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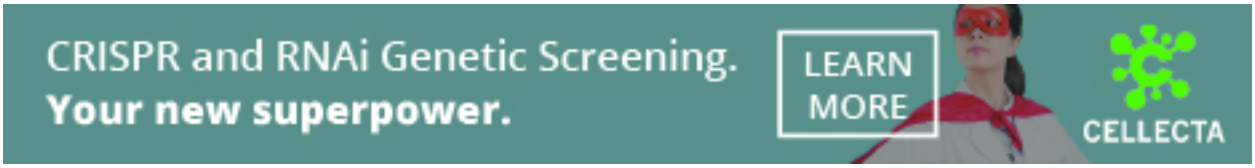

Characterization of Human AFLP Systems Apolipoprotein B, Phenylalanine Hydroxylase, and D1S80

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Methodology is presented for amplified fragment length polymorphism (AFLP) typing using a nonisotopic, PCR protocol. Human variable number tandem repeat (VNTR) loci used for identification in forensic and paternity testing were optimized for reaction and thermalcycling parameters. Loci analyzed were the apolipoprotein B (APOB) 3′ hypervariable region (HVR), phenylalanine hydroxylase 3′ HVR (PAH), and D1S80. Coamplification of a monomorphic β-globin fragment serves as an amplification control. Biotin is integrated into PCR amplicon through primer incorporation. AFLP products undergo agarose gel electrophoresis and Southern transfer to a nylon membrane. Amplicons were detected using a streptavidin–enzyme conjugate. Either colorimetric- or chemiluminescent-developed bands are genotyped using locus-specific allele ladders with known VNTR repeat numbers. Using this methodology, we have successfully typed >500 individuals from three population groups for each locus during data basing and casework.

The use of amplified fragment length polymorphism (AFLP) systems for human identification in forensic and parentage testing is expanding rapidly.[1–6] This report describes a systematic approach to human identity testing using nonisotopic PCR technology[7] and modified enzyme immunoassay detection. PCR reaction and thermal cycling parameters were optimized for three AFLP loci: apolipoprotein B 3′ hypervariable region (APOB HRV),[8–10] D1S80,[11–14] and phenylalanine hydroxylase 3′ HVR (PAH).[15,16] Coamplification of a monomorphic 1327-bp β-globin fragment[17] with each APOB and PAH reaction assured that amplifiable DNA was present and inhibitors were absent in each AFLP reaction. Parameters were optimized from those published previously by the following differences: use of biotinylated primer, direct detection of blots, reduction of genomic template DNA, β-globin coamplification, and sample temperature monitoring during thermal cycling. The parameters titrated for each locus were concentrations of $MgCl_2$ primers, *Taq* polymerase, and template DNA, along with annealing temperature, extension time, and number of PCR cycles. Standard conditions were established prior to AFLP data basing.

One objective of this study was to simplify and streamline the methodology for AFLP typing. This was done using an inorganic DNA extraction procedure, PCR amplification with biotinylated primers, agarose gel electrophoresis, Southern transfer, and direct detection with either a colorimetric or chemiluminescent end point. AFLP typing was done by comparison to locus-specific allele ladders (AL) containing known variable number tandem repeat (VNTR) numbers. The time required to process and type samples with one AFLP system was ~36 hr, with 4 hr of hands-on time.

Coamplification of the APOB primers described with an HLA–DQα kit was attempted on a limited number of known samples to assess whether both loci typed correctly. Specificity testing of APOB and PAH with nonhuman DNA under low- and high-stringency PCR was conducted. Future adjustments in AFLP methodology may include chemical prevention of amplicon carryover and sensitivity refinements if biotin ratios are altered.

MATERIALS AND METHODS

Genomic DNA Extraction

Human genomic template DNA samples were extracted using a commercially available, rapid inorganic extraction protocol (Super-Quik Gene Kit, AGTC). The procedure was modified slightly for forensic materials.[18]

DNA Quantitation

Human genomic DNA was quantitated either by spectrophotometry (A_{260}) or comparison to genomic DNA yield gel kit standards (YGK-75, AGTC). Verification of human DNA species and quantity was done by hybridization with the human-specific probe D17Z1 (Human DNA Quantitation Kit, Life Technologies, Inc.).

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PCR Primer Sequences

Oligonucleotide primers were synthesized and HPLC purified by anion exchange chromatography (Midland Certified Reagent Company). One oligonucleotide per PCR pair was 5′ biotinylated by phosphoramidite linkage using a DNA synthesizer (Applied Biosystems, model 391). All oligonucleotides were diluted to 10 μM with sterile deionized H_2O, mixed with the second primer as an equimolar set (5 μM stock), and stored at −20°C prior to use. The ratio of biotinylated to unlabeled primer was 1:1.

The sequence, terminology, and reference for each oligonucleotide PCR primer is as follows: APOB 2, 5′-biotin-GTTCCTCAGGATCAAAGTATGTAC-3′; APOB 3, 5′-GGAGAAATTATGGAGGGAAAT-3′;[8] PAH 2, 5′-biotin-GCTTGAAACTTGAAAGTTGC-3′; PAH 3, 5′-GGAAACTTAAGAATCCCATC-3′;[15] D1S80 A, 5′-biotin-GAAACTGGCCTCCAAACACT-3′; D1S80 B, 5′-GTCTTGTTGGAGATGCACGT-3′;[11] β-globin KM29, 5′-GGTTGGCCAATCTACTCCCAGG-3′; β-globin RS80, 5′-biotin-TGGTAGCTGGATTGTAGCTG-3′.[17] The two D1S80 oligonucleotides were derived from the original published sequences after removal of 3′-terminal GC nucleotides.

AFLP Reaction Conditions

Reactions were prepared in 100-μl final volumes in 0.65-ml tubes. PCR master mix (see Table 1) was prepared with the following components: 1× reaction buffer [10 mM Tris-HCl (pH 8.4), 50 mM KCl, and $MgCl_2$], AFLP primer set, 0.05 μM β-globin primers, 200 μM each dNTP (US Biochemical), and AmpliTaq polymerase (Perkin-Elmer Corp.). The $MgCl_2$ range tested was from 1.0 to 4.0 mM in 0.5-mM increments. Other variables titrated were *Taq* polymerase in 0.25-unit increments and primer pairs in 0.05-μM increments. A negative control tube (no template added) is amplified as the last tube to have master mix added prior to thermal cycling. At least one positive control (known AFLP genotype) is amplified with each set.

For coamplification of APOB with HLA-DQα (AmpliType kit, Perkin-Elmer), 0.5 μM of APOB primer set was added to each sample. The kit directions were followed precisely through amplification. A gel aliquot of PCR product was removed for AFLP analysis, as described below, while another aliquot was removed for HLA–DQα typing.

Thermal Cycling Parameters

Thermal cycling parameters optimized were number of PCR cycles, annealing temperature, and extension time (Table 1). Thermal cycling was done with a forced-air circulation oven (BioOven1, Biotherm, Inc.), which monitored sample temperature and set-point time. A 100-μl thermal probe blank with deionized H_2O was used for each amplification run.

Extended denaturation times (additional 1.5 min) were used for the first three PCR cycles. Denaturation temperatures of 93–94°C were used at an altitude (Denver, CO) where the boiling point of water is 96°C. Parameters for denaturation, annealing, and extension were as follows: APOB, 93°C, 15 sec/59°C, 15 sec/72°C, 15 sec; PAH, 93°C, 15 sec/54°C, 15 sec/72°C, 10 sec; and D1S80, 93°C, 15 sec/62°C, 15 sec/72°C, 15 sec. A total of 30 cycles were used for APOB and D1S80 while 27 cycles were used for PAH under the conditions specified.

AFLP AL Preparation

Standards with known numbers of VNTR units were determined by either DNA sequence analysis (APOB)[8] or molecular weight and data base frequency calculations (D1S80 and PAH). AL preparation used a specific set of genomic DNA samples genotyped for each locus. Genomic DNA samples from this set were amplified individually with locus-specific parameters (Table 1) without the β-globin control, so that only AFLP alleles were represented. An aliquot was removed for electrophoresis of individual template contribution. The remainder of PCR products were mixed in Centricon 50 microconcentrators (Amicon, Inc.) and dialyzed with TE buffer [10 mM Tris-HCl, 1 mM EDTA (pH 7.4)] to remove unincorporated primers and dNTPs. The microconcentrator is centrifuged at 3500 rpm for 10 min, washed once with one volume of TE buffer, centrifuged for 10 min, and collected via the retentate cup. The AL volume was then raised with TE buffer and 10× sample loading buffer (40% Ficoll, 2.5% bromphenol blue), so that AL and amplicon band intensities were comparable. Each AL preparation was coelectrophoresed with individual component products for quality control verification. ALs were stored at −20°C or 4°C and appeared to be stable for at least 1 year.

Agarose Gel Electrophoresis

Following thermal cycling, a 10- to 20-μl aliquot of each product was removed and mixed with 10× sample loading buffer. Samples were electrophoresed in 2% agarose gels (SeaKem LE, FMC Bio-

TABLE 1 Optimized AFLP Reaction and Thermocycling Parameters

	AFLP system		
Parameter[a]	APOB	D1S80	PAH
Template DNA (ng)	1–10	10–20	10–20
$MgCl_2$ (mM)	3.0	1.0	1.0
$MgCl_2$ coamp. (mM)	3.0	1.5	1.5
AFLP primer (μM)	0.50	0.20	0.35
Taq polymerase (units)	1.0	1.5	1.0
PCR cycles (no.)	30	30	27
Annealing temp. (°C)	59	63	54
72°C ext. time (sec)	15	15	10
VNTR (bp)	15	16	30
m.w. size range (bp)	480–1080	400–1000	370–760
Alleles (no.)	25	25	12
Alleles in AL (no.)	19	16	8
Allele repeat range	21–58	16–48	3–14
Electrophoresis (Vhr)	1600	1500	1450

[a]Parameters are for loci APOB, D1S80, and PAH. The $MgCl_2$ concentration for PAH and D1S80 coamplification with β-globin was raised to 1.5 mM. AmpliTaq polymerase was the enzyme utilized. Thermal cycling temperatures and times were based on sample monitoring. The number of alleles, repeat range, and size range were obtained during data basing shown in Table 2.

products) with dimensions 25×20×0.5 cm (Owl Scientific Plastics) using 1× TBE gel buffer. Gels and buffer contained 0.5 μg/ml of ethidium bromide and could be visualized at any point during electrophoresis under UV transillumination (365 nm). AFLP ALs were coelectrophoresed in the gel every 5 or 6 lanes. Following electrophoresis and photography, gels were trimmed to 14× 20 cm (length by width, respectively), centering the visualized bands, prior to amplicon transfer.

Southern Transfer

Agarose gels were treated with 1.5 M NaCl, 0.5 N NaOH, for 15–30 min to denature AFLP fragments. Capillary alkaline Southern transfer of DNA to charged nylon membranes (Biodyne B, Pall Corp.) was performed[19] in the same solution for 2–4 hr. Blots were air-dried prior to detection and either stored at room temperature or detected immediately. When neutral nylon membranes (Biodyne A, Pall Corp.) were used, a transfer buffer of 0.1 M Tris, 0.5 M NaCl, was used and blots were UV-cross linked (312 nm) for 0.3 J/cm^2.

Nonisotopic Detection of AFLP Blots

Biotinylated amplification products were detected by either colorimetric or chemiluminescent methods.

For colormetric detection, a commercially available kit was utilized (Southern Detection Kit, AGTC), using a streptavidin/alkaline phosphatase/NBT/BCIP system. Blots were incubated with stain in the dark for 0.5–4 hr, depending on band intensity. The colorimetric assay was terminated by rinsing blots in deionized water and air-drying.

For chemiluminescent detection, the same procedure was followed through the 1× stain wash. Blots were then coated with Lumi-phos 530 (Lumigen, Inc.) and sealed in a polyethylene bag. Sealed blots were exposed to X-ray film or print paper (Kodak polycontrast III RC) in a film cassette for 15 min to 2 hr and the film was developed by standard methods.[19]

Allele Typing and Data Analysis

With colorimetric detection, developed blots serve as a permanent record of the amplification run. Blots were sealed in plastic bags and labeled with sample number and AL repeat numbers. Unknowns are assigned AFLP repeat numbers by comparison with bands found in the AL.

AFLP alleles were then given statistical weight by use of allele frequencies determined in three population groups (U.S. Whites, Blacks, and Mexicans; see Table 2).

RESULTS

Reaction Parameters

Table 1 describes the optimized PCR re-

TABLE 2 AFLP Allele Frequency Distribution

APOB				D1S80				PAH			
Allele	White	Black	Mexican	Allele	White	Black	Mexican	Allele	White	Black	Mexican
21	0	0.0190	0.0037	16	0.0023	0.0082	0.0299	3	0.3161	0.0658	0.2896
24	0	0.0136	0	17	0	0.0299	0	4	0	0.0053	0
25	0	0.0054	0	18	0.2557	0.0543	0.2687	5	0.0021	0.0018	0
26	0	0.0027	0	19	0	0.01019	0.0075	6	0.0021	0	0
27	0	0.0027	0	20	0.0317	0.0571	0.0112	7	0.1322	0.0445	0.1175
28	0.0022	0.0136	0.0037	21	0.0249	0.1087	0.0299	8	0.3574	0.3327	0.3279
29	0	0.0245	0	22	0.0317	0.0625	0.0224	9	0.1426	0.3541	0.2295
30	0.0584	0.0625	0.0259	23	0.0158	0.0190	0.0149	10	0.0021	0.0801	0.0109
31	0	0.0027	0	24	0.3054	0.2147	0.2985	11	0	0.0249	0
32	0.0693	0.0679	0.0370	25	0.0452	0.0543	0.0522	12	0.0434	0.0641	0.0246
34	0.2338	0.1658	0.2185	26	0.0136	0.0082	0.0187	13	0.0021	0.0249	0
35	0.0087	0.0082	0.0185	27	0.0	0.0353	0.0075	14	0	0.0018	0
36	0.4069	0.2310	0.4037	28	0.0611	0.1196	0.0224				
37	0.0022	0.0027	0	29	0.0747	0.0489	0.0896	*n*	484	562	366
38	0.0260	0.0625	0.0148	30	0.0113	0.0109	0.0410	HET%	74.7	77.2	66.7
40	0.0173	0.0516	0.0222	31	0.0950	0.0679	0.0485				
42	0.0043	0.0815	0.0111	32	0.0023	0.0027	0.0149				
44	0.0043	0.0761	0.0259	33	0.0	0.0027	0				
45	0	0.0027	0	34	0.0090	0.0652	0.0075				
46	0.0758	0.0679	0.1222	35	0	0	0.0037				
48	0.0779	0.0190	0.0815	36	0.0023	0	0.0112				
50	0.0087	0.0109	0.0111	37	0.0045	0	0				
52	0.0043	0	0	39	0.0023	0	0				
54	0	0.0027	0	40	0	0.0109	0				
58	0	0.0027	0	48	0	0.0082	0				
n	462	368	270	*n*	442	368	268				
HET%	77.9	87.5	77.1	HET%	83.3	91.8	76.8				

Allele frequencies from population data basing of 550 individuals for APOB, 539 for D1S80, and 706 for PAH. Heterozygosity is also shown for U.S. Whites, Blacks, and Mexicans. Allele repeat designations were derived from references 8 (for APOB), 11 (D1S80), and 15 (PAH) as described in Materials and Methods.

action and thermal cycling parameters for APOB, PAH, and D1S80. Several concentrations were found to be uniform for each system: β-globin primer (0.05 μM); dNTPs (200 μM each), and agarose gel (2.0%). Key reaction variables were found to be template DNA amount and concentrations of $MgCl_2$, AFLP primer, and *Taq* polymerase. Lower and higher template DNA amounts (~80 pg–100 ng) have been amplified successfully on a limited number of samples (data not shown).

Systems PAH and D1S80 utilized a $MgCl_2$ optimum of 1.0 mM when amplified independently of β-globin, whereas APOB stringency was optimal with 3.0 mM $MgCl_2$ and remains unchanged with multiplex PCR. The coamplification of β-globin with PAH required an increase in $MgCl_2$ to 1.5 mM to get equivalent intensity of β-globin and AFLP bands, without appreciable loss of stringency. The β-globin signal was desensitized by reduction of primer concentration (0.05 μM) relative to APOB or PAH primers (Table 1). D1S80 was not routinely coamplified with β-globin but has been seen to yield stringent products with 1.5 mM $MgCl_2$.

Taq polymerase requirements varied between AFLP loci for maximum amplicon yield. APOB and PAH required 1 unit per 100-μl reaction, whereas D1S80 required 1.5 units. No additional *Taq* polymerase was required with β-globin coamplification.

Thermal Cycling Parameters

A forced-air oven was used for optimization and parameters may be instrument dependent. This system utilized sample temperature monitoring and eliminated the use of mineral oil overlay of samples. Denaturation, annealing, and extension times of 10–25 sec each were sufficient for synthesis of PCR products above 2 kb (data not shown). The extended denaturation times were utilized for the first three cycles of PCR to facilitate complete denaturation and stringent target selection as described.[20]

Thermal cycling parameters adjusted for optimal AFLP stringency were annealing temperature and number of PCR cycles. Nonspecific artifact bands were observed from lower annealing temperature by 2–3°C or increasing cycle number by 2 or 3. In addition to specificity, product yield diminished after 2°C variations in either annealing or denaturation temperatures.

Figure 1 shows agarose gel resolution of coamplification products for β-globin with APOB prior to Southern transfer. The sensitivity of blot detection was greater than gel visualization or photography (D. Latorra, C.M. Stern, and M.S. Schanfield, unpubl.). Blot sensitivity was estimated at picogram levels from dilution analysis, whereas amplicon was synthesized above nanogram levels for each allele with 30 cycles of PCR. Band intensity of homozygous alleles (two copies of the same allele) was roughly twice that of each heterozygous band.

Construction of an APOB allele ladder from individual AFLP template DNA components is shown in Figure 2. The initial APOB AL contained 19 alleles (of 25 discovered), the PAH AL contained 8 (of 12), and the D1S80 AL contained 16 (of 25). Gaps in the ladder were filled as rare alleles emerged from further population data basing and the genomic DNA source was added to the AL set. Table 1 shows the VNTR unit size of each allele, molecular weight size range of AFLP products, and number of alleles in each initial AL. All AFLP products observed for APOB, PAH, and D1S80 were smaller than the 1327-bp β-globin amplification control.

AL band intensity reflected population frequencies. More intense bands (e.g., 34, 36, 48 for APOB) were attributable to more copies of common alleles needed to include rarer alleles in the ladder.

The 25 APOB alleles found so far range ~480–1080 bp, as shown in Figure 2. The alleles ranged from 21 to 58 repeat units. Two AL components (genotypes 21,46 and 50,58) have alleles (21,58) that span the range identified from data basing and would serve as useful positive controls. The APOB primers described were coamplified with HLA–DQα on a limited number of knowns and typed correctly for both systems (data not shown). The DQα fragment was visible as a 239- or 242-bp band on the AFLP gel and biotinylated product on the detected blot. APOB amplicon apparently did not interfere with sequence-specific DQα reverse dot blot hybridization as correct types were determined.

PAH alleles range from 3 to 14 repeats (~370–760 bp). D1S80 alleles range from 16 to 48 VNTR, ranging from 400 bp to 1 kb. Table 2 illustrates the population frequencies of alleles for APOB, PAH, and D1S80 in three population groups in data basing conducted at AGTC (Denver, CO). The total number of alleles (*n*) were derived from 550 individuals (2*n*) for APOB, 539 for D1S80, and 706 for PAH.

Figure 2 illustrates the resolution of a single APOB repeat unit (15 bp) difference using the methodology presented. Two components of this AL (genotypes 28,29 and 35,36) differ by a single VNTR and can be distinguished easily as two discrete alleles in Figure 2.

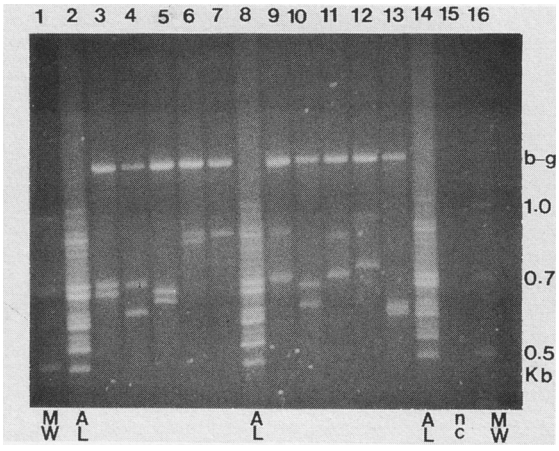

FIGURE 1 Agarose gel electrophoresis of β-globin and APOB coamplification products. DNA bands in the 2.0% gel were stained with ethidium bromide (0.5 μg/ml) and photographed under UV light (365 nm). (Lanes *1*, *16*) Molecular weight ladder with band sizes shown; (lanes *2*, *8*, *14*) APOB AL with alleles ranging from 21 to 58 repeats; (lanes *3–7*, *9–13*) individual samples show both β-globin (b-g) and APOB products; (lane *15*) a negative reaction control (NC). The DNA bands from this gel will undergo Southern transfer and blot detection prior to final typing.

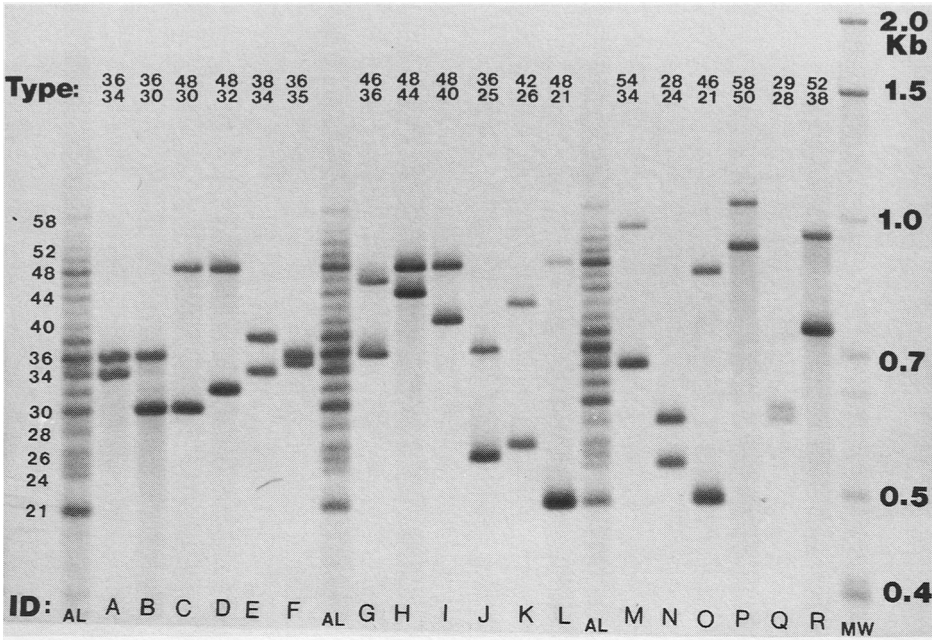

FIGURE 2 Detected blot of APOB AL components. Colorimetric detection of product aliquots from individuals *A–R*, along with the APOB AL, were electrophoresed in a 2.0% agarose gel, transferred, and detected as described in Materials and Methods. AL and molecular weight (MW) ladder bands are identified by repeat number and size in base pairs, respectively. Allele typing of each component sample is shown above each lane, using the nomenclature of Ludwig et al.[8] The 30, 34, 36, and 48 repeat alleles in the AL show higher intensity because of more component copies. A preferential amplification effect is seen with samples *L*, *M*, and *O*.

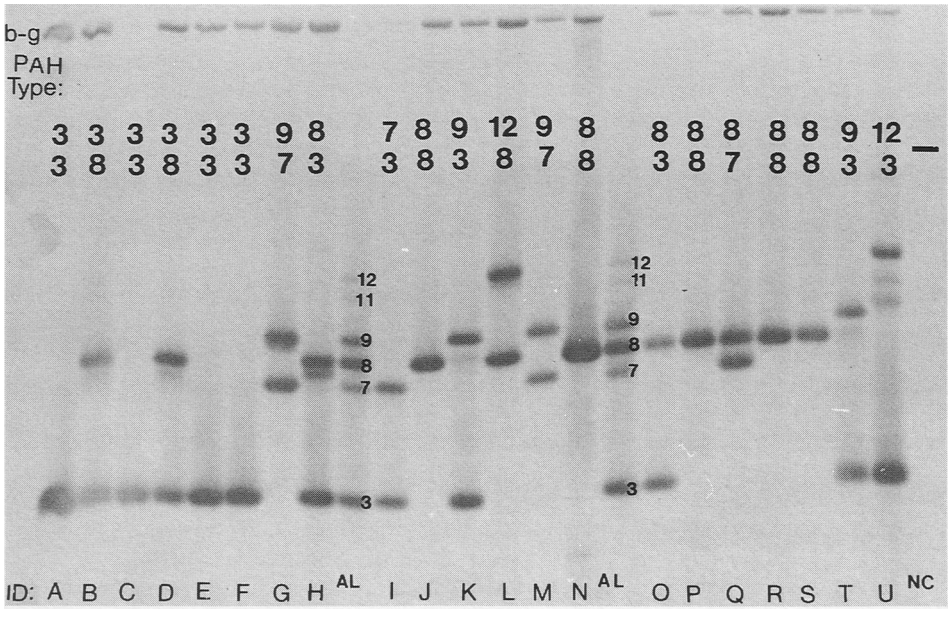

FIGURE 3 Detected blot of PAH and β-globin multiplex PCR. Colorimetric detection shows the 1327-bp monomorphic β-globin (b-g) band. Amplicon from individuals *A–U* was processed as described in Materials and Methods. PAH AL contains six alleles with the repeat numbers specified. A negative reaction control (NC) lane is shown. Products from individuals *H*, *K*, *T*, and *U* show evidence of artifact laddering, which appears to be sample dependent. These samples were reamplified from newly extracted samples to verify the types shown.

Figure 3 shows blot genotypes for PAH and β-globin multiplex PCR. Both the β-globin and PAH primer sets shown contain one biotinylated oligonucleotide for blot detection. Because of the relatively higher amplification yield, the β-globin primer concentration (0.05 μM) was 1/10 the APOB or 1/7 the PAH primer concentration.

PAH alleles (30 bp each) were resolved easily on agarose gels. The strong PAH band intensity attributable to high PCR yield was one reason that only 27 cycles of PCR were used. The adjustment of input genomic DNA and number of PCR cycles helped to maximize specific target yield and generate the highest PCR signal-to-noise (allele/artifact) ratio at each locus.

The D1S80 AL contains a comparable number of alleles as a commercially available D1S80 AL (Perkin-Elmer). However, the bands in this Perkin-Elmer AL are unlabeled and must be detected by ethidium bromide or silver staining of gels.

Preferential amplification of the smaller over the larger of two alleles was noted in these AFLP systems, for example, the 21,48 and 34,54 genotypes of APOB alleles in Figure 2. This was presumably attributable to a selective advantage of the smaller PCR target, as the only difference in DNA sequences is additional core repeats present in the larger allele. Both small and large alleles are well within the size range synthesized under the extension times programmed.

β-Globin coamplification (as shown in Figs. 1 and 3) was used to assess the presence of human target DNA and absence of PCR inhibitors in each sample. Although two independent loci were coamplified, this amplification control was useful to assess whether amplifiable target DNA was present, that is, a control for template and *Taq* polymerase integrity.

DISCUSSION

This report describes systems optimized for AFLP typing using a nonisotopic PCR amplification and detection procedure. This methodology utilizes ALs with defined numbers of VNTRs to assign genotype in forensic and paternity casework. Each AL described varies in number of alleles identified, repeat unit size, fragment size range, component DNA, and band intensity pattern.

The AFLP loci described here (APOB, PAH, and D1S80) have been optimized using the methodology described here. Modifications from previous publications were use of biotinylated PCR primer, colorimetric or chemiluminescent blot detection, reduction of genomic DNA target to 10 ng, β-globin control coamplification, and use of sample temperature monitoring for thermal cycling. Parameters were titrated to yield amplicon with optimal signal-to-noise ratios prior to population data basing and forensic validation studies.

Template DNA quality and precise quantity are factors when genomic DNA is limiting, as with some forensic casework. One goal of this study was to utilize ≤10 ng of genomic template DNA for each standardized PCR reaction. The inorganic extraction and human DNA quantitation described here was found to be reproducibly sensitive for the AFLP loci tested.

The monomorphic β-globin amplification control was coamplified with each polymorphic APOB and PAH sample. This demonstrated that amplifiable genomic DNA of sufficient size and quality was present and PCR inhibitors were absent in each sample. This 1327-bp control helped to assure that apparent homozygous types were not missing an allele because of genomic DNA degradation. Two copies of the same allele are amplified in a homozygous genotype, and the higher amplicon concentration is evident visually by band intensity.

The entire procedure described requires ~36 hr to complete and is not labor intensive. The methodology utilized 5′ biotinylated PCR primer oligonucleotides. A 1:1 ratio of biotinylated to unlabeled primer was used. Product sensitivity can be adjusted by altering the ratio of biotinylated oligonucleotide(s). APOB biotinylated primer concentrations were titrated and found to be detectable down to a biotin ratio of 1:200 (data not shown). Conversely, sensitivity could be further increased by biotinylation of both oligonucleotides per primer pair, although modification of number of PCR cycles and detection conditions would be needed. The use of biotin as the primer label affords reagent stability and choice of either colorimetric or chemiluminescent end point.

Poor quality of extracted genomic DNA or presence of PCR inhibitors has been implicated as a cause of amplification failure, especially in forensic casework where chemical and environmental insults to DNA may exist. In forensic AFLP testing, species origin and human genomic DNA concentrations were assessed by slot blot hybridization to the human-specific probe D17Z1. This step was important for quantitation of human template DNA added to AFLP reactions, as bacterial DNA can be copurified.

Genomic DNA amounts above 100 ng were observed to give reduced amplicon yield under the conditions described. Conversely, genomic DNA has been observed to yield products at 80 pg (D. Latorra, C.M. Stern, and M.S. Schanfield, unpubl.); however, it has yet to be titrated below this concentration.

PCR reactions were titrated for concentrations of $MgCl_2$, AFLP primers, β-globin primers, template DNA amount, annealing temperature, and number of PCR cycles at each AFLP locus. The PAH and D1S80 typing systems required increased $MgCl_2$ for coamplification of β-globin, mainly to keep AFLP and control band intensities equivalent. These AFLP loci amplify with either 1.0 or 1.5 mM $MgCl_2$, but the β-globin fragment is weak with 1.0 mM. No changes in APOB magnesium levels are required for β-globin coamplification.

We have observed PCR yield and stringency differences with 0.5 mM increments of $MgCl_2$, indicating the sensitive balance of PCR reactants. Concatomeric laddering patterns, with less intense artifact bands than main alleles, were associated with reduced stringency conditions, such as excess free magnesium ion. The mechanism for this pattern is unclear but has been discussed by others, especially with di-, tri-, and tetranucleotide repeats.[21,22]

Thermal cycling parameters were based on accurate sample temperature and time monitoring. Denaturation temperatures were held an additional 1–2 min in the first three PCR cycles to maximize correct target screening as described.[20] Annealing temperature was critical to product specificity and was different for all three loci, preventing coamplification of APOB, PAH, and D1S80 with the designated primers. The β-globin locus exhibited robust amplification efficiency and worked well at annealing temperatures used for PAH, APOB, and D1S80 (54°C, 59°C, and 62°C, respectively). The PAH locus amplified very strongly (based on band intensity) with 10–30 ng of genomic DNA, allowing the total number of PCR cycles to be reduced to 25–27.

These systems can resolve single VNTR differences among AFLP alleles for all three loci. Agarose gels and buffer contained ethidium bromide to visualize the products under UV light at any point during electrophoresis. The band-shifting effects of ethidium bromide seen with RFLP analysis of genomic DNA do not appear to affect discrete AFLP products as noted in template and amplicon concentration titrations (D. Latorra, C.M. Stern, and M.S. Schanfield, unpubl.).

The ability to view or photograph gels at any point during electrophoresis allows assessment of amplification success and band match or nonmatch. The monomorphic β-globin control allows electrophoretic migration to be analyzed with each gel run, making distortions or mobility shifts evident. From our observations, the β-globin amplification control band has been lost prior to AFLP alleles from degraded genomic DNA samples, although independent loci are involved. Because these three AFLP loci use the same 2% agarose gel matrix, coelectrophoresis of AFLP products and ALs from different loci on the same gel is straightforward.

Sensitivity of blot detection relative to gels appears to be increased. At a 1:1 biotinylated primer ratio, bands are evident after colorimetric detection that were not seen in gel visualization or photography. Preferential amplification of small versus large alleles may be exaggerated because of the sensitivity of colorimetric detection relative to ethidium bromide or silver staining of gels.

The AFLP ALs were derived from a locus-specific set of diluted genomic DNA. These standards with known VNTR numbers were amplified individually, purified, and coelectrophoresed with component samples for quality control validation.

Mutations or microsatellite variations in AFLP alleles may be detected as a mismatch not seen in the AL, and variations reported[23] with acrylamide gel electrophoresis may not apply here.

Best band resolution in AL preparations was seen with 50K molecular mass microconcentrators, although 30K and 100K were also used successfully. Purification of individual amplicon products

for use as allele-specific templates is obvious, but would also serve as a potent source of amplicon carryover contaminant.

In this study physical separation of areas for pre- and post-PCR processes was adhered to, and the use of plugged pipette tips has become standard practice.[24] Genotypes of all laboratory personnel were determined to assess possible sources of DNA contamination should they occur.

A lab-wide, chemical system for amplicon carryover prevention based on uracil-*N*-glycosylase (UNG)[25–28] was not implemented but is under study for incorporation with these AFLP loci. This method utilizes substitution of dUTP for dTTP in all PCR master mixes and degradation of any dUTP-containing amplicon (from previous amplification) with UNG. This modification may actually enhance PCR specificity by destroying nonspecific hybrids formed as reactions proceed from room temperature to initial denaturation.[28] The potential for incorrect allele typing in human identity testing attributable to the amplification power of PCR must be addressed as AFLP technology becomes instituted. Guidelines for PCR laboratory setup and prevention of amplicon carryover in forensic and paternity laboratories need to be implemented.

Data basing of samples from U.S. populations was done for APOB, PAH, and D1S80. Allele frequencies for >500 individuals from three groups are shown in Table 2 and compare favorably with previous studies with similar population groups.[8–16] These AFLP systems, when used in combination, yield a very low probability of two unrelated individuals having the same genotypes (1 in 1700 to 1 in 16,000, depending on population).

As part of our forensic validation testing, the PCR stringencies of the APOB and PAH test systems were artificially lowered and tested against human, primate, and other animal species (D. Latorra, C.M. Stern, and M.S. Schanfield, unpubl.). Testing of nonhuman DNA under high-stringency PCR demonstrated that only human DNA (with the exception of chimpanzee DNA) would generate bands in the AL range for these AFLP loci. Under low-stringency conditions, APOB and PAH generate "fingerprints," which appear species-specific and may be useful for evolutionary studies or wildlife forensics.

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