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David A. Moffet

Loyola Marymount University, dmoffet@lmu.edu

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Laboratory Exercises

From Gene Mutation to Protein Characterization

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David A. Moffett‡

From the Department of Chemistry and Biochemistry, Loyola Marymount University, Los Angeles, California 90045

A seven-week “gene to protein” laboratory sequence is described for an undergraduate biochemistry laboratory course. Student pairs were given the task of introducing a point mutation of their choosing into the well studied protein, enhanced green fluorescent protein (EGFP). After conducting literature searches, each student group chose the mutation they wanted to introduce into EGFP. Students designed their sequence-specific mutagenic primers and constructed their desired mutation. The resulting EGFP mutant proteins were expressed in *E. coli*, purified and characterized. This laboratory sequence connected the major concepts of molecular biology and biochemistry, while incorporating the thrill of novel discovery in an undergraduate-level biochemistry laboratory course.

Keywords: Enhanced green fluorescent protein (EGFP), mutagenesis, protein purification, protein characterization.

Biochemistry and molecular biology laboratory courses are often compilations of individual experiments targeted to proceed for 1–3 week. Although short, individual experiments are necessary for exposing students to the many topics in biochemistry and molecular biology, it can be challenging for students to conceptually connect what they have learned from one lab to the next. Additionally, students are expected to connect concepts from their molecular biology courses to their biochemistry courses. In an effort to unite the concepts of molecular biology and biochemistry, a seven-week laboratory sequence was developed to progress from plasmid DNA purification, gene mutagenesis, and DNA sequencing, through recombinant protein expression, protein purification, and finally protein characterization. The goal of this laboratory project was to provide continuity between each laboratory segment, connect the major themes of the many individual laboratory units performed throughout the year, and simultaneously provide students with a sense of scientific discovery.

An important distinguishing feature of this project is the fact that the students are responsible for designing their own experiment. Although each group follows the same day-to-day protocol, the specific mutation being introduced to the protein, and therefore the experiment being performed, is unique for each student group. Because each group is following the same protocol, the instructor can easily manage many projects simultaneously. Because each student group designs their own

mutation, those students are empowered to conduct their own scientific experiment by producing a protein sequence that may have never before existed.

The “gene to protein” laboratory sequence could be performed with any protein, but we chose to use the well-studied enzyme, enhanced green fluorescent protein (EGFP) [1, 2]. Because of its natural green color, this member of the green fluorescent protein family is an ideal protein to use in an undergraduate laboratory setting [3–5]. The green colored protein allows students (i) to visually monitor the expression of the protein in a recombinant host such as *E. coli*, (ii) to watch in real time the whereabouts of the protein during the purification process, and (iii) to easily characterize the protein with standard equipment found in a typical undergraduate laboratory [6, 7].

To start the sequence, each student pair was assigned the task of constructing an EGFP mutant protein of their choosing. Students were introduced to literature and information search engines, such as Pubmed Central (www.pubmed.gov), Google Scholar (<http://scholar.google.com/>), and the ExPASy proteomics server (<http://ca.expasy.org/>) to become familiar with the fluorescent protein sequences currently known in the literature. The students were given the option of reproducing previously reported EGFP mutations or attempting to produce novel EGFP mutations. In both cases, students were required to submit a short proposal to 1) describe their reasoning for making the intended mutation(s), 2) suggest what effect(s) their intended mutation might have on the protein, and 3) give a detailed description of how they would proceed to make the proposed mutant. Students were enthusiastic about creating protein mutants that have potentially never before existed. Students seemed eager to produce EGFP mutants that would “outshine” those of

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‡To whom correspondence should be addressed. 1 LMU Drive, Los Angeles, California 90045, United States. Tel.: 310-338-4400 E-mail: dmoffet@lmu.edu.

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atggtgagcaagggcgaggagctgttcaccgggggtggtgcccacccctgggtcgagctggac
M V S K G E E L F T G V V P I L V E L D
ggcgacgtaaacggccacaagttcagcgtgtccggcgagggcgagggcgatgccacctac
G D V N G H K F S V S G E G E G D A T Y
ggcaagctgaccctgaagttcatctgcaccaccggcaagctgcccgtgccctggccccacc
G K L T L K F I C T T G K L P V P W P T
ctcgtgaccaccctgacctacggcgtgcagtgcttcagccgctaccccgaccacatgaag
L V T T L T Y G V Q C F S R Y P D H M K
cagcagcacttctcaagtcgcccatgccgaaggtacgtccaggagcaccacatcttc
Q H D F F K S A M P E G Y V Q E R T I F
ttcaaggacgacggcaactacaagaccgcggcggaggtgaagttcgagggcgacaccctg
F K D D G N Y K T R A E V K F E G D T L
gtgaaccgcatcgagctgaagggcctcagcttcaaggaggacggcaacatcctggggcac
V N R I E L K G I D F K E D G N I L G H
aagctggagtagaactacaacagccacaacgtctatcatggccgacaagcagaagaac
K L E Y N Y N S H N V Y I M A D K Q K N
ggcatcaaggtgaacttcaagatccgccacaacatcgaggacggcagcgtgcagctcgcc
G I K V N F K I R H N I E D G S V Q L A
gaccactaccagcagaacacccccatcgggcagcggccccgtgctgctgcccgcacaaccac
D H Y Q Q N T P I G D G P V L L P D N H
tacctgagcaccagtcgccctgagcaaaagaccccaacgagaagcgcgatcacatggtc
Y L S T Q S A L S K D P N E K R D H M V
ctgctggagttcgtgaccgcccgggatcactctcggcattggacgagctgtacaagtaa
L L E F V T A A G I T L G M D E L Y K -

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FIG. 1. DNA and protein sequence of EGFP (Source: Protein databank and CLONTECH laboratories).

their peers, leading to extensive library and literature searches.

Students designed their mutagenic primers following the QuikChange Mutagenesis protocol (Stratagene). This protocol is extremely robust, allowing the students to incorporate a wide variety of mutations into any given gene. The major constraint with this mutagenesis protocol is that the mutagenic primers should be shorter than 45 nucleotides. With the QuikChange protocol with primers shorter than 45 nucleotides, it is possible for students to produce single or multiple mutations with one pair of mutagenic primers. The mutagenic primers were commercially synthesized (Iterative DNA Technologies, Coralville, IA). Upon successful construction and identification of the intended EGFP mutant gene (through DNA sequencing), students over-expressed their mutant proteins in BL21(DE3) *E. coli* (Stratagene). The mutant proteins were purified using a Ni-NTA affinity chromatography column (Qiagen). Students characterized their mutant proteins for comparison to wild-type EGFP. Although there are many characterizations one could perform, we used SDS-PAGE, absorbance spectroscopy, and fluorescence spectroscopy to compare the different protein mutants [6].

TABLE I

Weekly schedule for gene mutation to protein characterization

Week 1	Purify plasmid containing wild-type EGFP and order mutagenic primers
Week 2	Use primers to produce mutant genes
Week 3	Transform mutant into expression bacteria
Week 4	Purify mutant DNA to send for DNA sequencing
Week 5	Induce overexpression of mutant protein
Week 6	Purify the mutant EGFP protein
Week 7	Characterize mutant protein

COURSE SCHEDULE AND EXPERIMENTAL DETAILS¹

Session 1: Purification of p28EGFP Plasmid

The overall time table for the seven-week laboratory sequence is presented in Table I. Students, working in pairs, were given a 4 mL sample of BL21(DE3) *E. coli* cells harboring the p28EGFP plasmid² Fig. 1. These cells express a small amount of the EGFP protein under normal growth conditions, producing cells with a slight green color. Student pairs purified the p28EGFP plasmid using a plasmid purification miniprep kit (Qiagen) and following the manufacturers protocol. The purified plasmid DNA was eluted with 50 μ L of deionized H₂O. To verify successful purification of the plasmid, students performed diagnostic restriction digestions, where a small amount of the purified plasmid was incubated with the restriction enzymes *Nde*I and *Eco*RI as described in

¹The detailed experimental protocols given to the students can be obtained by contacting David Moffet (dmoffet@lmu.edu).

²The EGFP gene, and other similar fluorescent proteins, is commercially available from Clontech Laboratories. The EGFP gene was PCR amplified using the primers 5'-GAA CTG GAC CAT ATG GTG AGC AAG GGC GAG GAG-3' and 5'-GTT ACG CTG GAA TTC TTA CTT GTA CAG CTC GTC CAT GCC-3' which produce an *Nde*I restriction site at the 5' end of the gene and an *Eco*RI site at the 3' end of the gene. The PCR product was doubly digested with *Nde*I and *Eco*RI restriction endonucleases (New England Biolabs) and ligated into an analogously digested pET28a plasmid (Novagen). This recombinant plasmid, p28EGFP, yields an EGFP protein with an N-terminal hexa-Histidine tag. Figure 1 shows the complete gene and protein sequence of this construct.

TABLE II
Double digestion conditions for p28EGFP

	No digest (μL)	Double digest (μL)
dl H ₂ O	21	19
Purified p28EGFP vector	6	6
10 \times EcoR buffer	3	3
EcoRI endonuclease	0	1
NdeI endonuclease	0	1

Table II. The digested plasmids were separated on a 1% agarose gel containing 126 nM ethidium bromide (prepared by the instructor) and visualized on a transilluminator. Plasmid samples showing a band at \sim 5.5 kb (the pET28 plasmid) and a second band at \sim 750 bp (the EGFP gene) were kept for mutagenesis. Students performed all of the described work for this week, with the exceptions of preparing the overnight cultures of *E. coli* and the agarose gel (with ethidium bromide).

Student pairs were responsible for submitting their mutagenic primer sequences to the instructor. These primer sequences were sent electronically to IDT for synthesis. The synthetic primers were typically received within 48 hour of ordering.

Equipment List Session 1—Miniprep kit (Qiagen or Promega), restriction enzymes and DNA ladder (New England Biolabs or Promega), culture media (LB or 2xYT broth, Sigma), agarose gel containing ethidium bromide.

Session 2: Site-directed Mutagenesis

Students diluted their mutagenic primers with sterile H₂O to yield a final DNA concentration of 0.1 $\mu\text{g}/\mu\text{L}$. The mutagenic gene synthesis reagents and amplification conditions are described in Tables III and IV. After completing the gene synthesis reaction, students purified the mutated plasmid using a PCR clean-up kit (Qiagen). The students followed the directions of the manufacturer to purify their mutated plasmids, eluting their plasmid DNA with 30 μL dl H₂O. The purified mutated plasmid mix was stored at -20°C until the following session.

Equipment List Session 2—PCR reagents and thermocycler (New England Biolabs or Promega), PCR clean-up kit (Qiagen or Promega), mutagenic primers (Iterative DNA Technologies).

Session 3: Transformation of Mutated DNA into *E. coli*

The plasmid mix from the previous session was digested for 1 hour at 37°C with the restriction enzyme

TABLE III
Mutagenic gene synthesis reaction mixture

Reagent	Volume (μL)
Sterile deionized H ₂ O	37
10 \times Concentrated buffer	5
Purified p28EGFP	2
0.1 $\mu\text{g}/\mu\text{L}$ Forward primer	2
0.1 $\mu\text{g}/\mu\text{L}$ Reverse primer	2
10 mM dNTP	1
<i>Pfu</i> Ultra DNA polymerase	1

TABLE IV
Thermocycler conditions

# Cycles	Temp ($^\circ\text{C}$)	Time (min)
1	95	0.5
16	95	0.5
	55	1
	68	4.5
1	4	>2

Dpn I. This restriction enzyme selectively digests methylated parental DNA but leaves the mutated, nonmethylated DNA uncut. This digest was performed by adding 3.3 μL of the 10 \times concentrated digestion buffer (buffer 4 from New England Biolabs) and 1 μL of Dpn I restriction endonuclease (New England Biolabs) to the PCR product from the previous session. The digestion mixture was incubated at 37°C for 1 hour. Following the digestion, the plasmid DNA was purified using a Qiagen PCR clean-up kit and eluted with 30 μL dl H₂O. The students transformed their mutated plasmid DNA into electrocompetent *E. coli* (although chemically competent *E. coli* can also be used) [8]. This was performed by mixing 8 μL of the mutant plasmid DNA with 50 μL of electrocompetent BL21(DE3) *E. coli*. Immediately after pulsing the cells with 2.50 kV of electricity, the cells were rescued with prewarmed SOC media and incubated with shaking for one hour. The transformants were spread on LB plates containing kanamycin (the selective antibiotic for pET 28a plasmid) and grown overnight at 37°C . The following morning, plates containing colonies were stored at 4°C until the following session.

Equipment List Session 3—Dpn I restriction enzyme (Promega or New England Biolabs), competent cells (Stratagene), kanamycin (VWR), transporator.

Session 4: Purification of Mutated DNA for Sequencing

The day before lab, students selected three colonies from their transformation plates (from Session 3) and inoculated each of those colonies into a separate 4-mL LB media culture tube. Those cultures were grown overnight at 37°C in the presence of kanamycin antibiotic.

During the lab session, students prepared glycerol frozen stocks of each sample for permanent low-temperature storage. The frozen stocks were prepared by mixing 300 μL of sterile glycerol with 500 μL of each culture in a sterile cryo-vial. Those frozen glycerol stocks were labeled and stored at -80°C .

The remainder of each overnight culture was pelleted and the plasmid DNA was miniprep-purified (following the instructions of the manufacturer Qiagen). To ensure proper purification of plasmid DNA, each plasmid sample was digested as described in Table II and characterized on a 1% agarose gel containing 126 nM ethidium bromide (prepared by the instructor). The purified DNA samples known to contain plasmid DNA were sent for DNA sequencing (Davis Sequencing Facility). The samples were sequenced using the T7 forward primer (supplied free of charge by Davis sequencing facility). Sequencing

results were e-mailed to the instructor approximately two business days after sending the samples. Samples found to possess the desired mutation were kept.

Equipment List Session 4—Plasmid mini-prep kit (Qiagen or Promega), sequencing (Davis Sequencing Facility or Keck Sequencing—Yale University).

Session 5: Overexpression of Mutated Proteins in E. coli

The day before lab, the *E. coli* clones containing the sequence-verified mutated EGFP plasmids were grown overnight in 4-mL LB broth cultures containing kanamycin. Approximately 90 min before the lab, the instructor added 1 mL of the overnight culture to a fresh culture flask containing 250 mL of sterile LB media and kanamycin. The new cultures were incubated at 37 °C with vigorous shaking. During the lab, students recorded the O.D.₆₀₀ of their cultures over time. Cells were grown to an O.D.₆₀₀ of 0.7 (typically 2.5 hr after inoculation of the 250 mL culture). Upon reaching the O.D.₆₀₀ of 0.7, the students performed two tasks: 1) they pelleted 1 mL of the culture for future SDS-PAGE analysis and 2) they added sterile isopropyl-beta-D-thiogalactopyranoside (IPTG) to the 250 mL culture flask to yield an in-culture IPTG concentration of 2 mM. Students monitored the progress of IPTG induction by pelleting and freezing 1 mL aliquots of the cell culture every 30 min after IPTG induction. These pellets were saved for SDS-PAGE analysis in session 7. Approximately 90 min after IPTG induction, students pelleted the entire cell culture at $6,000 \times g$ for 10 min and discarded the supernatant. The cell pellets were stored at -80 °C.

Equipment List Session 5—Liquid media (LB or 2xYT – VWR), IPTG (VWR or Sigma).

Session 6: Protein Purification

The mutated protein can be purified by a variety of methods. We used Ni-NTA (Qiagen) resin to bind to the N-terminal Histidine tag of the expressed proteins. The bacterial pellets from session 5 were thawed by the students and resuspended in 5 mL of buffer A (0.1 M phosphate buffer, pH 7.2, 5 mM β -mercaptoethanol, and 20 mM imidazole). The cells were lysed with either 1 mL of Bugbuster solution (Novagen) or sonication. The resulting suspension was spun at $13,000 \times g$ for 5 min to pellet the cellular debris. The supernatant, which contained the soluble EGFP protein, was bright green in color (but will depend on the color each mutant protein produces). The entire green protein solution was passed through a Ni-NTA (Qiagen) affinity column at a flow rate of 1 mL/min. This affinity column was packed with 1.5 mL of Qiagen Ni-NTA resin equilibrated with three consecutive washes of 5 mL of buffer A. Upon mixing of the Ni-NTA beads with EGFP, the beads became green in color, whereas the supernatant became colorless. The column was washed three times with additions of 5 mL of buffer A. The mutated EGFP protein was eluted with 3 mL of buffer B (0.1 M phosphate buffer, pH 7.2, 5 mM β -mercaptoethanol, and 200 mM imidazole) which contains

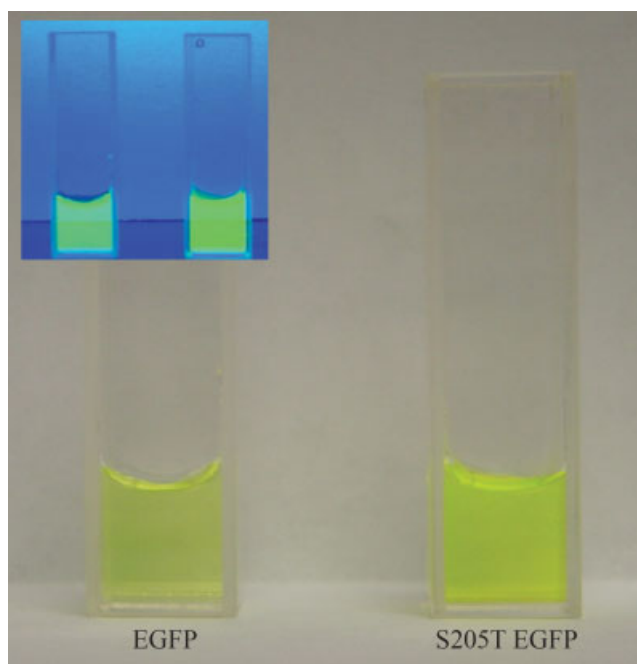


FIG. 2. **EGFP (left) and S205T EGFP (right) samples under white light.** Inset: EGFP and S205T illuminated using UV light.

a high concentration of imidazole buffer. The eluted protein solution, now a bright green color (Fig. 2), was stored at -80 °C.

Equipment List Session 6—Chromatography resin (Ni affinity resin Qiagen or Promega). Cell lysis solution (Bugbuster solution Novagen), sonicator.

Session 7: Protein Characterization

The students performed several characterizations on their mutant proteins. Each group ran an SDS-PAGE gel to show the IPTG induction and purification profiles of their protein. Figure 3 shows an SDS-PAGE gel obtained

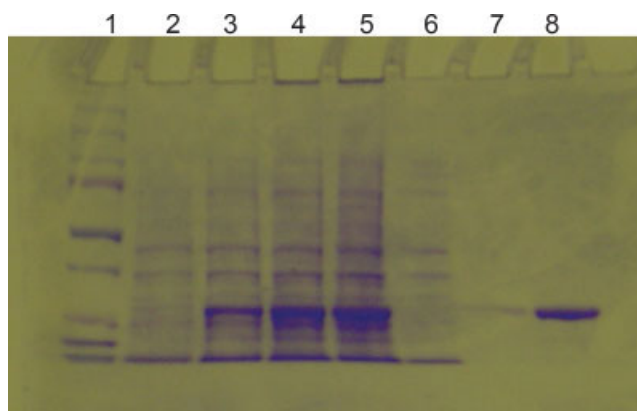


FIG. 3. **SDS-PAGE gel showing the IPTG-induced protein expression profile and the protein purification profile.** Lane 1: Precision plus protein standards (BIO-RAD). Lane 2: Cells before IPTG induction. Lane 3: Cells 30 min after IPTG induction. Lane 4: Cells 60 min after IPTG induction. Lane 5: Cells 90 min after IPTG induction. Lane 6: Ni-NTA flow through. Lane 7: Ni-NTA wash. Lane 8: Purified mutant protein. The pelleted cells from the IPTG time course (Lanes 2–5) were resuspended in 100 μ L of $\times 2$ loading buffer, were boiled for 5 min and 10 μ L loaded onto the 8% SDS-PAGE gel.

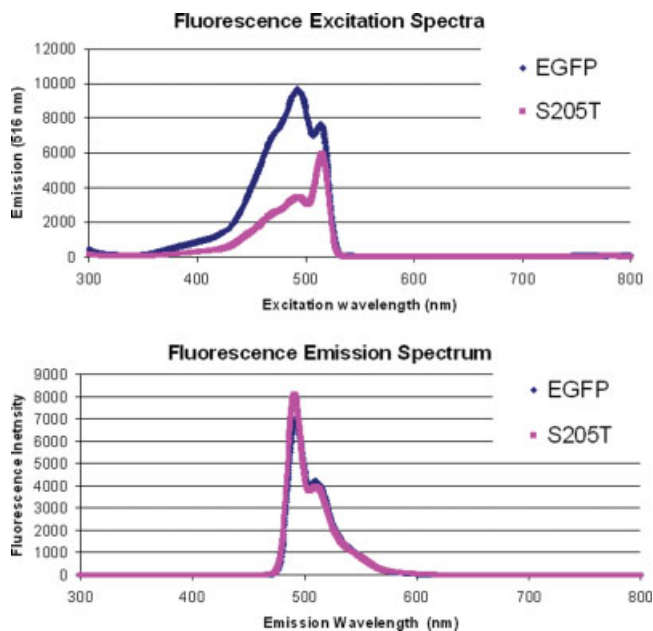


FIG. 4. Absorbance and fluorescence spectra of S205T EGFP. The emission spectra were recorded using an excitation wavelength of 490 nm. The excitation spectra were recorded by monitoring the emission at 516 nm.

by one student pair. To determine the concentration of their purified protein, each pair performed the Bradford assay [9,10]. For the final characterization, each student pair recorded the absorbance and fluorescence spectra for their protein, Fig. 4.

RESULTS AND DISCUSSION

Multiweek project-oriented courses should be popular additions to biochemistry and molecular biology laboratory courses [11]. These multiweek laboratory projects provide both procedural and conceptual continuity from one topic to the next. This continuity can be especially helpful for students who are expected to connect concepts from both biology and chemistry laboratory curricula. The gene to protein laboratory sequence described here provided the students with a continuous series of experiments from the purification of DNA to the characterization of a protein. Although any gene/protein can be used for such a study, EGFP proved to be an ideal system. EGFP is water soluble, expresses very well in *E. coli*, and has a bright green color that is easily monitored during the purification process.

One of the goals of a successful laboratory course is to prepare students for continued laboratory work in industrial, governmental, and academic settings. Although there are obvious benefits associated with teaching students classic techniques in DNA manipulation and protein purification, we chose to emulate the laboratory conditions likely to be encountered by students at their next level of laboratory work. To that end, several kits and protocols commonly used in industrial and graduate-level academic laboratories were used. Students were introduced to the theory behind each kit and protocol during prelaboratory lectures.

The mutation scheme leads to three possible outcomes: 1) Students were unable to produce the intended mutations. This outcome was an occasional occurrence. This result was likely due to problems with the designed mutagenic primers, such as inadvertent formation of DNA hairpins. An inability to construct the target mutation typically occurred when students were overly ambitious with the number of mutations they were attempting to make. Although inserting a single mutation worked every time, attempting to make more than two mutations using exceptionally long mutagenic primers rarely succeeded. Students unable to produce their targeted mutation simply continued with the lab sequence by purifying and characterizing wild-type EGFP. 2) Students produced the desired mutation, but the mutant displayed a loss-of function phenotype. This occurred in several of the groups. Mutations far from the active site rarely disturbed the fluorescent phenotype of the protein. However, mutations to the active site amino acids consistently abolished fluorescence activity. Examples of mutants produced by students that displayed loss of function were Y66H, F165Q, and E222W. In these instances, students purified their targeted mutant protein even if that protein was colorless and lacking fluorescent properties. 3) Students successfully produced their targeted fluorescent mutants. This occurred in the majority of groups. These mutants continued to show fluorescent properties. Examples of novel mutants possessing fluorescent properties produced by students were S205T and Y74W. Many other mutations were performed on EGFP that were also fluorescent, yet many of these were similar to previously published mutations with Green Fluorescent Protein [12–14]. Although students often attempted to drastically alter the spectroscopic characteristics of EGFP (such as to produce a red fluorescent protein), we have not yet produced a variant with significantly altered fluorescent properties [15].

An important factor to consider with a lab curriculum such as this is the cost of reagents and kits. Table V lists the major costs associated with this laboratory sequence. This table lists the materials specific for performing the 7-week sequence with 20 students (10 student pairs). This table does not include common materials (such as media, dNTP's, agarose) or equipment (such

TABLE V
Cost analysis of the 7-week laboratory sequence

Product	Approximate cost	Total cost (per 10 student pairs)
Mutagenic primers	\$10 per primer	\$200
PCR clean-up kit (Qiagen)	\$91 for 50 samples	\$37
Miniprep kit (Qiagen)	\$77 for 50 samples	\$62
Dpn I restriction enzyme (NEB)	\$58 for 20 μ L	\$29
<i>pfu</i> DNA polymerase (stratagene)	\$100 for 40 μ L	\$25
DNA sequencing (Davis)	\$12.50/reaction	\$375
BL21(DE3) competent cells	\$141 per 1 mL	\$71
Total		\$799

as a PCR thermocycler, incubator, gel boxes, or heat block). All costs considered, this half-semester project can be performed by 20 students for under \$1,000.

Learning Assessment

This course was developed for junior and senior-level science majors. Pre-requisites or corequisites for this course included organic chemistry, biochemistry lecture, and cell biology lecture. Each weekly meeting consisted of a 50-min lecture followed by a 3-hour laboratory period. A total of six quizzes were given at the beginning of each of the final six lab periods. Each quiz covered lecture material and laboratory protocols performed during the previous laboratory session. These quizzes amounted to 20% of the student's total grade for the 7-week laboratory sequence.

Before starting the laboratory sequence, student pairs worked together to select a target point mutation. The students were required to search the literature to find articles describing GFP and EGFP mutagenesis. Each student pair collaborated to write a formal research proposal that was formatted like a typical grant proposal. The guidelines for their proposals were those used by most scientific grant agencies, requiring a background and significance section, a proposed mutation section, a discussion section, and a references cited section. The students were not required to give a detailed account of methods for their proposal (as they were not prepared to do so at this point of the laboratory sequence).

Upon completion of the project, each student group worked together to write a single formal laboratory report. This report was written as if it was to be submitted to the journal *Biochemistry* for review and publication. In addition to the sections required for the proposal, the students were required to include a materials and methods section, a detailed results section and a discussion section. All data collected by the students during the 7-week sequence was incorporated into the report and analyzed by each student pair.

At the conclusion of the semester, students were surveyed with an anonymous questionnaire for their assessment of the semester-long course (with specific questions targeting the 7-week laboratory sequence). When asked if they would recommend this course to a friend, 94% of the students said yes. On a scale of 1–5 (one being lowest and five being highest) when asked if the proposal and final report were relevant for their understanding of the material 85% scored a five. The “additional comments” section of the student evaluations also gave strong indications of their great enthusiasm for their independent projects.

This project provided undergraduate science majors with a laboratory sequence that connected the major concepts of molecular biology and biochemistry. Students proceeded through a 7-week laboratory sequence that began with DNA purification, progressed through DNA manipulations such as PCR, restriction digestions, and plasmid transformation, and ended with the expression, purification, and characterization of a mutant pro-

tein. Students were introduced to scientific writing as it pertains to preparing scientific proposals and manuscripts. The students left the course with a strong sense of what to expect in a typical research laboratory while simultaneously experiencing the thrill of novel scientific discovery.

Hazards

E. coli BL21(DE3) is a nonpathogenic strain of bacteria. However, all containers used to incubate *E. coli* should be sterilized before discarding. Ethidium bromide may be harmful by inhalation, ingestion, or skin absorption and is believed to act as a mutagen. Chaotropic salts used to purify plasmid DNA can be mild skin irritants. 2-Mercaptoethanol can be toxic if absorbed through the skin and is harmful if swallowed or inhaled. Methanol/acetic acid mixtures may be skin and eye irritants, are flammable, and are toxic.

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