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Optimization of a Multiplex DNA Amplification of Three Short Tandem Repeat Loci for Genetic Identification

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Abstract Short tandem repeat (STR) loci have been used in the field of forensic science. There are literally hundreds of STR systems which have been mapped throughout the human genome. These STR loci are found in almost every chromosome in the genome. They may be amplified using a variety of PCR primers. In this study, a DNA genotyping system based on the multiplex amplification of highly polymorphic STR loci was developed. Three STR loci with nonoverlapping allele size ranges have been utilized in the multiplex amplifications including the Neurotensin receptor gene, D21S11, and Human tyrosine hydroxylase gene. The optimal condition for triplex PCR was obtained in a solution with a total volume of 25 μl containing 2.0 U of Taq polymerase, 3 mM of $MgCl_2$, 300 μM of dNTP, 10 pmole of each primer set, an annealing temperature of 62°C, and 35 cycles. The optimized condition was successfully employed in a family paternity test.

Key words: Short tandem repeat (STR), multiplex amplification, triplex PCR

In October 1993, the DNA Commission of the International Society of Forensic Haemogenetics (ISFH) recommended the nomenclature for STR systems which is commonly used today. Alleles are generally named according to the number of repeats which they contain [4]. The profiling of polymorphic short tandem repeat (STR) markers has been applied to human indentification, parentage testing, and genetic mapping [3]. Reliable genotyping of these markers are facilitated by polymerase chain reaction (PCR) amplification and high-resolution electrophoretic separation. PCR-based STRs have several advantages over conventional Southern blotting techniques of the larger variable number tandem repeats (VNTRs). Discrete alleles from STR systems may be obtained because of their smaller size, which places them in the size range where DNA fragments differing by a single basepair in size may be distinguished [1]. In addition, smaller quantities of DNA, including degraded DNA, may be typed using STRs. Thus, the quantity and integrity of the DNA sample makes less of an issue with PCR-based typing methods than with conventional restriction fragment length polymorphism (RFLP) methods [8]. In human identification, analysis of one STR locus is not always suitable for forensic applications. In a case where several STR loci were used, the uniqueness of each individual would become even more distinct. Moreover, considerable amounts of time and effort can be saved by simultaneously amplifying mutiple STR loci in a single reaction, a process that is referred to as mutiplex PCR [6]. In this study, we developed a triplex PCR system allowing a simultaneous single-tube amplification of three unlinked STR loci with nonoverlapping allele size ranges: Neurotensin receptor gene (339–431 bp), D21S11 (214–246 bp), and Human tyrosine hydroxylase (142–162 bp). This paper describes the effect of variation of amplification parameters on the efficiency and reliability of the triplex STR system.

Plucked hairs were taken in duplicate from each individual and used for DNA extraction by using the chelating resin method [2], which is summarized in Fig. 1. Three STR loci, NeuR, D21S11, and Humth01, were amplified using the specific primer sets described in Table 1. The multiplex PCR was carried out in a 25 μl of reaction mixture containing 3 U of *Taq* DNA polymerase, 400 μM of dNTP, 10 pmole of each of the primers, 2.5 μl of 10× PCR

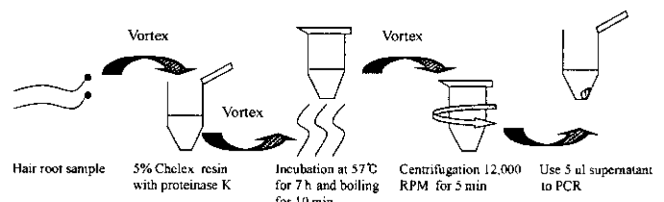

Fig. 1. DNA extraction using the chelating resin method.

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Table 1. Primer sequences used for Multiplex PCR.

Target	Primer sequence (5'→3')[a]	Repeat core size	Target size
Neurotensin receptor gene	forward CATCA gCTCA gAAgC AgATA gT reverse AgAgC AAgAA CTCCA TgTCT AAg	4 bp	340- 430 bp
D21S11	forward ATgTg AgTCA ATTCC CCAAg TgA reverse gTTgT ATTAg TCAAT gTTCT CCAg	4 bp	200- 240 bp
Human tyrosine hydroxylase gene	forward TgATT CCCAT TggCC TgTTC CT reverse AgCTC CCgAT TATCC AgCCT g	4 bp	140- 165 bp

[a]GenBank (http//www.ncbi.nlm.nih.gov/).

reaction buffer, and 5 μl of template DNA. Amplification was carried out using a Progene (Techne, England) PCR system as described previously [7]. The following cycling condition was applied for the amplification: 95°C for 5 min - initial denaturation; 94°C for 35 sec, 62°C for 1 min, and 72°C for 1 min, 35 cycles. Final extension was performed at 72°C for 5 min. PCR products were separated by 2% agarose gel electrophoresis. The DNAs on gels were stained with ethidium bromide and visualized under UV light. The gels were then photographed for a permanent record. PCR products amplified for paternity testing were separated by 8% high-resolution polyacrylamide gel electrophoresis (PAGE) prior to being silver-stained [1].

Results from the multiplex DNA amplification reaction performed under standard conditions are shown in Fig. 2. Enzyme concentration ranging from 2 U to 2.5 U appeared to be optimal, while higher concentrations (3 U to 5 U) resulted in a significant increase in NeuR product yield relative to other loci (Fig. 3). Interestingly enough, higher enzyme levels increased the occurrence of NeuR stutter bands. The effect of variation in Mg^{2+} concentration from 0.5 to 5 mM was examined. The magnesium concentration was shown to affect all of the following: primer annealing, strand dissociation temperatures of both template and PCR product, product specificity, formation of primer-dimer artifacts, and enzyme activity and fidelity [5]. The amplification efficiency for all 3 loci was severely reduced when the magnesium concentration was decreased to 0.5 mM. At 1.5 mM to 2.5 mM concentration, no amplification band was obtained for the NeuR and D21S11 loci, whereas Humth01 was not amplified at 5 mM (Fig. 3).

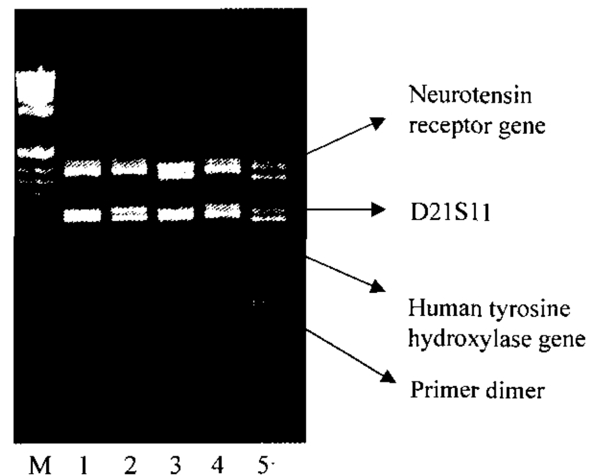

Fig. 2. Two-percentage agarose gel displaying multiplex PCR performed under standard conditions.
M, size marker; Lanes 1-5, samples for 5 unrelated Korean subjects.

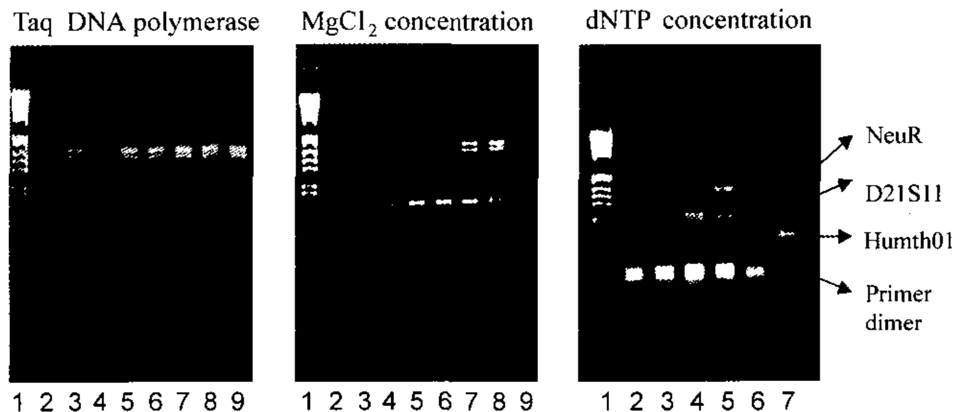

Fig. 3. Effects of enzyme, $MgCl_2$ and dNTP concentration on multiplex PCR.
Taq DNA polymerase: lane 1, size marker; lane 2, 0.5 U; lane 3, 1 U; lane 4, 1.5 U; lane 5, 2 U; lane 6, 2.5 U; lane 7, 3 U; lane 8, 4 U; lane 9, 5 U. $MgCl_2$ concentration: lane 1, size marker; lane 2, 0.5 μM; lane 3, 1 μM; lane 4, 1.5 μM; lane 5, 2 μM; lane 6, 2.5 μM; lane 7, 3 μM; lane 8, 4 μM; lane 9, 5 μM. dNTP concentration: lane 1, size marker; lane 2, 50 μM; lane 3, 100 μM; lane 4, 200 μM; lane 5, 400 μM; lane 6, 600 μM; lane 7, 800 μM.

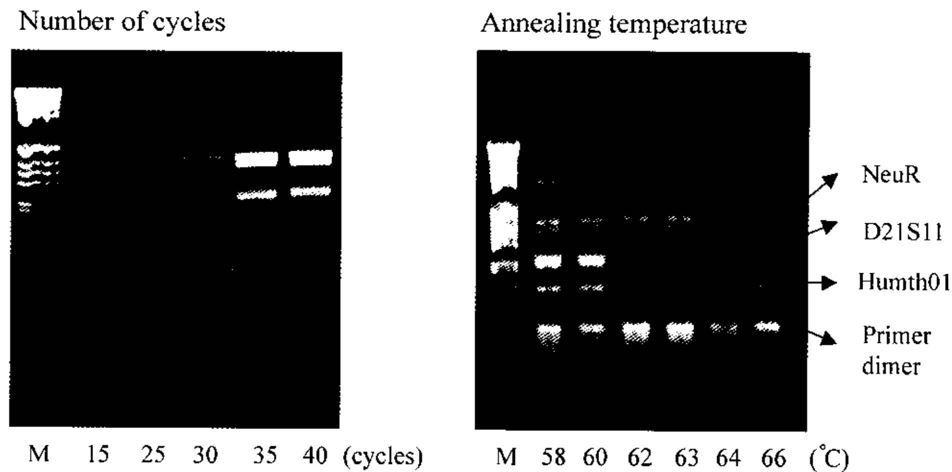

Fig. 4. Effects of annealing temperature and number of amplification cycles on multiplex PCR. M, size markers.

In a previous study, the band pattern obtained at 100 μM of dNTP exhibited the most distinctive and clean profile [8]. However, in our study, the concentration of dNTP was optimized for the multiplex PCR at 200 μM to 400 μM. At 50 μM concentration, only a weak Humth01 band was detected, whereas a strong Humth01 band was detected at 800 μM. At 600 μM concentration, the D21S11 band was not detected (Fig. 3). Annealing temperatures appeared to be one of the most critical parameters for the multiplex PCR. It has been reported that lower annealing temperatures resulted in decreased yield of the desired product and an increase in nonspecific amplification [11]. We also obtained similar results. All 3 loci were amplified efficiently at annealing temperatures of 62°C to 63°C. At temperatures lower than 62°C, an artifact band appeared near the NeuR locus. At 64°C, practically no amplification band was obtained from the Humth01 locus, whereas there was no amplification of the D21S11 locus at 65°C (Fig. 4). The effect of variation in thermal cycle numbers between 15 to 40 was examined. As expected, the total yield of PCR product was directly proportional to the number of PCR cycles: With 30 cycles all 3 loci were amplified. However, with more than 35 cycles, further amplification was obtained only on the NeuR locus (Fig. 4).

In summary, several different reaction conditions were optimized for triplex PCR reactions. The optimal condition for triplex PCR was obtained in a solution of 25 μl containing 2.0 U of Taq polymerase, 3 mM of $MgCl_2$, 300 μM of dNTP, and 10 pmole of each primer set, at a 62°C annealing temperature for 35 cycles. A DNA profiling system based on the multiplex amplification of highly polymorphic STR loci was developed. During the identification process of individuals, considerable amounts of time and effort can be saved by using this system. In a previous study, large DNA fragments were more difficult to amplify compared to the smaller ones [10]. To improve the visualization of these bands, we increased both the amount of primer and the extension time for the weakly amplified loci. These variations have been shown to increase the amplification of large fragments [10]. However, in our study, smaller DNA fragments were more weakly amplified than the large ones. Although we increased the amount of primer for the weakly amplified loci and also decreased the extension time to improve the visualization of these bands, the smaller DNA

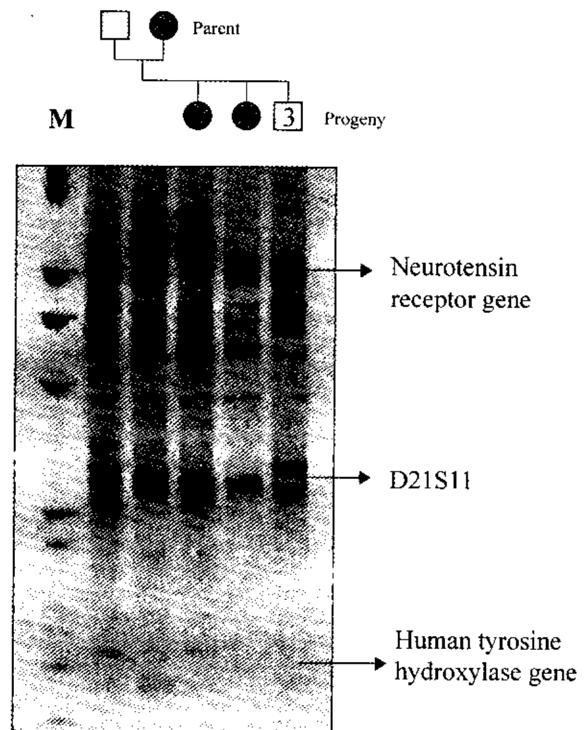

Fig. 5. A paternity testing for one family.
Silver-stained 8% PAGE displaying multiplex PCR products of three STR loci. M, size marker.

fragments were still weaker than the larger ones. Further studies are underway to improve the amplification of the suitable bands in our multiplex PCR system.

To examine the feasibility of the optimized multiplex PCR condition, we performed a paternity test on a family using the multiplex PCR. As shown in Fig. 5, three loci were successfully amplified in a single PCR. In the NeuR locus, one daughter (lane 1) inherited one of the father's allele (large size band) and one of the mother's allele (small size band) while one son (lane 3) inherited both father and mother's small size allele. In the D21S11 locus, two daughters (lanes 1 and 2) inherited one of the father's allele (small size band) and one of the mother's allele (small size band). One son (lane 3) inherited his father's allele (large size band) and his mother's allele (small size band). In the Humth01 locus, all progeny inherited the father's allele (large size band) and the mother's one allele.

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REFERENCES

1. Budowle, B., R. Chakraborty, A. M. Ginsti, A. J. Eisenberg, and R. C. Allen. 1991. Analysis of the VNTR locus by PCR followed by high resolution PAGE. *Am. J. Hum. Genet.* **48:** 137-144.
2. Erlich, H. A. 1989. Simple and rapid preparation of samples for PCR. pp. 31-38. *In PCR Technology, Principles and Applications for DNA Amplification.* Stockton Press, New York, U.S.A.
3. Gao Q., H. M. Pang, and E. S. Yeung. 1999. Simultaneous genetic typing from multiple short tandem repeat loci using a 96-capillary array electrophoresis system. *Electrophoresis* **20:** 1518-1526.
4. Henegariu, O., N. A. Heerema, S. R. Dlouhy, G. H. Vance, and P. H. Vogt. 1997. DNA recommendations: 1994 report concerning further recommendations of the DNA commission of the ISFH regarding PCR-based polymorphisms in STR system. *Int. J. Leg. Med.* **107:** 159-160.
5. Innis, M. A., D. H. Gelfand, J. J. Sninsky, and T. J. White. 1989. Optimization of PCRs. pp. 3-12. *In PCR Protocols. A Guide to Methods and Applications.* Academic Press, CA, U.S.A.
6. Miscicka-Sliwka D., T. Grzybowski, and M. Wozniak. 1997. Optimization of a hexaplex DNA amplification from short tandem repeat and amelogenin loci. *Electrophoresis* **18:** 1627-1632.
7. Park, Y. M. and J.-S. So. 1998. Quantitative counting of *Bifidobacterium* spp. in a sample mixed with *Lactobacillus acidophilus. J. Microbiol. Biotechnol.* **8:** 182-184.
8. Song, J. E., T. R. Heo, and J.-S. So. 1997. Optimization of PCR for RAPD analysis of *Bifidobacterium* spp. *Foods Biotechnol.* **6:** 30-33.
9. Sprecher, C. J., C. Puers, A. M. Lins, and J. W. Schumm. 1996. General approach to analysis of polymorphic short tandem repeat Loci. *Biotechniques* **20:** 226-276.
10. Thomas, E. and R. J. Herrera. 1998. Multiplex polymerase chain reaction of Alu polymorphic insertions. *Electrophoresis* **19:** 2373-2379.
11. Wittwer, C. T. and D. J. Garling. 1991. Rapid cycle DNA amplification: Time and temperature optimization. *Biotechniques* **10:** 76-80.