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Method Enabling Pyrosequencing on Double-Stranded DNA

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Pyrosequencing is a new nonelectrophoretic, single-tube DNA sequencing method that takes advantage of co-operativity between four enzymes to monitor DNA synthesis (M. Ronaghi, M. Uhlén, and P. Nyrén, *Science* 281, 363–365). Pyrosequencing has so far only been performed on single-stranded DNA. In this paper different enzymatic strategies for template preparation enabling pyrosequencing on double-stranded DNA were studied. High quality data were obtained with several different enzyme combinations: (i) shrimp alkaline phosphatase and exonuclease I, (ii) calf intestine alkaline phosphatase and exonuclease I, (iii) apyrase and inorganic pyrophosphatase together with exonuclease I, and (iv) apyrase and ATP sulfurylase together with exonuclease I. In many cases, when the polymerase chain reaction was efficient exonuclease I could be omitted. In certain cases, additives such as dimethyl sulfoxide, single-stranded DNA-binding protein, and Klenow DNA polymerase improved the sequence quality. Apyrase was the fastest and most efficient of the three different nucleotide degrading enzymes tested. The data quality obtained on double-stranded DNA was comparable with that on single-stranded DNA. Pyrosequencing data for more than 30 bases could be generated on both long and short templates, as well as on templates with high GC content.

Key Words: **pyrosequencing; DNA sequencing; template preparation; luciferase; apyrase; ATP sulfurylase; DNA polymerase; single-stranded DNA-binding protein; alkaline phosphatase; exonuclease I; double-stranded DNA; inorganic pyrophosphatase; nucleotides; luciferin.**

The ability to determine nucleic acid sequences has become increasingly important in light of efforts to sequence the large genomes of human and other organisms. The need to develop robust, high-throughput methods to replace the conventional DNA sequencing method by Sanger *et al.* (1) has led to the development of several new principles, e.g., array methods based on hybridization (2–4) and pyrosequencing (5). Pyrosequencing employs coupled enzymatic reactions to monitor the inorganic pyrophosphate (PP_i) released during nucleotide incorporation. Pyrosequencing has the advantages of accuracy, flexibility, parallel processing, and simple automation. Furthermore, the technique avoids the use of labeled primers, labeled nucleotides, and gel electrophoresis.

Although pyrosequencing is a simple method, the template preparation step prior to sequencing is still rather labor intense, mainly because single-stranded DNA (ssDNA)[2] is used as template. Direct use of double-stranded (dsDNA) would simplify the template preparation step since dsDNA can easily be prepared by polymerase chain reaction (PCR). The crucial point in the sequencing of double-stranded PCR products is purity. The excess amounts of primers and nucleotides after the amplification step must be removed prior to sequencing. Conventionally, interfering components of the PCR mixture are removed by filtration, chromatography, or gel purification methods. More recently, enzymatic methods have also been employed for removal of interfering components (6–10). For pyrosequencing, it is especially important that all PP_i is removed prior to sequencing as the method is based on detection of

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[2] Abbreviations used: PCR, polymerase chain reaction; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; PP_i, inorganic pyrophosphate; DMSO, dimethyl sulfoxide; SSB, single-stranded DNA-binding protein; CIP, calf intestine alkaline phosphatase; SAP, shrimp alkaline phosphatase; dNTP, deoxynucleosidetriphosphate; SNP, single-nucleotide polymorphism.

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PP_i. Another difficulty in sequencing double-stranded linear DNA is that hybridization of the primer to the template is interfered with with the reassociation of template strands.

Here we describe a simple and reliable procedure by which double-stranded PCR material can be sequenced by pyrosequencing.

MATERIALS AND METHODS

Synthesis and Purification of Oligonucleotides

The oligonucleotides AS-PCR-UP (5′-GCCTGGTG-GTGGGTTCGAGCC), AS-PCR-DOWN (5′-GGGCG-CCCTAGGCACAGCTG), RIT 28 (5′-AAAGGGG-GATGTGCTGCAAGGCGA), RIT 29 (5′-TGCTTCCG-GCTCGTATGTTGTGTG), 16S-388 (5′-CCARACTC-CTACGGRAGGCAGC-3′), 16S-593B (5′-biotin-GTT-YGATCCTGGCTCAGGAYDAACG), Pyro2 16S-256 (5′-GCCTAATACATGCATGTCGAGCGG), and SEQ-AS-DOWN (5′-GGGCGCCCTAGGCACAGCTG) were synthesized and HPLC purified by Interactiva (Ulm, Germany).

In Vitro Amplification

PCR reactions were performed using RIT 28 and RIT 29 for full-length amplification of the cloned *Escherichia coli* 16S rRNA gene (the 16S rRNA was a kind gift from Dr. Karl-Erik Johansson). Partial amplification of the 16S rRNA gene was performed using 16S-388 and 16S-593B. GC-rich human DNA was amplified using the primer pair AS-PCR-UP and AS-PCR-DOWN.

Solid-Phase Preparation of ssDNA Templates

Hundred-microgram streptavidin-coated super paramagnetic beads (Dynabeads M280-Streptavidin, Dynal A.S., Oslo, Norway) were washed three times by washing buffer supplied from the manufacturer. Fifty microliters of the biotinylated PCR product was added to the washed beads. The solution was incubated for 30 min at room temperature and nonbound DNA was removed by washing three times with 50 μl of washing buffer. Ten microliters of 0.10 M NaOH was added to the beads and the solution was incubated for 5 min. Subsequently, ssDNA was obtained by removing the supernatant. Immobilized ssDNA was dissolved in 10 μl 0.1 M Tris–acetate buffer, pH 7.75. Hybridization of sequencing primers to respective templates was carried out as described earlier (11). It is worth noting that both strands can be used as template for pyrosequencing.

Enzymatic Preparation of dsDNA Templates

For direct pyrosequencing on dsDNA, the PCR-amplified product was incubated with different combinations of enzymes for different time intervals and at different temperatures. The enzymes tested were (i) apyrase (Sigma Chemical Co., St. Louis, MO), (ii) HPLC-purified yeast inorganic pyrophosphatase (Sigma Chemical Co.), (iii) calf intestine alkaline phosphatase (Roche Diagnostics, Bromma, Sweden), (iv) shrimp alkaline phosphatase (Roche Diagnostics), (v) *E. coli* exonuclease I (Amersham Pharmacia Biotech, Uppsala, Sweden), and (vi) ATP sulfurylase (12). After the enzymatic treatment, the sample (5 to 10 μl) was heated to 94–100°C before the sequencing primer (2–10 pmol) was added. After incubation at 94–100°C for 1 to 5 min, the sample was immediately placed in an ice-water bath. In some cases, Klenow DNA polymerase (Amersham Pharmacia Biotech), dimethyl sulfoxide (DMSO), and single-stranded DNA-binding protein (SSB) (Amersham Pharmacia Biotech) were added individually or together to the reaction mixture to improve the sequencing quality.

Pyrosequencing

Pyrosequencing was performed at room temperature in a volume of 50 μl on an automated one-tube pyrosequencer prototype (kindly supplied by Pyrosequencing AB, Uppsala, Sweden, www.pyrosequencing.com). Briefly, 5 to 10 μl of a 50 μl PCR reaction (using enzymatic treatment or solid-phase preparation as described above) was used for an assay. Primed target DNA was added to the 50 μl pyrosequencing reaction mixture containing 8 U exonuclease-deficient (exo-) Klenow DNA polymerase (Amersham Pharmacia Biotech), 40 mU apyrase, 2.5 μg SSB, 0.1 μg purified luciferase (BioThema, Dalarö, Sweden), 25 mU purified recombinant ATP sulfurylase (12), 0.1 M Tris–acetate (pH 7.75), 0.5 mM EDTA, 5 mM magnesium acetate, 0.1% bovine serum albumin, 1 mM dithiothreitol, 5 μM adenosine 5′-phosphosulfate (APS), 0.4 mg/ml polyvinylpyrrolidone (360 000), and 100 μg/ml D-luciferin (BioThema). The sequencing procedure was carried out by stepwise elongation of the primer strand upon sequential addition of the different deoxynucleoside triphosphates (Amersham Pharmacia Biotech) and simultaneous degradation of nucleotides by apyrase.

Detection of Nucleotide Degradation Efficiency

Detection of nucleotide degrading activity was performed at room temperature in a volume of 50 μl on an automated one-tube pyrosequencer prototype. The three different nucleotide degrading enzymes tested were added to the assay mixture containing: 10 mM Tris–HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, 0.1% Tween 20, 1 mM dithiothreitol, 0.2 mM dNTP, 0.4 mg/ml polyvinylpyrrolidone (360 000), 1 ng purified

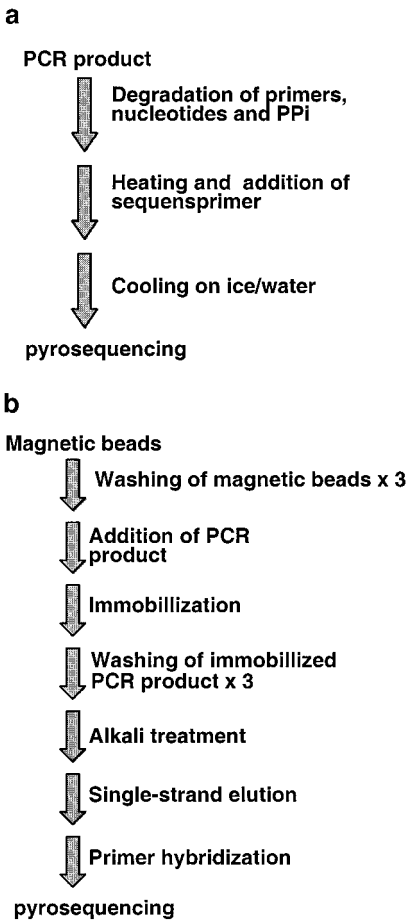

FIG. 1. A schematic representation of the principle of the DNA template preparation method for pyrosequencing on (a) dsDNA and (b) ssDNA. See the text for details.

luciferase, and 100 μg/ml D-luciferin. The reaction was started by the addition of enzyme, and the decrease in luminescence was continuously recorded.

Conventional DNA Sequencing

The pyrosequencing data was confirmed by semiautomated solid-phase Sanger sequencing (13).

RESULTS

Principle of Pyrosequencing on dsDNA

A schematic representation of the principle of the method for pyrosequencing on dsDNA is shown in Fig. 1a. PCR-amplified dsDNA is incubated with enzymes that degrade primers, nucleotides, and PP_i. After heating and addition of sequencing primer, the sample is cooled on ice. The heating step is necessary for heat inactivation of the primer-degrading activity and for assurance of efficient melting of the dsDNA before hybridization of the sequencing primer. Fast cooling is necessary for efficient primer hybridization.

In Fig. 1b the normally used (11) strategy for pyrosequencing on ssDNA is illustrated. Streptavidin-coated magnetic beads are used to prepare primed DNA template. This technology enables a biotinylated PCR product to be immobilized onto the magnetic beads. After sedimentation, the leftover from the PCR reaction is removed by washing, resulting in pure dsDNA. Single-stranded DNA is obtained by alkali treatment. Both strands can be used as template for pyrosequencing. From the schematic illustrations in Fig. 1, it is obvious that the ability to use dsDNA for pyrosequencing is a clear advantage, mainly due to the lower number of steps and the shorter hands-on time. The lower number of steps is especially important for future automation of the procedure.

Nucleotide and PP_i Degrading Enzymes

In pyrosequencing, four different nucleotides are added iteratively to the sequencing reaction and produced PP_i is continuously detected. Therefore, it is very important that all leftover nucleotides and PP_i from the PCR are degraded or removed before the sequencing procedure starts. Figure 2 illustrates the difference in efficiency of three nucleotide-degrading enzymes. The decrease in luminescence shown in Fig. 2 is due to degradation of dATP, which is a weak substrate for the firefly luciferase. Apyrase was the most efficient of the enzymes studied (Fig. 2). On unit basis, apyrase was more than 100 times as efficient as the two different alkaline phosphatases tested. The reason for the lower efficiency of the two alkaline phosphatases for degradation of nucleotides is the earlier observed (14) strong competitive inhibition by phosphate. The calf intestinal enzyme was the most efficient of the two alkaline phosphatases tested (Fig. 2).

Although apyrase is a very efficient nucleotide-degrading enzyme it cannot degrade the PP_i left over from the PCR. This is in contrast to the alkaline phosphatase enzymes that degrade not only nucleoside tri- and diphosphates, but also nucleoside monophosphates and PP_i. However, if apyrase is used in combination with enzymes, such as inorganic pyrophosphatase or ATP sulfurylase, PP_i can efficiently be removed. Inorganic pyrophosphatase hydrolyzes PP_i to phosphate, whereas ATP sulfurylase converts PP_i to ATP, which is hydrolyzed by apyrase.

Optimization of the Enzymatic Method for Template Preparation

Different combinations of enzymes were tested for the preparation of the dsDNA prior to pyrosequencing. The following combinations produced good pyrose-

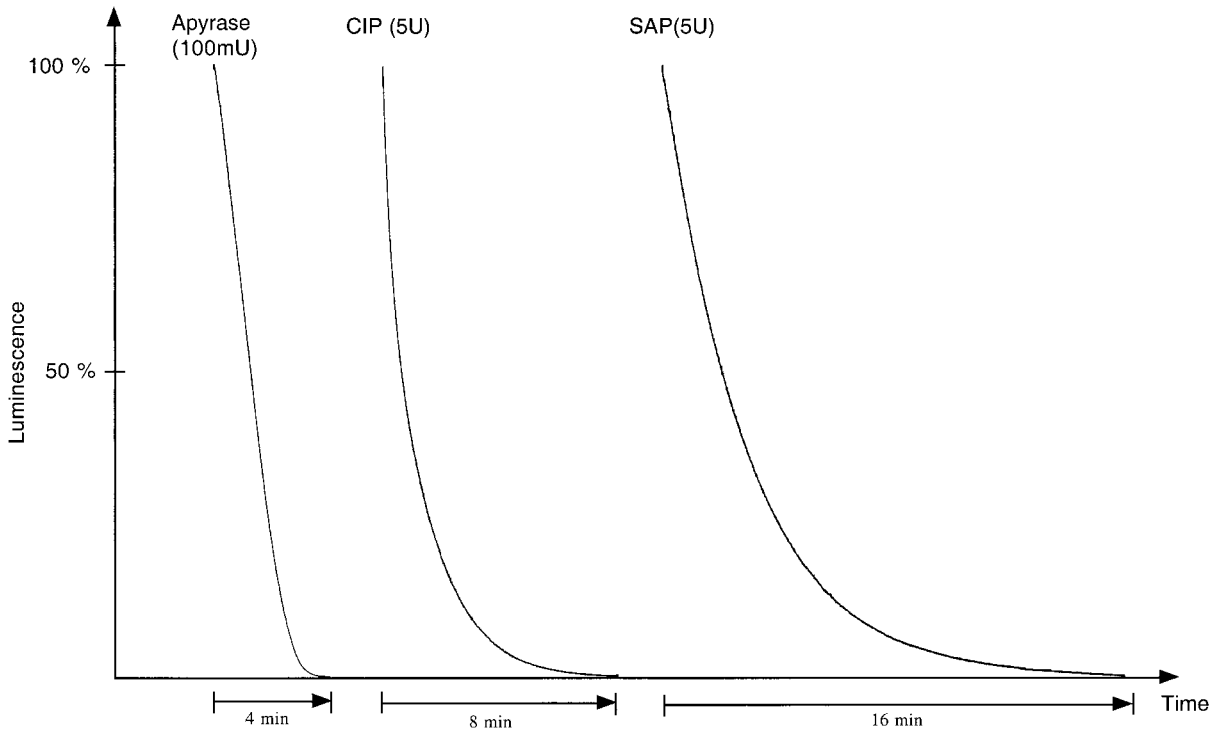

FIG. 2. Rate of nucleotide degradation for different nucleotide-degrading enzymes. The different reactions were started by the addition of the indicated enzymes and the decrease in luminescence was continuously monitored by the luminometer. CIP and SAP stand for calf intestine alkaline phosphatase and shrimp alkaline phosphatase, respectively. The reaction was performed as described under Materials and Methods.

quencing results: (i) shrimp alkaline phosphatase and exonuclease I (Fig. 3a), (ii) calf intestine alkaline phosphatase and exonuclease I, (iii) apyrase and inorganic pyrophosphatase together with exonuclease I, and (iv) apyrase and ATP sulfurylase together with exonuclease I. However, if the PCR was efficient and the primed template was incubated in the sequencing reaction for 10 min before the polymerase was added, there was no need for any additional enzymatic treatment prior to pyrosequencing (Fig. 3b). Although, we found that more robust results were obtained if exonuclease I was used, especially if higher amounts of primers were used in the PCR (Fig. 3c). When a combination of apyrase and inorganic pyrophosphatase was used for degradation of nucleotides and PP_i, the total procedure from PCR to sequencing could be performed within 5 min (Fig. 4).

In some cases, additives such as DMSO, single-stranded DNA-binding protein, and Klenow DNA polymerase decreased the background signals (Fig. 5). It is worth noting that in this study single-stranded DNA-binding protein was used as a standard ingredient in the pyrosequencing assay mixture. If the PCR products were not fully extended and/or the PCR primers formed primer dimers that were not degraded by the exonuclease I, sequence background was observed from the double-stranded template itself (Fig. 5d). This sequence background was not seen when the template was treated with Klenow DNA polymerase (Fig. 5e).

Pyrosequencing on GC-Rich and Long Templates

To investigate the feasibility of the template preparation procedure both a GC-rich and a long template were subjected to pyrosequencing. A 320-base-long template with a GC content of 64% (Fig. 6a) and a 1900-base-long template (Fig. 6b) were enzymatically prepared for pyrosequencing. Accurate data for more than 30 bases was generated (Figs. 6a and 6b).

Comparison between ssDNA and dsDNA as Template for Pyrosequencing

Traditionally pyrosequencing has been performed on ssDNA produced by a solid-phase approach (11). By this approach all leftover nucleotides and primers from the PCR are efficiently removed and clean sequencing data can be obtained. However, by using the developed enzymatic template preparation method, high quality sequence data on dsDNA can

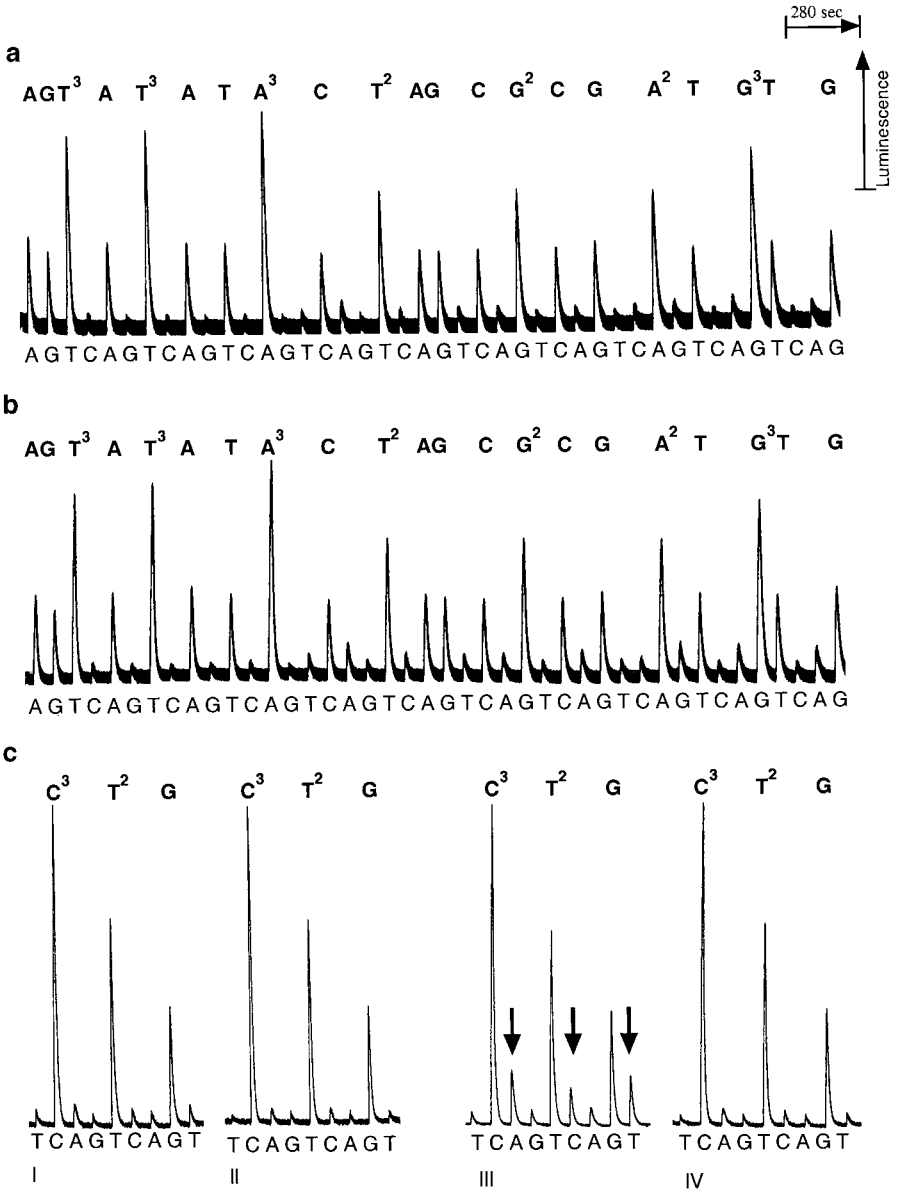

FIG. 3. The effect of exonuclease I treatment on pyrosequencing data obtained from two different templates. (a) Pyrosequencing was performed on 5 μl of a 310-base-long PCR-generated template from 16S rRNA gene enzymatically treated with 0.5 U shrimp alkaline phosphatase and 1 U exonuclease I for 20 min at 35°C and thereafter hybridized to 2.5 pmol sequencing primer (Pyro2 16S-256). (b) Pyrosequencing was performed without any prior enzymatic treatment directly on 5 μl of a 310-base-long PCR-generated template from 16S rRNA gene hybridized to 2.5 pmol sequencing primer (Pyro2 16S-256). The sequencing procedure was started with a short incubation of the primed template in the sequencing reaction mixture before the polymerase was added. (c) Pyrosequencing was performed on 10 μl of a 320-base-long fragment of a PCR-generated (5 pmol PCR primer (I and II) or 10 pmol PCR primer (III and IV) human gene with the sequencing primer SEQ-AS-DOWN. The template was enzymatically treated with 1 U shrimp alkaline phosphatase (I, II, III and IV) and 2 U exonuclease I (II and IV) for 20 min at 35°C and thereafter hybridized to 5 pmol sequencing primer. Note the increased background signals as indicated by arrows. The order of nucleotide addition is indicated on the bottom of the traces. The correct sequence is indicated above the traces. The pyrosequencing reaction was performed as described under Materials and Methods.

be obtained as well. In Fig. 7, a comparison between ssDNA and dsDNA templates is shown. The quality of the pyrosequencing data on dsDNA (Fig. 7a) was nearly as good as that generated on ssDNA (Fig. 7b).

DISCUSSION

Pyrosequencing is a newly-emerging technology for *de novo* DNA sequencing. This approach is particularly suited for short and medium-long DNA sequencing

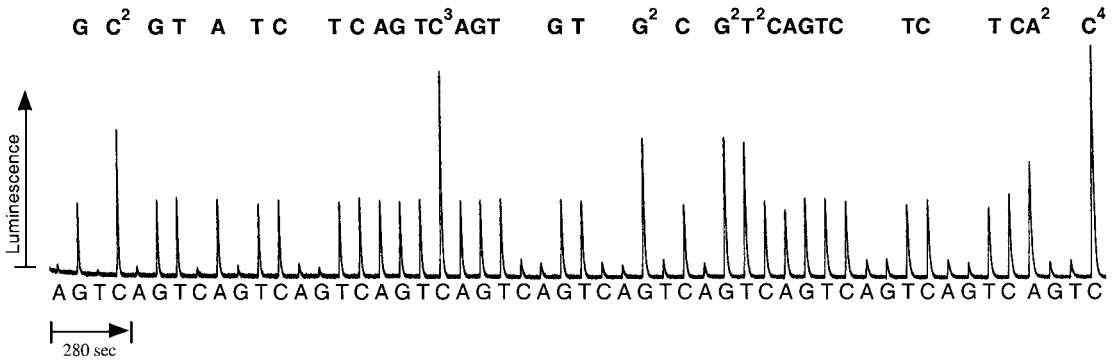

FIG. 4. Pyrosequencing data obtained on dsDNA after treatment with apyrase and inorganic pyrophosphatase. Pyrosequencing was performed on 10 μl of a 310-base-long (partial 16S rRNA gene) PCR-generated template enzymatically treated with 100 mU apyrase and 1 mU pyrophosphatase for 4 min at 37°C in the presence of 10 pmol sequencing primer, followed by heating to 100°C for 1 min, and finally cooling on ice. The order of nucleotide addition is indicated on the bottom of the traces. The correct sequence is indicated above the traces. The pyrosequencing reaction was performed as described under Materials and Methods.

projects, such as SNP genotyping and tag sequencing. Although the pyrosequencing method is simple and fast, it has so far been dependent on the use of ssDNA as template. In earlier experiments, streptavidin-coated magnetic beads were used to prepare ssDNA template (5,15,16). Although very high quality sequence data are obtained on ssDNA, there are several disadvantages in using solid support for template preparation, e.g., multiple steps for preparation, long preparation time, high cost, lower capacity for longer templates, and limitations in the product yields as low primer concentration must be used in the PCR step. Due to the simplicity of producing dsDNA by PCR, it would be a great advantage if dsDNA could be used as template for pyrosequencing. In this paper, different approaches for enzymatic treatment of PCR products enabling pyrosequencing on dsDNA were investigated.

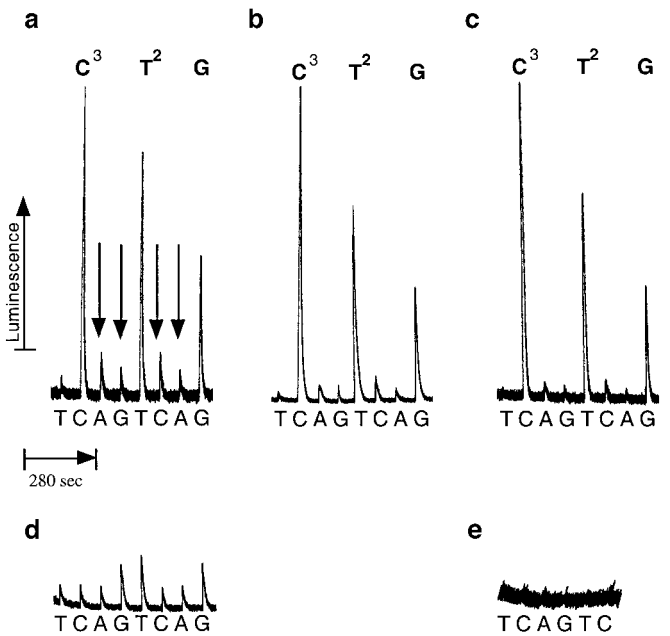

FIG. 5. The effect of additives on the pyrosequencing data quality. (a) Pyrosequencing was performed on 10 μl (a,b,c) or 5 μl (d,e) of PCR-generated template (320-base-long fragment) enzymatically treated with 1 U shrimp alkaline phosphatase and 2 U exonuclease I (a,b,c), 2 U Klenow DNA polymerase (b), 2 U Klenow DNA polymerase plus 7% DMSO and 0.25 μg SSB (c), and 2 U Klenow DNA polymerase (e), for 20 min at 35°C, and thereafter hybridized to 5 pmol sequencing primer (a,b,c). Note the increased background signals as indicated by arrows. The order of nucleotide addition is indicated on the bottom of the traces. The correct sequence is indicated above the traces. The pyrosequencing reaction was performed as described under Materials and Methods.

Four different enzyme combinations were shown to produce good results. The different combinations have different advantages and disadvantages. For instance, the simplest protocol used a combination of two enzymes, exonuclease I plus alkaline phosphatase. This combination requires a rather long incubation time as alkaline phosphatases are strongly inhibited by phosphate, which is a product in the nucleotide degradation reaction. A combination of apyrase with either inorganic pyrophosphatase or ATP sulfurylase allowed a much faster template preparation procedure. The most inexpensive procedure utilizes apyrase and inorganic pyrophosphatase together with exonuclease I. The apyrase procedure is also the most robust as the enzyme quickly and efficiently degrades all nucleotides to a low level. This is extremely important as any leftover nucleotides can induce unsynchronized extensions during the pyrosequencing procedure. If the PCR is efficient, the exonuclease I treatment can be omitted, allowing addition of sequencing primer plus PP_i and nucleotide-degrading enzymes in a single step.

Independent of which enzyme combination that was used for the template preparation step, good sequencing data could often be obtained. However, in some cases the sequencing data were of lower quality. The

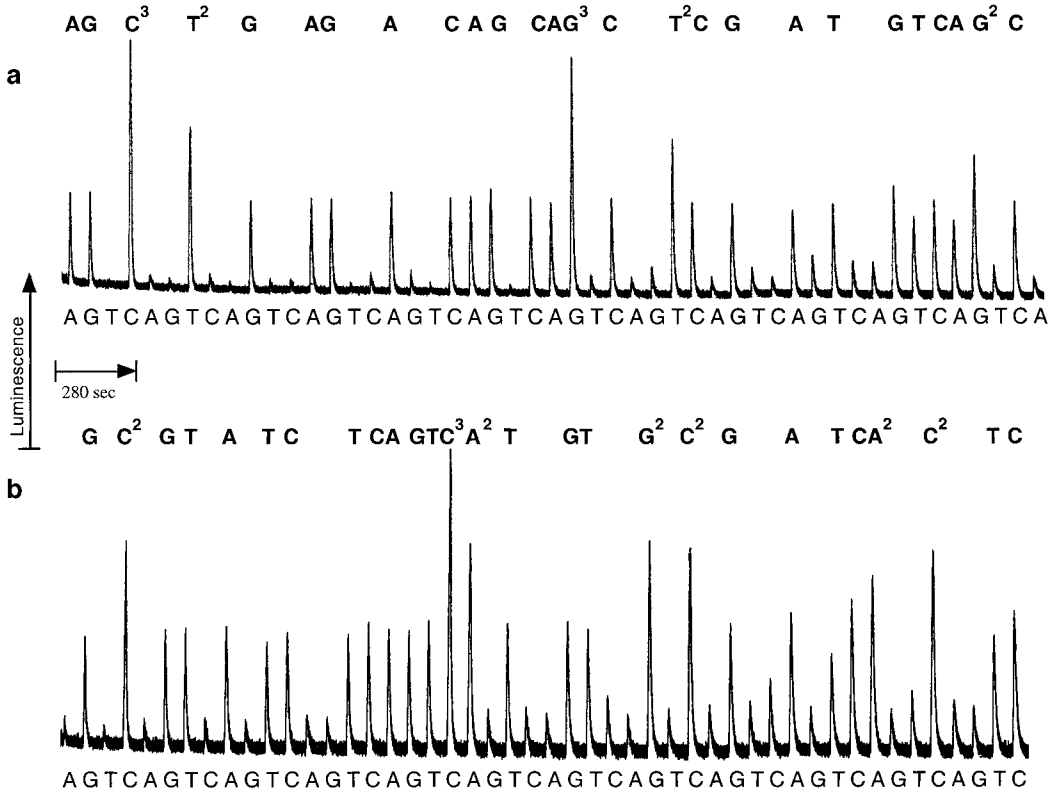

FIG. 6. Pyrosequencing data generated using GC-rich and long templates. (a) Pyrosequencing was performed on 10 μl of PCR-generated template (320-base-long with 64% GC content) enzymatically treated with 2 U Klenow DNA polymerase, 7% DMSO, 0.25 μg SSB, 1 U shrimp alkaline phosphatase, and 2 U exonuclease I for 20 min at 35°C. (b) Pyrosequencing was performed on 10 μl of PCR-generated template (1900-base-long 16S rRNA gene) enzymatically treated with 1 U shrimp alkaline phosphatase and 2 U exonuclease I for 30 min at room temperature. The order of nucleotide addition is indicated on the bottom of the traces. The correct sequence is indicated above the traces. The pyrosequencing reaction was performed as described under Materials and Methods.

main reasons for the lower data quality were (i) primer dimers produced in the PCR, (ii) primer dimers produced by the sequencing primer, (iii) unspecific priming, (iv) frayed PCR product, and (v) inefficient primer degradation by exonuclease I. Primer dimers and unspecific priming can be avoided by careful primer design, the application of stringent conditions, and the use of "hot-start" PCR. However, if high flexibility in the primer design is desired it is difficult to avoid the problem with primer dimers and unspecific priming. In such cases, addition of SSB and DMSO to the PCR reaction and/or to the sequencing reaction helped. If the PCR product was frayed, treatment with Klenow DNA polymerase improved the result by filling in "ragged" ends. We also observed that the template yield was increased if the PCR product was treated with Klenow DNA polymerase. This might be due to the stronger strand-displacement activity of Klenow compared to Taq DNA polymerase, which may allow for an additional amplification cycle in the presence of a double-stranded structure. A similar effect has been observed earlier (17). The PCR primer degradation, catalyzed by exonuclease I, was improved by addition of SSB and DMSO. To further improve the sequence data quality and the specificity of sequence data, a sequencing primer distinct from the PCR primers can be used.

If the enzymatic procedure for purification of dsDNA prior to DNA sequencing is used, several factors should be considered for optimal results. For instance, the amount of PCR product used is important, as a too large amount can affect the pH and salt concentration of the sequencing mixture. The pH of the PCR reaction is normally around 8.4, whereas the pH of the sequencing reaction is 7.75. Under our conditions 20 μl of the PCR product could be used in a 50-μl reaction volume without adjusting the pH. Another important factor is the type and amount of enzymes used in the template treatment step. It must be possible to inactivate the primer-degrading enzyme before the hybridization step to avoid degradation of the sequencing primer. Exonuclease I is thermosensitive and irreversibly inactivated at 95°C. Shrimp alkaline phosphatase, apyrase, and ATP sulfurylase are also inactivated irreversibly at high temperature, whereas both calf intestine alkaline phosphatase and yeast inorganic pyro-

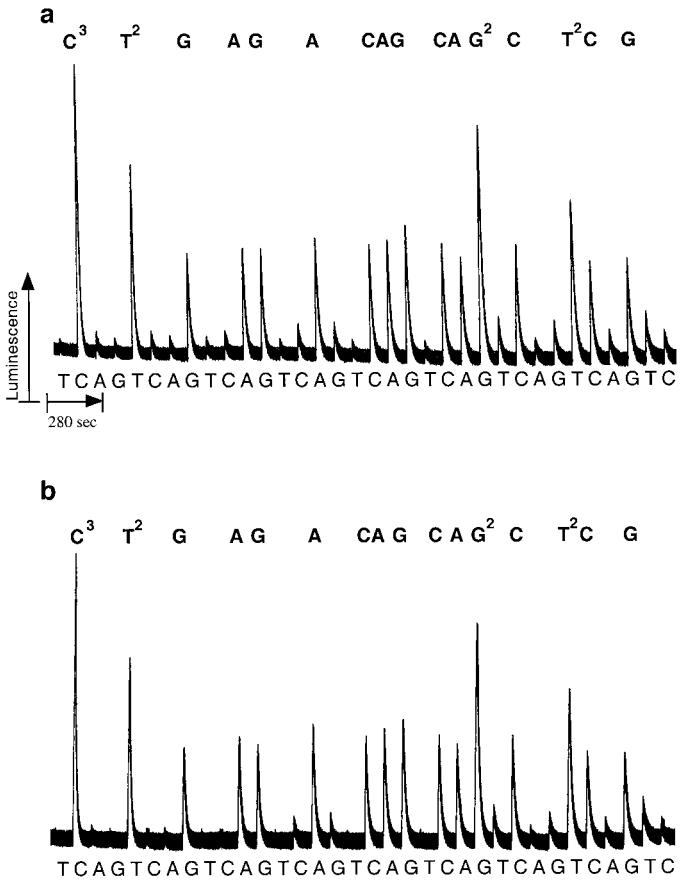

FIG. 7. Comparison between ssDNA and dsDNA as template for pyrosequencing. (a) Pyrosequencing was performed on 10 μl of PCR-generated template (320-base-long) enzymatically treated with 1 U shrimp alkaline phosphatase and 2 U exonuclease I for 20 min at 35°C. (b) Pyrosequencing was performed on ssDNA template (320-base-long) produced using solid-phase template preparation. The order of nucleotide addition is indicated on the bottom of the traces. The correct sequence is indicated above the trace. The pyrosequencing reaction was performed as described under Materials and Methods.

phosphatase retain some activity after heat treatment. However, some leftover activity of these enzymes is acceptable as long as the apyrase and ATP sulfurylase activities in the sequencing reaction mixture are dominant.

In conclusion, we have demonstrated the possibility of using double-stranded PCR products for pyrosequencing. The described procedure is simple and fast with a low number of steps and short hands-on time. The simplicity of the procedure makes it particularly suited for automation and high-throughput sequence-based analysis. An integrated, efficient, and cost-effective system for template preparation and pyrosequencing can be envisioned.

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