# DNA Sequencing

**(Revised 11/21/2006)**

Name_____Date _____Period _____Score

Background:

In 1977 two methods for sequencing DNA were developed, the chemical cleavage method and the chain termination method. The chemical cleavage method was developed by Allan Maxam and Walter Gilbert, at Harvard University and involves the specific chemical modification of each different nucleotide. The chemical change in the nucleotide allows cleavage at a specific site and identifies the nucleotide present at that position. The other method, chain termination method developed by Fred Sanger at the Medical Research Council's Laboratory of Molecular Biology in Cambridge, England has become the dominate method used and is described in detail here.

The Sanger method relies on two facts of DNA synthesis: 1. DNA polymerase will initiate synthesis of a new strand of DNA when a short DNA primer is hybridized to a single-stranded DNA template in the presence of the four dNTPs. 2. Dideoxynucleotide triphosphates (didNTPs) will stop DNA elongation when a didNTP is incorporated into the new DNA strand. This is because didNTPs lack a 3' hydroxyl group (-OH), which is necessary to form the phosphodiester linkage that joins adjacent nucleotides.

In the dideoxy sequencing protocol, four reaction tubes (A,T,C,G) are set up. Each of the reactions tubes contains a DNA template, a primer sequence, DNA polymerase, and the four deoxynucleotide triphosphates (dATP, dTTP, dCTP, and dGTP, one of which is radioactively labeled). A single type of didNTP is added to each of the four reactions—didATP (to tube A), didTTP (tube T), didCTP (tube C), or didGTP (tube G).

Working from the primer, the polymerase randomly adds dNTPs or didNTPs that are complementary to the DNA template. Each time didNTP is incorporated, synthesis stops, and a DNA fragment of specific size is created. Through this process After replication, there are millions of copies of the template DNA sequence terminated at each nucleotide position.

In Reaction A, for example, for every 100 thymine residues encountered on the DNA template, the polymerase will incorporate 99 units of the complementary nucleotide deoxyadenosine (remember that in DNA synthesis, A complements T in the opposite strand). In 1 instance out of 100, the polymerase will incorporate dideoxyadenosine, halting synthesis of the strand at that point. In other words, the DNA chain will be terminated with the thymine position in 1 % of all template molecules. Because millions of DNA molecules are present, Reaction A results in a collection of DNA fragments in which synthesis has been arrested to mirror the position of each thymine residue in the template DNA.

When the reactions are complete, formamide is added to denature the newly synthesized strands from the template DNA. Each reaction is loaded into a different lane of a polyacrylamide gel containing urea, which prevents the DNA strands from forming secondary structures during electrophoresis. The fragments *migrate* through the gel according to size, eventually resolving to form a "ladder" of bands, each composed of DNA molecules differing in length by a single nucleotide.

Following electrophoresis, the gel is placed in contact with X-ray film. The radioactively labeled nucleotides that were incorporated expose the X-ray film, producing a series of bands, indicating the length of fragments generated in the A, T, C, and G reactions. The gel is then "read" from bottom to top, beginning with the smallest DNA fragment, then scanning across the lanes to identify each successively larger fragment. Optical scanners were eventually introduced to read gels, because manual sequencing was far too slow to accomplish the goal of sequencing the human genome rapidly and accurately.

Automated sequencing was made possible by dye chemistry developed by Lee Hood and Lloyd Smith at the California Institute of Technology. In 1986, they paired a different fluorescent dye with each of the four didNTP reactions. The four sequencing reactions were added to a single lane of a sequencing gel, and the fluorescent labels were detected as the terminated fragments passed an Argon laser aimed at the bottom of gel. When struck by the laser light, each fluorescent terminator emits a colored light of a characteristic wavelength, which is then interpreted by the computer software as an A (green), T (red), C (blue), or G (yellow) at that position.

Hood then collaborated with Mike Hunkapiller, at Applied Biosystems, Inc. (ABI), to produce the first commercial instrument to read sequences from dye-labeled fragments. The ABI Model 370, first marketed in 1987, employed a polyacrylamide slab gel to resolve a ladder of DNA fragments. However, rather than hand or optical gel reading, it employed a laser to detect the fluorescently labeled nucleotides. The sequencer incorporated a computer program that builds a simulated gel image of colored DNA bands as they pass a scanning laser during electrophoresis. The final output took the form of an electropherogram, showing colored peaks corresponding to each nucleotide position. The ABI 370 DNA Sequencer, equipped with a 16-lane polyacrylamide gel, had the capacity to sequence as many as 20,000 nucleotides per day. Increasing the number of lanes to 32, 48, or ultimately 96 brought daily output to 80,000 nucleotides or more.

By allowing all four nucleotides to be analyzed in a single lane, Hood's fluorescent chemistry quadrupled the output of sequencing gels. Parallel improvements in DNA preparation further increased output. By the late 1980s, thermal stable polymerases (such as *Taq)* and DNA thermal cyclers were pressed into service to automate dye labeling, a hybrid method that became known as cycle sequencing. In 1987, Dupont introduced "dye terminators," which attach a different fluorescent dye directly to each of the four terminator nucleotides (didATP, didTTP, didCTP, or didGTP). This allowed all four nucleotides to be labeled simultaneously in a single reaction, streamlined sample preparation, and effectively quadrupled a technician's daily sequencing output.

The final phase in the development of high-throughput sequencing came by replacing the polyacrylamide slab gel with multiple capillary gels, whose feasibility was first demonstrated in 1990. New automated sequencing machines linked a 96-capillary array with a robot mechanism capable of automatically reloading samples up to 12 times per day. The capillary machines eliminated the time-consuming elements of pouring and loading sequencing gels, reducing human intervention to maintaining reagent levels and loading microtiter plates into the autoloader. As the race to complete the first draft of the human genome heated up, each of the major sequencing centers had become automated factories equipped with 30 or more of these sequencers, each churning out up to 400,000 nucleotides of sequence per day. The speed of sequencing had increased 400-fold since the start of the project.

Objectives: learn a technique of DNA sequencing through gel electrophoresis

Materials: 1 sheet of 24 Xerox copies of DNA sequence per team, colored pencils, scissors, large poster board, glue stick, mRNA/amino acid chart (DNA Science a First Course p. 54), and ruler.

Procedure:

Part A:

**1.** The following diagram represents a gel from the electrophoresis of segments of a DNA strand. Each letter at the top represents one of the four bases in a nucleotide of a DNA molecule. The marks under each of the bases represent segments of DNA that migrated through the gel. The numbers represent the relative distances traveled by the segments, with "1" being the furthest distance the segment traveled and "6" being the shortest distance. The smallest segments of DNA move the furthest while the longer segments of DNA move the shortest distance. When you read a gel, you read it from the bottom to the top as shown in the following example. This sequence is read as TTCGGA, T being the shortest segment and A being the longest segment.

| | G | A | T | C |
|---|---|---|---|---|
| 6 | | _____ | | |
| 5 | _____ | | | |
| 4 | _____ | | | |
| 3 | | | | _____ |
| 2 | | | _____ | |
| 1 | | | _____ | |

**2.** "Read" the DNA sequence for Watson from the bottom to the top of the diagram of a gel from the electrophoresis of segments of a DNA strand. Each line represents a segment of labeled DNA that migrated through the gel. The smallest pieces migrated the furthest and the larger ones migrated the shortest distance. Record your results in the Data section of this lab.

| | G | A | T | C |
|---|---|---|---|---|
| 12 | | | | |
| 11 | | | | |
| 10 | | | | |
| 9 | | | | |
| 8 | | | | |
| 7 | | | | |
| 6 | | | | |
| 5 | | | | |
| 4 | | | | |
| 3 | | | | |
| 2 | | | | |
| 1 | | | | |

Part B:

**1.** Working as a team, obtain 1sheet of the 24 single strands of DNA as shown below.


2. The scientists have attached a radioactive probe to the dATP's. Cut out the strands of DNA with the radioactive probe at each end. You will have 24 strands total.

3. Color six (6) strands of the DNA in each of the four colors. Yellow for Guanine, Green for Adenine, red for Thymine and Blue for Cytosine. This represents a fluorescent dye being with each of the four didNTP reactions during the cycling process.

4. In the dideoxy sequencing protocol, four reaction tubes (A,T,C,G) are set up. Each of the reactions contains a DNA template, a primer sequence, DNA polymerase, and the four deoxynucleotide triphosphates (dATP, dTTP, dCTP, and dGTP, one of which is radioactively labeled).

5. A single type of didNTP is added to each of the four reactions—didATP (to tube A), didTTP (tube T), didCTP (tube C), or didGTP (tube G).

6. When the reactions are complete, formamide is added to denature the newly synthesized strands from the template DNA.

7. Each reaction is loaded into a different lane of a polyacrylamide gel containing urea, which prevents the DNA strands from forming secondary structures during electrophoresis. The fragments migrate through the gel according to size, eventually resolving to form a "ladder" of bands, each composed of DNA molecules differing in length by a single nucleotide.

8. On your poster board, construct an electrophoresis gel template with the wells for G-A-T-C at the top of the "gel." Number the side from 1-21 because we are sequencing 21 nitrogen bases. "1" will represent the distance traveled of one nucleotide in the DNA segment, while "21" will represent the distance traveled of a 21 nucleotide DNA segment. Each number should be of equal distance from the previous one. Use a ruler to measure the scale. Make a grid as shown in the diagram. This will make it easier to move your DNA segments through the gel to their final position. Set the "gel" up as follows:

| | G | A | T | C |
|---|---|---|---|---|
| **21** | | | | |
| **20** | | | | |
| **19** | | | | |
| **18** | | | | |
| **17** | | | | |
| **16** | | | | |
| **15** | | | | |
| **14** | | | | |
| **13** | | | | |
| **12** | | | | |
| **11** | | | | |
| **10** | | | | |
| **9** | | | | |
| **8** | | | | |
| **7** | | | | |
| **6** | | | | |
| **5** | | | | |
| **4** | | | | |
| **3** | | | | |
| **2** | | | | |
| **1** | | | | |

5. On the strips colored yellow for G, make a cut AFTER the G, one from each strand. Make sure you cut after a different G on each of the strands. You will then result in pieces of several lengths from the probe end. You should have six different sized pieces of DNA, each starting with the probe and ending with a G. Throw away the portions of the DNA segment that do not contain the probe.
6. Repeat the procedure #5 for "test tubes" A, T and C. There may be extra strands for some of the treatments. These can be thrown away.

7. Take the segments for G that have a radioactive label attached to one end. The segments without the label would migrate through the gel but would not show up on the X-ray film. Do not use the unlabeled segments for this demonstration. From well G, move the segments through the gel according to their size. The smallest piece will move the furthest, the larger parts will move the shortest distance. In other words, a segment with only one nucleotide would move to position 1, one with two nucleotides to position 2, etc.
8. Repeat this for the other segments of DNA
9. Show your finished product to your teacher.

Data and Analysis:

Background:

1. How do the dideoxynucleotide triphosphates (didNTP's) stop the DNA elongation from occurring?

2. In the dideoxy sequencing protocol, what are the reactants placed in all four reaction tubes?

3. In the dideoxy sequencing protocol, what is the reactant that is unique to each reaction tubes?

4. Why is formamide added to the tube after the reactions are complete?

5. Why do scientists add urea to the polyacrylamide gel?

6. Explain how Lee Hood and Lloyd Smith changed the process of DNA sequencing.

Part A:

7. Record Watson's DNA sequence read from bottom to top.

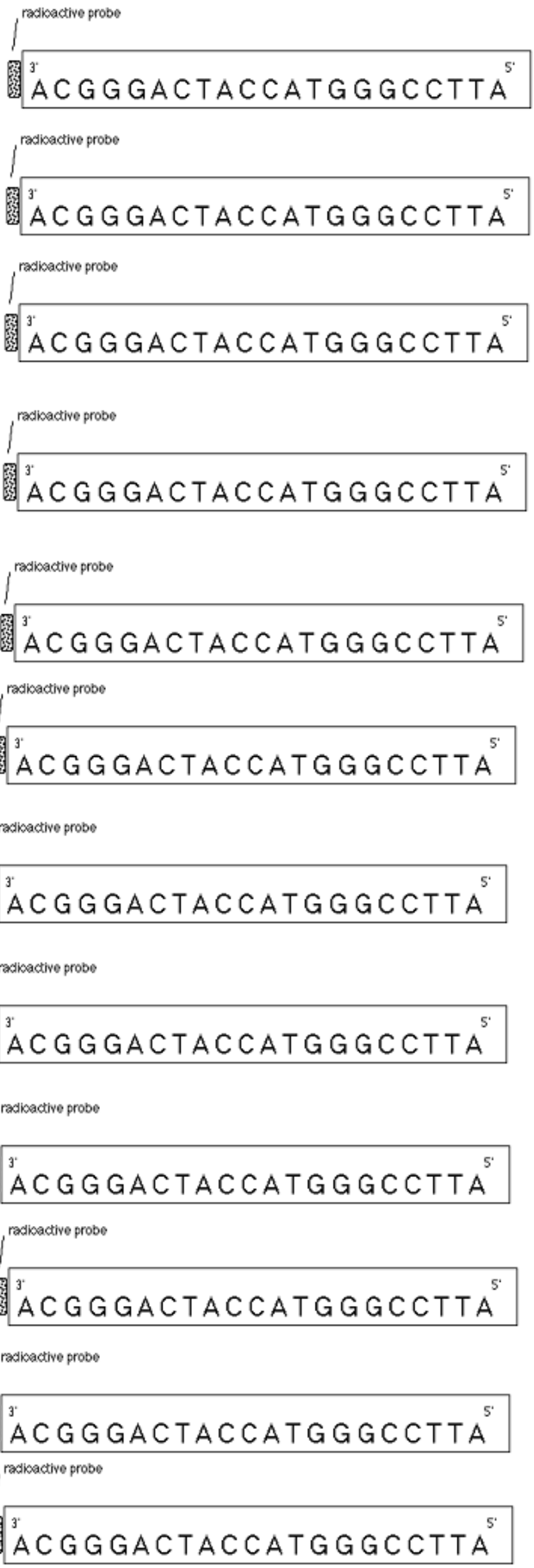
Part B:

8. Record the DNA sequence read from bottom to top that you produced with the DNA fragments.

9. What is the mRNA sequence that can be transcribed from the above DNA sequence?

10. Refer to the amino acid chart p.54 in DNA Science: A First Course. Give the amino acid sequence that is coded by the mRNA sequence.

11. What would happen if there were a mutation and the stop codon was found in the middle of the mRNA that coded for a protein?


radioactive probe
3'
ACGGGACTACCATGGGCCTTA
5'

radioactive probe
3'
ACGGGACTACCATGGGCCTTA
5'

Adapted From:

Ridenour, Nancy 2001 “DNA SEQUENCING LAB” Access Excellence: Activities Exchange 27 March 2003 <http://www.accessexcellence.org/AE/newatg/Ridenour/>

Micklos, David A., Greg A. Freyer, and David A. Crotty. DNA Science: A First Course. 2nd ed. Cold Spring Habor: Cold Springs Habor Laboratory Press, 2003.