General Approach to Analysis of Polymorphic Short Tandem Repeat Loci

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ABSTRACT

Polymerase chain reaction amplification products of 22 known polymorphic short tandem repeat (STR) loci were subjected to denaturing polyacrylamide gel electrophoresis and detected using a silver staining method. Loci that amplified efficiently and revealed the fewest amplification-related artifacts with this detection method were selected for development of allelic ladders. The combination of allelic ladders and silver stain detection provides an inexpensive and general non-isotopic analytical method for DNA identification. This approach has immediate application in forensic analysis, paternity determination, human cell line identification and monitoring of bone marrow transplants. It can also be adapted to more general applications of genetic analysis in human and other species including detection of genetic disorders and cancers.

INTRODUCTION

Short tandem repeat (STR) loci (8,9,37) consist of tandemly repeated 3- to 7-bp sequence motifs. The human genome may contain as many as one trimeric or tetrameric STR every 20 kb (3,8,9), and nearly half of the STR loci studied by Edwards et al. (9) are polymorphic in the number of repeats they contain, providing a rich source of genetic markers. The polymerase chain reaction (PCR) has been employed with unique flanking sequence primers to amplify DNA fragments containing STR loci. In recent years, the discovery and development of polymorphic STRs as genetic markers have stimulated progress in the elaboration of linkage maps, the identification and characterization of disease genes, and the simplification and precision of DNA typing (6,11,14,17,22,27). Short tandem repeat markers allow analysis of very small quantities of biological samples. They can be rapidly applied even with crude DNA purification methods, and results are easier to interpret than previous approaches such as detection of variable number of tandem repeat (VNTR) loci either by Southern hybridization or by analysis of amplification products (21,29).

In this work we describe a survey of 22 polymorphic STR loci to identify those most suited to rapid, simple and accurate analysis using silver-stain detection of PCR-amplified products to replace the more commonly used isotopic detection methods. In addition, allelic ladders (i.e., markers consisting of all or most of the known alleles for a particular locus) were constructed for nine selected systems to add precision and accuracy to the identification of alleles at each of these loci. Allelic ladders were applied to development of high-throughput methods allowing simultaneous evaluation of amplifications of several different loci or to multiple amplifications at a single locus in the same gel lane.

MATERIALS AND METHODS

DNA samples used to identify some of the allelic ladder components were kindly provided by individuals listed in the Acknowledgments. DNA isolation, PCR amplification, gel electrophoresis, silver-stain detection, DNA fragment purification and sequence analysis were performed as described previously (40,42). The oligonucleotide primer sequences, primer concentrations and amplification conditions for each locus are listed in Table 1.

RESULTS

In general, STR loci with trinucleotide and tetranucleotide repeats offer an advantage over dinucleotide repeat loci, as amplification products of the larger repeat motifs are less prone to the PCR artifact known as repeat slippage (25,44). This artifact manifests as the generation of extra amplification products that are one or more repeat units larger or smaller than the authentic alleles. Dinucleotide repeat loci frequently reveal multiple extra bands both larger and smaller than the authen-

Table 1. Primer Sequences, Primer Concentrations and Amplification Conditions for Loci Used in this Work

GenBank® Designation*	Primer Sequences	Primer Conc.	Amplification Protocol**	References
HSAC04 (ACTBP2)	Forward: AATCTGGGCGACAAGAGTGA Reverse: ACATCTCCCCTACCGCTATA	1 µM	2	11,22,36,49
HUMAPOA2 (APOA2)	Forward: GTGACAGAGGGAGACTCCATTAAA Reverse: GGAGCAGTCCTAGGGCCGCGCCGT	1 µM	2	11,22,24,52
HUMAPBM (APOB)	Forward: ACATTGATTCCTTGGAGTTTCTCTA Reverse: GGGCAAAAGAGCGAGACTCCATCTC	1 µM	2	51
HUMCRYGBC (CRYGBC)	Forward: CCGACAGAGTGAGACTCCATCTCA Reverse: ACAGGATCCTATCTTCTCAGGAGG	1 µM	2	15,38
HUMCSF1PO (CSF1PO)	*GenePrint*™ STR Systems - CSF1PO (Promega)	0.5 µM	1	14,40
HUMCYAR04 (CYAR04)	Forward: GGTGGGCAGGTACTTAGTTAGCTAC Reverse: GTTACAGTGAGCCAAGGTCGTGAG	1 µM	3	14
HUMSIRPODF (D7S460)	Forward: AATACCCCAAGGGGTGGTAA Reverse: CATTGATGAACAGTTCAAGCA	1 µM	2	18
HUMD21LOC (D21S11)	Forward: GTGAGTCAATTCCCCAAG Reverse: GTTGTATTAGTCAATGTTCTCC	1 µM	3	11,22,46
HUMF13A01 (F13A01)	*GenePrint* STR Systems - F13A01	0.1 µM	1	14,22,34,40,41
HUMBFXIII (F13B)	*GenePrint* STR Systems - F13B	1 µM	2	30,40
HUMFABP (FABP)	Forward: GTAGTATCAGTTTCATAGGGTCACC Reverse: CAGTTCGTTTCCATTGTCTGTCCG	1 µM	2	9,11,14,22,37
HUMFESFPS (FESFPS)	*GenePrint* STR Systems - FESFPS	1 µM	1,2	14,22,35,40
HUMFIBRA (FGA)	Forward: ATTATCCAAAAGTCAAATGCCCCATAGG Reverse: ATCGAAAATATGGTTATTGAAGTAGCTG	1 µM	2	28
HUMHPRTB (HPRTB)	*GenePrint* STR Systems - HPRTB	1 µM	2	9,11,14,16,40
HUMLIPOL (LPL)	*GenePrint* STR Systems - LPL	1 µM	2	14,40,53
HUMMYOPK*** myotonin protein kinase	Forward: GCTCGAAGGGTCCTTGTAGCCGGG Reverse: GATAGGTGGGGGTGCGTGGAGGAT	0.25 µM	1	12
HUMPLA2A1 (PLA2A1)	Forward: CTAGGTTGTAAGCTCCATGA Reverse: TTGAGCACTTACTCTGTGCC	1 µM	3	14,22,32
HUMRENA4 (RENA4)	Forward: AGAGTACCTTCCCTCCTCTACTCA Reverse: CTCTATGGAGCTGGTAGAACCTGA	1 µM	1	9,14
HUMTFIID (TFIID)	Forward: CAATGATGCCTTATGGCACTGGAC Reverse: TGTGAGAGTCTGTGAGTGGAAGAG	1 µM	2	33, ****
HUMTH01 (TH01)	*GenePrint* STR Systems - TH01	0.5 µM	1	9,11,22,39,40,42
HUMTPOX (TPOX)	*GenePrint* STR Systems - TPOX	0.2 µM	1	1,40
HUMVWFA31 (vWF)	*GenePrint* STR Systems - vWF	0.5 µM	2	11,22,23,26,31,40

*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, 70°C for 1.5 min.

Amplification program 3: 96°C for 2 min, then 10 cycles of 94°C for 1 min, 56°C for 1 min and 70°C for 1.5 min, followed by 20 cycles of 90°C for 1 min, 56°C for 1 min, 70°C for 1.5 min.

***myotonic muscular dystrophy

****Holly Hammond (personal communication)

tic allele (50). When separated by gel electrophoresis, the artifacts and true alleles in heterozygous individuals often overlap. Interpretation is difficult when the true alleles are of similar size. When observed at trinucleotide and tetranucleotide repeat loci, repeat slippage artifacts are usually limited to a single extra fragment one repeat length shorter than the dominant fragment representing the authentic allele (e.g., Figure 1, PLA2A1, RENA4 and vWF). The less complex pattern observed for PCR-amplified STR alleles provides confidence and speed in allele determination, especially when analyzing DNA of heterozygous individuals carrying alleles of similar size.

We have reviewed the quality of the STR amplification products generated for 5 individual systems carrying trinucleotide repeats (CRYGBC, FABP, myotonin protein kinase [myotonic muscular dystrophy], PLA2A1 and TFIID) and 17 individual tetranucleotide repeat loci. Each locus has been evaluated using one or more of the amplification protocols described in Table 1. An example of the typical amplification products for each locus is displayed in Figure 1.

Figure 1. Comparison of amplification products from 5 trinucleotide and 17 tetranucleotide repeat loci separated by denaturing polyacrylamide gel electrophoresis and detected by silver staining. In each case, DNA from two human cell lines, K562 and CCRF-SB, was amplified at the locus identified above the corresponding lanes and according to the protocols described in Table 1. Lanes marked (pGEM) contain pGEM™ DNA markers (Promega, Madison, WI, USA).

Amplified products were detected using a DNA silver-staining procedure described by Bassam et al. (2) after high-resolution separation by denaturing polyacrylamide gel electrophoresis (47). This is a very sensitive method that allows rapid evaluation of amplified STR fragments. As seen in Figure 1, this method sometimes reveals two fragments representing each allele (e.g., FESFPS and CYARO4). This is caused by differential strand migration for products of the same length but different sequence (10). The degree of differential migration of the opposing strands depends on the specific amplified sequences being separated. Thus, it varies with the locus being amplified, with the distance the fragments have been subjected to electrophoresis, with the concentration of the gel matrix and, to a certain degree, on the selection of primers for amplification. Some loci, such as CYAR04, FGA, TFIID and FESFPS, show significant differences, while others (e.g., CSF1PO and TPOX) reveal co-migration of the two fragments representing a single allele. Employing one radioactive- or fluorescent-labeled primer and one unlabeled primer allows the detection of only one of the two complementary strands (data not shown).

Silver-stain detection of STR loci reveals several artifacts. First, repeat slippage, described above, is seen to varying degrees with some of the tetranucleotide repeat loci (e.g., vWF, LPL, RENA4, FGA and TPOX). A second artifact appears as a light band one base below the primary allele fragment as seen with FABP and TPOX in Figure 1. Most likely this is caused by the terminal transferase activity of the *Taq* DNA polymerase adding a single nucleotide at the 3′ termini of the amplified PCR products as described by Clark (7) and also observed with STR amplification products by Kimpton et al. (22). The efficiency of this process varies among loci as indicated by the relative intensities of corresponding extra bands for the loci vWF, LPL and F13B.

Two artifacts that are not well understood are the production of extraneous high molecular weight bands associated with amplification of loci such as APOB, CRYGBC and PLA2A1 in

Table 2. Allelic Ladder Components

Locus	Chromosome Location	Repeat Sequence	Allele Size Range (bases)	Allelic Ladder Size Range (bases)	Allelic Ladder Component Names*
CSF1PO	5q33.5-34	AGAT	295–327	299–323	8,9,10,11,12,13,14
F13A01	6p24-25	AAAG	281–331	283–331	4,5,6,7,8,9,11,12,13,14,15,16
F13B	1q31-q32.1	AAAT	169–189	169–185	6,7,8,9,10
FESFPS	15q25-qter	AAAT	222–250	226–246	8,9,10,11,12,13
HPRTB	Xq26	AGAT	259–303	259–303	6,7,8,9,10,11,12,13,14,15,16,17
LPL	8p22	AAAT	105–133	105–133	7,9,10,11,12,13,14
TH01	11p15.5	AATG	179–203	179–203	5,6,7,8,9,10,11
TPOX	2p23-2pter	AATG	224–252	232–248	8,9,10,11,12
vWF	12p12-pter	AGAT	131–171	143–167	14,15,16,17,18,19,20

*Names of each allele represent number of repeats within the allele. Fourteen known alleles are not included in allelic ladders.

The TH01 allele 9.3 (198 bases), containing 9 full copies of the repeat plus 3 of the 4 bases of a tenth copy, is not included because it is only one base shorter than TH01 allele 10 (42).

The F13A01 allele 10 (307 bases) is not included because it is rare and its exclusion creates a gap which simplifies interpretation of the allelic ladder (41,43). The F13A01 allele 3.2 (281 bases) is not included because it is only 2 bases smaller than the F13A01 allele 4 (43).

The CSF1PO alleles 7 and 15, F13B allele 11, FESFPS alleles 7 and 14, TPOX alleles 6, 7, and 13 and vWF alleles 11, 13 and 21 are not included in these allelic ladders because they were not available at the time of ladder construction.

Figure 1 and the generation of several products shorter than the authentic allele that appear as extensive series of DNA fragments differing by one base (e.g., LPL, FABP). The first of these artifacts is often observed if too many cycles are included in the thermal cycling protocol, suggesting an illegitimate amplification event as the cause (19). The locus F13B sometimes displays these high molecular weight bands. When only one DNA strand is detected using a fluorescent analysis method, the extraneous high molecular weight bands have not been observed for this locus (data not shown). This suggests that either the unlabeled primer is involved or the high molecular weight bands do not contain a sufficient amount of fluorescent primer to be detected.

Several of the loci were selected for development of allelic ladders. An allelic ladder contains a mixture of many or all of the possible amplified alleles for a single locus. This mixture serves as a size standard allowing rapid and precise comparison of amplified sample DNAs with well-characterized allelic ladder components (41–43,45). Loci were chosen for ladder construction because *(i)* PCR-amplified alleles displayed few or no amplification artifacts; *(ii)* the alleles were easily detected by silver-stain analysis; and *(iii)* opposing PCR product strands of the same allele migrated close to one another in denaturing gels when compared with the strands of neighboring alleles. Nine loci located on separate chromosomes (Table 2) were selected because they are expected to be inherited independently in Mendelian fashion.

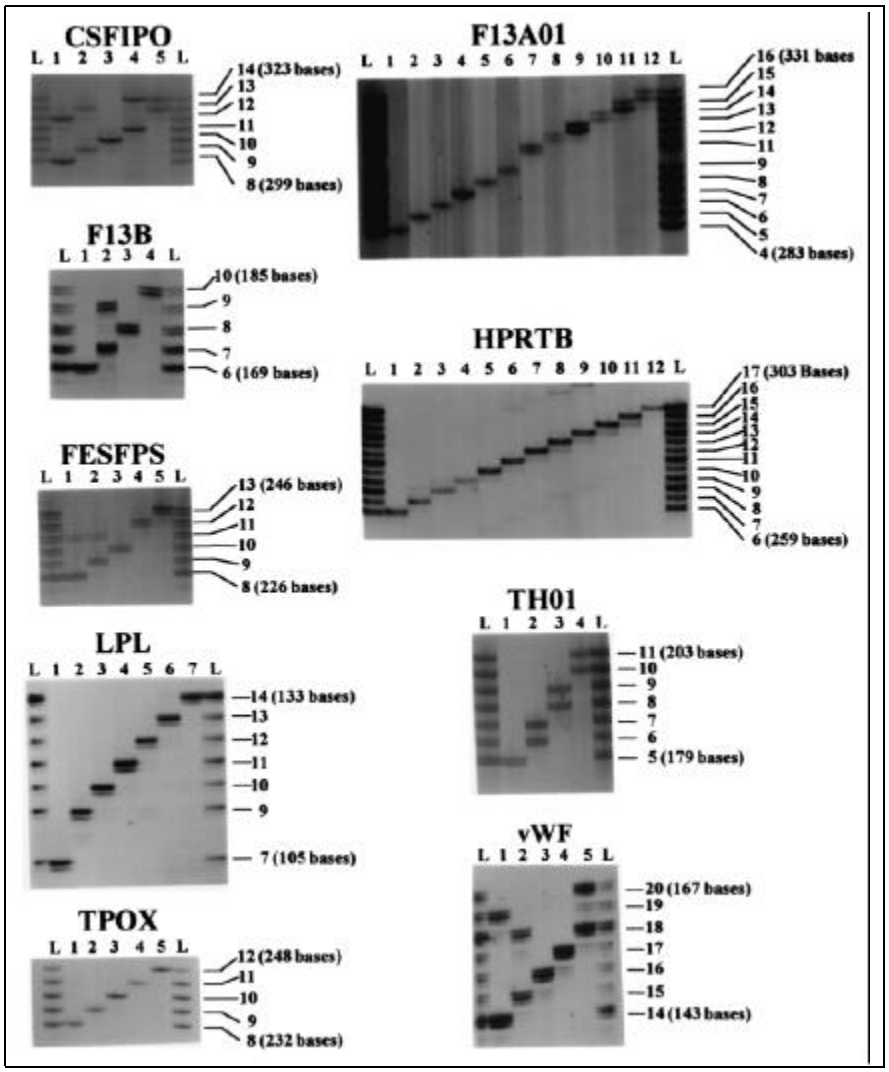

Figure 2. PCR-amplified fragments of allelic ladders and their components for nine STR loci separated by denaturing polyacrylamide gel electrophoresis and detected by silver staining. The name of each locus is associated with the corresponding photograph. Numbered lanes contain amplified material from DNA samples used to construct the respective ladders, while lanes labeled (L) contain the respective allelic ladders. The number of repeat units in each allele is displayed to the right of the corresponding allelic ladder. Two closely migrating fragments (doublets) are seen for each allele of the loci F13A01, F13B, FESFPS, LPL, vWF and TH01 due to sequence differences in their complementary DNA strands. Single fragments represent each allele for the STR loci CSF1PO, HPRTB and TPOX. The amplified alleles for the locus vWF sometimes display a third band that is probably created during PCR by addition of an adenine at the 3′ terminus of the PCR fragments by the terminal transferase activity of *Taq* DNA polymerase (7,22).

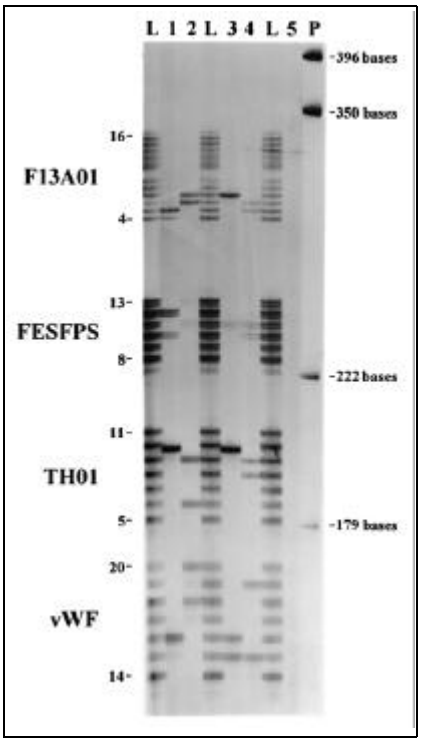

Figure 3. Simultaneous analysis of four separately amplified STR loci by denaturing polyacrylamide gel electrophoresis and silver stain detection. Genomic DNA samples, amplified separately at the loci F13A01, FESFPS, TH01 and vWF were mixed and simultaneously loaded onto the gel in lanes 1–4. A mixture of corresponding allelic ladders was loaded at the same time in lanes labeled (L). Lane (P) contains the pGEM DNA markers. Lane 5 contains a mixture of the negative control reactions (amplifications in absence of template DNA) for each locus. The sizes of the individual fragments of the pGEM DNA markers are shown to the right. Numbers next to each locus name identify the number of repeats in the largest and smallest components of the ladders.

Table 3. Characteristics of Loci Selected for Allelic Ladder Development

Locus	Number Tested		Heterozygosity (%)		PIC (4)		pM (20) Matching Probability		Typical PI		Power of Exclusion	
	Caucasians	Blacks	Caucasians	Blacks	Caucasians	Blacks	Caucasians	Blacks	Caucasians	Blacks	Caucasians	Blacks
CSF1PO	172	184	73	81	0.69	0.75	0.115	0.087	1.87	2.63	0.480	0.617
F13A01	174	175	67	78	0.69	0.78	0.114	0.069	1.50	2.24	0.379	0.557
F13B	207	220	75	73	0.67	0.71	0.143	0.101	2.03	1.86	0.516	0.479
FESFPS	182	164	71	73	0.65	0.71	0.148	0.106	1.75	1.82	0.451	0.469
HPRTB	80*	90*	66	70	0.74	0.73	0.086	0.086	1.48	1.67	0.373	0.428
LPL	189	174	71	72	0.62	0.71	0.166	0.099	1.75	1.81	0.451	0.467
TH01	186	185	81	80	0.74	0.72	0.098	0.106	2.58	2.50	0.611	0.599
TPOX	209	204	66	74	0.58	0.75	0.201	0.081	1.45	1.92	0.363	0.493
vWF	212	218	83	83	0.79	0.79	0.064	0.064	2.86	2.87	0.647	0.648

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) 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).

*Females only

Preference was also given to loci with frequencies of heterozygosity of at least 60% while displaying little or no known microheterogeneity (i.e., length variation that is smaller than one locus-specific repeat length). The presence of significant amounts of microheterogeneity can make calling of discrete alleles difficult. This negates one of the

major advantages of STR loci over classical VNTR loci. This point is illustrated with the locus ACTBP2 (49), which contains a four base repeat, AAAG, that is interrupted with a variety of sequences ranging in size from one to five bases (5,48). This locus also reveals alleles with deletions or insertions of variable length in the nearby flanking regions contained within the amplified segment. The result of all of this variation is that the amplified alleles of some loci do not always co-migrate with allelic ladder components, adding complexity and imprecision to the allele identification process (13).

Allelic ladders were constructed for each of the STR loci listed in Table 2 with the goal of including all known alleles with lengths corresponding to amplified fragments containing an integral number of copies of the tandemly repeated sequences. To identify the existing alleles, at least 75 DNA samples were surveyed. In some cases, DNA samples containing additional alleles were provided by individuals listed in the Acknowledgments. Amplified samples of homozygous or heterozygous individuals were mixed with gel-purified individual alleles in some cases to generate a mixture of alleles to be used as template for reamplification. In other instances, better results were achieved by amplifying subsets of the allele components and mixing just prior to use. The components of the allele mixtures for each of the nine allelic ladders are shown in Figure 2.

The nucleotide sequence of the repeat region of each amplified allele used in a ladder was determined. The characteristic repeat and the number of copies of the repeat in each ladder component are listed in Table 2, along with the allele size range for each locus and the size range of their allelic ladders. The lengths of the individual DNA fragments of the allelic ladders were determined by comparison with GenBank® sequence information. Constant lengths of both flanking regions for alleles from each locus were confirmed by sequencing of each allelic ladder component. For example, the A dideoxy termination reactions for the CSF1PO allelic ladder components were loaded onto adjacent lanes on a sequencing gel (43). Each allele differed from the next smaller one by the additional sequence pattern "A, gap, A, gap" representing the additional AGAT-repeat occurring in this allele (data not shown). No other differences were observed, indicating the lack of insertions or deletions in the sequences flanking the AGAT repeats. Similar results were obtained for the second flanking region of this locus and for both flanking regions of all allelic ladder components of the other eight loci.

Several aspects of genetic analysis are simplified with the application of allelic ladders. First, it is easier to call alleles with more confidence and precision. A quick inspection of Figure 2 illustrates the value of having a standard marker that consists of fragments generated by the same amplification process used for sample amplification. Alleles are called by rapid and direct comparison with the allelic ladder components rather than by measurement and calculation of fragment sizes. This method is also more accurate than comparison with classical size standards such as bacteriophage or plasmid markers. As we have already discussed, the speed of migration during electrophoresis is dependent upon sequence as well as size of the DNA fragment, so a single-size standard cannot accommodate the sequence variation present in all the loci being considered. In STR loci that display two fragments representing the opposing PCR product strands for each allele (e.g., Figure 2, F13B, FESFPS), interpretation is simplified because the same fragment pair is observed in both the individual DNA sample and the allelic ladder. As described above, the use of a single fluorescently labeled primer in amplification of samples or allelic ladders reveals a single fragment for each allele at each locus (data not shown) (45). This approach not only offers even greater clarity of interpretation but also requires expensive instrumentation for detection of the products. Locus-specific allelic ladders thus provide additional confidence for inter- and intra-laboratory comparisons that may include altered electrophoretic systems, buffer conditions or size standards.

Allelic ladders are also useful in the identification and characterization of microheterogeneity. The TH01 and F13A01 ladders were used to identify off-ladder alleles in several DNA samples. Sequence analysis confirmed the TH01 allele 9.3 was differentiated from allele 10 by a one-base deletion in the seventh copy of its AATG repeat, and the F13A01 allele 3.2 differed from allele 4 by a 2-base deletion outside, but adjacent, to the repeat unit (41–43). These alleles are easily distinguished from neighboring alleles when the appropriate allelic ladder is used as a standard.

These tools can also be applied in development of high-throughput analytical methods. For example, multiple samples amplified at a single locus can be amplified and loaded at different times with allelic ladders (40,41,43). Each sample co-migrates with the ladder loaded at the same time allowing for interpretation of many more samples on a single gel than would be possible with traditional marker systems. Likewise, the same sample may be amplified separately at several loci and the amplified samples mixed prior to gel electrophoresis. This mixture can be compared with a mixture of allelic ladders for the same loci. Interpretation is straightforward as long as the allele size ranges of the mixed loci do not overlap. An example displaying four loci, F13A01, FESFPS, TH01 and vWF, mixed following amplification and analyzed in a single gel is illustrated in Figure 3.

During preparation of this work, Holly Hammond (personal communication) at the Baylor College of Medicine (Houston, TX) and Steve Creacy and Robert A. Bever (personal communication) at Genetic Design (Greensboro, NC) have generated population data and provided us with genotype frequencies for the STR systems for which we have developed allelic ladders. Their data and other published data (14,30) were used to calculate the percentage of heterozygosity, polymorphism information content (PIC), matching probability (pM), typical paternity index (PI) and power of exclusion for these loci. These calculations are displayed in a summary format in Table 3 to allow a rapid evaluation of the different loci for use in forensic, paternity and linkage mapping

applications.

CONCLUSIONS

Although they are abundant in the human genome, not all STR loci are suitable for use in identity testing. Poor PCR product yield, PCR artifacts and sequence microheterogeniety can make the interpretation of results difficult. In our survey of 22 STR loci, only nine were considered for additional development. Allelic ladders for these nine loci were developed and characterized.

Allelic ladders allow rapid determination of alleles by direct comparison of the migration distances without a requirement for measurement or calculation. This improves the usefulness of these STR systems for forensic DNA analyses, parentage assessment, cell line authentication, bone marrow transplantation monitoring and linkage analyses.

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Correction

The ART® filtered pipet tips mentioned in "Use of Filtered Pipet Tips to Elute DNA from Agarose Gels" (BioTechniques *18*:980, 1995) were incorrectly referred to as a product of Continental Laboratory Products. In actuality, the ART tip is manufactured by and patented by Molecular Bio-Products, Inc. (San Diego, CA, USA). Additionally, the self-sealing feature of the ART tip is designed for use in *preventing* PCR carryover contamination, not for allowing the passage of DNA.