A New Method of Bipolymerase Sequencing Prevents "Stop-Bands"

Christopher C. Austin

Abstract

This article presents a relatively quick and cost-effective DNA sequencing method that prevents the formation of stop-bands. This method uses a combination of *Taq* and Sequenase that allows sequencing at both low and high temperatures. The ability to sequence at a high temperature appears to be the fundamental component in preventing stop-band formation in G + C rich regions.

Index Entries: Artifactual; DNA; polymerase; *Taq*; Sequenase.

Artifactual banding patterns, or stop-bands, are a common problem when sequencing DNA using the Sanger (dideoxynucleoside chain termination) method *(1)*. Radioactive labeling at the same position in all four lanes occurs frequently in G + C rich areas and is presumably the result of the stalling or pausing of the polymerase enzyme in regions of extreme secondary structure *(2)*. Stop-bands are problematic because they prevent the elucidation of the correct nucleotide sequence. Several methods have been proposed for eliminating these stop-bands *(2–5)*. Unfortunately these methods have met with only moderate and intermittent success in preventing stop-band formation.

Here I report a relatively quick and cost-effective method for eliminating stop-bands (Fig. 1). This method involves the combination of two polymerase enzymes, *Taq* and Sequenase Version 2.0 (United States Biochemical, Cleveland, OH), that, unlike other bipolymerase sequencing methods *(3)*, provides the benefit of being able to sequence both at low and high temperatures. The ability to sequence at a higher temperature appears to be the key determinant in eliminating the secondary structures that cause stop-bands. I have found that bipolymerase sequencing not only prevents the formation of hard stop-bands (Fig. 1, closed arrow), but also helps reduce ghost or background banding patterns (Fig. 1, open arrow), making the autoradiographs easier to read. This method has proven to be effective and consistent on diverse taxa (lizards and bacteriophage) and produces qualitatively better results than sequential sequencing techniques *(4)*.

Double-stranded plasmid DNA was prepared for sequencing by bringing up 2–10 µg of DNA in 19 µL of ddH_20 and 1 µL of 5*M* NaOH and incubating at 65°C for 5 min. Two microliters of 7*M* CH_3COONH_4, 9 µL of 1*M* CH_3COONa, and 14 µL of ddH_20 were added and mixed thoroughly. One hundred thirty microliters of 95% EtOH were added and this mixture was incubated at –20°C for 15 min. Samples were spun at maximum speed in a microfuge for 15 min after which time the supernatant was removed. One hundred thirty microliters of 70% EtOH were added and spun in a microfuge for another 15 min. The supernatant was discarded and the microfuge tube with the pelleted DNA was placed in a 37°C incubator for 5 min to evaporate off any remaining EtOH.

The dried samples were resuspended in 7 µL of ddH_20, 1 µL primer, and 2 µL 5X Sequenase Buffer, incubated for 2 min at 65°C, allowed to cool slowly to room temperature and then placed

Address to which all correspondence and reprint requests should be sent: Department of Zoology, University of Texas, Austin, TX 78712.

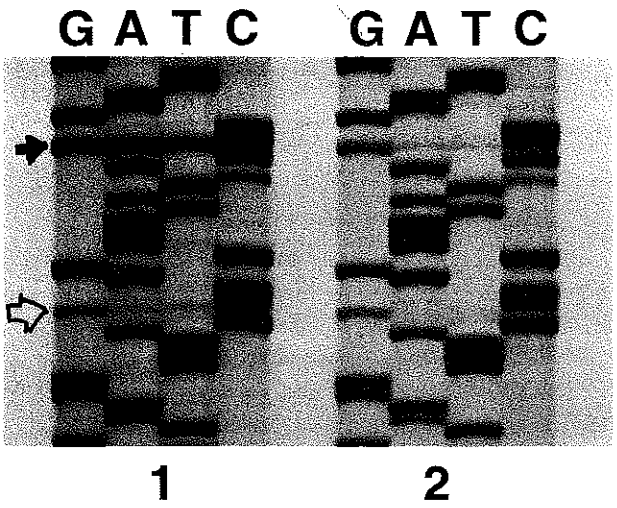

Fig. 1. Sequence gel autoradiograph of the mitochondrial cytochrome oxidase I gene from *Sphenomorphus solomonis* (Reptilia: Scincidae) showing the effects of bipolymerase sequencing. Reaction 1 is the result of normal sequencing following the Sequenase Version 2.0 T7 DNA polymerase sequencing protocol. Reaction 2 shows the effect that bisequencing has on hard stop-bands (closed arrow) and lighter stop-bands, or ghost patterns (open arrow). Reactions 1 and 2 were from an aliquot of the same DNA template and primer, and were run in parallel on an 8% acrylamide gel.

on ice (annealing reaction). One microliter of 0.1*M* DTT, 2 µL 1:5 Labeling Nucleotide Mix, 0.5 µL of ^{35}S-dATP, 0.25 µL Sequenase Version 2.0, 0.5 µL *Taq*, and 1.75 µL Enzyme Dilution Buffer were added to the annealed template-primer (note: this labeling reaction follows the Sequenase Version 2.0 T7 DNA polymerase sequencing protocol except that 0.5 µL *Taq* polymerase is added). The labeling reaction was incubated for 2–5 min at room temperature. After the labeling reaction was completed, 3.5 µL of the labeling reaction were added to each of the 4 termination tubes to which 2.5 µL of the appropriate dideoxynucleoside triphosphate (ddNTP) termination mixture had previously been added (termination reaction). These four termination reaction tubes were then incubated at 37°C for 5 min. The termination tubes were then heated at 95°C for 1.5 min, quickly spun in a microfuge, and placed in a 73°C water bath. Immediately after being placed in the 73°C bath, 2.0 µL of the appropriate dideoxynucleoside mix were again added to the termination reaction tubes. The termination tubes were then incubated at 73°C for 45 min after which time 4 µL of stop solution were added to each tube (Table 1).

Table 1
Protocol for the Elimination
of Stop-Bands Using Bipolymerase Sequencing

1. Follow the Sequenase Version 2.0 protocol for the annealing reaction.
2. Follow the Sequenase Version 2.0 protocol for the labeling reaction, except add 0.50 µL *Taq*.
3. After the labeling reaction is complete, add 3.5 µL of the labeling reaction to each of the termination tubes with 2.5 µL of the appropriate dideoxynucleoside (ddNTP) termination mixture. Incubate at 37°C for 5 min.
4. Place the four termination tubes in a 95°C bath for 1.5 min, remove and spin briefly, quickly add an additional 2.0 µL of the appropriate dideoxy mix to each tube, and then incubate at 73°C for 45 min. Add 4.0 µL of stop solution.

Acknowledgments

This work was supported by the National Science Foundation (IBN-9311139).

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