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On-line melting of double-stranded DNA for analysis of single-stranded DNA using capillary electrophoresis

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Abstract

Capillary electrophoresis (CE) is a convenient, fast and non-radioactive method with possibilities for automatization. To analyse single-stranded DNA molecules in a more automated way, we developed a heating device to melt double-stranded DNA fragments in the capillary during electrophoresis. In this study we used this device to obtain single-stranded DNA, necessary for the detection of point mutations in DNA using the single-strand conformation polymorphism technique. Results show that double-stranded DNA molecules can be melted on-line into single-stranded DNA molecules, although not for 100%. In an attempt to find universal electrophoretic conditions for the analysis of single-stranded DNA, we investigated the influence of several parameters on the yield of single-stranded DNA molecules and on the resolution of the single-stranded DNA peaks. We demonstrate that this heating device is a technical adjustment of CE which contributes to more automated analyses of DNA fragments.

Keywords: Capillary electrophoresis; DNA; Single-stranded DNA

1. Introduction

Since DNA mutations are involved in the pathogenesis of genetic diseases as well as in the multistep nature of cancer [1], efficient and reliable detection of those mutations is becoming more and more important for the diagnosis of these diseases. Often point mutations are involved, which are single nucleotide changes in both strands of the double-stranded DNA (dsDNA). A simple method to detect point mutations is the single-strand conformation polymorphism (SSCP) technique [2]. The technique is based on the fact that a single-stranded DNA (ssDNA) molecule folds itself into a conformation which depends on its sequence. A point mutation alters the sequence of a ssDNA molecule and therefore the conformation. Because the mobility of a ssDNA molecule in a non-denaturing gel depends on its conformation, mutant and wild-type DNA molecules can be separated.

Several electrophoresis protocols can be used for SSCP analysis [3]. Recently we described the application of SSCP to capillary electrophoresis (CE) using a 4% linear polyacrylamide gel [4]. SSCP using CE was shown to be a convenient and non-radioactive method for the detection of point mutations. Other methods for the detection of point

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mutations using CE are described, each with their own advantages and disadvantages [5–7].

For the SSCP method it is necessary to have ssDNA. Usually this is obtained by heating and immediate cooling of the sample before injection. To automate the existing SSCP method and to limit sample handling before injection, we developed a heating device to obtain ssDNA molecules in the capillary during analysis. Using this heating device a very small part of the capillary (4 mm) is heated to 95°C. When a dsDNA fragment migrates through the heated part of the capillary it melts into ssDNA during electrophoresis. Further migration of the ssDNA molecules leads to immediate cooling of the ssDNA molecules, because directly after the heating device the capillary is cooled again.

This device differs from the one used in the constant denaturant capillary electrophoresis method described by Khrapko et al. [5]. In their method a water jacket is used to heat a large part (10 cm) of the capillary to a temperature (30–40°C) where dsDNA heteroduplexes are only partially melted and dsDNA homoduplexes remain unmelted. Using this device a heating and annealing procedure of half an hour before injection is still necessary.

Since, at one electrophoretic condition, the melting of dsDNA depends on the length and the sequence of the DNA fragment [8], we tested this melting procedure on several DNA fragments using CE in a non gel sieving buffer (NGSB). Furthermore, we investigated the influence of the temperature of the heating device, the salt concentration of the running buffer and the injected amount of PCR fragment on the yield of single-stranded molecules.

To improve the separation of ssDNA molecules we tested several parameters known to have an influence on the migration of the single-stranded molecules, for one DNA fragment. Preliminary results of this work were presented previously [9].

2. Experimental

2.1. Material

DNA was isolated [10] from the cell lines CEM, MOLT-4 and SW480 and was used as template for, respectively, amplification of the P53 gene, the N-ras gene and the Ki-ras gene. A lysate from the Mycoplasma infected cell line CaCo2 was used to amplify the ribosomal RNA of the Mycoplasma.

2.2. Polymerase chain reaction

Oligonucleotides used for amplification were synthesised on a 391A DNA synthesizer (Applied Biosystems, Warrington, UK). The sequences and positions of the oligonucleotides are listed in Table 1 and the PCR conditions for each fragment are listed in Table 2. PCR was performed with a Perkin-Elmer Cetus thermocycler (Perkin-Elmer, Norwalk, CT, USA).

The *Taq* (*Thermus aquaticus*) DNA polymerase

Table 1
The sequences and positions of the oligonucleotides

Nr.	Sequence (5′–3′)	EMBL Genbank Accession Nr.	Position
1	GACTGAGTACAAACTGGTGG	M10055	3–22
2	TGGGCCTCACCTCTATGGT	M57430	190–198
3	GGCCTGCTGAAAATGACTGA	M54968	181–200
4	GTCCTGCACCAGTAATATGC	L00045	226–245
5	TTTCTTTGCTGCCGTGTTCCA	X54156	12973–12993
6	ATAAGATGCTGAGGAGGGGC	X54156	13325–13344
7	GCGGTGTGTACAAGACCCGA	M23931	1370–1389
8	GCGGTGTGTACAAA(AC)CCCGA	M24658	1369–1388
9	CGCCTGAGTAGTACGT(AT)CGC	U04657	517–536
10	TGCCTG(AG)GTAGTACATTCGC	L24103	847–866
11	CGCCTGGGTAGTACATTCGC	M24293	873–892
12	CGCCTGAGTAGTATGCTCGC	M23931	872–891

Table 2
The PCR conditions used for each fragment

	N-ras	Ki-ras	P53	Mycoplasma
Oligo	1+2	3+4	5+6	7 t/m 12
Amount each oligo (pmol)	30	30	30	80
Template DNA (μg)	0.5	0.5	0.25	20 μl lysate
Taq (units)	2.5	2.5	2.5	2.5
dNTP's (μM)	250	250	250	500
PCR volume (μl)	100	100	100	50
Number of cycli	35	40	35	40
Denaturation	30 s, 94°C	30 s, 94°C	60 s, 94°C	60 s, 94°C
Annealing	30 s, 55°C	30 s, 55°C	60 s, 56°C	60 s, 50°C
Extension	60 s, 72°C	60 s, 72°C	60 s, 72°C	120 s, 72°C
Length PCR fragment (bp)	118	162	372	518
% GC	50	40.7	58.1	46.1

was obtained from Life Technologies (Gaithersburg, MD, USA).

2.3. Instrumentation

An uncoated fused-silica capillary, 480 mm effective length and 0.075 mm I.D. (SGE, Ringwood, Australia) was mounted in a user-assembled cartridge and placed in a BioFocus 3000 CE instrument (Bio-Rad, Hercules, CA, USA).

For on-line melting the BioFocus user-assembled cartridge was adapted. The temperature range of the capillary cooling flow was extended from 15–40°C to 0–60°C by using an external thermostatic waterbath instead of the BioFocus 3000 capillary cooling. For local heating, this water-cooling flow was interrupted and diverted at 146 mm from the inlet side, and at right angles to this interrupted flow another water flow was applied, which can be heated to 95°C by another external waterbath (Fig. 1). The length of the heated capillary is 4 mm.

2.4. CE analyses

Prior to all analyses the capillary was rinsed, using a pressure of 100 p.s.i. (ca. 700 kPa), with water (60 s), 1 *M* NaOH (50 s), 0.1 *M* HCl (20 s) and with 1×TBE (89 m*M* Tris-borate, 0.2 m*M* EDTA, pH 8.3) (20 s) and then filled with 1:1 diluted Non Gel Sieving Buffer (kindly provided by Bio-Rad) for 50 s. PCR samples were pressure-injected (5 p.s.i.; ca. 34 kPa) without pre-treatment and separated at reversed polarity (cathode at the injection side) under constant current in a 1×TBE running buffer. UV absorption at 260 nm was used for detection.

3. Results and discussion

3.1. Polymerase chain reaction

The amplification of the N-ras gene of MOLT-4 DNA, the Ki-ras gene of SW480 DNA and normal DNA and the P53 gene of CEM DNA resulted in a PCR fragment of, respectively, 118 bp, 162 bp and 372 bp (Table 2). The amplification of the ribosomal RNA of Mycoplasma infected $CaCo_2$ lysate resulted in a fragment of 518 bp. Using these amplification reactions we had a broad range in length and GC content of the PCR fragments to test the on-line melting procedure.

3.2. On-line melting

We described the application of SSCP to CE using a 4% linear polyacrylamide gel in a coated capillary [4]. Under these conditions the capillary coating decayed when part of the capillary was heated to 95°C, resulting in a severe loss of resolution after repeated experiments. We therefore used a non gel sieving buffer (NGSB) in an uncoated capillary. The high temperature had no detectable negative effect on the resolution after repeated runs.

Fig. 2 shows the electropherograms of the am-

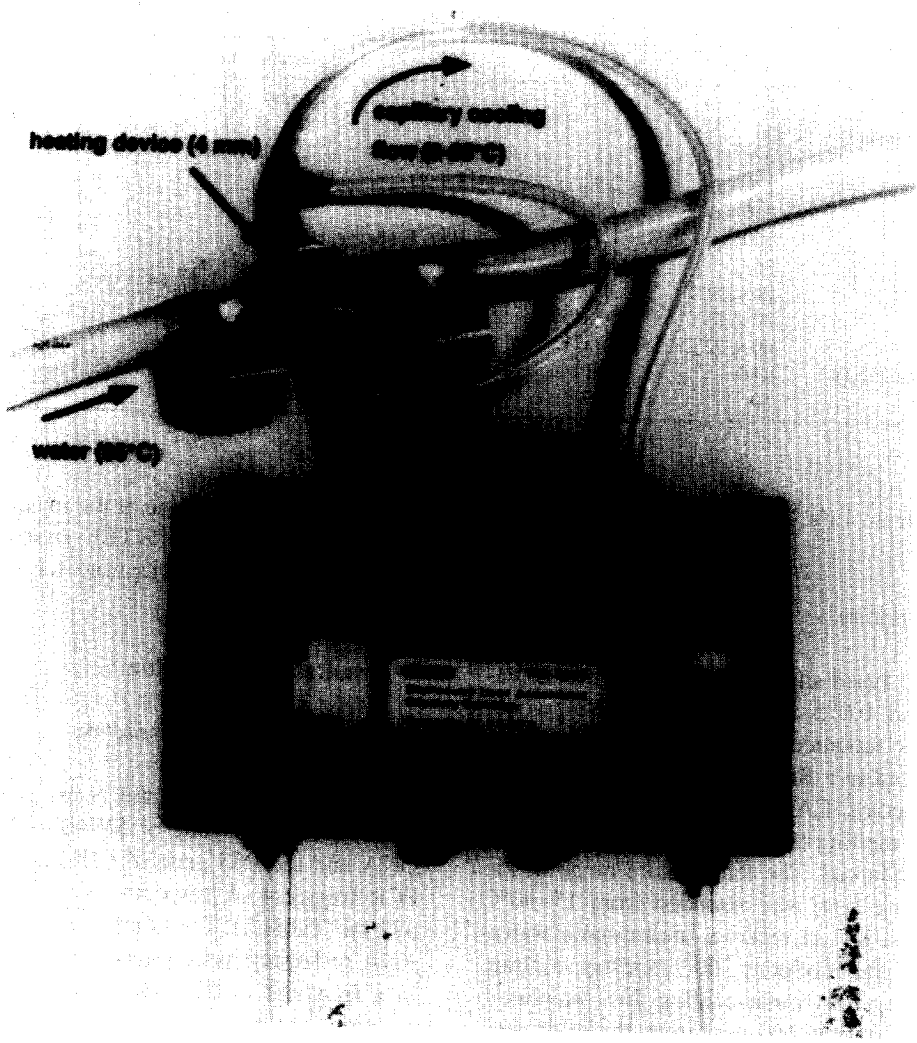

Fig. 1. The modified BioFocus user-assembled cartridge with the interrupted and diverted cooling flow and the heating device with the additional heating flow.

plified Ki-ras gene of normal DNA without (A) and with on-line melting (B). With the heating device at 65°C, only the dsDNA fragment is present. With the heating device at 95°C, part of the dsDNA molecules are melted into ssDNA molecules. Because of the complementary sequence of the single-strands of one DNA molecule, their folded conformation is different, which leads to different mobilities in the sieving buffer. This results in two different ssDNA peaks in the electropherogram. As is the case for external heating and cooling, the dsDNA peak is still present with on-line melting, due to incomplete denaturation. This dsDNA peak can be used as an internal standard because the mutant and normal dsDNA molecules have the same migration time. The shift of the dsDNA peak is a result of the effect of temperature on the migration time caused by the difference of the temperature of local heating (65°C versus 95°C).

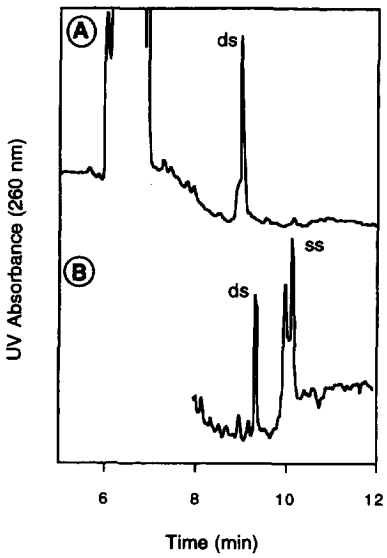

Fig. 2. Electropherograms of the Ki-ras PCR product of normal DNA with (A) the heating flow at 65°C and (B) the heating flow at 95°C. Conditions: pressure injection of 40 p.s.i.·s, constant running current of 15 μA and a temperature of the capillary of 5°C.

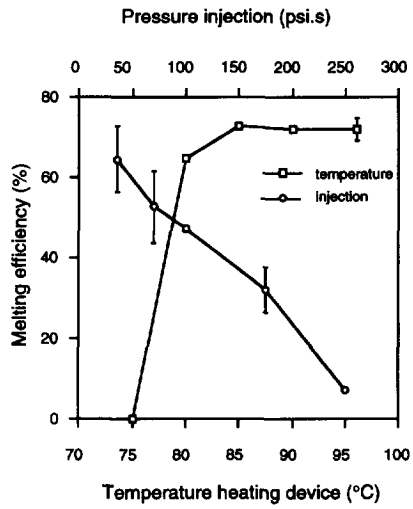

Fig. 3. Influence of the temperature of the heating device and the injected amount of PCR product on the yield of single-stranded molecules of the Ki-ras PCR product of normal DNA. Conditions: constant running current of 15 μA and a temperature of the capillary of 5°C.

Oligonucleotides, salt, enzyme and nucleotides used in the PCR migrate from 6 to 7.5 min (not shown in panel B).

3.3. Influence of parameters on the yield of single-stranded DNA

The UV absorbance of ssDNA molecules is different from the UV absorbance of dsDNA molecules [11]. We defined the DNA melting efficiency as the ratio of the area under the curve (AUC) of the two ssDNA peaks and the AUC of the ssDNA plus the dsDNA peaks.

Fig. 3 shows the influence of the temperature of the heating device on the yield of ssDNA molecules of the Ki-ras PCR product of normal DNA. Melting of the dsDNA molecules is well established using a heating device at more than 85°C, which is in agreement with the calculated melting temperature of this PCR fragment, which is 80.5°C [8]. Since the calculated temperatures of the other PCR fragments are 78.9°C (N-ras), 83.3°C (Mycoplasma) and 86.1°C (P53) we used a temperature of 95°C for further analyses to be sure of melting all different dsDNA samples used.

The influence of the injected amount of PCR product on the yield of ssDNA molecules of the Ki-ras PCR product of normal DNA is also shown in Fig. 3. Increasing the injected amount leads to a lower melting efficiency of dsDNA molecules probably as a result of a higher chance of renaturation. The yield of ssDNA molecules of different PCR products with the same concentration and the same injected amount differs for each PCR fragment, because also the length and the GC content of the PCR fragment influences the melting [8] and thus the yield of ssDNA molecules. Because detection becomes a problem when injecting at low amounts we used a pressure injection of 40 p.s.i.·s for further analyses.

To test the influence of the salt concentration of the running buffer on the melting efficiency, we used a 0.5×, 1×, 2× and 3×TBE running buffer. Increasing the salt concentration had a negative influence on the yield of single-stranded molecules. This can be

expected since increasing the salt concentration leads to a higher melting temperature and faster reannealing [8]. Further decreasing the salt concentration only resulted in longer migration times (data not shown). For further analyses we used a 1×TBE running buffer.

3.4. Influence of parameters on the resolution of the single-stranded DNA peaks

We investigated the influence of the voltage during the run, the concentration of the non gel sieving buffer and the temperature of the capillary on the resolution of the ssDNA peaks in order to find universal electrophoretic conditions for SSCP analysis. In this study we determined the resolution by dividing the difference in migration times between the ssDNA peaks by the migration time of the dsDNA peak.

The running voltage had no significant influence on the resolution. For further analyses we used a constant current of 15 μA. For the influence of the concentration of the NGSB we tested a 0.3×, 0.5×, 0.6×, 0.75× and a 0.8×NGSB diluted with 1×TBE. Decreasing the concentration to 0.5× had no influence on the resolution, but had a positive effect on the yield of ssDNA molecules. A further decrease had a negative influence on the resolution. For further analyses we used a 1:1 diluted NGSB (running voltage and NGSB data not shown).

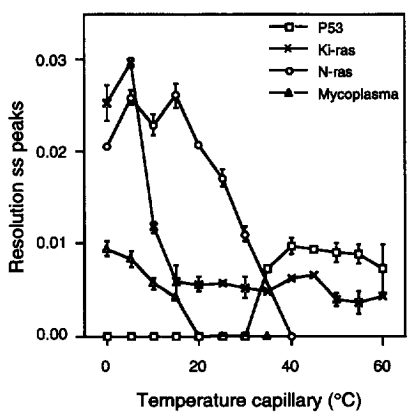

Fig. 4. Influence of the temperature of the capillary cooling flow on the resolution of the single-stranded peaks of different PCR products. Conditions: pressure injection of 20 p.s.i.·s and a constant current of 15 μA.

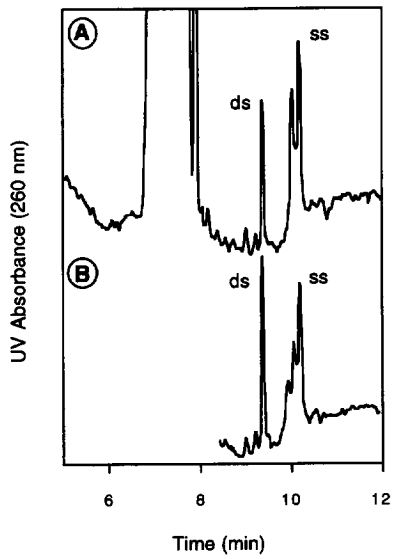

Fig. 5. Electropherograms of (A) the Ki-ras PCR product of normal DNA and (B) a 1:1 mixture of the PCR products of normal DNA and the homozygous cell line SW480. Conditions: pressure injection of 40 p.s.i.·s, constant current of 15 μA and a temperature of the capillary of 5°C.

The temperature of the capillary plays an important role in the resolution of the ssDNA peaks (Fig. 4). The optimal temperature is different for different PCR products. Not the length but the sequence of the DNA product is the reason for this effect.

For the resolution of the single-stranded molecules of the Ki-ras PCR product the optimal temperature of the capillary is 5°C. Fig. 5 shows that when this condition is used it is possible to separate the single-stranded peaks of SW480 DNA, which contains a homozygous point mutation, and normal DNA.

4. Conclusions

From the results we conclude that it is possible to melt dsDNA molecules into ssDNA molecules during analysis. The modification of the user-assembled cartridge is simple and easy to accomplish. The use of a NGSB in an uncoated capillary, instead of a 4% linear PAA gel with a coated capillary, makes it possible to use high temperatures, necessary to melt the dsDNA molecules, without decay of the capil-

lary. However, the resolution of the ssDNA molecules may be further improved by using other sieving polymers which can be used in uncoated capillaries. This, however, will be a topic for further studies.

We investigated the influence of several parameters on the yield and the resolution of the ssDNA molecules. The temperature of the heating device, the salt concentration of the running buffer, the voltage during the run and the concentration of the NGSB resulted in standard electrophoresis conditions for all PCR products. The optimal temperature of the capillary has to be determined separately for every individual PCR product. This is a disadvantage of the SSCP technique when used in an automated setting.

In this study we applied on-line melting for the detection of point mutations by means of the SSCP method, but this melting during analysis can probably also be used for other applications like on-line hybridisation of a probe to a single-stranded target DNA.

We feel that this on-line melting method contributes to a more automated and routine way of molecular diagnostics of human diseases.

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