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High-Fidelity Identification of Single Nucleotide Polymorphism by Type V CRISPR Systems

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Abstract

Accurate and sensitive detection of single nucleotide polymorphism (SNP) holds significant clinical implications, especially in the field of cancer diagnosis. Leveraging its high accuracy and programmability, the CRISPR system emerges as a promising platform for advancing the identification of SNPs. In this study, we compared two type V CRISPR/Cas systems (Cas12a and Cas14a) for the identification of cancer-related SNP. Their identification performances were evaluated by characterizing their mismatch tolerance to the BRAF gene. We found that the CRISPR/Cas14a system exhibited superior accuracy and robustness over the CRISPR/Cas12a system for SNP detection. Furthermore, blocker displacement amplification (BDA) was combined with the CRISPR/Cas14a system to eliminate the interference of the wild type (WT) and increase the detection accuracy. In this strategy, we were able to detect BRAF V600E as low as 10^3 copies with a sensitivity of 0.1% variant allele frequency. Moreover, the BDA-assisted CRISPR/Cas14a system has been applied to identify the BRAF mutation from human colorectal carcinoma cells, achieving a high sensitivity of 0.5% variant allele frequency, which is comparable to or even superior to those of most commercially available products. This work has broadened the scope of the CRISPR system and provided a promising method for precision medicine.

Graphical Abstract

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Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssensors.3c02158.

Detailed experimental procedures; sequences of crRNA and sgRNA; target DNA and FQ probes; primers and blockers for blocker displacement amplification; optimization of LbCas12a/gRNA-2 mixture concentration; detection of BRAF V600E dsDNA using the LbCas12a/gRNA-2 system; optimization of Cas14a/gRNA-3 mixture concentration; optimization of cycle number for BDA (PDF)

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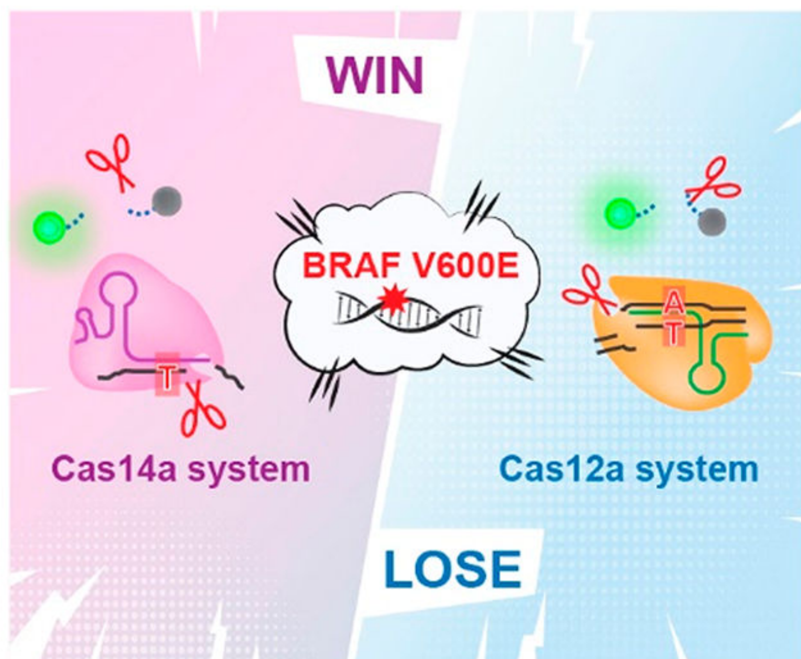

Keywords

CRISPR; BRAF V600E; Cas14; cancer diagnosis; single nucleotide polymorphism (SNP)

Single nucleotide polymorphisms (SNPs) are genetic variations occurring when a single nucleotide changes in the genome. In the past few years, genome-wide association studies have revealed that SNP mutations may directly affect human health by influencing DNA mismatch repair, cell cycle regulation, metabolism, and immunity.[1] Various SNPs have been reported to be associated with cancers.[2] A notable example is the V600E mutation in the BRAF gene, which is caused by the substitution of thymidine (T) with adenosine (A) and has been widely used as a biomarker for cancer diagnostics and treatment. This specific SNP mutation is tumorigenic to human cells by disrupting the normal growth cycle and interfering with extracellular signaling pathways. Therefore, the BRAF V600E mutation is often regarded as a biomarker of aggressive tumor growth in different cancers, such as metastatic melanoma, papillary thyroid cancer, and colorectal cancer.[3,4]

Due to its exceptional programmability and high sensitivity, the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) system has attracted much attention in recent years.[5] The type V CRISPR system (including Cas12a, Cas12b, Cas12f, etc.), renowned for its precise DNA recognition and *trans* cleavage capability, stands out from the crowd of CRISPR systems.[6–8] Currently, there have been only a few studies exploring gene editing using the type V CRISPR nucleases in the BRAF V600E mutation. For example, Cas12a has been exploited to achieve gene disruption of BRAF V600E in the HEK294T cell.[9] Later, structure-guided protein engineering was utilized to improve gene editing efficiency of Cas12a for BRAF V600E.[10] Despite the type V CRISPR system having

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made some progress in BRAF-related studies, most of them are mainly focused on *in vivo* applications, while the *in vitro* identification of the BRAF mutation has not been explored.

Here, we explored the potential of two type V CRISPR systems, CRISPR/LbCas12a and CRISPR/Un1Cas12f (CRISPR/Cas14a), for the identification of the BRAF V600E mutation. The two CRISPR systems share similar sensing elements: CRISPR nuclease, guide RNA (gRNA), and the ssDNA fluorophore-quencher (FQ) probe. Both systems follow an analogous sensing mechanism. In the presence of BRAF V600E, the nuclease/gRNA complex binds to the target, triggering the nuclease's *cis*-cleavage ability of DNA targets. Subsequently, the CRISPR systems indiscriminately cleave (*trans*-cleavage) the ssDNA FQ probes, releasing the fluorophores from the quenchers and generating a fluorescence readout. Therefore, the fluorescence intensity can accurately visualize the response of the CIRSPR system to the DNA target. Through investigating the performance of two type V CRISPR systems, we aim to establish the foundational knowledge for the mechanism of the CRISPR system and expand the potential applications of type V CRISPR nucleases in precision medicine.

As one of the most frequently used type V nucleases in CRISPR-based assays, we first chose LbCas12a to identify BRAF V600E. The LbCas12a system relies on a protospacer-adjacent motif (PAM) sequence to recognize the target region. As shown in Figure 1a, there are two PAM sites (5′-TTTN-3′) near the BRAF mutation. Based on the PAM sites, two gRNAs were designed for the CRISPR system: gRNA-1 (target bottom-strand of V600E, mutation at the 13th base of target region) and gRNA-2 (target top-strand of V600E, mutation at the 1st base of target region. The sequences of all gRNAs are supplied in Table S1. Furthermore, we tested the signal response of each LbCas12a/gRNA system toward V600E and the wild type (WT) using ssDNA or dsDNA targets. The concentration of targets is 10 nM. Phosphate-buffered saline (PBS) without any DNA was used as a negative control. It was found that both systems generated a strong signal response on target ssDNA and dsDNA, and the fluorescence signal of V600E saturated within 10 min (Figure 1b, d).

Next, the end-point fluorescence at 10 min was utilized to investigate whether the LbCas12 systems have the ability to distinguish V600E from WT. The LbCas12a/gRNA-1 system has a strong cleavage activation toward bottom ssDNA and dsDNA targets, but there is no significant difference in fluorescence between V600E and WT (Figure 1c). On the contrary, the LbCas12a/gRNA-2 generated a strong fluorescence response on top ssDNA and dsDNA targets (Figure 1e). It was found that there was a significant difference (p-value < 0.01) in dsDNA between V600E and WT during the first 10 min of reaction. According to previous literature, the CRISPR/LbCas12a system has lower mismatch tolerance within the initial 1st to 7th base of the target region from the PAM, which can be used to discriminate the SNP between the mutation and the WT.[11] Our results in Figure 1e confirm the previous conclusions, indicating that the LbCas12a system is more sensitive to the mismatch of dsDNA near the PAM site, and it is difficult for the LbCas12a system to distinguish the mismatch in the ssDNA substrate. We further investigated the LbCas12a/gRNA-2 system as a potential method to identify the BRAF V600E mutation. To achieve a good detection performance, the concentration of the LbCas12a/gRNA-2 mixture was optimized to 25 nM (Figure S1) and the limit of detection was determined to be 10 nM for the dsDNA target

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(Figure S2a–c). Afterward, we tested the LbCas12a/gRNA-2 system to detect V600E in a mixed solution of V600E and WT. The V600E concentration was fixed at 0.5 nM, and the WT concentrations were adjusted to achieve the different V600E/WT ratios ranging from 0.05% to 10%. It is interesting that all mixtures showed a higher fluorescence signal than the pure V600E in the first 10 min (Figure S2d–f), indicating that it is not feasible for the proposed LbCas12a/gRNA-2 system to resist interference from the WT. It is not an ideal system to be used for the V600E identification.

To find a better CRISPR system for mutation identification, we turned our attention to a smaller type V nuclease: Cas14a. Different from the LbCas12a system, the Cas14a system possesses a PAM-independent property, which makes it more flexible in the design of gRNA. As shown in Figure 2a, two gRNAs were programmed for the CRISPR/Cas14a system: the gRNA-3 aimed to match the bottom strand, while the gRNA-4 was designed to target the top strand. According to previous research, the Cas14a system has a lower mismatch tolerance when the mutation is in the middle of ssDNA target region.[12] To achieve higher sensitivity, both gRNAs were designed to contain the mutation site in the 11th base of the target region. Next, we investigated the cleavage kinetics of each Cas14a/gRNA system on ssDNA or dsDNA targets. Compared to Cas12a systems, Cas14a systems took a longer time to reach the plateau of the fluorescence signal (Figure 2b, d). Within 30 min, the Cas14a/gRNA-3 system displayed a significant fluorescence difference between V600E and the WT on the bottom ssDNA (Figure 2c), demonstrating its potential for further detection. On the contrary, the Cas14a/gRNA-4 system showed a higher mutation tolerance in the target sequence, resulting in a nonsignificant difference between V600E and the WT after 30 min (Figure 2e). The Cas14a/gRNA-4 system has a different but weak signal response on the V600E and WT dsDNA targets. This phenomenon can be explained by the fact that the Cas14a system can recognize dsDNA only in the presence of the PAM. But there is no PAM sequence designed downstream of our targets. Due to the weak fluorescence signals, the Cas14a/gRNA-4 system is not an ideal candidate for V600E detection.

Because of the ability to distinguish V600E from the WT, the Cas14a/gRNA-3 system was further studied to detect the bottom ssDNA targets (Figure 3a). The concentration of the Cas14a/gRNA-3 complex was optimized to be 200 nM (Figure S3). The detection range of the Cas14a/gRNA-3 system was determined to be from 0.5 to 100 nM by measuring the fluorescence intensities corresponding to the bottom ssDNA targets at different concentrations (Figure 3b–c). Subsequently, the time required for the Cas14a/gRNA-3 system to reach a better sensitivity was also investigated. Various concentrations of the WT were mixed with the ssDNA targets at 10 nM to obtain the variant allele frequency ranging from 0.05% to 10%. The Cas14a/gRNA-3 system was used to identify the V600E from the different mixtures. As shown in Figure 3d–f, Cas14a/gRNA-3 can reach a detection limit of 0.5% variant allele frequency (fraction of V600E) in 60 min. Therefore, further CRISPR reactions were performed within 60 min and the corresponding end-point fluorescence intensities were collected.

In clinical scenarios, it is extremely challenging to detect trace amounts of SNP in tumor cells and blood. Moreover, a large amount of the WT may interfere with the accuracy of the detection assay due to the similarity between SNP and its WT. Therefore, we designed a

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blocker displacement amplification (BDA) method combined with a Cas14a/gRNA-3 system to improve detection sensitivity and minimize WT interference. The BDA method is based on the hybridization affinity difference of the forward primer between the V600E and WT templates, leading to an uneven amplification efficiency in each cycle.[13] As shown in Figure 4a, the blocker oligo was completely complementary to the WT template and competed with the forward primer to bind the target template. On the other hand, because the blocker oligo has one base mismatch with the V600E template, the forward primer would easily displace the blocker and continue amplification in the V600E template. According to this principle, V600E and the WT could be unequally amplified and then used for downstream CRISPR/Cas14a reactions. In our study, the reverse primer was modified by phosphorothioate (PT) at the 5′ end, which can protect the bottom strand from degradation by T7 exonucleases and generate the bottom ssDNA as the target for the Cas14a/gRNA-3 system. The gel electrophoresis results indicated that the blocker effectively reduced the level of amplification of the WT during BDA (Figure 4b). The BDA products were further used for CRISPR/Cas14a-based detection (Figure 4c–d). The fluorescence intensities of the WT have a significant difference between the blocker-containing group and the blocker-free group, while the two V600E groups share similar signals. These results demonstrate that BDA visibly reduced the amount of the WT in the products, which in turn minimizes the interference of the WT on the downstream CRISPR assay. Besides, the amplification cycle was optimized to 40 cycles (Figure S4).

After constructing the BDA-assisted CRISPR/Cas14a system, we tested the limit of detection and sensitivity of the proposed system for V600E identification. To eliminate the influence of the baseline on the fluorescence signal, we adopted a normalization of the fluorescence signal. The synthetic V600E and WT dsDNA were amplified by BDA first and then degraded by T7 exonucleases to generate bottom ssDNA. Leveraging the precise recognition capabilities of the Cas14a/gRNA-3 system, the V600E ssDNA can be identified as low as 10^3 copies (Figure 4e). When mixing 10^4 copies of V600E with different concentrations of the WT, the sensitivity of detections can reach 0.1% variant allele frequency (Figure 4f), suggesting that the BDA-assisted CRISPR/Cas14 system can be utilized as an ideal method for BRAF V600E identification.

Moreover, to approach real-life clinical conditions, we tested the performance of the BDA-assisted CRISPR/Cas14a method to detect the BRAF gene extracted from human colorectal carcinoma cells. Colorectal cancer is the third most common cancer and the leading cause of cancer-related deaths worldwide. BRAF mutation occurs in around 10% of colorectal cancers.[3,14] Various concentrations of the BRAF WT gene were mixed with the BRAF V600E gene to obtain the variant allele frequency ranging from 0.01% to 10%. The mixtures were amplified using BDA to increase the fraction of BRAF V600E in the amplicons. Subsequently the BDA products were added into the Cas14a/gRNA-3 system and generated a fluorescence signal within 60 min. As shown in Figure 4g, the developed method can achieve a sensitivity of 0.5% for the V600E DNA in real samples. This sensitivity is much better than that of the most commercially available V600E identification kits, which normally have detection sensitivities above 1%.[15] The insufficient sensitivity has impeded the widespread adoption of early cancer screening and diagnosis. Therefore, our proposed

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method has great potential to be applied in the market for convenient and high-fidelity cancer screening and diagnosis in resource-poor areas.

In summary, we compared the analytical performance of two type V nucleases (Cas12a and Cas14a) for the identification of cancer-related SNP mutations (BRAF V600E). Although Cas12a offers higher reaction efficiency and lower detection limit, the PAM-dependent characteristic restricts the design of gRNA, and the high mutation tolerance makes it difficult to discriminate V600E from the WT. On the contrary, taking the gift of the PAM-independent characteristic and low mutation tolerance, the Cas14a nuclease is exceptionally well-suited for V600E identification. On this basis, we developed the BDA-assisted CRISPR/Cas14a system to achieve high-fidelity identification of the BRAF V600E mutation. By leveraging unequal amplification, it becomes possible to diminish the WT interference in the subsequent CRISPR assay, thereby enhancing the sensitivity of BRAF V600E identification. The total detection time was less than 2 h, which facilitates rapid cancer marker screening. The proposed detection system is comparable or even superior to some commercial SNP detection kits, which demonstrates the potential of CRISPR-based diagnosis technology for future biomedical diagnosis applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

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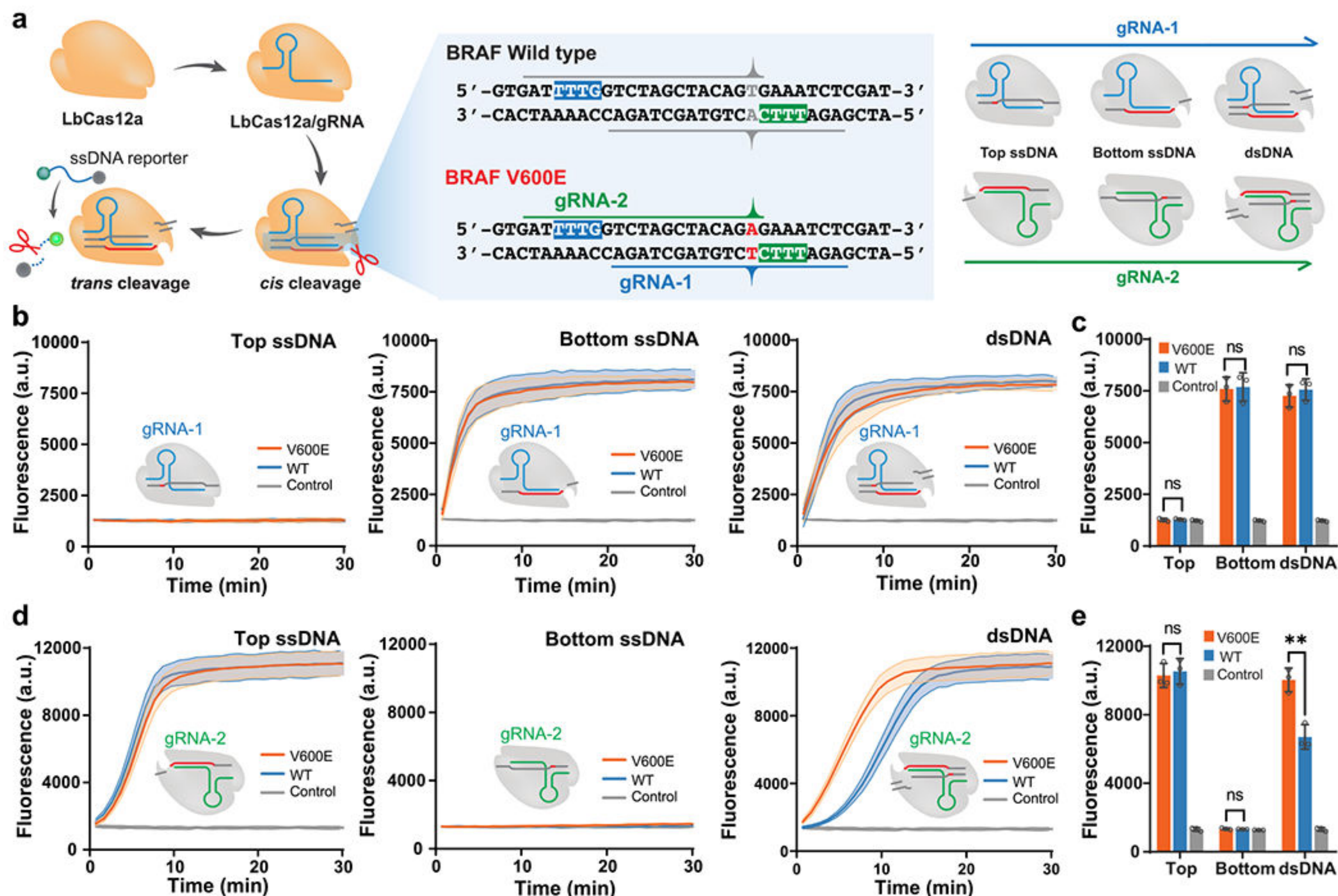

Figure 1.

Detection principle of the CRISPR/LbCas12a system for the discrimination of the BRAF gene. (a) Schematic illustration of CRISPR/LbCas12a activated by three types of substrate DNA: top-strand DNA, bottom-strand DNA, and double-strand DNA. gRNA-1 and gRNA-2 were designed to target bottom-strand DNA and top-strand DNA of the BRAF V600E mutation, respectively. (b) Cleavage kinetics for the LbCas12a/gRNA-1 system targeting top-strand DNA, bottom-strand DNA, and double-strand DNA. (c) End-point fluorescence at 10 min of each type of DNA using the LbCas12a/gRNA-1 system. (d) Cleavage kinetics for the LbCas12a/gRNA-2 system targeting top-strand DNA, bottom-strand DNA, and double-strand DNA. (e) End-point fluorescence at 10 min of each type of DNA using the LbCas12a/gRNA-2 system. All ssDNA and dsDNA concentrations were 10 nM.

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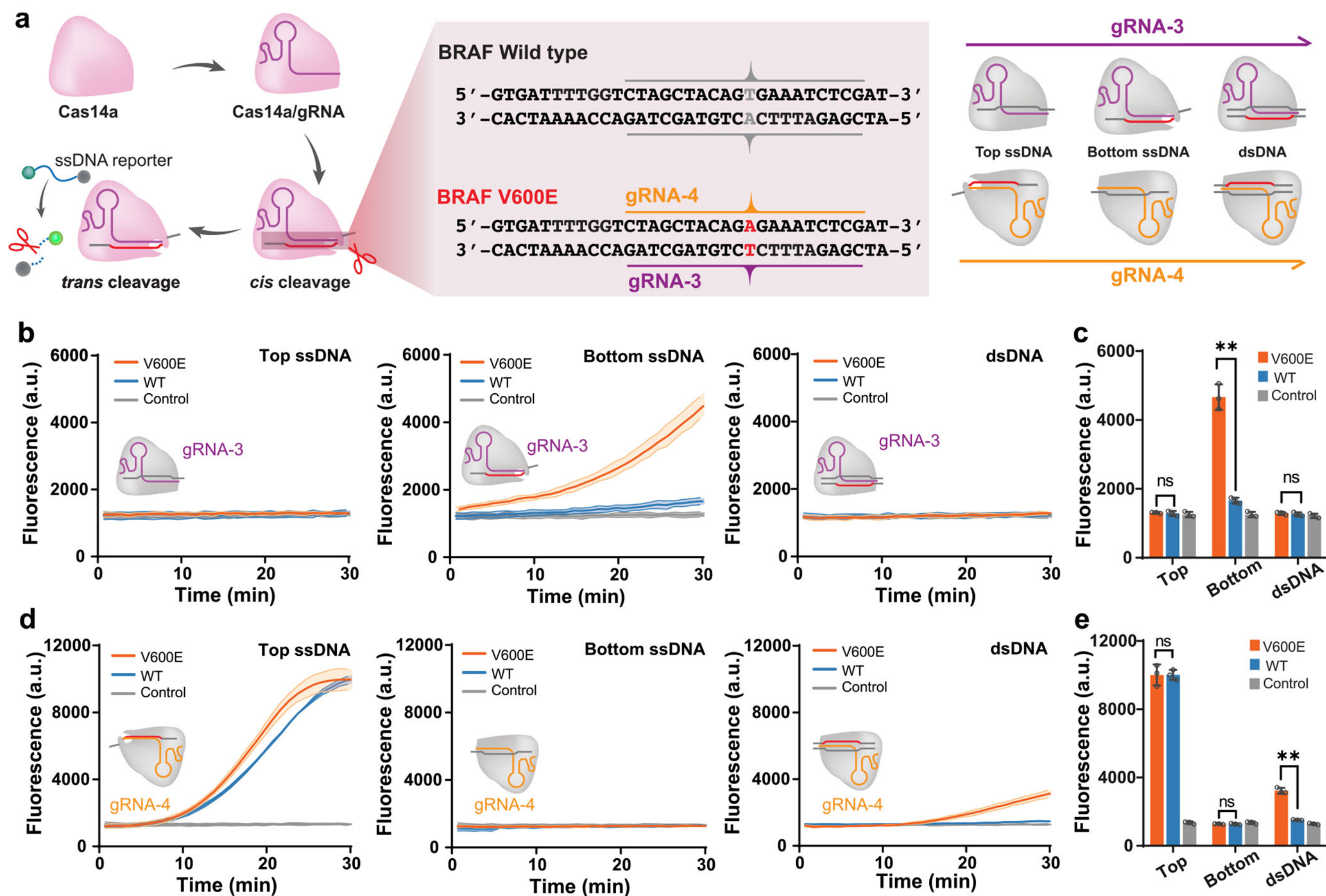

Figure 2.

Detection principle of the CRISPR/Cas14a system for the discrimination of the BRAF gene. (a) Schematic illustration of CRISPR/Cas14a activated by three types of substrate DNA: top-strand DNA, bottom-strand DNA, and double-strand DNA. gRNA-3 and gRNA-4 were designed to target bottom-strand DNA and top-strand DNA of the BRAF V600E mutation, respectively. (b) Cleavage kinetics for the Cas14a/gRNA-3 system targeting top-strand DNA, bottom-strand DNA, and double-strand DNA. (c) End-point fluorescence at 30 min of each type of DNA using the Cas14a/gRNA-3 system. (d) Cleavage kinetics for the Cas14a/gRNA-4 system targeting top-strand DNA, bottom-strand DNA, and double-strand DNA. (e) End-point fluorescence at 30 min of each type of DNA using the Cas14a/gRNA-4 system. All ssDNA and dsDNA concentrations were 10 nM.

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Figure 3.
Detection of V600E bottom ssDNA targets using the Cas14a/gRNA-3 system. (a) Schematic illustration of the Cas14a/gRNA-3 system. (b–c) Cleavage kinetics and end-point fluorescence at 30 min for the Cas14a/gRNA-3 system toward different concentrations of target ssDNA (The ssDNA concentration from low to high: 0, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50, and 100 nM). (d–f) End-point fluorescence of different ratio V600E/WT mixtures detected by the Cas14a/gRNA-3 system at 10, 30, and 60 min, respectively. The concentration of V600E ssDNA was fixed at 10 nM.

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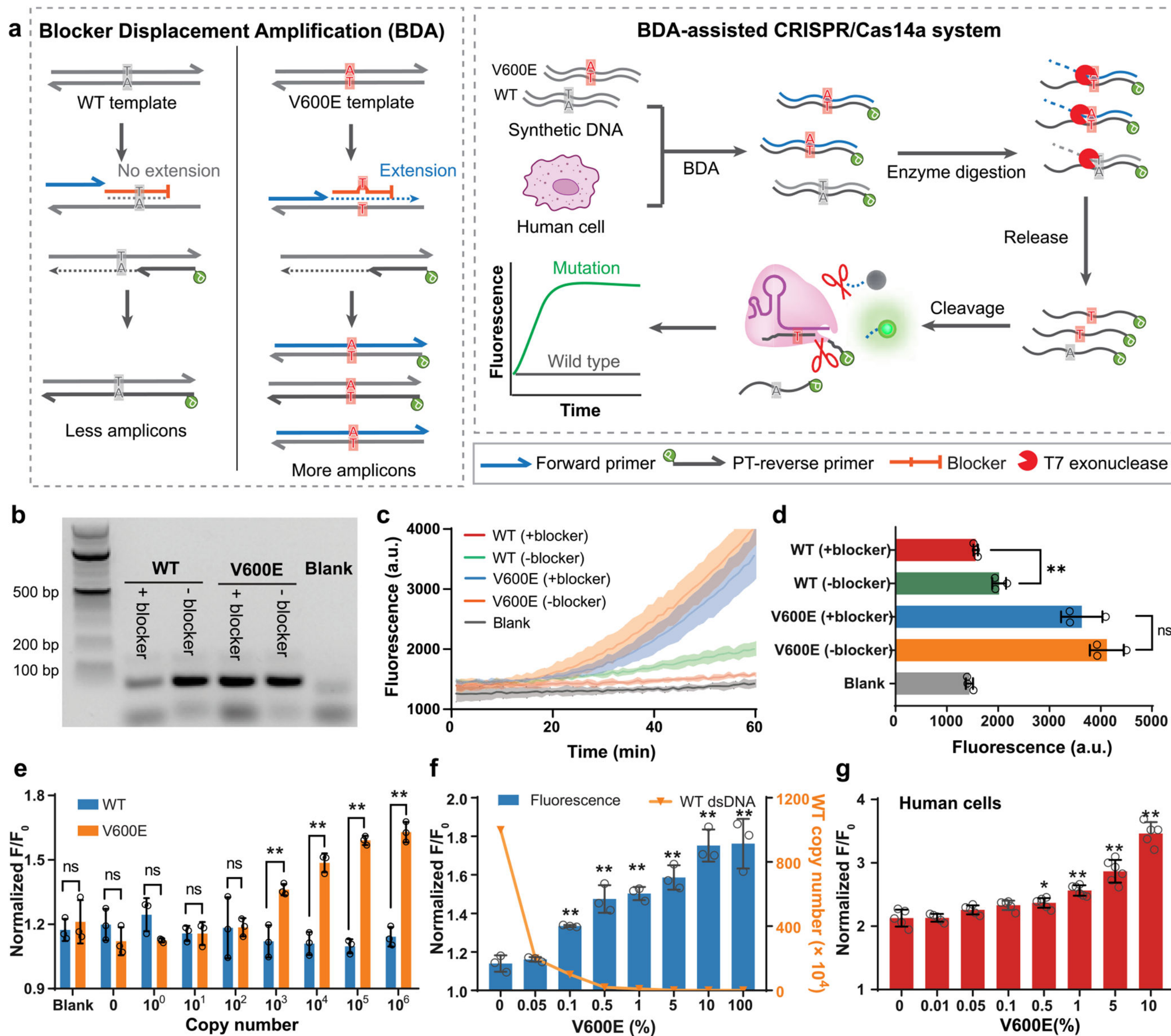

Figure 4.

Real sample detection. (a) Principle of blocker displacement amplification (BDA) and the BDA-assisted CRISPR/Cas14a system. (b) Gel electrophoresis results of BDA for the WT and V600E. (c–d) Cleavage kinetics and end-point fluorescence of BDA products. (e) BDA-assisted CRISPR/Cas14a system for the detection of the WT and V600E with different copy numbers. Normalized F/F_0 was calculated as the end-point fluorescence intensity divided by the initial fluorescence intensity. (f) Signal response of the BDA-assisted CRISPR/Cas14a system toward different ratios of V600E/WT mixtures. The V600E was fixed to 10^4 copies. (g) Detection limits for V600E in human cells using the BDA-assisted CRISPR/Cas14a system.