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A 26plex Autosomal STR Assay to Aid Human Identity Testing*†

ABSTRACT: A short tandem repeat multiplex assay has been successfully developed with 25 autosomal loci plus the sex-typing locus amelogenin for a total of 26 amplified products in a single reaction. Primers for the loci were designed so that all of the amplicons present were distributed from 65 base pairs (bp) to less than 400 bp within a five-dye chemistry design with the fifth dye reserved for the sizing standard. A multiplex design strategy was developed to overcome challenges encountered in creating this assay. The limits of the multiplex were tested, resulting in the successful amplification of a wide range of genomic DNA sample concentrations from 2 ng to as low as 100 pg with 30 cycles of PCR. The 26plex has the potential to benefit the forensic community for reference sample testing and complex relationship evaluation.

KEYWORDS: forensic science, short tandem repeat, DNA, miniSTR, multiplex, polymerase chain reaction, D1GATA113, D1S1627, D1S1677, D2S441, D2S1776, D3S3053, D3S4529, D4S2364, D4S2408, D5S2500, D6S474, D6S1017, D8S1115, D9S1122, D9S2157, D10S1248, D10S1435, D11S4463, D12ATA63, D14S1434, D17S974, D17S1301, D18S853, D20S482, D20S1082, D22S1045

Multiplex polymerase chain reaction (PCR) is commonly used for forensic DNA typing purposes. Simultaneous amplification of specific target regions of the genome enables "bar code"-like identification of individuals. Commercial short tandem repeat (STR) assays amplify the Combined DNA Index System (CODIS) STR loci that are required for data entry into the national level of the U.S. DNA database (1,2). Additional loci can play a useful role in the forensic community including situations involving relatives such as missing persons/mass disaster victim identification (3), immigration testing with limited reference samples (4,5), deficient parentage testing which is often needed if only one parent and child are tested (6), and in cases involving incest (7). Relationship testing labs are pushed to answer more difficult genetic questions and it is important that well characterized STR loci are available to address these issues.

A single amplification five-dye multiplex PCR assay has been developed to combine 25 autosomal STR loci plus the sex-typing marker amelogenin (AMEL) in one reaction to enable rapid analysis of reference samples. These new STR loci were described previously as mini-STR loci (8,9). These loci were selected because they span unused chromosomal locations across the 22 autosomes and have been characterized so that they may be combined without conflicting with the current 13 CODIS loci (8,9). This paper describes how to construct a large STR multiplex so that others can understand the technology and how to resolve difficulties that are encountered throughout the process.

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Materials and Methods

Multiplex Design

A two-dimensional multiplex schematic was created to illustrate the spatial (in base pairs, *x* axis) and spectral (four fluorescent dye labels, *y* axis) design of the multiplex. This assay was assembled according to a strategy previously described (10). There were eight loci that used the original miniSTR primer sequences in the initial multiplex design and did not require a redesign. The primers for the 18 remaining loci were redesigned to fit in the multiplex. Figure 1 displays the first generation design and Fig. 2 displays the final multiplex design.

The sex-typing locus AMEL was added to the multiplex for a total of 27 potential loci. The AMEL primers used in this multiplex were different from those commonly used in commercial kits. The new AMEL primers resulted in PCR product sizes at *c.* 80 and 83 bp for the X and Y chromosome, respectively (11). The addition of AMEL allowed the multiplex to be used for gender identification when testing different sets of samples.

Multiplex Design Software

LaserGene software (DNA STAR, Madison, WI) was used to map the genome sequence of the 26 loci. Primer3 software was used for primer design (12). Parameters were defined in Primer3 to allow for fixed PCR product sizes and narrow annealing temperature ranges (58–60°C are optimal) (10). All other settings were the default values. An alternate primer design program called Visual OMP, v6.2.2.0 (DNA Software, Inc., Ann Arbor, MI) was used for primer redesigns if Primer3 was unsuccessful. AutoDimer software was used to screen candidate primer sequences for interprimer compatibility (13). BLAST nucleotide (BLASTn) searches were performed to determine if the primer pairs were targeting the correct sequences in the genome (14).

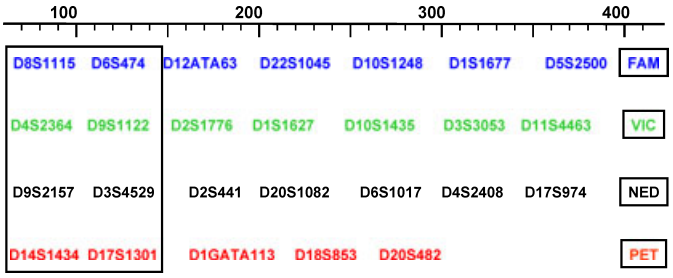

FIG. 1—*Initial 26plex design layout. The eight-boxed loci use the original miniSTR PCR primers. All unboxed loci use primers that were redesigned. Fluorescent primer labels are listed to the far right in boxes. STR, short tandem repeat. FAM = 6FAM.*

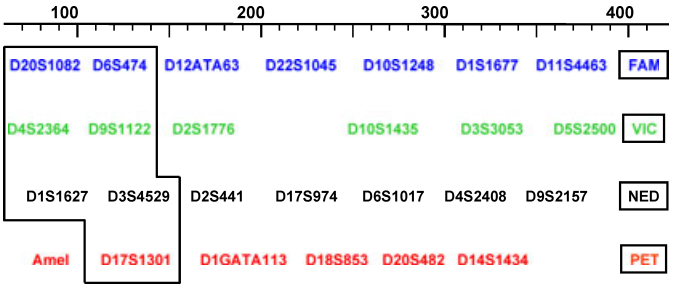

FIG. 2—*Final 26plex design layout. The seven boxed loci use the original miniSTR PCR primers. Amelogenin (AMEL) uses the published primers (11). All unboxed loci use primers that were redesigned. Fluorescent primer labels are listed to the far right in boxes. STR, short tandem repeat. FAM = 6FAM.*

The final primer sequences and concentrations are listed in Table 1. All of the forward primers were labeled at the 5′ end with either 6FAM™, VIC™, NED™, or PET™ (Applied Biosystems, Foster City, CA) fluorescent dyes as determined by the locus amplicon placement within the multiplex design. The reverse primers (Qiagen Operon, Alameda, CA) were unlabeled, with an additional guanine base (G) added to the 5′ end to promote full adenylation (3,15) (Table 1, noted in bold and underlined). The initial concentration of the forward and reverse primers was 0.2 μM; however, the amounts of several forward and reverse primers were empirically increased or decreased to generate balanced PCR products (as demonstrated by peak heights).

PCR Amplification

A set of in-house DNA samples that have been previously described (16–19) were used in these studies. PCR amplification was carried out on a GeneAmp® 9700 (Applied Biosystems) using 1 ng of sample DNA, 1× GeneAmp® PCR Gold buffer (Applied Biosystems), 2 mM $MgCl_2$ (Applied Biosystems), 250 μM dNTPs (USB Corporation, Cleveland, OH), ~0.2 μM dye-labeled forward (Applied Biosystems) and reverse (Qiagen Operon) primers (see Table 1 for primer sequences and exact concentrations), 1 Unit AmpliTaq Gold DNA polymerase (Applied Biosystems), and 0.16 mg/mL bovine serum albumin (Sigma-Aldrich, St. Louis, MO) in a 20 μL total reaction volume. The PCR amplification conditions using the GeneAmp 9700 were denaturation for 11 min at 95°C, amplification for 30 cycles of 45 sec at 94°C, 2 min at 59°C, and 1 min at 72°C, extension for 60 min at 60°C, and a final soak at 25°C.

Analysis on the ABI 3130xl Genetic Analyzer

Amplification products were diluted in Hi-Di™ formamide (Applied Biosystems) by adding 1 μL PCR product and 0.3 μL GS500-LIZ internal size standard (Applied Biosystems) to 8.7 μL of Hi-Di. The samples were analyzed on the 16-capillary ABI Prism® 3130xl Genetic Analyzer without prior denaturation of samples. Prior to testing, a 5-dye matrix was established under the "G5 filter" with the five dyes of 6FAM, VIC, NED, PET, and LIZ. POP™-6 (Applied Biosystems) polymer rather than POP™-4 was utilized for higher resolution separations on a 36 cm array (Applied Biosystems) with 1× A.C.E. buffer (Amresco, Solon, OH). Samples were injected electrokinetically for 10 sec at 3 kV. The STR alleles were then separated at 15 kV at a run temperature of 60°C. Data from the ABI 3130xl were analyzed using GeneMapper®*ID* v3.2 (Applied Biosystems). The bins and panels for the multiplex are available on STRBase (20).

Sensitivity Study

A sensitivity study was performed with the final 26plex using a highly characterized DNA sample. The conventional concentration of this sample was 52.4 ng/μL, verified using SRM 2372, Human DNA Quantitation Standard (21). Dilutions were made using distilled water (diH_2O) with the following amounts of template DNA and were evaluated in a 20 μL PCR reaction using the PCR parameters described previously: 2 ng, 1 ng, 750 pg, 500 pg, 400 pg, 300 pg, 250 pg, 200 pg, 100 pg, 50 pg, 25 pg, and 0 pg (diH_2O only). The prepared dilutions were amplified in triplicate using three different thermal cycling protocols of 28, 30, and 32 cycles.

Results and Discussion

Multiplex Design Challenges

The criteria for primer "failure" in this study is defined as those that produce profiles that exhibit incomplete adenylation, the presence of PCR artifacts, nonspecific products, low signal, or no PCR product at all. Initially, there were seven primer pairs out of a total of 26 that were potential failures in singleplex and had to be investigated further. The allele peaks for D2S1776 and D5S2500 had unusually low relative fluorescence unit (RFU) signal (<600 RFUs), the profile for D3S3053 had multiple nonspecific products with no obvious allele peaks present, and the allele peaks for D9S2157 had incomplete adenylation in singleplex PCR. The profiles for D14S1434, D17S974, and D20S1082 exhibited at least one artifact in addition to the allele peaks and were candidates for redesign.

There were several sets of primers that initially exhibited promising results in singleplex (19 total). These were combined together and tested in multiplex. This test 19plex indicated a high degree of baseline noise and many profiles displayed the features of failure. This was due to multiplex primer incompatibility. Thus, determining the maximum number of primers that would successfully amplify together in multiplex became the focus. This was established by grouping the primers by common forward dye labels and amplifying these in a smaller multiplex. This allowed identification of the primers causing difficulty in the larger multiplex. Once the successful primers were determined, they were combined together. In this assay development there were nine "core" sets of primers out of a possible 26 (D1S1677, D2S441, D3S4529, D4S2364, D4S2408, D6S1017, D9S1122, D10S1435, and D11S4463) that were initially compatible in multiplex. The remaining primer pairs were tested individually with the core multiplex to determine their

TABLE 1—*26plex PCR primers used in this study.*

Locus Name	Forward Dye Label	Primer Sequence (5′–3′)	Primer Concentration, μM	GenBank accession
D1GATA113	PET	F: acattaagcacatgcctctttgt	1	Z97987
		R: **G**atgaactcattggcaaaagga	1	
D1S1627	NED	F: catgaggtttgcaaatactatcttaac	2	AC093119
		R: **G**ttttaattttctccaaatctcca	2	
D1S1677	6FAM	F: gcagtcagcttgattgatcc	3	AL513307
		R: **GTTTCTT**agaatgcaatagcaaatatcagaatg	3	
D2S441	NED	F: caaaaggctgtaacaagggcta	1	AC079112
		R: **G**ttcactctccttcccaaatgtt	1	
D2S1776	VIC	F: ttacctgtgagtatgtgtgcgta	1.5	AC009475
		R: ggtgctaggtgtgctcagga	1.5	
D3S3053	VIC	F: tgacacaaatggaccaagaca	2	AC069259
		R: **GTTTCTT**gagagagcccttgaaatagca	2	
D3S4529	NED	F: cccaaaattacttgagccaat	0.75	AC117452
		R: **G**agacaaaatgaagaaacagacag	0.75	
D4S2364	VIC	F: ctaggagatcatgtgggtatgatt	0.75	AC022317
		R: **G**cagtgaataaatgaacgaatgga	0.75	
D4S2408	NED	F: agctgacatcttaccacatgttc	2	AC110763
		R: **G**tgcttggcatatattaagacactgta	2	
D5S2500	VIC	F: gtttactgataaaccaaatgatgtgc	2	AC008791
		R: **G**taacttaaagggtaaatgtttgcag	2	
D6S474	6FAM	F: ggttttccaagagatagaccaatta	1.5	AL357514
		R: **G**tcctctcataaatccctactcatatc	1.5	
D6S1017	NED	F: agatgggaacgatgcagaca	2	AL035588
		R: gcataaatggatgggtggat	2	
D8S1115	PET	F: gcaccatttcacatatcca	6	AC090739
		R: Gcagtctcctaagtagc	6	
D9S1122	VIC	F: gggtatttcaagataactgtagatagg	0.75	AL161789
		R: **G**cttctgaaagcttctagtttacc	0.75	
D9S2157	NED	F: gatcacgccacggta	5	AL162417
		R: **G**ttctccatttcaaagatcat	5	
D10S1248	6FAM	F: cagtaaaaagcaaacctgagca	1	AL391869
		R: gcttggcaaagagcagatg	1	
D10S1435	VIC	F: cacgttgggtttcctgactt	1	AL354747
		R: **G**cccagctacttgggatgcta	1	
D11S4463	6FAM	F: ctgtcccaaggctgagtgtt	3	AP002806
		R: **GTTTCTT**cgcagggcaaataaaaagaa	3	
D12ATA63	6FAM	F: aggtggcagtgagctgtaac	1	AC009771
		R: **GTT**tcttggattttgagggccta	1	
D14S14343	PET	F: ggctctgatttccaccactg	2	AL121612
		R: **G**caactcttggaagcccagtc	2	
D17S974	NED	F: ggaacacttgagcca	2	AC034303
		R: gtggactggggtaagg	2	
D17S1301	PET	F: aagatgaaattgccatgtaaaaata	2	AC016888
		R: **G**tgtgtataacaaaattcctatgatgg	2	
D18S853	PET	F: acatatataatgtgagaaaggaggagt	2	AP005130
		R: **G**ttaatgggtgcaacacacc	2	
D20S482	PET	F: ctccattctctcacacccaat	1	AL121781
		R: **G**cacttctggcttttctggttc	1	
D20S1082	6FAM	F: acatgtatcccagaacttaaagtaaac	1	AL158015
		R: **G**cagaagggaaaattgaagctg	1	
D22S1045	6FAM	F: ccctgtcctagccttcttatagc	1	AL022314
		R: **G**ctgtgcccaagttgagagaa	1	
Amelogenin	PET	F: ccctttgaagtggtaccagagca	2	M55418, M55419
		R: gcatgcctaatattttcagggaata	2	

The fluorescent dye labels are listed for the forward primers only. The reverse primers are unlabeled. The 5′ guanine (G) residue or ''PIGtail'' (GTTTCTT) sequence in each reverse primer was added to promote adenylation (3,15) and are underlined in bold print. The approximate primer concentrations are listed in μM (concentration in 20 μL total volume). The GenBank accession numbers were obtained from nucleotide searches through NCBI.

compatibility. Compatible primer combinations were added to the core if the profile was successful (Fig. 3*A*), or removed for redesign if the profile demonstrated a failure (Fig. 3*B*).

This ''adding to the core'' strategy was the basis of the empirical design of the multiplex. In this work, nine primer pairs (D1GATA113, D2S1776, D5S2500, D6S474, D10S1248, D12ATA63, D17S1301, D20S482, and D22S1045) were successfully added to the core multiplex for a total of 18 loci represented. Even though the allele peaks for D2S1776 and D5S2500 had low signal in singleplex, the primer pairs were compatible when tested in multiplex. There were eight sets of primers that were initially incompatible in multiplex. The allele peaks for D1S1627 appeared to exhibit extreme heterozygote imbalance, although this eventually turned out to be an artifact associated with a true homozygote. The primer pairs for D8S1115, D17S974, and D18S853 resulted in the presence of artifacts within the multiplex profile. The allele peaks for D9S2157 and D20S1082 displayed incomplete adenylation. The primers for D3S3053 created multiple nonspecific products, and the primers for D14S1434 caused absent signal from all alleles at all loci (including itself). Once all of the compatible primer pairs were added to the core multiplex, primers for the remaining eight loci were redesigned.

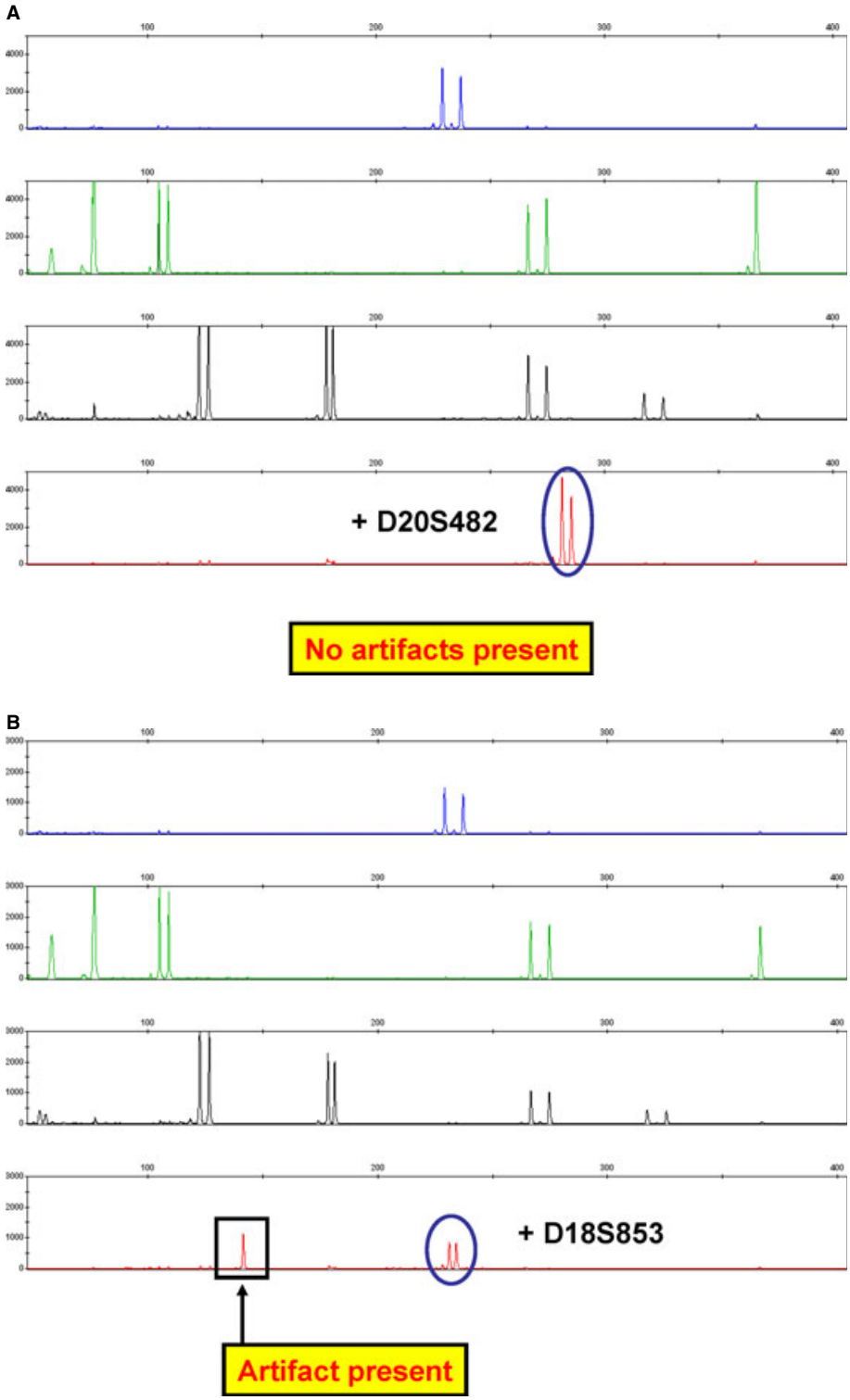

FIG. 3—*(A) Example of the core multiplex (nine loci) with the addition of D20S482 primers (D20S482 allele peaks are circled). These primers did not result in artifacts. (B) Example of the core multiplex (nine loci) with the addition of D18S853 primers (D18S853 allele peaks are circled). These primers created an artifact detectable in the PET channel (boxed).*

Primers were redesigned for three of the eight loci (D9S2157, D14S1434, and D17S974) in different PCR amplicon positions than the original design. The D1S1627 and D20S1082 amplicons were able to fit in the multiplex using the miniSTR primer sequences (8) with different dyes attached to the forward primers. The D3S3053 primers were redesigned but the amplicon size remained in the original position.

Incompatible primer sequences were the cause of one or more PCR artifacts present in the multiplex profile. The original primers for D18S853 and two sets of primers for D17S974 are examples. The incompatible primer combined with other primers in the multiplex and nonspecific binding to random parts of the genome occurred during amplification, ultimately causing the artifacts. To resolve this problem, the primer that is the origin of the artifact

was determined. The aberrant set of primers was established when added to the "core" multiplex, because this was when the artifact or multiple artifacts were observed. It is important to determine if it is the forward or reverse primer that is the cause. The forward and reverse primers were spiked into the multiplex primer mix separately. If the forward primer was the source of the artifact, the fluorescent dye was switched to the reverse primer to mask the artifact. The artifact was still present, but not fluorescently labeled and therefore not detected in the profile. If the reverse nonlabeled primer was the origin of the artifact, the primer set was redesigned.

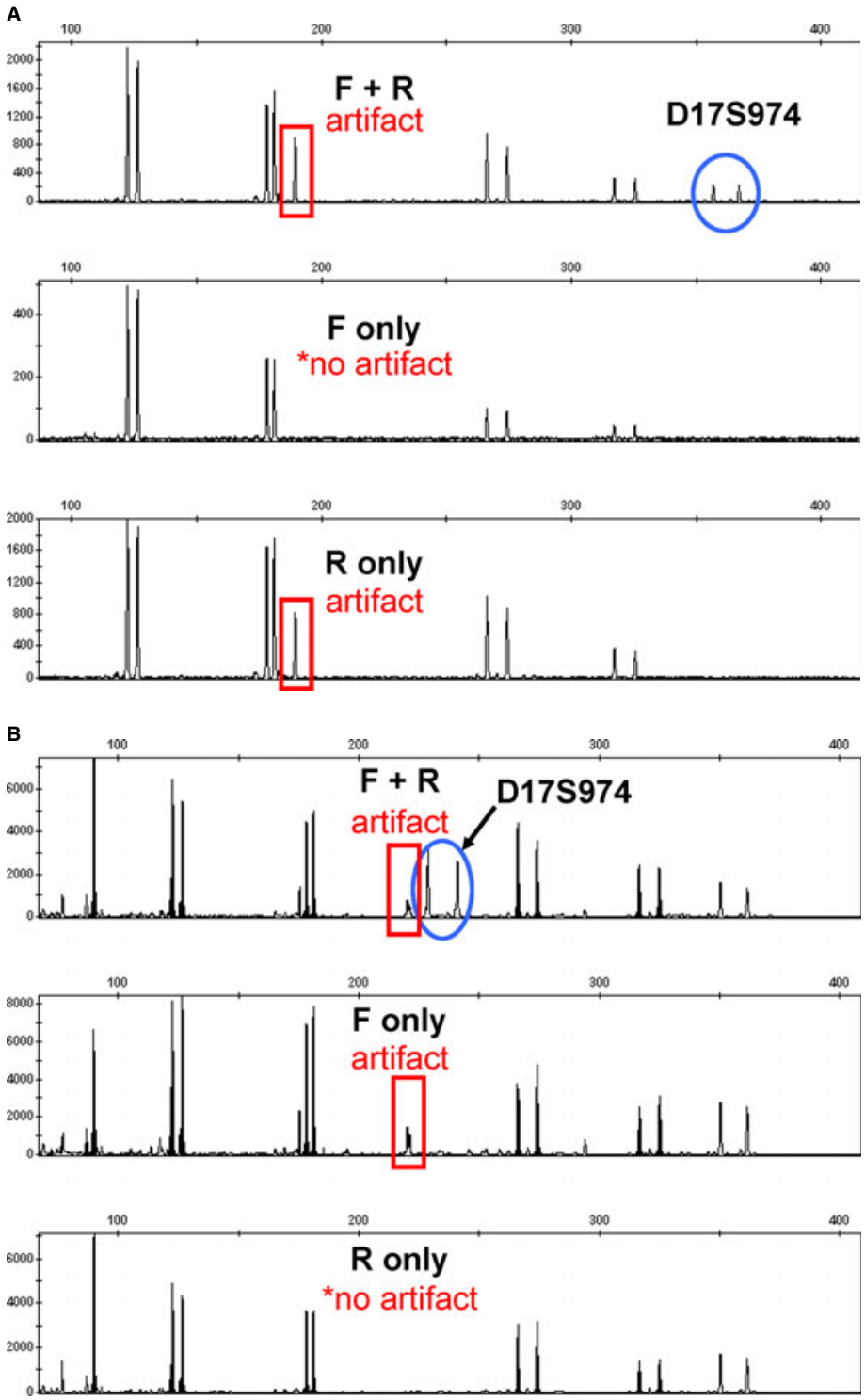

FIG. 4—*(A) Example of the D17S974 original primer set with an artifact caused by the reverse primer. The top panel shows the forward and reverse (F + R) primers added (D17S974 allele peaks are circled) with the artifact present (boxed). The middle panel shows the forward (F only) primer added with no artifact present. The bottom panel shows the reverse (R only) primer added with the artifact present (boxed). (B) Example of the D17S974 redesigned primer set with an artifact caused by the forward primer. The top panel shows the forward and reverse (F + R) primers added (allele peaks are circled) with the artifact present (boxed). The middle panel shows the forward (F only) primer added with the artifact present (boxed). The bottom channel shows the reverse (R only) primer added with no artifact present.*

An example of both situations is illustrated with the D17S974 locus (Fig. 4*A* and *B*).

If alleles from a locus were exhibiting incomplete adenylation, a "PIG-tail" (5′-GTTTCTT-3′) was added to the 5′ end of the reverse primer to help promote adenylation (3,15). There was success with four loci using the "PIG-tail" approach: D1S1677, D3S3053, D11S4463, and D12ATA63. Upon adding the additional bases to these reverse primer sequences, there were no further instances of incomplete adenylation with these loci.

When negative controls were amplified, the primer pairs exhibited dye artifacts in the electropherogram. The dye artifacts were identified because these peaks were fairly broad and possessed the spectrum of one of the dyes used for genotyping (3). Dye artifacts were removed post-PCR using gel filtration cartridges such as Edge Columns (Edge Biosystems, Gaithersburg, MD). In addition, reconstituting the fluorescently labeled primers using TE (10 mM Tris, 0.1 mM EDTA, pH 8) buffer as opposed to distilled water helped to reduce the number of dye artifacts present in the final multiplex.

Once the final primer sequences were optimized, empirical adjustments of primer concentration were performed for locus-to-locus balancing. Typically, the primers for the largest amplicons were increased to the highest concentrations. Conversely, the primers for the smallest amplicons were decreased to lower concentrations to avoid spectral bleed-through occurring in other dye channels. Due to lot-to-lot variation, it is valuable to quantitate the primers using UV–VIS spectroscopy when they are received to ensure reproducibility of preparing multiplex primer mixes. The primer concentrations listed in Table 1 serve as guidelines but may require further balancing. Table 2 provides a summary of the number of primer designs for each locus.

TABLE 2—*Primer designs for each locus used in the 26plex.*

Locus	No. Primers Tested Forward	No. Primers Tested Reverse	Total Primers Tested Per Locus
D1GATA113	1	1	2
D1S1627	2	2	4
D1S1677	2	4	6
D2S441	1	1	2
D2S1776	1	1	2
D3S3053	2	4	6
D3S4529	1	1	2
D4S2364	1	1	2
D4S2408	1	1	2
D5S2500	3	3	6
D6S474	1	1	2
D6S1017	1	1	2
D8S1115	**15**	**14**	**29**
D9S1122	1	1	2
D9S2157	7	7	14
D10S1248	1	1	2
D10S1435	1	1	2
D11S4463	2	2	4
D12ATA63	1	2	3
D14S1434	2	2	4
D17S974	7	7	14
D17S1301	1	1	2
D18S853	3	3	6
D20S482	1	1	2
D20S1082	9	10	19
D22S1045	1	1	2
Amelogenin	1	1	2
Total designed	70	75	145
Total used	26	26	52
Total unused	44	49	93

The primer pairs for 16 loci used the original primer pairs designed. The primer pairs for 11 of the loci had to be redesigned multiple times. The locus boxed and in bold print was ultimately not used in the 26plex (D8S1115).

The Final 26plex

In the final 26plex, there were seven loci that used the original miniSTR primer sequences. The primers for the 18 remaining loci were redesigned to fit into the multiplex. Amelogenin was added and D8S1115 was removed for a total of 26 loci (Fig. 2). The 26plex can be used routinely with 1 ng of DNA for 30 cycles of amplification (Fig. 5). Amplicon sizes ranged from ~65 to 400 bp and the forward primers were labeled with four fluorescent dye labels. It is a robust STR multiplex assay with balanced heterozygote peak heights and adequate interlocus peak balance. No PCR or fluorescent dye artifacts were present that could interfere in the correct genotyping of DNA samples.

Further Testing with the 26plex

To determine the minimal amount of DNA sample that can reliably be used with this multiplex assay, a sensitivity study was performed. For 28 cycles, the lowest DNA template amount where all peaks were detected above 50 RFUs was 200 pg. For 30 cycles, the lowest DNA amount under the same parameters was 100 pg and for 32 cycles it was 50 pg.

At 28 cycles, there was no evidence of spectral bleed-through with any of the DNA amounts. There was an artifact peak present within the D4S2364 allele range at ~70 bp (between 200 and 450 RFUs) that was not noted initially because optimization experiments were performed with 1 ng DNA template and 30 cycles. However, the artifact peak did not interfere with allele calling. The lower the amount of DNA, the more apparent the artifact peak became as the locus specific peak heights decreased. At 750 pg and lower amounts of DNA, the allele peaks at AMEL were lower than the peaks at other loci. AMEL was the first locus to drop-out at 100 pg.

At 30 cycles, there was spectral bleed-through between several loci with 2 ng DNA. At 400 pg or higher, the D4S2364 artifact peak was disproportionately lower than the allele peaks for this locus. Below 400 pg, the artifact signal was proportionate to the allele peaks. The AMEL peak heights were lower than the allele peaks of other loci with 300 pg or lower.

At 32 cycles, there was higher peak signal across many of the STR loci resulting in spectral bleed-through between dye channels from 2 ng to 750 pg. There was incomplete adenylation for several of the allele peaks for certain loci (D3S4529, D9S1122, D10S1248, D10S1435, D14S1434, and D20S1082). Poor adenylation efficiency was present until very low amounts of DNA were amplified, such as 100 pg or lower. The D4S2364 artifact peak was disproportionately lower than the allele peaks for this locus at all of the DNA amounts with 32 cycles. The AMEL peak signal was balanced with the allele peaks at all other loci for all DNA template amounts. Data and information from this sensitivity study could be found on STRBase (20).

Genotyping and allele sequencing were performed with SRM 2391b, PCR-based DNA Profiling Standard for all the components including 10 genomic DNA samples and 2 cell lines (12 samples total, No.: 1–12) using the 26 additional loci. Certified and reference values were assigned to all resulting alleles and the Certificate of Analysis was updated to include this new information (22). These values can serve as a way to calibrate the genotypes observed when analyzing data from the 26plex (23).

A modified version of the multiplex (with 22 autosomal loci and AMEL—23plex) was used in concordance evaluations with the miniSTR data (8,9) to determine the presence of null alleles, a mutation rate study with father/son sample pairs to examine

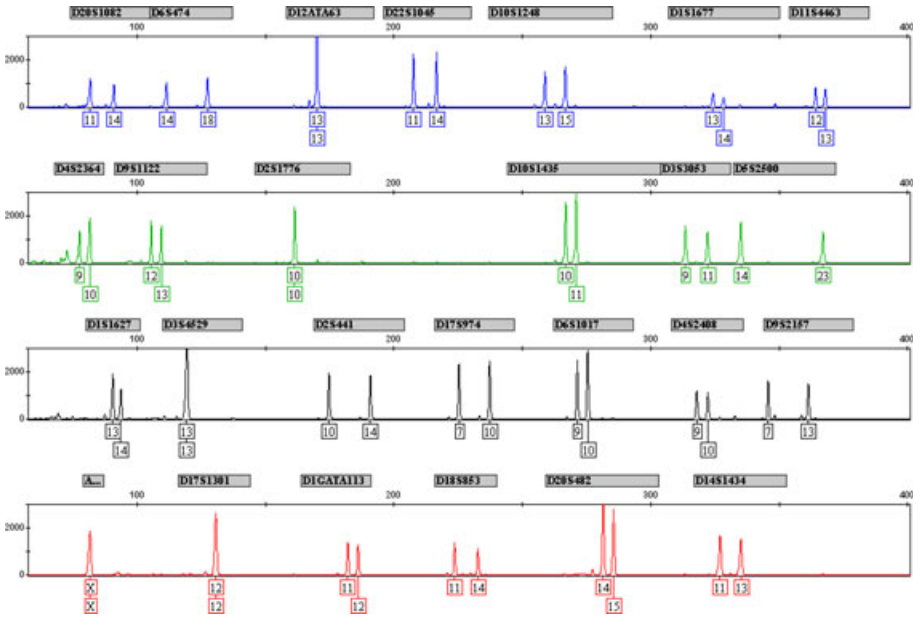

FIG. 5—*The final 26plex with 25 autosomal loci + the sex-typing locus amelogenin for a total of 26 loci. Positive control, 9947A, was used at 1 ng for 30 cycles.*

mutation rates with these primer pairs, and an extended family study to determine how extra-autosomal loci affected close family relationship forensic testing. The results from these studies were all discussed previously (24). All relevant data collected in our lab for the 26plex loci is available on STRBase (20).

Conclusions

This paper outlines the development of a single amplification five-dye multiplex that contains 25 unlinked autosomal loci plus the sex-typing locus AMEL. The main purpose of building this multiplex is for use with reference samples in a variety of situations such as paternity testing/kinship analysis, immigration testing, and for use in missing persons/mass disaster cases. Primers for a majority of the 25 autosomal STR loci were redesigned from the miniSTR loci described previously (8,9) and have already been established to be unlinked from the 13 CODIS loci and as being diverse with moderate-to-high heterozygosity values. They were comparable with the CODIS loci in terms of heterozygosities (8) with slightly lower mutation rates (24), suggesting that this STR multiplex will serve as a useful complement to the presently available commercial STR typing kits for the forensic analysis of DNA samples.

Multiple strategies were used to overcome various challenges that arose while building this multiplex such as incomplete adenylation, nonspecific products, artifacts, and simple primer incompatibility. To date, this multiplex represents the most STR loci typed together in a single PCR amplification assay. Working through the design process and optimization of the 26plex has allowed us to identify useful strategies for developing future large multiplexes.

The development of this 26plex assay provides support for the use of additional loci for challenging problems within the human identification community. Certified and reference values were assigned to all alleles of the 12 components of SRM 2391b for the 26 additional loci (23). The Certificate of Analysis was updated to include this information (22). This allows for the calibration of all genotypes resulting from analysis with the 26plex.

In the development of the 26plex, the effectiveness of the previously described miniSTR loci has been maximized by arranging and redesigning these loci into a single amplification multiplex. The use of additional loci with this approach can benefit the human identity testing community for reference purposes and relationship testing.

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