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Visual Detection of *ACE* I/D Polymorphism Using T-ARMS-PCR Combined with a Lateral Flow Assay and Its Clinical Application

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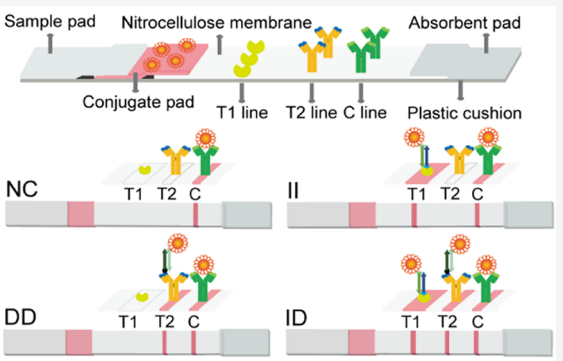
ABSTRACT: Insertions/deletions (indels) variations have been recognized as a promising marker for the development of various diseases. However, methods used for the genotyping of indels in studies were tedious, complicated, and required sophisticated or expensive instruments, as well as complex data analysis, which makes it difficult to meet the demand of point of care testing. Herein, we presented a fast and accurate biosensor (T-ARMS-PCR-LFA) by the combination of tetra-primer amplification refractory mutation system polymerase chain reaction (T-ARMS-PCR) and GoldMag lateral flow assay (LFA) for visual genotyping of *ACE* I/D polymorphism. *ACE* I/D can be distinguished by employing four primers in one PCR reaction, and genotyping results were presented by the visual inspection of colors on the nitrocellulose membrane of LFA strips within 5 min. And 50 of the human genomic DNA samples were used for the detection of *ACE* I/D to further validate the accuracy of the T-ARMS-PCR-LFA system. As a demonstration, we showed that *ACE* I/D could be genotyped using a low amount of DNA sample (25 ng) with an accuracy of 100%, without complicated operation steps and data analysis, which is better than that of the conventional method (agarose gel electrophoresis analysis after common PCR). In conclusion, the biosensor is highly applicable for genotyping specific large indel variants in clinical practices, which enables rapid clinical decision-making, improves the management of disease diagnosis, and facilitates personalized medicine.



INTRODUCTION

Insertions/deletions (indels) are length variations created by inserting or deleting one or more nucleotides in the genome.[1] As the second most common and functionally important type of genetic variation,[2] indels have been used as genetic markers to study ancestry affiliation,[3] population genetics, and forensic medicine.[4,5] It has been found that the importance of indels variations to human health is increasingly apparent. Numerous human genetic diseases and cancers have long been recognized to be strongly correlated with some indels polymorphism.[6−8] Considerate that the indels genetic markers, especially large indels, are implicated in many diseases and further association is yet to be discovered. There is a particular need to provide an accurate and rapid large indels detection method for facilitating conduction of function research and clinical diagnosis, treatment, and prognosis, which would offer prompt genetic information for the development of precision medicine.

There are some methods that can be used for the detection of indels such as next-generation sequencing (NGS), mass spectroscopy,[9] gene chip,[10] TaqMan qPCR assay,[11,12] and high-resolution melt analysis (HRMA).[13] NGS is commonly used in the screening of the human genomes sequence to discover new mutations, including SNPs and indels.[14] It is powerless to detect large indels, and only small indels can be detected well by NGS, which leads to leak detection of large indels.[15] Mass spectroscopy and a gene chip usually include tedious operation steps, complicated data analysis, and a longer test cycle of the clinical sample, which limit the application of these techniques for detection of indels in point of care testing (POCT). TaqMan qPCR assay and HRMA required expensive instruments and costly material, as well as professionals that may not be available in many hospitals or laboratories with limited resources. In addition, these high-throughput techniques are not suitable for the detection of a small sample size of indels.

Lateral flow assay (LFA) has received more attention in recent years due to its simplicity, low cost, and flexibility to provide POCT.[16−18] In our previous work, we have developed a method that combines an amplification refractory mutation

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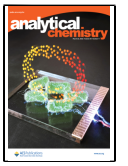

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system-PCR (ARMS-PCR) with LFA strips to detect delE746-A750.[19] The method is based on two separate PCR reactions with three primers to amplify a sample. Two LFA strips are used to detect PCR products and present small deletion genotyping results in the form of visualization, which greatly simplifies the operation steps and saves much time. However, the method can be further simplified. The tetra-primer amplification refractory mutation system polymerase chain reaction (T-ARMS-PCR) is a rapid way to detect gene polymorphism, which identifies two different alleles of an indel with four primers in a single PCR reaction,[20] while gel electrophoresis is required, which undoubtedly increases the workload and costs more time. Our previous study first combined T-ARMS-PCR and LFA to detect SNPs,[21] which is accurate, rapid, inexpensive, and scalable for genotyping.

ACE I/D polymorphism in the Alu repeat sequence of 16 introns was the most common variant of the angiotensin converting enzyme (ACE). *ACE* I/D is of great interest in clinical diagnoses due to its participation in the occurrence and development of many diseases,[22−24] such as knee osteoarthritis, renal disease, and especially essential hypertension (EH). Currently, few methods are suitable for the rapid clinical diagnosis of *ACE* I/D. In this study, we presented a new strategy that uses T-ARMS-PCR-LFA to detect *ACE* I/D polymorphism. The whole genotyping workflow is exhibited in Figure 1. Genomic DNA was extracted from the whole blood sample as the template, followed by T-ARMS-PCR amplification with specifically designed primers. The PCR products obtained from step 2 were added onto the sample pads of the LFA strips. Finally, genotypes were determined by the red bands on the LFA strips. In addition, a case-control study was also conducted to investigate the effects of *ACE* I/D polymorphism on EH to fill the gap in the correlation between *ACE* I/D polymorphism and EH among Han people in the Shaanxi Province.

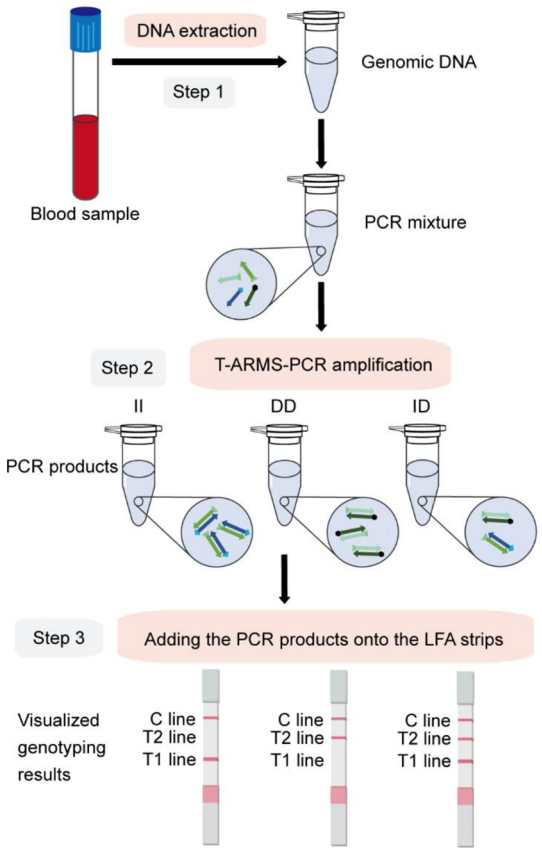

Figure 1. Workflow of the *ACE* I/D detection process. Major operation steps in a comprehensive workflow for *ACE* I/D detection are depicted in a simplified form. Genomic DNA was extracted from a whole blood sample (step 1) and then *ACE* I/D was amplified by T-ARMS-PCR with specifically designed primers (step 2). After PCR amplification, the PCR products were added onto the sample pads of the LFA strips. Genotypes were determined by the red bands on LFA strips (step 3). The T1 line was used to detect I-specific PCR products, the T2 line was used to detect D-specific PCR products, and the C line indicated that the chromatography system is reliable.

EXPERIMENTAL SECTION

Reagents and Materials. The whole blood genomic DNA isolation kit, poly(acrylic acid)-modified GoldMag nanoparticles (PGMNs), nitrocellulose (NC) membrane, sample pad, conjugate pad, and absorbent pad were offered by Xi'an GoldMag Nanobiotech Co., Ltd. (Xi'an, Shaanxi, PRC). The anti-digoxin antibody was bought from Meridian Life Science, Inc. (Saco, ME, U.S.A.). Streptavidin was purchased from Promega Biotech Inc. (Madison, WI, U.S.A.). Anti-Cy5 antibody was obtained from Sigma-Aldrich (Shanghai, PRC). Goat anti-mouse IgG was from Joey Bioscience Inc. (Shanghai, PRC). Primers were synthesized via Invitrogen (Shanghai, PRC). The 1× TE and 10× PCR buffer, HotMaster Taq DNA polymerase, 2× Taq PCR MasterMix, 6× DNA loading buffer, and deionized water were given by TIANGEN (Beijing, PRC). Uracil N-glycosylase (UNG) and dNTPs (dATP, dCTP, dGTP, dUTP) were from Shinegene (Shanghai, PRC). All chemicals were of analytical grade from reputable vendors. Buffers were prepared according to standard laboratory procedures.

Conventional Methods. The conventional technique was used to compare the accuracy and sensitivity of the T-ARMS-PCR-LFA system. Two primers that can be used to amplify the D and I alleles were designed for the first round of PCR. Concisely, the first round PCR amplification was performed with 2× Taq PCR MasterMix, forward primer 5′-AAGGAGAGGAGAGACTCAAGCAC-3′, reverse primer 5′-GGACCCAAGTGCCAGTGATGT-3′, and genomic DNA. The PCR procedure is as follows: 3 min at 94 °C, 31 cycles of 30 s at 94 °C, 30 s at 62 °C, and 1 min at 72 °C, and one step of 5 min at 72 °C. PCR products of 739 and 450 bp were obtained from the amplification of I and D alleles, respectively, and the obtained PCR products were detected by an agarose gel. A second independent PCR was conducted with DD genotype samples using another primer pair specific for I allele, and PCR products were also detected by an agarose gel. The second PCR process can avoid mistyping of ID as DD.

Another conventional PCR combined with agarose gel electrophoresis was used to select primers for constructing T-ARMS-PCR-LFA. Four sets of primers for the amplification of I and D alleles, respectively, that introduced different additional mismatches at the penultimate or antepenult nucleotide of the 3′ terminus of I-reverse primer and D-forward primer were designed. The same primer pair was added into two tubes for amplification of confirmed II and DD samples, separately. PCR products were then screened through agarose gel electrophoresis to obtain the best specific primer pairs. For analysis of I-specific primers, PCR amplification was performed with the I-forward primer, 5′-AAGGAGAGGAGAGAGACTCAAGCAC-3′, and I-reverse primer, 5′-TCGA-

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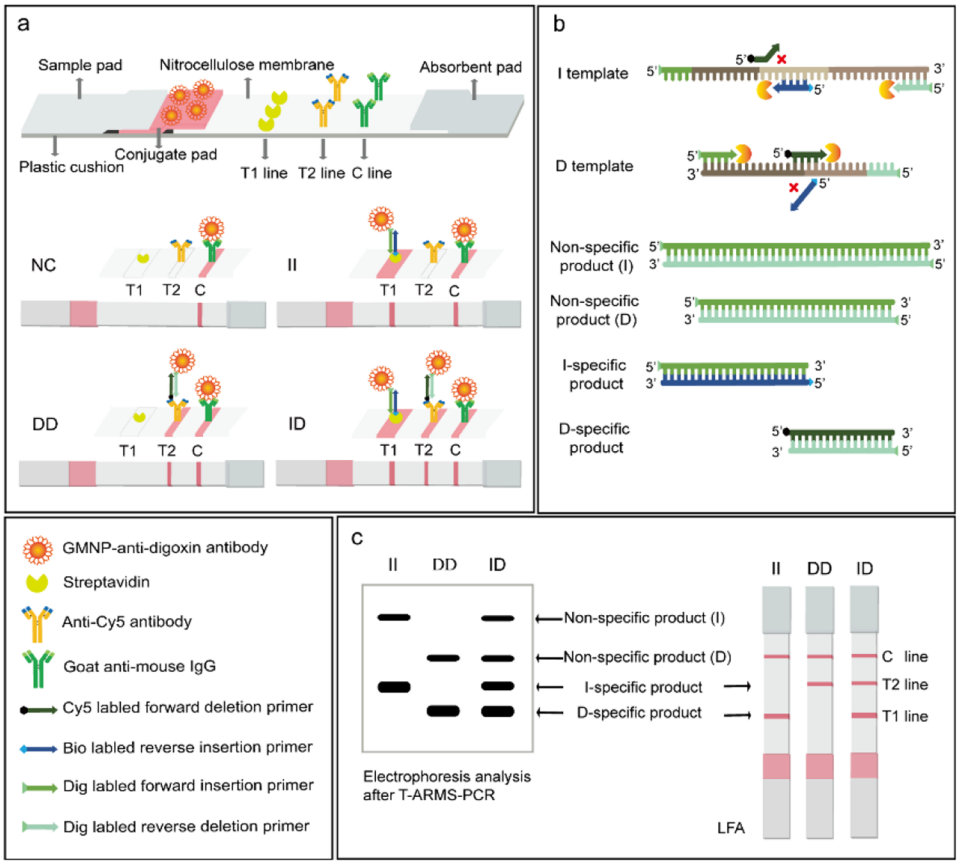

Figure 2. Schematic diagram of the tetra-primer ARMS-PCR LFA system. (a) The structure of lateral flow strip (upper) and the process of specific PCR products detected by LFA strips (lower). (b) The principle of T-ARMS-PCR for amplification of *ACE* I/D. (c) Genotyping results of *ACE* I/D polymorphism presented by gel electrophoresis after T-ARMS-PCR (left) and LFA strips (right). The T1 line was used to detect I-specific PCR products, the T2 line was used to detect D-specific PCR products, and the C line indicated that the chromatography system is reliable.

GACCATCCCGGCTAAAAC-3′. And for the analysis of the D-specific primers, PCR amplification was performed with the D-forward primer, 5′-GCTGCCTATACAGTCACTTT-TATGCGG-3′, and D-reverse primer, 5′-GGACCCAA-GTGCCAGTGATGT-3′. The sizes of the PCR products that were obtained from the amplification of I and D alleles detected with agarose gel were 380 bp (II) and 307 bp (DD), respectively.

Establishment of T-ARMS-PCR-LFA Method for Genotyping of *ACE* I/D. T-ARMS-PCR technique and LFA system were composed of the *ACE* I/D genotyping method. Four primers in a PCR mixture including two pairs of primers for amplification of I allele (Dig-F-I and Bio-R-I) and D allele (Cy5-F-D and Dig-R-D) were used to perform T-ARMS-PCR. The LFA strips were used to capture the target PCR products and present the genotyping results in the form of visualization.

PCR reaction for *ACE* I/D genotyping were performed in a final volume of 50 μL including 10× reaction buffer, 0.2 mM of each dNTPs, 0.5 U of UDG polymerase, 0.5 U of Hotmaster Taq DNA polymerase, 35 nM F-I primer: 5′-Dig-AAGG-AGAGGAGAGAGACTCAAGCAC-3′, 35 nM R-I primer: 5′-Bio-TCGAGACCATCCCGGCTAAAAC-3′, 45 nM F-D primer: 5′-Cy5-GCTGCCTATACAGTCACTTTTATG-CGG-3′, 45 nM R-D primer: 5′-Dig-GGACCCAAGTGC-CAGTGATGT-3′, and 3 μL of genomic DNA. PCR amplification was conducted with an initial denaturation at 50 °C for 2 min and 5 min at 95 °C, 31 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 62 °C, extension for 1 min at 65 °C, and a final extension for 5 min at 65 °C. All PCR reactions were performed by a 2720 Thermal Cycler (Applied Biosystems, Foster City, U.S.A.).

The structure of the LFA strip is shown in Figure 2a (upper). Gold-magnetic nanoparticles conjugated with anti-digoxin antibody (GMNPs-antibody) were dispensed on the conjugated pad. The synthesis of gold-magnetic nanoparticles and conjugation of anti-digoxin antibody have been described in detail previously.[25,26] Streptavidin, anti-Cy5 antibody, and goat anti-mouse IgG were sprayed on the nitrocellulose membrane as T1 line, T2 line, and C line, respectively, via a Biodot AirJet dispensing apparatus (BioDot, Irvine, CA, U.S.A.). After 12 h, when the conjugate pad and the membrane were dried, the whole LFA device was assembled and cut into 3 mm widths with an automatic strip cutter. Then placed LFA strips into sealed aluminum foil bags, storing with desiccant silica gel at room temperature until use.

Evaluation of T-ARMS-PCR-LFA Method. We performed a series of experiments to assess the accuracy, reproducibility, and sensitivity of the assay system. The accuracy of the genotyping method was determined with genomic DNA samples representing three genotypes of *ACE* I/D. Genotyping results were compared with those obtained by the conventional method. Reproducibility experiments were conducted to apply three tubes of PCR products with the same genotype to three batches of test strips and verified the stability of the assay system by the intensity change of the red bands in three

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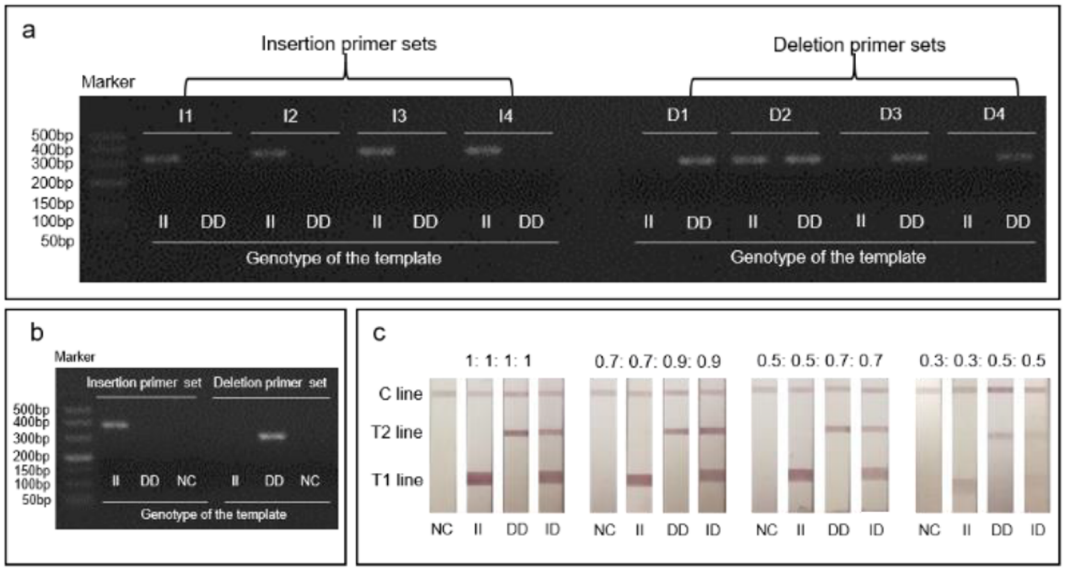

Figure 3. (a) Primers were screened by agarose gel electrophoresis and (b) the selected primers were reconfirmed. (c) Effect of the different concentrations of primers. NC = negative control, II = *ACE* II sample, ID = *ACE* ID sample, DD = *ACE* DD sample. The T1 line was used to detect I-specific PCR products, the T2 line was used to detect D-specific PCR products, and the C line indicated that the chromatography system is reliable.

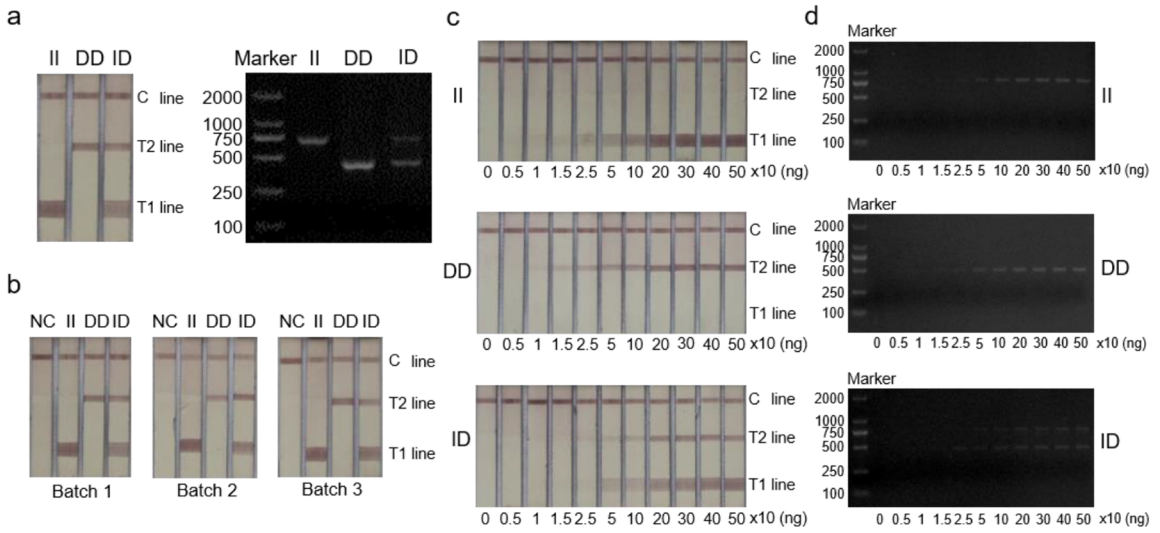

Figure 4. Evaluation of T-ARMR-PCR-LFA method. (a) The accuracy of the method confirmed by DNA samples of known genotypes and compared with the conventional method. (b) The genotyping results of T-ARMS-PCR-LFA system with three batches of strips. (c) The sensitivity of the method was confirmed by using DNA samples with different amounts. The T1 line was used to detect I-specific PCR products, the T2 line was used to detect D-specific PCR products, and the C line indicated that the chromatography system is reliable. (d) The sensitivity of the conventional method was confirmed by using DNA samples with different amounts.

batches of LFA strips. Genomic DNA samples of three genotypes were diluted using nuclease-free water and added into PCR reactions in final amounts of 500, 400, 300, 200, 100, 50, 25, 15, 10, and 5 ng to determine the limit of detection (LOD) of the assay system. Finally, 50 of the genomic DNA from clinical samples were simultaneously genotyped by both our method (T-ARMS-PCR-LFA) and the agarose gel electrophoresis analysis after common PCR to further verify the accuracy of the assay system.

Analysis of the Clinical Samples. Peripheral blood samples were collected from 135 essential hypertension cases and 189 healthy controls in Ankang Hospital of Traditional Chinese Medicine (AnKang, PRC). Patients with secondary hypertension, cardiac or renal failure, or other serious systemic diseases were excluded. The control group consisted of individuals without a history of hypertension, and their systolic and diastolic blood pressure was in the range of 90−140 and 60−90 mmHg, respectively. All extracted genomic DNA samples were detected by T-ARMS-PCR-LFA for genotyping of *ACE* I/D. A case-control study was conducted to find the relation of genotype and EH. Our protocol was approved by the ethic committee of the College of Life Sciences, Northwest University (Xi'an, China). All methods were performed in accordance with the approved guidelines.

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RESULTS AND DISCUSSION

Principle of T-ARMS-PCR-LFA for *ACE* I/D Detection. According to the principle of T-ARMS-PCR,[27] we designed four primers (F-D, R-D, F-I, and R-I) used for amplification of *ACE* I/D in a single PCR reaction. A primer pair of F-D and R-D was designed to extend the D allele of *ACE* I/D and the primer pairs of F-I and R-I were used for the amplification of the I allele of *ACE* I/D. In order to apply the four primers to the subsequent detection based on LFA strip, the Cy5 and biotin was used to label the 5′-end of F-D and R-I primers, respectively (Cy5-F-D and Bio-R-I), and the 5′-end of R-D and F-I primers were all labeled with digoxin (Dig-R-D and Dig-F-I). As shown in Figure 2b, I-specific PCR products labeled with biotin and digoxin were extended by Dig-F-I and Bio-R-I primers and D-specific PCR products labeled with Cy5 and digoxin were extended by Cy5-F-D and Dig-R-D primers. These specific PCR products would be captured by LFA strips (Figure 2a). Nonspecific PCR products labeled with digoxin at both ends were synthesized from Dig-F-I and Dig-R-D primers, which would not be captured by LFA strips having no impact on the specificity of the detection.

After PCR reaction, all the PCR product was put onto the sample pad of the LFA strip to perform a chromatographic reaction. As shown in Figure 2a (lower), with the fluid reaching the conjugate pad, the PCR product labeled with digoxin at one end will combine with the anti-digoxin antibody coated GMNPs forming PCR products−GMNPs composites. As the chromatography continues, if the other end of the PCR product was labeled with biotin, it will be captured by streptavidin at the T1 line. Otherwise, if the PCR product was labeled with Cy5 at the other end, PCR products−GMNPs composites will bind to the anti-Cy5 antibody which immobilized on the T2 line. In other words, I-specific PCR products are only captured by the T1 line and D-specific PCR products are only captured by the T2 line. The rest of the anti-digoxin antibody-coated GMNPs would be chromatographed to the C line and captured by goat anti-mouse IgG. Due to the optical property of GMNPs having a plasmon resonance peak at 520 nm, the aggregated GMNPs will form a red band when the composites are captured on the NC membrane. According to whether there is a red band on the T1 and T2 lines of the test strip, we can judge whether specific PCR products are produced so as to identify genotype. As shown in Figure 2c (right), the sample of II genotype appears as red bands on the T1 line and the C line of the LFA strip, the sample of the DD genotype shows red bands on the T2 line and the C line of the test strip, the sample of ID genotype displays red lines on the T1, T2, and C lines. If there was no production, a red band only shows on the C line, which confirms the effective performance of the strip. Compared with T-ARMS-PCR followed by gel electrophoresis (Figure 2c, left), our method significantly saves a lot of time and simplifies the operation steps that gel electrophoresis required by introducing LFA strips.

Table 1. Comparison of Accuracy between Two Methods for Genotyping of *ACE* I/D Polymorphism

	conventional PCR				
genotype	1′ PCR	2′ PCR	total	T-ARMS-PCR-LFA	agreement (%)
II	23		23	23	100
ID	22		22	22	100
DD	5	5	5	5	100

Results of Optimization of the T-ARMS-PCR with the LFA System. In order to obtain the best performance of T-ARMS-PCR-LFA, some parameters that influenced the specificity of T- ARMS-PCR, including primer concentrations, annealing temperatures, and cycle numbers, were optimized with the LFA system.

Primers play a very important role to ensure the specifics of the PCR system. To avoid mistyping of ID as DD, we designed four sets of primers that introduced different additional mismatches at the penultimate or antepenult nucleotide of the 3′ terminus of Bio-R-I and Cy5-F-D for the amplification of I and D alleles, respectively. The same primer pair was added into two tubes for amplification of II and DD samples, respectively. PCR products were then screened through agarose gel electrophoresis to obtain the best specific primer pairs for development of T-ARMS-PCR-LFA. Results of primer selection were shown in Figure 3a. Electrophoretic analysis of the PCR products presented a 380 bp fragment from the I allele amplified by the insertion primer and the 307 bp fragment from the D allele amplified by the deletion primer, respectively. We screened the insertion primer and deletion primer pairs separately. Four sets of primers designed for amplification of the I allele were able to accurately amplify the II genotype sample. The D2 and D3 primer pairs obtained the nonspecific products when detecting the II genotype templates, which showed poor specificity. Therefore, these

Table 2. Association Analysis between *ACE* I/D Polymorphism and the Risk of EH under Multiple Models of Inheritance[a]

					crude analysis		adjusted analysis	
gene	model	genotype	control	case	OR (95% CI)	*p* value	AOR (95% CI)	*p* value
ACE	codominant	II	83 (43.9%)	48 (35.6%)	1.00	0.14	1.00	
		ID	85 (45%)	63 (46.7%)	1.28 (0.79−2.08)		1.42 (0.79−2.55)	0.11
		DD	21 (11.1)	24 (17.8%)	1.98 (1.00−3.92)		2.36 (1.03−5.42)	
	dominant	II	83 (43.9%)	48 (35.6%)	1.00	0.13	1.00	0.094
		ID-DD	106 (56.1%)	87 (64.4%)	1.42 (0.90−2.24)		1.60 (0.92−2.78)	
	recessive	II-ID	168 (88.9%)	111 (82.2%)	1.00	0.089	1.00	0.085
		DD	21 (11.1%)	24 (17.8%)	1.73 (0.92−3.26)		1.96 (0.91−4.21)	
	overdominant	II-DD	104 (55%)	72 (53.3%)	1.00	0.76	1.00	0.66
		ID	85 (45%)	63 (46.7%)	1.07 (0.69−1.67)		1.13 (0.66−1.93)	
	log-additive				1.37 (1.00−1.90)	0.052	1.51 (1.02−2.23)	0.039

[a]*p* values were calculated by logistic regression analysis with adjustments for age, TC, TG, and HDL. AOR: odd ratio adjusted by age, TC, TG, and HDL. 95% CI: 95% confidence interval.

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Table 3. Comparison of Different Methods for Genotyping of *ACE* I/D Polymorphism

	conventional method[29]	real-time PCR[30,31]	T-ARMS-PCR[20]	gene chip[10]	T-ARMS-PCR-LFA
primers	4	3	4	2	4
probes	0	0	0	3	0
number of PCR rounds	2	1	1	1	1
total experimental steps	4	1	2	~9	2
total detection time	~240 min	90−120 min	~119 min	>253 min	~80 min
related instrument	PCR amplifier, electrophoresis apparatus, and gel imager system	GeneAmp 5700 sequence detection system	PCR amplifier, electrophoresis apparatus, and gel imager system	GeneAmp 9700 PCR system, OmniGridTM 100 TLC sample, GenePix 4000B laser confocal scanner, and GenePix Pro	PCR amplifier
genotyping results interpreted by a professional	no	yes	no	yes	no

two primer pairs were eliminated. Due to the poor amplification efficiency, the D4 primer pair were not suitable for detection of the DD genotype. The D1 primer pair showed a higher specificity and efficiency for genotyping of *ACE* DD. In order to make the PCR reaction more efficient, we selected specific and efficient primer pairs (I3 and D1, as shown in Figure 3a,b) for subsequent experiments. Here, there is no big difference between the lengths of the I and D products. Therefore, the efficacy of the amplification of the I and D alleles are almost same, which can avoid mistyping of ID as DD.

To avoid the mistyping of ID as DD genotypes and false positives that are caused by the different possible amplification efficiencies between I and D alleles, primers concentrations were optimized. Four primers (Dig-F-I/Bio-R-I/Cy5-F-D/Dig-R-D) with the same concentration (2.5 μM) was applied for the PCR reaction at 1:1:1:1 volume ratios, and PCR products were detected by LFA strips. Primers volume ratios were adjusted according to the intensity of the red bands on the strips. As shown in Figure 3c, the 1:1:1:1 volume ratio led to a weak signal of the T2 line for genotyping of *ACE* ID. To ensure generating an equivalent efficiency of I and D allele amplifications, we adjusted primer volume ratios according to the intensity of the red bands on the T1 and T2 lines when the *ACE* ID sample was detected. When the primer volume ratio was reduced to 0.7:0.7:0.9:0.9, there was no nonspecific PCR products, and the intensity of the red bands on the T1 and T2 lines was consistent for genotyping of *ACE* ID. The 0.5:0.5:0.7:0.7 and 0.3:0.3:0.5:0.5 volume ratios resulted in weak signals of T1 and T2 lines for genotyping of *ACE* II, DD, and ID. On the basis of the effect of genotyping results presented by LFA strips, 0.7:0.7:0.9:0.9 was selected as an optimal primer volume ratio for the detection of *ACE* I/D.

Furthermore, the effect of the annealing temperature and cycle number on specificity of the T-ARMS-PCR amplification was carried out for the genotyping of *ACE* I/D. Representative results are presented in Figure S1. Under the optimal primer concentrations, 62 °C of annealing temperature and 31 as the cycle number produced higher brightness at the T lines on the strip for genotyping of *ACE* I/D, which would render a better specificity in any resulting device. Therefore, 62 °C and 31 for the cycle number were selected as the optimal annealing temperature and cycle number for further use in the analysis.

Results of the Evaluation of the T-ARMS-PCR-LFA System. The evaluation of the T-ARMS-PCR-LFA system was conducted by accuracy, reproducibility, and sensitivity experiments under optimal experimental parameters. For accuracy, PCR products from three genotypes could be accurately distinguished on the LFA strips (Figure 4a, left). Homozygous genotype samples (II or DD) produced one red band in the T1 line (II) or in the T2 line (DD) and one red band in the C line. *ACE* ID genotype samples presented three red bands in the T1, T2, and C lines. Meanwhile, the genotyping results were coincident with those obtained by the conventional method (Figure 4a, right). The results demonstrated that there was no nonspecific amplification in this assay system, and the detection method accurately discriminates three genotypes of *ACE* I/D. For reproducibility, the experiments were conducted to apply three tubes of PCR products with the same genotype to three batches of test strips. As shown in Figure 4b, no significant difference in the brightness was found among the three LFA strips, which indicates that the detection system is stable. For sensitivity, three kinds of genotypes of genomic

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DNA were used to assess the sensitivity of the method. As is shown in Figure 4c, the intensity of the red bands increased with the amount of genomic DNA. For three genotypes of *ACE* I/D, 10 ng of II and DD DNA samples can be detected by our method. However, the ID genotype can be determined at 25 ng. Since the color development effect of the ID genotype is weaker than that of II and DD, the LOD of the ID genotype is determined as the LOD of the detection method. Therefore, 25 ng was used as the detection limit of the assay system. Figure 4d showed that, at the same number of cycles, the conventional method was capable of detecting at least 15 ng of II and DD DNA samples. Although 25 ng of the ID DNA sample was detected, only the D-specific PCR product was presented on the agarose gel, which resulted in the mistyping of ID as DD. Therefore, the LOD of the conventional method was 50 ng in this study. Compared to the conventional method, our biosensor is more sensitive.

To further verify the accuracy and clinical utility of the established genotyping system for detection of *ACE* I/D, 50 samples were detected by both the T-ARMS-PCR-LFA system and the conventional PCR method for genotyping of *ACE* I/D (Table 1). All the DD genotype obtained in the first round of the common PCR were detected by second PCR to avoid mistype of ID genotype (data not shown). As shown in Figure S2, we found that the genotyping results obtained from the T-ARMS-PCR-LFA system and agarose gel electrophoresis were fully coincident, which indicated that our method was accurate, reliable, and suitable for clinical application.

Clinical Application. The *ACE* DD genotype has been shown to be at a significantly higher risk for diseases such as myocardial infarction (MI) when compared with the ID or the II genotype. Discrimination of *ACE* I/D polymorphism for all patients with EH and normal samples was performed through the T-ARMS-PCR-LFA technique, and the obtained genotyping results were analyzed by logistic regression analysis under multiple models of inheritance (codominant, dominant, recessive, overdominant, and log-additive) to study the association between *ACE* I/D polymorphism and risk of primary hypertension (Table 2). Our result showed that there was significant association between D allele of the *ACE* I/D and EH after adjusting for age, TC, TG, and HDL (Table S1). Individuals with the D allele had a 51% increased risk of EH compared to individuals with the I allele in the log-additive model (OR = 1.51, 95% CI, 1.02−2.23; p = 0.039), which provide a reference for the study of association between *ACE* I/D polymorphism and EH in Shaanxi Han people.

Rigat et al.[28] first proposed a one round PCR followed by agarose gel electrophoresis for genotyping of the *ACE* I/D polymorphism. Shanmugam et al. found it might be mistyping and introduced the second independent PCR to recorrect this problem. This mistyping problem was caused by the big difference in the length between the I and D PCR fragments. DD and II have a high accuracy with one round of PCR. However, with regard to the ID genotype, the D allele is preferentially amplified, and amplification of the I allele is suppressed. Therefore, the ID sometimes was mistyped as DD. Shanmugam et al.[29] designed another primer pair used for the amplification of insertion, with the result of the DD samples being further confirmed by the second PCR. If there is no band in the gel, the result of the DD sample could be verified. This conventional method greatly improves the accuracy for genotyping of *ACE* I/D, and it is usually regarded as a standard method for comparing new techniques developed for the identification of *ACE* I/D variants.

In 2001, Lin et al.[30] and Hiratsuka et al.[31] provided strategies for detecting *ACE* I/D by a real-time PCR-based method, which shortened the detection times. In recent years, gene chip technology has been developed to perform a gene test, which was also used to distinguish I and D alleles. However, expensive/sophisticated instruments, complicated operation steps, and complex data analysis carried out by a professional hinder it from being applied to POCT in the clinic.

T-ARMS-PCR-LFA for genotyping of *ACE* I/D takes advantage of the T-ARMS-PCR and LFA system that allows distinguishing I and D alleles in a single PCR simultaneously and presents genotyping results in the form of visualization within 5 min. The presented assay system in our study has advantages over the existing methods that are used for genotyping of *ACE* I/D in clinical diagnosis and related studies (Table 3). First of all, we have solved the mistyping problem by designing two pairs of primers that amplify the I and D alleles separately and optimize the four primers concentration in one PCR reaction. High specificity and accuracy of genetic mutant detection are significant for the analysis of clinical specimens. Basically, primer design and primer concentration play crucial roles in the genotyping methods. In our study, an additional mismatch was introduced at the antepenult nucleotide of the 3′ terminus of the Cy5-F-D primer according to the principle of the mismatch amplification mutation assay to improve the specificity of amplification.[32] Subsequently, we optimized primer concentrations in one PCR reaction with the LFA strip to avoid mistyping of ID as DD. Moreover, our method significantly simplified the experimental procedure through a round of PCR and the introduction of the LFA strip. The presented T-ARMS-PCR-LFA enables us to detect *ACE* I/D polymorphism in a single PCR reaction and present a genotype by visual inspection of red bands on the LFA strip without complicated operation steps such as denaturation and gel electrophoresis, which is more convenient and time-saving compared with previous PCR-based methods. The detection system that just needs a common PCR amplifier is an affordable on-site technique for *ACE* I/D genotyping compared with currently techniques that usually run with expensive probes, special or sophisticated instruments that may not be available in many laboratories. Therefore, this method can be utilized even in low-tech laboratories. Furthermore, genotyping results are directly shown on the LFA strip without data analysis, which can be interpreted by average people.

To the best of our knowledge, it is the first time to detect *ACE* I/D using T-ARMS-PCR-LFA with high accuracy and quickness. The assay system would be facilitated to study the function of *ACE* I/D in diseases and provide a new opportunity for the diagnosis as well as treatment of diseases that are associated with *ACE* I/D. The advantages of this method and the potential to be applied to detect other indels by designing corresponding primers according to the target sequence would make it replacing conventional PCR methods to become a standard method for the detection of *ACE* I/D. In addition, the relation of *ACE* I/D and EH in the Han people of the Shaanxi province is for the first time to be studied, which will fill the gap and provide a reference for the study of the correlation between *ACE* I/D and EH to facilitate precision medicine.

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CONCLUSION

In this work, we used a method that combines T-ARMS-PCR and GoldMag lateral flow assay for genotyping of *ACE* I/D. This simple, convenient, and rapid method enables the accurate detection of *ACE* I/D in clinical samples, which is suitable for clinicians to diagnose the disease quickly. We demonstrated that the T-ARMS-PCR-LFA method possessed the ability to detect *ACE* I/D rapidly and more accurately than the conventional method. The time required for the conventional method with two independent PCRs, in combination with the corresponding gel electrophoresis, was significantly reduced by using our method that combined one round of T-ARMS-PCR with a LFA strip. The method in our study solved the mistyping problem that the conventional method arises from through one round of PCR combined with gel electrophoresis, which is very meaningful for accurate genotyping of *ACE* I/D as well as other large indels. In summary, the work in this paper represents a significant fundamental advance in the detection of *ACE* I/D polymorphism.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.1c04817.

Figure S1: Optimization of T-ARMS-PCR conditions with LFA strips; Figure S2: The genotyping results of *ACE* I/D; Table S1: Baseline characteristics of controls and EH cases (PDF)

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Notes

The authors declare no competing financial interest.

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