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Determination of mini-short tandem repeat (miniSTR) loci by using the combination of polymerase chain reaction (PCR) and microchip electrophoresis

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ABSTRACT

In this work, a simple and convenient method for the detection of mini-short tandem repeat (miniSTR) loci has been developed by the combination of polymerase chain reaction (PCR) and microchip electrophoresis (MCE). Degraded or inhibitor DNA greatly limited STR loci analysis. Therefore, The proper primers was designed as close as possible to the STRs region to produce smaller size STRs, and made the assay suitable for the destroyed samples. Two annealing temperatures were applied in one PCR procedure and the corresponding cycle numbers were studied to improve the sensitivity of PCR reaction. Under optimal conditions, 0.001 ng DNA templates were enough to generate miniSTRs. The relative standard deviations ($n=3$) of the size fifteen miniSTRs from DNA9947A ranged from 0.49% to 4.41%. The RSDs of concentrations were between 0.94% and 4.95%. Fifteen miniSTRs were also well produced from human hair, indicating that the method has great potential application in criminal identification and paternity testing.

1. Introduction

In the last decade, short tandem repeats (STRs) are widely applied to clinical diagnosis [1–3], paternity testing [4,5], forensic identification [6,7], agricultural [8,9], as the polymorphism allowed individual STR profiles to be generated [10,11]. Although more than 5 million profiles of STR have been characterized in human genome [5], the most common STRs for human identity testing are D2S1338, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D19S433, D21S11, vWA, CSF1PO, TPOX, THOI, FGA and D18S51 [4,12]. Multiplex STRs system has received more attention than single STR detection for saving time and cost [13]. The polymerization and cross reactions were one challenge in multiplex STRs system because they decreased the specificity of polymerase chain reaction (PCR) for many primers in one tube. By optimizing the annealing temperatures, many researchers improved the amplification efficiency and reduced the side reaction resulting from STR multiplex system. Another challenge for the determination of STRs was poor sample quality especially those from criminal investigations as their extensive DNA fragments might lead to a failure [14,15]. The damage of DNA also reduced Taq DNA polymerase fidelity and affected the PCR amplification efficiency [16]. Some groups have been developing single nucleotide polymorphism (SNP) analysis to get more information [17–19]. Some laboratories endeavored to develop better DNA extraction method for these inhibited samples [20,21]. Some people have adopted mitochondrial DNA with the hyper variable regions to obtain more information [22–24]. To date, the best approach to improve the PCR efficiency was to generate shorter amplicons by repositioning primers as close as possible to the STR region. These primers will produce smaller STRs termed "miniSTRs" which enhanced the amplification efficiency as smaller templates led to more potential template molecules [25].

The traditional STR assays are carried out by PCR method combined with electrophoresis separation [9,26], which has the disadvantage of time and reagent consuming. To address these issues, microchip electrophoresis (MCE) devices for forensic STR detection has been developed with many advantages such as high-throughput and rapid analysis with high sensitivity and resolution [27,28]. The use of MCE device requires less operation steps and is easy to realize automation, which reduces the risk of laboratory contamination and improves stability [20,29]. Fluorescent dye (SYBR Gold) with high affinity, low background and low toxicity was on-line labeled with PCR products in microchannels, which also decreased tedious steps, and then decreased the deviation [30,31]. Another improvement of MCE is that two markers as internal standards mixed with the samples were simultaneous analyzed to overcome the interference of sample matrix and were

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used to reduce the deviation [32]. Thus, the strategy of PCR coupled with MCE is well suitable for STR detection.

In this work, we report a rapid and sensitive method for multiplex STR detection based on polymerase chain reaction (PCR) and microchip electrophoresis. PCR was used to improve the sensitivity of STR analysis while microchip was used to realize rapid and high-throughput analysis. Moreover, fifteen STR loci were divided into four groups depending on their size and annealing temperature to reduce the interaction between DNA fragments. The proposed method was demonstrated to be suitable for real sample and will bring broad prospect to the application of forensic identification.

2. Materials and Methods

2.1. Materials and reagents

DNA samples were extracted from human hair by chelex-100. Human hair was donated from labmates thus had been exposed to the environment without any safeguard. Both DNA ladder solution and SYBR Gold solution were purchased from Invitrogen Corporation (Karlsruhe, Germany) and diluted with TE buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA). Hydroxyethyl cellulose was product of Sigma (HEC, 25000 Da, Sigma, USA). 2% HEC (w/v) was prepared with TE buffer and used as electrophoresis buffer. Thirty primers were synthesized by Beijing Dingguo Biotechnology Co. Ltd (Beijing, China). DNA 9947 A (Promega Corporation, USA) was used to optimize the amplification conditions. Taq DNA polymerase was purchased from New England Biolabs, USA. dNTP kit was obtained from Pharmacia Corporation (Stockholm, Sweden). Pure water used in this work was purchased from Wahaha Company (Hangzhou, China).

2.2. PCR amplification

Fifteen miniSTR loci were amplified by PCR and one PCR procedure had two annealing temperatures. β-globin was used as quality control. The fifteen different miniSTR loci were divided into four groups, A, B, C and D according to size and annealing temperature. Groups A, B, C and D are corresponded to A (D13S317, D21S11 and TPOX), B (D5S818, D16S539, D8S1179 and D7S820), C (D3S1358, FGA and CSF1PO), and, D (vWA, TH01, D19S433, D18S51 and D2S1338), respectively. Standard amplification of PCR mixture (20 μL) contained 13.5 μL pure water, 2.0 μL buffer, 2.00 μL of 1 mmol μL^{-1} dNTP, 0.5 μL of 0.5 U Taq DNA polymerase, 1.00 μL of 0.5 μmol μL^{-1} primers and 1.00 μL of 0.5 ng μL^{-1} DNA sample. The amplification was started at 94 °C for 5.0 min. After that, there were 27 cycles in the PCR procedure by using Mastercycler Gradient (Eppendort, Germany). The starting 10 cycles included a denaturation step at 94 °C for 45 s, an annealing step of primers at different temperature (*T*1, Table 1) for 45 s, and a chain extension at 72 °C for 45 s. The next 17 cycles contained 94 °C 45 s for all four groups; different temperatures (*T*2) for 45 s, and 72 °C for 45 s, respectively. A final elongation step was at 72 °C for 10 min (Figure S1-A).

Table 1
Different annealing temperature for four different groups of STR loci[a].

No.	Group A		Group B		Group C		Group D	
	T1(°C)	T2(°C)	T1(°C)	T2(°C)	T1(°C)	T2(°C)	T1(°C)	T2(°C)
1	**48.0**	**53.0**	50.5	55.6	52.4	57.4	**52.4**	**57.4**
2	50.5	55.6	**52.4**	**57.4**	**54.4**	**59.5**	54.4	59.5
3	52.4	57.4	54.4	59.5	56.6	61.5	56.6	61.5
4	54.4	59.5	56.6	61.5	58.8	63.9	58.8	63.9
5	56.6	61.5	58.8	63.9	60.8	65.9	60.8	65.9

[a] Application temperatures were adopted by the bold groups in Scheme1.

2.3. Microchip electrophoresis analysis

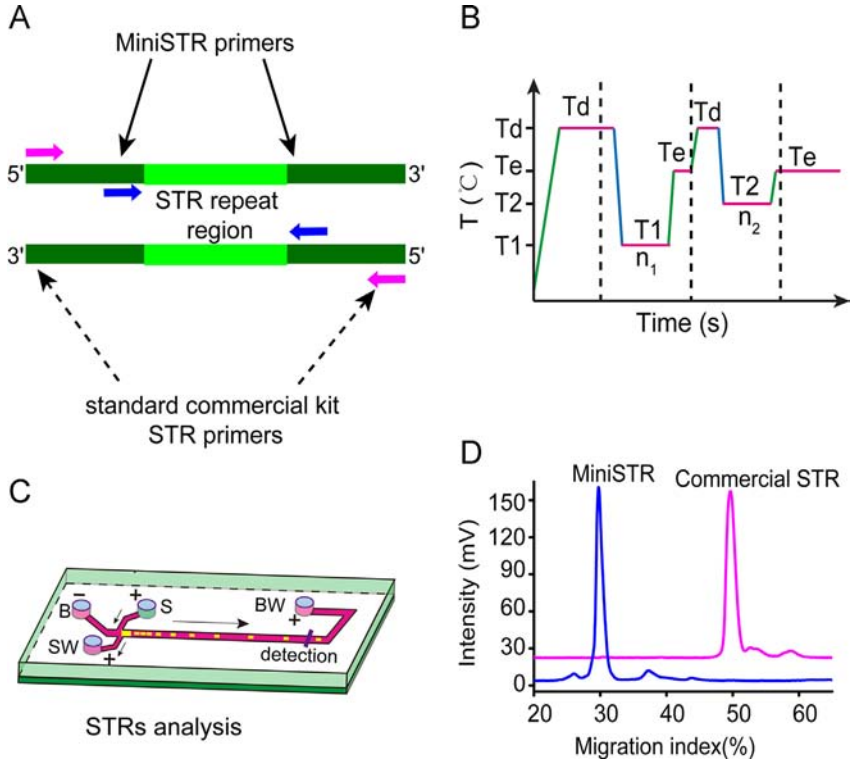
PCR products were on-line labeled with SYBR Gold and were separated in the microchip electrophoresis (MCE) by MultiNA 202 System (Shimadzu, Japan) (Figure S1-B). This system has a blue light emitting diode (LED) at 470 nm and the fluorescence detection at 525 nm. Four microchips with double-T injection were equipped in the apparatus for detection. The rectangular microchannel is 23 mm in length for separation, 88 μm in width and 50μm in depth. DNA ladder (0.313, 0.625, 1.25, 2.50, 5.00 and 10.0 ng μL^{-1}) was detected by the on-line labeling method. DNA template (0.001, 0.002, 0.010, 0.020 and 0.100 ng μL^{-1}) was used to assess the sensitivity of PCR-MCE system. Relative standard deviations of the size and the concentration were also detected for three times.

3. Results and discussion

3.1. Primers design and multiplex PCR amplification

DNA assay in forensic often suffers from low-quantity or poor-quality samples with environmental exposures, resulting in lots of DNA fragments. Excess DNA fragments can be produced from partial genetic profiles with allele and/or locus drop-out [14,24]. It had been reported that the most efficient approach was producing shorter miniSTRs to obtain DNA profiles. Therefore, we designed the primers to generate miniSTRs and these miniSTRs can convict different individual's DNA profiles (Scheme 1). In order to improve PCR efficiency, we divided fifteen miniSTR loci into four groups to reduce side reaction and the polymerization. Fig. 1 showed that not only single miniSTR locus but also multiplex miniSTRs were effectively amplified at standard conditions with the designed primers. The sizes of these fifteen STRs were showed in Table 2. D21S11 has been successfully produced although the sensitivity was low for its various alleles. D2S1338, TPOX and vWA have been reported to be hard to produce for their high mutation rate [5]. These miniSTRs were successfully produced because the new primers were used to generate smaller fragments. D19S433 primers were designed to avoid the high mutation site which often caused the silent allele [33,34]. D2S1338 was high variation rate for the false homozygosity and multiple fragments [4,35]. The good precision of vWA in our work was that the primers of vWA avoided binding site mutation which often made its alleles drop-out [4,5]. Although these STRs had some disadvantages in the report, they can be well produced in our work, indicating that the primers were suitable for the degraded or inhibitor samples as well as multiplex miniSTR loci system. The single STR was produced to quantify PCR products and can be used to evaluate the specificity of amplification system.

Primers were designed to keep robust signal intensity and balance peak heights in every group. Primers played a critical role in efficiency and specificity in PCR amplification. The concentration of primers affected not only the specificity of PCR products but also the yield of PCR products. Fig. 2A showed that the signals were not obviously changed when the concentrations of primers ranged from 0.02 μM to 0.10 μM. The maximum signal appeared at the 0.06 μM. The signal increased with the concentrations of primers up to 0.06 μM. However, the intensity of miniSTR loci decreased with the concentrations of primers higher than 0.06 μM due to the fact that the primers had cross reactions and the polymerase reaction was enhanced. Moreover, when the concentrations of the primers were



Scheme 1. Schematic diagram of PCR-MCE. (A) The principle of the miniSTR primers and conventional primers design. (B) MiniSTRs assay with two annealing temperatures in one PCR procedure. (C) MiniSTRs analysis conducted by microchip electrophoresis. (D)The sizes of miniSTR and conventional STR. Td indicated denaturation temperature. Te indicated extension temperature. *T*1 and *T*2 indicated two annealing temperature. n_1 indicated the cycle numbers, when *T*1 was used as the annealing temperature. n_2 indicated the cycle numbers, when *T*2 was used as the annealing temperature. S meant sample; SW was sample waste. B meant buffer solution; BW was buffer waste.

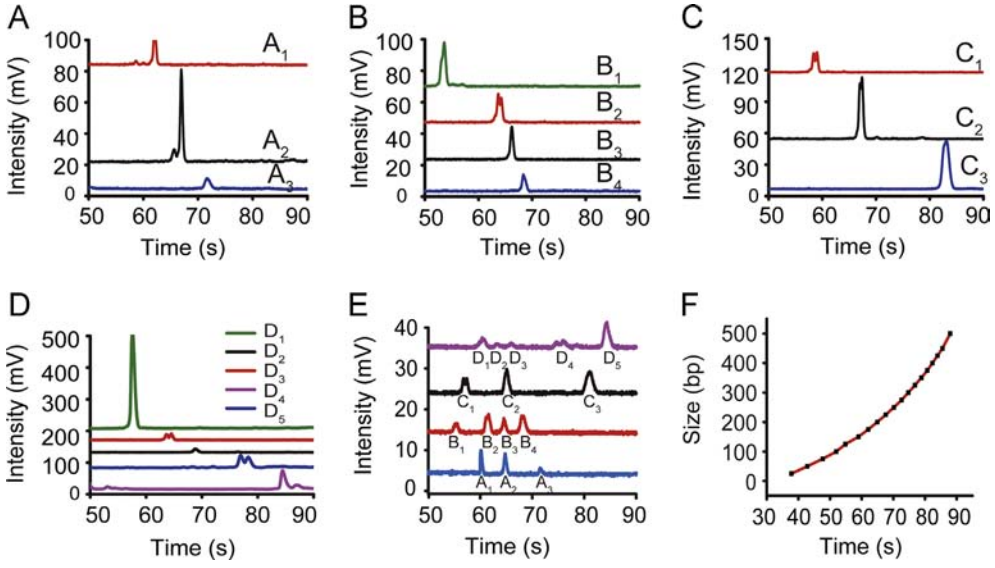


Fig. 1. Fifteen miniSTRs by PCR-MCE. (A) Simplex STR of Group A. (B) Simplex STR of Group B. (C) Simplex STR of Group C. (D) Simplex STR of Group D. (E) Multiplex PCR of 15 miniSTRs. (F) The relation between DNA size and migration time for STRs qualitative detection. A1, A2 and A3 were D19S433, D8S1179 and D7S820, respectively; B1, B2, B3 and B4 were D5S818, vWA, TH01 and FGA, respectively; C1, C2 and C3 were D3S1358, D21S11, and CSF1PO, respectively; D1, D2, D3, D4 and D5 were TPOX, D16S539, D13S317, D18S51 and D2S1338, respectively.

down to 0.02 μmol L^{-1}, D7S820 would not be successfully produced. It could illustrate that primers impacted the sensitivity. The phenomenon rarely happened to other miniSTR loci. Taking into consideration the sensitivity and reliable of all fifteen miniSTR loci, the primer concentration was set at 0.06 μM.

The damage of DNA and too many DNA fragments reduced the fidelity of Taq polymerase. The proper concentration of the Taq polymerase in the PCR system could improve the loyalty because base mismatch would be sharply decreased. The concentration also influenced the yield and the specificity [16]. Fig. 2B showed that the concentrations of Taq polymerase ranging from 1.0 U to 2.5 U had little effect on sensitivity. However, the intensity of A allele of TPOX with 0.5 U Taq polymerase was greatly stronger than that with other concentrations. Interestingly, the A allele of TPOX was the lowest size of TPOX even in the group and the other alleles of TPOX were decreased; moreover, D16S319 even

disappeared. Therefore, the reason for this phenomenon might be that the lowest size of a product have priority to be produced when the Taq polymerase was not sufficient. The specificity of PCR system was reduced with Taq polymerase concentrations higher than 1.5 U. The false peak appeared due to the enhancement of base mismatch with Taq polymerase. Other STRs was not influenced by Taq polymerase (The date not shown). In order to get reliable results, the concentration of Taq polymerase was selected at 1.5 U. Four kinds of dNTP were equivalently mixed to reduce base mismatch. All the STR loci were well amplified by using dNTPs in the concentration ranging from 200 μmol L^{-1} to 1000 μmol L^{-1}. 200 μmol L^{-1} of dNTPs was used in the following experiments for lower cost and less pollution.

Table 2
Precision of the size and concentration for fifteen miniSTR loci from DNA9947A in multiplex system ($n=3$).

Name	Commercial STR[a] (bp)	Size (bp)	RSD% ($n=3$)	Concentration (ng μL^{-1})	RSD% ($n=3$)
D19S433	92–150	92	0.86	0.44	3.70
D8S1179	157–209	180	0.63	0.78	2.97
D7S820	194–234	206	3.23	0.43	3.88
D5S818	129–178	151	1.98	1.06	0.94
vWA	123–183	142	4.37	1.45	4.95
THO1	146–190	173	0.59	0.64	3.53
FGA	158–314	192	1.66	2.60	4.45
D3S1358	97–145	134	1.20	0.77	3.77
D21S11	154–272	204	4.41	0.43	4. 18
CSF1P0	317–361	351	1.86	1.94	1.08
TPOX	98–302	117	1.13	2.05	3.39
D16S539	129–177	162	1.39	0.66	1.76
D13S317	157–205	189	1.19	1.55	3.85
D18S51	262–323	285	2.69	1.29	1.18
D2S1338	277–359	367	0.49	0.57	4.18

[a] The size of commericial STRs was from http://www.cstl.nist.gov/biotech/strbase/str_fact.htm.

3.2. Optimization of annealing temperature and cycle numbers

Annealing temperature for the renaturation of primers and DNA template was determined by the size and the concentration

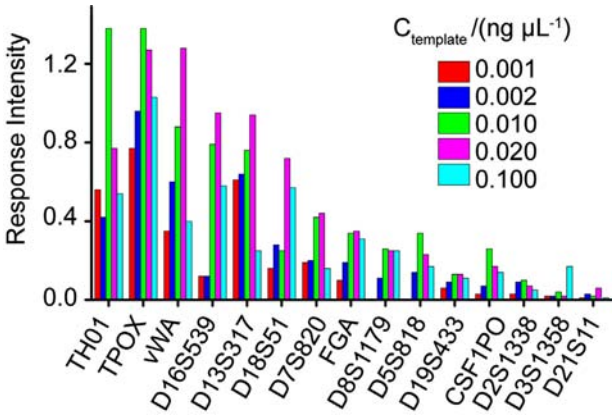


Fig. 4. Sensitivity of fifteen STRs loci in multiplex PCR system.

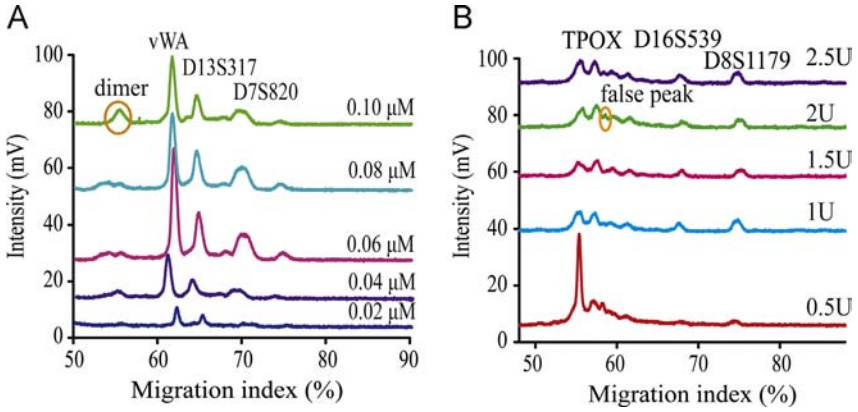


Fig. 2. Optimization of PCR conditions. (A) Effect of primer concentration on the multiplex STR loci; (B) The influence of Taq polymerase on the multiplex STR loci. The concentration of primers were 0.02, 0.04, 0.06, 0.08, 0.10 μmol L^{-1}. The concentrations of Taq polymerase were 0.5, 1.0, 1.5, 2.0, 2.5 U.

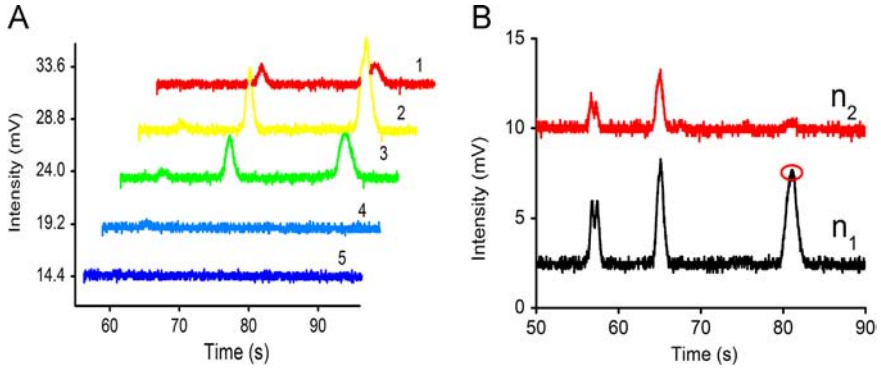


Fig. 3. Optimization of thermal cycle conditions for Group C loci. (A) Annealing temperature (T1 and T2) as mentioned in Table 1 with 10 cycles under T1 and 17 cycles under T2. (B) Cycle numbers of annealing temperature (T1=52.4 °C, T2=57.4 °C). 1, 2, 3, 4 and 5 indicated different annealing temperatures, corresponding to Table 1. n1 means 10 cycles under 52.4 °C and 17 cycles under 57.4 °C. n2 means 5 cycles under 52.4 °C and 22 cycles under 57.4 °C.

of primers. Annealing temperature was very important for PCR amplification of STR assay because every miniSTR locus had its particular annealing temperature. Inappropriate annealing temperature would decrease the yield of target DNA fragments and cause artifact peaks because of base mismatch [36,37]. In order to improve the amplification specificity and sensitivity, two annealing temperatures were designed in the PCR procedure (Scheme 1b). Fig. 3A showed that group C was greatly suitable with *T*1 at 54.4 °C and *T*2 at 59.5 °C. Group A was most suitable for *T*1 with 48.0 °C and *T*2 with 53.0 °C; Groups B and D were successfully amplified by *T*1 with 52.4 °C and *T*2 with 57.4 °C (Fig. S2). Other annealing temperatures decreased the intensity of STRs even mading their dropout, indicating that the inappropriate temperature made the primers of STRs not complemented to the DNA templates [38]. Hence, the optimal annealing temperature was chosen for the highest sensitivity and the best specificity.

The cycle number was defined as the degree of PCR amplification for miniSTR assay. As the cycle numbers depended on DNA templates, they were set at 27 cycles for the common of DNA template which we could get. Two different cycle numbers were adjusted at the corresponding annealing temperature in one program of amplification. Fig. 3B showed that for the group C, the sensitivity of 10 cycles for *T*1 and 17 cycles for *T*2 was better than that of 5 cycles for *T*1 and 22 cycles for *T*2. The optimization of cycle numbers of other groups was showed in Fig. S3. The signals of D8S1179, D3S1358, vWA, TH01 and D19S433 were enhanced when cycle numbers were increased at *T*1. As *T*1 was more suitable, the yield of PCR products was increased when the *T*1 cycle numbers were added [39,40]. Taking the balance of the sensitivity and specificity into account, the A, B, C and D groups adopted 10 cycles for *T*1 and 17 cycles for *T*2. The anneal temperatures of D group was 52.4 °C for *T*1 and 57.4 °C for T2.

3.3. The sensitivity of PCR amplification

The sensitivity of PCR-MCE was evaluated by the DNA template for every miniSTR locus. Single miniSTR locus was well amplified with DNA 9947A between 0.001 ng μL^{-1} and 0.1 ng μL^{-1} amplification system (the data not shown). Fig. 4 showed that miniSTR loci can be effectively amplified in multiplex system with 0.001 ng μL^{-1} to 0.100 ng μL^{-1} DNA templates. Although the signal/noise ratio value was high enough to detect PCR products, the yield of PCR products was significantly unbalanced. MiniSTR locus had its specific site of DNA template and needed different energy to generate miniSTR, so the yield of STR was different. Moreover, the increasing concentration of DNA template would not improve the intensity of fifteen miniSTRs in multiplex PCR as the side reaction and polymerization of oligonucleotides were enhanced when there was more DNA template [4,41]. The DNA template was taken as 0.02 ng μL^{-1} when the yield of most STR loci was the best.

3.4. Sample analysis

Qualitative analysis was based on the migration index/migration time of PCR products and markers. In order to achieve precise qualitative analysis and reduce interference between PCR reagents, two DNA markers as internal standards were used in microchip electrophoresis analysis to eliminate the discrimination among every injection [30]. To assess the potential application of PCR-MCE method, miniSTR loci were amplified by not only DNA 9947A but also human hair. Human hair was determined after its exposure in environment for a week. Table 2 showed the sizes and concentrations of miniSTR loci amplification from DNA 9947A. In the three experiments, the RSDs of sizes were in the range from 0.49% to 5.41% and the RSDs of the concentration were in the range from 0.94% to 7.95%. The good reproducibility indicated that this strategy of miniSTR assay was to some extend suitable for degraded sample. Fig. 5 showed the miniSTR maps from the four donors and the size of every miniSTR allele could be determined for its separation time. Some miniSTRs had multiplex peaks. One of the loci containing two or more than two alleles could produce multiplex peaks [4]. Hence, individual miniSTRs were composed of these multiplex peaks. Moreover, multiplex peaks or split peaks might be coming from incomplete adenylation, triallelic patterns, and variant alleles containing mutations in the repeat or flanking regions in biological sample especially the degraded and inhibited one [5]. Table 3

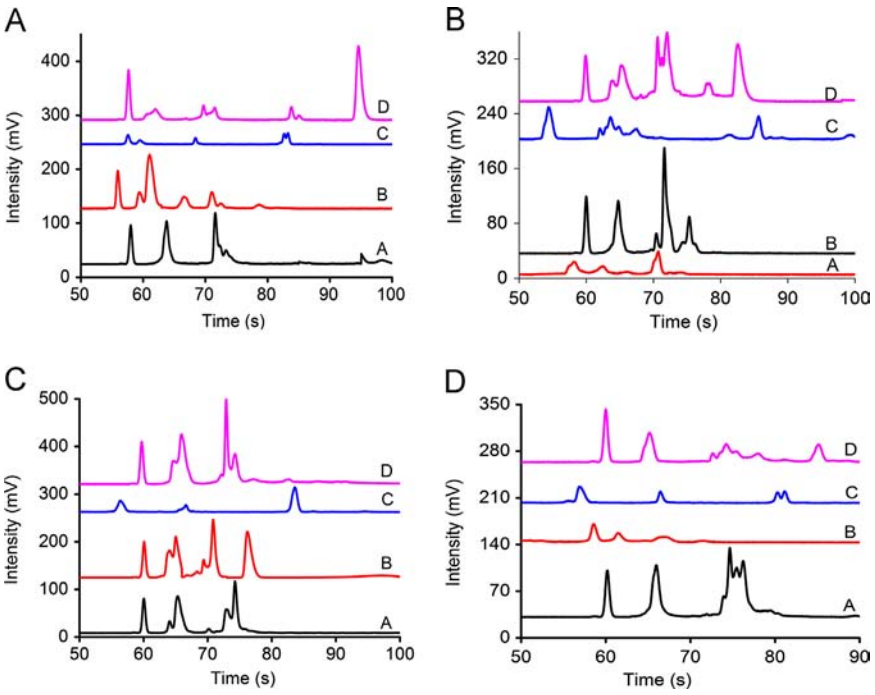


Fig. 5. Fifteen STR loci from human hair based on PCR-MCE. (a) The determination of 15 miniSTRs of real sample a. (b) Real sample b. (c) Real sample c. (d) Real sample d.

Table 3
The size and the concentration of fifteen STR loci from hair.

Name	Sample a (bp)	Concentration (ng μL^{-1})	Sample b (bp)	Concentration (ng μL^{-1})	Sample c (bp)	Concentration (ng μL^{-1})	Sample d (bp)	Concentration (ng μL^{-1})
D5S818	151	0.53	147	0.67	141	0.48	138	1.09
D8S1179	183	0.59	180	0.43	183	0.47	173	0.65
D13S317	176	0.28	192	0.69	187	1.12	168	0.60
FGA	191	0.30	196	0.27	189	1.35	174	1.08
D2S1338	413	0.78	351	0.43	369	0.12	367	0.35
THO1	172	0.32	172	1.17	168	0.36	167	0.21
D19S433	200	0.52	205	0.29	201	0.24	135	0.43
D7S820	221	0.62	224	0.18	299	0.16	206	0.29
vWA	158	0.76	152	0.61	139	0.64	146	0.36
CSF1P0	355	0.38	351	0.54	311	0.82	342	1.37
D3S1358	129	0.36	132	0.42	129	0.37	132	0.41
D16S539	164	0.33	161	0.56	161	0.34	161	0.28
D18S51	293	0.65	304	0.30	316	0.58	282	0.37
D21S11	204	0.21	178	0.26	205	0.27	258	0.54
TPOX	118	1.10	106	1.05	106	0.57	130	0.87

showed the sizes and the concentrations of miniSTR loci amplified from hair samples under standard conditions of PCR. All miniSTRs can be well produced although the sizes of D2S1338, CSF1P0, D18S51 were bigger than that from DNA9947A, indicating that the proposed method have potential application in human identification.

4. Conclusions

In this work, we have successfully developed a simple and sensitive assay for human identification by PCR-MCE. The proper primers could specifically generate miniSTR, leading to this strategy more suitable to poor-quality samples such as greatly degraded ones. Two annealing temperatures in one PCR procedure were applied to improve the amplification efficiency. The corresponding cycle numbers of different annealing temperatures were studied to enhance the intensity of miniSTR. A portable microchip system allowed for rapid and accurate analysis of miniSTR loci with low reagent consumption was developed. PCR products could be detected without any sample pretreatment and the assay was easy for automation. The method was simple, rapid and accurate for human identification, which greatly met community needs and was valuable for criminal identification especially in remote areas.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.04.012.

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