Laboratory Experiment on GENE SUBCLONING For Chemical Engineering Students

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Biochemical engineering is the application of chemical engineering principles to biological processes. Processes for the production of fine and commodity chemicals that are catalyzed by whole cells or enzymes are developed and analyzed through the principles of biochemical engineering. Surveys of the industrial employment of chemical engineers show a steady increase in the number of graduates entering the biotech area.[1] In addition, many chemical companies have new developments in industrial biocatalysis. Including the kind of course described in this paper in the undergraduate chemical engineering curriculum can encourage students to consider biotechnology companies as a career by increasing their exposure to the types of projects they may encounter in such a job.

To introduce students to the fundamentals of laboratory practice in biochemical engineering, we have developed a laboratory course that includes protein isolation and purification, basic molecular biology methods, microbial kinetics and energetics, enzyme kinetics, and operation of bioreactors. For the first five weeks of the 15-week course, students practice basic molecular biology procedures—microbial transformation, restriction digest, DNA ligation, plasmid preparation, DNA and SDS-PAGE electrophoresis, and aseptic techniques. The time block for the laboratory class each week is five hours long.

Five hours are needed each week for experiments, with time for 75- to 125-minute lectures while students are waiting for experiments to be completed. For example, the thermal cycler runs for close to two hours, so the lecture can be given during that time. The subcloning experiment involves the amplification of the gene for green fluorescent protein from a plasmid, followed by insertion into a separate plasmid designed for high levels of protein expression. The five-week sequence is summarized in this article, with presentation of data from the students' results where appropriate.

The model system used in the lab course is a green fluorescent protein (GFPuv) that has been shuffled to yield fluorescence that is enhanced from that emitted by the native protein found in *Aequorea victoria*.[2] This system is convenient for the students because by using a hand-held UV lamp, it is easy to detect the protein by observing the fluorescence produced. The use of a long-wave lamp avoids any harmful UV exposure.

We have found that students have a much better understanding of the process of DNA subcloning after concluding the experiment, which conforms to the general trend in engineering education of increasing opportunities for visual and hands-on experience. As stated in an NSF Report,[3] "Shaping the Future" with regard to undergraduate technical education,

> *It is important to assist them to learn not only science facts but, just as important, the methods and processes of research, what scientists and engineers do, how to make*

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informed judgments about technical matters, and how to communicate and work in teams to solve complex problems.

The laboratory course has proved to be valuable for students entering the biotechnology workforce, even when they do not continue to do molecular biology.

For process development projects involving recombinant host strains, chemical engineering graduates will likely work closely with molecular biologists for improvement of the desired biocatalyst. The experience gained in this course is targeted to assist them to work in teams with scientists and other engineers.

The objective of the experiment is to subclone the GFPuv gene into a plasmid that has been designed for high expression levels and to enable purification of the expressed protein after fermentation of the host organisms. The fermentation and protein purification experiments comprise eight of the remaining weeks of the course.

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The plasmid system chosen for this work is the pET plasmid from Novagen.[3-6] The pET plasmid has a T7 promoter that transcribes mRNA for the target gene in the presence of T7 RNA polymerase. The T7 transcription/translation system originated from a bacteriophage and works efficiently for the expression of very high levels of protein. *E. coli* that have a gene for the T7 RNA polymerase under the control of a lac promoter can express the polymerase in the presence of lactose or isopropyl-β-D-thiogalactoside (IPTG).

For the subcloning, the pET29a plasmid is cut with the same enzymes as the amplified GFPuv gene insert. The GFPuv gene can then be ligated into the plasmid and the presence of the insert can be identified by DNA electrophoresis. The pET29a-GFPuv plasmid can then be used to transform competent Bl21 *E. coli* containing a T7 RNA polymerase for enhanced expression of proteins from the pET expression system. The students do not need to prepare competent Bl21 *E. coli* cells because they are ready for transformation in the Novagen kit. Ampicillin is used as a selection agent for the pGLO plasmid and kanamycin is used with the pET29a plasmid.

MATERIALS AND METHODS

(Summary of the Experiment)

Chemicals and Materials

- Ampicillin
- Arabinose
- Kanamycin
- Yeast extract
- Tryptone
- NaCl
- Ethanol (200 proof)
- Agarose
- Ethidium bromide
- Tris
- Glacial acetic acid
- Ethylene diamine tetraacetic acid (EDTA)
- IPTG

were all purchased from Fisher. Primers for the polymerase chain reaction (PCR)

- Upstream TTTTGGGCTAGAAATAATTTTG
- Downstream ATCCCCGGGTACCGAGC

were purchased from Operon. Restriction enzymes

- KpnI
- XbaI

were purchased from Promega, and the Promega 10x multicore buffer was used for the digest.

Taq polymerase for the PCR was included with the Promega master mix. The **$MgCl_2$ solution and nuclease-free water** were supplied with the Taq polymerase. A **1-kb DNA ladder** was purchased from GeneChoice because it can be used to estimate the amount of DNA on the bands (Cat. #62-6108-01).

The **6x loading dye** for the DNA electrophoresis was purchased from Promega (G190A). **Petri dishes and all disposables** were purchased from Fisher, and the five-minute ligation kit was purchased from GeneChoice (Cat. #62-6104-20). The **pGLO plasmid** was purchased from Bio-Rad (pGLO Bacterial Transformation Kit; Cat. #166-0003EDU, approximately $50), which also contains dehydrated *e. coli* (strain H-101).

The **pET29a expression system kit** was given by Novagen free of charge to use in the class. Additional **competent cells** were purchased from Novagen (Novablue singles, Cat #70181, Bl21 (DE_3) singles, Cat #70235). **Jellyfish software** was purchased from Labvelocity on-line at <http://jellyfish.labvelocity.com/index.jhtml>. **Kits for plasmid purification and gel extraction** were purchased from Qiagen (Cat. #27104 and #28704, respectively).

Kanamycin and ampicillin stocks were prepared prior to the course with 30 mg/ml and 100 mg/ml, respectively. The solutions were sterile filtered and stored in 1-ml aliquots at -30°C.

Special storage conditions: The enzymes were stored at -30°C; the competent cells were stored at -80°C; frozen stocks of all strains were stored with 30% glycerol at -30°C.

Equipment

- Circulating waterbath (Brinkman LAUDA RM6)
- Incubator (Fisher Isotemp)
- Incubator shaker (New Brunswick G25)
- Eppendorf mastercycler gradient thermal cycler
- UVP PhotoDOC-IT with 4912 camera and software
- Agilent bioanalyzer 2100
- HP 8452A UV/visible spectrophotometer
- Turner Designs picofluor fluorometer
- Agarose gel electrophoresis chamber and power supply

Methods

The methods are described in the weekly laboratory session below.

- Week 1 -

LB agar (1% Bacto-tryptone, 0.5% yeast extract, 0.5% NaCl, 2% Difco agar in DI water, addition of 640 μ) was prepared, followed by autoclaving at 121°C for 15 minutes. Antibiotic was added to the agar from a 1000x stock prior to pouring. From an overnight culture of pGLO *E. coli,* 3 mls were distributed to each group and the plasmid was purified according to the Qiagen kit instructions. The bacteria were viewed under a microscope. In addition, the students were able to study the primer binding sites on the pGLO plasmid and the restriction cutting sites and insertion region on the pET29a plasmid using Jellyfish software. This software provides a numbered image of the plasmid and identifies the restriction sites for the user-selected enzymes. Information on the primers is also given to facilitate the PCR design. TAE buffer (0.04M Tris-acetate, 0.001M EDTA) was prepared for the following week.

- Week 2 -

PCR was performed to amplify the GFPuv gene. The PCR reaction mixture consisted of 25 μl of the Promega Mastermix, 1 μl of each primer (1 μM final concentration each), 3 μl of purified pGLO plasmid from the mini-prep of the previous week, additional $MgCl_2$ to make a 4 mM final concentration, and nuclease-free water added to make 50 μl total solution. The annealing temperature of 52°C is optimal. While the PCR reaction was running, a restriction digest on the pGLO plasmid purified the previous week was performed to check the length of the plasmid. The restriction digest was incubated at 37°C for one hour in a circulating water bath, where the reaction mixture comprised 10 μl of pGLO plasmid from mini-prep, 1 μl of KpnI enzyme, 2 μl of 10x Promega multi-core buffer, 0.2 μl of concentrated Bovine Serum Albumin (BSA, frozen stock solution comes with the restriction enzyme), and 6.8 μl of nuclease-free water to make the mixture 20 μl in total volume. The PCR product was run on a 1% agarose gel along with cut plasmid to check the concentration of the purified plasmid from the mini-prep and to check the length of the PCR product. A 1-kb ladder is adequate for both measurements. The PCR product bands were purified from the gel with the Qiagen gel-extraction kit according to the instructions.

- Week 3 -

A restriction digest was performed to cut the GFPuv gene from the PCR product with KpnI and XbaI. The reaction mixture contained 1 μl of each enzyme, 0.2 μl BSA, 2 μl 10x multi-core buffer, and 15.8 μl of the purified PCR product. The cut gene was eluted from the spin tubes with Tris buffer supplied with the gel extraction kit. At the same time as the cutting of the PCR product, the pET29a plasmid was cut with KpnI and XbaI. To prepare for the ligation and transformation, 1 μg of plasmid was used. For the plasmid cutting experiment, three separate restriction digests should be performed: 1) cut with KpnI only; 2) cut with XbaI only; 3) cut with both KpnI and XbaI. The digests were run on a 1% agarose gel and the bands from the double-cut PCR product and double-cut plasmid were cut out of the gel and cleaned with the Qiagen gel-extraction kit. The two single cuts are performed to ensure the enzymes are working properly, and should have identical bands on the gel that are the length of the full plasmid.

The ligation of GFPuv gene into the cut pET plasmid was performed with the five-minute ligation kit by Genechoice. The reaction mixture for the ligation included approximately 30 ng of cut PCR product and 50 ng of plasmid. The reaction was carried out at room temperature. Competent "Novablue" *E. coli* cells were transformed with the ligation product and with control plasmid provided in the Novagen kit according to instructions in the pET system manual. The transformants were plated on LB/kanamycin plates and incubated for 18 hours at 37°C.

- Week 4 -

Prior to the class, the TA prepared overnight cultures from the students' plates in LB broth with kanamycin. During the lab period, the students first performed a mini-prep of colonies that were grown in liquid culture and then checked for the insert in the plasmid by a restriction digest with KpnI and XbaI. The students again carried out the cutting with three separate restriction cuts: one with KpnI only, one XbaI only, and one with both enzymes to compare the length of the plasmids. The double-cut plasmid should run ahead of the single-cut plasmid due to the removal of the insert. A pET plasmid without the insert was also cut with KpnI and XbaI. The in-

sert including the GFPuv gene should be 778 bases long. The digests should be compared on a 1% agarose gel. Given that the pET-GFPuv plasmid was successfully prepared, as demonstrated by the presence of the insert, competent Bl21DE$_3$ *E. coli* cells were transformed with the pET-GFPuv plasmid and with the pET plasmid without the insert for a control.

- Week 5 -

The TA prepared overnight cultures of both the pGLO *E. coli* and the pET-GFPuv *E. coli*. For these cultures, 1 mM IPTG and 0.1% arabinose were used as inducers, respectively. During the lab period, the students compared the fluorescence and the optical density of both the pGLO *E. coli* and the pET-GFPuv *E. coli*. A determination of the fluorescence normalized with the optical density for both strains enables a quantitative comparison of the amounts of correctly folded protein per bacteria. A complete SDS-PAGE experiment may not be feasible for a five-hour lab session. It is possible to make this comparison if a microfluidic analysis can be performed, such as with the Agilent 2100 Bioanalyzer.

RESULTS AND DISCUSSION

The experiment begins with the bacterial culture that can be generated from the components of the Bio-Rad pGLO Bacterial Transformation Kit. The Biotechnology Explorer products are designed for high school students to get some hands-on experience with biotechnology, and the pGLO plasmid that comes with the kit serves as the starting point for the subcloning experiment performed in our course. The gene for the green fluorescent protein (GFPuv) is located in the

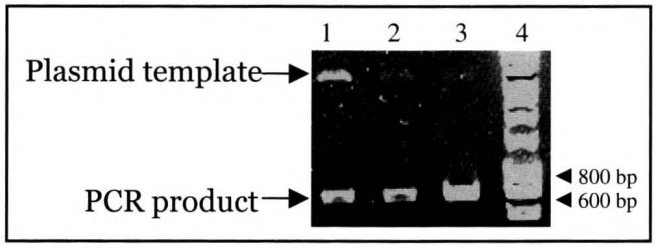

Figure 1. *Gel from lab session 2. The expected band of 795 was obtained by groups running in lanes 1-3.*

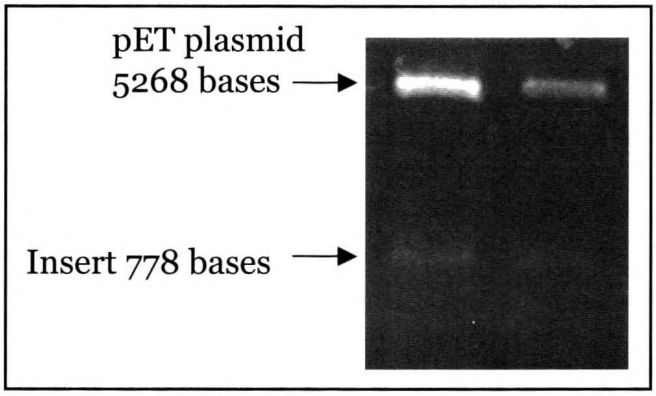

Figure 2. *pET-GFPuv plasmid cut with KpnI and XbaI showing the cut insert at 778 bases in length.*

pGLO plasmid downstream from the pBAD promoter, which can be induced with arabinose. The pGLO plasmid has both the ampicillin resistance gene as a selection marker for enabling only the growth of bacteria successfully transformed with the plasmid, as well as an arabinose promoter that enables user-control of the induction of the gene for GFP by addition of arabinose to the agar or broth. Transformation is necessary if the experiment will be performed in future courses, as the bacterial hosts provide a means to produce additional plasmid. The transformation should be performed ahead of the course and an overnight culture prepared prior to the first lab session.

In the second lab session, the students should be told that the order of the experiments is usually to check the results of the mini-prep with the restriction digest, followed by the agarose gel, prior to performing the PCR. Normally, one should be assured that the mini-prep was successful before running the PCR reaction, but the five-hour lab session does not permit this. If the students do not put correct amounts of the reagents into their tubes or do not follow steps accurately, their experiments will not work. Our philosophy is to maintain a stock of the necessary plasmid and gene products from the different experimental steps for use if any experiments fail. In that way the students can continue through the five weeks even if one of their experiments is not successful.

Figure 1 shows a section from the gel of a Week 2 lab session. The students are given the option of modifying the choice of annealing temperature and $MgCl_2$ concentration to further optimize conditions. The gel in Figure 1 shows three PCR reaction products (lanes 1-3), with the ladder in lane 4. The band between 600 and 800 bp is the desired PCR product containing the GFPuv gene. Because the students purified their own plasmids and chose different conditions for the PCR experiment, the brightness of the bands shows variations. Students whose bands are particularly bright are able to share some of their PCR product with other groups that got little or no band at the proper length.

The bacterial transformation of Week 3 is one of the most challenging steps in the five-week experiment. It is important to explain to the students that the times and temperatures listed in the protocol are critical, and if they change a step it will likely have a deleterious effect on their transformation. It has proven helpful to go over the procedure with them just prior to the actual experiment. The Novablue cells are used because they have a high transformation efficiency and lack the T7 RNA polymerase gene necessary for protein expression. After a ligation, it is favorable to use competent cells with a high transformation efficiency. Therefore, only after the Novablue cells are shown to contain the plasmid and the plasmid can be purified through a mini-prep are the Bl21(DE_3) cells transformed with the plasmid. While it is possible to include arabinose in the LB/Ampicillin plates to induce the

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expression of GFP after transformation with pGLO plasmid, the addition of IPTG to plates will prevent growth of the Bl21(DE_3) cells transformed with pET-GFPuv plasmid. The inducer should only be added after the cells have been successfully transformed. Figure 2 shows the pET29a-GFPuv plasmid cut with KpnI and XbaI. The insert runs close to 800 bases.

In the third-week experiments, it is helpful to note that the bases cut from the ends of the PCR product cannot be seen on the gel because they are too small to generate visible fluorescence. The failure of one or both of the enzymes to cut the ends, therefore, cannot be identified from the PCR product. It is instructive to check the activity of the enzymes by cutting the plasmid with the enzymes individually in two separate reactions. The single-cut plasmids should show bands of identical length provided the enzymes both cut efficiently. If this holds true, then the enzymes likely cut the PCR product successfully as well. It is also useful to explain to the students that as restriction enzymes are generally designed to cut longer pieces of DNA, they may not cut effectively close to the end of the gene, as with the PCR product. A useful reference is the New England Biolabs catalog, which can be viewed on-line at <http://www.neb.com/neb/frame_cat.html>. The section of "Technical Reference Literature" includes a table with optimal end lengths for commonly used restriction enzymes.

The fifth week is for analytical measurements of the protein expression in both folded and unfolded forms. The fluorescence emitted by the properly folded protein can be measured and normalized to the optical density. The difference between the normalized fluorescence of both the pGLO bacteria and the pET-GFPuv bacteria can be expressed as a ratio or percent increase, thus omitting the need for a GFP standard. The excitation light is ultraviolet light, and the amount of emitted fluorescence is measured with a fluorometer.

This is a challenging experiment for the students because they must dilute the culture until they are measuring in a linear range of both the fluorometer and the UV/vis spectrophotometer. Since two different expression systems are compared, ideally one would optimize the protein expression first and then make the measurements of fluorescence—but this has not been done. Instead, overnight cultures were prepared and the inducers were added at the initiation of the culture. When this approach is taken, the difference in fluorescence between the two cultures is minimal, being on the order of 15% higher for the pET-GFPuv culture. This also gives the students an opportunity to observe the difference in the fluorescence using the "wrong" induction method as compared with the "right" method that is used when the students run the fermentation. At that time, the culture is induced at the beginning of the lab period when the culture is in the exponential growth phase.

There has been very positive feedback about the lab. The enrollment has steadily increased, and there have also been students from local industries who have joined the class. Industry personnel who serve as advisors for the program or who have phoned for student references have been particularly positive about the content of the course. While they are interested in the fermentation and protein purification portions of the course, they have also praised the molecular biology portion. Because the chemical engineers often work closely with biologists for strain improvements, having hands-on experience with the DNA modifications enables them to follow the work of the biologists with greater understanding.

CONCLUSION

This paper describes a set of laboratory experiments that can be performed in five weeks for training undergraduate chemical engineering students in the basic steps in subcloning genes and molecular biology techniques. PCR, ligation, and the use of restriction enzymes are introduced with the lab, along with the use of gel electrophoresis to analyze changes in DNA. The use of green fluorescent protein enables fast determination of protein expression. Students who have performed the experiments have a better understanding of molecular cloning methods than students who have learned about it in the lecture course alone.

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