5. Heat to 100°C for 3 min in a thermal cycler and then plunge into an ice-water bath. Centrifuge briefly to collect contents, then store annealed template on ice until required (use within 4 h).
6. The product can be sequenced using the same labeling and termination reactions described in **Subheading 3.1., steps 5–8** or used as the template for automatic sequencing (*see* **Note 12**).

3.4.2.2. Gel Purification

1. Examine yield and specificity of PCR product by agarose gel electrophoresis. The whole PCR sample can be loaded directly onto the gel.
2. Use a scalpel blade or razor blade to excise the band required in the gel and place in a clean Eppendorf tube.
3. Follow the instructions supplied by the manufacturers with the Bio-101 GeneClean kit to extract the DNA sample from the agarose gel.
4. Determine the concentration of the purified DNA by agarose gel electrophoresis. Use the required amount of DNA in the automatic sequence reactions (*see* **Subheading 3.1.**).

4. Notes

1. For successful double-stranded sequencing, DNA should be clean and concentrated to approx 1 μg/mL. Check that the sample is free from RNA by electrophoresing on an agarose gel. The sample should give a smooth curve when analyzed by spectrophotometry on a spectrum between 200 and 300 nm; a convex slope in the 200 to 250-nm wavelength range indicates too much salt in the sample (to remove this, wash with 70% ethanol and dry thoroughly under vacuum); a rise at the very end of the profile indicates trace phenol: chloroform (repeat the phenol:chloroform, taking care not to carry over any into the aqueous layer).
2. It is best to carry on immediately with the labeling reactions, once the denaturing step has been completed.
3. For long reactions (to read more than 400–500 bp from the primer), a slightly different labeling reaction can be used to make sure that the polymerase extends to the region of sequence needed. Although the ^{T7}Sequencing and Sequenase kits employ different strategies for long reactions, there are two points common to both: first, that the incubation time of the labeling reaction should be increased from 5 min to 10 or even 15 min. Second, that the amount of ^{35}S αdATP used in the reaction should be increased to 1 μL.

   The ^{T7}Sequencing kit comes with two different types of termination reactions: read short and read long. These contain different molar amounts of dideoxynucleotides to alter the frequency of their incorporation. Use the read-long mixes in the termination reactions. The Sequenase kit has only one set of termination reactions, but here the labeling buffer is usually diluted to 1 × for short runs. For a long run, use the labeling buffer neat.