

AUTOMATED FLUORESCENT DNA SEQUENCER

Protocol for Texas Red dye primer sequencing of **single stranded DNA** templates: (using Sequenase fluorescent dye-primer sequencing kit)

1. In a microcentrifuge tube, set up a 21 μ l annealing reaction containing:
 - 1-2 μ g (0.4-0.8pmol) template ssDNA
 - 1.0-1.5 μ l Texas Red dye-primer (1.0-1.5pmol)
 - 1.5 μ l Reaction buffer
 - 1.5 μ l Manganese buffer
2. Heat for 5 minutes at 65°C and then anneal for a minimum of 10 minutes at room temperature.
3. Meanwhile, dispense 2 μ l of each of the four termination mixes into four separate microcentrifuge tubes or microtitre plate wells.
4. Prepare a solution of Sequenase enzyme at 3 units/ μ l by diluting the concentrated enzyme stock (nominally supplied at 13 units/ μ l) into ice-cold TE, pH8.0. Store the diluted enzyme on ice until needed and return the stock to the -20°C freezer immediately.
5. Add 2 μ l of diluted Sequenase enzyme (6 units) to the annealed primer/template. Mix by gently pipetting up and down four times and then dispense 5 μ l into each of the termination mix aliquots.
6. Incubate for 5-10 minutes (maximum) at 37°C.
7. Add 1/2 volume (3.5 μ l) of 7.5M ammonium acetate plus 3 volumes (approximately 30 μ l) of 100% ethanol to each reaction. Mix well and place on ice for a minimum of 10 minutes. Pellet the DNA at 13000 rpm for 10 minutes in a microcentrifuge.
8. Remove the supernatant carefully (do not use a vacuum line). Add 50 μ l-100 μ l of 70% ethanol (do not vortex). Centrifuge again at 13,000 RPM for 2-5 minutes before removing all residual traces of ethanol with a pipette. Allow samples to air dry completely for a few minutes at room temperature.
9. Add 2 μ l of loading dye and dissolve the DNA by gently flicking the tube to distribute the liquid evenly. Denature the DNA at 80°C for 3 minutes in a water bath. Load 1.5-2 μ l on to the sequencing gel.

Protocol for Texas Red dye-primer sequencing of **double-stranded DNA** templates: (using Sequenase fluorescent dye-primer sequencing kit)

1. In a microcentrifuge tube, set up an 18 μ l annealing mixture containing:
 - 1.5-3.0pmol dsDNA template (eg. approximately 4 μ g pUC18)
 - 3 μ l Texas Red dye-primer (3pmol)
 - Sterile deionized water to 18 μ l final volume
2. Denature for 5 minutes in a boiling water-bath then immediately snap-freeze by immersing the tube in a dry ice/isopropanol bath. Leave the tube in the dry ice until you are ready to continue.
3. Allow the primer/template mixture to thaw at room temperature. Spin the tube for a few seconds in a microcentrifuge to collect any condensate and then add:

1.5µl Reaction buffer

1.5µl Manganese buffer

4. Mix and incubate for 15 minutes at room temperature.
5. Meanwhile, dispense 2µl of each of the four termination mixes into four separate microcentrifuge tubes or microtitre plate wells.
6. Prepare a solution of Sequenase enzyme at 3 units/µl by diluting the concentrated enzyme stock (nominally supplied at 13 units/µl) into ice-cold TE, pH8.0. Store the diluted enzyme on ice until needed and return the stock to the -20°C freezer immediately.
7. Add 2µl of diluted Sequenase enzyme (6 units) to the annealed primer/template. Mix by gently pipetting up and down four times and then dispense 5µl into each of the termination mix aliquots.
8. Incubate for 5-10 minutes (maximum) at 37°C.
9. Add 1/2 volume (3.5µl) of 7.5M ammonium acetate plus 3 volumes (approximately 30µl) of 100% ethanol to each reaction. Mix well and place on ice for a minimum of 10 minutes. Pellet the DNA at 13000 rpm for 10 minutes in a microcentrifuge.
10. Remove the supernatant carefully (do not use a vacuum line). Add 50µl-100µl of 70% ethanol (do not vortex). Centrifuge again at 13,000 RPM for 2-5 minutes before removing all residual traces of ethanol with a pipette. Allow samples to air dry completely for a few minutes at room temperature.
11. Add 2µl of loading dye and dissolve the DNA by gently flicking the tube to distribute the liquid evenly. Denature the DNA at 80°C for 3 minutes in a water bath. Load 1.5-2µl on to the sequencing gel.

Reference:

Amersham DNA instrumentation systems fluorescent DNA sequencer kit instruction manual, 1994.