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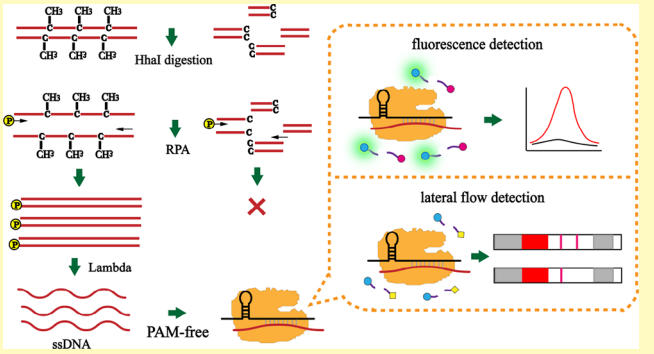
Endonuclease-Assisted PAM-free Recombinase Polymerase Amplification Coupling with CRISPR/Cas12a (E-PfRPA/Cas) for Sensitive Detection of DNA Methylation

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ABSTRACT: DNA methylation is considered as a potential cancer biomarker. The evaluation of DNA methylation level will contribute to the prognosis and diagnosis of cancer. Herein, we propose a novel assay based on endonuclease-assisted protospacer adjacent motif (PAM)-free recombinase polymerase amplification coupling with CRISPR/Cas12a (E-PfRPA/Cas) for sensitive detection of DNA methylation. The methylation-sensitive restriction enzyme (MSRE) is first used to selectively digest unmethylated DNA, while the methylated target remains structurally intact. Therefore, the methylated target can initiate the RPA reaction to generate a large amount of double-stranded DNA (dsDNA). To avoid the dependence of PAM site of CRISPR/Cas12a, one of the RPA primers is designed with 5′-phosphate terminuses. After treating with Lambda, the sequence with 5′-phosphate modification will be degraded, leaving the single-stranded DNA (ssDNA). The CRISPR/Cas12a can accurately locate ssDNA without PAM, then initiating its trans-cleavage activity for further signal amplification. Meanwhile, non-specific amplification can be also avoided under Lambda, effectively filtering the detection background. Benefiting from the specificity of MSRE, the high amplification efficiency of Lambda-assisted RPA, and the self-amplification effect of CRISPR/Cas, the E-PfRPA/Cas assay shows outstanding sensitivity and selectivity, and as low as 0.05% of methylated DNA can be distinguished. Moreover, the lateral flow assay is also introduced to exploit the point-of-care diagnostic platform. Most importantly, the proposed method shows high sensitivity for determination of genomic DNA methylation from cancer cells, indicating its great potential for tumor-specific gene analysis.



KEYWORDS: *PAM-free, RPA, CRISPR/Cas12a, MSRE, DNA methylation*

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INTRODUCTION

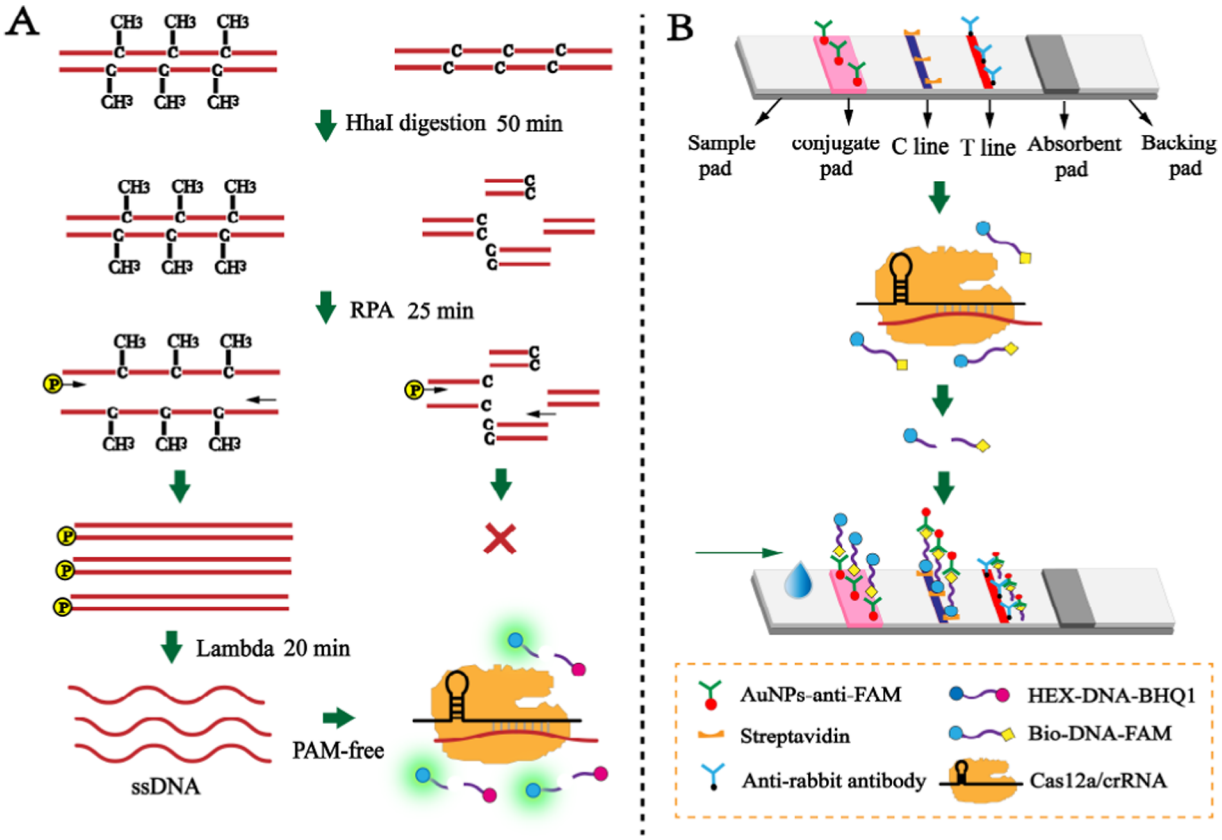
Many epigenetic changes occur early in tumorigenesis, and these changes are ubiquitous in different tumor types. Among them, DNA methylation is the most characteristic and common epigenetic modification, which is related with gene expression and gene stability.[1,2] Current advanced studies suggest that changes in DNA methylation levels in specific regions (regional methylations) are associated with different types of cancer, especially the hypermethylation in promoter regions.[3,4] For example, the hypermethylation of septin 9 gene in its promoter region is regarded as a promising biomarker for early diagnosis of colon cancer.[5] Therefore, the detection of methylation in specific regions is of great significance for early cancer screening and refined treatment.

Currently, a plethora of molecular detection approaches, including methylation-sensitive restriction enzyme (MSRE)-polymerase chain reaction (PCR),[6] bisulfite sequencing,[7] and mass spectrometry,[8] have been developed for analyzing DNA methylation. These methods are mainly based on the principle of bisulfite conversion or MSRE digestion.[9,10] The bisulfite-based conversion reaction is considered as a gold-standard method for determining DNA methylation, which is primarily to convert unmethylated cytosine (C) to uracil (U) by bisulfite. However, the process needs to be carried out under acidic and high-temperature conditions, which will cause DNA degradation and detection failure.[11,12] The MSRE-based method provides a bisulfite-independent way for determination of DNA methylation. The MSRE is a class of restriction endonucleases that are sensitive to methylated bases at their

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Scheme 1. Schematic Illustration of the E-PfRPA/Cas Strategy for Detection of DNA Methylation (A) and Its Application in LFA (B)



recognition sites, thereby distinguishing methylated from unmethylated sequences.[13,14] These endonucleases perform under mild conditions, which avoid DNA degradation.[15] However, the amount of methylated DNA from cancer cells presents as low as 0.1%, and a large amount of DNA is unmethylated.[16] Therefore, the sensitivity and accuracy for DNA methylation detection based on MSRE digestion should be further improved.

Nucleic acid amplification is a promising strategy for target signal amplification. Among the numerous amplification techniques, isothermal amplification, such as rolling circle amplification,[17] exponential amplification reaction,[18] and recombinase polymerase amplification (RPA),[19] have attracted increasing attention. Compared with conventional PCR-based detection which requires a thermal cycle process, isothermal amplification can be performed under isothermal conditions with a high amplification efficiency.[20] RPA is a highly sensitive isothermal amplification approach that works at 37−42 °C and can obtain a mass of amplification products less than 20 min.[21] Based on the above characteristics, RPA can be applied for developing point-of-care detection platforms for DNA methylation. It is worth noting that RPA is often combined with various tools to further improve its accuracy, sensitivity, and selectivity.[22,23] Among them, the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas system as a powerful diagnostic tool has attracted wide attention.[24] By combining nucleic acid amplification with the CRISPR/Cas system, the detection sensitivity and specificity are significantly improved. In addition, a variety of techniques are developed based on different CRISPR/Cas systems, such as SHERLOCK (Cas13),[25,26] DETECTR (Cas12 or Cas14),[27,28] and HOLMES (Cas12).[29,30] In the V-A type CRISPR system, CRISPR/Cas12a (Cpf1) exhibits specific recognition ability for double-stranded DNA (dsDNA) and can stimulate its trans-cleavage activity for target detection.[31] However, these usually require the protospacer adjacent motif (PAM) sites to locate the target region. Actually, many disease-related targets have no PAM sites, which greatly limits the application of the CRISPR/Cas system. In view of this, many studies have been devoted to develop PAM-free assays, such as μReaCH-PAD,[32] RACE,[33] and CUT-LAMP,[34] which are achieved by introducing with additional PAM sites upstream of the recognition fragments. However, these methods require strict primer design and are prone to false positive results. The search for the PAM-free nucleases may be a promising approach.[35] However, it still faces some problems, such as technical challenges, a prolonged target recognition time, and increased off-target tendency.[36] Therefore, it is necessary to develop a simple, accurate, and universal methylation detection method.

Herein, we exploit a novel assay based on endonuclease-assisted PAM-free RPA combined with CRISPR/Cas12a (E-PfRPA/Cas) for sensitive determination of DNA methylation. In the proposed method, RASSF1A methylation in its promoter region is regarded as an effective biomarker for breast cancer.[37,38] To detect RASSF1A methylation, methylation-sensitive restriction endonuclease is first applied to digest the unmethylated DNA. When the recognition site is methylated, the target DNA will not be cleaved and remain structurally intact, then triggering the RPA reaction to obtain abundant products within minutes. In order to realize PAM-free recognition of CRISPR/Cas12a, the reverse primers of RPA are designed with 5′ phosphate modification. Then

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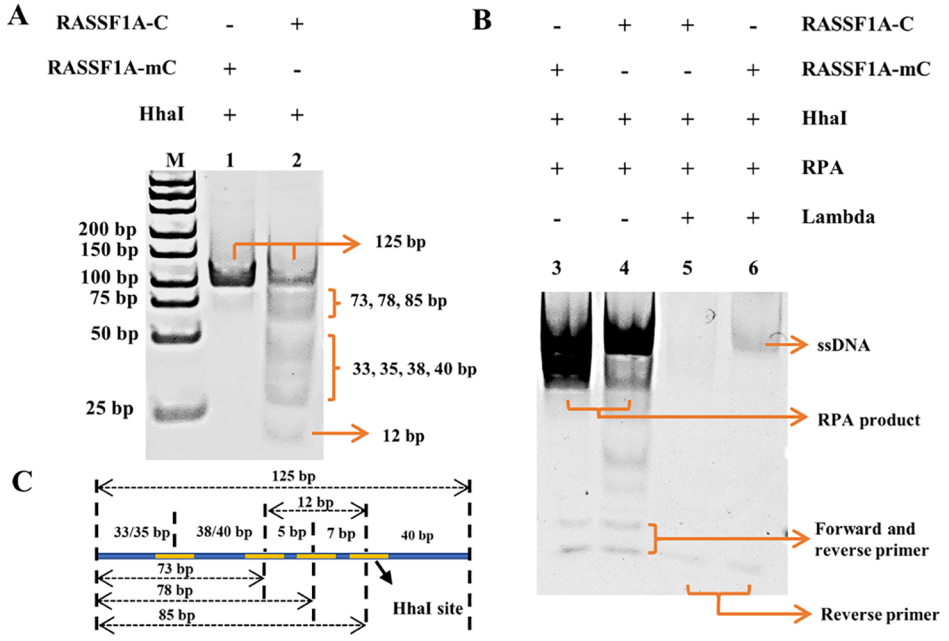


Figure 1. Feasibility verification of the proposed assay. (A) PAGE image of HhaI digestion; lane 1: RAFFS1A-mC/HhaI; lane 2: RAFFS1A-C/HhaI. (B) PAGE image of RPA and Lambda exonuclease treatment; lane 3: RAFFS1A-mC/HhaI/RPA; lane 4: RAFFS1A-C/HhaI/RPA; lane 5: RAFFS1A-C/HhaI/RPA/Lambda; lane 6: RAFFS1A-mC/HhaI/RPA/Lambda. (C) Recognition sites of HhaI in the RAFFS1A sequence.

Lambda exonuclease is used to release the mass of single-stranded DNA (ssDNA). The obtained ssDNA could be recognized by the CRISPR/Cas12a without PAM sites so as to activate its trans-cleavage activity for fluorescence detection and lateral flow strips. As a result, the E-PfRPA/Cas method shows high sensitivity and specificity and can be applied for actual sample detection. With such a simple design, CRISPR/Cas detection can be achieved without PAM sites, and non-specific signals can be effectively filtered to avoid false-positive results. More importantly, based on the programmability of crRNA, this method can be extended to the detection of other nucleic acid markers.

EXPERIMENTAL METHODS

All experiment procedures are presented in the Supporting Information, which include reagents, preparation of crRNA and the methylated target, amplification reaction process, CRISPR/Cas12a detection, polyacrylamide gel electrophoresis (PAGE) analysis and cell culture, and so on.

RESULTS AND DISCUSSION

Detection Principle of E-PfRPA/Cas Assay. The principle of detecting DNA methylation based on endonuclease-assisted PAM-free RPA coupling with CRISPR/Cas12a (E-PfRPA/Cas) is shown in Scheme 1, which incorporates the high amplification efficiency of HhaI-assisted RPA, efficient exonuclease activity of Lambda, and high recognition specificity of the CRISPR/Cas12a system. It can be seen from Scheme 1A that the methylated DNA and unmethylated DNA are first treated by HhaI exonuclease, which could identify and digest "-GCGC-" sites. Thus, unmethylated DNA will be digested by HhaI. In this work, RAFFS1A gene promoter contains DNA sites of −GCGC−. Thus, unmethylated RAFFS1A is fully digested by HhaI. In contrast, methylated RAFFS1A is unaffected and remains structurally intact. Then the intact DNA is used to trigger the next RPA reaction. In the PRA system, recombinant enzymes bind to primers to locate homologous sequences in the dsDNA and amplify the target region exponentially. In contrast, the amplification of unmethylated RAFFS1A will not occur due to the digestion of HhaI. Because a phosphate group is modified at the 5′ end of the reverse primer, all sense strands in the amplified duplex have the 5′-phosphate terminuses. Lambda exonuclease can degrade the sequence with 5′-phosphate modification in dsDNA. Therefore, large amounts of ssDNA are obtained via Lambda treatment. Meanwhile, non-specific amplification can be avoided under Lambda, effectively filtering the detection background. The produced ssDNA is partially complementary to crRNA so as to stimulate the trans-cleavage activity of Cas12a, which can indiscriminately cleave the nearby ssDNA reporter (HEX-DNA-BHQ1) to release the fluorescence signal with a high speed of thousands of turnovers per second.

In addition, the proposed assay can be incorporated into a lateral flow assay (LFA) for point-of-care detection by changing ssDNA reporter (Bio-DNA-FAM). As shown in Scheme 1B, biotin and FAM are modified at both ends of the ssDNA reporter so that the DNA could bind the AuNPs-*anti*-FAM. Due to the presence of streptavidin in the control line (C line), the AuNPs-*anti*-FAM/ssDNA reporter can be captured through biotin binding to the streptavidin. Thus, a red band appears on the C line. When there are methylation targets, they can initiate a series of reactions to produce the mass of ssDNA. Then the trans-cleavage activity of Cas12a will be activated to cut the ssDNA reporter. The cracked DNA with AuNPs can be captured by anti-rabbit antibodies on the test line (T line). As expected, a red band will appear on the T line at the same time. In contrast, only a red band is observed on the C line in the presence of the unmethylated target. Based on the detection principle of the sensor, DNA methylation can be successfully distinguished, and the method can be applied for point-of-care platforms.

Feasibility of the E-PfRPA/Cas Strategy. The feasibility of the proposed E-PfRPA/Cas strategy was verified by the

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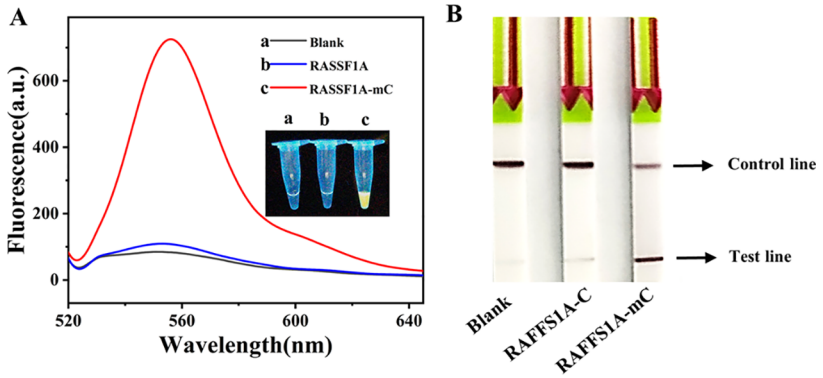


Figure 2. (A) Fluorescence spectra of different samples based on the E-PfRPA/Cas system. (B) LFA detection image of different samples based on the E-PfRPA/Cas system. Optimization of the experimental conditions.

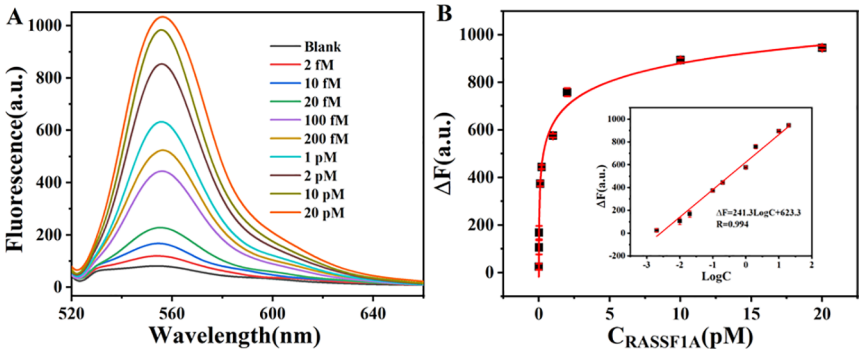


Figure 3. (A) Fluorescence spectra of the target RAFFS1A-mC at different concentrations from 0 fM to 20 pM. (B) Fluorescence response to different concentrations of RAFFS1A-mC from 2 fM to 20 pM; the inset is the linear relationship between fluorescence intensity (ΔF) and the logarithm of the RAFFS1A-mC concentrations in the range from 2 fM to 20 pM. Error bars represent the standard deviations of three independent measurements.

results of PAGE images, fluorescence measurements, and LFA detection. We first prepared the methylated and unmethylated dsDNA (125 bp) of the target fragment for PAGE validation. Figure 1A,B shows the methylated RAFFS1A and unmethylated RAFFS1A with a length of 125 bp, which contain "−GCGC−" sites of HhaI. After reaction with HhaI, unmethylated RAFFS1A is digested into different fragments. This can be seen from Figure 1A, where several bands of these fragments are observed in lane 2, which is consistent with that shown in Figure 1C. Due to the difficulty of dying short sequences, the bands of 5 and 7 bp cannot be seen in lane 2. The last band with low electrophoretic mobility is the incomplete digested RAFFS1A, corresponding to the methylated RAFFS1A in lane 1. These results indicate that methylated RAFFS1A and unmethylated RAFFS1A have been successful distinguished by the specific recognition of HhaI. Figure 1B shows the PAGE results of RPA reaction and Lambda exonuclease treatment. After amplification, an obvious band appears in lane 3, and this amplification band is relatively wide because of the interference from SSB and recombinase. The other two bands are related to forward and reverse primers. For unmethylated RAFFS1A, there is also RPA product in lane 4, which is due to incomplete digestion and non-specific amplification. However, when adding Lambda exonuclease, only the methylated target can generate ssDNA (lane 5 and lane 6), and the reverse primers with 5′-phosphate terminus are also digested by Lambda.

To further validate the DNA methylation detection based on CRISPR/Cas12a, fluorescence spectra and LFA results are shown in Figure 2A,B. From Figure 2A, only methylated RAFFS1A (RAFFS1A-mC) induces strong fluorescence (curve c). However, unmethylated RAFFS1A (RAFFS1A-C) (curve b) and the blank group (curve a) present negligible fluorescence. These above results indicate that RAFFS1A-mC can avoid HhaI digestion due to 5 mC modification, then initiating the RPA reaction to generate a mass of DNA duplex. After adding Lambda, the DNA sequence with a 5′-phosphate terminus can be recognized and degraded, leaving a large number of ssDNA. As a result, the obtained ssDNA can activate the trans-cleavage activity of Cas12a to release the fluorescence signal. In contrast, RAFFS1A-C is digested by HhaI, which cannot initiate the following series of reactions. For LFA detection, only the experiment group with RAFFS1A-mC can exhibit one obvious band in the T line; this is due to the conjugation of AuNPs-*anti*-FAM. Therefore, all these results indicate that the E-PfRPA/Cas method can successfully detect DNA methylation with low background interference, high sensitivity, and high accuracy.

To improve the sensitivity of the proposed strategy, several important parameters in this work were optimized, including cleavage time of Cas12a, digestion time of HhaI, amplification time of RPA, and concentration of Lambda. First, the cleavage time of Cas12a was investigated. As shown in Figure S3A, the fluorescence intensity enhances with the growing of cleavage time of Cas12a and reaches a peak at 30 min. Thus, 30 min is applied for the next following experiments. The HhaI is related with the background signal, and incomplete digestion will lead to a high background signal. The DNA target (RAFFS1A-mC

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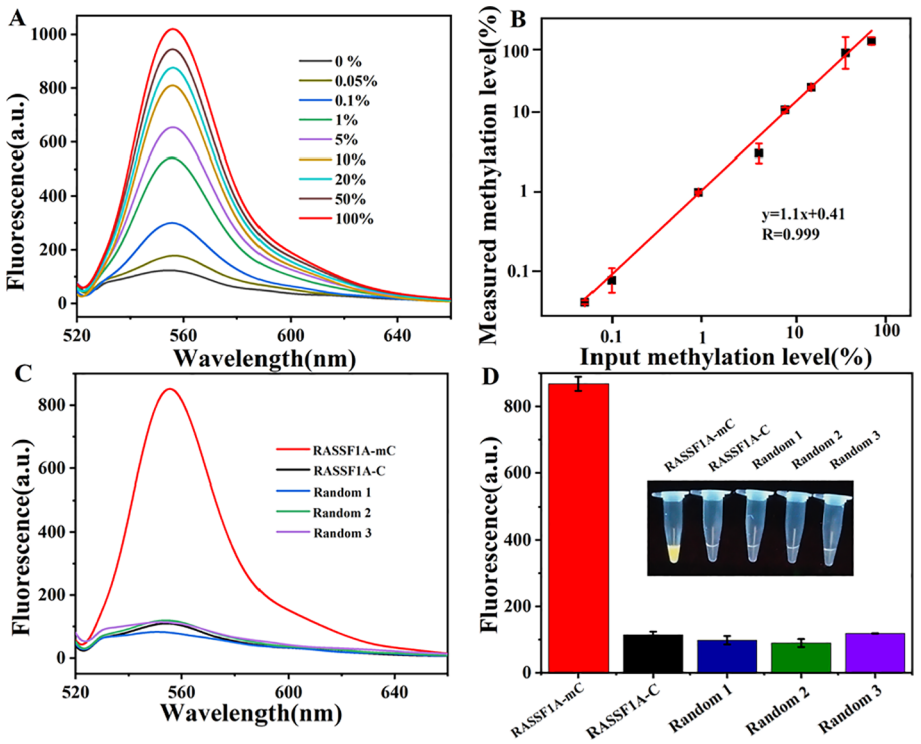


Figure 4. (A) Fluorescence spectra of the methylation level from 0 to 100%. (B) Linear fitting curve between the input methylation level and measured methylation level. (C) Fluorescence spectra of different targets (RASSF1A-mC, RASSF1A-C, random DNA) with a concentration of 2 pM. (D) Fluorescence response of different targets (RASSF1A-mC, RASSF1A-C, random DNA) with a concentration of 2 pM. Error bars represent the standard deviations of three independent measurements. Detection of DNA methylation in LFA strips.

and RAFFS1A-C) with a high concentration of 50 pM was set to ensure complete digestion. As depicted in Figure S3B, the background signal decreases as the digestion time increases from 10 to 70 min, and the fluorescence signal is completely quenched after 50 min of HhaI treatment. Therefore, the digestion of unmethylated DNA requires Hhal treatment for 50 min. The amplification time is also an important parameter, which is closely related to the sensitivity. The fluorescence intensity for RPA reaction is measured from 15 to 35 min; a saturated fluorescence signal appears after 25 min (Figure S3C). In addition, the concentration of the Lambda enzyme was also optimized for rapid detection. As shown in Figure S3D, the fluorescence response presents a concentration dependence on the Lambda and reaches saturation at 2 U/μL. Therefore, 2 U/μL of Lambda is chosen as the optimal condition.

Performance of the E-PfRPA/Cas Strategy for DNA Methylation Detection. To investigate the performance of the proposed method, a range of concentrations of RAFFS1A-mC were measured under optimal conditions. As depicted in Figure 3A, the fluorescence response enhances progressively with the growing of RAFFS1A-mC concentration from 0 fM to 20 pM. There is a good linear relationship between fluorescence intensity (ΔF) and the logarithm of RAFFS1A-mC concentration from 2 fM to 20 pM (the inset of Figure 3B). The fluorescence intensity (ΔF) refers to the value of sample fluorescence minus blank fluorescence. The linear fitting equation is $\Delta F = 241.3\ \mathrm{Log}C + 623.3$ ($R = 0.994$), and the limit of detection is calculated as 0.98 fM. Compared with the previously reported method for DNA methylation detection, the proposed sensor has a lower detection limit and a relatively wider detection range (Table S2). To further verify the efficient detection ability of the proposed method, we introduced the PAM site (TTTA-) into the forward sequence to construct a PAM-based system for methylation detection. Typically, the target was first treated with HhaI and then subjected to RPA reaction. The amplification products were identified by CRISPR/Cas12a under PAM site dependence. As shown in Figure S4B, compared with the E-PfRPA/Cas strategy (PAM-free system) (Figure 2A), unmethylated RAFFS1A (RAFFS1A-C) (curve b) and the blank group (curve a) of the PAM-based system present higher fluorescence. This high background is due to the non-specific amplification. Meanwhile, our method also shows a higher sensitivity (Figure S4C). These excellent performances may benefit from the following reasons: (1) the specific recognition of HhaI enables effective differentiation of methylated and unmethylated DNA. (2) The high amplification capability of RPA combined with Lambda enzyme can generate large amounts of ssDNA. The Lambda treatment can effectively eliminate the high background signal generated by non-specific amplification and performs well in the detection of low-concentration targets. (3) The obtained ssDNA can be recognized by Cas12a without PAM sites. The high specificity and self-amplification ability of CRISPR/Cas12a further improve the sensing performance. Based on the E-PfRPA/Cas strategy, the detection of multiple methylation sites can be achieved by changing the primers and crRNA sequences.

Researches have demonstrated that 5 mC accounts for 2−7% of cytosine in mammalian genomes; most of the genes are in an unmethylated state.[39] Therefore, it is necessary to distinguish methylated regions in the presence of a large number of unmethylated samples. First, the concentration of the unmethylated target (RAFFS1A-C) is set to 20 pM; then

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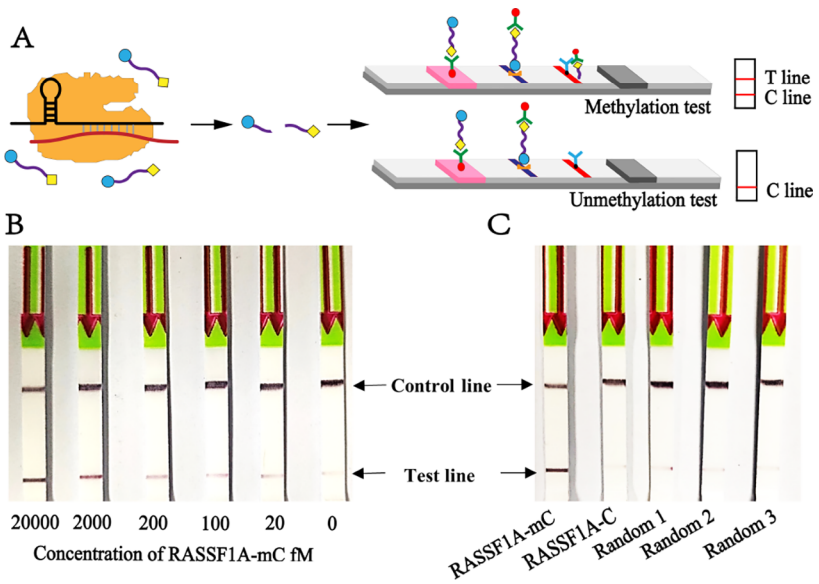


Figure 5. (A) Schematic illustration of LFA for detecting methylated DNA and unmethylated DNA. (B) LFA strip results of different concentrations of RASSF1A-mC from 20 fM to 20 pM. (C) LFA strip results of the different DNA targets.

different concentrations of methylated targets (RAFFS1A-mC) are added to the unmethylated (RAFFS1A-C) reaction system. As shown in Figure 4A, as the methylation level increases, the fluorescence signal gradually increases from 0 to 100%; besides, a good linear relationship is observed between the input methylation level and measured methylation level. The linear fitting equation is $y = 1.1x + 0.41$ ($R = 0.999$), where y refers to measured methylation level and x means input methylation level. It is noteworthy that as low as 0.05% of methylation level (10 fM) can be distinguished, indicating the high accuracy and sensitivity of the E-PfRPA/Cas strategy.

Selectivity and repeatability are crucial for a sensing system. Therefore, the selectivity of the sensor for site-specific methylation detection was first investigated by using RASSF1A-C and other random DNA as interferers. As seen from Figure 4C,D, a remarkable fluorescence peak appears with the existence of RASSF1A-mC. However, under the same concentration of RASSF1A-mC (2 pM), only weak fluorescence responses are observed relative to the RASSF1A-C and random DNA. This is due to the mismatch of other random DNA fragments with crRNA, making it impossible to activate the trans-cleavage activity of CRISPR/Cas12a. RASSF1A-C is digested by HhaI, so RASSF1A-C cannot activate the subsequent reactions. To further explore the detection ability of the E-PfRPA/Cas system, single-nucleotide polymorphism (SNP) mismatch analysis between the target and crRNA was also studied (Figure S5). Experimental results show that SNP mismatch between the ssDNA target and the crRNA has only a negligible effect on catalytic activation of Cas12a, which is consistent with that reported in the literature.[27] This may because single base mutations have little effect on the recognition between crRNA and ssDNA so that the cleavage activity of Cas is not affected. Based on the above research, our strategy has excellent selectivity for methylation detection. Future work on this project could focus on expanding the ability to detect other target substances.

Repeatability was then investigated by testing the fluorescence response of five parallel experimental and control groups (Figure S6). There is no significant fluctuation in fluorescence values between these groups. The relative standard deviation (RSD) is calculated as 2.28%. Last, the stability of the E-PfRPA/Cas strategy was also investigated by implementing three continuous tests at different RAFFS1A-mC concentrations (20 fM, 200 fM, and 2 pM) in parallel. All related reagents were stored at −20 °C. As shown in Figure S7, there is no significant fluctuation in the fluorescence intensity within 1 month; the assay shows a stable output trend; and the RSD of each group is 2.96% (2 pM), 3.06% (200 fM), and 4.26% (20 fM), respectively. The above results reveal that the proposed sensor holds remarkable repeatability and stability.

The lateral flow detection strip is intuitive and portable; it has good practical application potential for the establishment of a naked eye detection platform. Therefore, we integrated the E-PfRPA/Cas method into LFA strips. Figure 5A shows the details of LFA for detecting methylated DNA and unmethylated DNA. In the absence of amplification products, the trans-cleavage activity of Cas12a is inactivated, so the uncleaved ssDNA reporter (bio-DNA-FAM) will bind to Gold-NP anti-FAM in the conjugate pad. The bound product continues to move along the strip by capillary action and then is captured by streptavidin immobilized on the control line, which prevents its flow to the T line. Thus, only a red band appears on the C line. When the CRISPR-Cas12a effectors are activated, the ssDNA reporter (bio-DNA-FAM) will be digested. As a result, the separated ssDNA-FAM with Gold-NP anti-FAM can continue to flow forward and be captured by the T line antibody, which also appears as a red band. According to the detection principle of the strip, there are two red bands on the C line and T line in the presence of the methylated target (RASSF1A-mC). However, only a red band appears in the presence of the unmethylated target (RASSF1A-C).

The sensitivity of the LFA based on the E-PfRPA/Cas method was then investigated. As shown in Figure 5B, on the T line, with the increase of the RASSF1A-mC concentration from 0 fM to 20 pM, the color of the band shows a gradual change from colorless to dark. However, the color on the C line shows the opposite trend. As low as 20 fM of RASSF1A-mC can be obviously discriminated. Moreover, Figure 5B presents that only RASSF1A-mC can induce red color on the

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T line. The unmethylated target (RASSF1A-C) and other random DNA only induce red color on the control line. These results indicate the high sensitivity and selectivity of LFA based on the E-PfRPA/Cas method, which is promising for DNA methylation detection in resource-limited areas.

Real Sample Detection. In order to evaluate the practicability of the proposed E-PfRPA/Cas assay, 20-fold dilution of human serum was added to the target solution for simulating complex biological environments. The dilution target concentrations are 20 fM, 200 fM, and 2 pM, respectively. The dilution targets were measured based on the E-PfRPA/Cas assay. The results are shown in Table 1; the recovery rate ranges from 97 to 112.4%. The RSD is calculated from 2.05 to 4.66%. It indicates that the proposed strategy is suitable for complex sample detection. In addition, researches have demonstrated that the methylation of RASSF1A promoter is closely related with breast cancer; this methylation region (50340771 site−50340676 site) can affect cytokeratin 19 (CK19) mRNA expression, further resulting in tumorigenesis.[32] Therefore, to further evaluate the reliability of this method in practical applications, the E-PfRPA/Cas assay was applied in real genomic DNA samples. The genomic DNA of normal cells (LO2) and MCF-7 cancer cells are prepared as the test samples. As shown in Figure 6A,B, with the same amount of genomic DNA (200 ng/μL), the fluorescence response of the genomic DNA of MCF-7 cells is significantly higher than that of the genomic DNA of normal cells (LO2). Besides, the LFA strip results from Figure 6C also show remarkable differences between cancer cells and normal cells, and the genomic DNA of MCF-7 cells (500 ng/μL) can be detected, indicating its high sensitivity and specificity for determination of DNA methylation in cells.

Table 1. Proposed Biosensor for DNA Methylation Detection (RASSF1A-mC) in Human Serum Samples

target added	detected results	recovery (%)	RSD (%)
20 fM	19.4 fM	97	4.66
200 fM	224.7 fM	112.4	4.63
2 pM	2.18 pM	109	2.05

CONCLUSIONS

In conclusion, we develop a novel strategy based on endonuclease-assisted PAM-free RPA coupling with CRISPR/Cas12a (E-PfRPA/Cas) for sensitive detection of DNA methylation. Compared with traditional methylation-specific PCR, the HhaI-based RPA reaction can achieve rapid detection under mild conditions. Through primer specific design, a large number of ssDNA are generated by Lambda treatment, which not only avoids CRISPR's dependence on PAM sites but also filters the background signal. As a result, the proposed method exhibits excellent performance for determination of DNA methylation and can be combined with lateral flow strips to achieve POC detection. The fluorescence detection limit is 0.98 fM, and as low as 0.05% of methylation level can be distinguished. 20 fM of methylated DNA can be obviously identified with the naked eye by LFA. Moreover, the E-PfRPA/Cas assay shows good sensitivity toward genomic DNA. Based on the program ability of crRNA, the E-PfRPA/Cas-based method may be further used to establish a universal platform for multiple methylation sites and even nucleic acid detection.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssensors.2c01330.

Additional data including gel electrophoresis of crRNA, DNA sequences used in this work, plasmid map of RASSF1A-pUC-SP target, optimization experiments of the detection conditions, comparison of our sensor with other methods, comparison of our sensor with PAM-based assay, and repeatability and stability experiments (PDF)

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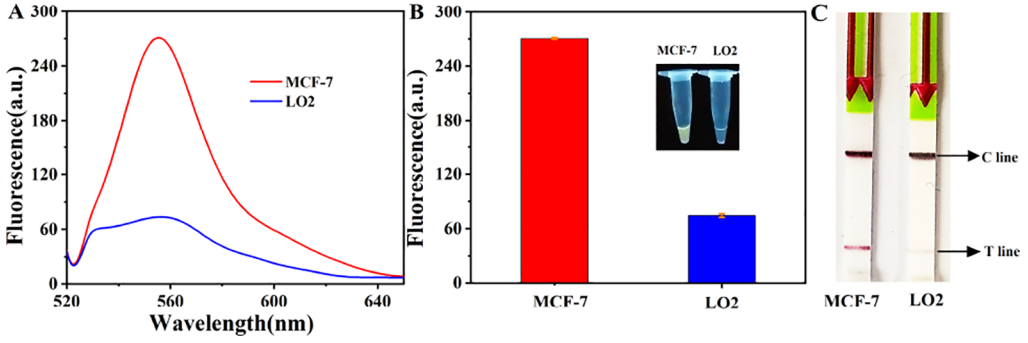


Figure 6. (A) Fluorescence spectra of the genomic DNA from MCF-7 and LO2 cells with a concentration of 200 ng/μL. (B) Fluorescence response of the genomic DNA from MCF-7 and LO2 cells with a concentration of 200 ng/μL. (C) LFA strip results of the genomic DNA from MCF-7 and LO2 cells with a concentration of 500 ng/μL. Error bars represent the standard deviations of three independent measurements.

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Author Contributions

The major experiments and manuscript writing were finished by S.Z. Some Cell culture and measurements were finished by J.D. and L.D. G.W., M.Y., and Y.W. provided editing and writing assistance. C.H. and D.H. provided guidance, fund support, instruments, and equipment for experiments.

Notes

The authors declare no competing financial interest.

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ACS Sens. XXXX, XXX, XXX−XXX