Chapter 3

Emerging Techniques for Thalassemia Gene Detection

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.75695

Abstract

Isothermal nucleic acid amplification is a simple process that rapidly and efficiently accumulates nucleic acid sequences at constant temperature such as 37 and 42°C. Isothermal nucleic acid amplification approach offers several advantages over temperature circle methods (such as PCR) including rapid assay results, cost-effectiveness, and portability. Two detection approaches based on circular strand-displacement polymerization reaction (CSDPR) were presented in this chapter for sensitive and specific thalassemia gene detection. One is a lateral flow strip biosensor based on CSDPR for semi-quantitative detection of thalassemia DNA. The other is a spectrophotometric DNA detection approach based on CSDPR for quantitative detection of thalassemia DNA.

Keywords: emerging techniques, isothermal amplification, thalassemia gene detection

1. Introduction

Thalassemia is a heterogeneous group of inherited disorders characterized by the reduced synthesis of one or more normal hemoglobin chains leading to imbalanced globin chain synthesis, which results in microcytic anemia [1–3]. Based on genetic disorders, thalassemia is divided into two main types, alpha-thalassemia and beta-thalassemia. Alpha- and beta-thalassemia occur when there is a reduction in alpha-globin chains and beta-globin chains, respectively. Symptom of anemia is variable ranging from none to severe.

Because the structural hemoglobin variants and thalassemias occur at high frequencies in some populations, both types of genetic defect can be found in the same individual such as $-^{SEA}/-\alpha^{3.7}$. The different genetic varieties of thalassemia and their combinations with genes for abnormal hemoglobins produce a series of disorders defined as thalassemia syndromes [4].

Until now, marrow transplantation is the only way in which they can be cured. Unfortunately, expensive fee and restricted marrow sources have hindered its application in thalassemia patients. Therefore, thalassemia carrier screening in premarital checkup is an effective way for defective child birth. Carrier screening for thalassemia can reduce the burden on individuals by identifying those at increased risk, thereby enabling individuals to receive information about their health, future health, and/or potential health of their offspring, so that they are informed and understand their reproductive risks and options [5].

Traditional diagnosis methods include osmotic fragility test and hemoglobin electrophoresis techniques. The decrease of osmotic fragility indicates the decrease of hemoglobin production. Although it is simple and convenient, osmotic fragility test is not specific for thalassemia. Hemoglobin electrophoresis was used to initially identify samples for common hemoglobinpathies such S and C. More complex electrophoresis techniques were then used to identify a great number of hemoglobin subtypes. Nevertheless, electrophoresis techniques are very complicated in experimental procedures, low in sensitivity, and difficult for quantitation.

Along with the advances in molecular biology, a variety of novel nucleic acid detection methods have been invented and applied in thalassemia gene detection, such as gap-PCR [6], reverse dot blot (RDB) [7], and DNA microarray [8]. However, these methods for thalassemia detection rely solely on PCR to amplify target genes. They are not only expensive and complicated in procedure, but also liable to contamination. On the other hand, as they operate at a constant temperature (mostly at 37°C), the isothermal nucleic acid amplification approach offers several advantages over temperature circle methods including rapid assay results, cost-effectiveness, and portability.

2. Emerging techniques for thalassemia gene detection

2.1. Types of globin

Globin, the protein portion of the hemoglobin molecule, is a tetramer consisted of two α and two non-α-globin chains. The structure and ontogeny of the hemoglobins are listed in **Figure 1**. The α-globin chains are encoded by two closely linked genes (α2 and α1) located on chromosome 16. The non-α genes, β, γ, and δ, are encoded by a cluster of genes on chromosome 11. All types of hemoglobins have similar protein structures. Each hemoglobin has a quaternary structure composed of two separate pairs of the same globin chains. The embryonic hemoglobins consists of two ζ or α chains and two ε or γ chains; all normal human hemoglobins consist of two α chains and two non-α chains. In hemoglobin A, the chains are composed of β chains ($\alpha_2\beta_2$); in hemoglobin A2, they are composed of δ chains ($\alpha_2\delta_2$); and in hemoglobin F, they are composed of γ chains ($\alpha_2\gamma_2$).

There are two main classes of thalassemia, and, in which α- and β-globin genes are involved. **Table 1** summarizes the different classes of α/β-thalassemia mutations. When only 1 of the 4 α-globin chain genes is defective, the patient has little clinical symptoms and this silent condition can only be detected at birth by the presence of a small amount of Bart hemoglobin.

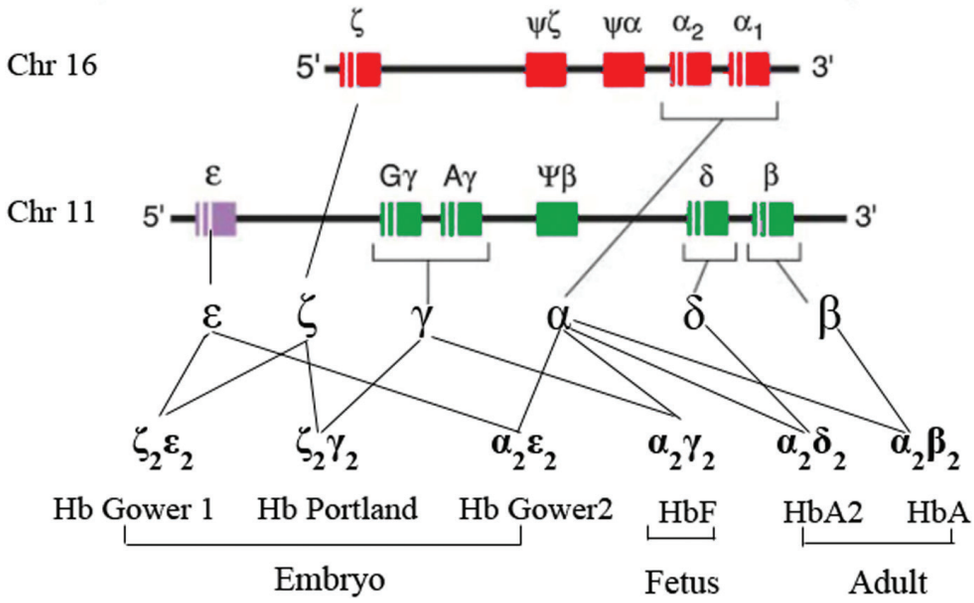

Figure 1. Schematic illustration for genetic control and protein structures of hemoglobin. The α- and β-globin are controlled by gene clusters located on chromosomes 11 and 16, respectively. Each hemoglobin has a quaternary structure composed of two separate pairs of the same globin chains.

α^0-thalassemia
Deletions involving both α-globin genes
Deletions downstream from α_2 gene
α^+-thalassemia
Deletions involving both α-globin genes
Point mutations involving α_2 or α_1 genes
mRNA processing
β^0-or β^+-thalassemia
Transcription
Deletions
Insertions
Promoter
5′UTR

Table 1. Classes of mutations that cause α/β-thalassemia.

α hemoglobin is made up of 4 γ-globin chains. Current high performance liquid chromatography (HPLC) techniques are relatively insensitive to the very low level of Bart hemoglobin with a single chain defect and are, therefore, not useful for the diagnosis of α-thalassemia carriers. Heterozygous α^0 and homozygous α^+ patients show microcytosis, hypochromia, and a

mild anemia, suggesting thalassemia minor. Patients with β-thalassemia minor due to either a gene deletion (β^0) or mutation (β^+) can be identified by the slight elevation in fetal hemoglobin and an increase in the hemoglobin A_2 level to between 4 and 7%.

2.2. Detection of thalassemia genes with traditional methods

Carrier detection approaches must be able to detect α- and β-thalassemias. The results of complete blood cell count (CBC) including the values of MCH, MCV, HbA_2, and HbF can provide clues to the hematological characteristics of the different types of thalassemia genes and their interactions. The most common thalassemia mutations and abnormal hemoglobins can be detected by PCR-based techniques such as RDB and gap-PCR. The main applications of molecular detection for carrier analysis are summarized as follows. 1. Heterozygous α-thalassemia and homozygous α-thalassemia can be discriminated by the analysis of α-thalassemia mutations using gap-PCR; 2. The identification of β-thalassemia mutations for patients requires prenatal diagnosis to predict the severity of the clinical phenotype of homozygous β-thalassemia; 3. The discrimination between δβ-thalassemia and hereditary persistence of fetal hemoglobin (HPFH) deletions can be carried out by gap-PCR [9].

Routine detection strategies for the detection of thalassemia gene are mainly based on PCR. Gap-PCR was developed for the detection of deletion types of thalassemia gene mutations. More than eight β-thalassemia deletions and two common α^+-thalassemia deletion genes, the $-\alpha^{3.7}$ and $-\alpha^{4.2}$ alleles, and five α^0-thalassemia deletion genes, the three Southeast Asian deletions ($-^{SEA}$, $-^{THAI}$ and $-^{FIL}$) and two Mediterranean deletions ($-^{MED}$ and $-\alpha^{20.5}$), can be detected by gap-PCR.

Meanwhile, allele-specific oligonucleotide hybridization (ASO) and RDB are applied to non-deletional α- and β-thalassemia mutations. ASO is used to detect single base mutations without the need of PCR or gel electrophoresis. For mutation analysis, a panel of ASO probes is employed to detect mutations in patients. For prenatal diagnosis and for genotyping homozygous patients, two labeled probes are employed to detect the mutation sequence, one complimentary to the normal gene DNA sequence and the other complimentary to the mutant DNA sequence at the same position. Generally, there is only one nucleotide difference in the two ASO probes. The genotype of the sample is observed by the absence or presence of the hybridization signal of two ASO probes.

In the RDB assay, the amplified target DNA is first fixed to a nylon membrane to form a filter-fixed DNA dot, the dot is then hybridized to ASO probes whose 5′ terminal was conjugated with either P-labeled deoxynucleoside triphosphates, biotin or horseradish peroxidase. The technique has been successfully applied in many laboratories, especially for populations with just one common mutation and a small number of rare ones. However, RDB was not suitable for the screening of a large number of different mutations because the method is time-consuming as it needs separate hybridization and washing steps for each mutation.

All these routine methods for thalassemia detection are tedious, expensive, and liable to contamination. To improve these methods, many PCR-derived methods have been developed

and applied to thalassemia gene assay, such as real time PCR, dissociation curve analysis, and droplet digital PCR. These methods are simple; however, expensive apparatus and need for experienced personnel hinder their broad application in basic level laboratories.

Recently, many amplification strategies have been developed to improve the sensitivity of DNA detection. Two commonly used methods are target amplification and signal amplification. PCR as well as several isothermal amplification techniques such as rolling circle amplification (RCA) and helicase-dependent amplification (HDA) are typical target amplification strategies. However, these conventional amplification methods for sequence-specific DNA detection suffer from the limitations of complicated procedures, easy contamination, and high cost, which have been hampering their application in many laboratories. Signal amplification techniques that are commonly applied to directly amplify the detection signal have been developed to overcome the disadvantages of traditional target amplification strategies. These amplification approaches including nicking enzyme signal amplification (NESA) and CSDPR [10], which are most commonly used for sequence-specific DNA amplification, provide several advantages over target amplification approaches including ease of use, portability, rapid assay results, and cost-effectiveness. Particularly, CSDPR combined with different detection platforms have attracted great attention due to its robustness and simplicity. CSDPR amplifies the DNA signal at an isothermal temperature that yields a large amount of DNA signal products that can be detected with different platforms.

2.3.1. Detection thalassemia gene with emerging techniques lateral flow biosensors

Based on CSDPR and gold nanoparticles (AuNPs), we have developed a lateral flow biosensor (LFB) for the visual detection of thalassemia genes [11]. This sensor is highly sensitive (the detection limit is 0.01 fM of nucleic acid), highly specific, and easy to use. It does not require the use of complex and expensive instrumentation. The development of this biosensor represents an important step toward point-of-care testing for genetic disorders. The Southeast Asia (SEA) deletion of α-thalassemia was detected with this lateral flow biosensor. The method has been successfully validated for the detection of the SEA type of α-thalassemia (100% concordance with the results obtained using PCR). In addition, this test can distinguish SEA from other types of thalassemia, such as $-\alpha^{3.7}$, $-\alpha^{4.2}$, and β-thalassemia.

As depicted in **Figure 2**, a CSDPR reaction comprising biotin-modified hairpin DNA, digoxin-modified primer, and DNA polymerase is initiated by target mutant DNA strand. The hairpin probe forms a stem-loop structure with biotin at the 5′ end of the stem region. The stem is a 10-nt sequence, and the loop is a 20-nt sequence that is complementary to the mutant target DNA. The 8-nt primer includes a digoxin tag at the 5′ end and is complementary to the stem region of the probe at the 3′ end. The biotin-modified hairpin recognizes and hybridizes with SEA mutant DNA when present, causing the hairpin probe to undergo a conformational change that leads to stem separation (1). The primer then anneals with the open stem (2) and triggers a polymerization reaction in the presence of dNTPs and polymerase Klenow exo- (3). During primer extension, the mutant DNA is displaced by the strand displacement activity of the polymerase, permitting the synthesis of complementary DNA and formation of a hairpin-DNA complex. To start the next cycle, the displaced target mutant DNA strand hybridizes with

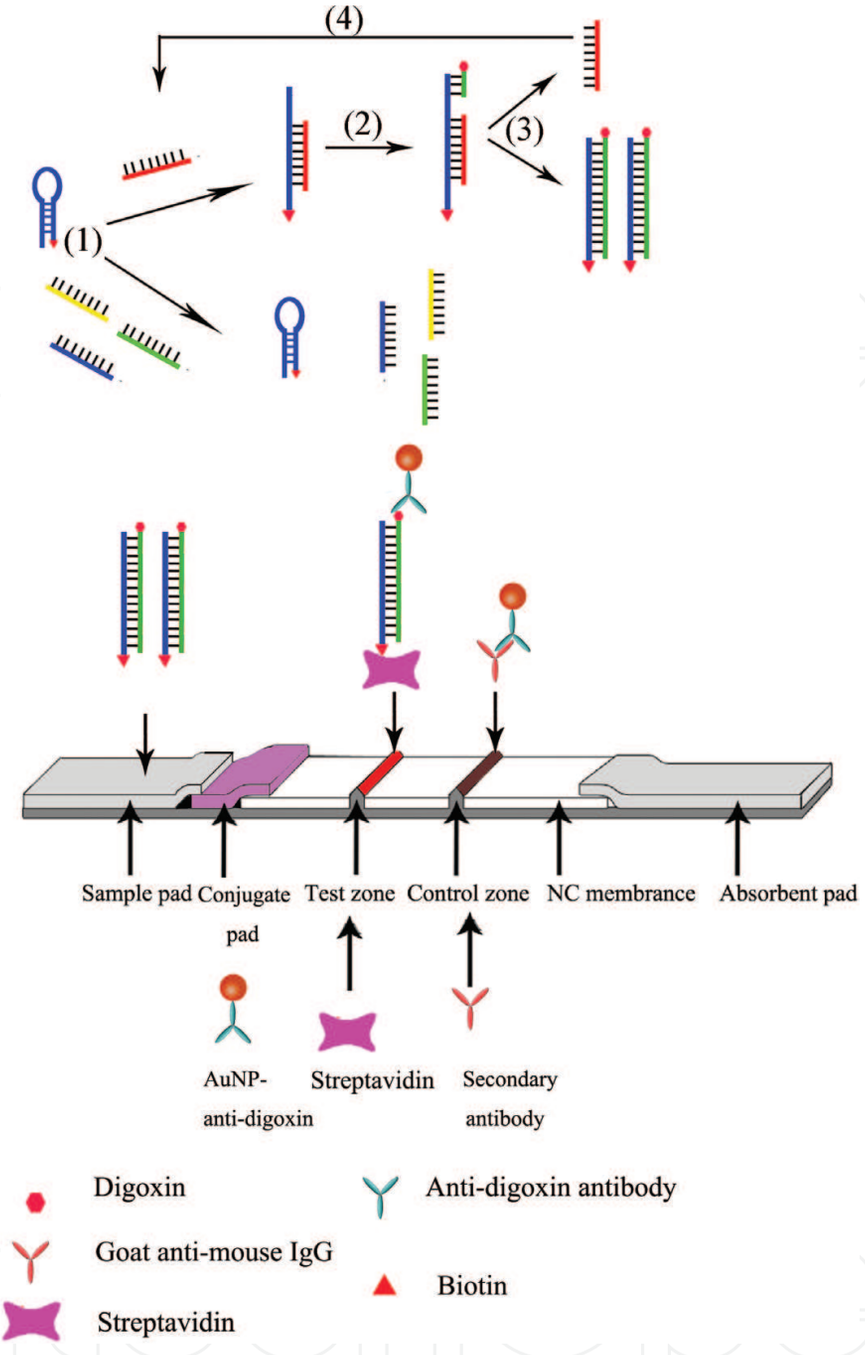

Figure 2. Illustration of the production of sequence tagged duplex DNA based on CSDPR and the structure of a lateral flow biosensor including four steps. (1) the biotin-modified hairpin recognizes and hybridizes with SEA mutant DNA when present, causing the hairpin probe to undergo stem and loop separation; (2) the primer then anneals with the open stem; (3) this triggers a polymerization reaction in the presence of dNTPs and polymerase Klenow exo-; (4) to start the next cycle, the displaced target mutant DNA strand hybridizes with another hairpin probe.

another hairpin probe, initiating another round of polymerization (4). Through this cyclical process, a large number of tagged-duplex DNA complexes are produced from a minute amount of target mutant DNA. The hairpin retains its original stem-loop structure in the presence of

wild-type or non-complementary DNA due to the weak hybridization between wild-type DNA and the hairpin probe. Therefore, the primer is unable to anneal to the hairpin to induce a polymerization reaction, and no tagged-duplex DNA complex is produced.

The large number of tagged-duplex DNA complexes produced by the CSDPR reaction is then detected using an LFB. As shown in **Figure 2**, the CSDPR products are mixed with a running buffer and migrate along the LFB by capillary action to the conjugate pad, where anti-digoxin–AuNP conjugates are deposited. The digoxin attached to the duplex DNA reacts with anti-digoxin on the AuNP surface to form a complex that consists of biotin-double strand DNA-digoxin–anti-digoxin–AuNP. The resulting complexes can be arrested by streptavidin at the test zone. The accumulation of gold nanoparticles on the test zone can be observed as an obvious red band. Superfluous free anti-digoxin-AuNP complexes keep on moving and accumulate at the control zone to form a second red band. In the absence of mutant SEA DNA or in the presence of wild-type DNA or non-complementary DNA, no duplex DNA is produced in the CSDPR; therefore, no anti-digoxin-AuNP conjugate is captured, and no red band is observed at the test zone. In this case, the observation of a red band at the control zone indicates that the LFB is working correctly.

This assay termed as lateral flow nucleic acid biosensor (LFNAB) can reach a sensitivity of 0.01 fM when using synthetic target DNA. **Figure 3** presents typical images together with the corresponding optical responses of the biosensors when loaded with various amounts of synthetic target DNA.

2.3.2. Spectrophotometric approach

LFNAB can qualitatively detect DNA by naked eye without expensive apparatus. It is fast and convenient. However, LFNAB is a qualitative or semi-quantitative detection method and the color observation may vary between different people. Therefore, we have developed a spectrophotometric DNA detection approach based on CSDPR for sensitive, selective, inexpensive, and quantitative detection of South East Asia (SEA) type of α-thalassemia [12]. As depicted in **Figure 4**, the signal amplification system is composed of a hairpin capture probe, a primer, polymerase, and streptavidin-horse radish peroxidase (SA-HRP). The structure of the hairpin capture probe consists of a biotin at 3′ end, a 11 base pair (bp) stem, a thiol group at 5′ end, and a 20-nt loop. The sequence of the loop is complementary to the target DNA. The primer (8-nt) is complementary to the stem region of the hairpin probe at 3′ end. The hairpin probe was first fixed on a gold nanoparticles (AuNPs) layer, which is coated by γ-globulin on a 96-well microtiter plate.

In the presence of target DNA, the loop region of the hairpin probe recognizes and hybridizes with the target DNA, causing conformational change of the hairpin probe to open the stem of the hairpin. The short primer then hybridizes with the open stem sequence and triggers a polymerization reaction in the presence of polymerase and dNTPs. Along with primer extension, the target DNA is released by polymerase with strand displacement activity, after which a longer

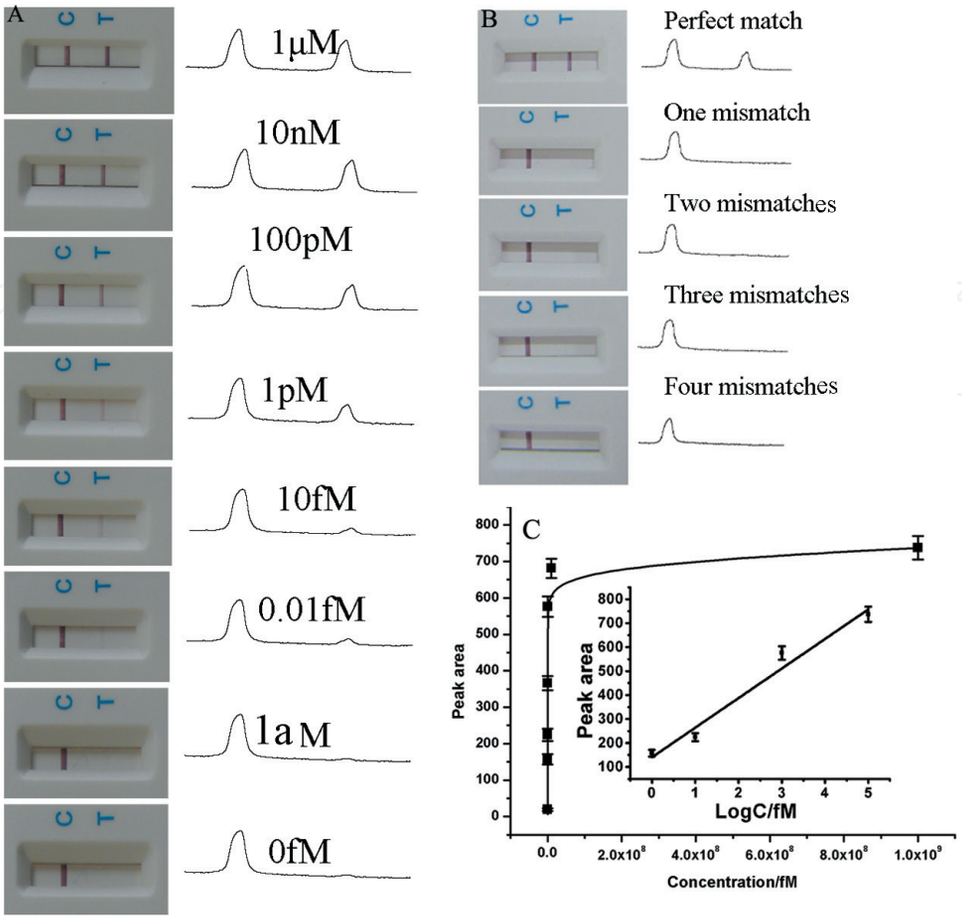

Figure 3. (A) Typical photographic images (left) and corresponding intensities (right) of an LFNAB in the presence of various concentrations of synthetic target DNA and the negative control. (B) Photographic images (left) and corresponding intensities (right) of the LFNAB tested with target DNA and target DNA including one or more mismatched bases. (C) Calibration curve of an LFNAB with various concentrations of target DNA. The error bars represent the standard deviations, $n = 3$.

complementary DNA is produced, forming a hairpin–DNA complex. The displaced target can hybridize with another immobilized hairpin probe to start the next cycle, which triggers yet another polymerization and target DNA release. Through this cyclical process, a great number of duplex DNA complexes with the biotin labeled are produced. The biotin groups on the duplex DNA products can conjugate with the SA-HRP. Finally, the target DNA can be sensitively detected via the HRP catalyzed substrate 3, 3′, 5, 5′-tetramethylbenzidine using a microtiter plate reader.

When the target DNA was absent or non-complementary DNA (such as wild-type DNA) was present, the fixed hairpin probe keeps the stem-loop structure and the reaction of biotin–SA-HRP would not take place as the steric effect. No signal would be detected.

Under optimal conditions, the sensitivity was calculated to be 8 aM and the dynamic range of the assay was from 0.1 fM to 10 nM. **Figure 5** presents the absorption spectrum and calibration curve of the assay.

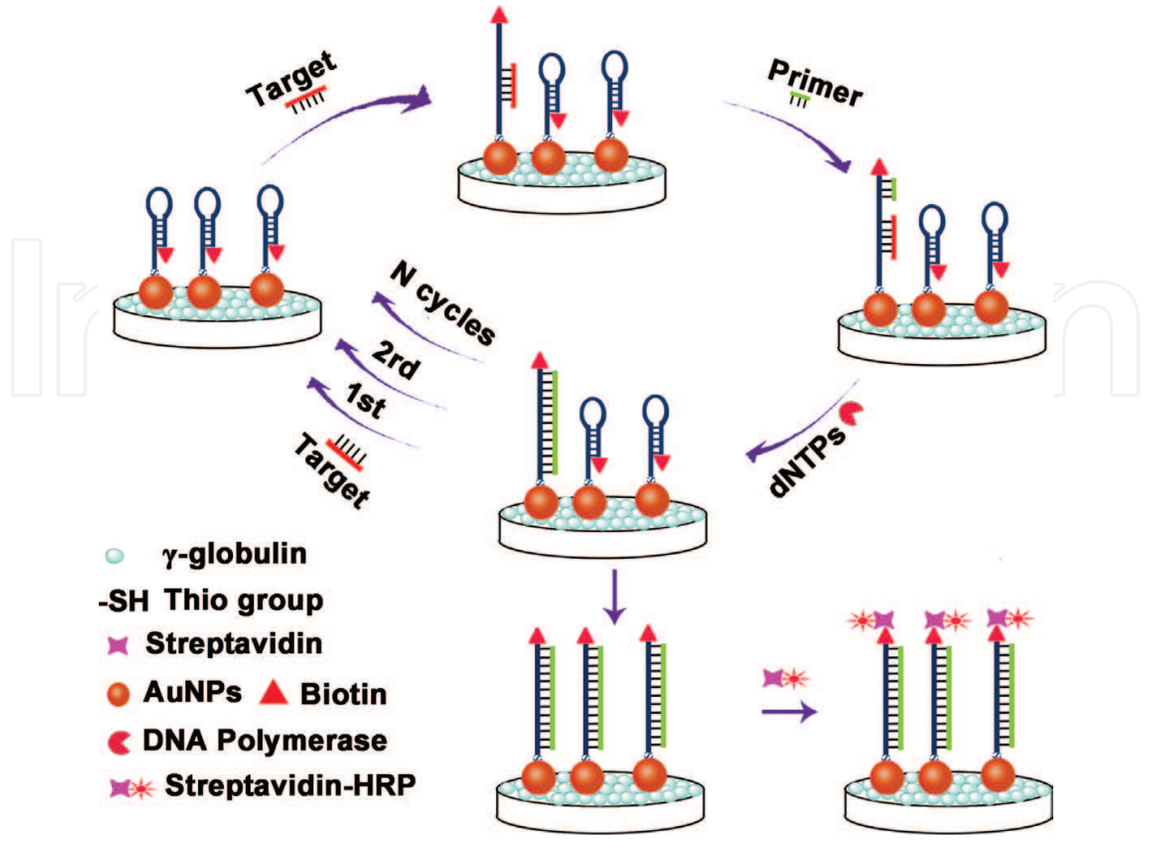

Figure 4. Schematic illustration for DNA detection based on CSDPR.

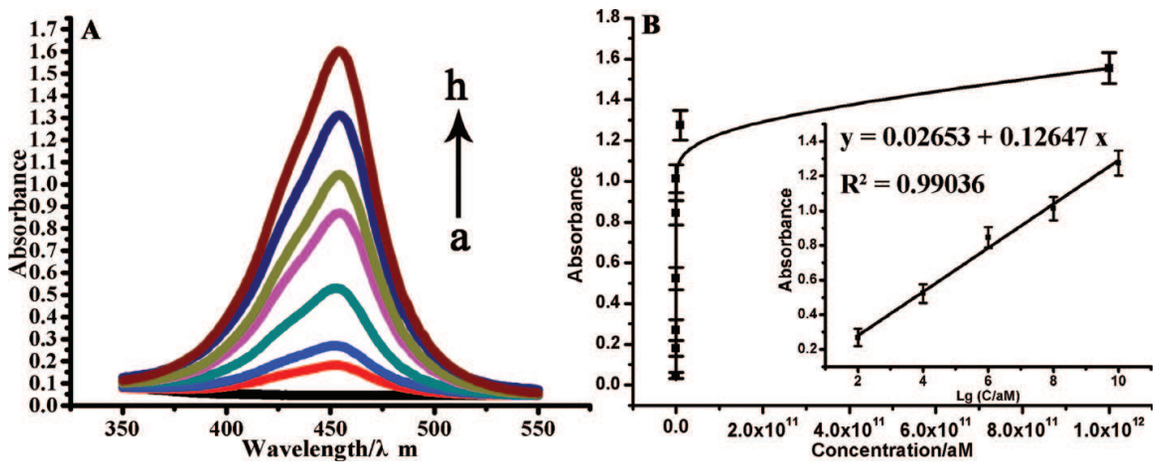

Figure 5. (A) Absorption spectrum response of the assay with different target DNA (0, 0.01 fM, 0.1 fM, 10 fM, 1 pM, 100 pM, 10 nM and 1 μM). (B) Calibration curve of the DNA assay with different concentrations of target DNA. Standard deviation was represent as error bars, $n = 3$.

CSDPR is a promising method for the detection of thalassemia genes. When combined with nucleic acid point-of-care testing methods such as DNA strip and spectrophotometric biosensors, this isothermal DNA amplification based approach could be widely applied in thalassemia carrier screening, especially in community hospitals.

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