NATIONAL CENTER FOR CASE STUDY TEACHING IN SCIENCE

The Colors that Do Magic:

How Fluorescence Enabled Automation in Nucleic Acid Sequencing

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Part I – Fluorescent ddNTPs

Sara, a college sophomore majoring in biological science, was both excited and worried. She had been awarded a research internship in a lab whose project was to find the species composition of a study site near campus. Her main activity would be the DNA sequencing of specific genes used for species identification. But it had been a whole year since she first learned the topic of DNA sequencing from her introductory biology class; how much did she really remember? So, on a Thursday afternoon, she opened her old notes and reviewed the paragraphs she had written about Sanger sequencing:

The most widely used DNA sequencing technique was invented by Frederick Sanger based in Cambridge, England, and his colleagues, Paul Berg and Walter Gilbert. They used the synthesized dideoxynucleotides to terminate the chain of DNA elongation. This new technique is based on the selective incorporation of chain-terminating dideoxynucleotides, also known as 2',3' dideoxynucleotides, and abbreviated as ddNTPs (ddGTP, ddATP, ddTTP and ddCTP). The extension reaction is divided between four different termination reactions, each containing a radioactive nucleotide that can be incorporated by the DNA polymerase during the extension step. Because the dideoxynucleotides lack a hydroxyl group on the 3´ carbon of the sugar ring, the addition of a nucleotide cannot continue, and this marks the end of the elongation. The resulting fragments can then be resolved on a high-resolution four-lane-wide and radioactive polyacrylamide gel that reveals the complementary sequence (Figure 1). Sanger, Berg and Gilbert won the Nobel Prize of Chemistry in 1980 for the "dideoxy method" also known as "Sanger method" or the "first generation sequencing" (see Sanger et al., 1977).

Later, Professor Hood's team at Caltech developed the technique, in which the radioactive nucleotide was replaced by fluorescent labels and included the use of a laser to detect the fluorescent colors. The electrophoretic process is connected to a computer for the display of the resulting sequence. They published their work in Nature (see Smith et al., 1986).

This work was further improved by the fluorescent labeling of ddNTPs by a team of researchers at the DuPont Research Station and the technique was described in an article led by Dr. James Prober in the journal Science (see Prober et al., 1987).

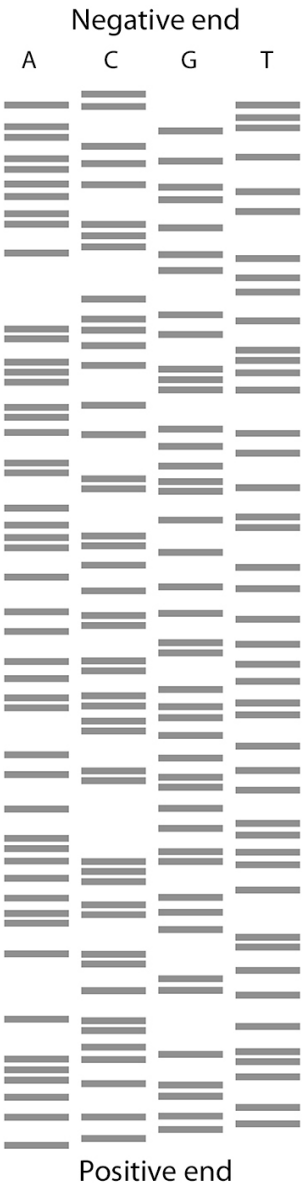

Figure 1. Autoradiograph from four separate dideoxy sequencing reactions.

On her first day of research work, Sara met with the postdoctoral researcher, Dr. Dana, who would go over the details of her tasks with her. Dr. Dana smiled at her new intern. "Welcome Sara! Are you excited for your first day in lab?"

"Hi, Dr. Dana," Sara replied. "I'm really excited to get started, although I'm a little worried because I've never done sequencing experiments before. Also, I've never used those sequencing machines, so I'm a bit anxious."

Dr. Dana nodded with understanding. "Well, I have good and bad news. I'll start with the bad; our sequencing machine is under routine maintenance today, but it should be working by tomorrow morning. The good news is that you will not waste your time today, because you'll be preparing the reaction tubes to be used tomorrow."

Sara received the protocol and the material from Dr. Dana and started her bench work. She wrote in her lab notebook the ingredients for the experiment. The DNA she was trying to sequence was 3′ATCGGTACAAGGTCG5′ with the first five bases used for primer binding.

Experiment 1:

- 0.5 pmole DNA template
- 0.5 pmole primer
- 1 µL of 10× *Taq* polymerase
- 80 µM dNTPs (equal portion of dATP, dTTP, dGTP, dCTP)
- 8 µM ddNTPs each
  - ddATP: red
  - ddGTP: blue
  - ddTTP: yellow
  - ddCTP: green

Total in 10 µL volume

As she gently added and mixed all of the components together Sara wondered what was actually happening within the tube. She went online and watched the following video to remind herself of the principles of DNA sequencing:

DNA Sequencing: The Chain Termination Method (Sanger Method). Running time: 3:00 min. Produced by Conor, 2012. <https://youtu.be/vK-HlMaitnE>

DNA Synthesis Group Activity

Imagine you are a DNA polymerase, and your classroom is one of the test tubes that Sara is preparing, with all the molecular components for a sequencing reaction. Follow the procedure below. Your instructor will provide you with the required materials.

Step 1: In this exercise you are provided with multiple possible primers. Choose the correct primer that is complementary to the beginning of the template strand.

Step 2: In the provided bag is a mixture of dNTPs and ddNTPs. You will take out one nucleotide at a time until you encounter one that is complementary to the template nucleotide. Put back in the bag all other nucleotides that are not complementary to the first position.

Step 3: Continue until you pick up a ddNTP that is complementary to the template nucleotide. Because the ddNTP does not allow you to attach any additional nucleotide, the elongation will be terminated at this nucleotide. You will now detach the fragment from the template. Set aside this single-stranded DNA.

Step 4: Continue with a new primer and repeat steps 2 and 3 above.

Step 5: Each time you take out a ddNTP and the reaction stops, detach the entire fragment from the template and put it at the side of your workspace.

Step 6: Organize your fragments from the shortest to the longest one as shown in Figure 2 (right).

Step 7: Read Questions 1 to 8 below before you start the group activity.

Step 8: Write in the spaces below the sequences of the fragments you have obtained. Include the primer sequence.

Fragment 1:_____

Fragment 2:_____

Fragment 3:_____

Fragment 4:_____

Fragment 5:_____

Fragment 6:_____

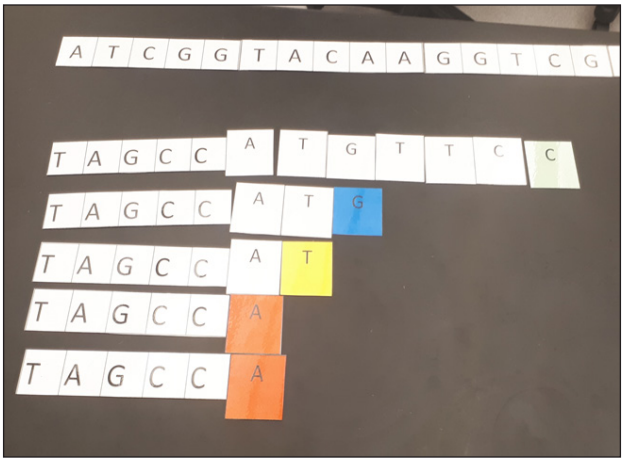

Figure 2. Synthesized single-stranded DNA organized on the workbench. The template sequence is at the top.

Questions

1. What would happen if you have sufficient or more than enough quantity of the primer?

2. Are the fragments DNA or RNA fragments?

3. Are the synthetized fragments single or double stranded?

**** Your instructor will collect the class data by combining the results from all groups. ****

4. Write down the sequence data collected from the entire class. Use appropriate colors for each nucleotide.

   5′_____3′

5. What is the minimum number of fragments needed to complete this sequence?

6. Using four different colors, draw the DNA bands in the gel in Figure 3 that Sara should obtain after running an electrophoresis with all the fragments in this activity. The numbers to the left of the image are size markers in bases.

**** Your instructor will summarize the DNA bands from the gel. Answer the following questions based on the results. ****

7. If the DNA template is read in the 3′→ 5′ direction from left to right, which direction should the primer be read?

8. Bases are added to which end of the primer, 5′ or 3′?

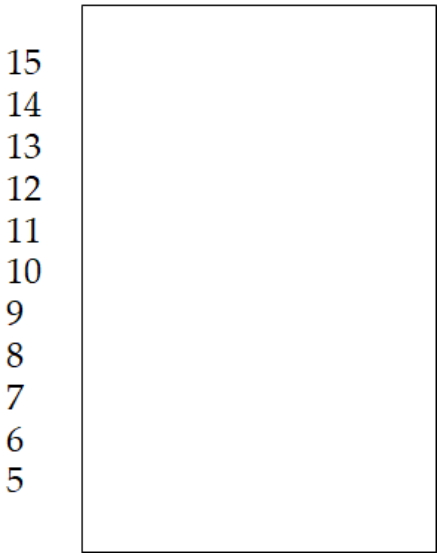

Figure 3. Resolved DNA bands with colors indicating fragments terminated at specific ddNTPs.

9. Where in the gel will you find the shortest fragment, near the wells or further away from the wells? Similarly, where will you find the largest fragments?

10. Which part of the gel corresponds to the 5′ position of the DNA sequence, near the wells or further away from the wells?

11. Write the sequence according to the bands from the bottom to the top of the gel and indicate the 5′→ 3′ direction.

12. Does this sequence correspond to the template strand or the complementary strand?

13. Write the sequence that is complementary to the one in Q12. Indicate the 5′→ 3′ direction.

14. Comparing the sequences obtained from this exercise, what is the sequence that you have synthesized? What is the complementary sequence to the strand you synthesized?

Part II – Traditional Sanger Sequencing

Sara called Dr. Dana to let her know that she had finished preparing the tube for cycle sequencing. Dr. Dana was clearly pleased. "That's great, Sara, you're fast! We'll put that on the thermal cycler and in the meantime run a traditional sequencing, just to get an idea of this short sequence. We'll then compare this to the chromatogram tomorrow once the machine is up and running to confirm the sequence. What do you think?"

"Sure," replied Sara. "But first, what do you mean exactly by 'traditional sequencing'?"

"Oh! I'm sure you've studied that before. Sequencing gels haven't always been colorful. In the original Sanger sequencing method, no one could tell one fragment apart from another. So, in order to be able to figure out the sequence, people had to make sure the four reactions were set up in four separate tubes and they had to know which tube had which dideoxynucleotide."

Sara nodded. "So, do you mean I'll have to prepare four different tubes and load them in four different wells later?"

"Exactly," replied Dr. Dana. "And please make sure you write everything you do in your notebook."

Sara headed back to the bench. She used the same template DNA 3′ATCGGTACAAGGTCG5′ with the first five bases used for primer binding. She started her work but was a little distracted by the music she was listening to. When she noticed that she had made a mistake in the procedure, she recorded the error in her notes:

Experiment 2:

- 0.5 pmole DNA template
- 0.5 pmole radioactive primer
- 10 µL of 10X Taq polymerase
- 80 µM dNTPs (equal portion of dATP, dTTP, dGTP, dCTP)
- 8 µM ddNTPs each, separately
  - ddATP Oops! I forgot to add ddATP in the A tube!
  - ddGTP
  - ddTTP
  - ddCTP

Total in 10 µL volume

Questions

15. How many reaction tubes does Sara need to prepare in this experiment?

16. What should each reaction tube contain?

17. Follow the example in the left panel of Figure 4 below to complete the gel picture in the right panel. The DNA bands should correspond to the fragment sizes shown on the left side of the panel and reflect the results from the absence of ddATP in one of the reactions.

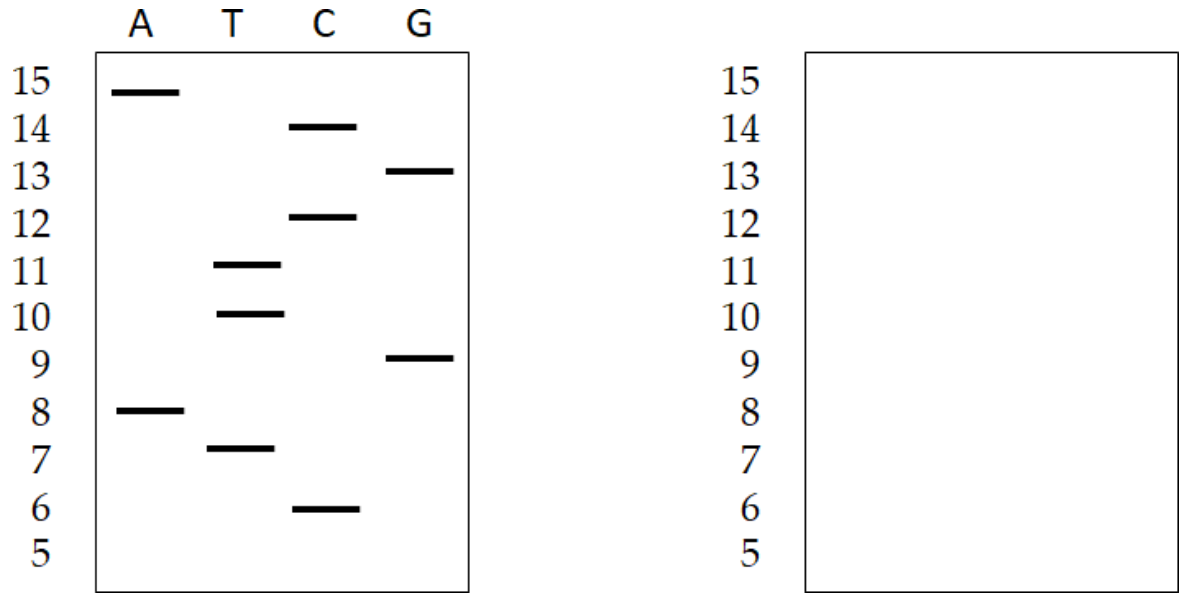

Figure 4. Gel image from Experiment 2. Use the left panel as an example for filling in the right panel.

18. Write the template sequence from 5′ to 3′ of this gel and include the primer. If the nucleotide position is unknown use a "?" mark to indicate it.

Experiment 2, Take Two

Sara realized that she still had some time before Dr. Dana came back. She waned to do it right this time, making sure to add all types of ddNTPs. When the reaction run completed this time, she produced the gel picture as in Figure 5.

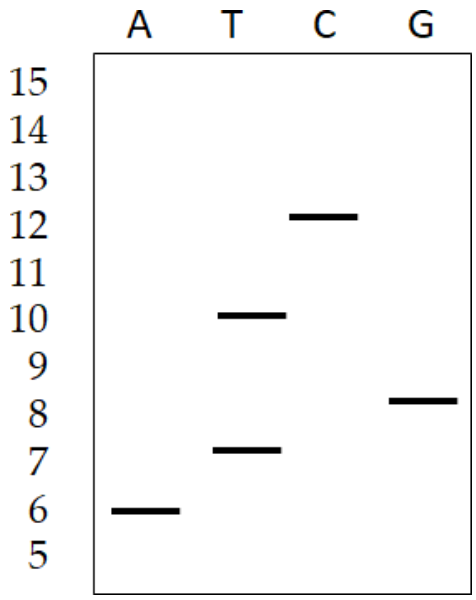

Figure 5. Gel image from Experiment 2.2.

Question

19. Knowing that Sara has added all the ingredients, what could be a reason for missing some of the bands? (*Hint:* consider the quantity of the ingredients.)

Sara showed her two gels to Dr. Dana, a little bit embarrassed that she hadn't been able to produce a correct gel with the traditional method.

Dr. Dana waved it aside by saying, "No worries! This type of mistake happens all the time. The most important thing is to keep taking good notes in your lab notebook so we can troubleshoot and understand what must have happened. I'm happy to see you did that. Please get some rest and I'll see you tomorrow morning as the machine is now ready."

The next day, Sara brought her reaction tube to run with the sequencing machine. The DNA fragments in Sara's tube were scanned by the machine and read by a computer. Figure 6 is the chromatogram obtained.

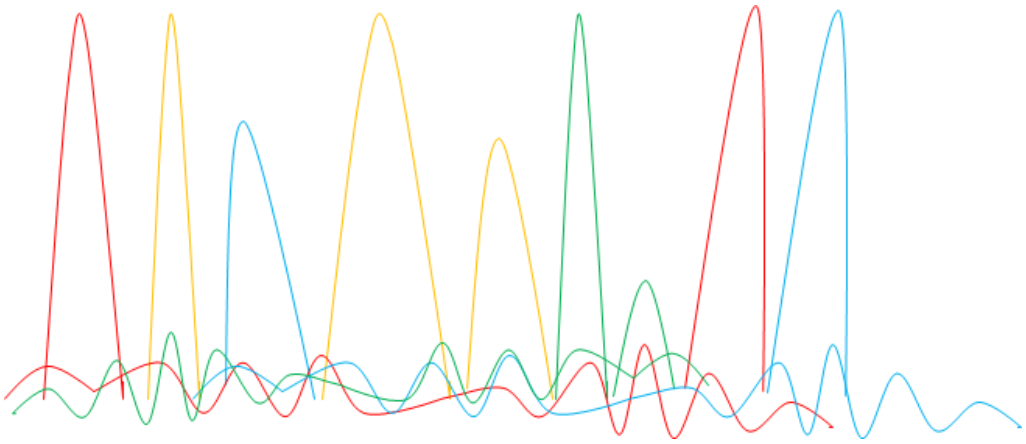

Figure 6. Chromatogram produced from Sara's experiment.

Questions

20. Is the sequence resolved from Figure 6 the template or the complementary sequence?

21. Is the direction of the chromatogram from left to right 5′ to 3′ or 3′ to 5′ of the sequence?

22. What does the height of a peak indicate? (*Hint:* consider the brightness or thickness of a DNA band in an electrophoretic gel.)

23 Do you think Sara was able to obtain the sequence of the primer from the dideoxy sequencing method? Explain.

24. The fluorescent label invention was developed by Professor Hood's group at California Institute of Technology. But they still had to carry out sequencing in four separate reaction tubes, until another team, in the DuPont Research Lab, further developed the technique. What specifics about the labeling allow sequencing to be conducted in a single reaction tube?

Conclusion

Dr. Dana was very pleased with the results and congratulated her new intern. "Well done Sara! We've got a nice chromatogram with minimum noise."

Sara smiled, relieved that she had been able to recover from the mistake she had made yesterday. She also now had a better understanding of how fluorescence allowed automation of sequencing, and how her work was greatly facilitated by the ability to carry out sequencing in a single tube. The initial worries she had felt about her new internship were gone, and now only excitement remained.

References and Recommended Resources

Bio-OER, LibreTexts. 2021. Sanger sequencing of DNA. [Webpage]. <https://bio.libretexts.org/@go/page/24787>

DNA Sequencing: The Chain Termination Method (Sanger Method). [Video]. Running time: 3:00 min. Produced by Conor, 2012. <https://youtu.be/vK-HlMaitnE>

Prober, J.M., G.L. Trainor, R.J. Dam, F.W. Hobbs, W. Robertson, R.J. Zagursky, *et al*. (1987). A system for rapid DNA sequencing with fluorescent chain-terminating dideoxynucleotides. *Science* 238(4825): 336–41. <https://doi.org/10.1126/science.2443975>

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Smith, L.M., J.Z. Sanders, R.J. Kaiser, P. Hughes, C. Dodd, C.R. Connell, *et al*. (1986). Fluorescence detection in automated DNA sequence analysis. *Nature* 321(6071): 674–9. <https://doi.org/10.1038/321674a0>

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