

Digestion of plasmid DNA pSpCas9(BB)-2A-Puro (PX459)
V2.0 by a restriction enzyme and agarose gel electrophoresis

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Abstract

The main goal of this experiment was to digest plasmid DNA pSpCas9(BB)-2A-Puro (PX459) V2.0, which was purified in the previous lab, using the restriction enzyme Pst I and then to run the agarose gel electrophoresis for digestion results analysis. Based on this, plasmids 3.2 and 3.4 demonstrated the best digestion, while plasmid 3.3 showed the worst result.

Introduction

The digestion of DNA, or restriction digestion, is one of the most essential biological methods for DNA analysis. During restriction digestion, nucleic acid sequences of DNA are cut into smaller pieces by restriction enzymes. These enzymes are important as they cleave the DNA at specific sites for further analysis. Several types of enzymes are used for restriction digestion. These examples include Eco R1, Hind III, Sai I, Hae III, and so on. The first 3 letters represent the species of bacteria from which the enzyme was isolated, the fourth letter describes the strain, and the numbers are used to show that more than one isolated restriction enzyme from the same bacteria is present. The restriction enzymes can lead to either blunt or stick ends. For example, a straight cut of restriction enzymes generates blunt ends, where both strands terminate in a base pair and have equal length, while non-adjacent digestion leads to sticky ends, where one strand is longer than another and has unpaired bases.

For this experiment, an enzyme Pst I was used. The Pst I enzyme cleaves DNA at recognition sequence 5'- CTGCA/G-3' generating fragments with 3-cohesive termini. This is a type II enzyme, isolated from gram-negative *Providencia stuartii*

bacteria, and yields sticky ends 4 base pairs long. Such enzymes are often used for cloning purposes and genetic therapies.

After digestion, the results are analyzed using the agarose gel electrophoresis. This helps to separate DNA fragments of different sizes varying from 100 bp to 25 kp.

The digestion resulting from the sample of DNA is loaded into the prepared advance wells in the gel, and a current is applied. This is used to allow the DNA fragments to migrate toward the positively charged side because the phosphate backbone of the DNA has a negative charge. Gel electrophoresis allows for the separation of the DNA by size where the smaller fragments migrate faster, while the larger ones stay on top, which helps to determine the amount of the base pairs.

However, it is hard to tell the exact number just by looking at the gel ladder. In this case, scenario, once the DNA separation is completed, the distance traveled by a segment is measured (in cm) and the approximate value of the base pairs is recorded. Then, using these numbers, the graph of distance vs log (bp) is created.

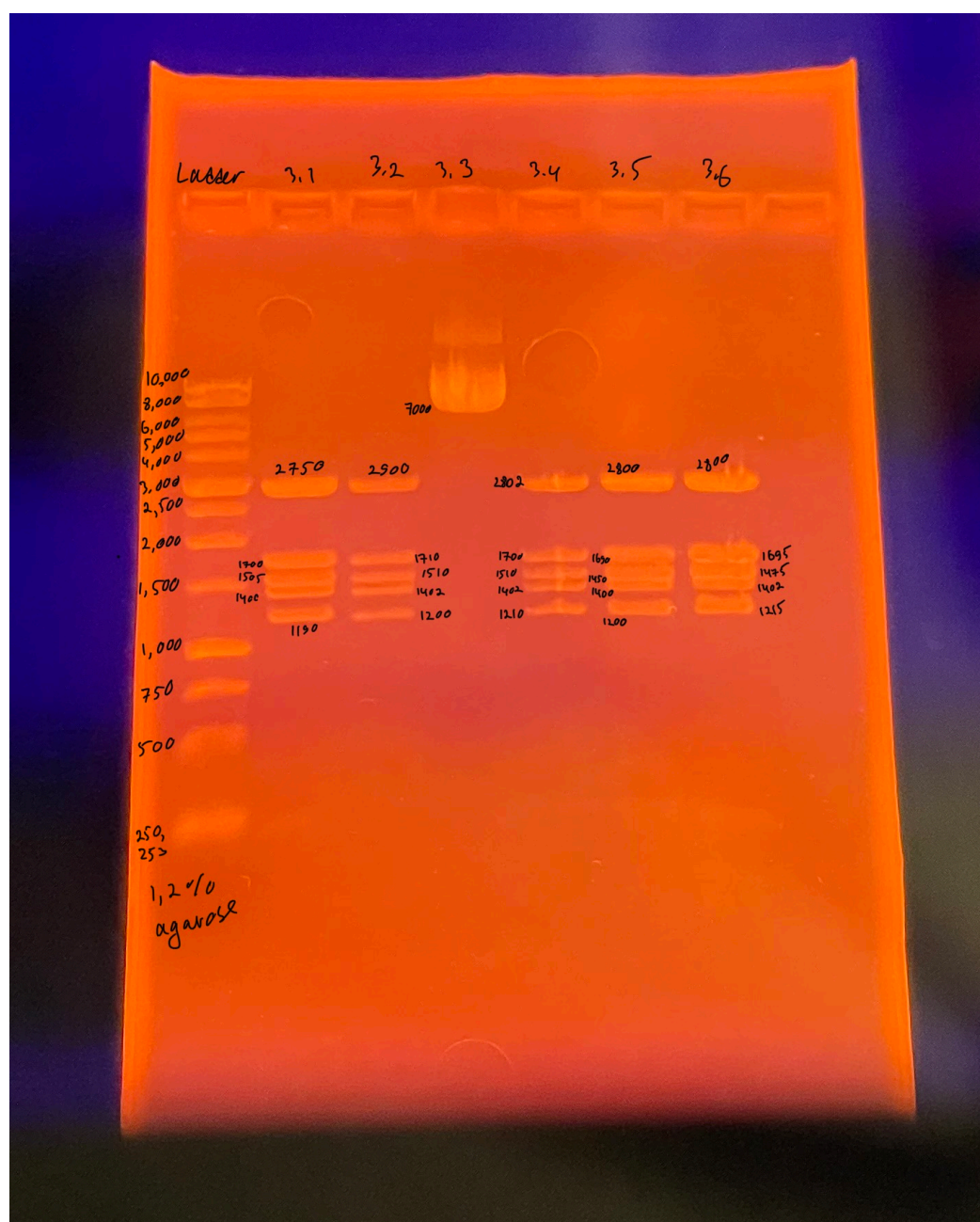
When the slope is obtained, a more accurate base pairs number can be found by plugging the distance into the equation of the lane.

Procedures

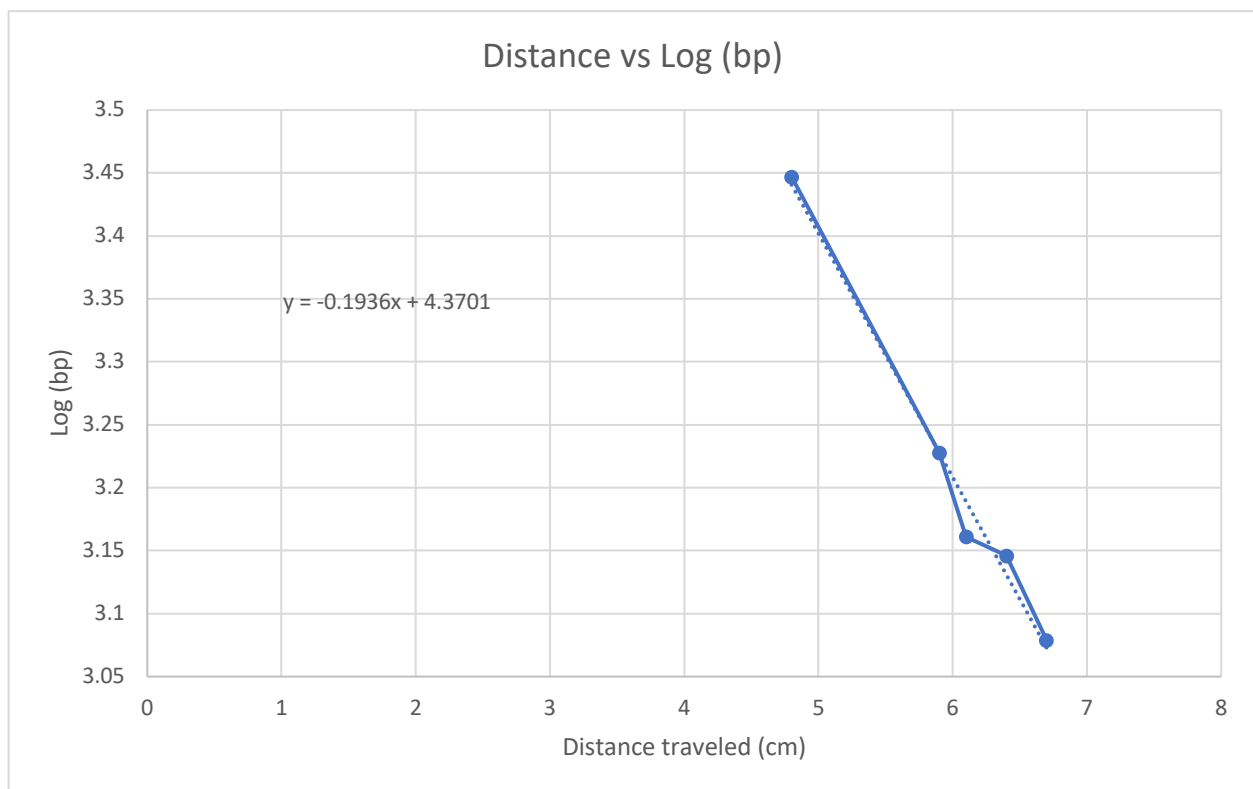
The very first step included the preparation of a buffer solution. To make 1 x TBE buffer (700 ml), 10x TBE buffer was used, which means that the concentration was ten times that of the regular concentration used. This buffer solution was used to prepare the 50 mL of a 1% agarose gel. The mixture contained 0.5 g of agarose, 50 mL of water, and ml of 10 x TBE buffer. Later, the mixture of agarose gel was heated in a microwave oven, and a homogeneous solution was formed. Once the gel cooled to approximately 60 – 70 degrees Celsius, it was poured into a gel tray with the comb and hardened for 20 minutes. Now, the mixture was ready to run the gel in a gel apparatus. The next step included the digestion of plasmid pSpCas9(BB)-2A-Puro (PX459) V2.0 itself. To commit the following action, a restriction endonuclease was used, which cleft the DNA in a very specific location. First, the digestion, which contained 5 microliters of pSpCas9(BB)-2A-Puro (PX459) V2.0, 2 microliters of Pst I, 2 microliters of 10 x Buffer, and 11 microliters of water was prepared. The total amount of the mixture was 20 microliters. The solution was gently mixed by tapping the bottom of the tube and then, incubated at 37 degrees Celsius for 30 minutes. Once the digestion was done, the gels were loaded, and the agarose gel ran at 120V. For the gel running, 10

microliters of the reaction mixture and 2 microliters of 6 x DNA loading dye were mixed, and 12 microliters of the sample were loaded in each well of an agarose gel. Later, the sample was stained with ethidium bromide, which allows for the detection of the location of the DNA on the gel by fluorescing only when it is intercalated into DNA.

Results



Segment (3.5)	Distance traveled (cm)	Base pairs	Log (bp)	Actual bp from the equation
1	4.8	2800	3.447	2754
2	5.9	1690	3.228	1698
3	6.1	1450	3.161	1549
4	6.4	1400	3.146	1349
5	6.7	1200	3.079	1175



Equation: $y = -0.1936x + 4.3701$

Substitute:

1) 4.8

$$y = -0.1936 (4.8) + 4.3701 = 3.44$$

$$10^{3.44} = 2754 \text{ bp}$$

2) 5.9

$$y = -0.1936 (5.9) + 4.3701 = 3.23$$

$$10^{3.23} = 1698 \text{ bp}$$

3) 6.1

$$y = -0.1936 (6.1) + 4.3701 = 3.19$$

$$10^{3.19} = 1549 \text{ bp}$$

4) 6.4

$$y = -0.1936 (6.4) + 4.3701 = 3.13$$

$$10^{3.13} = 1349 \text{ bp}$$

5) 6.7

$$y = -0.1936 (6.7) + 4.3701 = 3.07$$

$$10^{3.07} = 1175 \text{ bp}$$

Discussion/ Conclusion

Agarose gel electrophoresis is a very effective way of separating the DNA nucleic acids. These acids include Adenine, Thymine or Uracil, Guanine, and Cytosine.

Smaller DNA fragments can move quickly through the pores of a gel, while large fragments tend to migrate slower. However, the gel conditions, such as gel concentration, ionic strength of a buffer, current strength, and so on may affect the movement of a plasmid. For example, because of the net-like structure of the gel, plasmid DNA can be easily stocked into the agarose gel. That is why it is extremely important to perform every step of a gel preparation accurately.

Otherwise, it is hard to avoid contaminations and get the proper results. On the other hand, if all the procedures are followed correctly, many small bands can be seen as a result. These bands represent the stained DNA cut fragments of different sizes. The “bp” next to each number in the ladder show how many base pairs long the DNA fragment is. If there is only one large band, or no bands at all, on the gel, it means that the digestion was unsuccessful, and the plasmid DNA has not been cut as can be seen in sample 3.3. However, the rest of the samples – 3.1, 3.2, 3.4, 3.5, and 3.6 resulted in successful digestion as they all showed bands of different sizes, which serves as proof of separated DNA fragments of different sizes. In addition, based on the gel electrophoresis, the best digestion occurred in samples

3.2 and 3.4 since all the bands are relatively far from each other. Samples 3.1, 3.5, and 3.6 went through digestion as well, however, compared to samples 3.2 and 3.4, the bands are located closer to each other and, thus, the DNA fragments are about the same. The expected digestion should result in 30 bp, 204 bp, 1212 bp, 1368 bp, 1547 bp, 1743 bp, and 3016 bp, while the actual results are 1175 bp, 1349 bp, 1549 bp, 1698 bp, and 2754 bp. Some of the actual results are relatively close to the literature ones, others are mismatched. First, based on the expected results, gel electrophoresis should show 7 bands, however, there were only 5 bands on the gel presented. The next mismatch was in the location of the bands. The literature results had 2 bands at 30 bp and 204 bp, while there was nothing even close to those values on the actual gel plate. As follows, these 2 bands are mismatched ones, because the other bands' values are close to the expected results. This can be explained by the potential source of error during the gel preparation as the plate in which the gel was placed was cracked and some amount of the gel flowed out of it. Moreover, since the plasmid was used in the previous experiment, it could get contaminations during the preparation procedure. For example, the tip of the micropipette was not changed in a proper time manner.

Overall, the experiment was completed successfully, the goal was met, and the obtained results show is well – précised.

References

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Notebook

Lab 3

$\log(\text{DNA bp}) / \text{Distance graph}$
↓ ↓
y x

Use the equation for graph

- 1) Prepare Buffer
- 2) Agarose gel
- 3) Digestion

2ul PSI

37°C for 30min

Little crack in gel

- Measure the distance from the top of the ladder
- Use con