ONLINE INQUIRY & INVESTIGATION

An Integrated Molecular Biology Research Project for High School Students

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It is a common criticism among educators that laboratory experiments offered to students, either at the high school or college level, do not represent the way science is actually practiced. One problem is that the exercises, unlike true scientific explorations, have a predetermined outcome (Chinn & Moholtra, 2002). Another major objection is that many experiments are isolated: They cover a specific technique that is presented out of the context of a larger research problem (Lemke, 1992). However, when these techniques are consolidated into a larger "research module," in which students perform a series of exercises carrying over the results of one experiment to the next, it provides continuity towards a research goal. In this way, students are able to see how the various techniques work in concert. This approach makes it easier for the students to get a feel for experimental design. The experiments more closely match what actually goes on in a real laboratory.

An example of the lack of experimental continuity can be found in the molecular biology laboratory for the Advanced Placement (AP) Biology course (College Board). The AP Biology curriculum is designed to be the equivalent of a first year college biology course whose students plan to major in a biological or related field. One of the laboratory modules in the AP Biology course, Lab #6, covers some of the basic techniques in molecular biology and is divided into two sections. In the first section, students transform *E. coli*. In this exercise, a plasmid (a circular extrachromosomal DNA) that contains an ampicillin resistance gene (amp^r) is introduced into bacteria, and students select for cells that are able to grow in the presence of the antibiotic ampicillin. Cells that have successfully taken up the plasmid grow; those not acquiring the plasmid cannot grow. In the second section of this same laboratory exercise, students digest lambda bacteriophage DNA with a restriction endonuclease. They then electrophorese their samples on agarose gels, and determine the size of the fragments (College Board). In this section, the lambda DNA serves only as a piece of purified DNA. Any DNA is suitable for this purpose. Since the two techniques in AP Biology Lab #6 are presented out of context of a research goal, it is not clear how they relate to one another, and why a scientist would be utilizing these separate protocols.

We have designed a research project for high school and college students to provide an authentic research experience with an unknown outcome. The exercise, in which students transform a plasmid containing an insert of foreign DNA, purify that plasmid, and perform restriction digestion analysis to characterize the DNA insert, is more representative of the work of a practicing molecular biologist. Unfortunately, this exercise has one major drawback. It requires the students to grow the transformed bacteria in liquid culture, and then purify the plasmids (Sambrook & Russell, 2001). Purification of the plasmids requires a microcentrifuge, which is often prohibitive in cost ($500 - $1,500) for a high school or introductory college course laboratory.

In this article we demonstrate that the integrated experiment described above can be carried out inexpensively in a high school laboratory. Using a recently developed method, plasmid DNA is amplified and used for restriction digestion. This technique is integrated into a research project that is identical to what molecular biologists do routinely: characterizing a novel DNA fragment. The project combines both sections of the Advanced Placement Molecular Biology laboratory (6A and 6B) and fulfills the requirements for these sections.

The project has the following steps. First, the students transform *E. coli* using the standard kits available from several educational laboratory supply companies. However, instead of using the plasmid supplied in the kits, students transform

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the cells with DNA from a plasmid library of genomic DNA. A library is a collection of clones that contain inserts of random pieces of DNA. Molecular biologists often use plasmid libraries to isolate and characterize specific fragments of DNA (Sambrook & Russell, 2001).

Second, the students select for cells that have been transformed (such cells are resistant to ampicillin because the plasmid carries an ampicillin resistance gene), and perform a blue/white screen of the colonies to identify plasmid clones that have inserts.

Third, the students select one or more insert-containing colonies and amplify plasmid DNA using a protocol modified from the TempliPhi® Amplification Kit (Amersham Biosciences, a division of GE Healthcare) (Nelson et al., 2002). The TempliPhi® amplification was performed with equipment readily found in most high schools and introductory college laboratories.

Finally, the students map the plasmid DNAs using restriction endonucleases and gel electrophoresis. Since random DNA fragments were originally cloned into the library, each student works with a plasmid carrying a different insert and therefore each generates a unique restriction map. This modified TempliPhi® amplification protocol and research project were developed by high school biology teachers in the Waksman Student Scholars Program (WSSP), an intensive summer and academic year-long project based program involving 32 students and their teachers (http://avery.rutgers.edu/WSSP/) and tested in a high school setting at Montville High School, Montville, New Jersey.

Materials & Suppliers

- Hot plate or bunsen burner
- Pyrex beaker
- Coffee can lid for floating samples or a Temperature block for incubating samples at 95˚C and 65˚C
- Micropipetor

Figure 1.
A flow chart showing the steps in research project.

Experimental Flowchart

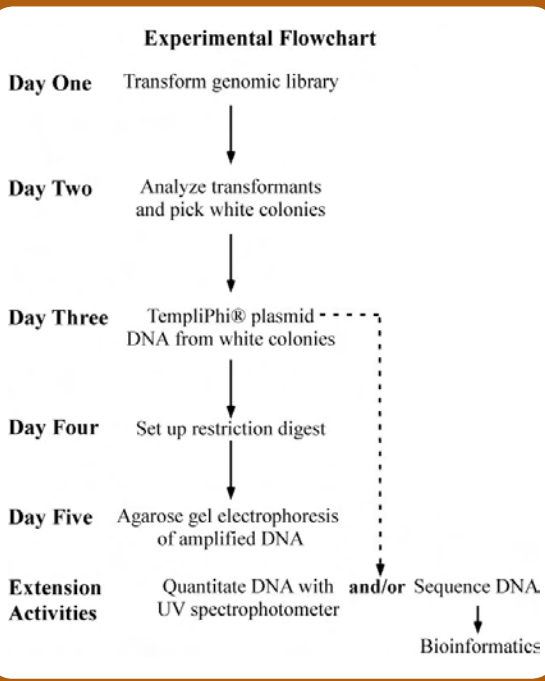|	
Day One	Transform genomic library
Day Two	Analyze transformants and pick white colonies
Day Three	TempliPhi® plasmid DNA from white colonies
Day Four	Set up restriction digest
Day Five	Agarose gel electrophoresis of amplified DNA
Extension Activities	Quantitate DNA with UV spectrophotometer **and/or** Sequence DNA → Bioinformatics

Figure 2.
A map of the vector used for the genomic library. A restriction map of the *Eco*RI and *Hind*III sites of the pRS313 vector with the position of the genes is shown. The genomic DNA fragments were cloned into the single *Eco*RI site within the vector.
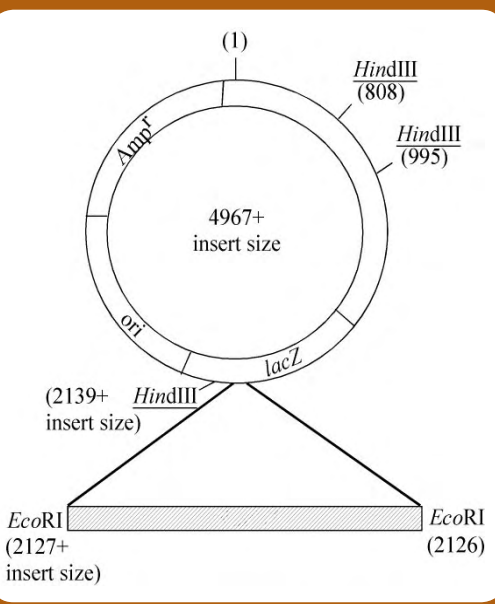
- Disposable Microcentrifuge Tubes (1.5 ml)
- Diposable pipet tips
- Bacterial Transformation kit (EDVOTEK #211 or Carolina #21-1145)
- TempliPhi® kit (Amersham Biosciences #25-6400-10)
- Restriction digest and gel electrophoresis kits (EDVOTEK #212 or BioRad #166-0002EDU)
- Agarose gel box and power supply

Genomic or cDNA plasmid DNA libraries are available from Genetic Stock centers such as American Type Culture Collection or from companies such as Invitrogen or Stratagene. These libraries can be expensive and are more complex than is needed for these assays. Research laboratories at local universities may be willing to provide a genomic or cDNA library. Educators may contact Sue Coletta of the Waksman Student Scholars Program (WSSP) (coletta@waksman.rutgers.edu) to request a sample of the library that was used in the experiments described here.

The Research Project

A flow chart of the steps for the research project is shown in Figure 1.

Day 1 (60 minutes)

Transformation of a genomic plasmid library into *E. coli* and plating of the cells on LB with ampicillin and X-gal. Bacterial transformation is simply defined as the process by which bacterial cells take up DNA molecules. For this step, standard protocols and reagents provided in transformation and blue/white colony color screen kits from educational supply houses, such as EDVOTEK (#211) or Carolina Biologicals (#21-1145), can be used. However, in place of the DNA provided by the kits, plasmid DNA carrying fragments of a genomic library should be added to the cells. The library we used was generated by digestion of chromosomal DNA from bakers yeast (*Saccharomyces cerevisiae*) with the *Eco*RI restriction endonuclease and ligation of the fragments into the plasmid pRS313 that was also digested with *Eco*RI (Figure 2). The pRS313 plasmid contains

a portion of the *lacZ* gene, which codes for the enzyme β-galactosidase. Expression of this protein produces a blue color in the presence of its chemical substrate X-gal (Figure 3). Expression of this protein is disrupted in cells transformed by a plasmid with a yeast DNA fragment inserted at the *Eco*RI site, and such cells form white colonies on the X-gal indicator plates. These white colonies are chosen for further analysis. In addition to transformation with plasmids carrying yeast DNA inserts, the students also separately transform cells with the vector plasmid, pRS313. Since pRS313 does not contain an insert, transformed cells should be blue and serve as a negative control for the blue/white color screen.

Day 2 (40 minutes)

Analysis of the Transformation & Preparation of Samples

A. Analysis of the Transformation

1. The students should examine their plates and determine if the transformation was successful. The efficiency of the transformation is determined by counting the number of colonies on the plate.

2. The plates should contain a mixture of blue and white colonies. The white colonies are cells transformed with a plasmid that contains an insert at the *Eco*RI site of the plasmid. Blue colonies were transformed with plasmids that self-ligated without incorporating a genomic DNA insert. Students determine the efficiency of inserting the random fragments into the vector by calculating the percentage of white colonies on the plate. Since the students use the same library DNA, the average insertion efficiency should be the same for all the groups.

B. Preparation of samples for plasmid amplification

Five transformed clones should be analyzed by each group of two students. Each student should choose two white colonies to be analyzed because some may contain inserts that are too small to be detected on stained agarose gels. Each white colony will contain a plasmid with a different insert and it is likely that at least one plasmid chosen will contain an insert of detectable size. As a negative control, each group should analyze one colony from the plate that was transformed with pRS313 that contains no insert. The specific steps for the modified plasmid amplification procedure are provided at the WSSP Web site http://avery.rutgers.edu/WSSP/. The amplification utilizes equipment available in most high school laboratories. In brief, the cells are heated and lysed

Figure 3.

A LB+amp+X-gal plated with *E. coli* cells transformed with DNA from the genomic plasmid library. The arrow designated (A) highlights a blue colony that was transformed with a plasmid lacking an insert, while (B) highlights a white colony that was transformed with a plasmid containing an insert.
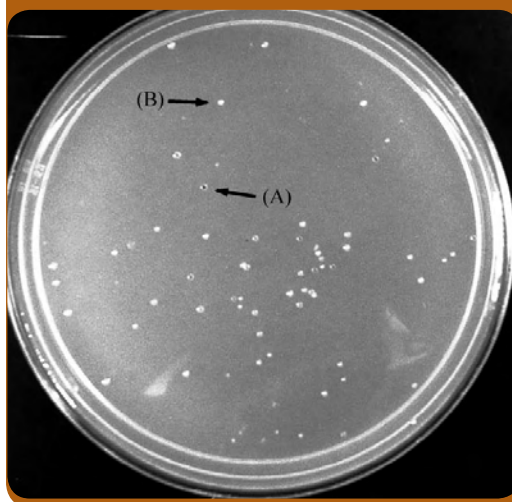
releasing the plasmid DNA. The TempliPhi® enzyme allows amplification of large quantities of DNA without the need for a thermocycler (Nelson et al., 2002). This DNA is suitable for analysis by restriction enzyme digestion.

Day 3 (45 minutes)

Set Up Restriction Digests

The DNA from the TempliPhi® amplification is heated to inactivate the enzyme. The amplified DNA is then diluted five-fold to provide a suitable concentration for the restriction digest.

1. Restriction Digestion of the Amplified DNA

Restriction enzymes are endonucleases that hydrolyze or digest the phosphodiester backbone at specific sites in a DNA sequence. The restriction digests of the amplified plasmid DNA can be performed using reagents from kits supplied by biological education supply companies, such as EDVOTEK (#212) or BioRad (#166-0002EDU), or with individual reagents purchased from other vendors, such as GE Healthcare Biosciences, New England Biolabs, or Fisher. The library that was used in these experiments was constructed by inserting random DNA fragments with *Eco*RI ends into the *Eco*RI site of the vector. Although a wide array of different restriction enzymes can be used to analyze the plasmid DNA, we recommend digesting the DNA with *Eco*RI to generate fragments corresponding to the insert and the linearized vector (Figure 2). If available, we also recommend performing a separate digest of the DNA with *Hin*dIII. This enzyme cuts multiple times in the plasmid vector and may or may not cut within the genomic insert (Figure 2). Analysis of the *Hin*dIII digests provides the students with the opportunity to perform more advanced mapping exercises and helps verify their prediction for the insert size they determined from analysis of the *Eco*RI digest. The digestions should be performed according to the instructions supplied in the kits that were purchased. In place of the DNA provided in the kit, students should substitute the same volume of the diluted TempliPhi® DNA in the reaction.

Day 4 (2 hours; 1 hour if agarose gels are prepared)

Run & Analyze the Agarose Gel of the Digested Samples

1. Digested and undigested samples should be electrophoresed on agarose gels according to methods

usually performed in the lab. Agarose gel electrophoresis and DNA staining kits from any of the Biological Education Supply houses would be suitable for this step. Be sure to run DNA markers of known size on each gel.

2. Typical results are shown in Figures 4 and 5. Samples of DNA containing amplified plasmid DNA without an insert from a blue colony and plasmid DNA with an insert from two white colonies are shown uncut and digested with either *Eco*RI or *Hin*dIII. The approximate sizes of the DNA fragments are determined by comparison with the marker bands. The *Eco*RI digestion of amplified pRS313 shows a single linear band migrating at approximately 5 kb (Figure 4, Lane 5). The amplification of the plasmid DNA from a blue colony produces a similar single linear fragment migrating at approximately 5 kb, suggesting the plasmid contained no detectable insert (Figure 4, Lane 5). The plasmids amplified from the white colonies designated as #1 and #2 both showed the same backbone fragment along with an additional band. The presence of this second band indicates the plasmid contains an insert of foreign DNA at the *Eco*RI site. The insert size for Plasmid #1 is approximately 1.0 kb (Figure 4, Lane 7). The insert size for Plasmid #2 was larger, with an approximate size of 3.0 kb.

Class Discussion Questions & Trouble-Shooting Suggestions

Day 2

The following are questions that should be considered while analyzing the transformation plates.

What was the transformation efficiency?

To answer this question the students count the number of colonies on the plate and calculate the efficiency as number of colonies/ μg of plasmid DNA.

Figure 4.

A picture of an ethidium bromide stained agarose gel with undigested (U) and *Eco*RI (E) DNA samples generated by the TempliPhi protocol. Lanes 1 and 10 are 1 Kb DNA marker (New England Biolabs) to serve as a size standard for the DNA fragments. The size of each fragment is indicated. Undigested (Lane 2) and *Eco*RI digested (Lane 3) of DNA that was amplified from a pRS313 colony. Note that the band in Lane 3 indicates the size of the plasmid vector without an insert. DNA prepared from a blue colony shows similar results as DNA amplified from pRS313 transformed cells (Lanes 4 and 5). Undigested (Lanes 6 and 8) and *Eco*RI digested (Lanes 7 and 9) DNA samples that were amplified from two white colonies, clone W1 (Lanes 6 and 7) and W2 (Lanes 8 and 9). Bands that are the same size as the band in Lane 3 is the plasmid vector backbone. The second band is the insert fragment and is a different size in for clone W1 (Lane 7) and W2 (Lane 9).
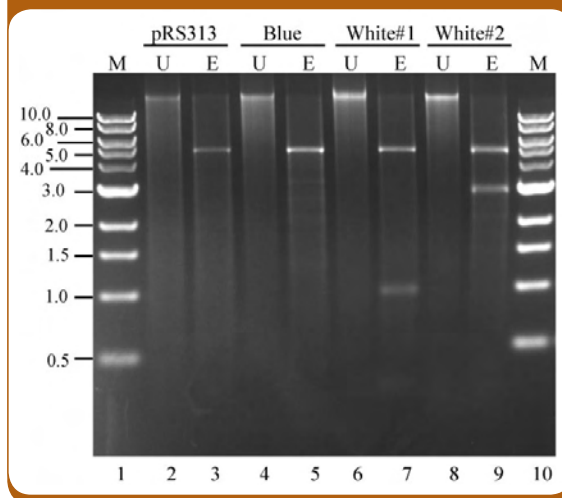
Why do differences occur in transformation efficiencies between groups?

Since the reagents are common to all the groups, differences in transformation efficiency are most likely the result of different experimental technique. How to improve the efficiency or increase the total number transformed cells could also be discussed.

Day 4

The following are questions that the students should consider while analyzing their gels.

Are the marker bands visible? Do they appear similar to the expected pattern?

This indicates if the students properly loaded, ran, and stained their gel (Figure 3, Lanes 1 and 10). If the marker is not present or appears different than expected, the student should trouble shoot which steps may have been a problem and how they would fix this if they repeated the experiment.

Is there DNA in the uncut lanes? What size is it?

Unlike a standard plasmid miniprep protocol, which produces multiple copies of individual plasmids, the Templphi® procedure produces long concatamers of the plasmid of varying lengths (Nelson et al., 2002). Therefore, instead of appearing as a single band on the gel, the uncut DNA amplified by the TempliPhi® protocol will appear as a large smear at the top of the gel (Figure 4, Lanes 2, 4, 6, and 8). If a smear is not present then it would suggest that the amplification reaction did not work. The most likely reason is that the enzyme reaction mix was not added to the sample. However, too many cells added to the reaction, inadequate lysis of the bacteria, inactivation of the enzyme, or improper buffer conditions (too much or too little) would also cause a decrease in yield or complete absence of amplification.

How many bands are in the Eco*RI digest of the DNA amplified from the blue colony? What size is this fragment?*

The plasmid DNA prepared from the pRS313 transformed colonies should contain vector without an insert. The *Eco*RI digest of this DNA should produce a single linear fragment that is the size of the plasmid vector (approximately 5 kb for the pRS313 vector

used in this experiment) (Figure 4, Lane 3). If the band is not present and the pattern of staining looks similar to Lane 2, then it indicates that the *Eco*RI digestion reaction did not work. This occurs if the digest was not set up properly or the *Eco*RI enzyme was inactivated.

How many bands are in the Eco*RI digest of the DNA amplified from the white colonies and what are the sizes of the fragments?*

The digested plasmid DNA from the white colonies should contain two fragments. One of the fragments is the plasmid vector itself, which should be the same size as the band in Lane 3. The other fragment is the genomic insert and should be different sizes in plasmids amplified from different colonies. The students estimate the size of the insert and draw a map of the plasmid indicating the position of the *Eco*RI sites and distance between each of the sites. It is possible that in some cases only one band appears in the digest. This occurs if the size of the insert is approximately 5 kb, the same size as the plasmid vector backbone. In this case the two fragments will migrate at the same position and therefore may appear as a single band on the gel. If the insert is less than 400 base pairs, it may not be detectable. Another potential problem is that one band appears that is greater than 5 kb or the digested sample may look similar to the undigested sample. This would occur if one or both of the *Eco*RI sites were mutated during the cloning process. Although it happens at a low frequency, it may be one explanation for the lack of two bands in the digest.

If Hin*dIII digests were performed, have the students determine the size and position of each band and draw the position of these sites on a map of the plasmid.*

The *Hin*dIII restriction enzyme digests three times in the plasmid vector backbone (Figure 2). There should be at least three fragments in each of the digests. For plasmids without an insert (blue library colonies or pRS313) there should be 3.6, 1.1, and 0.2 kb bands

Figure 5.

A picture of an ethidium bromide stained agarose gel with undigested (U) and *Hin*dIII (H) DNA samples generated by the Templifi protocol. Lane 1 and 10, 1 Kb DNA marker. Undigested (Lane 2) and *Hin*dIII digested (Lane 3) DNA that was amplified from a pRS313 transformed colony. Although there are three *Hin*dIII sites in the vector, Lane 3 contains two fragments because the 0.2 kb fragment was not detectable in this gel (see map in Figure 2). A blue colony shows similar results as the pRS313 cells (Lanes 4 and 5). Undigested (Lanes 6 and 8) and *Hin*dIII digested (Lanes 7 and 9) DNA samples that were amplified from the White #1 and White #2 colonies. The 3.6 kb band in Lanes 7 and 9 is a fragment from the plasmid vector backbone. The second band in Lane 7 corresponds to a fragment that contains the remaining 1.1 kb of the vector sequence and the 1.0 kb of insert fragment. In Lane 9 there are two other fragments in addition to the 3.6 kb band. This indicates that there is another *Hin*dIII site in the insert.
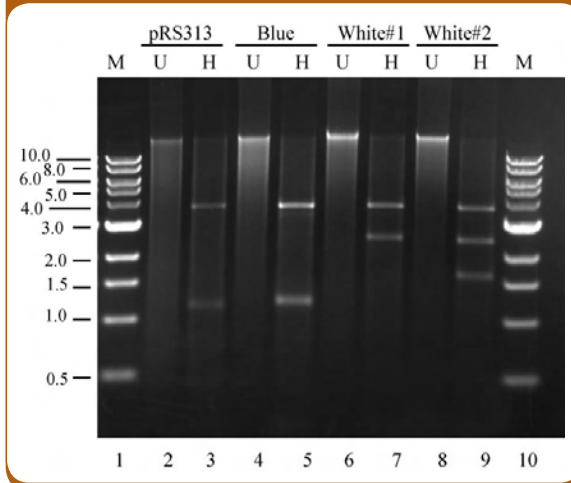
for the *Hin*dIII digest (Figure 2). Depending on the amount of DNA loaded and the stain used to visualize the DNA, the 0.2 kb fragment may not be visible (Figure 5, Lanes 3 and 5). For the white colonies, there should also be 3.6 and 0.2 kb bands, which correspond to fragments in the vector backbone (Figure 5, Lanes 7 and 9). However the 1.1 kb will not be present. Instead, there will often be a fragment that is larger than 1.1 kb that will contain the DNA corresponding to the 1.1 kb fragment from the vector and the DNA from the insert (Figure 5, Lanes 7 and 9). The *Eco*RI digest of DNA amplified from the White Colony #1 indicated the plasmid had an insert size of approximately 1 kb. We expect the *Hin*dIII digest to produce a 3.6 kb fragment as well as a 2.1 kb fragment in place of the 1.1 kb fragment. This is the pattern we observed (Figure 5, Lane 7).

It is also possible that there are *Hin*dIII sites in the genomic insert fragment. If this is the case, then an additional band will appear for each additional site in the plasmid (Figure 5, Lane 9). It is possible to determine the number of sites in the insert. The *Eco*RI digest of DNA amplified from the White Colony #2 indicated an insert size of 3.0 kb, which predicts the *Hin*dIII digest would produce a 4.1 kb fragment in place of the 1.1 kb fragment. However, in addition to the 3.6 kb vector fragment, the actual digest produced 2.5 and 1.6 kb fragments (Figure 5, Lane 9). If combined, the 2.5 and 1.6 kb fragments are the size of the expected 4.1 kb fragment, indicating that an additional *Hin*dIII site exists within the insert (Figure 5, Lane 9 and Figure 6B). Therefore, this restriction analysis confirmed the size of insert of foreign DNA and the existence of a *Hin*dIII site in the insert. The data from the restriction analysis can be summarized on a plasmid map as shown for plasmids #1 and 2 (Figure 6). Although the analysis of the restriction digest determined there was a *Hin*dIII site in the genomic insert of plasmid #2, the data allows for two models of its location (Figure 6B). Determining which of these models is correct requires additional experiments, such as an *Eco*RI/*Hin*dIII double digest of the DNA.

Discussion

The research module described in this paper gives students an authentic context in which to practice

science and an opportunity to interpret and apply data generated from real molecular biology experiments. The techniques and protocols used are identical to those currently performed by many biologists to isolate and analyze genes from a plasmid DNA library. The students perform a series of experiments with the single goal of understanding more about their novel DNA fragment, placing the individual protocols into a larger research framework. Although similar educational goals can be met by performing plasmid DNA preparations through standard procedures, these require expensive equipment that is often beyond the budget of a high school or introductory college level biology laboratory. The TempliPhi® protocol alleviates the equipment requirement by generating DNA samples directly from *E. coli* colonies and takes less classroom and hands-on time than standard plasmid miniprep procedures.

Molecular biologists, as in all scientific research, often encounter experiments that do not work or obtain results that are not expected. The scientist must therefore analyze the data, develop hypotheses to explain the results, and design experiments to test these models. Additional experiments may be required to explain the problem. Since the research module described in this paper does not have a single correct outcome, the students generate a range of results due to differences of the insert size. These differing outcomes require the students to individually analyze and interpret their results. For example, in the WSSP students observed a single 5 kb band in the *Eco*RI digested sample and proposed that the insert and vector were the same size. The students proposed that this model should be tested by performing a *Hin*dIII digest.

The TempliPhi® reaction is also advantageous in that it generates a large amount of DNA. Since only a small portion of the DNA is used for the restriction digests, other experiments can be performed with the DNA. One possible experimental extension is to use a UV spectrophotometer to determine the concentration of the DNA by measuring the absorbance at 260 nm and then calculate how much DNA was generated in the reaction. The amplified DNA is also an excellent template for DNA sequencing. DNA samples generated by the students could be sent to sequencing cen-

Figure 6.
Predicted restriction maps of the White #1 and White #2 Plasmids. The position of the sites was based on the available data shown in Figures 3 and 4. Note that there are two possible positions for the *Hin*dIII site in the insert of Clone White #2.
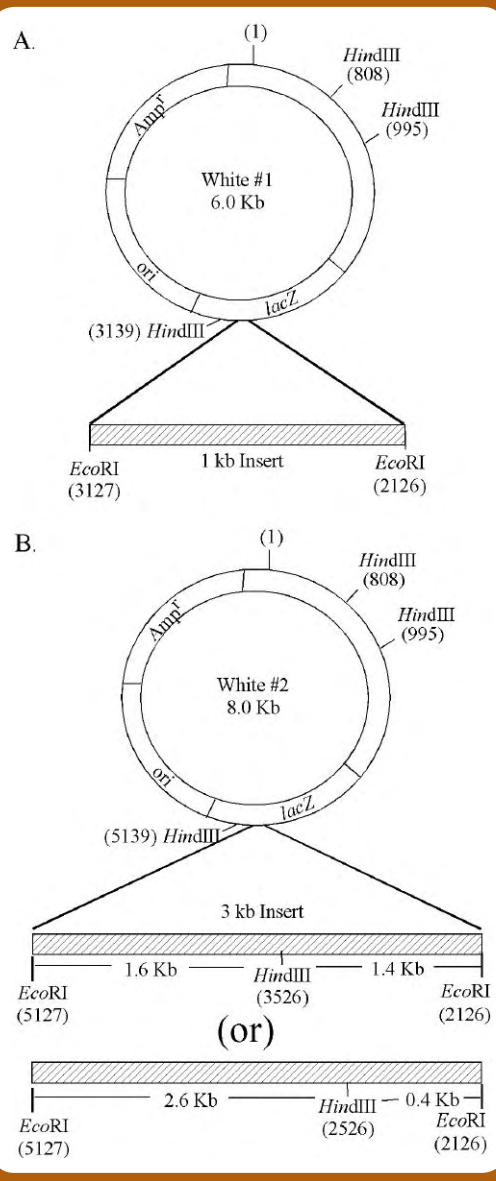
ters, such as those at Cold Spring Harbor Laboratory or local universities. The WSSP students performed this analysis and used the sequence information to search the international sequence databases to determine if there were matches to their DNA sequences and to explore topics such as the possible function of these genes and their evolutionary homologies and phylogenies.

Acknowledgments

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