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CircumVent[™] Thermal Cycle Sequencing and Alternative Manual and Automated DNA Sequencing Protocols Using the Highly Thermostable Vent_R[™] (exo⁻) DNA Polymerase

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ABSTRACT

CircumVent[™] thermal cycle and standard DNA sequencing protocols utilizing the cloned and highly thermostable $Vent_R^{TM}$ (exo⁻) DNA polymerase are described. The thermal cycle sequencing procedures are advantageous because they allow fast and simple semiautomation of the sequencing reaction; make possible the direct DNA sequencing of PCR products, bacterial colonies and phage plaques; require only femtomoles of template DNA; eliminate the requirement of an independent primer annealing step; remove the requirement of denatured plasmids for sequencing double-stranded templates; and use a highly thermostable DNA polymerase for sequencing through potential recalcitrant secondary structure domains and large linear double-stranded DNA templates such as λ derivatives. More standard methods of DNA sequencing (i.e., a one-step protocol and a labeling-termination protocol) are also presented. For each protocol, alternatives for choice of label and method of labeling are presented, including the use of 5' biotinylated primers for chemiluminescent DNA sequencing and fluorinated primers for automated sequencing using the BaseStation[™] Automated DNA Sequencer.

INTRODUCTION

The VentTM DNA polymerase (New England Biolabs, Beverly, MA) was isolated from *Thermococcus litoralis*, a novel species of extreme thermophile archaebacterium collected from a submarine thermal vent near Naples, Italy (3,11). The polymerase gene was cloned into *E. coli*, the DNA sequenced and characterized. *In vitro* mutagenesis was utilized to selectively remove the $3' \rightarrow 5'$ proofreading exonuclease activity from the Vent_RTM DNA polymerase, creating the modified enzyme, the Vent_R (exo⁻) DNA polymerase. The exonuclease deficient polymerase retains a 2-fold lower error rate than that of *Taq* polymerase and maintains other biochemical properties found with the native Vent DNA polymerase, with half-lives of >1 h at 100°C (H. Kong, R. Kucera, D. Comb, F. Perler and W. Jack, unpublished observations).

Methods for the use of the Vent_R (exo⁻) DNA polymerase for dideoxy chain-termination DNA sequencing (13) have been developed. The absence of inherent exonuclease activities, a high affinity for a DNA template and the high thermostability of the polymerase allow accurate Sanger dideoxy DNA sequencing information to be obtained from templates, including those which inhibit efficient polymerization at lower reaction temperatures due to complex secondary structure. The DNA sequencing protocols can be divided into three methods: a thermal cycle sequencing method using the CircumVentTM Thermal Cycle DNA Sequencing Kit (New England Biolabs), a standard one-step method and a labeling-termination method. For each, a protocol for radioactive label incorporation (either [α -³²P] dATP or [α -³⁵S] dATP) and a protocol for using a 5' end-labeled primer is presented. The end-labeled protocols are compatible with Uniplex™ (Millipore, Bedford, MA) or Phototope™ (New England Biolabs) chemiluminescent (nonisotopic) DNA sequencing (7) or automated DNA sequencing reactions using primers endlabeled with fluorescein. The one-step sequencing reaction is particularly useful for sequencing single-stranded DNA templates, while the labeling-termination reaction is useful for double-stranded, as well as, single-stranded templates. Thermal cycle sequencing is useful for both double-stranded and single-stranded templates and offers a fast, easy protocol requiring much smaller amounts of template in the sequencing reaction. This method eliminates separate primer annealing steps and the double-stranded template denaturation step before beginning the sequencing reaction. Thermal cycle sequencing enables direct sequencing from a single bacterial colony, phage plaque or PCR product.

MATERIALS AND METHODS

DNA Sequencing Using the Ventr (exo) DNA Polymerase

One-step sequencing protocol. The annealing reaction included 0.4 pmol single-stranded template DNA (ca. 1 µg single-stranded DNA 7.5 kb in length) or 0.8 pmol denatured double-stranded template DNA (ca. 2 µg double-stranded DNA 3.5 kb in length), 0.6 pmol primer for single-stranded template DNA (ca. 5 ng of a 24-mer oligonucleotide primer) or 1.2 pmol primer for double-stranded template DNA (ca. 10 ng of a 24-mer oligonucleotide primer), 1.5 µl 10× (exo⁻) DNA polymerase sequencing buffer and distilled water to a total volume of 11 µl. For single-stranded templates, reactions were incubated at 60°C for 6 min followed by incubation at 42°C for 20 min; for double-stranded templates, incubations were at 42°C for 20 min. After annealing, 2 µl of either $[\alpha^{-32}P]$ dATP or $[\alpha^{-35}S]$ dATP (500 to 3000 Ci/mmol, 10 mCi/ml; Du Pont NEN, Boston, MA) were added, followed by 1 μ l (2 units) of the Vent_R (exo⁻) DNA polymerase. This mixture $(3.2 \,\mu l)$ was then distributed to each of the 4 A, C, G, and T tubes, each containing 3 µl of the respective Vent_R (exo⁻) DNA polymerase deoxy/dideoxy termination mixture. The reactions were incubated at 72°C for 10 min before addition of 4 µl of stop/loading dye solution. Reactions using 5' end-labeled primers were performed identically, except that the annealing reaction was brought up to a final volume of 13 µl total volume, a 5' end-labeled primer was used and the labeled nucleotide was eliminated from the reaction.

Labeling-termination sequencing protocol. The annealing reaction was identical to that described in the one-step sequencing protocol, except the final volume of the annealing reaction was 9 µl. To the completed annealing reaction, the following were added: 2.0 µl of Vent_R (exo⁻) DNA polymerase labeling mixture V, 2 µl of either [α -³²P] dATP or [α -³²S] dATP (500 to 3000 Ci/mmol, 10 mCi/ml) and 1 µl (2 units) of Vent_R (exo⁻) DNA polymerase. The reaction was incubated for 5 min at 42°C, after which 3.2 µl were distributed to each of the 4 A,C,G,T reaction tubes containing 3 µl of the respective Vent_R (exo⁻) DNA polymerase deoxy/dideoxy termination mixture. Reactions were incubated at 72°C for 10 min before addition of 4 µl of stop/loading dye solu-

tion. Reactions using 5' end-labeled primers were performed identically, except that the annealing reaction was brought up to 11 μ l total volume, a 5' end-labeled primer was used, the labeled nucleotide was omitted from the final reaction and Vent_R (exo⁻) DNA polymerase labeling mixture VK was substituted for Vent_R (exo⁻) DNA polymerase labeling mixture V, in the labeling step.

CircumVent thermal cycle sequencing protocol. The following were combined in a 0.5-ml microcentrifuge tube: 0.04 pmol single-stranded template DNA (ca. 100 ng singlestranded DNA 7.5 kb in length) or 0.1 pmol nondenatured double-stranded template DNA (ca. 250 ng double-stranded DNA 3.5 kb in length); 0.6 pmol primer for single-stranded template DNA (ca. 5 ng of a 24-mer oligonucleotide primer) or 1.2 pmol primer for double-stranded template DNA (ca. 10 ng of a 24-mer oligonucleotide primer); 1.5 µl 10× Vent_R (exo⁻) DNA polymerase sequencing buffer; 1 µl 30× Triton[®] X-100 solution and distilled water to a total volume of 12 μ l. Two microliters of $[\alpha^{-32}P]$ dATP or $[\alpha^{-35}S]$ dATP (500-3000 Ci/mmol) and 1 µl (2 units) of Vent_R (exo⁻) DNA polymerase were then added. This reaction mixture (3.2 µl) was distributed to each of the 4 A,C,G and T tubes containing 3 µl of the respective Vent_R (exo⁻) DNA polymerase deoxy/dideoxy termination mixture. Each reaction was overlayed with a drop of sterile mineral oil and the tubes placed in the thermal cycler that had been preset with the reaction times and temperatures (see thermal cycle sequencing parameters below). After the thermal cycling was completed, 4 µl of stop/loading dye solution were added beneath the mineral oil in each reaction tube. Thermal cycle sequencing with 5' end-labeled primers was performed identically except that the template DNA amounts were reduced by 10-fold (i.e., 0.004 pmol single-stranded DNA or 0.01 pmol double-stranded DNA), an end-labeled primer was utilized and 2 µl water were substituted for the 2 µl labeled nucleotide in the reaction.

Thermal cycle sequencing reactions for the BaseStation[™] Automated DNA Sequencer (Millipore). The following were combined in a 0.5-ml microcentrifuge tube: 0.2 pmol single-stranded template DNA or 0.4 pmol nondenatured double-stranded template DNA, 2 pmol primer for single-stranded template DNA or 4 pmol primer for doublestranded template DNA, 1.5 µl 10× Vent_R (exo⁻) DNA polymerase sequencing buffer, 1 µl 30× Triton X-100 solution and distilled water to a total volume of 14 µl. Vent_R (exo⁻) DNA polymerase (2 μ l 4 units) was then added. This reaction mixture (3.5 µl) was distributed to each of the 4 A,C,G and T tubes containing 3 µl of the respective Vent_R (exo⁻) DNA polymerase deoxy/dideoxy termination mixture. Residual mineral oil contamination of the aqueous reactions can fluoresce and interfere with image analysis in the BaseStation; therefore, mineral oil was not used to cover the reactions for thermal cycling. Rather, a heating block, set to 115°C, was placed on the reaction tubes in the cycler. This effectively prevented condensation of the reactions on the walls and sides of the microcentrifuge tubes, maintaining the reaction volume and conditions. The tubes were placed in the thermal cycler that had been preset with the reaction times and temperatures (see thermal cycle sequencing parameters, below). After the thermal cycling was completed, the reactions were concentrated to approximately 1 µl by heating at 95°C for 4 min (with the microcentrifuge tubes open). Three microliters of a modified stop/loading dye solution (95% formamide, 3 mg/ml dextran blue) were added to each reaction tube. (The standard stop solution/loading dye contains bromophenol blue and xylene cyanol, which interfere with image analysis.) Primers were 5' end-labeled with 5- (and 6-) carboxyfluorescein with a 6carbon amine spacer (New England Biolabs Organic Synthesis Division or Genosys, The Woodlands, TX), allowing sequence information to be detected by a charge coupled device (CCD) camera with the ability to capture fluorescein excited light emission from the DNA in the sequencing gel. Gel electrophoresis and image analysis were performed as described in the BaseStation User manual. Data were collected and analyzed on a Sun SPARCstation computer (Sun Microsystems, Mountain View, CA).

Denaturation of double-stranded templates for the one-step and labeling-termination protocols. Double-stranded template DNA (0.8 pmol) was resuspended in a total volume of 20 μ l water. Two microliters 2 M NaOH, 2 mM EDTA were added and denaturation was allowed to proceed at 25°C for 5 min. Seven microliters of water, then 7 μ l 3 M sodium acetate (NaOAc), pH 6.0, were added to the mixture, and after ensuring neutralization by spotting 1 μ l on a piece of pH indicator paper, 75 μ l of ice-cold 100% ethanol were added. The mixture was placed at -70°C for 15 min. The precipitated DNA was pelleted in a microcentrifuge for 10 min, after which the supernatant was carefully removed. The pellet was washed with 500 μ l ice-cold 70% ethanol and dried in a vacuum concentrator (Savant Instruments, Farmingdale, NY) for 10 min. The DNA was stored at -20°C until use.

Radioactive end-labeling of sequencing primers with $[\gamma - 3^2 P]$ **rATP.** The following were combined in a 1.5-ml microcentrifuge tube: 13.5 µl water, 2.5 µl 10× T4 polynucleotide kinase buffer (500 mM Tris-HCl, pH 7.6, 100 mM MgCl₂, 50 mM dithiothreitol [DTT]), 7 µl $[\gamma - 3^2 P]$ rATP (3000 Ci/mmol; Du Pont NEN), 1 µl (10.5 pmol of 24-mer) of stock primer solution (stock = 2.5 µg primer resuspended in 30 µl of water) and 1 µl T4 polynucleotide kinase (New England Biolabs, 10 000 U/ml). The reaction proceeded at 37°C for 30 min before being placed at 95°C for 5 min. The reaction was briefly subjected to vortex mixing at room temperature, before storage at -20°C until use.

Template preparations. Single-stranded M13 DNA was obtained from New England Biolabs. Double-stranded template DNA was prepared as CsCl-purified DNA, by standard alkaline lysis (2), by boiling minipreparation protocols (12) or by Qiagen column chromatography (Qiagen, Chatsworth, CA). Cosmid templates were prepared as minipreparations or as CsCl-purified templates and were provided by D. Smith (Collaborative Research, Waltham, MA). Bacterial colony direct plasmid preparation was performed as follows (8,21): a colony from a fresh agar plate was resuspended by gentle vortex mixing in 12 µl of 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 50 µg/ml proteinase K. The suspension was incubated at 55°C for 15 min, then at 80°C for 15 min and finally on ice for 1 min. The reaction mixture was then microcentrifuged at high speed for 3 min, and 9 µl of the supernatant were directly used for DNA sequencing by the end-labeled primer Circum-Vent thermal cycle sequencing protocol. Sequencing from individual phage plaques (8) was performed identically to the bacterial colony sequencing protocol except that the top agar containing the plaque was utilized as the source for the template DNA. PCR products were directly sequenced using the ³²P end-labeled primer CircumVent thermal cycle sequencing protocol. The PCR products were alcohol-precipitated, resuspended in a 50- μ l volume of water and drop-dialyzed using a type VS 0.025- μ membrane (Millipore) (14) for 1 h each against 200 ml of sterile distilled water at room temperature. Based upon DNA estimation from agarose gel electrophoresis of the recovered DNA (>90% recovery), the appropriate amount of dialyzed solution to use in the sequencing reaction was determined.

Thermal cycle sequencing parameters. Thermal cycle sequencing for manual sequencing was performed on either a Techne PHC-2 (Princeton, NJ) or a Perkin-Elmer (Norwalk, CT) DNA Thermal Cycler 480 using the following conditions: 20 cycles of 95°C for 20 s, 55°C for 20 s, 72°C for 20 s. For the BaseStation reactions, 30 cycles were performed, utilizing the above conditions.

Gel electrophoresis and processing for autoradiography (manual sequencing analysis). Denaturing polyacrylamide gel electrophoresis was performed using 6% polyacrylamide gels containing 7 M urea and $1 \times TBE$ ($1 \times TBE = 0.1$ M Tris-HCl, 0.08 M boric acid, 0.001 M EDTA, pH 8.8) (15) (premixed reagents from American Bioanalytical, Natick, MA) on Owl Scientific Plastics (Cambridge, MA) Model S1S or S2S electrophoresis units. Some gels were precast and supplied with buffer by Tangent BioApplications (Beverly, MA). Radioisotopic gels (³²P and ³⁵S reactions) were processed for autoradiography by previously described methods (19). Gels containing chemiluminescent reactions were processed as previously described (7), although some were electroblotted using a vertical or horizontal electroblotter (Owl Scientific Plastics HEP or VEP) for 10 min at 1500 mA, 7 V (constant current), onto the Immobilon-S membranes (Millipore). DNA was cross-linked onto the membrane using a programmable heating cross-linker apparatus (Automated Biosystems, Model XL-254, Essex, MA) at 33 mW/cm². Detection procedures were carried out in a modified roller drum apparatus (Owl Scientific Plastics) on a roller (Beseler Model 8922, Linden, NJ), except for the final Lumiphos[™] (Lumigen, Detroit, MI) step, which was carried out in a sealed bag. Autoradiograms were manually processed.

Composition of DNA sequencing reagents:

Stop/Loading Dye Solution: Deionized formamide containing 0.3% bromophenol blue, 0.3% xylene cyanol FF, 0.37% disodium EDTA, pH 7.0.

 $10 \times Vent_{R}$ (exo⁻) DNA polymerase sequencing buffer: 100 mM KCl, 100 mM (NH₄)₂SO₄, 200 mM Tris-HCl, pH 8.8, 50 mM MgSO₄.

Vent_R (exo⁻) DNA polymerase labeling mixture VK: 25 μ M dATP, 25 μ M dCTP, 25 μ M dCTP, 25 μ M dTTP.

Vent_R (exo⁻) DNA polymerase labeling mixture V: 25 μ M dCTP, 25 μ M dGTP, 25 μ M dGTP.

30× Triton X-100: 3% Triton X-100.

Vent_R (exo⁻) DNA polymerase deoxy/dideoxy termination mixtures: A Mixture, 900 μ M ddATP, 30 μ M dATP, 100 μ M dCTP, dGTP and dTTP; C Mixture, 480 μ M ddCTP, 30 μ M dATP, 37 μ M dCTP, 100 μ M dGTP and dTTP; G Mixture, 400 μ M ddGTP, 30 μ M dATP, 37 μ M dGTP, 100 μ M dCTP and dTTP; T Mixture, 720 μ M ddTTP, 30 μ M dATP, 33 μ M dTTP, 100 μ M dGTP and dCTP. All mixtures also contained 5 mM MgSO₄, 20 mM Tris-HCl, pH 8.8, 10 mM (NH₄)₂SO₄ and 10 mM KCl.

RESULTS AND DISCUSSION

We have developed a series of DNA sequencing protocols utilizing the Vent_R (exo⁻) DNA polymerase. In the one-step protocol, all dNTPs and ddNTPs are present in a set of 4 primed synthesis reactions that occur at 72°C. Figure 1 shows single- and double-stranded template DNA sequencing utilizing this protocol, incorporating ³⁵S α -labeled dNTP or using primers 5' end-labeled with ³²P. It can be seen from the resultant autoradiogram that single-stranded DNA templates provide a strong signal after an overnight exposure. However, using this protocol, the signal for double-stranded templates are weaker, requiring 2 times longer exposure of the autoradiogram to achieve the same signal intensity as observed for single-stranded templates. For this reason, a set of protocols using a labeling-termination technique, modified from those developed for use with modified T7 DNA polymerase (Sequenase[™], United States Biochemical, Cleveland, OH) (16),



Figure 1. Autoradiograms of Vent_R (exo) DIVA polymetrus one only sequencing reactions as described in the text. Panel A: M13mp18 singlestranded DNA template (New England Biolabs [NEB] #404C) sequenced with the M13 forward universal primer (NEB #1224) and [α -³⁵S] dATP incorporation; Panel B: M13mp18 single-stranded DNA template (NEB #404C) sequenced with a³²P 5' end-labeled (kinased) M13 forward universal primer (NEB #1224); Panel C: pUC19 double-stranded DNA template (NEB #304-1) sequenced with the M13 forward universal primer (NEB #1224) and [α -³⁵S] dATP label incorporation. All lanes loaded left to right: G,C,A,T. All exposures overnight without an intensifying screen, except for Panel C, which was a 48-h exposure.

Figure 2. Autoradiograms of Vent_R (exo⁻) DNA polymerase labeling-termination sequencing reactions as described in the text. Panel A: pUC19 double-stranded plasmid template (NEB #304-1) sequenced with the M13 forward universal primer (NEB #1224) and $[\alpha^{-35}S]$ dATP incorporation; **Panel B:** pUC19 double-stranded DNA template (NEB #304-1) sequenced with a ³²P 5' end-labeled (kinased) M13 forward universal primer (NEB #1224). All lanes loaded left to right: G,C,A,T, except for the right panel which was loaded C,A,T,G. All exposures overnight without an intensifying screen.

were devised. In the first step of this procedure, primed DNA synthesis in the presence of limiting concentrations of all four dNTPs extends nascent DNA chains until the dNTP pool is depleted. In the second step, the addition of high concentrations of ddNTPs and additional low concentrations of dNTPs enables DNA synthesis to continue until termination occurs,



Figure 3. Autoradiograms of Vent_R (exo⁻) DNA polymerase thermal cycle sequencing reactions as described in the text. Panel A: pUC19 double-stranded DNA template (NEB #304-1) sequenced with the M13 forward universal primer (NEB #1224) with $[\alpha^{-35}S]$ dATP incorporation; Panel B: M13mp18 single-stranded DNA template (NEB #404C) sequenced with a ³²P 5' end-labeled (kinased) M13 forward universal primer (NEB #1224); Panel C: pUC19 double-stranded DNA template (NEB #304-1) sequenced with a biotinylated 5' end-labeled M13 forward universal primer (NEB #1224BT), followed by chemiluminescent detection (UniplexTM kit, Millipore/NEB #MBBL UNPO1). All lanes loaded left to right: G,C,A,T. All exposures overnight without an intensifying screen, except the chemiluminescent detection autoradiogram which is a 10-min exposure after a 20-min "ramp-up" time.

due to the incorporation of a ddNTP. Figure 2 shows autoradiograms of double-stranded DNA templates sequenced by the labeling-extension method, incorporating $[\alpha^{-35}S]$ dATP or using a ³²P 5' end-labeled primer. It should be noted that for single-stranded DNA templates, both the one-step and labeling-termination protocols that incorporate $[\alpha^{-35}S]$ or $[\alpha^{-32}P]$ dATP or which use ³²P 5' end-labeled primers give strong readable sequences after overnight exposure of the autoradiograms. For double-stranded templates, the labelingextension protocol provides a much stronger signal, compared with the one-step protocol, after an overnight exposure of the autoradiogram. End-labeled primer sequencing in the labeling-termination method may be useful for certain double-stranded templates that give less than optimal results with radiolabel incorporation in the labeling-termination method.

The thermal cycle sequencing protocol is modeled after that originally described by Carothers et al. (5) and as further modified (1,4,6,10,20). The cycling reactions are (essentially) linear amplifications in the presence of deoxyribonucleoside triphosphates (dNTPs) and dideoxyribonucleoside triphosphates (ddNTPs) using a single primer. Figure 3 shows the



Figure 4. Autoradiograms of $Vent_R$ (exo⁻) DNA polymerase thermal cycle sequencing reactions as described in the text. Panel A: DNA sequence obtained directly from a bacterial colony containing pUC19 double-stranded plasmid DNA (as described in the text) with a ³²P 5' end-labeled (kinased) M13 forward sequencing primer (NEB #1224); Panel B: DNA sequence obtained directly from an M13mp18 phage plaque (as described in the text) with a ³²P 5' end-labeled (kinased) M13 forward sequencing primer (NEB #1224); Panel C: DNA sequence obtained from 0.01 pmol double-stranded PCR template (purified as described in the text) with a ³²P 5' end-labeled (kinased) 20-mer primer; Panel D: DNA sequence obtained from 0.004 pmol (10 ng) M13mp18 single-stranded DNA template (NEB #404C) sequenced with the M13 forward universal primer (NEB #1224) and $[\alpha$ -35S] dATP incorporation; Panel E: DNA sequence obtained from 0.01 pmol (20 ng) double-stranded pUC19 double-stranded DNA template (NEB #304-1) sequenced with the M13 forward universal primer (NEB #1224) and $[\alpha^{-35}S]$ dATP incorporation; Panel F: DNA sequence obtained from a minipreparation of double-stranded cosmid DNA template with a ³²P 5' end-labeled primer. All lanes loaded left to right: G,C,A,T. All exposures overnight without an intensifying screen, except for Panels D and E, which were 48-h exposures.

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results of thermal cycle sequencing double- and singlestranded DNA templates incorporating $[\alpha^{-35}S]$ dATP or utilizing 5' end-labeled primers (either ³²P or biotinylated primers followed by chemiluminescent detection). The sequence signals are comparable with those obtained from the one-step and/or labeling-termination methods (Figures 1 and 2).

Using the ³²P-end-labeled primer CircumVent thermal cycle sequencing method, it is feasible to sequence plasmid DNA directly from bacterial colonies (Figure 4) or M13 or λ phage DNA directly from plaques (Figure 4) or λ phage lysates (data not presented). The protocol is also useful for direct sequencing of PCR products (Figure 4). Strong sequencing signals are obtained from as little as 0.004 pmol of single-stranded template DNA and/or 0.01 pmol doublestranded template DNA, including cosmid DNA, after an overnight exposure (no intensifying screen) (Figure 4). Even lesser amounts of template DNA can be utilized; we have sequenced 0.001 pmol of single-stranded template DNA, but exposure times, on the average, have been too long to be generally useful (2 days with an intensifying screen). Nevertheless, this may be useful in situations where DNA is limiting. Because of the ease, speed and versatility of the CircumVent thermal cycle sequencing method, it is utilized for all of our sequencing; it has been of enormous help in saving time and effort when simultaneously sequencing large numbers of templates or when only small amounts of template DNA have been available.



Figure 5. FluorogramsTM of Vent_R (exo⁻) DNA polymerase thermal cycle sequencing reactions as described in the text and as presented on the BaseStation Automated DNA sequencer. Left panel: double-stranded pGEM (Promega, Madison, WI) DNA template (with a 900-bp insert) sequenced with a 5'-fluorescein-labeled SP6 primer (New England BioLabs); center panel: M13mp18 single-stranded DNA template (with a 2-kb insert) sequenced with a 5'-fluorescein-labeled M13 forward universal primer (New England BioLabs); right panel: 833-bp double-stranded DNA template sequenced with a 5'-fluorescein 20-mer internal primer (Genosys). All lanes loaded left to right: A,C,G,T.

The CircumVent thermal cycle sequencing method is useful for automated, as well as, manual DNA sequencing. Figure 5 presents an actual computer screen display of three DNA templates (single-stranded, double-stranded plasmid and double-stranded PCR) on the BaseStation Automated DNA sequencer. The BaseStation uses a CCD camera to repetitively scan a 17-cm² area of the gel during electrophoresis; this provides an integrated Fluorogram[™] (Millipore) rather than intensity profiles. The CCD chip consists of 25 000 individual detectors, each able to register an individual photon of light emitted from the fluorescein-labeled DNA migrating in the gel. Each detector can distinguish 2¹⁶ different shades of gray, allowing high sensitivity in discrimination of false terminations (shadow bands). The data clearly indicates the usefulness of the Vent (exo-) DNA polymerase for generating sequence information in an automated DNA sequence format, with end-labeled fluorophores.

It is conceivable that different cycling parameters or other procedural modifications may be useful. As one example, it is often possible to eliminate the annealing (middle) step in the cycling reaction conditions. This can obviously be performed when the sequencing primer has a T_m above 72°C, but often is successful when the calculated T_m (9,17,18) is lower. For example, the universal M13 forward 24-mer primer (New England Biolabs catalog #1224) (15/24 G+C; T_m ca. 76°C) can be used in the thermal cycle sequencing reaction with cycle times and temperatures of 95°C for 20 s, 72°C for 20 s, 20 cycles. We have successfully used custom-synthesized primers with apparent T_m values as low as ca. 55°C with similar results (for example, an 18-mer primer 9/18 G+C, Tm ca. 54°C). The resultant band intensities on the autoradiograms are virtually identical to the band intensities of the reactions that were performed at the recommended cycle times and temperatures. When designing primers for sequencing projects, these results might be taken into consideration.

Concerning the use of the Vent_R (exo⁻) DNA polymerase, other helpful hints are worth mentioning. First, 7-deaza dATP or 7-deaza dGTP (Boehringer Mannheim, Indianapolis, IN) can be substituted in equal molar amounts, in lieu of dATP and dGTP, respectively. dITP is not recommended, as it is not incorporated efficiently by the polymerase. These substitutions may prove helpful in the resolution of sequencing or gel electrophoresis compressions.

The following sequencing patterns are characteristic of the Vent_R (exo⁻) DNA polymerase: a) The first C of a run of C's is darker than the following C's. b) The second A of a run of A's is darker than the preceding (or following) A's. c) G, following an A, tends to be darker than other G's. d) T following an A tends to be darker than other T's. These patterns differ from those of other polymerases and have been helpful in determining the sequence of difficult regions in some templates.

The use of ³³P, in lieu of ³⁵S (22) in any of the above sequencing protocols, provides a much darker signal with a shorter period of film exposure time while maintaining autoradiographic band sharpness and clarity. Despite the relatively weaker signal intensities than observed with ³²P (the β -emission is about 1/5 the intensity of ³²P), we have been able to obtain sequence directly from phage plaques, lambda phage lysates and plasmids from bacterial colonies, after overnight exposures of the autoradiograms without the use of intensifying screens (data not presented).

CONCLUSIONS

CircumVent thermal cycle sequencing with the Vent_R (exo⁻) DNA polymerase offers distinct advantages over more traditional methods. These include a simple, fast protocol; a substantial reduction of template DNA required for the sequencing reaction; the lack of a required "pre-reaction" primer annealing step; the lack of a required denaturing step for double-stranded DNA templates before initiation of the sequencing reaction; the use of a highly thermostable polymerase for sequencing through potentially recalcitrant secondary structure domains; and the versatility of sequencing PCR products, cosmids, other λ constructs, as well as DNA directly from bacterial colonies and phage plaques in both manual and automated formats. In addition to the thermal cycling protocols, we have also shown that more traditional DNA sequencing methods can be performed with the Vent_P (exo⁻) DNA polymerase when a thermal cycler is not available or when a more traditional approach is desired. We anticipate further developments with the (exo⁻) Vent_R DNA polymerase and its associated protocols to enable more rapid and easy DNA sequencing.

Note added in proof: Two additional references concerning the cloning, DNA sequencing and biochemical characterizations of the Vent_RTM polymerase family have recently been published:

Jack, W., R. Kucera and W. Kong. 1992. Biochemical characterization of VentR[™] and Deep VentR[™] DNA polymerases. The NEB Transcript (New England Biolabs) 4:4-5.

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