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Technical notes

Improved separation of PCR amplified VNTR alleles by a vertical polyacrylamide gel electrophoresis

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Summary. The effect of a stacking gel, the pH and crosslinking agent concentration on the resolution and sharpness of PCR amplified VNTR alleles in a vertical discontinuous polyacrylamide gel electrophoresis system was investigated. The experiments show that the use of a low crosslinking agent concentration, a stacking gel and a wide pH difference between the gel buffer and the electrophoresis buffer at the beginning of the electrophoresis resulted in reduced band width and increasing resolution in silver-stained polyacrylamide gels. The importance of sharp DNA fragments is especially emphasized when analyzing multi-allelic DNA loci, that exhibit alleles differing from only few bp to few dozen bp in length, such as variable number of tandem repeat (VNTR) or short tandem repeat (STR) loci.

Key words: VNTR-polymorphisms – STR-polymorphisms – Electrophoretic Resolution – Vertical PAGE

Zusammenfassung. Die Auswirkungen eines geschichteten Gels, des pH-Wertes und der Konzentration der Vernetzungs-Substanz auf die Auflösung und Schärfe der Bandendarstellung von PCR-amplifizierten VNTR-Allelen wurden in einem vertikalen diskontinuierlichen Polyacrylamidgel-Elektrophoresesystem untersucht. Die Experimente zeigen, daß die Verwendung einer niedrigen Konzentration der Vernetzungs-Substanz, eines geschichteten Gels und eines großen pH-Unterschiedes zwischen dem Gelpuffer und dem Electrophoresepuffer zu Beginn der Elektrophorese zu einer reduzierten Bandbreite führte, verbunden mit einer zunehmenden Auflösung in silbergefärbten Polyacrylamidgelen. Die Darstellung von DNA-Fragmenten in Form scharfer Banden ist besonders wichtig, wenn multi-allelische DNA-loci untersucht werden, welche Allele aufweisen, die sich im Bereich von wenigen Basenpaaren bis wenigen Dutzend Basenpaaren in der Länge unterscheiden, wie z.B. Loci mit einer variablen Anzahl von Wiederholungseinheiten (VNTR), besonders Loci mit kurzen Wiederholungseinheiten (STR).

Schlüsselwörter: VNTR-Polymorphismen – STR-Polymorphismen – Elektrophoretische Auflösung – Vertikale PAGE

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Introduction

At present the abundant polymorphism of VNTR or STR loci in the human genome is used for individual identification in both forensic casework and paternity testing [1, 2, 3]. In addition to its other applications, the polymerase chain reaction (PCR) [4, 5] is a novel tool for sample preparation in forensic medicine, since it enables specific amplification of the DNA at VNTR/STR loci from subanalytical quantities to a level at which several methods can be utilized for analysis.

Separation of PCR amplified DNA fragments is commonly performed by agarose gel electrophoresis. However, the resolution capacity of continuous, submarine agarose gel electrophoresis for analysis of small (< 2 kb) variable number of tandem repeat (VNTR) [6] or short tandem repeat (STR) [2] alleles, varying in length from only 2 bp to a few dozen bp, is not sufficient. Instead, a good resolution of small VNTR alleles can be achieved by employing polyacrylamide gel electrophoresis (PAGE). In PAGE, a discontinuous buffer system can be utilized to initially zone-sharpen the sample components prior to the electrophoretic separation of the DNA fragments [7]. The use of discontinuous PAGE has been demonstrated to result not only in better resolution, but also in sharper DNA fragments when compared to agarose gel electrophoresis [4, 8]. Both horizontal and vertical PAGE are suitable for the analysis of VNTR loci [1, 9].

The amplified fragment length polymorphism (AmpFLP) technique [1] is a convenient method for analysis

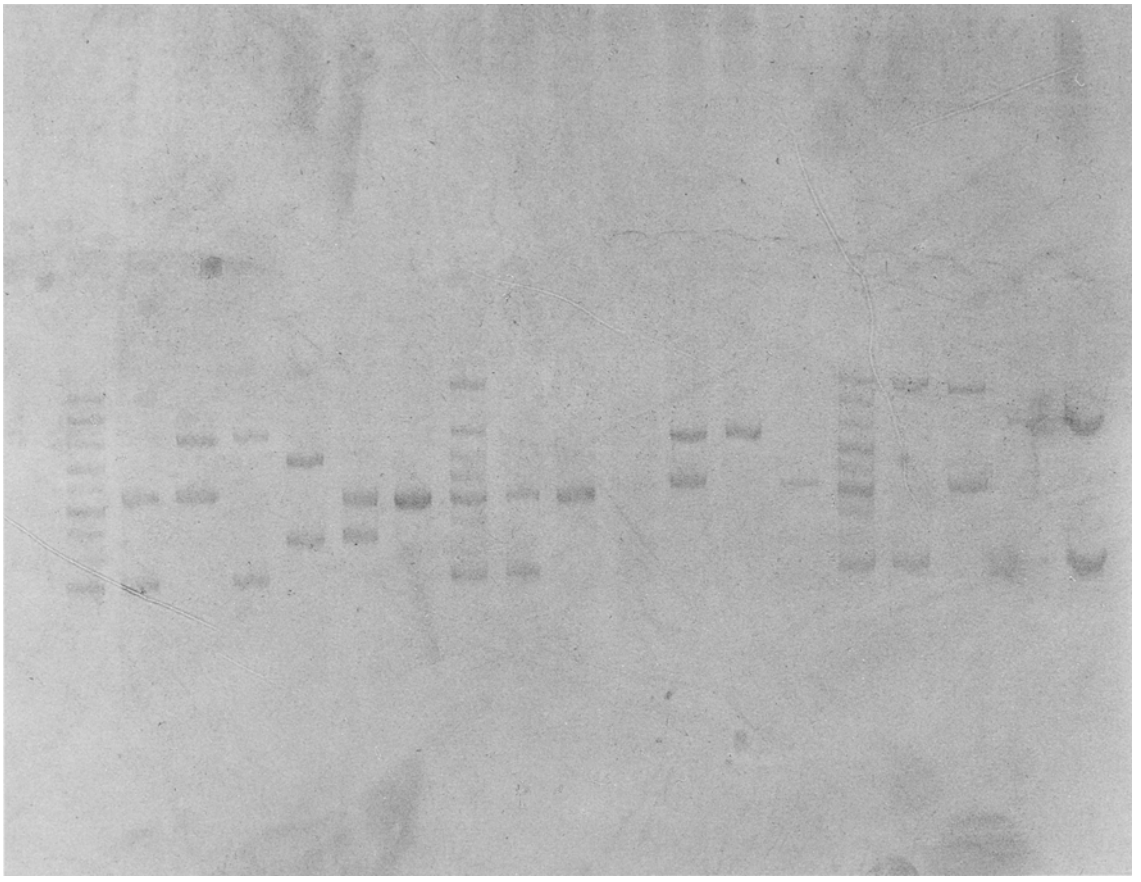

Fig. 1. Amp-FLP analysis of the D1S80 locus using 16 cm × 0.75 mm PAA gel 6% T, 3.2% C, in 33 m*M* Trizma-sulphate, pH 8.5, 7% glycerol. Electrophoresis buffer (TBE) at pH 8.5. Electrophoresis 2 h 50 min at 200 V. The ladders on lanes 1, 8 and 15 consist of alleles 18, 20, 22, 24, 26, 28, 31 (in lanes 1 and 15), 34, and 37. The size difference between the fragments in the ladder is 32 bp. The size of the allelic products range from approximately 430–750 bp

of amplified VNTR/STR loci. In this technique the amplified VNTR/STR alleles are separated by PAGE and subsequently visualized by staining with ethidium bromide [10] or silver nitrate [7].

In the present paper we demonstrate a modification of vertical discontinuous PAGE in the analysis of the forensically highly informative VNTR markers D1S80 (MCT118), D17S30 (YNZ22), and the hypervariable region close to 3' end of the apoB gene (apoB). The modification includes the use of a stacking gel, a wide pH difference between the gel and electrophoresis buffer at the beginning of the electrophoresis and a low concentration of crosslinking agent.

Materials and methods

Sample preparation. DNA was extracted from EDTA blood samples using the Chelex® resin-extraction approach described elsewhere [11, 12]. PCR amplification was performed for three different VNTR loci: D1S80 (MCT118) [13], D17S30 (YNZ22) [14] and the hypervariable region close to the 3' end of the apolipoprotein B gene (apoB) [15, 16]. The PCR was performed in 100 µl of reaction mixture containing 1–10 ng of extracted DNA in 50 m*M* Tris-HCl, pH 8.8, 15 m*M* $(NH_4)SO_2$, 1.5 m*M* $MgCl_2$, 0.01% gelatin, 0.1% Triton-X 100, 1.0 µ*M* of each primer, 200 µ*M* of each dNTP and 2.5 units of *Thermus aquaticus* (DNA-polymerase (Promega). PCR was carried out in a programmable thermal controller (MJ-Research, Inc.). Prior to the first cycle a "hot start" [17] was performed. The primer sequences and PCR time-temperature profiles were as described elsewhere [18].

Sample application. Ten µl of the amplified DNA sample was mixed with 2 µl of sample buffer containing 20% sucrose, 12.5% Ficoll (Pharmacia), 0.2% bromophenol blue in a solution containing 0.2 *M* Trisma Base, 0.1 *M* Na-acetate (anhydrous) and 1 m*M* EDTA at pH 8.3. A comb for 20 wells was used.

PAGE. A 16 × 14 × 0.075 cm vertical polyacrylamide (PAA)-gel was used to resolve the amplified DNA fragments. The electrophoresis was carried out in a LKB 2001 vertical electrophoresis unit at a constant voltage of 200 V and at ambient temperature using 100 m*M* Tris, 100 m*M* boric acid, and 2 m*M* EDTA as running buffer, pH 8.5 (TBE). The total acrylamide concentration (T) ranged from 6 to 10%, and the ratio of the cross-linking agent to the total acrylamide (C) from 1.6 to 3.2%. N,N-methylene bis-acrylamide (Kodak) was used as a cross-linking agent. The gels were prepared in 33 m*M* Trizma-sulfate (Sigma) containing 7% glycerol. The pH adjusted to 8.5 or left unadjusted at pH 4.5. The electrophoresis time was 2 h 50 min for MCT118, 3 h 45 min for apo B and 4 h for YNZ22. In each gel an allelic ladder consisting of a set of known alleles was run parallel to the samples to facilitate typing [17].

Stacking gel. To study the effect of a stacking gel on the resolution and sharpness of DNA fragments the 16 cm resolving gel was poured

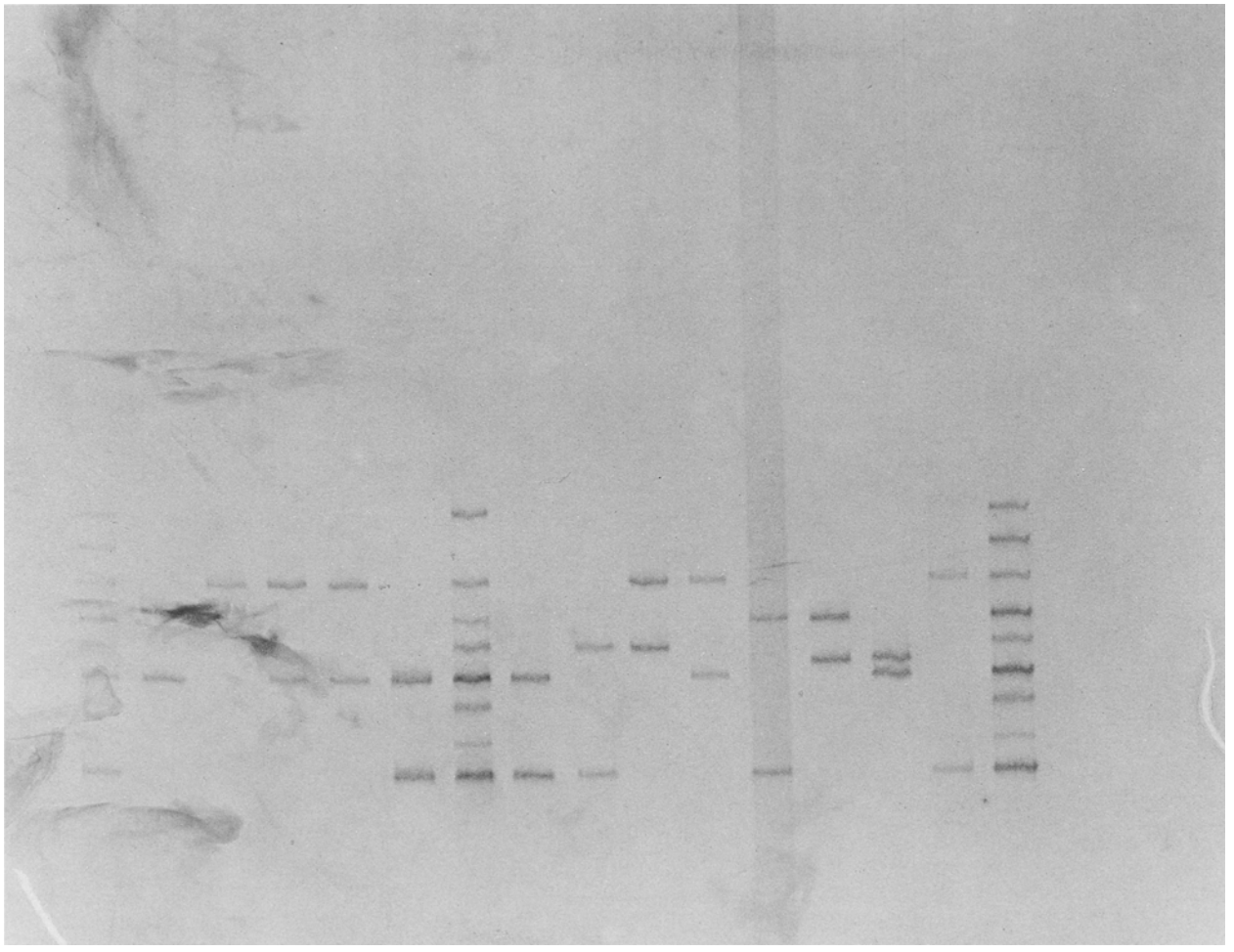

Fig. 2. Amp-FLP analysis of the D1S80 locus using 16 cm long PAA-gel 6% T, 1.6% C, in 33 m*M* Trizma-sulphate, 7% glycerol, pH 4.5. Electrophoresis buffer (TBE) at pH 8.5. Electrophoresis 2 h 50 min at 200 V. For the ladders on lanes 1, 7 and 16 see figure legend 1

to be a 12 cm × 0.75 mm resolving gel with a 4 cm × 0.75 mm stacking gel (3% T, 1.6% C) on top.

Silver staining. The detection of the separated DNA fragments was performed using silver staining as described earlier [5, 6].

Results and discussion

The discontinuous horizontal PAGE described by Allen et al. [7], can be efficiently used for the separation of PCR amplified DNA fragments in forensic medicine [1, 19]. Because of the previous experiences in vertical electrophoresis in our laboratory we wanted to modify this horizontal system to a vertical one.

Our modification to the vertical electrophoresis was to prepare a homogenous PAA gel (6% T, 3.2% C for markers D1S80 and apo B or 10% T, 3.2% C for marker D17S30) in 33 m*M* Trizma-sulfate, pH 8.5, containing 7% glycerol as gel buffer. TBE (pH 8.5) was used as a running buffer. After electrophoresis the alleles were separated, but the distance between the different alleles was not as desired and the individual alleles appeared diffuse (Fig. 1).

By lowering the concentration of bisacrylamide from 3.2% C to 1.6% C, a better separation of the alleles was achieved using the same electrophoresis conditions as above (data not shown). An additional improvement was seen when changing the pH in the gel buffer from 8.5 to 4.5. This resulted in sharper DNA bands after the same electrophoresis period and visualization of the alleles by silver staining (Fig. 2).

The most advantageous effect was demonstrated by applying a 4 cm long stacking gel on top of a 12 cm long PAA resolving gel, and using the lower cross-linking agent concentration (1.6% C) in the gel and the lowered pH (4.5) in the gel buffer. This resulted in extremely sharp DNA fragments in the silver stained gels (Fig. 3). The results were consistent for all three VNTR loci used in these experiments.

In this vertical PAGE system the preparation of the gel is convenient and the quality of the gels is constant from run to run. The sample loading is easy and also large volumes can be applied, if desired. Using this system the electrophoresis is free from disturbances such as uneven cooling and excessive humidity over the gel.

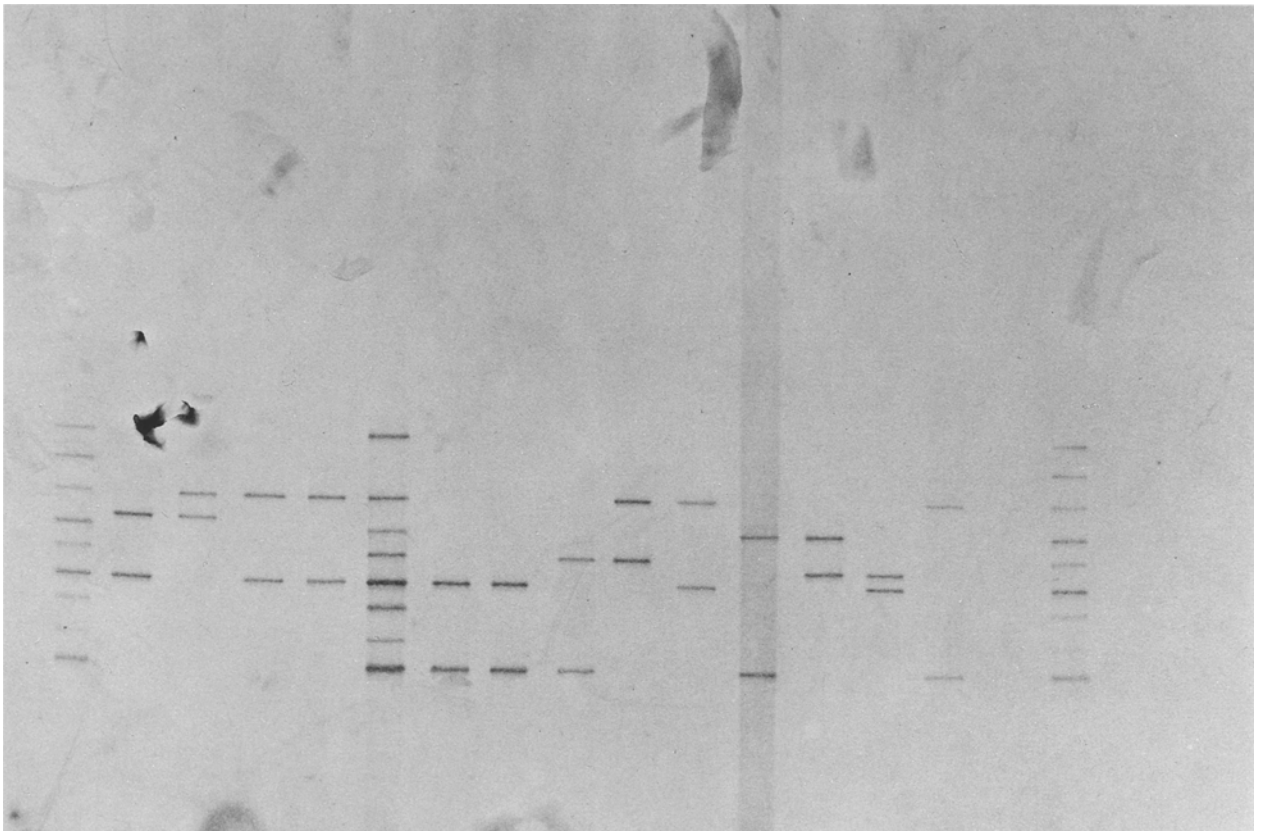

Fig. 3. Amp-FLP analysis of the D1S80 locus using 12 cm long resolving PAA-gel 6% T, 1.6% C and 4 cm long stacking PAA-gel 3% T, 1.6% C in 33 m*M* Trizmasulphate, pH 4.5, 7% glycerol. Electrophoresis buffer (TBE) at pH 8.5. Electrophoresis 2 h 50 min at 200 V. For the ladders on lanes 1, 6 and 17 see figure legend 1

Since altered migration, smiling or waving across the gel have not been encountered, the interpretation of the results can simply be done using a ruler and a light box. Furthermore, the silverstained gels can be easily dried between transparent films and used as a permanent record.

To conclude, the use of a stacking gel, a wide pH difference between the gel and running buffers, and a low cross-linking agent concentration in vertical discontinuous PAGE has an advantageous effect on the resolution and sharpness of the DNA fragments. This is an important factor in the interpretation of forensic DNA analyses utilizing the highly polymorphic VNTR or STR loci, where the difference in allele size can be as small as 2–4 bp. Since this vertical discontinuous PAGE is convenient to handle and has a good quality and reproducibility, it has been adapted to the routine forensic casework and paternity testing in our laboratory.

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