DNA SEQUENCING (using a Li-Cor 4200L automated DNA sequencer)

OBJECTIVE:

To label and separate DNA fragments varying by single nucleotides, in order to determine the sequence of nucleotides.

INTRODUCTION:

Determination of a DNA sequence is accomplished using one of two basic methods, and their derivations. Both methods were first described in 1977. The first method (Maxam and Gilbert 1977) is based on specific chemical degradation of the DNA. The DNA is first end-labeled using $^{35}$S or $^{33}$P, followed by separation of the two strands on a gel. Four aliquots of the desired strand are then subjected to a set of four DNA degradation reactions. One cuts the DNA at A residues, one at G residues, one at C residues and one at pyrimidine residues. Each of the four reactions is loaded into individual lanes of a thin (0.2 to 0.6 mm) polyacrylamide gel (5-15%). Electrophoresis is then performed at high voltage (usually 2,000 to 2,500 V, approximately 50-60 mA), for several hours, depending on the total length of the DNA fragment, and the portion of the sequence desired. If the fragment is short, and the sequence desired is close to the 5' end of that fragment, then the run time will be short (1-2 h). If the fragment is long, and the desired sequence is closer to the 3' end, then the run time will be longer (2-5 h).

The other method of sequencing DNA (Sanger 1977) uses a polymerization reaction (using DNA polymerase) in conjunction with mixtures of deoxynucleotide triphosphates (dNTPs) and dideoxynucleotide triphosphates (ddNTPs). Since dideoxynucleotides terminate the growth of the DNA polymer once they are incorporated (since the hydroxyl at the 3' position is absent), a series of fragments is produced dependent on the dideoxynucleotide used and the DNA sequence of the template. Since this is a polymerization reaction, the DNA is labeled during the reaction by the addition of an labeled nucleotide. Each of four reactions contains one of the four dideoxynucleotides, and is loaded into a separate well of a polyacrylamide gel (as above). This method has also been adapted for use with PCR. In this case, a small amount of DNA is used in conjunction with ddNTPs, dNTPs, and a labeled dNTP. This adaptation has also been used in automated sequencing. In this case, each of the ddNTPs is also labeled with a fluorescent dye (one dye for each dNTP in the ABI - Applied Biosystems Incorporated - automated sequencers; or one or two dyes total in the Li-cor automated sequencers). For ABI-type reactions, only one reaction mix is needed, since each of the nucleotides is labeled with a different colored dye. A
single lane is run on a polyacrylamide gel which is connected to a laser light source and a light sensor and computer. The laser excites the dye, which then emits light in the visible range. The light sensor and computer determine which color of light is being emitted, and record the corresponding nucleotide at that position. The DNA sequencer that we will use (Li-cor model 4200L) requires 4 reactions per DNA sequence, since each reaction is labeled using the same dye. In this case the dye emits in the infrared range (2 dyes are available, one emitting at 700 nm, and one at 800 nm). The advantages to this system over the ABI system are several. First, very long sequences (to over 1500 nucleotides) can be determined (the ABI limit is from 250 to 800 nucleotides, depending on the model). Second, the laser (a different type than in the ABI systems) has a longer life. Third, the laser and light emissions are such that less care in cleaning the glass surrounding the gel is needed. Fourth, the accuracy of nucleotide reading is greater, partly because each of the nucleotide lanes are run separately, and partly because the dye used in each reaction is exactly the same so that there are no effects caused by differences in the molecular weights of the dyes (as there are in the ABI systems). The disadvantages of the Li-cor systems (compared to the ABI systems) are: First, four reactions must be prepared for each DNA sequence to be determined (versus one in the ABI systems) and therefore four lanes on each gel must be used for each DNA sequence; Second, the sequencing apparatus is a bit more difficult to operate; Third, the chemistry and sequencing reactions are sometimes more troublesome.

DNA sequencing has been a molecular biology method that has developed from a technically difficult procedure for the elite labs, to a method that is indispensable for all molecular biology labs, as well as many other kinds of labs and classes. While automated DNA sequencing has become an increasingly popular choice for many labs, radio-labeled methods are still used in many cases where automated sequencing fails (for a variety of reasons).

**STEPS IN THE PROCEDURE:**

**General Steps:**

A. **Prepare or purify a suitable PCR product.**
B. **Perform Cycle Sequencing on the PCR product.**
C. **Prepare Polyacrylamide Gel**
D. **Initialize DNA Sequencer and Pre-Electrophorese Gel**
E. **Load and Run Gel**
F. **Clean Up Sequencer and Analyze Sequences**
A. Prepare or purify a suitable PCR product:

1. Use primers prITS4 and prITS5 in a 25 to 100 µl PCR reaction to amplify the desired DNA (starting with 5-10 ng). [For each sequencing reaction, from 50 to 100 ng of PCR product will be needed.]

2. Run 2 µl of this DNA on a 1.5% agarose gel to determine the result of the PCR reaction. If a single heavy band is seen, proceed to step 3. If not, go back to step 1. [Separation of the PCR product from the gel can be accomplished in several ways, including melting the band out of a low melting point gel and various column purification methods. In steps 3 - 14 below, a column purification method is described. In the Molecular Biology Laboratory Manual, a gel elution method is described using low melting point agarose gels. Both methods generally yield good results.]

3. Prepare a thick (double the standard volume) 1.0% low melting-point agarose gel (molecular biology grade). Be sure to use a comb that will allow for a marker lane, plus all of your samples with every other lane empty (e.g., three samples plus a marker lane can be run on an 8 well gel).

4. Add 12 µl of RS to 40 µl of the PCR reaction (avoiding the oil layer) for each well to be loaded.

5. Load the gel and start as usual, but the running voltage should be 75 V rather than 100 V (at 100 V the gel will heat up and melt).

6. Take a picture of the gel as usual. Using a fresh razor blade, turn on the UV source and cut around each band. Turn off the UV source, turn on the room lights, and transfer each of the bands into separate microfuge tubes using a clean spatula.

7. Place the microfuge tubes into a 65°C water bath until the gel slices melt. Add approximately 5 volumes of the PB buffer (Qiagen DNA purification kit) to each tube. In most cases this will be approximately 500 µl. Mix thoroughly and return to the water bath.

8. Place one Qiagen spin column into the Collection tube.

9. Load entire sample into the column.

10. Spin in a microfuge for 60 s.

11. Remove the Collection tube and discard the liquid.

12. Add 750 µl of PE buffer to the spin column. Spin in microfuge for 60 s.

14. Replace the Collection tube with a clean 1.5 ml microfuge tube labeled accordingly. Add 15 μl of sterile distilled water to the column and centrifuge again for 60 s. The tube will now contain the eluted DNA. Keep this on ice or store at -20°C.

15. Assay the DNA by running 2 μl of this in a single lane of a 1.0% standard agarose gel.

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B. Perform Cycle Sequencing on the PCR product.

Cycle (PCR) sequencing with the Li-cor system can be accomplished using a labeled nucleotide (usually A), or a labeled primer. Sequencing can be done in a single direction, using a single labeled primer, or it can be done simultaneously in both directions by using one primer labeled for the 700 nm channel dye (light emission at 700 nm), and the other primer labeled for the 800 nm channel dye. For this lab exercise, we will use labeled primers and sequence in one direction using a single labeled primer.

1. From the gel picture of the PCR amplifications, dilute the amplification products to 25 - 50 ng/μl.

Prepare the Reaction Mix as follows (sufficient for 1 set of sequencing reactions):
# Description
1 Upper buffer tank lid
2 Silicone tubing gasket
3 Upper buffer tank
4 Comb
5 Spacers
6 Back plate
7 Front plate
8 Left rail assembly
9 Right rail assembly
10 Lower buffer tank lid
11 Lower buffer tank
12 High voltage cable
13 Rail support pin
1 - 4 μl Template DNA (approximately 50 - 100 ng)
2.0 μl Labeled Primer (1.0 pmole/μl) *
7.2 μl Sequencing Buffer
1.0 μl EXCEL II DNA Polymerase
5.8 - 8.8 μl Distilled Water

20.0 μl TOTAL VOLUME

MIX WELL

* [Labeled primers are light sensitive. Limit exposure to light.]

2. Carefully label a set of four 0.5 ml microfuge tubes with A, G, C, and T, and include the template and primer information as well. [e.g., “A, V. faba, ITS4,” and “G, V. faba, ITS4,” etc.]

3. Add 2 μl of the A termination mix to the A tube(s), G termination mix to the G tube(s), C termination mix to the C tube(s), and T termination mix to the T tube(s).

4. Add 4 μl of the reaction mix to each of the tubes and mix well.

5. Add a drop of oil to the top if the thermal cycler lacks a heated lid.

6. Close the tubes and use the program called “CYCSEQ” to cycle sequence the DNA (program is 92°C for 2 min, then 30 cycles of 92°C for 30 sec, 50°C for 15 sec, and 70°C for 25 sec. the thermal cycler will then hold at 4°C for 48 hours).

7. At the end of the cycle sequencing add 3 μl of stop solution to each tube. Place on ice or at -20°C until ready to load samples.

C. Prepare Polyacrylamide Gel

1. Examine glass plates to be sure they are clean. Then, clean each plate (rectangular plate = rear plate, plate with "ears" at the top = front plate) by wiping with 100% isopropanol with a large Kimwipe.

2. In a small flask add 50 μl of bind silane to 10 ml of 100% ethanol (this stock solution can be stored at 4°C until needed). Add 100 μl of this solution to 100 μl of a 10% acetic acid solution.

3. Use a cotton swab to apply to the top part of the front plate and facing portion of the rear plate. This will ensure a complete bond between the gel and the glass plates in the well area, so as to avoid leaks. Allow several minutes to dry.
4. Lay back plate down flat. The plate has a small bevel or notch in one corner. The notched side should face downwards. Place one spacer on the left side, and one on the right side. Carefully position them such that they are precisely lined up with the edge of the glass plate.

5. Carefully place the front plate on top of the spacers. The notch should face upwards. Line the plates and spacers up together. Loosen screws on the rails and place them around the edges of the plates. Support knobs should be pointed outward. Notch for the upper buffer tray should be at the top. Carefully hand tighten the screws. Turn until just snug, and then one quarter turn more. DO NOT OVERTIGHTEN. Leave the top ones loose for now.

6. Select comb (for sequencing we will use the shark's tooth comb. [Note: The spacer thickness should match the comb thickness. For this gel, we will use the 0.25 mm thick spacers and comb.]

7. Make 1.0 ml of a 10% ammonium persulfate solution in deionized (or distilled) water.

8. Prepare a 4.0% acrylamide gel by mixing 25.2 g urea, 4.8 ml 50% acrylamide solution, 6.0 ml 10 X TBE, and water to make a final volume of 60.0 ml. Mix well. Filter solution by membrane filtration. [Note: Remember unpolymerized acrylamide is a neurotoxin!]

9. Place gel apparatus onto casting stand.
10. Add 400 μl of the 10% APS solution, and mix by swirling (do not introduce any bubbles).

11. Just before pouring gel, add 40 μl of TEMED (a catalyst for polymerization of the acrylamide).

12. Fill a 100 ml syringe with the gel solution. Begin squeezing out the gel solution by starting in the middle, then going from one side to the other only once. Then continue squeezing out the gel solution from the syringe at a rate that will keep a 1/4 inch bead of gel solution at the top of the front glass plate. This is so that no air bubble will be introduced. While you are squeezing out the gel solution tap the glass plates on both edges to keep the gel solution flowing smoothly on the edges. If this is not done, air bubbles will appear. Once formed these are difficult or impossible to remove.

13. Once the gel solution runs to the end of the glass plates, stop squeezing the syringe and lay the gel down flat. Place back end of shark's tooth comb into the top of the gel. Push it in 0.5 cm. Place casting plate into the notch at the top of the rails. Tighten the screws as before. DO NOT OVERTIGHTEN.

14. Rinse out syringe with water and leave with water inside the syringe chamber.

15. Allow gel to polymerize for 1 - 2 hrs.

16. When polymerized clean off excess acrylamide and clean the lower part of the back plate carefully with a Kimwipe and a spray of isopropanol (this part of the plate is where the laser and the detector are located).
17. Loosen the upper two knobs on the rails and remove the casting plate. Add a small volume of buffer to the notched area (over the comb). Remove the comb so that the buffer flows into the top part of the gel. Rinse the well area with a 20 cc syringe with a 22 gauge needle. Remove any pieces of acrylamide. Use a razor blade to remove any other excess pieces of acrylamide.

18. Insert the shark's tooth comb in the correct orientation such that the teeth are pressed onto the top of the gel. Press comb until the acrylamide deforms by about 0.5 mm, but the teeth should not puncture the gel.

19. Press white rubber gasket into the groove on the back of the upper buffer tank. Loosen the upper clamp knobs and slide the upper buffer tray into position. Tighten the screw clamp knobs (as before). Clean plates again.

20. Open the sequencer door and place the lower buffer tank into position.

21. Mount gel sliding the support arms into the hooks. The gel apparatus should slide easily into place. Do this slowly and carefully.

22. Fill the upper buffer tank to the Max Fill line (or below) with 1.0 X TBE. Do not splash the buffer. Fill the lower buffer tank with the same buffer. If any buffer runs down the front or rear plates, it must be removed completely prior to electrophoresis. Otherwise, a short can develop, which can cause the sequencing run to fail, and can be dangerous.

23. Clean the wells by squirting in and out of each gel with a 20 cc syringe with needle. Be very careful not to damage wells or splash buffer.

24. Check for any leaks. If there are none, place the lids onto the two tanks and connect the power cable from the tank to the sequencer. Close door.
D. Initialize DNA Sequencer and Pre-Electrophorese Gel

1. Turn on computer, printer, and sequencer (on back right side of sequencer). Computer will take several minutes to initialize. It will eventually show a "windows-like" screen with several icons (it is actually an OS/2 system). "BaseImagIR" is the set of applications that controls the sequencer, reads the bases, and analyzes the resultant sequences. "Data Collection" controls electrophoresis and "Image Analysis" recognizes and calls the bases.

2. Click on "Quick 4200" program, and configure the gel parameters. This will be a 66 cm gel, with a 64 well shark’s tooth comb.

3. Press "Start." This will start the focus routine, which focuses the laser and light detector on the gel.

4. Pre-Electrophoresis will start and will take about 15 minutes (after the focusing has been completed).

E. Load, Run, and Stop Electrophoresis

1. When pre-electrophoresis has completed, remove top tank lid and load samples. Load 0.6 to 0.7 µl from each tube into individual wells in the sequencing gel (a maximum of 1 to 1.5 µl is all that will fit into the wells). The preferred order of loading is ATGC (This is the default order. If you load in a different order, you need to manually adjust the order of the wells.)

2. Replace tank lid, close door press "interlock" button. Lights should come on, and sequencing will begin. For a 66 cm gel, the total time for electrophoresis is about 12 hours.

3. Stop run and click "OK."

F. Clean Up Sequencer and Analyze Sequences

1. Open door. Remove electrical cable on upper tray. Remove upper tank cover. Drain upper buffer tray with rubber tube. Leave the tube full of buffer and use it to drain the lower tray by using it as a siphon.

2. Remove upper buffer tray and gel. Remove lower buffer tray.

3. Remove upper tank from gel by loosening screws. Remove gasket and rinse with water.

4. Clean and rinse rails.


7. TA will go through the analyses with you.

SOLUTIONS:

PCR reagents:
(see previous section on PCR)

Low Melting Point Gel:
(See "Elution of DNA From Agarose Gels" section

Qiagen Column Purification:
QIAquick PCR Purification Kit:
QIAquick Spin Columns
PB buffer
PE buffer
Collection tubes

SequiTherm EXCEL II kit (Epicentre Technologies):
Sequencing Buffer
A Termination Mix
G Termination Mix
T Termination Mix
C Termination Mix
Stop/Loading Buffer
EXCEL II DNA Polymerase
Deionized Distilled Water
Control Template (plasmid)

REFERENCES:
