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SOLID PHASE PURIFICATION AND SSCP ANALYSIS OF AMPLIFIED GENOMIC DNA BY CAPILLARY ELECTROPHORESIS

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ABSTRACT

Detection and identification of point mutations in genomic DNA has proven increasingly important in biomedical research. A variety of methods for the analysis of single base substitutions have been proposed among which Single Strand Conformational Polymorphism (SSCP) quickly gained success due to its simplicity.

In this work we present an analytical on-line tool which combines the ease of solid phase purification of amplified genomic DNA, the simplicity of SSCP and the significant potential advantages offered by capillary electrophoresis (CE).

IINTRODUCTION

Several methods have been proposed since the introduction of DNA amplification for the identification of mutations in genomic DNA. Due to its simplicity single stranded conformational polymorphism (SSCP) is the most frequently encountered in recent literature (1).

Magnetic solid phase DNA purification has gained wide popularity for either fluorescent or radioactive DNA sequencing (4,5).

Capillary electrophoresis permits high speed separation of biopolymers including double stranded and single stranded DNA (see 2 for a recent review). This high resolution analytical technique is very sensitive and reproducible and in addition allows automated instruments to quickly explore several analytical conditions to enhance the separation in specific samples. SSCP analysis in capillary electrophoresis has been already proposed for detection of the p53 mutation (3).

This paper presents a procedure to detect point mutations in amplified DNA by solid phase magnetic purification (4), single stranded DNA separation, SSCP analysis carried out on an automated CE apparatus and fluorescent automated solid phase DNA sequencing of the positive samples. The human β globin gene was used as a test for this procedure.

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MATERIALS AND METHODS

Dynabeads M-280 Streptavidin were purchased from Dynal AS (Oslo, Norway). Ultrafree-MC filters were from Millipore (Bedford, MA). Methylcellulose (high viscosity) and other chemicals were from Fluka (Buchs, Switzerland).

PCR amplifications were performed as follows (6): 500ng of genomic DNA was added to a standard (100µl) PCR mix containing 2.5 U Taq polymerase (Finnzyme), 1.5 mM $MgCl_2$, 200µM dNTPs and 0.2µM primers (Ap1-bio and R94). The mixture was cycled 30 times at 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes. The PCR product (100µl) was incubated with 400µg of prewashed Dynabeads for 15 minutes. The supernatant was removed using a magnetic device and the beads were washed twice with Tris buffer, pH 8.0 and treated for 10 minutes with NaOH (0.1 M) at room temperature to denature the amplified product. The alkaline supernatant containing the non-biotinylated strand was transferred to a clean tube and neutralized with HCl, 0.1 M. Before loading on CE the samples were desalted with Ultrafree-MC filters (cut off 30kD). The final volume was about 50µl.

CE was performed on a BioFocus 3000 system (BioRad, Hercules, CA) (in the reversed polarity mode) using coated fused-silica capillary (50 cm x 50 cm or 36 cm x 50µm, BioRad). Samples were introduced into the capillary tube using electrokinetic injection (at 8 kV for 20 sec). Separation within the coated capillary tube was performed under a constant voltage. The CE buffer consisted of 100 mM Tris, 100 mM boric acid, 2 mM EDTA and methylcellulose (high viscosity) at concentrations ranging from 0.1 to 0.6% (w/v). On-column detection was performed by UV adsorption at 260 nm, and the temperature of the capillary was set at 15°C. Solid phase fluorescent DNA sequencing was performed on positive samples following a published procedure (6).

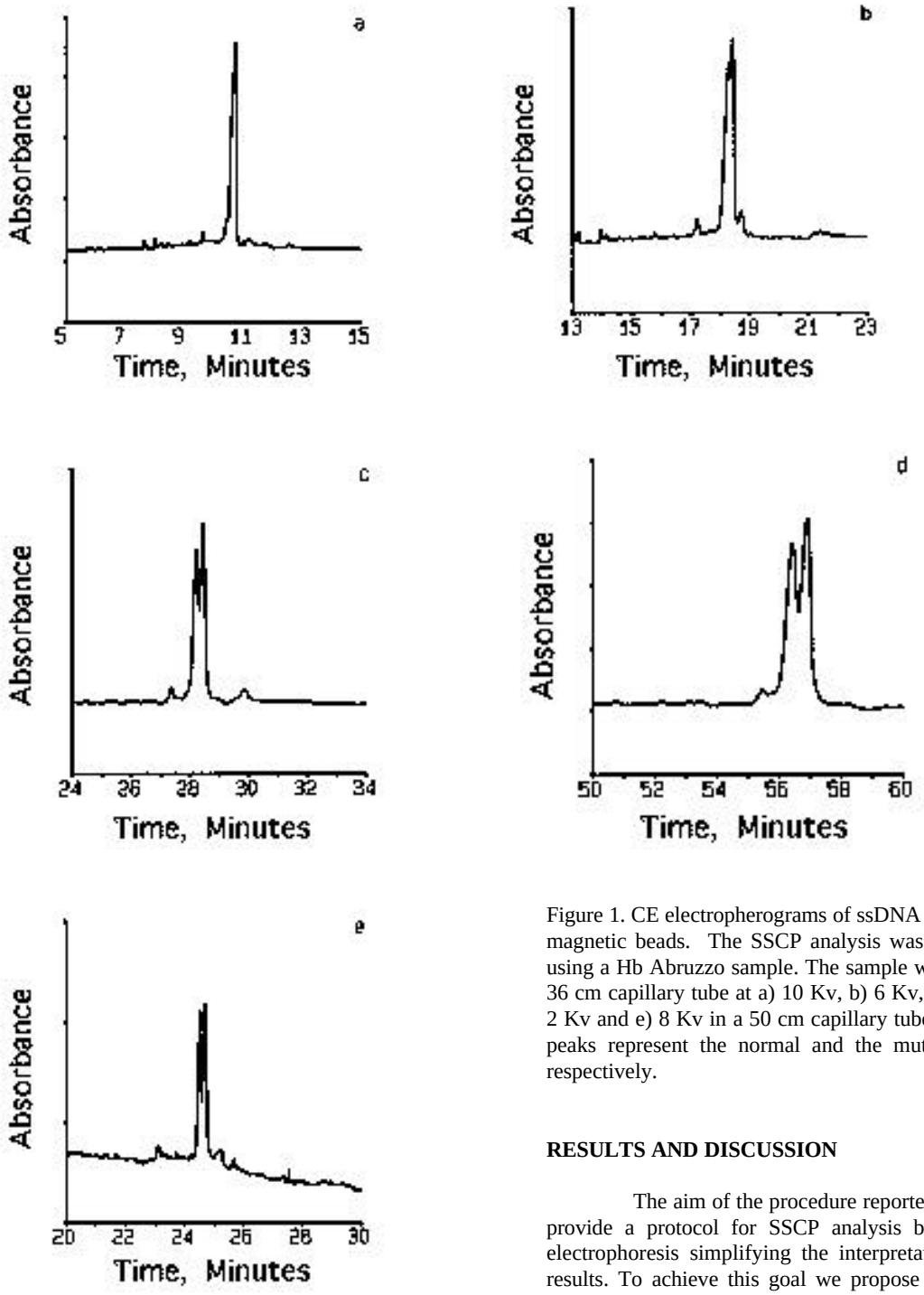

Figure 1. CE electropherograms of ssDNA purified by magnetic beads. The SSCP analysis was performed using a Hb Abruzzo sample. The sample was run in a 36 cm capillary tube at a) 10 Kv, b) 6 Kv, c) 4 Kv d) 2 Kv and e) 8 Kv in a 50 cm capillary tube. The two peaks represent the normal and the mutated DNA respectively.

RESULTS AND DISCUSSION

The aim of the procedure reported here is to provide a protocol for SSCP analysis by capillary electrophoresis simplifying the interpretation of the results. To achieve this goal we propose to amplify the target genomic region using a biotinylated primer. The resulting product could be purified from buffer, dNTPs and primers by a magnetic solid phase procedure. Then, single stranded DNA could be

rescued and analyzed by SSCP on a capillary electrophoresis apparatus running at a precise

temperature avoiding renaturation problems and interference from other PCR components (7). Positive samples could be sequenced using the immobilized DNA strand (5). In order to validate this hypothesis, we analyzed one sample that contained a heterozygous point mutation in the β globin gene (Hb Abruzzo (6)). A normal sample was used as a control.

We expected the presence of two peaks for the heterozygous sample and one peak for the normal sample. Separation was optimized by using two capillary tubes of different lengths and by varying the voltage of the runs. We also analyzed different concentrations of non-crosslinked gels made by methylcellulose. We tested glycerol as a possible additive (0-10%). However, we did not note any benefical effect on separation (data not shown). Figure 1 (a-d) shows results obtained on a 36 cm capillary tube. Separation improved by decreasing voltage from 10 Kv to 4 Kv. This change resulted in widening of the peaks. At 2 Kv, no improvement in resolution was evident. Using a 50 cm capillary tube the same sample gave a good resolution at 8 Kv in about 25 minutes (Fig. 1 e).

The single stranded DNA, rescued as explained, was used for SSCP analysis on a non-denaturing polyacrylamide slab gel under standard conditions (8). Silver staining showed two bands for the heterozygous sample and a single band for the normal one (data not shown). These findings demonstrate the feasibility of this approach for conventional SSCP analysis.

Many technical factors affect the sensitivity of SSCP including the type of the gel matrix, temperature and conditions of electrophoresis (9). Therefore to identify all possible mutations it is necessary to test several experimental conditions where a given set of conditions could allow detection of only a part of mutations (1). Automated capillary electrophoresis systems allow testing all possible experimental parameters in a rapid and automated way. It also allows precise control of the temperature of the run, one of the most critical parameters in conformational analysis. Furthermore, CE confers several advantages over more conventional techniques such as fast separation and use of only minute quantities of sample. This technique also allows replacement of the non cross-linked polymer matrix by introducing a new aliquot after each run, thus, ensuring a high run-to-run reproducibility. The precise control of the running temperature is crucial to the reproducibility of the analysis and to the stability of the different conformations of single stranded polynucleotides. The use of solid phase purification facilitates data interpretation and offers the possibility to sequence the DNA attached to the magnetic beads to verify the presence of the screened mutations. The proposed approach is suitable both for large scale automated SSCP screening of known mutations after method optimization and for optimizing SSCP analysis of unknown mutations.

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REFERENCES

1. I. Dianzani, C. Camascella, A. Ponzone & R.G.H Cotton: Dilemmas and progress in mutation detection. Trends Genetics. 9, 403-5 (1993)

2. C.A. Monnig & R.T. Kennedy: Capillary Electrophoresis. Anal Chem 66, 280R-314R (1994)

3. A.W.H.M. Kuypers, P.M.W. Willems, M.J. van der Schans, P.C.M. Linssen, H.M.C. Wessels, C.H.M.M. de Bruijn, F.M. Everaerts & E.J.B.M. Mensink: Detection of point mutations in DNA using capillary electrophoresis in a polymer network. J Chrom 621, 149-156 (1993)

4. S. Stahl, T. Hultman, A. Olsson & M. Uhlen: Solid phase DNA sequencing using the biotin-avidin system. Nucl Acids Res 16, 3025-38 (1988)

5. S.L. Thein & J. Hinton. 1991. A simple and rapid method for direct sequencing using Dynabeads. Br J Haematol 79, 113-5 (1991)

6. A. Mosca, R. Paleari, L. Zecca, F. M. Rubino, G. De Bellis, S. Debernardi, F. Baudo, D. Cappellini & G. Fiorelli: Hb Abruzzo β 143 (H21) His>Arg. A high oxygen affinity variant identified by mass spectrometry and DNA analysis. Hemoglobin 17, 261-8 (1993)

7. Q. Cai & I. Touitou: Excess PCR primers may dramatically affect SSCP efficiency. Nucl Acids Res 21, 3909-10 (1993)

8. P. J. Ainsworth, L. C. Surh & M. B. Coulter-Mackie: Diagnostic single strand conformational polymorphism, (SSCP): a simplified non-radioisotopic method as applied to a Tay-Sachs B1 variant. Nucl Acids Res 19, 405-6 (1991)

9. D. Glavac & M. Dean: Optimization of the Single strand conformation polymorphism (SSCP) technique for detection of point mutations. Hum Mut 2, 404-14 (1993)