# Journal Pre-proof


Simple and accurate visual detection of single nucleotide polymorphism based on colloidal gold nucleic acid strip biosensor and primer-specific PCR

Hai-Bo Wang, Li-Hong Ma, Ting Zhang, Kai-Chen Huang, Yuan-Di Zhao, Tian-Cai Liu

PII: S0003-2670(19)31133-X

DOI: https://doi.org/10.1016/j.aca.2019.09.048

Reference: ACA 237101

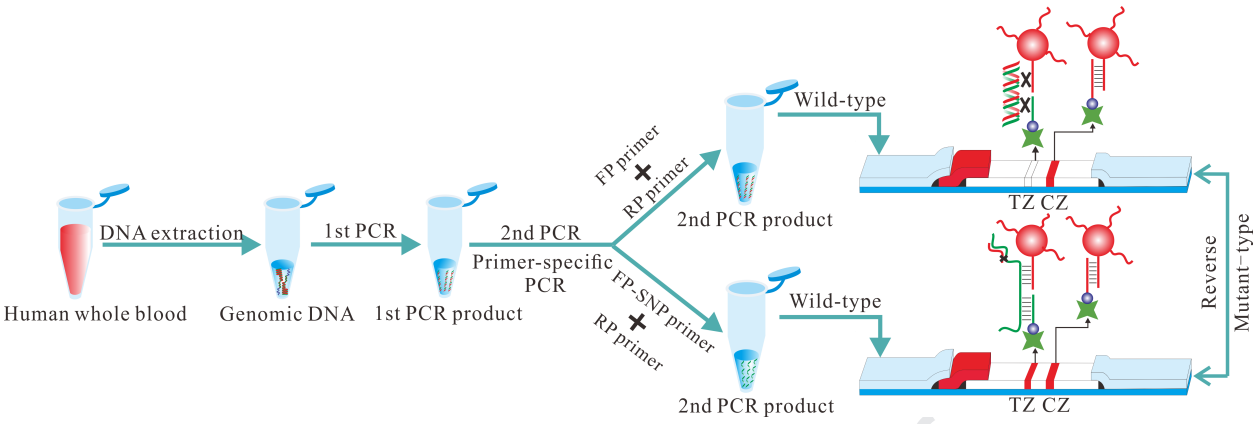
To appear in: *Analytica Chimica Acta*

Received Date: 26 June 2019

Revised Date: 10 September 2019

Accepted Date: 17 September 2019

Please cite this article as: H.-B. Wang, L.-H. Ma, T. Zhang, K.-C. Huang, Y.-D. Zhao, T.-C. Liu, Simple and accurate visual detection of single nucleotide polymorphism based on colloidal gold nucleic acid strip biosensor and primer-specific PCR, *Analytica Chimica Acta*, https://doi.org/10.1016/j.aca.2019.09.048.



# Simple and accurate visual detection of single nucleotide polymorphism based on colloidal gold nucleic acid strip biosensor and primer-specific PCR

Hai-Bo Wang[1,2], Li-Hong Ma[1], Ting Zhang[1], Kai-Chen Huang[3], Yuan-Di Zhao[1, 2]*, Tian-Cai Liu[3,4]*

[1] Britton Chance Center for Biomedical Photonics at Wuhan National Laboratory for Optoelectronics - Hubei Bioinformatics & Molecular Imaging Key Laboratory, Department of Biomedical Engineering, College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan 430074, Hubei, P. R. China

[2] Key Laboratory of Biomedical Photonics (HUST), Ministry of Education, Huazhong University of Science and Technology, Wuhan 430074, Hubei, P. R. China

[3] Institute of Antibody Engineering, School of Laboratory Medicine and Biotechnology, , Southern Medical University, Guangzhou 510515, Guangdong, P. R. China

[4] Guangdong Provincial Key Laboratory of Construction and Detection in Tissue Engineering, Southern Medical University, Guangzhou 510515, Guangdong, P. R. China

* Co-corresponding Author.

Tel: 86-27-87792235; Fax: 86-27-87792202. E-mail address: zydi@mail.hust.edu.cn (Y.D. Zhao); liutc@smu.edu.cn (T.C. Liu)

**Abstract:** Single nucleotide polymorphism (SNP) was associated with many human diseases, therefore, SNP detection was important for early diagnosis and clinical prognosis. Herein, a simple and accurate method for visual detection SNP sites (A/A, G/G, A/G) in CYP1A1 gene related to cancers based on colloidal gold nucleic acid strip biosensor and primer-specific polymerase chain reaction (PCR) was established. This method could directly distinguish SNP sites on strip biosensor by introducing twice PCR amplifications. The second PCR (primer-specific PCR) was performed using specific product of the first PCR as template, thus this twice PCR could reduce non-specific amplification greatly and obtain target product. In addition, single-strand or double-strand DNA (ssDNA or dsDNA) was accurately produced by introducing mismatched base at the 3' end of forward primers in primer-specific PCR. The designed strip biosensor could only combine with the ssDNA, thus visual detection of SNP could be achieved within 10 min by color difference of a pair of strips. 61 human blood samples by this method were identical with those of pyrosequencing. This method had the advantages of rapid, visual and low-cost and was expected to be applied in medical diagnosis.

**Key words** Primer-specific PCR; colloidal gold nucleic acid strip biosensor; single nucleotide polymorphism; cancer

## 1. Introduction

SNP refers to DNA sequence polymorphism caused by a single nucleotide variation at the genomic level. SNP mutations may have great impact on human health, and they are closely related to various diseases, in particular in various forms of cancer and hereditary diseases [1-4]. And the type of SNP mutation has significant influence on the success of personalized medicine or medical treatment [5]. Therefore, the detection of SNP mutation sites with high sensitivity and specific is of great significance for the early diagnosis, prevention and clinical prognosis of the disease. With the rapid development of biotechnology, accordingly, numerous SNP detection schemes have been developed, mainly including DNA pyrosequencing [6], fluorescent quantitative PCR (TaqMan PCR method) [7, 8], mass array method (Mass assay) [9, 10] and restriction fragment length polymorphism (PCR-RFLP method) [11], etc. These methods can achieve the detection of SNP mutations (or typing), however, it is difficult to be widely popularized because they all require expensive professional equipment and trained professionals. Therefore, it is of great significance to establish a fast, simple, sensitive and low-cost SNP detection technology.

CYP1A1 gene is human cytochrome P450 gene, which has polymorphism in the human population. The base A of position 4889 is replaced by G in the 7th exon (rs1048943). Therefore, there are three types in the human population, including homozygous wild-type (IIe/IIe or A/A), homozygous mutant type (Val/Val or G/G) and heterozygous mutant type (IIe/Val or A/G) [12-15]. This site mutation is associated with the occurrence of a variety of cancers, such as liver cancer, lung cancer and breast cancer, etc., when the gene mutation occurs, the aromatic hydrocarbon hydroxylase encoded by the cytochrome P450 gene reduces the detoxification ability of nitrosamines and benzidine, and thus the incidence of cancer is increased [14, 15]. Therefore, the detection of this gene

locus can help to assess the risk of cancer, in this way, the occurrence of cancer can be avoided or delayed by improving external causes.

Recently, colloidal gold strip biosensor as a solid-phase medium sensor has been widely used in clinical diagnosis (early pregnancy test, HIV test, etc.), due to the advantages of convenience, quick and low cost, visible detection with naked eyes. Colloidal gold nucleic acid strip biosensors for the detection of environmental pollutants and specific nucleic acid sequences have been realized [16-19]. However, when colloidal gold nucleic acid strip is used for SNP detection or screening, there will be false positive or false negative results, that is, the test zone (TZ) of the strip pairs all have visible color or no visible color, thus, the detection of SNPs in actual samples with colloidal gold nucleic acid strip biosensors is a great challenge. In general, colloidal gold nucleic acid strip biosensors can only be combined with ssDNA and not be combined with dsDNA. So how to get the desired ssDNA or dsDNA in the actual sample is a technical difficulty. PCR is simple and convenient and can specific exponential amplification to get the desired product. In order to distinguish SNP sites, primers that are completely complementary to the template and mismatched with the template at the 3’ end, respectively, were introduced. When the same samples were amplified, mismatched primer could not be combined with template, so the target ssDNA was obtained, while fully complementary primers could bind to the template to produce dsDNA. However, in the amplification of SNP samples, the mismatched primer would inevitably exhibit non-specific amplification because of complexity of genomic DNA (gDNA), thus the target ssDNA would not be obtained. Hence, twice PCR were introduced, after the first PCR, specific dsDNA was obtained, and the primer-specific PCR was performed using the first PCR product as template, in this way, non-specific amplification could be reduced greatly, thus, the target ssDNA or dsDNA appeared in product. And ssDNA could

combined with designed AuNP-DNA probe on strip biosensor, thus TZ of strip biosensor had visible band. Thereby, this method realized the visual detection of SNP in actual samples. Compared with PCR-RFLP and amplification refractory mutation system-PCR (ARMS-PCR) [20, 21], visible detection of colloidal gold nucleic acid strip biosensor could remove the post-treatment step (e.g., gel electrophoresis, data analysis, etc.), achieve fast, simple and low-cost SNP detection, therefore, it could be conveniently used for screening of SNPs related to diseases. This method only required twice ordinary PCR reactions, and then PCR products could be directly applied to the strip biosensor to distinguish SNP mutation. Therefore, it was expected to play an important role in medical diagnosis in the future.

## 2. Experimental

### *2.1. Apparatus*

Conjugate pad, TZ and control zone (CZ) were obtained with HM3030 dispenser (Shanghai Kinbio Tech., China). Strip was cut by a programmable strip cutter (Shanghai Kinbio Tech., China). PCR was amplified with T100 thermal cycler (Bio-rad, USA). Gel electrophoresis analysis was empolyed PowerPac$^{TM}$ Basic electrophoresis apparatus (Bio-rad, USA) and Sub cell GT horizontal electrophoresis tank (Bio-rad, USA), gel was imaged by UVP imaging system (UVP, USA). UV-vis absorption spectra were performed with UV 2550 spectrophotometer (Shimadzu, Japan). Ultrapure water (≥18.2 M·cm) was produced by the Milli-Q system (Millipore, USA).

### *2.2. Materials and reagents*

Chloroauric acid ($HAuCl_4 \cdot 4H_2O$), trisodium citrate, sodium chloride, trisodium phosphate, Tween 20, sucrose, polyethylene glycol octylphenyl ether (Triton X-100), sodium dodecyl sulfate (SDS), polyethylene glycol (PEG-4000), disodium hydrogen phosphate ($Na_2HPO_4 \cdot 12H_2O$), sodium

dihydrogen phosphate ($NaH_2PO_4 \cdot 2H_2O$), tris(hydroxymethyl)aminomethane (Tris), acetic acid and hydrochloric acid were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Tris(2-carboxyethyl)phosphine (TCEP) was purchased from Aladdin Biochemical Technology Co., Ltd (Shanghai, China). Streptavidin was purchased from BBI Life Sciences Corporation (Shanghai, China). All chemicals were of analytical-reagent grade. Premix Taq™ (TaKaRa Taq™ Version 2.0) was purchased from Takara Biomedical Technology Co., Ltd. (Beijing, China). Genecolour I nucleic acid dye was purchased from Beijing Jinboyi Biotechnology Co., Ltd. (Beijing, China). Low MW DNA Marker-A (25~500 bp) was purchased from Sangon Biotech Co., Ltd. (Shanghai, China). High-purity low-osmosis agarose was purchased from Beijing Tsingke Biotechnology Co., Ltd. (Beijing, China). 1×TAE running buffer with pH 8.0 was prepared by Tris (40 mM), acetic acid (20 mM) and EDTA (2 mM). The suspension buffer with pH 7.4 was prepared from $Na_3PO_4$ (20 mM), BSA (5 %), Tween 20 (0.25 %) and sucrose (10 %). Absorbent paper (SX42), glass fiber (CB06), PVC plastic adhesive backing (SMNF31-25) were obtained from Shanghai Kinbio Tech. Co., Ltd (Shanghai, China). Nitrocellulose membrane (CN140) was purchased from Sartorius (Goettingen, Germany). All blood samples were taken from Wuhan Commercial Workers Hospital and the study was approved by the Ethics Committee of Huazhong University of Science and Technology, and the type of SNP mutation (or typing) was confirmed by sequencing at Wuhan Tsingke Innovation Biotechnology Co., Ltd. (Wuhan, China). The sequences of the oligonucleotides used in this study were shown in Table 1. Modified nucleic acid sequences were synthesized and purified (HPLC) by Sangon Biotech Co., Ltd. (Shanghai, China) and ordinary nucleic acid sequences were synthesized and purified (PAGE) by Wuhan Tsingke Innovation Biotechnology Co., Ltd. (Wuhan, China).

**Table 1.**

*2.3. Preparation of AuNP*

AuNP was prepared by a previously reported trisodium citrate reduction method [22]. Briefly, 100 mL $HAuCl_4$ solution (0.01 %) was heated to boiling and then added 2 mL trisodium citrate solution (1 %) rapidly. The color of solution turned from yale yellow to wine-red, and after heating for another 10 min, the solution was slowly cooled to room temperature, filtered with 0.22 μm Millipore syringe filter to remove large particle, the filtrate was stored in refrigerator at 4 ºC for future experiments.

*2.4. Preparation of AuNP-DNA probe*

AuNP-DNA probe was prepared according to the protocol reported previously with slight modifications [23]. Briefly, TCEP was added to the thiol-modified DNA (1 OD, 100 μM) with a final concentration of 10 mM to destroy disulfide bond. The treated thiol-modified DNA was added into 14 mL AuNP and incubated for 16 h under gentle shaking and dark environment. Then 100 mM phosphate buffer (PB) and 10 % SDS were added into the above solution to achieve the final concentration of 9 mM and 0.1 % respectively, and incubated for 30 min with gentle shaking. The solution was aged for 1 d after adding 2 M NaCl (final concentration of 0.2 M) and then centrifuged at 4 °C for 20 min with 7200×g, the supernatant was discarded to remove unbounded DNA, and the red precipitate was resuspended in 1 mL suspension buffer, this procedure was repeated three times. Finally, the red precipitate was resuspended in 700 μL suspension buffer (20 times concentrated). The obtaining AuNP-DNA probe was stored at 4 °C for future use.

*2.5. Preparation of the strip biosensor*

The area of strip biosensor from left to right according to the direction of migration was composed of four parts: sample pad, conjugate pad, nitrocellulose membrane and absorption pad. The sample

pad and conjugate pad was obtained from the glass fiber and soaked with the buffer (0.5 % Triton X-100, 1 % BSA, 2 % glucose, 2 % PEG 4000 and 10 mM PB, pH 7.4), then it was dried at 37 ºC for 1 h and stored at room temperature. The conjugate pad was prepared by spraying 5 μL/cm AuNP-DNA probe 1 onto the processed glass fiber with HM3030 dispenser, it was dried at 37 ºC for 1 h and stored at 4 ºC. The biotinylated DNA probe T (Biotin-DNA probe T) and probe C (Biotin-DNA probe C) were mixed with streptavidin (1 mg/mL) by 1:1 (v/v) respectively, and incubated for 2 h at room temperature. The streptavidin-biotinylated DNA probe T (SA-biotin-DNA probe T) and the streptavidin-biotinylated DNA probe C (SA-biotin-DNA probe C) were dispensed on the nitrocellulose membrane of TZ and CZ with HM3030 dispenser, respectively. The distance between the TZ and CZ was around 5 mm. Finally, all the parts were assembled on the PVC plastic adhesive backing, each part was overlapped 2 mm to ensure migration of the sample solution during testing process. The strip biosensor was cut to width of 4.0 mm with programmable strip cutter, and stored at 4 ºC in desiccator.

*2.6. Preparation of PCR samples*

The human blood gDNA was extracted by blood extraction kit (Tiangen Biotech, Beijing, China), and the gDNA was used as template for PCR reaction. In the first PCR, 50 μL PCR reaction mixture containing 25 μL 2×Premix Taq$^{TM}$, 2 μL forward primer FP-L (10 μM), 2 μL reverse primer RP-L (10 μM), 2 μL blood gDNA, and 19 μL sterilized water. The thermal cycling protocol included an initial activation of Taq polymerases at 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 58.8 °C for 15 s, and 72 °C for 20 s, and a final extension step at 72 °C for 10 min. PCR product was used for sequencing to obtain SNP mutations and also used as the template for the second PCR. The primer-specific PCR was performed in two tubes, 2 μL forward primer FP with 3’ end of T which

was complementary to the wild-type A site was added to the tube 1, and in the tube 2, 2 μL forward primer FP-SNP with the 3' end of C which was complementary to the mutant type G site was added. 50 μL second PCR reaction mixture was composed of 25 μL 2×Premix Taq™, 2 μL reverse primer RP, 2 μL FP or FP-SNP, 2 μL first PCR product and 19 μL sterilized water. The second PCR thermal cycling was 94 °C for 30 s, 63.2 °C for 15 s and 72 °C for 12 s with a total of 30 cycles, the initial denaturation and final extension steps were extended to 5 and 10 min, respectively. All PCR products were analyzed by agarose gel electrophoresis at 3 % (containing 0.01 % Genecolour I nucleic acid dye) under the conditions of 100 V for 40 min. The gel was then imaged by a UVP imaging system.

*2.7. SNP detection with strip biosensor*

50 μL second PCR product was directly added to the sample pad of the strip biosensor, a clear band was observed on the TZ of strip biosensor by chromatographic reaction after 10 min, then strip biosensor was photographed and the band intensity of TZ was analyzed by Image J.

**3. Results and discussion**

*3.1. Principle of colloidal gold nucleic acid strip biosensor combined with primer-specific PCR for SNP detection*

In principle, this strategy was suitable for the detection of any SNP site. For the proof of concept study, SNP sites (A/A, A/G, G/G) in CYP1A1 gene related to multiple cancers was chosen as a target model. The strip biosensor could effectively distinguish the SNP sites of CYP1A1 gene associated with multiple cancers, and the principle detection was schematically illustrated in Figure 1. Human gDNA was extracted from whole blood, which was performed as template for twice PCRs. After the first PCR, a 246 bp dsDNA was obtained, and the primer-specific PCR was performed in two tubes using the first PCR product as template. Both tubes were added with the reverse primer RP, which

was completely complementary to the template. In addition, a forward primer FP with the 3' end T corresponding to the wild-type site A and a forward primer FP-SNP with the 3' end C corresponding to the mutant type site G were added to the tube 1 and 2, respectively. When the primer is fully complementary to the template, dsDNA was generated. However, when the 3' end of primer was mismatched with the template, the chain extension reaction of DNA polymerase could not be extended to form double strand due to the obstacle in the formation of 3', 5'-phosphodiester bond, and the target ssDNA was appeared. The designed AuNP-DNA probe could only combine with target ssDNA, thus AuNP was accumulated on TZ through the sandwich structure, so TZ would appear visible red band. Based on this, two tubes of PCR products were loaded on two strips, respectively, for the homozygous wild-type (A/A), TZ of strip biosensor with tube 2 had visible but no visible band for tube 1, for the homozygous mutant-type (G/G), TZ of strip biosensor with tube 1 had visible but no visible band for tube 2, for the heterozygous mutant-type (A/G), TZ of strip biosensor with both tubes had no visible band. Thus, the type of SNP could be judged by the color of TZs of a pair of strip biosensors.

**Figure 1.**

*3.2. Characterization of AuNP*

AuNP-DNA probe was the key component of strip biosensor, so its quality was critical. The prepared AuNP and AuNP-DNA probe were characterized by UV-visible spectroscopy and gel electrophoresis. AuNP had an absorption peak at 519 nm (Figure 2), and its concentration was calculated to be about 0.9 nM according to the literature [24]. The absorption peak of AuNP-DNA probe was red shifted to 523.5 nm, which was due to the increase of colloidal gold hydration radius and gold particle aggregation after thiol DNA modification. Comparing with wine red of colloidal

gold (Figure 2, inset I), the AuNP-DNA probe was dark red (Figure 2, inset I), which was consistent with the absorption spectra. At the same time, the intensity of absorption peak was decreased, which was caused by the loss during the purification of gold-labeled probe. Agarose gel electrophoresis showed that naked colloidal gold agglomerated in the well (Figure 2, inset II) because it could not tolerate the TAE electrophoresis buffer, while a clear band was observed for AuNP-DNA probe (Figure 2, inset II), indicating that the DNA had been modified on the surface of the colloidal gold to withstand the TAE electrophoresis buffer. After this, the effect of spraying amount of AuNP-DNA probe on conjugate pad (i.e. the concentration of AuNP-DNA probe on conjugate pad) on the color development of strips was examined (Figure S1). It could be seen that the band of TZ was weak when the AuNP-DNA probe was 1, 2 or 3 μL/cm, respectively. However, the band intensity of TZ was stable when the AuNP-DNA probe was 4, 5 or 6 μL/cm, at this time, the amount of AuNP-DNA probe was excessive. Therefore, the optimal spraying amount of AuNP-DNA probe with 5 μL/cm was immobilized on conjugate pad (Figure S1).

**Figure 2.**

*3.3. Effect of annealing temperature on different SNP samples in primer-specific PCR*

In order to distinguish SNP sites quickly and easily, we had tried to obtain the target ssDNA directly by only PCR once. Primer-specific PCR was tried obtaining the target single-strand DNA using the extracted genomic DNA as template directly. For homozygous samples, the ideal PCR result should present the great difference of the PCR amplification result (or efficiency) between the two forward primers of FP and FP-SNP. Therefore, FP-1 and FP-SNP-1 were used as the representative to investigate the PCR amplification results with different SNP locus at different annealing temperatures. FP-1 and FP-SNP-1 were primers that were completely complementary to

homozygous wild-type (A/A) and homozygous mutant type (G/G), respectively, and amplicon of the primer-specific PCR was 100 bp (Figure S1). For homozygous wild-type samples, the intensity of target band amplified by FP-1 primer at all temperatures were greater than that of FP-SNP-1 primer amplification (Figure 3A), due to the fact that FP-1 could be completely complementary to the template sample, and the mismatch between the C base at the 3' end of FP-SNP-1 primer and the A at the corresponding position of wild-type sample (Table 1) decreased PCR amplification efficiency. With the increase of annealing temperature (56.6 to 64.2 °C ), the difference between the intensities of the target bands of these two primers was greater, which might be due to the primer containing the mismatched base was more difficult to bind the template as the increase of annealing temperature, resulting in further reduce in amplification efficiency. However, the opposite result was obtained for the homozygous mutant sample (Figure 3B). Since the T base at 3' end of the FP-1 primer was mismatch with G base at the corresponding position of the mutant template, while the FP-SNP-1 primer was completely complementary to the template, thus the intensity of the PCR product with the FP-1 primer was weaker than that of the FP-SNP-1 primer at different annealing temperatures. For heterozygous mutant samples, since the genomic DNA samples could be completely complementary to the FP-1 primer and the FP-SNP-1 primer, the electrophoresis band intensities of PCR products of two primers were similar at different annealing temperatures (Figure 3C). The results of PCR product electrophoresis of three SNP site samples also showed as increasing annealing temperature (56.6 to 63.2 °C), the band intensities of both FP-1 and FP-SNP-1 primer amplification products were reduced. However, this decreasing trend was not obvious after 63.2 °C, and the intensity difference between the FP-1 and FP-SNP-1 amplification product bands was maximized (Figure 3A and B). Therefore, the optimal annealing temperature for primer-specific PCR

was 63.2 °C. However, it was disappointed that when the above PCR products was loaded on the strip biosensor, TZ of all the strip biosensors had no visible color, indicating that only one base mismatch at the 3' end of primer could not generate enough target ssDNA. Therefore, in order to effectively distinguish SNP, it was considered more mismatches were introduced to obtain ssDNA. Then the other base mismatch at the reciprocal 2nd or 3th base of the 3' end of forward primer was introduced (Table 1). Compared with FP-1 and FP-SNP-1, FP-2~4, FP-SNP-2~4 and FP-5~7, FP-SNP-5~7 were introduced different mismatch bases at the reciprocal 2nd position and 3rd position of the 3' end of the primers (Table 1). 7 group primers (a total of 14 primers) were performed in primer-specific PCR using gDNA samples of different SNP site as templates directly**,** and all PCR products were loaded on strip biosensors. However, it was regrettable that all TZs of the strip biosensors had no visible band, indicating that primer-specific PCR using gDNA as template directly was still unable to detect SNP sites on the strip biosensor, even if one mismatch base at 3' end of primer was introduced. This might be due to the complexity of gDNA, and the direct amplification of primer-specific PCR could not obtain the target ssDNA effectively. Therefore, the twice PCR method was considered to distinguish SNP effectively, while gDNA was amplified by the first PCR to obtain a 246 bp DNA duplex, which was used as a template for primer-specific PCR. It should be noted that 63.2 °C annealing temperature was also chosen as the second primer-specific PCR.

**Figure 3.**

*3.4. Effect of different annealing temperatures on the first PCR reaction*

Since the 246 bp DNA duplex obtained by the first PCR (Figure S2) would be used as the template for the primer-specific PCR, whose amplification quality was critical. In order to obtain

ssDNA to ensure the specificity of the primer-specific PCR template, the effect of different annealing temperatures on the specificity of the primer-specific PCR product was investigated. When the annealing temperature was between 47.8 and 50.7 °C (Figure 4), there were certain non-target bands besides the 246 bp target band. When the annealing temperature was between 54.2 and 60 °C, the PCR products were specific target band. Therefore, the annealing temperature of 58.8 °C was chosen for the first PCR reaction.

**Figure 4.**

*3.5. Effect of different primers on primer-specific PCR and strip biosensor detection*

The purpose of the twice PCR method was to get target ssDNA by the second PCR, which used the single and high concentration double-stranded DNA obtained by the first PCR as the template. In the 7 groups of primers, the primers with multiple mismatch bases might result in enough target ssDNA, and primers with few mismatch bases might lead to insufficient target ssDNA. Therefore, AuNP-DNA probe 1 and AuNP-DNA probe 2 were prepared to detect the target single-strand DNA, who could bind to them in different positions. When the target ssDNA was present, the two AuNP-DNA probes would be agglomerated and discolored because of formation of the bridge structure. When the target single strand DNA was absent, the two AuNP-DNA probes did not change color due to their independent existence. In addition, the products of the second primer-specific PCR was also characterized by agarose gel electrophoresis to verify the test results (Figure S3). For the samples of three SNP sites (A/A, A/G, G/G), after FP-1 and FP-SNP-1, FP-3 and FP-SNP-3, and FP-4 and FP-SNP-4 were amplified by PCR respectively, it was found no visible band appeared on TZ after loading the products onto strip biosensors, and the color of AuNP-DNA probe remained unchange (Figure 5A-D), indicating that the PCR reactions involved in these primers did not produce

enough target single-strand DNA. Gel electrophoresis also proved this point, their products of the second primer-specific PCR had only three dsDNA electrophoretic bands (Figure S3A, B and C, lane 1, 2, 5, 6, 7 and 8). For the homozygous wild-type (A/A) and heterozygous mutant (A/G) samples, the products of FP-3 had only two bands (Figure S3A and C, lane 5), this might be caused by insufficient sensitivity of the agarose gel. Since 3' end of FP-1 and FP-SNP-1 had only one base mismatch to the samples, therefore, they had little effect on PCR amplification efficiency. The reciprocal 1st and 2nd base at the 3' end of FP-3 and FP-SNP-3 as well as FP-4 and FP-SNP-4 were mismatched to the template, however, the mismatch of G/T or C/T at these two positions did not affect the efficiency of PCR amplification and the generation of dsDNA.

After the amplification of FP-2 and FP-SNP-2, FP-5 and FP-SNP-5, there were obvious red bands on their TZs of strip biosensors, and the color of AuNP-DNA probes turned blue-violet (Figure 5A-D), indicating that PCR of both groups of primers produced sufficient ssDNA. Meanwhile, almost four gel electrophoresis bands showed their products of the second primer-specific PCR had both dsDNA and target ssDNA (Figure S3A, B and C, lane 3, 4, 9 and 10). Although not all four bands in these lanes were clear, this might be due to the low concentration of ssDNA produced by PCR amplification and it could not detected by agarose gel. Since the mismatch base of T was introduced at the reciprocal 2nd or 3rd position at the 3' end of two groups of primers respectively, thus it was inferred that base mismatch of T/T could strongly influence the binding of the primer to the template, whether it occurred at the reciprocal 2nd or 3rd position of the 3' end of primer. Therefore, the amplification efficiency of PCR was greatly reduced, and sufficient single-strand DNA was formed in PCR products. However, it was not the ideal result we wanted, and it was impossible to distinguish the SNP types when both TZs had obvious red bands or without obvious

red bands.

FP-6 and FP-SNP-6, FP-7 and FP-SNP-7 primers showed different results for the three SNP samples. For the homozygous wild-type (A/A) samples, the TZs of the amplification products from FP-SNP-6 and FP-SNP-7 primers had obvious red bands (Figure 5A), the color of the AuNP-DNA probe also turned blue-violet; while the TZs of the strip biosensors for FP-6 and FP-7 primers had no visible red bands, and the color of AuNP-DNA probe solution remained unchange (Figure 5A). Moreover, gel electrophoresis showed FP-SNP-6 and FP-SNP-7 primers produced target ssDNA (Figure S3A, lane 12 and 14), however, FP-6 and FP-7 primers did not produce target ssDNA (Figure S3A, lane 11 and 13). For the homozygous mutant (G/G) sample, the detection result of strip biosensor was opposite. TZs of FP-6 and FP-7 primers had clear red bands, and AuNP-DNA probe turned blue-violet (Figure 5B), and the products of FP-6 and FP-7 primers had target ssDNA (Figure S3B, lane 11 and 13), however, the TZs of FP-SNP-6 and FP-SNP-7 primers had no visible bands, and the color of AuNP-DNA probe did not change (Figure 5B). Gel electrophoresis also showed FP-SNP-6 and FP-SNP-7 primers did not produce target ssDNA (Figure S3B, lane 12 and 14). For heterozygous mutant (A/G) samples, four TZs of FP-6 and FP-SNP-6, FP-7 and FP-SNP-7 primers did not appear red bands, and the color of AuNP-DNA probe remained unchange (Figure 5C). Gel electrophoresis showed these primers also did not produce target ssDNA (Figure S3C, lane 11-14). Therefore, the following conclusion was drawn: the second G or C base mismatch was introduced at the reciprocal 3rd position of 3’ end of primer if 1st reciprocal position at the 3’ end had been mismatched, the primer would be difficult to bind the template, resulting in significant reduction in the efficiency of PCR amplification. Efficiency of PCR amplification was not affected when the reciprocal 1st position of the 3’ end of primer was completely complementary to the corresponding

position of template, and C/T or G/T mismatch between the reciprocal 3rd position at 3' end of the primer and the template (Table 1). Therefore, FP-6 and FP-SNP-6, FP-7 and FP-SNP-7 could effectively distinguish three types of SNP (A/A，G/G，A/G). For homozygous type (A/A and G/G) samples, the band intensity difference between FP-6 and FP-SNP-6 primer was better than that of FP-7 and FP-SNP-7 primer (Figure 5D), so FP-6 and FP-SNP-6 could distinguish SNP sample more effectively, thus they were selected to detect SNP sample in following experiment.

**Figure 5.**

*3.6. Investigation of strip biosensor detection under different sample concentrations*

In order to ensure the good amplification of twice PCR for low concentration of sample, the effect of different sample concentration on the strip biosensor detection was investigated. After the first PCR amplification of gDNA samples at different SNP site with concentrations of 100, 50 and 10 ng, it was found that the concentrations of the PCR amplified products of the three samples (A/A, G/G, and A/G) were similar and the electrophoresis bands were single (Figure 6A). After primer-specific PCR, it was found that for homozygous wild-type (A/A) samples, the AuNP-DNA probe turned blue-violet and the TZ of strip biosensor had a clear red band (Figure 6B) caused by target single-strand DNA of FP-SNP-6 primers at three concentrations, however, FP-6 primers did not generate the target single-strand DNA, and their TZs of strip biosensors had no visible color (Figure 6B). For homozygous mutant (G/G) samples, FP-6 primers at three concentrations all produced the target single-strand DNA, the TZs of strip biosensors all had obvious bands (Figure 6C), and the color of AuNP-DNA probe was blue-violet, while FP-SNP-6 primer did not produce target single-strand DNA, so the AuNP-DNA probe remained unchange and the TZs of strip biosensors had no visible color (Figure 6C). For heterozygous mutant (A/G) samples, the TZs of strip biosensors

under the PCR products of FP-6 and FP-SNP-6 primers at three concentrations presented colorless (Figure 6D). In summary, the primer-specific PCR amplified with different concentration samples achieved satisfactory effect, which was very practical for the extraction of samples with lower concentration.

**Figure 6.**

*3.7. Real samples detection*

Based on the above results, twice PCR amplification and strip biosensor detection were performed on 61 human blood samples, and the results were compared with those of pyrosequencing (gold standard method for SNP detection). The results showed that there were 30 A/A site samples, 5 G/G site samples and 26 A/G site samples among them (Table S1), the results of typical No. 25, 31 and 59 samples were showed in Figure 7. The strip biosensors could clearly determine the type of SNPs, which were in good agreement with the sequencing results. It was indicated that the strip biosensor could effectively distinguish SNP sites as long as the quality of twice PCR were ensured. Therefore, the colloidal gold nucleic acid strip biosensor combined with primer-specific PCR had good practicability and good market application prospect for SNP sites detection.

**Figure 7.**

## 4. Conclusions

In summary, a cancer-related SNP site detection method had been developed based on colloidal gold nucleic acid strip biosensor combined with primer-specific PCR. In brief, twice PCR were adopted, the first PCR provided single template with high concentration for the following primer-specific PCR, this twice PCR strategy could obtain the target DNA and reduced the non-specific amplification greatly, based on this, the visual detection of SNP on strip biosensor could

be realized. In addition, mismatched base at 3' end of primer was introduced in primer-specific PCR, and the PCR product (ssDNA or dsDNA) was first studied by AuNP-DNA probe. This method could remove the complicated post-treatment steps because the visual detection of SNP by loading the product of twice PCR directly on the strip biosensor. The method was fast, simple, accurate, low-cost and obtained satisfactory results for the detection of 61 human blood samples, importantly, it could be extended to detect any other SNP sites easily. We believe that this method was expected to have great application potential in future medical diagnosis.

**Conflicts of interest**

There are no conflicts to declare.

**Declaration of interests**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

**Acknowledgements**

This work was supported by the National Key Research and Development Program of China (2017YFA0700402), the National Natural Science Foundation of China (Grant No. 81771878, 81971658), and the Fundamental Research Funds for the Central Universities (Hust: 2016YXMS253, 2017KFXKJC002, 2018KFYXKJC048). We also thank the Analytical and Testing Center (HUST), the Research Core Facilities for Life Science (HUST) and the Center for Nanoscale Characterization & Devices (CNCD) at WNLO of HUST for the help of measurement. Prof. T.C. Liu also thanked the support of the National Natural Science Foundation of China (21575058).

**References**

[1] J. N. Hirschhorn, Genetic approaches to studying common diseases and complex traits, Pediatr. Res. 57 (2005) 74R-77R.

[2] S. Kim, A. Misra, SNP genotyping: technologies and biomedical applications, Annu. Rev. Biomed. Eng. 9 (2007) 289-320.

[3] D. J. Hunter, P. Kraft, K. B. Jacobs, D. G. Cox, M. Yeager, S. E. Hankinson, S. Wacholder, Z. M. Wang, R. Welch, A. Hutchinson, J. W. Wang, K. Yu, N. L. J. Chatterjee, N. Orr, W. C. Willett, G. A. Colditz, R. G. Ziegler, C. D. Berg, S. S. Buys, C. A. McCarty, H. S. Feigelson, E. E. Calle, M. J. Thun, R. B. Hayes, M. Tucker, D. S. Gerhard, J. F. Fraumeni Jr, R. N. Hoover, G. Thomas, S. J. Chanock, A genome-wide association study identifies alleles in FGFR2 associated with risk of sporadic postmenopausal breast cancer, Nat. Genet. 39 (2007) 870.

[4] M. Krawczak, E. V. Ball, I. Fenton, P. D. Stenson, S. Abeysinghe, N. Thomas, D. N. Cooper, Human gene mutation database-a biomedical information and research resource, Hum. Mutat. 15 (2000) 45-51.

[5] K. K. Jain, Personalized medicine, Curr. Opin. Mol. Ther. 4 (2002) 548-558.

[6] R. H. Fakhrai, N. Pourmand, M. Ronaghi, Pyrosequencing™: an accurate detection platform for single nucleotide polymorphisms, Hum. Mutat. 19 (2002) 479-485.

[7] K. J. Livak, Allelic discrimination using fluorogenic probes and the 5′ nuclease assay, Genet. Anal. Biomol. Eng. 14 (1999) 143-149.

[8] K. Fujii, Y. Matsubara, J. Akanuma, K. Takahashi, S. Kure, Y. Suzuki, M. Imaizumi, K. Iinuma, O. Sakatsume, P. Rinaldo, K. Narisawa, Mutation detection by TaqMan-allele specific amplification: Application to molecular diagnosis of glycogen storage disease type Ia and medium-chain acyl-CoA dehydrogenase deficiency, Hum. Mutat. 15 (2000) 189-196.

[9] J. Tost, I. G. Gut, Genotyping single nucleotide polymorphisms by mass spectrometry, Mass Spectrom. Rev. 21 (2002) 388-418.

[10] Z. Fei, L. M. Smith, Analysis of single nucleotide polymorphisms by primer extension and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, Rapid Commun. Mass Spectrom. 14 (2000) 950-959.

[11] S. Fukuen, T. Fukuda, H. Maune, Y. Ikenaga, I. Yamamoto, T. Inaba, J. Azuma, Novel detection assay by PCR-RFLP and frequency of the CYP3A5 SNPs, CYP3A5* 3 and* 6, in a Japanese population, Pharmacogenet. Genomics 12 (2002) 331-334.

[12] K. Kawajiri, K. I. Nakachi, Z. K. Imai, J. Watanabe, S. I. Hayashi, The CYP1A1 gene and cancer susceptibility[J]. Critical reviews in oncology/hematology, Crit. Rev. Oncol. Hemat. 14 (1993) 77-87.

[13] T. L. McLemore, S. Adelberg, M. C. Liu, N. A. McMahon, S. J. Yu, W. C. Hubbard, M. Czerwinski, T. G. Wood, R. Storeng, R. A. Lubet, J. C. Eggleston, M. R. Boyd, R. N. Hines, Expression of CYP1A1 gene in patients with lung cancer: evidence for cigarette smoke-induced gene expression in normal lung tissue and for altered gene regulation in primary pulmonary carcinomas, J. Natl. Cancer Inst. 82 (1990) 1333-1339.

[14] S. Garte, Carcinogenesis, The role of ethnicity in cancer susceptibility gene polymorphisms: the example of CYP1A1, 19 (1998) 1329-1332.

[15] Y. S. Hong, J. H. Chang, O. J. Kwon, Y. A. Ham, J. H. Choi, Polymorphism of the CYP1A1 and glutathione-S-transferase gene in Korean lung cancer patients, Exp. Mol. Med. 30 (1998) 192.

[16] J. Chen, S. Zhou, J. Wen, Disposable strip biosensor for visual detection of $Hg^{2+}$ based on $Hg^{2+}$-triggered toehold binding and exonuclease III-assisted signal amplification, Anal. Chem. 86 (2014) 3108-3114.

[17] D. Mazumdar, J. Liu, G. Lu, J. Zhou, Y. Lu, Easy-to-use dipstick tests for detection of lead in paints using non-cross-linked gold nanoparticle-DNAzyme conjugates, Chem. Commun. 46 (2010) 1416-1418.

[18]X. Mao, Y. Ma, A. Zhang, L. Zhang, L. Zeng, G. Liu, Disposable nucleic acid biosensors based on gold nanoparticle probes and lateral flow strip, Anal. Chem. 81 (2009) 1660-1668.

[19]X. Mao, H. Xu, Q. Zeng, L. Zeng, G. Liu, Molecular beacon-functionalized gold nanoparticles as probes in dry-reagent strip biosensor for DNA analysis, Chem. Commun. 21 (2009) 3065-3067.

[20]E. Chiapparino, D. Lee, P. Donini, Chiapparino E, Lee D, Donini P. Genotyping single nucleotide polymorphisms in barley by tetra-primer ARMS-PCR, Genome 47 (2004) 414-420

[21]N. Okayama, K. Fujimura, J. Nakamura, Y. Suehiro, Y. Hamanaka, Y. Hinodaet, Evaluation of a new efficient procedure for single-nucleotide polymorphism genotyping: tetra-primer amplification refractory mutation system-polymerase chain reaction, Clin. Chem. Lab. Med. 42 (2004) 13-16.

[22]G. Frens, Controlled nucleation for the regulation of the particle size in monodisperse gold suspensions, Nature 241 (1973) 20-22.

[23]H. D. Hill, C. A. Mirkin, The bio-barcode assay for the detection of protein and nucleic acid targets using DTT-induced ligand exchange, Nat. Protoc. 1 (2006) 324-336.

[24]W. Haiss, N. T. K. Thanh, J. Aveyard, D. G. Fernig, Determination of size and concentration of gold nanoparticles from UV-vis spectra, Anal. Chem. 79 (2007) 4215-4221

Figure 1. Schematic diagram of colloidal gold nucleic acid strip biosensor combined with primer-specific PCR for SNP detection. (A) All steps of SNP sites detection. Genomic DNA extracted from human whole blood was used as template for twice PCR. The 2nd PCR was amplified in two tubes, dsDNA product could not bind the AuNP-DNA probe, test zone (TZ) had no visible color, while single-strand DNA could bind AuNP-DNA probe, so TZ had visible color. (B) SNP sites (A/A, A/G, G/G) in CYP1A1 gene was chosen as target model, SNP site sample was amplified with primer-specific PCR in two tubes, 3' end of FP primer and FP-SNP primer was fully complementary to A site sample and G site sample, respectively, dsDNA was formed when the primer was fully complementary to the template, however, single-strand DNA was formed when the primer was mismatched with the template. SNP type was determined by observing the TZ color of a pair of strip biosensors.

Figure 2. UV-visible absorption spectra of AuNP (a) and AuNP-DNA probe (b). Inset: their photo (I) and gel electrophoresis (II); agarose gel concentration: 1%; electrophoresis condition: 90 V, 10 min.

Figure 3. Agarose gel electrophoresis of primer-specific PCR products with different SNP site samples at different annealing temperatures. agarose gel concentration: 3%; electrophoresis condition: 100 V, 40 min.

Figure 4. Agarose gel electrophoresis of the first PCR product at different annealing temperatures. The agarose gel concentration and electrophoresis condition were same as Figure 2

Figure 5. Photo images of AuNP-DNA probe, strip biosensor and histogram of corresponding TZ

peak area (D) of the primer-specific PCR products of 7 groups of primers using A/A site (A), G/G site (B) and A/G site (C) samples

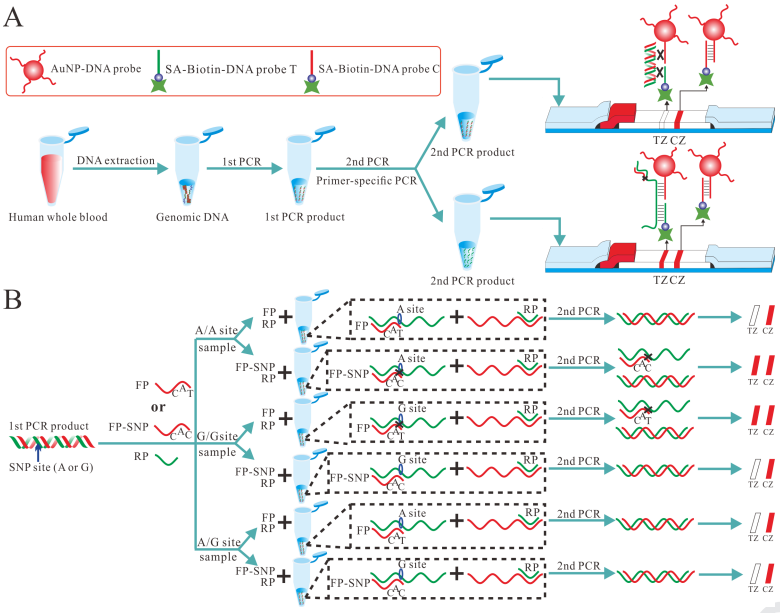
Figure 6. Agarose gel electrophoresis (A) of the first PCR amplification products of 100, 50 and 10 ng A/A (lanes 1~3), G/G (lanes 4~6) and A/G (lanes 7~9) site gDNA samples; photo images of AuNP-DNA probe, strip biosensor and histogram of the corresponding TZ peak area of the twice PCR products of FP-6 and FP-SNP-6 primers using 100, 50 and 10 ng A/A (B), G/G (C) and A/G (D) site gDNA samples. The agarose gel concentration and electrophoresis condition were same as Figure 2

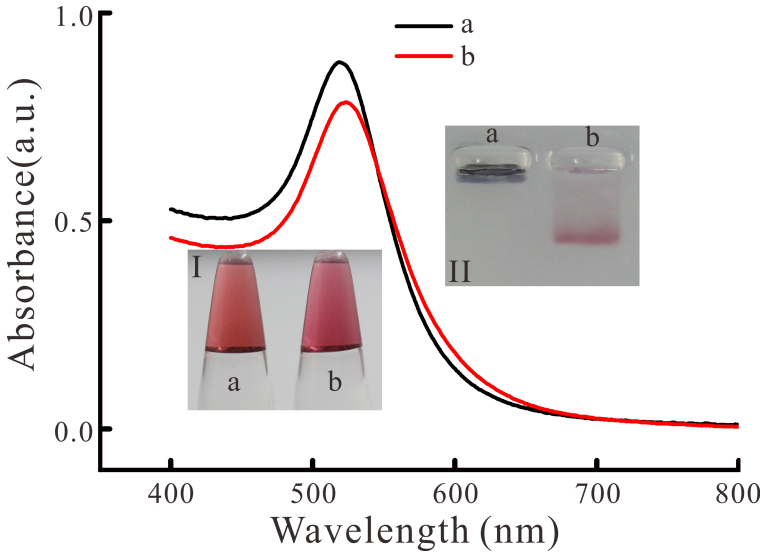
Figure 7. Three SNP types of actual samples on strip biosensor detection and corresponding pyrosequencing results (single peak: one allele; double peak: two alleles; purple R: base A/G).

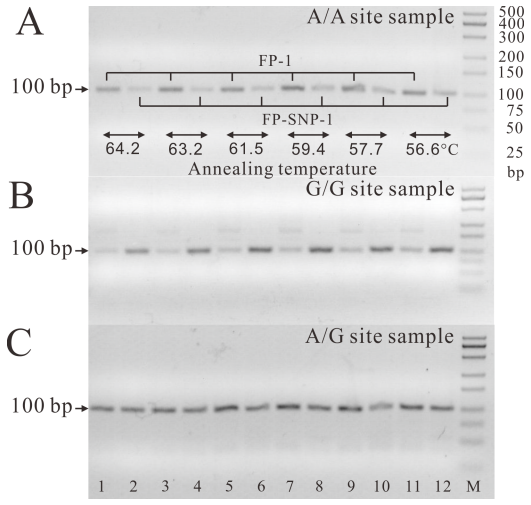
Table 1. Sequences of oligonucleotides used in the study.

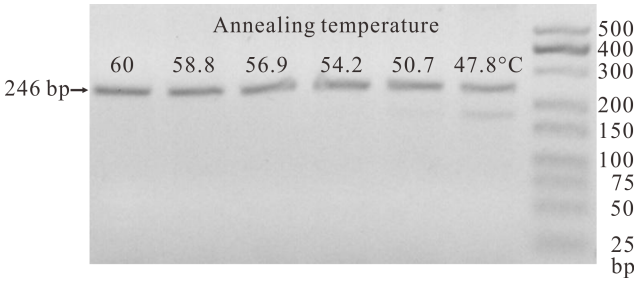
| Name | Sequence (5'- 3') |
|---|---|
| FP-L | CTGCATTTGGAAGTGCTC |
| RP-L | CTACCTGAACGGTTTCTCAC |
| FP-1 | AACTCCCAGCGGGCAA*T* |
| FP-SNP-1 | AACTCCCAGCGGGCAA*C* |
| FP-2 | AACTCCCAGCGGGCA*TT* |
| FP-SNP-2 | AACTCCCAGCGGGCA*TC* |
| FP-3 | AACTCCCAGCGGGCA*GT* |
| FP-SNP-3 | AACTCCCAGCGGGCA*GC* |
| FP-4 | AACTCCCAGCGGGCA*CT* |
| FP-SNP-4 | AACTCCCAGCGGGCA*CC* |
| FP-5 | AACTCCCAGCGGGC*T*A*T* |
| FP-SNP-5 | AACTCCCAGCGGGC*T*A*C* |
| FP-6 | AACTCCCAGCGGGC*C*A*T* |
| FP-SNP-6 | AACTCCCAGCGGGC*C*A*C* |
| FP-7 | AACTCCCAGCGGGC*G*A*T* |
| FP-SNP-7 | AACTCCCAGCGGGC*G*A*C* |
| RP | CACCCCTGATGGTGCTATC |
| AuNP-DNA probe 1 | SH-GCCAAAGATAATCAC |
| AuNP-DNA probe 2 | SH-CTTCTCACTTAACAC |
| Biotin-DNA probe T | CTTCTCACTTAACAC-Biotin |
| Biotin-DNA probe C | Biotin-GTGATTATCTTTGGC |

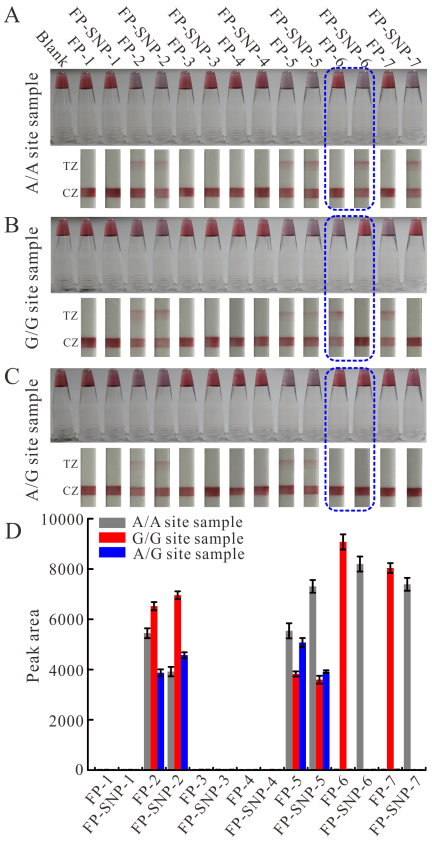
FP: Forward primer; RP: Reverse primer; single underline: the bases of FP-X and FP-SNP-X were matched with A/A site and G/G site, respectively; double underline: mismatched base

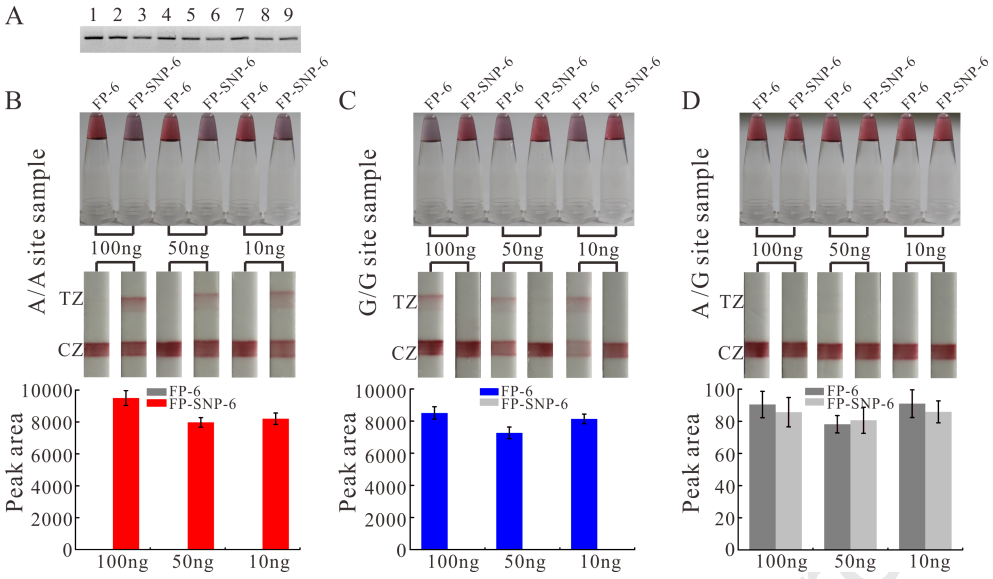


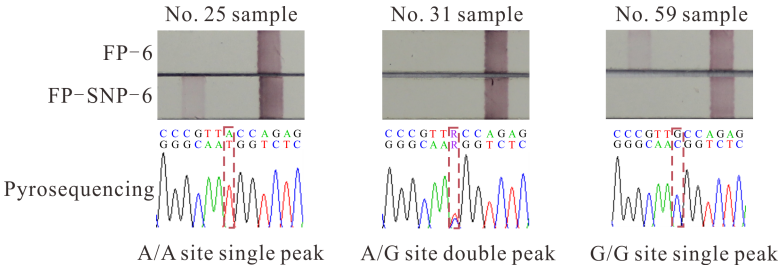












- SNP site detection is achieved based on strip biosensor and primer-specific PCR
- Twice PCRs are introduced to reduce non-specific amplification greatly
- The detection of CYP1A1 gene SNP site is visible within 10min using strip biosensor
- The results of 59 human blood samples by this method are identical with sequencing
- This method can be applied to the detection of any other SNP site

**Declaration of interests**

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Conflict of interest statement

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled