of nuclease activity of LbCas12a, AsCas12a, and SuCas12a2 in terms of the types of targets, *cis*-cleavage substrates, and *trans*-cleavage substrates. Our discovery, marked in red, is that a PFS-free ssRNA can serve as the activator to initiate the nuclease activity of both LbCas12a and AsCas12a, a characteristic previously unique to Cas13a orthologs.

Finally, drawing from the experimental results elaborated above and previous literatures, we have summarized the properties of three type V single-effector Cas nucleases. These include two Cas12a orthologs (i.e., LbCas12a and AsCas12a) commonly used for DNA-activated nucleic acid detection and one newly discovered RNA-activating nuclease, SuCas12a2[15]. We delineate their effective nucleic acid types as activators, *cis*-cleavage substrates, and *trans*-cleavage substrates (Figure 1g). For LbCas12a, both ssDNA and dsDNA can initiate the nuclease activity, leading to degradation of the DNA activators themselves and collateral ssDNA or dsDNA substrates[3, 7]. AsCas12a possesses the same nuclease activity as LbCas12a, with the exception of the *trans*-cleavage on dsDNA substrates[7]. Unique from LbCas12a and AsCas12a, SuCas12a2 requires a protospacer flanking site (PFS) on its RNA activator (5′-GAAAG-3') to trigger *trans*-cleavage activity, and this collateral degradation applies to ssDNA, dsDNA, and ssRNA[15a]. Because this protein lacks the Nuc domain involved in DNA target strand loading, DNA activators may not be capable of initiating the nuclease activity[15b]. In addition, the degradation of RNA target with Cas12a2 was mild enough to exhibit a distinct difference from the *cis*-cleavage of DNA targets with Cas12a[15b]. Taken together, the intriguing observations from this study revealed that the ssRNA target could initiate the *trans*-cleavage activity of both AsCas12a and LbCas12a, albeit

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with a lower magnitude observed with LbCas12a. Meanwhile, the ssRNase activity was consistently weaker than the ssDNase activity for both orthologs.

RNA activation improves the detection specificity of Cas12a

Following our observation of the intrinsic RNA-targeting ability of Cas12a, we investigated whether there might be a compromise in target recognition specificity when switching the target from DNA to RNA. To this end, we designed a series of 20-nt targets containing continuous two-base mismatches, where the mismatches were the complementary bases to the fully matched ones (Figure 2a). In our experiment, we determined the specificity of target recognition by calculating the relative fluorescence enhancement rate between a mismatched target and a fully matched target. A lower relative fluorescence enhancement rate indicates better specificity because the mismatched target is more likely to be distinguished. The concentration of ssDNA or ssRNA targets was maintained at 30 nM for all enzymes tested. From the results shown in Figure 2b-e, mismatched ssRNA targets generally had a weaker fluorescence response compared to mismatched ssDNA targets with all four Cas12a enzymes tested. Specifically, only one group of mismatched ssRNA targets (mismatches #7–8) exhibited similar or higher relative fluorescence enhancement rates compared to their ssDNA counterparts (Figure 2b, c, and d). Additionally, for the ssDNA targets, there were several cases in which the fluorescence response for a mismatched target was even stronger than that for the fully matched one, indicating a failure to identify a mismatched ssDNA target. However, the fluorescence responses for the fully matched ssRNA target were consistently higher than those for the mismatched ssRNA targets with all enzymes tested, indicating that the mutated RNA targets could always be distinguished. Specificity tests on single-base mismatches yielded the same conclusions (Figure S11). Therefore, our experimental results suggest that RNA activation improves the specificity of *trans*-cleavage with both Cas12a orthologs, rendering it a more ideal option for nucleic acid detection, especially when a higher level of specificity is required.

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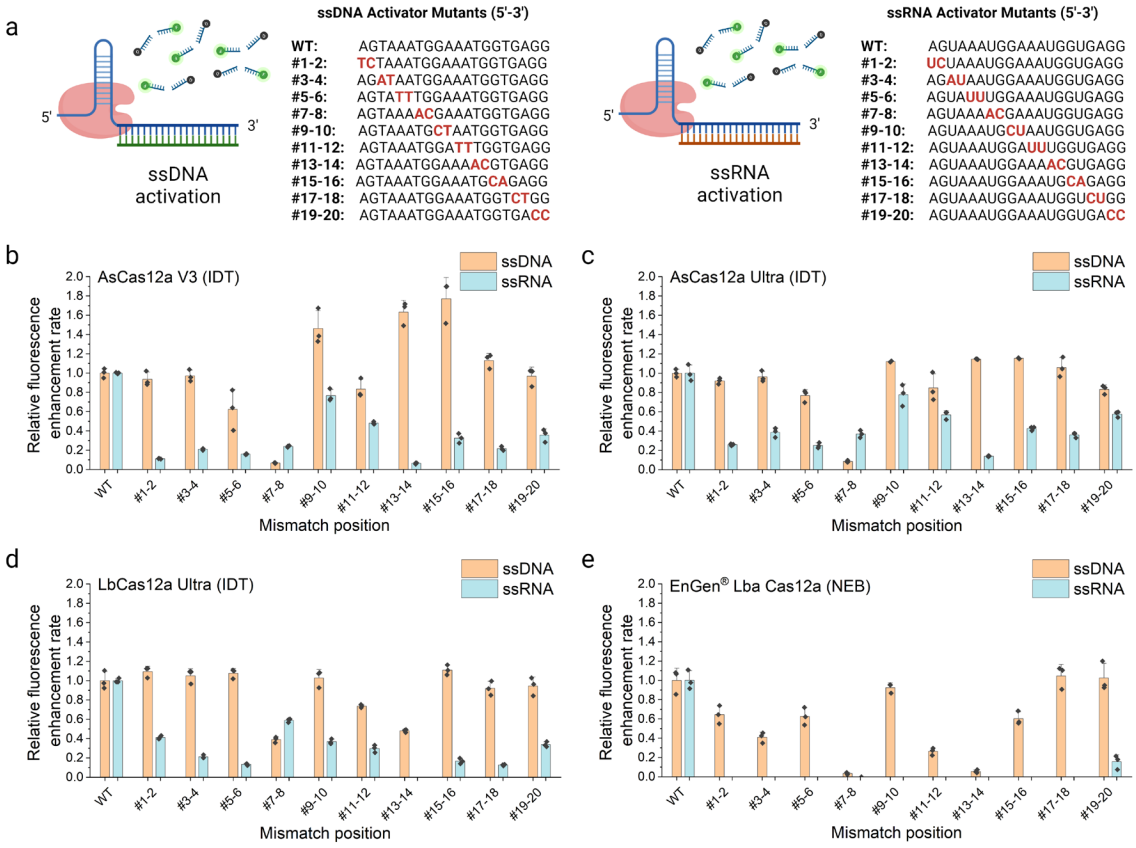


Figure 2. Specificity comparison of *trans*-cleavage activity with Cas12a by ssDNA and ssRNA targets with double-base mutations. a) Schematic illustration of ssDNA- and ssRNA-activated *trans*-cleavage activity of Cas12a and the mutated target sequences used to investigate the specificity of *trans*-cleavage activity with ssDNA and ssRNA targets. Wild-type (WT) sequences refer to target sequence fully complementary to the spacer of crRNA. Mutation positions are marked in red. b-e) Measurements were carried out with b) AsCas12a V3 (IDT), c) AsCas12a Ultra (IDT), d) LbCas12a Ultra (IDT), and e) EnGen® Lba Cas12a (NEB) by calculating the relative fluorescence enhancement rate to that for the fully matched target. The points represent individual measurements, and the error bars represent means ± standard deviation (s.d.) from three technical replicates (n = 3). The experimental groups with negative fluorescence enhancement rates, indicating no reactivity to a mismatched target, were left blank in the figure.

Highly sensitive PAM-free nucleic acid detection using UNIVERSE

Intrigued by the *trans*-cleavage activity of Cas12a triggered by RNA targets, we aimed to develop a universal nucleic acid detection method by combining T7 transcription of DNA amplicons with

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the RNA-activated *trans*-cleavage feature possessed by Cas12a. Insights into the nuclease activity of Cas12a revealed that PAM, while required for strand separation in double-stranded targets, is not necessary for successful recognition of single-stranded targets[3, 7]. Considering that transcripts are released from the enzyme at the termination site of the DNA template[16], the product of T7 transcription is expected to be ssRNA. ssRNA can be recognized without PAM restriction, thus allowing us to establish a PAM-free CRISPR-based detection scheme. In the meantime, T7 transcription unwinds the dsDNA amplicon and produces displaced ssDNA, which also contains the target sequence and contributes to the *trans*-cleavage signal (Figure S12). Similar to the pre-amplification step in SHERLOCK[17] which used LwaCas13a and AsCas12a, we first amplified nucleic acid template through an RPA reaction, in which the forward primer was tagged with a T7 promoter and the amplicons were subjected to T7 transcription for production of RNA targets. Because the 20-nt target in the RPA amplicon lacked the PAM sequence, LbCas12a and AsCas12a were not able to efficiently degrade the dsDNA amplicons as the template for RNA synthesis[6c, 18], therefore enabling T7 transcription and CRISPR-based detection to proceed in one pot. We termed this reaction scheme "Universal Nuclease for Identification of Virus Empowered by RNA-Sensing" (UNIVERSE). Presumably, without T7 transcription, the PAM-less dsDNA target may not induce significant fluorescence enhancement from the *trans*-cleavage activity of the used Cas12a orthologs. On the contrary, the fluorescence response is expected to dramatically improve with the incorporation of a T7 transcription step (Figure 3a). We performed denaturing gel electrophoresis to verify the production of transcripts by T7 transcription. Our results showed that RPA amplicons, in the presence of T7 transcription, were almost completely transcribed to RNA by T7 RNA Polymerase (T7 RNAP), although this process generated numerous by-products, which could potentially be transcripts from non-specific RPA amplicons (Figure 3b).

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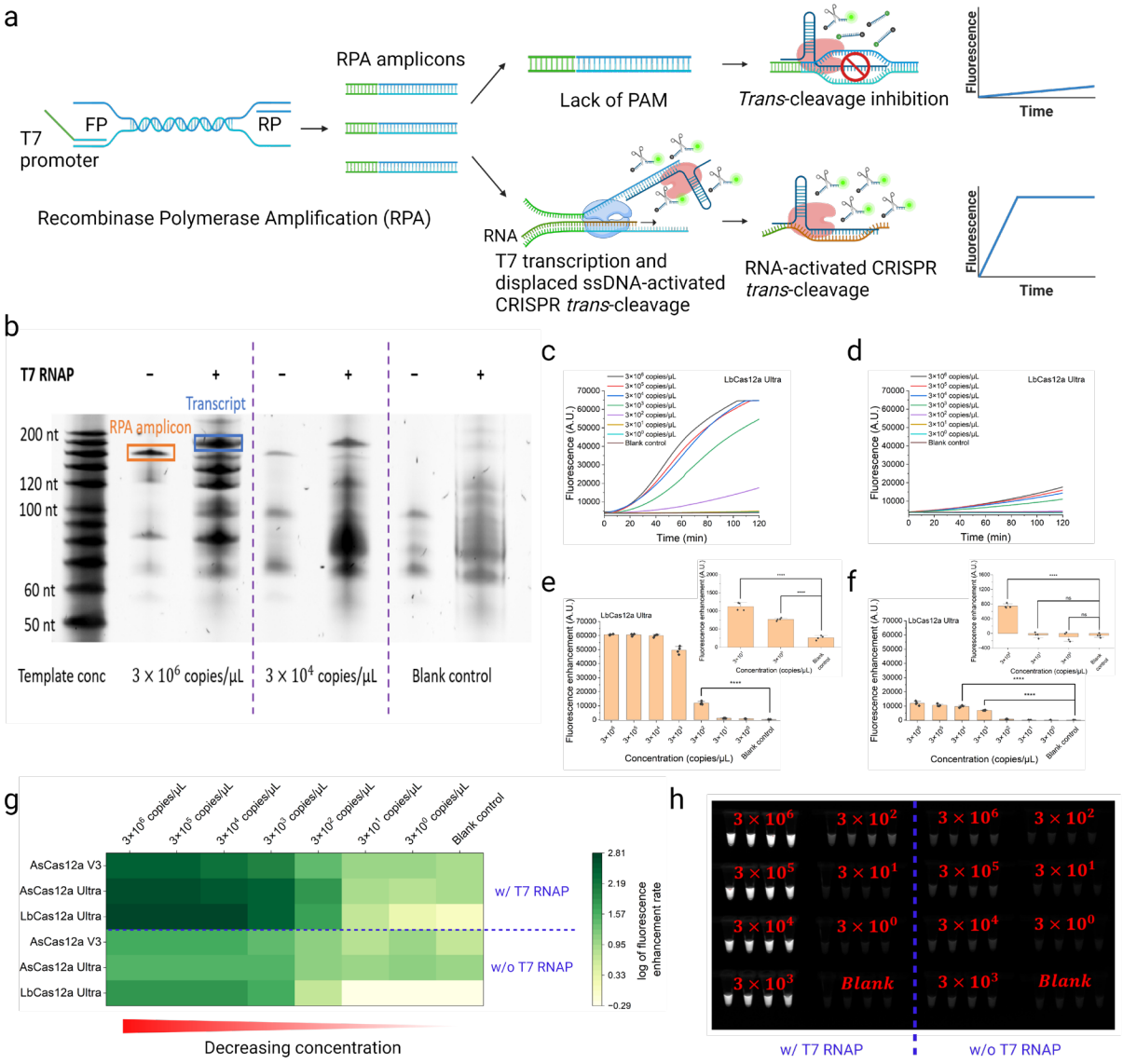


Figure 3. The UNIVERSE assay for highly sensitive PAM-free nucleic acid detection. a) Schematic illustration of the UNIVERSE assay for detection of a sequence lacking PAM. In the RPA step, T7 promoter was brought into the amplicon by prefixing it to the forward primer. T7 RNAP recognizes the T7 promoter in RPA amplicons which includes a 20-nt target sequence complementary to the spacer of crRNA, and starts synthesizing RNA strands. During T7 transcription, the amplicons are unwound, allowing the displaced ssDNA containing the target sequence to initiate the *trans*-cleavage activity of Cas12a. Meanwhile, the synthesized RNA strands can be released in single-stranded form and continue to trigger the *trans*-cleavage. If directly using the RPA amplicon (dsDNA target) in the conventional CRISPR reaction, the fluorescence signal is much weaker than that of T7-transcribed amplicons (RNA target) since the target lacks PAM[3], which will be shown in Figure 3c-f. b) Denaturing PAGE gel electrophoresis for identification of the RPA

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amplicons and the T7-transcribed amplicons. The template is HIV p24 plasmid at two different concentrations. The positions of the full-length RPA amplicon and transcription product, which are expected to be 161 nt, are determined by the position of the ssDNA ladder and marked in colored boxes. For uncropped gel scan see Figure S24 in Supporting Information. c-d) Real-time fluorescence kinetics of PAM-free detection with c) the UNIVERSE assay and d) the conventional RPA/CRISPR-Cas12a assay. e-f) Endpoint fluorescence enhancement at 120 min for PAM-free detection with e) the UNIVERSE assay and f) the conventional RPA/CRISPR-Cas12a assay. The detection was performed on 10-fold serially diluted HIV p24 plasmid starting at 3×10^6 copies/μL. The inset figures are zoomed-in views when template concentrations were low. The points represent individual measurements, and the error bars represent means ± standard deviation (s.d.) from four technical replicates ($n = 4$). The unpaired two-sample t-test was used to analyze the statistical significance. ****: $p < 0.0001$. ns: not significant. g) Comparison of fluorescence enhancement rates in PAM-free detection of HIV p24 plasmid using the UNIVERSE assay with T7 RNAP and the conventional RPA/CRISPR-Cas12a assay without T7 RNAP. To facilitate the comparison, the intensity of the *trans*-cleavage activity was represented by the logarithm of the average fluorescence enhancement rate from four technical replicates ($n = 4$) to base 10. h) Fluorescence image of reactions of the UNIVERSE assay and conventional RPA/CRISPR-Cas12a assay using LbCas12a Ultra (IDT). The image was captured after a 120-min reaction detecting the PAM-free sequence. The concentrations of 10-fold serially diluted HIV p24 plasmid template are marked in red with a unit of "copies/μL". The experiment was run in quadruplicate ($n = 4$) for each concentration.

Next, we adapted UNIVERSE to carry out a *trans*-cleavage assay by monitoring the real-time fluorescence generated from degradation of ssDNA-FQ. We examined the performance of the UNIVERSE assay with a plasmid containing the p24 gene of the HIV genome. The CRISPR target was a 20-nt segment within the RPA amplicon, where no PAM sequence was found upstream or downstream. Because EnGen® Lba Cas12a exhibited relatively weaker *trans*-cleavage activity when activated by ssRNA, it was not considered a candidate enzyme to develop our UNIVERSE assay. For the other three enzymes tested, all assays showed near-saturated fluorescence signal within 120 min for a template concentration greater than 3×10^3 copies/μL (Figure 3c, Figure S13). However, for the conventional RPA/CRISPR-Cas12a assay detecting the same PAM-less target without T7 RNAP, the fluorescence signals remained weak even if high template concentrations were applied (Figure 3d, Figure S14), which may be attributed to the lack of PAM in the CRISPR target sequence. In the UNIVERSE assay, we consistently observed background signals from blank

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controls with all enzymes tested (Figure 3e, Figure S13c and d), which may be a result of non-specific amplification of RPA[19] (Figure S15). Among the three Cas12a enzymes used in the tests, LbCas12a Ultra, which showed intermediate RNA-initiating *trans*-cleavage activity in the basic CRISPR assay, exhibited the strongest signal in the UNIVERSE assay compared with the other two AsCas12a enzymes, which may be attributed to the contribution to signal by displaced ssDNA.

Finally, we evaluated and compared the PAM-free detection sensitivity of the UNIVERSE assay and the conventional RPA/CRISPR-Cas12a assay. For all three enzymes tested with the UNIVERSE assay, a statistically significant fluorescence enhancement between plasmid samples and blank control was observed at a template concentration of 30 copies/μL with AsCas12a V3 and AsCas12a Ultra (Figure S13). The UNIVERSE assay achieved a sensitivity as low as 3 copies/μL with LbCas12a Ultra (Figure 3e). However, with the conventional RPA/CRISPR-Cas12a assay, the sensitivity deteriorated to 300 copies/μL for all enzymes tested (Figure 3f, Figure S14). To facilitate further comparisons, we calculated the base 10 logarithm of the average fluorescence enhancement rates within the first 60 min of the reaction (Figure 3g). Consistent with the above conclusion, the addition of T7 RNAP significantly accelerated the fluorescence enhancement in the RPA/CRISPR-Cas12a assay. More importantly, the UNIVERSE assay generated much stronger endpoint fluorescence signals compared to the conventional RPA/CRISPR-Cas12a assay, enabling direct visual detection (Figure 3h). It is noteworthy that detection of a PAM target was more efficient with the conventional RPA/CRISPR-Cas12a assay when the template concentration was high. However, at low template concentrations typically encountered in real-world viral testing, the UNIVERSE assay detecting a PAM-less target exhibited similar efficiency (Figure S16). Overall, these results highlighted the superiority of the UNIVERSE assay in nucleic acid detection in terms of magnified signal intensity and improved detection sensitivity.

UNIVERSE enables unrestricted selection of target sequences

Cas12a-based nucleic acid detection has been inherently limited by the requirement of PAM or suboptimal PAM sequences. However, since the activator in the UNIVERSE assay is expected to be ssRNA[16] and displaced ssDNA, which does not necessitate a PAM sequence to initiate the *trans*-cleavage response of Cas12a, theoretically, any sequence from the amplicon can be viable in the UNIVERSE assay. Hence, we hypothesized that the UNIVERSE assay could eliminate the

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need for PAM or suboptimal PAM sequences, allowing unrestricted selection of target sequence in an amplicon. To test this hypothesis, we arbitrarily chose six 20-nt CRISPR target sequences from the amplicon of the HIV detection mentioned above. These targets were screened to exclude canonical and suboptimal PAM sequences. The positions of the targets are labeled in Figure 4a. Using nucleic acids extracted from the HIV RNA-positive plasma control, we performed both the conventional RT-RPA/CRISPR-Cas12a and the UNIVERSE assay. In the conventional RT-RPA/CRISPR-Cas12a assay, all six targets generated negligible or very weak fluorescence signal over a 3-h incubation. However, the fluorescence signal was remarkably enhanced in the UNIVERSE assay, with a 17.7-fold to 153.4-fold change in fluorescence enhancement compared to the conventional RT-RPA/CRISPR-Cas12a assay (Figure 4b-g).

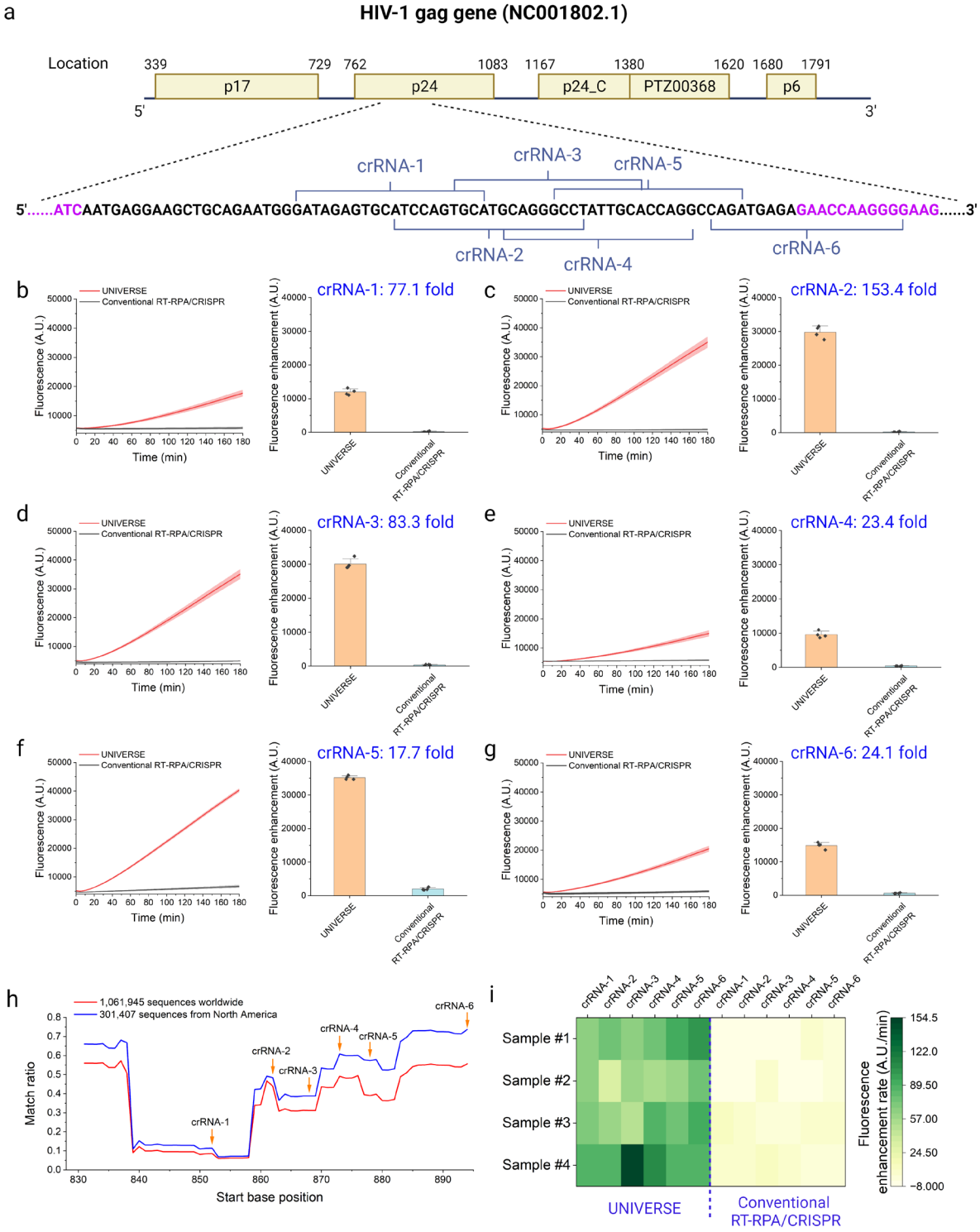
Next, we investigated whether these target sequences are more homologous by comparing the selected sequence to 1,061,945 HIV sequencing records worldwide from the NCBI Virus Database[20] (Figure S17). Considering that the RPA reaction remains effective in the presence of a single-base mutation, insertion, or deletion not located close to the 3' ends of the primers[21], we obtained 43,320 valid amplicons from the sequencing records using the primer pair employed in this study. Within these amplicon sequences, we then searched for every 20-nt segment within the amplicon sequences and counted the records with a perfect match to the sequence of the selected CRISPR target. The homology was represented by a match ratio, indicating the proportion of matching records to the total number of valid amplicons. Given that our HIV clinical samples were collected in North America, and considering the possibility of geographical specificity for viral mutations[22], a screening of records in North America was also conducted, with 301,407 samples. We observed that most of the selected targets were in regions with higher homology (Figure 4h), which established the basis of using the corresponding crRNAs for clinical detection. We then performed detection on clinical HIV-positive samples, validating the clinical effectiveness of the UNIVERSE assay through unrestricted selection of CRISPR targets. Similar to the detection of the HIV-positive control, there was a significant improvement in fluorescence response compared to the conventional RT-RPA/CRISPR-Cas12a assay (Figure 4i). Thus, the UNIVERSE assay not only broadens the range for target sequence selection, but also increases the fluorescence detection signals. This enhancement enables more sensitive, robust, and reliable nucleic acid detection.

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346

347 Figure 4. UNIVERSE enables detection of undetectable sequences under the conventional RPA/CRISPR-
348 Cas12a assay. a) Locations of the crRNA targeting sites in the amplicon of the HIV RNA used for the
349 detection. The purple bases refer to a segment of targeting sites for RPA primers. Six crRNA targeting sites

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were selected in the black amplicon sequence and marked with their indexes. b-g) Real-time fluorescence detection with UNIVERSE and conventional RT-RPA/CRISPR-Cas12a using the six selected crRNAs and the fold changes of fluorescence enhancement between these two assays with 3 h of reaction. The figures indicate the results for b) crRNA-1; c) crRNA-2; d) crRNA-3; e) crRNA-4; f) crRNA-5; and g) crRNA-6. The graphs of real-time fluorescence represent means (bold line) ± standard deviation (s.d.). The points in bar charts represent individual measurements and the error bars represent means ± standard deviation (s.d.) from four technical replicates (n = 4). The fluorescence fold changes are marked in each subfigure. h) The homology for every CRISPR target in the amplicon with the indexes of selected targets marked in the figure. The homology was measured by calculating the proportion of 20-nt CRISPR targets completely matching the reference (NC_001802.1), observed in valid RPA amplicons from sequencing records of HIV from the NCBI Virus Database. i) Comparison of detecting clinical HIV-positive samples with the UNIVERSE and the conventional RT-RPA/CRISPR-Cas12a assays using the six selected crRNAs.

Viral nucleic acid detection in clinical samples using UNIVERSE

Before testing HIV clinical samples, we assessed the detection sensitivity using an HIV-positive control in human plasma. To enable accurate sensitivity evaluation, we first quantified the HIV RNA copy numbers in the HIV-positive control samples using digital RT-PCR (Figure S18 and S19). In addition, we employed real-time RT-qPCR, the gold standard for HIV detection, to detect HIV in serially diluted HIV-positive control samples (Figure 5a). Then, we applied UNIVERSE to detect HIV RNA in these serially diluted HIV-positive samples, achieving the same sensitivity (2 copies/μL ≈ 3.3 aM) as the RT-qPCR assay (Figure 5b and c). While achieving a similar sensitivity at the attomolar level, UNIVERSE integrates the strengths of the previously reported DETECTR[3] and SHERLOCK[17] assays by enabling PAM-free detection with a more prevalent Cas12a system (Table S1).

Next, we explored the potential utility of the UNIVERSE assay in detecting viral nucleic acids extracted from clinical samples. We tested ten clinical plasma samples, including four HIV-positive and six HIV-negative samples confirmed by standard RT-qPCR (Figure 5d). With the UNIVERSE assay, we could detect and differentiate all HIV-positive samples from negative samples, obtaining consistent results with the RT-qPCR assay (Figure 5e). All the positive samples showed significantly higher fluorescence enhancement compared to that of the negative samples.

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As expected, the endpoint fluorescence results of clinical HIV detection could be easily visualized using a fluorescence imager, allowing clear differentiation between positive and negative results (Figure 5f). To demonstrate the versatility, we further validated UNIVERSE in detecting DNA viruses such as HPV 16 from clinical cervical swab samples. We tested ten clinical cervical swab samples, including five HPV 16-positive samples and five HPV 16-negative samples determined by RT-qPCR (Figure 5g). After 2 h of incubation, the UNIVERSE assay generated consistent and clearly distinguishable positive and negative results (Figure 5h), which could be visually recognized under a fluorescence imager (Figure 5i).

From the real-time fluorescence kinetics, a clear differentiation between positive and negative results was achieved at around 75 min for the detection of clinical HIV samples, and as early as 30 min for the detection of clinical HPV 16 samples (Figure S20). Although the difference in Cq values was trivial, the positive fluorescence response with the DNA virus was apparently stronger than that observed with the RNA virus. We speculated that this phenomenon might be related to a variety of factors within the reaction, such as compromised reactivity of T7 polymerase or Cas12a enzyme resulting from less compatible buffering conditions introduced by solvents in reverse transcriptase or RNase H, inconsistent efficiency of RPA amplification, or differences in *trans*-cleavage activity associated with the sequences of crRNAs and their targeting sites. Overall, these clinical results demonstrate that the UNIVERSE assay provides a simple, universal, PAM-free CRISPR-based method for nucleic acid detection, expanding the toolbox for CRISPR-based molecular diagnostics.

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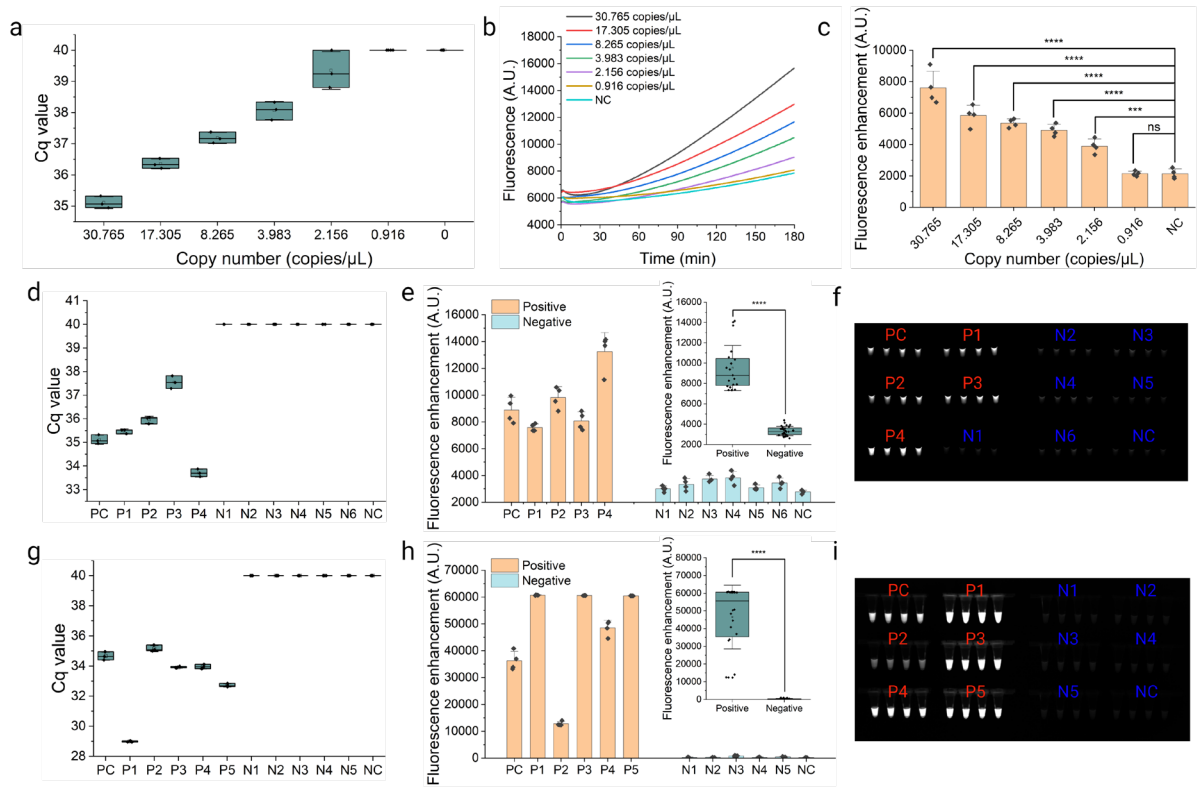


Figure 5. Clinical validation of the UNIVERSE assay for HIV and HPV 16 detection. a) Cq values for detection of HIV RNA-positive control with the RT-qPCR assay. b) Real-time fluorescence kinetics for detection of HIV RNA-positive control with the UNIVERSE assay. c) Fluorescence enhancement at the endpoint of detection of HIV RNA-positive control with the UNIVERSE assay. Copy numbers in a-c) were determined by digital RT-PCR. d) Cq values for detection of clinical HIV-positive and HIV-negative samples with the RT-qPCR assay. e) Fluorescence enhancement for HIV clinical sample detection with the UNIVERSE assay after 180 min of incubation. The inset compares fluorescence enhancement of all positive samples to all negative samples. f) Fluorescence image of UNIVERSE reactions for clinical HIV sample detection. The image was captured after 180 min of reaction. The sample names are marked above each set of tubes. For d-f), the positive control (PC) was undiluted HIV RNA-positive control, and the negative control (NC) was extractions from healthy human plasma. g) Cq values for detection of clinical HPV 16 positive and negative samples with the qPCR assay. h) Fluorescence enhancement for HPV 16 clinical sample detection with the UNIVERSE assay after 120 min of incubation. The inset compares fluorescence enhancement of all positive samples to all negative samples. i) Fluorescence image of UNIVERSE reactions for clinical HPV 16 sample detection. The image was captured after 120 min of reaction. The sample names are marked above each set of tubes. For g-i), the PC was 1 fM of plasmid containing HPV 16 L1 genome fragment, and the NC was AE buffer from the DNeasy Blood and Tissue Kit used for HPV 16 DNA

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extraction. For a), d) and g), the center line represents the median value, the bounds of the box indicate the interquartile range, and the whiskers indicate means ± standard deviation (s.d.) of three technical replicates (n = 3). For samples not detectable within 40 cycles, the Cq values were designated as 40. For c), the points represent individual measurements, and the error bars represent means ± standard deviation (s.d.) from four technical replicates (n = 4). The unpaired two-sample t-test was used to analyze the statistical significance. ***: $p < 0.001$. ****: $p < 0.0001$. ns: not significant. For e) and h), the points represent individual measurements and the error bars represent means ± standard deviation (s.d.) from four technical replicates (n = 4) measuring each sample. In the inset of e) and h), the unpaired two-sample t-test was used to analyze the statistical significance. ****: $p < 0.0001$.

Conclusion

In this study, we found that the tested Cas12a orthologs could be directly activated by a fully complementary RNA target without the need for additional DNA activators. Interestingly, we observed noticeable differences in the *cis*-activity, wherein the DNA target was prone to degradation while the RNA target exhibited much higher resistance. In addition, the specificity towards RNA targets was greatly improved compared to DNA targets. These features inspired us to develop the UNIVERSE assay for universal nucleic acid detection with improved sensitivity and specificity, simply by incorporating T7 transcription to convert dsDNA amplicons to ssRNA. This unique detection modality eliminates the need to search for a canonical PAM or a suboptimal PAM sequence, or to resort to the complicated experimental design of artificially introducing a PAM. With the UNIVERSE assay, we successfully detected multiple PAM-less sequences in HIV clinical samples that were undetectable by the conventional RPA/CRISPR-Cas12a assay. Although targets with a PAM may exhibit accelerated reaction kinetics and should warrant priority in target selection, in some cases the PAM-flanked target might not be present within highly conserved regions, potentially compromising reliability in clinical detection.

We validated the clinical feasibility of the UNIVERSE assay by detecting HIV in clinical plasma samples and HPV 16 in clinical cervical swab samples, achieving a consistent performance with that of the RT-PCR/PCR method. Given that the UNIVERSE assay can be conducted at steady temperatures, it is superior to the RT-PCR/PCR method in terms of potential applicability in resource-limited settings where a thermocycler is unavailable. Compared to the Cas13a system, a

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prevalent technique in nucleic acid detection that utilizes a similar *trans*-cleavage mechanism upon RNA targeting, the UNIVERSE assay with Cas12a can be accomplished with shorter RNA guides[23] and more durable ssDNA probes. As the synthesis of RNA oligos is expensive, these properties will also contribute to reducing the overall cost of the assay. We envision that the integration of microfluidics technology will further advance UNIVERSE toward a valid point-of-care testing tool[5b, 5d, 24].

Although we have demonstrated the RNA-activated cleavage reactivity of Cas12a through substantial experimental observations, a more detailed study, particularly from a microscopic perspective, is needed to unravel the in-depth mechanisms, such as the allosteric regulation of Cas12a induced by RNA targets[6a, 25]. Moreover, the Cas12a enzymes used in this study may have experienced complex protein engineering to enhance their functionality. Therefore, the protein structure may exhibit critical differences from already structurally characterized Cas12a orthologs, resulting in exceptional characteristics in terms of RNA activation capability. In the future, additional studies focusing on the cleavage mechanism and conformational activation pathway of RNA-activated Cas12a could unveil new opportunities for developing engineered Cas12a enzymes for RNA detection with an improved performance.

Overall, we anticipate that the study of RNA-activated *trans*-cleavage with Cas12a and the UNIVERSE assay will have broader applications in molecular diagnostics, potentially leading to a further shift in the understanding of CRISPR-Cas biochemistry.

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Keywords: CRISPR-Cas12a • RNA-activation • *trans*-cleavage • nucleic acid detection • protospacer adjacent motif (PAM)-free

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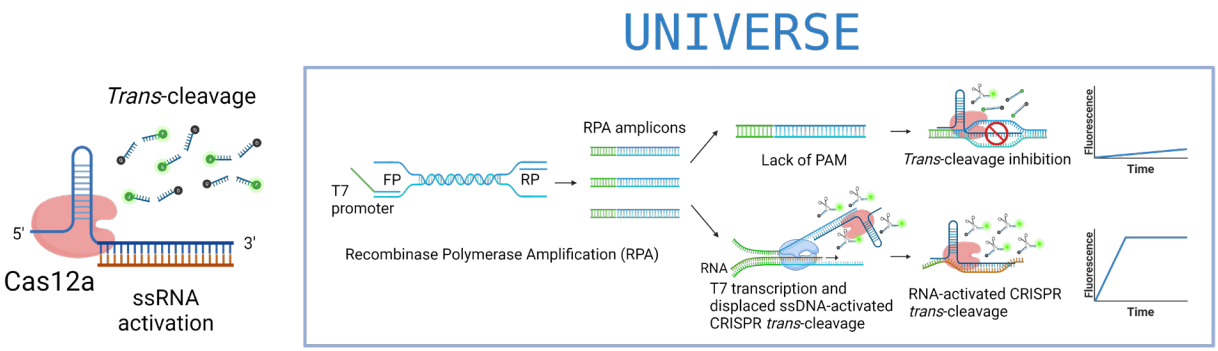
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We investigated the intrinsic RNA-activated cleavage activity of Cas12a and developed the "Universal Nuclease for Identification of Virus Empowered by RNA-Sensing" (UNIVERSE) assay for protospacer adjacent motif (PAM)-free nucleic acid testing with improved specificity. We validated its clinical utility by detecting both HIV RNA and HPV 16 DNA in clinical samples, achieving comparable performance with the PCR/RT-PCR method.

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