

BioEngineering Lab Techniques: A Novel Lab Course for Protein Expression in Bacterial and Mammalian Cells

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Abstract

BioEngineering Lab Techniques is an interdisciplinary lecture + lab course that is designed to give undergraduate students hands-on experience with cutting edge molecular biology and cell culture techniques that are utilized for recombinant protein production. Specifically, the students learn and perform every step necessary to express fluorescent proteins (e.g. GFP, mCherry) and chromoproteins (e.g. aeBlue, tsPurple, eforRed, et al.) in bacterial and animal cells by completing the following modules.

Module 1: Molecular Genetics & Cloning

The course begins with an overview of DNA and different cloning techniques, including PCR and Circular Polymerase Extension Cloning (CPEC). The first module includes 5 labs, in which the students amplify the gene for a colorful chromoprotein with PCR (Lab 1), clone that gene into an expression plasmid (Lab 2), transform the new plasmid into *E. coli* (Lab 3), then extract (Lab 4) and sequence (Lab 5) the plasmid.

Module 2: Bacterial Fermentation – Expression of Chromoproteins

In Module 2, the students use the expression plasmids from Module 1 to express their recombinant chromoproteins in *E. coli* (Lab 6), then purify the protein with either chitin affinity chromatography (Lab 7) or immobilized metal affinity chromatography (Lab 8). The purified protein is then characterized to determine its purity (PAGE), concentration (BCA assay), and absorbance spectrum in Lab 9, while Lab 10 is a virtual exercise that introduces the students to protein modeling software (FoldIt, SwissPDB).

Module 3: Animal Cell Culture – Genomic Integration of Fluorescent Proteins

As an alternative to bacterial protein expression, Module 3 shows students how to integrate genes into mammalian cell chromosomes to create stable transgenic animal cell lines for protein expression. Specifically, students begin by learning to passage adherent cells (Lab 11), then they prepare plasmids for the genomic integration of an mCherry gene (Lab 12) and transfect those plasmids into animal cells (Lab 13). Transgene expression is detected with fluorescent microscopy (Lab 14) and the course concludes with a virtual demonstration of transfectant selection & expansion of the transformed cells in a WAVE bioreactor (Lab 15).

In summary, this course teaches students every step in recombinant protein production from start (plasmid construction) to finish (protein purification & characterization). Student feedback to surveys is highly positive and graduates indicate that the course helped them prepare for and acquire their current jobs.

Course materials are available upon request from the instructor.

Introduction

According to a survey conducted by the American Institute of Chemical Engineers (AIChE) in 2015, a significant fraction of chemical engineering (ChE) graduates pursue careers in biotechnology and/or pharmaceuticals (see Figure 1).[1] Specifically, of the 48.9% of ChE graduates that go into industry, ~12% are initially placed in biotechnology and pharmaceuticals, 9% in food and consumer products, and 3% in environmental engineering. If other non-biological fields are excluded (e.g. fuels, chemicals, etc.) to specifically analyze the initial placement of biochemical engineering (BioChE) students, it is revealed that approximately 50% of BioChE students work in the biotechnology and pharmaceutical industries.

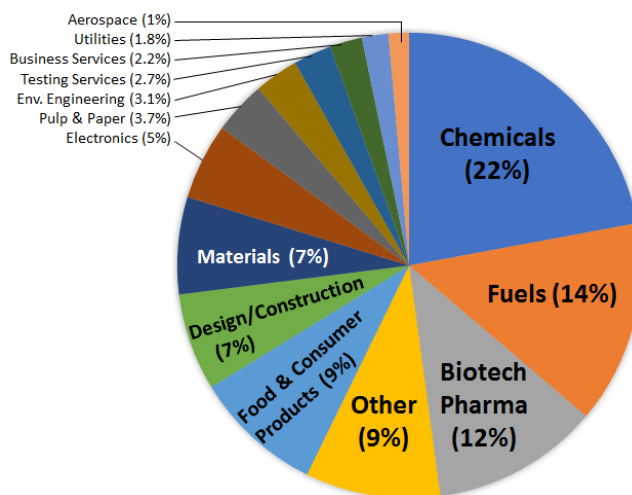


Figure 1 – Initial job placement of ChE/BioChE students in industry after graduation with a B.S. in ChE. Survey conducted by AIChE in 2015; this chart only reflects industry job placement (48.9% of students) and does not include the 22.9% of students that enter graduate school.

Since such a large fraction of BioChE students pursue jobs in biotech/pharma, it is important that we prepare our graduates for those fields by training them with a wide range of modern biotechniques. For example, many jobs in the biotech sector require engineers to culture bacterial or animal cells, manipulate DNA to synthesize new genes or sequence existing genes, and purify pharmaceutical proteins (e.g. antibodies, clotting factors, etc.). In addition, since the FDA approval of recombinant T cells (*Kymriah*TM, tisagenlecleucel) for the treatment of acute lymphoblastic leukemia (ALL) in 2017, many companies (Novartis, Johnson & Johnson, etc.) have started specifically recruiting graduates for the isolation, genetic engineering, and manufacturing of human cells as a therapeutic product. Consequently, BioChE students must be trained in molecular genetics, protein purification/characterization, and cell culture techniques to be successful in the modern biotech/pharma industry. This paper describes the development of a ChE elective lab course that gives students hands-on experience with all these highly desirable skills to prepare them for jobs in biotech/pharma.

Course Overview: BioEngineering Lab Techniques

BioEngineering Lab Techniques (BELT) is a course that has a lecture component with two classes a week (50 min/class) and a lab session that meets once a week (3 hours/lab) to conduct experiments. The lecture component focuses on a wide range of bioengineering techniques, including the experiments that are conducted during the weekly lab session. The semester is split into 3 modules which have the following goals:

- **Module 1 – Molecular Genetics (Figure 2)**
 - **Goal:** Students will know how to manipulate and analyze DNA.
 - **Outcome 1:** When asked to construct a new plasmid, students will be able to use PCR (or alternative cloning techniques) to create the new plasmid.
 - **Outcome 2:** Analyze the sequence and relative concentration of a DNA or RNA sample using PCR, Sanger Sequencing, and other next-generation methods.
- **Module 2 – Bacterial Fermentation (Figure 3)**
 - **Goal:** Students will know how to produce recombinant proteins in bacterial cells.
 - **Outcome 1:** When asked to produce a recombinant protein, students will be able to transform and grow *E. coli* cells to produce large amounts of the protein.
 - **Outcome 2:** Evaluate and compare different purification methods for the isolation of the desired recombinant protein from bacterial cell lysate.
- **Module 3 – Animal Cell Culture (Figure 4)**
 - **Goal:** Students will know how to produce recombinant proteins in eukaryotic cells.
 - **Outcome 1:** When asked to integrate a gene into a host cell genome, students will be able to design and construct plasmids for Cas9-mediated genomic editing.
 - **Outcome 2:** Aseptically culture, transfect, and select adherent animal cell lines that are genetically engineered to express a recombinant protein.

In addition to these goals and outcomes, two virtual labs are conducted in Modules 1 & 2 to introduce the students to bioinformatics techniques (e.g. BLAST, Lab 5) and protein structure simulations (e.g. Swiss PDB Viewer, Lab 10). Altogether, these experiences cover every step in the production of recombinant proteins, from start (genes) to finish (pure protein).

The proteins used in this course include colorful chromoproteins that are expressed in *E. coli* and fluorescent proteins that are expressed in eukaryotic cells (see Table 1). These proteins do not have any therapeutic value, but their ability to be observed by the naked eye allows students to readily see their expression inside the cell and track their purification on chromatography columns. The chromoprotein genes used in Module 2 were generously provided by Erik Gullberg of Uppsala University in Sweden [2], while plasmids containing the fluorescent protein genes (and Cas9) were purchased from Addgene.org (Plasmids #80492 and #48138).

In addition to lessons and lab experiments, students are also required to complete 3 lab reports that summarize the experiments performed in lab and a written course project that describes how the techniques used in lab could be used to produce a therapeutic protein that treats a disease

(e.g. insulin for diabetes). Each of these assignments goes through a draft/revision process in which a first draft is submitted, marked up by the instructor for technical content and spelling/grammar, and then the students have 1 week to prepare the final draft of the report. To emphasize the importance of brevity and clarity, each report is limited to only 2 pages of text, with figures and references included on additional pages.

Protein	Organism	Color	Supplier	Cat#
GFP	<i>Aequorea victoria</i>	Ex: 489 nm (blue) Em: 589 nm (green)	AddGene	48138
mCherry	<i>Discosoma anemone</i>	Ex: 587 nm (yellow) Em: 610 nm (orange)		80492
aeBlue	<i>Actinia equina</i>	Blue	Uppsala University iGEM Team	BBa_K864401
amilGFP	<i>Acropora millepora</i>	Yellow		BBa_K592010
asPink	<i>Anemonia sulcata</i>	Pink		BBa_K1033927
eforRed	<i>Echinopora forskaliana</i>	Red		BBa_K592012
tsPurple	<i>n/a</i>	Purple		BBa_K1033905

Table 1 – Proteins used in this course.

Note: The following sections provide an overview of the lessons and lab experiments in the course, but the author is willing to share a more detailed lab manual and slide decks.

Module 1 – Molecular Genetics and Cloning

The objective of the first module is to familiarize students with several different molecular biology techniques (e.g. PCR, digests, ligation, etc.) that can be used to clone genes into plasmids for expression in *E. coli* or other bacteria. The advantages and disadvantages of many different cloning techniques are discussed in lecture, including traditional restriction-based methods, recombinase systems, overlap extension PCR (OEPCR), circular polymerase extension cloning (CPEC), and Gibson Assembly. Subsequent lessons focus on the optimization of gene expression, transformation of bacterial and plant cells, next-generation sequencing, and bioinformatics.

This first module also includes 5 lab sessions in which the students amplify one of the chromoprotein genes with PCR (Lab 1), clone that gene into an expression plasmid using CPEC (Lab 2), transform the new plasmid into *E. coli* (Lab 3), then extract (Lab 4) and sequence (Lab 5) the plasmid. Two types of expression plasmids are used to express the chromoproteins (see Figure 2). In the pCBD-I-CP plasmid, the 5' end of the chromoprotein gene is fused to a purification tag with a chitin binding domain (CBD) that selectively binds chitin beads and a self-cleaving intein that cleaves the tag from the chromoprotein at pH 6.2. In the pHIS-EK-CP plasmid, the chromoprotein gene is fused to a different purification tag with 6 histidines that bind to metal ions (e.g. Ni^{2+}) immobilized on chromatography resin and an enterokinase linker that can be cleaved with the protease enterokinase (EK) to release the native chromoprotein. These plasmids are also used in Module 2 to express the fusion proteins and then compare the ease, cost, and efficacy of purification with chitin beads and nickel chelate resin.

Lab 1 - PCR

The goal of the first lab is to use the polymerase chain reaction (PCR) to amplify a chromoprotein gene from a storage plasmid that lacks a promoter. Students design and optimize their own PCR primers in class, but use primers that were pre-ordered ahead of time by the instructor. In the lab, the students set up 5 aliquots of the same PCR reaction, which are then put through different PCR thermal cycler routines with different annealing temperatures (T_a). Running these reactions on an agarose gel (Figure 2, top right) reveals that reactions run with a T_a less than or equal to the predicted melting temperature ($T_a \leq T_m$) of the primers are successful, while reactions performed at higher temperatures ($T_a > T_m$) fail because the primers cannot anneal to the template. The successful amplicons (bands on the agarose gel) are then excised and extracted with a Gel Extraction Kit for use in Lab 2.

Lab 2 - CPEC

In the second lab, the chromoprotein gene that was amplified and extracted in Lab 1 is cloned into the two expression plasmids with N-terminal purification tags shown in Figure 2 using a PCR-based method called Circular Polymerase Extension Cloning (CPEC).[3] CPEC stands out

from other cloning techniques because (1) it does not require specific sequences (e.g. restriction sites), (2) it only requires 1 enzyme (Phusion Polymerase), and (3) it is a relatively quick and mostly simple process, and (4) the new plasmid can be visualized on an agarose gel. To prepare for CPEC, the instructor must perform a PCR reaction that uses bipartite primers (i.e. 5' ends complimentary to the target gene and 3' ends complimentary to the expression plasmid) to amplify the everything in the expression plasmid (ca. 6,000 bases) except for the old gene (MBP) that is being replaced. This results in an expression plasmid backbone amplicon that is flanked by sequences that are complimentary to the target gene. The students then mix the gene amplicon from Lab 1 with the plasmid backbone amplicon (supplied by the instructor) in a 2' PCR reaction, in which the overlapping sequences anneal to each other and extend in the 3' direction, resulting in a (nicked) expression plasmid that contains the new gene (see Figure 2). However, since this reaction is not 100% efficient, multiple PCR cycles are required to fuse all the genes to the plasmid backbones. As shown in Figure 2, one cycle of 2' PCR produces only low amounts of the plasmid (upper dim band), with mostly unreacted gene amplicons leftover (lower band). More of the plasmid is produced with each subsequent cycle and nearly 100% conversion is achieved after 10 cycles. Students investigate this phenomenon by running CPEC reactions for 1, 5, and 10 cycles. A portion (5 uL) of these reactions is run on a gel to determine which reaction produced the most plasmid, then the rest of the optimum reaction (45 uL) is frozen until Lab 3.

Labs 3-5 - Transformation and Miniprep and Sequencing

Once plasmid production is confirmed in Lab 2, the corresponding CPEC reaction is used to directly transform chemically competent DH5 α *E. coli* in Lab 3. The transformants are spread onto ampicillin plates for selection, incubated overnight at 37°C, and then the instructor stores the plates in the refrigerator (4°C). On the day before Lab 4, the instructor uses colonies on the plate to inoculate Luria-Bertani (LB) broth cultures containing ampicillin, which are incubated at 37°C overnight. This provides turbid bacterial cultures by the next morning for Lab 4, in which the students extract the plasmid from the transformed cells. The purified plasmids are then sent off for sequencing (using T7 promoter and T7 terminator primers) to confirm that the gene was cloned into the expression plasmid without any mutations. The raw sequencing data is then analyzed using NCBI BLAST in Lab 5. In addition, students are also shown how to use a few other bioinformatics tools, including Clustal Omega for multiple alignments, Primer3 for PCR/rt²PCR primer design, and the NCBI Entrez database for the retrieval of gene/protein sequences.

Lab Report

After Module 1, students are required to submit a lab report that describes the cloning process and the optimization of annealing temperature during the 1' PCR reaction and the cycle number during the 2' PCR reaction. In addition, the students also discuss the transformation efficiency achieved in Lab 3 (e.g. how many colonies grew on the agar plates) and the sequencing results that were analyzed in Lab 5 (presence of the gene and any mutations).

Module 1: Molecular Genetics & Cloning

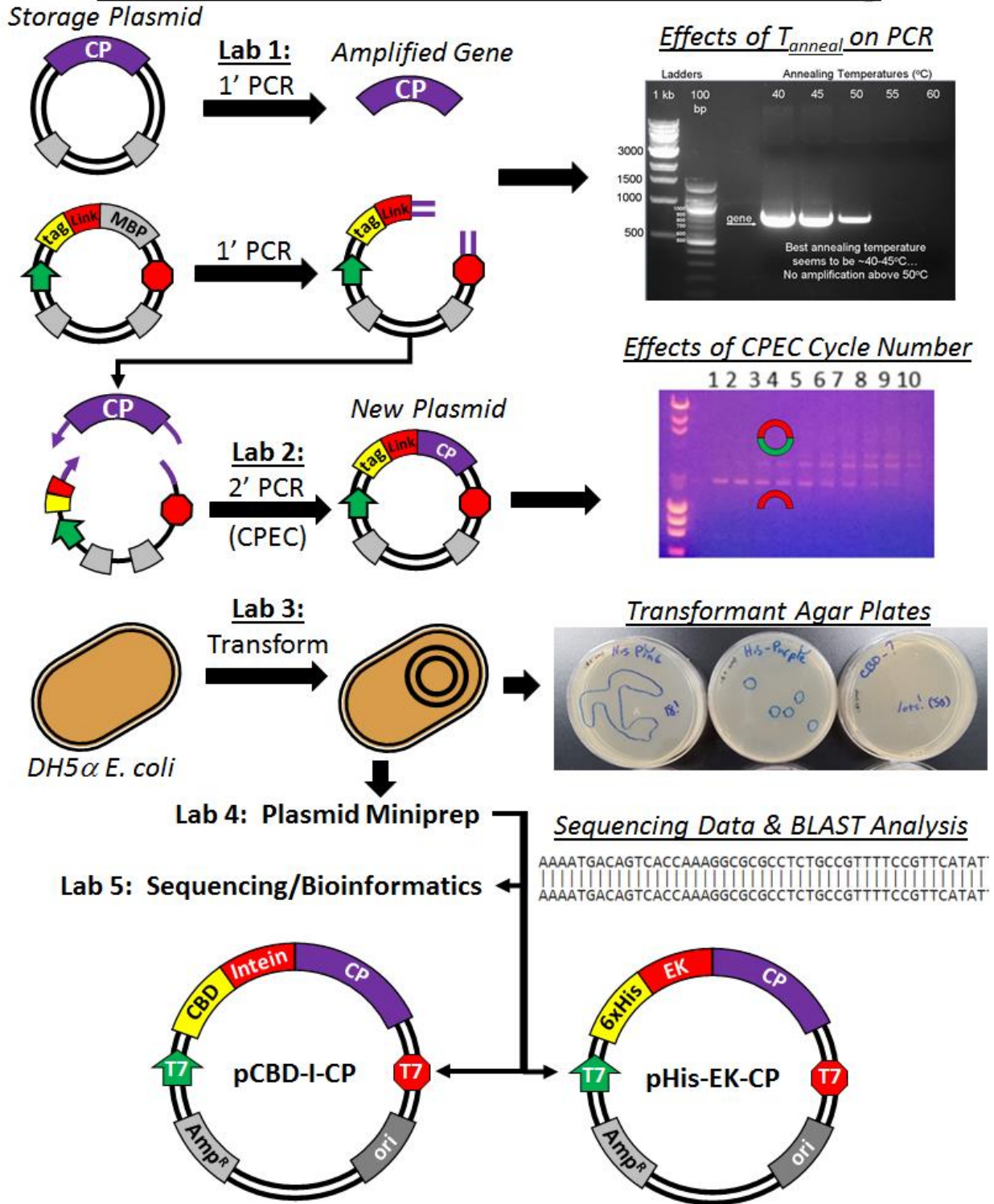


Figure 2 – Overview of Module 1. In this module, students use CPEC to clone a chromoprotein gene into expression plasmids with N-terminal purification tags. PCR reactions are verified on gels and the new plasmids are sequenced & analyzed with BLAST.

Module 2 – Expression of Chromoproteins in Bacteria

The objective of the second module is to teach students how to express recombinant proteins in *E. coli* and isolate them using one or more different purification techniques. The lectures begin with the basics of bacterial cell growth and optimization of protein expression, then several lessons are devoted to comparing and contrasting different purification techniques and chromatography resins. The design of automated fast performance liquid chromatography (FPLC) systems is also discussed. In the last few lessons of the module, 3 different software packages that simulate protein folding and 3D structure (i.e. FoldIt, Swiss PDB Viewer, and SWISS-MODEL) are demonstrated in class and the students are required to use these programs to generate 3D structures that are included in their lab reports (i.e. “virtual” lab 10).

While the first module focused on cloning techniques, the experiments performed in Module 2 are designed to show students the differences between protein purification techniques (see Figure 3). Specifically, each group expresses two types of chromoprotein with different purification tags (a chitin binding domain/intein tag and a 6xHistidine/EK linker tag) in *E. coli* in Lab 6. The chromoprotein with the CBD/I tag is then purified with chitin beads in Lab 7, while the chromoprotein with the His/EK linker is purified with a Nickel-NTA resin in Lab 8. The purity of the protein samples is then analyzed using PAGE, while their overall yield (mg protein/L culture) is estimated using a BCA assay in Lab 9.

Lab 6 – Bacterial Fermentation

To prepare for this lab, the plasmids that were transformed into DH5 α *E. coli* in Module 1 must be transformed into BL21 *E. coli*, since their T7 promoters are only active in the BL21 strain. The instructor must then inoculate shaker flasks with the transformed BL21 *E. coli* to provide cultures that are turbid by the start of the lab session. The students then aseptically split their flasks into smaller shaker flasks (e.g. 50 mL in a 250 mL flask), which are induced with IPTG to activate gene expression and then incubated in separate shakers at different temperatures (e.g. 25°C, 30°C, and 37°C) to determine the optimum conditions for chromoprotein expression. For example, the chromoproteins should change the color of the bacterial cells as they express (e.g. high expression of aeBlue will cause the cells to appear dark blue instead of the usual tan). In our experience, the chromoproteins tend to be highly stable and optimum expression levels are obtained at 37°C with a post-induction incubation time of 4 hours. If necessary, the instructor can then use the optimized culture conditions to repeat the fermentation with larger flasks (~200 mL culture in a 1 L flask) to prepare a large number of cells/chromoprotein for Labs 7/8.

Lab 7 – Affinity Purification with Chitin Beads

In this lab, chitin beads are used to isolate the CBD-intein-chromoprotein fusion. First of all, students lyse the cells by resuspending them in a lysis buffer containing Triton X-100 and sonicating them (20% power, 10 sec on/10 sec off for 10 minutes). **It is important to note that these cells cannot be lysed with lysozyme, since the lysozyme will degrade the chitin beads used for purification.** The cell lysate is clarified via centrifugation and 0.22 μ m filtration, then

the lysate is added to chitin beads that have been equilibrated in a solution of 20 mM Tris/1 mM EDTA (pH 8.0). At this point, the CBD-intein-chromoprotein fusion binds to the beads, while all the other *E. coli* host cell impurities are rinsed out of the column with additional buffer, thereby providing 100% purity in a single step. The chromoprotein is then retrieved by equilibrating the beads in 20 mM MES buffer at pH 6.2. At this lower pH, the intein undergoes a conformational change that allows it to cleave at its C-terminus, thereby releasing the native chromoprotein. Unfortunately, the intein cleavage process is slow and may take several days at room temperature (or weeks at 4°C). Nonetheless, the pure chromoprotein can then be eluted from the column and frozen until it is analyzed in Lab 9.

Lab 8 – Immobilized Metal Anion Chromatography (IMAC)

As an alternative to chitin bead purification, IMAC is used to bind the 6xHis-EK-chromoprotein fusion in this lab. Specifically, a spin column containing Nickel-NTA resin is equilibrated in 20 mM Tris at pH 7, then the bacterial lysate is passed through the column to bind the fusion chromoprotein. The resin is then washed with increasing amounts of imidazole (a compound that competes with histidine to bind the immobilized Ni²⁺) to remove impurities that are weakly bound. Finally, the column is rinsed with 250 mM imidazole to elute the fusion protein. In contrast to chitin bead purification, this protocol yields only a partially purified chromoprotein that is still tagged, since the 6xHis/EK tag is not cleaved off and many other *E. coli* proteins have a strong affinity for the Nickel-NTA resin, allowing them to bind and elute alongside the chromoprotein. If a native chromoprotein with higher purity is desired, the purification tag can be cleaved by adding an enterokinase enzyme to the column to cleave the EK linker over 4-8 hours at room temperature. Once the tag is cleaved, pure chromoprotein can be eluted from the column while the other impurities remain bound to the resin. However, the enterokinase enzyme is highly expensive, so we do not use it in our lab.

Lab 9 – Characterization of the Purified Proteins

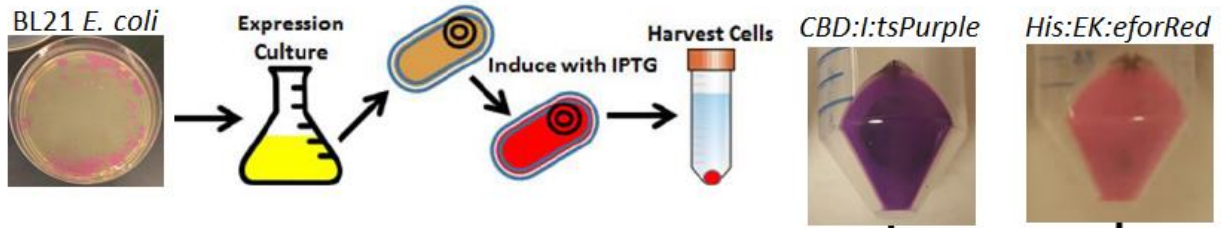
In the final lab of the module, the chromoproteins purified in Labs 7 and 8 are characterized with PAGE, UV-Vis Spectroscopy, and a BCA assay. The PAGE gel (see Figure 3) allows students to analyze the purity of samples taken throughout each purification process. As expected, the lysates contain many proteins, while the chitin-purified chromoprotein is 100% pure. In contrast, the IMAC-purified chromoprotein sample contains some residual impurities. The concentration and yield of the chromoprotein is then measured using a BCA assay and/or UV-Vis spectroscopy to measure the absorbance spectrum of the chromoprotein sample.

Lab 10 –Protein Simulation Software – FoldIt, SwissPDB, and SWISS-MODEL

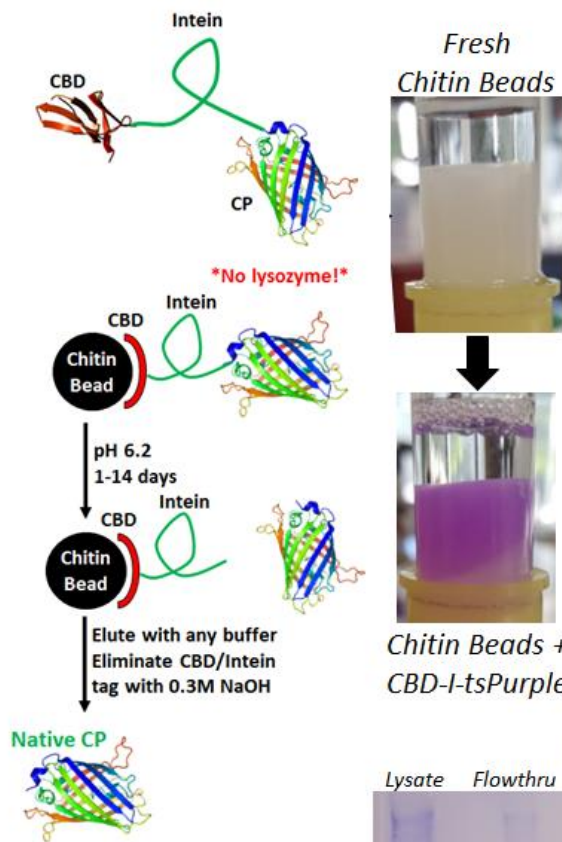
During the final week of the module, two lecture periods are devoted to tutorials of 3 different software programs that simulate protein folding (FoldIt) and 3D structure (Swiss PDB Viewer and SWISS-MODEL). The FoldIt program (available at <https://fold.it>) effectively gamifies protein folding simulations, allowing users to “play” with protein structures until they achieve a high score, which reflects a conformation with high stability/probability. Students can

Module 2: Expression of Chromoproteins in *E. coli*

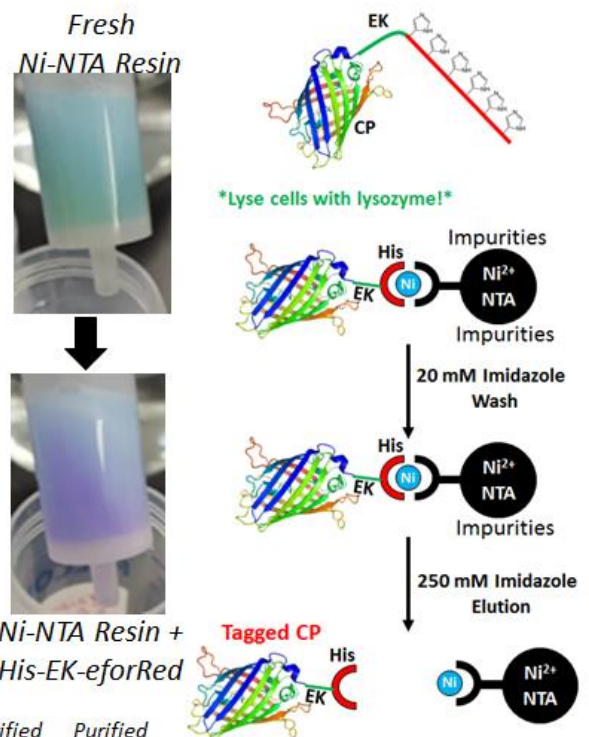
Lab 6: Bacterial Fermentation & Recombinant Protein Expression



Lab 7: Affinity Purification (*chitin beads*)



Lab 8: IMAC Purification (*Ni-NTA resin*)



Lab 9: PAGE + BCA:

Protein purity and concentration

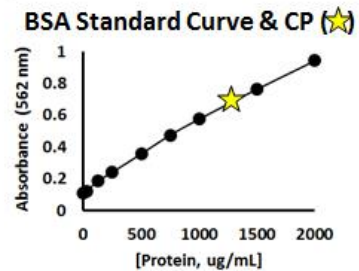
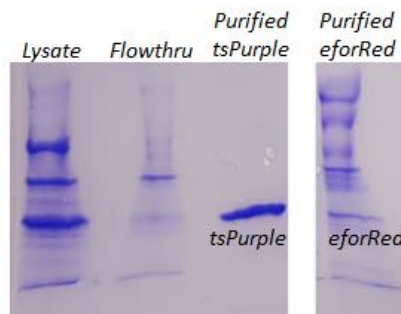


Figure 3 – Overview of Module 2. In this module, students use the plasmids that they constructed in Module 1 to express recombinant fusion proteins in *E. coli*. The fusion proteins are then purified with chitin beads or IMAC resin, then protein purity is analyzed with a PAGE gel and protein yield/concentration is measured with a BCA assay and/or UV-Vis spectroscopy.

simply complete the training modules in the FoldIt program or they can be challenged to complete more advanced puzzles and challenges (which can be designed by the instructor, if desired).

The students are also required to use Swiss PDB Viewer (available at <https://spdbv.vital-it.ch/disclaim.html>) to obtain a 3D structure of their chromoprotein that is included in their lab report. For proteins that do not have known 3D structures, students are also shown how to use the SWISS-MODEL program to prepare a homology model of their protein. Specifically, students upload the amino acid sequence of their protein to the SWISS-MODEL website (<https://swissmodel.expasy.org>), which then searches the protein data bank for proteins with similar sequences and known structures to predict the structure of the target protein.

Lab Report

The focus of the second lab report is on maximizing recombinant protein yields. Specifically, the students discuss the effects of incubation temperature on chromoprotein expression levels in Lab 6 and then compare/contrast the chromoprotein yields and purities obtained with chitin bead purification and IMAC. The students are also prompted to include a short discussion on the correlation between the absorbance spectrum of the chromoproteins and their observed color, along with an analysis of the 3D structures prepared with SWISS-MODEL.

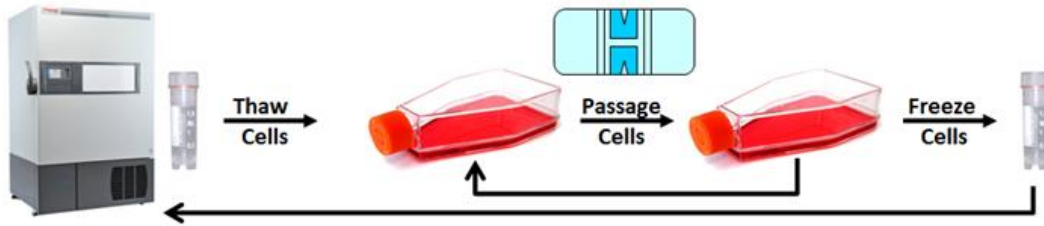
Module 3 – Expression of Fluorescent Proteins in Eukaryotes

The objective of this final module is to train students how to culture animal cells and use them to express recombinant proteins, since eukaryotic cells have several advantages over prokaryotic cells. Indeed, while making recombinant proteins in *E. coli* is relatively easy, it is not feasible for proteins that require complex post-translational modifications (e.g. disulfide bonds or glycosylation) that can only be performed in eukaryotic cells. The lessons in this module begin with a range of animal cell culture techniques (e.g. flasks, WAVE bioreactors) and then transition to genomic editing with the Cas9 enzyme, which the students use in lab to integrate a gene for the red fluorescent protein mCherry into the genome of a human cell line. Transfection techniques (e.g. viruses, lipids, etc.) are also discussed, along with different types of microscopy and an overview of pre-clinical and clinical trials of experimental drugs.

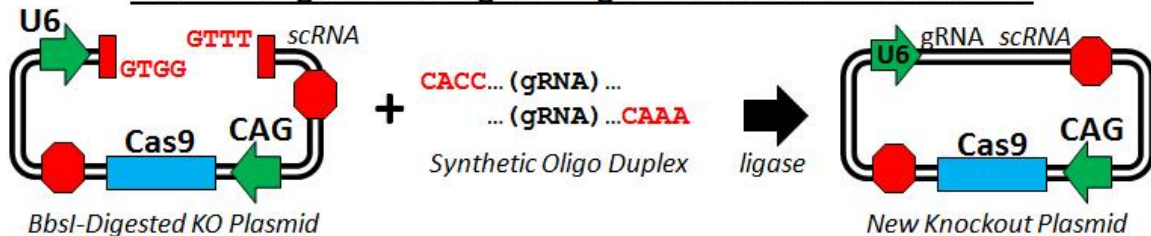
The lab sessions in Module 3 begin with an introduction to animal cell culture techniques, including the thawing, passaging, and freezing of adherent cell lines (e.g. PC-3 or NIH-3T3) in Lab 11 (Figure 4). Once the students have initiated their cell lines, the students prepare a plasmid for Cas9-mediated genome editing in Lab 12. Specifically, they produce a “knockout” plasmid that expresses a Cas9-GFP fusion and a gRNA sequence that targets Cas9 to cut a specific location in the genome. The students then use Lipofectamine™ to transfect cells in Lab 13 with the knockout plasmid and a second “knockin” plasmid, which contains an expression cassette for mCherry that is integrated at the Cas9 genomic cut site. Finally, fluorescent microscopy is used in Lab 14 to detect transfected cells, which fluoresce green (GFP) and/or red (mCherry). If time permits, cells that integrated the mCherry gene can then be selected for with puromycin and cultured to produce the protein in either flasks or a WAVE bioreactor (Lab 15).

Module 3: Animal Cell Culture & FP Expression

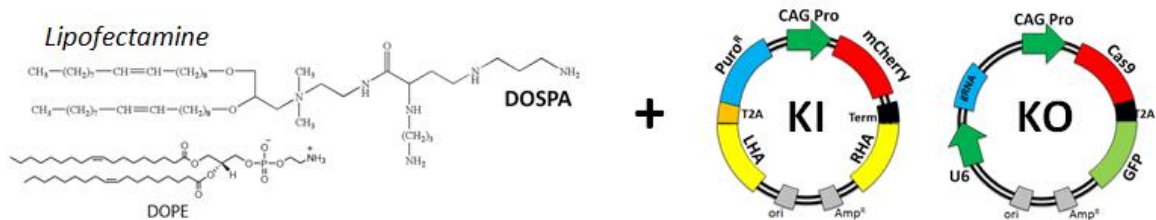
Lab 11: Animal Cell Culture Techniques (Thawing, Passaging, and Freezing Cells)



Lab 12: Oligo Annealing Cloning of a Cas9 Knockout Plasmid



Lab 13: Transfection of KO and KI Plasmids into Animal Cells with Lipofectamine



Lab 14: Fluorescent Microscopy (Visualizing GFP and mCherry Expression)



Lab 15: Expansion of Cells in the WAVE Bioreactor for Protein Expression

Selection of transfected cells with Puromycin and demonstration of WAVE

Figure 4 – Overview of Module 3. In this module, students learn how to culture animal cells, then use Lipofectamine to transfect them with a pair of plasmids that express a Cas9-GFP fusion that specifically cuts the genome, allowing for the integration of an mCherry expression cassette.

Lab 11 – Animal Cell Culture

Students begin this module by learning how to culture adherent animal cells in Lab 11. In a single lab period, they thaw out frozen cells to inoculate a tissue culture flask with RPMI media, then passage cells that were previously inoculated by the instructor 3-7 days in advance. Excess cells from the passaging are then frozen in DMSO. Students perform all these tasks in a biosafety cabinet after the instructor personally trains them on proper aseptic technique.

Lab 12 – Oligo Annealing Cloning (KO Plasmid)

While the students' cells are recovering from the freeze/thaw process and starting to grow, they use oligo annealing cloning (OAC) to prepare a knockout plasmid (see Figure 4) that expresses sgRNA with a U6 promoter and a Cas9-GFP fusion with a CAG promoter. Specifically, the instructor first digests an existing KO plasmid with the restriction enzyme BbsI, which creates a linear plasmid backbone with sticky ends. The students then mix together two synthetic complimentary oligonucleotides corresponding to a gRNA sequence that is complimentary to the desired cut site in the genome. The oligonucleotides are also designed to have sticky ends that are complimentary to the digested plasmid, such that mixing the oligo duplex with the digested plasmid allows the two fragments to anneal and then ligate after adding T4 DNA Ligase. The ligation mixture is then transformed into bacteria at the end of the lab session. In the following days, the instructor then cultures the transformants, isolates the new plasmid, and sends it off for sequencing, such that the new plasmid is validated prior to the next lab.

Lab 13 – Animal Cell Transfection

After the KO plasmid is sequenced, students co-transfect the KO plasmid with a knockin (KI) plasmid into eukaryotic cells using various amounts of Lipofectamine™. Once inside the nucleus, the KO plasmid expresses sgRNA and a Cas9-GFP fusion that can be visualized via fluorescent microscopy (Lab 14). The Cas9-sgRNA complex translocates to the nucleus and cuts genomic sequences that are complimentary to the sgRNA (and on the 5' end of a NGG PAM).

The KI plasmid carries an expression cassette for a puromycin resistance gene and mCherry that is flanked by regions that are homologous to the Cas9 cut site. Consequently, the KI plasmid can then participate in a homology-dependent recombination (HDR) reaction with the genomic site that was cut by Cas9, thereby inserting the mCherry expression cassette and making the cells resistant to puromycin. It is important to note, however, that in this system mCherry will be expressed before and after genomic integration, since it has its own CAG promoter.

Lab 14 – Fluorescent Microscopy

In the final lab of Module 3, students determine the efficiency of the Lipofectamine™ transfections by examining the cells with a fluorescent microscope. In this context, green fluorescence indicates that cells were transfected by the KO plasmid and expressed Cas9, while cells that fluoresce red were successfully transfected with the KI plasmid. Any cells that fluoresce both green and red may have the Puro/mCherry expression cassette successfully integrated into the genome, but subsequent selection via exposure to puromycin can confirm genomic integration.

In addition to GFP and mCherry fluorescence, the cells can also be stained with dyes, including Hoechst 3342 (binds DNA to fluoresce blue), Ethidium Bromide (binds DNA in dead cells and fluoresces red), Calcein AM (fluoresces green in live cells), or a variety of other fluorescent dyes.

Lab 15 – Virtual Lab: WAVE Bioreactors

After transfection, the next step required to produce recombinant proteins in eukaryotic cells would be to select the positively transfected cells (e.g. with puromycin) and then expand the culture to a larger volume to obtain a high number of cells to produce the protein. However, the selection and expansion steps require a long time and prohibitively expensive materials (e.g. cell culture media). Therefore, instead of performing these steps in the lab, they are simply discussed in class. The students are also brought into our cell culture facility for a demonstration of a WAVE bioreactor, which is commonly used to culture animal cells for protein production.

Lab Report

The experiments in Module 3 are straightforward and only one variable is investigated (the ratio of Lipofectamine™ to DNA in Lab 13), so the last lab report is relatively short. However, to make up for the lack of experimental variables, students are prompted to include a detailed description of how the KO and KI plasmids would be prepared for Cas9-mediated genomic integration. They are also required to carefully analyze the fluorescent microscopy images obtained in Lab 14 and use them to estimate transfection efficiency.

Practical Considerations

Student Feedback

At the end of each semester, a uniform survey was administered in this course and all the other courses offered in the Department of Chemical Engineering and College of Engineering. The survey contained three questions regarding student satisfaction with the course, which are shown in Figure 5. The feedback on these surveys was highly positive, with the students indicating that they “learned a great deal” in the course and found the course “intellectually stimulating.” Indeed, the values recorded for each of these metrics in this course were in the top quartile for the Department and significantly higher than other courses in the College of Engineering.

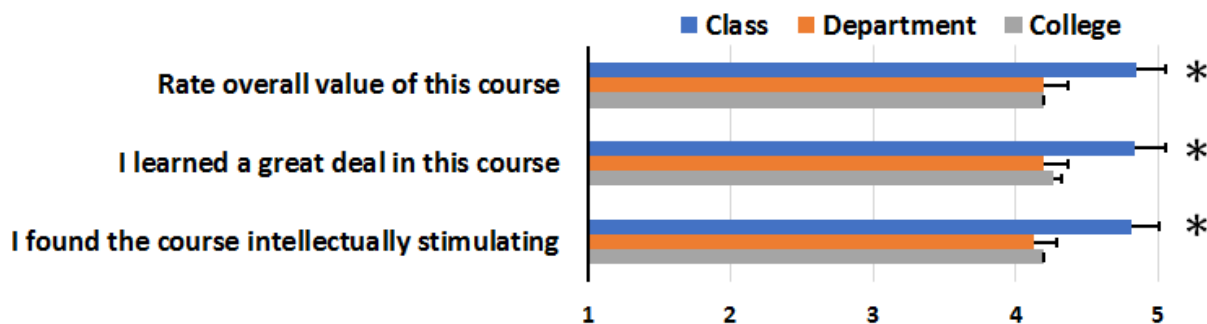


Figure 5 – Asterisks (*) indicate that this ranking was in the top quartile compared to other courses in the department. Data are averaged over the course of 3 years with 27 total students.

In addition to the University-mandated survey shown in Figure 5, the instructor also recently administered a survey that was only sent to students that had graduated to determine if the class helped them to prepare them for a career in the biotech/pharmaceutical industry (see Figure 6). The results of this survey were also highly positive, with 100% of graduates indicating that they would take the course again, while 93.8% indicated it was one of the top 5 classes they took (12.5% ranked it the best course they took as an undergrad). Approximately one third (31.3%) of the respondents also indicated that the course helped them to secure their current job (50% indicated that it might have helped). A large fraction of the respondents also indicated that the course helped them decide to pursue a career in biotechnology (37.5% responded “Yes”, 56.3% responded “To some degree”).

To determine if the course directly prepared students for their current positions, the last question on the survey asked students to indicate which of the techniques covered in class that they use in their current jobs (if any). Only 9 alumni completed this section of the survey, but out of those 9, almost all of them (8/9) indicated that they used animal cell culture techniques. Other frequently utilized techniques included bacterial cell culture (6 of 9 respondents), gel electrophoresis (6 of 9 respondents), and PCR (5 of 9 respondents).

These survey data show that the course does include techniques that are widely used by (bio)chemical engineering graduates. However, in an attempt to determine if additional techniques should be added to the course to better prepare our students, the survey also asked the participants to recommend additional techniques that they currently use in their jobs to add to the course. A wide variety of techniques were suggested, but the most common responses (submitted by at least two students) were flow cytometry, immunostaining, and qPCR. While immunostaining might be cost-prohibitive since antibodies are relatively expensive, flow cytometry and qPCR could be easily incorporated into the course, if the FACS and qPCR instruments are available.

Instructor Workload

For those interested in implementing this course, this section contains a brief description of the effort required by the instructor to prepare each lab. The first time this course is run, the instructor will spend a large amount of time generating all the plasmids and cell lines. However, the author is willing to share most of the materials, which can then be produced in large quantities and frozen until subsequent semesters to save time. Consequently, the largest amount of effort is involved in the labs that require live cells, since the instructor must inoculate and culture the cells days or weeks in advance. There are also several buffers and reagents that must be prepared fresh for some of the labs, so a teaching assistant would be very helpful, if available. Finally, the primary author is also willing to share an instructor’s guide that contains preparation instructions, order lists, and other helpful references to help instructors prepare for the course.

Budget/Costs

Compared to other chemical engineering labs, this course is relatively expensive to run, since it requires a variety of expensive enzymes, reagents, and instruments. Specifically, consumable costs typically vary from \$2,00-\$2,500 each semester and the initial capital costs for equipment (e.g. incubators, thermal cyclers, fluorescent microscope) may exceed \$50,000.

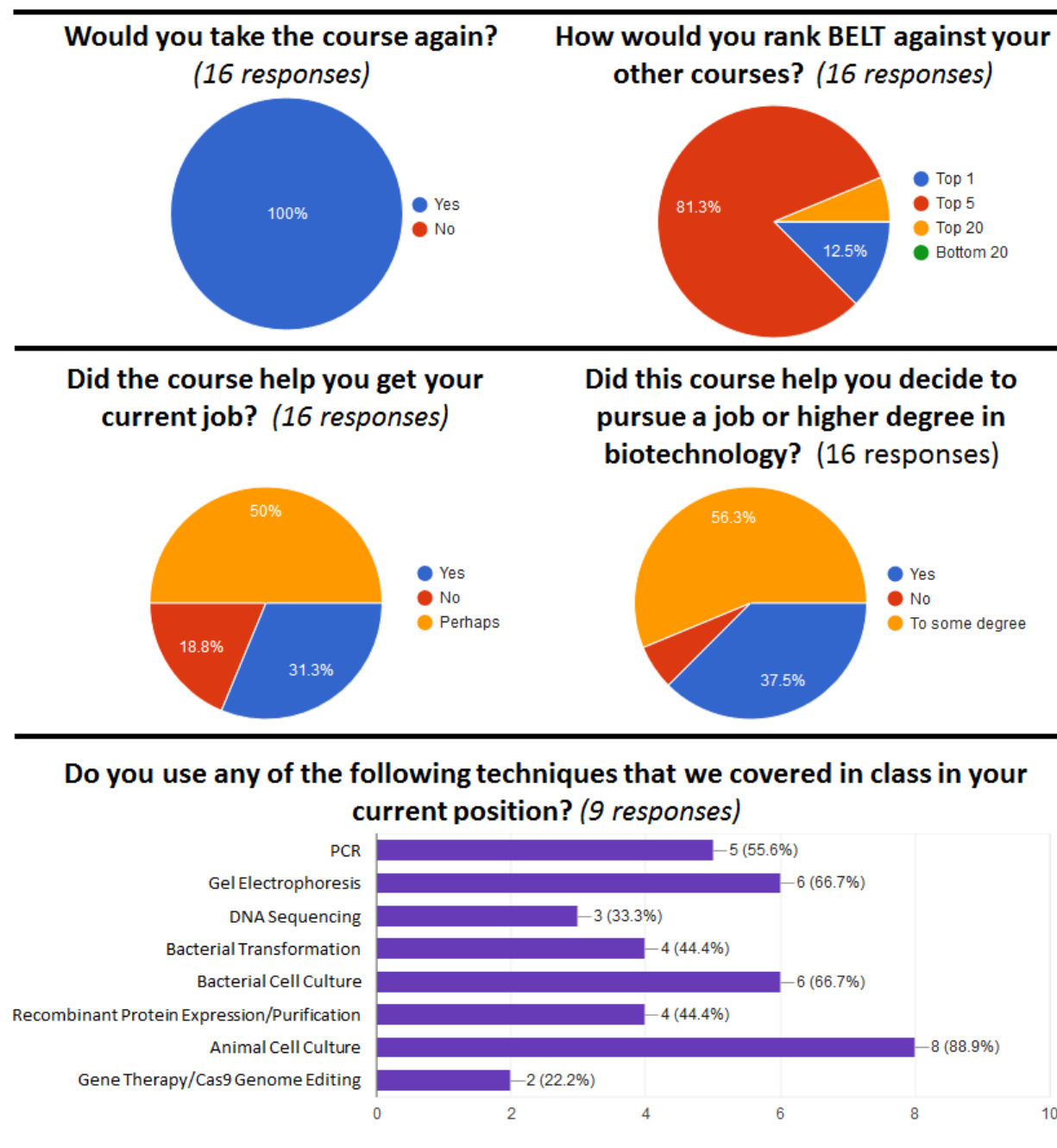


Figure 6 – Results of a survey administered to former students 1-2 years after graduation. The survey was submitted to a total of 22 graduates, but only 16 replied to the survey and only 9 completed the final question.

References

1. AIChE's Initial Placement Survey: Where is the Class of 2015? Available online: <https://www.aiche.org/resources/publications/cep/2015/december/cep-news-update/aiches-initial-placement-survey-where-class-2015> (accessed on Jan 1, 2018).
2. Collections/Uppsala Chromoproteins - parts.igem.org Available online: http://parts.igem.org/Collections/Uppsala_Chromoproteins (accessed on Jan 30, 2018).
3. Quan, J.; Tian, J. Circular Polymerase Extension Cloning of Complex Gene Libraries and Pathways. *PLoS One* **2009**, *4*, e6441, doi:10.1371/journal.pone.0006441.