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Separation of D1S80 Alleles by Vertical Electrophoresis Through a Two-Tier Resolving Gel

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ABSTRACT: The clarity of VNTR allele profiles generated by vertical polyacrylamide electrophoresis is improved by the addition of a stacking gel layer. However, the size of the resolving gel must be reduced to provide space for the stacking gel; therefore, resolving power is conceded in order to improve clarity. The procedure described in this paper delivers near maximum allele separation while maintaining excellent band clarity. This has been achieved by reducing the length of the stacking gel and dividing the resolving gel into two layers with a small increment in acrylamide in the lower layer.

KEYWORDS: criminalistics, D1S80, AMP-FLP, vertical electrophoresis, PCR

Recent advances in molecular biology, particularly DNA technology, are providing forensic investigators with new and powerful tools to help solve criminal and paternity issues. In particular, the polymerase chain reaction (PCR) [*1,2*], allows polymorphic DNA loci to be specifically targeted and amplified up to 10 million-fold, providing the accuracy, specificity, and sensitivity required for forensic analyses. There are millions of polymorphisms on the human genome that are potentially suitable for discriminating between individuals. By far the most useful DNA polymorphisms are the VNTR (variable number tandem repeat) loci, which are highly polymorphic because of the variable number of tandemly repeated core sequences present at a particular locus [*3,4*]. There are thousands of different VNTRs with core sequences that range from only a few base pairs (bp) to several thousand base pairs in length. The most useful VNTRs for forensic purposes are the AMP-FLPs (amplified fragment length polymorphisms) and STRs (short tandem repeats), which are small enough to be amplified by PCR [*5–9*]. AMP-FLP core sequences range from about 16 to 70 bp while STR repeat sequences are generally 2 to 5 bp in length.

The D1S80 AMP-FLP is well suited for use in forensic practice. The core sequence, which is 16 bp in length, is quite well conserved [*5*] making this locus particularly amenable to accurate allele assignment by comparison with a ladder of known alleles. Additionally this locus appears to be less prone to dropout of higher molecular weight alleles during amplification than other AMP-FLPs [*10*].

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Materials and Methods

Sample Preparation and PCR Amplification

DNA was extracted from biological samples using the chelex extraction methods recommended by Cetus [*11*]. Samples were amplified by PCR using the Perkin-Elmer *Ampli*FLP D1S80 PCR Amplification Kit, which was used in accordance with manufacturer's instructions.

Polyacrylamide Gel Electrophoresis of Amplified DNA

The D1S80 alleles were analyzed by electrophoresis using the BioRad Protean II (20 × 16 × 0.1 cm) vertical polyacrylamide gel apparatus with a discontinuous buffer system. The polyacrylamide gels were cast in a three layer format that had a two-tier resolving gel and a stacking gel as specified in Table 1. Gels were made by successive preparation of each layer. The resolving gel layers were poured and overlaid with distilled water and allowed to polymerize for 10 to 20 min; the interface was washed after polymerization to remove any unpolymerized acrylamide. The stacking gel layer was poured on top and the comb inserted to a depth of 1 cm. The gel was allowed to polymerize for a further 45 to 60 min before loading. Routinely, samples of 5 μL amplified DNA were mixed with 5 μL distilled water and 2 μL loading buffer (20% sucrose, 0.1% bromophenol blue) for loading; however, sample volumes up to 25 μL could be loaded with no loss of clarity. The D1S80 allelic ladder (Perkin-Elmer) was prepared for electrophoresis by mixing 5 μL D1S80 ladder with 5 μL PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM Mg_2Cl, 200 μM dNTPs) and 2 μL loading buffer. The samples were prerun at 125 v with 33 mM Tris-sulphate pH 6.5 in the upper (cathodal) buffer tank and 90 mM Tris-borate pH 8.5, 2 mM EDTA (TBE) in the lower buffer tank. After 10 min the upper tank buffer

TABLE 1—*Polyacrylamide gel design.*

Lower resolving gel: 8% T, 1.6% C
7% glycerol
33 mM Tris-sulfate (pH 6.5)
Length: 8 cm
Upper resolving gel: 7% T, 1.6% C
7% glycerol
33 mM Tris-sulfate (pH 6.5)
Length: 9.5 cm
Stacking gel: 5% T, 1.6% C
7% glycerol
33 mM Tris-sulfate (pH 6.5)
Length: 1.5 cm below comb; 1 cm well size

TABLE 2—*Silver staining protocol.*

Step[a]	Time
40% methanol[b]	10 min
200 mg/L sodium thiosulfate	1 min[c]
1% nitric acid	6 min[c]
Distilled water (×2)	1 min
2 g/L silver nitrate; 0.75 mL/L 37% formalin	30 min
Distilled water (×2)	1 min
60 g/L sodium carbonate; 0.5 mL/L 37% formalin; 4 mg/L sodium thiosulfate (require three or four changes)	The solution should be changed once it begins to turn brown. Develop to the desired intensity but for no longer than 5 min.
Distilled water (×2)	1 min
50% methanol; 12% acetic acid	2 h[d]
Distilled water[e]	30 min

[a]Use volumes of approximately 500 mL for each change of solution.
[b]If silver staining cannot be performed immediately gels can be stored overnight at 4°C.
[c]Timing is essential.
[d]The gels may be stored for several days in the dark at 4°C without loss of image.
[e]This step is performed prior to drying to rehydrate the gels which contract due to the high concentration of methanol.

was replaced with TBE buffer and electrophoresis continued at constant voltage (125 V) for 18 h at 10° C. Routinely two gels were run simultaneously in the BioRad Protean II apparatus. Upon completion of electrophoresis the upper resolving gel and stacking gel layers were detached and discarded, unless otherwise stated, and the lower resolving gels placed in 40% methanol for immediate silver staining or stored at 4° C overnight.

Silver Staining of the Polyacrylamide Gels

Gels were stained by a modification of the silver staining method of Allen, et al. 1989 [*12*] as presented in Table 2. Solutions used for silver staining were prepared fresh prior to staining and unless otherwise stated all manipulations were performed at room temperature. Staining was performed in pyrex dishes with gentle agitation. Following staining, the gels were rehydrated in distilled water. Commonly the final size of the rehydrated gel is slightly larger than that of the original.

Allele Assignment

Genotypes of samples were assigned by comparison of unknowns with either the 15-allele or 27-allele D1S80 ladders provided in the *Ampli*FLP D1S80 PCR Amplification Kit (Perkin-Elmer). To assist in accurate allele assignment each sample was run adjacent to an allelic ladder. Each amplification batch included a known positive and negative control to monitor the efficiency of amplification and contamination.

Results and Discussion

The clarity of alleles separated by vertical electrophoresis is improved by the use of a stacking gel. The stacking gel used in this system is not *per se* those developed for protein electrophoresis such as the Ornstein-Davis, 1964 procedure [*13,14*], never-theless the two systems are analogous and perform the same function. The alleles are primarily stacked due to the discontinuous buffer system [*12*] where molecules are concentrated between the leading and trailing ions at the moving boundary. The sharp increment in acrylamide concentration between the stacking and resolving gels also has the effect of further stacking the molecules. The alleles fall behind the moving boundary due to the sieving effect of the acrylamide and subsequently separate by zone electrophoresis in a continuous buffer. Ideally the stacking gel should be a low acrylamide concentration so that the DNA molecules are not retarded by molecular sieving and should be at least twice as deep below the well as the depth of the sample in the well to allow sufficient space for effective stacking [*15,16*]. Sajantila and Lukka, 1993 [*17*] have recently published a procedure that is based upon this design, using a 12 cm 6% T resolving gel with a 4 cm 3% T stacking gel. These conditions provide excellent clarity [*17*; this laboratory, unpublished data] but at a substantial loss of separating power since the length of the resolving gel must be reduced in order to construct the lengthy stacking gel. Using this design alleles 14 to 40 are separated by approximately 4.5 to 5 cm after staining.

The procedure described in this paper is designed to maximize the separation of alleles, yet maintain the band clarity. The stacking gel, at a concentration of 5% T, has sieving capacity, hence, separation begins soon after the samples enter the matrix. The stacking gel length is reduced in order to maximize the length of the resolving gel. Band clarity is primarily obtained from the combined effects of the discontinuous buffer system and the 2% T increment in acrylamide concentration between the stacking and upper resolving gels. The second increment of 1% T in the lower resolving gel further enhances the allele clarity but without causing excessive stacking, hence, the separation of the alleles is not significantly effected; the distance separating alleles 14 to 40 is reduced by approximately 3–5 mm by the 1% T increment in acrylamide concentration in the lower layer (unpublished data). Figure 1 shows a typical D1S80 gel separated using the two-tier resolving gel design. The resolving power is maximized providing a separation of 7–7.5 cm of alleles 14 to 40 after staining. Yet the allele profiles are clear and uniform across the width of the gel allowing accurate allele assignment by well-to-well (unknown-to-ladder) comparisons. The 8% T lower resolving gels are more durable than 6% T or 7% T gels and are more able to withstand the rigors of staining and drying. There is little difference in the allelic separation of

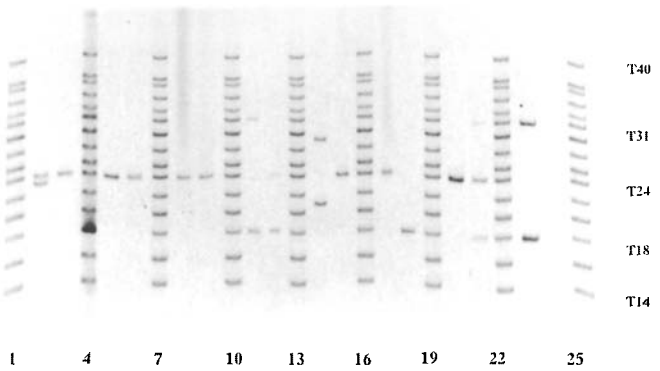

FIG. 1—*Silver stained D1S80 gel displaying profiles of forensic specimens that have been amplified by PCR and analyzed using vertical electrophoresis through the two-tier resolving gel. The known positive (T18/T31) and negative controls are present in lanes 23 and 24, respectively. The allelic ladder in lane 4 is spiked with known PCR product to allow the identification and orientation of the gel after silver staining. The D1S80 15-allele ladder consists of the following alleles: T14, T16, T18, T20, T22, T24, T25, T27, T29, T31, T32, T34, T36, T37, T40.*

8% T and 6% T gels except that a longer running time is required for the higher acrylamide concentration (unpublished data). Furthermore the 5% T stacking gel is more suitable for the preparation of smaller wells allowing more samples to be run on each gel.

This design has the added advantage of retaining most heteroduplexes and other non-specific high molecular weight fragments in the upper resolving gel, hence they are not visualized. However, D1S80 alleles from 13 to 53 repeat units in length, or approximately 330 bp to 1000 bp in size, are observed thereby detecting all known alleles [*18,19*]. The upper layers were retained and stained for the purpose of validation and the construction of population databases to ensure that any high molecular weight alleles in excess of 50 repeat units were not omitted. However, no D1S80 alleles greater than 45 repeat units have been observed in over 800 Australian individuals of caucasian, aboriginal or asian origin. Only one allele greater than 41 repeat units in length has been observed and this was typed from an asian individual. With only 21 unrelated asians typed to date this may be significant since alleles greater than 41 repeat units in length have been observed in asian populations [*19*]. Population data from 683 unrelated caucasians and 78 unrelated aboriginals show no excess of homozygotes.

This system utilizes both a buffer differential and a pH differential of 2 pH units (gel buffer, pH 6.5) to aid the separation and clarity of allele profiles. Sajantila and Lukka, 1993 [*17*] use the same buffers but with a wider pH differential of 4 pH units (gel buffer, pH 4.5). However the gel buffer is preferably used at pH 6.5 for three main reasons.

First, this is the lowest pH at which Tris has buffering capacity. Second, at pH 6.5 all nucleotides have a constant net charge whereas at pH 4.5 the charge of the nucleotides differs slightly [*20*] consequently separation could be affected by base composition. Third, there is no visible difference in allele clarity or separation whether the gel buffer is at pH 4.5 or pH 6.5 (unpublished data).

One of the chief advantages of vertical electrophoresis systems is that composite gels, such as the design described in this paper, are very easy to cast and run. There are literally thousands of combinations of buffer, pH, acrylamide and viscosity differentials, and other additives, running temperature and voltage that could be tried to improve separation and clarity. It is expected that similar composite designs may also be useful for the manual separation of STR alleles. Such tests are under way.

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