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Original Paper

Experiment Examining the Progress of the GFP DNA Sequence

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Abstract

In this experiment, two recombinant plasmas containing the coding sequence (CDS) for the green fluorescent protein (GFP) have been cloned into the commercial cloning vector pJET1.2 that we use. We used three different molecular methods to determine which way the GFP DNA sequence was going. Extending a gene's expression by means of amplification using polymerase chain reaction. It was discovered that bacteria streaking allowed us to see and compare the variation in fluorescence level expressed in a regular plasma vector and an expression vector. As a result of their widespread utility, restriction enzymes have established themselves as a staple in the toolkit of each self-respecting molecular scientist. Different restriction enzymes are more suited for certain jobs than others due to subtle distinctions in their recognition sites and cutting mechanisms.

Keywords

fluorescent protein, αGFP DNA insert, E. coli

1. Introduction

The majority of the studies performed in contemporary molecular biology involve the manipulation of DNA using enzymes. Restriction enzymes, also known as endonucleases, have the ability to restrict, or cut DNA at specified locations. This can bind to specific DNA sequences and then break the DNA either at the binding site itself or next to it. Ligase enzymes are responsible for reassembling DNA fragments. In the cell, there are enzymes called DNA methylases that work to stop restriction enzymes from breaking down the cell's own DNA (Loenen & Raleigh, 2013). Methylases are enzymes that alter the DNA at specific locations, thereby preventing restriction enzymes from attaching to or cleaving the DNA at those locations. In the early 1970s, Smith and Nathans were the first people to successfully isolate the restriction enzyme known as endonuclease R (Owens, 2017).

There is a wide variety of restriction enzymes, each of which recognizes a particular DNA location. Both type I and type III restriction enzymes can bind to restriction sequences, but because they break DNA in an unpredictable manner, molecular biology does not make use of them. DNA can be manipulated through the use of type II restriction enzymes because they cleave certain DNA sequences at or close to the restriction sequence. The amount of restriction enzyme necessary to thoroughly digest 1 g of DNA in one hour at the prescribed buffer and temperature conditions (generally 37 degrees Celsius) is typically defined as one "unit" of restriction enzyme. The successful restriction of DNA is dependent on adjusting the concentrations of both the DNA and the enzyme, as well as making use of the appropriate buffer and incubation conditions.

The purpose of the experimental work is to demonstrate that the orientation of the GFP insert can be determined using a variety of methods, as well as to prepare pure plasma preparations for both pGFP-1 and pGFP-2 (a simple restriction digest, PCR amplification, or DNA sequencing). Additionally, we will look into how DNA methylation affects the plasmids' ability to withstand restriction digestion, and we will use the Golden Gate cloning technique to transfer the GFP CDS into an expression vector in order to achieve high levels of GFP expression in E. coli. Finally, we will research the utilization of several E. coli strains that have been genetically altered to produce various colored (chromogenic) and fluorescent proteins as a "paint pallet" in order to create living art.

We are able to identify and isolate particular fragments by using a technique called gel electrophoresis, which separates fragments according to their size. With the help of a technique known as restriction mapping, it is possible to produce a diagram that shows how restriction sites are scattered throughout the entirety of a DNA sequence. In order to accomplish this, we first cleave the DNA using restriction enzymes both on their own and in combination, and then we measure the fragments that are left over. It is possible to ascertain, using this method, the specific location of restriction sites that are sought inside a DNA fragment.

2. Results

We first determined the orientation of the αGFP DNA insertion using the PCR amplification method. Primer 1 (binds upstream of the EcoRV site), Primer 2 (binds onto GFP DNA sequence), and Primer 3 (binds downstream of the EcoRV site) were used in a PCR reaction on both pGFP plasmas. The PCR products, which should be of differing lengths according to primer 2′s facing direction, may be compared to the known BP lengths of the amplified area on each of the two insertion vectors, indicating the orientations of the GFP insertion. To further examine the size distinction between the two PCR products, gel electrophoresis was performed. We acquired two bands in lanes 1 and 2, one between 600 and 700 BP and the other between 900 and 1000 BP. We were able to tell that the PCR product in lane 1 corresponded to the amplified sequence of pGFP-1 and that the PCR product in lane 2 corresponded to the amplified sequence of pGFP-2 based on their respective positions in the gel electrophoresis result. Depending on the direction in which the primers are inserted, primer 2 may either face primer 1 or primer 3,

respectively. Both pGFP plasmids were treated with restriction enzymes (NcoI and HindIII) to establish the GFPs′ orientation. The next step was gel electrophoresis. As we saw from figure 1, the NcoI lanes showed three bands, whereas the HindIII lanes showed two. The effect of stellar activities was also suspected to have caused the appearance of many hazy bands around 2000-3000BP.

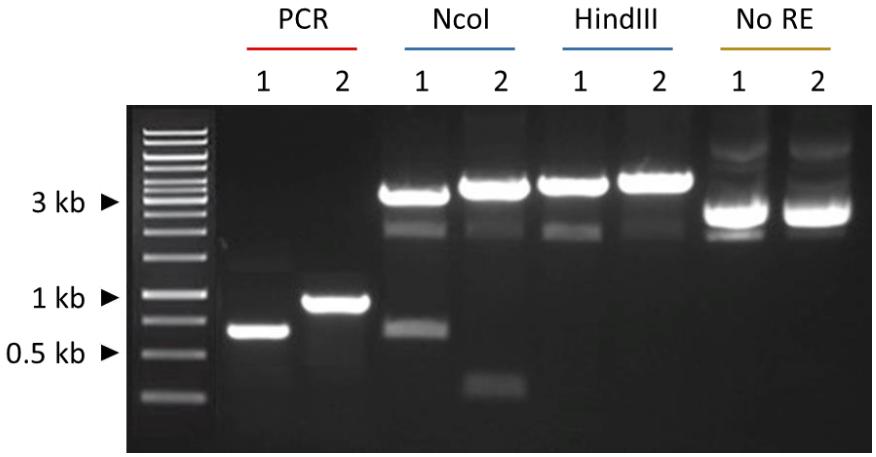

Figure 1. PCR Analysis and Restriction Digest Analysis of Plasmid pGFP-1 and pGFP-2

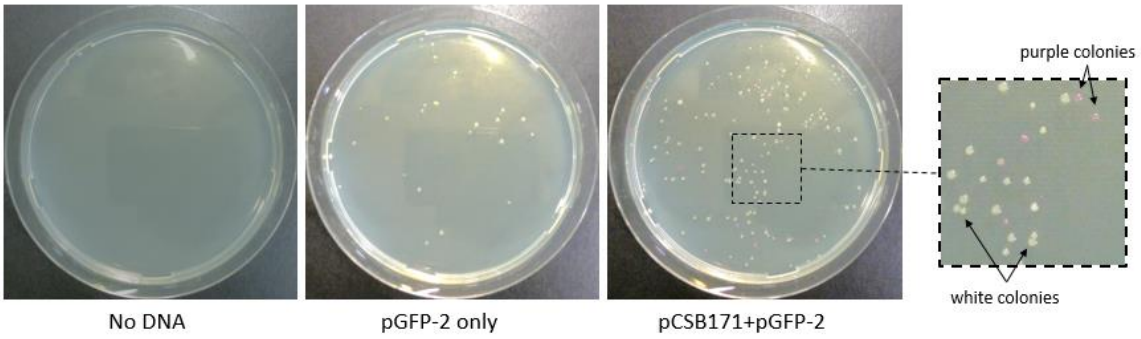

Figure 2. Results of E.coli Colonies Growth

We examined the difference in fluorescence of E. coli colonies growing on Luria broth agar (LBA) plates supplemented with ampicillin (50g/ml) between those having no plasmas (NP group), plasma pGFP-2 (G alone group), and plasma pCSB171 (171+G group). As we saw in figure 2, we found no bacterial colonies in the NP category. Our results showed that ampicillin in LBA is effective at killing bacteria because this control group did not contain either the pGFP- 2 or pCSB171 plasmas, which would have conferred ampicillin resistance on E. coli. The NP group showed no signs of GFP fluorescence. Approximately 30 E. coli colonies were found to be widely dispersed within the G alone group. In order to objectively determine the amount of pGFP-2 plasmas that have avoided having their GFP sequences cleaved off by Sap1 restriction enzymes, we counted the number of bacterial colonies that were not destroyed by the enzymes (Only uncut plasma sequences would be retained by E.coli and provide them

with ampicillin resistance). The weak GFP fluorescence observed in all other colonies was traced back to those containing the pGFP-2 standard plasma vector. This low level of fluorescence expression could be caused by the presence of random upstream sequences that function as weak promoters.

As compared to the G-only group, the 171+G group contained more widely spread E. coli colonies. Both uncut pCSB171 and SapI cut GFP inserted pCSB171 plasmas have been linked to increased bacterial survival (both of which carry ampicillin resistance genes in E. coli). Most of the E. coli colonies seemed to be white. This might be because some colonies were harboring pCSB171 plasmas with full length. TsPurple expression would explain the purple color of these bacteria.

3. Discussion

In the NP group, we did not find any evidence of bacterium colonies. The purpose of this group was to serve as a control, and the results showed that the ampicillin in the LBA medium is effective in killing bacteria. Specifically, it killed bacteria that did not have the plasmas pGFP-2 or pCSB-171, both of which would have conferred ampicillin resistance on E. coli. The NP group did not exhibit any fluorescence of the GFP protein.

In the G alone group, there was a smattering of E. coli colonies (about 30), which was noted. The number of bacteria colonies that were able to survive is a quantitative indication of the amount of pGFP-2 plasmas that were able to avoid the cutting out of GFP sequences that was performed by the Sap1 restriction enzymes. A low degree of GFP fluorescence was found, which can be attributable to the fact that some of the colonies carried the pGFP-2 standard plasma vector. All of the colonies that were examined seemed to be white colonies. It's possible that random upstream sequences are to blame for this poor fluorescence expression since they function as weak cryptic promoters.

In comparison to the G-only groups, the 171+G group was found to have a noticeably higher number of scattered E. coli colonies (approximately 120), which was observed. It is possible that E. coli has acquired either uncut pCSB171 or SapI cut GFP inserted pCSB171 plasmas (both of which carry ampicillin resistance genes), which has led to an increase in the number of bacterial colonies that have managed to survive. White colonies appeared to make up the vast majority of the E. coli colonies that were examined. However, there were also some purple colonies found, which could be attributable to the bacteria that possessed the pCSB171 plasmas harboring the uncut TsPurple-expressing plasmids.

To conclude, restriction enzymes have a wide range of applications and are an indispensible tool for a molecular biologist. Variation in restriction enzyme recognition sites and cutting mechanisms makes different restriction enzymes better suited for particular endeavours. This experiment effectively demonstrated the ability of restriction enzymes to fragment DNA and the usefulness of identifying restriction sequences

References

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Owens, R. (2017). *Microbial Biotechnology, lecture 5, BI311*. Maynooth University.