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Research Article

CE combined with rolling circle amplification for sensitive DNA detection

Here we describe an assay which combines CE with rolling circle amplification (RCA) for sensitive DNA detection and quantification. RCA is an isothermal DNA replication technique that generates a long ssDNA with tandem repeats. It requires simpler temperature control in reaction and offers higher sequence specificity and greater quantitation capability compared to other amplification technologies. In this study, RCA amplified the DNA target *via* a circular template, and the product was digested into monomers for CE analysis. Less than 2 fmol of the DNA target could easily be detected using this RCA-CE assay and the assay has a dynamic range of two orders of magnitudes. Moreover, simultaneous detection of both the target DNA and the internal standard was achieved by designing two padlock probes with different sizes, which could significantly improve the quantification accuracy. The RCA-CE assay is easy to perform, readily adaptable for detection of multiple targets because of the high resolution power of CE, and is compatible with other applications employing RCA as a signal amplification tool. Additionally, this assay can be used with a capillary array system to perform sensitive, high-throughput genetic screening.

Keywords:
CE / DNA detection and quantification / Enzyme digestion / Rolling circle amplification
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1 Introduction

Rolling circle amplification (RCA), an isothermal DNA replication technique, has been proved very useful for the detection of target nucleic acids and proteins [1, 2]. In RCA, a DNA polymerase with strand displacement activity amplifies a circular template using a forward primer. The resulted product is a long ssDNA molecule that contains thousands of repeats of the complementary sequence to the template, meaning that thousands of detection sites are generated from each template [3]. The circular template for RCA can be formed from a linear ssDNA, the padlock probe. The 5′- and 3′-termini of the padlock probe first hybridize precisely onto the target and then are joined by a DNA ligase [4]. RCA offers several advantages over other amplification technologies. The linear amplification process of RCA allows better quantification compared to the exponential process in regular PCR/RT-PCR. The stringent requirement of ligation on strand matching increases the specificity of RCA in DNA detection. The isothermal reaction condition eliminates the requirement for a temperature cycler and makes RCA readily adaptable to routine clinical use with less issues concerning quality control of the instrument. Furthermore, the amplification signal can be confined locally if the amplification primer is immobilized onto a solid support. Because the DNA polymerase acts equally on circular probes with different sequences, the amplification is independent of strand sequences, simplifying the design of multiplexed assays. All these unique properties of RCA facilitate its applications in different research and molecular diagnosis areas like *in situ* detection, microarray, immunoassay, SNP, *etc.*

Because ligation is very sensitive to base-pair mismatch between the padlock probe and the target, ligation of the padlock probe followed by RCA can be used to detect SNP [5, 6]. Mutant strands mixed with excess wild-type alleles at a ratio of 1:10 000 were detected combining PCR for target amplification and RCA for SNP discrimination [7]. Most frequently, RCA was employed to detect nucleic acids. RCA resulted in around 8000-fold signal enhancements over simple hybridization on a DNA microarray as demonstrated by Nallur *et al.* [8]. A few copies of DNA extracted from a bacterial pathogen, *Vibrio cholerae*, were detected by counting individual amplification products under a confocal fluores-

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Abbreviations: miRNA, microRNA; **RCA,** rolling circle amplification

cence microscope after circle-to-circle amplification (C2CA), a cascade RCA technique [9]. A microRNA (miRNA) detection system developed by Kjems and co-workers [10] amplified several specific miRNAs from a few nanograms of total RNA by RCA. The products were transferred to a membrane and labeled with radioisotopes for detection. The padlock hybridization enabled unambiguous discrimination of the closely related miRNAs [10]. On the other hand, RCA was applied to detect proteins as well. The RCA primer can be linked covalently to the reporter antibody and RCA can improve the sensitivity of the immunoassays hundreds or even thousands of times [2]. For example, RCA facilitated the multiplexed measurement of cytokines on microarrays with femtomolar sensitivity [11].

In the RCA-based assays, target quantification is done through the quantification of the RCA products. Different techniques have been utilized for this purpose. For amplifications performed on solid surfaces like the glass arrays, the RCA products hybridized to multiple fluorescent, complementary oligonucleotide probes, and the fluorescence was measured by a conventional microarray scanning device [6, 8, 11]. For solution-based reactions, a confocal microscope or a flow cytometer was used to count individual RCA products, because they generated local enrichment of the DNA binding fluorophores and induced distinguishable fluorescence difference from the background [7, 9, 12]. A slot-blot system was employed for miRNA detection and quantification after RCA [10]. These detection schemes usually require the usage of advanced laboratory equipments and complicated data acquisition techniques. Due to the wide application range of RCA, it is still in demand to develop more simple and rapid methods to monitor RCA products.

Because RCA products are large, megabase fragments and fold into micrometer-sized random coils in solution, it is not practical to analyze the products directly using gel electrophoresis [13]. PFGE could be a solution, but smear bands may be produced due to the various fragment sizes generated in the one-primer amplification process, making it difficult to perform quantification. Compared to slab gel electrophoresis, CE is simpler, faster, consumes much smaller amount of samples, and offers higher separation efficiency. CE can separate DNA molecules with different sizes or even different conformations. CE-LIF with intercalating dyes in both the running buffer and the separation matrix offers effortless and sensitive detection of DNA fragments, no chemically modified detection probes needed. However, to the best of our knowledge there has been no attempt in applying CE to analyze the RCA products so far. Hence, in this study we explored the possibility of using CE to detect and quantify synthetic DNA targets *via* RCA. We proved that CE was a simple and sensitive detection platform for RCA, and it was possible to use the RCA-CE assay to detect multiple targets within one sample. The duplexed detection could be used for accurate relative quantification of DNA or RNA targets in biological samples.

2 Materials and methods

2.1 Reagents

PVP (average molecular weight of 1 300 000), Tris, boric acid, disodium ethylenediamine tetraacetate (EDTA), hydrochloric acid (HCl), and methanol were purchased from Fisher Scientific (Fairlawn, NJ, USA). SYBR® Green II RNA gel stain (10 000 × concentrate in DMSO) was obtained from Invitrogen (Carlsbad, CA, USA). All solutions were prepared in deionized water (18 MΩ) from a Milli-Q™ water purification system (Millipore, Billerica, MA, USA). Oligonucleotides and the ssDNA ladder (20–100 nt) were from Integrated DNA technologies (Coralville, IA, USA). RepliPHI™ Phi29 DNA polymerase and mixture of deoxyribonucleotides (dNTPs) were purchased from Epicentre (Madison, WI, USA). *Hha*I restriction enzyme and *E. coli* DNA ligase were from Promega (Madison, WI, USA) and Takara (Shiga, Japan), respectively. Magnesium chloride hexahydrate ($MgCl_2 \times 6H_2O$), albumin from bovine serum (BSA), and β-nicotinamide adenine dinucleotide hydrate (NAD^+) were purchased from Sigma–Aldrich (St. Louis, MO, USA). CentriSpin 20 columns were from Princeton Separations (Adelphia, NJ, USA).

2.2 Instrumentation

All CE experiments were performed in a P/ACE™ MDQ Glycoprotein CE System equipped with a 488 nm laser (Beckman Coulter, Fullerton, CA, USA). Sieving matrix for DNA separation was 2 or 4% PVP solutions prepared in 1 × Tris-borate-EDTA (TBE) buffer and stained with 1 × SYBR Green. The 50 cm fused-silica capillary (75 μm id, 365 μm od; Polymicro Technologies, Phoenix, AZ, USA) with an effective length of 40 cm was rinsed at 30 psi for 3 min with 0.1 M HCl, deionized water, methanol, and PVP solution sequentially prior to injection. Injections were either done at −5 kV for 5 s (normal injection) or at −2 kV for 60 s (stacking injection). All CE separations were done at −15 kV in 1 × TBE with 1 × SYBR Green. Peak area calculation was done by the 32 Karat software provided with the CE instrument.

2.3 RCA templates, probes, replication primers, and reaction conditions

Two synthetic oligonucleotides served as the arbitrary targets, and were detected by the RCA-CE assay using two padlock probes and one amplification primer in the present study. The sequences of the reaction substrates can be found in Table 1. Target 1 contained the sequence on the human K-*ras* gene with a G12A mutation, and target 2 was derived from the human TP53 gene with an R273H mutation [14, 15]. Even though these two targets were originally designed for detection of DNA point mutations, the current study was dedicated to the detection of specific DNA targets instead of SNP. Probe 1 was complimentary to target 1 and probe 2 was

Table 1. Oligonucleotides used in this study[a)]

Replication primer	5′-TGC GCT ATC TTC A-3′
Probe 1	5′-p T GGC GTA GGC AAG ATA GAA TGA AGA TAG CGC ATC GTA GGA CTT ATT TCG TAG GAC TTA GGT AGT TGG AGC TGT-3′
Probe 2	5′-p TGT TTG TGC CTG TCC TAG AAT GAA GAT AGC GCA TGA GGT GTT ACA GCT TTG AGG TGC A-3′
Target 1	5′-TCT TGC CTA CGC CAA CAG CTC CAA CTA CC-3′
Target 2	5′-GG ACA GGC ACA AAC ATG CAC CTC AAA GCT GT-3′

a) Target 1 and probe 1 used in this paper were designed according to ref. [14] with some modifications.

to target 2. (i) A 40 μL of reaction mixture in solution A (30 mM Tris-HCl (pH 7.8), 0.1 M KCl, 0.2 mM NAD^+, 4 mM $MgCl_2$), which contained the target oligonucleotide and the padlock probe, was heated up to 65–70°C and then incubated in a 45°C water bath (Isotemp® 2150-B5; Fisher Scientific) for 1.5 h. No target DNA was added to the blank reaction mixture. Then, 2.26 μL of 1.2 U/μL *E. coli* DNA ligase and 1 μL of 2% BSA were added to each reaction mixture. Incubation in a 37°C water bath for 15 min was required for ligation. (ii) RCA was carried out with the addition of 1.7 μL of 10 μM primer solution, 1.6 μL of 0.2 M $MgCl_2$, 6.4 μL of 25 mM dNTPs, 6.4 μL of 50 mM Tris-HCl (pH 7.5), and 2 μL of 100 U/μL Phi29 DNA polymerase to each reaction mixture followed by incubation at 37°C for 45 min. A denaturing step at 65–70°C for 15 min was necessary before digestion. (iii) 0.5 μL of 10 μg/μL acetylated BSA and 1.3 μL of 10 U/μL *Hha*I were added to perform a 2 h digestion at 37°C. Reaction mixtures and blanks were denatured at 65–70°C for 15 min and then cleaned-up by spin columns to remove salts, dNTPs and other low-molecular-weight impurities.

3 Results and discussion

3.1 Initial investigation of the RCA reaction with CE

The first step in our investigation was to explore the possibility of using CE to analyze the RCA products directly. Padlock probe 1 hybridized onto target 1 and formed a circular probe by the *E. coli* ligase, which was then amplified by the replication primer and the Phi29 polymerase. Because the amplification is linear, the product amount should be proportional to that of the target. Previous studies have confirmed that RCA generates DNA fragments with very high molecular weights, which cannot be separated in polyacrylamide gels under traditional electrophoresis conditions [3, 13, 16, 17]. Based on the biased reptation model that depicts the electrophoretic migration of DNA in a polymer network, as the DNA coil becomes more elongated than 20 000 base pairs, the size dependence of the electrophoretic mobility μ disappears [18]. Beyond this limit, pulsed-field techniques must be used for effective size separation of the ultralarge DNA molecules. Another factor that prevents direct gel electrophoretic separation of the RCA products probably lies in their large gyration radius. Because the elongated DNA fragment is single-stranded, it forms a random coil in free solution. It was once estimated that the gyration radius of a RCA product containing 1000 repeats of a 93 nt template was 775 nm [12]. Once the product was hybridized to the fluorescently labeled oligonucleotide probes, the diameter of the random coil could go up to 1 μm [9]. Such a large gyration radius would cause great difficulties for the RCA product to enter the gel. Due to the same reasons, CGE or CE with entangled polymer solutions would not be feasible, either.

We proved this hypothesis by injecting the amplification product into a capillary filled with 1% PVP solution. No peak showed up within 60 min except the background peaks from the padlock probe (73 nt) and the amplification primer (13 nt) (data not shown). On the other hand, if the same reaction mixture was analyzed in an open capillary coated with 0.05% polyethylene oxide (8000 kDa molecular mass), the electropherogram showed multiple narrow peaks (spikes), indicating the presence of submicrometer- and even micrometer-sized large DNA conglomerates with different size-to-charge ratios (Fig. S1, Supporting Information) [19]. The spikes were irreproducible in both peak height and migration time, which could be attributed to the irregular shape and size of the RCA products and the low data acquisition rate (4 Hz) of the commercial CE system. The high electric field may introduce further deformation of the random coils and promote entanglement among the DNA coils, increasing their size and shape variations. Another contribution to the size variation is from the reaction itself, which does not have a mechanism to generate DNA fragments with defined fragment lengths. The multiple and irreproducible spikes are not useful at all in target DNA quantification *via* RCA, and a different approach has to be taken.

3.2 Enzyme digestion of the RCA product

Since the RCA product is actually a concatemer with repeated sequences arranged end-to-end, it can be digested into identical fragments by restriction enzymes if we incorporate a digestion site to the template sequence. An oligonucleotide complementary to the RCA product is added to form a double-stranded portion for restriction enzyme digestion. The much smaller monomer can then be analyzed easily by CE. The monomerization of RCA products has been successfully employed in C2CA to obtain higher amplifica-

tion folds by repeated circularization and polymerization [16, 20]. To increase digestion efficiency and simplify the procedure, a sequence of 5′-GCG^C-3′ that can be cleaved by *Hha*I was introduced to both the padlock probes and the replication primer. *Hha*I not only works within the double-stranded region, but also cuts the single-stranded region *via* the transiently formed double-stranded hairpin structures at a relatively fast reaction speed compared to other endonucleases like *Cfo*I [21]. We found out that incubating the reaction mixture at an elevated temperature like 65–70°C prior to enzyme digestion was necessary to obtain optimal reaction yield. This denaturing step deactivates the Phi29 enzyme and terminates the polymerization reaction to ensure high digestion efficiency. It may also loosen up the coiling structure of the single-stranded product, expose more binding sites, and consequently allow better attachment of the replication primer and the digestion enzyme.

The schematic illustration of the RCA-CE assay is displayed in Fig. 1, and the results from enzyme digestion of the RCA products are shown in Fig. 2. *Hha*I digestion was performed after RCA of the target oligonucleotide. Then the reaction mixture was first cleaned-up by phenol extraction and ethanol precipitation, and then analyzed on the 20% denatured polyacrylamide gel (Fig. 2a). It can be seen that the product has a fragment size similar to that of the padlock probe (73 nt). The additional product band may be a ghost band because it did not show up in CE. No linear padlock probe was seen in the trace for blank reaction. Nevertheless, this result demonstrated that the enzyme digestion step resulted in a much smaller reaction product suitable for CE analysis.

Next, the reaction mixture was separated by CE. In order to keep the whole assay simple, we performed all reactions in one tube. Reaction substrates were simply mixed and enzymes were added sequentially. No wash procedure was involved, and no extra effort except simple desalting was taken to purify the final product. The SYBR Green II RNA gel stain was used to label the single-stranded oligonucleotides so that they could be detected by LIF. The CE was done under a native condition, because we found that the denatured condition of 7 M urea lowered the fluorescence intensity in detection. This phenomenon indicates that the ssDNA product may contain some double-helix structures along the molecule which bind better to the intercalating dyes. Therefore, more sensitive intercalating dyes for dsDNA detection, such as the Yo-Pro, SYBR Gold, and SYBR Green I, can be tested in the future to enhance the assay sensitivity. Since the reaction substrates and intermediates (padlock probe, replication primer, circular probe, products from enzyme digestion of the padlock probe and the primer) were coinjected into the capillary with the final product, we optimized the CE conditions to resolve the final product from the background oligonucleotides. Different PVP concentrations and running voltages were compared, and the 4% PVP solution and the running voltage of −15 kV were chosen. The electropherograms of the positive and blank reactions are shown in Fig. 2b. The residue circular probe was eluted later than the digested RCA product in CE. Surprisingly, if we spiked the padlock probe into the reaction mixture, the digestion product migrated slower than the padlock probe under our native running condition. We suspected the conformation of the digested RCA product was different from that of the original padlock probe. CE is well known for its capability to separate ssDNA with different conformations in native running conditions [22]. In addition, by monitoring the products from every step of the whole process using CE, we found out that most of the padlock probes were digested by *Hha*I if coincubated with excess amount of the replication primer. Both phenomena indicated that the padlock probe imposed no interference to the analysis of the final product. The background peaks were resulted from residue reaction substrates, enzyme digestion of the replication primer and the

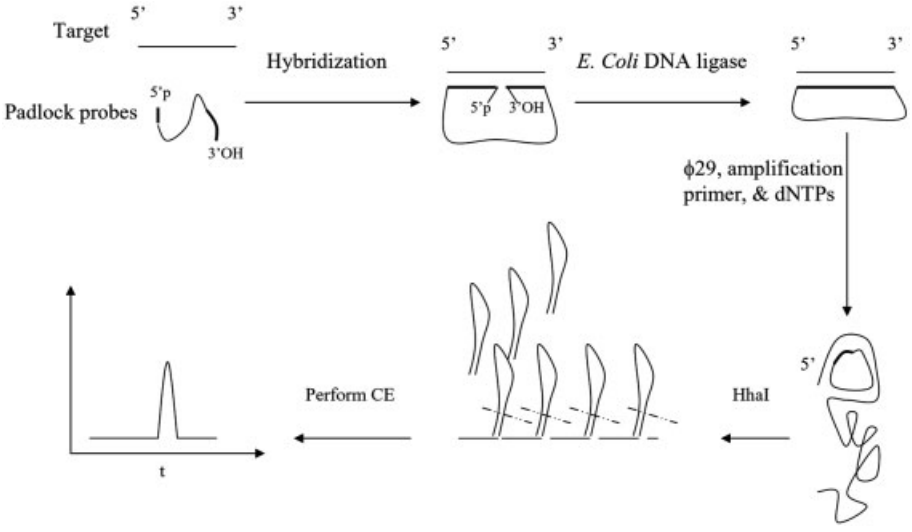

Figure 1. Schematic illustration of the RCA-CE assay.

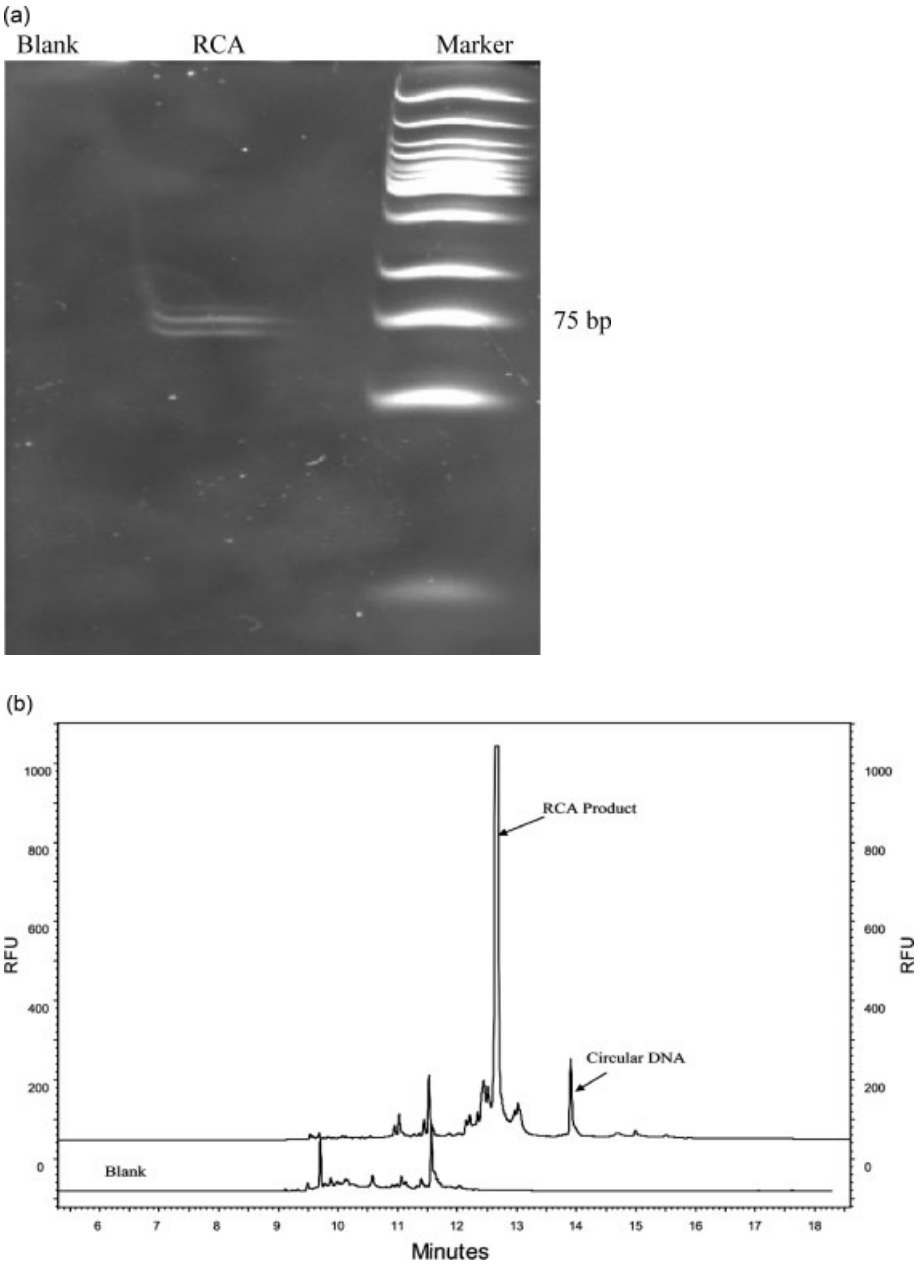

Figure 2. (a) Size confirmation of the digestion product by gel electrophoresis. Blank, blank reaction without any target DNA; RCA, positive reaction with 80-fmol target 1; Marker, low-molecular-weight DNA ladder (25–766 bp; New England Biolabs, MA, USA). Gel electrophoresis conditions: 20% polyacrylamide gel with a size of 10 cm × 10 cm, 1 × TBE, 200 V, stained with ethidium bromide. (b) Electropherograms of digestion products from blank and positive reactions. Separation conditions: uncoated fused-silica capillary (total length 50.0 cm, effective length 40.0 cm, 75 μm id); temperature, 25°C; separation at −15 kV; injection at −5 kV for 5 s; sieving matrix: 4% PVP in 1 × TBE, with 1 × SYBR Green II RNA gel stain; LIF excitation at 488 nm.

linear padlock probe, and other reaction intermediates, which were well separated from the digested RCA product (Fig. 2b). The separation was rapid, taking less than 15 min after the enzymatic reaction.

3.3 Reaction optimization

Once we confirmed that CE could be used to monitor the RCA product, we carried out investigations on the reaction conditions to optimize the RCA-CE assay. We first tested the padlock probe circularization conditions because it determined the successful linear amplification of target DNA. The circular probe was eluted later than the linear padlock probe in CE when using 2% PVP as the sieving matrix (Fig. 3). The peak area of the circular DNA can be used as an indicator for the reaction yield. Figure 3 superimposed the electropherograms of ligation products generated with different hybridization duration. It can be seen that, the reaction yield increased with the hybridization duration, but the augmentation leveled off once the duration was longer than 1.5 h. The long hybridization time ensures that more target molecules can anneal to the padlock probes to obtain higher reaction yields. Other reaction conditions like the initial concentration of the padlock probe and the ligation duration

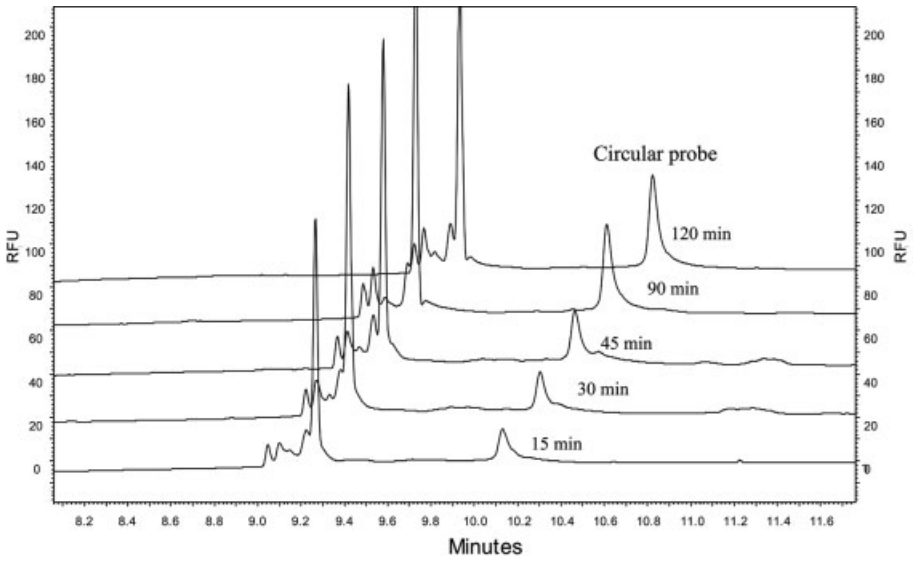

Figure 3. Electropherograms of circular DNA formed by ligation with different hybridization durations. Traces moved to increase visual clarity. Separation conditions as in Fig. 2b, except that PVP concentration was 2%.

were also investigated using the same strategy. Higher concentration of padlock probe did not increase the yield of circularization once the concentration ratio of the padlock probe to the target was larger than 1. Ligation was very efficient if enough time was given for hybridization, taking only 15 min to reach the optimal yield.

Different RCA durations were compared as well and the results are shown in Fig. 4. The digested RCA product was coinjected with an ssDNA ladder (20–100 nt with a 10 nt increment), and the product peak area (*A*) was normalized against that of the first peak of the ladder (*A*1) to eliminate random variations in different CE runs. Optimal RCA duration was found to be at 45 min from the plot of normalized peak area (*A*/*A*1) *versus* RCA duration (inserted graph in Fig. 3). Longer RCA duration than 45 min actually decreased the product signal. We suspected that as the RCA product grew too long and formed a very compact random coil, some of the digestion sites might be embedded too deep inside the coil and become inaccessible to the digestion enzyme. The phenomenon that the supercoiled DNA was a poor substrate for primer and enzyme binding was also observed in using real-time PCR for mitochondrial DNA quantification [23]. The digested product migrated later than the 100 nt peak from the ssDNA ladder in our case, even though it should have a similar size as the padlock probe (73 nt). This further confirms that there may some double-helix structures formed along the product molecule.

The co-injection with an ssDNA ladder allowed us to study the reproducibility of the CE runs. The RSDs of peak areas and migration times of each ladder fragment within these four injections were calculated to be between 1 and 3%, respectively, which confirmed the practicability of using migration time and peak area to identify and quantify the RCA product.

3.4 Target quantification and duplexed assay

Next we applied this RCA-CE assay to detect and quantify the artificial DNA targets. We performed reactions with different amounts of target 1 (40, 16, 8, 4, 1.6, and 0 fmol in 40 μL reaction mixtures) and analyzed the products by CE using 4% PVP solution stained with SYBR Green II as the sieving matrix. The injection was done at −2 kV for 60 s to increase the injected amount. Preliminary investigations found that such a stacking injection could enhance the product signal by 40-fold with our experimental condition. The product peak from the reaction with 1.6 fmol target was clearly distinguishable from the background. The electropherograms and the titration curve of target amount *versus* peak area are displayed in Fig. 5. Since CE consumes very small amount of sample for each analysis, we can further minimize the total reaction volume or use SPE columns to reduce the final product volume during sample cleanup. Either way should result in even lower detection limit of the RCA-CE assay. A relatively narrow dynamic range of two orders of magnitudes was obtained in our study because higher target amount than 80 fmol would saturate the detector in our commercial CE instrument using the stacking injection.

Till now, we demonstrated that the RCA-CE assay could be used to detect a low amount of target oligonucleotides, but the quantification was entirely relied on one single peak from the digested RCA product. It is not a reliable quantification method because many variations would arise during the whole process. For instance, slight fluctuation in the reaction conditions, like the incubation temperature and the concentrations of the starting materials, may be amplified during reaction and introduce big errors to the final quantification. The running and dye staining conditions of CE may vary from run-to-run as well, leading to irreproducible peak intensities. To improve the accuracy of target quantification,

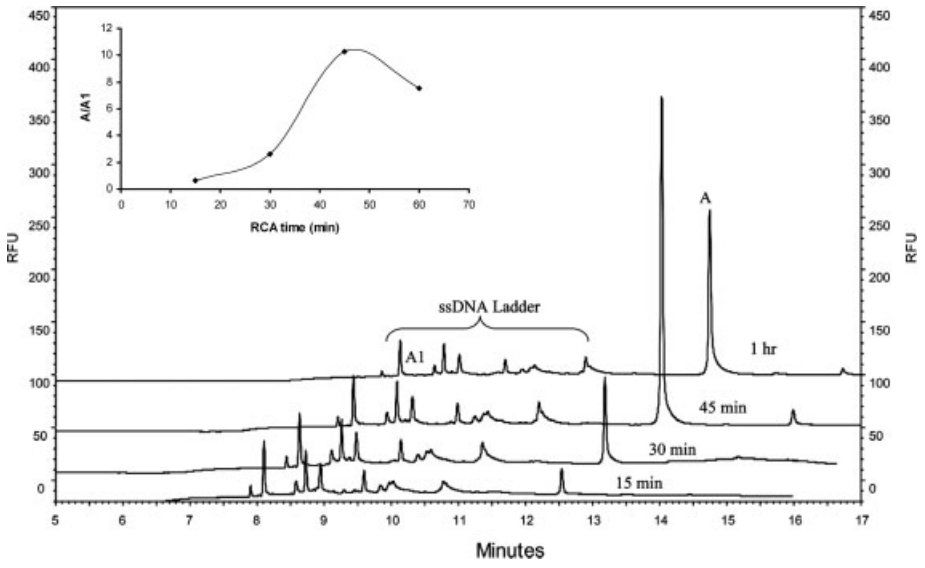

Figure 4. Overlaid electropherograms of digested RCA products with different RCA durations. Separation conditions as in Fig. 2b. Inserted: plot of normalized peak area ratio (*A*/*A*1) *vs.* RCA time.

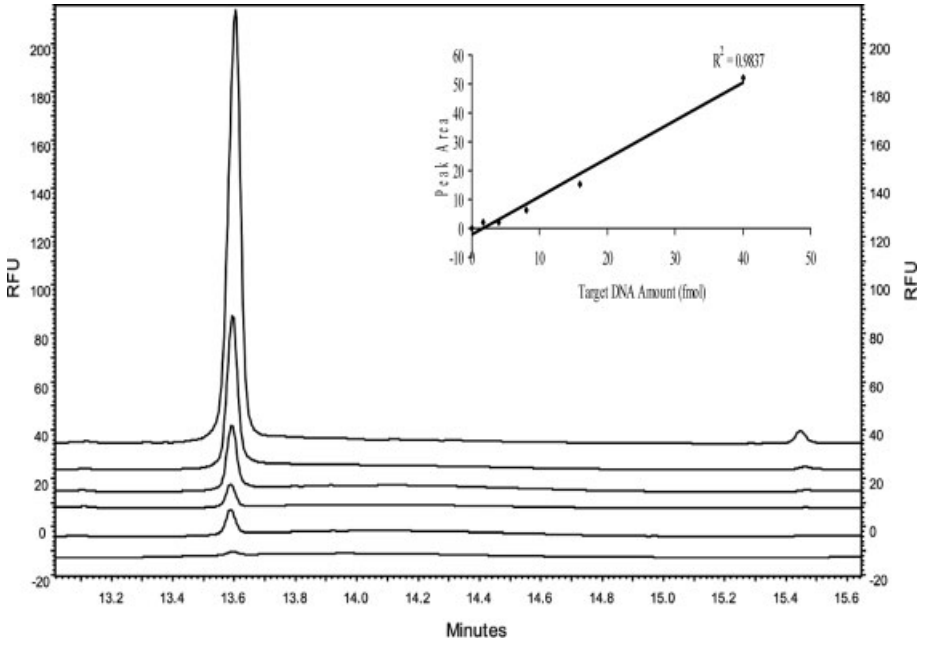

Figure 5. Electropherograms of reaction products from different amounts of target 1. From top to bottom, reaction containing 40, 16, 8, 4, 1.6, 0 fmol target 1. Inserted: the plot of product peak area (original reading from the 32 Karat software divided by 10^6) *vs.* target amount. Separation conditions as in Fig. 2b, except that injections were done at −2 kV for 60 s.

duplexed detection of two DNA targets was tested in our study, one of the targets being viewed as the internal standard (IS). A second padlock probe (probe 2) was designed with a shorter fragment length than probe 1, and used to amplify target 2 in this demonstration. Since the enzyme digestion product has a fragment size similar to the padlock probe, the products from these two targets can be separated quite well by CE. The internal standard (target 1 in this case) and the target (target 2) are amplified simultaneously in one reaction tube and the resulted products are analyzed together in one capillary. Therefore, the peak area ratio should accurately reflect the quantity ratio of the starting materials. In this way, we can normalize the signal intensity obtained from the target to that of the IS and improve the quantification accuracy. Such a normalization concept has been applied widely in microarray analysis [24]. Figure 6 displays the electropherogram from the amplification of both target 2 and IS mixed at a ratio of 1:1. Other mole ratios (target 2/IS) of 1:10, 1:5, 1:2, 2:1, 5:1, and 10:1 were also tested, keeping the amount of IS constant. The product peak area ratio (product 2/IS) was plotted against the target ratio, which showed a good linearity of $R^2 = 0.992$ (Fig. 6).

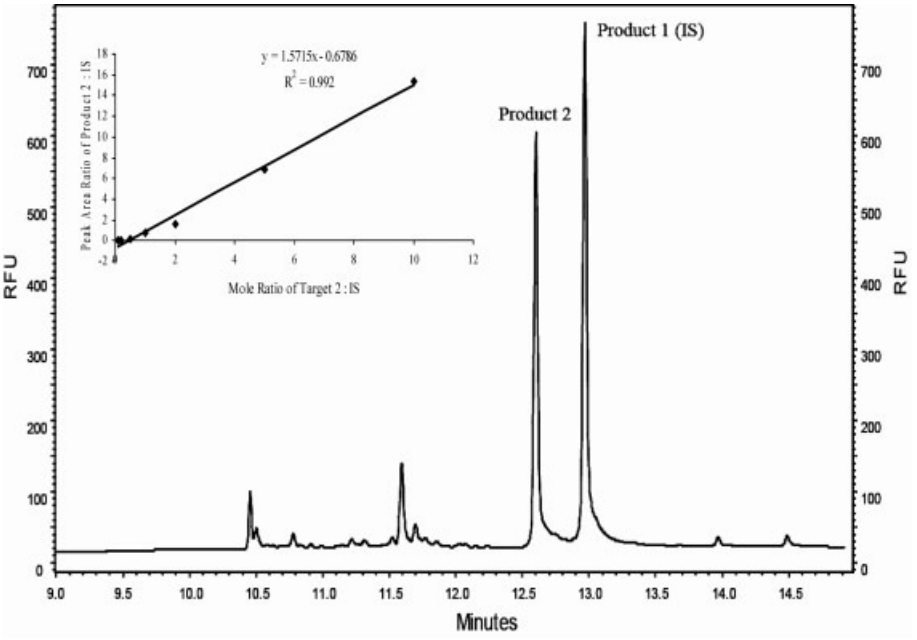

Figure 6. Electropherogram of reaction with target 2 and IS at a mole ratio of 1:1. Inserted: plot of peak area ratio of product 2/IS *vs.* target mole ratio of target 2/IS.

However, the function between the peak area ratio and the target ratio in Fig. 6 is not an identity function. The plot has an overall slope larger than 1. It indicates that the reaction kinetics is different for the two padlock probes. The longer probe (probe 1, 73 nt) seemed to have a faster reaction rate than the shorter probe (probe 2, 58 nt), because the peak area ratio of product 2/product 1 was smaller than 1 when the two targets were mixed at a mole ratio of 1:1. Since the amplification procedure is sequence independent, the kinetics difference should be from the ligation reaction. The shorter probe may need to overcome higher free energy barrier in folding because of its short strand length. The ligation kinetics may also be concentration dependent. As the concentration of target 2 increased in the reaction mixture, the product peak area ratio became larger than the target mole ratio. Such a deviation from the true value should not affect the relative quantification because the relationship between the peak area ratio and the target mole ratio is linear. A precircularized probe instead of a padlock probe can be used in the amplification reaction to reduce the difference brought in by the ligation reaction. Anyhow, simultaneous detection of both the DNA target and the IS eliminates the variations caused by random differences in the reaction or analysis procedure and improves the quantification accuracy. Therefore, expression levels of genes or small RNAs can be assessed correctly using our assay if an internal standard that has a constant concentration level could be found.

4 Concluding remarks

In this study, we demonstrated that RCA-CE assay is a fast and effective technique for sensitive detection of target nucleic acids. Because the RCA products have very large and different fragment sizes not suitable for direct electrophoretic separation, a specific sequence is introduced into the padlock probe for restriction enzyme digestion of the products. The digested fragments are subject to CE separation using 4% PVP solution as the sieving matrix under native running condition. The RCA-CE assay is a low cost and simple technique for detection of target nucleic acids. The separation process takes less than 15 min; no fluorescent dye- or radioisotope-labeled probes are needed; no sophisticated instruments are involved. A detection limit of a few femtomole was achieved after simple desalting of the final product in our study. Decreasing the reaction volume and removing the reaction substrates after RCA should be able to improve the detection limit further. Simultaneous detection of multiple targets was also demonstrated for more accurate target quantification. We coamplified the target with an internal standard and the relative quantity of the target to the IS was achieved. More targets can be detected simultaneously with the RCA-CE assay because RCA is independent of the template sequence. This is one big advantage of using CE as the detection method for RCA compared to other real-time detection techniques with molecular beacons [25, 26]. Multiple padlock probes with one or more nucleotides difference in length can be designed for different targets and the products can be well resolved in CE due to its high separation efficiency. Additionally, the single-column RCA-CE assay can be easily adapted to a capillary array system to enhance the sample throughput hundreds of times. Short, single-stranded oligonucleotides like small RNAs that are hard to be analyzed with real-time or regular PCR due to their small fragment sizes can be amplified and detected with our RCA-CE assay, because RCA can extend the small

RNAs directly from their 3′-end upon hybridization to the circular probe and does not require any amplification primers. For other types of nucleic acids, the reaction process can certainly be modified to be compatible with different targets, and this assay should be able to detect SNP or proteins (*via* immuno-RCA or aptamer) like other RCA techniques. The isothermal reaction condition makes it simple in designing an on-column or on-chip reactor to minimize the reaction volume, integrate the analysis procedure, and enhance the assay sensitivity. High-throughput screening of small RNA expression in biological samples using this RCA-CE assay is under development in our laboratory.

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The authors have declared no conflict of interest.

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