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Simultaneous separation and genetic typing of four short tandem repeat loci by capillary electrophoresis

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Abstract

Genetic typing based on the four short tandem repeat (STR) loci vWF, THO1, TPOX and CSF1PO by capillary electrophoresis and laser-induced fluorescence detection has been demonstrated. It was necessary to separate these alleles in single-stranded form and to prevent rehybridization and eliminate secondary structures. Separation under both denaturing and non-denaturing conditions is discussed. Poly(ethylene oxide) (PEO) (1.6% 8 000 000 M_r) and PEO (1.5% 600 000 M_r) were dissolved in 1×TBE (0.54 g Tris, 0.275 g boric acid and 0.100 mmol disodium EDTA, diluted to 50 ml with deionized water), 3.5 *M* urea buffer was the separation matrix used for denaturing condition. A matrix composed of 1.9% 8 000 000 M_r PEO and 1.8% 600 000 M_r PEO in 1×TBE was used for the non-denaturing condition. Elevated temperature was required when the separation was performed without urea. Single-base resolution was obtained in both cases. Non-denaturing electrophoresis results in faster separations. Future adaptation to multiple-capillary array systems should allow fast genotyping of large numbers of samples simultaneously.

Keywords: DNA

1. Introduction

STR (short tandem repeat) loci consist of short, repetitive sequence elements of 3–7 base pairs (bp) in length [1–3]. These abundant repeats are well distributed throughout the human genome and are a rich source of highly polymorphic markers which often may be detected using the polymerase chain reaction (PCR). Alleles of these loci are differentiated by the number of copies of the repeat sequence contained within the amplified region, and are typically distinguished from one another using radioactive, silver stain, or fluorescence detection following slab gel electrophoresis [4]. Since STRs have shorter repeat units than VNTR repeats such as D1S80 [8], the resulting 100–500 base-pair PCR products are more tolerant of partial degradation of the DNA samples than other genotyping methods such as Southern hybridization, RFLP or PCR methods involving VNTR systems [5,6]. Potential applications related to STR include human identification, which is very popular in current forensic testing, sample-source determinations in transplantation, cell-line authentication and construction of genetic linkage maps. There are important applications also in disease diagnosis; for example, the increased number in the trinucleotide repeats over normal results in Huntington's disease [19].

It is thus of great interest to find a rapid, accurate method to perform genotyping based on an STR system. In a previous study, we have demonstrated a precise genetic typing method by capillary electrophoresis with co-injection of a ds-allelic ladder as an absolute standard [9]. In this study, we demonstrate

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genetic typing based on four STR loci according to the same principle. The pooled ss-allelic CTTv ladder serves as an absolute standard. An individual sample including specific alleles in each locus was amplified by PCR and was co-injected with the allelic ladder to determine the genotypes. The four STR loci studied are vWA (formerly vWF), THO1, TPOX and CSF1PO. The core repeats are AGAT, AATG, AATG and AGAT, respectively. The main goal of this study is to show several strategies to achieve the separation of single-stranded fragments with single-base resolution and with good reproducibility. Separation under both denaturing and non-denaturing conditions is discussed.

2. Experimental

2.1. Capillaries and reagents

Capillaries (Polymicro Technologies, Phoenix, AZ, USA) with 75 μm I.D. and 365 μm O.D. were used. Tris base, boric acid and EDTA were obtained from Sigma (St. Louis, MO, USA). Poly(ethylene oxide) (PEO) was obtained from Aldrich (Milwaukee, WI, USA). Urea was purchased from ICN Biomedicals (Aurora, OH, USA). A dry-bath incubator was purchased from Fisher Scientific.

2.2. Separation matrix

The polymer (powder) with the molecular mass needed was weighed, then added slowly to 10 ml stirred 1×TBE (0.54 g Tris, 0.275 g boric acid and 0.100 mmol disodium EDTA, diluted to 50 ml with deionized water) buffer. Stirring was maintained at room temperature until the matrix was homogeneous and transparent (about 1 day). No degassing was needed. The matrix preparation procedures were similar for both denaturing and non-denaturing separation except 3.5 *M* urea was added in 1×TBE buffer in the case of the denaturing matrix.

2.3. DNA sample and materials

The vWA, THO1, TPOX and CSF1PO allelic ladders were purchased from Promega (Madison, WI, USA). The PCR products of an individual sample were obtained from the US Department of Justice, Berkeley, CA, USA. Both the ladder and the sample were labeled with fluorescein dye on one of the primers in each pair.

2.4. CE–laser-induced fluorescence (LIF) detection system

An air-cooled argon ion laser (Uniphase, San Jose, CA, USA; Model 2213-150ML) with multi-line emission was used. The 488-nm line was separated with a glass prism for excitation. The experimental set-up is similar to that previously described [9]. A 520-nm edge filter (Ealing Electro-Optics, Holliston, MA, USA) and a 525-nm long-pass filter (Edmund Scientific, Barrington, NJ, USA) were combined to reject the scattered light. The total length of the capillary was 60 cm, with 50 cm from the injection end to the detection window. The running voltage was −12 kV.

2.5. Experimental procedure

A new capillary was flushed with 1 *M* HCl for 1–2 h [10]. The matrix was forced into the capillary by a 50 μl syringe. The capillary column was prerun for 10 min at the running voltage. The CTTv ladder was made by mixing the four loci together according to the ratio 2:2:1:1. A 2.0 μl-sample of this DNA mixture was transferred into a 1.5-ml microcentrifuge tube. Then, 3.5 μl of a mixture of formamide and 5% EDTA (5:1, v/v) was added to the tube. The PCR products of an individual sample were treated in the same way, but were mixed with diluted ladder (1:1) for coinjection. The DNA sample was injected at −12 kV for 20 s. Injection was carried out immediately after the sample was heated to 95°C for 2 min. The high temperature runs were performed by immersing the capillary into a 1-l beaker filled with hot water. The beaker was protected by a jacket to maintain the temperature during the run. Also, a layer of mineral oil was added on top of the water to minimize evaporation. After separation, the matrix was pushed out by water in a 50-μl syringe. The capillary was then flushed by 1 *M* HCl for about 30 min, after which it was ready for next run.

2.6. Sample treatment and injection

It was found that the mixture of alleles from the four loci was very difficult to denature. It is important to keep the DNA fragments in the denatured state at injection. Otherwise extra peaks appeared easily because the denaturing power of the matrix in the column is in general not sufficient to denature any rehybridized DNA. Several strategies can be employed during sample treatment and injection. (1) One can add an appropriate amount of formamide and EDTA to the sample. The ratio used in this study was DNA–formamide–5% EDTA (1:2:0.5, v/v/v). (2) The temperature of the DNA sample can be kept above 80°C before injection. (3) One can chill the sample on ice immediately after heating at 95°C for 2 min, and then inject the sample as soon as possible. (4) The ionic strength of the DNA sample is critical, and a low ionic strength is recommended to keep DNA in the denatured state for a longer time.

3. Results and discussion

3.1. Characteristics of STR alleles

The four corresponding base-pair ranges for vWA, THO1, TPOX and CSF1PO are 139–167, 179–203, 224–252 and 295–327 respectively. They are not overlapping. The alleles of an individual are defined according to the number of complete iterations of core repeat units present. The CTTv allelic ladder contains all the alleles of the four loci observed so far, which are the 13–20 alleles in CSF1PO, 5–11 alleles in THO1, 6–13 alleles in TPOX and 13–20 alleles in vWA. A particular case is that the locus THO1 has a common 9.3 allele which is a single-base deletion of the allele 10 [7]. This requires high resolution in the separation. Since we have successfully separated the double-stranded D1S80 locus (16 bp repeats) by 2% 8 000 000 M_r PEO matrix (in 1×TBE) without urea [9], we first tried this matrix for the CTTv allelic ladder separation. The DNA standard sample was directly injected without any pretreatment. However, the electropherogram obtained showed a continuous distribution of unresolved peaks. It was even impossible to distinguish each locus because the peaks were not ordered. The reason is that these alleles have similar and highly repeated sequences. This characteristic in base composition means that hybridization occurs not only between complementary strands, but also easily among non-complementary strands. Since THO1 and TPOX have the same repeated sequence AATG, it is also possible that non-specific annealing occurs across the two loci. In addition, heteroduplex [15] or secondary structure of DNA fragments (caused by self-hybridization or folding back) are present. It is therefore difficult to separate the STR alleles especially when a large number of them are mixed.

3.2. Separation using denaturing conditions

As discussed above, it is important to denature the individual allelic fragments during sample preparation and to keep the single-stranded DNA denatured during separation. In DNA sequencing analysis, usually a high concentration of urea is necessary to keep the DNA fragments from renaturing. Formamide has also been added in the gel and the buffer as an additional denaturant in room-temperature runs [16]. Recently, the denaturing agent in the running buffer has been modified from the typical 7 *M* to 3.5 *M* with [17] or without formamide [10]. Here, we tried the separation of STR alleles by a matrix designed for DNA sequencing. The composition is 1.6% 8 000 000 M_r PEO and 1.5% 600 000 M_r PEO mixed in 1×TBE buffer with 3.5 *M* urea. The DNA sample was denatured and then injected. The three electropherograms which are derived from (a) the allelic ladder only, (b) the PCR products of an individual sample including known alleles in each of the four loci and (c) a mixture of the ladder and an individual sample, are shown in Fig. 1. Clean separation of the allelic ladder was achieved. The 9.3 allele in THO1 is one base away from allele 10. The genotype was determined as homozygous 16 in vWA, homozygous 9.3 in THO1, heterozygous 8 and 9 in TPOX, and heterozygous 9 and 10 in CSF1PO. The conclusion is that good separation can be obtained by using typical sequencing matrix. No extra peaks due to secondary structures were observed.

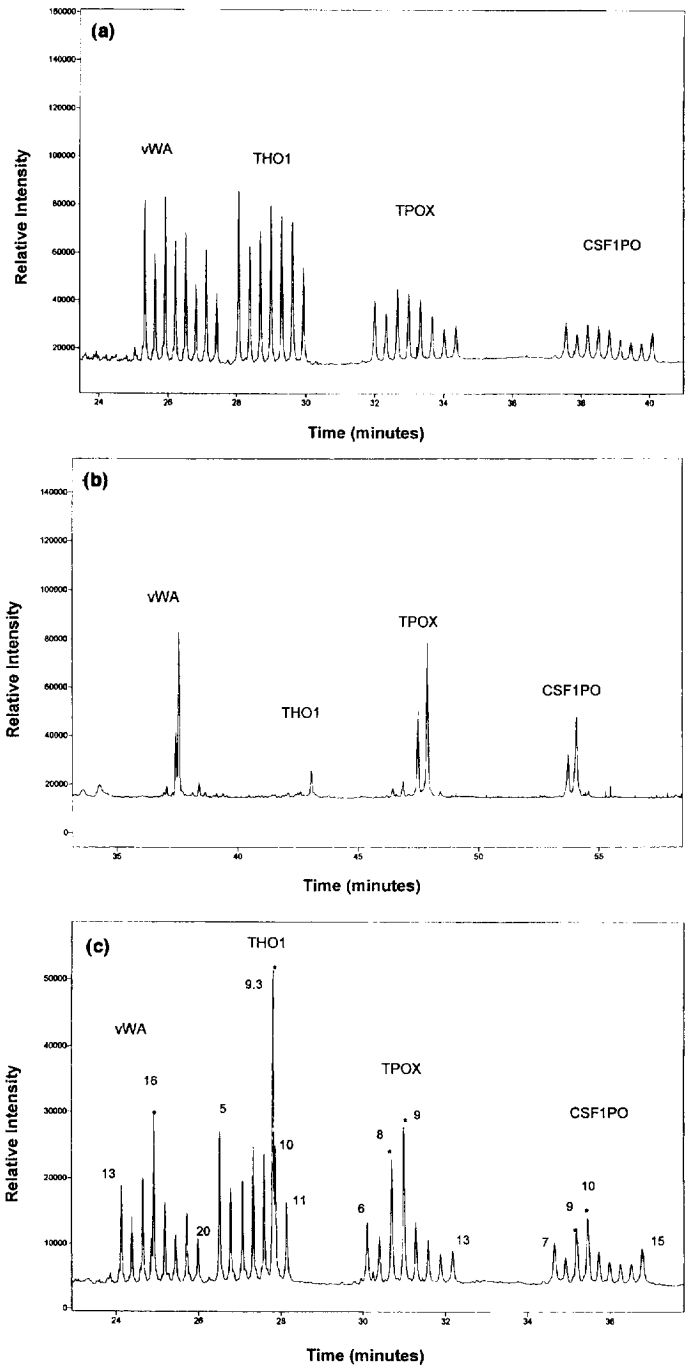

Fig. 1. (a) Electropherogram of CTTv standard ladder only. (b) Electropherogram of the PCR products of an individual sample. (c) Electropherogram of the co-injected mixture of CTTv ladder and individual sample. The genotype is determined by those peaks marked with * as shown in (c). Separation matrix: 1.6% 8 000 000 M_r PEO and 1.5% 600 000 M_r PEO dissolved in 1×TBE with 3.5 M urea. Running buffer: 1×TBE with 3.5 M urea. Voltage: −12 kV. Denaturing: 95°C for 1 min. Injection: −12 kV for 25 s. Capillary: 75 μm I.D., 60 cm total length, 45 cm effective length. Detection: 488-nm argon ion laser was used for excitation. The fluorescence signal was detected by a PMT with a 520-nm long-pass filter.

3.3. Separation using non-denaturing conditions

Separation under non-denaturing conditions has several advantages. First, it was found in the previous study [9] that PEO matrix without urea is very friendly to the fused-silica capillary column. The column can be repeatedly used for a long time. On the other hand, denaturing agents such as urea reduce the column lifetime. Second, the presence of high concentrations of denaturing agents increases the viscosity of the matrix so that it makes column filling and flushing more difficult [17]. Third, if the STR systems can only be analyzed by a denaturing matrix, it reduces the throughput of genotyping based on capillary electrophoresis. This is because the analysis of both VNTR (which needs non-denaturing conditions) and STR loci cannot be combined in one run.

Since no urea is needed when preparing the non-denaturing matrix, it is possible to use a higher percentage of polymer to improve the resolution. The optimal separation matrix was found to be composed of 0.19 g 8 000 000 M_r PEO mixed with 0.18 g 600 000 M_r PEO dissolved in 10 ml 1×TBE buffer. When the separation was performed using the same conditions as when a denaturing matrix was used, the separation was good but was not reproducible. The most obvious feature was that one peak was missing in the vWA locus. Also, the peaks were not equally spaced as expected. The problem was most serious in vWA. A possible explanation is the GC content in this locus is higher than the other loci. In addition, some extra peaks were observed. The electropherogram is shown in Fig. 2. We tried to denature the sample thoroughly, but without any improvement. The implication is that the urea-free matrix cannot prevent rehybridization and formation of secondary structure in the DNA fragments during separation. So, it is not possible to achieve good separation at room temperature without a denaturant in the matrix.

3.4. Separation at elevated temperatures

It is known that elevated temperatures can help denature DNA [11]. One concern is bubble formation inside the capillary when the temperature is increased. Another concern is whether the HCl-treated capillary wall (to eliminate the electroosmotic flow) can tolerate the high temperature. For these experiments, the urea-free matrix was stirred for a longer time (about 30 h) than normal matrices used in room temperature. The matrix was forced into the capillary by pressure, then most of the length of the capillary was immersed into hot water. After

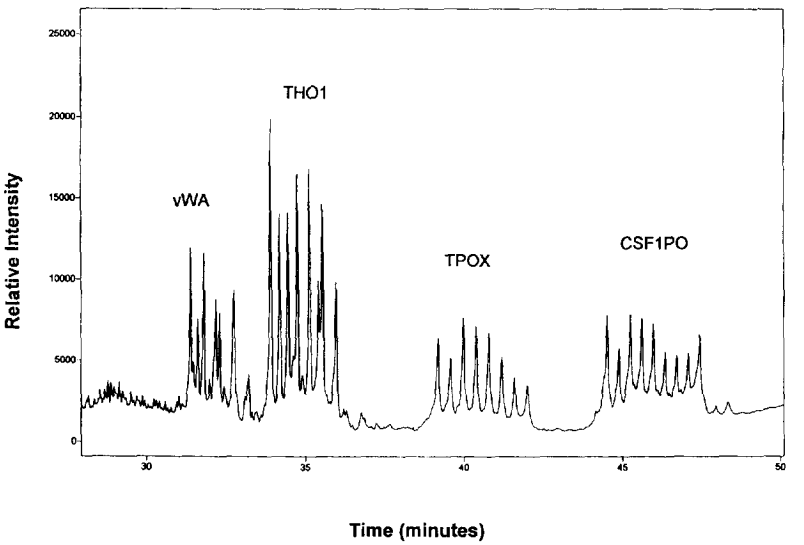

Fig. 2. Electropherogram of the sample in Fig. 1c obtained by using a urea-free matrix at room temperature. Separation matrix: 1.9% 8 000 000 M_r PEO and 1.8% 600 000 M_r PEO in 1×TBE. Other conditions are the same as in Fig. 1.

equilibration at running voltage for about 5 min, the denatured DNA sample was injected. Surprisingly, both the matrix and the capillary wall can tolerate high temperatures. Since the water bath cools over the course of the separation, the temperature changed from 70–55°C. The electropherogram obtained at 70–55°C is shown in Fig. 3. The high temperature electropherogram did not show rehybridization or the formation of secondary structure. The 9.3 allele is almost baseline resolved from allele 10 which has one additional base. Another advantage is that the separation time was shortened by ~20 min compared with the time needed at room temperature (Fig. 3 vs. Fig. 2). Experiments at 80°C were also attempted, with separation performance very similar to that of 70–55°C. However, slight peak broadening was observed when the temperature was increased to 85°C. This observation is in agreement with that of other groups [18]. The high temperature data shows that the entangled PEO matrix is very flexible, and that it can tolerate much higher temperatures than crosslinked polyacrylamide gel. This is because expansion of the gel matrix at elevated temperature destroys the covalent bonding of the gel with the capillary wall in the case of crosslinking, damaging its structure [8]. Also, because urea is absent, there is no decomposition, even at these high temperatures, to cause bubble formation.

4. Conclusions

We found that a 1.6% 8 000 000 M_r and 1.5% 600 000 M_r PEO mixture dissolved in 1×TBE buffer with 3.5 M urea provided single-base resolution and reproducible separation of STR alleles. The genotype of an individual can be readily determined by coinjection of the PCR products of the individual sample with the CTTv allelic ladder. It was also found that STR alleles can be separated successfully by a non-denaturing matrix at high temperatures. The composition of the non-denaturing matrix was 1.9% 8 000 000 M_r and 1.8% 600 000 M_r PEO in 1×TBE buffer. To avoid extra peaks due to heteroduplex formation, it was necessary to inject the DNA samples immediately after denaturation. The high temperature not only increased the separation speed but also improved the peak shape. Future adaptation to a multi-capillary array system [12–14] should allow rapid genotyping with high throughput.

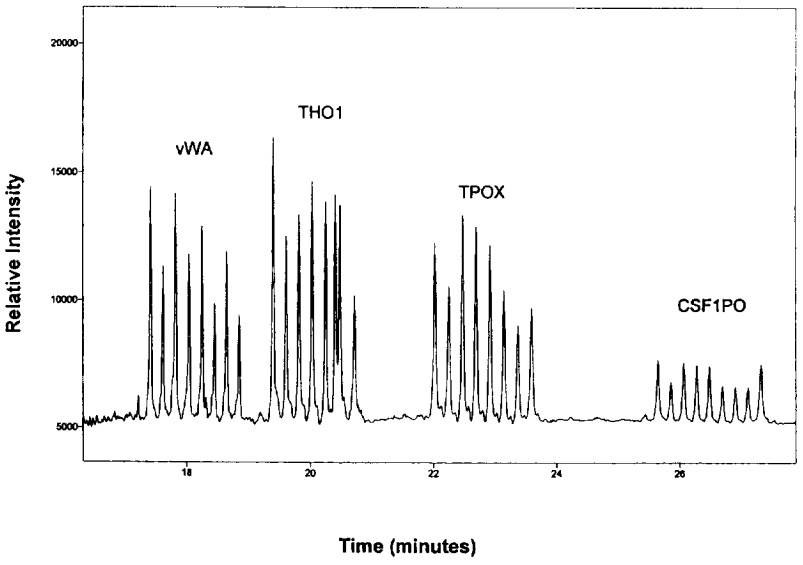

Fig. 3. Electropherogram of the sample in Fig. 1c obtained at high temperature by using a urea-free matrix. Buffer: 1×TBE. Temperature: 70°C (start) to 55°C (end). Other conditions are similar to those in Fig. 1.

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