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Received August 21, 2012
Revised September 5, 2012
Accepted September 5, 2012

Research Article

Determination of PCR products by CE with contactless conductivity detection

The use of CE with contactless conductivity detection for the determination of PCR products is demonstrated for the first time. The separation of specific length PCR products according to their size could be achieved using 5% PVP as a sieving medium in a separation buffer consisting of 20 mM Tris and 20 mM 2-(cyclohexylamino)ethansulphonic acid (pH 8.5). A fused silica capillary of 60 cm length and 50 μm id and an applied separation voltage of –15 kV were employed and separations could be completed within 20–50 min. PCR amplified DNA fragments of different sizes obtained from different bacterial plasmid templates as well as a fragment from genomic DNA of genetically modified soybeans could be successfully identified.

Keywords: Capillary electrophoresis / Contactless conductivity / Detection / Genetically modified organisms / PCR fragments
DOI 10.1002/jssc.201200800

1 Introduction

CE has a number of advantages compared to planar gel electrophoresis. In case of CE, higher voltages can be applied and therefore faster separation times are obtained; staining with toxic chemicals such as ethidium bromide is not required for detection and the CE process can easily be automated. For these reasons, CE is now used routinely for DNA sequencing. On the other hand, other forms of DNA analysis, such as the determination of restriction fragment length polymorphism and the simple analysis of PCR-amplified DNA fragments, are often still carried out by planar electrophoresis and have only partly been replaced by capillary methods.

In both formats, planar electrophoresis and CE, DNA fragments are separated by their size in a sieving matrix. Conventionally, cross-linked polyacrylamide gels have been used very frequently in planar electrophoresis. In contrast, in CE, entangled not cross-linked polymer solutions are now commonly used as the sieving material [1]. The polymer solutions should have low viscosity for easy handling, and should have the ability to coat the capillary wall in order to suppress the EOF and prevent adsorption of analytes. Many different polymers have been studied for the separation of DNA fragments such as linear polyacrylamide, PVP, poly-N,N-dimethylacrylamid, polyvinylalcohol, polyethyleneoxide, and various cellulose derivatives such as hydroxyethyl cellulose, hydroxypropyl cellulose. For details see these review articles [2, 3].

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Abbreviation: C^4D, capacitively coupled contactless conductivity detection

Detection for DNA in CE is usually carried out via UV-absorption as described previously [4–7] or for sequencing and when low detection limits are required by fluorescence as described previously [8–11]. However, labeling is generally required for the latter method.

Capacitively coupled contactless conductivity detection (C^4D) is an attractive alternative detection method. It is inherently much simpler than optical detection and suitable for any ionic species without requiring derivatization. The technique also enables the design of inexpensive and compact, portable, battery-operated CE instruments [12–16]. The standard UV detection, on the other hand, is not readily implemented for such instruments due to the high power consumption of conventional UV lamps. For details on C^4D, recent review articles are available [17–20].

A few instances of the detection of DNA in CE by conductivity measurements have been reported. Galloway et al. [21] used conventional contact conductivity measurements in 2002 for the detection of PCR products in electrochromatography. In 2005, Abad Villar et al. [22] demonstrated the suitability of C^4D for the detection of a DNA fragment on a lab-on-chip device. Xu et al. [23] used a potential gradient detector (a form of indirect conductometric detection) for determining DNA ladder segments and this was followed by a paper using carbon nanotubes in the sieving medium and C^4D [24]. Mühlberger et al. [25] reported the separation and detection of a ladder on a microfluidic chip using contactless conductivity measurement. Although these studies indicated the possibilities, practical uses of CE-C^4D for DNA analysis have not yet been shown. In this paper, the application of the determination of PCR products from plasmid DNA and also from genomic DNA is demonstrated. Different fragments obtained from bacterial plasmid templates were first determined. This application may be useful, for example, for the identification of particular microorganisms in

Table 1. List of primers used for PCR amplification of the DNA fragments

	Primer name	Primer sequence (5′–3′)
1	B1[&]HisDELYodASKA (Forward primer)	CGATTCGTCTTTACAAACTGGCTGTTGCTTTAGGTGTC
2	B2[&]HisDEL-BlcASKA (Forward primer)	CGCCTGCTGCCGCTGGTGGCGGC
3	R[$]ASKAseq, cloning (Reverse primer)	CAGGTCGACCCTTAGCGGCCGCATAGGCC
4	F-pCM655 (Forward primer)	GAGCGGATAACAATTTCACACAGGAAACAG
5	F-SEQ pCM655 (Forward primer)	GCGTACGTCGCGACCGCGGACATGT
6	R-SEQ pCM655 (Reverse primer)	CACCGCGCTACTGCCGCCAGGCA
7	Soy-F (Forward primer)	GAAGCAACCAAACATGATCCTC
8	Soy-R (Reverse primer)	ATGGATCTGATAGAATTGACGTTA

cell cultures. The second application concerned the identification of genetically modified soybeans. In many jurisdictions, food derived from genetically modified organisms must be specially labeled or is even banned completely, and therefore simple means for their detection are desired. The developed method for the analysis of PCR products is suitable for implementation on portable CE-C^4D instruments, and has the potential for field measurements.

2 Materials and methods

2.1 Chemicals

PVP (MW 1 300 000) was obtained from Sigma-Aldrich (Buchs, Switzerland), Tris and CHES were obtained from Fluka (Buchs, Switzerland). The DNA mass ladder was purchased from Invitrogen (Carlsbad, CA, USA) that is composed of an equimolar mixture of six low-mass ladder DNA fragments of 100, 200, 400, 800, 1200, and 2000 bp (base pairs). DNA Taq polymerase with 10× Thermopol reaction buffer and deoxynucleoside triphosphates (dNTP's) were obtained from New England Biolabs (Maine, USA) and oligonucleotide primers from Mycrosynth (Balgach, Switzerland). The DNA template for genetically modified organism soybean flour was obtained from the Cantonal Laboratory of Basel-City and the genomic DNA was extracted by using the genomic extraction REDExtract-N-Amp™ Seed PCR Kit obtained from Sigma (St. Louis, Missouri, USA).

2.2 PCR

Plasmid DNAs (pCA24N-YodA, pCA24N-Blc, and pCM655Empty) were transformed and isolated from the XL1-Blue strain by using a Wizard plus Miniprep Kit from Promega (Madison, WI, USA) and genomic DNA of genetically modified soybeans (soybean template Roundup Ready GM-Soybean-EX961053A) by using the REDExtract-N-Amp™ Seed PCR Kit. These DNA sources were used as templates for the production of PCR fragments of the specific sizes of 180, 400, 489, 555, and 674 bp by using corresponding primers (Table 1). All PCRs were performed on an Eppendorf PCR Thermal Cycler (Hamburg, Germany). The PCR reactions were carried out in a total volume of 50 μL, containing 300 nM of each primer (forward and reverse), 4% DMSO, 1.6 mM dNTP's (0.4 mM each), 5 U Taq polymerase, 10× Thermopol reaction buffer, 100 ng of template for genomic DNA, and 50 ng of plasmid DNA. The PCR reaction consisted of an initial denaturation step at 95°C for 3 min followed by 40 PCR cycles, each cycle consisting of denaturation at 95°C for 3 min, annealing at 59.5°C for 1 min, and extension at 72°C for 3 min. The final elongation was for 10 min at 72°C to ensure full extension of all amplified fragments. The PCR products were purified on Wizard SV PCR-Gel cleanup minicolumns (Promega) and eluted with 10 mM Tris-HCl (pH 7.5) containing 1 mM EDTA. This buffer was selected as it corresponds to the buffer in which the DNA mass ladder was supplied.

2.3 Instrumentation

Two CE instruments with contactless conductivity detectors were used for the measurements: a commercial as well as an in-house constructed system. The commercial instrument was a PrinCE 500 (Prince Technologies, Emmen, the Netherlands) and used for the work presented in Figs. 1–3. The unit built by us was based on the design of a portable, battery-powered instrument described earlier [16], but utilized a high-voltage supply from Spellman (CZE 2000R, Spellman, Pulborough, UK) capable of delivering up to 30 kV and was mains powered. It was used for the measurements presented in Figs. 4 and 5. The detectors employed with both instruments were also constructed in-house according to a design reported earlier [26]. The signals were monitored and recorded with an e-corder data acquisition system (eDAQ, Denistone East, NSW, Australia).

2.4 CE

Fused silica capillaries of 60 cm length, 50 μm id, and 365 μm od (Polymicro Technologies, Phoenix, AZ, USA) were used for the CE measurements. New capillaries were conditioned by rinsing with 0.1 M NaOH for 20 min and

then with deionized water for 20 min, followed by 0.1 M HCl, and again with deionized water for 20 min. Before each run, a capillary was rinsed with the background buffer for 3 min. The system was then equilibrated by applying the separation voltage until a stable baseline was obtained. For the analysis of DNA ladder and PCR samples, hydrodynamic injection was used. On the instrument constructed internally this was carried out by siphoning, through lifting the injection part of capillary up to 30 cm height, and on the commercial instrument, a pressure of 13.8 kPa was applied. In both cases, the injection time was 45 s. The background buffer consisted of 20 mM Tris, 20 mM CHES (pH 8.5), and 5% PVP (except where stated otherwise).

3 Results and discussion

3.1 Selection of sieving polymer and choice of the buffer

As sieving matrix a PVP-based medium reported by Xu et al. [23] and used in their work using potential gradient detection was adopted. The polymer was employed by Xu et al. [23] for its relative low viscosity and its ability to coat the inner capillary surface and thereby minimize the EOF. Important for application in CE-C^4D is also its electrical neutrality. As background electrolyte, a buffer composed of 20 mM Tris/CHES (pH 8.5) was used. This was also adopted from Xu et al., but used at a lower concentration to improve baseline stability.

Initially, the experiments were carried out with a DNA mass ladder containing fragments of 100, 200, 400, 800, 1200, and 2000 bp that covers the broad range of interest for the PCR products to be detected. The effect of the concentration of PVP for the range from 1 to 6% w/v on the separation is shown in Fig. 1. Note that due to the responsiveness of C^4D to all ionic species, several peaks caused by constituents of the buffer solution in which the ladder is contained are also observed. As expected, an increase in the concentration of the polymer led to increased delay times for the fragments and increasingly improved separation between the peaks. For concentrations of PVP from 1 to 3%, the separation was not adequate due to strong interference of the buffer matrix. For the higher concentrations of the polymer, the ladder components were well separated and removed from peaks due to the matrix alone. However, in contrast to the normal tasks of CE it is not just necessary to achieve baseline separation of the fragments, but normally the spacing of the peaks should be adequate to allow distinction of the PCR products of interest for a task at hand. The concentration of 5% was adopted for the subsequent work. Solutions of higher concentrations (6% and more) were not suitable, as flushing of the capillary was found exceedingly difficult due to the higher viscosity. Note that, this concentration is higher than that used by Xu et al., who reported 2%. Also tested was the influence of the applied separation voltage between 12 and 21 kV using the buffer containing 5% PVP and the results are shown in Fig. 2. Clearly, variation of the high voltage is a convenient method to achieve the desired compromise between the required separation (spacing of the peaks) and the analysis time.

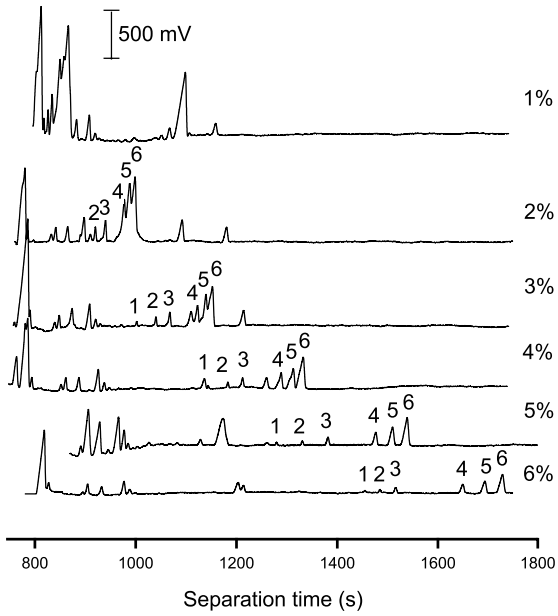

Figure 1. Electropherograms of low-mass DNA ladder fragments of (1) 100 bp, (2) 200 bp, (3) 400 bp, (4) 800 bp, (5) 1200 bp, and (6) 2000 bp in different concentrations of PVP w/v. Experimental conditions: fused silica capillary of 60 cm total and 55 cm effective length with 50 μm id. Buffer: 20 mM Tris, 20 mM CHES (pH 8.5). Separation voltage: –16 kV.

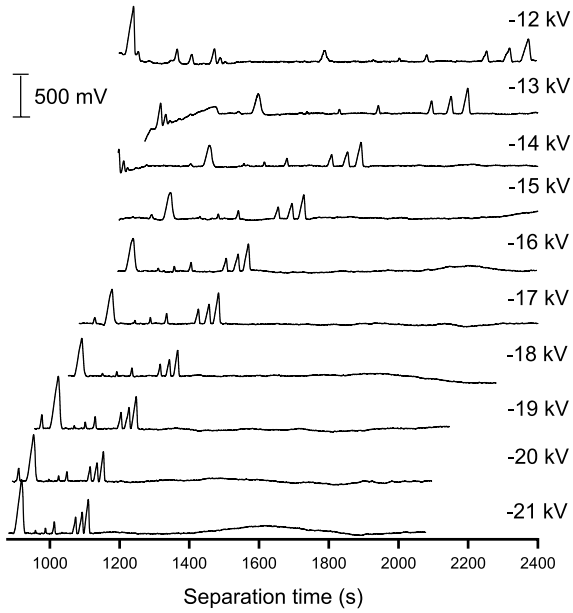

Figure 2. Electropherograms of low-mass ladder DNA fragments in a buffer consisting of 20 mM Tris, 20 mM CHES, and 5% PVP at different separation voltages. Other conditions as for Fig. 1.

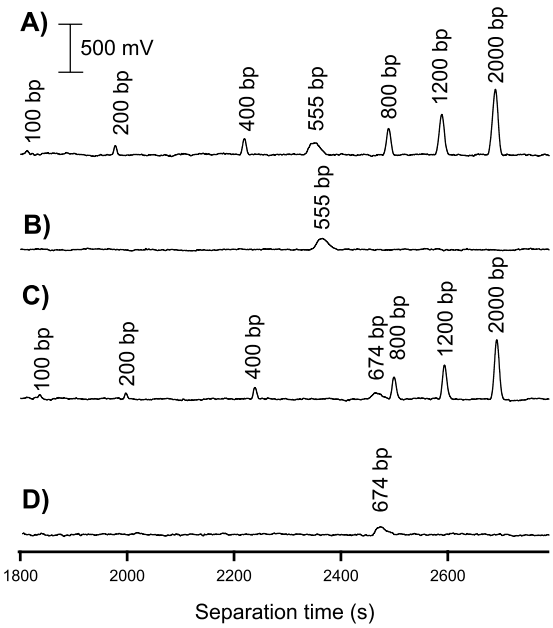

Figure 3. Electropherograms of PCR products from bacterial plasmids pCA24N-Blc, pCA24N-YodA with and without low-mass DNA ladder. (A) PCR fragment of pCA24N-Blc bacterial plasmid of 555 bp length with the low-mass DNA ladder, (B) PCR fragment of 555 bp, (C) PCR fragment of pCA24N-YodA bacterial plasmid of 674 bp length with the low-mass DNA ladder, (D) PCR fragment of 674 bp. Separation voltage: –10 kV. Other conditions as for Fig. 2.

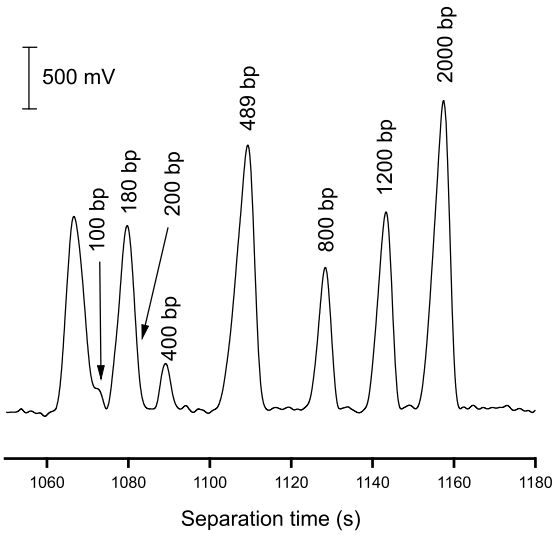

Figure 4. Electropherogram of PCR products from bacterial plasmid pCM655-Empty of 180 bp and 489 bp length with the low-mass DNA ladder. Capillary: 56 cm total length and 51 cm effective length. Separation voltage: –20 kV. Other conditions as for Fig. 2.

3.2 Analysis of PCR samples

3.2.1 Bacterial plasmid DNA

The first experiments were carried out using the plasmid DNA isolated from *Escherichia coli* bacteria. Two PCR reactions were carried out; one PCR reaction using primers 1 and 3 and plasmid DNA, pCA24N-YodA, as template and another PCR reaction using primers 2 and 3 and plasmid DNA, pCA24N-Blc, as template yielding fragments of 674 bp and 555 bp, respectively. The PCR products were injected hydrodynamically, because they contain a high salt concentration that prevents electrokinetic injection without prior to desalting of the sample. Hydrodynamic injection allows direct analysis of PCR products without further purification of the PCR product. The resulting electropherograms are shown in Fig. 3. The products of 674 and 555 bp could be clearly detected and are positioned between the peaks for the fragments of 400 and 800 bp of the low-mass DNA ladder. However, the peaks for the PCR products showed a relatively low sensitivity. Note that a relatively low separation voltage of –10 kV was employed in order to achieve baseline separation, resulting in relatively long electropherograms.

In the second experiment, another plasmid DNA template pCM655-Empty was used as a PCR template with two pairs of primers: primers 4 and 6 and primers 5 and 6 (Table 1), to yield two fragments of 489 bp and 180 bp, respectively. In order to obtain a higher concentrations of the PCR products and thus to improve detection sensitivity, the number of PCR cycles was increased to 40. The product was mixed with the ladder consisting of the six entities of 100, 200, 400, 800, 1200, and 2000 bp fragments, and then separated by the CE-C^4D procedure. The resulting electropherogram is shown in Fig. 4. As expected, the new tall peak corresponding to the 489 bp fragment is located nicely between the 400 and 800 bp ladder peaks. This was possible with the relatively high separation voltage of –20 kV leading to a considerably faster separation run, about 20 min duration, than for the previous separation. Under these conditions, the second PCR product of 180 bp, with its comparatively high concentration, overlaps with the smaller peak of the 200 bp fragment of the ladder. The even smaller peak of the ladder fragment of 100 bp is also found to overlap with an unidentified peak (left most peak in the electropherogram) due to a background ion in the sample matrix. An improved separation of these early peaks, if necessary, would be possible by a reduction of the separation voltage.

3.2.2 Genetically modified soybeans

After successful detection of PCR product of the plasmid DNA, the detection of PCR product from genomic DNA of genetically modified soybeans was investigated as a prominent representative of an application of PCR for the identification of food samples. Roundup Ready GM-Soybean-EX961053A was used as a genomic DNA template. The forward Soy-F and reverse Soy-R primers from Table 1 were designed in order to obtain a PCR product of 400 bp length. The annealing temperature of the primers was optimized to Ta = 55.5°C and the number of PCR cycles was 30. The resulting capillary

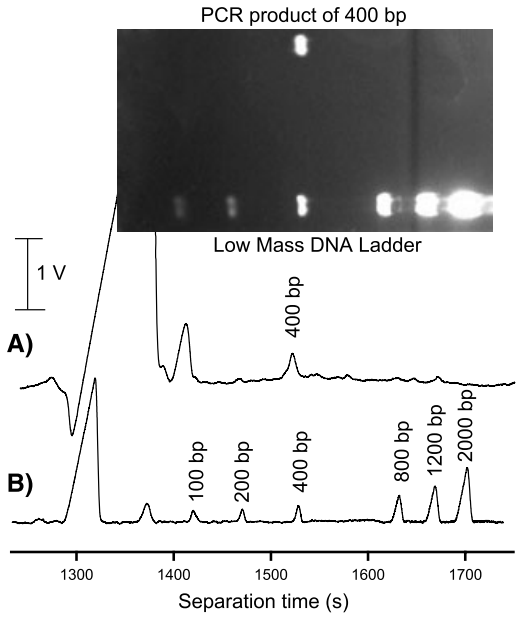

Figure 5. Electropherograms of the PCR product from Roundup Ready GM-Soybean-EX961053A of 400 bp length (A) and low-mass ladder (B). Separation voltage: –15 kV. Other conditions as for Fig. 2. The inset is a picture of the ethidium bromide stained agarose gel also showing both separations.

electropherograms of PCR product and ladder are shown in Fig. 5 along with a picture of a conventional 1% agarose slab gel electrophoresis. Note that this conventional agarose gel electrophoresis separation required approximately 2 h, whereas the separation by CE could be completed in less than 30 min using optimized conditions. The left most peaks in the capillary electropherogram are again due to unidentified matrix elements from the sample.

4 Concluding remarks

It has been demonstrated that CE-C^4D is suitable for the determination of PCR products and may be employed in the detection of DNA fragments amplified from varied sources. The method is more easily automated than the currently still widely used slab gel separations. The separation could be achieved in a much shorter period of time, and further optimization in this regard is possible. CE-C^4D does not require sample preprocessing and there is no need for labeling. A further advantage is the simplicity of the method and the potential for implementation of the method in portable instruments. This is demonstrated by the results presented in the last two figures, which were obtained on the quasifield portable instrument built in house, and are undistinguishable from the measurements carried out on the conventional commercial instrument.

The authors would like to thank Zhang Ling, and Gong Xiao Yang for some preliminary tests and Marc Creus for assistance with the PCR experiments. This work has been supported through grant nos. 200020–126384/1 and 200020–137676/1 from the Swiss National Science Foundation.

The authors have declared no conflict of interest.

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