Chapter 27

A Lateral Flow Biosensor for the Detection of Single Nucleotide Polymorphisms

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Abstract

A lateral flow biosensor (LFB) is introduced for the detection of single nucleotide polymorphisms (SNPs). The assay is composed of two steps: circular strand displacement reaction and lateral flow biosensor detection. In step 1, the nucleotide at SNP site is recognized by T4 DNA ligase and the signal is amplified by strand displacement DNA polymerase, which can be accomplished at a constant temperature. In step 2, the reaction product of step 1 is detected by a lateral flow biosensor, which is a rapid and cost effective tool for nuclei acid detection. Comparing with conventional methods, it requires no complicated machines. It is suitable for the use of point of care diagnostics. Therefore, this simple, cost effective, robust, and promising LFB detection method of SNP has great potential for the detection of genetic diseases, personalized medicine, cancer related mutations, and drug-resistant mutations of infectious agents.

Key words Lateral flow biosensor, Point-of-care test, Nucleic acid biosensor, Gold nanoparticle, SNP genotype, Isothermal amplification

1 Introduction

A single nucleotide polymorphism (SNP*See* Single nucleotide polymorphism (SNP)) is a single base DNA mutation occurring at a specific position within a genome. SNPs cause or increase susceptibility to many health problems such as genetic diseases, cancers, and drug resistance of infectious agents. SNPs also provide key information in support of precision medicine. Therefore there is great need for convenient and accurate SNP detection methods for clinical diagnosis and research.

Many techniques have been developed to meet the need for SNP detection. Such techniques include the polymerase chain reaction (PCR) [1], SNP microarray [2, 3], fluorescence resonance energy transfer (FRET) [4, 5] and electrochemical detection [6]. These techniques have been widely applied for SNP detection and

Ben Prickril and Avraham Rasooly (eds.), *Biosensors and Biodetection: Methods and Protocols, Volume 2: Electrochemical, Bioelectronic, Piezoelectric, Cellular and Molecular Biosensors*, Methods in Molecular Biology, vol. 1572,
DOI 10.1007/978-1-4939-6911-1_27,

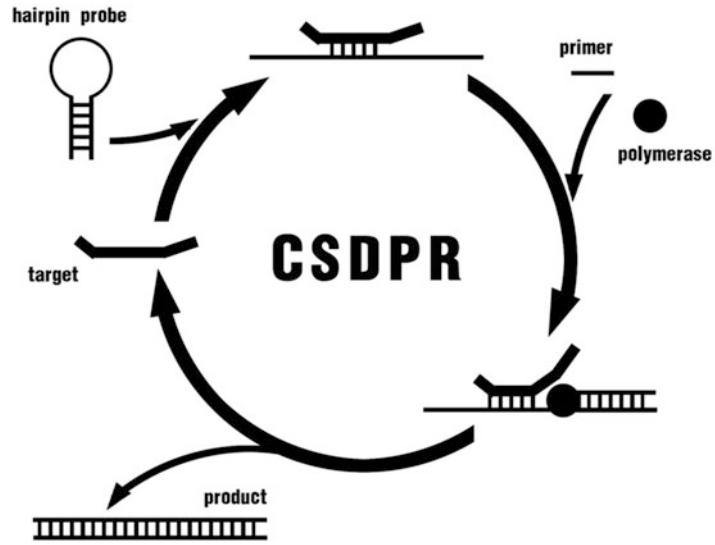

Fig. 1 Principle of CSDPR. Target nucleic acid hybridizes to the DNA probe and causes the conformational change of DNA probe leading to exposure of primer binding site. The primer then hybridizes to DNA probe and is extended by DNA polymerase. Due to strand displacement the target nucleic acid is displaced and released rather than degraded. The released nucleic acid triggers next cycle of reaction, catalyzing transformation of more DNA probe into dsDNA product

are effective. But these techniques are time-consuming and require expensive instruments and highly trained personnel.

As a new method for nucleic acid detection, circular strand displacement reaction (CSDPR*See* Circular strand displacement reaction (CSDPR)) is simple and convenient [7]. The principle of CSDPR is shown in Fig. 1. First, target nucleic acid hybridizes to the DNA probe (usually hairpin DNA) and causes conformational change of the DNA probe leading to the exposure of a primer binding site. The primer hybridizes to the DNA probe and then is extended by a DNA polymerase with strand displacement activity. Because of strand displacement activity, the target nucleic acid is displaced and released rather than degraded. The released nucleic acid can trigger the next cycle of reaction and change more DNA probe into dsDNA product. Compared to traditional PCR, CSDPR can be carried out at a constant temperature and requires no special instrumentation. In addition, ligation reactions with DNA ligase can be used to detect SNPs with high specificity [8, 9]. Ligase can covalently join two adjacent probes on the SNP site with high fidelity.

Recently, lateral flow biosensors (LFBs*See* Lateral flow biosensors (LFBs))*see* Lateral flow biosensors (LFBs) have attracted a great deal of research interest because they offer a promising approach to realizing point-of-care nucleic acid detection. As shown in Fig. 2, a LFB is usually composed of four parts: sample

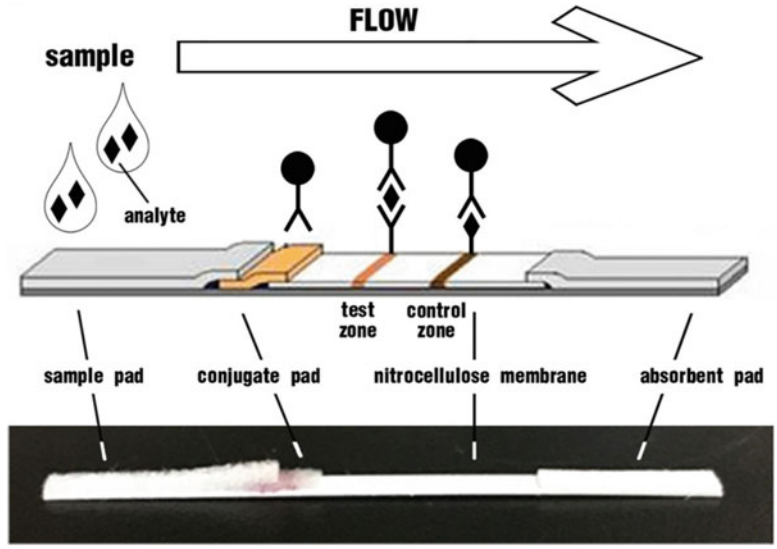

Fig. 2 Principle of LFB. A LFB is composed of four parts: sample pad, conjugate pad, nitrocellulose membrane, and absorbent pad. Analyte is applied to the sample pad. The conjugate pad stores the reporter molecules (usually AuNP–protein or AuNP–DNA). Capture molecules of analyte and capture molecules of reporter are printed on the nitrocellulose membrane to form test zone and control zone, respectively. In the assay, sample is applied to the sample pad and driven by capillary force to the absorbent pad. For sandwich assays both reporter and capture molecules on test zone recognize target analyte. The presence of analyte causes aggregation of reporter molecules on the test zone leading to appearance of a visible line on the test zone. Reporter capture molecules on the control zone can directly interact with the reporter producing a visible line

pad, conjugate pad, nitrocellulose membrane, and absorbent pad. The sample pad is pretreated with buffer and can offer suitable pH and ion strength for the detection. The conjugate pad is used for the storage of reporter molecules (usually AuNP-protein or AuNP-DNA). Capture molecules of analyte and capture molecules of reporter are printed on nitrocellulose membrane to form test zone and control zone, respectively. In the LFB assay, the sample is directly applied onto the sample pad. Driven by capillary force, sample solution would automatically flow from sample pad to absorbent pad. For sandwich assay, both reporter molecules and capture molecules on test zone recognize target analyte. The presence of analyte causes aggregation of reporter molecules on the test zone, leading to appearance of a visible line on the test zone. Reporter capture molecules on the control zone can directly interact with reporter molecules, and thus the appearance of a visible line on the control zone demonstrates that the biosensor works properly. The whole assay (from loading of sample to readout of result) can be accomplished in 20 min. Compared to ELISA assay with similar principle, LFB has several advantages. First, LFB needs only a sample loading operation, avoiding multiple incubation and washing steps necessary for ELISA assay, which is time-saving and labor-saving. Second, LFB excludes the use of reagents such as

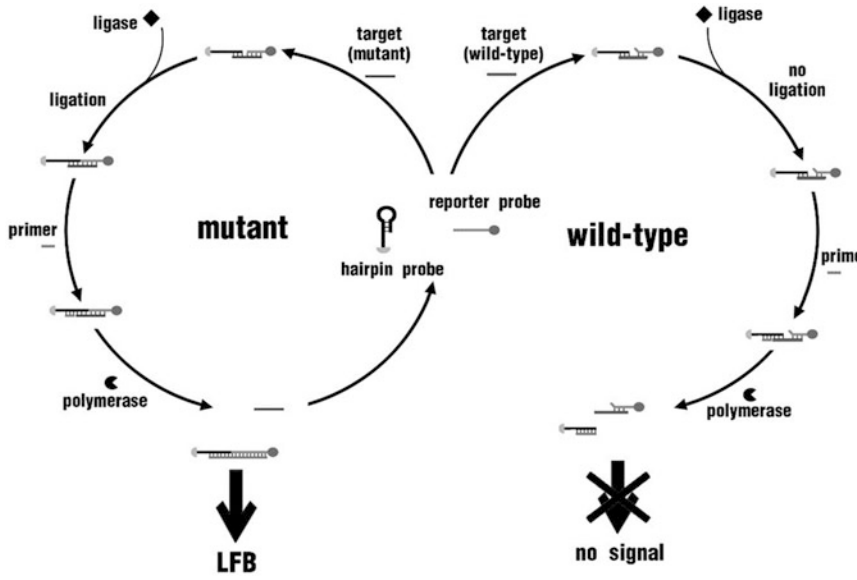

Fig. 3 Schematic illustration of CSDPR used in the assay

washing buffer and substrate solution, greatly reducing the cost of package and transportation. Finally, the result readout of LFB can be accomplished by naked eye, thus requiring no complicated machines such as a microplate reader. Thus LFB is more suitable for point-of-care detection. In the past 2 years, we have developed time- and labor-saving LFBs for the detection of nucleic acids [10], proteins [11], cancer cells [12], and copper ion [13].

Taking advantage of high fidelity of T4 DNA ligase, excellent signal amplification by CSDPR, and the special optical properties of gold nanoparticles (AuNPs), we developed an LBF for the SNP analysis of codon 12 in K-ras oncogene sequence [14]. The assay is composed of two parts: isothermal amplification and LFB detection. As shown in Fig. 3, the isothermal amplification system consists of biotin modified hairpin DNA probe, a short primer, digoxin-labeled reporter DNA probe, T4 DNA ligase, Klenow polymerase exo-, dNTPs, and buffer solution. In the presence of mutant target DNA (Fig. 3, route mutant), the hairpin probe is opened. After hairpin probe and the digoxin-labeled reporter DNA probe annealing to the mutant target DNA, T4 DNA ligase covalently joins the two adjacent probes that flank the mutation site by 5′-PO_4 of hairpin probe and 3′-OH of reporter probe. Then the short primer anneals to the open hairpin probe and starts a polymerization reaction with polymerase and dNTPs. The reaction product, a biotinylated digoxin-modified DNA sequence is synthesized while the target is displaced, which triggers a new round of polymerization reaction. This repeating process generates large number of biotinylated digoxin-modified DNA sequences.

The biotinylated digoxin-modified DNA sequence joins to AuNP–anti-digoxin antibody conjugates (AuNP-anti-dig) and is then captured by streptavidin on the test zone of the nitrocellulose membrane. Polyclonal goat anti-mouse antibody on the control zone can capture the superfluous AuNP-anti-dig. The red line at the control zone indicates that the biosensor works properly, and the red line at the test zone indicates the presence of mutant target nucleic acid. Qualitative analysis is performed by observing the color change caused by aggregation of AuNPs on the test zone, and semi-quantitative detection can be realized by recording the optical density of the test line with a portable strip reader. While in the presence of wild-type target DNA (Fig. 3, route wild-type), because of the specificity of T4 DNA ligase, 5′-PO4 of hairpin probe and 3′-OH of reporter probe cannot be ligated. The short primer triggers a polymerization reaction in the presence of dNTPs and polymerase and the target is displaced from hairpin probe. No biotinylated digoxin-modified DNA sequence is synthesized. Therefore no test line but control line is observed when the reaction product is loaded to the LFB.

2 Materials

1. All DNA sequences used in the experiment are listed in Table 1. DNA is dissolved in H_2O to 10 μM and stored at −20 °C.
2. Rinsing buffer is prepared by dissolving 1% BSA, 8% sucrose, and 0.05% NaN_3 in 50 mL H_2O. Rinsing buffer is stored at 4 °C.
3. Sample pad buffer is prepared by dissolving 0.5% BSA, 5% Triton X-100, and 0.05% NaN_3 in 50 mL 0.01 M PBS pH 7.8. Sample pad buffer is stored at 4 °C.
4. Adhesive plate: PVC plate, obtained from Vinostech, Shanghai, China.
5. Nitrocellulose membrane: AE99 25 mm × 50 m 1/pk, product code: 10,548,081. Obtained from GE Healthcare, Shanghai, China.

Table 1
DNA sequence used in the test

Hairpin probe	**PO_4-TGGCGTAGGCAAGAGTGCCCTGCCTACGCCATTCC-$(T)_{13}$-biotin**
Reporter probe	digoxin- $(T)_9$-CTTGTGGTAGTTGGAGCTGT
Primer	GGAATGGCG
Mutant target	TTTTGGGCACTCTTGCCTACGCCAACAGCTCCAACTACCACAAGCCCC
Wild-type target	TTTTGGGCACTCTTGCCTACGCCACCAGCTCCAACTACCACAAGCCCC

6. Fiberglass: Obtained from Shanghai Jie Ning Biotechnology, Shanghai, China. Ref: 800,305.
7. Absorbent paper: Obtained from Shanghai Jie Ning Biotechnology, Shanghai, China. Ref: 800,201.
8. Dispenser: XYZ dispenser HM3030, obtained from Shanghai Kinbio, Shanghai, China.
9. Cutter: Automatic Strip Cutter ZQ2000, obtained from Shanghai Kinbio, Shanghai, China.
10. Strip reader: Test Strip Reader DT2032, obtained from Shanghai Kinbio, Shanghai, China.
11. $HAuCl_4$: Product code: G4022-1G. Obtained from Sigma-Aldrich Corporation.
12. Sodium citrate: Trisodium citrate dihydrate, Product code: 10019490. Obtained from Sinopharm Chemical Reagent Co., Ltd., Shanghai, China.
13. K_2CO_3: obtained from Guangzhou Chemical Reagent Factory, Guangzhou, China.
14. Monoclonal mouse anti-digoxin antibody: Product code: D8156. Obtained from Sigma-Aldrich Corporation.
15. Streptavidin: Product code: bs-0437P. Obtained from BEIJING BIOSYNTHESIS BIOTECHNOLOGY CO., LTD., Beijing, China.
16. Polyconal goat anti-mouse antibody: Product code: C020201. Obtained from CELLWAY-LAB, Luoyang, China.
17. T4 DNA ligase and buffer: Product code: EL0011. Obtained from Thermo Scientific.
18. Polymerase Klenow fragment exo-: Product code: M0212S. Obtained from New England Biolabs.
19. dNTPs: Product code: 4019. Obtained from TAKARA BIOTECHNOLOGY (DALIAN)CO.,LTD.
20. PCR tube: Product code: PCR-02-C. Obtained from Axygen.

3 Methods

3.1 Preparation of AuNP–Anti-digoxin Antibody Conjugates

1. Preparation of AuNPs: AuNPs are prepared using citrate reduction method. Briefly, 8 mL 1% sodium citrate is added to 400 mL of a rapidly stirred and boiling aqueous solution of 0.01% $HAuCl_4$. After turning red, the solution is boiled for 10 additional minutes, then cooled to room temperature (*see* **Note 1**).
2. Optimization of antibody amount: Three microliters of 0.1 M K_2CO_3 is added to 1 mL of the AuNP solution and shaken

gently for 5 min at room temperature. Then various volume (for example 5, 10, 15, 20, 25, 30 μL) of 0.5 mg/mL monoclonal mouse anti-digoxin antibody is added to the AuNP solution and shaken gently for 0.5 h at room temperature. Finally 100 μL 10% NaCl is added to antibody coated AuNPs and antibody coated AuNPs would turn purple or black if antibody is not enough. The least antibody-consuming condition (20 μL 0.5 mg/mL in our experiment) with which the mixture stayed red after NaCl addition is chosen. If the mixture turned purple or black, this step is repeated with increasing volume of antibody.

3. Preparation of AuNP–anti-digoxin antibody conjugates: Three microliters of 0.1 M K_2CO_3 is added to 1 mL of the AuNP solution to adjust the pH of solution and shaken gently for 5 min at room temperature. After pH adjustment, 20 μL (chosen in "Optimization of antibody amount") 0.5 mg/mL monoclonal mouse anti-digoxin antibody is added to the AuNP solution and shaken gently for 0.5 h at room temperature. The antibody coated AuNPs are subjected to "blocking" by adding 100 μL 10% BSA. The solution is shaken gently at room temperature for 0.5 h. Particles are centrifuged (12×10^3 rpm, 20 min) to remove any unbound antibody. The red pellet is re-suspended in 100 μL of rinsing buffer (1% BSA, 8% sucrose, and 0.05% NaN3) and then stored in a refrigerator at 4 °C until use.

3.2 Construction of Lateral Flow Biosensor

1. Nitrocellulose membrane is attached to the central part of an adhesive plate. Streptavidin (8 mg/mL) and polyconal goat anti-mouse antibody (2 mg/mL) are dispensed onto the nitrocellulose membrane at the speed of 0.6 μL/cm and 0.8 μL/cm simultaneously, to form a test zone and a control zone with a dispenser (Fig. 4a). Dispensing speed is a parameter of the dispenser and would affect the width of the test and control zones. The membrane is then dried at 37 °C for 12 h and stored at room temperature until use (*see* **Note 1**).
2. Strips of fiberglass (16 mm in width) are used as sample pads after being soaked in sample pad buffer. The sample pads are dried and stored in low-humidity at room temperature.
3. Conjugate pads are made by spraying AuNP–anti-digoxin antibody conjugates on fiberglass of 6 mm in width at a speed of 9 μL/cm with a dispenser (Fig. 4b), and then dried and stored in low-humidity at room temperature.
4. Absorbent pads are strips of thick absorbent paper of 17 mm in width. A strip of sample pad, conjugate pad, nitrocellulose membrane, and absorbent pad is attached along the long axis of an adhesive plate with an overlap of 2–3 mm as shown in Fig. 4c. The plate is then cut into 0.3 cm-wide strips using a cutter.

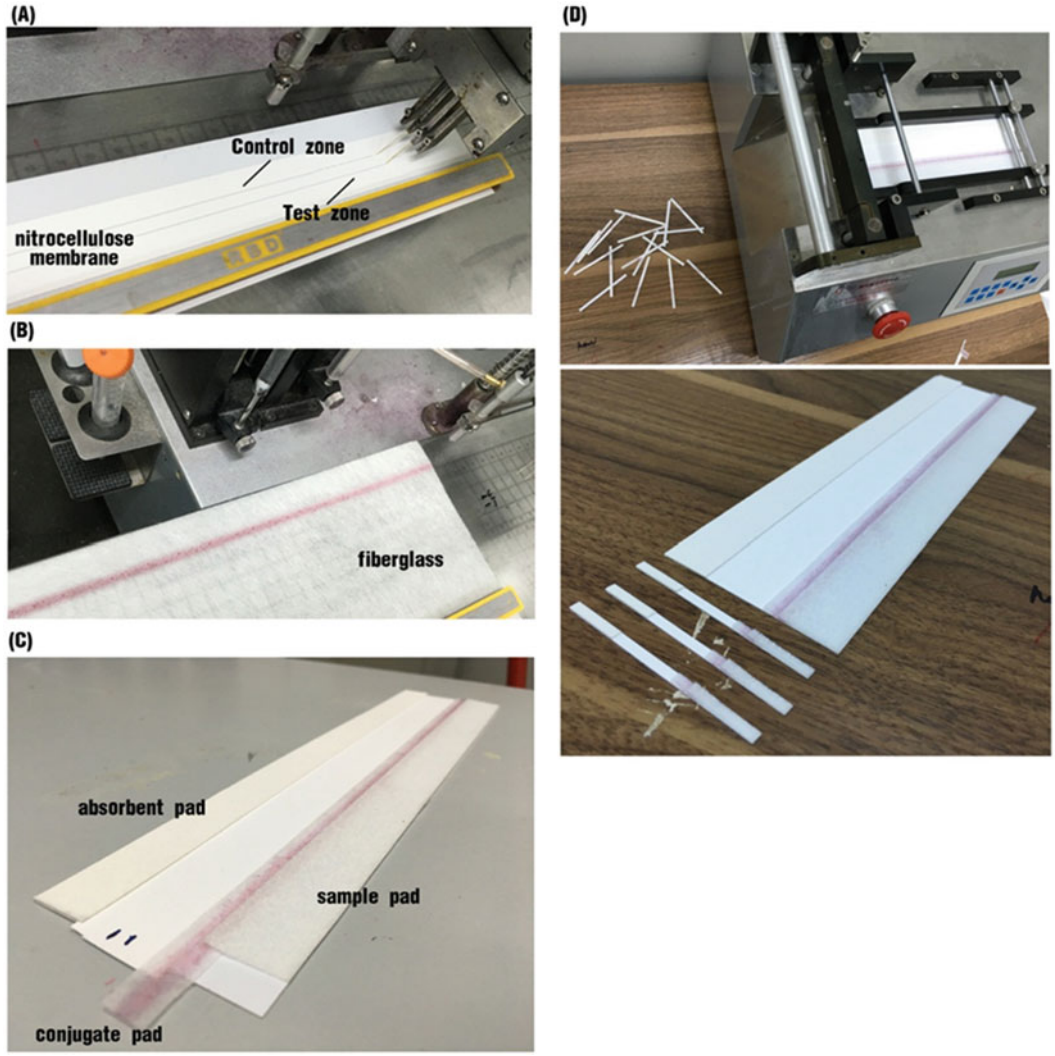

Fig. 4 Photos of lateral flow biosensor construction. (**a**) Drawing of control zone and test zone. (**b**) Spraying AuNP–anti-digoxin antibody conjugates on fiberglass. (**c**) Attachment of absorbent pad, conjugate pad and sample pad to the adhesive plate. (**d**) Cutting the plate into strips

3.3 DNA Probe Design

All DNA probes are analyzed with OligoAnalyzer Tool from Integrated DNA Technologies (http://sg.idtdna.com/calc/analyzer). A 40-base (SNP site, upstream 20 bases and downstream 19 bases) sequence is chosen as the target sequence. The 3′ terminus of reporter probe is complementary with the 3′ terminus of target (20 bases, including the SNP site). The 5′ terminus of hairpin probe (including 5′ arm and loop of the hairpin) is complementary to the 5′ terminus of target (20 bases). The length of the stem is about 11 bp. Melting temperature of the hairpin probe is about 65–75 °C. The primer is complementary to the 3′ arm of the

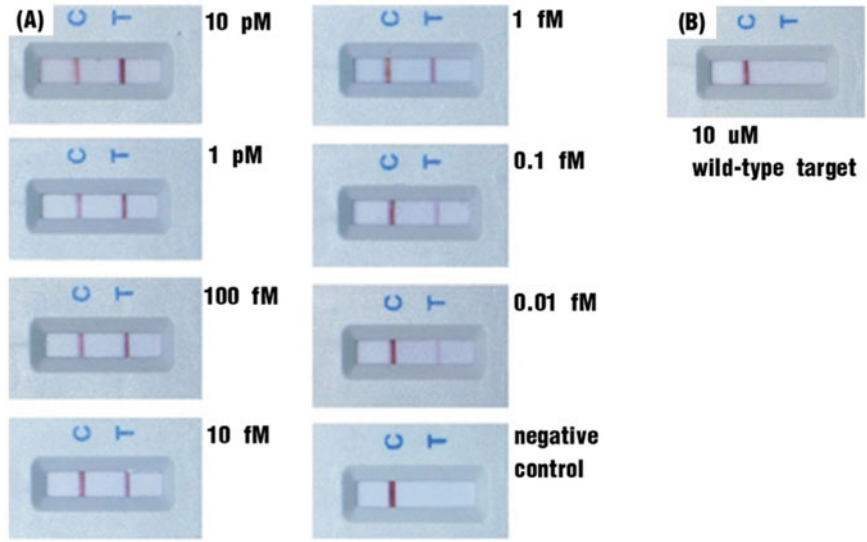

Fig. 5 Results of (**A**) different concentrations of mutant target and (**B**) 10 μM of wild-type target

hairpin probe. A nine-base primer is long enough for efficient amplification. All probes are analyzed to confirm that there would be no self-dimer formation (*see* **Note 2**).

3.4 Isothermal Amplification of Nuclei Acid

For isothermal amplification, 1 μL of synthetic mutant target or wild-type DNA is added to 20 μL reaction solution [1.2 μM hairpin probe, 12 nM primer, 9.6 nM reporter, 10 U T4 DNA ligase, 5 U polymerase Klenow fragment exo-, 50 μM dNTPs, and 1× T4 DNA ligase reaction buffer (40 mM Tris–HCl pH 7.8, 10 mM DTT, 10 mM $MgCl_2$,)] in a 200 μL PCR tube. Reaction solution is incubated at 25 °C for 2 h either in conventional water bath or PCR machine and then detected by lateral flow biosensor.

3.5 Detection of Amplification Product by Lateral Flow Biosensor

To detect amplification product by lateral flow biosensor, all amplification product is loaded onto the sample pad of the biosensor. Then, 60 μL of PBS is loaded to the sample pad. The biosensor is scanned using a strip reader 20 min later.

For the detection of synthetic mutant target DNA, the limit of detection is as low as 0.01 f. (Fig. 5a). On the other hand, as much as 10 μM of wild-type DNA cannot lead to the presence of red line in test zone (Fig. 5b).

4 Notes

1. The affinity interactions used in the assay (streptavidin–biotin, anti-digoxin antibody–digoxin) can be replaced by other antibody–antigen pairs, and the condition of AuNP antibody conjugation and test zone preparation should be adjusted.

2. This method can be used for analysis of other SNP sites. In that case, hairpin probe should be carefully optimized, including the length of (1) stem, (2) loop, (3) complementary sequence, and (4) primer.

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