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Composite PAGE: an alternate method for increased separation of amplified short tandem repeat alleles

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Summary. Genetic typing of short tandem repeat (STR) loci may require electrophoretic separation techniques which, depending on the locus, can resolve alleles differing in size by only 2, 3 or 4 base pairs (bps). Many such loci can be separated by horizontal, discontinuous polyacrylamide gel electrophoresis (hd-PAGE). However, some loci present particular separation challenges. A composite (or step) gel system consisting of 2 zones, anodal and cathodal, which differ by pore size and ionic strength, has been developed to augment resolution of DNA fragments without a need for additional electrophoretic equipment. Depending on pore size, as well as ionic strength, initial migration can be increased or decreased to effect resolution. When compared with hd-PAGE, this composite gel electrophoretic system can result in enhanced resolution of PCR amplified STR alleles.

Key words: DNA – VNTR – STR – PAGE – Composite gels

Zusammenfassung. Die genetische Typisierung von Short Tandem Repeat (STR)-Loci erfordert elektrophoretische Trenntechniken, welche in Abhängigkeit vom Locus Allele unterscheiden können, welche sich in der Größe durch nur 2, 3 oder 4 Basenpaare unterscheiden. Zahlreiche solcher Loci können mit Hilfe der horizontalen, diskontinuierlichen Polyacrylamidgel-Elektrophorese (hd-PAGE) aufgetrennt werden. Einige Loci stellen jedoch eine besondere Herausforderung an die Trenntechnik. Ein zusammengesetztes (oder Schritt-)Gelsystem, welches aus zwei Zonen besteht – einer anodischen und einer kathodischen –, welche sich durch die Porengröße und Ionenstärke unterscheiden, wurde entwickelt, um die Auftrennung von DNA-Fragmenten zu verbessern, ohne daß eine zusätzliche elektrophoretische Ausstattung erforderlich ist. In Abhängigkeit von der Porengröße und von der Ionenstärke kann die anfängliche Wanderungsgeschwindigkeit verstärkt oder verlangsamt werden, mit Auswirkung auf die Auflösung. Im Vergleich zur hd-PAGE kann dieses zusammengesetzte gelelektrophoretische System zu einer gesteigerten Auftrennung von PCR-amplifizierten STR-Allelen führen.

Schlüsselwörter: DNA – VNTR – STR – PAGE – zusammengesetzte Gele

Introduction

With the advent of DNA analysis, the field of legal medicine gained a valid and robust technology for analyzing biological materials (Jeffreys et al. 1985, 1988). The polymerase chain reaction (PCR; Saiki et al. 1985) augmented the utility of DNA typing technology by affording greater specificity and sensitivity of detection of particularly small quantities of biological materials. Initially, the PCR was used to amplify loci containing simple sequence polymorphisms, such as HLA-DQα (Saiki et al. 1985). Since then a number of different polymorphic loci have been subjected successfully to PCR. One class of DNA markers that could be quite useful for genetic characterization with PCR are the highly polymorphic variable number of tandem repeat (VNTR) loci (Boerwinkle et al. 1989; Budowle et al. 1991; Horn et al. 1989; Kasai et al. 1990; Wolff et al. 1988). These genetic markers have been termed AMP-FLPs for amplified fragment length polymorphisms (Budowle et al. 1991).

At first, the AMP-FLP VNTRs of interest contained repeat sequences ranging in size from approximately 16 (e.g. D1S80) to 70 base pairs (e.g. D17S5) (Kasai et al. 1990; Budowle et al. 1991; Horn et al. 1989; Boerwinkle et al. 1989; Wolff et al. 1988). While not a concern for D1S80, preferential amplification of the larger of the 2 alleles in a heterozygote for some of these loci (e.g. D17S5) can occur. Consequently, short tandem repeat

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(STR) loci (Edwards et al. 1991, 1992; Polymeropoulos et al. 1992a, b; Kimpton et al. 1992), which are less susceptible to preferential amplification, are being sought as potential AMP-FLP systems for forensic identification. The difference in size between alleles of STR loci generally ranges from 2 to 5 base pairs. Therefore, depending upon the STR, resolution approaching that of sequencing gels may be necessary. To facilitate technology transfer and/or accomodate high-volume laboratories, simple, rapid and effective procedures for typing AMP-FLPs are desired.

Many variations on the horizontal, discontinuous polyacrylamide gel electrophoresis (hd-PAGE) format described by Allen et al. (1989) have been used to improve electrophoretic resolution of DNA fragments. These include the addition of matrix modifiers (Allen et al. 1989, 1993; Budowle et al. 1991), using different trailing ions (Allen et al. 1993; Rand et al. 1992), altering the pH of the gel (Sajantila et al. 1992), and the use of vertical gels (Hochmeister et al. 1991; Sajantila et al. 1992; Allen 1974). Sajantila and Lukka (1993) were the first to report the use of a composite (or step) gel system for augmenting resolution of AMP-FLPs and is essentially a vertical d-PAGE (Allen 1974) patterned after the stacking gel format (Ornstein 1964; Davis 1964). The gel was divided into 2 portions (both containing Tris-Sulfate, pH 4.5), a more open pore cathodal gel of approximately 4 cm in length and a tighter pore resolving gel that could vary in length. The procedure, however, requires additional electrophoretic equipment than that used by the current authors for hd-PAGE.

This paper describes a technique using a composite (or step) discontinuous polyacrylamide gel run horizontally on a cooled surface for separation of STR alleles. The STRs selected for evaluating the effectiveness of a composite d-PAGE (cd-PAGE) system are MBP (myelin basic protein: Polymeropoulos et al. 1992a) and SE-33 (human beta-actin related pseudogene: Polymeropoulos et al. 1992b). The technique described in this paper can separate STR alleles differing in size by as little as 2 base pairs. The system uses the same equipment and reagents used for separation of larger repeat sequence AMP-FLPs. Therefore, converting to an alternate electrophoretic regime, in many cases, may be unnecessary for typing STRs.

Materials and methods

DNA was isolated by organic extraction (Brinkmann et al. 1992) from whole venous blood from unrelated Spanish Caucasians living in Andalucia (southern Spain).

Polymerase chain reaction

MBP amplification. The primers employed were: 5′-GGACCTCGTGAATTACAATC-3′ and 5′-ATTTACCTACCTGTTCATCC-3′ (Polymeropoulos et al. 1992a). The components of the PCR were 1–3 ng of genomic DNA, 1 µl of each dNTP (10 m*M* stocks), 5 µl of 10 × PCR buffer (Perkin-Elmer), 1 µl of each primer (20 µ*M* stocks), and 2.5 units of Taq polymerase (Perkin-Elmer). The final volume was brought to 50 µl with sterile double distilled H_2O. The PCR was carried out in a Perkin-Elmer 9600 thermal cycler. The cycles were 95°C for 30 s for denaturing, annealing at 64°C for 30 s, and primer extension at 72°C for 30 s. The total number of cycles was 27.

SE33 amplification. The primers employed were: 5′-AATCTGGGCGACAAGACTGA-3′ and 5′-ACATCTCCCCTACCGCTATA-3′ (Polymeropoulos et al. 1992b). The components of the PCR were 1–3 ng of genomic DNA, 1 µl of each dNTP (10 m*M* stocks), 5 µl of 10 × PCR buffer (Perkin-Elmer), 0.25 µl of each primer (100µ*M* stocks) and 2.5 units of Taq polymerase (Perkin-Elmer). The final volume was brought to 50 µl with sterile double distilled H_2O. The PCR was carried out in a Perkin-Elmer 9600 thermal cycler. The cycles consisted of denaturation at 94°C for 10 s, annealing at 60°C for 10 s, and primer extension at 72°C for 60 s for an initial 10 cycles; the final 16 cycles consisted of denaturation at 90°C for 10 s, primer annealing at 64°C for 10 s and primer extension at 72°C for 60 s.

PAGE electrophoresis. MBP and SE33 products were separated initially by hd-PAGE gels (21 cm long). For MBP, the polyacrylamide gels were 8.5% T and 3.3% C (piperazine diacrylamide as cross-linker) and contained 80 m*M* formate, pH 9.0. SE33 alleles were separated in 7% T and 3.3% C (piperazine diacrylamide as cross-linker) polyacrylamide gels that contained 60 m*M* formate, pH 9.0.

In all cases, 4 µl of amplified sample were surface loaded via fiber glass applicator tabs (Pharmacia N1850-901) that were placed

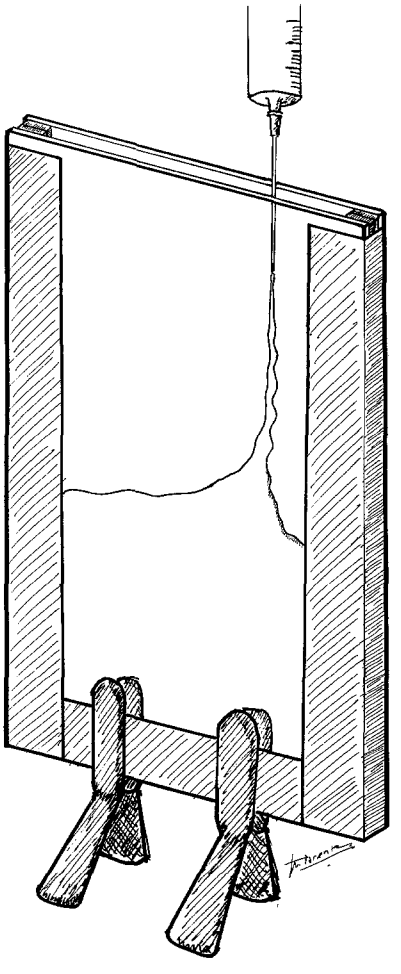

Fig. 1. Composite-gel casting: vertical array

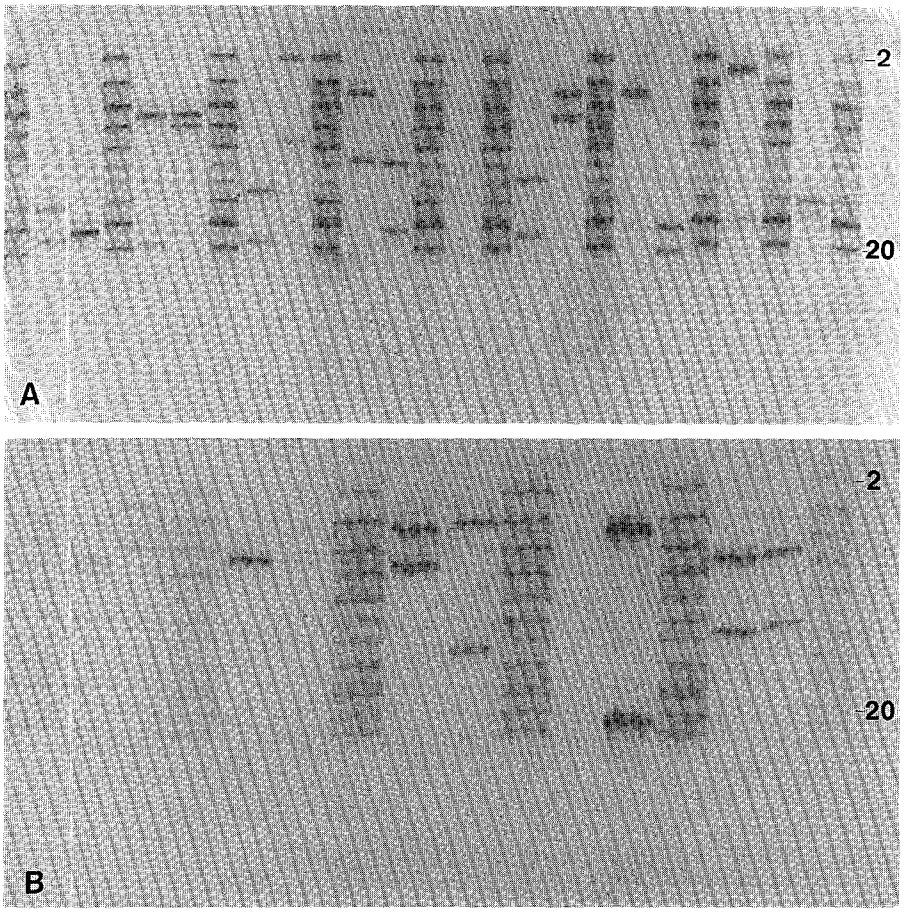

Fig. 2. A SE33: standard hd-PAGE. **B** SE33: composite-gel (cd-PAGE)

1 cm from the cathode. The electrode buffer was 0.28 *M* tris-borate, pH 9.0, which also contained 0.01% bromophenol blue to serve as a dye marker. The electrophoresis power supply was set at 600 V, 20 mA and 20 W. The electrophoresis temperature was maintained at 15°C. The electrophoretic process was stopped when the moving boundary migrated to the anodal plug. The alleles were visualized by silver staining (Budowle et al. 1991).

Composite-PAGE electrophoresis. Many variations of composite gels can be used to enable resolution of AMP-FLP alleles. As examples for improving the resolution of MBP and SE33 alleles obtained with hd-PAGE described above, the following composite gel systems were employed: a cathodal (or sample origin) gel and anodal gel.

	SE33	MBP
Cathodal gel:	6% T, 4% C Formate, pH 9.0: 55 m*M* Length: 11 cm	9% T, 4% C Formate, pH 9.0: 90 m*M* Length 12 cm
Anodal gel:	7% T, 2% C Formate, pH 9.0: 50 m*M* Length: 10 cm	10% T, 2% C Formate, pH 9.0: 84 m*M* Length 9 cm

Composite gels were cast on Gel-Bond sheets (FMC) in a vertical array between 2 glass plates sealed with adhesive tape (Scotch-brand electrical 3M) or with 3% agarose (Fig. 1). Gel thickness was dictated by a gasket composed of 2 layers of Dymo tape (Esselite Pendaflex Corporation, Augusta, Georgia). After pouring the amount of acrylamide necessary for the first zone (cathodal or anodal, whichever is preferred), polymerization was allowed to proceed for 10 min. The second zone was poured and the composite gel was allowed to polymerize for at least an additional 2 h. Electrophoresis was carried out as described above for hd-PAGE.

Results and discussion

High resolution PAGE (e.g. sequencing gels) can resolve DNA molecules which differ in size by one base pair. These gels are usually run in a vertical format, and the bands are developed using radioisotopic or chemiluminescent detection techniques. Long vertical gels can require longer running times, require alternative equipment, and can be more cumbersome for casting and staining procedures. However, the use of horizontal gels, with a discontinuous buffer system, can reduce run times and labor times. The hd-PAGE format was modified to augment the resolving capability of this technique by including a composite gel format.

The STR polymorphic loci, MBP (Polymeropoulos et al. 1992a) and SE33 (Polymeropoulos et al. 1992b), have been shown to be amenable to PCR and potentially could be applicable to identity testing. Therefore, these 2 STRs were used for the study described in this paper. Discontinuous buffer composite polyacrylamide gels run in a horizontal format were used to demonstrate that separation of MBP and SE33 alleles can be improved. Figures 2a–b and 3a–b show comparisons of SE33 ladders and MBP ladders, respectively, typed using hd-PAGE and cd-PAGE with the same size gels both run horizontally on the same electrophoretic apparatus. The separation of the SE33 allelic ladder (i.e. the distance between alleles 2 and 20) increased from 2.2 cm

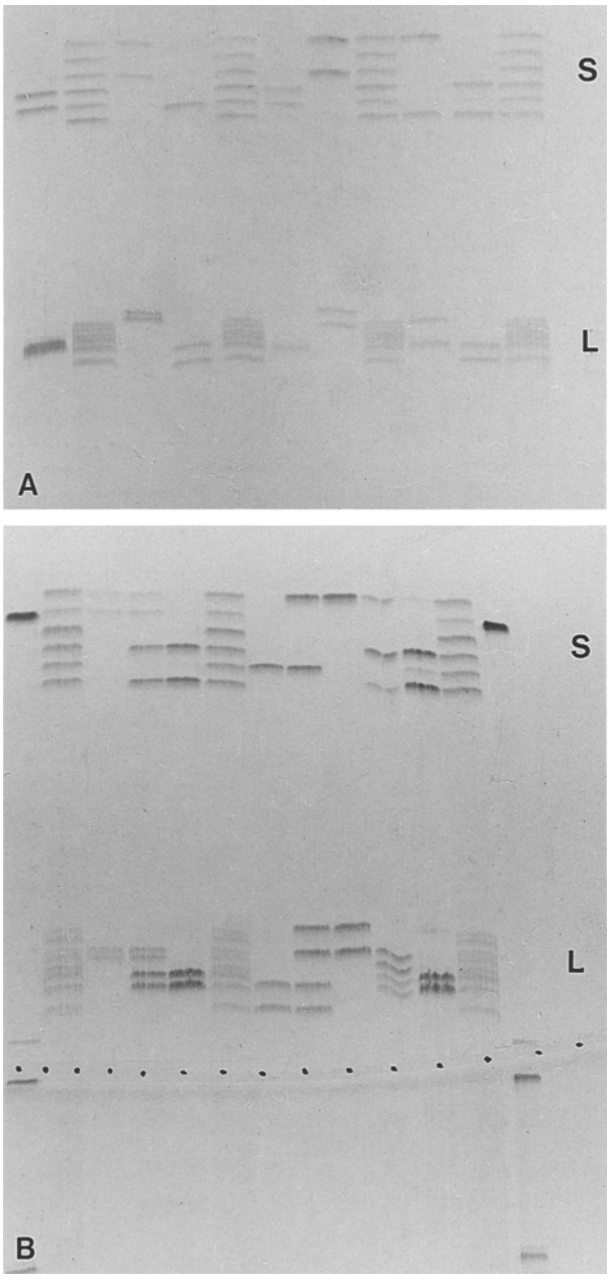

Fig. 3. A *MBP:* standard hd-PAGE. **B** *MBP:* composite-gel (cd-PAGE); *s:* shorter locus – *l:* longer locus; ·····: boundary between two type of gels

with hd-PAGE to 2.8 cm with cd-PAGE, and there was only a slight inrease in band width. This enhanced separation with cd-PAGE was sufficient to resolve consecutive alleles at the SE33 locus.

The resolution of alleles at the MBP locus presents an additional challenge to just separating alleles differing by 4 base pairs. The STR alleles for MBP result from 2 tandemly repeated loci that differ in size from one another by approximately 100 base pairs, since one set of repeats is flanked by the two MBP primers and the second set of repeat regions is itself duplicated at the 3′ end. The cd-PAGE approach enabled separation of alleles at both MBP loci (Fig. 3b), which was not readily possible with hd-PAGE in a 21 cm gel (Fig. 3a). Since alleles at the larger locus were able to be resolved without compromising the separation of the alleles of the smaller locus, the composite gel approach also may be applicable to multiplex AMP-FLP typing.

It is hypothesized that the difference in pore size between the cathodal and anodal portions of the composite gel enables increased separation of alleles compared with hd-PAGE. In the cathodal zone the pore size is that generally used to separate DNA molecules based on size differencess with hd-PAGE. However, the anodal zone consists of a larger pore size. When the separate molecules reach the boundary between the two gels, the smaller alleles arrive prior to the larger alleles. Therefore, the smallest allele has a longer time period in which it can migrate faster in the less restrictive anodal gel. The increased separation observed with the composite gel is a result of the longer period of time each smaller allele migrates in the anodal zone compared with each successive larger allele.

Although not shown here, many variations in % T, % C, ionic strength of the gel buffer, and the lengths of the anodal and cathodal gels were tried. The cd-PAGE conditions described in this paper for typing MBP and SE33 are one of many approaches that can be utilized. However, after empirically testing many parameters of cd-PAGE, certain trends were apparent: 1) empirical testing is required for determining the most effective cd-PAGE approach for AMP-FLP typing of each STR; 2) to obtain good separation of STR alleles without concomitant band diffusion, the cathodal zone should contain more cross-linker than the anodal gel. However, only differences of 1–2% C can be tolerated before band widths tend to increase to undesirable levels; 3) while differences in ionic strength between the cathodal and anodal gels did improve allele separation, large differences in ionic strength between the 2 gel zones resulted in excessive band diffusion; and 4) cd-PAGE can also be used to improve AMP-FLP typing in longer (32–40 cm) vertical gel formats (data not shown).

In conclusion, the composite gel system can increase the separation of PCR amplified STR alleles differing by 2, 3, or 4 base pairs, obviating the necessity of employing different electrophoretic separation systems for STRs and larger VNTR AMP-FLPs. This technique also may be applicable in other fields where electrophoretic separation of molecules, with only slight differences in molecular weight, is necessary.

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