ANALYTICAL BIOCHEMISTRY **231,** 359–365 (1995)

# Analysis of Double-Stranded DNA by Capillary Electrophoresis with Laser-Induced Fluorescence Detection Using the Monomeric Dye SYBR Green I

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Received April 10, 1995

**The monomeric fluorescent dye, SYBR Green I, was investigated and compared with the dyes YO-PRO-1 and thiazole orange (TO) for their application in capillary electrophoresis (CE) with laser-induced fluorescence (LIF) detection of double-stranded DNA (dsDNA). DNA fragments were injected by hydrodynamic pressure and separated in a replaceable matrix of hydroxypropyl methylcellulose. For all 3 dyes, optimal concentrations were established and efficient separations of DNA fragments ranging in size from 75 to 12216 bp were obtained. The most promising results in terms of linear detection range were achieved with SYBR Green I. At the optimal dye concentration, fluorescence intensity versus DNA concentration was linear over more than three orders of magnitude (4 pg/$\mu$l to 30 ng/$\mu$l). Limit of detection (LOD) with SYBR Green I was approximately 80 fg of dsDNA (240 zmol of a 200-bp fragment). Similar LOD was obtained with YO-PRO-1, whereas TO resulted in lower sensitivity. Precision in both fluorescence intensity and migration time was high (relative standard deviation, RSD $< 3.6\%$; $n = 10$) for dsDNA fragments complexed with SYBR Green I. In conclusion, SYBR Green I is a fluorescent dye well suited for efficient separation and quantitative, sensitive, and precise determination of dsDNA by CE–LIF.**



Capillary electrophoresis (CE)[2] represents a significant improvement in electrophoretic analysis of nucleic acids in terms of speed, resolution, quantitation, reproducibility, and automation compared with conventional gel electrophoresis systems (1,2). Highly efficient separations of single- and double-stranded DNA ranging in size from a few nucleotides to millions of base pairs have been obtained by CE (3,4).

Due to the minute sample volumes injected in typical CE analyses (nanoliter range), the practical application of CE is highly dependent on sensitive detection systems. Detection by uv absorbance is commonly used in CE analyses of DNA, but confers limited sensitivity. Extremely high sensitivity can be obtained by laser-induced fluorescence (LIF) detection (5). This approach requires fluorescent DNA derivatives obtained by either covalent binding of fluorophores or physical absorption of fluorogenic dyes.

Characteristics of an ideal fluorescent dye for CE–LIF analysis of DNA have been summarized by Schwartz and Ulfelder (6) as (1) excitation maximum of the DNA–dye complex close to a available laser wavelength, (2) low intrinsic fluorescence of dye not complexed with DNA, (3) large fluorescence enhancement upon binding of dye to DNA, and (4) high fluorescence quantum yield of the DNA–dye complex. Additional important characteristics for quantitation of DNA in unknown samples are (5) uniform and not sequence specific binding, (6) constant fluorescence intensity and electropherographic profile over a wide range of DNA–dye ratios, and (7) large linear detection range.

A series of new fluorescent dyes with improved physical and spectroscopic properties have recently been developed (7–10). The dyes can be classified as monomeric and dimeric. The dimeric dyes have several favorable features, including high affinity for and large fluorescence enhancement upon binding dsDNA (10). However, efficient separations are only obtained over

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[2] Abbreviations used: CE, capillary electrophoresis; LIF, laser-induced fluorescence; YO-PRO-1, 1-(4-[3-methyl-2,3-dihydro-(benzo-1,3-oxazole)-2-methylidene]-quinolinium)-3-trimethylammonium propane diodide; TO, thiazole orange; LOD, limit of detection; DMSO, dimethyl sulfoxide; HPMC, hydroxypropyl methylcellulose.

0003-2697/95 $12.00

a narrow range of DNA–dye ratios, and broad and distorted peaks attributed to multiple complex formation modes are obtained if not proper measures are taken (11,12).

Among the monomeric dyes, the intercalator, ethidium bromide, has been found to improve peak shape and resolution in CE of dsDNA (13). Recently, the monomeric dyes 1-(4-[3-methyl-2,3-dihydro-(benzo-1,3-oxazole) - 2 - methylidene] - quinolinium) - 3 - trimethylammonium propane diodide (YO-PRO-1) and thiazole orange (TO; 4-[3-methyl-2,3-dihydro-(benzo-1,3-thiazole)-2-methylidene]-quinolinium iodide) were evaluated (6,14). TO intercalates dsDNA at a ratio of one dye molecule per two base pairs of DNA (8). High sensitivity detection and high resolution of dsDNA fragments have been obtained with both fluorescent dyes (6,14).

SYBR Green I is the trade name of a new ultrasensitive monomeric dye developed for the detection of dsDNA in agarose and polyacrylamide gels. SYBR Green I has several attractive properties, including excitation maximum close to the 488 nm line of the argon ion laser, extremely low intrinsic fluorescence, high fluorescence quantum yield of the DNA–dye complex, and 100-fold higher affinity for dsDNA than ethidium bromide (7).

In the present work we demonstrate for the first time the applicability of SYBR Green I for separation and quantitation of dsDNA by CE–LIF. Important features of SYBR Green I are compared with those of the cyanine dyes YO-PRO-1 and TO. The most prominent advantages of SYBR Green I are large linear detection range of dsDNA and high resolution of small dsDNA fragments.

## MATERIALS AND METHODS

### *Chemicals*

SYBR Green I (in dimethyl sulfoxide (DMSO); concentration not given) and YO-PRO-1 (1 mM in DMSO) were purchased from Molecular Probes Inc. (Eugene, OR). TO was a gift of Molecular Probes, and is at present not commercially available. TO was dissolved in methanol (Rathburn Chemicals Ltd., Walkerburn, Scotland) to a final concentration of 2 mM.

Working stock solutions of SYBR Green I (1:100 dilution of stock), YO-PRO-1 (10 $\mu$M) and TO (10 $\mu$M) in 20% DMSO (E. Merck, Darmstadt, Germany) were prepared daily, and stored in the dark at room temperature.

Phenylmethylpolysiloxane-coated fused silica capillaries (DB-17, 100 $\mu$m i.d., 0.1 $\mu$m film thickness) were purchased from J & W Scientific (Folsom, CA). Wizard PCR Preps DNA Purification System was purchased from Promega Corp. (Madison, WI). Acrodisc PVDF filters (0.45 $\mu$m pore size) were from Gelman Sciences Inc. (Ann Arbor, MI) and MF-Millipore membrane filters (0.025 $\mu$m pore size, 10 mm diameter) were from Millipore Corp. (Bedford, MA). Hydroxypropyl methylcellulose (HPMC, 4000 cP, 2% aqueous solution, 25°C) and other chemicals (molecular biology grade) were obtained from Sigma Chemical Co. (St. Louis, MO). Solutions containing HPMC were prepared as described elsewhere (15). Reagents and reaction tubes (Thin-Walled GeneAmp) for PCR were purchased from Perkin Elmer (Norwalk, CT). Water, double distilled and purified on a Milli-Q Plus Water Purification System (Millipore), was used for preparation of all aqueous solutions.

### *Capillary Electrophoresis Instrumentation*

Capillary electrophoresis was performed on a Beckman P/ACE System 2100 equipped with P/ACE LIF detector (Beckman Instruments, Palo Alto, CA). Laser excitation was at 488 nm (argon ion laser, 3 mW, Beckman Instruments). The emitted light was collected by passage through 488 nm blocking and 520 nm bandpass filters (bandwidth 10 nm) (Beckman Instruments).

Total capillary length was 27 cm (20 cm effective length). Capillary electrophoresis was performed in the reversed polarity mode with capillary temperature set at 18°C.

Beckman System Gold software (version 7.12) was used for system control, data collection, and processing. Electropherograms for preparation of figures were transferred as DIF files to an Apple Macintosh computer and redrawn by Sigma Plot (version 4.16; Jandel Scientific GmbH, Erkrath, Germany).

### *DNA Molecular Size Standards*

The 1 kb DNA Ladder (1.04 $\mu$g/$\mu$l) and DNA Mass Ladder (117.5 ng/$\mu$l) were from Life Technologies Inc. (Gaithersburg, MD). The 1 kb DNA Ladder contains 21 DNA fragments ranging in size from 75 to 12216 bp, whereas the DNA Mass Ladder is an equimolar composition of 100-, 200-, 400-, 800-, 1200-, and 2000-bp DNA fragments. The DNA standards were diluted to appropriate concentrations in purified water and stored at 4°C.

### *Polymerase Chain Reaction and Purification of the Product*

A 210-bp segment originating from N-terminus of the human chromosomal *mut* gene (16) was amplified using the following protocol: A 100-$\mu$l reaction volume containing 100 pg of the MCM26 plasmid (16), 100 pmol of each primer, 200 $\mu$M dNTPs, 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin and 2 U AmpliTaq DNA Polymerase (Perkin Elmer) was subjected to 36 temperature cycles on a DNA Thermal Cycler 480 (Perkin Elmer). PCR ther-

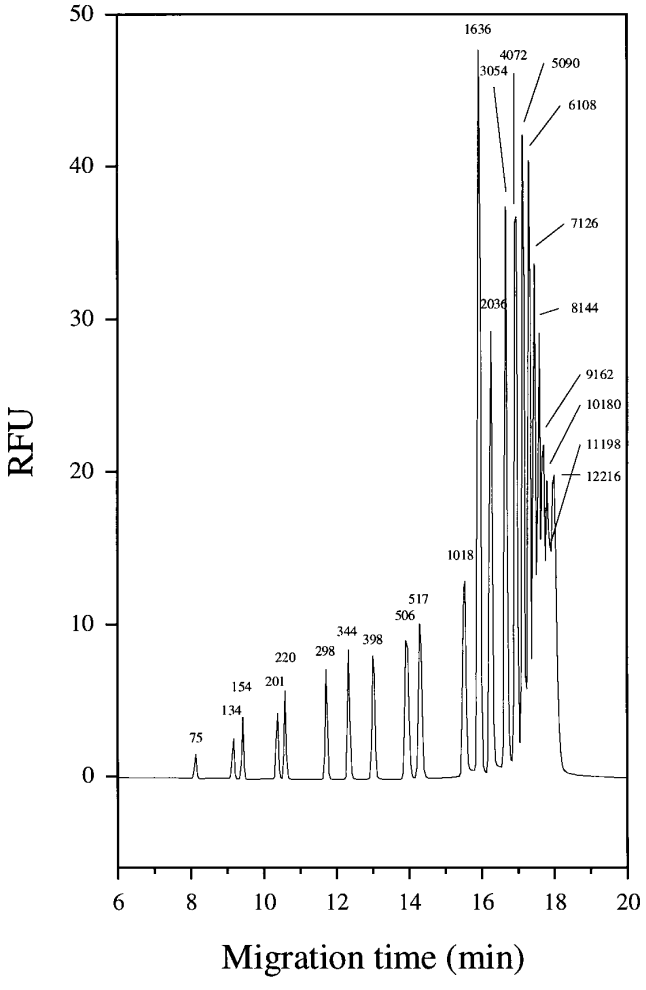


**FIG. 1.** Electropherogram of the 1 kb DNA Ladder complexed with SYBR Green I. The concentration of SYBR Green I in electrophoresis and separation buffers corresponds to a 1:30,000 dilution of stock solution. Total DNA concentration, 10 ng/$\mu$l (corresponding to 200 pg of DNA injected). Other experimental conditions as described under Materials and Methods. RFU, relative fluorescence units.

mocycling program: 1 min at 94°C (initial denaturation), followed by 36 temperature cycles of 1 min at 94°C (denaturation), 1 min at 45°C (primer annealing), and 1 min at 72°C (primer extension). Final primer extension was 7 minutes at 72°C.

If not otherwise stated, PCR samples were purified by Wizard PCR Preps DNA Purification System (Promega) and dialyzed on a MF-Millipore membrane filter (Millipore) for 20 min (17) prior to analysis by capillary electrophoresis. For determination of linear detection ranges, the PCR product was initially quantitated by uv spectrophotometry and then diluted in purified water to appropriate concentration.

*Capillary Electrophoresis*

Electrophoresis buffer was 89 mM Tris–borate, 2 mM EDTA, pH 8.3 (1× TBE). Separation buffer was electrophoresis buffer containing 0.5% (w/v) HPMC. All buffers were passed through Acrodisc PVDF filters and degassed by sonication for 10 min prior to CE. A significant adsorption of dye to filter matrix was observed if dye was added before filtration. Fluorescent dye was added to both electrophoresis and separation buffers.

The capillary was filled with separation buffer by high-pressure injection at 137 kPa (20 psi) for 30 s, followed by a short immersion in purified water (to prevent carryover of buffer salts and HPMC). Samples were injected by low pressure at 3.44 kPa (0.5 psi) for 10 s and separation performed at an electric field strength of 180 V/cm. After each electroseparation, the capillary was flushed with 1× TBE for 60 s by high pressure. For direct analysis of PCR reaction mixtures, purified water was injected (low pressure, 10 s) prior to the PCR sample. This procedure has been advocated to prevent matrix effect (18).

In order to obtain quantitative data from the on-line detection system, fluorescence intensity from analytes having different mobilities were normalized by using the product of fluorescence intensity and mobility for each of the analytes. All data presented here were normalized by the System Gold software.

## RESULTS AND DISCUSSION

*Capillary Electrophoresis System*

The aim of this study was to investigate the properties of monomeric SYBR Green I as a fluorescent dye for quantitative analysis of dsDNA by CE–LIF and compare SYBR Green I with the two monomeric dyes, YO-PRO-1 and TO.

Hydrodynamic injection of sample was used since this allowed quantitative introduction of dsDNA fragments of different sizes dissolved in matrices of variable ionic strength (18,19). Using the Poiseuille equation, we estimated that a 10-s low-pressure injection introduced approximately 20 nl of sample into the capillary. Hydrodynamic injection is only possible with replaceable separation matrices. Preliminary experiments demonstrated that a HPMC concentration of 0.5% in the separation buffer and an electric field

**TABLE 1**

Excitation and Emission Maxima of SYBR Green I, YO-PRO-1, and TO Complexed with DNA

| Fluorescent dye | Excitation maximum (nm) | Emission maximum (nm) |
|---|---|---|
| SYBR Green I | 494 | 521 |
| YO-PRO-1 | 491 | 509 |
| TO | 509 | 525 |

strength of 180 V/cm gave acceptable separation of DNA fragments ranging in size from 75 to 12216 bp (Fig. 1), and that resolution in the lower and higher size range could be further improved by increasing (to 1%) or decreasing (to 0.2%) the concentration of HPMC, respectively.

The DNA–dye complexes have excitation maxima (494 nm, SYBR Green I; 491 nm, YO-PRO-1 (9); 509 nm, TO (8)) close to the 488-nm line of the argon ion laser used in these experiments. The emission maxima of these dyes are in the range of 509–525 nm (Table 1). The emitted light was directed through a bandpass filter (520 ± 5 nm) which collected light with intensity 50–100% of the emission maxima.

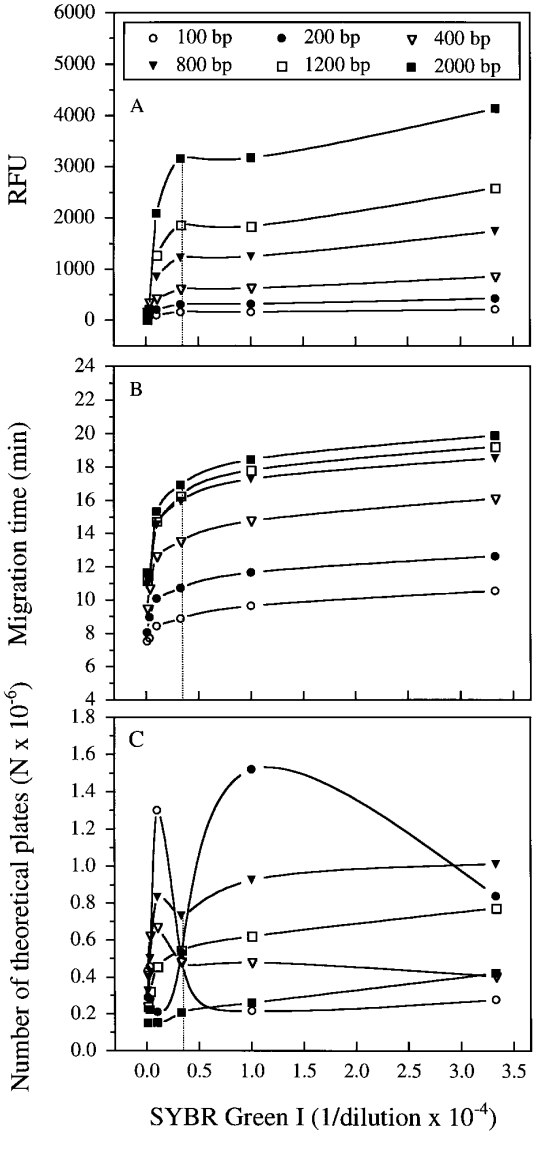


**FIG. 2.** Effect of SYBR Green I concentration on fluorescence intensity, migration time, and plate numbers for dsDNA fragments. The DNA Mass Ladder at a total DNA concentration of 10 ng/$\mu$l was subjected to CE–LIF in the presence of increasing concentrations of SYBR Green I in electrophoresis and separation buffers. Other experimental conditions were as described in the legend to Fig. 1. SYBR Green I concentration corresponding to a 1:30,000 dilution of stock solution is indicated by a vertical dotted line. The fluorescence intensity was normalized for the analytes. RFU, relative fluorescence units.

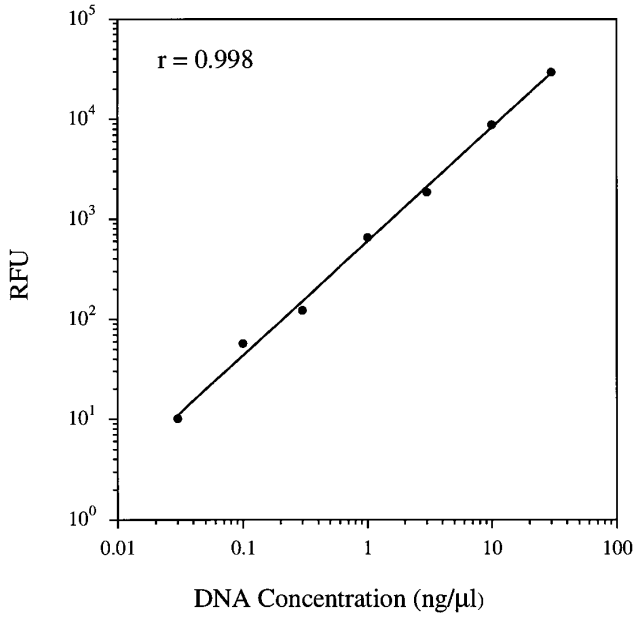


**FIG. 3.** Linear detection range. A 210-bp PCR amplified segment of the *mut* gene was subjected to CE in the presence of SYBR Green I concentration corresponding to a 1:30,000 dilution of stock solution. The concentration of DNA varied between 0.025 and 25 ng/$\mu$l, as indicated on the abscissa. Other experimental conditions were as described in the legend to Fig. 1. RFU, relative fluorescence units.

### *Optimization of Dye Concentration*

Absorption of monointercalators induces structural and electrostatic changes in dsDNA (20), resulting in altered migration dynamics when the complexes are subjected to electrophoresis. Separation characteristics depend on the stoichiometric relations between DNA base pairs and intercalating dye molecules (13). Consequently, an optimal DNA–dye ratio in terms of peak resolution, $R_S$, and plate number, $N$ will exist.

We investigated the effect of increasing fluorescent dye concentrations on fluorescence intensity, migration time, and separation characteristics in CE–LIF of DNA molecular weight standards.

The fluorescence intensity and migration time for DNA fragments ranging from 100 to 2000 bp increased with SYBR Green I concentration up to a dilution of 1:100,000–1:30,000. Higher dye concentration caused only small further increase in these parameters (Figs. 2A and 2B). Shift in migration times was positively correlated to fragment size (Fig. 2B).

**TABLE 2**

Limit of Detection and Linear Detection Range of DNA Analysis with SYBR Green I, YO-PRO-1, and TO

| Fluorescent dye | Limit of detection (fg)[a] | Linear detection range (ng/$\mu$l) |
|---|---|---|
| SYBR Green I | 80 | 0.004–30 |
| YO-PRO-1 | 80 | 0.004–12 |
| TO | 850 | 0.042–12 |

[a] Determined for a 200-bp DNA fragment of the DNA Mass Ladder.

The plate numbers increased for all fragments up to a SYBR Green I dilution of 1:100,000. At dilutions between 1:100,000 and 1:3000, the plate numbers for the different DNA fragments showed a complex relation to dye concentration, as depicted in Fig. 2C. We routinely used SYBR Green I at a dilution of 1:30,000 which, in addition to high fluorescence intensity, resulted in the highest resolution ($R_S$) of DNA fragments up to 1000 bp ($R_S$ = 1.5 for the 506/517 bp fragments). Under these optimized conditions, we obtained baseline separation of the 134/154, 201/220, and 506/517 bp doublets (Fig. 1).

The fluorescence intensities and migration times for DNA fragments ranging from 100 to 2000 bp increased with YO-PRO-1 and TO concentrations up to 300 nM (for both dyes) and then leveled off (data not shown). The increment in migration times, attributed to combined mass and charge effects due to complex formation, was most pronounced for the higher molecular weight fragments.

The dye concentration 300 nM was also found to be optimal with respect to plate numbers for YO-PRO-1 and TO (data not shown). Increasing the dye concentrations beyond the optimal level caused the loss of system efficiency. At TO concentrations higher than 1 $\mu$M, there was a marked deterioration of peak shape and a significant reduction in plate numbers for DNA fragments smaller 400 bp (data not shown). This observation is essentially in agreement with published data (6).

At low concentrations of SYBR Green I (diluted >1:100,000) and YO-PRO-1 (<100 nM), we observed a selective loss of fluorescence from DNA fragments larger than 400 bp and a significant reduction in their migration times (data not shown). This phenomenon has previously been described for YO-PRO-1, and is attributed to depletion of dye by complex formation with the faster migrating fragments (14). Almost quantitative extraction of dye from the separation buffer at low concentrations of SYBR Green I and YO-PRO-1 probably reflects their high affinity for dsDNA. This phenomenon was not observed with TO which have lower affinity for dsDNA.

### *Linear Detection Range*

Optimal concentrations of SYBR Green I (dilution 1:30,000), YO-PRO-1 (300 nM) and TO (300 nM) were established at a total DNA concentration of 10 ng/$\mu$l. We further investigated the fluorescence intensity over a large concentration range of dsDNA (0.1 ng to 100 ng/$\mu$l) at optimal concentrations of the fluorescent dyes. The DNA Mass Ladder and a 210-bp PCR amplified

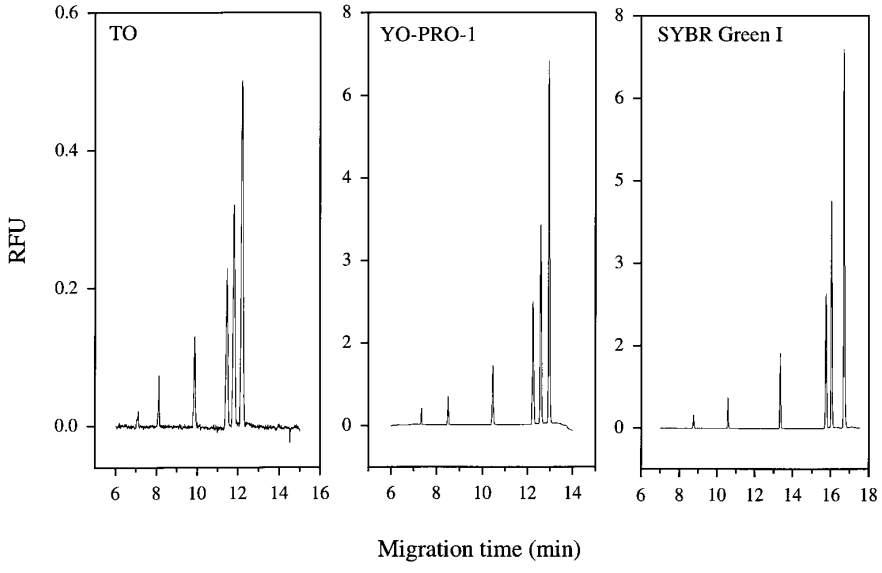


**FIG. 4.** Electropherografic profiles obtained with TO, YO-PRO-1, and SYBR Green I. The DNA Mass Ladder at a total concentration of 1 ng/$\mu$l was subjected to CE in the presence of optimal concentrations of TO (300 nM), YO-PRO-1 (300 nM), or SYBR Green I (a 1:30,000 dilution of stock solution). Other experimental conditions were as described in the legend to Fig. 1. RFU, relative fluorescence units.

**TABLE 3**

Precision of DNA Analysis with SYBR Green I, YO-PRO-1, and TO

| Fluorescent dye | DNA fragment size (bp) | RSD, migration time (%)[a] | RSD, fluorescence intensity (%)[a] |
|---|---|---|---|
| SYBR Green I | 200 | 0.46 | 3.3 |
| | 800 | 0.48 | 3.2 |
| | 2000 | 0.49 | 3.6 |
| YO-PRO-1 | 200 | 0.95 | 5.6 |
| | 800 | 1.14 | 8.4 |
| | 2000 | 1.15 | 8.6 |
| TO | 200 | 0.46 | 9.9 |
| | 800 | 0.63 | 11 |
| | 2000 | 0.62 | 12 |

[a] Data were obtained from 10 repetitive injections of a single sample ($n$ = 10).

DNA segment of the *mut* gene were used for these experiments.

The fluorescence intensity of DNA fragments complexed with SYBR Green I increased linearly as a function of DNA concentration over three orders of magnitude. Figure 3 illustrates the large linear range (25 pg/$\mu$l to 25 ng/$\mu$l) for detection of the 210-bp PCR product. Notably, the linear ranges obtained with YO-PRO-1 and TO were smaller (Table 2). The migration times and separation characteristics were essentially constant within these DNA concentration ranges (data not shown).

The linearity in fluorescence intensity versus DNA concentration observed for the monomeric dyes investigated here is explained by a constant amount of dye per fragment over a wide range of DNA–dye ratios. This contrasts to the variable intensity obtained when using constant concentration of dimeric dyes at different DNA–dye ratios (11).

In conventional gel electrophoresis, the fluorescence intensity from dsDNA fragments complexed with intercalating dyes of weak base specificity has been found to be linearly related to the fragment size. We made analogous observations with SYBR Green I and YO-PRO-1. For these dyes, the fluorescence intensity per base pair was independent of DNA fragment size, indicating uniform binding to dsDNA. In contrast, using TO as intercalator, the fluorescence intensity per base pair was highest for the smallest fragments and decreased with fragment size. This phenomenon was observed with both DNA molecular size standards analyzed and over a wide range of DNA concentrations (0.1 to 100 ng/$\mu$l) (data not shown).

The relation between DNA fragment size and fluorescence intensity can have practical implications for the construction of quantitative methods for dsDNA based on CE–LIF. The fluorescence intensity per base pair is not dependent on the size of dsDNA fragments complexed with SYBR Green I, thus providing greater freedom in selection of internal standard (in terms of molecular weight).

*Performance of DNA Analysis*

Limits of detection (signal to noise ratio of 3:1, 3$\sigma$) of a 200-bp DNA fragment were approximately 80 fg

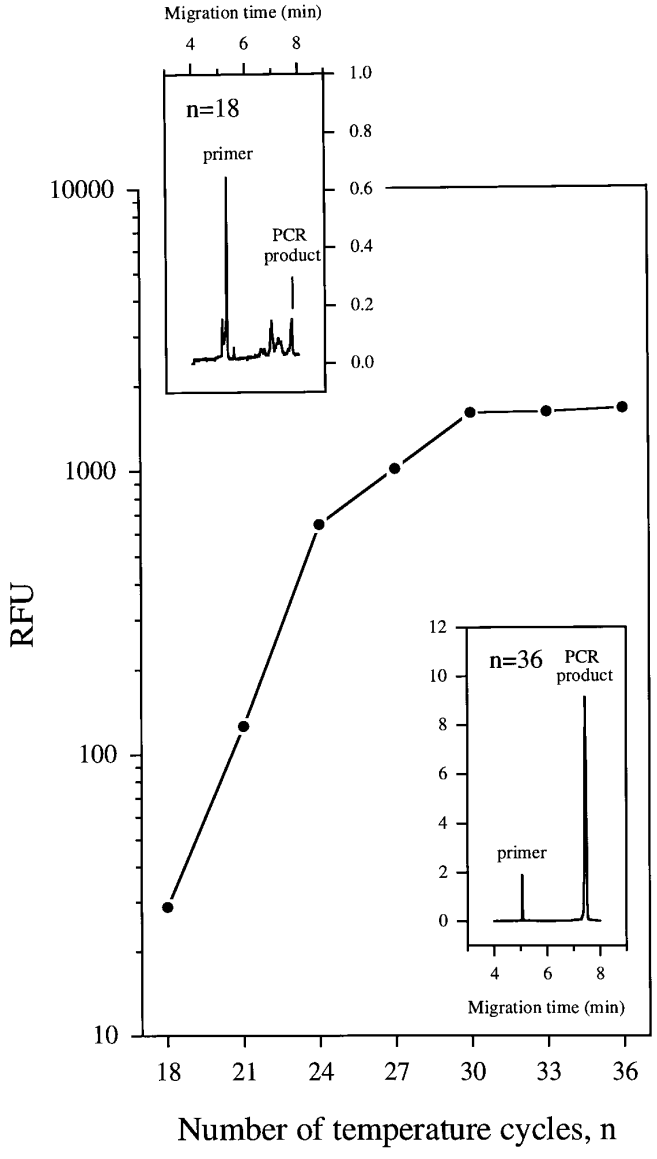


**FIG. 5.** Monitoring PCR amplification. A 210-bp segment of the *mut* gene was amplified by PCR as described under Materials and Methods. Samples from parallel PCR reactions were injected by hydrodynamic pressure (10 s at 3.44 kPa) without prior purification. CE was performed with SYBR Green I (a 1:30,000 dilution of stock solution) in the electrophoresis and separation buffers. The main panel illustrates the progress curve for the PCR reaction, whereas the insets illustrate electropherograms of reaction mixtures after 18 and 36 temperature cycles, respectively. RFU, relative fluorescence units.

with SYBR Green I and YO-PRO-1 and approximately 850 fg with TO (Table 2). The higher sensitivity obtained with SYBR Green I and YO-PRO-1 is also demonstrated by comparing electropherograms of the DNA Mass Ladder (total DNA concentration 1 ng/$\mu$l) (Fig. 4).

Construction of the LIF detection system is an obvious determinant of sensitivity. We used a commercial detector which does not fully exploit the potential of LIF detection (21). Previous studies have shown that the cyanine fluorophores TO and the YO-PRO-1 analog, YO, give 10-fold higher sensitivity than ethidium and its derivatives, propidium-2 and propidium-3 (11). Thus, SYBR Green I seems to compare favorably with established intercalating dyes previously used in CE–LIF (11,12).

Precision of fluorescence intensity and migration time was determined by repetitive ($n$ = 10) hydrodynamic injections of the DNA Mass Ladder. The relative standard deviation was between 3.3 and 12% for fluorescence intensity and 0.46 and 1.2% for migration time (Table 3).

### *Monitoring PCR Amplification of the mut Gene*

We monitored PCR amplification of a 210-bp segment of the human *mut* gene through 36 temperature cycles by analyzing the reaction products by CE–LIF. Samples were analyzed without prior purification and detected by complex formation with SYBR Green I. The product, detected after 18 cycles, was efficiently separated from unreacted primers and interfering material (Fig. 5). The results demonstrated rapid, quantitative, and precise determination of PCR products detected by the use of SYBR Green I.

## CONCLUSION

Excitation maximum of the dsDNA–SYBR Green I complex is close to the 488-nm line of the argon ion laser. SYBR Green I has low intrinsic fluorescence and large fluorescence enhancement upon binding to dsDNA. At optimal concentrations (dilution 1:30,000) of SYBR Green I, efficient separation and sensitive detection of dsDNA fragments are obtained by CE–LIF over a wide range of DNA–dye ratios. YO-PRO-1 and TO also serve as useful dyes for dsDNA analysis by CE–LIF. Compared with these dyes, the most prominent advantages of SYBR Green I are the high resolution of DNA fragments 100–1000 bp in size and a large linear detection range. The latter feature is a prerequisite for quantitative measurement of DNA samples.

## ACKNOWLEDGMENTS

This work was supported by grants from the Norwegian Cancer Association. The technical assistance of Gry Kvalheim is highly appreciated. We also thank Dr. Singer at Molecular Probes for useful discussions.

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