Quantitative Capillary Electrophoretic Analysis of PCR Products Using Laser-Induced Fluorescence Detection

Kathi J. Ulfelder
Beckman Instruments, Inc.

Abstract
A more sensitive approach to compete with slab gel electrophoresis and autoradiography for low-level detection and quantitation of PCR*-amplified DNA fragments is demonstrated using capillary electrophoresis (CE) with laser-induced fluorescence (LIF) detection. In the first system, addition of a fluorogenic intercalator to the buffer was used to identify and quantify the PCR products. In a modification of this technique, fluorescein-labeled primers were used to create fluorescently-labeled PCR products. The DNA was analyzed by CE using coated capillaries and a replaceable polyacrylamide buffer. CE detection was on-line LIF using the 488 nm line of an argon-ion laser as the excitation source. Quantitation of PCR product was possible through the generation of a standard curve showing linearity between the amount of DNA template present prior to amplification and the peak area of resulting PCR product. The enhanced resolution and sensitivity observed with CE, together with the ease of quantitation, make it a powerful alternative to slab gels for the separation and quantitation of PCR products.

Introduction
While excelling at amplification of genomic material, the polymerase chain reaction (PCR) suffers from difficulties in quantitation of the reaction products. One reason for this is the method currently used for separation and detection of PCR products: slab gel electrophoresis in conjunction with autoradiography. In addition, the method cannot be easily automated. Recently, capillary electrophoresis (CE) has been used successfully for the separation of DNA restriction fragments and PCR products with high reproducibility and efficiency.(1-3) With the introduction of laser-induced fluorescence (LIF), the sensitivity of CE has increased a thousand-fold,(4,5) which is required if one wishes to compete with autoradiography for low-level detection. Given these advances in CE methodology, CE was used as the method for separation and quantitation of PCR products.

In previous studies,(6,7) RNA has been reverse transcribed and PCR amplified, with the resulting DNA products analyzed by CE-LIF. As a first step towards quantitation of the PCR products in unknown samples, it was possible to generate a standard curve demonstrating a linear relationship between amount of template and corrected peak area.
Identification of the products based on size was possible through the use of co-injected DNA size standards.

In the present study, two methods of CE fluorescence detection and quantitation are compared. Bacteriophage lambda DNA was used in a PCR, producing a 500 base-pair (bp) fragment. The reaction product was generated using one primer of the set fluor-labeled, thus producing a fluorescent PCR product. The DNA was analyzed by CE using LIF and compared to UV detection. In addition, unlabelled PCR product was also analyzed by CE-LIF, using a fluorescent intercalator in the buffer system. A curve demonstrating linearity between amount of template DNA and corrected peak area could be generated for both LIF methods.

**Materials and Methods**

**Template**
Whole bacteriophage lambda DNA was obtained from the GeneAmp PCR Reagent Kit (Perkin-Elmer, Norwalk, CT) at a concentration of 1 µg/mL. Used as the template, this DNA was titrated from 1000 pg to 0 pg.

**Primers**
Both primers were synthesized on an Oligo 1000 DNA Synthesizer from Beckman at the 30 nmol scale of synthesis. The *FAST* Synthesis method with final detritylation and the Ultra*FAST* Cleavage and Deprotection chemistries were used. The primers were dried in a SpeedVac (Savant, New York, NY), resuspended in deionized, sterile water, and used without further purification. Final concentration in the PCR was 0.5 µM (50 pmol per 100 µL reaction volume).

One primer (#1) was labeled at the 5’ end with fluorescein. This primer was prepared in a single step by using fluorescein-labeled CED-phosphoramidite (Glen Research, Sterling, VA) in the last coupling step.

**Primer #1:**
5’- FL - GAT GAG TTC GTG TCC GTA CAA CTG GG - 3’

**Primer #2:**
5’- GGT TAT CGA AAT CAG CCA CAG CGC CC - 3’

**PCR amplification**
The PCR amplification reaction was performed using 0.2 mL microcentrifuge tubes in a 50 µL reaction mixture with no oil overlay on a PE-9600 thermocycler (Perkin-Elmer). Taq polymerase and dNTPs were obtained from Perkin-Elmer and used at the concentrations recommended in the GeneAmp PCR Reagent Kit.

The buffer was modified as follows:
**Buffer**
(Final Composition): 50 mM Tris-HCl, pH 9.0
20 mM (NH₄)₂SO₄
2 mM MgCl₂
0.00% Tween-20

The temperature profile was also modified:
**Initial Denaturation:** 94°C for 2 minutes
**Cycling:**
94°C for 45 seconds
50°C for 30 seconds
72°C for 1 minute
(25 cycles)
**Final Extension:** 72°C for 5 minutes

**Capillary Electrophoresis**
All CE analyses were conducted on either a P/ACE™ 2200 or a P/ACE 5010 from Beckman. Separations were carried out in the reversed polarity mode (anode at detector side). UV detection was at 260 nm; LIF detection used an argon-ion laser source (Beckman) with excitation at 488 nm and emission at 520 nm (for fluorescein) or 530 nm (for Enhance intercalator). Data was collected and analyzed using Gold™ software, version 8.1, from Beckman with the Molecular Weight option.

Analysis of primers was accomplished using the eCAP™ ssDNA 100 Kit: a linear polyacrylamide-filled capillary (100 µm i.d., 37 cm), in a Tris-borate, 7 M urea buffer. Run temperature was set at 30°C. Oligonucleotides were injected electrokinetically for 3–5 s at 7 kV following sample resuspension. Separations were carried out at 300 V/cm.

Analysis of PCR products was conducted using the eCAP dsDNA 1000 Kit: a coated capillary (100 µm i.d., 47 cm), filled with Tris-borate-EDTA containing replaceable linear polyacrylamide. To visualize unlabeled PCR products, the fluorescent intercalator Enhance was added to the buffer system at 0.4 µg/mL. Run temperature was set at 20°C. PCR products were injected hydrodynamically for 10 s at 0.5 psi, directly following amplification and without further sample preparation. This results in a 7.8 nL injection of sample into the capillary. In some analyses, the PCR sample and DNA size markers—200 and 1000 bp fragments combined, 1 µg/mL each in water (GenSura, Del Mar, CA)—were injected sequentially and allowed to co-migrate in the capillary. Separations were carried out at 200 V/cm.
Results and Discussion

For most applications, synthetic oligonucleotides do not require purification. However, since quantitation of labeled PCR products will depend greatly on the degree of primer labeling, purity assessment of primer #1 was performed. CE was carried out under identical conditions using either UV or LIF detection. Figure 1 shows a comparison of the two detection methods. At first inspection by UV (Figure 1A), there appear to be many failure sequences, especially one resembling a large N-1-mer. By LIF, it appears that most of the failure sequences do not fluoresce and therefore are not labeled (Figure 1B); however, there is still present the large N-1-mer. In fact, this fragment is not a failure sequence but rather the N-mer labeled with a diastereomer of the fluorescein phosphoramidite. A positional isomer of fluorescein in the phosphoramidite may also account for additional fluorescent peaks.

In Figure 2, a 500 bp product was generated with 500 pg lambda template DNA and using the fluorescein labeled primer, but was detected by UV for comparison to LIF methods. The electrophrogram showed many peaks, including dNTPs, primers, and primer–dimer.

In an electrophrogram, the area under the peak for a particular DNA fragment can be correlated to the quantity of that fragment. Correction of peak area becomes critical when one realizes that the peak area of a DNA fragment is related to its residence time in the detector. Slower migrating (large) fragments will remain in the detector window longer than a faster migrating (small) fragment, generating a larger area that is not representative of the true quantity of DNA passing through the detector. The Gold algorithm that corrects for area compensates for this anomaly by normalizing analyte velocity. This corrects for differences in fragment mobility.

**Figure 1.** CE separation of labeled primer.

**Figure 2.** CE-UV separation of the PCR product generated from 500 pg of template lambda DNA.
The corrected peak area for a DNA fragment can be compared to a standard concentration curve to determine absolute amount. Alternatively, a ratio of the unknown’s peak area relative to that of an internal standard can be determined (e.g., for competitive PCR). To develop this standard curve for quantitation of product in a PCR reaction with an unknown amount of template, a serial dilution of lambda DNA template was performed. In this reaction, only unlabeled primers were used, with LIF detection through the use of a fluorogenic intercalator. The replaceable buffer system has been formulated to contain a “dye,” EnhanCE, which specifically interacts with DNA (and RNA). This molecule acts as a mono-intercalator, inserting itself between every two base pairs of DNA. Intercalation changes the molecular length, conformation, and charge on the DNA molecules, resulting in a change in electrophoretic behavior. Moreover, this DNA-dye complex will fluoresce at 530 nm when excited by the 488 nm line of an argon-ion laser, whereas the dye alone (as well as non-DNA sample components) will not. Figure 3 shows CE-LIF analysis of the PCR products using decreasing amounts of template. The limit of detection was determined to be 10 pg template or $\approx 190,000$ copies. When the results obtained were compared to UV detection, LIF with intercalator produced a $>100$-fold increase in sensitivity. In addition, the PCR mixture contained components such as dNTPs and Taq polymerase, which are detected by UV methods and can complicate pattern interpretation. These peaks are not detected by LIF with intercalator since only the complex of fluorogenic intercalator + oligonucleotide will fluoresce.

An estimation of relative molecular size is possible by comparing an unknown sample’s mobility to a standard size curve (log of base pair number vs. migration time) generated from the mobility of fragments from a marker diluted in water. However, since DNA fragments from restriction digests and the PCR typically are contained in a high salt matrix, their mobility will vary depending on sample salt concentration. Therefore, as with slab gel analysis, size determination compared to a standard in water is not accurate. To correct this, a double injection technique is employed, whereby an unknown and standard are injected sequentially and allowed to co-migrate in the capillary. Gold software corrects for any migration time variations from run-to-run by use of reference peaks. Corrected migration times for the co-injected marker fragments generate the molecular size curve, which is then used to determine the relative size of the unknown DNA fragment. Using the co-injected 200 and 1000 bp marker fragments as reference, the lambda PCR product was determined to be 496 bp. Alternatively, an internal standard may be included in the sample (such as PCR co-amplification of a second target sequence, or addition of a known quantity of DNA to each sample).

Peak area for the 500 bp lambda product was corrected for transit time through the detector by Gold software and plotted as a function of the

![Figure 3. CE-LIF separation of unlabeled lambda PCR product using EnhanCE intercalator.](image-url)
amount of DNA used in the PCR. A linear relationship is observed from 10 to 1000 pg DNA template. PCR with >1000 pg template was not studied (Figure 4).

In Figure 5, the same labeled PCR products as in Figure 2 were analyzed by CE-LIF, in this case detecting only the fluor from the PCR product. With increasing amounts of DNA template, peak height and area of the product also increased up to a point, whereupon the “plateau effect”—the leveling off of the rate of PCR amplification—occurs. The limit of detection was determined to be 1 pg template or ≈19,000 copies. Note the increase in primer and primer–dimer peaks as template availability decreases. Unfortunately, PCR product size could not be determined without the use of similarly labeled DNA size standards.

Peak area for the labeled 500 bp lambda product was again corrected for transit time through the detector and plotted as a function of the amount of

![Figure 4. Quantitation of unlabeled lambda PCR product with EnhanCE intercalator. Plot of corrected peak area vs. DNA template amount.](image)

![Figure 5. CE-LIF separation of labeled lambda PCR product.](image)
DNA used in the PCR (Figure 6). A linear relationship is observed up to 250 pg DNA template. Inclusion of data points with DNA template >250 pg demonstrates PCR plateauing. When compared to CE-LIF with the use of intercalators in the buffer, CE-LIF of fluor-labeled PCR products shows >10-fold lower detection and quantitation limits. Although it seems unusual that a single fluor molecule would display greater signal that a molecule intercalated with many fluors, there are three possible explanations for this better sensitivity.

First, fluorescence intensity is directly proportional to the product of a fluor’s molar extinction coefficient ($\varepsilon$) for absorbance and its quantum yield ($\Phi$) for fluorescence (see reference 9 for a review of fluorescence). Under the conditions used for PCR product separation and detection, both $\varepsilon$ and $\Phi$ are greater for a single fluorescein molecule than for the Enhance + DNA complex.\textsuperscript{10}

Second, although Enhance intercalator has a high quantum yield when complexed to DNA, it still exhibits some low background fluorescence. At high DNA concentrations (>1 µg/mL), this background appears negligible; however, at the DNA levels in the present investigation (producing a signal <1 relative fluorescent unit full scale), background fluorescence becomes significant for low-level quantitation. While it is possible to reduce the dye concentration in the buffer in order to decrease background, this will affect the signal linearity for the highest DNA concentrations (>100 µg/mL) due to insufficient amount of intercalator.

Third, the presence of excess dye in the buffer may cause self-quenching—the reduction in fluorescence intensity caused by interactions between individual fluorophores. Again, although signal reduction is insignificant at high DNA concentrations, it cannot be neglected for the DNA levels in this study. Removal of the dye from the buffer and prestaining of samples with a higher affinity intercalator such as TOTO-1 or YOYO-1\textsuperscript{10} requires that the sample concentration be known \emph{a priori} since the correct DNA bp:dye ratio is critical for signal linearity. For samples of unknown DNA concentration, this is not a viable option. Because of this reduction in signal-to-noise ratio, sensitivity may be compromised for low-level DNA, especially when compared to single fluor molecule detection without the use of intercalators.

\textbf{Summary}

Using this separation buffer system, together with CE-LIF, no sample preparation is required for CE-LIF analysis. Marker standards may be used in migration time correction for confirmation of peak identity and size determination. CE-LIF of PCR products with the use of intercalators in the buffer shows >100 times lower detection and quantitation limits than CE with UV detection and no added dye. Furthermore, CE-LIF of fluor-labeled PCR products gives an additional ten-fold greater sensitivity. This may be due to background fluorescence or quenching of the dye in the buffer. However, linearity of the standard curve for both CE-LIF detection methods suggests its feasibility for automated PCR quantitation.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure6.png}
\caption{Quantitation of labeled lambda PCR product. Plot of corrected peak area vs. DNA template amount.}
\end{figure}
Acknowledgment
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References

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