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Microchip Electrophoresis for Specific Gene Detection of the Pathogenic Bacteria *V. cholerae* by Circle-to-Circle Amplification

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We have developed a new method for a fast and precise analysis of circle-to-circle amplification (C2CA) product for specific gene detection by microchip electrophoresis. In this method, we have added a new enzymatic step to the C2CA reaction, which could be carried out isothermally at 37°C. Compared to the original single-stranded DNA, the double-stranded DNA that is produced by this enzymatic reaction is more reliable for analysis by microchip electrophoresis. C2CA product was detected within 55 s with high reproducibility by this method which was successfully applied to the detection of 10-ng genomic DNA of the pathogenic bacteria *Vibrio cholerae* within 110 s. Purification was found to be an indispensable step for the analysis of the C2CA product of genomic DNA samples.

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Introduction

With completion of the total human genome sequencing^{1,2} and many other organisms and the availability of huge nucleotide variant databanks, two main challenges has appeared. The first is providing smart technologies to select or detect the desired sequence in the whole genome at single-nucleotide resolution;³ the second is developing more efficient and faster techniques to detect data produced from the first challenge. The aim of lab-on-a-chip technology then becomes to combine the techniques of these two challenges to meet today's medical diagnostic needs for developing more reliable and faster detection methods for human diseases that originated from a mismatch in the genome or microorganisms.⁴⁻⁶ The emerging field of microchip electrophoresis (μ -CE) technology has proven to be very promising for the analysis of biological molecules.^{7,8} High-throughput abilities, short analysis times, small amounts of samples and reagents, and low-costs have made this technology ideal for lab-on-a-chip uses. One of the methods that suggests

high sensitivity and specificity in the detection of nucleotide sequence variants is the padlock probe detection method. Circularizing oligonucleotide probes (padlock probes) are molecular tools that can connect tightly to the target DNA strands.⁹ Padlock probes are linear oligonucleotide probes with two ends complementary to the target strand. After hybridization of the padlock probe and the target strand, the two probe ends are brought in juxtaposition and can be joined by DNA ligase. The dual recognition combined with the ligation reaction ensures specificity of detection. So padlock probes have been introduced as excellent detectors of genomic variants.³

Circle-to-circle amplification (C2CA) is a precise amplification method for small circular DNA using padlock probing technology that amplifies DNA circles in a highly controllable manner and is based on rolling circle amplification (RCA).¹⁰ To date, the reported techniques for the detection of C2CA products have included microarrays, real-time monitoring using molecular beacons, denaturing acrylamide-gel electrophoresis and single molecule detection.^{10,11} Although all of the described methods are precise, they are not well suited for the demands of routine laboratory work. Detection based on molecular beacons and single molecule detection is fast, but needs the designing of additional probes. Microarrays need long post-amplification handling and expensive labeling

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procedures. Denaturing acrylamide gel electrophoresis is the most suited technique for routine laboratory work, but its low detection speed and labor-intensive character have limited its applications. A method with automation ability and simple applicability to detect C2CA product faster with acceptable precision is desirable. We gave a preliminary report on an analysis method for C2CA product using μ -CE.¹² Because of the clinical importance of C2CA-based amplified padlock probes, a method development for trustworthy analysis of this product using commercially available μ -CE devices is necessary. Also, careful distinguishing of this product from the possible by-products is essential. Here, we describe the features for microchip electrophoretic analysis of C2CA product in more detail that would be applicable in routine clinical diagnostic laboratories using low-cost and high-throughput microchip devices.

Experimental

Reagents and chemicals

DNA fragments (HPLC purified) were ordered from Sigma Genosys (Japan). φ 29 DNA Polymerase, *RsaI* and *E.coli* DNA Polymerase I Klenow fragment (klenow fragment) were purchased from New England Biolabs (USA). T4 DNA ligase was ordered from Amersham Bioscience (USA). PNK enzyme was ordered from Fermentas (Lithuania) and Ampligase was ordered from Epicentre (USA). TO-PRO-3 and SYBR Green I dyes were purchased from Molecular Probes (USA). Poly(ethylene oxide) (PEO) with different molecular weights of (8×10^6 , 1×10^6 , 4×10^5 , 1×10^5), hydroxypropyl methylcellulose (HPMC)-4000 and TBE 10X buffer (89 mM boric acid, 2.5 mM Tris-EDTA, pH 8.3) were purchased from Sigma-Aldrich (USA). A 10-bp DNA ladder was purchased from Gibco BRL (USA). A PCR product DNA purification kit was purchased from Qiagen (Germany).

Apparatus

An Agilent 2100 Bioanalyzer (Germany) that uses epifluorescent detection with a semiconductor laser that emits at 630 nm was used in this experiment. The microchips had 12 sample wells and 3 gel-dye mix wells, and one well for an external marker. The microchips were made from soda lime glass with a depth of 10 μ m, a width of 50 μ m and an effective separation length of 15 mm. A DNA-500 assay kit, which included dye, gel, markers and a DNA ladder, were bought from Agilent Technologies. Furthermore, for analysis of a sample using a plastic microchip (SV1100 and SV1210 Hitachi μ -CE Systems (Japan)) were used. The SV1100 instrument was equipped with a blue-light emitting diode (LED), emitting at 470 nm and using a photodiode detector, while the SV1210 instrument was equipped with a diode laser emitting at 635 nm. A plastic injection molded microchip (*i*-chip 3; Hitachi, Japan) made of poly(methyl methacrylate) (PMMA) with channels 100 μ m wide and 30 μ m deep was used for those instruments (Hitachi SV1100 and SV1210).

Polymer solution preparation and microchip electrophoresis condition for a PMMA microchip

After a 0.7% HPMC-4000 polymer solution was prepared by a literature method,¹³ SYBR GreenI dye was mixed with it according to the manufacturers' instructions. A poly(ethylene oxide) (PEO) mixed polymer solution was prepared by a literature method¹⁴ with some modifications of the polymers' percentages. A polymer solution was prepared by mixing PEO polymers having different molecular weights (M_w) with

percentages of 0.1% (M_w , 8×10^6), 0.6% (M_w , 1×10^6), 1.1% (M_w , 4×10^5), and 1.2% (M_w , 1×10^5) in TBE 1X buffer. The TO-PRO-3 intercalating dye was mixed with the solution (according to the manufacturer's instructions) which was left at 4°C for 6 h to ensure that all air bubbles were removed. Before electrophoresis using a PMMA microchip, all of the microchannels were infused with a polymer solution. The same polymer solution then loaded into the sample waste, buffer reservoir and analysis reservoirs and the sample was loaded into the sample well of the microchip. Sample injection was carried out by applying 300 V at the sample waste reservoir and grounding the other three reservoirs for 60 s. Separation for the SV1100 Hitachi μ -CE system was carried out for 180 s by applying 130 V at the sample and sample waste reservoirs, while the buffer reservoir was grounded and 750 V was applied to the analysis reservoir. Separation using the SV1210 Hitachi μ -CE system was carried out for 180 s by applying 140 V at both the sample and sample waste reservoirs, while the buffer reservoir was grounded and 780 V was applied at the analysis reservoir. Every time a 10- μ l portion of the dsDNA C2CA product was loaded into the sample well.

Preparation of DNA circle

A padlock probe was designed using the Probe Maker software.¹⁵ The padlock probe (5'-TAG GTT GAG CCC AGG GAC TTC TAG AGT GTA CCG ACC TCA GTA GCC GTG ACT ATC GAC TTG CGT CTA TTT AGT GGA GCC CAA ATG CGA TTC C-3') was phosphorylated and circularized on a synthetic target (5'-CCC TGG GCT CAA CCT AGG AAT CGC ATT TG-3') with a previously described method.¹²

C2CA reaction

We used two kinds of DNA targets for preparing the DNA circle using the padlock probe: a synthetic target (for the first cycle of the C2CA reaction) and a bacterial genomic DNA (for the second cycle of the C2CA reaction). Circularized padlock probe was replicated in the C2CA reaction using the described method.¹² In brief, for the first cycle of the C2CA reaction, RCA of the padlock probe was performed by the priming of 10 nM replication oligonucleotide with negative polarity (RO-) (5'-TAC TGA GGT CGG TAC ACT CT-3'), 100 μ M dNTP and 4 mu/ μ l φ 29 DNA polymerase and 0.2 μ g/ μ l BSA in 1X φ 29 DNA polymerase buffer in a total volume of 50 μ l. The monomerization step was carried out by adding reagents with concentrations of 10 units *RsaI*, 182 nM replication oligonucleotide with positive polarity (RO+) (5'-AGA GTG TAC CGA CCT CAG TA-3'), 0.2 μ g/ μ l BSA and 1X φ 29 DNA polymerase buffer in a 5- μ l total volume. The reaction was then incubated at 37°C for 10 min, followed by heat inactivation at 65° for 10 min. Then, the monomerized C2CA product was directed to the duplication step of C2CA, which was carried out by adding a mixture of reagents containing 0.2 μ g/ μ l BSA, 1X φ 29 DNA polymerase buffer, 38 μ M dNTP mix and 5 units of Klenow fragment in a total volume of 5 μ l, and incubating this for 10 min at 37°C. To generate the second cycle dsDNA C2CA product, genomic DNA was prepared as described before,¹² and only a lower concentration of initiated genomic DNA (10 ng) was used in this study. The C2CA reaction was directed to the ligation step of the first cycle of the C2CA reaction. The ligation reaction was carried out by adding 1 unit of T4 DNA ligase, 833 μ M ATP and 0.2 μ g/ μ l BSA and 1X φ 29 DNA polymerase buffer in a total volume of 5 μ l and incubating at 37°C for 10 min followed by an enzyme heat inactivation at 70°C for 10 min. The second cycle of the C2CA reaction was started by adding a reagents' mixture containing

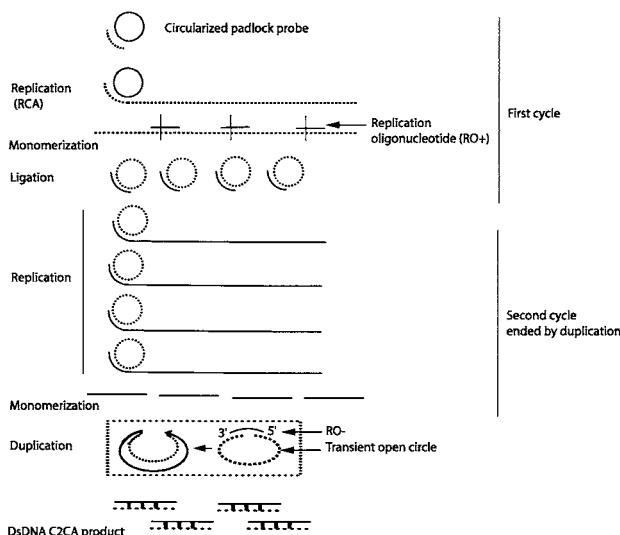


Fig. 1 Schematic drawing of the two cycles of C2CA ended with a duplication step and the mechanism of duplication. Each cycle includes three steps: replication, monomerization and ligation. The second cycle monomerized product was directed to produce dsDNA C2CA product in the duplication step.

33 μM of $\phi29$ DNA polymerase, 0.2 $\mu\text{g}/\mu\text{l}$ BSA, 38 μM dNTP and 1X $\phi29$ DNA polymerase buffer in a total volume of 5 μl and incubating at 37°C for 10 min, followed by heat inactivation at 65° for 10 min. The monomerization step of the second cycle was carried out using the following concentrations of the reagents: 571 nM RO-, 10 units *RsaI*, 0.2 $\mu\text{g}/\mu\text{l}$ BSA and 1X $\phi29$ DNA polymerase buffer in a total volume of 5 μl and incubating at 37°C for 10 min, followed by heat inactivation at 65°C for 10 min. The duplication step for the second cycle of C2CA reaction was carried out by adding reagents with the following concentrations of 0.2 $\mu\text{g}/\mu\text{l}$ BSA, 1X $\phi29$ DNA polymerase buffer, 33 μM dNTP mix and 5 units of Klenow fragment, in a total volume of 5 μl followed by 10 min incubation at 37°C and a heat inactivation at 75°C for 20 min. The product was then purified using a PCR product purification kit.

Results and Discussion

C2CA

C2CA is a kind of DNA amplification based on RCA, which was originally designed for the amplification of padlock probes. Figure 1 shows a schematic illustration of the C2CA reaction ending with a duplication step. Each cycle of this reaction includes an RCA, a monomerization step for digestion of the concatemeric RCA product, and circularization of the monomer product (ligation). Each of the circularized product can then initiate a new cycle of this reaction starting from RCA. Using the efficient $\phi29$ DNA polymerase it is possible to produce ≈ 1000 monomers in a 1-h RCA reaction. That means that a billion-fold amplified product could be achieved after three cycles of C2CA reaction. Basically, in this reaction starting padlock probe is considered to have positive polarity, so the amplified padlock has negative polarity, and this feature will be saved in all cycles of the reaction.

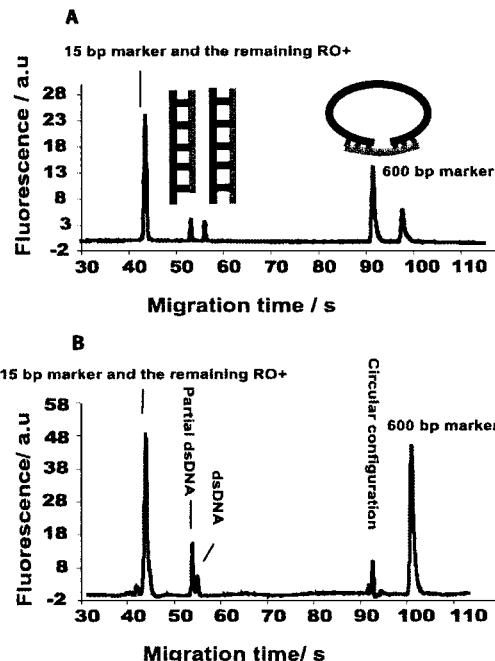


Fig. 2 (A) Analysis of the dsDNA C2CA product using $\phi29$ DNA polymerase (the product of the first cycle). The three peaks were thought to correspond to partial dsDNA, full dsDNA and circular configuration between an RO+ and a linear C2CA product. The 15 and 600 bp fragments are internal standards. A schematic drawing of the product is shown over the peaks. (B) Peaks' patterns appearing in the dsDNA product generation reaction using $\phi29$ DNA polymerase in the presence of 2-fold less amounts of the RO+ (compare to part (A)).

Experiments for dsDNA C2CA product generation (using the Agilent 2100-Bioanalyzer)

DsDNA C2CA product generation using $\phi29$ DNA polymerase. Before producing dsDNA product, we confirmed the production of the usual single-stranded DNA (ssDNA) product of the C2CA reaction using μ -CE. For consistency in a comparison of dsDNA and ssDNA product, we always produced ssDNA in parallel with dsDNA. Every time, ssDNA product were loaded on one of the sample wells, and run in the same chip as dsDNA product, and the generation of ssDNA product was further confirmed by agarose gel electrophoresis (not shown). First experiments to generate dsDNA C2CA product were performed using $\phi29$ DNA polymerase; its selection was based on available data for the amplification mechanism of $\phi29$ bacteriophage (the original source of this enzyme), which indicated that the $\phi29$ DNA polymerase is supposed to produce a dsDNA product in nature,¹⁶ and the C2CA reaction buffer is compatible with this polymerase (1X $\phi29$ DNA polymerase buffer). Figure 2A shows the analysis of dsDNA C2CA product generated by $\phi29$ DNA polymerase. As can be seen in this figure, two close peaks appeared at sizes around our expected size of dsDNA after the first cycle (98 bp). Another peak appeared at about 90 s. These products were not well-defined. We guessed that the first peak might be related to partial dsDNA, the second peak to full dsDNA and the latter peak to a configuration formed by the hybridization of linear C2CA product and the uncut RO+. A schematic drawing of appearing fragments is provided over each peak in Fig. 2A. To identify the peaks, we arranged a similar experiment with a reduced concentration of the RO+ (two-times reduction). Since the RO is essential factor for dsDNA production and in the C2CA reaction should be present in excess to achieve complete

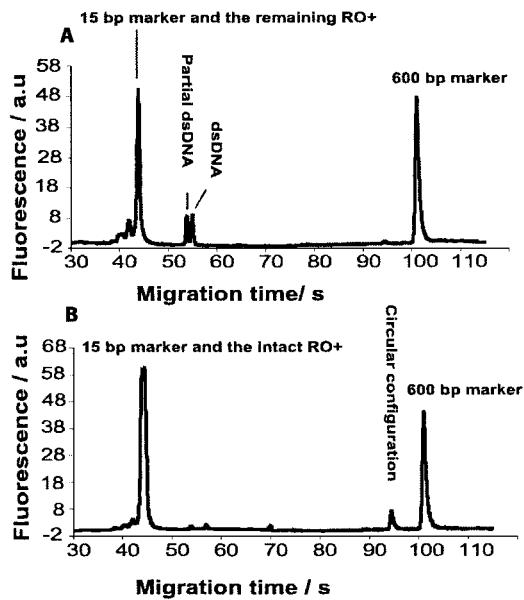


Fig. 3 Analysis of the dsDNA C2CA product using (A) higher amounts of $\phi 29$ DNA polymerase or (B) in the absence of this enzyme. Details: (A) 5-fold higher quantities of $\phi 29$ DNA polymerase were added to the reaction; (B) only the peak for the circular configuration product appeared. Mili-Q water was added instead of the enzyme. In this reaction the added RO+ remained intact due to absence of the enzyme. The 15 and 600 bp fragments are internal standards.

concatemeric product digestion. We expected that the lower concentrations of RO+, may cause the right peak (which we supposed might be related to dsDNA product), to shift toward the left peak. In other words, we expected to observe fewer dsDNA product to appear. The results are given in Fig. 2B confirmed this expectation and our primary idea about the identities of the left and right peaks. In this figure we could also observe semi-circular configuration. The next experiment to obtain dsDNA C2CA product was performed with higher concentrations of $\phi 29$ DNA polymerase (five-fold increase). As can be seen in Fig. 3A, both peaks of partial dsDNA and full dsDNA appeared again, despite increasing the enzyme quantity. In both Figs. 2 and 3A, the two peaks appear between 50 and 60 s. The exact size of the generated partial dsDNA product is not clear, which may explain the slight difference in the migration time of this product between the two figures. The differences in peak intensities between Figs. 2 and 3A can be explained by the fact that in the experiment for Fig. 3A, higher enzyme quantity existed and consequently higher amounts of the product and higher peak intensities are expected compared to Fig. 2. Then, the next C2CA reaction was performed in the absence of $\phi 29$ DNA polymerase to see if possible fragments would appear in this reaction in the absence of the enzyme (Fig. 3B). As can be seen in this figure, the semi-circular configuration is observable at about 95 s, confirming that the generation of the circular configuration was independent of the presence of the enzyme. In this figure, the RO+ can be seen at higher intensity (since it is not supposed to be consumed in the absence of the enzyme).

DsDNA C2CA product generation using Klenow fragment. To obtain dsDNA product more efficiently in this reaction, we decided to change the $\phi 29$ DNA polymerase to another enzyme by filling the end ability and compatibility with the C2CA reaction buffer. The Klenow fragment of DNA polymerase I was chosen for this purpose. Figure 4 shows the generation of

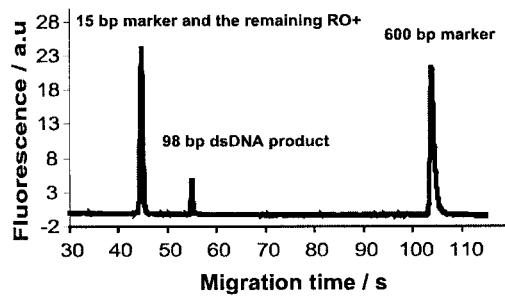


Fig. 4 Detection of full dsDNA C2CA product using the Klenow fragment in the duplication step of the C2CA reaction. The 15 and 600 bp fragments are internal standards.

full dsDNA C2CA product using this enzyme. Undesirable circular configuration was eliminated, and a single peak of dsDNA C2CA product appeared. The product was checked using agarose gel electrophoresis (not shown). The migration time of the product was compared with the 15–600 bp DNA ladder, which was available in the DNA-500 assay kit (not shown). The migration time was about 55 s and the RSD of the migration time was calculated to be 3.6% ($n = 6$) confirming the reproducibility of dsDNA production as well as the detection method. Using this reaction we could set up the production of dsDNA C2CA product with good compatibility to the reaction condition of the C2CA experiment. In some analysis of the dsDNA product generation by the Klenow fragment, we could observe a small peak for circular configuration, but it has not affected the dsDNA production (not shown).

Mechanism of dsDNA C2CA product formation and a size increase for the final dsDNA C2CA product

The mechanism for dsDNA formation using Klenow fragment for the first cycle of the C2CA reaction, can be described as below. In the duplication step (an additional step to C2CA), first the intact RO+, which is in excess, can be hybridized onto the both ends of the linearized (monomerized) product in an intra-molecular hybridization bonding reaction, creating an “open circular” structure. This structure is similar to the one that they form before the ligation step of the C2CA reaction. Then, duplication using Klenow fragment can be started from both the 3' end of the monomerized product and the 3' end of RO+. In both process the 5' end of the attached RO+ will be displaced and the ssDNA fragment will be filled, making dsDNA product with blunt end. However, the length of the finally produced dsDNA will increase slightly. The mechanism for the generation of dsDNA C2CA product of the second cycle will be the same. The only difference would be the converted polarity of the DNA fragments (RO- will be used in the second cycle instead of RO+ in the first cycle). The length of dsDNA product depends on the cycle in which DNA is amplified and on the position to which the RO has a complementary sequence with the linearized product since the polarity of the product switches in each cycle. For example in the first cycle the polarity of the product is negative and the RO+ fragment in our fragment (depending on RO and padlock probe designs) has a 13 nt complementary part with the 5' end of the linearized C2CA product. As a result, a 7-nt overhang length will be available at the 3' end of the RO+. However in the second cycle of the C2CA reaction, the length of the overhang segment will be increased to 13 b. Thus, in the first cycle of the C2CA reaction, we will have a 7-bp increase in the size of the final product, meaning that the final size of the dsDNA C2CA

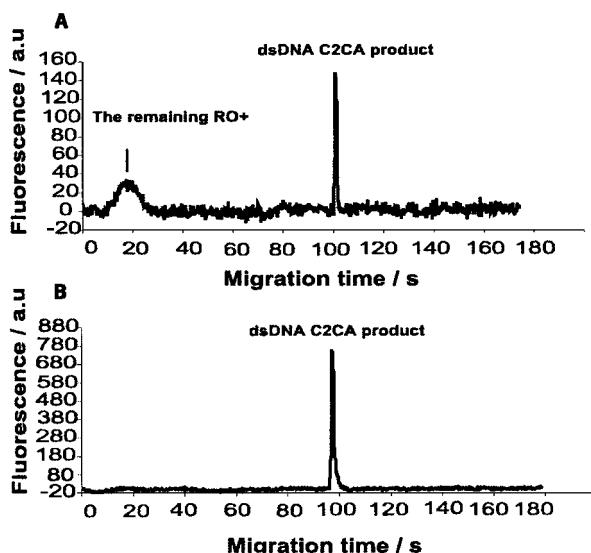


Fig. 5 Analysis of the first cycle dsDNA C2CA product using a HPMC-4000 0.7% and SYBR Green I mixed polymer-dye solution. (A) Unpurified product of C2CA was separated from the remaining RO+. (B) The purified C2CA product with higher intensity is observable in this electropherogram.

product will be 98 bp. On the other hand, the final size of this product at the second cycle of C2CA will be 104 bp. In the third cycle of C2CA, the polarity of the DNA fragments is the same as the first cycle, and as a result the size of the dsDNA product will be the same as in the first cycle. A schematic diagram of the mechanism is provided in Fig. 1.

Analysis of the first cycle of the C2CA product on a plastic microchip

Plastic microchips are promising for future applications in disposable microchips, since they are substantially less expensive, and offer higher speed than a glass one (the total detection time using the utilized plastic microchip is 180 s, compared to 600 s in a glass one used in this study). Therefore, we decided to use a plastic microchip for the next analyses. An analysis of the first cycle dsDNA C2CA product on a PMMA microchip was performed using the Hitachi SV1100 μ -CE system; 0.7% HPMC-4000 polymer mixed with SYBR Green I was used as a separation matrix. Figure 5A shows the separation of dsDNA C2CA product from the remaining RO+ in the unpurified reaction solution. Figure 5B shows detection of the purified dsDNA C2CA product using the same separation matrix. A considerable increase in the peak intensity for the purified product was observed (\approx 5 fold increase). During the purification process, RO+ fragment was washed away from the solution.

Analysis of the second cycle of the C2CA product on a plastic microchip (detection of bacterial genomic DNA)

For the analysis of the second cycle dsDNA C2CA product, a bacterial genomic DNA was used as a sample. For a correct size measurement of the amplified padlock designed for the species-specific detection of the bacterial genome, a polymer with a higher resolving power was chosen. PEO polymer has been introduced as polymers with a self-coating ability. A mixed PEO solution has been used to separate a 20-bp ladder and PEO polymers with M_w s of 4×10^5 and 8×10^6 reported to be important for the separation of small and large DNA

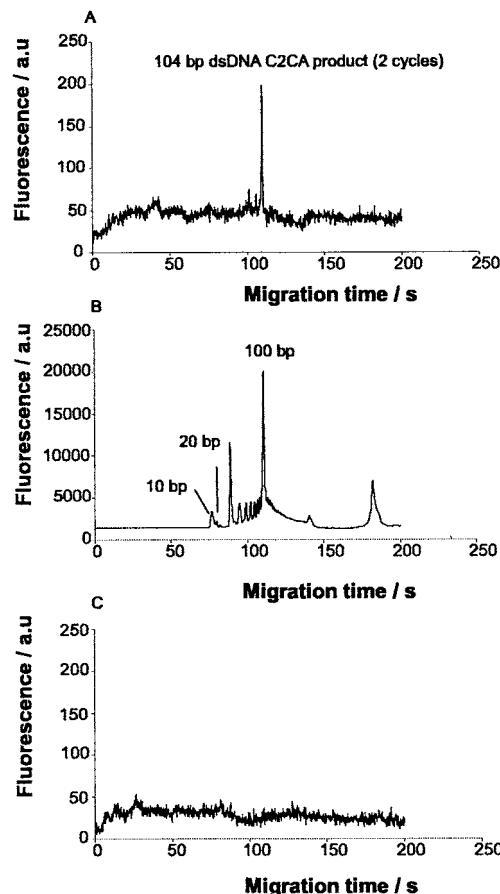


Fig. 6 (A) Analysis of a 10-ng genomic DNA sample from pathogenic bacteria (*V. cholerae*) after two cycles of the C2CA reaction, ending with the duplication step. (B) Separation of a 10-bp ladder. (C) Analysis of a purified negative control after two cycles of the C2CA reaction. Milli-Q water was used instead of the DNA circles in this reaction. Separation was performed using a modified PEO mixed polymer solution (see text) mixed with TO-PRO-3 dye.

fragments, respectively.¹⁵ In this study we found that using a modified mixture of these PEO polymers (a reduced percentage of the $M_w 8 \times 10^6$ by 3-times and an increased percentage of the $M_w 4 \times 10^5$ by 0.12 times), is useful for resolving of the 10 bp ladder using μ -CE (see Experimental section). The dsDNA C2CA product of a 10-ng genomic DNA was successfully detected using this separation matrix. Figures 6A - C shows a comparison between the second cycle dsDNA C2CA product, separation of a 10-bp ladder and a negative control, respectively, confirming the generation of the correct size product from an amplified padlock probe circularized to genomic DNA.

Conclusions

Features for the analysis of C2CA product using three available microchip electrophoresis instruments (the Agilent 2100 Bioanalyzer (using a glass microchip), the Hitachi SV1100 and SV1210 μ -CE systems using PMMA microchip), were explained here as a fast alternative for the analysis of C2CA product. The product of the duplication step was analyzed using those μ -CE instruments. This paper differs from our previous report¹² in that it presents more details through the process of generating dsDNA C2CA product, providing more

clear reasons for why the Klenow fragment is better than φ29 DNA polymerase for this purpose, and explaining the length increase of the dsDNA product in the second and third cycles of C2CA for μ-CE application. In addition a modified PEO mixed polymers solution for the separation of a 10-bp ladder was introduced, enabling a higher resolution, which is promising for the analysis of dsDNA C2CA product, and especially multiplexed ones for future applications. Also 2.5-times reduced concentration of genomic DNA has been detected compared to our previous report. The peak intensities for the direct analysis of the dsDNA C2CA product by μ-CE, regardless of the kind of chip (glass or PMMA), were found to be moderate. This moderate intensity can be explained by the properties of the buffer components in the C2CA reaction, and having several addition steps. However, even for a normal PCR, a purification step was recommended, especially for higher accuracy analysis.¹⁷ Although the detection of synthetic sample is possible after the first cycle of C2CA using μ-CE, directly without purification, for the detection of a genomic sample using μ-CE, at least two cycles of the C2CA reaction, followed by a purification step are necessary. With the ability of the padlock probes to be amplified in a multiplexed format,¹⁸ we think that by providing higher resolution polymer matrixes and optimizing a good laddering system, this method could be applicable to the detection of different sizes of padlock probes amplified in the same reaction.

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