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New Restriction Analysis Exercise for Introductory Biology

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ABSTRACT

To improve the learning experience for students in the first-year biology course (BIOL160) at Messiah College, restriction endonuclease cleavage sites within the bacterial plasmid pGLO were sought that would allow students to diagnose the orientation of the GFP-encoding insert. Such sites were found in pGLO using New England Biolabs NEBcutter 2.0, and the presence of the predicted sites and suitability of the resulting restriction patterns were confirmed using agarose gel electrophoresis. Recombinant plasmids containing the GFP gene insert in the reverse orientation (relative to pGLO) or lacking the GFP insert were generated. Three recombinants were cleaved with two suitable endonucleases (HindIII or BamHI) to produce restriction patterns appropriate for structural comparison and demonstration. These findings were used to revise the restriction endonuclease module of BIOL160.
INTRODUCTION

The pGLO plasmid, also known as pBAD-GFPuv, consists of an ampicillin resistant gene ($amp^R$), an origin of replication ($ori$), a Green Fluorescent Protein (GFP) insert, an $araC$ gene, an arabinose-sensitive $P_{BAD}$ promoter, a prokaryotic transcriptional termination sequence ($rrnB$), and several recognition sequences for a variety of restriction endonucleases.

Bacterial colonies that contain pGLO fluoresce when grown on medium that contains the sugar arabinose while colonies that contain the empty pBAD vector do not. In the exercise currently used in BIOL160, students compare the structures of plasmids extracted from glowing vs. non-glowing colonies. pGLO and pBAD are cleaved with $Hind$III. $Hind$III cleavage of pGLO yields two fragments: the vector and the GFP insert and this is compared to the single band “vector-only” pattern observed following digestion of pBAD with $Hind$III. Digestion with $Hind$III and separation via agarose gel electrophoresis can be used to determine whether an insert is present in the plasmid extracted from glowing colonies (Figure 1).

Though $Hind$III can be used to detect the presence of a GFP fragment, it cannot be used to determine whether a plasmid which has a GFP fragment contains that fragment in the proper or reverse orientation (pGLO-rev). Reverse orientation clones should not cause the bacterial colonies to glow and therefore such colonies should appear indistinguishable from non-fluorescent colonies that contain the empty vector. Students will have the opportunity to compare plasmids of different structures that are isolated from non-fluorescent colonies. To do so, a second restriction endonuclease that cleaves pGLO and pGLO-rev differently needed to be found.
MATERIALS and METHODS

Analysis of pGLO sequence

Michael Shin provided us with the DNA sequence of pGLO. This sequence was entered into a web freeware from New England Biolabs, NEBcutter 2.0, to find appropriate restriction endonucleases cleavage sites in pGLO. The restriction endonucleases sought were those that contained a cleavage site within the vector close to the GFP fragment and a cleavage site within the GFP fragment far from the center of the fragment. Such restriction endonucleases would produce cleavage products that are easily distinguishable between clones with normal vs. reversed GFP orientations. The expected cleavage results of endonucleases that showed promise were mapped, and a predicted restriction pattern was drawn.

Verifying presence of restriction sites

Digestion reactions were carried out using pGLO and a number of restriction endonucleases that seemed to produce cleavage products that are easily distinguishable between clones with normal vs. reversed GFP orientations. The cleavage products were then separated using agarose gel electrophoresis to verify that the restriction sites did exist. Once the restriction sites were verified in these restriction endonucleases, the most affordable endonuclease was selected.

Generating reverse clones

To generate reverse or empty recombinants, pGLO was cleaved with HindIII. The two DNA fragments that resulted, a large “vector” fragment and a small “insert” fragment, were separated using electrophoresis on a preparative agarose gel and extracted from gel slices. The
ends of the vector were dephosphorylated with calf intestinal phosphatase (CIP) to reduce the incidence of self-ligation, which would have resulted in recovery of an over-abundance of self-ligated vectors. The inserts were then ligated into the vector at random using DNA ligase. The recombinants were transformed into amp$^S$ bacteria which were then plated on media containing ampicillin and arabinose. Non-glowing colonies were chosen because they should contain empty vector (pBAD) or vector containing the GFP insert, but in the reverse orientation. Cleavage with BamHI and subsequent agarose gel electrophoresis were used to distinguish between these candidates.

*Revising the Lab Module*

The three plasmid recombinants were cleaved with *Bam*HI and electrophoresed in adjacent lanes, and it was found that the restriction pattern did indeed allow the three recombinants to be distinguished. Multiple experiments were conducted on 8-lane, 15-lane, and 21-lane gels containing 1% or 2% agarose to determine the optimal conditions for separation.
RESULTS

Analysis of pGLO sequence

The analysis of the pGLO sequence using freeware NEBCutter 2.0 suggested that there were several restriction endonucleases that would produce cleavage products that are easily distinguishable between clones with normal vs. reversed GFP orientations. Among those restriction enzymes were BamHI, AflIII, NdeI, MluI, and PstI. The expected cleavage results of endonucleases that showed were mapped in pGLO and pGLO-rev, and some of them are shown in Figure 2. Using the plasmid maps, the sizes of the fragments that would result from cleavage with each enzyme was calculated, and a predicted restriction pattern was drawn for some of the enzymes (Figure 3).

Verifying presence of restriction sites

Digestion reactions were carried out using pGLO and a number of restriction endonucleases that seemed to verify the presence of the restriction sites found using NEBcutter 2.0. Agarose gel electrophoresis showed that all of the enzymes tested, except for NdeI, cut pGLO into the expected number and sizes of fragments.

Generating reverse clones

Cleavage of pGLO with HindIII and separation via preparative agarose gel electrophoresis were successfully. The two fragments were extracted from gel slices. The dephosphorylation of the vector ends and the ligation of the inserts to the vector were also successful. Amp\(^\text{\textsuperscript{\textregistered}}\) bacteria were successfully transformed with the plasmids. Both non-glowing and glowing colonies were found growing on the plate. Screening these colonies by cleavage
with *BamHI* and subsequent gel electrophoresis showed that pGLO-rev had successfully been synthesized. The plasmids pGLO and pBAD were also found in some of the colonies.

**Revising the Lab Module**

Different experiments in which the three plasmid recombinants were cleaved with *BamHI* and electrophoresed in adjacent lanes were conducted in different types of gel to determine the optimal conditions for separation. The best separation occurred using a 15-lane, 2% agarose gel.
DISCUSSION

The purpose of this experiment was to improve the restriction analysis module in the BIOL160 course at Messiah College by finding a restriction endonuclease that cleaves pGLO and pGLO-rev in such a way that the two plasmids would be easily distinguishable through agarose gel electrophoresis. It was also necessary to generate pGLO-rev plasmids.

From the analysis of the pGLO sequence using freeware NEBcutter 2.0, it was found that multiple restriction endonucleases fitted our criteria, namely, they cleaved pGLO and pGLO-rev in ways that were easily distinguishable because they had at least one restriction site on the vector and one site on the GFP insert far from the center. If the restriction site on the GFP insert had been close to the center of the insert, similar-sized fragments would be obtained whether the insert was in the proper or reverse orientation, and the two plasmids would not have been easily distinguished.

The digestion reactions that were carried out using pGLO and a number of the candidate restriction endonucleases and the subsequent agarose gel electrophoresis showed that the pGLO plasmid used in this experiment did indeed have the restriction sites as predicted using NEBcutter 2.0. Sometimes spontaneous mutations occur over time in plasmids, and if a nucleotide in any of the recognition sites had been mutated, cleavage would not have occurred. On the other hand, if nucleotides outside of the recognition sites had been mutated, it could have added an extra cleavage site. The digestion of pGLO using NdeI was not successful. However, since BamHI was the most affordable restriction endonuclease, it did cleave pGLO in the expected manner, and only one restriction endonuclease was needed for the project, it was not necessary to perform the NdeI digestion again.
The generation of the pGLO-rev clone was successful. Since the insertion of the GFP fragment into the vector using DNA ligase was done at random, the transformed bacteria that were plated on media with arabinose and ampicillin were relatively equally divided between those that glowed and those that did not. Other than the three plasmids that we were looking for, pGLO, pGLO-rev, and pBAD, another plasmid which contained two GFP inserts in the reverse direction was also found. However, this plasmid was not needed, and it was put in storage for further experiments.

The different experiments used to revise the lab module showed that a 2% agarose gel was able than a 1% agarose gell to separate the two smallest fragments that resulted from cleavage of pGLO-rev with BamHI. It was also noted that an 8-well gel did not provide enough lanes to do the necessary comparisons between the three transformants and the three treatment methods. It was therefore concluded that a 15-lane, 2% agarose would be most appropriate in the revised lab module.

Overall, the experiment can be considered a success because the restriction endonuclease sought was found in BamHI, and the plasmid recombinants with GFP inserted in the reverse orientation (pGLO-rev) was successfully generated. BamHI, pGLO-rev, and the 15-lane, 2% gel can be used in a restriction endonuclease module for future BIOL160 classes.
REFERENCES

**Invitrogen.** 1 Kb Plus DNA Ladder and 100 bp Ladder. Invitrogen Catalog.

**Mylin, L.M.** 2010. Laboratory Manual for Molecular Biology. Messiah College Department of Biological Sciences.

**NEB.** New England Biolabs NEBcutter 2.0.

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FIGURE LEGENDS

**Figure 1.** The current exercise. Plasmid maps and agarose gel demonstrating *HindIII* cleavage sites in pBAD and pGLO and the restriction pattern that results after cleavage.

**Figure 2.** Plasmid maps that show predicted cleavage sites for restriction endonucleases that should distinguish between pGLO and pGLO-rev (pBAD containing the GFP insert in the reverse orientation).

**Figure 3.** Predicted restriction patterns that would result from cleaving pGLO and pGLO-rev with some of the restriction endonucleases that showed promise. Price listed is per 10,000 units of enzyme. *BamHI* was the least expensive of these endonucleases.

**Figure 4.** An agarose gel demonstrating that the restriction endonucleases of interest cleaved pGLO in the expected manner. Since *BamHI* was the least expensive of these endonucleases, it was selected as the endonuclease to incorporate into the new BIOL160 lab module.

**Figure 5.** A flow chart that shows how reverse and empty clones were generated.

**Figure 6.** A 15-lane, 2% agarose gel that contains the three different recombinant plasmids either cleaved with *HindIII* or *BamHI*, or not cleaved. In the no enzyme set, the empty plasmid travels the furthest, as expected. In the *HindIII* set, the recombinants with the GFP fragment in the proper and reverse orientations yield the same restriction pattern. In the *BamHI* set, the two
recombinants with the GFP insert are easily distinguished. The plasmid maps beside the gel show that pGLO and pGLO-rev are cleaved identically by HindIII but differently by BamHI.
Figure 1
Figure 3
GFP
Amp
R
ori

pGLO

Desphosphorylate ends of vector
Ligate insert into vector “random”
Capture individual recombinants
Transform Bacteria to Amp

Amp colonies (glowing or not)

Preparative separation of fragments

Screen plasmids from individual non-glowing colonies

4.7 kb fragment “vector”
650 bp fragment “insert”

Figure 5