TECHNICAL NOTE

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Multigenerational Amplification of a Reference Ladder for Alleles at Locus D1S80

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ABSTRACT: The interlaboratory typing of DNA specimens that have been amplified at the D1S80 locus necessitates the use of a standard allelic reference ladder. This communication describes a technique in which individual, amplified alleles are isolated, combined, and amplified by PCR to produce a functional reference ladder composed of many of the alleles that occur at this locus. The amplified ladder can serve directly as a template source for production of the next generation of reference ladder. This process, in which each amplified ladder serves as the template for the next has been carried through multiple generations.

KEYWORDS: pathology and biology, Allelic ladder, locus D1S80, Polyacrylamide gel electrophoresis

The use of the polymerase chain reaction (PCR) [*1,2*] for the amplification of variable number tandem repeat (VNTR) sequences [*3–5*] promises to be a valuable technique that can be used for the forensic examination of biological evidence [*6–10*]. A favorable attribute of the amplified fragment length polymorphism (AMPFLP) typing approach is that loci amenable to this analysis possess alleles that can be resolved readily by gel electrophoresis into more discrete fragment bands than can be achieved by the restriction fragment length polymorphism analysis technique currently used for the examination of crime scene biological evidence [*11*].

The visual determination of specimen phenotype with discrete alleles is enabled through direct reference to allele ladders that are in adjacent lanes on the electrophoretic gel. Allelic ladders that contain many or all of the general allele types found in the relevant population can be constructed by amplifying genomic DNA obtained from individuals who carry the desired types and combining these separate allele products into a ladder array [*8,12*]. Unfortunately, this process of genomic DNA amplifications and allele mixing

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must be repeated each time the supply of ladder is exhausted. In addition, since some alleles occur more frequently, an excess number of copies of these common alleles accumulates in a combined ladder, which can lead to undesirably broad bands and uneven allele intensities.

Because the FBI Laboratory is engaged in the validation of methods for the detection of VNTR alleles that occur at the D1S80 locus [*13*,*14*], it was necessary to produce an amplifiable reference ladder that would be suitable for facilitating the classification of D1S80 alleles. Sajantila et al. [*15*] recently have reported the feasibility of reamplification of a composite D1S80 ladder. Their ladder was a direct combination of amplified allelic products and contained eight alleles. The D1S80 reference ladder produced in this study has been reamplifiable through three generations and contains 23 alleles that represent the major electrophoretic forms of each allele type. An undesirable buildup of common alleles was avoided by selective purification of alleles used to compile the ladder.

Materials and Methods

Amplification and Recovery of Specific D1S80 Alleles

Genomic DNA was prepared from liquid blood samples [*16*] and quantified by UV spectrophotometry. Donor individuals had been previously identified as carrying D1S80 alleles of interest. DNA also was isolated and purified by the Chelex extraction procedure [*17*] from a bloodstain prepared from the blood of an individual carrying allele number 27. DNA yield from this stain was quantified by a slot blot hybridization procedure [*18*]. Each genomic DNA sample was amplified in a 50 μL reaction mixture containing: 10 ng template DNA, 1 nmole each dNTP, 5 μL 10X reaction buffer (Perkin Elmer Corp., Norwalk, CONN.; consisting of 100 mM Tris-HCl, pH8.3; 500 mM KCl; 15 mM $MgCl_2$; 0.01% gelatin), 12.5 pmoles each primer [*14*], and 2.5 units TAQ polymerase (Perkin Elmer Corp.). Amplifications were carried out in a Gene Amp PCR System 9600 (Perkin Elmer Corp.) for 10 s at 95°C, 10 s at 67°C, and 30 s at 70°C for 27 cycles. The amplified products were separated on 0.4 mm thick acrylamide gels (7.5%T/2.0% C) prepared on Gelbond (FMC Corp., Rockland, Maine) by the flap technique [*12*]. The gel buffer was Tris-formate, pH 9.0 (60 mM with respect to formate ion). Four μL of each amplified sample was applied individually to a 3 mm × 5 mm fiberglass application tab (Pharmacia LKB Biotechnology, Inc., Piscataway, N.J., catalog no. 1850-901) that had been placed on the gel surface one cm from the cathodal plug. Approximately 1.5 cm × 1.5 cm plugs of 2% agarose extending across the width of the gel and containing electrode buffer (Tris-borate, 280 mM with respect to borate ion, pH 9.0) and bromophenol blue dye (50 μg/100 mL buffer) were situated at opposite ends of the gel. The electrode apparatus rested upon the gel plugs. Electrophoresis was initiated at 600 V, 20 ma, and 12 W and continued until the dye front, which marked the leading edge of the moving boundary, reached the agarose plug at the anodic end of the gel. To isolate the allele of interest, each gel was cut into three sections after marks had been placed on the Gelbond backing to enable realignment. The outside gel sections were silver stained [*12*] to reveal the location of the specific alleles. The stained sections were then realigned with the unstained, center portion of the gel, and the area of the unstained gel, corresponding to the positions of each desired alleles, was scraped from the gelbond and placed in a 1.5 mL microcentrifuge tube. The DNA was eluted from the gel matrix by adding 400 μL 10 mM Tris-0.1 mM EDTA, pH 7.5 (TE) and incubating the mixture overnight at 37°C. After a 5-min centrifugation at 18 000 × G, the supernatant fluid was pipetted into a clean tube, and the DNA precipitated by the addition of ammonium acetate and cold absolute ethanol [*16*]. The precipitate was washed with 70% ethanol and the DNA resolubilized in TE. Four μL of each resolubilized allele was subjected to electrophoresis

under the conditions described above to confirm that the desired allele had been segregated completely from its companion allele. For the initial and the confirmatory electrophoresis steps, an allelic ladder that was a combination of amplified genomic DNAs was used as reference for assessing allele size.

Allele Nomenclature

Allele designations are according to Sajantila, et al. [*15*,*19*].

Amplification of Combined D1S80 Alleles

After confirmation that the desired alleles had been isolated, the individual alleles were mixed by combining 3 μL each of alleles 16 to 34; 5 μL each of alleles 35 through 37; and 15 μL of allele 41. This solution was diluted 1:250 000-fold with deionized H_2O and reamplified in a volume of 100 μL composed of 20 μL template DNA, 2 nmole each dNTP, 10 μL Perkin Elmer Cetus 10X reaction buffer (consisting of 100 mM Tris-HCl, pH8.3; 500 mM KCl; 15 mM $MgCl_2$; 0.01% gelatin), 100 pmoles of each primer [*14*], and 10 units TAQ polymerase. The combined alleles were amplified for 27 cycles of 95°C for 30 s, 67°C for 30 s, and 70°C for 60 s. Amplified product was subjected to electrophoresis under the conditions described, with electrodes situated 21 cm apart, followed by silver staining. The product of this amplification is referred to as first generation allelic ladder. Alleles below 16 and alleles 38 to 40 have not been identified in this study and were not included in these ladders.

Reamplification of Amplified D1S80 Ladder

First generation allelic ladder was diluted with deionized H_2O in serial 10-fold steps from 270 000 to 27 000 000-fold. Twenty μL of each dilution were used as a template source in a 100 μL reaction volume to determine if the amplified allelic ladder could be reamplified. After assessment of initial amplification results, the optimal template dilution was further refined by serial two-fold titration on either side of the chosen 10-fold dilution. Twenty μL of each dilution were used as template. The amplification solution was identical to that used for amplification of the combined ladder alleles. The product of this study is referred to as second generation allelic ladder.

The same scheme of titration and amplification was carried out with reamplified ladder to produce what is referred to as third generation allelic ladder.

Results

Confirmation of Specific Allele Recovery

Genomic DNA samples from 20 individuals, heterozygous at locus D1S80, were amplified and the allele(s) of interest carried by each individual was recovered by elution from acrylamide gel sections. Confirmation that the alleles needed for assembly of the D1S80 allelic ladder had been isolated and recovered from the gels is shown in Fig. 1. Lanes 1, 6, 11, 12, 17, 22, and 28 contain the temporary allele reference ladder that was assembled by combining amplified genomic DNAs. The temporary ladder points out the undesirability of directly combining amplified genomic DNAs to achieve a reference ladder: excessive allele buildup and commensurate broadening of bands. Alleles 18 and 24, which occur frequently in the population, are notable examples of this buildup. In this figure the following alleles can be seen: (allele/lane number) 16/2; 17 and 18/3; 19/4; 20/5; 21/7; 22/8; 23/9; 24/10; 25/13; 26/14; 28 and 31/16; 29/18; 30/19; 32/20; 33/21; 34/24; 36/25; and 37/26.

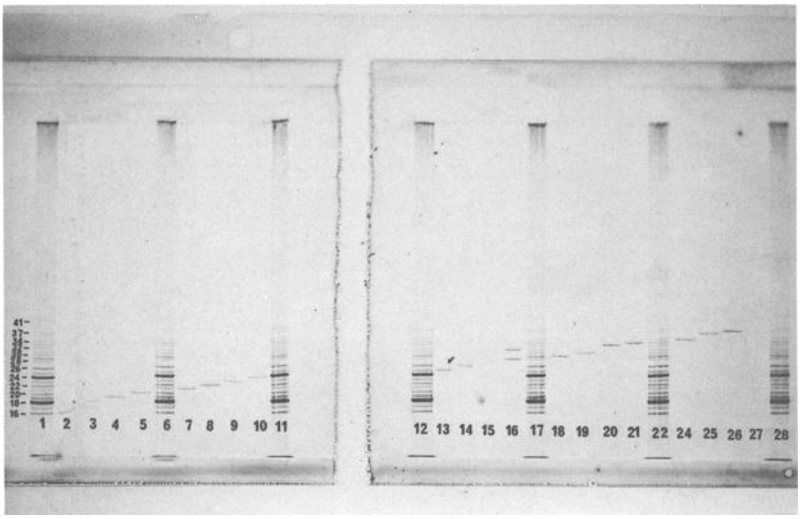

FIG. 1—*A polyacrylamide electrophoretic gel that confirms the isolation of desired D1S80 alleles. Lanes 1, 6, 11, 12, 17, 22, and 28 contain temporary allele reference ladders composed of combined amplified genomic DNAs. Alleles purified from amplified genomic DNA are shown in the remaining lanes. Allele designations are given to the left of the ladder in lane 1.*

Alleles 27, 35 and 41 are absent from this gel for they failed the isolation procedure on the initial attempt. These alleles were isolated successfully in a subsequent experiment (data not shown).

Amplification of Combined Alleles

Isolated alleles were combined directly to form a template source for amplification. In pilot experiments, it was observed that the larger D1S80 alleles consistently failed to amplify under conditions utilized for ladder amplification to the same extent as smaller alleles. In an attempt to overcome this limitation, it was necessary to add greater quantities of some of the larger alleles to the allele mixture template. Despite the increase in concentration of the larger alleles, it also was found necessary to alter the amplification conditions from those used for amplification of genomic DNA. The times for denaturation and primer annealing were increased from 10 seconds to 30 seconds for each of these steps. In addition, the primer extension time was increased to 60 seconds and the quantity of TAQ polymerase was increased from 2.5 U/reaction to 10 U/reaction.

Lanes 1, 2, 11, and 12 of Fig. 2 illustrate the products of amplification of the combined ladder template after electrophoretic separation. All alleles in this first generation allelic ladder are resolved clearly and are of even intensity after silver staining.

Second and Third Generation Ladders

First generation ladder could be diluted and reamplified to provide second generation ladder (Figure 2: lanes 3, 4, 9, and 10). In turn, second generation ladder was determined to be a suitable template source for production of a third generation ladder for D1S80 (Figure 2: lanes 5, 6, 7, and 8). The alleles in the second generation ladder were only minimally reduced in intensity; however, in the third generation ladder, some diminution of allele intensities for alleles greater than number 30 could be discerned. Although

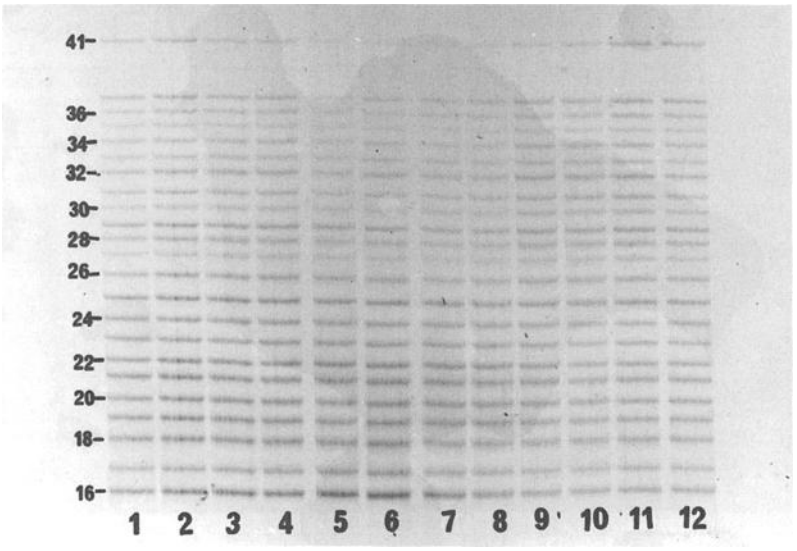

FIG. 2—*A polyacrylamide electrophoretic gel showing several generations of the D1S80 allele reference ladder. Lanes 1, 2, 11, and 12 contain first-generation ladder product amplified from isolated alleles that had been amplified. Lanes 3, 4, 9, and 10 contain amplified second-generation product using first-generation ladder as the template. Lanes 5, 6, 7, and 8 contain third-generation amplified product using second-generation ladder as the template. Allele designations are given to the left of the ladder in lane 1 (alternate alleles not designated).*

attempts to ameliorate this loss of intensity in third generation ladders were unsuccessful, they still can be readily used for allele classification.

It was observed in some experiments that the range of dilutions of first and second generation ladders that could serve as templates for the production of acceptable second and third generation ladders was rather narrow. After defining the approximate optimum dilutions of these templates using serial tenfold steps, the optimal dilution was determined using two-fold serial dilutions. For the second and third generation allelic ladders shown in Fig. 2, their respective template sources had been diluted 1:10 000 000.

Discussion

There is considerable interest in the potential inclusion of AMPFLP analysis into crime laboratory protocols for the examination of biological evidence. Among the most promising in this regard has been the AMPFLP analysis of allelic variation at the D1S80 locus [*8–10,12,14*]. An integral part of the qualification process of new genetic markers for forensic use is the determination of allele distributions in relevant population groups. In this regard, it is essential that all laboratories engaging in the exchange of allele frequency data at various loci utilize common arrays of reference alleles. In addition, the planned interchange of DNA typing data among crime laboratories through the computer-based Combined DNA Index System [*20*], compels the use of common reference materials.

In constructing the D1S80 allele reference ladder, consideration was given to the observation that electrophoretic variants occur for a number of D1S80 alleles [*6*]. These variants, which presumably differ from neighboring alleles by less than a full repeat unit of 16 base pairs, or by differences in sequence, migrate anodically or cathodically to the alleles represented in the reference ladder. It was important to insure that the alleles placed in the ladder represented the forms of the alleles that occurred most frequently

in the general reference populations. Early ladder constructs contained less common variant alleles at several positions, and these were replaced by the most common allele form in the final version of the ladder described in this paper. The current D1S80 ladder has been used to identify the alleles carried by more than 2000 individuals and has been found to contain the predominant form of each of the alleles (manuscript in preparation). Other D1S80 ladders [*12,15*] are direct combinations of amplified genomic DNA samples. In order to include less-frequently occurring alleles, genomic DNAs were used that contain common alleles in combination with rarer alleles. As a result, these ladders could suffer from buildup and commensurate broadening of the more frequent allele bands. Through purification of single alleles from polyacrylamide gels, the accumulation of excess copies of high frequency alleles in the ladder has been avoided. While we attempted to make the allele intensities more equal in the current ladder, it would be possible to selectively highlight certain alleles in the ladder for visual registration purposes by increasing the quantity of specified alleles to a degree in the template mixture.

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