2.4. Automatic Sequencing

2.4.1. Sequenase PCR Product Sequencing Kit

Sequenase PCR product sequencing kit.

2.4.2. Gel Purification of PCR Products

1. Bio 101 gene clean kit.
2. Agarose.
3. 1X TAE: 400 m*M* Tris, 12% acetic acid, 10 m*M* EDTA.

3. Methods

3.1. Double-Stranded Sequencing

Double-stranded sequencing produces reliable sequencing if source DNA is clean (*see* **Note 1**). A cesium-chloride (CsCl) prep is not necessary, in fact, sequencing can be carried out from miniprep quantities of DNA. However, when more than one reaction per sample is required, it is convenient to make a stock of DNA by using an alkali-lysis maxiprep method. This method is outlined using the ^{T7}Sequencing kit; an alternative kit, Sequenase, by US Biochemicals, has a very similar method; instructions supplied with both kits are easy to follow.

1. Use 2–5 μg of DNA and make up to a volume of 32 μL with SDW.
2. Add 8 μL of freshly made 2*M* NaOH to denature the DNA. Vortex and leave at room temperature for 10 min.
3. Add 4 μL of SDW, 7 μL of 3*M* NaOAc, and 120 μL ice-cold ethanol to precipitate the denatured DNA. Place at –70°C for 15 min. Microcentrifuge at 12,000*g* for 15 min to pellet the DNA. The pellet will be very small and may not even be visible. Wash the pellet very gently in 70% ice-cold ethanol and microcentrifuge for 15 min at 12,000*g*.
4. Dry the pellet under vacuum and resuspend in 10 μL SDW (*see* **Note 2**).
5. Add 2 μL of primer and 2 μL of annealing buffer and incubate at 65°C for 5 min. Transfer to a 37°C water bath for 10 min. Place at room temperature for 5 min. During this time, label four tubes for each reaction A, C, G, and T. In each tube, place 2.5 μL of the appropriate termination mixture (A, C, G, or T).
6. Once the incubation period is over, add 3 μL labeling buffer, 0.5 μL of ^{35}S αdATP, and 2 μL of diluted T7 enzyme to the DNA and primer mixture (*see* **Note 3** for different method for long runs).
7. Incubate the labeling mix at room temperature for 5 min. The termination reactions must be placed at 37°C for at least 1 min before the labeling mixture is added.
8. Transfer 4.5 μL of labeling mixture to each of the four termination reaction tubes A, C, G, and T. Incubate for 5 min at 37°C. Add 5 μL of stop mixture and mix by pipeting. The reactions are now ready for loading onto a gel. They can be stored temporarily at –20°C or long-term at –70°C.