Multiplex Sets for the Amplification of Polymorphic Short Tandem Repeat Loci — Silver Stain and Fluorescence Detection

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ABSTRACT

Multiplex PCR amplification systems were developed using well-characterized, polymorphic short tandem repeat (STR) loci. Eight loci utilized in the multiplex amplifications included HUMCSF1PO, HUMTPOX, HUMTH01, HUMVWFA31, HUMF13A01, HUMFESFPS, HUMBFXIII and HUMLIPOL. From this list, three or four non-overlapping loci were simultaneously amplified, separated by denaturing polyacrylamide gel electrophoresis and visualized using silver stain or fluorescence detection. The multiplex PCR amplification systems offer a non-isotopic method for rapid, simple and accurate analysis of STR loci. This high-throughput method for DNA identification has immediate and valuable application in forensic analysis, paternity determination, tissue culture strain identification and bone marrow transplantation studies.

INTRODUCTION

Short tandem repeat (STR) loci (9,10,27–29) consist of tandemly repeated 3 to 7 base pair sequences. They provide a rich source of polymorphic markers resulting from variation in the number of copies of the repeated motif. In this respect, they are similar to previously described polymorphic sites such as variable number of tandem repeats (VNTR) loci (25) and minisatellite loci (15) which contain longer repeat units, and microsatellite or dinucleotide repeat loci (3,21,36,38) containing shorter repeats.

STR loci display several qualities that make them attractive genetic markers. They are very plentiful, averaging one trinucleotide or tetranucleotide tandem repeat locus every 15 kb in the human genome (3,9), and they are amenable to polymerase chain reaction (PCR) amplification using flanking sequence primers. The resulting amplification fragments of the individual STR loci range from 100 to 400 base pairs. STR systems carrying trinucleotide or tetranucleotide repeats produce fewer PCR artifacts than seen with dinucleotide repeat loci (3,35,38), especially the extra band production described as repeat slippage or stuttering (20,34).

Many STR systems yield precisely defined PCR amplification products that can be readily and accurately analyzed using well-characterized allelic ladders [i.e., standard markers consisting of amplified fragments of the same lengths as several or all of the known alleles for the locus (31–33,35)]. For these systems, population analysis does not require the use of arbitrarily defined fixed bins (6); therefore, statistical analysis of populations, which is based upon accurate, precise and confident allele determination, is simplified.

In recent years, the application of STR polymorphisms has stimulated progress in the elaboration of linkage maps, identification and characterization of disease genes, and simplification and precision of DNA typing. Recently constructed human genetic maps have placed thousands of dinucleotide repeat and STR loci (1,23) in relation to previously localized VNTR loci (8). In addition, several inherited disorders have been associated with trinucleotide repeat expansion within the gene responsible for the disorder (5,18,19, 22,24,26,39).

The characterization of a large number of highly polymorphic STR loci (9, 10,13,14,17,35,37), along with the construction of well-defined allelic ladders for several of the most easily interpreted loci (31–33,35), will allow increased use of these systems in forensic analysis, paternity determination, tissue culture strain identification and bone marrow transplantation analysis. In this work, we describe the development of multiplex PCR amplification systems to allow the simultaneous amplification and analysis of three or four polymorphic STR loci. In this work, we describe the development of multiplex PCR amplification systems to allow the simultaneous amplification and analysis of three or four polymorphic STR loci. The amplified fragments are separated by denaturing polyacrylamide gel electrophoresis and detected using a nonisotopic method. The silver stain detection method is suitable for laboratories that desire an inexpensive, high-throughput analysis. Fluorescence detection offers advantages of speed and convenience. With the appropriate software, the fluorescent sample data may be automatically analyzed, printed and stored in a database. Both detection methods offer rapid, simple and accurate analysis of multiple STR systems.
MATERIALS AND METHODS

DNA isolation and PCR amplification were performed essentially as described previously (30,33). Locus nomenclature and amplification protocols used in this work are included in Table 1. The sequence or source of oligonucleotides for each locus was detailed by Sprecher et al. (35). All oligonucleotides were synthesized using an Applied Biosystems DNA Synthesizer Model 380B or Model 394 (Perkin-Elmer/Applied Biosystems Division, Foster City, CA, USA). Amplification products generated with unlabeled primers were detected using silver stain as described by Bassam et al. (2). In the case of fluorescence detection, one of the two primers utilized in the reaction was labeled at the 5′ terminus with fluorescein amidite. Multiplex configurations contained a mixture of either three or four separate primer pairs for simultaneous amplification of multiple loci in a single reaction tube. Amplification products were subjected to gel electrophoresis in a 4% denaturing polyacrylamide gel containing 7 M urea and 0.2% bisacrylamide as the cross-linker.

Fluorescence detection was accomplished using the FluorImager™ SI (Molecular Dynamics, Sunnyvale, CA, USA). The FluorImager scans the gel with a 488-nm argon ion laser beam that sweeps through the gel using a galvanometer-controlled mirror. The laser light activates fluorescent molecules in its path, and they, in turn, emit light of higher wavelength. An internal optical filter prohibits passage of the original laser light, but allows collection of the emitted light by a fiber-optic collector.

RESULTS AND DISCUSSION

The STR loci originally identified and characterized by Sprecher et al. (35) were chosen as candidates for multiplex development. In that work, nine STR systems were selected based on the favorable display of the following criteria: 1) good amplification product yield, 2) high degree of polymorphism, 3) little or no PCR artifacts arising from nonspecific amplification and 4) ease of allele determination. The criteria were used as guidelines, not as absolutes, for the selection of loci. First, loci that produced poor yields upon amplification were rejected. In the second case, a moderate to high degree of polymorphism at the locus was required. Third, loci were evaluated for PCR amplification artifacts that could be generated by repeat slippage (20, 34), primer dimer formation or addition of a terminal nucleotide to the PCR product at less than 100% efficiency (7,17). Amplification of some STR loci produced high molecular weight fragments of undefined origin. All of these artifacts were undesirable and were either reduced by the selection of a different primer sequence or simply avoided by choosing a different locus altogether. Finally, each locus was examined to determine the ease of allele determination. It is difficult to assign alleles using denaturing polyacrylamide gel electrophoresis followed by silver stain detection if there is a large difference in electrophoretic mobility of complementary strands of an individually amplified allele in a system (35). While the opposing DNA strands of a single allele are the same length, they may migrate differentially due to sequence differences (12). If the variance in electrophoretic mobility of complementary strands is large, a situation can occur in which the slower migrating strand of one allele overlaps the faster migrating strand of the next larger allele during electrophoresis. Allele designation becomes more difficult and confusing when observing these “overlapping alleles”. For this work, loci were selected in which the complementary strands from each individual allele co-migrate or migrate close to one another relative to the neighboring alleles. In addition, loci showing a high degree of repeat length microheterogeneity (variant alleles containing deletions or...
insertions in a portion of the repeat motif) were rejected. Since it is difficult to define a standard, precisely characterized allelic ladder when using a locus with extensive microheterogeneity, binning of alleles is generally necessary. Only two of the eight loci used in this work contain variant alleles. The F13A01 locus contains one variant allele, designated allele 3.2, which is two bases shorter than the allele containing 4 repeat units (32) (Figure 1, Panel B, lane 9). The TH01 locus displays the relatively frequent allele 9.3, which is one base shorter than allele 10 (33) (Figure 1, Panel A, lane 2). If two alleles differing by one base cannot be routinely distinguished after gel electrophoresis, binning of the two alleles may be necessary. Sample 5 in Panel A of Figure 1 is an allele 10, 9.3 heterozygote for the TH01 locus. When denaturing gel electrophoresis is used in conjunction with silver stain detection, the bottom strand of allele 10 and the top strand of allele 9.3 actually co-migrate and appear as a dark band with lighter bands above and below. Using fluorescence detection, only one strand of each allele is visualized (Figure 3, Panel A, lane 5) and the two alleles are resolved.

To construct multiplex sets, individual loci having compatible amplification protocols and allele size ranges that do not overlap were simultaneously amplified in one reaction tube, separated by denaturing polyacrylamide gel electrophoresis and detected by silver stain or fluorescence. The selected loci in each multiplex set must not overlap to allow for independent interpretation of each system. Following amplification, samples were evaluated for amplification yield and PCR artifacts. To correctly analyze all loci in the multiplex, it is desirable to have similar amplification yields for the different loci. Thus, multiplex sets were designed to permit amplification of each locus without generating new PCR artifacts, such as those which could be produced by utilizing primers from different loci. Several multiplex amplification systems that failed to produce acceptable yields for one or more of the involved loci, or yielded spurious DNA fragments in undesirable regions of interest, were observed and rejected (data not shown).

After evaluating numerous locus combinations and amplification conditions, two triplex systems were identified and developed for silver stain detection (Figure 1). The first triplex (Panel A) includes the co-amplification of the CSF1PO, TPOX and TH01 loci; the second triplex (Panel B) contains the F13A01, FESFPS and vWF loci. In both gels, amplified DNA samples were subjected to electrophoresis adjacent to a mixture of allelic ladders for the respective loci. All three loci in each of the two multiplexes amplify well while generating very few artifacts. Figure 2 displays the template sensitivity for the CSF1PO, TPOX and TH01 triplex (Panel A) and F13A01, FESFPS and vWF triplex (Panel B). For both multiplexes, amplification was successful when using as little as 0.5 ng template DNA. Consistent interpretation of alleles generated by amplification with these triplexes can be made using from 0.5 to 250 ng of template DNA. This approach allows rapid, simple and precise identification of sample alleles by visual comparison with components of the allelic ladder, without the need for specialized equipment. A gel electrophoresis unit, power supply, three large tubs for staining and film for the development of a permanent record are all that is required.

Two STR multiplexes were developed for a second nonisotopic procedure that involved the detection of fluorescently tagged amplification products using the FluorImager SI. For this detection method, the amplifications are performed with the inclusion of one 5'-fluoresceinated primer and one

![Figure 2. Template sensitivity for multiplexes detected by silver stain.](image-url)
unlabeled primer for each locus in the multiplex. Thus, only one of the amplified complementary DNA strands is fluorescently labeled and subsequently detected. Separation of the amplified material is achieved in the same fashion as with the silver stain method except that low-fluorescence glass plates are used for electrophoresis. Following electrophoresis, the glass/gel/glass sandwich is inserted into the instrument, which scans the gel, digitizes the data and produces a hard-copy printout as shown in Figure 3. This approach achieves higher throughput and more rapid analysis than the silver staining detection method.

Two quadriplex amplification systems were developed for fluorescence detection (Figure 3). Panel A displays the fluorescent products from the co-amplification of the CSF1PO, TPOX, TH01 and vWF loci, while Panel B contains the amplification products of the multiplex containing the F13A01, FESFPS, F13B and LPL loci. For both gels, amplified DNA samples were subjected to electrophoresis adjacent to a mixture of fluorescent allelic ladders for the corresponding STR loci.

Figure 4 demonstrates the template sensitivity of the CSF1PO, TPOX, TH01 and vWF quadriplex (Panel A) and the F13A01, FESFPS, F13B and LPL quadriplex (Panel B). For both multiplexes, amplification is successful with as little as 0.5 ng template DNA. As is true for the previously described triplexes, amplification of 0.5 to 250 ng of template DNA is readily and consistently interpreted. The fluorescent quadriplex STR analysis method using fluorescent allelic ladders allows for rapid, simple, precise and reproducible identification of alleles for each locus.

**Figure 3. Fluorescent detection of multiplex amplification systems.** Panel A displays the fluorescent amplification products of human DNA samples at the polymorphic loci CSF1PO, TPOX, TH01 and vWF (CTTv Quadriplex). Panel B displays the fluorescent amplification products of human DNA samples at the polymorphic loci F13A01, FESFPS, F13B and LPL (FFFL Quadriplex). One of the primers for each locus was labeled with fluorescein. The amplification products were separated in a 4% denaturing polyacrylamide gel and detected using a FluorImager SI. Lanes labeled (L) contain a mixture of allelic ladders for the respective loci in each multiplex; lanes 1–11 contain individually amplified DNA samples; and lanes labeled (N) contain negative control amplification reactions (i.e., no template DNA). For each locus, the largest and smallest alleles of the corresponding allelic ladder are labeled to the right with the number of tetranucleotide repeats they contain.
The results are presented in Figure 5. The most noticeable variation in detection is that the alleles for the largest locus, CSF1PO, are oversaturated with silver, while no alleles are detected for the smallest locus, vWF. This illustrates that the amplification conditions for the fluorescent quadriplex are not compatible with silver stain detection. Silver stain detection is based on mass of the amplified products, while fluorescence detection is dependent on moles of incorporated fluorescent primer. Thus, if products in Figures 1 and 3 reveals that the silver stain and fluorescence detection methods produce the same allele designation for each sample at each locus. However, inspection of the two figures shows that the fluorescence detection method produces an overall cleaner appearance than the silver stain detection method. There are several reasons for this difference. First, with fluorescence detection, only one of the amplified product strands is labeled and displayed. This simplifies interpretation in that one fragment per allele is observed with fluorescence rather than the two fragments per allele often seen with silver stain detection. Some of the allelic ladders, in particular the F13A01 ladder, benefit significantly from this simplification. We have recently observed that the two-band pattern displayed for the vWF alleles (Figure 4, Panel A) can be reduced to a one-band pattern by selecting the opposing primer for fluorescent labeling (data not shown).

Second, the entire gel is exposed to silver during the silver stain reaction and is prone to silver deposition which causes a significant increase in general background. With the fluorescent reporter, this background haze is not observed because only the primer is labeled and unincorporated primers migrate out the bottom of the gel prior to detection.

Third, some artifact bands of the amplification reaction contain a significant amount of mass, but a relatively small amount of primer, and are thus detected efficiently using the silver stain procedure. Since the fluorescent method detects only products with the labeled primer, some of these artifacts are not detected. This explains why we have developed multiplex reactions containing four loci for fluorescence detection, but only three loci for the silver stain method. To illustrate this point, we analyzed fluorescent CSF1PO, TPOX, TH01 and vWF multiplex amplification products using silver stain detection instead of fluorescence. The results are presented in Figure 5. The most noticeable variation in detection is that the alleles for the largest locus, CSF1PO, are oversaturated with silver, while no alleles are detected for the smallest locus, vWF. This illustrates that the amplification conditions for the fluorescent quadriplex are not compatible with silver stain detection. Silver stain detection is based on mass of the amplified products, while fluorescence detection is dependent on moles of incorporated fluorescent primer. Thus, if

<table>
<thead>
<tr>
<th>Locus (GenBank® Name)</th>
<th>Chromosome Location</th>
<th>Heterozygosity (%)</th>
<th>Matching Probability (pM)</th>
<th>Typical Paternity Index (PI)</th>
<th>Power of Exclusion (PE)</th>
<th>Amplification Protocola</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSF1PO (HUMCSF1PO)</td>
<td>5q33.5-34</td>
<td>81</td>
<td>0.087</td>
<td>2.63</td>
<td>0.617</td>
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<tr>
<td>F13A01 (HUMF13A01)</td>
<td>6p24-25</td>
<td>78</td>
<td>0.069</td>
<td>2.24</td>
<td>0.557</td>
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<tr>
<td>F13B (HUMBFXIII)</td>
<td>1q31-q32.1</td>
<td>73</td>
<td>0.101</td>
<td>1.86</td>
<td>0.479</td>
<td>1</td>
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<tr>
<td>FESFPS (HUMFESFPS)</td>
<td>15q25-pter</td>
<td>73</td>
<td>0.106</td>
<td>1.82</td>
<td>0.469</td>
<td>1</td>
</tr>
<tr>
<td>LPL (HUMLIPOL)</td>
<td>8p22</td>
<td>72</td>
<td>0.099</td>
<td>1.81</td>
<td>0.467</td>
<td>1</td>
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<tr>
<td>TH01 (HUMTH01)</td>
<td>11p15.5</td>
<td>80</td>
<td>0.106</td>
<td>2.50</td>
<td>0.599</td>
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<tr>
<td>TPOX (HUMTPOX)</td>
<td>2p23-2pter</td>
<td>74</td>
<td>0.081</td>
<td>1.92</td>
<td>0.493</td>
<td>2</td>
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<tr>
<td>vWF (HUMVWF3A1)</td>
<td>12p12-pter</td>
<td>83</td>
<td>0.064</td>
<td>2.87</td>
<td>0.648</td>
<td>1</td>
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<td>CTT Triplex</td>
<td>-</td>
<td>-</td>
<td>7.5 × 10^4</td>
<td>12.62</td>
<td>0.922</td>
<td>2</td>
</tr>
<tr>
<td>FFv Triplex</td>
<td>-</td>
<td>-</td>
<td>4.7 × 10^4</td>
<td>11.70</td>
<td>0.917</td>
<td>1</td>
</tr>
<tr>
<td>CTTv Quadriplex</td>
<td>-</td>
<td>-</td>
<td>4.8 × 10^5</td>
<td>36.23</td>
<td>0.973</td>
<td>1</td>
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<tr>
<td>FFFL Quadriplex</td>
<td>-</td>
<td>-</td>
<td>7.3 × 10^5</td>
<td>13.72</td>
<td>0.935</td>
<td>1</td>
</tr>
</tbody>
</table>

The information displayed in this table was calculated from genotype data obtained from Holly Hammond (personal communication) at the Baylor College of Medicine (Houston, TX, USA) for all loci except F13B, TPOX and vWF. Information for loci F13B, TPOX and vWF was calculated from genotype data obtained from Steve Creacy and Robert A. Bever (personal communication) at Genetic Design (Greensboro, NC, USA).

a Amplification program 1: 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.

Amplification program 2: 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.
a fragment is large but contains very little labeled primer, the fragment will be heavily stained using silver, but will fluoresce in relation to the amount of fluorescein-labeled primer that is present. For the best results, the development of multiplex sets and amplification conditions must be optimized with the detection method being employed.

Table 1 reviews the characteristics of the individual loci and lists relevant data for the four multiplex systems. The alleles for each system are inherited in Mendelian fashion and are present on different chromosomes, suggesting independent inheritance. The common statistical information used in forensic and paternity laboratories has been determined for African-American and Caucasian-American populations using the CSF1PO, TPOX and TH01 triplex (CTTv Triplex); F13A01, FESFPS and vWF triplex (FFv Triplex); CSF1PO, TPOX, TH01 and vWF quadruplex (CTTv Quadruplex); and the F13A01, FESFPS, F13B and LPL quadruplex (FFFL Quadruplex). The power of these multiplexes is clearly illustrated in Table 1. While each individual locus provides limited information, more significant discriminatory power is achieved by combining three or four loci in a multiplex. Thus, the use of STR multiplexes generates more information and requires less time and reagents than using multiple monoplex systems.

The characteristic often measured in forensic analysis to determine the
discriminatory power of a genetic system is the average matching probability \((pM)\) (16). The average \(pM\) values calculated for the CTT Triplex, FFv Triplex, CTTv Quadriplex and FFFL Quadriplex for African-American and Caucasian-American populations are listed in Table 1. The combined \(pM\) values for the CTT Triplex and FFv Triplex are \(3.5 \times 10^{-7}\) and \(2.5 \times 10^{-6}\) for African-American and Caucasian-American populations, respectively. The chance that two individuals match at all eight loci contained in the two quadriplexes is \(3.5 \times 10^{-9}\) for African-Americans and \(5.6 \times 10^{-8}\) for Caucasian-Americans.

A measure of discrimination used in paternity analyses is the paternity index \((PI)\), which is a means for presenting the genetic odds in favor of paternity given the genotypes for the mother, child and alleged father (4). The typical \(PIs\) for each locus and all multiplexes are listed in Table 1. The combined \(PI\) for the CTT Triplex and FFv Triplex is 147.65 and 52.57 for African-American and Caucasian-American populations, respectively. The combined typical \(PI\) for the two quadriplexes is 497.08 and 186.69 for African-American and Caucasian-American populations, respectively. A second measure of discrimination often used in paternity analyses is power of exclusion \((PE)\), which measures the ability of a genetic test to exclude a falsely accused individual \((4,11)\). The combined power of exclusion for the loci in the CTT Triplex and FFv Triplex is 0.994 for African-Americans and 0.985 for Caucasian-Americans. The combined \(PE\) for the eight loci in the two quadriplexes is 0.998 and 0.996 for African-American and Caucasian-American populations, respectively.

Non-isotopic multiplex amplification systems have many advantages over other methods of DNA identification. They allow for the rapid, simple and accurate analysis of STR loci. Simultaneous amplification and detection of three or more loci greatly increase throughput. However, these multiplex systems do not approach the limits of high-throughput analysis using this gel format. Separate amplification products of additional loci may be loaded onto the gel either at the same time, before or after loading of the multiplexes to increase the number of loci that can be detected simultaneously. Furthermore, it should be possible to produce multiple fluorescent multiplex sets, each tagged with a different fluorescent moiety. These could be run simultaneously in the same lane and detected by multiple consecutive scans employing optical band-pass filters to visualize only one multiplex set at a time. We are exploring these and other possibilities for high-throughput analysis of STR loci.

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**REFERENCES**


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