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A rapid and sensitive CRISPR/Cas12a based lateral flow biosensor for the detection of Epstein–Barr virus†

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Nasopharyngeal carcinoma (NPC) is one of the most common malignant tumors in the world, and several studies have associated Epstein–Barr virus (EBV) with NPC occurrence and development. EBV-PCR (polymerase chain reaction), *in situ* hybridization and immunoassays are the most common methods for NPC identification. However, these approaches have drawbacks, which include tedious procedures and false results. Therefore, a rapid, accurate, and sensitive clinical diagnostic method for the prognosis of EBV-related diseases is needed. In this study, we developed a simple and sensitive approach for EBV detection based on the combination of CRISPR-Cas12a and a lateral flow biosensor (LFB). Cas12a exhibits collateral cleavage propensity of both target DNA and any single-stranded(ss) DNA in the vicinity (herein referred to as a reporter). The LFB test line contained an ssDNA probe complementary to the reporter. In the presence of the target, Cas12a *trans*-cleaved the ssDNA reporter, which resulted in the inability of cleaved sequences to bind the LFB test line. With a PCR pre-amplification of the target (45 min), the assay achieved a sensitivity of 7.1×10^{-14} M (~42 000 copies per μl) both in plasmid and plasmid-spiked samples. The assay attained a high specificity in the presence of various bacteria and applicability in EBV Burkitt's lymphoma serum samples. This method could be applied for the detection of EBV and other infectious diseases.

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Introduction

Epstein–Barr virus (EBV), also known as human herpesvirus IV, belongs to a lymphofollicular virus of the Herpesviridae family. EBV was the first oncovirus to be discovered and its infection rate remains high with a serological positive of up to 90%.[4] EBV infection is associated with NPC Hodgkin's lymphoma, Burkitt's lymphoma, gastric cancer, and other malignancies.[1–3,5–9] According to the International Agency for Research on cancer, EBV is a class 1 carcinogen.[9] It is closely related to NPC, which is one of the most common multiple malignant tumors with high incidence worldwide.[10] Early-stage NPC is more sensitive to radiotherapy by up to 91%.[11] However, due to NPC's hidden location, the symptoms are difficult to distinguish from other benign diseases; thus, most middle and late-stage NPC cannot be accurately diagnosed. Therefore, early detection of the causative agent can be an effective way to improve the treatment of NPC and other EBV related diseases.

The most common methods for EBV detection are enzyme-linked immunosorbent assay (ELISA),[12] *in situ* hybridization[13] and polymerase chain reaction (PCR).[14] ELISA is labor-inten-

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sive and time-consuming because of multiple steps and long incubation time, and because it detects EBV-antibodies in the blood, sometimes a false-positive may occur. Although PCR provides high specificity, sensitivity, and efficiency, it often involves the more laborious steps of gel electrophoresis and analysis and remains susceptible to cross-contamination.

To overcome these drawbacks, diagnostic procedures that make use of the CRISPR/Cas system have been developed. For example, DNA Endonuclease Targeted CRISPR *Trans* Reporter (DETECTR),[15] Specific High Sensitivity Enzymatic Reporter UnLOCKing (SHERLOCK),[16,17] and a one-HOur Low-cost Multipurpose highly Efficient System (HOLMES)[18,19] detection systems rely on the collateral cleavage of the target and any single nucleic acid in the vicinity (reporter). In the presence of the target, fluorescence signals are detected upon the CRISPR/Cas system *trans*-cleavage of the fluorophore–quencher labeled reporter. To increase the sensitivity of CRISPR diagnostics, the target gene is amplified by specific amplification methods such as PCR or LAMP (for HOMES), while the SHERLOCK and DETECTR employed Recombinase Polymerase Amplification (RPA). RPA is a new isothermal amplification of nucleic acid amplification technology. Its most significant advantages are that it can amplify specific nucleic acid sequences at a constant temperature (25–43 °C), and the results can be readout within 20 minutes. Although these approaches are sensitive and specific, their reliance on fluorescence apparatuses increases the cost and time. They are inconvenient for resource-limited settings. Therefore, the development of DNA-probe based LFBs could be an alternative approach for sensitive, specific, and inexpensive detection.

A lateral flow biosensor (LFB) is a biosensor in which the results can be analyzed with the naked eye.[20,21] Its structure mainly includes five parts: sample pad, conjugate pad, nitrocellulose membrane, and absorbent pad assembled on a plastic backing pad. It has the advantages of simple operation, high sensitivity and specificity, and low cost. Hence, it has been widely used in clinical diagnosis and environmental monitoring.

In this study, we present a CRISPR/Cas system based LFB for the detection of EBV-DNA. The LFB principle is that in the presence of Epstein–Barr virus nuclear antigen 2 (EBNA-2) coding DNA (hereafter referred to as the EBV-DNA target),[22] the *lachnospiraceae bacterium* Cas12a (LbaCas12a) cleaves an ssDNA reporter and the target collaterally. The *trans*-cleaved ssDNA reporter cannot bind to a complementary DNA immobilized on the LFB test line. In contrast, the presence of a test line indicates the absence of a target (Fig. 1). Based on the PCR pre-amplification and specific crRNA design, the LFB successfully detected EBV-DNA with high sensitivity and specificity. This assay shows great promise in practical applications for infectious disease detection.

Materials and methods

Reagents and chemicals

All oligonucleotides, including EBV-DNA specific target sequences, primers, crRNAs (designed using benching), reporters, and LFB capture probes were synthesized by Sangon Biotech (Shanghai, China) (Table 1). Purified LbaCas12a was purchased from New England Biolabs (New England, USA). Taq polymerase premix was purchased from Takara (Takara, China).

$HAuCl_4 \cdot 3H_2O$, trisodium citrate, and SA were purchased from Sigma-Aldrich (Steinheim, Germany). Absorbent and fiberglass papers and nitrocellulose membranes (NC) were purchased from Sartorius AG (Gottingen, Germany). A dispenser for antibody, probe DNA, conjugates, and a nitrocellulose membrane cutter were purchased from Shanghai Kinbio (Shanghai, China). Biotin-labeled goat anti-mouse IgG (H+L) was purchased from Beyotime Biotechnology (Shanghai, China). A portable strip reader was purchased from Goldbio Technology Co. (Shanghai, China). Whole blood genome and plasmid extraction kits were purchased from Qiagen (Hilden, Germany) and Tiangen Biotech (Beijing, China), respectively. All buffers used in this study were prepared in our laboratory.

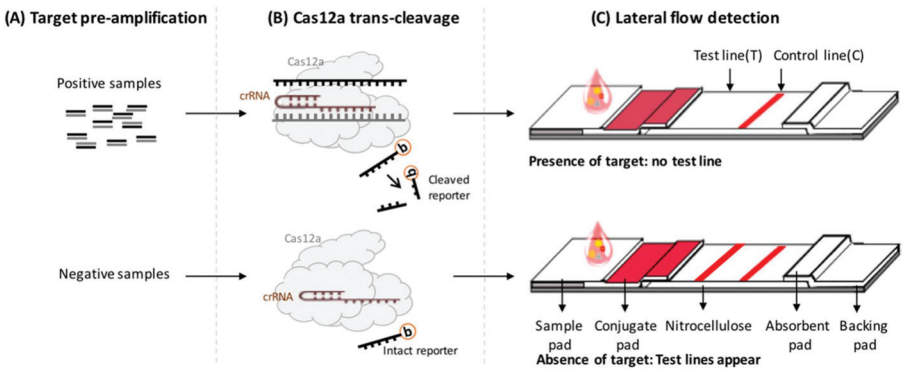

Fig. 1 Schematic representation of the CRISPR based LFB for EBV-DNA detection. (A) Genomic DNA is extracted from samples and PCR pre-amplification. (B) Cas12a reaction using the PCR product. (C) The product in (B) is loaded onto the LFB containing a DNA capture probe and biotinylated IgG immobilized on the test and control zones, respectively. The positive samples exhibit no test line as a result of reporter *trans*-cleavage, while negative ones display a test line.

Table 1 Oligonucleotide sequences used in this study

Oligonucleotide	Sequence (5′-3′)
Reporter (8TATT)	B-TTTTTTTTATT
Test line capture probe	AATAAAAAAAAAAAAAAAAAATAAAAAAAAAAAAAAAA
EBV-DNA	TGGAAACCCGTCACTCTCAGTAATTCCCTCGAATCCCTACCAGGAACAACTGTCAGACACTCCATTAATTCCACTAACAATCTTT GTTGGGGAAAACACGGGGGTGCCCCCACCACTCCCACCACACCCCCCCCACCACCACACCCCCACCACACCCCCACCAC CCCCACCACCCCCACCACCTCCACCACCTTCACCACCACCCCCCGCCCCCACCACCCCCACCACCTCAGCGCAGGGATGCCTG GACACAAGAGCCATCACCTCTTGATAGGGATCCGCTAGGATATGATGTCGGGCATGGACCTCTAGCATCTGCTATGCGAATGC TTTGGATGGCTAATTATATTGTAAGACAATCACGGGGTGACCGGGGCCTTATGTTGCCACAAGGCCCACAAACAGCCCCTCAG GCCAGGTTGGTACAGCCACATGTCCCCCCTCTACGCCCGACAGCACCCACCATTTTT**GTCACCTCTGTCACGACCGAGGCT**TA CCCCTCCACAACCACTCACGA
Forward primer	CCACCTCCACCACCTT
Reverse primer	TCGTGAGTGGTTGTGGA
crRNA	UAAUUUCUACUAAGUGUAGAU**GUCACCUCUGUCACGACCGAGGCU**
Activator	AGCACCCACCATTTTT**GTCACCTCTG**

B stands for 5′ biotinylation. Underline represents the reporter's capture probe sequence (repeated twice). Bold represents the crRNA targeting sequence.

Other chemicals were purchased from commercial sources with standard analytical grade purity.

Preparation of AuNPs and conjugates

AuNPs of 15 nm average diameter were prepared by the citrate reduction method[23] with slight modifications. Briefly, 350 ml of 1 mM aqueous gold chloride solution ($HAuCl_4$) was boiled, stirred in a flask, and 3.5 ml of 1% sodium citrate solution was subsequently added to it. The colorless mixture turned to wine red after boiling for 10 min and then it was cooled to room temperature for further use.

To prepare the AuNP–SA conjugate, 1 ml of prepared AuNP solution was collected by centrifugation (12 000 rpm, 25 min), concentrated four times, and subsequently mixed with 100 μl of suspension buffer (20 mM Na_3PO_4, 5% BSA, 0.25% Tween-20, 10% sucrose, and 0.1% NaN_3). Then, 0.5 mg ml^{-1} SA was added, gently shaken for 3 hours at 4 °C, centrifuged (12 000 rpm, 25 min), and then rinsed three times with suspension buffer to remove unbound SA. The red pellet was resuspended in 100 μl of suspension buffer before dispensing on the conjugate pad.

Construction of LFB

As shown in Fig. 1, the LFB is composed of three main components: sample pad, NC membrane, and absorbent pad. The capture probe 3′-16A-T-AA-5′ (100 μM) and biotinylated rabbit polyclonal anti-IgG antibody (0.7 mg ml^{-1}) were dispensed on the LFB NC membrane (25 mm in width) to form the test and control zone, respectively; by keeping the distance between T-line and C-line approximately 5 mm. The membrane was exposed to UV light for 15 min in a laminar flow cabinet, dried at 25 °C for 12 h and stored at 4 °C until use. To assemble the LFB, a fiberglass sample pad (16 mm in width) was immersed in sample pad buffer (1% Triton, 1% BSA, 2% glucose, and 50 mM boric acid; pH 8.0), dried and stored in a desiccant container. The conjugate and absorbent pads (6 mm and 17 mm in width, respectively) were used without buffer treatment. Then, all pads were sequentially assembled along the adhesive part of the NC with an overlap of 2 mm and then cut into 0.4 cm-wide strips.

Target DNA design, cloning, and amplification

The EBV specific DNA sequence herein used is an EBV conserved region of EBNA-2 gene[22] cloned into a customized pUC57 vector and the resultant recombinant plasmid was used as a model for PCR amplification and CRISPR/Cas12a reaction. For PCR amplification, 25 μl PCR premix was composed of 0.3 μM forward and reverse primers, 12.5 μl 1× rTaq Premix, and 1–2 μl of extracted genomic DNA (200 ng) and ddH_2O was added to the final reaction volume. The reaction mixture was run in a thermocycler for 45 min with 95 °C, 5 min for denaturation; 95 °C, 10 s for each cycle; 62 °C, 10 s for annealing; 72 °C, 30 s for elongation; 35 cycles. PCR products were analyzed by using 2.5% agarose gel electrophoresis.

Target cleavage assay

Cas12a cleavage assay was carried out according to a previously described method[15] with slight modifications. Briefly, the Cas12a-crRNA complex was preassembled with 6 μl of cleavage buffer (150 mM KCl, 10 mM $MgCl_2$, 1% glycerol, 0.5 mM DTT, and 20 nM HEPES (pH 7.5)), 3 μl of crRNA (300 nM) and 1 μl of LbaCas12a (1 μM) incubated at 37 °C for 15 min. The preassembled complex was then mixed with 2 μl of activator (400 nM), 2 μl of biotinylated ssDNA reporter (600 nM), and 2 μl of target DNA or real heated samples, and then 25 μl of nuclease-free water was added to the final reaction volume. Finally, the reaction was incubated at 37 °C and analyzed using the LFB after adding 35 μl of ddH_2O water prior to sample pad loading.

For fluorescence analysis of the *trans*-cleavage reaction, the Cas12a reaction set up was prepared as mentioned above with the 5′-FAM and 3′-TAMRA labeled ssDNA reporter instead of the biotinylated ssDNA reporter. The reaction was incubated at 37 °C in a 96-well microplate and then read using the Mithras[2] LB 943 plate reader (Berthold Tech, Germany). The fluorescence from the cleavage of the reporter was measured from 0 to 1 hour (excitation at 495 nm and emission at 520 nm).

LFB detection of reporter sequences

Prior to sample analysis, the 11 base biotinylated reporter (5′-biotin-TTTTTTTTATT-3′) was used for the Cas12a cleavage assay, which in the absence of cleavage can hybridize with the 38 base capture probe (AATAAAAAAAAAAAAAAAAAATAAAAAAAAAAAAAAA) immobilized on the LFB test line. To analyze the LFB reporter cleavage after the Cas12a reaction, 25 μl of the reaction mixture was mixed with 35 μl of ddH2O and then loaded onto the sample pad. After 5 min, the biosensor was read using a portable LFB reader. The peak area intensities of the test lines were plotted after LFB photo analysis using ImageJ (NIH Research Services, US), which provided similar intensities between each analyzed triplicate. The Cas12a cleavage performance was considered positive when the reporter sequence was entirely *trans*-cleaved by Cas12a (no test line signal), and the control line containing the biotinylated rabbit polyclonal anti-IgG antibody captured the excess of AuNP–SA complex from the conjugate pad (Fig. 1). Therefore, the development of DNA-probe based LFBs could be an alternative approach for sensitive, specific, and inexpensive detection.

Preparation and analysis of EBV plasmid spiked and clinical samples

EBV-negative/positive serum samples were used in our assay. Two hundred μl of each negative sample was spiked with different concentrations of EBV-DNA containing plasmid and was then subjected to extraction using the whole blood genome extraction kit. EBV-DNA from frozen clinical serum samples were similarly extracted without pretreatment. Two μl of each extracted DNA was subjected to PCR and then the Cas12a cleavage assay was applied, as mentioned above.

Results and discussion

Establishment of CRISPR based LFB biosensor for EBV detection

Two main components orchestrate the CRISPR/Cas12a based LFB: (i) the ssDNA reporter, which is *trans*-cleaved by Cas12a providing a signal upon target recognition and (ii) LFB probes immobilized on the LFB test line to capture the reporter. Therefore, as we previously reported,[24] reporters and probes of different lengths of bases were used to monitor reporter *trans*-cleavage (Table 1). On the LFB, the biotinylated reporter binds to the AuNPs–SA complex at the conjugate pad and migrates to hybridize with its complimentary test-line probes. As shown in Fig. 2A, weak test lines were detected when 100 nM 11 base reporter (8TATT) dissolved in double-distilled water was applied to the LFB with a similar length probe on the test line. The increase of the test line probe to 38 bases strongly detected the 11 base reporter. Therefore, the 11 base reporter was used as the Cas12a substrate to obtain no test line upon *trans*-cleavage, while the full-length ssDNA reporters were hybridized with the test-line probes in the absence of *trans*-cleavage. We further tested different concentrations of the optimized reporter (25, 50, and 100 nM) on the LFB (Fig. S1†). The test line was detected at a reporter concentration above 10 nM, which became prominent with a subsequent increase in the concentration (Fig. S1†). The 50 nM reporter provided a strong test line in the absence of the target, and a non-detectable test line signal in the presence of the target (Fig. 2B). The concentration of 50 nM and the reporter length of 11 bases were then used for subsequent Cas12a cleavage experiments. Moreover, this system also requires crRNA, Cas12a, and an activator DNA,[15] which is a short sequence partially comp-

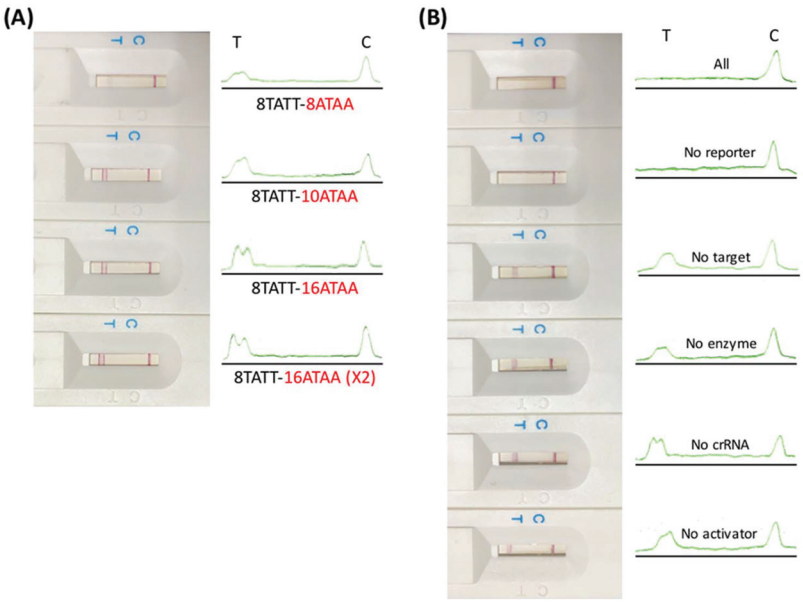

Fig. 2 Reporter length optimization and controls for CRISPR based LFB. (A) LFB images (left) and intensity chromatograms (right) corresponding to the 100 nM reporter (8TATT, in black) subjected to different lengths of test line probes immobilized on the test line (in red). (B) LFB images of the CRISPR based LFB for EB detection in the presence of its all reaction components (All) or absence of one component.

lementary to the crRNA. The activator triggers the Cas12a enzyme to *trans*-cleave the ssDNA reporter in the presence of the target (Fig. 2B).

Sensitivity of the biosensor for EBV detection

The sensitivity of the biosensor was assessed using different concentrations of serially diluted EBV recombinant plasmid (7.1×10^{-9}, 7.1×10^{-10}, 7.1×10^{-11}, 7.1×10^{-12}, 7.1×10^{-13}, 7.1×10^{-14}, 7.1×10^{-15}, and 7.1×10^{-16} M) applied to the CRISPR based LFB with and without PCR pre-amplification. There was no detectable test line at 7.1×10^{-14} M and above for PCR pre-amplified target plasmid (45 min), while strong test lines were observed at $\leq 7.1 \times 10^{-15}$ M (Fig. 3 and S2†). Without PCR pre-amplification, our assay detected 7.1×10^{-9} M alone (Fig. S3†). These results suggested that the developed LFB had high sensitivity for EBV testing. Although this sensitivity is less than previously reported isothermal amplification-based CRISPR diagnostics[15,16] and nested PCR,[25] it is still comparable to PCR based CRISPR approaches.[18,19,26] However, our assay was accomplished without tedious gel electrophoresis, sophisti-

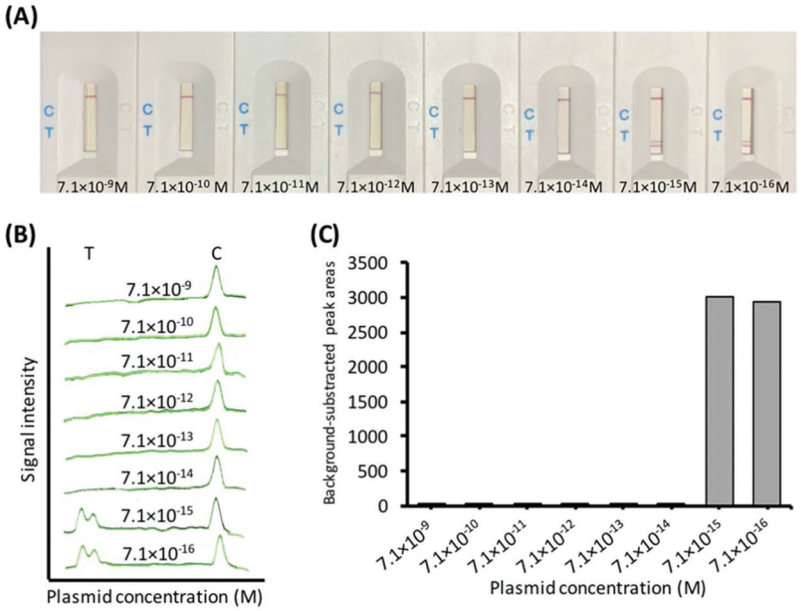

Fig. 3 Sensitivity of the CRISPR based LFB assay. (A) From left to right, the concentration of EBV recombinant plasmid subjected to PCR and then to CRISPR based LFB. (B) Peak intensities corresponding to the LFB in (A). (C) Calculated peak areas corresponding to the LFB test lines in (A).

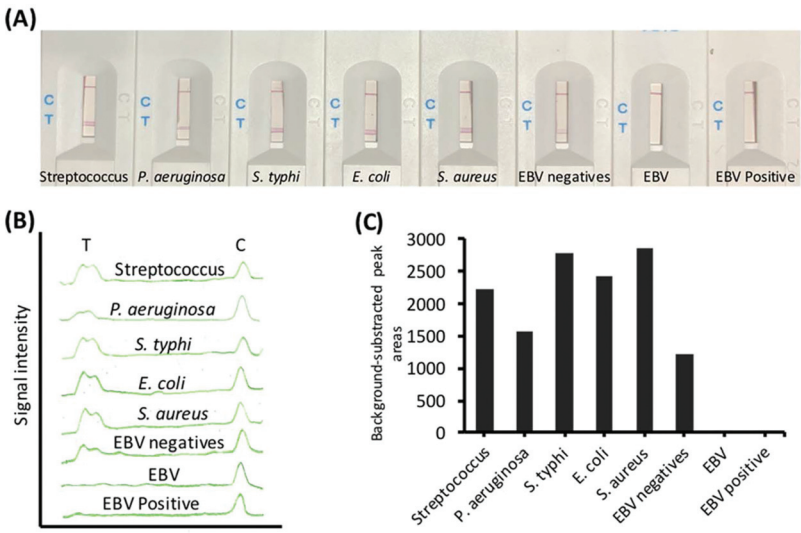

Fig. 4 Specificity of the CRISPR based LFB. (A) LFB images (left) and intensity chromatograms (right) corresponding to human DNA, different bacterial strains (10^4 cfu ml^{-1}), EBV recombinant plasmid (EBV), and clinical samples. (B) Peak intensities corresponding to the LFB in (A). (C) Calculated peak areas corresponding to the LFB test lines in (A).

cated PCR, or fluorescence reader apparatus, reducing susceptibility to false positives that can be generated from fluorescence background. Notwithstanding that, one can combine our LFB with other pre-amplification methods such as RPA, LAMP, *etc*. to enhance the sensitivity to a single copy of EBV-DNA.

Clinical sample and selectivity of the biosensor for EBV detection

EBV can cohabit with other microbiomes in the bloodstream, other body fluids such as saliva, and even can spread *via* objects or human interaction.[27] Thus, the sensor's selectivity was tested using various bacterial strains (*Streptococcus* sp., *P. aeruginosa*, *S. typhi*, *E. coli*, and *S. aureus*), 10 EBV negative serum, 1 available EBV positive serum and EBV recombinant plasmid as a positive control (EBV). Fig. 4A shows the typical photo images of the biosensor. Except for the absence of EBV, which showed similar detectable test lines, the distinctive cleavage was only observed in the presence of EBV (Fig. 4A). These results were confirmed by the detected peak intensities and areas (Fig. 4B and C).

Conclusion

In summary, a lateral flow biosensor based on CRISPR-Cas12a combined with PCR was successfully constructed for the detection of EBV. CRISPR-Cas12a provided high specificity derived from its crRNA, while the LFB made the assay fast, inexpensive, and straightforward by avoiding gel electrophoresis processes, which reduced in part cross-contamination. The short period of PCR pre-amplification enriched the target and improved the LFB sensitivity, which is higher than the standalone PCR. The high corroboration of PCR and CRISPR based LFB also demonstrated a high specificity as shown in both EBV recombinant plasmid, clinical samples, and various bacteria. Our established CRISPR based LFB could be applied to the on-site diagnosis of infectious diseases by using their specific gRNAs and the introduction of isothermal amplification approaches.

Author contributions

O.M. and L.Z. contributed to the concept and biosensor design, fabrication, and manuscript preparation. T.Y. and O. M. performed the experiments and wrote the manuscript. Z.L., W.C., Y.Z., J.D.D.H., Y.Z., R.Z. and C.N. provided experimental support and performed manuscript proofreading. Z.H. and L. Z. directed and supervised this work.

Conflicts of interest

There are no conflicts to declare.

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