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Cas12a/blocker DNA-based multiplex nucleic acid detection system for diagnosis of high-risk human papillomavirus infection

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ABSTRACT

Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) proteins are an innovative tool in molecular diagnostics owing to their high specificity and modularity for target nucleic acid sequences. However, the sequence-indiscriminate *trans*-cleavage activity of the Cas protein renders multiplex detection challenging. In this study, we developed a Cas12a-based multiplex detection system by designing blocker DNA complementary to reporter DNA, which enables the simultaneous detection of two genes with a single Cas protein in a single reaction. As a proof of concept, we chose high-risk human papillomavirus (HPV) 16 and 18 as the model targets and incorporated recombinase polymerase amplification (RPA) and transcription reactions to achieve high accuracy and sensitivity. Using the proposed system, we detected the genes of both HPV 16 and 18 down to 1 aM within 80 min under isothermal conditions. We validated the performance of the system in detecting genomic DNA from various cell lines and clinical samples from cervical cancer patients with high specificity. The proposed system facilitated rapid multiplex detection of high-risk HPVs in a single reaction tube with only Cas12a, thus representing a more user-friendly and economical alternative to previous Cas protein-based multiplex detection assays. The proposed system has considerable potential for point-of-care testing and could be expanded to detect various nucleic acid biomarkers.

1. Introduction

Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) proteins play a vital role in the adaptive immune system of bacteria and archaea (Barrangou et al., 2007; Bhaya et al., 2011): Fragments of invading viral nucleic acids are incorporated into CRISPR arrays located in the genome. Subsequently, the CRISPR/-Cas proteins recognize and cleave the viral nucleic acid when cells are reinfected with the same virus (Li and Peng, 2019). Owing to its high specificity and modularity to target nucleic acid sequences, the CRISPR/Cas system has become an essential tool in various fields, including genome editing (Doudna and Charpentier, 2014; Hwang et al., 2013; Strecker et al., 2019; Xu et al., 2021), gene expression modulation (Bikard et al., 2013; Luo et al., 2015), cell imaging (Qin et al., 2017; Zhou et al., 2017), and detection of nucleic acids (Aman et al., 2020; Gong et al., 2021; Gootenberg et al., 2017; Han et al., 2023; Li et al., 2019; Shin et al., 2022; Wang et al., 2020; Zhao et al., 2021; Zhou et al., 2020), proteins (Han et al., 2022; Zhang et al., 2021), and exosomes (Ding et al., 2022; Li et al., 2021; Zhao et al., 2020). Among the Class 2 CRISPR/Cas systems, CRISPR/Cas12a and CRISPR/Cas13a have *cis*-cleavage and unique *trans*-cleavage activities, which indiscriminately degrades neighboring nucleic acids following *cis*-cleavage of the target nucleic acid (Chen et al., 2018; Liu et al., 2017). Given the persistent *trans*-cleavage activity of these proteins (Chen et al., 2018; Zhou et al., 2021), they can be utilized as signal amplifiers in biomolecular detection using short oligonucleotides (i.e., reporters) labeled with a fluorophore and quencher.

Detection of multiple genes, biomarkers, or pathogens is often necessary for in vitro diagnostics (Kim et al., 2022; Savvateeva et al., 2021; Shin et al., 2022; Tian et al., 2022), which increases the demand for multiplex systems (Dincer et al., 2017). The research and development on multiplex nucleic acid detection systems using CRISPR/Cas proteins have peaked in recent years (Bruch et al., 2021; Gootenberg et al., 2018; Li et al., 2022; Tian et al., 2022; Xiong et al., 2021; Zhang

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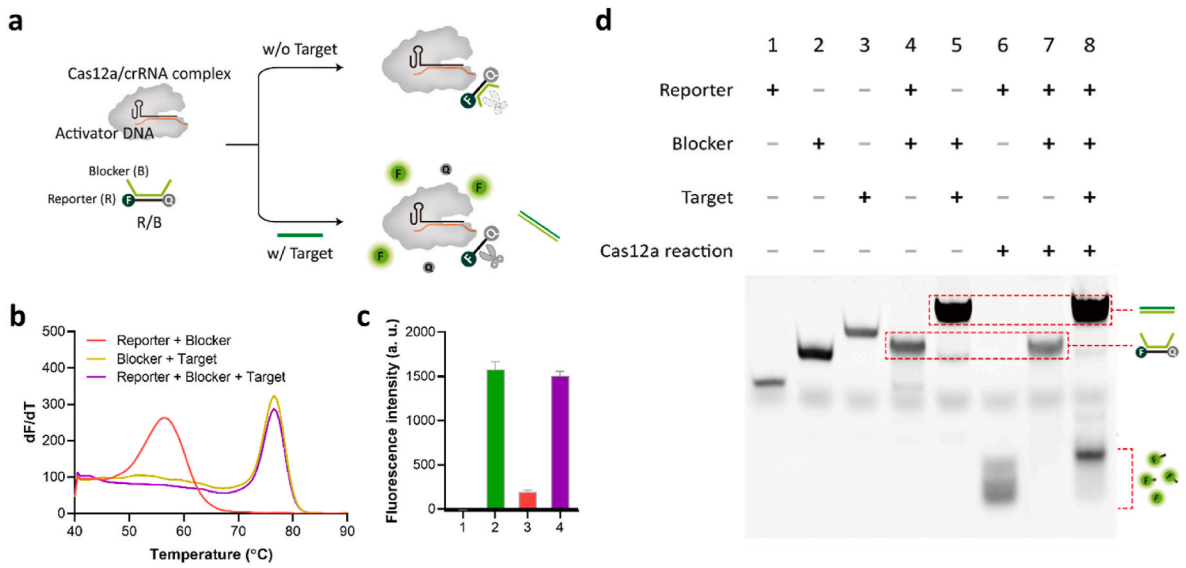


Fig. 1. (a) Schematic illustration of blocker DNA-assisted modulation of Cas12a *trans*-cleavage activity. (b) Melting curve analysis of blocker (1 μM), reporter (1 μM), and target (1 μM) interactions. (c) Fluorescence intensities of Cas12a-catalyzed *trans*-cleavage, calculated as the difference between initial fluorescence intensity and that at 20 min (1: Reporter + Cas12a/crRNA complex, 2: Reporter + Cas12a/crRNA complex + Activator DNA, 3: R/B + Cas12a/crRNA complex + Activator DNA, 4: R/B + Target + Cas12a/crRNA complex + Activator DNA) (d) PAGE analysis of the blocker-assisted modulation of Cas12a *trans*-cleavage activity. "Cas12a reaction" refers to the incubation with Cas12a/crRNA complex and activator DNA. The corresponding oligonucleotides are presented. When the reporter is cleaved by *trans*-cleavage activity of Cas12a, it is fragmented into very short oligonucleotides having high mobility and thus it appears at the bottom of the gel after the gel electrophoresis.

et al., 2021). Current strategies based on the *trans*-cleavage activity of Cas proteins include using two or more different Cas proteins or separating spaces specific to different targets. Although these strategies facilitate the multiplex detection of nucleic acids, the assays are complex, and the analysis is expensive, which is ascribed to the intrinsic sequence-indiscriminate *trans*-cleavage activity of Cas12a and 13a. Therefore, novel methods should be designed to overcome the limitations of previous CRISPR/Cas-based multiplex systems and exploit their capacity for signal amplification.

Human papillomavirus (HPV) is a double-stranded DNA (dsDNA) virus involved in the pathogenesis of various diseases and cancers, particularly cervical cancer. HPV can cause high- and low-risk infections; low-risk HPV infections only cause genital warts, whereas persistent infections with high-risk HPV are likely to induce precancerous lesions in the cervix. High-risk HPVs are thought to be closely related to cervical cancer (Gong et al., 2021). They are prevalent in over 90% of patients with cervical cancer, with HPV 16 and 18 accounting for approximately 70% of high-risk cases (Crosbie et al., 2013). Cytological screening (i.e., the Pap smear test) is the most prevalent method for detecting cervical cancer. However, this requires skilled experts and takes approximately 1–3 weeks to obtain results. Visual inspection of cervical cancer with acetic acid (VIA) is a simple, fast, and economical alternative; however, it is limited by high false-negative and false-positive rates (Hegde et al., 2011). Notably, HPV type cannot be determined using either the Pap smear or VIA test. In contrast, HPV DNA testing can help identify the HPV type and is suitable for cervical cancer screening owing to its high sensitivity, specificity, and accuracy (Ying et al., 2014). Concordantly, the recently revised guidelines of the World Health Organization recommend screening through HPV DNA or mRNA tests rather than by Pap smear or VIA testing (World Health Organization (WHO), 2021). The current gold standard for HPV screening is the Cobas® HPV test (Roche, Basel, Switzerland), which can help screen for 14 HPV types. Since this test is based on polymerase chain reaction (PCR), it requires specialized equipment and controlled reaction temperatures, which limits its application in facility-limited or point-of-care (POC) settings. Therefore, there is an urgent need to develop accurate POC HPV DNA tests that can be performed under isothermal conditions.

In this study, we developed and validated the performance of a Cas12a/blocker DNA-based multiplex detection (CASTI) system that enables cost-effective multiplex nucleic acid detection and HPV-type classification with a single Cas protein. In principle, the blocker DNA is designed to be partially and fully complementary to the reporter and the target, respectively. Thus, the presence of target that preferentially binds to the blocker DNA converts the dsDNA reporter into the single-stranded DNA (ssDNA) one, enabling Cas12a to exert the effective *trans*-cleavage activity and generating high fluorescence signal. We also combined the CASTI with isothermal recombinase polymerase amplification (RPA) and transcription reactions (R-CASTI), which can be operated at 37 °C with high sensitivity and accuracy. We validated the efficacy of R-CASTI system in simultaneously detecting HPV 16 and HPV 18 in various cancer cell lines as well as clinical samples from cervical cancer patients. We believe our study would help demonstrate the potential of this multiplex platform in diagnosing high-risk HPV infections using a single Cas protein in a single reaction tube at isothermal temperatures, contributing to the accurate HPV screening in a facility-constrained environment.

2. Materials and methods

This section with details is presented in supporting information.

3. Results and discussion

3.1. Blocker DNA-assisted modulation of Cas12a trans-cleavage activity

Our proposed strategy of multiplex nucleic acid detection using a single CRISPR/Cas12a protein relies on the fact that the *trans*-cleavage activity of Cas12a is much more effective on ssDNA than dsDNA (Chen et al., 2018). Specifically, the blocker DNA complementary to the fluorophore- and quencher-labeled reporter DNA at the 5′ and 3′ ends was designed such that the dsDNA structure (R/B) suppresses the *trans*--cleavage activity of Cas12a complexed with crRNA and activator DNA (without target), generating a negligible fluorescence signal. However, in the presence of a target that binds to the blocker DNA, the reporter

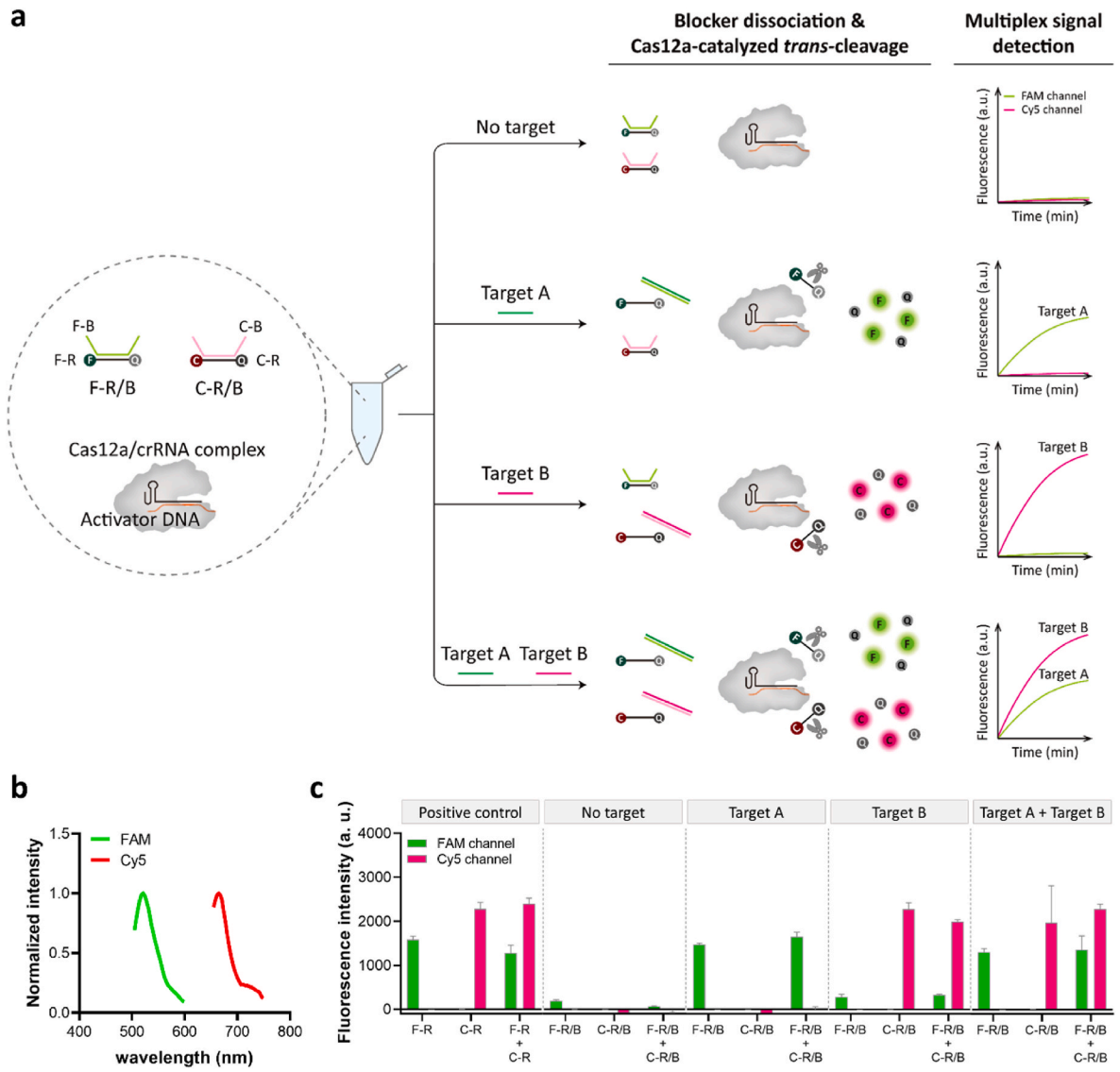


Fig. 2. Cas12a/blocker DNA-based multiplex detection (CASTI) system. (a) Schematic illustration of the CASTI system. (b) Normalized fluorescence emission spectra of FAM and Cy5. Fluorescence intensities were normalized against the maximum value. (c) Fluorescence signals from the F-R/B and C-R/B in the absence and presence of Target A or B. Fluorescence intensities were calculated as the difference between the initial measurement and that at 20 min.

DNA is released as ssDNA, activating the *trans*-cleavage activity of Cas12a complexed with the crRNA and activator DNA (with target). Consequently, the reporter DNA is cleaved, and the distance between the fluorophore and quencher increases to generate a high fluorescence signal (Fig. 1a).

First, we investigated the interactions between blockers, reporters, and targets. In the presence of the target, the melting temperature (Tm) of the R/B was changed to that of dsDNA consisting of the blocker and target (termed B/T; Fig. 1b), confirming that the blocker binds with higher affinity to the target than the reporter. We also observed that Cas12a complexed with crRNA and activator DNA exerted effective *trans*-cleavage activity on the reporter to generate a strong fluorescence signal (1 and 2 in Fig. 1c). The presence of a reporter and blocker resulted in a low fluorescence signal; however, when the target was present, the fluorescence signal was restored to that observed in the absence of the blocker (3 and 4 in Fig. 1c). Gel electrophoresis analysis supported the preferential binding of the blocker to the target over the reporter, and the effective *trans*-cleavage activity of Cas12a (Fig. 1d). The reporter and blocker bound together generated a new R/B band (4) and exhibited no bands at the bottom of the gel (7), suggesting that the R/B is not a substrate for Cas12a-catalyzed *trans*-cleavage. In the presence of the target (8), the R/B band disappeared, and a new B/T band appeared; bands corresponding to the cleaved reporter were observed at the bottom of the gel. These results demonstrate that the blocker effectively modulated the *trans*-cleavage activity of Cas12a.

3.2. Principle and feasibility of the CASTI

The CASTI system was designed with an F-blocker (F-B) that binds to a FAM and BHQ1-labeled reporter (F-reporter, F-R) and a C-blocker (C-B) that binds to a Cy5 and BHQ2-labeled reporter (C-reporter, C-R) for the simultaneous detection of targets A and B, respectively. Thus, the presence of Targets A and B separates F-B and C-B from the corresponding reporters (F-R and C-R) to release the ssDNA forms of F-R and C-R; the Cas12a-catalyzed *trans*-cleavage of these ssDNAs then results in the emission of strong fluorescence signals of FAM and Cy5, respectively. We assessed the functioning of the CASTI in four contexts (Fig. 2a): (i) no fluorescence signal is generated when neither nucleic acid targets exists; (ii) in the presence of Target A but not Target B, only the FAM fluorescence signal is generated; (iii) in the presence of Target B

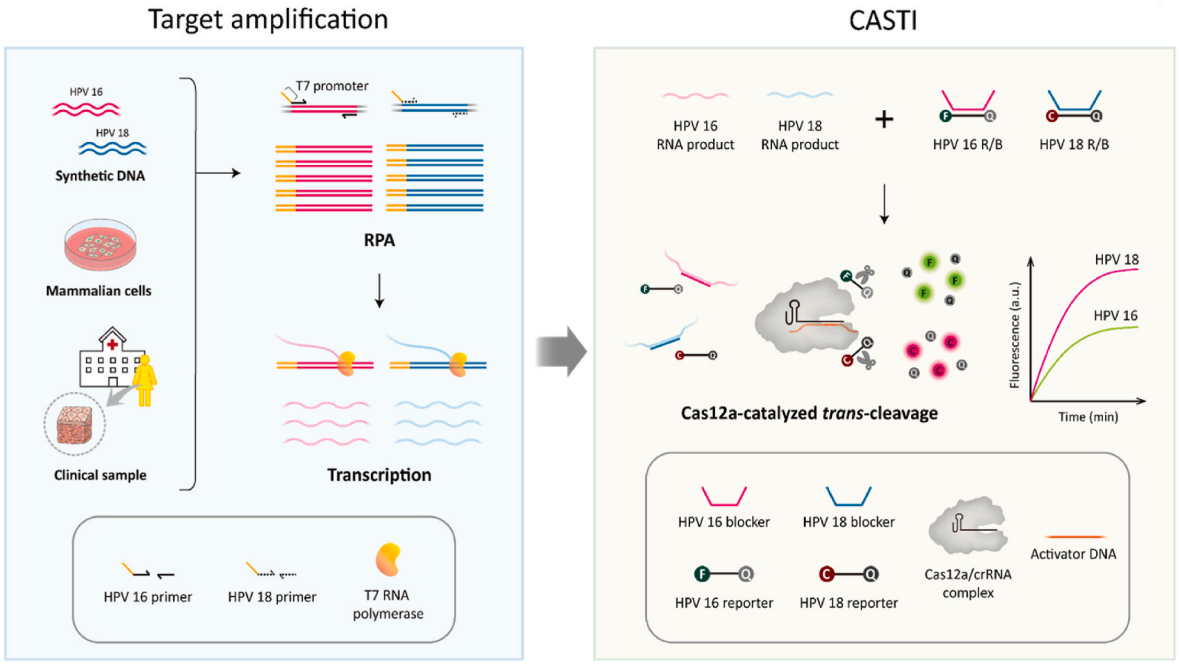


Fig. 3. Schematic illustration of the RPA/transcription-CASTI (R-CASTI) system. In target amplification, RPA with T7 promoter-anchoring primers and transcription reaction amplify gene of HPV 16 or 18 from different analysis sample types, such as synthetic DNA, mammalian cell lines, and clinical samples from cervical cancer patients. In subsequent CASTI reaction, RNA product from the amplification binds to its corresponding blocker, generating high fluorescence signal by Cas12a-catalyzed *trans*-cleavage.

but not Target A, only the Cy5 fluorescence signal is generated; and (iv) in the presence of both targets, both FAM and Cy5 fluorescence signals are generated. In these contexts, each target is designed to interact with its complementary blockers without cross-reactions, and the fluorophores do not exhibit spectral overlap. Therefore, the CASTI system can identify two or more target nucleic acids in one reaction tube with one Cas protein (Cas12a) through rationally designed blocker sequences and appropriately selected fluorophores with distinct spectral peaks.

To demonstrate the feasibility of the CASTI system for multiplex nucleic acid detection, we measured the fluorescence emission spectra of FAM and Cy5. The results in Fig. 2b showed that they produced distinct emission fluorescence spectra without interference. We then performed the CASTI in the presence or absence of Targets A or B, mimicking the four contexts described in Fig. 2a. In the absence of both targets, the F-R/B and C-R/B sets exhibited minimal fluorescence; however, in the presence of Target A only, FAM signals were generated from the F-R/B, whereas Cy5 signals were not generated, regardless of the addition of the C-R/B (Fig. 2c). Conversely, in the presence of Target B alone, the Cy5 signal was generated from the C-R/B; no FAM signal was observed even when the F-R/B was added. In the presence of both Targets A and B, high FAM and Cy5 fluorescence signals were observed for the F-R/B and C-R/B. Notably, the fluorescence signals restored by the specific targets were comparable to those of the corresponding positive controls, in which Cas12a-catalyzed *trans*-cleavage occurred without a blocker. These results confirm that Targets A and B specifically bind to F-B and C-B, respectively, releasing the ssDNA form of the reporter and inducing a high fluorescence signal without cross-reactivity, confirming that the CASTI system can detect multiple nucleic acid targets.

3.3. Development of the R-CASTI system for the detection of high-risk HPV

The sensitivity of CRISPR/Cas-based nucleic acid detection systems is often insufficient for practical application and thus has been combined with different nucleic acid amplification reactions, such as PCR (Shin et al., 2022; Yang et al., 2022), LAMP (Broughton et al., 2020; Mahas et al., 2021; Nguyen et al., 2020), RPA (Chen et al., 2018; Ding et al., 2020; Kellner et al., 2019), NASBA (Pardee et al., 2016), SDA (Zhou et al., 2018), and RCA (Qing et al., 2021; Tian et al., 2020; Xu et al., 2020). We incorporated RPA, an isothermal nucleic acid amplification method, to improve the sensitivity of the CASTI system and integrated the transcription reaction to generate ssRNA complementary to the blocker DNA. Notably, ssRNA is less prone than DNA to degradation by Cas12a-catalyzed *trans*-cleavage (Chen et al., 2018) and is more accessible than dsDNA to displace blocker DNA (Lesnik and Freier, 1995). The principle of the R-CASTI is that, during the RPA reaction, HPV 16 and HPV 18 genes are recognized and amplified with RPA primers anchoring the T7 promoter sequence at the 5′ end, and a T7 RNA polymerase then proceeds with transcription using the RPA product containing the T7 promoter sequence as a template (Fig. 3; target amplification). Subsequently, the HPV 16 and HPV 18 RNA products bind orthogonally to the HPV 16 and HPV 18 blockers, respectively, separating them from each reporter (Fig. 3; CASTI). Consequently, Cas12a-catalyzed *trans*-cleavage leads to the generation of strong FAM and Cy5 signals from the HPV 16 and 18 reporters, respectively.

We performed gel electrophoresis in the absence or presence of HPV 16 to validate the RPA/transcription reaction. As shown in Fig. S1, the HPV 16 blocker band remained unchanged when the products of the RPA/transcription reaction obtained in the absence of HPV 16 were incubated with the HPV 16 blocker, whereas the band shift of the HPV 16 blocker in the upper position of the gel was observed when the products of RPA/transcription reaction obtained in the presence of HPV 16 were incubated with the HPV 16 blocker (Fig. S1a). These results confirm that the HPV 16-specific RPA primer anchoring the T7 promoter sequence at the 5′ end effectively generates ssRNA products that bind to the HPV 16 blocker. Next, we investigated the applicability of RPA/transcription in the CASTI by gel electrophoresis. Only when HPV 16 was present in the R-CASTI, the HPV 16 blocker was dissociated from the HPV 16 R/B, leading to the disappearance of the HPV 16 R/B band and formation of the cleaved reporter by Cas12a-catalyzed *trans*-cleavage at the bottom of the gel (Fig. S1b). These results demonstrate that the ssRNA products obtained by the RPA/transcription reaction effectively

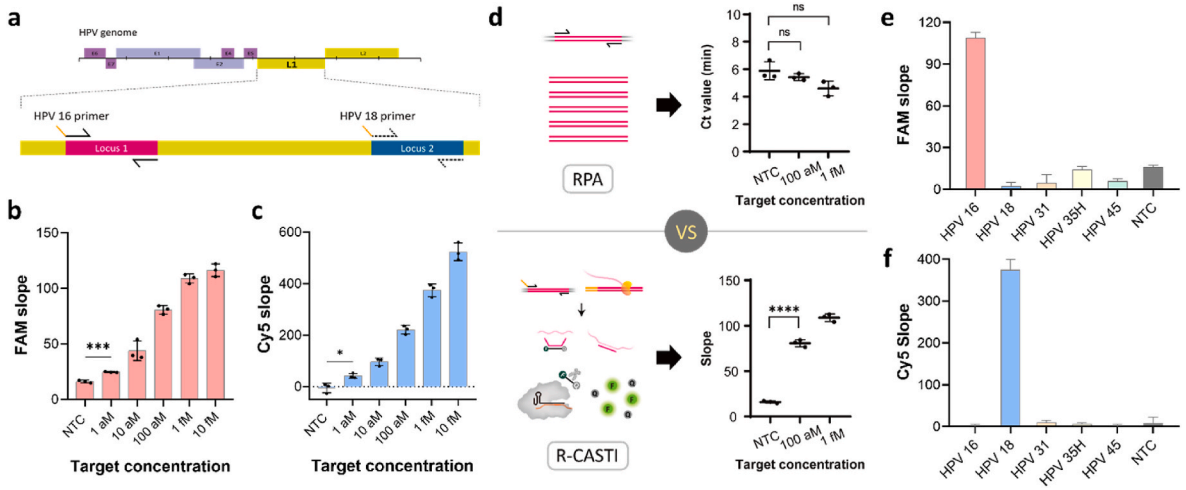


Fig. 4. Detection of HPV 16 or 18 using the R-CASTI system. (a) Schematic illustration of the HPV genome map depicting the selected loci for RPA primers. (b and C) Quantitative analysis of HPV 16 (b) and 18 (c) (two-tailed Student's *t*-test: $*p < 0.05$; $***p < 0.001$). (d) Comparison of RPA and the R-CASTI system for HPV detection. (e) Specificity of the R-CASTI system for HPV 16 (1 fM), targeting locus 1. (f) Specificity of the R-CASTI system for HPV 18 (1 fM), targeting locus 2. In (e) and (f), the concentration of other low-risk HPV types was 10 fM.

displaced the blocker from the R/B and triggered Cas12a-catalyzed *trans*-cleavage to generate a strong fluorescence signal.

To maximize the performance of the R-CASTI system, we optimized the conditions for the RPA and transcription reactions. When different RPA primer concentrations were used, we observed that the highest signal-to-noise ratio occurred at an RPA primer concentration of 300 nM (Fig. S2a). The RPA and transcription reaction times were also optimized to be 7 min and 30 min, respectively, because the background-noise signal increased thereafter (Figs. S2b and c).

3.4. Detection performance of the R-CASTI system on high-risk HPV 16 and 18

We evaluated the sensitivity and specificity of the R-CASTI system for the detection of HPV 16 or 18 under optimal conditions. Based on the multiple sequence alignment of the L1 gene of various HPV types, we selected loci 1 and 2, which are specifically recognized by the HPV 16 and 18 primers, respectively (Fig. 4a and Fig. S3). We examined the fluorescence signals in the presence of HPV 16 or 18 at different concentrations using the R-CASTI system with the selected RPA primer sets. The R-CASTI system detected both HPV 16 and HPV 18 with high sensitivity (Fig. 4b and c), with a limit of detection (LOD) of 1 aM (approximately 0.6 copies/μL), which is the lowest target concentration for producing a signal greater than three times the standard deviation of the no-template control (NTC) signal ($m + 3 \times SD$, where m and SD represent the mean and standard deviation of the NTC signal, respectively). The R-CASTI system effectively distinguished low target concentrations, such as 100 aM and 1 fM. However, RPA alone did not, indicating that the R-CASTI system has better sensitivity than that of RPA (Fig. 4d). Moreover, high fluorescence signals were observed only

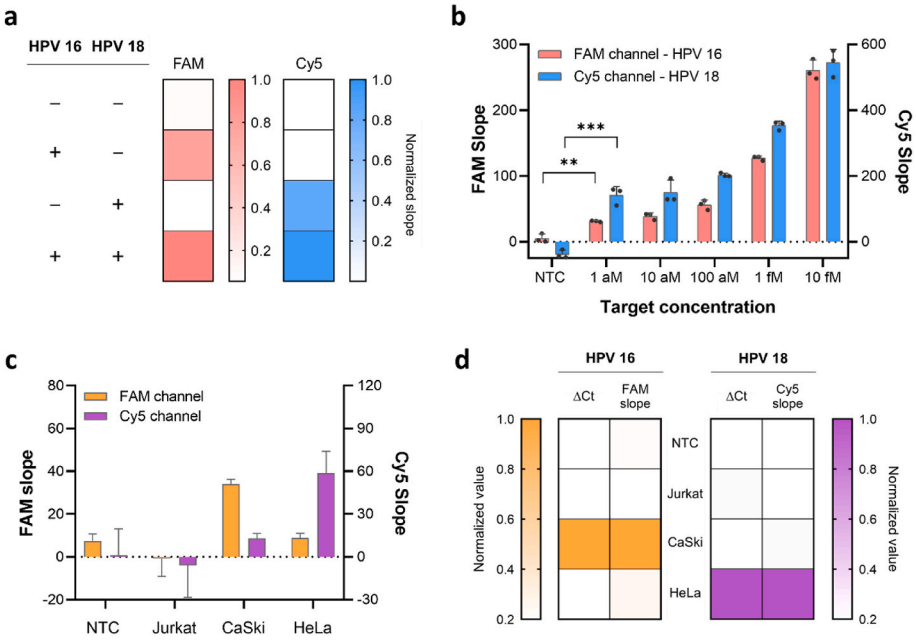


Fig. 5. Multiplex detection of HPV 16 and HPV 18 using the R-CASTI. (a) Fluorescence signals of the R-CASTI in the absence and presence of HPV 16 and/or HPV 18 (10 fM). (b) Quantitative analysis of HPV 16 and 18 (two-tailed Student's *t*-test: $**p < 0.01$; $***p < 0.001$). (c) R-CASTI signals in three cell lines and the no-template control (NTC). (d) Heat-map analysis of the normalized ΔCt values by qPCR and fluorescence slopes by the R-CASTI for HPV 16 and 18 in (c).

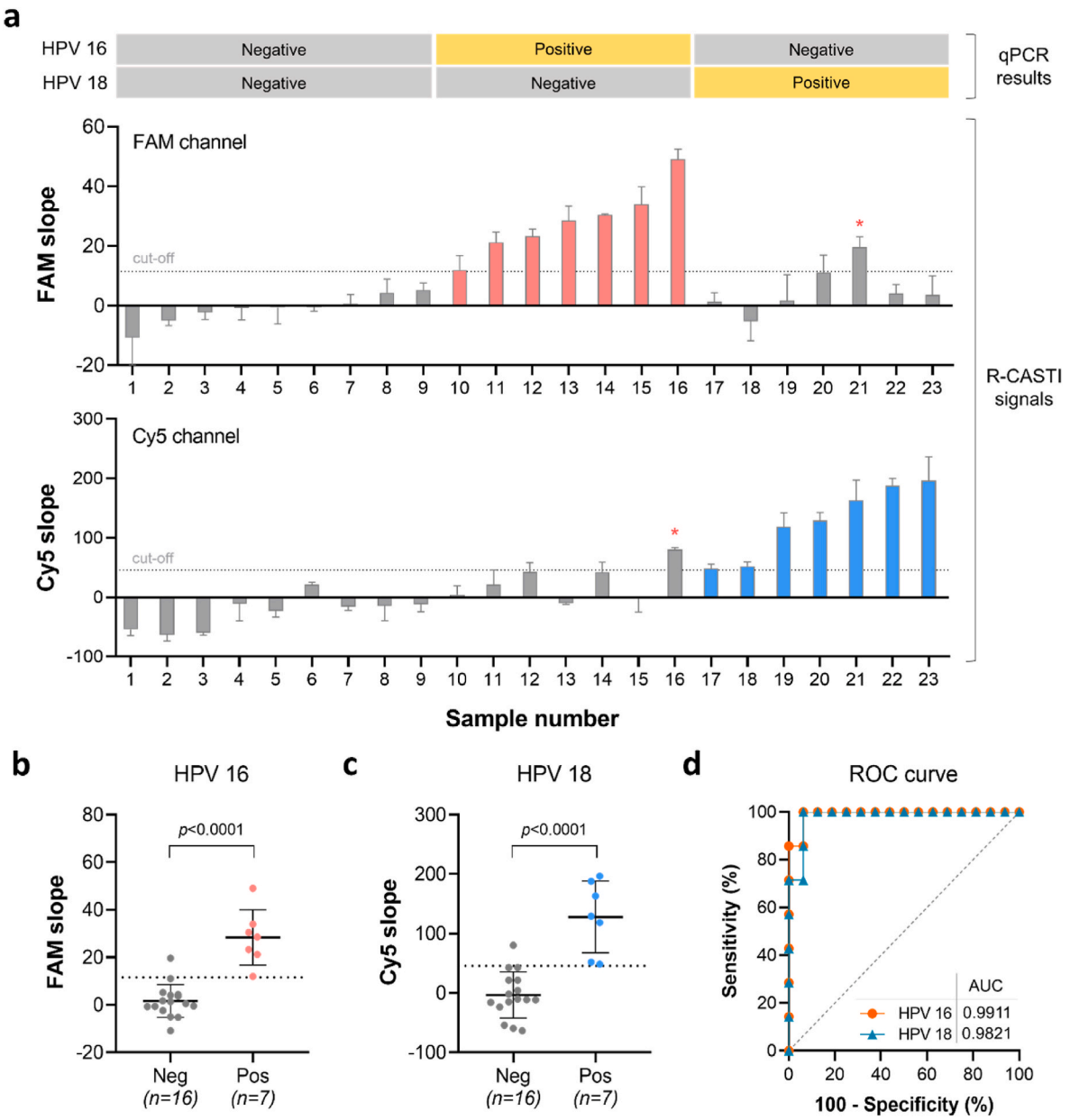


Fig. 6. Clinical sample analysis using the R-CASTI. (a) qPCR results and R-CASTI signals for HPV 16 and HPV 18. Cut-off values in each channel were calculated from the receiver operating characteristic (ROC) curve (11.5 for and 45.5 for FAM and Cy5 slopes, respectively). (b and c) Scatter plot showing the R-CASTI signals of 16 negative and 7 positive clinical samples for HPV 16 and 18, respectively, in the corresponding channel. (d) ROC curve analysis of R-CASTI signals.

in the presence of HPV 16 or 18. In contrast, other low-risk HPV types (HPV 31, 35H, and 45) elicited negligible signals, which confirms the high specificity of the R-CASTI system for high-risk HPVs (Fig. 4e and f).

We investigated the feasibility of the R-CASTI system in multiplex nucleic acid detection. We first optimized the activator DNA concentration because the reporter amount for Cas12a-catalyzed *trans*-cleavage is higher than that for single-plex detection. Both FAM and Cy5 fluorescence signals were saturated at 6 nM activator DNA in the presence of both HPV 16 and 18 (Fig. S4), representing the highest signal-to-noise ratio and optimal activator DNA concentration. Then, simultaneous detection of HPV 16 and 18 using the R-CASTI system was performed in a single tube. The presence of HPV 16 and HPV 18 selectively generated FAM and Cy5 fluorescence signals, respectively, and did not induce non-specific fluorescence signals (Fig. 5a). By measuring FAM and Cy5 fluorescence signals at varying concentrations of HPV 16 and HPV 18, we determined that the LOD of the R-CASTI system was 1 aM (Fig. 5b). This result is superior or comparable with that of other multiplex CRISPR/Cas-based detection systems (Bruch et al., 2021; Chang et al., 2019; Gootenberg et al., 2018; Tian et al., 2022), which confirms the high sensitivity of the R-CASTI system in detecting multiplex nucleic acid targets. Finally, we used three cell lines (Jurkat: HPV 16–/18–; CaSki: HPV 16+/18–; and HeLa: HPV 16–/18+) to validate the effective detection of high-risk HPVs in human genomic DNA (gDNA). The Jurkat cell line presented no signal for either FAM or Cy5, the CaSki cell line generated only the FAM signal, and the HeLa cell line generated only the Cy5 signal (Fig. 5c). In contrast, negligible FAM and Cy5 signals were observed in the absence of gDNA (NTC). These results are consistent with those of qPCR, the gold standard for nucleic acid detection, confirming the reliability of the R-CASTI system (Fig. 5d).

3.5. Clinical sample analysis

Following preclinical validation, we applied the R-CASTI to human clinical samples from cervical cancer patients whose HPV 16 or 18

infection was confirmed by qPCR. The positive or negative status of the 21 samples determined by our assay for HPV 16 and 18 was consistent with that of the qPCR results, with two exceptions (Fig. 6a): false positives for HPV 16 (sample #21) and 18 (sample #16). Furthermore, the R-CASTI revealed distinct signals ($p < 0.0001$, two-tailed Student's *t*-test) between positive and negative groups for both HPV 16 and 18. Based on the ROC curve analysis, the clinical sensitivity and specificity of the developed assay were 100% and 93.75%, respectively, for both HPV 16 and 18, and the AUC was 0.9911 for HPV 16 and 0.9821 for HPV 18 (Fig. 6d), validating the satisfactory clinical performance of the R-CASTI. Collectively, these results suggest that the proposed assay is an effective diagnostic tool for high-risk HPV infections.

4. Conclusions

In this study, the CASTI system was designed to modulate the *trans*-cleavage activity of Cas12a by blocking reporter DNA with its complementary sequence. The R-CASTI system, which integrates RPA and transcription reaction into the CASTI, enabled the simultaneous detection of HPV 16 and 18 down to 1 aM with high specificity. The proposed system was applied to gDNA from cell lines and clinical samples for HPV 16 or HPV 18 with optimal sensitivity and specificity. Our study provides a novel strategy to control Cas12a-catalyzed *trans*-cleavage using blocker DNA, enabling rapid multiplex detection in a single reaction tube with one Cas protein (Cas12a), which overcomes the inherent complexity of previous CRISPR/Cas-based multiplex detection assays using more than two types of Cas proteins or separate reaction spaces. With high-risk HPV types as model, we simultaneously detected multiple nucleic acids with high sensitivity and specificity, making the R-CASTI system a promising HPV testing platform.

As summarized in Table S2, the R-CASTI system exhibits unique advantages over reported CRISPR/Cas-based multiplex detection systems, including cost-effectiveness, low complexity, and relatively short assay time. By elaborately designing context-specific blocker DNA, more than five target nucleic acids can be detected, which indicates the opening of a new way for multiplex detection using the CRISPR/Cas system. However, the detection of target DNA in cell lines and clinical samples was less efficient than with the synthetic DNA, and thus not only further optimization to avoid the interference from other DNA or RNA in the sample but also rigorous clinical validation in a large cohort of patient samples remain to be performed in future studies. Nevertheless, the proposed assay has considerable potential for POC testing, where the synchronous detection of multiple nucleic acids under isothermal conditions is desirable. Furthermore, our detection system could be expanded to the detection of other clinically important biomolecules, such as cancer biomarkers (e.g., miRNA), 16s rRNA of pathogenic bacteria, or other viral RNA.

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CRediT authorship contribution statement

Jinjoo Han: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft. **Jiye Shin:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft. **Eun Sung Lee:** Methodology, Validation. **Byung Seok Cha:** Methodology, Validation. **Seokjoon Kim:** Methodology, Validation. **Youngjun Jang:** Methodology, Investigation. **Seokhwan Kim:** Methodology, Investigation. **Ki Soo Park:** Writing – review & editing, Funding acquisition, Resources, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bios.2023.115323.

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