DNA SEQUENCE ANALYSIS OF PCR PRODUCTS

DNA sequencing is commonly performed by the so-called "dideoxy method" (also called "chain termination", or "Sanger" method) using specific primers, purified template DNA, a DNA polymerase and 2',3'-dideoxy dNTPs which cause termination of the new DNA strand (pp. 140-143 in Route Maps in Gene Technology). You may also want to consult a contemporary genetics text for a description of these structures and their use in DNA sequencing. We will use an adaptation of the traditional method utilizing a commercial kit (USBiochemicals Thermo Sequenase Radiolabeled Terminator Cycle Sequencing Kit). This protocol uses [α-P^{33}]ddNTPs, a thermo-stable DNA polymerase, and a PCR cycling apparatus. In the first lab, we will carry out the reaction; in the second lab, we will separate products on a denaturing polyacrylamide gel. Products will be visualized by autoradiography.

Template Preparation: *To eliminate unreacted nucleotides, primers, and other single-stranded DNAs from your PCR reactions, we will treat the reaction mixtures with a combination of shrimp alkaline phosphatase and exonuclease I. After the digestion, these two enzymes will be themselves inactivated by heat.*

1. To a microtube that contains 2 μl exonuclease I/shrimp alkaline phosphatase mixture, transfer 5 μl of your PCR reaction to a new microtube, avoiding mineral oil. Mix and incubate 15 min at 37°C.

2. Inactive the enzymes by incubating 15 min at 80°C. This is your template DNA.

Sequencing Reactions

3. Briefly centrifuge the microtube containing template DNA, then add in order: 10 μl water, 2 μl Reaction Buffer mix, 1 μl primer (2 pmol), 2 μl Thermo Sequenase polymerase (8 u). Mix this reaction mixture and hold on ice.

4. Termination mixes will be prepared by the TAs: G (dNTPs + [α-P^{33}]ddGTP); A (dNTPs + [α-P^{33}]ddATP); T (dNTPs + [α-P^{33}]ddTTP); C (dNTPs + [α-P^{33}]ddCTP). These will be provided to you as aliquots, 2.5 μl per tube.

5. Add 4.5 μl of your reaction mixture (prepared in step #3) to each termination mix tube. Use a new pipet tip for each transfer and gently mix. Overlay with a drop of mineral oil.

6. Place the tubes in the Robocycler and carry out 30 cycles of the following program: 95°C for 30 sec, 50°C for 30 sec, 72°C for 2 min.

7. Add 4 μl Stop Solution to terminate each reaction. We will freeze the samples until the next lab.

Denaturing PAGE analysis

8. A 6% denaturing polyacrylamide gel will be prepared the day before it is to be used. The gel will be placed in the apparatus with a "shark's tooth" comb; samples will be loaded between the teeth of the comb.

9. To ensure that DNA fragments are thoroughly denatured, incubate them 2-10 min at 70°C, immediately before loading.

10. Load 5 μl of denatured sample, creating lanes G, A, T, C.

11. Run the gel at 1500V for two hrs, or until the most rapidly migrating marker approached the bottom of the gel.

12. The gel will be carefully transferred to a sheet of 3MM paper, placed in a heated gel dryer, and dried.

13. Autoradiograph and/or subject to phosphorimaging.

SOLUTIONS

Reaction buffer mix (concentrate): 260 mM Tris-Cl, pH 9.5; 65 mM $MgCl_2$

Termination mixes (each containing a unique ddNTP): 6 μM dATP, 6 μM dCTP, 6 μM dGTP, 6 μM dTTP, 0.06 μM [α-P^{33}]ddNTP (1500 Ci/mmol; 0.09 μCi/μl)

Stop solution: 95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF

10X TBE buffer: 0.44 M Tris base, 0.44 M boric acid, 10 mM EDTA

6% sequencing gel: 5.7% acrylamide, 0.3% N,N'-methylenebisacrylamide, 1X TBE, 8.33 M urea, 0.08% ammonium persulfate, 0.05% TEMED