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Methods

Improved DNA Sequencing Accuracy and Detection of Heterozygous Alleles Using Manganese Citrate and Different Fluorescent Dye Terminators

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The use of dideoxynucleotide triphosphates labeled with different fluorescent dyes (dye terminators) is the most versatile method for automated DNA sequencing. However, variation in peak heights reduces base-calling accuracy and limits heterozygous allele detection, favoring use of dye-labeled primers for this purpose. We have discovered that the addition of a manganese salt to the PE Applied Biosystems dye-terminator sequencing kits overcomes these limitations for the older rhodamine dyes as well as the more recent dichloro-rhodamine dyes (dRhodamine and BigDyes). Addition of manganese to reactions containing dRhodamine-based dye terminators produced the highest base-calling accuracy. This combination resulted in the most uniform electropherogram profiles, superior to those produced by BigDye terminators and published for dye primers, and facilitated detection of heterozygous alleles.

The most versatile means of automated DNA sequencing uses dye terminators, which are dideoxynucleotide triphosphates (ddNTPs) labeled with different fluorescent dyes. This allows performing the sequencing reactions simultaneously in a single tube and electrophoresing the products in a single lane (Prober et al. 1987). However, the variability in electropherogram peak heights reduces base-calling accuracy and hinders heterozygous mutation detection. Alternatively, sequencing primers can be individually labeled and used in separate sequencing reactions to produce more even peak heights, but this demands a more cumbersome and expensive procedure (Rosenblum et al. 1997).

Thermostable *Thermus aquaticus* (Taq) DNA polymerase strongly discriminates against ddNTPs in a manner dependent on sequence context (Tabor and Richardson 1989, 1990, 1995). Tabor and Richardson (1995) identified and altered a specific amino acid residue in DNA polymerases responsible for this discrimination. Two thermostable, genetically engineered DNA polymerases incorporating this mutation (phenylalanine 667 to tyrosine; F667Y) are now commercially available—AmpliTaq DNA polymerase FS from PE Applied Biosystems (PE/ABI; Foster City, CA) and Thermo Sequenase from Amersham Life Science (Arlington Heights, IL; Reeve and Fuller 1995; Samols et al. 1995; Vander Horn et al. 1997). Use of these mutant polymerases and dye-labeled primers produces electropherograms with very similar peak heights along

the entire length of the readable sequence (McDonald et al. 1995; Samols et al. 1995; Vander Horn et al. 1997). However, peak heights are considerably less uniform when using these DNA polymerases in dye-terminator sequencing reactions.

A common problem with ddNTPs labeled with rhodamine-based dyes occurs where a G follows an A. The size of the dye-labeled G peaks is reduced (i.e., “suppressed”) to less than both the average height of G peaks and the height of the preceding A peak in the sequence trace. The G peak sometimes is obscured not only by these A peaks, but also by the peak following a suppressed G, which can be much taller than average. Although Parker et al. (1995, 1996) and Zakeri et al. (1998) have analyzed the context dependency of peak height variations to improve data interpretations, this variability still limits the usefulness of the dye terminators. To improve base calling of automated fluorescent sequencing, PE/ABI has developed two alternatives to the rhodamine-based dye-terminators: 4,7-dichloro-rhodamine-based dyes (dRhodamine dyes) and energy-transfer variants of the dRhodamine dyes (BigDyes) (Lee et al. 1997; Rosenblum et al. 1997). Dideoxynucleotides labeled with these dyes show more uniform peak heights and less G suppression (Rosenblum et al. 1997; Zakeri et al. 1998). However, these improvements to both the DNA polymerases and the labeling dyes do not produce sequencing results as uniform as those with dye-labeled primers (Rosenblum et al. 1997) or with the wild-type phage T7 DNA polymerase and the mutant enzyme Sequenase 2.0 (Tabor and Richardson 1989; Ju et al. 1995).

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We recently reported a significant improvement of automated cycle sequencing that uses addition of MnCit (an equimolar combination of manganese chloride and the metal buffer, trisodium citrate) to rhodamine-based dye-terminator cycle-sequencing reactions containing magnesium and the F667Y variant *Taq* DNA polymerases (Korch and Drabkin 1999). In the present report, we characterize the effects of MnCit addition on all three fluorescent dye-terminator cycle-sequencing procedures from PE/ABI. The uniformity of peak heights is dramatically improved, especially for the rhodamine- and dRhodamine-based sequencing kits, and is applicable to all four PE/ABI sequencing instruments: models 373A, 373A XL, 377, and 377 XL. MnCit addition, together with other modifications of the DNA-sequencing protocol, significantly increases base-calling accuracy at minimal cost. Furthermore,

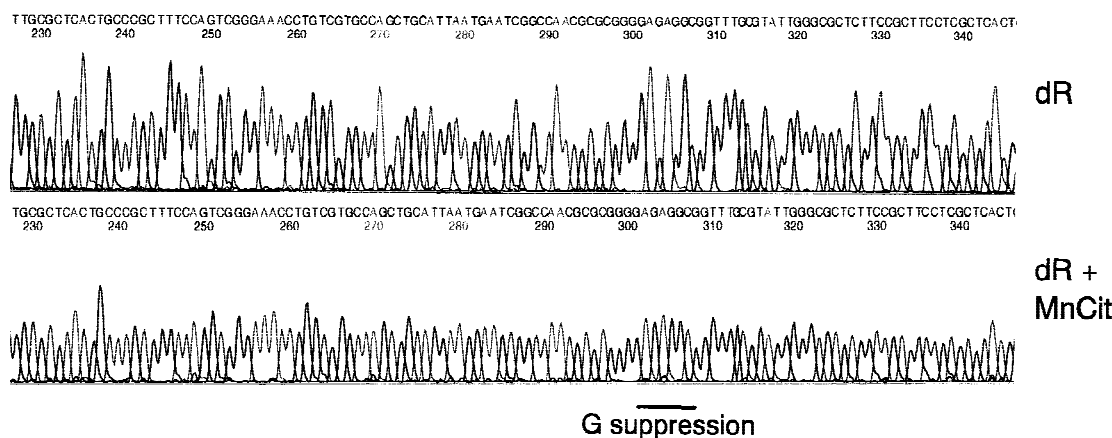
the addition of manganese improves the detection of heterozygous nucleotide differences.

RESULTS

MnCit Improves Peak Height Uniformity with Rhodamine, dRhodamine, and BigDye DNA-Sequencing Kits

As described earlier, the addition of MnCit to a reaction containing 1.5–2.0 mM magnesium improved the uniformity of peak heights produced by AmpliTaq DNA polymerase FS with the standard rhodamine dye terminators (Korch and Drabkin 1999). Figure 1 compares the effect of adding optimal amounts of MnCit to DNA cycle-sequencing reactions based on the newer dye-terminator kits: dRhodamine and BigDye. Using the control primer and pGEM3Zf(+) DNA template in-

dRhodamine



BigDye

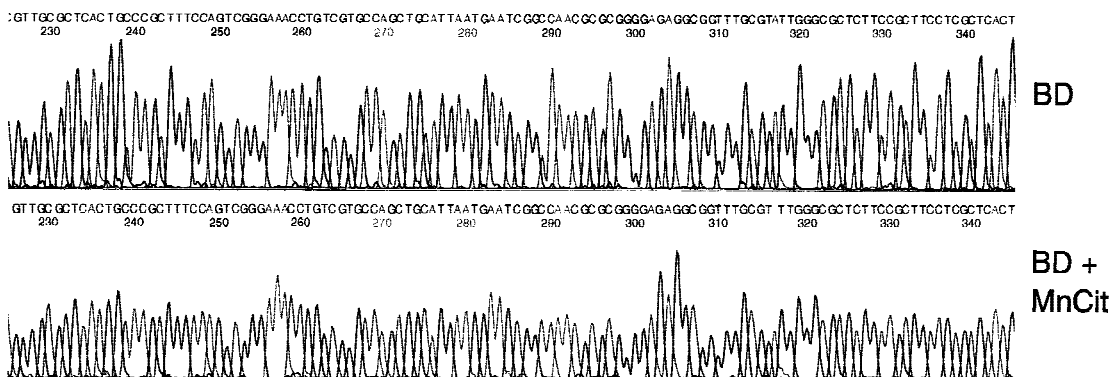


Figure 1 Addition of MnCit reduces peak height variations in DNA-sequencing electropherograms produced using different PE/ABI dye-terminator sequencing kits. Shown are electropherograms of DNA-sequencing reactions utilizing ddNTPs labeled with dyes based on dRhodamine and BigDye. The pGEM3Zf(+) template and the primer (–21/pUC or M13F) were either from the PE/ABI rhodamine dye terminator Ready Mix sequencing kit, which were dissolved in TE (BigDye \pm 0.8 mM MnCit results), or prepared by us and dissolved in water (dRhodamine \pm 0.3 mM MnCit results). The underlined region of nucleotides 300–310 illustrates how the presence of MnCit reduces the suppression of G peaks that follow A peaks.

cluded in the sequencing kits, MnCit addition produced distinctly more uniform peak heights with the dRhodamine kit as we showed earlier with the rhodamine kit, whereas its addition to the BigDye kit showed only a slight improvement of peak height uniformity. The apparent reduction in peak height of the dRhodamine + MnCit results is relative to the abnormally strong T peak in the 5'-GATC-3' sequence near the primer, but not included in the electropherogram panel in Figure 1 (also see below). Using the rhodamine kit in the presence of 0.5 mM trisodium citrate, addition of different manganese salts (chloride, sulfate, and acetate) all produced comparable improvements on peak height variability (data not shown).

Most earlier papers describing the effects of manganese on sequencing used the metal buffer trisodium isocitrate at concentrations of 4–25 mM (Fuller 1989; Tabor and Richardson 1989, 1990, 1995; Kristensen et al. 1990; Zimmermann et al. 1990). We found that both citrate and isocitrate could be used, but the optimal concentrations were ~0.5 mM using the DNA template and primer controls from the kits. The improvement by MnCit was optimal at equimolar ratios of MnCl_2 and trisodium citrate (data not shown). From the relative intensities of banding patterns in fluorescent gel images and the sum of the average signal strengths for the bases, concentrations >2 mM trisodium citrate or 2.5 mM trisodium isocitrate inhibited the sequencing reactions (data not shown). This loss of "activity" was caused by chelation of the enzymatic cofactor Mg^{2+} , as addition of 1 mM and 2 mM MgCl_2 to the reaction mixtures restored activity. MnCit concentrations >1.0 mM also caused the unincorporated dye terminators to precipitate more easily and to be more difficult to remove from the reactions by ethanol precipitation prior to loading on gels (data not shown). MnCit concentrations <0.7 mM also inhibited incorporation of dye terminators (data not shown), but (1) increasing the amount of DNA template, (2) increasing the number of cycles used in the sequencing reactions, (3) loading a larger portion of the sequencing reactions on the gel, or (4) a combination of these measures could compensate for these smaller losses of signal strength.

The effect of increasing amounts of MnCit on profile variations was quantified by measuring the height of 100 consecutive peaks. We found that increasing MnCit concentrations from 0.1 mM to 1.0 mM decreased peak height variation sharply (as measured by the coefficient of variation, CV, of peak heights, Fig. 2). The CV values for the different kits in the absence of MnCit were very similar to those values reported by Rosenblum et al. (1997). The rhodamine kit in the absence of MnCit showed the highest CV values (56%–59%, Fig. 2; see Korch and Drabkin 1999). Addition of MnCit to the dRhodamine dye-terminator cycle-

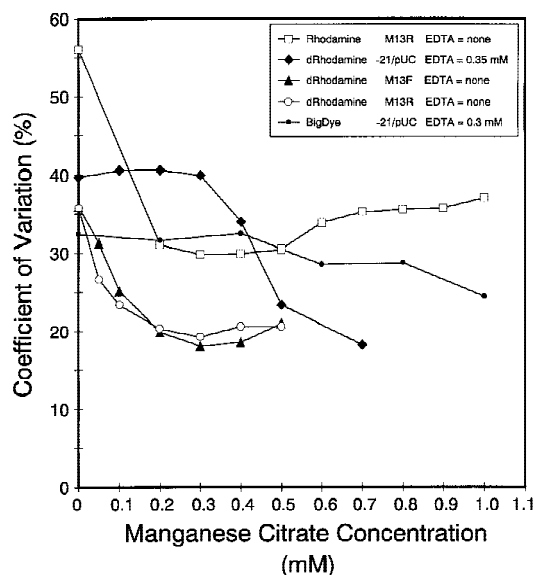


Figure 2 Increasing MnCit concentrations decrease the CVs of peak heights of different PE/ABI dye terminator sequencing kits. The data were obtained by sequencing with the kits, templates, and primers indicated. The curves of the CV values using the –21/pUC, M13F, M13R primers were determined by measuring the height of 100 nucleotide peaks as described in Methods, except the BigDye kit results with the –21/pUC primer are based on the fifty nucleotides 55–104. The dRhodamine and the BigDye sequencing results that contain the indicated amounts of EDTA were produced using the control –21/pUC primer and DNA template from the kits; whereas, the other results were obtained using our preparations of primers and pGEM3Zf(+) DNA that were dissolved in water.

sequencing reactions produced electropherograms with the lowest CV values (18%). The CVs of peak height for all primers with a specific kit were very similar (e.g., compare the dRhodamine results with the M13F and M13R primers in Fig. 2). In contrast, the BigDye sequencing results showed the least amount of peak height variation in the absence of MnCit, but addition of MnCit to these dye terminators only reduced the CV values from 32% to 24% (Fig. 2). Despite these minor improvements in peak height uniformity, addition of ~0.4 mM MnCit (in the presence of ~0.3 mM EDTA from solvent of kit controls; see below) caused a dramatic change in the color of the fluorescent gel image of the BigDye sequencing products. This color shift was due to reducing the relative strength of the G signals ~2.5-fold and increasing the relative strength of the C and T signals ~1.5-fold, making these dyes predominate (data not shown).

The optimal MnCit concentration for each of the kits depended on the template and primer solvent. Using the –21/pUC primer and pGEM3Zf(+) template controls included in all three sequencing kits, peak height variability reached a minimum at ~0.6 mM MnCit (Fig. 2). In contrast when the primers and DNA were dissolved in water, the optimal concentration of

MnCit was ~ 0.3 mM MnCit. This discrepancy is due to the $-21/pUC$ primer, and pGEM3Zf(+) template controls included in these sequencing kits are dissolved in a Tris-EDTA buffer (TE), according to the manufacturer. However, this fact is not mentioned in the kit data sheets/instructions. Consequently, the final concentration of EDTA in the sequencing reaction would vary between 0.3 and 0.35 mM, depending on the amounts of each of the kit controls (primer and DNA) required for sequencing with the different kits (see legend to Fig. 2 and Methods). This amount of EDTA raised the minimal concentration of MnCit required to minimize peak height variability from ~ 0.3 mM (Fig. 2, \blacktriangle , \circ , \square) to ~ 0.6 mM (Fig. 2, \blacklozenge , \bullet). For consistent results, we currently dissolve primers and DNA templates in either water or 1–10 mM Tris-HCl (pH 8) and add 0.2–0.3 mM MnCit to the sequencing reactions.

MnCit Improves Sequencing Accuracy

Using pGEM3Zf(+) with either the M13F or $-21/pUC$ primer, we tested the effect of adding MnCit on the accuracy of sequence determinations by comparing unedited base calls with the known sequence of this plasmid. Optimal concentrations of MnCit significantly increased the accuracy of base calling by the computer algorithm. Figure 3 illustrates this effect on the number of miscalled bases for the two newer dye-terminator sequencing kits. Earlier we noted that with the rhodamine kit, MnCit significantly increased the total number of correctly called bases (on average, by 40 within the first 750 nucleotides) and extended the length of sequence with equivalent base-calling accuracy by ~ 50 –150 nucleotides (Korch and Drabkin 1999). The dRhodamine and BigDye sequencing kits produced more accurate base calls than the rhodamine kit (cf. Figs. 3 and 2 of Korch and Drabkin 1999). Addition of MnCit increased accuracy with the dRhodamine kit, but not with the BigDye kit. In reflection of the MnCit effect on peak height uniformity, the dRhodamine kit with MnCit addition produced the most accurate sequence determinations (<11 miscalled bases between nucleotides 51 and 750), exceeding the results seen with the BigDye kit (Fig. 3). Because of weak signal strength in the first 50 nucleotides from the primer and decreased resolution by the gel matrix above 500–600 bases, all three kits showed elevated base-calling error rates in these regions.

Generally, the 5'-AG-3' dinucleotides are the most problematic for accurate base calling because of the so-called "suppression" of Gs that follow As. Additions of optimal amounts of MnCit significantly reduced G suppression for both the rhodamine (shown earlier) and dRhodamine results. In many cases, the G peak following an A was as tall or taller than the A peak (see nucleotides 300–310 in Fig. 1). With the templates

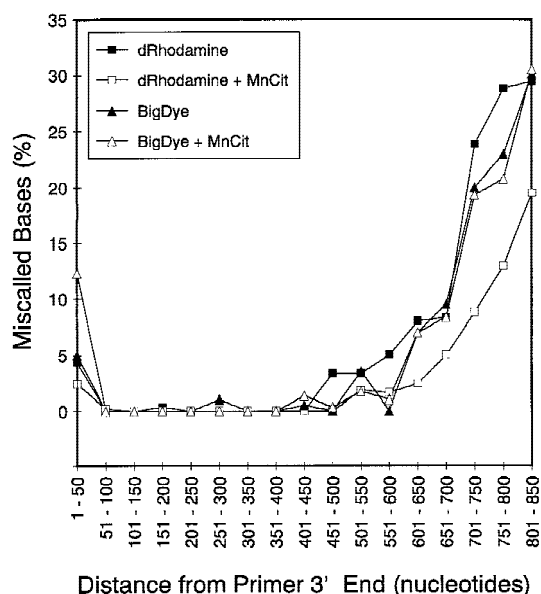


Figure 3 MnCit improves mean base-calling accuracy for the rhodamine and dRhodamine dye-terminator sequencing kits, but not for the BigDye kit. The template pGEM3Zf(+) was sequenced with either the M13F or the $-21/pUC$ primer. The unedited base calls obtained using the different sequencing kits (without or with the addition of the optimal concentration of MnCit) were aligned with the known sequence of pGEM3Zf(+). The percent miscalled bases was determined for each 50-nucleotide increment, as measured from the 3' end of the sequencing primer. In the increment 1–50, the error rate calculations do not include bases not detected, but only those incorrectly called. The mean dRhodamine values are based on 6 and 10 sequences in the absence (\blacktriangle) and presence (\triangle) of MnCit, respectively. The mean BigDye values are based on four and six sequences in the absence (\blacksquare) and presence (\square) of MnCit, respectively. Error bars are not shown for clarity.

we used, no other systematic base-calling errors were observed.

MnCit Improves Detection of Genetic Heterozygosities

The discovery that addition of MnCit to the rhodamine- and dRhodamine-sequencing reactions greatly reduced peak height variations suggested that MnCit-modified dye-terminator sequencing reactions could be used in the analysis of heterozygosity in PCR-amplified products. On comparing the sequencing results in the absence and presence of MnCit for an $\sim 1:1$ mixture of two plasmids differing at specific nucleotide positions, it is clear that MnCit makes it easier to visually detect the presence of two bases at the indicated positions of the mutations (Fig. 4). For example, in the mixed template sequence ("heterozygous" condition) in Figure 4A, the site of the mutation appears as a suppressed G in the absence of MnCit (compare with the height of the suppressed G peaks surrounding the mutation). Addition of MnCit produces peaks of more uniform height except at this site, where the peaks are distinguishable and about one-half their average

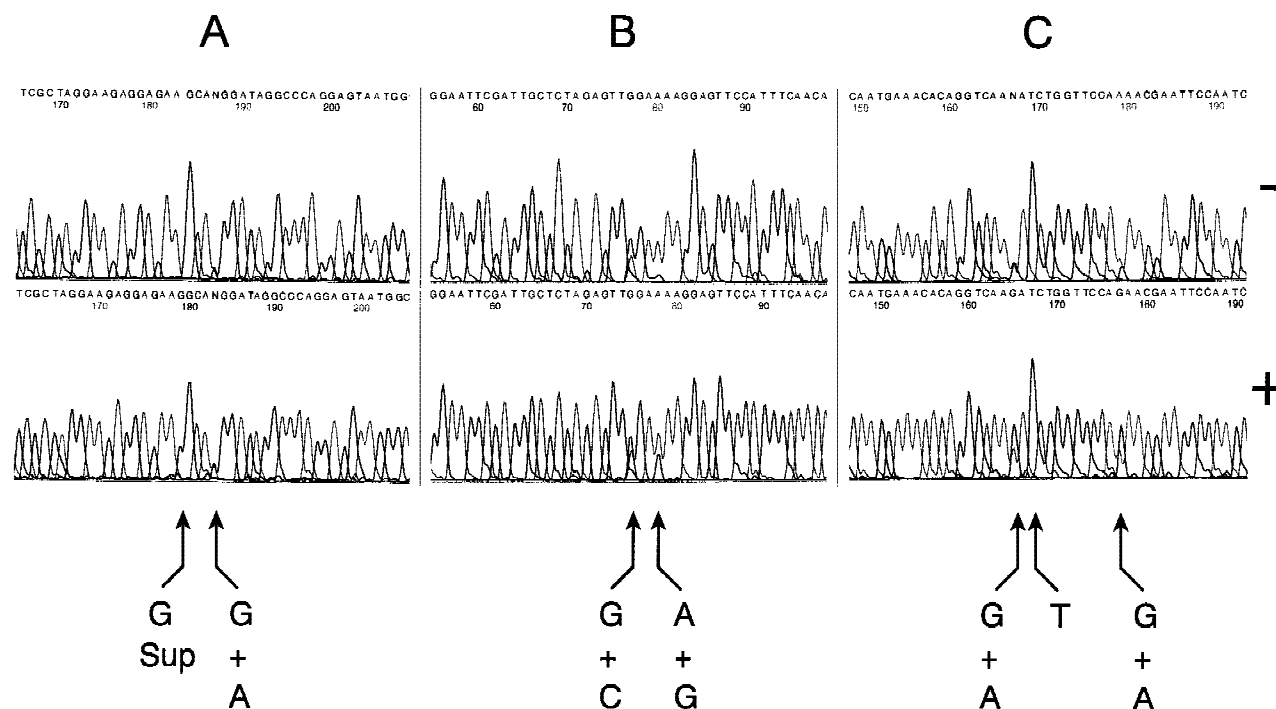


Figure 4 MnCit addition improves detection of heterozygous mutations. Compared are DNA sequence electropherograms produced by sequencing different pairs of plasmids with dRhodamine reagents in the absence (-) and the presence (+) of 0.2 mM (A) or 0.3 mM (B,C) MnCit. The templates were approximately equimolar mixtures of two plasmids containing segments of DNA that differed by single nucleotides. The arrows and letters indicate the sites of a suppressed G (G sup); mixed peaks are attributable to the DNA sequences corresponding to heterozygous mutations (e.g., G + A), with the upper letter being the taller peak; and a T peak (T) that is strong in the template with the sequence 5'-GATC-3', but not in the template with the sequence 5'-AATC-3' (data not shown).

height. By visual examination of the Figure 4A electropherograms, the site of mixed bases in the presence of MnCit can be seen as a depression in the uniform peak profile, making it easier to identify those regions of potential heterozygosity that need to be confirmed by sequencing of the complementary DNA strand. Also, base calling is improved by MnCit addition as is evident upstream of the mutation in Figure 4A, where there are two Gs that in the absence of MnCit appear as one because of G suppression.

However, MnCit addition does not even out all peak heights to produce this depression effect. Instead, MnCit addition increased the strength of some weak peaks so that the heterozygous site is more clearly evident as a peak of mixed bases (Fig. 4B,C). Moreover, higher than optimal MnCit concentrations produce three sequence profile anomalies with the rhodamine- and dRhodamine-sequencing kits. Unlike most G peaks, the strength of G peaks in the sequence 5'-CTG-3' increased disproportionately with increasing concentrations of Mn²⁺. This effect was stronger if the G preceded pyrimidines rather than purines (data not shown). In a complement of this sequence (i.e., 5'-CCAG-3'), elevated levels of Mn²⁺ caused a slight misincorporation of G dye terminator instead of A dye terminator, giving rise to an A peak with an underlying

G peak in these sequences (data not shown). In addition, although optimal amounts of MnCit produced very uniform peak heights with the dRhodamine dye-terminator kits, Ts in the sequence 5'-GATC-3' were clearly stronger than the surrounding signals. This effect, evident in Figure 4C because one of the two variant templates has the sequence 5'-GATC-3', produced a strong T peak in the mixed templates. Therefore, to avoid mistaking a heterozygosity in the sequence with one of these anomalies and to use them to flag potential sites of mixed bases, the optimal MnCit concentration should be determined for nonmutant DNA template and primer combinations, with both dissolved in water.

DISCUSSION

Tabor and Richardson (1989, 1995) compared the ability of three DNA polymerases (from phage T7, *Escherichia coli*, and *T. aquaticus*) to discriminate against ddNTPs in the presence of either 5 mM Mg²⁺ or 2 mM Mn²⁺. Substitution of manganese for magnesium reduced the discrimination against ddNTPs by 4- to 100-fold. As a result, manganese with trisodium isocitrate has been reported to improve peak height uniformity using T7 and *E. coli* DNA polymerases (Fuller 1989; Tabor and Richardson 1989, 1990) and the thermo-

stable DNA polymerases *Taq* (Brandis et al. 1996) and *Tub* (Rao and Saunders 1992). In the case of the *Taq* and *Tub* polymerases, variability in peak heights is much greater than that seen with wild-type T7 DNA polymerase and the mutant Sequenase. Tabor and Richardson (1995) reported that substitution of manganese for magnesium had no significant effect on ddNTP incorporation by the F667Y mutant *Taq* DNA polymerase (i.e., the enzyme in the PE/ABI kits). In contrast, Brandis et al. (1996) reported that KlenTaq (a genetically engineered form of *Taq* DNA polymerase lacking the 5' to 3' exonuclease domain) was strongly inhibited by manganese in the absence of magnesium and isocitrate or citrate. They did not report testing the combined effect of manganese in the presence of magnesium. Although manganese reduced peak height variability with KlenTaq, they concluded that the improvements were insufficient to warrant its routine use, because of the severe inhibition of enzymatic activity.

Recently, we demonstrated that the uniformity of sequencing peak heights could be improved by addition of MnCit to a DNA cycle-sequencing reaction based on rhodamine dye terminators with a F667Y mutant *Taq* DNA polymerase (Korch and Drabkin 1999). Here, we compare the improvements produced by MnCit with the rhodamine-based, dRhodamine, and BigDye dye-terminator sequencing kits in a reaction mixture. With these kits, the most dramatic effect of Mn²⁺ addition is to reduce the discrimination by this DNA polymerase against all dye terminators, especially increasing the incorporation of dye-labeled ddGTPs following incorporation of an unlabeled dAMP in the sequence (see Fig. 1). Rao and Saunders (1992) noted a related phenomenon when they determined with *Tub* DNA polymerase that the least amount of manganese was needed to significantly increase incorporation of ddGMPs as opposed to the incorporation of other dideoxynucleotides (0.2 mM vs. 0.4–0.6 mM).

In the absence of metal chelators such as EDTA, the optimal Mn²⁺ concentration is ~0.3 mM. Addition of equimolar amounts of the trisodium salts of either citrate or isocitrate further improves the uniformity. To obtain the most consistent MnCit optimization, it is critical not to dissolve the DNAs and primers in buffers containing metal chelators, such as EDTA. Normally, differences in DNA concentration between samples are compensated by adjusting the sample volume to obtain optimal signal strengths in DNA sequencing reactions. As a consequence, the amount of EDTA added to the reaction would vary and therefore, different concentrations of MnCit would be required to minimize peak height variability. To avoid this complication, the DNAs and primers should be dissolved in water or a weak buffer (1–10 mM Tris-HCl, pH 8). To compensate for the inhibitory effect of MnCit and to avoid produc-

ing artifacts resembling heterozygous sequences, it may be necessary to determine the optimal concentrations of MnCit, DNA, and primers for different applications.

Although the newer dRhodamine- and BigDye-based sequencing methodologies produce less variation in peak heights than the rhodamine-based method (Zakeri et al. 1998; this paper), they cannot be used on the PE/ABI 373A sequencer without a costly upgrade of the filter wheel, which because of spectral incompatibilities cannot be used with the dyes on genotyping primers. As we pointed out earlier (Korch and Drabkin 1999), addition of MnCit to the rhodamine-based dye-terminator sequencing reactions can therefore eliminate the need for this upgrade and retain the versatility of this sequencer. Using this instrument with 48-cm [well-to-read (WTR)] 5% Long Ranger gels and the rhodamine kit with MnCit addition, we found the first N in the sequence usually appeared after 650 nucleotides from the primer.

Furthermore and significantly, the dRhodamine-based dye-terminator sequencing chemistry with the addition of MnCit produces electropherograms with the most uniform peak height, even more uniform than those produced by the energy-transfer dyes (BigDyes), resulting in the most accurate sequence determinations. In fact, the dRhodamine kit with 0.3 mM MnCit yielded a CV of 18%. In comparison, Rosenblum et al. (1997) obtained a CV of 26% for sequencing the same DNA with dye-labeled primers. Also, we calculated a CV of 35% from dye-primer data from McDonald et al. (1995). Consequently, the MnCit improvement of the dRhodamine sequencing kit produces the most uniform peak heights, making it ideal for the identification of heterozygous mutations in mixed templates.

METHODS

Template and Primer Preparation

The DNA template used for most of these studies, pGEM3Zf(+) (Promega Corporation, Madison, WI), was either used as provided in the sequencing kits by PE/ABI (dissolved in TE buffer: 10 mM Tris-HCl at pH 8, 1 mM EDTA) or prepared as described earlier (Korch and Drabkin 1999) and dissolved in water. The sequencing primers, listed below, were synthesized by GIBCO/BRL Life Technologies (Gaithersburg, MD) and dissolved in water, with the exception of the –21/pUC primer, which has the same sequence as the M13 forward (M13F) primer, but was supplied by PE/ABI in the sequencing kits and is dissolved in a TE buffer. The sequences of the primers tested in this study are (5' → 3'): –21/pUC and M13F (TGTA AACGACGGCCAGT); T7 (TAATACGACTCACTATAGGG); M13R (TCACACAGGAAACAGCTATGAC); M13R GC+ (GCTATGAC-CATGATTACGCC); M13 GC– (GGAAACAGCTATGACCATGATTA); SP6 (GCTATTAGGTGACACTATAGAA); SP6 GC+ (CCAAGCTATTTAGGTGACAC); and T3 (ATTAACCTCAC-TAAAGGGA). The T3 primer was tested by sequencing plas-

mids based on pBlueScript SK(–) from Stratagene (La Jolla, CA).

DNA Sequencing Protocol

DNA template (400 ng) was mixed with sequencing primer (0.8–50 pmoles) and 3 μ l of AmpliTaq DNA polymerase FS mixture in a total volume of 10 μ l. Dye-terminator cycle sequencing was performed as recommended by the manufacturer and described earlier (Korch and Drabkin 1999). We tested the dye-terminator sequencing kits from PE/ABI using ddNTPs labeled with (1) rhodamine-based dyes (rhodamine kit; PE/ABI Prism Dye-Terminator Cycle-Sequencing Ready Reaction kit with AmpliTaq DNA polymerase FS); (2) dichloro-rhodamine-based dyes (dRhodamine kit; PE/ABI Prism dRhodamine Terminator Cycle-Sequencing Ready Reaction Kit with AmpliTaq DNA polymerase FS); and (3) energy-transfer dichlororhodamine-based dyes (BigDye kit; PE/ABI Prism BigDye Terminator Cycle-Sequencing Ready Reaction Kit with AmpliTaq DNA polymerase FS). Reactions using the dRhodamine and BigDye terminators contained 600 and 200 ng of pGEM3Zf(+) DNA, respectively.

MnCit solutions (2–10 mM) were made by mixing equal volumes of a 100 mM solution of manganese acetate tetrahydrate, manganese chloride tetrahydrate, or manganese sulfate monohydrate with a 100 mM solution of either trisodium citric acid dihydrate or trisodium DL-isocitrate monohydrate and diluting to make mixed stock solutions with final concentrations of 2–10 mM of each component and stored at –20°C. An aliquot (0.5–4 μ l per 10- μ l final reaction volume) of one of the mixed stock solutions was added to the sequencing reaction mixtures to produce the desired final concentration.

Excess dye-labeled terminators and buffers were removed by ethanol precipitation at room temperature. To each 10- or 20- μ l reaction volume, 50 μ l of 0.3 mM sodium acetate (pH ~ 5, room temperature) was added and the mixture was transferred to a microcentrifuge tube containing 125 μ l of 100% ethanol (room temperature) and mixed by vortexing. The mixtures were stored at room temperature for 20 min and centrifuged in a microcentrifuge at maximum speed at room temperature. The supernatant was slowly decanted to remove the supernatant and the last drop was blotted onto paper towels. The pellets were rinsed with 1 ml of 70% ethanol (vol/vol in water), vortexed, and centrifuged as above for 10 min. Subsequently, the supernatant was decanted very slowly so the film of 70% ethanol formed as few droplets as possible on the side of the centrifuge tube. The final droplets were removed by tapping the tubes upside down on paper towels. For the Big Dye reactions, an additional rinse with 70% ethanol was required. Less than 5 μ l of rinsing solution should be left in the tube. The samples were dried in a Speed-Vac (Savant Instruments, Holbrook, NY). The pellets were resuspended by vortexing with 3 or 4 μ l of loading buffer (5:1 mixture of 100% freshly deionized formamide/2.5% blue dextran in 25 mM EDTA at pH 8). Samples were heated for 2–3 min at ~95°C, cooled on ice, and 1.75 μ l was applied to a 34-cm (WTR), 0.2-mm-thick gel of the PE/ABI 377. Alternatively, a 3- μ l sample was applied to a 48-cm WTR, 0.3-mm thick gel of an PE/ABI 373A. The PE/ABI 377 (34-cm) gels were made with 1 \times TBE (89 mM Tris, 89 mM borate, 2 mM EDTA), 6 M urea with either 4.2% polyacrylamide/bis-acrylamide (19:1) or using 5.0% Long Ranger polyacrylamide matrix (FMC Bioproducts, Rockland, ME). The PE/ABI 373A (48-cm) gels were made of 8.3 M urea, 1 \times TBE, and either 4.75% polyacrylamide/bisacrylamide (19:1) or 5% Long Ranger matrix.

Analysis of Sequence Data

Data were collected on the different instruments with the PE/ABI Prism 373 DNA-Sequencing System Data Collection (v. 1.2.1) and the PE/ABI Prism 377 DNA Sequencer Data Collection (v. 1.1). Sequence analysis was done with the PE/ABI Prism DNA-Sequencing Software (v. 2.1.1). Peak height variability was determined either by visual inspection of the electropherogram or by measuring the height of 50 or 100 individual peaks on the printed electropherograms. For the –21/pUC, T7, and M13F primers, these peaks corresponded to nucleotides 55 (base 1 of the *Xba*I site) through 104 or 154 nucleotides from the 3' end of the –21/pUC or M13F primer on the plasmid pGEM3Zf(+) (GenBank accession no. X65306). For the M13R primer, the peaks corresponded to bases 135–234 from the 3' end of the primer on the same template DNA. Coefficients of variations (CVs = 100% \cdot [s.d./mean]) of the peak heights were determined for all bases in these regions as well as for each of the four bases. Base-calling accuracy was determined by comparing the sequence called by the PE/ABI algorithm to the known sequence of pGEM3Zf(+) as described earlier (Korch and Drabkin 1999). The relative incorporation of dye-labeled terminators was estimated by summation of the average strength of peak signals for the four bases. These estimates of “activity” are subject to inaccuracies in loading small sample volumes.

Mixing of plasmid DNAs containing variants of different genes (obtained from R. Calvo and H. Drabkin) were performed to test for the effect of MnCit on the detection of heterozygous mutations. The DNA concentrations were quantified by (1) visual estimations of band intensities after electrophoresis on agarose gels containing ethidium bromide and (2) sequencing the individual plasmids and using the sum of the average strength of peak signals for the four bases. Sample volumes containing equivalent amounts of DNA were mixed and sequenced with the T7 primer together in either the absence or presence of MnCit.

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