Methods for Typing the STR Triplex CSF1PO, TPOX, and HUMTHO1 That Enable Compatibility Among DNA Typing Laboratories

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INTRODUCTION

Typing polymorphic loci at the DNA level has become a routine procedure in the paternity and identity testing fields. Originally, highly polymorphic variable number of tandem repeats (VNTR) loci were characterized by restriction fragment length polymorphism (RFLP) analysis (Wyman and White 1980; Jeffreys et al. 1985a; Jeffreys et al. 1985b). A subgroup of these VNTR loci is the short tandem repeats (STR) loci. These loci are highly polymorphic and are abundant in the human genome (Edwards et al. 1991; Edwards et al. 1992). The STR loci are composed of tandemly repeated sequences of 2-7 base pairs in length. Because the allele size of STRs is generally less than 350 base pairs, they are amenable to amplification by the polymerase chain reaction (PCR) (Saiki et al. 1985; Edwards et al. 1991). Therefore, STRs can be typed with a high degree of specificity and sensitivity, in a relatively short time period, and without the need for isotopic detection methods. Moreover, the amplified products of STRs can be resolved at least to a single repeat unit by separation on denatured polyacrylamide gels (Edwards et al. 1991; Hochmeister et al 1995; Huang et al. 1995). Thus, more discretized allelic data can be obtained for the loci than was possible with VNTRs typed by RFLP analysis.

The typing of STR loci for human identity testing has been facilitated by the ability to amplify two or more STR loci simultaneously in one PCR by a procedure known as multiplex PCR (Edwards et al. 1991; Edwards et al. 1992; Gill et al. 1992; Sullivan et al. 1992). The advantages of a multiplex system are that less sample DNA is consumed than when analyzing each locus independently, less reagents are required, and the time needed to perform population studies on several loci is greatly reduced. The amplified STR products of a multiplex PCR can be separated by polyacrylamide gel electrophoresis, and the amplicons are detected by silver staining or by laser excitation of a fluor attached to the 5' end of one primer of each of the primer sets. The fluor-labeled PCR products can be detected in real time (using the ABI 373A or ABI 310, Perkin-Elmer) or using a decoupled detection system after gel electrophoresis (FluorImager SI or Hitachi FM BIO). Both detection schemes - manual silver staining and automated fluor detection - can be used to obtain reliable data for STR typing. The silver staining approach is simple and does not require expensive equipment for detecting the amplified STR products. However, in order to obtain unequivocal typing of the various loci in the multiplex their

sizes cannot overlap. In contrast, because of the advent of different colored fluors, the loci in the fluor-labeled multiplex need not be of different size.

The loci CSF1PO, TPOX, and HUMTHO1 are STRs containing tetranucleotide repeat sequences. The tetranucleotide STR loci were selected for analysis because the alleles, based on the repeat sequence, generally can be resolved by polyacrylamide gel electrophoresis (Hochmeister et al 1995; Huang et al. 1995), and these loci generally exhibit less stutter (or shadow) bands than STR loci containing smaller repeat size sequences. Also, the size of the largest allele in the triplex (allele 15 in the CSF1PO locus allelic ladder) is less than 300 bp in size (GenePrint Technical Manual 1994). Thus, forensic biospecimens that contain substantially degraded DNA may be more readily typed than when analyzing VNTR loci by RFLP typing or by typing loci such as D1S80. Some characteristics of the CSF1PO, TPOX, and HUMTHO1 loci are displayed in Table 1 (GenePrint Technical Manual 1994).

Table 1. Information on CSF1PO, TPOX, and HUMTHO1 loci.

Locus	Chromosome Location	Repeat Sequence	Non-Four Base Repeat alleles	K562 Types
CSF1PO	5q33.3-34	AGAT	NA	10,9
TPOX	2p13	AATG	NA	9,8
HUMTHO1	11p15.5	AATG	9.3	9.3,9.3

This paper describes procedures for typing a commercially-available STR triplex - CSF1PO, TPOX, and HUMTHO1 - that can be performed using either silver staining or automated laser fluorescence detection. Thus, the multiplex STR typing can be implemented into most human identity testing laboratories, enabling greater compatibility among laboratories for sharing casework results and population data.

MATERIALS AND METHODS

Sample Preparation

Whole blood was obtained in EDTA vacutainer tubes by venipuncture or by fingerprick and placed on cotton cloth and air-dried. The DNA was extracted by the phenol-chloroform method and washed using microcon 100 filters (Amicon) according to the method of Comey, et al. (1994). The quantity of extracted DNA was estimated using the slot-blot procedure described by Waye, et al. (1989) using chemiluminescent detection (Budowle et al. 1995).

Multiplex PCR

The coamplification of HUMTHO1, TPOX, and CSF1PO was performed using the GenePrint kit (Promega Corporation, Madison, WI) according to the following conditions. The PCR was carried out in 25 or 50μl reaction volumes containing 0.1-5 ng template DNA and 1.5 units of Taq DNA polymerase per 50μl

reaction. The primers for the STRs were described previously (Huang et al. 1995). The reactions were placed into a Perkin Elmer 9600 thermal cycler and were subjected to denaturation at 95°C for 30 seconds, primer annealing at 67°C for 30 seconds, and primer extension at 70°C for 30 seconds, for a total of 28 or 30 cycles, depending on the initial quantity of template DNA.

Typing

Three different methods were used to type the STR triplex. Method I: Three μl of loading dye (10 mM NaOH, 95% formamide, 0.05% bromophenol blue, and 0.05% xylene cyanol FF) were mixed with 3 μl of PCR product. The samples were denatured for 2 minutes in a Perkin Elmer Model 480 DNA thermal cycler and 5 μl were loaded onto the cathodal end of the gel. A discontinuous denaturing polyacrylamide gel was used to separate the STR amplicons. These polyacrylamide gels (6%T, 2.%C; cross-linker was piperazine diacrylamide; 31 cm long and 0.4 mm thick) contained 7M urea and 60 mM Tris-formate, pH 9.0 (with respect to the formate ion). The gel was permitted to polymerize for a minimum of one hour at ambient temperature. The gel was placed in a SA 32 apparatus (GIBCO-BRL, Gaithersburg, MD) and the electrode buffer was 90 mM Tris-borate, pH 8.3 (90 mM with respect to the borate ion). Electrophoresis was performed initially at 80 W for approximately five minutes and then continued with settings of 25 W at ambient temperature. The run was allowed to continue until the xylene cyanol tracking dye migrated to the top of the lower reservoir buffer (approximately three hours). The gels were stained with silver according to the method of Budowle et al. (1991).

Method II: Two μl of loading dye (10 mM NaOH, 95% formamide, 0.05% bromophenol blue, and 0.05% xylene cyanol FF) were mixed with 4 μl of PCR product. The samples were denatured for 2 minutes in a Perkin Elmer Model 480 DNA thermal cycler and 5.5 μl were loaded onto the cathodal end of the gel. The denaturing polyacrylamide gels (4%T, 5%C; cross-linker was bisacrylamide; 31 cm long and 0.4 mm thick) contained 7 M urea and 0.5X (or 90 mM) Tris-Borate-EDTA buffer, pH 8.3 (TBE). The electrode reservoir also was 0.5X TBE buffer. Electrophoresis was carried out on an SA 32 Electrophoresis Apparatus (BRL, Gaithersburg, MD). The conditions for electrophoresis were set at a constant power of 40 watts at ambient temperature. Electrophoresis was stopped when the xylene cyanol dye migrated 6 cm from the anode (approximately 75 minutes). After electrophoresis, the fluor labeled amplicons were detected using the FluorImager SI with the PMT set at 1000 (Molecular Dynamics).

Method III: With this method only 1 ng of DNA template was used in the PCR, and the PCR was for 25 cycles. Four μl of loading dye (10 mM NaOH, 95% formamide, 0.05% bromophenol blue, and 0.05% xylene cyanol FF) and 0.5 μl of the GS 500 internal standard were mixed with 1.5 μl of PCR product. The samples were denatured for 2 minutes in a Perkin Elmer Model 480 DNA thermal cycler and 5.5 μl were loaded onto the cathodal end of the gel. The denaturing polyacrylamide gels

(6%T, 5%C; cross-linker was bisacrylamide; 28.5 cm long and 0.4 mm thick; 12 cm well to read distance) contained 7.5 M urea and 1X Tris-Borate-EDTA buffer, pH 8.3 (TBE). The electrode reservoir was 0.5X TBE buffer. Electrophoresis was carried out on an ABI 373A. The conditions for electrophoresis were set at 50W, 1200V, and 50mA at ambient temperature. During electrophoresis, the fluor labeled amplicons were detected in real time with the PMT set at 585.

Allele designations were determined by comparison of the sample fragments with those of the allelic ladders supplied with the GenePrint kit. Allele designations were made according to recommendations of the DNA Commission of the International Society of Forensic Haemogenetics (1994).

RESULTS AND DISCUSSION

This study demonstrates that the STR triplex CSF1PO, TPOX, and HUMTHO1 can be typed using several different electrophoretic separation and detection strategies. Figs. 1-3 show that typing results for the multiplex were obtained using discontinuous formate-borate denaturing gels and silver staining, discontinuous formate-borate denaturing gels and automated fluor detection (using the FluorImager SI), and continuous TBE denaturing gels and automated fluor detection (using the ABI 373A), respectively.

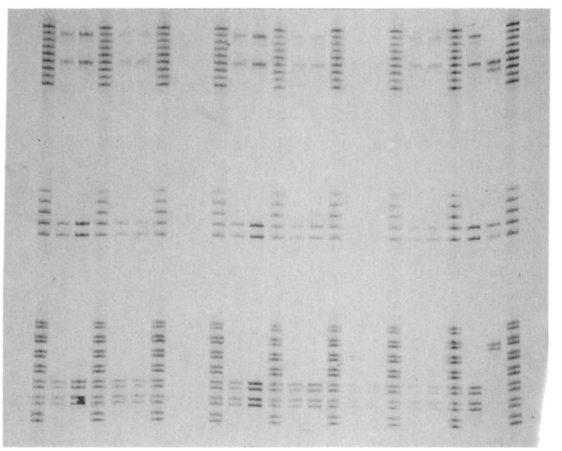

Figure 1. A silver stained discontinuous gel displaying STR triplex types from semen stains that have resided in sunlight or shade up to 10 weeks.

PCR Conditions

The PCR conditions used in this study differ from those recommended by the manufacturer. The temperatures and times for each step of the PCR cycle are designed for the Perkin-Elmer 9600 thermal cycler, while those advocated by the manufacturer are designed for the Perkin-Elmer 480 thermal cycler. Sensitivity of detection of DNA samples was increased using the PCR conditions described in this study. Samples containing template DNA from 4 ng to as little as 125 pg can

be typed readily.

In addition, for the triplex kit, where amplicons are detected by silver staining, the annealing temperature was raised from 64°C to 67°C. The higher annealing temperature did not compromise typing, and it could be anticipated, with a higher stringency, that artifact bands would be reduced. Since the introduction of the silver staining triplex kit, a fluorescently-tagged quadplex kit has been developed. The quadplex contains the same three loci as the triplex, and the locus VWF has been added. In order to obtain amplification of all four STR loci, the annealing temperature for the PCR was reduced to 60°C. This was done to accommodate the VWF locus. Currently, we have decided to maintain the annealing temperature at 67°C, which will not enable typing at the VWF locus. If another locus is to be added to the triplex, the PCR annealing temperature for that locus could be similar to that of the other triplex loci.

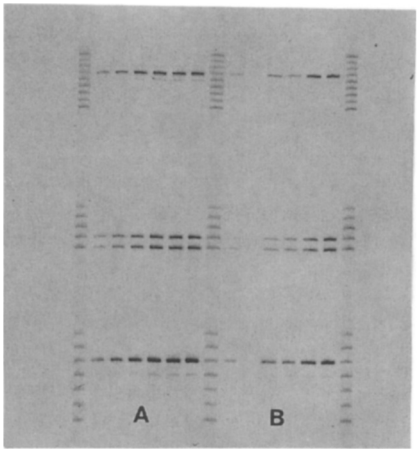

Figure 2. A FluorImager SI generated discontinuous gel image displaying the STR triplex. The samples in Region A were amplified for 30 cycles and contained template DNA (from left-to-right) of 125 pg, 250 pg, 500 pg, 1 ng, 2 ng and 5 ng and in Region B were amplified for 28 cycles and contained template DNA (from left-to-right) of 125 pg, 250 pg (failed amplification), 500 pg, 1 ng, 2 ng and 5 ng.

Size of STR Loci

The feature of this STR triplex that enables compatibility among laboratories is that the loci CSF1PO (295-327 bp), TPOX (232-248 bp), and HMUTHO1 (179-203 bp) do not overlap in size. Thus, this triplex can be used in DNA typing laboratories that are equipped with high-technology apparatuses, as well as in laboratories with limited resources.

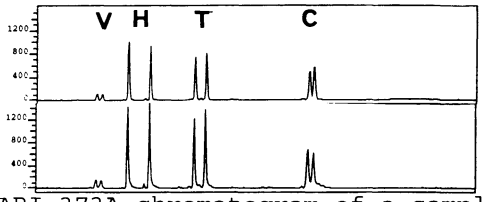

Figure 3. An ABI 373A chromatogram of a sample run on a continuous gel displaying an STR triplex profile. The top chromatogram is a sample amplified for 25 cycles and the bottom was amplified at 26 cycles. The template DNA was at 1ng. V = VWA (note that their is low level VWA detected even though the annealing temperature was at 67°C); H = HUMTHO1; T = TPOX; C = CSF1P0.

Separation of Alleles

All samples were separated electrophoretically in a denatured environment. The separation distances between fragments differing by one repeat unit were augmented under denaturing conditions compared with native gel conditions (data not shown). With the formate-borate system, the distance between CSF1PO alleles 7 and 15 was approximately 1.5 cm, between TPOX alleles 8 and 12 was approximately 1.2 cm, and between HUMTHO1 alleles 5 and 11 was approximately 2.5 cm. All alleles in the triplex were resolved to one repeat unit (i.e., four base pairs). Moreover, the HUMTHO1 9.3 allele, which is a relatively common allele in Caucasians (Edwards et al. 1992; Hochmeister et al. 1995; Lorente et al. 1994, Puers et al. 1993), is one base pair smaller in size than the 10 allele, and could be resolved. The ability to type unequivocally the 9.3 allele demonstrates the resolving capacity of the electrophoretic system used in our study for typing HUMTHO1.

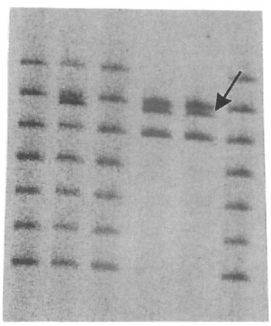

Figure 4. A FluorImager SI generated image of a discontinuous gel displaying resolved 9.3 and 10 alleles (indicated by arrow).

Differences Between Silver Staining and Fluor Detection

There were some notable differences between the silver stain detection approach and the fluor detection method using, for example, the FluorImager SI. With fluor detection only one of the two strands from the denatured duplex can be observed; in contrast, silver staining will enable detection of both strands. With the denatured gel systems used in this study

only the denatured strands at the HUMTHO1 locus are resolved. Detection of STR amplicons as single bands or as separated double bands does not compromise typing. The use of the FluorImager SI was less laborious than silver staining. There was no need to separate the glass plates after electrophoresis for fluor detection. Thus, there were no additional steps for staining. In addition, there was no requirement for photography to record the image; the image can be printed on an inexpensive paper medium. The advantage of silver staining is that there is no requirement for expensive equipment. Finally, the most sensitive detection system was fluor detection using the ABI 373A; it generally provided at least an order of magnitude more sensitivity than the FluorImager SI or silver staining.

CONCLUSION

Using multiplex PCR, separation of the PCR products on denaturing polyacrylamide gels, and silver staining or fluor detection, rapid typing with high resolution of the loci CSF1PO, TPOX, and HUMTHO1 can be obtained. The multiplex analytical procedures described above are relatively simple and can be implemented into most application-oriented laboratories.

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