Sequencing

Materials

- Scissors
- clear cellophane tape
- 4 paper cups
- 4 colored pencils (red, blue, green and yellow)
- 1 copy of nucleotides (A, T, C, G) and dideoxynucleotides (ddA, ddT, ddC, ddG) sheet
- 1 copy of the DNA template and primer sheet
- a computer with Internet access

Procedure

1. Cut out the following:
   - 20 of each free nucleotide (A, T, C, G) = 80 dNTPs;
   - 5 of each dideoxynucleotide (ddA, ddT, ddC, ddG) = 20 ddNTPs;
   - 1 template DNA (10 nucleotides long); and
   - 5 primers (5 nucleotides long).
2. Label each of the four paper cups: A, T, C, and G.
3. Place the dideoxynucleotides (ddNTPs) and the nucleotides (dNTPs) into the appropriate cups. For example, the A's and ddA's should be mixed together in cup "A."
4. Go to www.dnai.org > **Manipulation > Techniques > sorting and sequencing.**

   Click on the 2-D animation called "Cycle sequencing." This animation shows how a section of DNA is sequenced.
5. Work through this animation to find out the reagents required to perform a cycle sequencing reaction. (Hint: you'll need to hit "continue" three times to see all the reagents).

   You should have paper versions of most of these reagents in front of you.

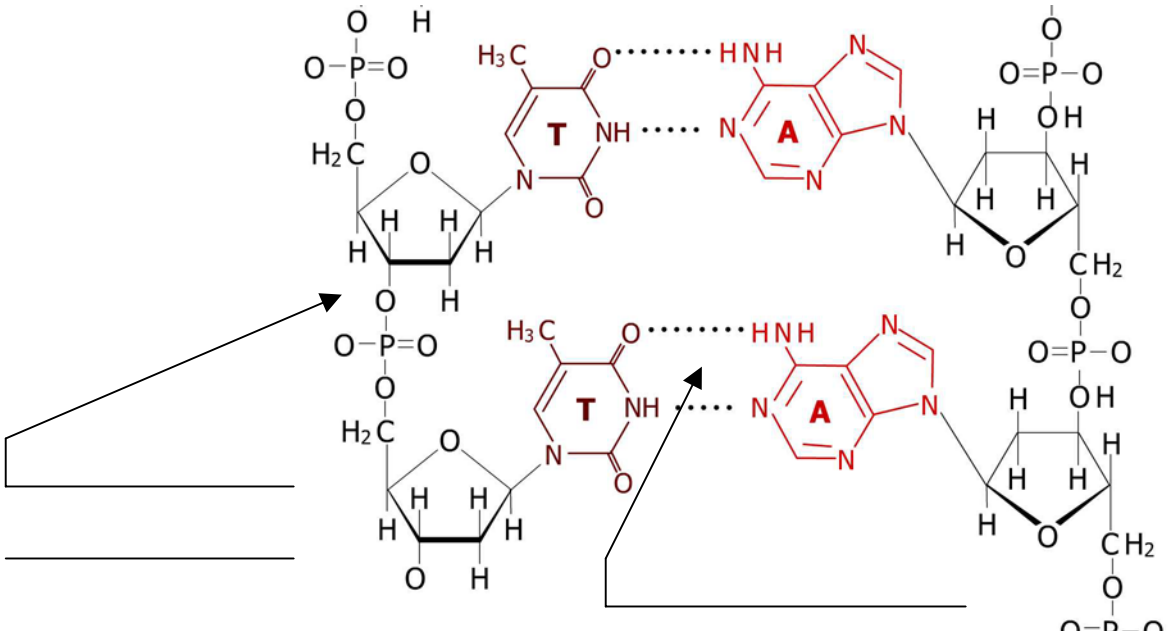
   Which reagent don't you have? _____

   You will act out the part of the missing reagent during this exercise.

The sequencing reaction

In this "reaction," you will be simulating making and breaking two types of bonds between the nucleotides.

On the diagram below, label the chemical bonds (hydrogen or phosphodiester) that hold the molecules in DNA together.



6. Each cycle of the sequencing reaction has distinct three steps, each performed at a different temperature. What are those three temperatures? Briefly state which action is performed at each temperature.

First Temperature: _____ Action: _____

Second Temperature: _____ Action: _____

Third Temperature: _____ Action: _____

7. Perform one cycle of the sequencing reaction. All three steps make one cycle.

Use the animation and the information below (Steps I, II, and III) as a guide to modeling this process with your paper cutouts.

Step I. Denaturing

Use the scissors to cut the double-stranded template DNA along the dotted line.

What causes the DNA to denature in a sequencing reaction?

_____

Step II. Annealing

Line up one of the primers with its complementary sequence on a strand of the template DNA.

What type of bonds form between the complementary bases?

_____

Step III. Extending

The enzyme DNA polymerase adds complementary nucleotides to a growing strand of a partially double-stranded DNA molecule in the 5' to 3' direction. Here, you will mimic the action of that enzyme.

A. Choosing the appropriate nucleotide

Start at the end of the double-stranded region (where the primer is now bound) and read the next base on the single stranded DNA template.

Decide which of the four nucleotides is complementary to this base.

B. Forming a phosphodiester bond

Shake the cup labeled with this letter and randomly pick out a slip of paper.
Like DNA polymerase, you shouldn't be able to distinguish between free nucleotides (dNTPs) and dideoxynucleotides (ddNTPs) before you pull them out of the cup.

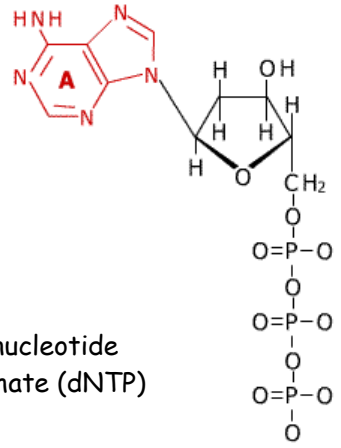
Place your new nucleotide at the end of your growing strand. Eventually, you will use a piece of scotch tape to attach the new slip of paper, simulating a phosphodiester bond.

Was the nucleotide you just added a free nucleotide or a dideoxynucleotide?

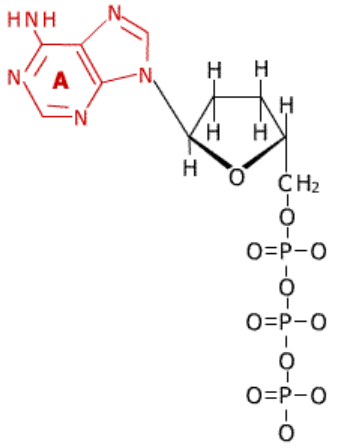
Look at the structures of a free nucleotide (also called deoxynucleotide triphosphates or dNTP) and a dideoxynucleotide (also called dideoxynucleotide triphosphate or ddNTP) below.

Circle the difference(s) between the structures.

Direction of DNA synthesis



A deoxynucleotide triphosphate (dNTP)



A dideoxynucleotide triphosphate (ddNTP)

Why do you think a ddNTP would be unable to form a phosphodiester bond?

_____

C. Determine whether or not to terminate the cycle

If you added a free nucleotide (A, C, G, or T), then DNA polymerase will continue to synthesize the growing strand of DNA. Repeat **Step III: Extending**.

If you are able to complete the strand before encountering a ddNTP, just empty the pieces back into the cups and start step III again.

If you added a dideoxynucleotide (ddA, ddC, ddG, or ddT), the synthesis of the new strand has ceased. The missing hydroxyl group on a ddNTP will not allow the DNA polymerase to add a new nucleotide to the growing strand.

Use pieces of scotch tape to stick the primer and nucleotide(s) together, and put the strand you made aside.

8. Perform another cycle.

Step I: Denaturing	Begin again with your original template.
Step II: Annealing	Use a new primer to bind to the single stranded template DNA.
Step III: Extending	Follow the same decision process, adding free nucleotides until you encounter a ddNTP that stops the synthesis.

9. Complete a total of five cycles. Follow the above steps (I, II, and III) until all five primers have had new nucleotides added to them (taped on). Other groups in your class will also do five cycles each, making a total of approximately 25 cycles. Scientists typically use 25 cycles to sequence a segment of DNA.

10. Place all the different size fragments of DNA produced into a single container for the class.

Sorting the pieces

11. As a class, perform an electrophoresis that models the workings of automated sequencing machine. Electrophoresis sorts different sized fragments of DNA.

    To develop a better understanding of how electrophoresis works, go to **www.dnai.org > Manipulation > Techniques > sorting and sequencing.**

    Click on the *Gel Electrophoresis* 2-D animation.

12. Use the blackboard or an empty section of wall to simulate the electrophoresis process.

    The top of the board = the positive pole of an electrophoresis setup.
    The bottom of the board = the negative pole.

    In a sequencer, all of the fragments produced by the 25 cycles are run in a single lane.

    Organize the fragments on the board (use masking tape to hold them up) in the order that they would run through the gel in a DNA sequencer.

    (Hint: Smaller fragments will move through the gel more quickly than larger fragments. If there are multiple fragments of the same size, place the same size fragments on top of each other. In a real gel there are thousands of fragments of the same size.)

Seeing the sequence

13. In a sequencing reaction, the dideoxynucleotides (ddA, ddT, ddC, and ddG) are labeled with four different-colored fluorescent dyes.

    Use the "Cycle Sequencing" animation to learn which colors represent each nucleotide.

    List the colors that correspond with each dideoxynucleotide.

Dideoxynucleotide	Color
ddG	_____
ddA	_____
ddT	_____
ddC	_____

14. Imagine that you are the laser capable of distinguishing between the different wavelengths of light (colors) for each of the four different nucleotides. You will always read only the dideoxynucleotide (the last one on the fragment).

    Create a graph that shows how your fragments would read. (The computer attached to an automated DNA sequencer usually performs this task.) Remember the smallest fragments will run past the laser first; so read the fragments from smallest to largest. Draw a peak in the color that corresponds with that letter along the timeline below.

    For example, if the dideoxynucleotide on the shortest fragment is ddA, then draw a peak from the "NORMAL" line at the bottom to the "PEAK" line at the top at point "1" in ddA's color (green).

    Use a different colored pencil for each letter and work on only one letter at a time.

    0 1 2 3 4 5

    PEAK_____

    NORMAL_____

    0 1 2 3 4 5

    The graph you have drawn is called an electropherogram.

    Using the electropherogram, you can determine the sequence of your template DNA.

15. Based upon your sequencing data above, what is the sequence?

    _____

16. If your template strand is 10 nucleotides long, how long should your sequence be?

17. Why is your final sequence different from your original template sequence?