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Validation of Multiplex Polymorphic STR Amplification Sets Developed for Personal Identification Applications*

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ABSTRACT: Polymorphic short tandem repeat (STR) loci, which typically consist of variations in the number of 3–7 base pair repeats present at a site, provide an effective means of personal identification. Typing can be accomplished by amplification of genomic DNA using the polymerase chain reaction (PCR) and locus-specific primers, separation of amplified alleles using gel electrophoresis and their display using silver staining or fluorescent detection. Primers for several STR loci can be combined in a single multiplex reaction so typing of multiple loci can be accomplished rapidly and with less DNA than required if each locus were analyzed separately. Before such multiplex systems are used in forensic or paternity applications, it is desirable that they undergo testing for their reliability.

This study evaluates the performance of two STR triplex systems, one containing the loci HUMCSF1PO, HUMTPOX, and HUMTH01, and the other containing HUMHPRTB, HUMFESFPS, and HUMVWFA31. Protocols for amplification of these two triplexes, and their corresponding monoplexes, were evaluated for sensitivity of detection, resistance to changes in the annealing temperature of the amplification protocol, and the ability to identify the minority contributor in amplification of mixed samples. In addition, five laboratories determined the alleles of twenty DNA samples, each extracted by one of four different extraction methods. The results illustrate that the two STR triplex systems and the monoplex systems contained within them can be used with as little as 0.25 ng of DNA template. Both triplexes amplified with 100% success using the Perkin Elmer Model 480 thermal cycler. With the GeneAmp 9600 System, the CTT triplex amplified with 100% success and the HFv triplex in 95.6% of attempts. These experiments meet many requirements for use in validation of DNA typing systems for forensic cases and paternity identification.

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KEYWORDS: forensic science, short tandem repeat (STR), polymerase chain reaction (PCR), validation, multiplex, amplification, microsatellite, paternity, polymorphism

Genetic loci that contain dinucleotide repeat or short tandem repeat (STR) 3–7 base pair motifs are abundant and well-distributed in the human genome providing a rich source of polymorphic markers (1–3). Loci comprising tetranucleotide repeats are particularly attractive for forensic and paternity analyses because amplified fragments containing these repeats are easily resolved by electrophoresis (4–8). Loci can also be selected from this class of repeat elements, which display very few artifacts from PCR amplification (7). In particular, the extra fragments generated by repeat slippage (9,10), which are so prevalent with dinucleotide repeat locus amplification (11,12), are greatly diminished or even absent with many tetranucleotide repeat loci (7).

Combinations of STR loci that can be amplified as multiplexes and detected following denaturing polyacrylamide gel electrophoresis using either radioactivity (13) or fluorescence (4,5,14,15) have been described. Recently, nine STR loci have been developed along with allelic ladders (that is, size standards consisting of most or all known amplified alleles for the locus) (7,16) for separation in the same matrix, and detection using a silver strain method (17). Primers for six of these loci have been formulated into two amplification systems each capable of simultaneous amplification of three individual loci (14,18,19).

A study among five laboratories to evaluate performance characteristics of these six individual amplification systems and the two corresponding triplex systems is described. At least one representative from each of the five laboratories involved with the study came to a single location (Promega Corporation, Madison, WI) to standardize methods and coordinate planning of the work. The combined effort has fulfilled many of the requirements for validation of these systems for use in forensic applications as defined by the "Guidelines for a Quality Assurance Program for DNA Analysis" of The Technical Working Group on DNA Analysis Methods (TWGDAM) (20). These validation studies will also assist paternity laboratories in meeting standards to be set forth by the American Association of Blood Banks (AABB) (21).

Materials and Methods

Genomic DNA Isolation and Quantitation

Genomic DNA was isolated by four different methods chosen by the individual participating laboratories. DNA samples 1 through 5

were isolated from bloodstains from random individuals using the organic extraction method of Comey et al. (22), samples 6 through 10 were isolated from liquid blood from random individuals using the inorganic method of Grimberg et al. (23), samples 11 through 15 were isolated from tissue culture strains RAJI, IM-9, HL-60, CCRF-SB, and CCRE-CEM, respectively, using the salt precipitation method of Miller et al. (24), and samples 16 through 20 were isolated from liquid blood of random individuals by the method of Walsh et al. (25) using the anion-exchange resin, Chelex® 100 (BIO-RAD, Richmond, CA). Human tissue culture strain K562 DNA was obtained from Promega Corporation (Madison, WI). Quantitation of DNA samples was performed using the dot blot hybridization method of Waye et al. (26) using a D17Z1 human-specific alkaline phosphatase-conjugated probe.

Polymerase Chain Reaction Amplification of STR Loci

Amplification protocols of each locus and the triplexes are listed in Table 1. The primers and amplification conditions used are those provided in the GenePrint™ STR Systems (Promega Corporation, Madison, WI). Monoplex PCR amplifications were performed in either 25 μL or 50 μL volumes using 25 ng template unless otherwise stated, 0.01 U *Taq* DNA Polymerase/μL, 1X STR Polymerase Buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25°C C), 0.1% Triton X-100 and 1.5 mM $MgCl_2$) and 200 μM each of dATP, dCTP, dGTP and dTTP using a DNA Thermal Cycler 480 (Model 480) or GeneAmp PCR System 9600 (Model 9600)(Perkin-Elmer Corp., Norwalk, CT) as noted in the text and tables. Amplification protocols for the triplexes were the same as for the individual loci which comprise them, except that 0.03 U *Taq* DNA Polymerase/μL was used in the reaction. With samples 1 through 5, 1.6 μg/μL BSA was included in the amplification reactions. Prior to use, the thermal cyclers were calibrated for proper temperature control using the Perkin Elmer Temperature Verification System.

Detection of Amplified Products

Amplification products were separated by electrophoresis through a 0.4 mm thick 4% T, 5% C denaturing polyacrylamide gel containing 0.5 × TBE and 7 M urea and was chemically cross-linked to one glass plate (29). Two and a half microliters of each DNA sample was mixed with 2.5 μL of loading solution (10 mM NaOH, 95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol), denatured at 95°C for 2 min, and chilled on ice prior to loading 3 μL of the mixture. Electrophoresis was performed at 60 W with the S2 gel apparatus (Gibco BRL, Bethesda, MD) or 40 W with the SA32 gel apparatus (Gibco BRL, Bethesda, MD) in 0.5× TBE for 1–2 h. The gels were subjected to electrophoresis for 30 to 75 minutes prior to loading of the samples. Following electrophoresis, the DNA was detected by the silver staining method of Bassam et al. (17). Permanent images were obtained by exposure to Electrophoresis Duplicating Films (EDF) (Promega Corporation, Madison, WI).

Results and Discussion

Validation Study Organization

The goal of this study was to evaluate the reliability and robustness of six monoplex STR systems and two triplex systems. The monoplex systems include the loci CSF1PO, TPOX, TH01, HPRTB, FESFPS, and vWF. The first of the triplexes, designated CTT, includes the first three of these loci, while the second triplex, HFv, includes the last three monoplex systems. Except when specifically noted, all evaluations in the analysis were performed with each monoplex system and each triplex. The evaluation of amplification sensitivity, variation of annealing temperatures, and of mixtures containing 25 ng total DNA were carried out using the Model 480 at one site (Promega Corporation, Madison, WI) by representatives from each of the five different laboratories participating in this study. Subsequently, evaluation of unknown samples prepared using four different extraction methods were performed in each individual laboratory, with each laboratory using either the Model 480, the Model 9600, or both thermal cyclers.

Amplification Sensitivity

Both triplex systems, as well as the monoplex systems that comprise them, were evaluated for sensitivity using the recommended amplification protocol (Table 1) and tissue culture strain

TABLE 1—*Locus-specific and multiplex information.*

GenBank Designation*	Chromosome location	Amplification Protocol†	Alleles of standard cell lines		Reference
			K562	CCRF-SB	
HUMCSF1PO (CSF1PO)	5q33.5-34	1	10,9	12,10	13
HUMFESFPS (FESFPS)	15q25-qter	2	12,10	12,12	13
HUMHPRTB (HPRTB)	Xq26	2	13,13	12,—‡	27
HUMTH01 (TH01)	11p15.5	1	9.3,9.3	10,9	27
HUMTPOX (TPOX)	2p23-pter	1	9,8	8,8	unpublished observations
HUMVWFA31 (vWF)	12p12-pter	2	16,16	18,18	28
(CSF1PO, TPOX, TH01 multiplex)		1			14,19
(HPRTB, FESFPS, vWF multiplex)		2			14

*Locus names used in this publication are in parentheses.

†Amplification program 1: 96°C for 2 min., then 10 cycles of 94°C for 1 min., 64°C for 1 min., and 70°C for 1.5 min., followed by 20 cycles of 90°C for 1 min., 64°C for 1 min., 70°C for 1.5 min.

§Amplification program 2: 96°C for 2 min., then 10 cycles of 94°C for 1 min., 60°C for 1 min., and 70°C for 1.5 min., followed by 20 cycles of 90°C for 1 min., 60°C for 1 min., and 70°C for 1.5 min.

‡Cell line CCRF-SB was obtained from a human male. Therefore, it is hemizygous for the HPRTB locus.

K562 DNA as template. Figure 1*A* displays the results with the CTT triplex. Amplification of varying amounts of templates, from 500 ng to as little as 0.1 ng of DNA, were tested. All results in this study are based upon observation of the original films. All alleles for the three loci tested were detected using as little as 0.1 ng of DNA as template. Furthermore, the pattern of the predominating amplified fragments representing alleles was constant throughout the full 5000-fold range of template tested. Smearing of silver deposition and minor extra bands were detected when more than 25 ng of template DNA was amplified. However, even with the most intense signals at these high template concentrations, the ratio of intensities of the authentic STR alleles to minor band components remains constant with those elicited with lower template concentrations. Dilution of amplified products from reactions with excess genomic DNA decreases smearing and minor band components (data not shown). Amplification of each of the DNA concentrations using individual CSF1PO, TPOX, and TH01 primer sets generated the identical sensitivity results as when the samples were amplified using the CTT triplex except that the TH01 products were visible using as little as 0.25 ng of template (data not shown).

Similar results for the HFv triplex system were observed (Fig. 1*B*). HFv alleles were detected at DNA concentrations as low as 0.25 ng or lower depending on the locus. The HPRTB, FESFPS, and vWF monoplex systems showed sensitivities of 0.1 ng, 0.5 ng, and 0.1 ng, respectively (data not shown). In general practice, 5 to 25 ng template are used to generate amplification products similar in yield to components of the allelic ladders.

The detection of denatured amplification products using silver stain sometimes reveals two distinct fragments. These represent the opposing strands of the PCR product that are the same length, but migrate differentially because they contain different DNA sequences (30,19). The loci CSF1PO and TPOX display a single visible band per allele in the samples while the loci TH01, HPRTB, and FESFPS display two bands per allele (Fig. 1). The locus vWF also displays the two-band form, but in addition, shows a phenomenon known as repeat slippage characterized by the occasional loss of a 4-base repeat unit resulting in the display of extra bands below the authentic alleles. This is especially prevalent when more than 25 ng of template is used (Fig. 1*B*). The presence of repeat slippage products, sometimes referred to as stutter bands, are more strongly correlated with particular loci than with primer selection or amplification protocols (16). With the exception of the vWF locus, the STR loci in this study were originally selected, in part, because they generate amplification products with very little stutter.

Variation of the Annealing Temperature in Amplification Protocols

Amplification of three DNA samples (K562, CCRF-SB, and HL-60) using the CTT triplex with the recommended amplification protocol (Table 1, 64°C annealing temperature) were compared with protocols in which the annealing temperature was set to 62°C or 66°C (Fig. 2*A*). Robust amplification is observed with all samples at all three loci using any of these annealing temperatures in the amplification protocol. Comparison to an identical experiment in which the STR systems are amplified at the individual loci is displayed in Fig. 2B. As expected, each of the individual loci, CSF1PO, TPOX, and TH01 produces the same alleles in separate amplifications as in the CTT multiplex in which the

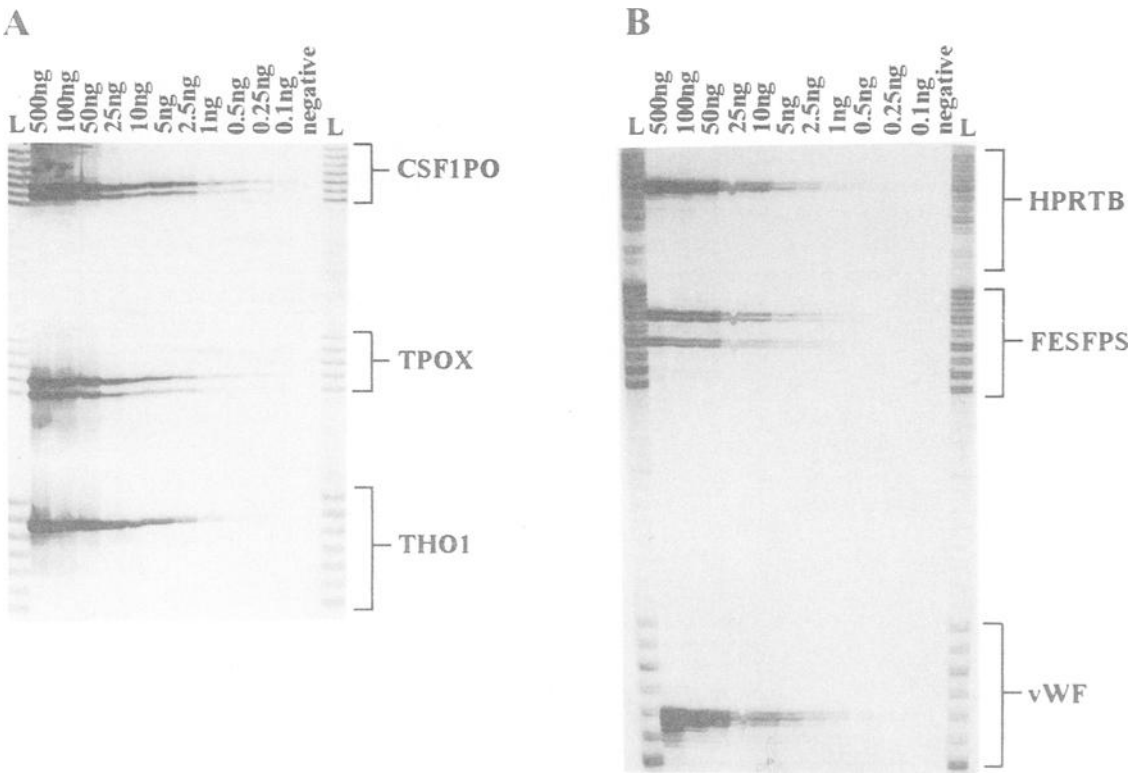

FIG. 1—*Sensitivity of analysis using CTT and HFv STR triplex systems with silver stain detection. K562 DNA ranging from 500 ng to 0.1 ng template was amplified simultaneously either at the CSF1PO, TPOX, and TH01 loci* (A), *or the HPRTB, FESFPS, vWF loci* (B). *The range of alleles for each locus is listed to the right of each panel. The amount of template used in the amplification is noted above each lane. Lanes labeled L contain a mixture of the CTT allelic ladders* (A) *or HFv* (B) *allelic ladders. Lanes labeled "negative" display the amplification products in the absence of template DNA.*

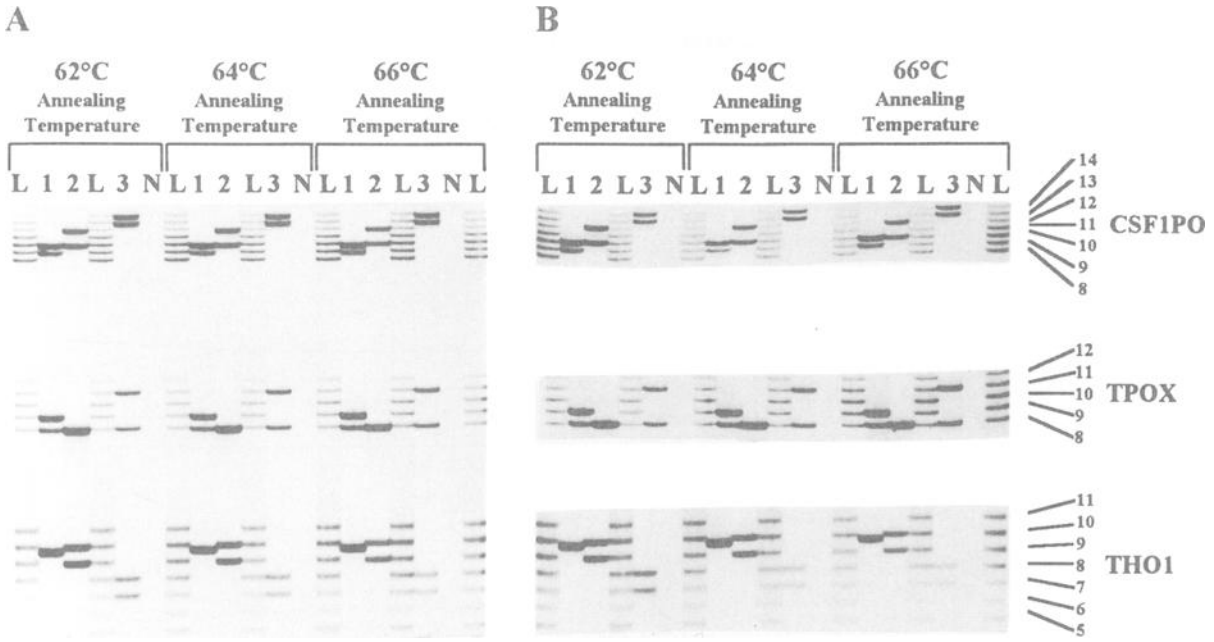

FIG. 2—*Effects of varying annealing temperature in amplification protocols—CSF1PO, TPOX, TH01. The CTT triplex* (A) *or each component monoplex system* (B) *was used to amplify three template DNAs either with the amplification protocol recommended by the manufacturer or with an annealing temperature two degrees above or two degrees below that which was recommended. The annealing temperature used in each reaction is specified above each panel. The specific loci used are indicated in* (B). *Lanes labeled L contain allelic ladders of the loci being analyzed. Numbers to the right of the allelic ladders in* (B) *indicate the number of repeats in each component of the ladder. Lanes labeled 1, 2, and 3, contain amplified DNA from cell lines K562, CCRF-SB, and HL-60, respectively. The lanes labeled* N *contain the amplified products for the triplex or each monoplex when no DNA template was present.*

loci are amplified simultaneously, and the amplification is equally robust at each annealing temperature.

Figure 3 illustrates a similar experiment for the HFv triplex (Fig. 3*A*) and its component loci (Fig. 3*B*). In this case the recommended amplification protocol includes an annealing temperature of 60°C (Table 1). The amplification products of the same three DNA templates generated with this protocol were compared with those made using annealing temperatures of 58°C or 62°C, respectively. All three loci performed well with annealing temperatures of 58°C or 60°C in both the triplex or as monoplex amplifications. However, less amplification product is observed for the HPRTB locus in both the triplex and the monoplex amplifications when an annealing temperature of 62°C is used. Thus, it is important to calibrate the instrument and use the recommended protocol for proper evaluation of the HPRTB locus.

In all cases the pattern of alleles produced in the monoplex format was identical for every locus as the pattern observed with the triplex format. Thus, these formats may be used interchangeably as needed.

Evaluation of Mixed DNA Samples

Genomic DNA from the cell lines K562 and CCRF-SB were mixed in ratios of 100:0, 99:1, 95:5, 90:10, 80:20, 50:50, 20:80, 10:90, 5:95, 1:99, and 0:100 (Fig. 4*A*). Each reaction contained a total of 25 ng of template DNA. As noted in Table 1, K562 DNA contains CSF1PO alleles 10 and 9, TPOX alleles 9 and 8, and TH01 allele 9.3, while the corresponding alleles for CCRF-SB DNA are 12 and 10 for CSF1PO, 8 for TPOX, and 10 and 9 for TH01. The minor contributor to each amplification is detectable at a different sensitivity varying with the locus being analyzed. For example, with the CSF1PO locus, the contribution of allele 12 from CCRF-SB can be detected when present at 5% of the total DNA. On the other hand, the TPOX allele 9 in K562 DNA is detected readily with 5% and marginally with as little as 1% representation, and the TH01 allele 9 from the CCRF-SB DNA is seen when present as 10% of the amplified material. Individual monoplex systems produced the same results (data not shown).

DNA ratio experiments as described earlier, were also conducted using HPRTB, FESFPS, and vWF, as monoplex systems and the HFv triplex system. With the HFv triplex, the minor allele for each locus was observed when present as 10%, 50% (marginal at 20%), and 1%, respectively (Fig. 4*B*). Theoretically, the existence of the stutter band below each vWF allele could complicate the interpretation of mixtures. This would be a concern especially if the minority DNA component contained both alleles 4 bases smaller than those of the majority species. By contrast, the example displayed in Fig. 4*B* allows identification of the authentic alleles and stutter bands contributed by each DNA sample. Despite the presence of stutter bands, the vWF locus allows more sensitive detection of the minority component than do the HPRTB and FESFPS loci in these mixed samples. The individual loci reveal the contribution of the minor component when it is present as 10%, 20%, or 1% respectively of the total material.

Many specimens analyzed in forensic laboratories are the result of body fluid mixtures of two or more individuals. The most common mixtures include blood with blood, saliva with blood, or vaginal cells with semen stains. Among PCR-based methods available for forensic analysis, the reverse dot blot protocol (31,32), involving hybridization of a PCR product with sequence-specific oligonucleotides immobilized on a test strip, is an important investigative aid in DNA identification. However, interpretation of the reverse dot blot results is limited to presence or absence of specific

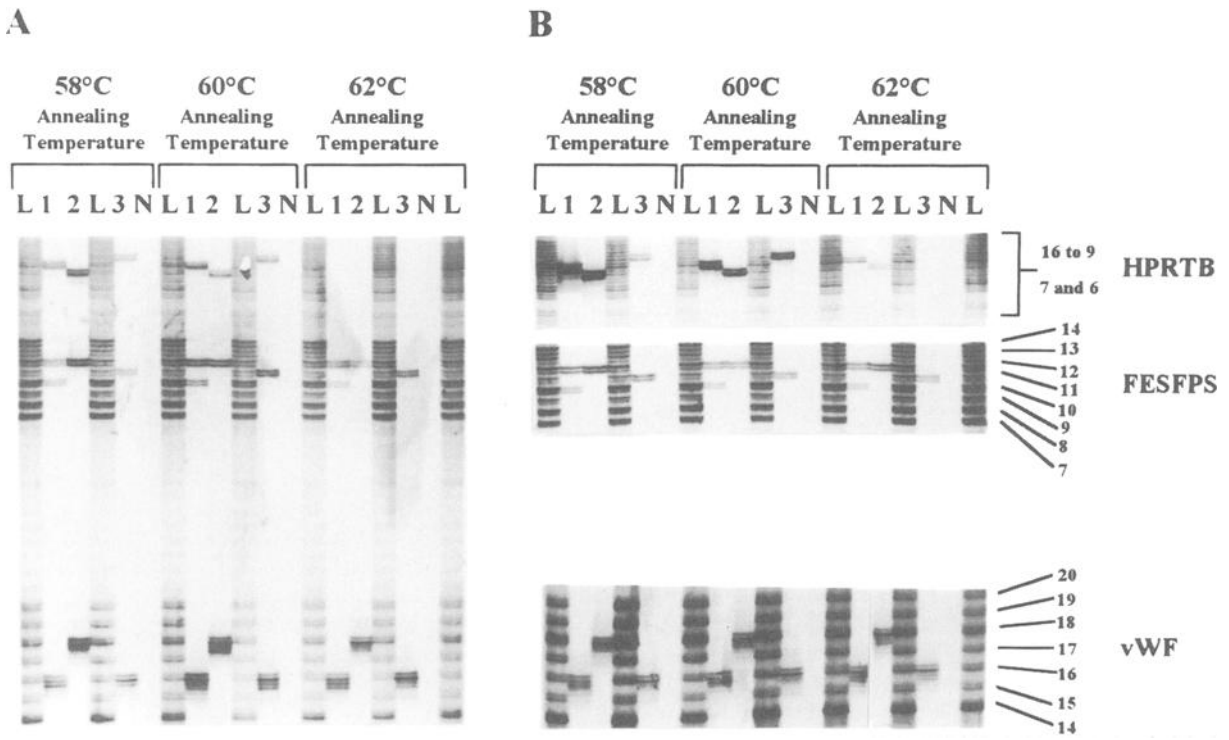

FIG. 3—*Effects of varying annealing temperature in amplification protocols—HPRTB, FESFPS, vWF. The HFv triplex* (A) *or each component monoplex system* (B) *was used to amplify three template DNAs either with the amplification protocol recommended by the manufacturer or with an annealing temperature two degrees above or two degrees below that which was recommended. The annealing temperature used in each reaction is specified above each panel. The specific loci used are indicated in* (B). *Lanes labeled L contain allelic ladders of the loci being analyzed. Numbers to the right of the allelic ladders in* (B) *indicate the number of repeats in each component of the ladder. Lanes labeled 1, 2, and 3, contain amplified DNA from cell lines K562, CCRF-SB, and HL-60, respectively. The lanes labeled* N *contain the amplified products for the triplex or each monoplex when no DNA template was present.*

hybridization events and comparison of their relative intensities. When DNA mixtures are present in the sample, interpretation becomes complex often leading to less statistically significant results. In sharp contrast, the STR systems described in this report and the amplifiable D1S80 system (33,34) offer a major advantage in evaluation of mixtures due to the electrophoretic separation and subsequent detection of more alleles present at each locus.

Amplification Reliability

To determine the reliability of amplification and consistency in assigning alleles in the six monoplex systems and the two triplex systems, 21 DNA samples, including K562 DNA as a positive control, were distributed to five laboratories for analysis. The specifications of the DNA samples and the isolation procedures used to prepare them are discussed in the Materials and Methods. Twenty-five nanograms of template DNA was used in 25 μL for all amplification reactions except those with Chelex-100-purified DNA as template, in which cases, 5 ng template was amplified in 50 μL reactions. Three laboratories amplified all combinations using the Model 480 while four locations performed amplification using the Model 9600 thermal cycler. Data collected included the number of amplification failures, the intensity of the amplification product, and the allele assignments for each sample/locus/thermal cycler combination.

The success rate of the 882 amplification attempts in producing typable results with the CTT triplex and its component monoplexes is shown in Table 2. Only three DNA samples were not defined due to failed amplification of the sample. Each of these failures occurred in a monoplex system and was successfully amplified upon a second amplification attempt. Thus, there was 100% success in amplifications of the CTT triplex in all participating laboratories. Further, there were only five individual cases of poor product yield or weak silver stain signals reported for specific amplification attempts with these systems. In each of these cases, alleles were correctly identified.

Of the 882 reactions attempted with the HFv triplex and its component monoplexes (Table 3), 26 reactions failed to amplify (last row of Table 3). Of the 378 reaction attempts using the Model 480, only two samples failed to amplify. These two were successfully amplified in repeat attempts. Of the 504 reaction attempts using the Model 9600 thermal cycler, 24, or 4.8%, failed to amplify. Eleven of the amplification failures were successfully amplified in repeat attempts. The Model 9600 thermal cycler-related failures were most often observed in attempts to amplify the HPRTB locus as a monoplex and the FESFPS locus when included in the triplex. These amplification systems also tended to produce a higher proportion of amplification products which were lower than the average product yields, but still strong enough to allow allele determination (data not shown).

Similar results were achieved when 5 ng of DNA template was used instead of the 25 ng used, above, with each triplex system for the samples extracted by methods other than Chelex® 100. Amplifications were performed in both the Model 480 and the Model 9600. Fourteen of fourteen samples amplified using the CTT triplex in each thermal cycler. With the HFv triplex, 12 of 12 amplified using the Model 480, and 11 of 12 using the Model

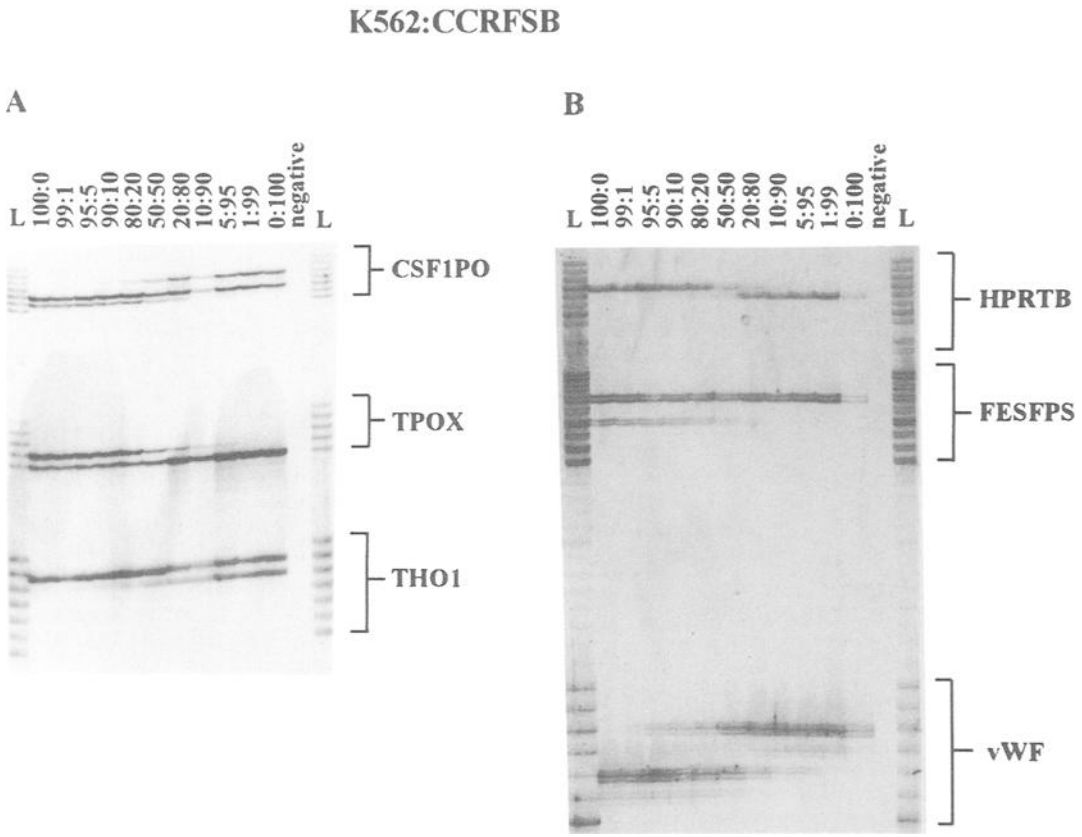

FIG. 4—*Evaluation of mixed DNA samples. Lanes labeled with ratios of numbers contain amplification product of 25 ng of total template amplified with the CTT STR triplex system* (Panel A) *or the HFv STR triplex system* (Panel B). *The ratios indicate the relative amounts of K562 DNA and CCRF-SB DNA included in these amplifications. Lanes labeled L contain allelic ladders of the three loci contained within the CTT or HFv triplex, respectively. The lanes labeled "negative" display the amplification products in the absence of template DNA.*

TABLE 2—*Number of successful amplification reactions versus number of amplification attempts for CSF1PO, TPOX, and TH01 monoplex amplifications and CTT triplex amplifications. Percentage of successful amplifications is in parentheses for each case.*

Thermal cycler Amplification format	Model 480 monoplex	Model 480 multiplex	Model 9600 monoplex	Model 9600 multiplex	Total
Samples 1–5	45/45(100)	45/45(100)	60/60(100)	60/60(100)	210/210 (100)
Samples 6–10	45/45(100)	45/45(100)	58/60(96.7)	60/60(100)	208/210 (99)
Samples 11–15	45/45(100)	45/45(100)	60/60(100)	60/60(100)	210/210 (100)
Samples 16–20	45/45(100)	45/45(100)	60/60(100)	60/60(100)	210/210 (100)
Sample K562	8/9(88.9)	9/9(100)	12/12(100)	12/12(100)	41/42 (97.6)
All samples	188/189 (99.5)	189/189 (100)	250/252 (99.2)	252/252 (100)	879/882 (99.7)
Amplification failures	CSF1PO-sample K562	none	TPOX samples 6,9	none	

TABLE 3—*Number of successful amplification reactions versus number of amplification attempts for HPRTB, FESFPS, and vWF monoplex amplifications and HFv triplex amplifications. Percentage of successful amplifications is in parentheses for each case.*

Thermal cycler Amplification format	Model 480 monoplex	Model 480 multiplex	Model 9600 monoplex	Model 9600 multiplex	Total
Samples 1–5	45/45(100)	45/45(100)	60/60(100)	56/60(93.3)	206/210 (98.1)
Samples 6–10	45/45(100)	45/45(100)	56/60(93.3)	58/60(96.7)	204/210 (97.1)
Samples 11–15	45/45(100)	45/45(100)	54/60(90.0)	56/60(93.3)	200/210 (95.2)
Samples 16–20	43/45(95.6)	45/45(100)	58/60(95)	59/60(98.3)	205/210 (97.6)
Sample K562	9/9(100)	9/9(100)	11/12(96.7)	12/12(100)	41/42 (97.6)
All samples	187/189 (98.9)	189/189 (100)	239/252 (94.8)	241/252 (95.6)	856/882 (97.1)
Amplification failures (Numbers in parentheses indicated for failure in more than one laboratory)	HPRTB sample 12; FESFPS sample 14	none	HPRTB samples 7(2),12,13,15, 16,18,K562; FESFPS samples 7,11,14,15; vWF sample 7	HPRTB samples 3,5; FESFPS samples 3,5,6,9,11,13,15(2),17	

9600. (There was insufficient template DNA available to test all 15 samples.)

The CTT triplex and its related monoplexes performed with excellent reliability (Table 2). Nearly 99.7% of amplification attempts were reported as successful using either thermal cycler. Repetitions of the three monoplex failures produced 100% success on the second attempt. Reliability of the HFv multiplex and its related monoplexes (Table 3) was 99.5% using the Model 480 and 95.2% using the Model 9600. No significant correlation between amplification success rate and particular DNA samples, the DNA purification method, or the tissue source employed was observed for any of the eight systems tested (that is, multiplexes or monoplexes). With the exception noted in the following, amplifications were generally successful and the rare failures cannot be ascribed to anything other than random events. A significant number of amplification failures were observed primarily with the HPRTB and FESFPS loci when used in combination with the Model 9600. It should be noted that amplifications protocols for both multiplexes and all monoplexes were developed using the Model 480. Future protocol modifications may improve reliability of the HPRTB and FESFPS loci with the Model 9600.

Identification of Alleles in DNA Sample Unknowns

The alleles present at six independent STR loci were determined for twenty DNA samples (plus the K562 DNA control template) in this study. Of the 120 sample-locus combinations, identical results were obtained by all five laboratories in 117 of these combinations. Each of the three discordant determinations, listed in Table 4, represent a particular caution which must be considered in the use of STR loci for DNA typing. The individual cases of discordance and possible explanations for each case are discussed below.

The first case of discordant allele determination is illustrated in Fig. 5. Amplification products from the CSF1PO locus are shown for samples 6 through 10 and 15. Samples 6, 7, and 8 are typical heterozygotes not derived from cell lines, and all demonstrate a characteristic relatively equal band intensity between the two alleles present at the CSF1PO locus in each DNA sample. Samples 9 and 10 are single-banded patterns representing homozygotes for the CSF1PO locus. The amplification products of sample 15 show an intermediate result, with a strong upper allele relative to the lower allele. This pattern, which is not characteristic for CSF1PO amplification products, caused confusion in that some individuals interpreted the result as an 11/11 homozygote with an overly strong shadow band in the position of allele 10, while others assigned both alleles, designating sample 15 as an 11/10 heterozygote.

This discordance may result from the fact that sample 15 is derived from the cell line CCRE-CEM and there may be more than two copies of the CSF1PO region in this sample. We have observed, although rarely, the presence of three alleles with STR loci in two previous cases. These were the cell line K562 DNA with the D21S11 locus and an anonymous human sample with the TH01 locus (data not shown). Therefore, it is possible that the CSF1PO locus is duplicated on one of the chromosomes such that three copies are present in the sample, with two copies of allele 11 and one of allele 10.

A second possibility is that a different DNA sequence is present at the site of one of the CSF1PO primers in the K562 allele 10 resulting in reduced product yield for that allele. Alternatively, a third explanation is that a larger region of chromosome 5 has been duplicated. To test this third possibility, we amplified six separate loci on chromosome 5 (5p loci—D5S807 and D5S819; 5q loci—D5S815, D5S818, D5S820, and D5S1456) to look for differences in the amplification product intensities of alleles present in normal human DNAs (samples 6 through 10 of this study) and the cell line CCRE-CEM DNA. This was performed using one fluorescently labeled primer for each locus, and detection was achieved by scanning the gel with the Molecular Dynamics Fluorimager™ 575

TABLE 4—*Discordant determinations.*

Locus	Sample	Common allele determinations		
		Most common	Second most common	Third most common
CSF1PO	15	11/11 (9 cases)	11/10 (5 cases)	none
TH01	7	10/9.3 (12 cases)	9.3/9.3 (2 cases)	none
HPRTB	9	11/11 (6 cases)	11+/11 (4 cases)	12/11 (4 cases)

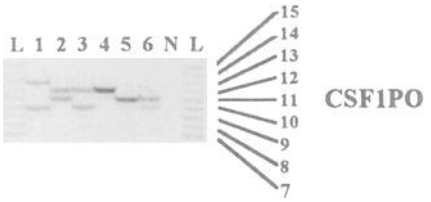

FIG. 5—*Evaluation of discordant allele determinations relating to locus CSF1PO. Lanes 1 through 6 contain products amplified at the CSF1PO locus from samples 6, 7, 8, 9, 10, and 15. Lanes labeled* L *contain the CSF1PO allelic ladder. The lane labeled* N *displays the amplification products in the absence of template DNA.*

(Sunnyvale, CA). Thus, it was possible to quantitate the amount of amplification product present in each allele. No significant differences were observed between the normal DNAs and the CCRE-CEM DNA except for the D5S818 locus, in which case the ratio of allele intensities observed was greater than twice that observed with the two normally heterozygous DNA samples 6 and 10. Of the loci tested, the D5S818 locus is most terminal on the long arm of chromosome 5 and located closest to the site of CSF1PO. This result is most consistent with the suggestion that a chromosomal region larger than the CSF1PO locus, but smaller than the long arm of chromosome 5, is present in more than two copies.

The second case of discordant allele determinations was seen with amplification of the TH01 locus using sample 7. In 12 cases, this sample was designated a 10/9.3 heterozygote, while in the 2 remaining cases (both with the monoplex TH01 system), it was designated a 9.3/9.3 homozygote. Alleles 10 and 9.3 differ in length by a single base (6). Thus, we saw that in the two homozygote assignments that either the band intensities of the two distinct alleles were too great causing them to be identified as one band, or the electrophoresis of the gel in which they were separated did not allow complete separation of the two alleles. This points out that individual laboratories which use the TH01 system will have to validate their own level of confidence in separating these two alleles. Inclusion of the TH01 allele 9.3 in the same lane as the TH01 allelic ladder may assist in this evaluation.

The final case of discordance was observed with sample 9 at the HPRTB locus. The confusion in this case resulted from the existence of a rare off-ladder allele in the test population. This microvariant which migrates more quickly than allele 12, but more slowly than allele 11 (data not shown), was originally described by Edwards et al. (27). Most often, validation participants designated this allele 12 (6 cases) or allele 11 (4 cases), rather than correctly as a variant of allele 11 (4 cases). This result emphasizes the need to examine results carefully when working with STR loci. New or unusual results must be identified and reported accurately. The existence of rare alleles does not negate the use of these loci for identification testing. Matches can still be made and statistical approaches exist to properly evaluate rare alleles (35).

The remaining 117 cases of allele determinations for the 20 DNA samples at the six loci tested showed no discordances in interlaboratory allele determinations. Overall, the use of the STR systems evaluated in this work is extremely effective for making accurate allele assignments in DNA samples of unknown allele content.

Except as previously noted for the distinction of the TH01 alleles 10 and 9.3, there were no significant differences observed between allele determinations following amplification of individual STR systems as monoplexes or amplification of the same systems when included in multiplexes. Furthermore, the selection of DNA purification method or thermal cycler did not affect the interpretation of results.

Conclusions

The experiments described in this paper show that the two triplex STR systems examined, CTT and HFv, are sensitive and robust. Both triplexes amplified with 100% success using the Model 480 thermal cycler. In addition, the CTT triplex amplified with 100% success and the HFv triplex with 95.6% success using the Model 9600. These experiments meet several of the TWGDAM guidelines for validation of forensic DNA typing tests (20) and will also assist paternity laboratories in meeting standards to be set forth by the American Association of Blood Banks (AABB) (21). Comparison of all test results between monoplexes and triplexes shows that analysis of genomic DNA using multiplex systems is in no way compromised as compared with analysis using individual monoplexes. Genomic DNA in the range of 0.1–0.25 ng can be detected with each system. The CTT system can tolerate more variation in annealing temperature than the HFv system can. Proper quality checks of thermal cyclers are critical in any laboratory performing PCR.

The typing of samples blindly by five laboratories demonstrates that identifying alleles using allelic ladders is highly reliable. Each of the discordant allele designations observed in this study can be explained by direct examination or from previous literature. Thorough knowledge of off-ladder alleles present in the population and careful comparison with allelic ladders assists in identification of alleles. Inclusion of microvariant alleles such as the HUMTH01 9.3 allele as a standard control may provide additional assistance in typing unknown samples. Studies are currently underway to validate these systems further for forensic and paternity use. These include population studies, species specificity studies, co-amplification with a gender identification locus, and typing of samples exposed to a variety of environmental conditions. Old evidentiary samples are being collected for analysis as well.

Work is also underway to explore the possibility of replacing the HUMHPRTB locus in the HFv triplex with the HUMF13A01 locus. The HUMF13A01 locus is an autosomal locus, in contrast to the X-linked HUMHPRTB locus. Thus, this substitution will provide stronger matching probabilities with male DNA samples and will provide additional information in paternity analyses

involving male offspring. It is also anticipated that this substitution will help overcome the small number of amplification failures observed with the HFv triplex when using the Model 9600 thermal cycler.

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