Quantification of Polymerase Chain Reaction Products in Agarose Gels with a Fluorescent Europium Chelate as Label and Time-Resolved Fluorescence Spectroscopy

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We have 5'-end-labeled one polymerase chain reaction (PCR) primer with the europium chelator 4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA). After performing PCR in the presence of another unlabeled primer, we separated the 362 bp PCR product with 2% low melting point agarose gel electrophoresis. The gel was then immersed into a Eu^{3+} solution. During soaking, Eu^{3+} diffuses into the gel and associates with BCPDA to form a fluorescent complex of long fluorescence lifetime. This complex can be quantified by scanning the gel with a time-resolved fluorometric reader. Because BCPDA and Eu^{3+} are not fluorescent by themselves, background signals are very low. The detection limit was about 5 ng of DNA. We have also shown that the BCPDA-labeled product could be blotted and detected on the membrane by using an anti-BCPDA antibody. These two technologies may find applications other than in PCR, e.g. in fluorescence-based DNA sequencing and in solution hybridization.

INTRODUCTION

The polymerase chain reaction, PCR, has gained wide acceptance as an exponential nucleic acid amplification technique and is used very frequently for research and clinical applications.[1,2] PCR is currently used mainly as a qualitative tool. However, for certain applications, there is a need for obtaining semiquantitative or even quantitative information. Although quantitative PCR has been described,[3,4] it is not used frequently because of its relative complexity. As a compromise, many are using semiquantitative approaches. In these protocols, PCR products become radioactive by incorporating either radioactive primers or radioactive nucleotides.[5,6] Similarly, others have incorporated biotin or digoxigenin.[7,8] These haptens can be detected by linking them to detection systems, e.g. enzymes, through streptavidin or antibodies, respectively. More recently, high-performance liquid chromatography has been used to quantify the PCR products.[9,10] Within a relatively narrow working range, it can be shown that the amount of PCR product generated is roughly proportional to the amount of the initial template concentration.[10]

Ethidium bromide staining is currently the standard method for the qualitative assessment of DNA in agarose gels, including PCR products.[11] Glazer et al. have quantified DNA embedded in agarose gels using fluorometric measurements. Their method is based on the ability of double-stranded DNA to form stable fluorescent complexes with ethidium homodimer. Background readings remain low because the fluorescent complex is formed before electrophoresis; excess dye and stained DNA move in opposite directions during electrophoresis.[12]

In this paper, we describe a new method for the quantitative assessment of PCR products directly in agarose gels. The method involves labeling of the 5'-end of one PCR primer with the europium chelator 4,7-bis(chlorosulfophenyl)-1,10-phenathroline-2,9-dicarboxylic acid, BCPDA.[13] After PCR, the products are separated by agarose gel electrophoresis. The gel is then immersed into an aqueous Eu^{3+} solution. During soaking, Eu^{3+} diffuses into the gel and associates with BCPDA to form a fluorescent complex of long fluorescence lifetime.[14,15] This complex can be quantified in the gel with scanning time-resolved fluorometry.[16] A feature of this method is that neither BCPDA nor Eu^{3+} are fluorescent by themselves and thus, background readings are very low. Additionally, native short-lived fluorescence from the gel or other sources and scattered light are eliminated by using pulsed-excitation time-resolved measurements.[15] This newly devised method has been successfully applied in a model system.

We have also shown that BCPDA-labeled PCR products can be blotted to nylon membranes and subsequently visualized by using anti-BCPDA antibodies and an alkaline phosphatase-based detection system. These results suggest that BCPDA may be a useful hapten for DNA labeling and may find applications similar to those of digoxigenin, a hapten that is detected in diverse applications using labeled antibodies.[17]

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MATERIALS AND METHODS

Instrumentation. We have used the CyberFluor 615 immunoanalyzer, a time-resolved fluorometer available from CyberFluor Inc., Toronto, Canada. A special software was written which transforms this instrument from a microplate reader to a high-resolution time-resolved fluorometric scanner. This special software is also available through CyberFluor. The performance of this scanner was previously described.[16] Absorbance measurements were carried out with a Model UV160U UV–vis double-beam spectrophotometer from Shimadzu Corp., Kyoto, Japan. PCR experiments were performed with a thermal cycler from Perkin-Elmer Corp., Norwalk, CT 06859. Electrophoresis was performed with equipment from Pharmacia, Piscataway, NJ 08855. High-performance liquid chromatography (HPLC) was performed with equipment from Shimadzu and a DEAE-TSK weak anion-exchange column (4.6 mm × 3.5 cm) from Perkin-Elmer. On-line detection was with a UV monitor at 260 nm.

Materials. The PCR primers used in this study were described by Hsia et al.[18] In addition to these primers, we also synthesized primers of the same sequence which contain a free amino group at their 5′-ends. This was accomplished with the TFA–amino linker amidite, available from Pharmacia LKB Biotechnology. The amino linker was added after the final 5′-nucleotide of each primer and the 6*N*-(trifluoroacetyl) moiety was removed by treatment with concentrated ammonia. After ammonia evaporation, the 5′-amino-containing primers were purified with gel filtration chromatography on Sephadex G-25 with 0.01 mol/L sodium borate as the mobile phase. The primer-containing fractions were combined and evaporated to dryness. BCPDA was synthesized as previously described.[13] As PCR target, we used a pUC19 plasmid containing a cytomegalovirus DNA isert of 2260 base pairs (bp). The insert was cloned with use of Eco RI and Kpn I restriction enzymes. Additionally, we inserted a 210 bp sequence from the ϕX174 phage with use of the Bal I restriction enzyme. This plasmid, when amplified with the primers of Hsia et al.[18] gives a PCR product of 362 bp. All chemicals used were molecular biology grade or reagent grade. Europium chloride hexahydrate was from Aldrich Chemical Co., Milwaukee, WI.

Labeling and Purification of PCR Primers with BCPDA. The 5′-amino-containing primers were dissolved in a 0.01 mol/L borate buffer, pH 9.1, at a concentration of 2 mg/mL. To this solution, we added a 10-fold molar excess of a freshly prepared ethanolic solution of BCPDA. The volume of the BCPDA ethanolic solution added was less than 10% of the total reaction volume. The labeling reaction was allowed to proceed at room temperature for 2 h. The reaction mixture was then loaded on a PD-10 disposable gel filtration column (Pharmacia) which was equilibrated and eluted with 0.1 mol/L sodium bicarbonate solution. Eluate fractions of 0.5 mL were collected. The fractions were checked by absorbance measurements at 260 and 325 nm (where BCPDA absorbs) and also by time-resolved fluorometry as follows: Ten-microliter fractions were added in microtiter wells and mixed with 100 μL of a 10^{-5} mol/L solution of $EuCl_3$ in a 0.1 mol/L Tris buffer, pH 7.80. The fluorescence of the solution, which is due to the formation of the BCPDA–Eu^{3+} complex, was then measured by time-resolved fluorometry with the 615 immunoanalyzer.

Fractions containing BCPDA-labeled primers eluted near the void volume of the column, and they were pooled, evaporated to dryness with a vacuum centrifuge (Savant Instruments, Farmingdale, NY 11735), reconstituted in distilled water, and used for the PCR experiments.

Assessment of the Extent of BCPDA Labeling of Primers. The extent of labeling of the BCPDA-derivatized primers was assessed by absorbance measurements at 325 nm. At this wavelength, BCPDA, with an extinction coefficient of 1.5×10^4 $mol^{-1} \cdot L\ cm^{-1}$ [19] is the only absorbing species.

Polymerase Chain Reaction Experiments. PCR experiments were carried out in a 100-μL total reaction volume. Ninety microliters was comprised of primers (500 ng each), buffer (50 mmol/L KCl, 10 mmol/L Tris, pH 8.3), nucleotides (200 μmol/L) and Taq polymerase (2.5 units). The sample volume was 10 μL. The PCR mixture was preheated at 94 °C for 6 min before adding the primers (hot start protocol). The cycling was as follows: denaturation at 94 °C for 1 min, annealing at 55 °C for 2 min, extension at 72 °C for 2 min. The number of cycles was 30.

Detection of BCPDA-Labeled Primers and PCR Products in Agarose Gels. We have constructed a special gel casting tray which is identical in size to a microtiter plate with dimensions of 8 × 12.5 cm. On this casting tray we marked lines corresponding to the positions of the eight rows of the plate and used a special plastic comb which creates wells in each row of the casting tray.

We used 70 mL of a 2% agarose solution in Tris–acetate buffer to cast the gels and loaded between 1- and 25-μL sample volumes. PCR products generated with unlabeled primers were stained with ethidium bromide as suggested.[11] PCR products generated with BCPDA-labeled primers were best detected in 2% low melting point agarose gels. Gels were stained with Eu^{3+} solution as follows. After electrophoretic separation, the gel is immersed into a $EuCl_3$ solution (10^{-4} mol/L in 10^{-2} mol/L HCl) for 30 min at room temperature. Subsequently, the gel is washed twice with distilled water. BCPDA-labeled primers were detected on agarose gels with the same staining procedure.

Detection of PCR Products by High-Performance Liquid Chromatography. The PCR product was spiked with 40 ng of a HindIII-linearized pUC 19 plasmid as an internal control, and 20 μL of the sample was then injected into the HPLC system with an autosampler. The HPLC gradient elution system used was as follows: Buffer A was a 0.025 mol/L Tris buffer, pH 9.0, containing 1 mol/L NaCl. Buffer B was a 0.025 mol/L Tris buffer, pH 9.0. The HPLC column was equilibrated with 50% each of buffers A and B before injection. Starting at injection time (0 min), buffer A was increased linearly to 68% in 6 min. At 6.1 min, buffer A was increased to 100%. At 9 min, the buffer composition returned to 50% each. The next injection was at 15 min, allowing column re-equilibration for 6 min.

The absorbance of the eluent was continuously monitored at 260 nm. Data processing and quantitative area calculation of each peak was automatic.

Development of Polyclonal Antibodies to BCPDA. Rabbits were immunized withe a BCPDA–thyroglobulin conjugate prepared as previously described.[19] Injections with complete and incomplete Freund's adjuvant were given following standard protocols as described.[20] Good titers of antibodies were obtained after about 4 months. The BCPDA antiserum was used without prior purification.

Southern Blot Experiments. PCR products were separated by electrophoresis on 2% agarose gels. Gels were blotted to Hybond-N nylon membranes (Amersham) using standard procedures.[11] After UV fixing of the nucleic acids to nylon, membranes were processed in one of two ways: (a) The membrane was washed with water and then immersed into a $EuCl_3$ solution, 10^{-4} mol/L, in 10^{-3} mol/L HCl for 10 min. After washing with water, the membrane was dried in an air-oven (45 °C) and scanned with the time-resolved fluorometer. (b) The membrane was incubated in a blocking solution, commercially available from Boehinger-Mannheim, for 1 h at room temperature and then immersed into a solution containing rabbit anti-BCPDA antiserum diluted 8000-fold in the blocking solution.

After 30 min, the membrane was washed twice with a wash solution (100 mmol/L Tris, 150 mmol/L NaCl, pH 7.5) and incubated by shaking in a solution containing alkaline phosphatase-labeled goat anti-rabbit antibody (from Jackson Immunoresearch, West Grove, PA 19390) diluted 5000-fold in the blocking solution. After a 30-min incubation and subsequent membrane washing as above, the membrane was immersed into a developing solution containing bromochloroindolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) as described.[11] After color development for 15–30 min or longer as required, the reaction was stopped by removing the membrane.

Extraction and Quantification of PCR Products in Microtiter Wells. PCR products generated with BCPDA-labeled primers were separated in 2% agarose gels. The bands

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Table I. Labeled and Unlabeled Primers Used in This Study

primer	stock solution, μg/mL	absorbance ratio A_{260}/A_{280}	molar ratio, BCPDA/primer	base sequence
1401	68.9	1.83		5′-GGCAGCTATCGTGACTGGGA-3′
1401* [a]	34.6	1.61[b]	0.86	
1402	66.0	1.81		5′-GATCCGACGACCCATTGTCTAAG-3′
1402*	61.2	1.62[b]	1.05	

[a] Asterisk indicates primers labeled with BCPDA. [b] Ratio decreases due to the presence of BCPDA.

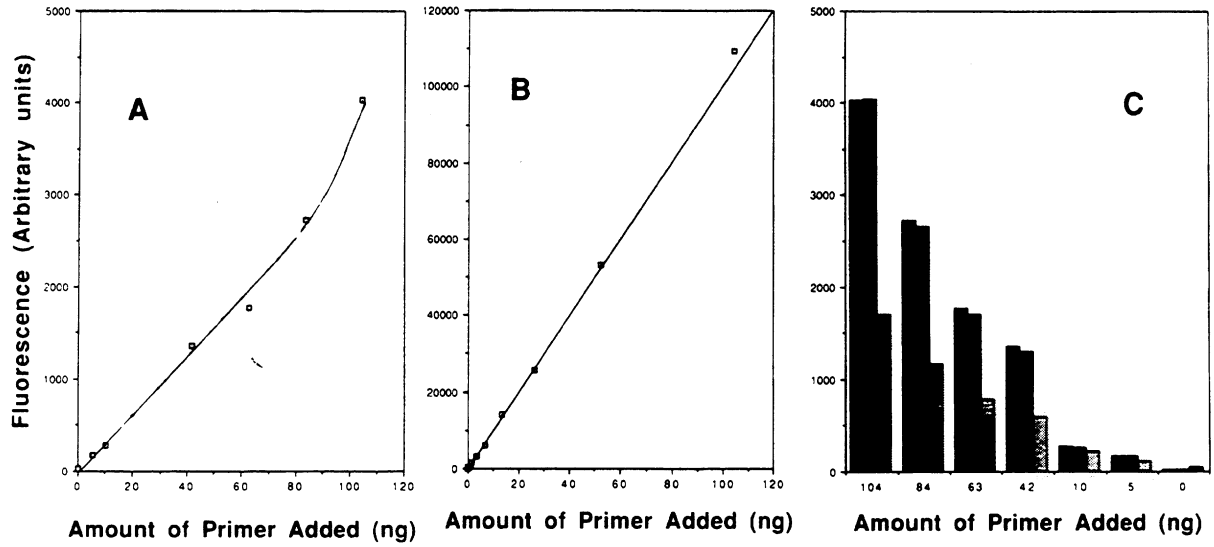

Figure 1. (A) Plot of fluorescence versus the amount of BCPDA-labeled PCR primer 1402* present in 2% low melting point agarose gels. These gels were stained with Eu^{3+} and scanned with the time-resolved fluorometer. The reproducibility of each point between various experiments was 10–15%. (B) The same amount of primer was measured in microtiter wells in the presence of excess Eu^{3+}. Each point is a mean of three replicates with CV's between 2 and 3%. (C) Effect of washing the gels containing BCPDA-labeled PCR primer after staining with Eu^{3+}. First bar: no wash. Second bar: 15-min soaking. Third bar: 2-h soaking.

were visualized by ethidium bromide staining, excised from the gels and DNA extracted as follows: (a) From agarose gels with the Geneclean kit (Bio 101, Inc., La Jolla, CA 92038-2284) which utilizes glass beads as the solid-phase. (b) From low melting point agarose gels using the enzyme gelease (Epicentre Technologies, Madison, WI 53713) to digest the agarose. In both cases, the manufacturer's instruction were followed. The final extract from a, 10–20 μL, was transferred to white opaque microtiter wells (from Dynatech laboratories), and 100 μL of a 10^{-4} mol/L Eu^{3+} solution in 0.1 mol/L Tris buffer, pH 7.80, was added. The final extract from b, about 100 μL, was also transferred to microtiter wells and the Eu^{3+} solution added as above.

In both cases, the fluorescence of the solutions, which is directly proportional to the amount of BCPDA present in the sample, was quantified with the 615 immunoanalyzer working as a microplate time-resolved fluorometric reader.

RESULTS AND DISCUSSION

The unlabeled and BCPDA-labeled primers used in this study are described in Table I. The sequences have been described elsewhere.[18] Figure 1 shows data for the detection of labeled primer 1402* in 2% low melting point agarose gels. For this experiment, we used a stock 1402* solution of 5 μg/mL and loaded sample volumes of 1–20 μL (5–100 ng). After a short electrophoresis for 5 min, to allow entrance of the primers into the gel, we stained the gel with Eu^{3+} solution as described. The gel was then scanned at various times to assess the stability of the signal. During this period the gel was stored at 4 °C. Detectable changes, presumably due to diffusion effects, appear after 6 h of storage or longer. The detection limit for primer 1402*, defined as the amount of primer that could be distinguished from zero with 95% confidence, was about 0.3–0.5 ng. When we constructed a calibration curve by plotting fluorescence readings versus amount of primer loaded, we obtained the plot shown in Figure 1A. A direct relation exists, but the response is not linear with loadings greater than 80 ng.

When the same amounts of primer were added in microtiter wells and measured in the presence of excess Eu^{3+}, the response was linear and the fluorescence readings were much higher (Figure 1B). This finding is expected for various reasons (a). In microtitration wells, about 50–60% of the measured fluorescence is reaching the detector through reflections at the sites of the wells; this signal is not collected in gel scanning measurements (b). The excitation of the band in the gel is not optimal. Because of its linear shape, part of the band is not excited at all. With measurements in microtitration wells, the excitation beam is optimized to focus in the center of the well (c). The presence of the gel above the fluorescent band poses a barrier to both excitation and emission radiation and may also contribute to scattering.

The effect of washing the gels after staining with Eu^{3+} was studied with gels containing labeled primer 1402*. Unwashed gels and gels soaked in water for up to 15 min show similar readings. Extensively soaked gels (2 h or more) start to lose fluorescence, presumably through leakage of Eu^{3+} from the complex (dissociation) (Figure 1C). Unwashed and briefly washed gels have similar backrounds which are 15–20 arbitrary fluorescence units.

Preliminary PCR experiments with labeled primers which were not purified from excess unreacted BCPDA have shown that no PCR products were obtained. Presumably, this is due to Mg^{2+} chelation by the excess BCPDA. Excess BCPDA was removed with gel filtration chromatography on PD-10 disposable columns (Pharmacia) as shown in Figure 2. Free BCPDA has affinity for the Sephadex matrix[19] and elutes much later in comparison to BCPDA-labeled primers. The primer-containing fractions were pooled and concentrated by vacuum centrifugation.

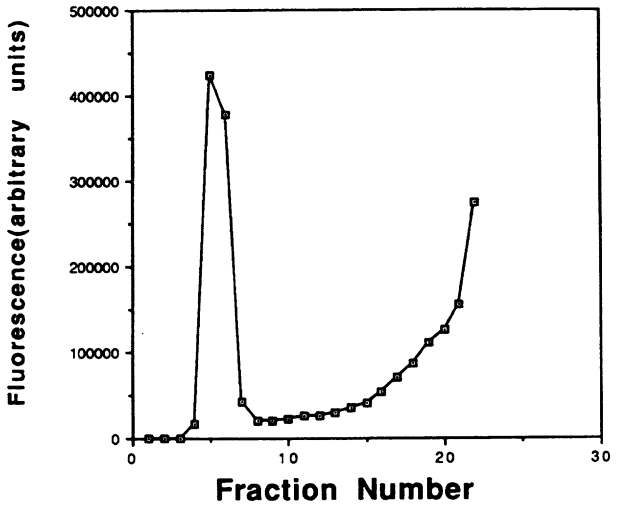

Figure 2. Elution of BCPDA-labeled primer 1402* and unreacted free BCPDA from a disposable PD-10 Sephadex column. The column was eluted with a 0.1 mol/L sodium bicarbonate solution. Pure BCPDA-labeled primer elutes near the void volume of the column (fractions 4–7, 0.5 mL each). Each fraction was measured in triplicate in microtiter wells in the presence of excess Eu^{3+}-CV's were between 2 and 3%.

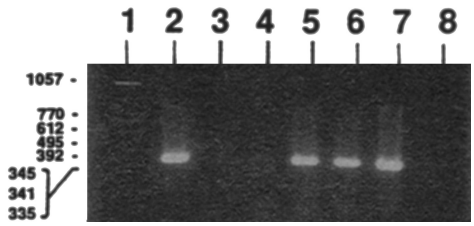

Figure 3. Ethidium bromide-stained 2% agarose gel containing PCR products generated from 1 ng of plasmid target, in the presence of various combinations of PCR primers described in Table I. Lane 1: Molecular weight markers ϕX174, Hinc II digest. The length of the markers in bp is shown on the left side. Lane 2: Primers 1401–1402. Lane 3: 1401*–1402. Lane 4: 1401*–1402*. Lane 5: 1401–1402*. Lanes 6 and 7 are repeats of lanes 2 and 5, respectively. Lane 8: Negative control without PCR target. The primers with an asterisk after the number are labeled at their 5′-end with BCPDA.

The molar ratio of BCPDA to primer was calculated with absorbance measurements at 325 nm where only BCPDA absorbs[19] and at 260 nm where both components absorb. The calculated molar ratio was close to the theoretically expected (Table I).

The effect of soaking time with Eu^{3+} on the fluorescence readings was studied with gels containing labeled primer 1402* at varying amounts. Soaking time ranged from 5 to 75 min. It was found that plateau in fluorescence is obtained with soaking times of about 20–30 min. No backround increase was observed after extensive soaking (75 min) with Eu^{3+}.

In Figure 3 we present data for various PCR reactions using different combinations of labeled and unlabeled primers. We used 1 ng of plasmid target in all cases. Specific PCR product, 362 bp long, was obtained when unlabeled primers were used (lane 2) or when unlabeled primer 1401 was used in combination with labeled primer 1402* (lane 5).

No products were detected when labeled primer 1401* was used either with unlabeled (lane 3) or labeled (lane 4) primer 1402. For all subsequent experiments, we used the primer combination 1401–1402*. The reasons for the absence of PCR products when labeled primer 1401* was used were not examined further.

Figure 4 shows time-resolved fluorometric scans of PCR products generated with primers 1401–1402*, separated on agarose gels as shown in Figure 3 and stained with Eu^{3+}. The two peaks of Figure 4A correspond to the 362 bp PCR product and the excess primers, respectively. The ratio of signal/background for the first peak is about 27 (3220/120). The same PCR product was run on a 2% low melting point agarose gel and scanned as above (Figure 4B). The scan is qualitatively similar to that of Figure 4A, but the resolution is improved and the backround signal was reduced to almost undetectable levels (less than 30 arbitrary fluorescence units). Thus, low melting point agarose gel electrophoresis is the preferred separation method for the proposed application. Scanning of gels stained with Eu^{3+}, containing PCR products generated with unlabeled primers or molecular weight standards, give no appreciable fluorescence or peak patterns.

The quantitative nature of the proposed method was established by separating varying amounts of a PCR product generated with primers 1401–1402* on 2% agarose gels and scanning, after Eu^{3+} staining. Figure 5 is an ethidium bromide-stained gel which is loaded with varying amounts of a PCR product generated with 1401–1402*. The gel was stained with Eu^{3+} and scanned with the proposed method. There is a direct relationship between fluorescence and sample volume, but as also shown in Figure 1, the response is not linear.

Figure 6 shows PCR products generated from varying amounts of the plasmid template and stained with ethidium bromide. The 1401–1402* primer set was used. Some data, with target plasmid amounts less than 100 pg, are not shown because the PCR product was not visible on ethidium bromide gels. The same gels were stained with Eu^{3+} and the lanes scanned with the time-resolved fluorometer. The peak heights, plotted against the initial target plasmid amount, are shown in Figure 6B. There is a direct relationship between signal and target up to about 1 ng of target plasmid. Above that level, the plateau effect of PCR is evident. A PCR product, generated with primers 1401–1402*, was quantified by using high-performance liquid chromatography with a weak anion-exchange column. This product was used to calculate the detection limit of the scanning method in gels which is about 3–5 ng of PCR products. This detection limit is similar to that achieved by HPLC and about 5–10-fold better in comparison to ethidium bromide staining with visual inspection.

Sets of PCR products were quantified by HPLC and by the gel scanning method. The signals observed, in arbitrary units, correlated well with each other (Figure 7).

We also made efforts to extract BCPDA-labeled PCR products from gels and quantify them in microtiter wells. For these experiments, we used two different strategies: (a) After cutting the gel containing the PCR band, we dissolved the gel with enzyme digestion using the Gelease technique. (b) We used the Geneclean kit. These methods exhibited high backround signals and lower signal to backround ratios in comparison to the direct gel scanning technique and for this reason were not examined further.

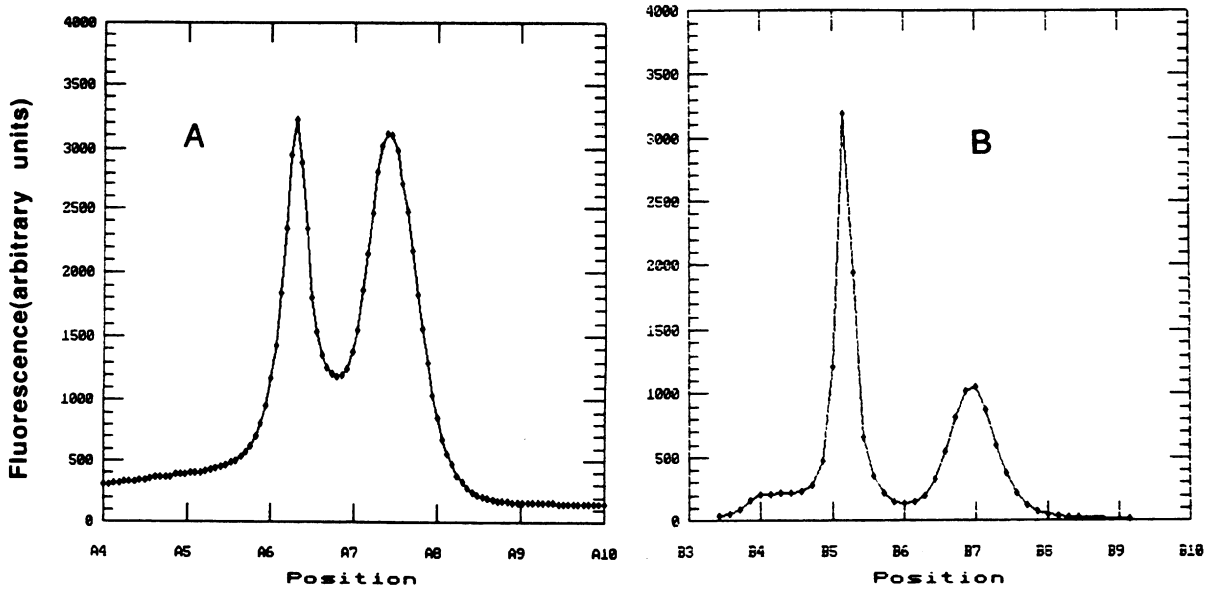

Figure 4. Time-resolved fluorometric scan of a 2% gel containing BCPDA-labeled PCR product (first peak) and BCPDA-labeled unreacted primers (second peak). The gel was stained with Eu^{3+}. (A) Regular agarose gel. (B) Low melting point agarose gel. The relative position of each peak is printed by reference to microtiter well row (A and B) and well number.

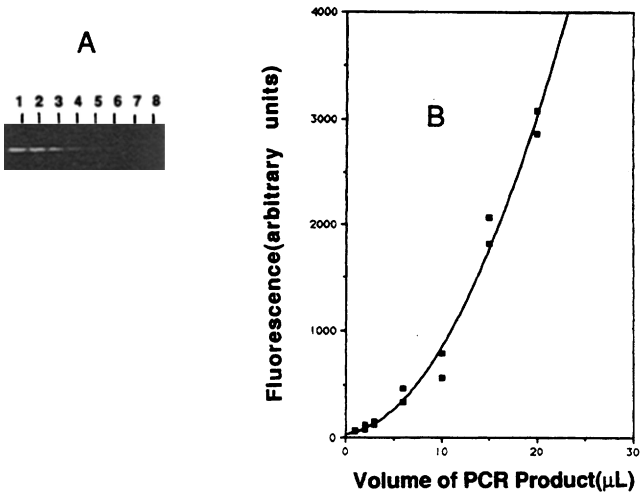

Figure 5. (A) Ethidium bromide-stained 2% agarose gel containing varying amounts of a PCR product generated with primers 1401–1402*. Volume loaded was 20, 15, 10, 6, 3, 2, 1, and 0 μL in lanes 1–8, respectively. The same gel was stained with Eu^{3+} and scanned with the time-resolved fluorometer. The peak height was plotted as a function of volume loaded as shown in B. Each volume was loaded in duplicate.

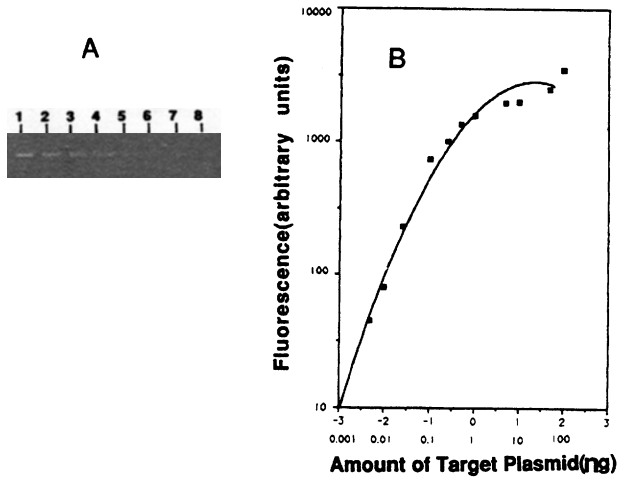

Figure 6. (A) Ethidium bromide-stained 2% agarose gel containing PCR products generated with primers 1401–1402*. The amount of target plasmid DNA used (ng) was 100 (lane 1), 50 (lane 2), 10 (lane 3), 5 (lane 4), 1 (lane 5), 0.5 (lane 6), 0.25 (lane 7), 0.1 (lane 8). Lower amounts down to 5 pg were also used but are not shown. (B) The gel was stained with Eu^{3+} and scanned with the time-resolved fluorometer. The fluorescence of each lane (peak height, singleton measurements) was plotted against the target plasmid amount used, in a double logarithmic plot. The plateau effect is evident at target concentrations above 1 ng.

We have also examined the possibility of using the Southern blot technique to transfer the BCPDA-labeled PCR products to nylon membranes and subsequently stained them with Eu^{3+}, by immersing the membranes in Eu^{3+} solutions. The membranes could then be scanned with the time-resolved fluorometer.

Some data are shown in Figure 8. Higher fluorescence readings are observed with the membrane scanning methods but backround readings were also higher in comparison to direct gel scanning (1500 vs less than 30 arbitrary fluorescence units, respectively). Moreover, saturation effects appear relatively quickly presumably due to membrane saturation at higher levels of DNA blotted. Comparatively, gel staining and scanning is the preferred method because the detection limits and dynamic range are better and the method is less time-consuming.

We have used the BCPDA-labeled PCR products immobilized on nylon membranes to devise another new method for their detection, using polyclonal antibodies against BCPDA. These antibodies were raised in rabbits using a BCPDA–thyroglobulin conjugate as immunogen. The immobilized PCR product was reacted with the BCPDA antibodies. Subsequently, a goat anti-rabbit secondary antibody, labeled with alkaline phosphatase was added. The alkaline phosphatase activity was then detected with the BCIP/NBT substrate. Obviously, other substrates for alkaline phosphatase could also be used. Some results are shown in Figure 9. Lane 2 contains a PCR product generated with unlabeled 1401 and 1402 primers. No peak is detected with the proposed method. Lanes 3, 4, 5, 6, and 7 contain PCR products labeled with BCPDA through primer 1402*. With ethidium bromide staining the PCR product is seen up to lane 6. With the immunological procedure, which utilizes anti-BCPDA antibodies, the PCR product in lane 7 is easily detectable. The detection limit of the immunological procedure is about 100 pg of specific PCR product, which is superior to the method of direct gel scanning and HPLC.

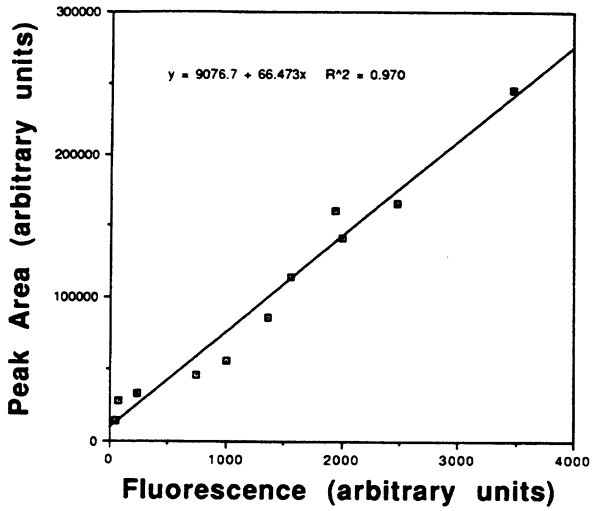

Figure 7. Correlation of signals obtained with high-performance liquid chromatography (*y*) and fluorometric scanning in agarose gels (*x*). PCR products were analyzed by both methods at varying dilutions. For more details see text.

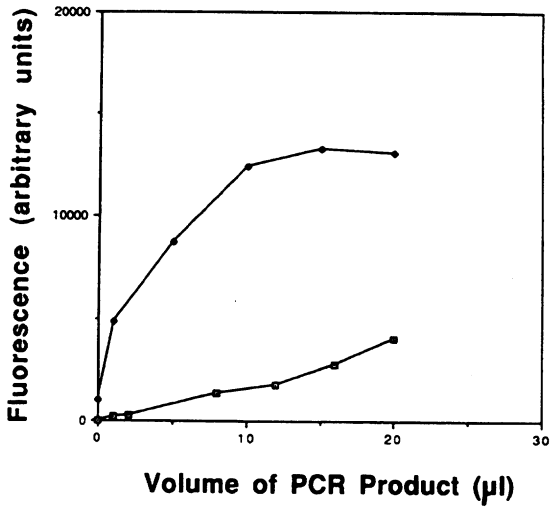

Figure 8. Fluorometric scanning of PCR products generated with primers 1401–1402*. The product was loaded in gels at varying amounts. The agarose gel was stained with Eu^{3+} and scanned (⊡). The same gel was Southern blotted on nylon membranes. The transferred PCR products were then stained with Eu^{3+} and scanned (◆). All measurements were in singleton.

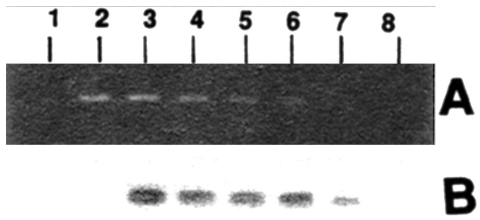

Figure 9. (A) Ethidium bromide-stained gel containing a PCR product generated with unlabeled primers 1401–1402 (lane 2) or with 1401–1402* (BCPDA-labeled 1402 primer) (lanes 3–7). The PCR product was loaded at varying amounts as follows. Lane 1: molecular weight markers, now shown. Lane 2: 20 µL. Lane 3: 20 µL. Lane 4: 15 µL. Lane 5: 10 µL. Lane 6: 5 µL. Lane 7: 1 µL. In lane 8 no target was added (negative control). The gel in A was blotted to nylon by the Southern method and stained with use of anti-BCPDA antibodies. (B) No signal was obtained in lane 2 as expected. The band in lane 7 is not visible with ethidium bromide staining but is easily detectable with the proposed method.

Although this method is qualitative, it does give sharp bands and exhibits very low nonspecific staining (Figure 9).

We here propose a new technology for the direct quantification of nucleic acids in agarose gels. The method is based on the use of a europium chelate, BCPDA–Eu^{3+}, which is fluorescent and can be quantified with microsecond scanning time-resolved fluorometry. In the proposed method, BCPDA is covalently bound to nucleic acids, i.e. at the 5′-end of a PCR primer.

The fluorescent complex can be formed by the diffusion of Eu^{3+} ions into the gel from an aqueous solution. In this case, neither BCPDA nor Eu^{3+} are fluorescent by themselves. Thus, the backround signal observed due to the reagents used is minimal. Additionally, any other backround signal due to scattering or short-lived fluorescence from other chemicals present is eliminated by using the time-resolved fluorometric principle.[14] This technology could find applications other than PCR. For example, 5′-end fluoresceinated primers are used for automated DNA sequencing.[21] BCPDA-labeled primers could also be similarly used. We are currently investigating the possibility of synthesizing BCPDA-labeled nucleotides for general molecular biology applications. These reagents could be detected in gels or after blotting to membranes. In addition to using the time-resolved fluorometric scanner described here, a time-resolved camera recently constructed, (available by Kronem Systems, Toronto, Canada) could also be applicable in obtaining gel or filter pictures.

The development of a BCPDA antibody that recognizes BCPDA on blots and in microtiter well assays makes BCPDA an attractive hapten similar to digoxigenin for DNA applications.[17] In this regard, BCPDA could be used as one of two haptens in systems employing solution hybridization strategies. In such systems, one hapten is used to link the hybrid to a solid phase and another one to link the hybrid to a detection system. These possibilities are currently under investigation.

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