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Genomic DNA detection using cycling probe technology and capillary gel electrophoresis with laser-induced fluorescence

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Abstract

Cycling probe technology (CPT) is an isothermal DNA analysis method that has been shown to be useful for identifying genetic markers of antibiotic-resistant bacteria in clinical settings. CPT assays have previously employed several assay methods that include polyacrylamide gel electrophoresis and magnetic beads for separations and radioisotopic and colorimetric detection for detection. Capillary gel electrophoresis with laser-induced fluorescence (CGE-LIF) is an alternative separation and detection method that offers attributes such as low sample consumption, short analysis times, no radiation hazards and potential for high throughput. We report on the development of a CGE-LIF CPT assay for genomic DNA from *Erwinia herbicola* and the comparison of this assay with a conventional ^{32}P radioisotopic PAGE CPT assay. Separation and detection of intact and cleaved fluorescein-labeled CPT probe molecules by CGE-LIF was achieved in under 4 min through a gel-filled capillary (7 cm separation length to detector). Total time, from setup to detection, was about 1 h for the complete assay versus several hours (3–12 h) for the radioisotopic PAGE CPT assay. Similar detection limits of 10^5–10^6 copies of genomic target DNA were observed with each assay method. This study demonstrated that CGE-LIF CPT is a suitable analysis method for the detection of genomic DNA sequences.

Keywords: DNA; Cycling probe technology; Capillary gel electrophoresis

1. Introduction

Cycling probe technology (CPT) is an isothermal DNA sequence detection reaction [1–12] whereby the presence of the target molecule is determined by enzymatic cleavage of a sequence-specific nucleic acid probe (Fig. 1). The probe is a single-stranded polynucleotide approximately 25–30 bases in length containing a short run of four to six ribonucleotides flanked by deoxynucleotides. The CPT reaction is carried out at a single elevated temperature (55–65 °C) in the presence of thermostable RNase H, an enzyme that cleaves the phospho-ribonucleotide bond of the probe-target hybrid. The cleaved probe fragments, hybridized to the target sequence, have lower thermal stability (melting temperature) than that of the intact probe. At the reaction temperature, the probe fragments melt (dissociate) from the target sequence leaving the target free to hybridize to another probe molecule. The cleavage event can be observed by a variety of methods, most commonly by separating and detecting the cleaved product(s) from the intact probe by slab polyacrylamide gel electrophoresis (PAGE) using radioisotope-labeled probes [1–4]. Antibody-mediated colorimetric enzyme assays [5–7], test strips [8] and magnetic separations [9] have been described for CPT-based detection as well. CPT offers an alternative method to other DNA detection methods such as PCR. The assay can

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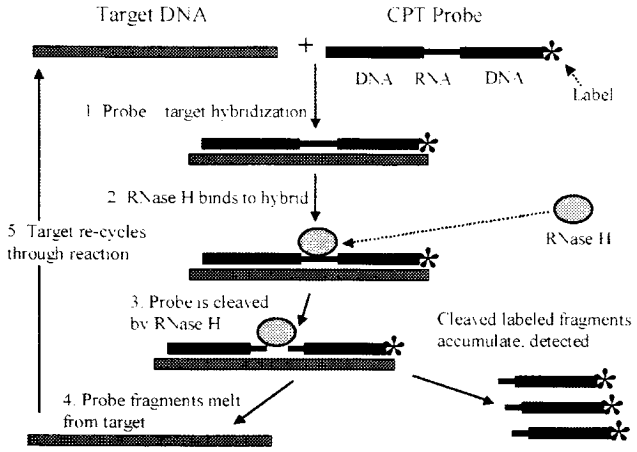

Fig. 1. Cycling Probe Technology. A sequence-specific single-stranded probe (approx. 25–30 nucleotides) contains an internal stretch of 4–6 ribonucleotides (RNA) flanked by deoxyribonucleotides (DNA). One end of the probe is labeled (*). The labeled probe is incubated with target DNA. The probe hybridizes with the target sequence and forms a segment of RNA/DNA duplex (1). Thermostable RNase H (2) binds to the RNA/DNA duplex region and cleaves the RNA segment (3). Since the thermal stability of the resulting cleaved products is lower than the intact probe, the products melt (dissociate) from the target sequence (4) and the target sequence then becomes available to hybridize with another intact CPT probe (5). Accumulated cleaved labeled probe is separated from other reaction products and detected.

show a linear response under certain conditions facilitating easier quantitation [13]. The isothermal reaction simplifies the assay process.

Capillary electrophoresis has emerged as a powerful tool for bio-analysis providing high-resolution separation, low sample consumption and short analysis times [14]. Capillary gel electrophoresis (CGE) is routinely used in DNA analysis for sequencing [15] and sizing [16]. Detection limits of fluorescently labeled bio-molecules have been demonstrated in the picomolar range for capillary electrophoresis with laser-induced fluorescence (CE-LIF) [17] and are comparable to those of conventional radioisotopic assays.

Previously the CPT assay was successfully developed with sufficient sensitivity in a radioisotopic format to identify as few as 250 organisms of *Mycobacterium tuberculosis* [3]. It has also been used successfully to identify vancomycin-resistant enterococci [7,10] and methicillin-resistant *Staphylococcus aureus* [5,11,12] in clinical samples using radioisotope and colorimetric-based detection in microtitre plates. Free zone (non-gel) CE-LIF [13] and CGE-LIF [18] analyses of CPT reactions have been demonstrated using synthetic DNA as the target. Here we demonstrate the utility of the CPT assay for detection of genomic DNA from *Erwinia herbicola* using CGE-LIF. This paper describes the development of a CGE-LIF format using fluorescein-labeled probes and polyacrylamide gel-filled columns and compares this format with a conventional radioisotopic format in terms of sensitivity and reproducibility.

2. Materials and methods

2.1. CPT probe design strategy

Candidate probe sequences were selected from published *E. herbicola* gene sequences in the Genbank Sequence Database (National Center for Biotechnology Information, National Institutes of Health, Bethesda MD, USA). The criteria for probe design were: (i) 26–31 nucleotides in length containing four consecutive centrally located adenosine residues; (ii) low formation of intra- and inter-strand secondary structure; (iii) melting temperatures (T_m) of the hybridized probe fragments 5–10 °C lower than the T_m of the hybridized intact probe. Candidate sequences were analyzed for secondary structure formation and T_m using the OLIGO Program Version 5.1 for Macintosh (NBI/Genovus Inc., Plymouth MN, USA). Sequences meeting the probe design criteria were screened in silico against the Genbank database for homology to *E. herbicola* using either Internet BLAST searches [19] or FINDPATTERNS searches (GCG Wisconsin Package Release 9.1, July 1997, Genetics Computer Group, Madison WI, USA). From this procedure, nine probe sequences representing three separate genes (beta-glucosidase, dimethylallyltransferase and tyrosine phenol-lyase) were identified as candidate probes and were custom synthesized by Integrated DNA Technologies (Coralville IA, USA) with a 5′ fluorescein label. Several candidate probes were also synthesized with a 5′ Cy5 label. Probes were made to 200 μM in sterile nuclease-free water (Promega, Madison WI, USA) and stored at −20 °C. Probes were analyzed for purity by CGE using ultraviolet (UV) and laser-induced fluorescence (LIF) detection and ranged in purity from 95–100%.

2.2. 3′Radiolabeling of CPT probes

CPT probes used for radioisotopic assays were radiolabeled at the 3′ terminus by reacting 2 pmol of 5′fluoresceinated probe with 25 μCi (alpha) ^{32}P-ddATP and 48 units of terminal deoxyribonucleotidyl transferase, at 37 °C for 15 min, following the manufacturer's directions (Amersham Pharmacia Biotech Canada, Baie d'Urfe QC, Canada). The labeling reaction was stopped by adding 0.5 M EDTA to a final concentration of 50 mM. Probes were purified on NICK® Sephadex® G-50 oligonucleotide separation columns (Amersham Pharmacia Biotech, Canada) using nuclease-free water. The final radiolabeled probe concentration was 5 nM. A 10× saline-citrate solution (1.5 M NaCl; 0.15 M citrate) was added to a final concentration of 0.1× [20]. Purity of radiolabeled probes was determined by PAGE and autoradiography.

2.3. Target DNA

Complementary synthetic DNA sequences were synthesized on a Beckman Oligo 1000 DNA synthesizer

(Beckman–Coulter Inc., Fullerton CA, USA) using standard Beckman reagents and protocols. Synthetic DNA concentrations were adjusted to 100–200 μM in sterile nuclease-free water. Purity of the synthetic DNA (10 μM) was assessed by CGE-UV. Synthetic DNA sequences typically ranged from 60–100% purity as determined from 20 to 40mer oligonucleotide reference standards (Beckman–Coulter) and were used for probe screening without further purification.

Genomic DNA was isolated from *E. herbicola* (American Type Culture Collection, Rockville MD, USA catalogue # 33243) by standard methods [20]. The DNA was sheared by 10 passages through a 21-gauge needle and resuspended in sterile nuclease-free water. Genomic DNA was sonicated to lengths of 0.5–2 kb and the concentration adjusted to 0.1 μg/μL. Sample dilutions were made in nuclease-free water from these stock concentrations. The quantitative expression of genomic DNA in terms of gene copies or organisms in this paper assumed one chromosomal copy per organism having a chromosomal molecular weight of approximately 6×10^9 Da (National Center for Biotechnology Information, National Institutes of Health, Bethesda MD, USA).

2.4. Capillary gel electrophoresis

All CGE was performed on a Beckman P/ACE System 5010 CE unit (Beckman–Coulter) with on-column LIF or UV detectors at a constant column temperature of 30 °C. Separations were carried out using eCAP100ssDNA polyacrylamide gel columns (Beckman–Coulter) with an internal column diameter of 100 μm. The separation buffer was eCAP100ssDNA column buffer (44% TRIS/56% borate/7 M urea pH 8.3, Beckman–Coulter). Buffers were filtered through a 0.22 μm filter and degassed under vacuum for 15–20 min prior to use. Samples were electrokinetically injected onto the column at 7.5 kV for 3 s resulting in a calculated injection volume of approximately 20 nl. Separation occurred at a field strength of 300 V/cm. The fluorescein label was excited at 488 nm using the Beckman P/ACE System Laser Module 488 unit with an emission wavelength of 520 nm. The Cy5 label was excited with a 635 nm diode laser module (Beckman–Coulter) with an emission wavelength of 670 nm. Beckman P/ACE Station software (Version 1.2) for Windows was used for data collection, analysis and instrument control. Signals were measured in relative fluorescence units at a data collection rate of 5 Hz. For data analysis in either UV or LIF modes, migration times in minutes were determined for each detectable peak (signal/background ratio ≥ 2). Peak areas were calculated and expressed as a percentage of the total area.

The purity of probe and synthetic target was assessed by CGE with UV detection using a column length of 20–40 cm to the detector in reverse polarity mode. UV absorbance was measured at 254 nm. Probe purity was also assessed by CGE-LIF using a column length of 20–30 cm to the detector in reverse polarity mode. For CPT assays, samples were separated in the reverse direction on the column leading to a column length of 7 cm to the detector. For these separations the voltage was applied in the normal polarity mode. The 7-cm column length provided baseline separations and decreased the run time significantly.

2.5. Radioisotopic CPT assays

Radioisotopic CPT assays using synthetic DNA were performed in a total assay volume of 10 μl. Each sample contained 20 mM TES (Sigma-Aldrich Ltd, Oakville ON, Canada) buffer pH 6.8, 0.05% (w/v) Triton X-100 (Sigma-Aldrich), 4 mM $MgCl_2$ (Sigma-Aldrich), 0.01 μg/μl *Thermus thermophilus* RNase H (ID Biomedical, Bothell WA, USA) and 2 fmol of radiolabeled probe (0.2 nM probe in the reaction). Synthetic DNA concentrations ranged from 300 to 3×10^6 molecules in 10-fold increments. Reactions were incubated at 60 °C for 30 min and stopped by the addition of 10 μl of gel loading dye (8 M urea, 100 mM EDTA, 0.25% (w/v) bromphenol blue). Samples were denatured at 95 °C for 5 min and cooled on ice prior to electrophoresis.

Radioisotopic CPT assays using genomic DNA were carried out in similar fashion to that described for synthetic DNA. In addition, EDTA and spermine were added to the cocktail mix to final concentrations of 50 μM and 2 mM, respectively, to reduce background from non-specific probe-DNA interactions [4]. To accommodate higher DNA concentrations in genomic samples, the enzyme concentration was increased from 0.01 to 0.05 μg/μl. Finally, the reaction volume was increased to 20 μl. The probe concentration in the assay was 0.1 nM. Genomic target concentrations ranged from 1000 to 10^8 copies of chromosomal DNA. Assays were performed in triplicate.

Gel electrophoresis of radiolabeled probes and radiolabeled CPT assay products was carried out in duplicate on denaturing 20% polyacrylamide/7 M urea gels in 1× TBE (Tris-borate–EDTA) buffer [20] at room temperature and 40 V/cm, using a Bio-Rad Mini-Protean II (Bio-Rad, Hercules CA, USA) apparatus. Gels were exposed overnight on Kodak XAR-5 film (Interscience, Markham ON) with a phosphor screen at −20 °C to obtain autoradiograms. For data analysis, autoradiograms were scanned using a HP ScanJetIIcx scanner (150×150 dpi). Densities of intact and cleaved probe bands were calculated using the public domain program NIH Image v1.61 for Macintosh (National Institutes of Health, Bethesda MD, available on the Internet at http://rsb.info.nih.gov/nih-image/). The extent of probe cleavage (% cut) was determined from the band densities (pixel counts). The total pixel count in each lane was normalized to the mean total count of all lanes according to NIH Image protocol.

2.6. CGE-LIF CPT assays

CPT assays using CGE-LIF detection were carried out essentially as described for CPT assays with ^{32}P labeled probes, with the following modifications. Fluorescent probe was used in place of radiolabeled probe at a final concentration of 10 nM (200 fmol in 20 µl reaction volume). Phosphate buffer (10 mM pH 7.0) was used in place of TES buffer as it was found to improve electrokinetic injection of the sample onto the column. The CPT reaction was quenched with 10 mM EDTA in place of gel loading dye. For CGE-LIF assays, genomic samples were not heat-denatured prior to CGE analysis so as not to degrade the fluorescent probe. In fact, denaturation did not improve signal detection (data not shown). Probe cleavage was calculated by integration of the electropherograms and expressed as the percentage of the total peak areas of cleaved and intact probe for each target concentration. Probe cleavage values were corrected for C2 activity (see below). Assays were performed in duplicate.

2.7. Assay controls and limit of detection

Two controls were employed in the CPT assays. One of the controls, C1, contained all reagents except RNase H and target. C1 was used to measure the amount of non-enzymatic hydrolysis of the probe. Its purpose was to verify that the probe molecules remained intact prior to the assay, i.e. during post-synthesis storage or on the bench in a CPT reagent cocktail. Typically C1 activity accounts for less than 1% (0.1–0.5%) of the intact probe. The C2 control contained all reagents except target. Its purpose was to determine the extent of probe-mediated cleavage resulting from intra- and inter-probe interactions. The amount of C2 observed was dependent on a number of factors such as probe sequence, probe concentration, end labels, reaction temperature and electrolyte concentration. The C2 controls were verified (i.e. the observed cleaved probe product was indeed due to formation of double-stranded structures of probe molecules and subsequent cleavage by RNase H) by the addition of EDTA (100 mM final concentration) to the C2 samples. The added EDTA sequestered the Mg^{2+} ions in the reaction mixture and inhibited RNase H activity associated with C2. C2 generally had greater variation than C1. Probe design and reaction conditions were optimized to maintain low C2 values, typically 5% or less. In this work, C1 was always observed in an acceptably low range, less than 0.5% of the intact probe. Specific values of C1 are not reported here. All reported values of probe cleavage were corrected for C1 and C2. In this study, the lower limit of detection (LOD) was defined as the lowest amount of target DNA that could be detected above background cleavage (C2 activity) with a signal-to-background ratio of two or greater.

3. Results and discussion

3.1. Assessment of CPT probes

As described in Section 2, nine CPT probe candidate sequences (Table 1) for *E. herbicola* were selected for suitability in CPT assays based on computer analysis with respect to guanine-cytosine (GC) content, melting temperatures and capacity for self-hybridization. These were subsequently synthesized for testing. The probes were initially tested by radioisotopic CPT reactions for production of cleaved probe fragments in the presence of genomic and synthetic DNA targets. Control reactions (C2) that contained probe plus reagents in the absence of target DNA were also tested. Successful candidate probes should yield relatively high amounts of cleaved product in the presence of target and low amounts in the control. The results of the probe screening are given in Table 2. All probes were initially predicted to have a low probability of intra-strand and inter-strand interactions using the OLIGO™ program and hence low C2. However, experimentally, several probes exhibited C2 activities greater than 10% probe cleavage; EH5 and EH10 showed high C2 activities of 25 and 36%, respectively. The addition of EDTA, an RNase quencher, to the C2 samples for these probes reduced the probe cleavage to less than 1% indicating that the observed C2 activity was due to the actual enzymatic

Table 1
CPT probe descriptions

Genbank name	Designation	Sequence[a]
Beta-glucosidase		
EHBGL279-28	EH1	5′ tga tca gta cct (aaaa) gat att cag ctg
EHBGL610-30	EH2[b]	5′ gtt gaa gtt gca ac (aaaa) atc atg gcc gag
EHBGL1044-31	EH3[c]	5′ tat tcg cca tca ga (aaaa) tgc ttc aga agt c
EHBGL1468-31	EH4	5′ atc atc cgt ggt g (aaaa) tat cag tga tgt tg
Dimethylallyltransferase		
EHETZGENE858-30	EH5[d]	5′ tat aat tat tgg ct (aaaa) agc aga gtg cta
EHETZGENE1175-26	EH6[e]	5′ ttg cag atg caa (aaaa) att gga ttt g
Tyrosine phenol-lyase		
ERWTPL1224-28	EH8[f]	5′ acg cgg ggc gg (aaaa) cct gct ctc gca g
ERWTPL1158-28	EH10	5′ cta cca tct cga (aaaa) acg gtg aaa gag
ERWTPL1751-26	EH11[g]	5′ tct cag cgg c (aaaa) gag ttg gtt gtc

[a] Bracketed nucleotides are ribonucleotides.
[b] Hairpin with loop $T_m = 33$ °C (not involving ribo region).
[c] Weak hairpin with loop $T_m = 5$ °C (1 A of ribo region involved).
[d] Hairpin $T_m = 40$ °C.
[e] Hairpin $T_m = 64$ °C.
[f] Hairpin $T_m = 52$ °C.
[g] Ribo region is in weak hairpin ($T_m = 11$ °C).

Table 2
Detection limits of radioisotopic CPT assays using synthetic and genomic target

Probe	% C2	No. trials	LOD using synthetic target (copies)	LOD using genomic target (chromosomal copies)
Beta glucosidase				
EH1	5	4	3.00×10^{3}	1.00×10^{7}
EH2	13.3	3	3.00×10^{3}	1.00×10^{7}
EH3	6.4	4	3.00×10^{3}	1.00×10^{7}
EH4	12.8	3	3.00×10^{3}	1.00×10^{5}
Dimethylallyl transferase				
EH5	24.8	3	3.00×10^{5}	1.00×10^{7}
EH6	14	4	3.00×10^{4}	1.00×10^{7}
Tyrosine phenol lyase				
EH8	4	6	3.00×10^{4}	1.00×10^{4}
EH10	36.3	4	N/D	N/D
EH11	4	2	N/D	1.00×10^{7}

cleavage of the probe in the absence of target (data not shown) and not due to degradation of the probe by non-enzymatic processes (C1). This activity was considered true C2 activity. Probes that exhibited high C2 activity were subsequently re-analyzed in silico, using temperatures and sodium ion concentrations closer to the actual reaction conditions. The re-analysis suggested that EH5 and EH10 formed stable secondary structures that promoted non-specific cleavage of the ribonucleotide region by RNase H (data not shown) and were deemed not suitable.

Based on the results shown in Table 2, one probe sequence, EH8, exhibited acceptably low C2 activity (less than 5%) and good target recognition for both synthetic and genomic target DNA in radioisotopic assays and was subsequently used in the comparative CGE-LIF studies in this work. The CPT reaction methods were essentially the same in each technique. The only difference apart from the post-reaction analysis was the enzymatic 3′-end labeling of the probe with ^{32}P. Minimal C2 activity was observed for the EH8 probe in radioisotopic assays and it was used subsequently for the capillary electrophoresis assays reported here. Computer analysis predicted EH5 and EH10 to have lower C2 than EH8, which was not the case. This observation revealed that probes should be tested empirically to determine performance suitability in CPT assays. A representative radioisotopic CPT assay with *E. herbicola* genomic DNA and the EH8 probe is shown in Fig. 2.

3.2. CGE-LIF CPT

Reproducible migration patterns were observed as two major bands separated to the baseline by about 1.5 min using fluorescein-labeled probe and genomic DNA (Fig. 3a–f). The intact probe appeared as a slower migrating single sharp peak. For the control electropherogram (Fig. 3a), the signal-to-noise ratio of the intact peak was 65. The faster migrating labeled cleaved probe fragment, on close inspection, was seen as four partially resolved peaks (Fig. 3d) each separated by about 0.20 min, likely representing fragments varying in length by a single nucleotide due to cleavage at different sites within the probe. The greatest amount of the cleaved probe appeared in the peak at 0.9 min, indicating a preferred hydrolysis site in the 4-base ribonucleotide section of the probe. This behavior of RNase H to preferentially cleave at a site within ribonucleotide region has been reported by Hogrefe et al. [21]. Since detection was by LIF, the unlabeled probe fragment was obviously not observed in the electropherograms.

Separation of intact and cleaved products was usually achieved in under 3 min for fluorescein-labeled probe using a 7-cm capillary and a constant voltage of 300 V/cm. For fluorescein-labeled probe, the mean migration times for a set of consecutive runs ($n=6$) were 2.213 ± 0.026 min for the intact probe and 0.719 ± 0.013 min for first cleaved probe. For the same set, the mean migration time difference between intact and first cleaved probes was 1.494 ± 0.014 min. Over the course of 30–40 runs, there was a decrease in migration time by about 5%, however, the time difference between peaks remained relatively constant, less than 2%, indicating reproducible behavior.

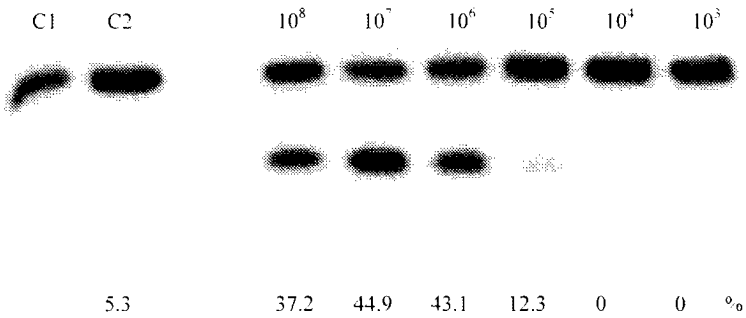

Fig. 2. A representative isotopic CPT assay with *E. herbicola* genomic DNA and EH8 probe. Assays were performed using 2 fmol probe/reaction (0.1 nM) as described in the text. Samples contained 10^{8}–10^{3} copies of chromosomal DNA. Values beneath autoradiograms indicate % cleavage above C2 background.

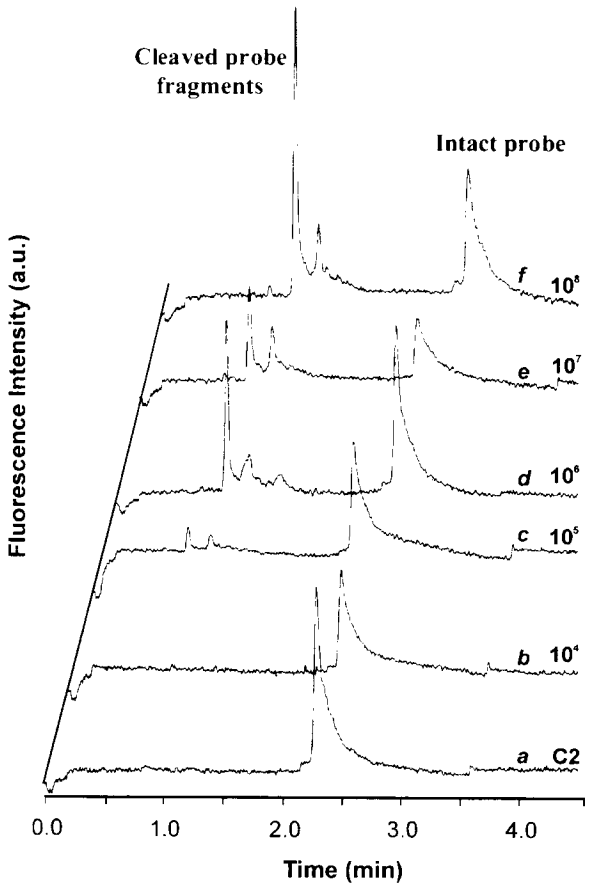

Fig. 3. Electropherograms of representative CGE-LIF CPT assays using *E. herbicola* genomic DNA and 200 fmol fluorescein EH8 probe/reaction (10 nM). The migration times of the intact and the first cleaved probe were 2.213 ± 0.026 and 0.719 ± 0.013 min, respectively. (a) C2 control (0.5%); (b) 10^4 copies (1.7%); (c) 10^5 copies (7.7%); (d) 10^6 copies (40.9%); (e) 10^7 copies (62.5%); (f) 10^8 copies (50.0%). Values in parentheses are percent probe cleavage based on peak areas.

3.3. Comparison of isotopic and CGE-LIF detection for CPT using EH8 probe

For genomic target, the radioisotopic and CGE-LIF CPT assays routinely yielded lower limits of detection (LOD) of 10^5 molecules (see Table 3). At higher concentrations of genomic target (above 10^7 copies), there was a decrease in the amount of cleaved probe detected. This phenomenon was observed in both radioisotopic and CGE assays and

Table 3
Comparison of radioisotopic and CGE-LIF assays using EH8 CPT probe and genomic target DNA

	Radioisotopic assay (^{32}P)	CGE-LIF (fluorescein)
LOD (copies of genomic target)	1×10^5	1×10^5
Probe molecules	1.2×10^9	1.2×10^{11}
Reaction volume	20 μl	20 μl
Volume used for analysis	10 μl	~20 nl
Detection limit of probe	0.15 fmol	0.18 fmol

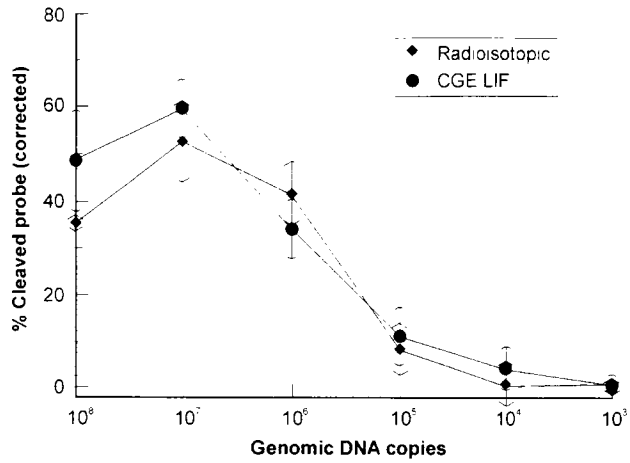

Fig. 4. Comparison of genomic CPT assays with radioisotopic and CGE-LIF detection. Assays were performed as described in the text. Each point represents six separate assays performed in duplicate (radioisotopic detection ◆) or 10 separate assays performed in duplicate (CGE-LIF detection ●). Error bars represent standard deviations.

likely a result of inhibition of the CPT reaction in the presence of high total DNA concentration, rather than a CGE artifact. Modrusan et al. reported similar observations at high DNA target concentrations [4]. The calculated percentages of cleaved probes over the range of target molecules tested were similar for the radioisotopic and CGE-LIF assays (Fig. 4). Slightly greater inter-assay variance was observed between trials for CGE-LIF CPT assays (including the C2 control) than for the radioisotopic assays.

For synthetic target, the lower LOD by radioisotopic assay was 3×10^4 molecules (0.05 attomole). By CGE-LIF assay, using Cy5-labeled probe, the LOD was an order of magnitude higher, approximately 3×10^5 molecules. This difference in LOD between the radioisotopic and CGE-LIF assays persisted even when different lots of synthetic target DNA were used. The C2 activity for the synthetic target was typically 2–4% in radioisotopic assays and 5–6% in CGE-LIF assays. The increase in C2 activity for the CGE-LIF assays raised the LOD cut-off.

The total time required to complete the CPT assay with CGE-LIF detection was under 1 h from setup through 30-min reaction time to detection, using a bench top CE unit equipped with a single gel-filled capillary. The analysis of CGE data was quick using manufacturer-supplied analysis software. The radioisotopic method was considerably longer (3–12 h), more tedious and more labor intensive.

Although the CPT assay was optimized for the reagents used in the CGE experiments herein, the CPT process has the potential for further enhancement of the sensitivity. Judicious choice of the target sequence is one option. For example, targeting a direct repeat sequence, as found in *M. tuberculosis*, enabled CPT detection sensitivity to be enhanced to as few as 250 cell equivalents [3]. Targeting a sequence present on high-copy-number plasmids could also improve sensitivity. Generally the limiting factor for LOD

was the C2 control. Thus reaction conditions that alleviate inter- and intra-molecular interactions of the probe and thereby reduce unwanted RNA-DNA double stranded character will lower the C2 background.

The high levels of sensitivity achieved in PCR, i.e. detection of 1–10 genomic copies [22,23], are not feasible with CPT but it is important to note that not all sequence detection assays are conducted in the regime of low numbers of analyte molecules. For applications where low copy number is not an issue, CPT can be advantageous since it is not highly susceptible to sample carryover and subsequent false positive results. CPT and CGE-LIF provide a useful combination. The assay is easy to perform since it requires a single temperature in the range of 58–63 °C. Temperature ramping and high temperature denaturations are not required, thus simplifying the assay process and equipment needed.

In this work, CPT reactions were quenched by addition of EDTA. However, it has been shown that lowering the temperature to below 20 °C effectively quenched the CPT reaction [13]. This simplifies the process by eliminating the need to add quenching agent. The fact that CPT is not highly susceptible to sample carryover is a useful feature for the design of automated instrumentation. Assays can be prepared, run and analyzed in close proximity. In fact, Tang et al. [13] were able to perform a series of complete CPT assays on a single reusable fluidic glass microchip.

CGE-LIF analyses were performed using gel-filled columns in this study. We observed very minor variations in migration times from run to run that we attributed to gel matrix effects. Gel-filled columns are not ideally suited for long-term repeated use because of potential matrix degradation and reproducibility issues [24,25]. Some of the problems associated with gel-filled capillaries could be alleviated by using refillable gels as is the case with many of the commercial CE-based DNA sequencers. Another option is to eliminate the gel and perform free zone CE-LIF analysis. The constant linear charge density of DNA yields electrophoretic mobilities that are independent of molecular weight [26]; hence DNA does not separate by size in free zone CE. However, if the CPT probe is chemically modified at one end so that the cleaved fragment and the intact probe have different charge-mass ratios, then free zone CE is possible. This approach was demonstrated in the microchip CPT-CE assay of synthetic DNA target whereby the 3′-end of the probe was labeled with biotin, and the 5′-end with fluorophore [13]. In that work, near-baseline separations of the intact probe and cleaved fragment were obtained by microchip CE-LIF although the single base resolution of cleaved probe fragments, as seen in our study, was not observed. Greater separation of probe and product could be achieved by the addition of streptavidin (post reaction) to probes containing terminal-labeled biotin molecules. The intact probe would have a greater effective mass as a result of streptavidin binding, compared to fluorescent cleaved product that lacks a biotinylated terminus. Free zone CE studies in our laboratory with biotinylated CPT probes and streptavidin have demonstrated that the intact and cleaved species were easily separable (data not shown).

4. Conclusions

This work demonstrates the use of CPT for genomic DNA sequence detection using conventional CGE-LIF instrumentation. The combination of CPT and CGE provides a powerful yet relatively simple method of detecting specific DNA sequences.

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