

2008

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Recommended Citation

Muth, Gregory W., and Joseph W. Chihade., "A Streamlined Molecular Biology Module for Undergraduate Biochemistry Labs". *Biochemistry and Molecular Biology Education*. 2008, 363: 209-216. Available at: <https://doi.org/10.1002/bmb.20170>. Accessed via Faculty Work. Chemistry. *Carleton Digital Commons*. https://digitalcommons.carleton.edu/chem_faculty/3
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Laboratory Exercises

A Streamlined Molecular Biology Module for Undergraduate Biochemistry Labs

Received for publication, October 22, 2007, and in revised form, December 17, 2007

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Site-directed mutagenesis and other molecular biology techniques, including plasmid manipulation and restriction analysis, are commonly used tools in the biochemistry research laboratory. In redesigning our biochemistry lab curricula, we sought to integrate these techniques into a term-long, project-based course. In the module presented here, students use structural data to design a site-directed mutant and make the mutation using the Kunkel method. A second, silent mutant, that creates or removes a restriction site, is simultaneously introduced. Restriction digestion and agarose gel electrophoresis are used to assess the success of mutagenesis. Placing these procedures in the context of continuous, student-driven project serves to create a “research style” laboratory environment.

Keywords: Biochemistry, molecular biology, mutagenesis, undergraduate.

The potential advantages of an open-ended, project-based, laboratory curriculum for enhancing student learning are well established [1–4]. Such an approach is endorsed by the “BIO2010” review of science curricula [5] both as a way of providing students with exposure to realistic scientific questions and as an opportunity to introduce the inherent interdisciplinary nature of experimental science. In redesigning our respective biochemistry laboratory courses for junior and senior chemistry and biology majors at St. Olaf College and Carleton College to be project-based, we sought to create a curriculum that teaches fundamental laboratory techniques as part of a term-long investigative process in a research style environment. We define a “research style” experience as involving the generation of a clear and unique hypothesis, collecting data, and interpreting the data in a manner that either supports or refutes the model proposed. The process we arrived at is continuous, not only from week to week but also from year to year. Results generated in previous offerings of the course are openly discussed and the models generated from those results are used as the basis for new hypotheses.

An important component of the courses, we envisioned, was an introduction to fundamental techniques in molecular biology. Although these techniques are commonly used tools in biochemistry research, they are less frequently employed in undergraduate level biochemistry laboratory courses. Our goal was to provide an opportu-

nity in which the technical procedures of DNA manipulation, site-directed mutagenesis, restriction digestion, agarose gel electrophoresis, and bacterial transformation were pursued in an efficient manner and in a context in which students have significant ownership of experimental design and analysis of results. Perhaps more importantly, we wanted this molecular biology module to be an integral part of the term-long project.

The project-based courses that we devised involve the creation and characterization of student-designed site-directed mutants in an enzyme of interest, in our case *E. coli* cystathionine- β -lyase (CBL). The courses are built around three multiweek modules: 1) Site-directed mutagenesis, during which students design and create the mutant enzyme to be analyzed. 2) Isolation, during which students use affinity chromatography to purify their mutant and use standard SDS-PAGE and Bradford assays to gauge purity and yield. 3) Characterization, during which students use a colorimetric assay to determine Michaelis-Menten parameters for their mutants and compare these to values obtained for both the wildtype enzyme and mutants created by their classmates. The order in which the modules are executed can be varied. At St. Olaf, students work through the modules in the order presented above, thus they design the mutants that they characterize later in the term. At Carleton, in part to align the lab more closely with the associated lecture course, students begin by isolating and characterizing a mutant designed by students who took the course in previous years, and take their results into account in designing new mutants to be analyzed in subsequent years. The molecular biology module, as the portion of our laboratory courses that

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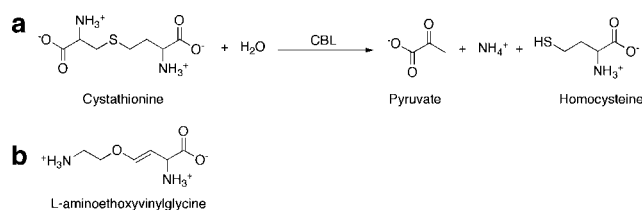


FIG. 1. **Reaction catalyzed by cystathionine β -lyase.** (a) Overall reaction. (b) Structure of L-aminoethoxyvinylglycine, a substrate based inhibitor of CBL.

differs most from a traditional biochemistry lab, is our focus in this article.

At the onset of the three laboratory periods comprising the molecular biology module, we begin by focusing on creating the mutation. Students analyze both the proposed mechanism of the enzyme-catalyzed reaction (Fig. 1a), a pyridoxal phosphate dependant β -elimination, and the crystal structure of the enzyme bound to a substrate-based inhibitor, L-aminoethoxyvinylglycine (Fig. 1b). In our case, this analysis includes access to related publications [6–8] as well as information generated by students in previous iterations of the course. With this information, each group of students must create and clearly state a hypothesis regarding the role of a particular residue in the catalytic process. Our goal is to create a situation where rather than answering a black-and-white “how does it work” question, students are required to utilize critical thinking skills and synthesize their own statement; “I think it works this way.”

For students to generate reasonable hypotheses, they of course need to have a reasonable level of comfort with enzymology. This background takes two main forms common to both our courses, though we provide this information in different ways. One important focus is on the chemical reaction catalyzed by the enzyme we are studying. Students are reminded of arrow pushing formalism and the applicability of tools learned in organic chemistry in the analysis of biochemical catalysis. In this context, students are also reminded of the theories of catalysis (acid–base, metal ion mediated, proximity, covalent catalysis). A second aspect of enzymology that is crucial to the students’ success is a familiarity with protein structure, especially the use molecular modeling software to visualize the enzyme in three dimensions and focus on particular attributes of amino acids in and around the enzymatic active site. A third exercise to increase student comfort with enzymology could be a pedagogical assignment in reading the primary literature related to CBL and similar PLP containing enzymes. This assignment could certainly bracket our proposed module, nicely complementing the technical attributes we currently cover.

Once each group of students has arrived at a mutation they will make, they are motivated to learn the molecular biology required in order to create it. Although the QuikChange[®] method of mutagenesis is more commonly used in modern research laboratories, and has been used by others [9] in teaching laboratories, we have chosen to use the older Kunkel method [10, 11], which we

feel has pedagogical advantages in addition to being more economical. In particular, we find that we can fit the individual steps of the Kunkel method – annealing, elongation-ligation, and bacterial transformation—into a single laboratory period in which students are actively involved. In contrast, the very time-consuming PCR step in the QuikChange[®] protocol necessitates work outside of scheduled lab time while simultaneously reducing students’ active participation. Use of the Kunkel method provides us the opportunity to discuss, from a biochemical perspective, the action of DNA polymerases, ligases, and repair enzymes. We also take advantage of an opportunity to introduce the use of mutant bacterial strains in molecular biology and biochemistry, in this case the repair deficient *dut*[−] *ung*[−] *E. coli* strain CJ236. Using the Kunkel method does not require the generation of a single-stranded template DNA (ssDNA). As all of the students are making mutations to the same parent plasmid, we have found it most efficient for the instructor to prepare the ssDNA outside of the scheduled lab time. In our case, a single preparation generated sufficient ssDNA for use at both colleges over the past several years.

The final portion of the molecular biology module involves verifying that the intended mutation has been created. We wanted to maximize the exposure to molecular biology concepts and methods in the course and to minimize both the costs and “hands-off” aspect associated with off-site sequencing. To achieve these goals, we have adopted another relatively “old-fashioned” yet hands-on technique. In addition to the codon-changing site-directed mutation, students also introduce a second, silent mutation to create or remove a restriction site alongside (or near by) the intended functional point mutant. This exercise provides another opportunity for student-driven experimental design; the lab groups must use bioinformatics software to locate potential sites for silent mutation and must weigh their potential options, taking into account variables including primer design, restriction site frequency, and restriction enzyme cost. In the final week of the module students then use restriction digestion and agarose gel electrophoresis to determine whether plasmid DNA from several bacterial cultures derived from the previous week’s work harbor the desired mutations based on *in silico* predictions and comparison to controls.

Previous reports [9, 4] have illustrated the learning value of an investigative laboratory experience utilizing site-directed mutagenesis. However, in these instances student enrollments were quite high, so the resources available limited the range of mutations investigated. Because we have fewer students in our laboratory sections, we have been able to expand the realm of our student-generated hypotheses and thus have expanded the number and type of potential mutants for functional analysis. While this approach harbors the potential for “naïve” hypotheses with potentially uninformative results, we have found that unusual choices can lead to some rather unique data sets and open up avenues for new models and interpretation. Restriction fragment analysis has also been used by others [12] whether as a stand-alone experiment or as part of a multiweek project. Presenting

this procedure in the context of site-directed mutagenesis helped us meet our goal of creating a continuous, “research-style” laboratory experience.

THE MODULE

Week 1—Structure Analysis and Mutant Design

The first week of the molecular biology module consists entirely work done on the computer. To maximize student comfort and efficiency during this session, we usually conduct it in a dedicated computer lab, rather than in the usual laboratory space. Internet access is required in order to access several of the sequence analysis tools used.

The first portion of the lab period is dedicated to using molecular modeling software to examine the structure of the inhibitor bound protein. With some prompting from the instructor and teaching assistants, students need to focus on regions of interest within the structure, most importantly the active site. Using the research article in which the crystal structure was originally described [6] and the results of previous mutagenesis experiments as a guide, the students relate the three dimensional positioning of residues to their potential roles in catalyzing the reaction. This process takes about an hour. The groups then work to develop their hypotheses and to propose mutations that will test them. In part because of our emphasis on the mechanism of this pyridoxal phosphate dependant enzyme, most students focus on the active site or proximal cofactor-binding site. In recent years we have attempted to widen the range of potential mutations by pointing out, mostly in individual conversations during the lab period, other interesting portions of the structure, in particular the dimer interface and comparisons with related pyridoxal phosphate-dependant enzymes that catalyze different reactions. In all cases, students must make and orally defend their hypotheses to the instructors before continuing. We do not allow multiple groups to propose the same mutation (although we do allow multiple mutations of the same residue). Students are similarly prohibited from proposing a mutation that has been made in a previous year.

There are several molecular-modeling packages available at no cost for use in academic settings, including RasMol [13], MDL Chime [14] and the Chime-dependant Protein Explorer [15], Jmol [16], and several Jmol-dependant tools, such as FirstGlance [17] and the Jena-Lib viewer [18], MBT Protein Workshop [19], UCSF Chimera [20], Pymol [21], and VMD [22]. The software features we have found to be most important include a straightforward means of highlighting or restricting the display to a subset of selected residues, as well as the ability to select residues within a given distance from a point of interest. Although all of the programs are functional, we found the University of Illinois’ VMD package [23] to have the best combination of features and usability. Students start the lab period already familiar with the features of VMD. At St. Olaf, this preparation involves a graded pre-lab exercise, while at Carleton, students have used the package to complete one or more problem sets in the accompanying lecture course. Not surprisingly,

many students tend to explore the program a bit more deeply and gain additional expertise as they use it to answer the open-ended question we pose.

After students have formulated a hypothesis, decided on a mutation to make, and discussed their plans with the instructor, they proceed to designing a primer. The major set of tools that we use in this process are part of the EMBOSS suite of software, which we access through one of the several web-based servers running the EMBOSS Explorer implementation [24]. We provide a set of instructions that outlines the use of particularly useful elements of the software package, as described below. Students begin with a text file containing the DNA sequence of the pET21b-based expression vector in which the CBL protein coding sequence has been highlighted. The expression vector as well as the sequence file is available upon request from either author. The EMBOSS “showseq” tool is used to produce an annotated translation in which the DNA sequence correlated to the amino acid sequence of the protein. Using this output, the students identify the codon corresponding to the residue they will vary to test their hypothesis and what changes in the DNA sequence are needed to accomplish the desired mutation.

Students use a different set of EMBOSS tools to accomplish the more challenging task of designing an appropriate silent mutation. The goal of this exercise is to introduce or remove a restriction site without changing the amino acid sequence of our protein. To determine which of the codons nearby their mutation are amenable to silent mutation, students select a portion of the plasmid sequence encompassing 9–12 nucleotides on either side of the functional mutation site. It is important that this input sequence includes changes to the codon of interest that create the functional mutation and that the input sequence begins in the correct reading frame. This sequence is inputted into the EMBOSS tools “silent,” to show potential sequence alterations that can result in the addition of a restriction site, or “recoder,” to show sequence alterations that can result in the removal of a restriction site. Output from the EMBOSS tools consists of tables that list all of the potential site changes that can be made to yield a silent mutation and the corresponding restriction enzymes (Fig. 2).

Students are then challenged with evaluating the tables to determine which silent mutation would be the most practical. They make their decisions based on three criteria. Most importantly, they must consider the number and position of pre-existing sites for the restriction enzyme in the plasmid sequence (determined using another EMBOSS tool, either “remap” or “restrict”). As an example, the enzyme AflIII cuts pET21b-CBL only one time (so the supercoiled circular DNA plasmid is cut to make a linear piece of DNA). So, adding an AflIII site would lead to a plasmid that is cut twice (leading to two pieces of linear DNA), which is easy to detect, and removing the AflIII site would lead to a plasmid that was not cut by this enzyme (remaining circular and supercoiled), and also easy to detect. On the other hand, the enzyme AclI cuts pET21b-CBL 94 times (leading to 93 pieces of linear DNA). Differentiating between 92, 93,

Start	End	Dir	EnzymeName	RS-Pattern	Base-Posn	AAs	Silent	Mutation
2	6	.	BthCI	GCNCG	6	G.G	Yes	G->C
2	6	.	Fnu4HI	GCNCG	6	G.G	Yes	G->C
4	9	.	MspB4I	GGYRCC	6	G.G	Yes	G->Y
2	6	.	Fsp4HI	GCNCG	6	G.G	Yes	G->C
5	8	.	RsaI	GTAC	6	G.G	Yes	G->T
5	8	.	PlaAII	GTAC	6	G.G	Yes	G->T
4	9	.	BspT107I	GGYRCC	6	G.G	Yes	G->Y
4	9	.	Aha88I	GGTACC	6	G.G	Yes	G->T
5	8	.	Csp6I	GTAC	6	G.G	Yes	G->T
4	9	.	PfaAI	GGYRCC	6	G.G	Yes	G->Y
5	8	.	CviQI	GTAC	6	G.G	Yes	G->T
5	8	.	CviRII	GTAC	6	G.G	Yes	G->T
4	9	.	Eco64I	GGYRCC	6	G.G	Yes	G->Y
5	8	.	AfaI	GTAC	6	G.G	Yes	G->T
2	6	.	TauI	GCSCG	6	G.G	Yes	G->C
2	6	.	FbrI	GCNCG	6	G.G	Yes	G->C
4	9	.	Asp718I	GGTACC	6	G.G	Yes	G->T
4	9	.	HglCI	GGYRCC	6	G.G	Yes	G->Y
4	9	.	HglHI	GGYRCC	6	G.G	Yes	G->Y
4	9	.	BanI	GGYRCC	6	G.G	Yes	G->Y
2	6	.	Uur960I	GCNCG	6	G.G	Yes	G->C
4	9	.	SthI	GGTACC	6	G.G	Yes	G->T
4	9	.	KpnI	GGTACC	6	G.G	Yes	G->T
5	8	.	HpyBI	GTAC	6	G.G	Yes	G->T
2	6	.	ItaI	GCNCG	6	G.G	Yes	G->C
4	9	.	Acc65I	GGTACC	6	G.G	Yes	G->T
4	9	.	BbvBI	GGYRCC	6	G.G	Yes	G->Y
4	9	.	BshNI	GGYRCC	6	G.G	Yes	G->Y
2	6	.	BsoFI	GCNCG	6	G.G	Yes	G->C
4	9	.	AccBII	GGYRCC	6	G.G	Yes	G->Y
2	6	.	Bsp6I	GCNCG	6	G.G	Yes	G->C
2	6	.	SatI	GCNCG	6	G.G	Yes	G->C
7	10	.	MaeII	ACGT	9	T.T	Yes	C->G
7	10	.	HpyCH4IV	ACGT	9	T.T	Yes	C->G
7	10	.	TscI	ACGT	9	T.T	Yes	C->G
7	10	.	Tsp49I	ACGT	9	T.T	Yes	C->G
7	10	.	TotI	ACGT	9	T.T	Yes	C->G
4	10	.	SamDI	GGWCCC	10	L.L	Yes	T->C
4	10	.	SseI82SI	GGWCCC	10	L.L	Yes	T->C
10	17	.	PacI	TTAATTAA	12	L.L	Yes	G->A

FIG. 2. Typical output from the EMBOSS Silent module. A portion of the output generated from a thirty nucleotide input sequence is presented. "Start" and "End" refer to the restriction site, using numbering from the input sequence. "Dir" refers to the direction in which the sequence is translated, with "." meaning the forward direction and "rev" the reverse. For a correctly inputted sequence, only the forward direction is relevant. The enzyme name and restriction pattern to be created are listed, along with the nucleotide position ("Base-Posn") to be mutated. The amino acid encoded before and after the nucleotide change is noted, and the silent nature of the mutation confirmed. In the final column, the particular nucleotide mutation to be made is indicated. Note that several possible restriction sites can often be created by the same mutation and that isoschizomers receive separate entries.

and 94 pieces of DNA would be very difficult, so adding or removing an Acil site is of little utility. Similarly, introducing a new restriction site less than 100 basepairs from an existing site for the same enzyme would typically result in a fragment too small to be practically detected by agarose gel electrophoresis. As they begin their search, students are reminded that the longer the DNA recognition sequence for a particular restriction enzyme, the higher the selectivity in the cleavage of plasmid. (Recognition sites are conveniently listed in the output tables from "silent" and "recoder" under the "RS-pattern" column.) This stage of the experimental design is usually quite challenging for the students, as it requires them to predict and compare potential experimental outcomes. A tool that helps students visualize restriction patterns is "NEB Cutter 2.0," found on the New England Biolabs homepage [25]. Using the "custom digest" and "view gel" options allows students to generate and compare *in silico* digests of wildtype and potential mutant plasmids to determine whether the resulting patterns will be sufficiently different to be diagnostic of successful mutagenesis.

The second criterion students must take into account is the proximity of the two point mutations to each other. In

our experience mutagenesis is most successful, and the primer needed is more affordable, when the two changes to the DNA sequence are close to each other. Finally, students are required to ascertain the availability and price of the enzyme. Once, the students have chosen an appropriate enzyme based on all three criteria, they go on to design an appropriate primer—taking into account the T_m , G/C content, directionality, and terminal nucleotides based on a set of specific guidelines we provide. At the end of the lab period, they communicate their experimental design to the instructor by completing a report form that shows the 20–40 nucleotide sequence of their primer, the changes they designed to create the functional and silent mutations, and the restriction enzyme needed to complete the analysis. Ordering the student-designed primers at a 25 nmol scale (the smallest scale that is commercially available) provides a more than sufficient excess of material for subsequent manipulations.

Week 2—Mutant Plasmid Synthesis and Bacterial Transformation

Prior to the second laboratory session in the module, the instructor or a technician must complete the first *in vivo* step of Kunkel mutagenesis by preparing the single-stranded, uracil-containing DNA template (ssDNA). This process involves the initial transformation of the expression plasmid into competent *E. coli* K12 CJ236 cells (NEB). We have been successful in generating single stranded DNA from the resulting transformants by following the manufacturer's protocol that accompanies the M13K07 helper phage provided by New England Biolabs. To obtain single stranded DNA, it is crucial that the expression plasmid contains an f1 origin of replication. This was one of our major motivations in using a pET-based expression system.

A second step that we carry out in advance of the laboratory session is the phosphorylation of each of the mutagenic primers. This procedure is straightforward, as we simply follow the protocol that accompanies T4 polynucleotide kinase purchased from NEB, and is performed outside of the lab only to save time. Another alternative might be to purchase 5' phosphorylated primers directly, but this would significantly increase costs.

Students are provided with single stranded DNA template and their phosphorylated primers upon arrival in lab. Kunkel mutagenesis is performed essentially as originally described [10]. Annealing is achieved by combining the ssDNA (1 μ g) and the 5' phosphorylated primer (10 pmol) in 1X PE1 buffer (20 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 50 mM NaCl, 1 mM dithiothreitol) to give a final reaction volume of 10 μ L. This mixture is heated to 85 °C for 5 minutes and allowed to cool slowly to room temperature. While the mixture is cooling the students prepare a second tube containing components of the extension/ligation reaction mixture: 1X T7 DNA polymerase buffer (20 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM dithiothreitol), 50 μ g/mL BSA, 1 mM ATP, 0.1 mM each dNTP. This tube is stored on ice until combined with the room temperature annealing reaction, followed by addition of 1 μ L

T7 DNA polymerase (10 units) and 1 μ L T4 DNA ligase (400 units). The reaction mix is incubated on ice for 5 minutes, at room temperature for 5 minutes then at 37 $^{\circ}$ C for 2 hours. This long incubation time provides a good opportunity to provide some more background on the mutagenesis method and site-directed mutagenesis methods in general.

Following incubation, aliquots of the extension/ligation reactions are used to transform competent *dut⁺ ung⁺ E. coli* cells either by electroporation or heat shock [26]. The transformed cells are plated (Luria broth-ampicillin, 50 μ g/mL) and incubated overnight at 37 $^{\circ}$ C. We typically remove the plates from the incubator ourselves, but if students are able to access the lab outside of class hours, they could perform this step. Because the presence of CpG and *dam* mediated methylation can significantly alter restriction patterns, we have begun to use the methylation deficient *E. coli* strains ER2925 (NEB) and SCS110 (Stratagene) in the transformation step. The plasmids isolated from cultures of these strains in the subsequent lab period can be digested by methylation sensitive restriction enzymes, significantly increasing the number of choices available for making a diagnostic silent mutation.

Week 3—Plasmid Isolation and Restriction Digest

At the beginning of the third lab period in the module, students are provided with pellets or liquid culture grown from up to six individual colonies selected from their transformed bacteria. The instructor or a technician prepares overnight cultures (5 mL, Luria broth-ampicillin, 50 μ g/mL) prior to the laboratory session. Again, students could do this step if they are given access to the lab on the previous day. Students use a standard miniprep DNA plasmid isolation kit (Qiagen or Promega) to isolate the plasmid DNA. Isolating a larger number of individual colonies reinforces the principle of comparison when analyzing the results of the subsequent gel, as well as increasing the likelihood of finding the desired mutation. Students also isolate the unmutated parent plasmid from a bacterial culture, which they will use for comparison in the subsequent digestion step.

Manufacturer's protocols should be followed whenever possible for restriction digests. A typical reaction recipe would combine 10 μ L miniprep DNA (\sim 2 μ g), 2 μ L 10 \times reaction buffer (specific for each enzyme), 0.5 μ L 100 \times bovine serum albumin (not required for all enzymes), water to a final volume of 20 μ L and 1 μ L restriction enzyme. The reactions are generally incubated at the specified temperature for 30–60 minutes. Following digestion, 4 μ L of 6 \times glycerol loading buffer (30% v/v glycerol, 0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol) is added to each reaction. About 10–20 μ L aliquots and a broad range marker are loaded into a 1% agarose gel. Electrophoresis proceeds for about 1 hour (depending on the gel apparatus used) in 1 \times TAE or 1 \times TBE buffer. Gels are stained with SybrGold or GelStar and visualized using a gel documentation system. Students generally do an initial interpretation of their results

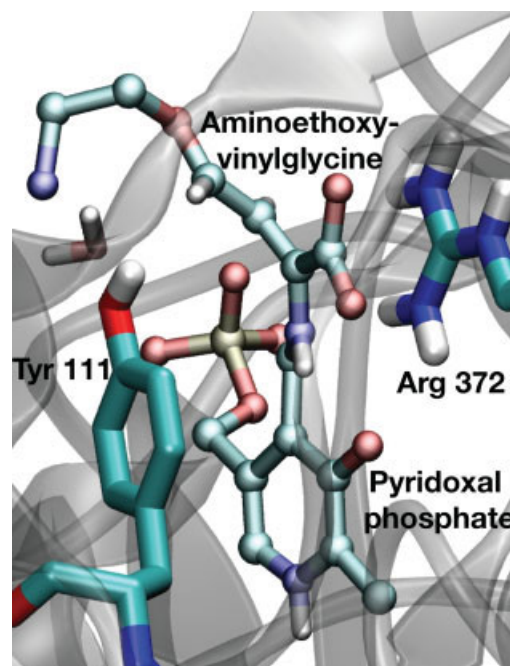


FIG. 3. Active site of cystathionine β -lyase bound to L-aminoethoxyvinylglycine. This view of the structure, PDB ID 1CL2 [7], shows the inhibitor covalently bound to the pyridoxal phosphate cofactor. Interactions of Tyr 111 and Arg 372 with the inhibitor are emphasized. (Figure was created using VMD [22].)

immediately, but are also required to do a more thorough analysis in a graded lab report in which they prepare a properly labeled figure as it may appear in a journal, including proper labels and a written caption. The report also includes a brief paragraph interpreting the information present in the figure. The writing assignment is graded using a rubric based on correct information (labels, values etc.), accurate and thorough comparisons, clear and concise writing and insight of interpretation.

RESULTS

The three-week, molecular biology module has been implemented in the biochemistry laboratory curriculum at Carleton College for three terms (Biological Chemistry Laboratory, taught once per year) and St. Olaf College for four terms (Experimental Biochemistry, taught twice per year). During this time, a total of 87 students have successfully created 18 point mutants of CBL. To illustrate the step-by-step process of primer design, restriction fragment prediction and agarose gel analysis, we present results generated by two different student lab groups.

A group of St. Olaf students (Chrissie Chow, Rachel Dyer and Larissa Nordstrom) in the Spring of 2006 wanted to mutate Tyr111 in CBL, which appears to interact with a terminal amine in the cystathionine substrate through a water-mediated hydrogen bond (Fig. 3). By changing this residue to a phenylalanine, the interaction would be removed, which the students hypothesized would result in weaker substrate binding and an increase in K_M . (In fact, subsequent experiments showed that k_{cat}

is significantly more affected by this mutation, providing a nice opportunity for students to reformulate their hypothesis in light of experimental data.) A portion of the DNA coding sequence flanking the relevant codon is presented below. The TAT codon for Y111 is underlined.

ACC AAC ACC GCC TAT GAA CCG AGT CAG GAT

Creating the Y111F functional mutation requires only a single A to T point mutation to make:

ACC AAC ACC GCC TTT GAA CCG AGT CAG GAT

Inputting this sequence into the EMBOSS “silent” module produces 162 potential silent mutation/restriction enzyme combinations. Many of these are due to isoschizomers or overlapping, so the actual number of unique silent mutations that are possible is only eight. Of these, only one, TTTGAA → TTCGAA would introduce a site for a six-base cutter (in this case BstBI and its isoschizomers). Rather than choosing to make that mutation, this particular group of students decided to introduce a change two codons away, converting the codon for Pro113 from CCG to CCT in order to create a restriction site for Bfal and its isoschizomers. The doubly mutated sequence would then be:

ACC AAC ACC GCC TTT GAA CCT AGT CAG GAT

Mutations are noted in bold and the new restriction site is italicized. Although we would normally discourage students from using a four-base cutter, in this case Bfal was a very reasonable choice. There are seven Bfal sites in the unmutated pET21b-CBL expression vector. Complete digestion yields fragments of lengths: 1,902, 1,481, 1,316, 1,104, 335, 253, and 161 bp. These fragments can be relatively easily resolved by agarose gel electrophoresis. More importantly, introducing another site at the Pro113 codon would cause a significant change in the restriction pattern, cutting the 1,316 bp fragment into 938 and 378 bp fragments. Giving students the opportunity to discover and take advantage of this sort of serendipitous arrangement of restriction sites is one of the benefits of student-driven nature of the module.

Kunkel mutagenesis in our system requires an oligonucleotide that is the reverse complement of the coding sequence, therefore the following primer was ordered:

5'-ATC CTG ACT AGG TTC AAA GGC GGT GTT GGT-3'

Students performed the mutagenesis reactions and used them to transform ER2925 cells, and several colonies were obtained. Overnight cultures were grown from four of these. Students isolated DNA from the cultures and digested the purified plasmids and an aliquot of unmutated pET21b-CBL with Bfal. The digestions were then separated on a 1% agarose gel. As shown in Fig. 4, DNA from one of the four colonies (Lane 5) showed the expected pattern for successful mutagenesis, lacking a 1,316 bp fragment, but exhibiting a band at 938 bp. In this case, several incomplete digestion products are visible, but their relatively weak intensity makes them distinguishable from the diagnostic bands. Having students think through the origin of these unexpected bands can present a nice teaching opportunity. Subsequent sequencing of the plasmid with an altered restriction pattern confirmed the presence of both the silent mutation and the functional mutation (data not shown).

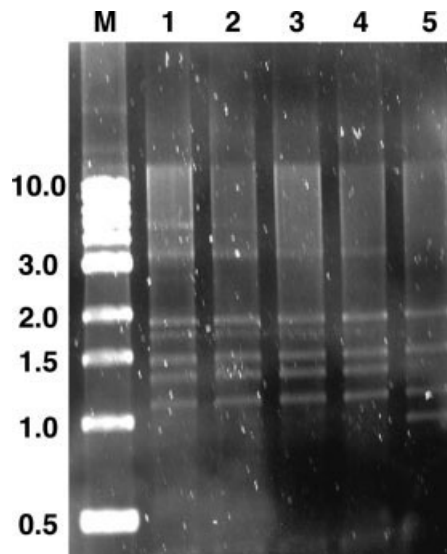


FIG. 4. Agarose gel of Bfal digested plasmid DNA. M: 1kb DNA ladder, Lane 1 parent pET21b-CBL plasmid (no intended mutation), Lanes 2–5 plasmid DNA isolated from colonies transformed with mutant DNA.

A second example of a student-selected mutant is the conversion of Arg372 to Lys as designed by St. Olaf students Tony Hoff and Andrew Keay in the spring of 2007. Arg 372 forms a salt bridge with the α -carboxyl group of cystathionine (Fig. 3). These students wondered whether the precise positioning of this interaction would be important, and decided to substitute a different positively charged amino acid to probe the flexibility of this interaction. The portion of CBL coding sequence flanking the R372 codon is shown below:

AGC GGG ACC TTG ATT CGC CTG CAT ATT GGT CTG

The R372K functional mutation requires substitution of all three codon nucleotides. The students could use either an AAA or AAG lysine codon. Using AAA gives the sequence:

AGC GGG ACC TTG ATT **AAA** CTG CAT ATT GGT CTG

Inputting this sequence into “silent” yields 173 potential silent mutation/restriction site combinations, with seven distinct mutations. There are two possibilities for introducing a six-base cutter (SthI or BclI and their isoschizomers) site, in addition to potential sites for seven- (SanDI) and eight-base (PacI) cutters. Using the criterion that the silent mutation should be as close as possible to the functional one, the students opted to introduce a BclI site. Thus the sequence they wanted to create was:

AGC GGG ACC TTG **ATC** AAA CTG CAT ATT GGT CTG

Mutations are noted in bold and the new restriction site is italicized. The DNA primer ordered, corresponding to the reverse-complement of this sequence, was:

5'-CAG ACC AAT ATG CAG TTT GAT CAA GGT CCC GCT-3'

Again, site-directed mutagenesis was performed, and several of the resulting colonies were used to grow overnight cultures producing purified plasmid DNA. The unmutated pET21b-CBL contains only one BclI site, so

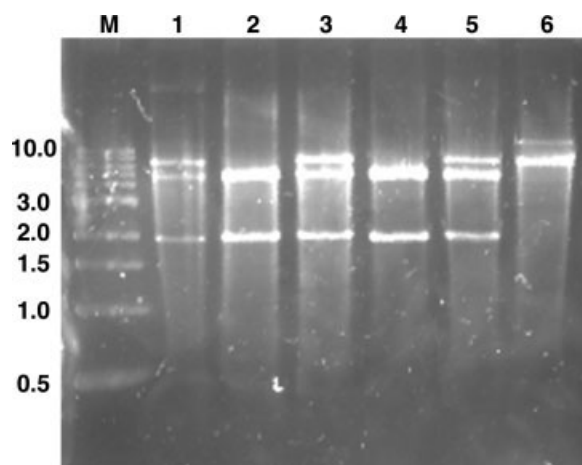


FIG. 5. Agarose gel of *BclI* digested plasmid DNA. M: 1kb DNA ladder, Lanes 1–5 plasmid DNA isolated from colonies transformed with mutant DNA, Lane 6: parent pET21b-CBL plasmid (no intended mutation).

digestion results in a single linear 6,552 bp fragment. Introduction of the second site results in a two-band digestion pattern, with fragments of 4,600 and 1,952 bp. Figure 5 illustrates the students' results for the restriction digest analysis for plasmid DNA isolated from five different cultures. Lane 6 shows the expected band of linearized pET21b-CBL plasmid. While Lanes 2 and 4 show the expected digestion pattern for the designed mutant with an additional *BclI* site, Lanes 1, 3 and 5 show a combination of both patterns, suggesting either incomplete digestion or that these cultures contained a mixture of mutated and unmutated plasmid. Although it might have been of interest to differentiate between these possibilities, the group decided (as most researchers would) to simply use a plasmid preparation that gave the clearest results. Thus, the DNA from Lane 2 was subsequently sequenced and clearly showed the presence of both the silent mutant and the desired functional mutant (data not shown).

STUDENT REACTION

Although there is some frustration expressed during the course, when students realize that there is not necessarily one "right answer" or they realize that the instructor may have as little data as they do about a particular issue, overall student reaction has been positive. Because we have tended to conduct course evaluations at the end of the term, it is difficult to disaggregate reaction to the molecular biology module from reaction to the course as a whole. In general, students are very appreciative of the continuous nature of the experiments over the course of a term. Some typical student comments include: "I really liked following one project the entire term, and I like the continuation over several years." "My favorite thing was designing the mutation using bioinformatics tools and pictures of the active site of CBL...I loved doing a term long project."; "I liked the lab, especially the independence we had to do our experiments and design the mutation."; and "This was the most extensive lab that I taken at Carleton. I enjoyed the

carryover between labs, as the overall lab section focused on one problem."

We have been most pleased by our ability to replicate a research environment. Those students who have gone on to do further research enthusiastically report that the overall design of the laboratory and the skills utilized during the molecular biology module are immediately transferable and that they are as prepared to interpret data and troubleshoot experiments as many of the graduate students they encounter.

DISCUSSION

While we have had success using CBL as our enzyme of interest, we feel the molecular biology module we have presented here is adaptable to any enzyme or functional protein that is amenable to a biochemical assay. The only constraints are: 1) the protein has been cloned into an expression vector suitable preparation of ssDNA. (This preparatory step involves relatively straightforward cloning and could easily be accomplished on site.) 2) the protein's crystal structure has been solved and is available and 3) there is some basis for formulating testable hypotheses connecting structure and function, whether that involves the arrow pushing mechanism of an enzyme catalyzed reaction, the requirements for ligand binding, the quaternary structure of the protein, or any other measurable property.

To fit this module into the context of a continuous investigative semester-long project, it is, of course, also important to consider the purification methods that will be used to isolate expressed protein and the assays that will be used to determine its functional properties. We intentionally chose to use metal affinity chromatography with a His₆-tagged protein and chose an enzyme for which a simple colorimetric assay was available in an attempt to avoid potential technical difficulties.

A final issue we considered was the biological relevance of the enzyme chosen with respect to student interest. Cystathionine β -lyase is part of the activated methyl cycle and methionine biosynthesis in bacteria. In introducing the lab, we compare this cycle to the mammalian system, particularly to its relevance in clinical cases where patients have developed arteriosclerotic clots. Genetic or dietary deficiencies that lead to an inefficient methionine pathway can result in elevated homocysteine levels and a corresponding increase in reactive oxygen species [27]. This connection appeals to many of our students with postgraduate biomedical aspirations.

The number and relative complexity of the tasks to be completed during the first week of the module can seem daunting, but in general we have found that the students have been able to work efficiently and with complete the lab with relatively little difficulty. A key element in this success seems to be working in teams of two or three students. In both of our courses, students arrive with rather uneven backgrounds. At St. Olaf, approximately equal mixture of biology and chemistry majors are enrolled in the course. At Carleton, enrollment in the lab consists mainly of chemistry majors. Some of these have considerable background in biology, having taken an

introductory genetics course and in some cases a molecular biology seminar, while a few others have taken no college level biology courses. We have tried to capitalize on this mixture of backgrounds by treating the lab as an opportunity for students to help their peers with the disciplinary concepts where they have the most strength. Those with strong chemistry backgrounds tend to be better at developing mechanism-based hypotheses, whereas those with more biology experience are better at the mechanics of creating the mutant and looking for appropriate restriction sites. We like to think that having a mixture of students working together to formulate a hypothesis allows students to hone communication skills in addition to producing stronger work. The largest bottleneck we have encountered during the first lab period of the module is the desire of some students to seek “the right answer” or a single mutation that will illuminate all of the catalytic mechanistic details in one experiment. We have tried to use this bottleneck as a vehicle to informally discuss how multiple pieces of data are needed in order to formulate and support models.

Expense is, of course, a major consideration in adopting a new laboratory curriculum. Because each group of students designs and orders both a DNA oligonucleotide and may need a unique restriction enzyme, the module does come at a relatively significant expense for larger sections of the course. However, the level of competition among suppliers of custom oligonucleotides has significantly lowered prices. We typically obtain primers for \$8–12 each. Purchasing the smallest available amount—usually for \$50–70—can contain restriction enzymes prices. Because students typically choose to make mutations in the same regions of the protein, one restriction enzyme will often serve several different groups, significantly decreasing the number of distinct enzymes that need to be purchased. Finally, if stored properly, restriction enzymes can be used for several years. Keeping a detailed inventory of those enzymes that are already on hand is another important way to reduce costs.

A final area of concern is the success rate of the site-directed mutagenesis process. Others have noted that mutagenesis failures can be high in novice student hands [9]. In fact, although very efficient transformations and relatively low occurrences of parent plasmid contamination have been reported with Kunkel mutagenesis [10, 11], in our students’ work we have observed quite a range of efficiencies and contaminations. In some cases, all isolated plasmids contained the desired mutation, while in others, none did. In several cases, no colonies were obtained upon transformation of competent cells with the mutagenesis reaction mix. The results described in the results section above, in which some, but not all of the isolated plasmids contain the desired mutant, and some may contain mixtures of mutant and wildtype DNA, are most typical. Not having a definite explanation for any particular result presents the opportunity for students to interpret their gels, provide plausible explanations in their writing assignments and offer possible troubleshooting schemes if they were to repeat the experiments, which they sometimes do. By not having a completely

“clean” result, we feel we are more accurately illustrating a research style environment where results can be somewhat ambiguous and require careful consideration. Because multiple groups are participating in the experiment, students who do not obtain their desired mutation are typically invited to join up with another group for the remainder of the module. In general, students have succeeded in obtaining the desired mutants frequently enough that the overall morale of the class has not been significantly affected.

Acknowledgments—We acknowledge all of the St. Olaf and Carleton students who bravely participated in these courses in 2005–2007. We also acknowledge able technical assistance from Brian Mars and Julie Karg (Carleton) and Michelle Howe (St. Olaf).

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