1144–1148 *Nucleic Acids Research, 1996, Vol. 24, No. 6*

Cassette labeling for facile construction of energy transfer fluorescent primers

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Received October 31, 1995; Revised and Accepted February 1, 1996

ABSTRACT

DNA primer sets, labeled with two fluorescent dyes to exploit fluorescence energy transfer (ET), can be efficiently excited with a single laser line and emit strong fluorescence at distinctive wavelengths. Such ET primers are superior to single fluorophore-labeled primers for DNA sequencing and other multiple color-based analyses [J. Ju, C. Ruan, C. W. Fuller, A. N. Glazer and R. A. Mathies (1995) *Proc. Natl. Acad. Sci. USA* 92, 4347–4351]. We describe here a novel method of constructing fluorescent primers using a universal ET cassette that can be incorporated by conventional synthesis at the 5′-end of an oligonucleotide primer of any sequence. In this cassette, the donor and acceptor fluorophores are separated by a polymer spacer (S_6) formed by six 1′,2′-dideoxyribose phosphate monomers (S). The donor is attached to the 5′ side of the ribose spacer and the acceptor to a modified thymidine attached to the 3′ end of the ribose spacer in the ET cassette. The resulting primers, labeled with 6-carboxyfluorescein as the donor and other fluorescein and rhodamine dyes as acceptors, display well-separated acceptor emission spectra with 2–12-fold enhanced fluorescence intensity relative to that of the corresponding single dye-labeled primers. With single-stranded M13mp18 DNA as the template, a typical run with these ET primers on a capillary sequencer provides DNA sequences with 99% accuracy in the first 550 bases using the same amount of DNA template as that typically required using a four-color slab gel automated sequencer.

INTRODUCTION

Four-color DNA sequencing with fluorescent primers or terminators is the most commonly used method in high-throughput DNA sequencing laboratories (1–3). The fluorophores used in the previous studies are typically fluorescein and rhodamine derivatives. Recently, near-infrared fluorophores have also been developed for DNA sequencing in a one-color, four-lane format (4). In our studies, we have exploited fluorescence resonance energy transfer (ET), a well known and useful spectroscopic phenomenon (5–13), to optimize the spectroscopic properties of the fluorescent tags. This led to the development of multiple-color fluorescent ET primers which are superior for DNA sequencing and PCR fragment analyses (14–17). The fluorescent ET primers we designed carry a fluorescein derivative at the 5′-end as a common energy donor and other fluorescein and rhodamine derivatives attached to a modified thymidine within the primer sequence as acceptors. These primers all have strong absorption at a common excitation wavelength (488 nm) and well separated fluorescence emission maxima of 525, 555, 580 and 605 nm, respectively. The acceptor emission intensity of the ET primers is dependent on the spacing between the donors and the acceptors. The electrophoretic mobilities of the primers also vary with the spacing. Sets of four different primers can be selected where the number of nucleotides between the donor and acceptor is chosen to maximize the acceptor fluorescence emission and where the electrophoretic mobilities of the single base extension DNA fragments generated with the primers are closely matched. High precision sequencing and mapping with the ET primers requires much less DNA template than previously available procedures (14–17).

We describe here a method for the synthesis of fluorescent primers labeled with an ET cassette and their application in DNA sequencing. The ET cassette is constructed with a polymer spacer carrying donor and acceptor fluorophores, which is not constrained by the requirement of complementarity to a particular target DNA sequence, or by the spacing between a 5′-terminal base and a modified thymidine residue within the DNA primer sequence. Sets of primers modified with such ET cassettes can be efficiently excited with a single laser line and display well-separated acceptor emission spectra. Fluorescence emission intensities of primers labeled with ET cassettes are 2–12-fold greater than those of corresponding primers labeled with only the acceptor dye. These primer sets allow high precision DNA sequencing by the Sanger dideoxy method (18). The development of these universal cassettes provides a simple and practical method to fluorescently tag oligonucleotides of any sequence as well as other target molecules with ET coupled dyes.

MATERIALS AND METHODS

Design and synthesis of ET cassette-labeled oligonucleotide primers

Chemicals were purchased from Applied Biosystems (Foster City, CA) unless otherwise stated. Sequenase Version 2.0 T7 DNA polymerase and other DNA sequencing reagents were obtained from Amersham Life Science (Cleveland, OH). Oligodeoxynucleotides

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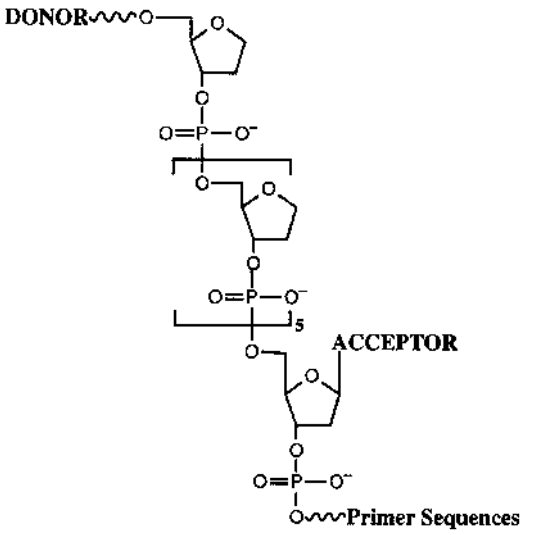

Figure 1. Design of ET cassette-labeled primers.

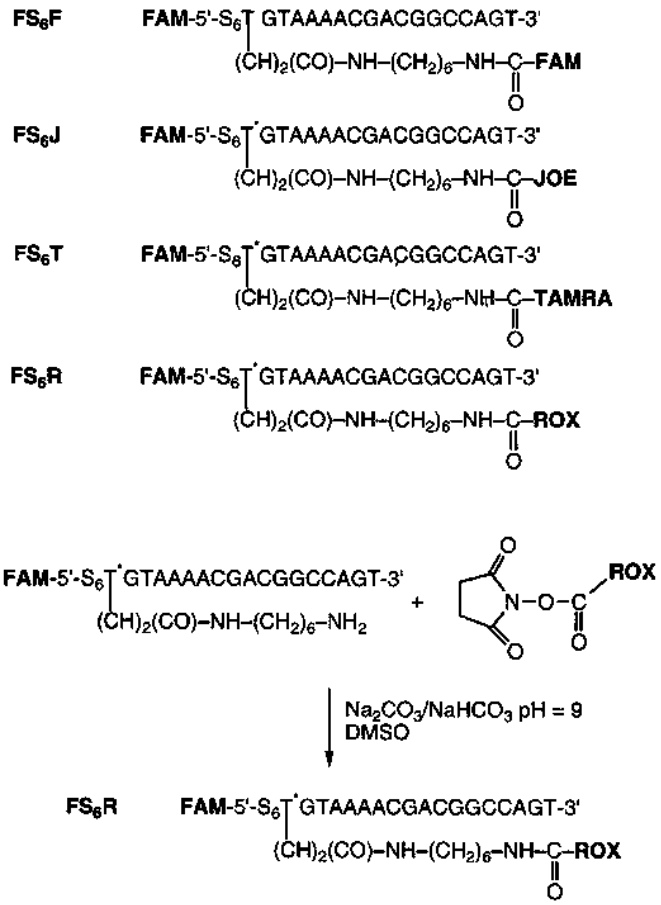

Figure 2. Structures of the four ET primers (FS_6F, FS_6J, FS_6T and FS_6R) and a synthetic scheme for the introduction of the acceptor (ROX) in FS_6R.

were synthesized by the phosphoramidite method on an Applied Biosystems 392 DNA synthesizer. Figure 1 presents a schematic drawing of the components of ET cassette-labeled primers. The structures of the four ET cassette labeled primers used in this work and a representative synthetic reaction scheme are presented in Figure 2. The M13 (–21) universal primers containing 18 nucleotides (nt) and a spacer (S_6), with the sequence 5′-S_6T*GTAAAACGACGGCCAGT-3′, were synthesized with donor–acceptor fluorophore pairs separated by a polymer linkage (S_6) formed by six 1′,2′-dideoxyribose phosphates (S). The 1′,2′-dideoxyribose phosphates were introduced using 5′-dimethoxytrityl-1′,2′-dideoxyribose-3′-[(2-cyanoethyl)-(*N*,*N*-diisopropyl)]-phosphoramidite (dSpacer CE Phosphoramidite, Glen Research, Sterling, VA) (19,20). The oligomer contains a modified base T* introduced by the use of 5′-dimethoxytrityl-5- [*N*-(trifluoroacetyl aminohexyl)-3-acrylimido]-2′-deoxyuridine, 3′-[(2-cyanoethyl)-(*N*,*N*-diisopropyl)]-phosphoramidite (Amino-Modifier C6 dT, Glen Research) which has a protected primary amine linker arm at the C-5 position. The T* was attached to the 5′ end of the nucleotide sequence and 3′ to the spacer. The donor dye was attached to the end of the spacer on the 5′ side of the oligomer, and the acceptor dye was attached to the primary amine group on the T*. The ET primers were purified as previously described (14), quantified by their 260 nm absorbances corrected for the dye absorptions, and then stored in 10 mM Tris–HCl, 1 mM EDTA, pH 8.0 at a final concentration of 0.4 pmol/µl for DNA sequencing reactions.

The primers are designated as D-S_6-A, where D is the donor, A is the acceptor and S_6 indicates the number of spacing sugars between D and A. In all the primers prepared, D = FAM (F); A = FAM (F), JOE (J), TAMRA (T) or ROX (R) which are fluorescein or rhodamine derivatives (2). It is likely that alternative ET primer sets with a wide variety of other dyes can be synthesized. For example, we have recently shown that 5- or 6-carboxyrhodamine-6G can be used in place of JOE to form ET primers with excellent results (Hung,S-C., Ju,J., Mathies,R.A. and Glazer,A.N., manuscript in preparation). Primers of any sequence can be labeled with ET coupled dyes simply by introducing a T* using Amino modifier dT or other phosphoramidite such as Amine-VN Phosphoramidite (CLONTECH, Palo Alto, CA) at the 5′ end of the oligonucleotide sequence, and then six 1′,2′-dideoxyribose phosphates using dSpacer Amidite. Donor and acceptor dyes can be easily attached to this scaffold forming ET cassette-labeled primers.

The absorption spectra of the primers were measured on a Perkin-Elmer Lambda 6 UV-visible spectrophotometer and the fluorescence emission spectra on a Perkin-Elmer model MPF 44B spectrofluorimeter. Samples were dissolved in 1× TBE buffer. ET efficiency was calculated by measuring the fluorescence intensity of the donor in the presence and absence of the acceptor (21).

DNA sequencing

Sequencing was performed using M13mp18 template DNA and modified T7 DNA polymerase on a four-color capillary electrophoresis (CE) DNA sequencer designed in our laboratory (15). Four reactions were run, one for each dye/ddNTP combination. The reactions containing ddCTP were run with the FS_6F primer, ddATP with the FS_6R primer, ddGTP with the FS_6T primer, and ddTTP with the FS_6J primer. The preparation of the sample for sequencing is similar to that previously described with 7-deaza-dGTP or dITP in place of dGTP (14,15). The sequencing of samples using 7-deaza-dGTP was performed in Mn^{2+}-containing buffer. Samples were introduced into a 50 cm long (30 cm effective

separation length) 3% T, 3% C polyacrylamide gel-filled capillary (J&W Scientific, Folsom, CA) by electrokinetically injecting for 45 s. Electrophoresis was performed at 200 V/cm in 100 mM Tris-borate–7 M urea, pH 8.3 (room temperature). A matrix transformation was performed on the raw data to correct for the crosstalk between the four channels (15) to generate the four-color CE sequencing profile.

RESULTS AND DISCUSSION

We have established previously that when regular nucleotides are used as spacers between the donor and acceptor fluorophores the fluorescence emission intensity of the ET primers increases as the number of spacing nucleotides is increased in the order 1, 2, 3, 4, 10 (15). ET primers with a 6 nt spacing, designed for multiple color PCR-based DNA typing, exhibited up to 10-fold fluorescence enhancement compared with the corresponding single dye-labeled primers (16). On the basis of these observations, each of the ET cassette-labeled primers described here was synthesized with six 1′,2′-dideoxyribose phosphates as a spacer. The fluorescence intensities of these primers are comparable to those of the optimized set of ET primers we previously described (15). The use of such spacers is advantageous in several aspects: (i) the spacer will not hybridize to any sequences within the DNA template and therefore false priming is avoided; (ii) the linkage of the spacer maintains the natural nucleic acid phosphate functionality, which avoids possible anomalies in electrophoretic mobility; and (iii) the elimination of the aromatic base groups on the deoxyribose rings in the spacer may reduce the likelihood of fluorescence quenching.

Figure 3 presents the absorption and emission spectra of the ET primers. Each primer exhibits the characteristic absorption of FAM at 496 nm, as well as strong absorption at 525 nm due to JOE in FS_6J, at 555 nm due to TAMRA in FS_6T, and at 585 nm due to ROX in FS_6R. The fluorescence emission spectra of the ET primers are dominated by the acceptor emission, indicative of efficient energy transfer. While the emission maximum of FS_6F is at 525 nm, the emission of FS_6J with 488 nm excitation is Stokes-shifted to 555 nm, that of FS_6T is shifted to 580 nm, and that of FS_6R is shifted to 605 nm. For FS_6R, the Stokes shift is >100 nm. Figure 3 also presents emission spectra of the single dye-labeled primers, measured at the same molar concentration as that of the corresponding ET primers. Since the common donor (FAM) in all four ET primers efficiently captures the excitation energy at 488 nm and then efficiently transfers the energy to the long-wavelength absorbing acceptors, substantial enhancement of the ET primer acceptor emission intensity is observed compared with that of corresponding single dye-labeled primers. The fluorescence intensity improvements derived from the data in Figure 3 are: FS_6F = 1.7× FAM; FS_6J = 3× JOE; FS_6T = 10× TAMRA; FS_6R = 12× ROX. In the case of FS_6F, where the donor and acceptor are identical, the fluorescence intensity increases ~2-fold compared with single FAM-labeled primers, as expected. The ET efficiency was calculated to be 83% for FS_6J, 84% for FS_6R and 85% for FS_6T.

The ET cassette-labeled primers were next used for DNA sequencing to determine whether they are good substrates for DNA polymerase and introduce no anomalies. Figure 4 presents an analyzed four-color DNA sequencing run obtained using the FS_6F, FS_6J, FS_6T and FS_6R primer set on a CE sequencer. Although the sequence can be called without applying any mobility shift adjustment, the data in Figure 4 have been corrected by applying a 5 s mobility shift (offset) of the FS_6F trace. This experiment was performed using equal amounts of primer (0.4 pmol) and 0.4 µg (0.2 pmol) of template DNA for each base extension. The separations extended to 600 bases in 140 min with strong signals. The sequence can be called up to 550 bases with 99% accuracy. This performance is comparable to that achieved with the previous ET primer set (15). The analysis of the data using more sophisticated peak calling software should lead to even better base calling and longer reads. This demonstrates that the presence of the ET cassette does not inhibit the hybridization of primers to the DNA template or the polymerase extension reaction. Some compressions are observed especially from nt 42 to 53. This region is known to cause compression even using 7-deaza-dGTP in capillary gel electrophoresis at room temperature (22). These compressions are completely resolved by using dITP in place of

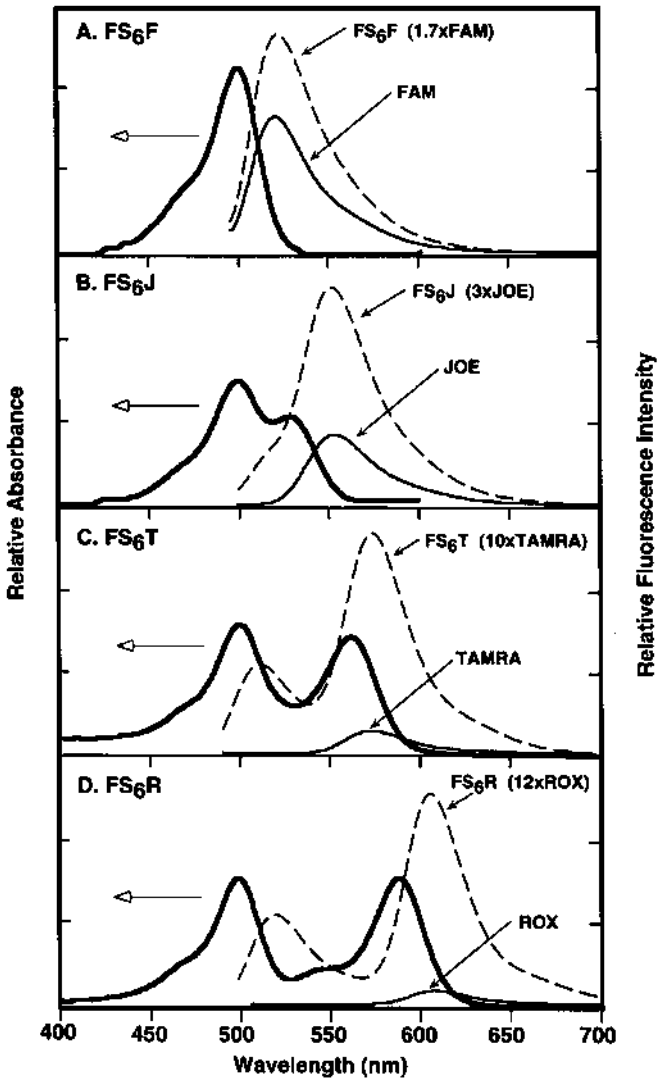

Figure 3. Comparison of the fluorescence emission intensity of the four energy transfer (ET) primers (FS_6F, FS_6J, FS_6T and FS_6R) with the corresponding single dye-labeled primers with excitation at 488 nm (1× TBE, 7 M urea). The thick lines indicate the absorption spectra of the ET primers. (**A**) FS_6F versus FAM, (**B**) FS_6J versus JOE, (**C**) FS_6T versus TAMRA and (**D**) FS_6R versus ROX. The emission spectra for each primer pair were determined with solutions at the same molar concentration.

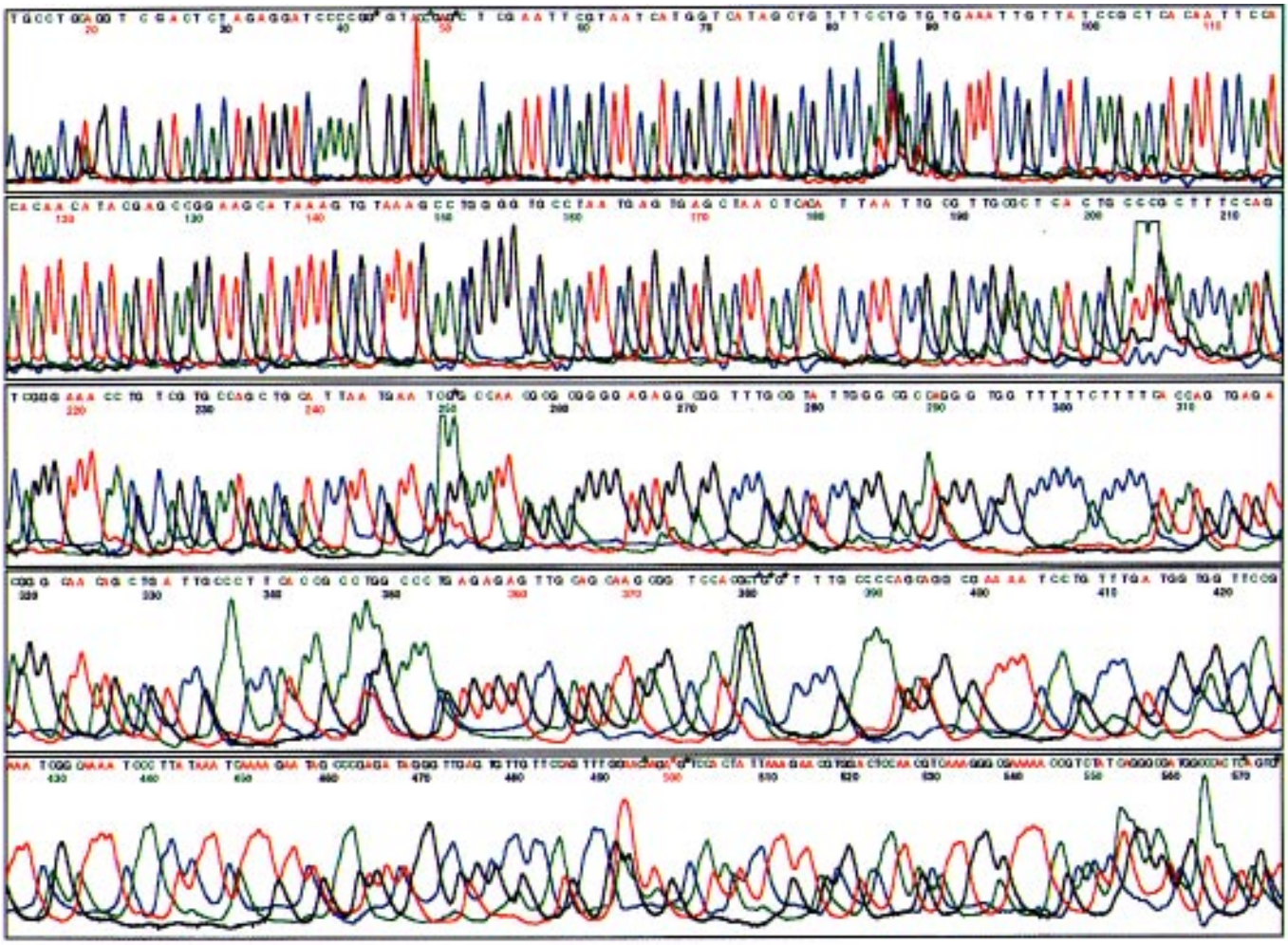

Figure 4. Analyzed four-color CE DNA sequencing profile of M13mp18 DNA using the ET primers FS_6F, FS_6J, FS_6T, FS_6R and Sequenase 2.0. Primer concentration: 0.4 pmol; DNA template: 0.4 µg (0.2 pmol) for each base extension. Asterisks indicate the misscalls due to compressions. The total electrophoresis time is 140 min.

dGTP, indicating that the compressions are not due to the use of ET primers.

We have shown here that the ET cassette method provides a new and general approach for labeling biopolymers or other target molecules with high sensitivity fluorescent tags that, unlike our earlier work, does not depend on the sequence of the labeled oligonucleotide. With the ET cassette method, it is now possible to easily label all the oligonucleotide primers and probes for DNA sequencing, PCR and hybridization, etc., with ET coupled dyes. Such classes of donor–acceptor labeled molecules should find wide application in multiple-color multiplex genetic analyses because of the enhanced sensitivity that results from optimizing the spectroscopic properties of these labels as well as the concomitant opportunity for reduced instrumentation cost and complexity.

ACKNOWLEDGMENTS

We thank the members of the Berkeley High-Sensitivity DNA Analysis Project, John Chase and Carl W. Fuller from Amersham Life Science Inc. for many valuable interactions. This research was supported by the Director, Office of Energy Research, Office of Health and Environmental Research of the US Department of Energy under contract DE-FG-91ER61125. J.J. was supported by a DOE Human Genome Distinguished Postdoctoral Fellowship sponsored by the US Department of Energy, Office of Health and Environmental Research and administered by the Oak Ridge Institute for Science and Education. Financial support from Amersham Life Science Inc. is also gratefully acknowledged.

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