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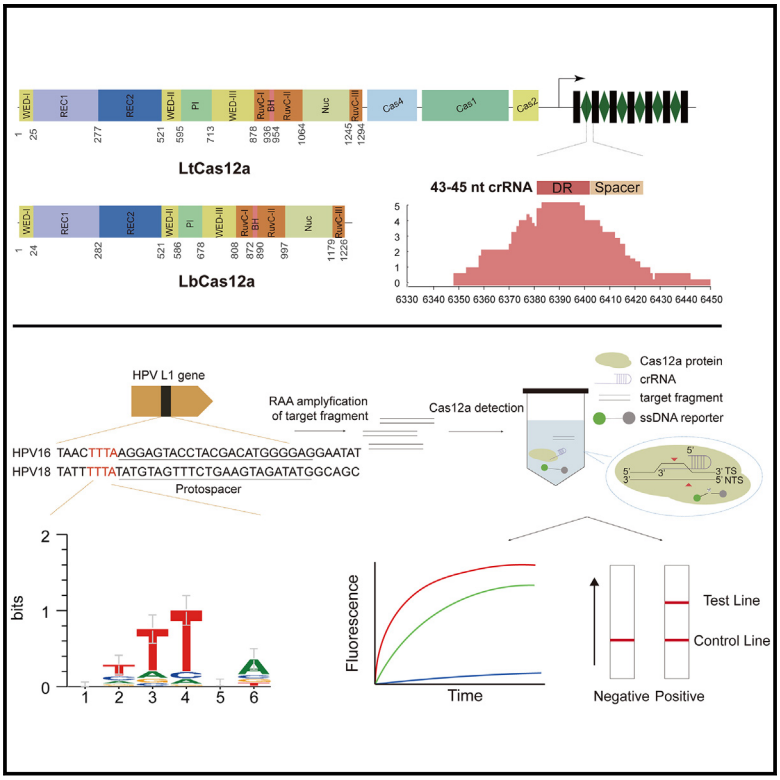
A Cas12a ortholog with distinct TTNA PAM enables sensitive detection of HPV16/18

Graphical abstract



Highlights

- Identification of a Cas12a ortholog—LtCas12a—with a distinct TTNA PAM
- Equivalent editing efficiency and fidelity to LbCas12a in mammalian cells
- LtCas12a enables HPV detection with high specificity and comparable sensitivity to qPCR
- Potential to broaden clinical diagnostic applications of Cas12a enzymes

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In brief

Chen et al. identify and characterize a Cas12a ortholog that recognizes a distinct TTNA PAM. They utilize LtCas12a for HPV gene detection, demonstrating high sensitivity and specificity in detecting the HPV16/18 L1 gene.

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A Cas12a ortholog with distinct TTNA PAM enables sensitive detection of HPV16/18

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MOTIVATION CRISPR-Cas9 with NGG PAM has been well known and widely applied in the field of gene editing. In addition, the Cas12a family represented by LbCas12a and AsCas12a has also been intensively investigated due to its unique characteristics including requiring only a single crRNA guide RNA and the high fidelity of its editing performance. However, AsCas12a and LbCas12a exert cleavage activities by recognition of TTTV PAM, which restricts the targeting scope. Here, we utilized human gut samples to identify a LtCas12a that utilizes distinct TTNA PAM with equivalent cleavage ability and specificity, thereby providing the potential for development of new therapeutics and diagnostics.

SUMMARY

CRISPR-associated (Cas) nucleases are multifunctional tools for gene editing. Cas12a possesses several advantages, including the requirement of a single guide RNA and high fidelity of gene editing. Here, we tested three Cas12a orthologs from human gut samples and identified a LtCas12a that utilizes a TTNA protospacer adjacent motif (PAM) distinct from the canonical TTTV PAM but with equivalent cleavage ability and specificity. These features significantly broadened the targeting scope of Cas12a family. Furthermore, we developed a sensitive, accurate, and rapid human papillomavirus (HPV) 16/18 gene detection platform based on LtCas12a DNA endonuclease-targeted CRISPR *trans* reporter (DETECTR) and lateral flow assay (LFA). LtCas12a showed comparable sensitivity to quantitative polymerase chain reaction (qPCR) and no cross-reaction with 13 other high-risk HPV genotypes in detecting the HPV16/18 L1 gene. Taken together, LtCas12a can broaden the applications of the CRISPR-Cas12a family and serve as a promising next-generation tool for therapeutic application and molecular diagnosis.

INTRODUCTION

Bacteria and archaea utilize CRISPR-Cas (clustered regularly interspaced short palindromic repeats and CRISPR-associated nuclease) as a natural defense system for resisting invading bacteriophages and plasmids.[1] The acquisition of exogenous genome fragments, creating a memory bank of infection, helps to defend against repeated viral attacks.[2] Particularly, class 2 CRISPR-Cas systems are suitable for the genome-manipulation technologies for their single effector enzymes.[3] RNA-guided Cas9, which comes from type II CRISPR systems, has extensive applications in biological research, therapeutic development, and diagnostic tools for correcting genetic defects in various organisms.[4–9]

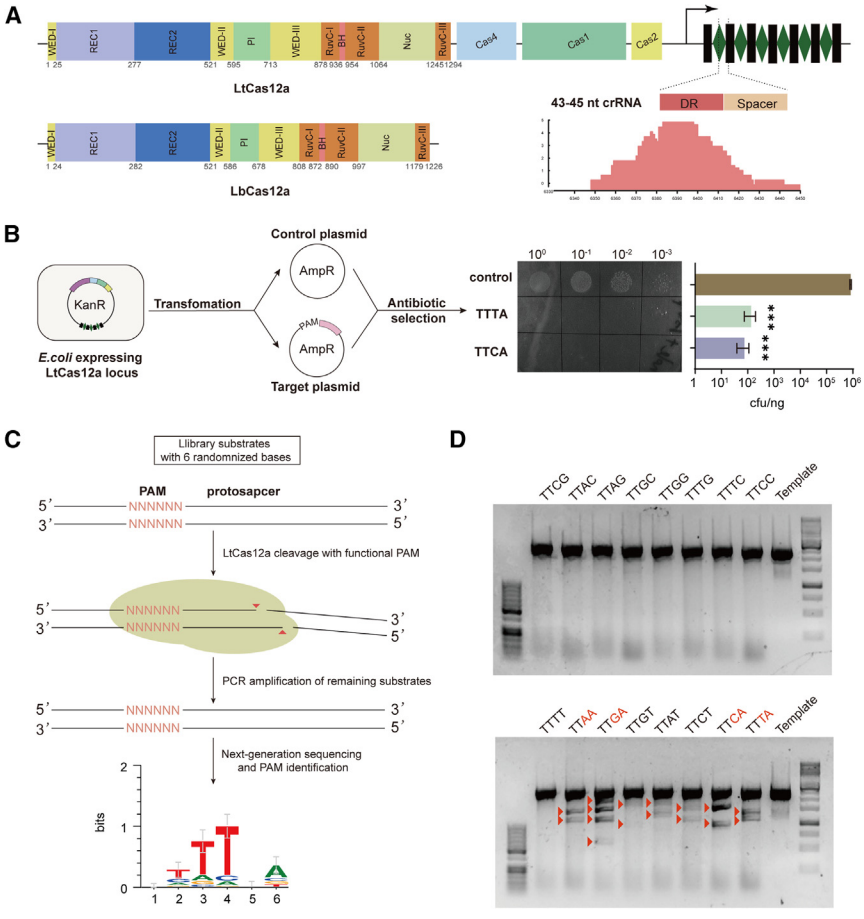

Figure 1. LtCas12a was a functional gene editing tool in prokaryotes and *in vitro*

(A) Organization of LtCas12a loci. LtCas12a protein, Cas4 (blue), Cas1 (light green), Cas2 (yellow), direct repeat (black), spacer (dark green), and small RNA-seq of *E. coli* were exhibited. Domain distributions of LtCas12a and LbCas12a were also shown.

(B) Schematic illustration of plasmid interference experiments. Pink rectangle represented protospacer. Data are shown as mean ± SEM (n = 3).

(C) Schematic illustration of *in vitro* cleavage assay for confirming PAM sequence and Weblogo for the LtCas12a PAM.

(D) DNA cleavage of LtCas12a with TTNN PAM *in vitro*.

In addition to Cas9, two orthologs of Cas12a from *Acidaminococcus sp* (AsCas12a) and *Lachnospiraceae bacterium* (LbCas12a) have been investigated and widely applied.[10–12] However, AsCas12a and LbCas12a utilize TTTV as a canonical protospacer adjacent motif (PAM),[10] which restricts the targeting scope. Therefore, additional CRISPR enzymes with a distinct PAM but equivalent cleavage ability and specificity would be desirable.

One important application of CRISPR-Cas12a systems is for pathogen nucleic acid detection. Combining rapid amplification with simple readout methods, including fluorescence assay and lateral flow assay (LFA), makes the Cas12a-based nucleic acid detection practical and user friendly.[13–15] However, the detection sensitivity in different pathogens varies greatly. Further improvement could be achieved by engineering original Cas12a enzymes or discovering novel CRISPR enzymes.

In this study, we discovered three Cas12a orthologs and characterized in depth LtCas12a (≤80% sequence similarity compared with LbCas12a and AsCas12a,[10] 1,296 amino acids, MW: 152.7 kDa) (Table S1). We demonstrate a promising application of LtCas12a for the detection of the human papillomavirus (HPV) 16/18 gene.

RESULTS

A Cas12a candidate recognizing distinct TTNA PAM *in vitro*

We used CRISPRCasFinder to search the Cas12a nucleases in collected human gut samples and identified three CRISPR-Cas12a loci that had ≤80% sequence similarity with reported Cas12a. Then, we synthesized and cloned them into *E. coli* expression plasmids (Figures 1A and S1). Since Cas12a had a conserved T-rich PAM,[16] we tested the cleavage activity of these 3 candidates by plasmid interference assay[17] with target plasmids containing canonical TTTA and non-canonical TTCA PAMs. We successfully screened out one functional Cas12a nuclease named LtCas12a (Figures 1B and S2A). Small RNA sequencing (see STAR Methods) showed that mature CRISPR RNA (crRNA) consisted of 22 nt direct repeats (DRs) and 21–23 nt spacers (Figure 1A). Based on small RNA sequencing (sRNA-seq) result, we tried different truncation combinations of DRs and spacers and found that LtCas12a required at least 22 nt DRs and 19 nt spacers to achieve a cleavage effect (Figure S2B). The cleavage activity of LtCas12a was further investigated with other possible non-canonical PAMs (Figure S3). According to interference assay analysis, several functional non-canonical PAMs have been identified, including TCTA, TTGC, TTAC, TTCC, TTGG, GTTG, TTAG, GTTC, TTCG, and GGTA.

Furthermore, we purified LtCas12a and explored the specific PAM requirements by *in vitro* depletion assay.[16] After incubation of the LtCas12a-crRNA complex and 740 bp library substrates (containing 6 randomized bases at the 5′ end of protospacer), the uncut substrates were extracted and prepared for deep sequencing (Figure 1C). The data showed that LtCas12a recognized the TTNA (N represented A, T, C, G) PAM sequence, which was slightly different from the TTTV PAM from LbCas12a, AsCas12a, and FnCas12a in the third and fourth positions (Figure 1C). Furthermore, we designed 16 crRNA pools (TTNN

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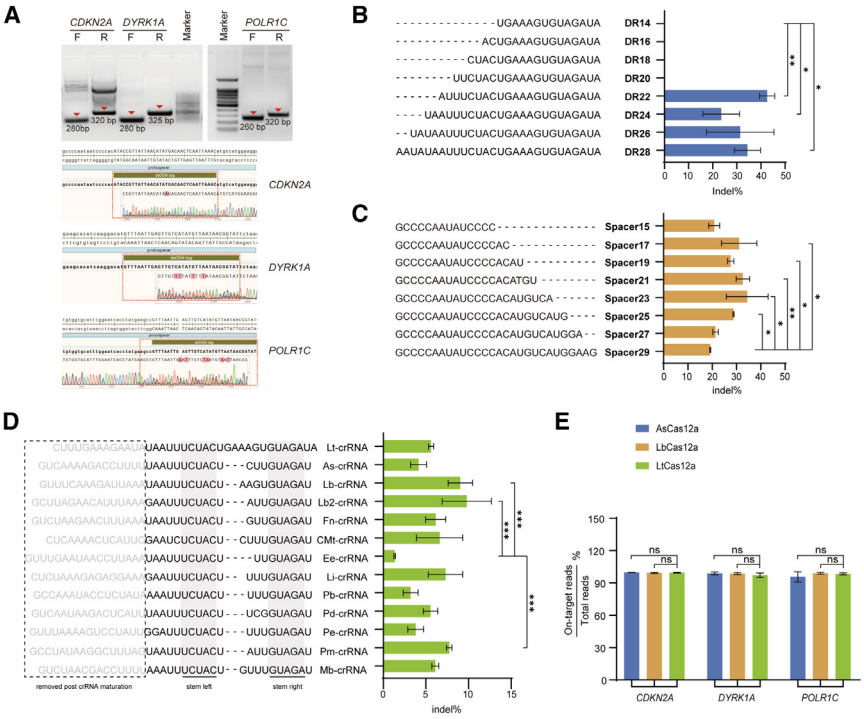

Figure 2. LtCas12a was active in mammalian cells

(A) dsODN breakpoint PCR and respective Sanger sequencing result of integration sites induced by LtCas12a targeting endogenous loci *CDKN2A*, *DYRK1A*, and *POLR1C*.

(B and C) Targeting loci indels were induced by LtCas12a with various lengths of direct repeats (DRs) (B) and spacers (C). Respective editing efficiency was assessed by deep sequencing. Data are shown as mean ± SEM (n = 3). Kruskal-Wallis and Bonferroni test. *p < 0.05, **p < 0.01, ***p < 0.001, ns, not significant.

(D) Targeting loci indels were induced by LtCas12a with 12 Cas12a orthologs crRNAs, bearing similar stem structures. Respective editing efficiency was assessed by TIDE assay. Data are shown as mean ± SEM (n = 3). Kruskal-Wallis and Bonferroni test. *p < 0.05, **p < 0.01, ***p < 0.001, ns, not significant.

(E) Parallel comparison of on-target efficiency of LtCas12a, LbCas12a, and AsCas12a in mammalian cells. On-target efficiency targeting *CDKN2A*, *DYRK1A*, and *POLR1C* were analyzed based on GUIDE-seq data. Data from biological replicates are shown as mean ± SEM (n = 3). One-way ANOVA and Bonferroni test, *p < 0.05, **p < 0.01, ***p < 0.001, ns, not significant.

PAM at the 5′ end of target sequence), and each pool consisted of 10 crRNAs targeting different human genomic loci (*CDKN2A*, *DYRK1A*, *CTLA4*, *RNF2*, *DNMT1*, *GRIN2B*, *FANCF*, *RUNX1*, *EMX1*, and *CCR5*, respectively). In TTNA pools, LtCas12a exhibited robust cleavage efficiencies, while in TTGT, TTAT, and TTCT pools, LtCas12a displayed modest cleavage efficiencies (Figure 1D). Therefore, LtCas12a specifically recognized the 5′-TTNA PAM sequence.

Then, to explore the kinetics of LtCas12a, LtCas12a-based cleavage assays were performed with varying substrate concentrations (Figure S4). The initial enzymatic reaction rate (V_0) was measured and displayed in the Michaelis-Menten curve (Figure S4A). To confirm the kinetic analyses, Hanes plots ([S]/V versus [S]) were also drawn and found to be linear (Figure S4B). Further, the reaction maximal velocity (V_{max}) and Michaelis constant (K_m) were calculated and are presented in Figure S4C (V_{max} = 96.88 RU s^{-1}, K_m = 15.6 nM).

Referring to previous reports,[16,18] it is established that AsCas12a and LbCas12a generally recognized canonical TTTV PAM and non-canonical C-containing PAM (CTTV, TCTV, TTCV, CCTV, TCCV, and CCCV). Alternatively, LtCas12a with TTNA PAM could target sites that were not editable by other CRISPR-Cas12a and therefore had significance for gene editing and therapy.

Gene editing with LtCas12a in mammalian cells

To verify whether LtCas12a could produce double-strand breaks (DSBs) in mammalian cells, we synthesized human codon-optimized LtCas12a and crRNA and then cloned them into mammalian expression vector PX330. We tested the cleavage activity of LtCas12a in three genomic loci, *CDKN2A*, *DYRK1A*, and *POLR1C*, in HEK293T cells[19] by electroporating double-stranded oligodeoxynucleotides (dsODNs) (Table S2; STAR Methods). dsODNs could be integrated into DSBs and detected by dsODN breakpoint PCR as well as Sanger sequencing. LtCas12a was observed to induce DSBs in all three sites and therefore could be harnessed for gene editing in mammalian cells (Figure 2A).

To optimize the crRNA structure of LtCas12a, we conducted truncation assays in *CDKN2A* loci using different lengths of DR sequences (14, 16, 18, 20, 22, 24, 26, and 28 nt). LtCas12a showed the highest cleavage efficiency with a 22 nt DR (mean = 42.56%) and became inactivate with DRs ≤ 20 nt (Figure 2B), which was consistent with prediction of LtCas12a crRNA secondary structure and our findings in prokaryotes (Figure S2B). Then, we explored the optimal spacer sequence length by combining the 22 nt DR with various guide lengths (15, 17, 19, 21, 23, 25, 27, and 29 nt). The insertion or deletion (indel) frequencies with 17–25 nt spacer lengths were significantly higher than those with wide-type guide sequence lengths (29 nt) (Figure 2C). Previous study has reported that exchange of crRNA from other Cas12a orthologs might enhance cleavage efficiency.[20] Then, we combined the LtCas12a enzyme with 12 different crRNAs that shared the same stem regions and detected their cleavage function in mammalian cells (As-5-UCUU-3, Lb-5-UAAGU-3, Lb2-5-UAUU-3, Fn-5-UGUU-3, Cmt-5-UCUUU-3, Ee-5-UUU-3, Li-5-UUUU-3, Pb-5-UUUU-3, Pd-5-UUCG-3, Pe-5-UUUU-3, Pm-5-UAUU-3, and Mb-5-UGUUU-3). Indel frequencies were assessed by tracking of indels by decomposition (TIDE) assay and indicated that different crRNAs had various performances on gene editing efficiency. LtCas12a with EeCas12a crRNA produced modest cleavage efficiency

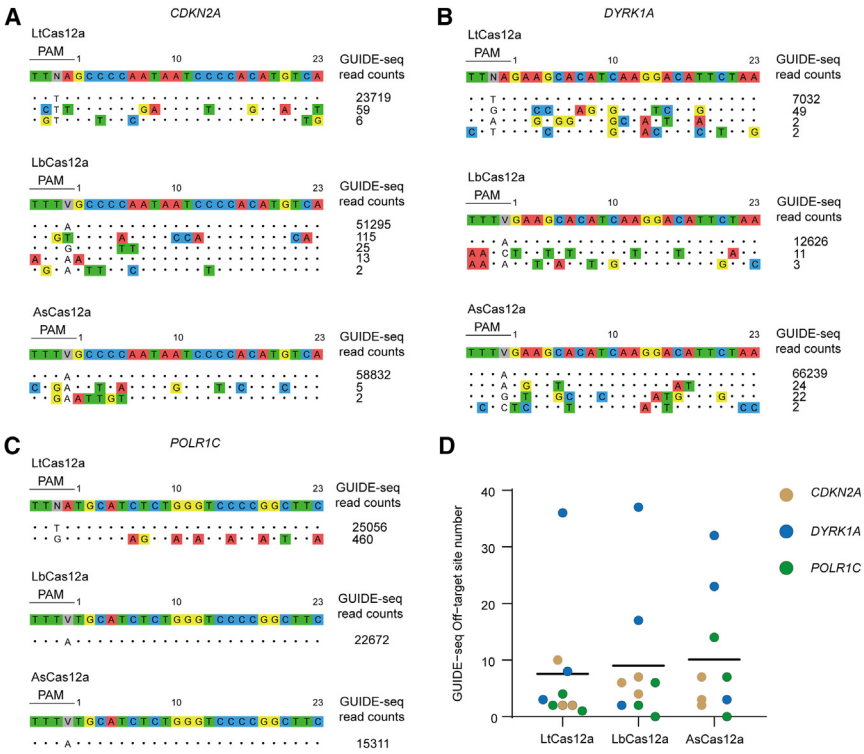

Figure 3. Identified LtCas12a specificity *in vitro* using GUIDE-seq

(A–C) On-target and off-target sites for LtCas12a, LbCas12a, and AsCas12a with respective crRNA targeting *CDKN2A* (A), *DYRK1A* (B), and *POLR1C* (C) loci investigated by GUIDE-seq *in vitro*. The first lane represented on-target site, while mismatch sites were marked in different colors. Respective on-target and off-target read counts were listed on the right.

(D) Scatter diagram illustrated counts of off-target sites for LtCas12a, LbCas12a, and AsCas12a by GUIDE-seq.

(mean = 1.33%), while LtCas12a with crRNA from LbCas12a (mean = 9.03%), Lb2Cas12a (mean = 9.80%), and PmCas12a (mean = 7.73%) produced significantly higher editing efficiency (Figure 2D).

To assess the gene editing ability of LtCas12a, we conducted parallel comparisons with LbCas12a and AsCas12a in 3 endogenous loci (*CDKN2A*, *DYRK1A*, and *POLR1C*), in HEK293T cells (Table S3). According to next-generation sequencing (NGS) data, in terms of on-target efficiency (on-target reads/total reads), LtCas12a (*CDKN2A* = 99.55%, *DYRK1A* = 97.25%, and *POLR1C* = 98.48%) had comparable cleavage performance with LbCas12a (*CDKN2A* = 99.36%, *DYRK1A* = 98.77%, and *POLR1C* = 99.02%) and AsCas12a (*CDKN2A* = 99.85%, *DYRK1A* = 98.99%, and *POLR1C* = 95.63%) (Figure 2E). Together, LtCas12a showed equivalent editing performance to LbCas12a and AsCas12a in mammalian cells.

Specificity investigation of LtCas12a using GUIDE-seq

Generally, Cas12a nucleases, such as LbCas12a and AsCas12a, have high editing specificity in genomic scale.[21,22] We evaluated and compared the specificity of LtCas12a with LbCas12a and AsCas12a *in vitro* by genome-wide, unbiased identification of DSBs enabled by sequencing (GUIDE-seq) assay in three different human genomic loci, *CDKN2A*, *DYRK1A*, and *POLR1C* (Figures 3A–3D).

As illustrated (Figures 3A–3C), the cleavage efficiency of LtCas12a varied greatly in different gene loci. For *CDKN2A* and *DYRK1A*, LbCas12a (on-target reads: *CDKN2A* = 51,295, *DYRK1A* = 12,626) and AsCas12a (on-target reads: *CDKN2A* = 58,832, *DYRK1A* = 66,239) had better performances than LtCas12a (on-target reads: *CDKN2A* = 23,719, *DYRK1A* = 7,032). In contrast, in terms of *POLR1C*, LtCas12a (on-target reads: *POLR1C* = 25,056) exhibited a superior performance compared with LbCas12a (on-target reads: *POLR1C* = 22,672) and AsCas12a (on-target reads: *POLR1C* = 15,311).

According to GUIDE-seq data, off-target activity has been fully assessed in three different human genomic loci (*CDKN2A*, *DYRK1A*, and *POLR1C*) (Figure 3). On average, as illustrated in Figure 3D, fewer off-target sites were observed in LtCas12a than LbCas12a and AsCas12a. Overall, LtCas12a exhibited comparable or even higher specificity than LbCas12a and AsCas12a in all these three loci.

Detection of HPV16/18 DNA with LtCas12a-based DETECTR

Cas12a has been reported to cleave ssDNA without discrimination once it recognizes the ssDNA/dsDNA substrates complementary to crRNA.[15,23] Based on this characteristic, development of CRISPR-Cas12a-based nucleic acid detection was applied in diverse pathogen diagnosis.[15,24–26] To confirm the collateral cleavage ability of LtCas12a, we chose M13 phage ssDNA as a non-specific cleavage substrate.[15] LtCas12a catalyzed M13 phage ssDNA breaks only in the presence of a complementary ssDNA activator and thus generated diffuse fragments according to the agarose gel electrophoresis (Figure 4A). These findings indicated that LtCas12a was capable of robust, non-specific ssDNA *trans*-cleavage.

Then, we explored whether LtCas12a could be programmed as a nucleic acid detection platform for various pathogens. Taking HPV as an example, there were over 528,000 new reported cervical cancer cases in 2020.[27] HPV16 and HPV18, as the principal risk factors for the tumorigenesis and development of cervical cancer, contribute to approximately 70% of cases of cervical cancer.[28,29] Therefore, developing a rapid and sensitive diagnosis system for HPV is in urgent need. We chose 5 target sequences located next to TTNA PAM for HPV16/HPV18 L1 gene, respectively (Table S2). Functional crRNA was selected by LtCas12a-based DNA endonuclease-targeted CRISPR *trans*

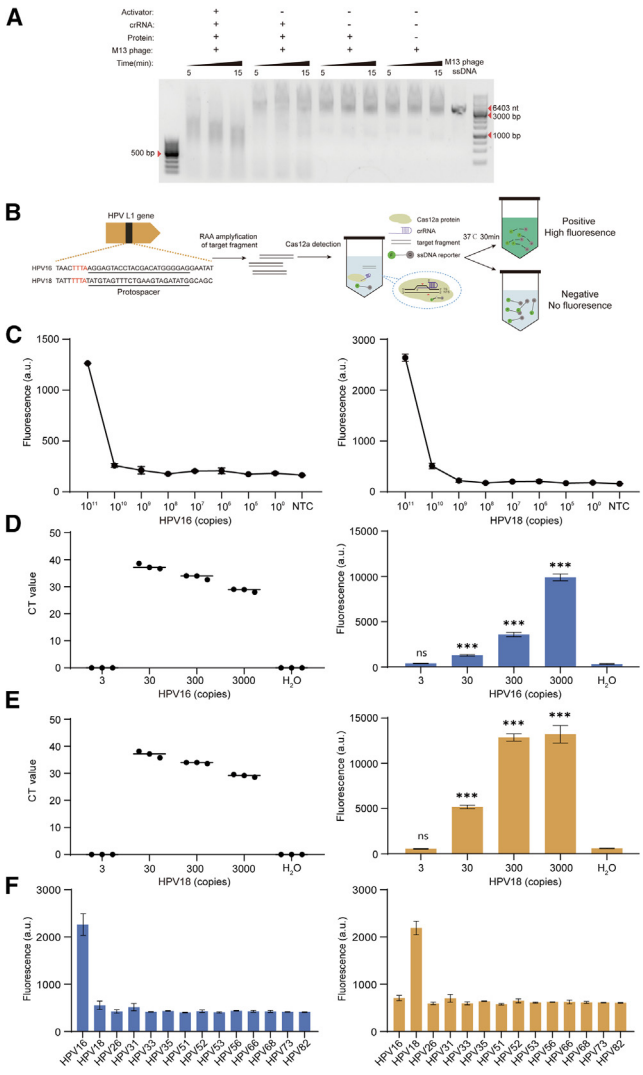

Figure 4. Sensitivity and specificity of HPV16/18 detection with LtCas12a-based DETECTR assay

(A) Time courses of LtCas12a collateral cleavage in different groups.

(B) Schematic illustration of LtCas12a DETECTR assay.

(C) Detection limitation of LtCas12a-based DETECTR assay. Serially diluted HPV16 and HPV18 plasmids was used as template.

(D and E) Parallel comparison of detection limitation of qPCR and LtCas12a-based DETECTR with RAA for HPV16 (D) and HPV18 (E) was shown.

(F) Specificity analysis of LtCas12a-based detection of the HPV16 and HPV18 L1 gene. Fluorescence signal was recorded by qPCR machine under 528 nm light. Fluorescence data from biological replicates are shown as mean ± SEM (n = 3).

reporter (DETECTR) assay (Figure 4B). In particular, 2 crRNAs targeting the HPV16 L1 gene and 4 crRNAs targeting the HPV18 L1 gene exhibited robust fluorescence signals and were used for the following study (Figure S5A–B). The threshold of fluorescence readout of HPV16 was 10^{11} and of HPV18 was 10^{10} copies (Figure 4C). To improve the sensitivity, we combined the LtCas12a-based DETECTR and recombinase-aided amplification (RAA) and then conducted a parallel comparison with the quantitative polymerase chain reaction (qPCR) method. Generally, the limit of detection (LOD) of the LtCas12a-based DETECTR assay was 30 copies for HPV16 and HPV18, showing equal sensitivity to the qPCR method (30 copies for HPV16 and HPV18) (Figures 4D and 4E). Moreover, LtCas12a exhibited high specificity, showing no fluorescence signal in cross-reaction with 13 other high-risk HPV genotypes (Figure 4F). To better evaluate the specificity of the LtCas12a detection assay, we introduced defined mismatches to the spacer sequence (Table S2). The fluorescence signal analysis showed that introduction of mismatches would have a significant impact on the cleavage activity of LtCas12a, suggesting high specificity of the LtCas12a-based DETECTR assay (Figure S6).

Detection of HPV16/18 DNA with LtCas12a-based LFA

For simple readout, we combined LtCas12a and LFA (see STAR Methods) to detect the HPV16/18 L1 gene, which had the advantage of simple operation, rapidity, and low technical requirements. The reaction system was similar to that of DETECTR except that the reporter was changed into 5-FAM-TTATT-biotin-3. TTATT-biotin-3 was collaterally cleaved and captured on the control line, while the rest of the reporter 5-FAM-TTATT with golden nanoparticles (GNPs) was captured by an anti-GNP antibody to generate a visible test line.[30,31] The LOD of lateral flow strip readout was approximately 30 copies HPV16/18 L1 DNA per reaction with RAA (Figures 5A and S7A). LtCas12a-based LFA showed great specificity in distinguishing HPV16/18 from other high-risk HPV genotypes (Figures 5B and S7B). To assess whether LtCas12a could detect the HPV gene in more complex sample, crude DNA from SiHa and HeLa cell lines was extracted after 10 min lysis at room temperature and then added into the LtCas12a-based lateral flow strip reaction. An increasing density of test band indicated that LtCas12a clearly identified HPV16/18 in the supernatants of SiHa (Figure 5C) and HeLa (Figure S7C) cell lines, respectively. Furthermore, LtCas12a-based LFAs were explored with diverse concentrations of primers, ranging from 400 to 800 nM (Figure 5D). According to the grayscale analysis, the density of the test band increased, accompanied by a higher primer concentration.

Furthermore, we developed an all-in-one assay for detecting the HPV L1 gene. All components for RAA and LtCas12a-based nucleic acid detection were thoroughly mixed in one tube.[24] Of note, the all-in-one system could avoid repeated opening tubes' caps and potential sample cross-contamination, which was critical for the demand of high-throughput clinical tests.[32] Then, we optimized the LtCas12a-based all-in-one assay by adjusting the input of MgOAc and designed new target sequences without PAM limitations.[23,24] Both the separated system and the all-in-one system could identify the HPV16 L1 gene within 45 min (Figure S8).

Detection of HPV16/18 DNA with LtCas12a-based DETECTR in clinical samples

We assessed the performance of LtCas12a-based DETECTR in HPV clinical samples that we have already characterized in qPCR. A total of 36 patients aged 36.75 ± 8.15 years (ranging from 19 to 45 years) were enrolled for sample collection. All

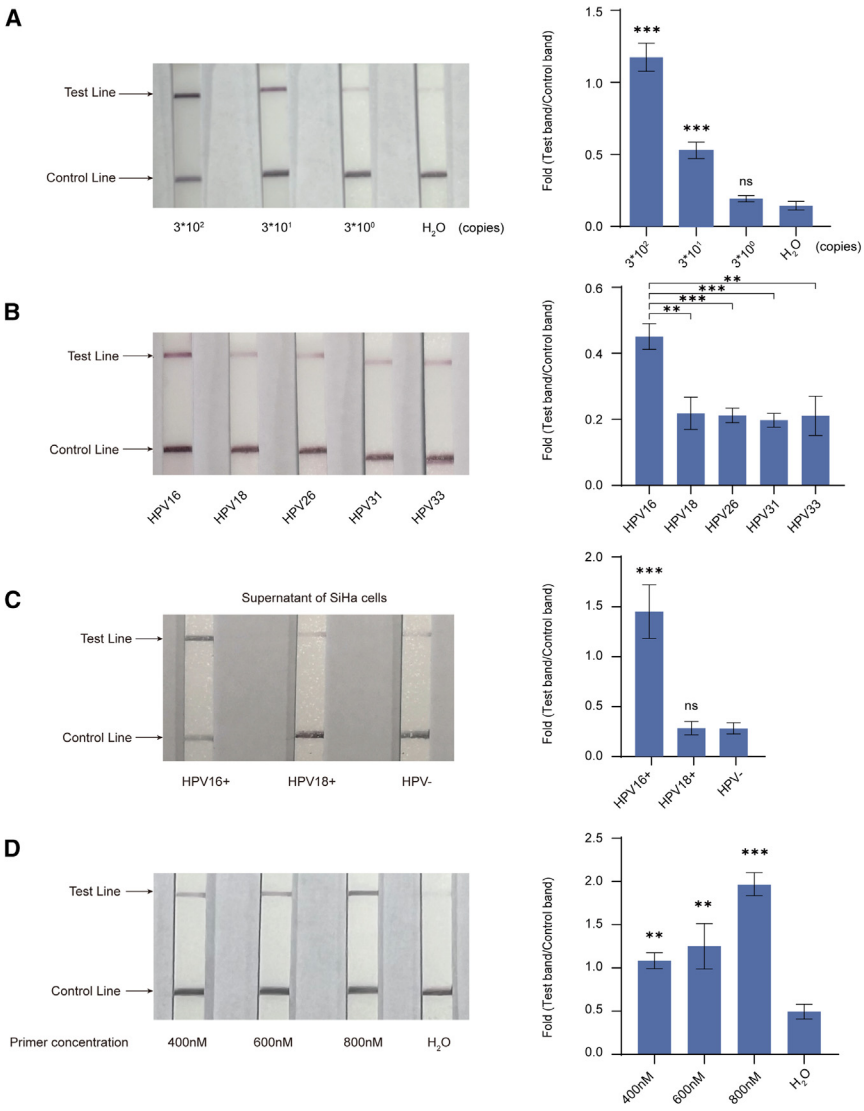

Figure 5. Sensitivity and specificity of HPV16/18 detection with LtCas12a-based lateral flow strip

(A) Detection limitation of LtCas12a-based lateral flow strip assay. Serially diluted HPV16 plasmid was used as template.

(B) Specificity analysis of LtCas12a-based lateral flow strip assay.

(C) LtCas12a-based lateral flow strip detection of HPV16 in SiHa cells.

(D) All-in-one lateral flow strip detection of HPV16 L1 gene with different primer concentrations. The visualization of sample bands intensity is shown, and the corresponding adjusted band intensity was analyzed by ImageJ.

Data from biological replicates were shown as mean ± SEM (n = 3). One-way ANOVA and Bonferroni test, *p < 0.05, **p < 0.01, ***p < 0.001, ns, not significant.

participants provided their sanitary napkins during their menstrual period for HPV detection. Among them, one cervical intraepithelial neoplasia grade 1 (CIN1), one cervical intraepithelial neoplasia grade 2 (CIN2), and two cervical cancer cases were diagnosed based on pathological results. HPV DNA samples were extracted from menstrual blood spots in sanitary pads for DETECTR. According to qPCR analysis, there were 15 samples that were HPV16 or HPV18 positive, with a wide range of Ct values (16.51–31.73). LtCas12a-based DETECTR had a sensitivity of 90% and a specificity of 96.2% for HPV16 detection and a sensitivity of 90% and a specificity of 92.3% for HPV18 detection (Figure 6).

DISCUSSION

Distinct features of Cas12a make it a promising candidate for CRISPR gene editing. First, Cas12a utilizes T-rich PAMs at the 5′ end of targeted sequences,[10] different from the G-rich PAM at the 3′ end of targeted sequence for SpCas9. Second, Cas12a was reported to have higher specificity than Cas9 enzymes in human cells.[21,22] Third, Cas12a has non-specific ssDNA cleavage after activation by specific target binding, which was utilized as a nucleic acid detection tool.[13,15,23] In this study, we demonstrate that LtCas12a is a programmable single RNA-guided DNA nuclease functioning both *in vitro* and in mammalian cells. LtCas12a is identified to utilize TTNA PAMs at the 5′ end of the targeted sequences (Figure 1D). Therefore, LtCas12a could achieve a wider range of targetable loci. Taking HPV for instance, 140 and 136 previously inaccessible candidate loci could be targeted by LtCas12a for gene therapy or pathogen diagnosis for HPV16 and HPV18, respectively (Figure S9).

An ideal diagnosis method should meet the criteria of ASURED (affordable, sensitive, specific, user friendly, rapid, robust, equipment free, and deliverable to end users) from the World Health Organization.[13] Therefore, CRISPR-Cas12a-based nucleic acid detection is a hopeful next-generation diagnosis tool.[26,31,33] In this study, we describe a sensitive, accurate, and rapid LtCas12a-based assay for the detection of HPV16 and HPV18. Regarding the sensitivity, the LOD of LtCas12a detection is 30 copies (Figures 4D and 4E), which is comparable to the qPCR method (30 copies; Figures 4D and 4E). In terms of accuracy, LtCas12a could distinguish HPV16/18 from 13 other high-risk HPV genotypes (Figure 4F). For simple operation and elimination of contamination, we develop an LtCas12a-based all-in-one system in this study (Figure S8). This assay is, to the best of our knowledge, the first system that contains all components to be incubated in one single tube for detecting the HPV16/18 gene. This LtCas12a-based all-in-one system has the following

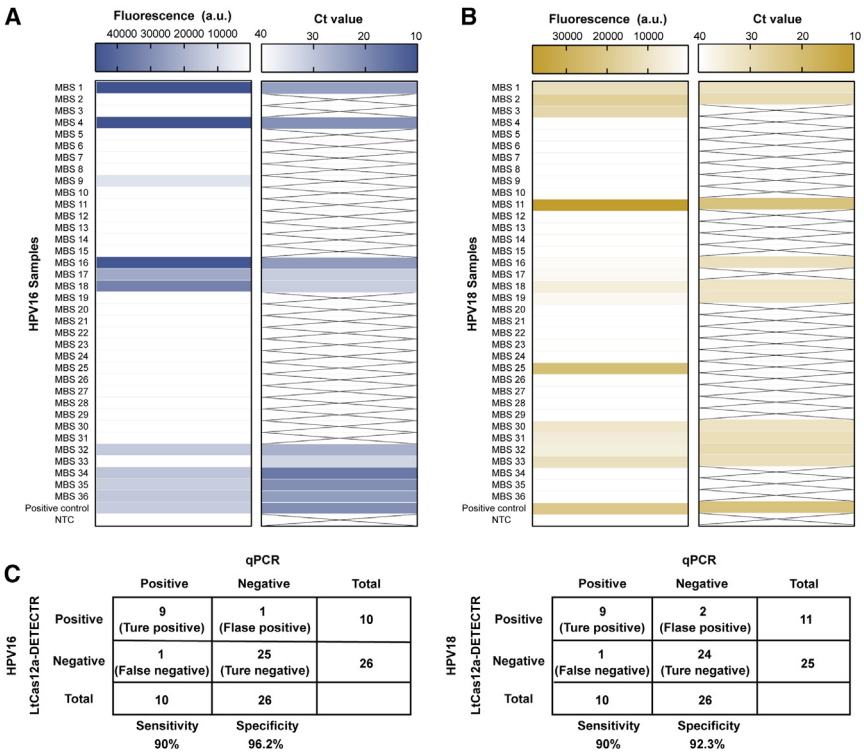

Figure 6. Detection of HPV16/18 DNA using LtCas12a-based DETECTR in clinical samples

(A) Identification of HPV16 in 36 patient samples by qPCR. ×, negative samples; NTC, no template control.

(B) Identification of HPV18 in 36 patient samples by qPCR.

(C) Concordance table between LtCas12a-based DETECTR and qPCR for clinical samples.

advantages. First, the components of RAA and the Cas12a reaction are set in one single tube, therefore avoiding repeated tube opening and cross-contamination. Second, the all-in-one components could be easily freeze dried for long-term transport and storage.[34] Third, a lateral flow strip is utilized for result readout, which we think would be safer for the eyes than previously reported blue LED or UV light.[35–37] Using LtCas12a-based methods, patients could complete HPV nucleic acid detection at home without special equipment or operation, therefore avoiding the tedious inspection and a long wait for laboratory results. Easy detection accelerates the coverage of HPV screening in medical resources-limited regions, which is critical for the prevention and treatment of cervical cancer.[38]

Taken together, LtCas12a with a unique TTNA PAM displays equivalent efficiency and specificity with widely used LbCas12a and AsCas12a, thus expanding the targeting scope of Cas12a family. The LtCas12a-based lateral flow strip has great potential for sensitive, accurate, and rapid HPV detection. We believe that our CRISPR-Cas12a system could serve as a promising tool for both gene therapy and pathogen diagnosis.

Limitation of the study

In this work, LtCas12a has been investigated for cleavage performance in mammalian cells, showing the equivalent editing ability and specificity to representative LbCas12a. Next, combining DETECTR fluorescence technology and LFA, we further developed a sensitive, accurate, and rapid LtCas12a-based HPV gene detection platform with comparable sensitivity to qPCR (LOD, 30 copies). Apart from the *in vitro* exploration, applications in eukaryotic settings including establishing disease models and therapeutic approaches have not yet been carried out. Additionally, canonical PAM and non-canonical PAM have been examined at prokaryotic and *in vitro* levels. Also, we made an initial attempt at all-in-one DETECTR fluorescence assays for HPV detection with non-canonical PAM. In the next step, it will be necessary to display an in-depth comparison between canonical PAM and non-canonical PAM in detail. For the clinical diagnostic value, a preliminary exploration of LtCas12a has been conducted in patient samples with or without HPV16/18 infection. However, the detection performance in other high-risk HPV genotypes remains to be investigated in the future.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.crmeth.2023.100444.

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AUTHOR CONTRIBUTIONS

Investigation, Y.C., L.M., B.L., and Y.W.; validation, Y.C. and L.M.; methodology, Y.C., L.M., B.L., L.L., and Z. Hu; writing – review & editing, Y.C. and Z. Hu; writing – original draft, L.L. and Z. Hu; formal analysis, B.L. and L.L.; visualization, J.L. and Z. Huang; resources, X.T.; project administration, R.T. and Z. Hu; supervision, R.T. and Z. Hu; conceptualization, Z. Hu; funding acquisition, Z. Hu. All authors have read and approved the final manuscript.

DECLARATION OF INTERESTS

The authors declare that they have no conflict of interest.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Trans5α Chemically Competent Cell	Trans	CD201-01
BL21(DE3) Chemically Competent Cell	Trans	CD601-02
Chemicals, peptides, and recombinant proteins		
2xMultiF Seamless Assembly Mix	ABclonal	RK21020
RNA-easy Isolation Reagent	Vazyme	R701
HiScript Q RT SuperMix for qPCR	Vazyme	R122-01
2xPhanta Max Master Mix	Vazyme	P515-01
VAHTS DNA Clean Beads	Vazyme	N411-01
IPTG Solution	Sangon	B541007-0001
Sodium chloride	Sangon	A610476
HEPES	Sangon	A600264
Magnesium chloride hexahydrate	Sangon	A601336
Glutathione reduced	Sangon	A600229
Proteinase inhibitor	Sigma	P8340
TEV Protease	Sigma	T4455-10KU
Molecular sieve	Sigma	208620
NEBuffer 2.1	NEB	B7202V
Dulbecco's Modified Eagle Medium	GIBCO	C11995500CP
Fetal bovine serum	GIBCO	10091148
X-tremeGENE HP DNA Transfection Reagent	Roche	6366546001-1
Green Taq Mix	Vazyme	P131-02
PowerUp™ SYBR™ Green Master Mix	ThermoFisher	A25742
Critical commercial assays		
TransNGS® rRNA Depletion Kit	Trans	KD201-11
VAHTS Small RNA Index Primer Kit for Illumina	Vazyme	N813
SDS-PAGE Gel Preparation Kit	Yeasen	20328ES50
HiScribe T7 High Yield RNA Synthesis Kit	NEB	E2040S
Monarch RNA Cleanup Kit	NEB	T2040L
EasyPure® Quick Gel Extraction Kit	Trans	EG101-01
SF Cell line kit	Lonza	V4XC-2012
EasyPure® Genomic DNA Kit	Trans	EE101-11
RAA Nucleic Acid Amplification Kit	biolifesci	M20901-0048
Lateral flow strip kit	Milenia	MGHD 1
Dried blood spot DNA extraction kit	TIANGEN	DP334-03
Deposited data		
Amino Acid Sequence of LtCas12a	This paper-Table S1	N/A
Kinetic enzyme parameters	This paper-Figure S4	N/A
Target sequences	This paper-Table S2	N/A
Primer sequences	This paper-Table S3	N/A
Bacteria RNA Sequencing	This paper	NCBI SRA: SRR23698764

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
GUIDE-seq data	This paper	NCBI SRA: SRR23515679, SRA: SRR23515681, SRA: SRR23515680, SRA: SRR23515682, SRA: SRR23515697, SRA: SRR23515684, SRA: SRR23515685, SRA: SRR23515690, SRA: SRR23515691, SRA: SRR23515693, SRA: SRR23515694, SRA: SRR23515683, SRA: SRR23515686, SRA: SRR23515687, SRA: SRR23515688, SRA: SRR23515689, SRA: SRR23515692, SRA: SRR23515696, SRA: SRR23515700, SRA: SRR23515701, SRA: SRR23515703, SRA: SRR23515704, SRA: SRR23515698, SRA: SRR23515699, SRA: SRR23515702, SRA: SRR23699155
Experimental models: Cell lines		
HEK293T cell line	ATCC	CRL-3216
SiHa cell line	ATCC	HTB-35
HeLa cell line	ATCC	CRM-CCL-2
Oligonucleotides		
5′-FAM-ssDNA (TTATT)-Biotin-3′ reporter	Sangon	N/A
Double-stranded oligodeoxynucleotide	GENWIZ	N/A
Recombinant DNA		
pET28a-MED11	Addgene	#15414
pUC19	Addgene	#50005
6His-MBP-TEV-huAsCpf1	Addgene	# 90095
pX330-SpCas9-HF1	Addgene	#108301
Software and algorithms		
GraphPad Prism v8.3.1 for MacOS	GraphPad software	N/A
SnapGene v4.2.2 for MacOs	SnapGene software	N/A
Tracking of Indels by DEcomposition	Brinkman et al. (2014)[39]	http://shinyapps.datacurators.nl/tide/
SPSS Statistics 20 for MacOS	SPSS software	N/A
WebLogo	Crooks GE et al. (2004)[40]	https://weblogo.threeplusone.com/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Hu Zheng (huzheng1998@163.com).

Materials availability

The ID numbers of plasmids and reagents are provided in the key resources table.

Data and code availability

- GUIDE-seq data and sRNA-seq data reported in this paper are available at the Sequence Read Archive (SRA). Accession numbers are listed in the key resources table.
- This paper did not generate original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

HEK293T cell line

HEK293T cells were obtained from the American Type Culture Collection (ATCC) (#CRL-3216).

Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (GIBOC, #C11995500CP) supplemented with 10% FBS (GIBCO, #10091148).

Detachment of HEK cells for passaging was performed using the Trypsin reagent (GIBCO, # BS-00-0784).

Cells were cultured at 37°C, 5% CO2 in a humidified atmosphere.

SiHa cell line

SiHa cells were obtained from the American Type Culture Collection (ATCC) (#HTB-35).

Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (GIBOC, #C11995500CP) supplemented with 10% FBS (GIBCO, #10091148).

Detachment of SiHa cells for passaging was performed using the Trypsin reagent (GIBCO, # BS-00-0784).

Cells were cultured at 37°C, 5% CO2 in a humidified atmosphere.

HeLa cell line

HeLa cells were obtained from the American Type Culture Collection (ATCC) (#CRM-CCL-2).

Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (GIBOC, #C11995500CP) supplemented with 10% FBS (GIBCO, #10091148).

Detachment of HeLa cells for passaging was performed using the Trypsin reagent (GIBCO, # BS-00-0784).

Cells were cultured at 37°C, 5% CO2 in a humidified atmosphere.

HPV16/18 clinical sample collection

The clinical samples detected in this study were approved by the Central Hospital of Wuhan Ethics Committee. 36 female patients aged 36.75 ± 8.15 years (ranging from 19 to 45 years) were enrolled for sample collection. Among them, one cervical intraepithelial neoplasia grade 1 (CIN1), one cervical intraepithelial neoplasia grade 2 (CIN2), and two cervical cancer cases were diagnosed based on pathological results. Participants provided sanitary napkins during their menstrual periods for HPV detection. Based on our previous study,[41] HPV DNA was extracted from menstrual blood spots using Tiangen dried blood spot DNA extraction kit (Tiangen Biotech) and prepared for LtCas12a-based DETECTR.

METHOD DETAILS

Construction of Cas12a prokaryotic plasmid

The CRISPR-LtCas12a loci was synthesized (Genewiz, China) and cloned into pET28a-MED11 (Addgene, #15414). We made the following modifications to the CRISPR array: (1) The spacer sequences with the top 5 highest frequency in wild-type CRISPR array were selected and synthesized; (2) We synthesized a target sequence between the first and second direct repeat for the downstream interference assay; (3) A strong heterologous promoter J23119 was added to the upstream of CRISPR array to promote the expression.

Plasmid interference assay

Target sequence downstream the TTTA/TTCA PAM was cloned into pUC19 plasmid backbone using Gibson assembly (ABclonal, China). Plasmids with/without target sequence were transfected into Electrocompetent *E.coli* harboring LtCas12a loci, respectively. Electrocompetent *E.coli* was recovered for 1 h at 37°C, serially diluted, and incubated in LB agar containing both carbenicillin (100 mg/L) and kanamycin (50 mg/L) at 37°C overnight. The counts of *E.coli* clones from experiment and control group were collected.

Bacteria RNA sequencing

Target plasmid was transfected into Electrocompetent *E.coli* harboring LtCas12a loci. Electrocompetent *E.coli* were incubated overnight at 37°C and all the cells were harvested. Total RNA was purified with RNA-easy Isolation Reagent (Vazyme, China) and ribosomal RNA was removed by TransNGS® rRNA Depletion Kit (Transgen, China). RNA length ranging from 15nt to 120nt was selected for further RNA library preparation. 1 μg rRNA-depleted and length-selected total RNA was 3′ and 5′ universal adaptor ligated using VAHTS Small RNA Index Primer Kit (Vazyme, China), and was followed by reverse transcription, PCR amplification, and magnetic bead purification. The prepared cDNA libraries were sequenced on NocaSeq6000.

Purification of LtCas12a protein

Protein purification was conducted as previously described[10] with modification. LtCas12a protein was codon-optimized for *E. coli* and cloned into 6His-MBP-TEV-huAsCpf1 (Addgene, #90095). The protein expression plasmid was transformed into BL21(DE3) Chemically Competent Cell (Transgen, China) and recovered at 37°C overnight. The culture cells were then inoculated to LB growth media at 37°C until the cell density reached 0.6 OD600. 500 μM final concentration of IPTG was added to induce protein expression at 18°C for 14–18 h. All the cells were harvested and resuspended with high-concentration buffer (500 mM NaCl, 20 mM HEPS, 1 mM $MgCl_2$, pH = 7.5), along with proteinase inhibitor (sigma, China). The bacteria resuspension was fully lysed by sonication and centrifuged at 14000 rpm, 30 min at 4°C. The lysate was filtered through 0.8 μM membrane (sigma, China) and mixed with GST fusion protein agarose beads (GE, China) at 4°C and incubated for 1 h. The mixture was passed through a gravity column (GE, China) and washed with low-concentration buffer (200 mM NaCl, 20 mM HEPS, 1 mM $MgCl_2$, pH = 7.5) and elution buffer (200 mM NaCl, 20 mM HEPS, 1 mM $MgCl_2$, 15 mM GSH, pH = 7.5). TEV Protease (sigma, China) was added and eluted product was digested overnight at 4°C. Cas12a nuclease was run on SDS-PAGE gel to observe whether there was an expective size. The sample was

loaded into a molecular sieve (sigma, China) and the fractions containing Cas12a were confirmed by SDS-PAGE and then stored at −80°C.

PAM depletion assay *in vitro*

Linear library sequence containing 6 random nucleotides upstream of spacer was constructed into pACYC184 plasmid by 2X MultiF Seamless Assembly Mix (ABclonal, China), and then 740 bp dsDNA substrates were amplified by PCR. Negative-strand oligo transcription template was synthesized (GENEWIZ, China), which contains spacer, direct repeat, and T7 promoter sequence. Then transcription template was annealed with positive-strand T7 promoter and synthesized with HiScribe T7 High Yield RNA Synthesis Kit (NEB, England), and purified with Monarch RNA Cleanup Kit (NEB, England) according to instructions of the manufacturer. *In vitro* cleavage mixture containing final concentrations of 0.3 μM LtCas12a, 0.3 μM crRNA, and 10x NEBuffer 2.1 was incubated at room temperature for 10 min. 200 ng dsDNA substrates were added and digested at 37°C for 15 min. Reaction was purified with EasyPure® Quick Gel Extraction Kit (Transgen, China) and added with adaptor for deep-sequencing for Illumina, so did undigested dsDNA as a negative control. Depleted PAM sequences were identified and were used to generate a WebLogo (https://weblogo.threeplusone.com/).[40]

Kinetics experiments of LtCas12a

The initial reaction rate was calculated at 10 μM LtCa12a with varying substrate concentrations. The reaction constants (Km, Vmax), Michaelis-Menten curve, and Hanes plots ([S]/V versus [S]) were generated using GraphPad Prism Version 8.0 (GraphPad Software, San Diego, CA).

Construction of Cas12a eukaryotic plasmid

The human codon-optimized LtCas12a sequence was synthesized (GenScript, China) and cloned into pX330-SpCas9-HF1 (Addgene, #108301) along with crRNA element.

Cell culture, electroporation, and transfection

Human Embryonic Kidney 293T (HEK293T), SiHa, and HeLa cell lines were purchased from American Type Culture Collection (ATCC), cultured in Dulbecco's Modified Eagle Medium (GIBCO, America) supplemented with 10% Fetal bovine serum (GIBCO, America) and incubated at 37°C and 5% CO_2. For cell electroporation, Double-stranded oligodeoxynucleotide (dsODN) was synthesized (GENWIZ, China). Cells were transfected using SF Cell Line 4D-Nucleofector™ X Kit (Lonza, Germany) according to the manufacturer's protocol. 2.5 μg plasmid containing Cas12a and crRNA and 150 pmol dsODN were co-transfected into 12-well plates. All the transfected cells were incubated for 72 h after incubation. For cell transfection, cells were seeded 1 day prior to transfection at a density of approximately 1.0×10^5 cells per 24-well. A total of 1 μg plasmids containing Cas12a and crRNA were delivered with X-tremeGENE HP DNA Transfection Reagent (Roche, Switzerland) according to the manufacturer's instruction.

dsODN breakpoint PCR and sanger sequencing

Genomic DNA was extracted by EasyPure® Genomic DNA Kit (Transgen, China). Appropriate PCR primers were designed upstream or downstream of the targeted loci (generally no more than 500 bp), and the other primer was located in dsODN sequence, serving as an anchor. dsODN breakpoint PCR was conducted by Green Taq Mix (Vazyme, China) according to the manufacturer's protocol. PCR product was visualized by 1.5% agarose gel electrophoresis. Sanger sequencing using the primer located in genome loci re-confirmed the dsODN integration.

Deep-sequencing of target sites

Appropriate pairs of primers were designed around target loci with 150–250 bp in length. The PCR fragment was purified with VAHTS DNA Clean Beads and added with adaptors for deep-sequencing for Illumina. Library was sequenced in Illumina Hiseq2500 platform and generated sequencing data.

TIDE assay

100 ng extracted genomic DNA after transfection was used for amplifying genomic fragments surrounding target sites whose length should be 500–700 bp and target sites should locate at about 200–300 bp downstream the sequencing primer, according to the instruction from the manufacturer (Table S2). TIDE-sequencing results were generated with website tool Tide[39] (http://shinyapps.datacurators.nl/tide/).

GUIDE-seq experiments and analysis

The double-stranded oligodeoxynucleotide (dsODN) was purchased from Sangon Company (Sangon Biotech, Shanghai, China). First, 150 pmol dsODN and 2.5 μg plasmid containing Cas12a and crRNA were co-transfected into HEK293T cell line using SF Cell Line 4D-Nucleofector™ X Kit (V4XC-2024, Lonza, Germany) according to the manufacturer's instructions. After 72 h, DNA extraction was implemented immediately after cell harvest and prepared for dsODN breakpoint PCR (verification of effective cleavage). Referring to the reported study,[42] GUIDE-seq libraries were constructed. In brief, the DNA went through shearing, adding

Y adapters, and two rounds of PCR, and then was finally sequenced using MGISEQ-2000RS sequencer with customized settings for 16 bp UMI. Data was then demultiplexed and analyzed with guideseq v1.1.[43] The sequences of targets and primers were provided in Tables S2 and S3.

DETECTR assay

DETECTR assay was performed as previously described,[15] including 200 ng Cas12a protein, 25 nM FAM-ssDNA-BHQ (Sangon, China), 1 μM crRNA, 2 μL sample, and 10x NEBuffer 2.1 in a 20 μL reaction. QPCR machine was used for recording fluorescence signal at 37°C (5′-FAM-ssDNA-BHQ-3′ reporter, λex: 485 nm; λem: 535 nm). LtCas12a-based all-in-one system combined the RAA reaction with Cas12a cleavage in a one-pot reaction. The 50 μL system consisted of 200 ng Cas12a protein, 25 nM FAM-ssDNA-BHQ (Sangon, China), 1 μM crRNA, 4 μL NEBuffer 2.1, 0.36 μM forward and reverse primers, and 1.06 μL of MgOAc.

RAA amplification

The RAA amplification was performed using RAA Nucleic Acid Amplification Kit (Qitian, China), according to the instruction. A 50 μL RAA reaction system was prepared with 25 μL of 2× Reaction Buffer, 17.5 μl Enzyme-free water, 2 μL RAA primer F (10 μM), 2 μL RAA primer R (10 μM), 1 μL of template DNA, and 2.5 μL of Magnesium acetate into one Reaction Tube containing enzyme and dNTPs powder. And it was incubated in the tube at 37°C for 25–30 min.

Lateral flow assay (LFA)

Lateral flow assay reaction system was similar to that of DETECTR assay except for the 5′-FAM-ssDNA (TTATT)-Biotin-3′ reporter (Sangon, China). 4ul of the RAA product was added into the CRISPR/Cas12a reaction mixture, containing 2 μl NEBuffer 2.1 (10 x), 2 μl LtCas12a (10 uM), 4 μl crRNA (10 uM), 0.7μl ssDNA reporter (5′-FAM-TTATT-Biotin-3′) (Sangon, China) (10 μM), and 7.3 μl nuclease-free water. The above mixture was incubated at 37°C for 30–50 min. The 20 μl reaction volume was finally added with 80ul strip buffer and then loaded onto the strips (Milenia Biotec GmbH, German). Photograph and record the result after incubating at room temperature for 5 min. Gray scale values of bands were read by ImageJ software.

Quantitative PCR (qPCR)

The qPCR was performed using the Bio-Rad CFX96 system with PowerUp™ SYBR™ Green Master Mix (A25742, Applied Biosystems, USA). Threshold-cycle value (Ct value) was used for analysis for each qPCR reaction. The primers involved were displayed in Table S3.

Crude cell DNA preparation

3–4*10^4 SiHa or HeLa cells were harvested, resuspended by 25 μL DPBS and mixed with 25 μL lysis buffer containing 2% SDS at room temperature for 10 min. 5 μL of crude DNA sample was used for RAA amplification and nucleic acid detection.

Polymerase chain reaction

Each reaction system was conducted by Green Taq Mix (Vazyme, China) according to the manufacturer's protocol, containing 1x Green Taq Mix, 1 μL serial diluted HPV plasmid, and specific amplification primer (Table S3) in a total 25 μL volume. 5 μL of PCR product was visualized by 1.5% agarose gel electrophoresis.

QUANTIFICATION AND STATISTICAL ANALYSIS

The experimental data was analyzed with IBM SPSS Statistics 20. The comparisons of indel frequency and fluorescence strength were performed using Kruskal-Wallis and Bonferroni test, One-way ANOVA and Bonferroni test (n = 3) respectively.

Data from biological replicates were shown as mean ± s.e.m. (n = 3). p values were reported using GraphPad style: not significant (ns), $p \geq 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.