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Imperative Strictures in the Construction of Miniplex primer Sets for Short Tandem Repeats to Aid in the Genotyping of Challenged DNA Samples

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Abstract

A forensic analyst routinely encounters variety of challenging samples- biological and non-biological at the crime scene. Deoxyribose nucleic acid (DNA) extraction and its profiling are used in forensic science for establishing origin of biological fluids found at the crime scene. In many of the biological fluids samples DNA is highly degraded. This degraded fragmented DNA leads to poor amplification of large sized short tandem repeats (STR) loci. To get DNA profile from these challenged forensic samples, mini-STR analysis is considered useful. In mini-STRs, primers are located close to the repeat regions; hence shorter amplicons are generated by PCR. This improves PCR efficiency and provides better DNA profiles.

This study reports the results of individually amplified five short tandem repeats (STRs) of Lipoprotein lipase gene, Tyrosine hydroxylase gene, Thyroid peroxidase gene, Hypoxanthine phosphor ribosyl transferase and D5 (LPL, TH01, TPOX, HPRT and D5S818) loci as well as that of all five loci amplified together on gel. Importantstrictures in the construction of miniplex are discussed in details. ABI 3100 Genetic Analyzer (Applied Biosystems) results for three loci (LPL, TH01 and TPOX) are also discussed.

A meaningful DNA profile can be obtained from degraded biological samples by using mini-STRs or miniplexes. Mini-STRs using 3 to 5 loci multiplexed together were increasingly useful in the analysis of fragmented DNA.

Keywords: Forensic identification; Degraded DNA; Short tandem repeats (STRs); Polymerase chain reaction (PCR); Miniplex.

Introduction

Ceaseless efforts are being made to avert and detect crimes to track down criminals, in order to protect innocent and law abiding citizens. This is achieved by police personals enforcing law with the help of forensic scientists. Forensic science is a dynamic field. Its main help to police during the course of investigation is in the collection of right evidence material from scene of crime, analyze the evidence scientifically and opine on the results of analysis. This in turn helps the judges and juries to solve various medico legal issues involved in criminal cases and other legal matters in civil cases. Over the period of last 30 years forensic science has gained tremendous importance as far as evidential values are concerned. This was particularly possible due to a biological tool namely the analysis of DNA.[1] This has revolutionized forensic investigations.

DNA profiling refers to the identification of individuals through their DNA. It is used to identify, confirm or eliminate a suspect. DNA profiling has also become a particularly beneficial tool in exonerating those wrongfully convicted.[2,3] In the early 1990's DNA profiling by using short tandem repeats (STRs) were first described as

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an effective tool for human identification.[4] The tandem repeated sequences of two to five bases long were more compatible with the use of multiplex polymerase chain reaction (PCR). Since then, this method has been successfully applied in many areas of DNA testing, including analysis of deletions[5, 6], mutations[7] and polymorphisms[8], or quantitative assays[9], reverse transcription PCR[10], forensic science for individual identification[11, 12] and in solving disputed paternity cases.[13]

The current commercially available multiplex kit amplifies more than 20 STR loci and generates amplicon sizes from 100-450 base pairs. In degraded forensic samples, very often, DNA is fragmented. Hence, STR loci with larger amplicon sizes over 300 base pairs are either not amplified or the amplicon size decreases proportionally. Efforts have been taken to increase the efficiency of STR markers in degraded biological samples.[14,15] Mini STRs were developed, by making new primers which are close to STR repeat region, thus reducing the size of amplicon (80-300).[15, 16, 17] The small size of amplicons increases the amplification efficiency in fragmented DNA. Besides this, mini-STRs amplify three to five loci with two or three fluorescent dyes, which make them much cheaper than the commercially available kits. For a successful miniplex PCR assay, the relative concentration of the primers of the loci amplified, the concentration of the PCR buffer, the cycling temperatures and the balance between the deoxynucleotide concentrations are most important. Therefore, a study of these imperative parameters influencing the amplification was initiated to design a standard miniplex PCR protocol. Many papers and manuals have discussed in details about the quality of PCR but very few papers are published about the construction of miniplex PCR. This study reports the results of individually amplified as well as amplified in various combinations five short tandem repeats (STRs) of LPL, TH01, TPOX, D5S818 and HPRT loci on gel using Genei's Hot start Taq polymerase assay buffer (10X) and Hot-start Taq polymerase enzyme. Same protocol was followed for miniplexing the three (LPL, TH01, TPOX) loci on ABI 3100 genetic analyzer for comparison. This protocol would be useful also to those using PCR technology in the clinical laboratories as well as to the researchers.

Materials and Methods

Sample collection

Blood samples were collected from 114 healthy, unrelated subjects. Each donor's age and sex was recorded. Bloodstains were prepared on cotton cloth and dried at room temperature. The stains were labeled and were placed in paper envelope and stored at 4 c for further analysis.

DNA extraction

DNA was extracted by organic extraction method using lysis buffer and proteinase K for digestion of proteins present in the blood.[18] Digested proteins were separated by adding phenol and the aqueous layer was separated in fresh tubes and DNA was precipitated by adding ethanol. Submerged electrophoresis using 1% agarose gel was carried out to separate DNA bands. DNA bands were observed under Bio Rad UV trans illuminator.

PCR amplification

PCR was performed on Gene Amp 9700 (Applied Biosystems) Thermal cycler.[19] The PCR conditions were standardized by varying template concentration, annealing temperature, by adding different quantities of forward and backward primers, enzyme Hot-start Taq polymerase etc. Table 1 shows PCR components used for miniplex.

PCR Protocol

The components of the reaction were added in following order: water was added first, then buffer, followed by magic solution, dNTPs and primers. This was termed as master mix. All the components of the mix as well as the master mix were kept in ice bath during these operations. Aliquots of 24µl were placed in each PCR tube placed on ice bath in which the template DNA was added. The vials were transferred to the thermal cycler.

Standardized PCR cycle condition

Hot start Taq polymerase enzyme was activated at 94°C for 5 min. Each PCR cycle consisted of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min; and a final extension at 72°C for 7 min. 28 such cycles were run for DNA amplification.

Analysis of PCR Products

The PCR products were separated by electrophoresis on 3% Agarose Gels to which ethidium bromide (0.05mg/ml) was added in 1XTAE [0.04 M Tris-acetate; 0.001 M EDTA (pH 8.0)] at room temperature using voltage gradients of 7 V/cm. For any given gel analysis, an equivalent volume of PCR products was loaded in each gel slot. Results

were visualized under UV trans illuminator.

Genotyping and Data Analysis

Sequencing gels (6% polyacrylamide /7 M urea) were used for separation of the PCR products. The equivalent of about 10µl PCR product was loaded in each gel lane, after mixing with the loading buffer. These gels were run in 0.6 X TBE at 1800–2000 V (60 A) for about 2 h and stained by silver staining.

Amplification products were also electrophoresed on the ABI 3100 Genetic Analyzer (capillary electrophoresis). For the ABI PRISM 3100 Genetic Analyzer 20, 211.5 µl of amplicons and 0.3 µl of ROX-500 Internal Lane Size Standard were added to 12µl of deionized formamide and denatured at 95°C for 3 min. This mixture was loaded and electrophoresed at 15 kV in Performance Optimized Polymer 4 (POP4™). Results were analyzed using the Genemapper analyzing software application.

Table 1: A Standardized Miniplex PCR protocol.

Reagents	Amount/ Volume
Genomic DNA	100 ng (~10 µl)
dNTP mix (2.5 mM each)	1.0 l
Primer mix (2 Nos.-10 Nos.)	50 ng each (1.0 µl)
Genei Hot-start Taq pol assay buffer (10X)	2.5 µl
Hot-start Taq polymerase Enzyme	5U (0.5 µl)
MAGIC solution (2.5X)	1X (10 µl)
Glass distilled water	volume to 25 µl

Table 2: Chromosomal Location, Size of alleles of the selected loci and the primers used for the experiments.

Locus	Locus Definition	Chromosomal location	Size range in base pairs	Primers selected
HPRT	Hypoxanthine-guanine phosphoribosyl transferase	Xq26	259-303	5'- ATG CCA CAG ATA ATA CAC ATC CCC-3'
				5'-CTC TCC AGA ATA GTT AGA TGT AGG-3'
TPOX	Thyroid peroxidase gene	2p25.3	216-264	5'-ACT GGC ACA GAA CAG GCA CTT-3'
				5'-GGA GGA ACT GGG AAC CAC ACA GGT-3'
TH01	Tyrosine hydroxylase gene	11P15-15.5	171-215	5'-GTG GGC TGA AAA GCT CCC GAT TAT -3'
				5'-ATT CAA AGG GTA TCT GGG CTC TGG-3'
D5S818	_____	5q23.3-32	115-163	5'-GGT GAT TTT CCT CTT TGG TAT CC-3'
				5'-AGC CAC AGT TTA CAA CAT TTG TAT CT-3'
LPL	Lipoprotein lipase gene	8p22	105-133	5'-CTG ACC AAG GAT AGT GGG ATA TAG-3'
				5'-GGT AAC TGA GCG AGA CTG TGT CT-3'

Selection of primers

The forward primer is upstream of the ROA (region of amplification) and is complementary to the lower strand. The sequence of the forward primer is identical to that of the upper strand of template DNA. The reverse primer is downstream of the ROA and is complementary to the upper strand. Primers selected were between 18-24 nucleotides in length and contained 50-60% guanidine and cytosine (G+C). Each primer pair had dissociation temperature (Tm) between 52-60°C. Longer primers (28-30 bp) allowed the reaction to be performed at a higher annealing temperature and yielded less unspecific products.[22] In forensic cases, very of ten, the extracted DNA is in denatured state. If longer primers are used then the challenged DNA samples of shorter nucleotide length could not be amplified. Table 2 shows the details of the locus studied and primers selected and used in the experiments.

Single locus PCR

Before construction of a miniplex, selected primers of every locus were individually electrophoresed. Figure 1 shows the results of the electrophoresis of each monoplex PCR using the set of two single stranded primers (forward and reverse).

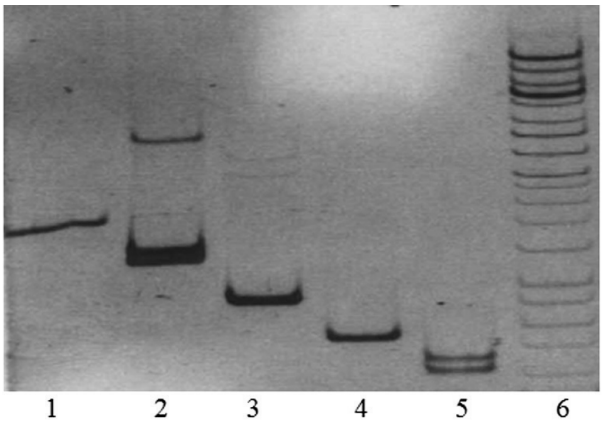

Fig. 1: PCR products with str marker (monoplex)

For any given gel analysis, an equivalent volume of PCR products was loaded in each gel slot. Results

were visualized under UV transilluminator.

Lane 1: Human HPRTB locus

Lane 2: Human TPOX locus

Lane 3: Human TH01 locus

Lane 4: Human D5S818 locus

Lane 5: Human LPL locus

Lane 6: 20 bp ladder (Genei)

Miniplex PCR

In designing a miniplex PCR, the first important step was choosing the primer pairs which might be combined and amplified simultaneously. Therefore, the amplification of every single locus was tested individually along with the miniplex to design a PCR program which would allow optimal amplification of all loci. Combining the primers in various mixtures and amplifying many loci simultaneously required alteration and optimization in the amount of primers to be added.

Optimization of Extension temperature and time

Extension time and temperatures plays an important role in the outcome of a miniplex reaction. Experiments were carried out by varying the extension temperature and time. In general, there was a higher concentration of amplicons when the extension temperature was 72°C. At higher extension temperatures, a decrease in the amplification of loci was observed whereas, at lower temperatures amplification of unspecific products were seen.

In miniplex PCR, as more loci are simultaneously amplified, the pool of enzyme and nucleotides becomes a limiting factor and more time is necessary for the polymerase molecules to complete synthesis of all the products. The above experiments also illustrated the influence of PCR on the extension time. It was observed that increasing the extension time in a miniplex PCR increased the amount of longer products and visibly higher yields of PCR products were obtained for all loci when a longer extension time was used. In the present studies, five loci were miniplex simultaneously, hence extension time of 2 minutes was found to be ideal for the complete amplification of all the five loci.

Annealing time and temperature

During denaturation, the template's double stranded DNA gets separated into two single strands. Two different primer sequences anneal to their complementary sequences. This annealing temperature of 550^0C-580^0C was calculated by using the formula [Tm = 40 (G+C) +20 (A+T)]. Modification of the annealing time from 0.5 min to 2 min did not alter the amplification efficiency, but a change in the annealing temperature affected the results and hence it was one of the most important and critical strictures. Each individual locus amplified at 57°–60°C, our experiments showed that in a miniplex lowering the annealing temperature by 2°–3°C was required for all the five loci to be co amplified together. At 54°C, unspecific amplification occurred. To overcome the unspecific amplification, annealing was carried out at 55°C, 1 min.

Number of PCR cycles

Number of PCR cycles and final extension were kept constant (28 cycles) for all the experiments. However once the parameters for annealing and extension were standardized using this protocol 30, 35 and 40 cycles were also carried out to see the changes in the products of amplification. Figure 2 shows the changes observed for 35 cycles and figure 3 for 40 cycles.

Amplicons should a gradual increase in the concentration of bands when the number of cycles was increased. The most obvious variation in the amount of products was for 35 and 40 cycles. Twenty eight to thirty cycles are usually sufficient for a successful miniplex PCR reaction. By increasing the cycle number up to 40 for DNA samples which were completely degraded were amplified successfully. But, 40 cycles are of little use in normal samples, as they showed non specific bands and stutters when checked on capillary electrophoresis.

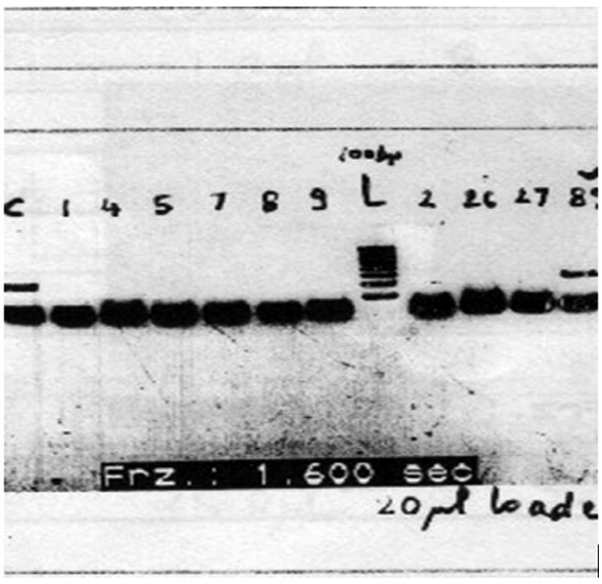

Fig 2: Checking for successful PCR 20 µl of all PCR products loaded for 35 cycles.

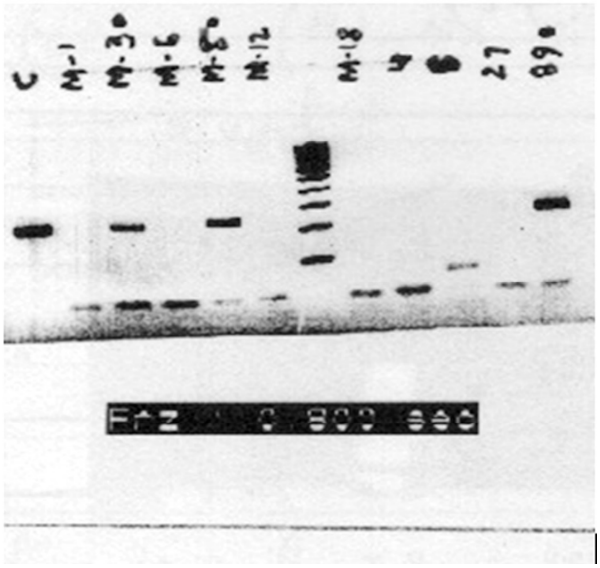

Fig. 3: Checking for successful PCR for 40 cycles 10 µl of all PCR products loaded.

Amount of primer:

Initially primer concentrations of 0.2–0.4 µM each were used in the miniplex PCR, but there was uneven amplification, of some of the products. To overcome this problem, different concentrations of primers were tried out in the reaction mixture, with an increase in the amount of primers for the "weak" loci and a decreased in the amount for the "strong" loci. Figures 4 and 5 shows the amplified PCR products obtained with different concentration of primers. The final concentration of the primers used was 50ng/µl each.

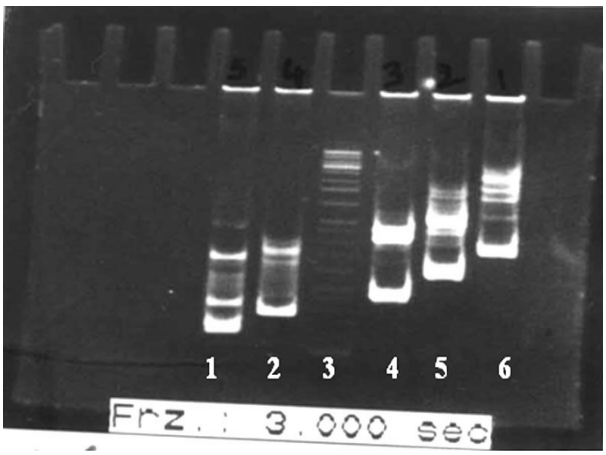

Fig. 4: Photograph Showing the PCR products with varying concentration of primers (Used 200 ng of each primer).

Lane1: Human HPRT locus
Lane 2: Human TPOX locus
Lane 3: 20 bp ladder (Genei)
Lane 4: Human TH01 locus
Lane 5: Human D5S818 locus
Lane 6: Human LPL locus

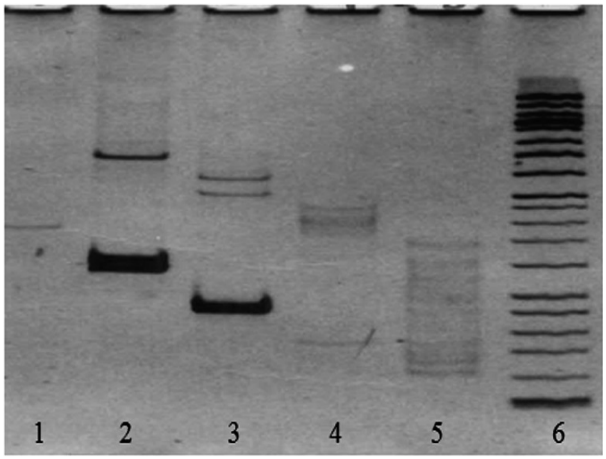

Fig. 5: Photograph Showing the PCR Products with varying concentration of primers (Used 100 ng of each primer).

Lane1: Human HPRT locus
Lane 2: Human TPOX locus
Lane 3: Human TH01 locus
Lane 4: Human D5S818 locus
Lane 5: Human LPLlocus
Lane 6: 20 bp ladder (Genei)

dNTP concentrations

The dNTP concentration was increased stepwise from 0.5–3.5 mM each. The best results were observed at 2.5mM of each dNTP. Lower dNTP concentration (0.5mM) allowed PCR amplification but amounts of products were not visible. Stock solutions of dNTP were sensitive to thawing/ freezing cycles. Therefore, small aliquots (1ml, 10–20 reactions) of dNTP (2.5 mM /µleach) should be made and kept frozen at -20°C and centrifuged before use.

PCR buffer concentration

2.5 µl of 10X Genei Hot-start Taq polymerase assay buffer was used for finalized miniplex PCR. Using higher concentration of buffer improved the efficiency of the miniplex reaction.

Amount of template DNA

Mixture component in the PCR has a limited supply of enzyme and nucleotides, and all products compete for the same pool of supplies. Therefore addition of right quantity of DNA template was equally important along with other parameters involved in a miniplex PCR set up.

Use of too much or too little template of DNA in miniplex STR amplifications can cause problems and necessitate reanalysis in order to generate interpretable results. Accurate quantitation of DNA

template is necessary to produce a well-balanced on scale STR profiles. Addition of excess of DNA causes split peaks due to incomplete adenylation or off-scale peaks that produce bleed-through between dye colors.[11] Too little DNA causes loss of alleles due to stochastic PCR effects.

DNA template quantities between 50 and 300ng/1µl had showed no significant differences. However, below 50 ng the amplification of HPRT locus decreased. Signal loss was observed as PCR product size increases because the number of full-length, intact molecules around a STR locus is reduced when the DNA sample has been damaged. Reducing the primer size and moving it more close to the repeat region of interest can improve amplification with degraded DNA samples.[15] When the amount of template DNA was in picogram quantity, efficient and specific amplification was obtained by further lowering the annealing temperature.

Taq Polymerase Enzyme

Different concentrations of Hot-start Taq Polymerase enzyme (Genei) were tested using primer mixture (Figure 6). The most efficient enzyme concentration seemed to be around 5 U (0.5 µl).

Use of adjuvants

Various authors recommended that addition of Dimethyl sulphoxide (DMSO) and glycerol improves amplification efficiency and specificity (no unspecific products) of PCR, when used in concentrations varying between 5%-10 % (v/v).[23] Henegariu and coworkers showed conflicting results with the addition of these adjuvants in the PCR reaction. They showed that addition of 5% DMSO improved the amplification of some products and decreased the amount of others, whereas some loci were not influenced at all. Similar results were obtained when 5% glycerol was used. In the present studies we added a commercial adjuvant called Magic solution (2.5X) (Genei) and it worked like a magic, especially for degraded and fragmented DNA samples. Figure 6 shows the results with addition of magic solution 1X (10 µl) and figure 7 shows the PCR products without the use of magic solution.

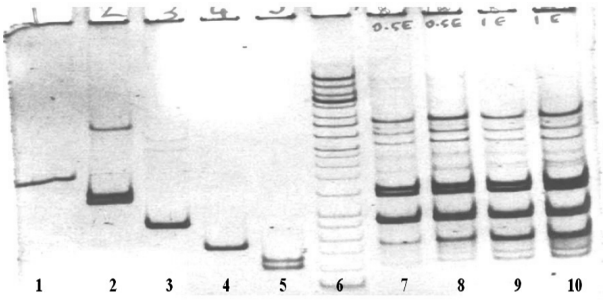

Fig. 6: PCR products with\ final standardized conditions with magic solution.
Lane 1: Human HPRTB locus
Lane 2: Human TPOX locus
Lane 3: Human TH01 locus
Lane 4: Human D5S818 locus
Lane 5: Human LPL locus
Lane 6: 20 bp ladder (Genei)
Lane 7: Miniplex reaction with all 5 loci amplified, 1.5U enzyme used, and 8µl loaded
Lane 8: Miniplex reaction with all 5 loci amplified, 1.5U enzyme used, and 16µl loaded
Lane 9: Miniplex reaction with all 5 loci amplified, 3U enzyme used, and 8µl loaded
Lane 10: Miniplex reaction with all 5 loci amplified, 3U enzyme used, 16 µl loaded

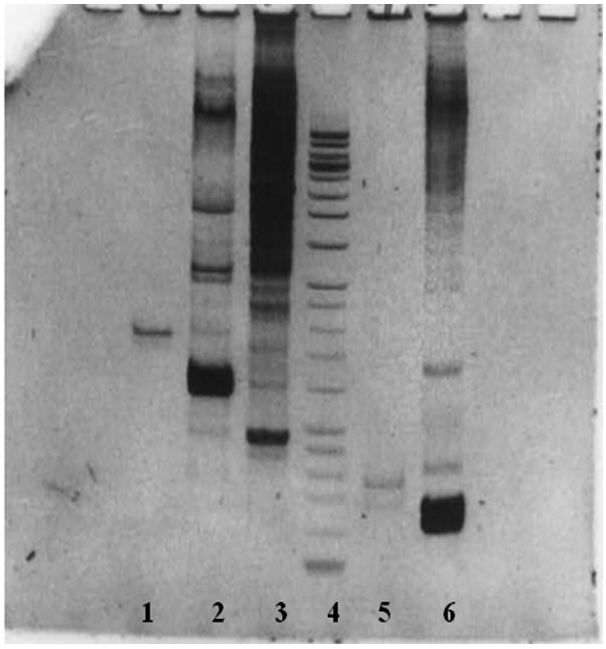

Fig. 7: Photograph showing the PCR products without use of magic solution
Lane1: Human HPRT locus
Lane 2: Human TPOX locus
Lane 3: Human TH01 locus
Lane 4: 20 bp ladder (Genei)
Lane 5: Human D5S818 locus
Lane 6: Human LPL locus

Separation of Amplicons

Miniplex PCR products, differing from each other by 30–40 bp in length could be conveniently separated on 3% agarose gels. Overnight separation of products at lower voltage gradients notably decreased the sharpness of individual PCR bands, especially when the products were smaller than 400–500 bp. To separate PCR products differing in only a few bp in length, 6%–10% PAA gels are used. Non-denaturing PAA gels work very well for non polymorphic loci. In the present studies, the loci tested were highly polymorphic and a higher resolution was required, hence 6% PAA/7 M urea denaturing gels were used.

However, this needs to be stained with silver nitrate or exposed to autoradiogram. Silver staining is time consuming method and takes about one day to get results. It is also difficult to count between lanes from allele to allele on overexposed auto radiograms or highly stained gels. It is difficult to read alleles which are closely placed (similar base pairs). The faint artifact bands that arise during the PCR cause false reading of alleles. There is no differentiation in the color of staining different loci; hence capillary electrophoresis technique was considered the best for STRs.

Multiplex PCR that utilizes fluorescently labeled PCR primers for detection and incorporation of an internal size standard was developed by Edwards et al, Ambach et al and Butler et al.[4,24,25]

LPL has 105-133 base pairs and D5S818 has 115-163 base pairs i.e. these two loci have overlapping size range. Loci with overlapping base pairs are difficult to read on gel, as the resolution was less. HPRT and TPOX loci were also overlapping, HPRT has 259-303 base pairs and TPOX has 216-264 base pairs. Also, amplification of HPRT and D5S818 loci was not uniform in a pentaplex for degraded DNA samples and also in some non-degraded DNA samples.

Hence further studies by capillary electrophoresis using ABI 3100 genetic Analyzer (Applied Biosystems) was carried out for single Monoplex as well as for a triplex of LPL, TH01 and TPOX loci. Figures 8, 9, 10 shows the Monoplex for each locus and figure 11 shows the miniplex of the three loci. LPL and TPOX loci were labeled with FAM, a blue colored fluorescent dye whereas, locus TH01 was labeled with HEX, a green colored fluorescent dye. ROX 500 was used as an internal size standard for all genotyping reactions.

Time Considerations

STR samples can be typed now in a matter of hours with most of the time being spent in PCR thermal cycling. STRs have been typed in less than one hour using rapid cycling methods.[24,25] With an ABI 3100, an individual can easily generate data from more than one hundred extracted DNA samples in a single day.

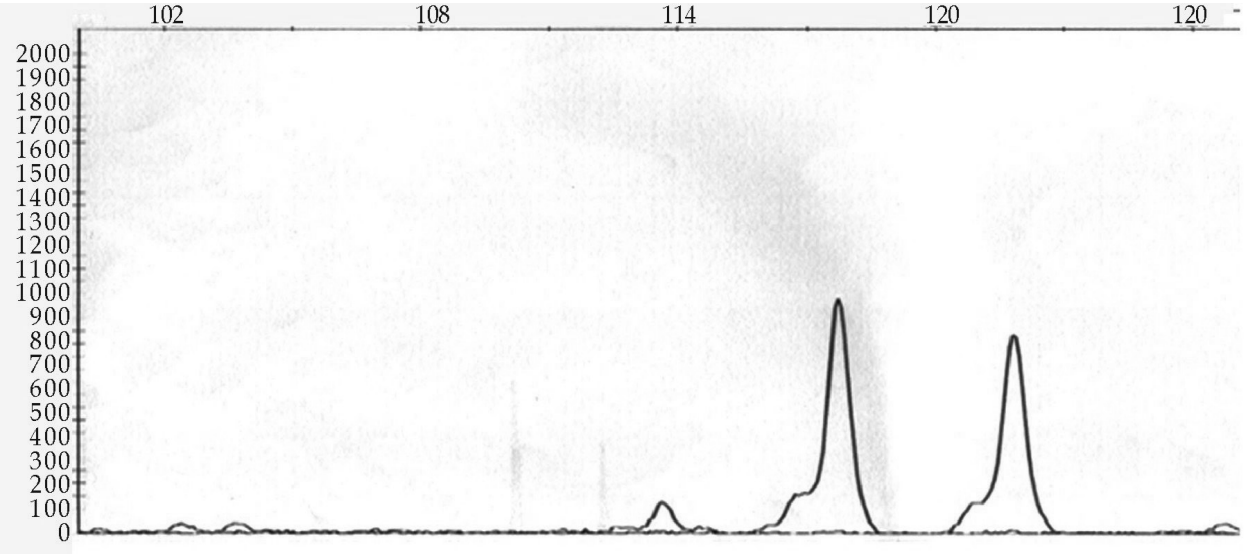

Fig. 8: LPL locus analysis by ABI Genetic Analyser.

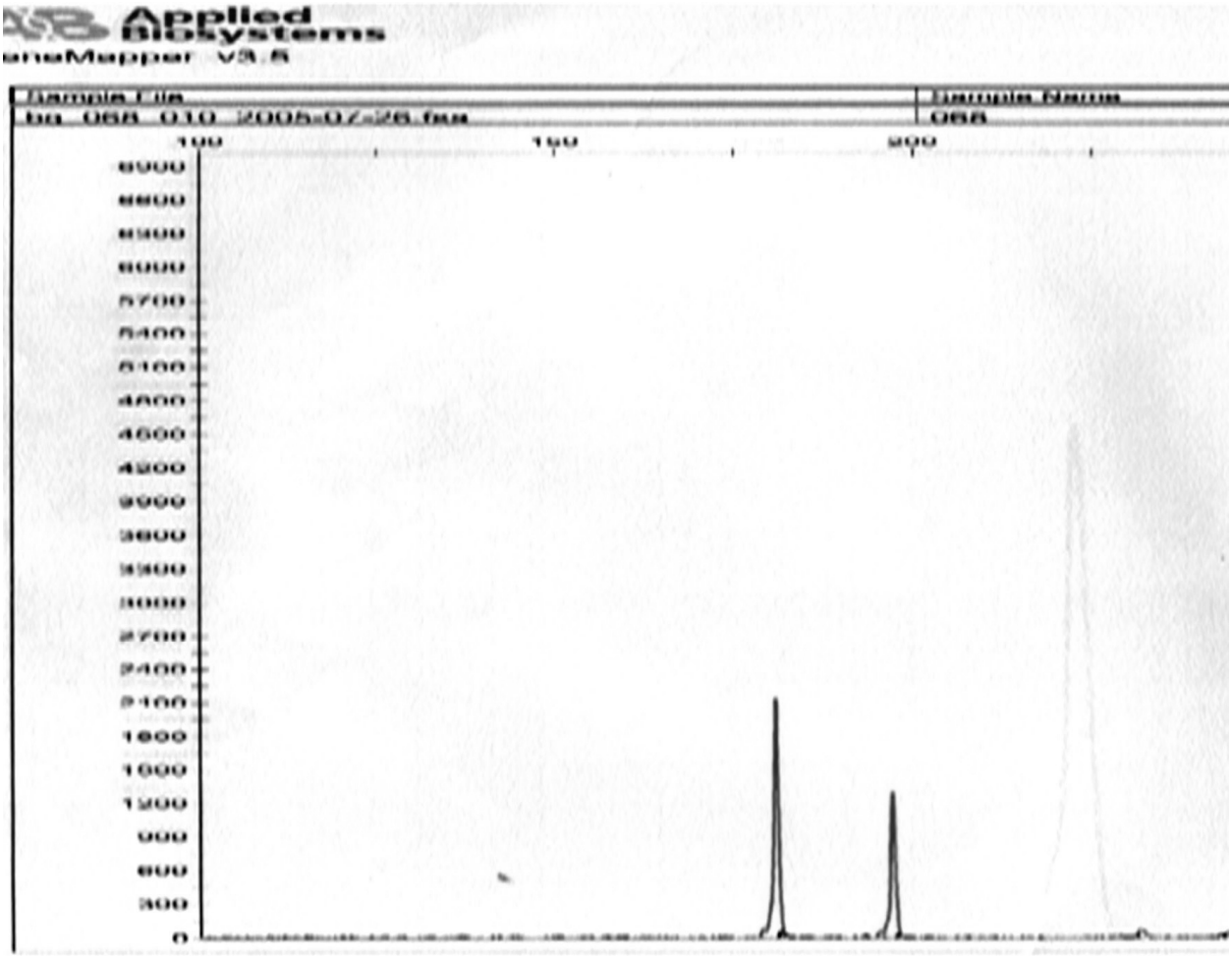

Fig. 9: TH01 locus analysis by ABI Genetic Analyser.

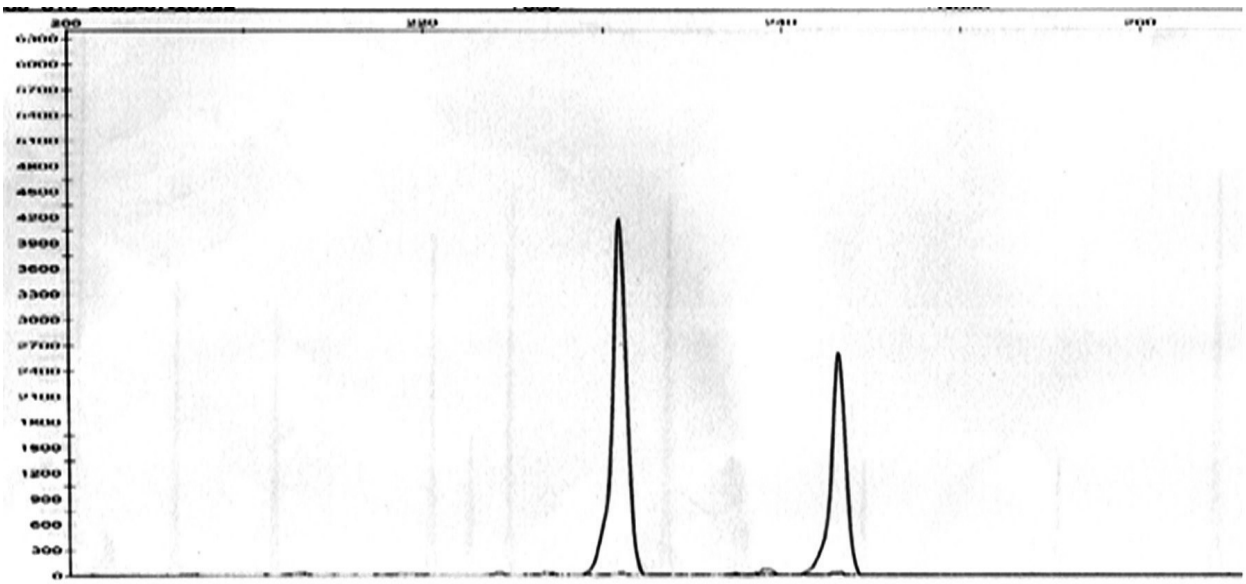

Fig. 10: TPOX locus analysis by ABI Genetic Analyser.

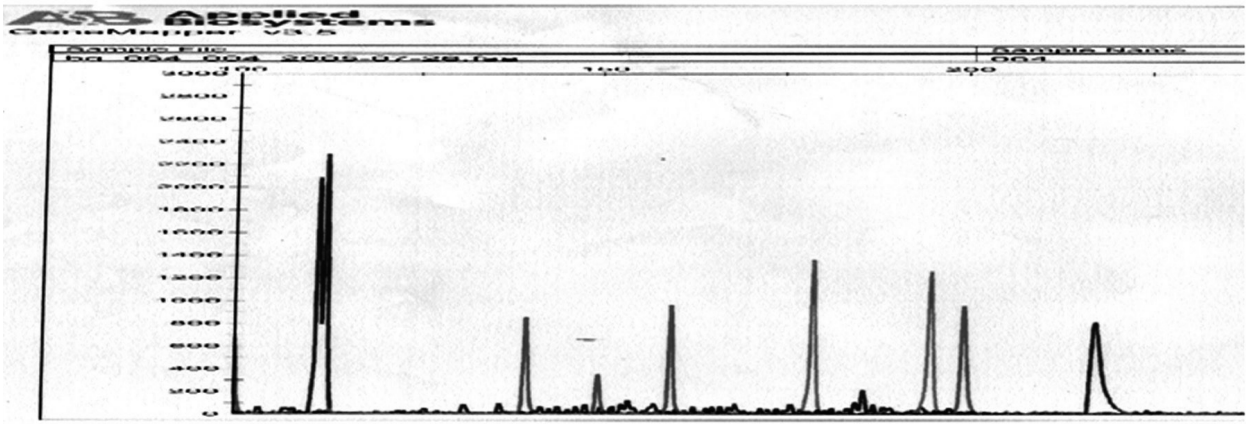

Fig. 11: Mini-str (LPL, TH01 and TPOX) loci analysis by ABI Genetic Analyser..

Conclusions

We have presented a series of examples of testing various strictures to optimize miniplex STR PCR. Optimal combination of two of these strictures, annealing temperature and concentration of template DNA, is essential in any PCR to obtain highly specific amplification products. In miniplex, adjusting primer amount for each locus is also essential. Nevertheless, optimization of the parameters as presented in this work should provide a basic way of approaching some of the common problems of miniplex PCR.

The availability of commercial STR kits enables reliable PCR amplification of STR markers. Reagents for standard reaction volumes of 50 µl or 25 µl can cost a user about $25-30 per sample. Therefore, to reduce the cost of DNA profiling some simple miniplexes needs to be developed. The present study would certainly be of great help in this direction.

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