



Research Paper

Optimizing denaturing HPLC as a robust technique for identification of Short Tandem Repeats (STR) in forensic medicine

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ABSTRACT

Introduction: Short Tandem Repeats (STRs) are defined as short lengths of 2–7 base pairs spreading through human genome which due to their highly diverse individually distribution are widely applied for identity detection and other forensic medicine purposes. Burdening considerable costs by the conventional methods such as capillary electrophoresis, we aimed to compare concomitant usage of multiplex PCR and denaturing high-performance liquid chromatography (DHPLC) as cheap, fast, highly accurate, and more accessible methods, with capillary electrophoresis (CE) to evaluate their potential for early screening of STRs.

Materials and methods: The present study randomly included 20 blood samples from the subjects referred to forensic medicine of Semnan, Iran. According to the size and allele frequency, we selected 8 major STR loci including CSF1PO, VWA, D18S51, TPOX, Amelogenin, FGA, SE33, and Penta D. A quad-STR multiplex PCR was performed for each locus and the PCR products were then analyzed using DHPLC machine and compared with the basic genetic properties obtained by capillary electrophoresis.

Results: By optimizing the PCR and DHPLC conditions, our findings suggest this strategy as an effective method for STR detection. The genotypes were determined using size of loci which led to comparable results with capillary electrophoresis confirming an insignificant variation in the detection of TOPX, Amelogenin, CSF1PO, and D18S5 (p = 0.331), but discrepant results for FGA and VWA loci (p = 0.002).

Conclusion: Our study proposed DHPLC method as an effective screening method to characterize TOPX, Amelogenin, CSF1PO, and D18S51 as frequently used STR loci during identity detection in forensic medicine.

1. Introduction

For decades, DNA sequence analysis for forensic purposes has been widely used for analyzing the criminal cases in which the identity of suspected individuals are under query. This necessitates, therefore, designing and optimizing robust methodologies capable to accurately recognize the particular loci in genomic DNA of individuals with unique population distribution.¹ In this regard, several lines of studies have reported the Short Tandem Repeats (STRs) as small segments of 2–7 or more base pairs in length scattering through the human genomic regions. Due to their higher mutation rate than the other regions of DNA resulting in high genetic diversity among the individuals, several STR loci have been growingly applied in forensic medicine for the identity detection purposes.² These segments possess a high level of

polymorphisms which are simply detected using PCR method.³ Moreover, the short length of these sequences (up to 200bp) provides this possibility to be evaluated using tiny amounts of basal DNA material even from the very low quality samples. These characteristics, thus, has turned them as sensitive and accurate platform in the identity detection.⁴

Since the STR typing offers a cost-effective and non-intensive strategy for genetic diversification of the individuals, it has engrossed a substantial consideration.¹ According to the recent advances in promoting conventional methodologies, as well as building novel techniques, it requires comparative analysis of various techniques which are basically used as routine experimentation or those under evaluation.

Nowadays, a remarkable change has been emerged in using high-throughput DNA experimentation for forensic medicine using high-tech

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methods such as Genetic Analyzer devices.⁵ However, having a high demanding process such as requiring precious laboratory kits and expensive prices of the genetic analyzers, further scrutiny is needed to replace them with cheaper methods such as DHPLC. Alongside, we aimed to analyze the results obtained by STR genetic analysis in terms of accuracy using DHPLC method and its comparison with capillary electrophoresis (CE) as the mostly conventional method used for this purpose.

Herein, we highlighted that DHPLC-based technique can be applied to detect at least four STR loci with high levels of accuracy resembled to that gained from CE. Due to its lower needed costs and offering acceptable accuracy, DHPLC might be an ideal candidate to replace the expensive genetic analyzer devices for early screening of genetic diversity among individuals.

2. Materials and methods

2.1. Subjects and sample preparation

In the current study, we randomly selected 20 subjects for further analysis. The venous blood samples were collected from the participants following a written consent. The project was confirmed by the ethic committee of Tehran University of Medical Sciences (TUMS). To perform the experiment, the blood samples were collected using a sterile K2-EDTA vacuum tube as the base material for STR typing using DHPLC. Also, 5 punches of the blood samples (1.2 mm) were put on Flinders Technology Associates (FTA TM) which is used to simplify the steps of DNA collection, transportation, purification and storage. In this study, the FTA paper was utilized to determine the genetic profiles using CE as the gold standard.

2.2. Genomic DNA extraction

Genomic DNA samples were isolated using PrimePrep™ Genomic DNA isolation Kit (GENET BIO, Daejeon, South Korea) according to the manufacturer's instructions. To confirm the adequate purity and concentration, the DNA samples were analyzed by a Nanodrop spectrophotometer (Ratios of A260/A280 and A260/A230). The samples were also resolved on 1.5% agarose gel and electrophoresed to ensure the total integrity.

2.3. Primer design for STR loci

The corresponding sequences (detailed information of the loci is listed in Table 1) were derived from STRBase database (<https://strbase.nist.gov/>). Based on the characteristics such as the number of polymorphisms, distribution of alleles in different populations, novelty,

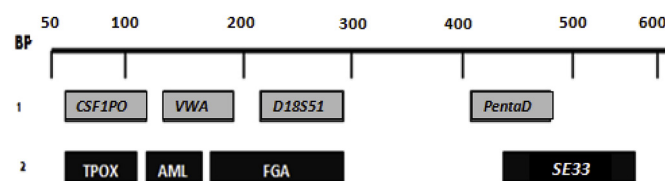


Fig. 1. Designing two sets of quadruplex PCR based on fragments' size and the scale of BP. There is no overlap between the sizes of the product and any of the primer pairs.

length and genomic region, and the PCR product size; the loci were categorized into two groups of four loci each to enter into two distinct multiplex PCR, so that avoiding overlap in the size of products or primer pairs (Fig. 1). The primers were selected as previously described by A. Odriozola et al.⁶ The primers were then reanalyzed using Primer-BLAST tool for the target specificity.

2.4. PCR, gel electrophoresis, and data analysis

Prior to entering into PCR reactions, to verify their functions, the received primers were primarily applied in a Monoplex PCR reaction and checked whether they form the desired products. The PCR cocktail for this reaction contained DNA as template (50 ng), 1X PCR buffer, MgCl₂ (1.5 mM), forward and reverse primers (0.6 mM), dNTP (200 mM, Taz, Germany), and Taq Polymerase (Native Fermentas, Germany) were added into a 0.2 ml tube.

Due to the variability in the thermal conditions; magnesium, and primer concentration; we performed the reactions for two groups of each four loci: CSF1PO, VWA, D18S51, PentaD; and TPOX, Amelogenin, FGA, SE33. The PCR reactions were performed using a thermal cycler (BioRad -Germany). The PCR thermal condition followed by: the initial denaturation step (95 °C for 3 min), 35 cycles of denaturation (94 °C for 30 s), annealing (64 °C for 45 s), and extension (72 °C for 45 s). Finally the reaction was terminated by an extension step for 10 min in 72 °C.

PCR products were then resolved and visualized on an agarose gel (2.5%). The precise product size and other genotyping characteristics were ultimately measured by the ABI 3130 genetic analyzer (Applied Biosystems®, Foster City, CA, USA).

2.5. DHPLC analysis

To separate fragments of PCR products, a non – denaturing method was used. Prior to the sample injection, the column was first washed using Buffer A containing 5% triethylammonium acetate and 95% water for 15 min with velocity of 75 ml/min. Then, to prepare the PCR

Table 1

STR locus name and some characteristics of the STR loci under examination in this study.

Locus	Genebank accession N°	Allele range	PCR fragment (bp)	Primer sequence (5–3)	TM
CSF1PO	X14720	6–15	75–111	F: ACTGCCTTCATAGATAGAAGAT R: GCCCTGTCTCAAGTACTTCCT	56.6 59.4
TPOX	M68651	6–16	61–101	F: CTTAGGGAACCTCACTG R: GCAGCGTTTATTGCCCCAA	64.89 65.99
FGA	M64982	16-51/2	151–293	F: CTCACAGATTAAACTGTAACCA R: TTGCTGTAAATTGCCAGC	58.20 57.96
VWA	M25858	11–24	121–173	F: TCAGTATGTGACTTGGATTGA R: GTAGGTTAGATAGATAGACAGA	55.5 62.5
D18S51	X91254	7–27	213–293	F: GTCTCAGTACTTGCAGG R: GGAGATGTCTTACAATAACAGTTG	56.11 60.51
SE33	V00481	10–34	450–523	F: AATCTGGGCGACAAGAGTGA R: ACATCTCCCTACCGCTATA	58.4 58.4
PentaD	AP001752	30–45	400–429	F: GAAGGTGCAAGCTGAAGT R: ATTAGAATTCTTTAATCTGGACACAAG	57.33 60.75
Amelogenin	M55418 M55419	X, Y	121,127	F: CCCTGGGCTCTGTAAAGA R: AGGCTTGAGGCCAACCAT	56.1 56.1

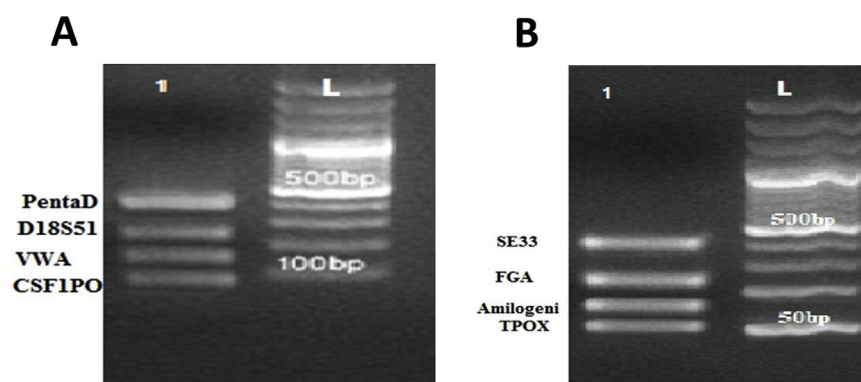


Fig. 2. Gel electrophoresis of the QuadruPlex products (1*1) of a sample under study. The names of the STR loci in this sample are annotated in each panel. The size marker proper to each group loci (Fermentas) with product number 0321 SM and 0371 SM for A and B, respectively are shown on the right side of each gel and the band size is specified at the top of each. Bands are in the expected size range.

Table 2

Optimized conditions for calculating the size of PCR fragments in DHPLC.

Optimization steps	Optimized conditions
Mode	Nondenaturing
Volume of PCR Product	5–10 μ l
Temperature	55 °C
Loading Duration	0.1 min
Loading Drop	5%
Gradient Slope	2% Buffer B/min
Gradient Duration	4 min
Clean Duration	0.5 min
Equilibration Duration	1 min
Flow Rate	0.9 mL/min
Retention Time	20 min

products (10 μ l) and to eliminate the possible interfering components, they were added separately into micro-tubes and quickly centrifuged. The samples were then processed by DHPLC device using a DNAsep Cartridge (Transgenomic® WAVE® System 4500, Glasgow, UK). Briefly, the sample injection was carried out with cautious and to avoid possible carry over, the column was washed between the injections, respectively using the following solutions:

- 1) 5 mL of Buffer B containing 5% triethylammonium acetate, 25% acetonitrile, and 70% water; 2) 3–5 ml acetic acid (40–20%) at 60 °C; and 3) reappassing 5 ml of buffer B. The syringe used to inject the samples was also frequently washed with 40–20% acetic acid and buffer B, respectively, between each injection.⁵

The harvesting percentage of the device was determined 65% and 35% from the buffers A and B, respectively, through the entire run time. The absorption rate of samples was read at the wavelength of 260 nm. Teak curves as well as the retention time were provided by the Wave Software.

2.6. Capillary electrophoresis

In order to check whether the CE technique as gold standard method verifies the DHPLC results, we perfumed the CE on the products using the ABI PRISM 3130 Genetic Analyzer (Applied Biosystems®, Foster City, CA, USA). Briefly, 2 μ l of each PCR product were analyzed, and mixed with 9 μ l of Hi-Di™ Formamide (Applied Biosystems®, Foster City, CA, USA) and 0.5 μ l of GeneScan™ 500 LIZ® Size Standard (Applied Biosystems®, Foster City, CA, USA). Following denaturation (6 min at 95 °C), they were cooled (4 min at 4 °C) and separated in the CE device. The CE results were then analyzed by the Genmapper software, version 4.0, (Applied Biosystems®, Foster City, CA, USA).

2.7. Statistical analysis

To compare the results achieved from DHPLC and CE experiments, the Chi-square test was used. The SPSS software, Version 20, was applied for this purpose.

3. Results

To examine the primer's functionality, we considered a series of factors for primer design including melting temperature, the product size visualized on the electrophoresis, and the banding efficiency of the primer pairs associated with the quadruple. Owing to these characteristics, we set up two distinct systems of QuadruPlex covering eight STR loci including CSF1PO, VWA, D18S51, PentaD and TPOX, Amelogenin, FGA, SE33. Following the optimization of QuadruPlex conditions, based on the individual Monoplex conditions, a full procedure was performed for the associated QuadruPlex analysis; however, several modifications were conducted to optimize the ultimate conditions.

3.1. Optimal setup conditions were designed for the multiplex systems

Our experiments showed that the temperatures of 62–66 °C and the magnesium concentration of 1.5 mM, allow all the STR markers under study to be detectable similar to those observed with Monoplex PCR. Besides, it was shown that where the primers concentration and the related temperatures are analyzed using an unchanged magnesium load, the bands for CSF1PO and D18S51 markers are weaker than expected.

We confirmed that by adding the Taq polymerase to the tubes at 94 °C with a common buffer and fixed concentration of Mg against variable primer load and temperatures, the Multiplex PCR bands can be readily observed (Fig. 2).

3.2. DHPLC results

In this study we aimed to evaluate the STR-based identity detection using the DHPLC method. Expectedly, we showed that using the proper temperatures to the columns and various buffer concentrations offer efficient work conditions. Accordingly, the thermal conditions ranging from 50 to 60 °C which ascends two degrees per injection was used for the tests optimization. Finally, following multiple injections, the temperature of 56 °C was shown to be optimal. Then, according to the band sizes, the amplified loci were eluted from the columns. A summary of different optimized parameters is listed in Table 2.

Figs. 3 and 4 show the DHPLC chromatograms for the two sets of Quadriplex. The chromatogram provides a comparison of graph achieved from test samples with that taken from the standard sample of DHPLC device. The different sizes of each peak are also illustrated in the Figures. As it is indicated in Figs. 3 and 4, the chromatograms of TPOX, Amelogenin, FGAa, D18S51, and CSF1PO are homoduplex;

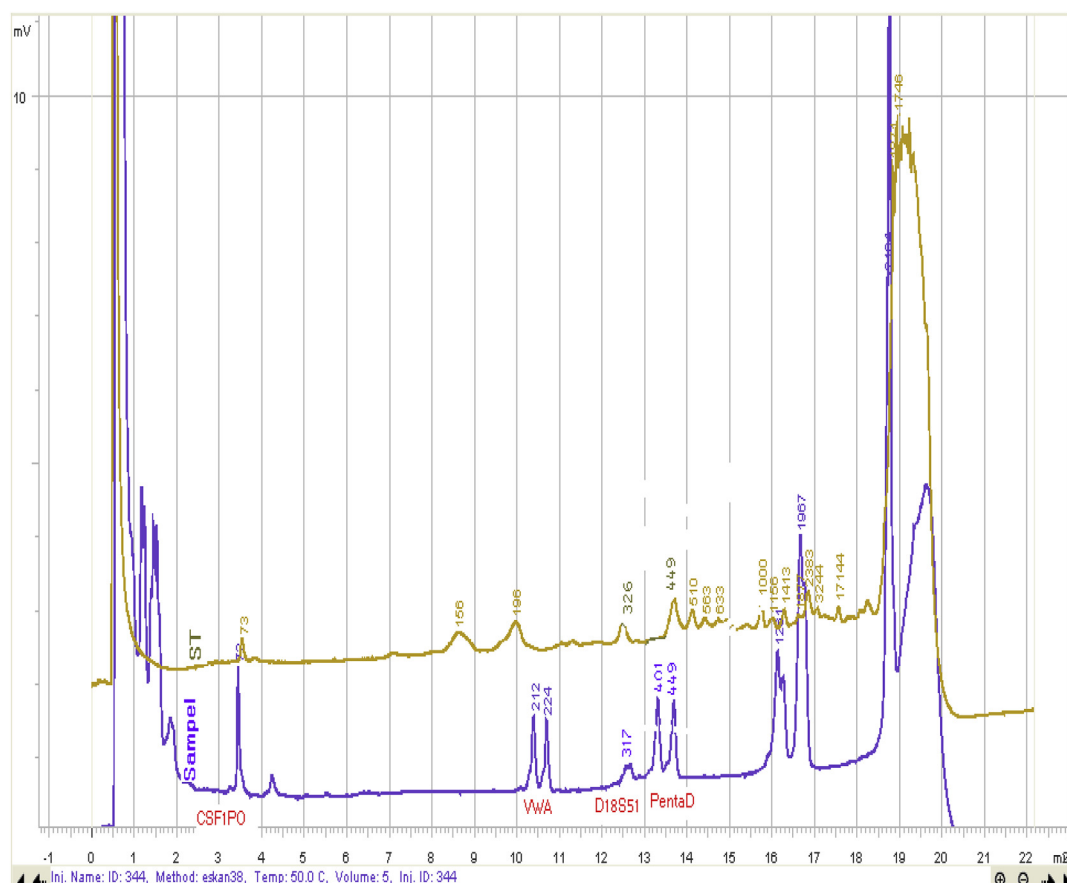


Fig. 3. DHPLC chromatogram of Quadriplex 1 in one sample. The chromatogram illustrates the sizes and standard comparison of the four loci: CSF1PO, VWA, D18S51, PentaD. Loci are in the expected range. As it is clear, in this sample chromatogram the loci of D18S51 and CSF1PO are homoduplex; while the VWA and PentaD loci are heteroduplex.

while VWA, PentaD, and SE33 loci show a heteroduplex.

We then calculated the number of alleles per each locus. This can be achieved by measuring the size of fragment lengths resulted from the desired locus which is calculated as follows: The number of nucleotides per each primer pair is subtracted from the product size obtained by the DHPLC device for each locus. The resulted number was then divided by 4 as the loci contained four nucleotides, except PentaD having 5 nucleotides which is divided by 5. The final number is considered as individual's allele numbers.

3.3. CE provides consistent results with DHPLC for TOPX, amelogenin, CSF1PO, and D18S5 loci

To evaluate whether the DHPLC results corroborate the genotyping characteristics of the loci, we performed the CE genetic analyzer as the gold standard. The chromatograms were analyzed using the Gene Mapper software (Fig. 5). Finally, a comparison analysis was conducted between the two methods to reveal the possible variations (Table 3). Our findings showed a meaningful variation between VWA and FGA loci in the comparative results; nevertheless, the other loci revealed a high level of consistent results achieved by either CE or DHPLC. This variation can be attributed to the PCR conditions, other methodologies and set up conditions in this study. In the case of PentaD and SE33 loci, it should be noted that there is not a pre-optimized procedure to analyze them in commercial kits, thus we assessed them based on a DHPLC procedure using an on-site optimization (Figs. 4 and 5).

4. Discussion

For many years, DHPLC has become a promoting tool for the separation of STR fragments amplified by PCR, providing potential platform for identity detection and other forensic medicine purposes. In order to clarify the unprecedented applications of DHPLC in this context we aimed to investigate the possible indication of this technique to detect some common STR loci used in forensic medicine. In the current study, therefore, we first manually set up a multiplex PCR system in which 7 STR loci and one amelogenin locus were evaluated. Our assays included 6 cases of loci in CODIS system as well as a MiniSTRs and SE33 locus recently developed by Germany National Database (GCL). Interestingly, our findings suggest the possible candidacy of DHPLC to replace the conventional CE, at least to detect four out of 8 loci, including TOPX, Amelogenin, CSF1PO, and D18S5.

CE is commonly regarded as the most routine analytical method for STR identification used in clinical setting. However, the detection methods are switching to other rapid and cost effective methodologies. This is due to several barriers regarding the use of high expensive methods, including necessities of specific materials, costly multiplex PCR kits, and requiring fluorescent primers.⁶

In the current study, in order to enhance the sensitivity of PCR technique, we took advantage of Non-denaturing method at 50 °C and measured the PCR product size of the STR loci.^{7,8} In this context, a previous study made by Pental et al., showed that in the multiplex analyses using DHPLC device, Non-denaturing method offered a higher accuracy and easily performed comparing to the fully denaturing method.⁷ In addition, Ellis et al. compared the sequencing analysis of the fragmented DNA using HPLC. By examining the HPLC modes, they

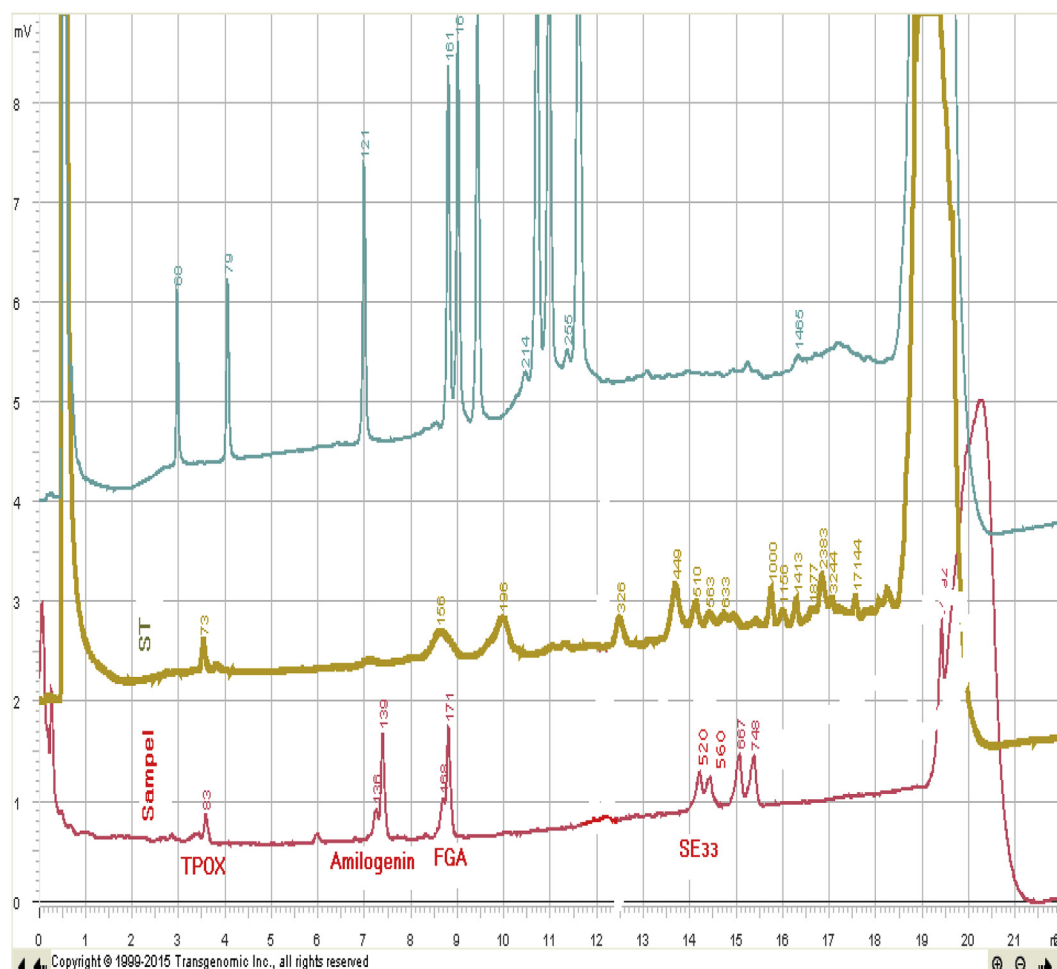


Fig. 4. DHPLC chromatogram of Quadriplex 2 in one sample. The chromatogram obtained from the injection of Quadriplex 2 PCR products of four loci including *TPOX*, *Amelogenin*, *FGA*, and *SE33* were specified in the image and were compared with the standard and the size of each locus was determined. Loci are in the expected range. As it is indicated, the chromatograms of *TPOX*, *Amelogenin*, and *FGA* have homoduplex chromatogram; while *SE33* shows a heteroduplex.

indicated that the Non-denaturing method served a high efficacy in separating DNA fragments based on their sizes.⁸ Our findings were shown to be consistent with their results proposing the technique as a major platform for distinguishing STR loci using Non-denaturing method.

In our set up, we also used the DNasep column (Transgenomic® WAVE® System 4500, Glasgow, UK) consisting the poly styrene-divinyl benzene particles. In a previous study by Herbert Oberacher et al., a comparison was made between the efficiency of Silica, Octadecylsilica, and poly styrene-divinyl benzene columns to separate the oligonucleotides in ion-pair reversed-phase high-performance liquid chromatography (IP-RP-HPLC) method.⁹ They ultimately concluded that the PS-DVB columns offer the highest efficacy in separating the oligonucleotide segments, hence introducing that as a robust tool for STR identification.³

In the present study, based on the manufacturer's instruction for the DNA fragment analysis, we considered the size of PCR product fragments between 50 and 1000bp, which was also previously reported in the study of A.Odriozola et al. Loci to be varied in size from 60 bp to 500 bp and selected as ideal candidates for our study. Furthermore, based on the study of A.Odriozola et al. and to avoid the STR overlapping, the fragments were selected based on their product lengths which then divided into two groups of Quadriplex.⁶ By studying 49–279 bp of 15 STR loci using DHPLC technology, A. Odriozola suggested this technique as a method with various advantages over expensive methodologies in terms of speed, and cost effectiveness during primary

screening.⁶ Since the optimization methods were resembled to those made by A.Odriozola et al., such as PCR and primer characteristics as well as the product sizes, consistent results were made in our PCR experiments and DHPLC chromatograms.¹⁰ Moreover, it is indicated that the elution occurs faster at the same temperatures for the shorter amplified fragment lengths. Our study also concluded the same, as the smaller fragments eluted faster than those with larger lengths at a fixed temperature.¹¹

To analyze the individual Monoplex loci, a standard temperature was applied according to the protocol proposed by Stanford University Website with some minimal modifications. A previous study by Xio et al. showed that nonspecific temperatures during STR identification result in misidentification of heteroduplex which can also remove the chromatogram for some short fragments. They also argued that the temperature indicated on the instrument is not always align with that required optimally, as the probes measuring thermal conditions recorded two degrees deviations from optimal temperature.¹² Consistently, our study concluded the same strategy, and according to the predicted temperature suggested by the Stanford University website, we used 56 °C in the present study following the application of 2 °C changes, and assessed their chromatograms. Our data were also in agreement with the study of Jones AC et al. where they confirmed that by arising the column temperature, the samples elute faster.¹³ Indeed, we demonstrated that by assigning the temperature at 50 °C, the *TPOX* locus fragment is identifiable upon 5 min of injection, while at 58.5 °C of column temperature it can be detected after 4 min.

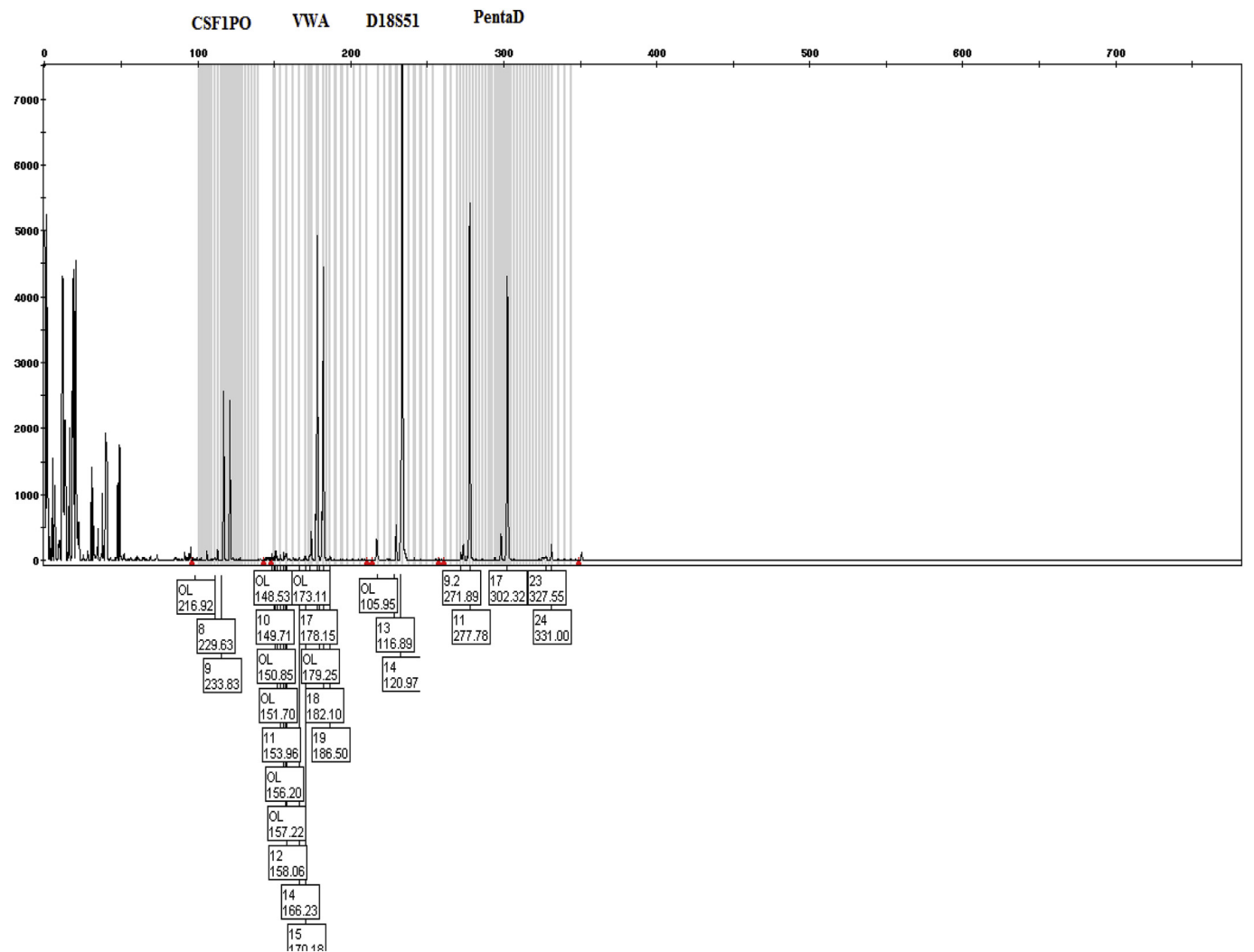


Fig. 5. Genetic profile image shows one subject under study in STR loci (CSF1PO, vWA, D18S51, PentaD) with CE method.

Table 3
Comparison of DHPLC and CE methods in the analysis of studied loci.

Parameter	Result															
	CSF1PO		VWA		D18S51		PentaD		TPOX		Amelogenin		FGA		SE33	
	+ ^a	- ^b	+ ^a	- ^b	+ ^a	- ^b	+ ^a	- ^b	+ ^a	- ^b	+ ^a	- ^b	+ ^a	- ^b	+ ^a	- ^b
CE	95	5	95	5	95	5	–		95	5	95	5	95	5	–	
DHPLC	90	10	50	50	75	25			80	20	90	10	55	45		
P	0.548		0.001		0.077				0.151		0.548		0.003			
X ²	0.360		10.157		3.137				2.0157		0.360		8.533			

^a Separation of alleles on DHPLC and CE.
^b Lack of separation of alleles on DHPLC and CE.

In a previous analysis, GR Taylor et al. used DNasep column for the separation of STR Loci. It was noted in their study that the device column was a non-polar stationary alkylating phase and the buffer is liquid with the polarized phase of acetonitrile. Due to the negative charge that phosphate groups give to the DNA molecules, there is a need for organic cations (giving positive charge) to connect the stationary phase (column) with DNA. In this regard TEAA seems to constitute the most frequent organic compound, which due to its positive charge has a high chance to bind with DNA molecules. Additionally, there is also the possibility of linking non-polar fragments to the stationary phase. Having triethyl fragments, TEAA can also link with the

stationary phase, thus DNA molecules weakly connect with acetonitrile, and the small fragment lengths pass through the column faster than larger fragments. In this study, acetonitrile and TEAA buffers were used in the DHPLC device and it was demonstrated that all the loci can be eluted from the column respectively from large to small fragments.¹⁴

In another effort made by Shi et al. discussing the optimal percentage of the gradient buffers, it was shown that in the conditions with inappropriate buffers used in the device, the loci with small sizes are removed, thus cannot be identified during their passage through the column. According to the above-mentioned study the proper conditions of temperature and buffer gradient were selected in our study, leading

to four optimized DHPLC chromatograms.¹⁵

We have also used WAVER Maker software in our analysis to decipher the chromatogram sizes, offering coherent results with those achieved by Joseph.M et al.

It was concluded in their study that besides PCR amplification, determining the precise data on genotyping requires further separation steps, thus the heterozygous alleles of STRs can be distinguished from non-specific components resulting from PCR-associated hetero-duplex formation.¹⁶ In our assessment, we were able to purify the STR-related hetero-duplex alterations using WAVER System in HPLC, proposing this technique as a robust tool for STR identification.

Acceptable findings were achieved by optimization of multiplex PCR and DHPLC experimentation in the mentioned loci. However, the Chi-square analysis showed that although DHPLC offers accurate individual loci determination, its potential to identify some loci was not adequately acceptable. This may be due to the PCR conditions, gene expression profile, or other procedural conditions in the study.

5. Conclusion

Taken together, our results suggest that the DHPLC technique has several advantages over CE such as its lower cost for DNA analysis and therefore, it was performed in this study as a novel method to detect STR loci fragments for the genetic identification purposes.

This strategy was studied for the first time in Iran to determine the genetic characteristics of STR loci for forensic medicine purposes. Showing high levels of efficacy, the DHPLC technique was proposed in our study possibly as the best candidate to replace the expensive traditional methods for identity detection. Further studies, however, are required to optimize this method for a wider range of genetic loci commonly used for identity detection. Moreover, studying other STR loci with higher sample size, determining the genetic profile using DHPLC on mtDNA samples, and genetic profile identification of sensitive DNA samples or on the paraffinized samples to check the sensitivity of DHPLC methods are highly suggested.

Conflicts of interest

There is no conflict of interest.

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