EXERCISE
4

DNA Sequencing

Background

Chain termination with dideoxynucleoside triphosphates (ddNTPs) is used in automated sequencers and for sequencing at the bench. Dideoxy sequencing occurs in a set of 4 reactions, each of which contains the DNA template to be sequenced; an oligonucleotide primer complementary to DNA 3' of the area to be sequenced; DNA polymerase; the 4 deoxynucleoside triphosphates (dNTPs), 1 of which is radioactively labelled to allow detection; and a small amount of *1* of the 4 ddNTPs. Either single-stranded template is used or double-stranded DNA is denatured to allow the primer to anneal. During primer extension a ddNTP will sometimes incorporate instead of the corresponding dNTP. Because the ddNTPs lack a 3'-OH, which is required to add the next nucleotide, incorporation of a ddNTP terminates a growing chain at that position. The result is a mixture of DNA chains of different lengths, complementary to the template, each of which was terminated at the position of a specific nucleotide in the sequence. When the 4 reactions are loaded in 4 wells of a denaturing polyacrylamide gel and separated by electrophoresis, the dideoxy-terminated segments form a ladder of bands. The gel is exposed to X-ray film, and the sequence (complementary to the template) is read off the film.

Automated sequencing reactions are labeled with fluorescent dyes, which are detected as they travel past a laser beam during electrophoresis. Four reactions must be run, with 4 different dye labels, 1 for each dNTP. The fluors are attached either to the primer or to the ddNTPs.

You will sequence the putative mutant *virD2* gene created in your site-directed mutagenesis experiment using α-^{35}S-labelled dATP.

Steps of the experiment are

A. Polyacrylamide sequencing gel eletrophoresis

B. Sequencing reactions

C. Automated sequencing

D. Introduction to databases and gene sequence analysis

Safety Precautions

Sulfur 35 can be safely used at the bench. However, once it has been added to the sequencing reaction, all tips and tubes that contact reactions must be discarded in radioactive waste. Line a beaker with plastic wrap and use it to dispose of radioactive trash; empty it into the radioactive waste barrel at the end of the day. When you are done with sequencing, use the Geiger counter to scan your fingers, Pipetman dispensers, lab coat, bench, the floor, and anything you touched, and clean up as needed.

Notes

A. POLYACRYLAMIDE SEQUENCING GEL ELECTROPHORESIS

Introduction

Polyacrylamide sequencing gels contain urea to denature DNA fragments produced during the sequencing reactions. Glass plates for sequencing gels are 20 × 40 cm. The spacers and combs are 0.4 mm thick.

Safety Precautions

Acrylamide is a neurotoxin that should only be handled with gloves. When weighing out dry acrylamide powder to prepare stock solutions, wear gloves, lab coat, and mask.

Unincorporated ^{35}S-dATP will migrate into the buffer in the bottom well. Treat this buffer as radioactive waste.

Technical Tips

Use freshly prepared ammonium persulfate (APS).

Acrylamide begins to polymerize when tetramethyl ethylenediamine (TEMED) and APS are added, so add these immediately before you pour the gel.

Protocol

1. Obtain a set of 2 glass plates. Clean both sides of the plates with ethanol. When dry, assemble plates and spacers. Tape the sides and bottom of the plates with Scotch Brand 3M yellow electrical tape. Put 3 heavy (bulldog) clips on each side of the plates.

2. Mix: 40 mL sequencing acrylamide
   32 μL TEMED
   140 μL 10% APS

3. Using an electric pipettor, dispense the acrylamide mix into the prepared plates, down one side, at a rate such that the acrylamide does not pool at the top and flows steadily between the plates, which are held almost vertical. Avoid forming bubbles. Fill the gel plates to 1.5 inches below the top. Allow bubbles to rise to the top, then lower to a nearly horizontal position with the top end supported by a rubber stopper. The acrylamide solution will then fill and slightly overflow the glass plates. Insert the flat edge of the comb about 6 mm from the top of the short plate. Place 3 large clips over the comb, pressing the plates firmly over the comb. Allow gel to polymerize about 1 hour. Remove clips over comb, drip a little 1× NNB buffer on comb, or for longer storage cover comb with buffer-saturated filter paper, and wrap with plastic until use. This gel will keep for 1 day.

4. Immediately before use, remove the plastic wrap and filter paper, wash salts and urea off plates, invert comb and reinsert with teeth **just touching** the top surface of the acrylamide. Attach the gel to the electrophoresis device and fill with 1× NNB buffer.

5. Sequencing reactions already contain load buffer. Load 1 μL of each sequencing reaction in the following order: CATGC. Make a record of the loading order. Make sure the gel is not bilaterally symmetrical (so you can tell which side is which when it is exposed to film).

6. Apply 30 W (constant power) until the bromphenol blue dye nears the bottom (slightly less than 2 hours). Turn off power and load a 2nd set of the same reactions (in different lanes). Continue electrophoresis until the bromphenol blue in these lanes nears the bottom.

7. Prepare a tray with enough gel fix in the bottom to cover the gel (approximately 2 cm). Remove gel from electrophoresis device. Slit the tape with a razor and remove it. Remove comb and spacers and **carefully** separate the 2 plates by inserting a metal spatula down one side where the spacer was. The gel will adhere to 1 of the plates. Lower this plate, gel side up, into the gel fix and soak gel for 0.5 hour.
8. Remove gel from the remaining plate by blotting onto 3 MM paper. Dry gel approximately 1.5 hours (dry to touch) in a gel dryer under a vacuum and place in a light-tight cassette on X-ray film.

Solutions for Acrylamide Sequencing Gels

Sequencing acrylamide: 0.5× NNB buffer, 8% acrylamide, 8 *M* urea
For 300 mL, mix 144 g urea, 48 mL 50% acrylamide–2.5% bis-acrylamide, 15 mL 10× NNB buffer, and 126 mL ddH_2O. Filter.
If using purchased acrylamide solution, use 60 mL 40% acrylamide (19:1 acrylamide:bis-acrylamide), 144 g urea, 15 mL 10× NNB buffer; bring to 300 mL with ddH_2O; filter.

10× NNB buffer:
162 g Tris base
27.5 g boric acid
9.3 g EDTA–Na_2
870 mL ddH_2O

Gel fix: 10% methanol, 10% acetic acid, 2% glycerol

Notes

B. DIDEOXY SEQUENCING

Introduction

Sequencing a single-stranded template produces the best results. Single-stranded templates are commonly isolated from clones in M13 or phagemids. However, it is also possible to denature a double-stranded plasmid immediately before sequencing it, for example by treatment with sodium hydroxide or by boiling. The quality of sequence obtained from a double-stranded template depends on the quality of the template; plasmid DNA purified by cesium chloride density gradient centrifugation gives better results than DNA prepared by the small-scale alkaline lysis method. Commercial kits also yield good-quality DNA for sequencing.

You will sequence a double-stranded template, the putative mutant *virD2* gene created in your site-directed mutagenesis experiment. Immediately before sequencing, you will denature the plasmid with sodium hydroxide, neutralize, and precipitate with ethanol. You will use a commercially available kit (Sequenase, U.S. Biochemical) because the balance between the dideoxynucleotides and the deoxynucleotides must be precise.

Sequencing is often done with ^{32}P-labeled primers. These primers are 5'-end-labeled with γ-^{32}P, and they have a short shelf-life. Instead, you will label with α-^{35}S-dATP, which DNA polymerase will incorporate into nascent DNA. (Several kits are available for the non-isotopic detection of sequencing reactions.) You will perform an extra "C" reaction. This makes it much easier to read the sequence.

Safety Precaution

Use gloves and lab coat when handling radioactivity.

Protocol

This protocol is a checklist. Fill out the amounts needed and check off each step as you do it.

1. To 2 to 3 μg of plasmid DNA from a putative mutant, add 1 *M* NaOH to a final concentration of 0.2 *M*. Incubate for 5 minutes at room temperature.
2. Neutralize by adding 0.4 volumes of 5 *M* ammonium acetate (pH 7.5). Mix immediately.
3. Precipitate the DNA with 4 volumes ethanol. Incubate on ice for 15 minutes. Centrifuge at top speed in microfuge for 10 minutes. Wash the pellet in 70% ethanol, air dry, and redissolve in 7 μL ddH_2O. Store on ice.
4. Start 37 and 65°C heating blocks, incubators, or water baths.
5. Prepare annealing mixture:

DNA (approx. 2 to 3 μg)	__ μL
H_2O	__ μL
5× Reaction buffer	2.4 μL
Primer (0.5 pmol/μL)	1.2 μL
Total	12 μL

6. Heat annealing mixture to 65°C for 2 minutes and then cool slowly to less than 35°C for 15 to 30 minutes. Chill on ice.
7. While the annealing mix is cooling, label tubes "C", "C", "A", "T", "G". Fill with 2.5 μL of each termination mix and cap tubes. Place them at 37°C.

ddC/dNTPs	2.5 μL
ddC/dNTPs	2.5 μL
ddA/dNTPs	2.5 μL
ddT/dNTPs	2.5 μL
ddG/dNTPs	2.5 μL

8. Dilute labeling mix 1:10 with ddH_2O. **[TAs will do this step for the class]**

9. Dilute Sequenase enzyme 1:8 in ice-cold enzyme dilution buffer (or TE). **[TAs will do this step for the class]**

10. Labeling reaction: to annealed primer-template, add **in the order listed, making sure to add the enzyme last:**

Dithiothreitol, 0.1 *M*	1.2 μL
Diluted labeling mix	2.4 μL
[^{35}S]dATP	0.6 μL
Diluted Sequenase enzyme	2.4 μL

    Mix, incubate at room temperature 2 to 5 minutes.

11. Transfer 3.5 μL of labeling reaction to each termination (ddN/dNTP) tube, mix, and incubate at 37°C for 5 minutes.

12. Stop the reactions by adding 4 μL of stop solution to each tube; store at −20°C.

13. Before loading on a gel, denature at 75°C for 2 minutes.

Solutions for Dideoxy Sequencing

Sequenase kit (U.S. Biochemical, Cleveland, OH)

Sequencing primer: M13 F (universal)

α-^{35}S-dATP

Notes

C. AUTOMATED SEQUENCING

Automated sequencing is frequently carried out using "cycle sequencing", which uses a thermally stable DNA polymerase. Like PCR, the reaction is repeatedly raised to 95°C, allowed to drop to the temperature at which the sequencing primer anneals, and then raised to the correct temperature for the polymerase. This denatures double-stranded DNA, allowing double-stranded plasmids to be directly sequenced. You will use this method to sequence your putative mutant *virD2* and compare the sequence obtained in this way with the sequence you did at the bench.

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D. INTRODUCTION TO DATABASES AND GENE SEQUENCE ANALYSIS

The purpose of this exercise is to introduce some of the options that are available to search genetic databases and analyze nucleic acid and protein sequences. To take full advantage of the laboratory techniques covered in this course, you will need to access genetic databases. You may need to design PCR primers or hybridization probes, or retrieve a gene sequence. You may also need to analyze your own sequences.

It is outside the scope of this course to demonstrate all the computer tools available to molecular biologists, just as in the lab we could not introduce all important protocols. Instead, as in the lab, we will give you a hands-on introduction to a few of them, to give you a place to start and an idea of what can be done.

You have just sequenced a gene. Imagine that you do not know what it is, and you want to identify it. Where do you start?

1. Use Netscape to reach http://www.ncbi.nlm.nih.gov/
2. Select ("click on") BLAST.
3. Select Basic BLAST Search.
4. Leave the Program window set to blastn to search for a nucleotide sequence. Leave the Database window set to nr to search all databases. (If you want an explanation of these options, click the Program or Database buttons. However, **you do not need to do anything here for this project.**)
5. Enter your sequence in the box under Sequence in FASTA format. You can type the sequence by hand, or you can copy a file (from a word processor, for example) and paste it into this box. Enter only nucleotide sequence; numbers and other text are not permitted,

although blank spaces within the sequence do not matter.

6. Click Submit Query.

7. To perform literature searches, use Netscape to reach http://www.ncbi.nlm.nih.gov/ then select Entrez; next, select Literature-PubMed and enter your search terms.

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EXERCISE 4. STUDY QUESTIONS

1. What is the role of the ddNTPs in sequencing reactions?

2. You perform a sequencing reaction and separate the products by electrophoresis on a polyacrylamide gel, allowing the bromphenol blue to migrate to the bottom of the gel. When you expose the gel to film and develop the film, you see bands only at the bottom of the gel, not at the top. What caused this? What if the bands were at the top of the gel instead?

3. You have just isolated a new thermostable DNA polymerase from a bacterium that grows at 90°C in marine hydrothermal vents. You plan to sequence the gene that encodes this polymerase. What problems might you encounter? Why? Can you suggest approaches to overcome these problems?

4. You loaded the 4 dideoxy reactions in separate lanes on the sequencing gel. In the automated sequencer, the 4 dideoxy reactions were loaded in the same lane of the gel. Why?