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Development of a Pentanucleotide STR Marker for Human Identity Testing

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ABSTRACT

Design of an amplification technique and assessment of a pentanucleotide tandem repeat, with locus of D6Sp57, for human identity testing in forensic sciences were investigated in this study. This short tandem repeat (STR) has a repeating sequence of CACAG. The sequence repetition and the size of STR product are specific to each person. The selective amplification of this locus in different individual genomes by polymerizes chain reaction (PCR) technique needs the optimal processing condition of thermocycler and the master mix. This optimization can be done by the correct choice of the primers and the optimization of PCR protocol. The STR of five blood samples from different individuals was amplified by PCR under the optimal condition. The products of the reaction were electrophoretically separated on a polyacrylamide gel. The results have shown the specificity of the marker to different individuals and approved the applicability of this pentanucletide marker for forensic purposes.

Key words: Pentanucleotide tandem repeat, DNA amplification, Human identity testing, Polyacrylamide gel, Electrophoresis

1. INTRODUCTION

Short tandem repeat, mitochondrial deoxunuclotide acid (DNA), and Y-chromosome are important genetic markers for human identity testing, mapping studies, and disease diagnosis (1-3). Short tandem repeats (STR) or microsatellites contain repeat units of 2 to 7 base pair in length can be readily amplified with polymerize chain reaction (PCR) (4). This property along with occurrence in between genes where a high degree of variability is tolerated made them effective genetic markers for human identity testing in forensic caseworks and paternity testing. About 10 core STR loci, in Uinted States of America, United Kingdom, and Europe, together with their commercial kits were developed to make and map DNA profiles (1). The information resources on commonly used STR DNA markers have compiled and maintained in an internet database, so called STRBase (5). Petannucleotide repeat markers are of much interest and importance for forensic science due to exhibiting high variability and low amounts of stutter in electrophoretic patterns (1, 6). The pentanucletode tandem repeats were first discovered and characterized by Promega scientists (7, 8). Penta D and Penta E core loci have been described by Promega and their commercial kits were released for forensic purposes (7). The D10S2325 and D6S957 loci were described as forensic DNA markers in Korean population and in Germans and Chinese societies, respectively (6, 9). The applicability of the latter pentanucleotide for forensic purposes was investigated in this study.To this end, an amplification strategy was developed at first and the specificity of the DNA marker in five different Iranian individuals was shown later by the development of electrophoretic patterns. The specificity was revealed by the distinct electrophoretic patterns of the STR marker.

2. MATERIALS AND METHODS

2.1. Materials

The chemicals for agarose and polyacrylamide gels including acryl-amide, bis-acrylamide, ammonium persulfate, agarose powder were all purchased from Merck (Darmstadt, Germany) and used as received without any further purification. The biochemicals for DNA template amplification including the primers, deoxy-nucleotide

triphosphates (dNTPs), Taq DNA polymerase and those for Tris/Borate/EDTA (TBE) preparation were bought from Sina-Jen (Tehran, Iran). All the other Chemicals were purchased from Sigma-Aldrich (Schnelldorf, Germany). Blood samples from five donors were collected and preserved in EDTA-antocoagulated Vacutainer blood collection tubes. The 1.2 mm punches were taken from each blood sample using a puncher and placed in 200 µL amplification tubes. The DNA extraction from each sample and protection from environmental damage was done by the punch cards according to the FTA punch-in protocol (10).

2.2. Design of primers

Primer design for the D6S957 locus was done by gaining all the information on this marker from Genome Browser UCSC (11). According to the exon number of the gene, the internet database suggests a few common primers for its amplification. To have a successful and specific DNA amplification, the primers were designed and assessed with the aid of Primer3 and Oligo Analyzer, respectively. The forward and reverse primers of the marker are respectively CTTTCCTCCTCTGCCTCTCA and GGAAAAGGAGCACGAACA with the respective annealing temperatures of 60.47 and 59.02.

2.3. Preparation of Tris/Borate/EDTA buffer

The stock solution of TBE buffer was prepared in 10X concentration and diluted to 1X during the experiments. To this end, 26 g Tris-base powder was dissolved in 50 mL deionized water. This solution was further mixed with 1.86 g EDTA and 13.75 g Boric acid. The deionized water was then added to the solution to reach a volume of 250 mL. This stock solution was kept in the refrigerator at 277 K for future use.

2.4. PCR amplification of the DNA marker

The DNA amplification of each blood sample was done by placing one dried punch card in a 0.2 mL PCR tube. The master mix and water were then added to the tube to be amplified using a GeneAmp® PCR System 9700. The successful amplification of the DNA marker from a biological sample needs the optimization of the parameters involved in PCR amplification process. The effect of parameters including magnesium ion concentration, the number of extension cycle, the quantity of DNA sample in the electrophoresis well on the efficiency of the PCR process was studied at first. This study resulted in an optimal master mix solution for the DNA marker amplification by PCR. The components of the optimal master mix, prepared in 1X concentrations, were presented in Table 1.

Table 1. The components of the master mix in the PCR optimization protocol

Component	Concentration
Taq DNA polymerase	5 $u.\mu L^{-1}$
Deoxy nucleotide triphosphates (dNTPs)	3 mM
Mg^{2+}	2.5 mM
TBE buffer	1X

The PCR amplification strategy was then optimized regarding the primers concentration (0.1, 0.25, and 0.5 µM), the annealing temperature (50, 51, 53, and 54°C), and the DNA sample concentration (0.1, 0.5, and 1 mM). The total volume of the sample for use in thermocycler is 30 µL with the components presented in Table 2.

Table 2. The components of the PCR cocktail

Component	Sample volume, µL
Forward Primer	0.75
Reverse Primer	0.75
Extracted DNA sample	1
Master Mix (2X)	15
Deionized water	13.5
Total	30

The vials in which the specimen was prepared for PCR amplification and optimization were placed in the thermocycler and amplified under the thermal program shown in Figure 1. The extension cycles were shown to be 30 in Figure 1 but it should be noted that this is an optimization parameter.

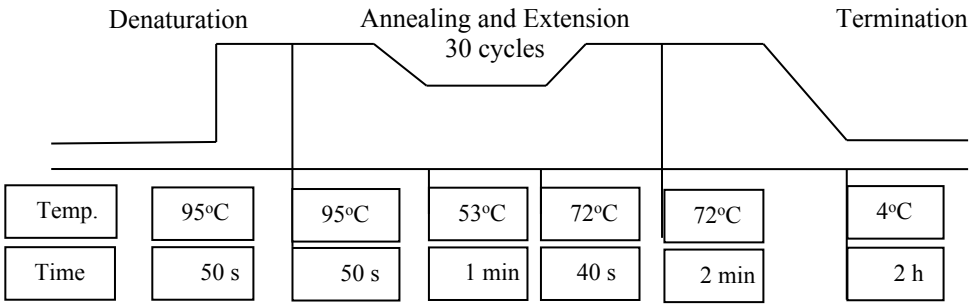

Figure 1. The thermal protocol of thermocycler for PCR amplification of the DNA marker

In the first step, the specimens' temperature was raised to 95 °C during 50 s so as to make single stranded DNA. The second step involves denaturation, annealing, and extension of the DNA strands at 95 °C in 50 s, 53 °C in 1 min, and 72 °C in 40 s for 30 cycles, respectively. The samples were then incubated at 72 °C for an additional 3 min to ensure that the final DNA extension was complete. Thereafter, the polymerization process terminates and the forth step begins in which the specimens temperature decreases to 4 °C within 2 h. This thermal cycling program is the result of an optimization strategy that maximizes both the yield and specificity.

2.5. Polyacrylamide gel electrophoresis

Following the STR amplification, the products were size separated via polyacrylamide gel electrophoresis of 10 µL DNA samples mixed with 5 mL formamide containing TBE buffer, carried out on a slab gel apparatus similar to the one described by (12). The DNA markers were examined on slab gels (20 cm × 20 cm × 30 cm) in TBE buffer with 1X concentration. The same buffer was used in the electrophoresis reservoirs. To prepare the 8% polyacrylamide gel, 15.13 mL deionized water, 25.6 mL of 30% acrylamide solution, 230 mL of 10% ammonium persulfate, and 5 mL of EDTA solution in TBE with a concentration of 5X were mixed together. After degassing the solution, the pre-gel solution was polymerized by the addition of 5 µL tetramethylene diamine as the catalyst. After gel preparation, the electrophoresis well was cleansed with TBE buffer and the digestion enzyme was added to the sample wells. The cleansing media in the sample wells was replaced with electrophoresis buffer immediately prior to sample applications. The electrophoresis was carried out at room temperature for 2.5 h at 80 V.

3. RESULTS AND DISCUSSION

The study of the parameters involved in successful amplification of a DNA with PCR increases our understanding on the effect of magnesium ion and the DNA sample concentrations, the annealing temperature, and the number of extension cycles. The addition of other components, *e.g.* proofreading enzymes, to the PCR cocktail was not considered suitable for this study, which results in no improvement in the STR amplification signal (13). There are diverse PCR applications throughout the research community and a single protocol would not be appropriate for all situations (14). Consequently, optimization of the parameters for maximization of yield and specificity of the desired product is an essential prerequisite. The optimization of magnesium ion concentration at a fixed number of extension cycles is beneficial because of the effects on primer annealing, strand binding strength, the specificity of the desired product, the formation of artifacts, and enzyme activity and fidelity (14). A high and low magnesium ion concentration led to low amplification due to hardness of the solution and decreased primer sticking to the DNA strand, respectively (15). The magnesium ion concentration was studied in the range of 1 to 5 mM based on some experimental investigations. Cobb and Clarkson also investigated the influence of magnesium ion concentration within the same range on PCR performance (16). They found that the magnesium ion concentration altered the specificity of the reaction and the amplification of spurious products was promoted at high concentrations. The results of our investigation have shown that an increase in magnesium ion concentration give a better amplification at high annealing temperature due to an increase in the hardness of the solution. The initial sample quantity and the magnesium ion concentration have no noticeable interaction with each other so that similar electrophoretic patterns were obtained under PCR amplification of the different sample quantities with various Mg^{2+} concentrations. This experimental investigation resulted in an optimal concentration of Mg^{2+}, *i.e.* 2.5 mM, for the preparation of the master mix. The other components were taken as the recommended values for DNA amplification using PCR (14). A low deoxyribunucleotide concentration (0.75 mM each) was adopted in the present work so as to minimize mispriming at non-target sites and to reduce the possibility of misincorporated nucleotide extension. The experimental results in the form of electrophoretic patterns were not brought in here due to brevity. The specificity of the marker in different individuals was investigated by PCR amplifying the DNA marker under an optimized PCR mixture. The mixture was optimized regarding the primer concentration, the annealing temperature and the sample DNA concentration. The range of the parameters under investigation is presented in Table 3. The master mixture

for this study is the one optimized and brought in Table 1. The best solution regarding successful amplification and electrophoretic separation is a primer concentration of 0.25 µM, an annealing temperature of 53 °C, and the DNA template concentration of 1 mM.

Table 3. The range of the parameters involved in the D6S957 marker amplification study

Parameter	Variability			
Primer Concentration (µM)	0.1	**0.25**[a]	0.5	
Annealing temperature (°C)	50	51	**53**	54
DNA template concentration (mM)	0.1	0.5	**1**	

[a] Numbers in bold indicate the optimal values for PCR amplification

The effect of annealing temperature on PCR performance was studied in four temperatures of 50, 51, 53, and 54 °C to find the optimal one, estimated to be within this range (17). The annealing temperature is a function of base length, composition, and the primers concentration so that a typical value cannot be assigned for all situations (17). The annealing temperature of 53 °C yielded the best result while the higher and lower values resulted in no distinct bands and blurred bands, respectively. The extension temperature of 72 °C for about one minute was suggested suitable for DNA sequences with a moderate A+T content (18). The recommended denaturation time and temperature for DNA sequences with a moderate G+C content is about 30 s and 95 °C (14). Accordingly, the optimal values of 95 °C and 50 s were used for the PCR amplification of the target product. A primer concentration of 0.25 µM yielded the best result. The PCR amplification of the target DNA at high primer concentrations resulted in unspecific products possibly because of template independent artifacts. Similar observation was reported by Henegariu et al. (19) during multiplex PCR of mixtures Y-1 to Y-4. Ruijter et al. (20) also observed an increase in the fluorescence baseline of the PCR product due to formation of artifacts. The optimum number of PCR cycles is a function of the starting concentration of the target DNA provided that the other parameters were optimized. Figure 2 provides a useful guide in the choice of the number of cycles appropriate for the target copy number.

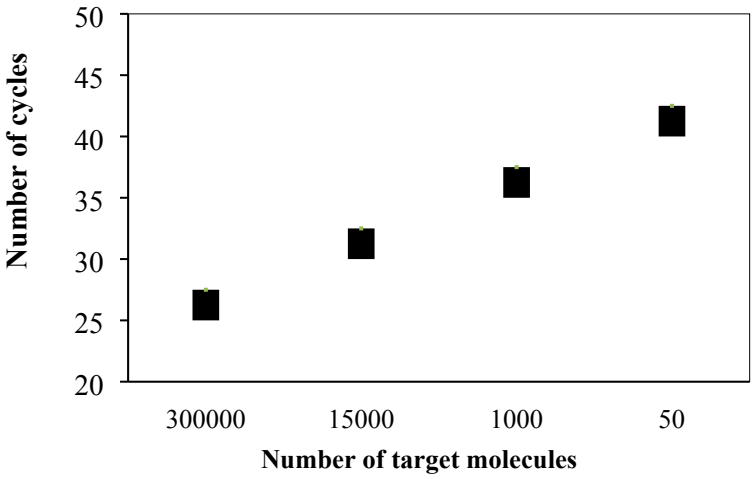

Figure 2. The optimum number of PCR cycles at different starting concentration of the target DNA

After these optimization studies, the specificity of the marker in different individuals was investigated for human identity testing in forensic sciences. To this end, the extracted DNA from the blood of five different persons was amplified with PCR and the amplified specimens were the electrophoretically separated in 8% polyacrylamide gel. The bands relevant to different specimens were shown in Figure 3. The different sizes of the amplified marker clearly show the specificity of this pentanucleotide STR marker in these persons.

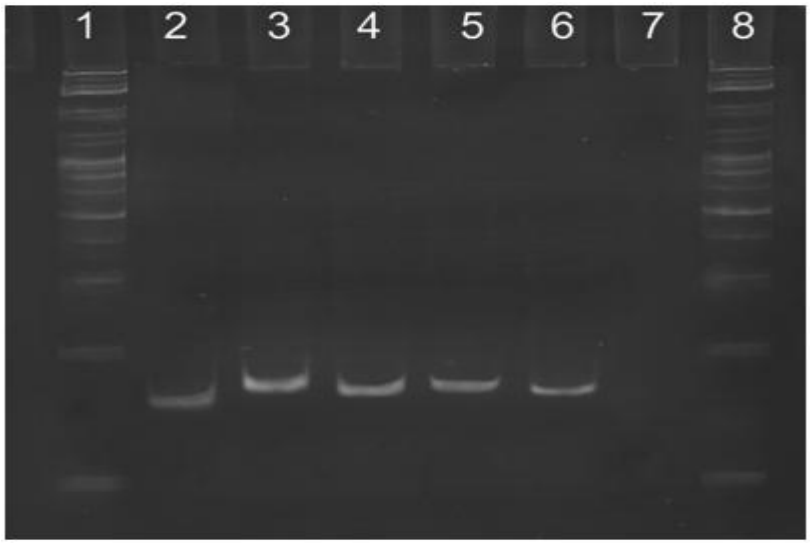

Figure 3. The product of PCR amplification of five different blood samples on 8% polyacrylamide gel. 1 and 8 are the pattern of the ladder; 2, 3, 4, and 5 are the electrophoretic patterns of the five different specimens. 7 is the pattern of negative control specimen

4. CONCLUSION

The PCR amplification of pentanucleotide tandem repeat marker with D6S957 locus was studied and the parameters of high importance were recognized. The optimization of the magnesium ion and the DNA sample concentrations, the annealing temperature, and the number of extension cycles was found essential in the maximization of both yield and the specificity. The optimal PCR amplification protocol was successfully exploited in the assessment of the marker applicability for human identity testing. The specificity of the DNA marker in five different Iranian individuals was approved by the development of distinct electrophoretic patterns

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AUTHORS CONTRIBUTION

This work was carried out in collaboration among all authors.

CONFLICT OF INTEREST

The authors declared no potential conflicts of interests with respect to the authorship and/or publication of this article.

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