

New energy transfer dyes for DNA sequencing

L. G. Lee*, S. L. Spurgeon, C. R. Heiner, S. C. Benson, B. B. Rosenblum, S. M. Menchen, R. J. Graham, A. Constantinescu, K. G. Upadhy and J. M. Cassel

PE Applied Biosystems, 850 Lincoln Centre Drive, Foster City, CA 94404, USA

Received March 27, 1997; Revised and Accepted May 22, 1997

ABSTRACT

We have synthesized a set of four energy transfer dyes and demonstrated their use in automated DNA sequencing. The donor dyes are the 5- or 6-carboxy isomers of 4'-aminomethylfluorescein and the acceptor dyes are a novel set of four 4,7-dichloro-substituted rhodamine dyes which have narrower emission spectra than the standard, unsubstituted rhodamines. A rigid amino acid linker, 4-aminomethylbenzoic acid, was used to separate the dyes. The brightness of each dye in an automated sequencing instrument equipped with a dual line argon ion laser (488 and 514 nm excitation) was 2–2.5 times greater than the standard dye-primers with a 2 times reduction in multicomponent noise. The overall improvement in signal-to-noise was 4- to 5-fold. The utility of the new dye set was demonstrated by sequencing of a BAC DNA with an 80 kb insert. Measurement of the extinction coefficients and the relative quantum yields of the dichlororhodamine components of the energy transfer dyes showed their values were reduced by 20–25% compared with the dichlororhodamine dyes alone.

INTRODUCTION

Four-color automated DNA sequencing instruments have traditionally utilized single dyes as fluorescent labels (1–3). Recently, several groups have described the use of oligonucleotide backbones as linkers in energy transfer dyes which exhibit brighter fluorescence and longer Stokes' shifts than the individual dye components (4–8). These labeled oligonucleotides can be used in automated sequencing as dye-labeled primers and provide brighter signal than the set of four acceptor dyes alone. However, the 10mer oligonucleotide backbone that separates the donor-acceptor pair prohibits the use of these energy transfer compounds as labels for small molecules such as deoxy- and dideoxynucleotides.

In 1988 we examined a compound named 'bifluor-1', a dye dimer consisting of 5-carboxytetramethylrhodamine linked to 4'-aminomethyl-5-carboxyfluorescein (Fig. 1). Bifluor-1 was a gift from Richard Haugland of Molecular Probes Inc. (Eugene, OR). The absorbance spectrum of bifluor-1 appeared to be the additive absorbance spectra of both donor and acceptor dyes and its emission spectrum appeared to be only of the longer wavelength

acceptor dye, tetramethylrhodamine. The fluorescence of the fluorescein component, the donor dye, was completely quenched. It appeared that bifluor-1 was an energy transfer dye which could prove useful in DNA sequencing. However, bifluor-1 was no brighter than tetramethylrhodamine alone. Our goal was to improve the brightness and spectral properties of the bifluor type of energy transfer dye.

MATERIALS AND METHODS

Instrumental methods

Absorbance measurements were performed on a Perkin-Elmer Lambda-Bio UV-Vis spectrophotometer. Fluorescence measurements were performed on a Perkin-Elmer LS-50B fluorescence spectrophotometer. High pressure liquid chromatography (HPLC) was performed with a Waters 660E pump equipped with a 996 photodiode array detector. Separations utilized an Applied Biosystems Aquapore RP-300 C-8 column.

DNA sequencing reactions were performed using a Perkin-Elmer GeneAmp PCR system 9600 thermal cycler and analyzed using an ABI PRISM 377 DNA sequencer. The standard dye-primer set used 10 nm virtual filters at 536, 559, 586 and 615 nm. The d-rhodamine and fluorescein-d-rhodamine sets used 10 nm filters centered at 540, 570, 595 and 625 nm.

Preparation of dR110

A mixture of 3-dimethylaminophenol (0.25 g, 2.3 mmol), 3,6-dichlorotrimellitic anhydride (0.33 g, 1.3 mmol) and sulfuric acid (1 ml) were combined and heated to 190°C for 12 h. Water (10 ml) was added to the reaction and the black solid separated by filtration. The solid was extracted with acetonitrile (10 ml). The orange solution was concentrated to dryness and the residue dissolved in 250 mM carbonate/bicarbonate buffer, pH 9 (10 ml). The solution was acidified with concentrated HCl and the red precipitate collected by centrifugation and dried in a vacuum centrifuge. A red solid was obtained (32 mg, 5% yield).

Preparation of dR6G, dTMR and dROX

The three dyes, dR6G, dTMR and dROX, were each prepared by condensation of substituted 3-aminophenols with 3,6-dichlorotrimellitic anhydride in polyphosphoric acid. A representative preparation of dR6G is described below.

*To whom correspondence should be addressed. Tel: +1 415 638 5990; Fax: +1 415 572 2743; Email: leelg@perkin-elmer.com

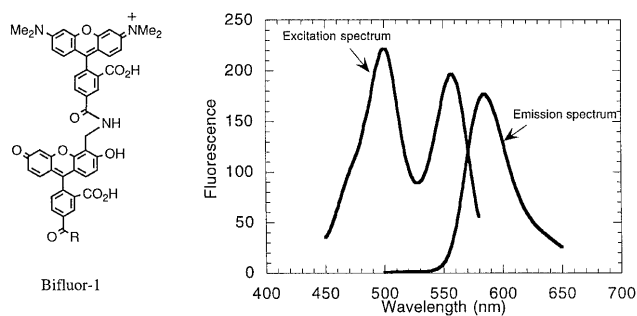


Figure 1. Bifluor-1: structure and absorbance and fluorescence emission spectra. The dye solution was diluted to an $OD_{480\text{ nm}}$ of 0.05 in 40% acetonitrile, 0.1 M TEAA buffer.

A mixture of 3-aminomethyl-*p*-cresol (100 mg, 0.66 mmol), 3,6-dichlorotrimellitic anhydride (100 mg, 0.38 mmol) and polyphosphoric acid (0.5 g) were combined and heated to 180°C for 12 h. Water (10 ml) was added to the red-brown reaction and the mixture filtered. The solid was taken up in 1 M carbonate/bicarbonate buffer, pH 9 (25 ml), and filtered. The filtrate was acidified with concentrated HCl and the solid collected by centrifugation and dried. The solid was extracted with acetonitrile (10 ml) and the extracts were concentrated to a sticky solid. The solid was dissolved in dimethylformamide (0.6 ml). The yield was estimated by diluting an aliquot into 40% acetonitrile, 0.1 M triethylammonium acetate (TEAA) buffer and measuring the absorbance in a UV/visible spectrophotometer. Using an extinction coefficient of 50 000/cm/M at the absorbance maximum of 540 nm the yield was estimated to be 9%.

Identities of 5- and 6-d-rhodamine isomers were inferred from HPLC retention times. All ^1H NMR-confirmed 6-carboxy isomers of xanthene dyes, e.g. 6-carboxyfluorescein and 6-carboxyrhodamines, elute faster than the 5-carboxy isomers on reverse phase HPLC. The isomers of 5- and 6-carboxy-4,7-dichloroxanthene dyes could not be assigned based on ^1H NMR spectral data since there is no splitting of the lone 5- or 6-hydrogen. We assumed the 5- and 6-carboxy-4,7-dichloroxanthene dyes exhibited the same relative HPLC elution order as the 5- and 6-carboxyxanthene dyes.

Preparation of succinimidyl esters of 5- or 6-carboxy-d-rhodamines

Activation of 5- and 6-carboxy-d-rhodamines to the succinimidyl esters was performed on the crude mixtures using *N*-hydroxysuccinimide and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride. A representative preparation of dR6G-NHS is described below.

A solution of dR6G in dimethylformamide (6 μmol in 0.1 ml) was combined with *N*-hydroxysuccinimide (22 mg, 0.2 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (12 mg, 0.06 mmol). Reaction progress was monitored by thin layer chromatography (TLC) on silica gel using a mixture of dichloromethane, methanol and acetic acid (600:60:16) as eluant. After the starting material was consumed, the reaction was diluted with dichloromethane (10 ml) and washed with 5% HCl (10 ml). The material which was insoluble in both phases was discarded. The organic phase was washed successively with 250 mM carbonate/bicarbonate (10 ml) and with 5% HCl (10 ml). The

solution was dried (MgSO_4) and concentrated to a dark oil. The residue was taken up in dimethylformamide (0.2 ml) and stored at -20°C .

After 3 days at -20°C gold crystals had formed in the dimethylformamide solution. The supernatant was decanted and discarded. The residue was dissolved in methylsulfoxide (0.1 ml). An aliquot was diluted into 40% acetonitrile, 0.1 M TEAA. Using an extinction coefficient of 50 000/cm/M at the absorbance maximum of 548 nm the yield was estimated to be 7%.

Preparation of fluorescein-rhodamine dimers

Preparation of fluorescein-rhodamine dimers with either the standard rhodamines or the d-rhodamines were performed by reaction of the rhodamine succinimidyl esters (dye-NHS) with 4'-aminomethyl-5-carboxyfluorescein. Preparation of the fluorescein-rhodamine dimers with the linker compound, 4-aminomethylbenzoic acid (B), required reaction of the rhodamine succinimidyl ester with 4-aminomethylbenzoic acid followed by activation of the derivatized rhodamine. Reaction with 4'-aminomethyl-5-carboxyfluorescein followed by another activation reaction provided the succinimidyl ester of the fluorescein-B-rhodamine dimer. A representative synthesis of 5CFB-5TMR and its activation is described below.

Synthesis of B-5TMR

A mixture of 4-aminomethylbenzoic acid (3 mg, 19 μmol), 5TMR-NHS (5 mg, 9 μmol) and triethylamine (20 μl) was suspended in dimethylformamide (DMF, 200 μl) in a 1.5 ml Eppendorf tube. The mixture was heated to 60°C for 10 min. Reaction progress was monitored by TLC on silica gel with elution with a 400/30/10 mixture of dichloromethane, methanol and acetic acid. The insoluble 4-aminomethylbenzoic acid was separated by centrifugation and the DMF solution was decanted into 5% HCl (1 ml). The insoluble B-5TMR was separated by centrifugation, washed with 5% HCl (2×1 ml) and dried in a vacuum centrifuge. The product was dissolved in DMF (200 μl) and used to prepare B-5TMR-NHS.

Synthesis of B-5TMR-NHS

A solution of B-5TMR in DMF (125 μl), diisopropylethylamine (10 μl) and disuccinimidylcarbonate (10 mg) was combined in a 1.5 ml Eppendorf tube and heated to 60°C. Reaction progress was monitored by TLC on silica gel with elution with a 600/60/16 mixture of dichloromethane, methanol and acetic acid. After 5 min the reaction appeared to be complete. The solution was diluted into methylene chloride (3 ml) and washed with 250 mM carbonate/bicarbonate buffer, pH 9 (4×1 ml), dried (Na_2SO_4) and concentrated to dryness in a vacuum centrifuge. The solid was dissolved in DMF (100 μl). The yield was determined by diluting an aliquot into pH 9 buffer and measuring the absorbance at 552 nm. Using an extinction coefficient of 50 000/cm/M, the concentration of 5TMR-B-NHS was 4.8 mM. Yield from 5TMR-NHS was 8%.

Synthesis of 5CFB-5TMR

A solution of B-5TMR-NHS (1 μmol in 250 μl DMF) was combined with a solution of 4'-aminomethyl-5-carboxyfluorescein (5CF, 2.2 μmol in 100 μl DMSO) and triethylamine (20 μl) in a 1.5 ml Eppendorf tube. The reaction was monitored by HPLC

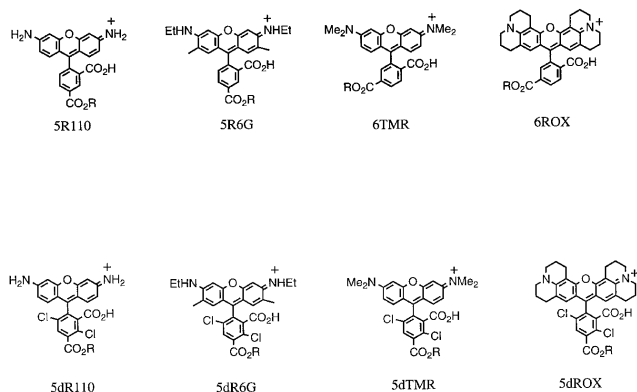


Figure 2. Structures of the standard rhodamine dye set (top) and the d-rhodamine dye set (bottom).

using a C8 reverse phase column with an elution gradient of 15–35% acetonitrile versus 0.1 M TEAA. HPLC analysis indicated that 5TMR-B-NHS was consumed, leaving the excess, unreacted 5CF. The reaction was diluted with 5% HCl (1 ml) and the product separated by centrifugation, leaving the unreacted 5CF in the aqueous phase. The solid was washed with 5% HCl (4×1 ml), dried in a vacuum centrifuge and taken up in DMF (300 μ l). The yield was quantitative.

Synthesis of 5CFB-5TMR-NHS

A solution of 5CFB-5TMR (0.6 μ mol in 100 μ l DMF), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (DEC, 2 mg) and *N*-hydroxysuccinimide (4 mg) were combined in a 1.5 ml Eppendorf tube. The mixture was sonicated briefly and heated to 60°C. The reaction was monitored by TLC on silica gel with elution with a 600/60/16 mixture of dichloromethane, methanol and acetic acid. The reaction was complete in 30 min and diluted with 5% HCl. The product was separated by centrifugation and dried in a vacuum centrifuge. The activated dye was dissolved in DMF (20 μ l).

Preparation of dye-labeled oligonucleotides

Both d-rhodamine labeled primers and fluorescein–d-rhodamine labeled primers were synthesized by reaction of succinimidyl esters of the dyes with 5'-aminoethyl-derivatized universal primer (–21 M13). A representative preparation of dR6G-labeled primer is described below.

A solution of 5'-aminoethyl-functionalized oligonucleotide (10 μ l, 1 mM) and dR6G-NHS (10 μ l, 12 mM in methylsulfoxide) and carbonate/bicarbonate buffer (2 μ l, 1 M) were combined. After 10 min at room temperature the solution was subjected to gel filtration on Sephadex G-25 to separate free dye. The fraction containing dye-labeled oligonucleotide and unlabeled oligonucleotide was collected and subjected to HPLC purification on a reverse phase column. The unlabeled oligonucleotide and each dye isomer of dye-labeled oligonucleotide were separated using an elution gradient of 10–30% acetonitrile versus 0.1 M TEAA. The solutions containing dye-labeled oligonucleotide were concentrated in a vacuum centrifuge and redissolved in TE buffer.

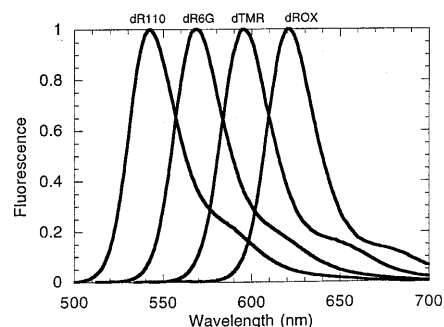
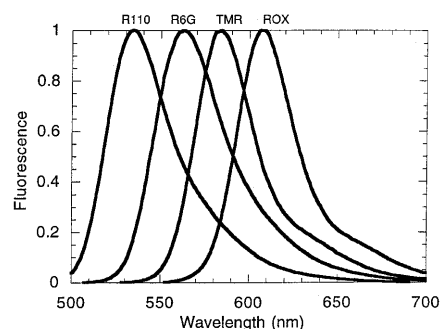


Figure 3. Normalized fluorescence emission spectra of oligonucleotides labeled with the standard rhodamine dye set (top) and the d-rhodamine dye set (bottom). Spectra were measured in 8 M urea, 1 \times Tris–borate EDTA, pH 8 (1 \times TBE).

DNA sequencing

Dye-primer cycle sequencing was performed using AmpliTaq DNA polymerase, FS, according to the *ABI PRISM 377 DNA Sequencer User's Manual* (revision B, January 1995, Chapter 2; Perkin-Elmer Corp., Foster City, CA).

RESULTS AND DISCUSSION

Donor dyes

A necessary feature of the energy transfer fluorescein–rhodamine dye is the bifunctionality of the donor dye. One functional group is used to attach the acceptor and the other functional group is used to attach the oligonucleotide or nucleotide. Fluorescein and its derivatives are ideal as bifunctional donor dyes because their structure is readily modified both before and after construction of the chromophore. The 4'-aminomethyl group of bifluor-1 is added to the fluorescein dye component by a Mannich reaction of hydroxymethylacetamide and 5'-carboxyfluorescein (9,10). The resulting acetamide is hydrolyzed by HCl to provide the amine.

Acceptor dyes

The original bifluor-1 dye used tetramethylrhodamine (TMR) as the acceptor dye in the energy transfer system. TMR is a member of a set of four spectrally resolved rhodamine dyes which are useful in automated DNA sequencing, particularly as part of a set of dye-labeled dideoxynucleotides. We will refer to this set as the

'standard' set of rhodamine dyes, consisting of R110, R6G, TMR and ROX (11). The dyes are attached to the dideoxynucleotide or oligonucleotide by either the 5- or 6-carboxy isomer of each dye. Both isomers provide the same fluorescent emission spectra, but a pure isomer must be used because of differences in electrophoretic mobility between the two isomers. The structures and normalized fluorescence emission spectra of the set of rhodamine dyes attached to oligonucleotides are shown in Figures 2 and 3.

Four-color sequencing relies on the spectral resolution of the set of four dyes. The degree of resolution of the standard rhodamine dyes can be seen in the points of intersection of their normalized emission spectra. A perfectly resolved set of dyes would intersect at an ordinate value of 0 and have zero overlap of emission spectra.

The standard rhodamine dyes have emission spectra that intersect at values of 0.75–0.85. The original dye set for dye-primer sequencing, FAM, JOE, TMR and ROX, includes two of the standard rhodamine dyes, TMR and ROX, and has a similar set of fluorescence emission spectra. Bodipy dyes have intersection values of 0.35–0.6 (12). Most of the useful fluorescent dyes (fluoresceins, rhodamines, bodipies) have Stokes' shifts of 15–25 nm and absorbance spectra which are mirror images of the emission spectra. Therefore, dye sets which are better resolved and have narrow emission spectra also have lower extinction coefficients at excitation wavelengths useful for exciting the shortest wavelength dye of the set. However, in energy transfer systems the absorbance at shorter wavelengths is provided by the donor dye. Energy transfer systems which utilize well-resolved acceptor dyes should provide both bright and well-resolved signals.

Xanthene dyes with narrow fluorescence emission spectra can be obtained by the addition of chlorides to the phenyl group. For example, the emission spectra of 4,7-dichlorofluorescein dyes are 10–15% narrower and have emission maxima at 10–15 nm longer wavelengths than the unsubstituted fluoresceins (13). By adding the 4,7-dichloro- substitution to the rhodamine dyes, we synthesized a set of four dichlororhodamine dyes, which we refer to as d-rhodamines, which have narrower emissions and emission maxima at longer wavelengths than the standard rhodamine set. The structures and normalized fluorescence emission spectra of the d-rhodamine dyes are shown in Figures 2 and 3. The intersection values of the d-rhodamines are 0.65–0.68, an improvement over the standard rhodamine set.

Energy transfer dyes

In an initial attempt to improve the brightness of bifluor-1 we synthesized several fluorescein–tetramethylrhodamine adducts with different linking groups between the fluorescein and rhodamine components. Two non-rigid amino acid linkers, glycine and lysine, produced fluorescein–tetramethylrhodamine dimers with decreased brightness compared with bifluor-1 (data not shown). A rigid linker, 4-aminomethylbenzoic acid, produced energy transfer compounds with increased brightness.

A comparison of the brightness of fluorescein–rhodamine dimers with variable acceptors, acceptor isomers and linkers is shown in Figure 4. Energy transfer fluorescein–rhodamine dyes were constructed using both isomers of 4'-aminomethyl-5 (or 6)-carboxyfluorescein (5CF or 6CF) as the donor dye and 5-carboxy-R6G (5R6G) or 5- or 6-carboxy-dR6G (5dR6G or 6dR6G) as the acceptor dye. The linker, if used, was 4-aminomethylbenzoic acid (B). The fluorescence emission spectra were measured using dye solutions with equivalent absorbances at the acceptor dye

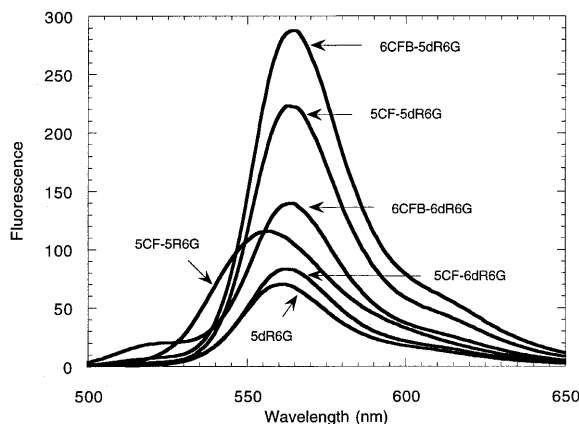


Figure 4. Fluorescence emission spectra of fluorescein–R6G and fluorescein–d-R6G dimers. The donor dyes were 5- or 6-carboxy-4'-aminomethylfluorescein (5CF or 6CF), the acceptor dyes were 5-carboxy-R6G (5R6G) or 5- or 6-carboxy-d-R6G (5dR6G or 6dR6G) and the linker, if used, was 4-aminomethylbenzoic acid (B). Spectra were measured in 40% acetonitrile, 60% 0.1 M TEAA, pH 7. Equimolar solutions were approximated by assuming that the extinction coefficient of the R6G or dR6G component was equivalent among the different dimers and for free 5dR6G.

absorbance maxima. Assuming equivalent losses in extinction coefficients, the relative peak heights can be regarded as the relative molar brightness of the various energy transfer compounds.

No difference in brightness was observed between the isomers of 4'-aminomethyl-5 (or 6)-carboxyfluorescein (data not shown). The 5-carboxy isomers of the d-rhodamines gave energy transfer compounds with a brighter signal than the 6-carboxy isomers. The 4'-aminomethylbenzoic acid linker B improved the brightness of both d-rhodamine isomers. The effect of the dichloro groups could be seen in the narrower and redder emissions of dR6G versus R6G. The d-rhodamine energy transfer compounds showed emission spectra which were virtually indistinguishable from the d-rhodamines alone. The combination of the 5-carboxy-d-rhodamine, the 4'-aminomethylbenzoic acid linker and 4'-aminomethyl-5 (or 6)-carboxyfluorescein appeared to provide an improved, bifluor-type dye.

Dye-labeled primer sequencing

The choice of dye sets affects three parameters in automated dye-primer sequencing: the mobility shift, the multicomponent matrix and the overall signal. The mobility shift refers to the effect of the dye on the relative electrophoretic mobility of oligonucleotide fragments. Attachment of a dye causes a change in the mobility of the fragment compared with unlabeled DNA and the relative change in mobility among the set of four dyes is the basis of the mobility shift correction. The correction can be a constant, or 'parallel', shift or it can be a variable, or 'curved', shift. A parallel mobility shift implies a constant displacement for every fragment length labeled with one dye, relative to the same fragment labeled with another dye. A curved mobility shift applies a greater correction to the shorter fragments than the longer ones. Dye sets composed of dyes of a single chemical class are usually corrected with parallel shifts. A collective mobility shift of less than half a base pair for all four dyes will not require new mobility corrections for small changes in gel composition, temperature or read length.

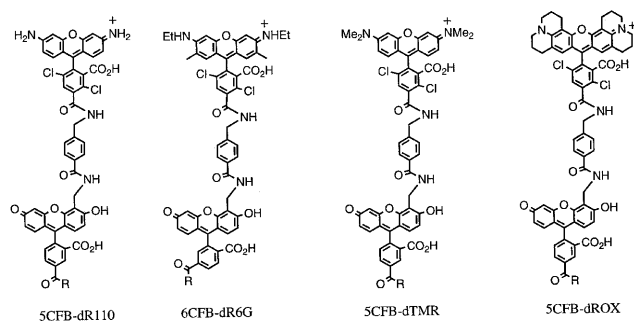


Figure 5. Structures of fluorescein-d-rhodamine dyes for dye-primer sequencing.

The multicomponent matrix refers to the 4×4 matrix which is applied to the fluorescence signal to quantitate the contribution of each dye. The matrix contains the relative contributions of each of the four dyes to each of the four 'bins', or virtual filters, of collected light. Each 'bin' is a 10 nm wide window of light collection by the CCD detector. Since the 'bins' can vary among instruments, the matrix is both machine and dye set dependent. A useful metric to compare matrices is the condition number, which is the ratio of the largest and the smallest eigenvalues of the inverse matrix (14). The condition number can vary between 1 and ∞ , where a value of 1 indicates a perfect matrix, which could be obtained with a dye set with ordinate intersection values of 0. The multicomponent noise, or the artifacts which arise after application of the 4×4 matrix to the raw data, is proportional to the condition number (15).

Finally, the brightness of each dye in the instrument is reflected in the overall signal. The brightness is a product of the extinction coefficient and the quantum yields of each dye at the wavelengths of excitation of the instrument. Dye-labeled primer sequencing is an ideal method to measure the relative brightness of dye sets, because the primer is used in molar excess of the template. In an experiment in which all the parameters are constant except the dye-primers themselves, the fluorescence signal is dependent only on the brightness of the dyes.

We constructed the set of eight d-rhodamine energy transfer dyes using the 4-aminomethylbenzoic acid linker with both the 5- and 6-carboxy isomers of fluorescein. By testing all the combinations in four-color sequencing, we found that the 6-carboxy isomer had a faster mobility than the 5-carboxy isomer and the energy transfer dyes with dR6G had slower mobility than the other d-rhodamines in the set. The combination of four energy transfer d-rhodamine dyes with the least mobility correction is shown in Figure 5. The mobility correction was a constant shift. The largest correction among the dye-labeled primers was 4/10 of a base, within the half base requirement for minimal artifacts.

The fluorescence emission spectra of the four fluorescein-d-rhodamine dyes are shown in Figure 6. The same multicomponent matrix was used for the fluorescein-d-rhodamine dyes and for the d-rhodamine dyes alone. The values of the condition number for the fluorescein-d-rhodamine dyes on three sequencing instruments varied from 2.6 to 2.8. The values of the condition number for the standard dye-primer dyes, FAM, JOE, TMR and ROX, varied from 5.4 to 6.0. The effect of the 2-fold improved condition number was observed as decreased multicomponent noise in the sequencing electropherograms.

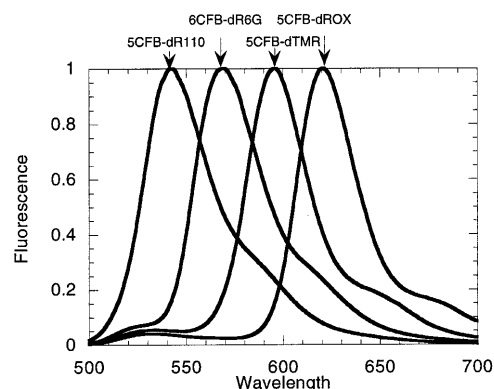


Figure 6. Normalized fluorescence emission of -21 universal primer labeled with the fluorescein-d-rhodamine dye set. Spectra were measured in 8 M urea, $1 \times$ TBE.

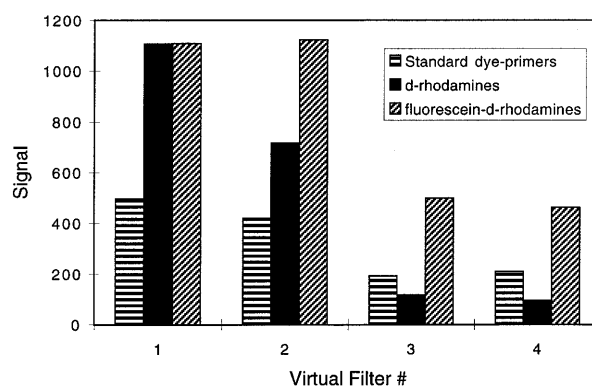


Figure 7. Comparison of signal strength of three dye sets: the standard dye-primer dye set (FAM, JOE, TMR, ROX), the d-rhodamine dye set and the fluorescein-d-rhodamine dye set. Sequencing of M13 was performed on the same instrument, with virtual filters optimized for each dye set. Signals are the average values of between seven and nine runs, with a standard deviation of 7–22%. The signals for filters 3 and 4 were divided by two to account for the double reactions. The matrix for the standard dye set had a condition number of 5.6 and the matrix for the d-rhodamine and fluorescein-d-rhodamine sets had a condition number of 2.9.

Four-color sequencing with dye-labeled primers was carried out with three sets of dye-primers: the standard dye-primer set, the d-rhodamine set and the fluorescein-d-rhodamine set. A comparison of signal numbers for each of the dye sets is shown in Figure 7. The signals for the d-rhodamine dyes and the energy transfer dyes can be compared directly, but the comparison between the energy transfer dyes and the standard dye-primer dyes does not reflect the decreased multicomponent noise of the energy transfer dyes. By multiplying the 2- to 2.5-fold increase in signal by the 2-fold improvement in condition number, the signal-to-noise improvement of the energy transfer d-rhodamine dye set compared with the standard set was determined to be 4- to 5-fold.

The utility of the energy transfer dye-primers is shown in Figure 8. BAC end-sequencing has become a popular method for large scale genomic sequencing. Sequencing the insert ends of BAC clones is useful in their mapping and high quality sequence data can be used to provide primer sequences for new sequence-

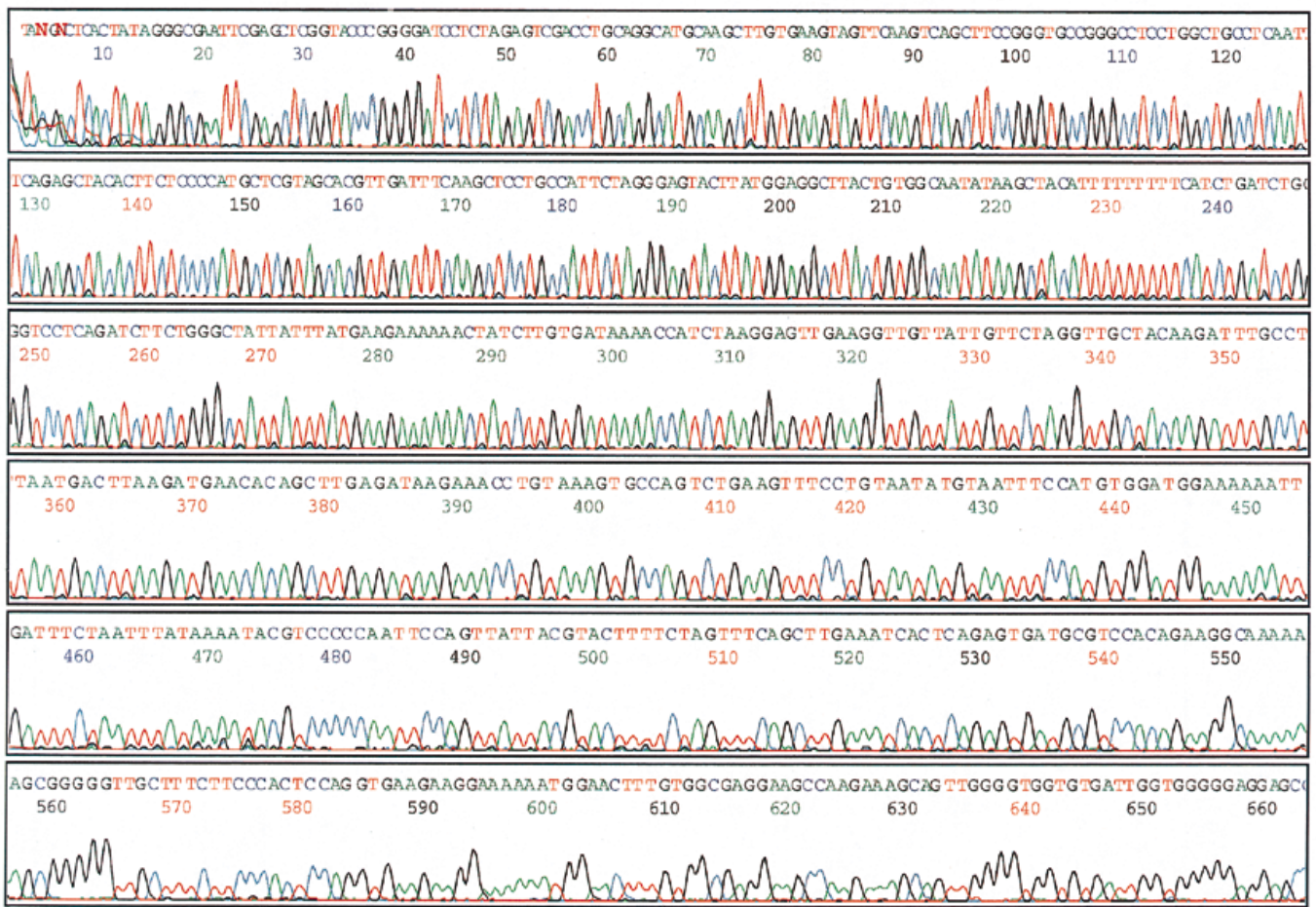


Figure 8. BAC sequencing with the fluorescein–d-rhodamine dye-primers. A BAC containing an 80 kb insert was sequenced using Taq FS and –21 M13 primers labeled with the fluorescein–d-rhodamine energy transfer dyes. For the A, C and T reactions, 0.4 pmol primer was used. For the G reaction, 0.8 pmol primer was used. The BAC was diluted to 40 ng/μl and 2 μl were used for the A, C and T reactions and 3 μl for the G reaction. The pooled reaction was resuspended in 4 μl formamide and half was loaded on the 377 DNA sequencer, a total of ~200 ng BAC DNA. The insert sequence began at 70 bp and the first ambiguous base was called at 765 bp.

tagged sites. BAC clones typically contain inserts of 40–200 kb. Brighter dyes allow successful automated sequencing, despite the low molar amount of template in the sequencing reaction (16).

Relative quantum yields and extinction coefficients

The d-rhodamine and fluorescein–d-rhodamine primers were used to compare the extinction coefficients and quantum yields of the dye components (Table 4). Extinction coefficients of the fluorescein and d-rhodamine components of the energy transfer dyes could be calculated using the nucleic acid absorbance at 260 nm as a measure of the concentration of the primers. The extinction coefficient of the dR110 component of the fluorescein–dR110 energy transfer dye could not be determined because the energy transfer dye gave only one broad absorbance. Ratios of extinction coefficients of the d-rhodamine component of the energy transfer dye to d-rhodamine alone were calculated [$\epsilon(\text{ET})/\epsilon(\text{d-Rhod})$]. Solutions of d-rhodamine and fluorescein–d-rhodamine dye-primers which gave equal absorbances at the d-rhodamine absorbance maxima were excited at the d-rhodamine absorbance maxima and

the fluorescence emission measured to determine their relative quantum yields [$\phi(\text{ET})/\phi(\text{d-Rhod})$].

Each energy transfer dye had both lower extinction coefficients and lower relative quantum yields than the corresponding d-rhodamine dye alone. These results show that although the energy transfer dyes are effectively brighter than the d-rhodamine dyes with 488/514 nm excitation and although the fluorescein component appears to be completely quenched, the energy transfer system is only ~60% of maximal brightness. Additional combinations of orientation and linker composition could produce energy transfer systems with increased brightness.

Our energy transfer system provides enhancements in sequencing data that compares favorably with the brightness, spectral resolution and mobility corrections of energy transfer systems based on oligonucleotide backbones. The use of a compact dye allows labeling of any primer sequence, regardless of the availability of appropriately spaced linker arm thymidines. Perhaps our biggest advantage, however, is the utility of these energy transfer dyes in labeling small molecules. Future papers will describe the use of the d-rhodamine dyes and the fluorescein–d-rhodamine energy transfer dyes on dye-labeled terminators.

Table 4. Extinction coefficients and relative quantum yields of d-rhodamine and corresponding fluorescein–d-rhodamine dyes^a

Dye	λ (nm)	ϵ (per cm/M)	$\epsilon(\text{ET})/\epsilon(\text{d-Rhod})$	$\phi(\text{ET})/\phi(\text{d-Rhod})$
dR110	522	92 000		
5CFB-dR110	504	115 000	–	0.78
dR6G	548	120 000		
6CFB-dR6G	502	80 000		
	548	89 000	0.74	0.82
dTMR	577	120 000		
5CFB-dTMR	499	80 000		
	577	90 000	0.75	0.76
dROX	602	144 000		
5CFB-dROX	501	87 000		
	605	115 000	0.80	0.79

^aDye extinction coefficients were determined in 1× TE buffer, pH 8.0. The d-rhodamines were assumed to contribute an extinction coefficient of 20 000/cm/M at 260 nm. The fluorescein–d-rhodamines were assumed to contribute an extinction coefficient of 25 000/cm/M at 260 nm. These numbers were estimated by measuring the ratio of absorbances in the UV and visible regions of pure solutions of free dye. The extinction coefficients of both d-rhodamine dye-primers and fluorescein–d-rhodamine dye-primers increased by 10% in 8 M urea, 1× TBE. Quantum yield comparisons were determined in 8 M urea, 1× TBE. The quantum yield increase of the fluorescein–d-rhodamine dye-primers in 8 M urea was much greater than the quantum yield increase of the d-rhodamine dye-primers.

ACKNOWLEDGEMENTS

We thank Kip Connell and John Shigeura for help in defining and discussing the use of condition numbers and Muhammad Sharaf and Dan Allison for help in matrix and mobility file corrections. We are grateful to the following scientists for their help in optimizing the syntheses and use of the fluorescein–d-rhodamine dyes: Paul Kenney, Brian Evans, Mary Fong, Pete Theisen, Sandy Koepf, Iqbal Zaidi and David Yu. Finally, we would like to thank Richard Henfrey for his enthusiasm in the early stages of the project.

REFERENCES

- Smith, L.M., Sanders, J.Z., Kaiser, R.J., Hughes, P., Dodd, C., Connell, C.R., Heiner, C., Kent, S.B.H. and Hood, L.E. (1986) *Nature*, **321**, 674–679.
- Connell, C., Fung, S., Heiner, C., Bridgham, J., Chakerian, V., Heron, E., Jones, B., Menchen, S., Mordan, W., Raff, M., *et al.* (1987) *BioTechniques*, **5**, 342–348.
- Prober, J.M., Trainor, G.L., Dam, R.J., Hobbs, F.W., Robertson, C.W., Zagursky, R.J., Cocuzza, A.J., Jensen, M.A. and Baumeister, K. (1987) *Science*, **238**, 336–341.
- Ju, J., Ruan, C., Fuller, C.W., Glazer, A.N. and Mathies, R.A. (1995) *Proc. Natl. Acad. Sci. USA*, **92**, 4347–4351.
- Ju, J., Kheterpal, I., Scherer, J.R., Ruan, C., Fuller, C.W., Glazer, A.N. and Mathies, R.A. (1995) *Anal. Biochem.*, **231**, 131–140.
- Ju, J., Glazer, A.N. and Mathies, R.A. (1996) *Nucleic Acids Res.*, **24**, 1144–1148.
- Hung, S.-C., Ju, J., Mathies, R.A. and Glazer, A.N. (1996) *Anal. Biochem.*, **243**, 15–27.
- Mineno, J., Ishino, Y., Ohminami, T. and Kato, I. (1993) *DNA Sequence J. DNA Sequencing Mapping*, **4**, 135–141.
- Shipchandler, M.T., Fino, J.R., Klein, L.D. and Kirkemo, C.L. (1987) *Anal. Biochem.*, **162**, 89–101.
- Fino, J.R. and Shipchandler, M.T. (1986) European patent application 86105340.3.
- Bergot, B.J., Chakerian, V., Connell, C.R., Eadie, J.S., Fung, S., Hershey, N.D., Lee, L.G., Menchen, S.M. and Woo, S.L. (1994) US patent 5,366,860.
- Metzger, M.L., Lu, J. and Gibbs, R.A. (1996) *Science*, **271**, 1420–1422.
- Menchen, S.M., Lee, L.G., Connell, C.R., Hershey, N.D., Chakerian, A., Woo, S. and Fung, S. (1993) US patent 5,188,934.
- Pettofrezzo, A.J. (1978) *Matrices and Transformations*. Dover, Englewood Cliffs, NJ.
- Kalivas, J.H. (1986) *Anal. Chem.*, **58**, 989–992.
- Marra, M., Weinstock, L.A. and Mardis, E.R. (1996) *Genome Res.*, **6**, 1118–1122.