

New dye-labeled terminators for improved DNA sequencing patterns

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ABSTRACT

We have used two new dye sets for automated dye-labeled terminator DNA sequencing. One set consists of four, 4,7-dichlororhodamine dyes (d-rhodamines). The second set consists of energy-transfer dyes that use the 5-carboxy-d-rhodamine dyes as acceptor dyes and the 5- or 6-carboxy isomers of 4'-aminomethylfluorescein as the donor dye. Both dye sets utilize a new linker between the dye and the nucleotide, and both provide more even peak heights in terminator sequencing than the dye-terminators consisting of unsubstituted rhodamine dyes. The unsubstituted rhodamine terminators produced electropherograms in which weak G peaks are observed after A peaks and occasionally C peaks. The number of weak G peaks has been reduced or eliminated with the new dye terminators. The general improvement in peak evenness improves accuracy for the automated base-calling software. The improved signal-to-noise ratio of the energy-transfer dye-labeled terminators combined with more even peak heights results in successful sequencing of high molecular weight DNA templates such as bacterial artificial chromosome DNA.

INTRODUCTION

Sanger dideoxy DNA sequencing is the most commonly used method for DNA sequencing, particularly in large scale genomic sequencing (1). Automated DNA sequencing uses fluorescent dyes for the detection of the electrophoretically resolved DNA fragments. Two variations of automated DNA sequencing have evolved: dye-labeled primer sequencing (2–4), in which the fluorescent dyes are attached to the 5' end of the primer oligonucleotide, and dye-labeled terminator sequencing, in which the dyes are attached to the terminating dideoxynucleoside triphosphates (5,6). Each sequencing method has advantages and disadvantages.

Dye-labeled primer sequencing has benefited from the development of DNA polymerases which do not discriminate between deoxy- and dideoxynucleotides (7,8). These polymerases provide sequencing electropherograms with very even peak heights. Base-calling is easy and reliable, and the ability to call

heterozygotes can be based on peak heights as well as the presence of two bases at a position (9,10). The major disadvantage of the dye primer method is the requirement for four separate extension reactions and four dye-labeled primers for each template.

The major advantages of dye-labeled terminator sequencing are convenience, since only a single extension reaction is required for each template, and the synthesis of a labeled primer is unnecessary, allowing the use of preferred hybridization sites. In addition false terminations, in which the DNA fragments are terminated by a deoxynucleotide rather than a dideoxynucleotide, are not observed as these products are unlabeled. The major disadvantage of dye-labeled terminators is that with every polymerase the pattern of termination with dye-labeled terminators has been found to be less even than for dye-labeled primers. The presence of very small or very large peaks can result in errors in automated base-calling.

We have evaluated the use of two new dye sets, 4,7-dichloro-substituted rhodamines (d-rhodamines), and a set of energy-transfer dyes that were previously described for use on dye-labeled primers (11). Use of the new terminators, d-rhodamine terminators and BigDye™ terminators, using the energy transfer dyes, required optimization of the linker attaching the dyes to the nucleotides, the dye isomer used, and the choice of each dye on a particular nucleotide. A major objective with both dye-sets was to obtain more even peak patterns compared with current DNA sequencing terminators. The energy-transfer dyes also offer the advantage of increased signal. The improved peak evenness found in both new dye sets allows greater accuracy in base-calling, longer reads and the ability to use dye-labeled terminators for heterozygote analysis.

MATERIALS AND METHODS

Dye-labeled terminators

The dye-labeled terminators were prepared by methods previously described (12). Briefly, the succinimidyl ester of each dye was mixed with the nucleoside triphosphates at pH 9. The products were purified on HPLC by anion-exchange chromatography to remove excess dye, followed by reverse-phase chromatography to separate unlabeled triphosphates and to separate dye-isomers.

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Table 1. Linkers, fluorophores and final terminator concentration for each nucleotide of the three terminator sets: rhodamine, d-rhodamine and BigDye

| Terminators Nucleotide | Rhodamine | | | d-Rhodamine | | | BigDye | | | |
|---------------------------|---------------------|---------|------------------|-------------|----------|------------------|--------|-----------|--------------|------------------|
| | Linker ^a | Dye | Final conc. (μM) | Linker | Dye | Final conc. (μM) | Linker | Donor dye | Acceptor dye | Final conc. (μM) |
| ddATP | PA | 5-R6G | 0.02 | PA | dR6G-2 | 0.02 | PA | 6-FAM | dR6G-2 | 0.11 |
| ddCTP | PA | 6-ROX | 0.13 | EO | dTAMRA-2 | 0.12 | EO | 6-FAM | dROX-2 | 0.16 |
| ddGTP | PA | 5-R110 | 0.01 | EO | dR110-2 | 0.01 | EO | 5-FAM | dR110-2 | 0.10 |
| ddTTP | PA | 6-TAMRA | 0.23 | EO | dROX-1 | 0.18 | EO | 6-FAM | dTAMRA-2 | 1.12 |

^aThe linkers are PA, propargylamino and EO, propargyl ethoxyamino.

DNA sequencing

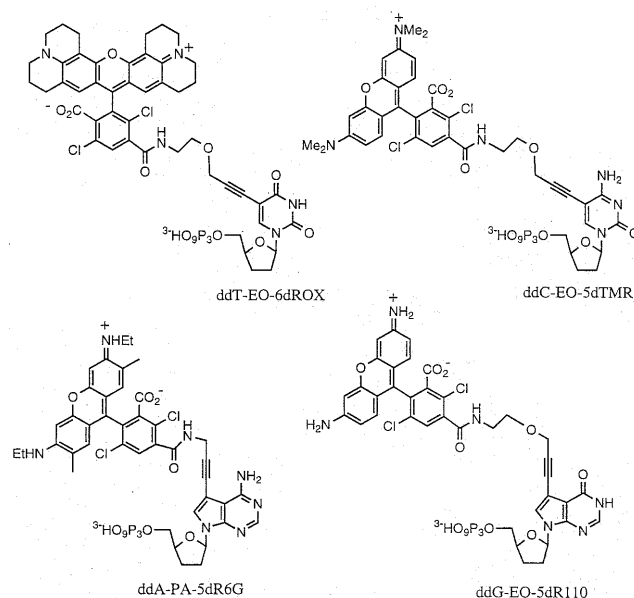
Dye-labeled terminator cycle sequencing with d-rhodamine and BigDye terminators, using the energy-transfer dyes, was performed using AmpliTaq DNA polymerase, FS, according to the ABI PRISM™ sequencing manual (PE Applied Biosystems, Foster City, CA). With the BigDye terminators, dUTP was substituted for dTTP at the same concentration in the dNTP mix. The concentrations of the dNTPs in the reactions were 100 μM for dATP, dCTP and dTTP (or dUTP) and 500 μM for dITP. The concentrations of the terminators used in the reactions were determined by titrating each terminator in single-color terminator reactions and selecting the concentration which maximized the signal of the 700th nucleotide (12). The concentrations were adjusted according to the relative brightness of each dye in order to obtain approximately equivalent signal for each color in the four-color reaction (Table 1). The d-rhodamine and BigDye terminator sets used 10 nm virtual filters on the CCD camera of the ABI PRISM 310 and 377 centered at 540 or 545 (ABI PRISM 310), 570, 595 and 625 nm.

Bacterial artificial chromosomal (BAC) DNA was purified by an alkaline lysis protocol (13) and was sequenced with BigDye terminators with a slight modification of the terminator protocol. Table 2 shows the reagents used per reaction for BAC sequencing. BAC samples were cycled in a Perkin-Elmer 9600 thermocycler according to the following protocol: initial denaturation at 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, 55 °C for 20 s, and 60 °C for 4 min. Excess terminators were removed using Centri-Sep spin columns (Princeton). Samples were vacuum dried and then resuspended in 2 or 4 μl of formamide, heated to 95 °C for 2 min and 2 μl of the sample loaded on the ABI PRISM 377. The BAC clone, bW XD342, used in these studies contains an insert, 169 kb in length, from the human X chromosome, locus Xq21.3.

Table 2. Reagent mix used for BAC sequencing

| Reagent | Per reaction |
|---------------------------------|--------------|
| Terminator mix | 8.0 μl |
| 5X sequencing buffer | 4.0 μl |
| Amplitaq FS for dye terminators | 0.5 μl |
| Primer | 4.0 pmol |
| BAC DNA | ~400 ng |
| Distilled water | to volume |
| Final volume | 40.0 μl |

Single color analysis of the d-rhodamine and BigDye terminators was performed using the ABI PRISM 310 Genetic Analysis system. Single color sequencing reactions were prepared as described earlier, except that in all cases excess terminators were removed using Centri-Sep spin columns (Princeton). Samples were resuspended in

**Figure 1.** Structure of the d-rhodamine dye-labeled terminators.

Template Suppression Reagent (TSR; PE Applied Biosystems, Foster City, CA) and heated to 95 °C for 2 min. A computer program has been developed to determine peak heights and to calculate the mean, standard deviation and relative error, where the relative error is the ratio of the standard deviation to the mean.

RESULTS AND DISCUSSION

Dichlororhodamine terminators

In order to optimize the performance of the new d-rhodamine terminators, we synthesized and tested 39 out of a possible 64 combinations of the four dichlororhodamine dyes (both 5- and 6-carboxy isomers), propargylamino (PA) or propargyl ethoxyamino (EO) linker, and nucleotide terminators ($8 \times 2 \times 4 = 64$). The structure of both the dye and the linker between the nucleotide and the dye affected the pattern of termination (manuscript in preparation). We chose the dye set which maximized the evenness of the peaks in the sequencing pattern. The final d-rhodamine terminator set had mobility shifts within the half base requirement for minimal artifacts. (The set of all 32 possible rhodamine dyes with the EO linker was tested but a 4-dye set was not found that had acceptable mobility characteristics.) The structures of the d-rhodamine dye-labeled terminators are shown in Figure 1. Only the A-terminator retains the propargylamino linker of the original terminators (Table 1).

BigDye terminators

We have previously reported on a set of energy-transfer dyes for dye-labeled primer sequencing that uses the 5-carboxy-dichlororhodamine dyes as acceptor dyes and the 5- or 6-carboxy isomers of 4'-aminomethylfluorescein as the donor dye. These dyes show both improved spectral resolution and improved brightness compared with the standard dyes used for dye-primer sequencing (11). Here, we have investigated the use of the energy-transfer dyes on dye-labeled terminators.

We synthesized and tested 18 out of a possible 64 combinations of energy-transfer dyes (four 5-carboxy-d-rhodamines with both 5- and 6-carboxy isomers of 4'-aminomethylfluorescein), propargyl-amino (PA) or propargyl ethoxyamino (EO) linker, and four nucleotide terminators ($8 \times 2 \times 4 = 64$). Again, we chose the dye set which maximized the evenness of the peaks in the sequencing pattern and minimized the dye-related mobility effects. The final BigDye terminator set had mobility shifts within the half base requirement for minimal artifacts. The structure of one of the four BigDye terminators, the ddT-EO-6CFB-dTMR, is shown in Figure 2. In addition to varying the terminator structure, we found that varying the structure of the dNTPs also affected the pattern of termination. By substituting dUTP for dTTP the termination pattern for energy-transfer dye-labeled ddT terminators was improved for each of the seven different energy-transfer dye/linker/ddT compounds tested.

A feature of the BigDye terminator set is that the total signal is increased compared with either the rhodamine or d-rhodamine dye sets. BigDye terminators can be used with advantage in cases when template molar equivalents are limited. BAC DNA fragments typically have very high molecular weights and cannot

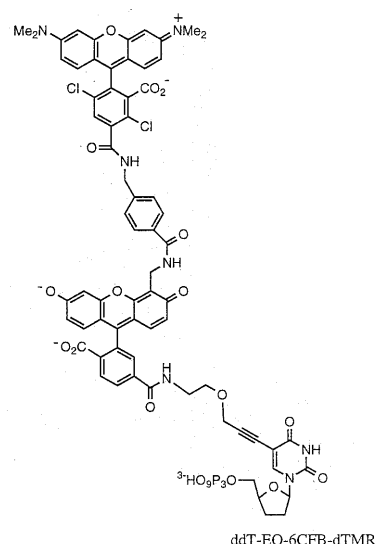


Figure 2. Structure of the ddT-BigDye terminator.

be sequenced with single dyes. Energy-transfer dye-labeled primers (9,14–17) have proven useful for these templates (13). Here we demonstrate the signal advantage of energy-transfer dyes combined with the convenience of dye-labeled terminators. Figure 3 shows a sequencing pattern for a bacterial artificial chromosome template (clone:bWXD342; sequencing reaction Table 2) using the BigDye terminators.

Table 3. Comparison of number of sequencing accuracy, read length and total signal for different templates with the three dye terminator chemistries

| Template | %GC | Errors to 720 bases | | | Read length at 98.0% accuracy | | | Signal strength | | |
|----------------------|------|---------------------|-------|--------|-------------------------------|-------|--------|-----------------|-------|-------------------|
| | | Rhod | dRhod | BigDye | Rhod | dRhod | BigDye | Rhod | dRhod | BigDye |
| 349, -21M13 | 65.3 | 50 | 14 | 16 | 394 | 704 | 683 | 1453 | 732 | 1422 ^a |
| 349, -21M13 | | 45 | 15 | 34 | 543 | 688 | 613 | 2812 | 1182 | 2993 |
| 4009, -21M13 | 71.4 | 44 | 18 | 26 | 586 | 692 | 646 | 1098 | 333 | 2056 ^a |
| 4009, Reverse M13 | | 28 | 14 | 10 | 616 | 701 | 741 | 693 | 397 | 2692 ^a |
| ABD 114, Reverse M13 | 59 | 16 | 7 | 7 | 691 | 781 | 733 | 2830 | 1413 | 3698 ^a |
| pGEM, -21M13 | 50.8 | 11 | 4 | 8 | 718 | 796 | 723 | 3478 | 1447 | 4865 |
| pGEM, -21M13 | | 21 | 6 | 6 | 654 | 784 | 776 | 3548 | 1374 | 3834 ^a |
| pGEM, Reverse M13 | | 12 | 9 | 0 | 785 | 727 | 831 | 3202 | 1374 | 4268 ^a |
| pcDNA, Reverse M13 | 33 | 4 | 4 | 2 | 790 | 790 | 843 | 3929 | 1366 | 5184 ^a |
| DJ2, Reverse M13 | 30 | 7 | 3 | 2 | 743 | 798 | 790 | 1874 | 724 | 4434 ^a |
| ABD 116, -21M13 | 37.3 | 12 | 6 | 1 | 744 | 791 | 810 | 2268 | 948 | 2750 |
| ABD 116, -21M13 | | 25 | 11 | 12 | 612 | 736 | 769 | 1974 | 502 | 1346 ^a |
| ABD 116, Reverse M13 | | 6 | 2 | 2 | 788 | 788 | 776 | 2397 | 1090 | 4385 |
| ABD 116, Reverse M13 | | 3 | 6 | 5 | 807 | 740 | 807 | 2102 | 650 | 2384 ^a |
| ABD 100, -21M13 | 48 | 26 | 8 | 2 | 605 | 785 | 813 | 877 | 375 | 1118 ^a |
| ABD 100, Reverse M13 | | 21 | 12 | 6 | 659 | 727 | 758 | 1336 | 498 | 1606 ^a |
| ABD 90, -21M13 | 36.3 | 33 | 18 | 4 | 364 | 671 | 835 | 1172 | 442 | 1884 ^a |
| ABD 90, Reverse M13 | | 3 | 8 | 12 | 834 | 773 | 720 | 1064 | 602 | 1552 ^a |
| MEAN | | 20 | 9 | 9 | 663 | 748 | 759 | 2117 | 858 | 2915 |

^aBigDye terminator separations were performed with half the sample load compared with rhodamine separations. Signal value has been normalized by doubling the signal value for the BigDye terminators for these separations.

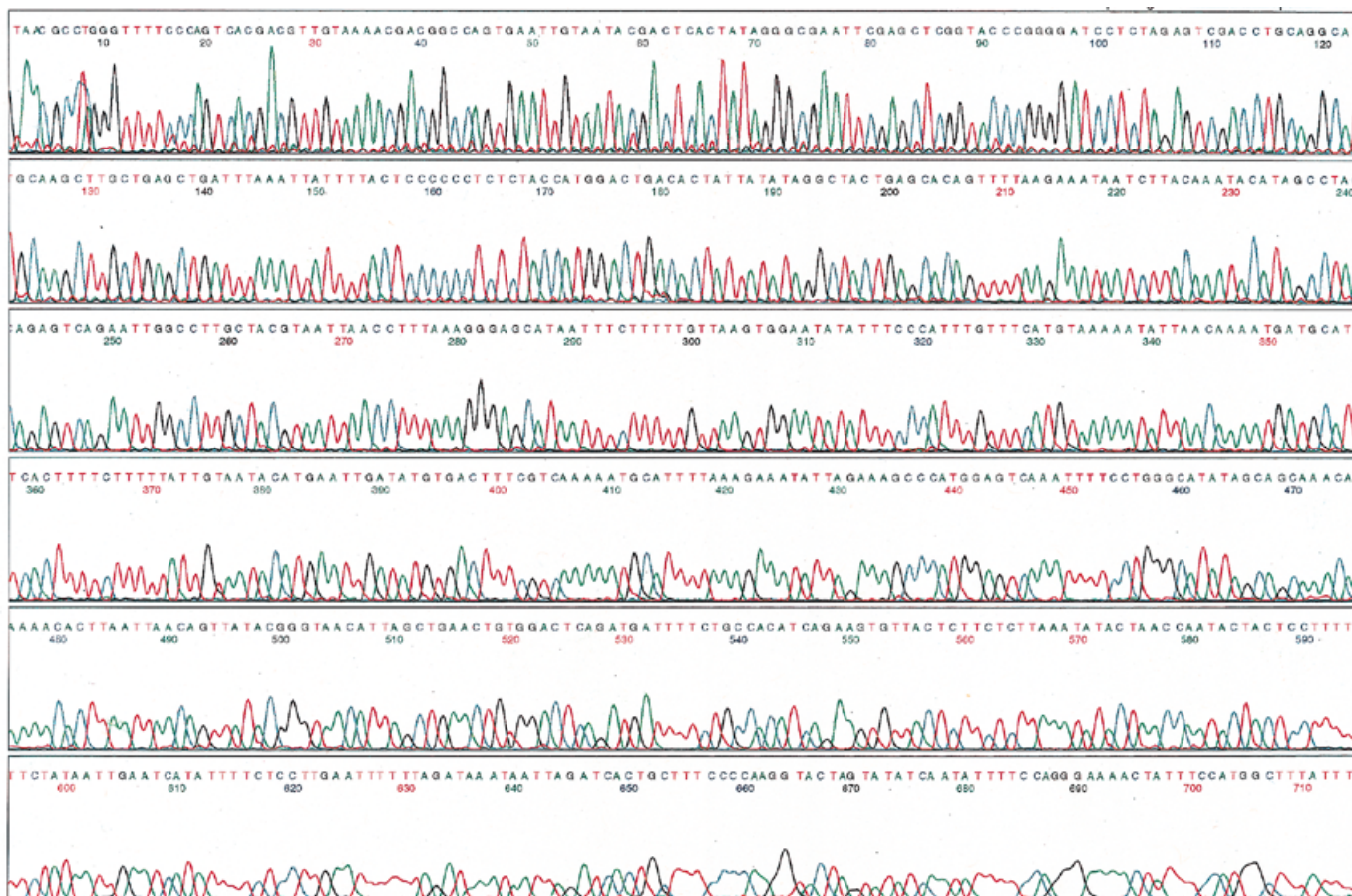


Figure 3. Sequencing of BAC DNA (clone:bWXD342) with BigDye terminators.

Comparison of the three terminator sets

Table 1 shows the matching of linker and fluorophore for the original rhodamine, the d-rhodamine, and the BigDye terminators. Table 3 compares the sequencing accuracy, read-lengths and signal for the three terminator sets for different templates. As a result of the better peak evenness of the d-rhodamine and BigDye terminator sets both base-calling accuracy and read-lengths are improved. Although the total signal for the d-rhodamine terminators is reduced compared with the rhodamines (equal amounts of template were loaded on the sequencing gels), the multicomponent noise is also reduced due to the better spectral resolution of the d-rhodamine dye set. The signal strength for the BigDye terminators with most of the templates is higher than the signal strength for the rhodamine terminators as expected based on the brightness of the energy-transfer dyes (11). The 2-fold reduction in multicomponent noise for the d-rhodamine and BigDye terminators compared with the rhodamine terminators is not reflected in the signal number (11).

We have analyzed a series of different templates with three sets of dye-labeled terminators, rhodamine, d-rhodamine and BigDye, to compare peak evenness and to identify sequence context effects. The sequencing patterns of a portion of template DJ2 using the three sets of dye-terminators are shown in Figure 4. In the rhodamine set, very weak G peaks after A peaks are observed, with some weak G peaks after C peaks. There are also some very strong peaks that result in the smallest peaks being >10-fold

smaller than the largest peaks, with a small peak frequently appearing just before or after these large peaks. In both the d-rhodamine and the BigDye terminator patterns, the peaks are of more even heights, so that in general the adjacent peaks are <5-fold different in size. The small G peaks after A peaks or C peaks has also improved, with the BigDye terminators showing no weak G peaks and the d-rhodamines showing a few weak G peaks after A peaks or C peaks. These small G peaks are reliably called by the automated software due to the better balance in the peak heights. The BigDye terminators show weaker T peaks after G peaks, but again, because the overall pattern is more balanced, these T peaks are called by the automated software.

Table 4. Relative errors^a of peak heights for bp 10–315 in pGEM with the three terminator sets and dye primers

| | Dye-primer | Rhodamine | d-Rhodamine | BigDye dT | dU |
|---------|------------|-----------|-------------|--------------|------|
| A | 0.27 | 0.83 | 0.49 | 0.23 | 0.23 |
| C | 0.26 | 0.60 | 0.33 | 0.28 | 0.31 |
| G | 0.31 | 0.83 | 0.42 | 0.37 | 0.31 |
| T | 0.22 | 0.52 | 0.36 | 0.55 | 0.40 |
| Average | 0.26 | 0.69 | 0.40 | 0.36 | 0.32 |

The dye-primer reactions use ϵ^7 dGTP in the dNTP mix, while the terminator reactions use dTTP in the dNTP mix instead of dGTP.

^aThe relative error is the ratio of the standard deviation of the peak heights to the mean of the peak heights for a selected group of peaks.

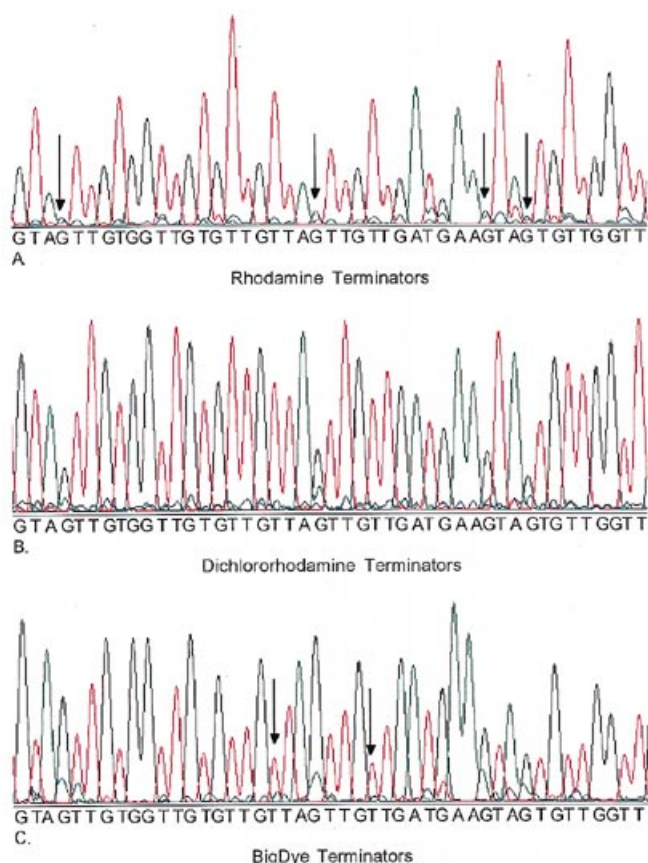


Figure 4. Comparison of sequencing patterns of rhodamine, d-rhodamine and BigDye terminators on an AT rich template, DJ2. Arrows in (A) are G peaks with weak signal. Many of these G peaks are similar in size to noise peaks under adjacent peaks. In (B) and (C), all of the G peaks are much larger than any noise peaks. In (C), the arrows indicate weaker T peaks following G peaks. These T peaks are larger than any noise peaks and are called by the automated software.

The peak patterns can be quantitatively evaluated by measuring the peak heights of a given sequence and calculating an average and standard deviation. The data are normalized by defining the relative error as the ratio of the standard deviation to the average peak height. A completely uniform series of peaks would yield a relative error value of 0. This is unlikely to occur for any type of Sanger sequencing except over a very short group of peaks, because of the exponential decay of the terminal events with increasing fragment size (12), results in decreasing peak height with increasing fragment length over a large group of bases. Thus, for a group of >100 bases, a value of 0.15–0.3 would be expected for dye primer sequencing (Table 4). Table 4 shows the results of peak height evaluation for the rhodamine, d-rhodamine and BigDye terminator sets, along with the relative error values for dye-labeled primer sequencing for the same region.

CONCLUSIONS

We have developed two new dye-terminator sets that are both improvements over previous dye-terminators. The peak patterns of these chemistries are nearly as even as dye-labeled primer sequencing patterns. The genome sequencing community requires data with a high confidence of base-calling, and data generated by two different sequencing approaches in areas with single-orientation coverage (18–22). In the past this has meant that the bulk of the data

were generated using dye-primers, supplemented by dye-terminators only when necessary. These requirements may now possibly be met with the two new dye-terminator chemistries, eliminating the need for dye primers. Sequencing for heterozygote analysis may be able to be performed with these new dye-terminators rather than dye-primers. Future work in enzyme engineering and dye synthesis to further enhance the performance of dye-labeled terminators will likely render obsolete traditional dye-labeled primer sequencing.

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