Name _____ **Period** _____

Ms. Foglia **Date** _____

AP LAB 23: SANGER SEQUENCING

It is a bit of a conceptual leap from the discovery of the structure of DNA to the sequencing of the human genome, but one leads directly to the other. The double-helix model of DNA led to an understanding of how the DNA is duplicated as cells grow and divide. This process of DNA replication was then harnessed as a tool for the Sanger method of determining the sequence of a piece of DNA.

Modern DNA sequencing technology is based on the method of controlled interruption of DNA replication developed by Fred Sanger in 1977, for which he was awarded his second Nobel Prize in 1980 shared with Walter Gilbert and Paul Berg (he won his first unshared Nobel in 1958 for his work on the structure of proteins, especially that of insulin).

Sanger combined the natural DNA replication machinery of bacterial cells (with a bit of recombinant DNA technology and some clever biochemistry) to create a system in which a cloned fragment of DNA is copied, but some of the copies are halted at each base pair along the sequence.

Natural DNA replication uses the DNA polymerase enzyme which copies a template DNA sequence (one half of the DNA double helix) and creates a new DNA polymer, complementary to the template, by joining nucleotides into a growing DNA chain. Remember that the replication reaction also requires a primer—a short piece of DNA that is complementary to the template—to which the polymerase can affix the first added base (see Figure 1).

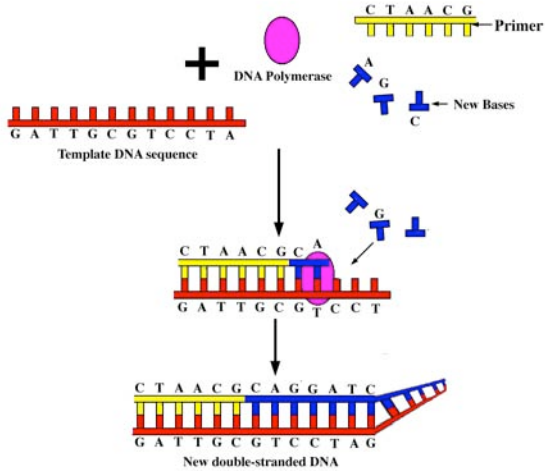

Figure 1. DNA replication: DNA polymerase uses a primer and free nucleotides to synthesize a complementary strand of DNA from the original template strand. Sanger sequencing builds on this natural process.

The Sanger Method

The Sanger sequencing method builds on this natural copying process. This laboratory technique makes use of specially modified nucleotides — dideoxynucleotides. DNA is called deoxyribonucleic acid because the ribose sugar part of the molecule is lacking an oxygen atom found in normal ribose. Dideoxy bases lack a second oxygen atom that is required to extend the growing DNA chain. This means that when a dideoxy base is incorporated into a DNA molecule, the chain stops or terminates.

The reactions are set up so that there is a mix of “normal” bases (G, A, T, C) and dideoxy bases (ddG, ddA, ddT, ddC) as well as the other components necessary for replication: primers and DNA polymerase enzyme. In this way, the reaction is set up so that it doesn't work all the time — we don't want a perfect, complete copy of the DNA. At any position, either a normal base will be added, so the chain can continue to grow, or a dideoxy base will be added, so the chain terminates. After many cycles of copying, all the possible chain-termination molecules are produced: the reaction has stopped chains at every base (see Figure 2).

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In the original technique developed by Sanger, the fragments are separated by length using polyacrylamide gel electrophoresis, with one gel lane for each of the four different dideoxynucleotide reactions: a G lane, an A lane, a T lane, a C lane (see Figure 3 and Figure 5a). The synthesized DNA segments are sorted by size: short ones travel further than long ones. An autoradiogram is made of the gel, and the DNA sequence is visualized and interpreted.

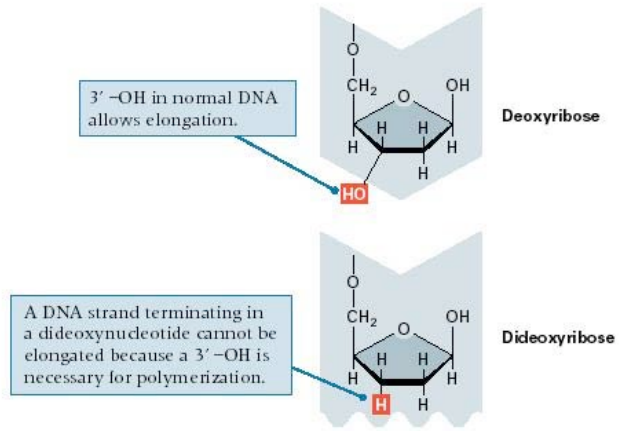

Figure 2. Sanger sequencing using dideoxy-nucleotides to terminate growing DNA chains.

DNA fragments of a specific length form a distinct band on the gel, so there is one band for each base in the template sequence. Then the gel, which contains the radioactively labeled DNA fragments, is placed on top of a sheet of X-ray film so that the radioactive bands of DNA can expose it (see Figure 4). Finally, the sequence is manually read off of the X-ray film from the positions of the bands and typed into a computer.

The value of determining DNA sequences was immediately obvious to many biologists, but the laboratory techniques of the Sanger method, while being ingenious, are both laborious and technically demanding. DNA sequencing became a rite of passage for many molecular biology graduate students in the 1980s and early 1990s. Initially, some kits were developed to simplify and standardize the biochemistry. These kits eventually included superior types of polymerase enzymes, and minor improvements were made in the polyacrylamide gel apparatus; however, the essential technique remained unchanged for about 15 years.

Automated DNA Sequencing

The first major innovation to improve DNA sequencing was Leroy Hood's development of fluorescently labeled nucleotides in 1985 to replace the standard radioactive labels. Most commonly, each dideoxy base is given a different "colored" tag: we can imagine that A is green, G is yellow, C is blue, and T is red. Now, the fluorescent labels could be measured directly in the polyacrylamide gel as DNA fragments passed by a laser detector, thus eliminating both the radioactivity and the X-ray film. In addition, using the four differently colored fluorescent labels meant that the four sets of fragments could be run in a single lane of a polyacrylamide gel and the identity of the correct base could be determined

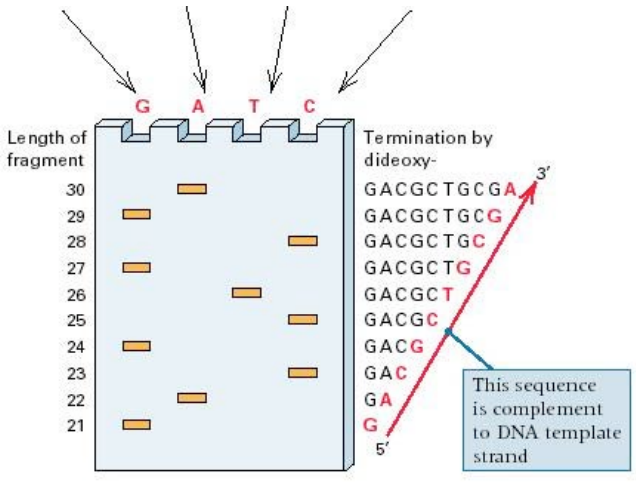

Figure 3. A sequencing gel showing bands in four gel lanes, representing the DNA fragments produced by the four different dideoxy sequencing.

automatically by the color of the end base on each fragment (see Figure 5a). Hood also directly connected the fluorescent detector to a computer so that the fluorescent signal was automatically collected and converted to a DNA sequence (see Figure 5b). Together with Lloyd Smith, Michael Hunkapiller, and Tim Hunkapiller, Hood founded Applied Biosystems, Inc. (ABI), which manufactures a commercial version of this automated fluorescent sequencer, which became available in 1986.

Since 1986, ABI (in cooperation with the PerkinElmer Corp.) has consistently improved their machines and has dominated the commercial marketplace for automated sequencers. Essentially all of the Human Genome Project and absolutely all of Celera Genomics' sequencing was done on ABI machines. However, ABI machines still have many of the limitations of the original Sanger method. They still rely on DNA polymerase to copy a template DNA sequence and on polyacrylamide gel electrophoresis to separate the fragments.

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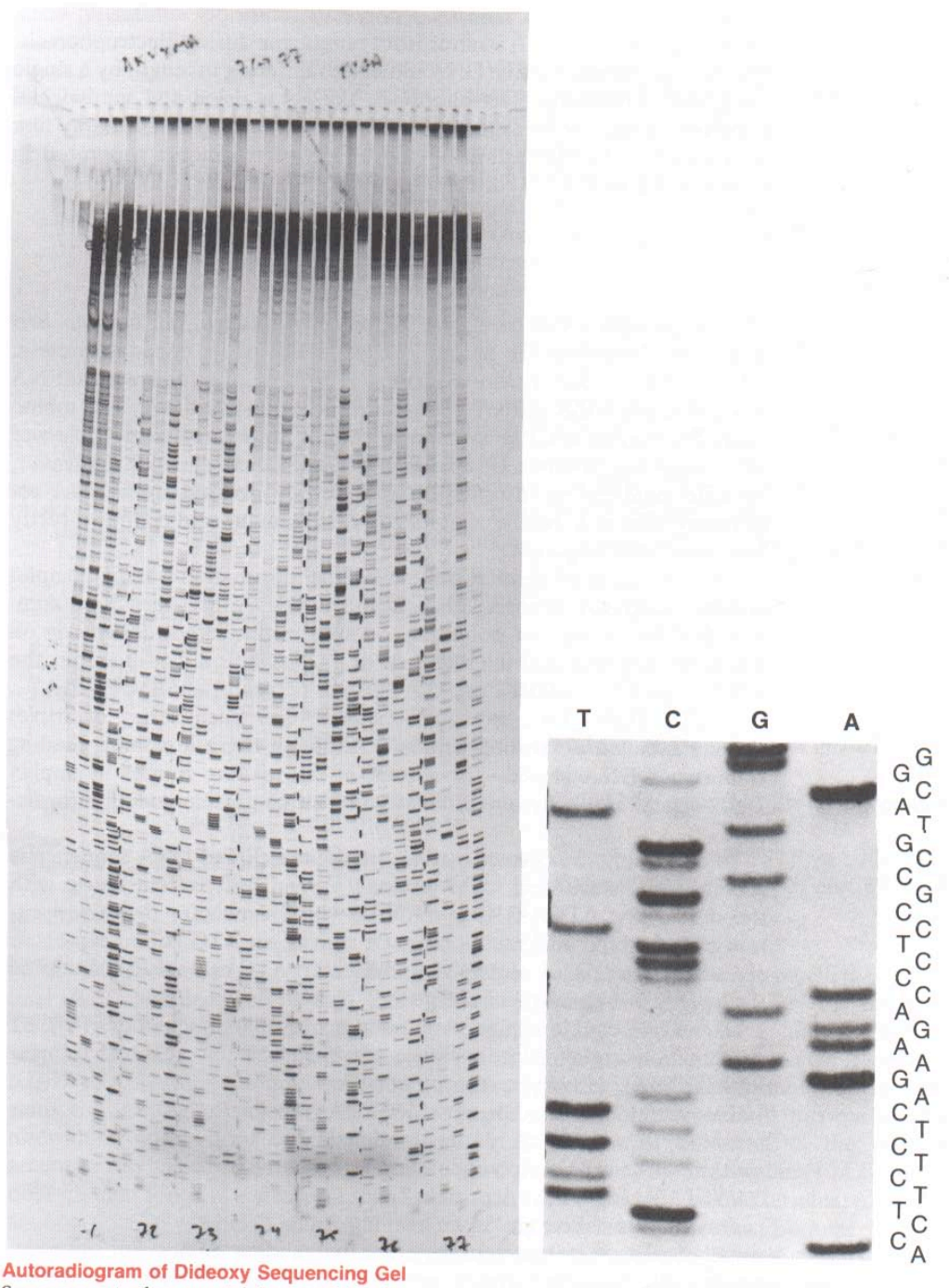

Autoradiogram of Dideoxy Sequencing Gel

Seven separate clones containing restriction fragments were sequenced in side-by-side reactions and coelectrophoresed on a sequencing gel. Each set of four columns (T,C,G,A reactions) on this autoradiogram are the sequence data from a different clone. A portion of the sequence data for clone 5 is shown in detail. Performed in the laboratory of Fred Sanger, who developed the dideoxy sequencing method. (Courtesy of W. Herr, Cold Spring Harbor Laboratory.)

Figure 4. An autoradiogram (X-ray film) of a DNA polyacrylamide sequencing gel. Each sequence requires four lanes, one for each base.

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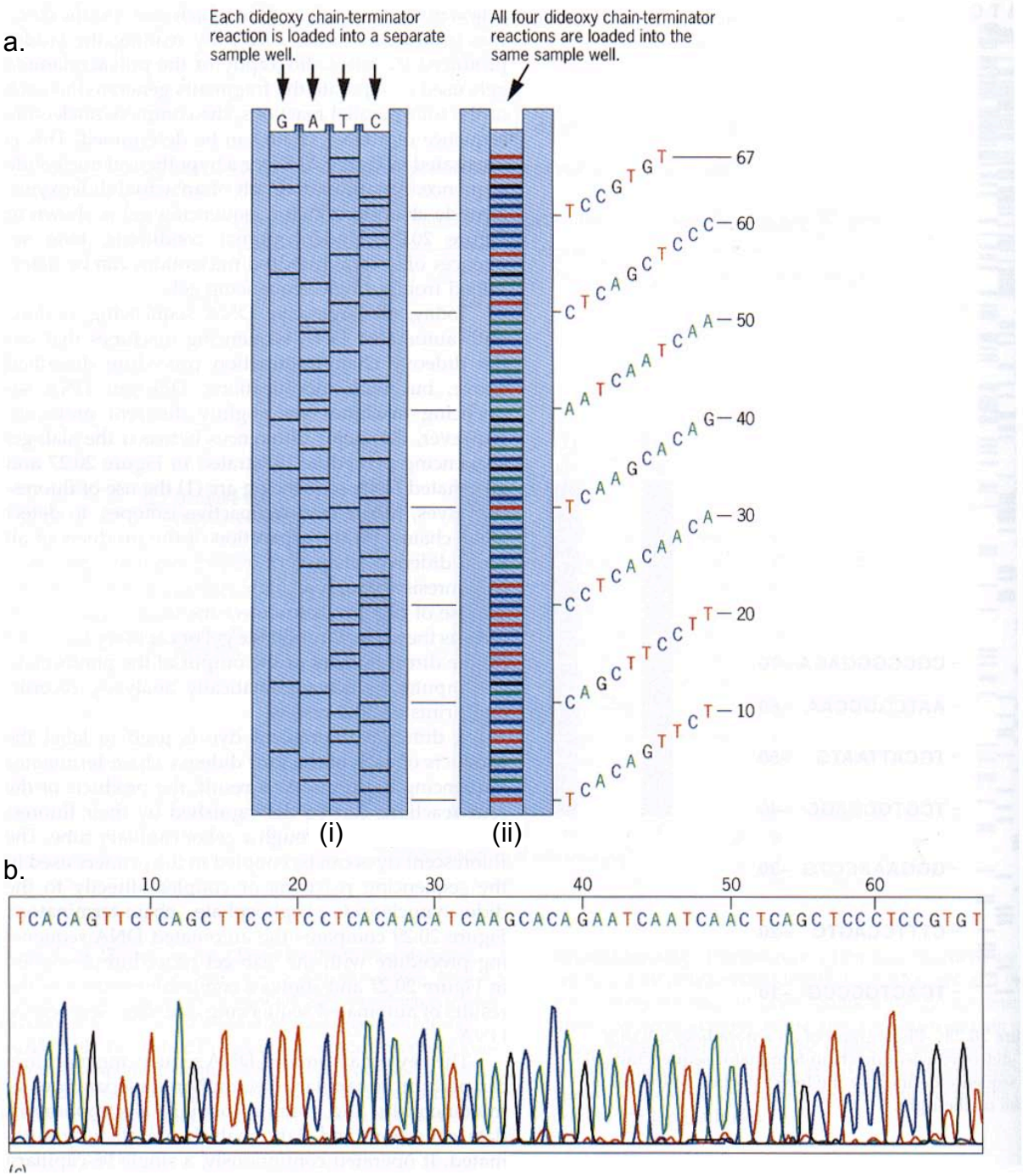

Figure 5. ABI fluorescent sequencers allow all four bases to be sequenced in a single gel lane and includes automated data collection directly into a computer.

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AP LAB 25: SANGER SEQUENCING SIMULATION

Objective:

This lab will simulate the Sanger method of sequencing DNA, both visually and kinesthetically; it uses colored pop-beads, which represent nucleotides and dideoxynucleotides. The dideoxynucleotides have their "stubs" cut off to simulate the inability of these molecules to extend the DNA chain beyond them.

Materials:

- A segment of DNA
- Reaction containers with pop-beads representing the 4 different DNA bases:
    - green = adenine
    - blue = cytosine
    - yellow = guanine
    - red = thymine

      note some of the pop-beads have their ends cut off to represent the radioactive dideoxynucleotides
- Masking tape

The masking tape is used to tape off a table top in the outline of an electrophoresis chamber with four wells, labeled A, C, G, and T respectively, to run your reaction mixtures

Teacher Guide:

1. Divide the class into 4 groups.

2. The following reaction container mixtures need to be prepared by the instructor. Each group of students will only receive **one** reaction container mixture.

Group / Color	A	C	G	T
Adenine (green)	50, 25 dideoxy	75	75	75
Cytosine (blue)	75	50, 25 dideoxy	75	75
Guanine (yellow)	75	75	50, 25 dideoxy	75
Thymine (red)	75	75	75	50, 25 dideoxy

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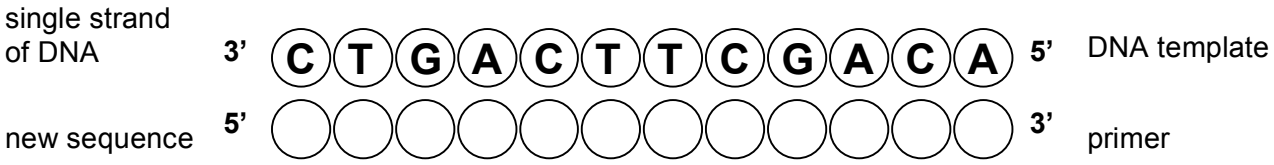
Procedure

1. Below is the section of DNA that you will be sequencing. Obviously if this were a true sequencing situation, you would not know this sequence. You would be using this procedure to determine the sequence of this unknown segment of DNA. You get to see this sequence now, because, in this part of the lab, you will be playing the role of **DNA polymerase** reading the DNA template.

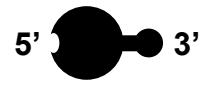
Figure 1.



2. Fill in the open half of the DNA strand in Figure 1 above with the appropriate nucleotide letter. Check that these are correct, as these will form the single-stranded nucleotide chains in your DNA sequencing.

3. Obtain a "reaction mixture" container of nucleotide & dideoxynucleotide pop-beads. There are four different bags. Each lab group will prepare sequences from **only one** "reaction mixture" container. Class results will be combined in the "giant electrophoresis chamber," which is taped on one of the lab tables.

4. Start building nucleotide chains as complementary strands to the DNA sequence in Figure 1. The "nub" on the pop-bead is the 3' end of the nucleotide. Remember, DNA can only be built by adding bases to the 3' end of a growing DNA strand. Select nucleotides out of your "reaction mixture" container without looking at whether you have selected a nucleotide or a dideoxynucleotide pop-bead. Remember, you are DNA polymerase so you must always construct your sequences from 5' to 3'. Build each strand until you link a dideoxynucleotide pop-bead to the chain. This always terminates the sequence. (You will quickly see it is physically impossible to add anymore nucleotides after a dideoxynucleotide has been bonded to the chain.) You will end up with a variety of DNA sequences of different lengths. Continue until you have 10-12 DNA segments, or until you run out of nucleotides.

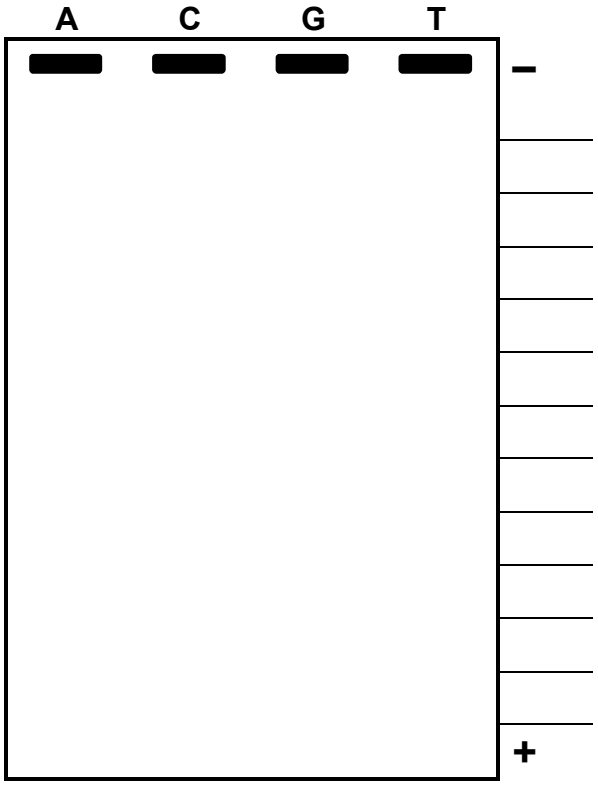


5. Take your DNA segments to the "giant electrophoresis chamber," at the specially marked lab bench. Place your sample in the proper "well" of the chamber. For example, the group which works with the reaction mixture containing dideoxyadenine will put its segments into "well A." Turn on the current (i.e., use your hands and mind!) and move your DNA segments their proper distance along the "sequencing gel". Remember: Smaller segments migrate farther than larger segments.

6. When all groups have run their reaction mixture through the "sequencing gel," sketch the DNA sequence bands in the chamber illustration on the next page. Fill in the proper bases (letters) on the blanks to the right of the illustration These bases signify the last base — the

dideoxynucleotide base — that terminated the strand. Since smaller segments of DNA migrate farther on the gel, the sequence is then read from **bottom to top**.

DNA Sequencing Gel



Questions

Complete the following questions by **typing** your answers on a separate sheet. You may hand in the document as a print-out or you may send it to me as an attachment to an e-mail.

1. What figurative role did people play in the sequencing technique?

2. What function does the gel play in the sequencing process?

3. Why was the use of colored fluorescent tagging a significant advancement over the use of radioactively tagged nucleotides?

4. Discuss 3 examples in which a person might want to make use of DNA sequencing technology.

5. Briefly explain how many advancements in basic biological are enabled only by advancements in technology.