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Activity

Manipulative-based Activity Using Pop Beads for Demonstration of Sanger Sequencing

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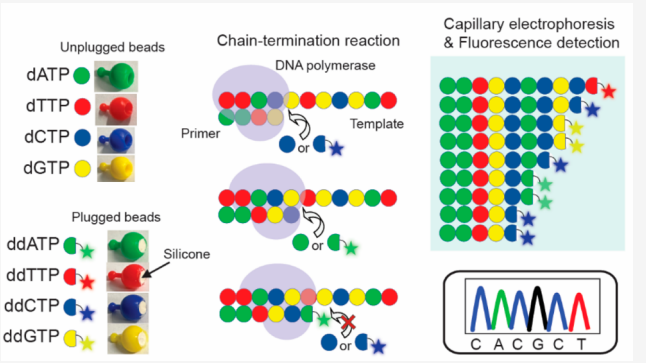
ABSTRACT: Sanger sequencing, also known as dideoxy sequencing, is a widely used method for DNA sequencing, particularly for cloned plasmids and clinical samples. This technique requires a combination of essential biochemistry skills, such as a chain-termination reaction, gel electrophoresis, and fluorescence detection. Unfortunately, there is a lack of activities that replicate the Sanger sequencing process for students to learn and practice these skills. To address this issue, a manipulative-based Sanger sequencing activity was developed that incorporates colorful pop beads to demonstrate a chain-termination reaction, separation of products, and fluorescence detection. The beads represent deoxynucleotides and dideoxynucleotides, allowing for a visual representation of DNA fragment generation. This kinesthetic learning activity offers a high visual impact for students, aiding in their understanding of the Sanger sequencing process, and can also be used to illustrate polymerase chain reaction (PCR)-based techniques.



KEYWORDS: *Sanger (dideoxy) sequencing, upper-division undergraduate, biochemistry, collaborative learning, chromatography, and nucleic acid/RNA/DNA*

INTRODUCTION

Sanger sequencing, also known as dideoxy chain-termination sequencing, is a widely used DNA sequencing method developed in the 1970s.[1] This method is still commonly employed for sequencing recombinant DNA constructs and validating variant calls generated by Next-Generation sequencing methods.[2−6] DNA sequencing is a valuable tool for teaching biochemistry concepts and techniques to university students.

Sanger sequencing consists of the chain-termination reaction, fragment separation, and detection. In the chain-termination reaction, it starts by synthesizing a new strand of DNA from a template strand using a DNA polymerase, a single oligonucleotide primer, deoxynucleotides (dNTPs), and fluorescently labeled dideoxynucleotides (ddNTPs). The reaction terminates when a ddNTP is incorporated onto the 3′-end of the new strand, creating a mixture of DNA fragments of different lengths. The ratio of dNTPs to ddNTPs determines the frequency of the ddNTP incorporation. Thus, this variation of sequencing is termed the chain-termination method. The fragments generated are sorted by size through capillary gel electrophoresis. The sequence of the DNA is then determined by observing the fluorescent colors of the incorporated ddNTPs in the separated DNA fragments. The detection of fluorescence is performed by an automated machine equipped with a laser and detector, providing an accurate determination of the DNA sequence.

Although Sanger sequencing is an important topic for students to learn, access to Sanger sequencing machines in colleges is limited, as are other relevant hands-on activities.[7] To overcome this challenge, we created a hands-on activity to simulate the Sanger sequencing process. This activity utilizes colorful pop beads to enable visualization of the chain-termination reaction with dNTPs and ddNTPs, DNA fragment separation, and template sequencing using fluorescence detection. Our survey and pre/post-test results showed that this activity had a strong visual impact that helped students to better understand the entire Sanger sequencing process. Students in lab classes grasped the principles of Sanger sequencing prior to examining actual Sanger sequencing data from their cloning results. This method can also be used as an effective lesson to teach polymerase chain reaction (PCR), real-time quantitative PCR (RT-qPCR), and site-directed mutagenesis.

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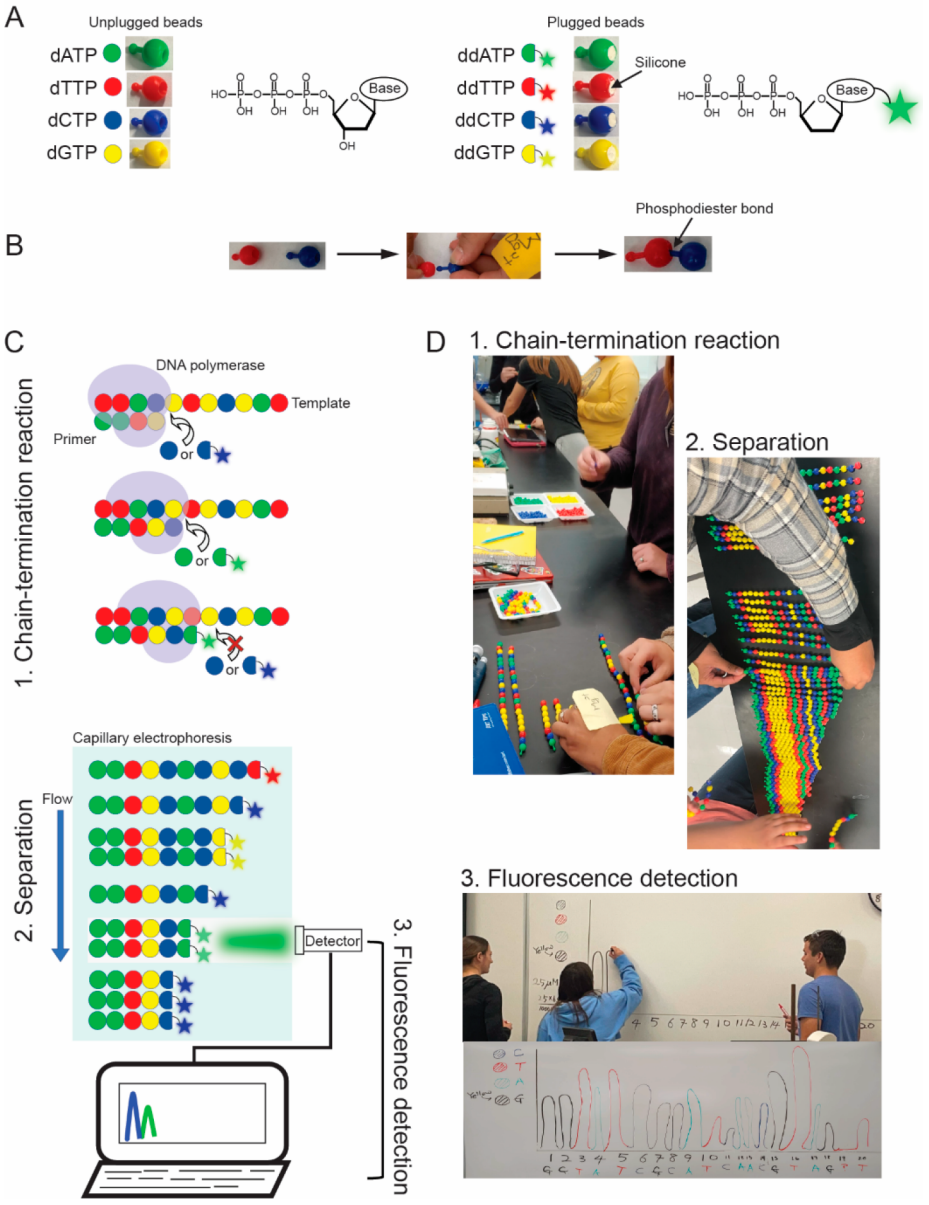

Figure 1. Activities mimic Sanger sequencing using beads. (A) Unplugged and plugged beads represent dNTPs and ddNTPs, respectively. Plugged beads were sealed with silicone. The chemical structures of both dNTPs and ddNTPs are shown next to their respective bead images. A star represents a fluorophore. (B) An example of a reaction between nucleotides. The hand represents a DNA polymerase with its cofactor, Mg^{2+}. The connection between a hole of dTTP and a peg of dCTP represents a phosphodiester bond. (C) Schematic of the reactions mimicked by the activities. In the chain-termination reaction, a DNA strand is synthesized from a template using a DNA polymerase. The synthesis is stopped once ddNTP is added to the newly synthesized DNA strand. In the separation step, the fragments synthesized during the chain-termination reaction are separated by size in a capillary electrophoresis column. The fluorescence detection step involves reading and recording the fluorescent color of the ddNTP in each fragment. (D) Photographs showing students' activities in each step.

DESIGNING THE ACTIVITY

The activity is divided into three parts: the chain-termination reaction, the separation of DNA fragments produced, and fluorescence detection (Figure 1).

Preparation of Materials for Chain-Termination Reaction Simulation

Each reagent in the chain-termination reaction, DNA polymerase, a primer, a template, dNTPs, ddNTPs, and buffer, is represented within the activity. Colorful beads represent each dNTP. Red, blue, green, and yellow beads represent dTTP, dCTP, dATP, and dGTP, respectively (Figure 1A). Each bead has a hole and a peg for connecting to other beads, demonstrating the reaction between nucleotides (Figure 1B). These beads are available for purchase from online retail platforms, including Amazon. The peg end represents the 5′-triphosphate group, and the hole end represents the 3′-hydroxyl group of a dNTP. To help the students understand the formation of the phosphodiester bond, we explain that the reaction is energetically favorable due to the relief of repulsion between the negatively charged alpha and beta phosphates when they are separated and the formation of additional resonance structures in the released pyrophosphate. This results in a negative change in the free energy, which drives the reaction forward. The beads that represent ddNTPs are the same colors as those representing the dNTPs, but the hole of

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the bead is blocked (by injection of silicone), preventing further connections (Figure 1A). Unplugged beads are linked together to represent the template and primers. A buffer creates the optimal environment for the maximum activity of DNA polymerases. Because students' hands played the role of a DNA polymerase, we prepared a relaxing Lo-Fi soundtrack as a buffer to create an optimal environment for student activity. A Mg^{2+} sticker is also used to illustrate the Mg^{2+} cofactor required for the DNA polymerase activity (Figure 1B).

Optimization of Template Length for Chain-Termination Reaction Simulation

Under ideal conditions, the chain-termination reaction will produce a nested set of fragments from a DNA template of any length. As our goal was to complete the activity within 1 h, the template needed to be short. To maximize the number of fragments generated for a short template, our experiments used a 9:1 ratio of dNTP beads to ddNTP beads. This ratio is lower than what is typically used in Sanger sequencing (10:1 to 300:1).[7] Our initial faux reaction conditions to optimize a template size included 54 identical five-bead-long primers, a template of 29 complementary nucleotides with the last five nucleotides at the 3′-end serving as the primer binding site, and 400 beads per nucleotide with the 9:1 ratio between unplugged and plugged beads. We extended the primer on the template strand by randomly selecting a bead from a pool of beads that contained unplugged and plugged beads of complementary colors until a plugged bead was selected, which inhibited the fragment from extending further. The data on the lengths of synthesized fragments are presented in Figure 2. Our results showed that when the fragment length exceeded

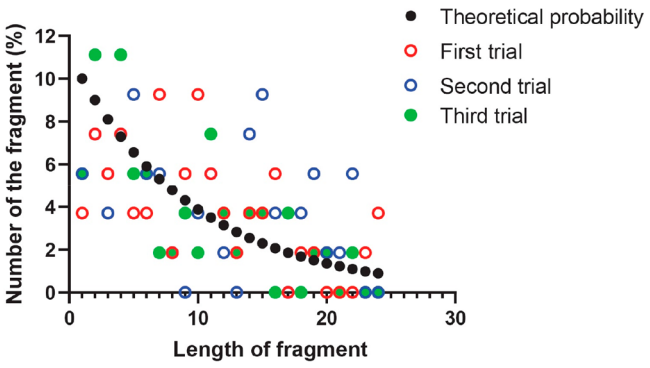

Figure 2. Optimization of template length to minimize missed fragments in chain-termination reactions. The open red and blue circles and the closed green circles represent the results from three separate trials of the chain-termination reaction using a 9:1 ratio between dNTP and ddNTP. X and Y axes denote fragment length and number of fragments produced in the reactions, respectively. The closed black circle represents the theoretical probabilities calculated using eq 1 to produce each fragment. The template size used in this test was 29 nucleotides including a primer binding site of 5 nucleotides, and a total of 54 primers were used.

20 nucleotides, there was an increase of the number of missing fragments (Figure 2). This is due to the reduced probability of generating longer fragments. The formula for calculating the probability of forming each fragment is given below:

$$\%\text{Chance}_n = 0.1 \times 0.9^{(n-1)} \times 100 \quad (1)$$

where the definition of % Chance_n is the probability of making a fragment with a length of n nucleotides ending with a 3′ ddNTP. Based upon these simulations, we reduced the template size to 25 nucleotides to minimize missing fragments. We also increased the number of primers to 80 because 80 primers allowed us to finish the activity in 1 h when the class size was 20 students or fewer. More primers can be used, depending on the class size, to ensure that each student is able to synthesize at least four strands.

Preparation for the Separation Step

The second step of this activity focuses on explaining the separation of DNA fragments generated from the chain-termination reaction. For this part, we utilized an expired capillary electrophoresis column from the Western South Dakota Genetics and Genomics Core (WestCore) at Black Hills State University (BHSU) to demonstrate the real column (ABI 3500 Capillary Array, Applied Biosystems) used in the process. In the case that instructors cannot acquire a column, they can use any alternative to demonstrate the concept of capillary electrophoresis.

Preparation for the Fluorescence Detection Step

The final activity involves the detection of DNA fragments by using fluorescence. In a real Sanger sequencing setup, a machine reads the colors of the fragments and records the results on a computer. To simulate this, we used a whiteboard as a computer screen and markers of four distinct colors to represent the fluorescent color on each ddNTP.

PRE-LAB ASSIGNMENT

The key elements for Sanger sequencing are the ddNTPs. Fluorophores attached to them enable a reading of the sequences. Students were prepared for the lab activity through prelab questions aimed at familiarizing them with these elements and the detection of DNA fragments. The students were also given a link to a video that explains Sanger sequencing, which can be found at https://www.youtube.com/watch?v=e2G5zx-OJIw.

ACTIVITY

The goal of this activity is to give students a comprehensive understanding of the three key aspects of Sanger sequencing: the chain-termination reaction using ddNTPs to generate DNA fragments, the separation of these fragments, and the detection of the fragments through fluorescence.

Activity Setup

The setup for the activity involved creating a 25-nucleotide template DNA strand with approximately equal numbers of each color of bead. The last five nucleotides at the 3′-end of the template serve as the primer binding site. The students were informed that, in real experiments, primers are typically at least 18 nucleotides long. Based on the outcome of the optimization activity, a total of 80 primers complementary to the 3′-end of the bead template were constructed to mimic the optimal concentration of primer in the faux reaction. Supplementary Table S1 outlines the required number of beads in a biochemistry lab class (CHEM464L).

This activity was conducted to help students grasp the principles of Sanger sequencing prior to examining actual Sanger sequencing data from their cloning results in CHEM464L with 16 students and in a genomics techniques class (BIOL724L), a graduate course with six students. The students worked in groups of three or four and were given all the necessary materials, including four containers with each

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Table 1. Rules of the Activity

Step	Rules
Chain-termination reaction	When simulating the role of a DNA polymerase, students must put a Mg^{2+} sticker on their hand to mimic the DNA polymerase enzyme cofactor necessary for optimal enzyme activity. Students playing the role of a DNA polymerase must choose a bead complementary to the template without looking. A DNA fragment is completed when a plugged bead is added to a newly synthesized strand.
Separation	Students playing the role of a column gather all the fragments produced from the reaction. They will then align the fragments based on their size. The larger fragments are placed at the top, near the negative electrode, and smaller fragments are placed at the bottom, near the positive electrode.
Fluorescence detection	Students playing the role of a detector read the color of a plugged bead in each fragment. These students will then verbally convey the color information to the students playing the role of a computer. The computer students will record a chromatograph using colorful markers and interpret colors to the corresponding nucleotide sequences.

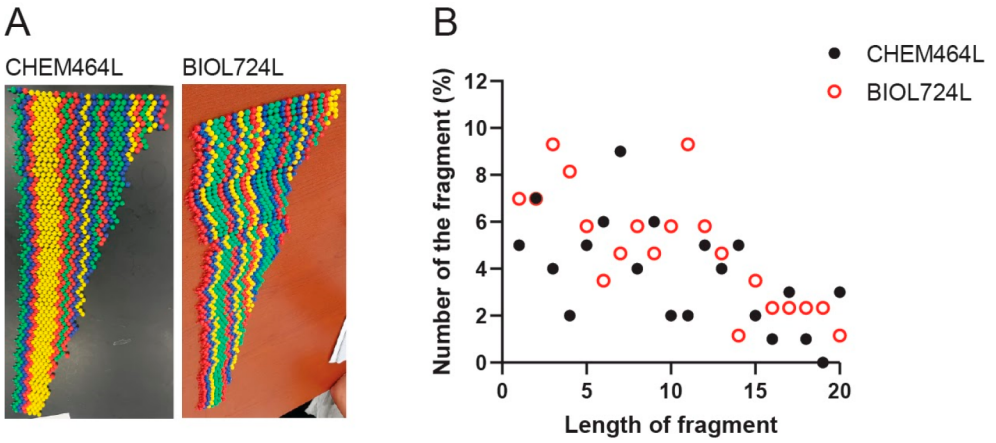

Figure 3. The results of the chain-termination reactions and separation experiments conducted in CHEM464L and BIOL724L classes. (A) Separated fragments by size. (B) Fragment counts for each length.

color of unplugged (i.e., dNTP) and plugged (i.e., ddNTP) beads with a 9:1 ratio, a template DNA, 80 primers, and a Mg^{2+} sticker. As a final preparation step, we selected a music playlist that served as a buffer and adjusted the audio controls appropriately. This way, students can appreciate the music without it becoming overly distracting.

Before beginning the activity, it is recommended that students have a background in the chain-termination reaction, electrophoresis, and fluorescence. However, if they lack this knowledge, the instructor should provide a brief explanation. To ensure a thorough understanding, the instructor should explain the purpose and procedure of Sanger sequencing using Figure 1. Additionally, to establish clear guidelines for the activity, the instructor introduces the rules outlined in Table 1 to the students.

Part 1: Chain-Termination Reaction

In the CHEM464L and BIOL724L classes, each group of 3−4 students was provided with a DNA template. We prepared 400 beads per nucleotide with a 9:1 ratio between unplugged and plugged beads. The beads and 80 primers were also evenly distributed in each group. The activity started with the separation of the double-stranded DNA template into single strands, which is a process known as denaturation. In the following annealing and extension stages, the students placed the primer next to the template strand with complementary colors aligned and extended the newly synthesized DNA strand, acting as the DNA polymerase (Figure 1D). The extension was done by randomly selecting a bead from a pool of beads that contained the ratio of unplugged and plugged beads. Students continued to extend the strand until they selected a plugged bead, which inhibited the fragment from extending further. The data on the lengths of the synthesized fragments are presented in Figure 3. During the CHEM464L activity, one fragment was missing out of 20, providing a learning opportunity for students to contemplate the possibility of missed fragments in real-life chain-termination reactions. However, the instructor and students estimated that approximately 1.5×10^{13} primers would be used in real Sanger sequencing, greatly reducing the likelihood of missing fragments.

Part 2: Separation

In this activity, each group had one student playing the role of a capillary electrophoresis column. The instructor demonstrated the working principle of capillary electrophoresis using a real column and explained it based on the principle of agarose gel electrophoresis. The students collected all of the fragments and arranged them by size, with the smallest fragments at the bottom (positive electrode) and the largest fragments at the top (negative electrode), as seen in Table 1. The separation results from both the CHEM464L and BIOL724L classes are displayed in Figure 3A.

Part 3: Fluorescence Detection

Four students, each assigned to a different ddNTP, are stationed at the whiteboard, which acted as a virtual computer screen. The whiteboard displays the sequence using colors to represent the four nucleotides: red for thymine, green for adenine, yellow (black in the electropherogram) for guanine, and blue for cytosine. The students separating the fragments by size announced the nucleotide, and those on the whiteboard used this information to draw peaks (Figure 1D). One student wrote out the sequence based on the peak colors, which was then matched with the template sequence.

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■ ASSESSMENT

The goal of this activity was to provide students with a comprehensive understanding of the three crucial elements of Sanger sequencing: termination of reactions with ddNTPs, separation of the generated DNA fragments, and detection of the fragments through fluorescence. To assess the effectiveness of the activity, students in CHEM464L were asked to answer questions about the key concepts before and after participating in the activity (Figure 4, top). Despite being assigned to watch a video on Sanger sequencing as a preparatory laboratory assignment and answering associated questions, the pretest results indicated that many students lacked a clear understanding of the concepts. However, the post-test results showed a significant improvement in the students' understanding of Sanger sequencing, demonstrating the efficacy of the activity (Figure 4, bottom).

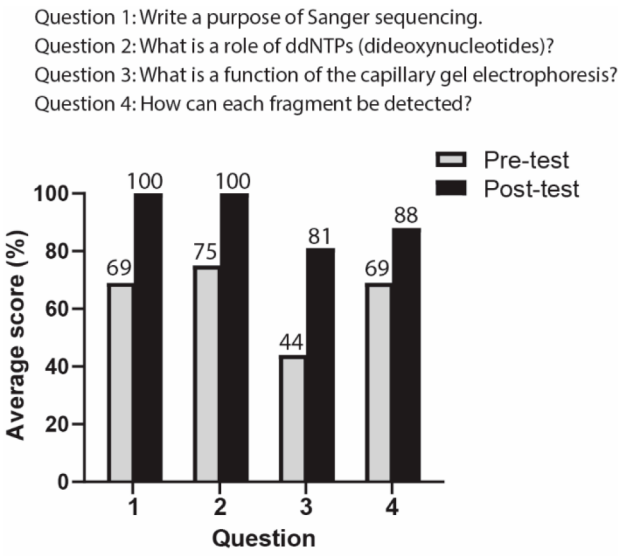

Figure 4. Results of pre- and post-tests from 16 students in CHEM464L. The numbers on the bars are the average score of the question.

To gauge the impact of the activity, an anonymous survey questionnaire was distributed to students in CHEM464L and BIOL724L to determine if the activity helped improve their understanding of Sanger sequencing. In response to the question, "Were the Sanger sequencing activities helpful to you to understand Sanger sequencing?", 88% of students in CHEM464L and 100% of students in BIOL724L responded that they either strongly agreed or agreed (Figure 5). When asked why the activity was helpful, students cited the hands-on experience of all of the steps as key in their learning process. They also mentioned that the visual representation of each step using colorful beads kept them engaged and interested. The pre- and post-tests and the anonymous survey results indicate that the Sanger sequencing activity achieved its learning goal by providing students with hands-on experience and visually appealing representation, resulting in high engagement and interest.

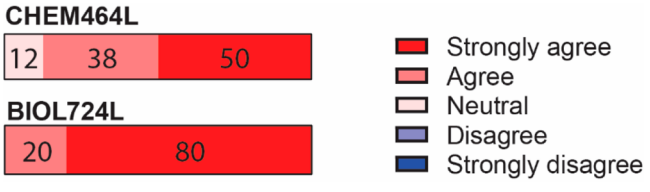

Figure 5. Survey results of CHEM464L and BIOL724L. The bar graph shows the percentage of 16 students in CHEM464L and 6 students in BIOL724L to the question, "Were the Sanger sequencing activities helpful to you to understand Sanger sequencing?".

■ DISCUSSION

An overwhelming majority of the students from both classes reported that the activity improved their understanding of Sanger sequencing. However, there are still areas where the activity could be improved. Two students reported confusion about the separation and fluorescence detection steps. To enhance the students' understanding, the instructor could carry out a demonstration using agarose gel electrophoresis with two dyes with distinct molecular weights. This would illustrate the concept of separation based on size and provide a color-coded visual aid. This demonstration would occur concurrently with the bead activity.

Students can accidentally add the wrong beads when they generate new DNA fragments. Students involved in the optimization of the chain-termination reaction and in CHEM464L made this mistake (Supplementary Figure S1), which brought up two discussion topics with students. The first is whether DNA polymerase can also make mistakes. The error rate of Taq DNA polymerase is between 0.02% and 0.002%.[8,9] The second discussion topic is polymorphism of template DNA.[10] The sequences of genes from different individuals can be different. If a mixture of DNA templates from different individuals is used, it is possible that sequencing results can show polymorphism.

This activity utilizing beads to illustrate the principles of Sanger sequencing has demonstrated success in improving students' understanding of the process before they delve into the analysis of real sequencing data. This activity can be adapted for various educational settings, such as a high school biology course or a class for undergraduate students not majoring in science, serving as a practical tool to elucidate DNA sequencing basics. Furthermore, this approach can also be extended to teach similar techniques, such as PCR, RT-qPCR, and site-directed mutagenesis. By visualizing the products generated during PCR using beads, this activity provides an effective way for students to grasp the fundamental principles of these techniques, where primers are utilized to amplify target regions of DNA.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available at https://pubs.acs.org/doi/10.1021/acs.jchemed.3c00177.

Additional activity photograph and table of bead counts (PDF, DOCX)

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Author Contributions

Kylie Light and Jordan Brooks made equal contributions to this project.

Notes

The authors declare no competing financial interest.

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