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Undergraduate research in a biotechnology teaching laboratory: expression and purification of a tyrosine kinase

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The class consisted of senior molecular biology majors who had previously taken cell biology, microbiology, biochemistry and molecular biology lecture courses but who had little or no previous lab experience. These students were asked to design and create an expression vector and purify the expressed protein. This project provides the students with the opportunity to appreciate interconnections between experiments while learning the necessary techniques. *Journal of Industrial Microbiology & Biotechnology* (2000) **24,** 359–363.

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Introduction

The first goal of any biotechnology laboratory course is to teach students the required techniques of the field. The instructor should allow the students to work with the standard equipment of the molecular research laboratory and become familiar with commonly used protocols. In addition, given that certain techniques are more easily mastered by students than others and that some techniques depend on previously learned skills, a teaching laboratory should present the laboratories in an appropriate manner. A complete teaching laboratory includes other learning requirements, such as the ability to integrate multiple experiments and the ability to predict which experiments are necessary for a given problem [4,11]. Unfortunately, many laboratory manuals divide protocols and techniques into individual laboratories and do not discuss connections between the experiments or emphasize the importance of completing one experiment successfully before the next step in a process can begin. In addition to portraying a false view of laboratory research, this approach fails to prepare students fully for a career in biotechnology. Our goal has been to provide a laboratory that teaches the necessary techniques in the appropriate order and which teaches the necessity of connecting experiments and the intellectual challenges encountered in biotechnology research.

RET is a receptor tyrosine kinase that consists of an extracellular domain, a single transmembrane domain and a consensus cytoplasmic tyrosine kinase domain [14]. *ret* was originally identified as an oncogene in a transfection assay used to isolate potential oncogenes from a transformed cell [12]. Although the original identification of *ret* as an oncogene was due to an artifact in the assay, the receptor was eventually identified as a potential oncogene in many papillary tumors and as the gene responsible for familial medullary thyroid carcinoma (FMTC), multiple endocrine neoplasia (MEN) 2A and MEN 2B [3,6,7]. FMTC and MEN 2A are caused by a mutation in the cysteine-rich region of the extracellular domain which causes constitutive dimers [1]. MEN 2B is caused by a mutation at amino acid 918 of the cytoplasmic domain [10]. The mechanism by which the MEN 2B mutation alters RET function is not known. Inactivating mutations throughout the *ret* gene have been shown to cause Hirschsprung's disease, a disease state characterized by loss of enervation of the lower intestinal tract [8,9]. In normal cells, RET functions as a receptor for glial cell line-derived neurotrophic factor (GDNF), a molecule implicated as a treatment for Parkinson's disease [13,15,16]. Further research on the kinase, specifically the analysis of RET kinase activity, will help in understanding the role of RET in these disease states.

In this project, the students were asked to express and purify the cytoplasmic domain of RET in bacteria beginning with a mammalian expression vector containing the full length *ret* gene, and the bacterial expression vector pGexKG [5]. The students were required to create a new plasmid, purify this plasmid, amplify DNA, grow bacteria, express the *ret* gene, purify the *ret* protein and analyze expression of the protein product. The goal of this project was to make the laboratory flexible, topical and interesting to the students from a biomedical perspective. The following research project has been performed in our molecular biology laboratory and can be performed in other molecular biology teaching laboratories. The research project will be presented as it was performed although some possible modifications will be presented. All plasmids and antibodies used in the following laboratory are available from the author.

Materials and methods

Laboratory organization

This project was conducted in the upper division molecular biology course at Montclair State University, Biol 434 and the graduate molecular biology course Biol 548. Biol 434 is a 3-credit semester course that meets 4 h per lab session, twice a week. Requirements for the course include cell biology, microbiology, genetics, biochemistry and molecular lecture. This course is offered primarily for molecular

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biology majors although biochemistry and biology majors also enroll. Biol 548 meets twice a week, 3 h per lab session, and requires a molecular biology lecture course. The laboratory experience of the students varies but few of the students have had significant laboratory experience beyond lower division lab classes.

Production of stored competent cells

DH5αF′ and BL21/DE3 (Novagen, Madison, WI, USA) cells were grown overnight in Luria Broth (LB), shaking cultures at 37°C. Bacterial cultures were diluted and grown to an OD A_{600} of 0.3. The bacteria were then centrifuged for 5 min at 5000 × *g* and the pellet resuspended in 1/5 volume competent cell buffer (60 mM $CaCl_2$, 15% glycerol, 10 mM PIPES, pH 7.4). Cells were kept on ice for 30 min before spinning them again for 5 min at 5000 × *g*. Pelleted bacteria were resuspended in 1/10 volume competent cells buffer, aliquoted and stored at –80°C for the duration of the semester. Competence was determined by transformation with 1 μg of pGexKG plasmid [5]. Cells were checked for contamination by transforming bacteria in the absence of plasmid and plating the bacteria on LB-amp plates (LB + 50 μg ml^{-1} ampicillin).

Amplification of RET DNA

Two sets of primers were used for polymerase chain reaction (PCR) amplification of *ret* (Accession Nos X12949, X15262) for preparation of the GST-RET fusion protein. The 5′ primer corresponding to nucleotides 1973–1989 of human *ret* (Primer 1: GGTAGTCGACACTGCTACC ACAAGTTT) was paired with a 3′ primer corresponding to nucleotides 3327–3345 (Primer 2: GTGAAAGC TTTTAACTATCAAACGTGTCC) for amplification of the DNA coding for the complete cytoplasmic domain of human RET [14]. The 5′ primer corresponding to nucleotides 2176–2193 (Primer 3: CGTCGAATTCCGCTT GGAAAAACTCTAGGA) was paired with the 3′ primer corresponding to nucleotides 3026–3042 (Primer 4: GAGGCTCGAGCTAGTCTCTCCTCTTAACCA) for amplification of the DNA coding for the kinase domain of human RET. Human full-length RET in the pCDNA3 mammalian expression vector (Stratagene, La Jolla, CA, USA) was used as a template for both PCR reactions. Reaction conditions for both PCR reactions were 35 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min using a Perkin Elmer Gene Amp 2400 PCR machine. Control PCR reactions in the absence of template were also performed and appropriate amplification was detected by agarose gel electrophoresis and ethidium bromide staining. The PCR reactions were performed using 10 ng of each primer, 1 ng of template (pCDNA3-RET [16]), 1× PCR buffer (Promega, Madison, WI, USA), 1.5 mM $MgCl_2$, 0.2 mM dNTP (Promega), and 5 units of Taq polymerase (Promega) in a 50-μl reaction.

Construction of the GST-RET construct

Five microliters of the PCR product and 1 μl (1 mg ml^{-1}) of the pGex-KG [5] were digested with *Sal*I and *Hind*III (product from primers 1 and 2) or *Eco*RI and *Xho*I (product from primers 3 and 4). The pGexKG vector was then incubated with calf intestinal phosphatase (Promega) for 15 min at room temperature. Both the digested vector and digested product were run on a 0.8% low melting agarose gel to purify the appropriate DNA fragments and the appropriate bonds isolated from the gel. Agarose gel slices were then heated to 65°C. The melted agarose slices containing the digested vector (1 μl) and amplified DNA (6 μl) were ligated in a 50-μl reaction and transformed into DH5αR′ cells.

Expression and purification of GST-RET fusion proteins

GST-RET was expressed in BL21/DE3 cells as described previously [5] with some modifications. Specifically, 500 ml of a culture bacteria containing the GST-RET vector were grown to an A_{600} of 0.6 at which point expression of GST-RET was induced in the presence of 100 μm IPTG. Bacteria were grown for an additional 2 h at which point the bacteria were centrifuged for 5 min at 5000 × *g*. The bacteria were resuspended in lysis buffer (10 mM tris, pH 7.5, 50 mM NaCl and 2 mM DTT), sonicated for three 30-s bursts (Ultrasonics Cell Disruptor, Plainview, NY, USA) and then centrifuged for 30 min at 10 000 × *g* in an SS34 rotor. The supernatant was added to a new tube and mixed with tween-20 (Sigma) to a concentration of 0.05%. Bacterial extract was incubated with glutathione agarose beads (Sigma, St Louis, MO, USA) for 30 min at 4°C. The beads were washed with wash buffer (lysis buffer + 0.05% Tween-20) and GST-RET protein was eluted in elution buffer (wash buffer + 2 mM glutathione). The glutathione eluate was run on a 10% SDS-PAGE gel [2] and stained with Coomassie. Western blot [2] using anti-RET antibody [16] was used to detect the presence of the RET protein.

Results

Introduction to the laboratory

At the beginning of the semester, students were divided into groups. Groups were organized by a mixture of student preference and experience as determined by a questionnaire on the first day of the lab. Students were asked to provide a quick summary of previous molecular lab experience, if any. Roughly one quarter of the students had previous experience outside of the university or through independent research within the university. Each group was assigned micropipets and freezer and refrigerator space. Students were presented with the general research project: The class was introduced to *ret* as a gene that codes for a receptor tyrosine kinase responsible for four different clinical diseases when mutated. Their assigned goal was to express and purify the cytoplasmic domain of the receptor in the hope of further understanding the enzymes function. The students were also told that the kinase had been expressed previously but that the expression was poor and the purification incomplete.

After presenting the overall goal of the project, students were asked to make the solutions required for the experiments. Although students might be expected to know the basics of media preparation and making solutions from previous classes, these skills were deemed sufficiently important to warrant reinforcement. Therefore, our technical laboratory began with discussing the required solutions and the methods used to make these solutions. Individual

groups were assigned specific solutions to be made including LB, LB-agar and LB-amp. Measurements for all solutions were approved by the instructor.

After making media, another basic requirement of a biotechnology teaching laboratory is the use of aseptic techniques and maintaining bacterial cultures. Although some institutions, including ours, require a microbiology course before taking the biotechnology laboratory, bacterial manipulation was also felt to be an essential component of modern molecular laboratories that should be reinforced early in the laboratory course. Students were provided with a protocol, given a plate of DH5αF′ cells and asked to make competent cells. The cells were prepared in a manner that would allow them to be frozen at –80°C for use throughout the semester (see Methods).

Plasmid isolation and analysis

In the next set of experiments, students transformed competent DH5αF′ bacteria with pGexKG vector [5] or a plasmid containing the full-length *ret* gene that had been isolated previously. Students were taught how to transform bacteria, test the viability and quality of competent cells, and transform a plasmid to be used in later experiments. All four groups produced useful competent cells and obtained the expected results with the bacterial transformation. Specifically, transformation with the provided plasmid produced over 100 colonies per plate whereas the control transformation procedure without plasmid produced no colonies.

Students recorded their results from the bacterial transformation and proceeded to isolate the plasmid DNA from the appropriate bacteria and analyse the isolated plasmids. The students learned how to purify plasmid DNA, the theory behind a plasmid preparation and the practical aspects of restriction enzymes and restriction digests. The mini-prep and restriction digest were performed at this point because of the critical role of these techniques in molecular biology. This experiment also proved useful in either increasing the student's confidence in laboratory research or demonstrating serious problems in learning the procedures. A standard mini-prep protocol [2] was provided using basic lysis of DNA and phenol/chloroform purification of the plasmid DNA. Introduction of a commercial kit (Promega) was used later in the semester. Students analyzed the digested plasmid using agarose gel electrophoresis [2]. They determined the predicted fragment sizes and compared the expected sizes with the actual results. The importance of a DNA ladder was explained and the groups were asked to determine the exact sizes of the fragments by graphing the results and comparing the digested fragments to a standard curve derived from known fragments [2] (Gibco-BRL, Rockville, MD, USA, 1-kb ladder). Once it was determined that the correct plasmid had been isolated, a large-scale culture of the bacteria was grown and a large-scale preparation of the plasmid was completed by each group. The students purified DNA using an affinity chromatography column (Qiagen, Valencia, CA, USA) in part because of its convenience and partly because of time constraints that limited the ability to perform a CsCl purification.

After the plasmid DNA had been purified, students were asked to determine the amount of DNA isolated and the purity of the DNA using A_{260}/A_{280} spectrophotometry readings. Each group determined the amount of DNA produced and then digested the purified *ret* DNA and a plasmid in which the *ret* gene would be inserted. Although this project was not required for the later experiments in which the appropriate region of *ret* would be amplified by PCR, it was believed that performing these experiments more than once and performing these experiments relatively early in the semester would be beneficial. The groups isolated the relevant fragments from a low melting agarose gel [2] in preparation for ligation.

Once the appropriate DNA fragments were isolated, these fragments were ligated and transformed into the competent cells produced previously. This experiment ran into some unforeseen problems both with the isolation of DNA fragments (inability to see their DNA on the gel) and ligation of the isolated DNA (no increase in the number of colonies in the vector + insert samples in comparison to the bacteria transformed with digested vector alone). Since a RET construct had been successfully transformed into bacteria previously in the laboratory, it was concluded that the difficulty in the experiment involved improper ligation. As previously noted however, the experiment was not essential to continue the research project and was used simply to allow the students to become familiar with these techniques. The previously isolated pCDNA*ret* plasmid was used to continue the research project.

DNA amplification and plasmid construction

Due to the significance of computers in the modern biotechnology laboratory, it was felt that students should become familiar with the process of DNA analysis, alignments and sequence searches. Also, because of the importance of DNA amplification in modern biotechnology, the theory behind primer design and the analysis of amplified DNA was incorporated into the class. Although new techniques, these experiments were placed early in the course due to the simplicity of the experiments. Students were introduced to the capabilities available on the computer, including NCBI, BLAST, and the MacVector sequence analysis program. They were then asked to design PCR primers that would be used for the PCR reaction. They were instructed to design primers that would produce a PCR product with the following constraints: (1) the PCR product must be digested by the appropriate enzymes; (2) the digested PCR product must be in frame with the GST gene when placed into the pGexKG sequence; (3) primers should have equivalent melting temperatures; (4) each group must amplify either the entire cytoplasmic domain of RET or the kinase domain alone. Students were asked to design the primers themselves although assistance was provided when necessary.

Although it would have been beneficial at this point to allow each group to order their designed primers, delays in ordering and the expense of ordering made it necessary to use previously-synthesized primers. Each group performed a PCR reaction with these primers using their own purified plasmid as a template and analyzed the results by running the PCR samples on an agarose gel. All of the students were successful in obtaining a PCR fragment although not

all groups produced a PCR fragment of the appropriate size. Once the PCR product was obtained and analyzed, it was ligated into the pGEMT vector (Promega). The ligation reaction was transformed into DH5αF′ cells, the appropriate bacteria grown and the purified plasmid analyzed. Although the pGEMT vector was expected to contain the portion of the *ret* gene coding for the cytoplasmic domain, none of the groups obtained the expected results. Thus, a plasmid previously produced that contained a gene coding for glutathione S-transferase was provided for future experiments.

Expression and purification of GST-RET

Although protein expression is not taught in traditional molecular biology teaching laboratories at Montclair State University, it was felt that the common use of bacterial protein expression in molecular research was sufficient reason to include this section in the course. Students transformed the GST-RET product into BL21 cells. The bacteria were grown in LB-amp and lysed using sonication. The extract was clarified by centrifugation and the extract incubated with glutathione agarose beads [5]. GST protein was then eluted with glutathione and the product run on an SDS-PAGE gel. The acrylamide gel was stained with Coomassie blue but the expressed protein could not be detected. Analysis of the control samples also run on the gel demonstrated that the GST-RET protein could not be seen in the crude extract or in the supernatant isolated from the cell extract. The students concluded correctly that their protein was either not expressed or was expressed weakly. In an attempt to determine if RET was expressed at all, more of the purified protein (and the original extract) was loaded onto an SDS-PAGE gel and a Western blot [2] was performed using anti-RET antibodies. The students learned how to analyze protein expression using gel electrophoresis and antibodies specific for their protein. Because no RET standard was loaded onto the gel, it could not be determined if there was a lack of protein expression or if there were mistakes in performing the Western blot.

Discussion

Upper division molecular biology laboratories need to incorporate the concepts of research as well as teaching techniques required in a biotechnology laboratory. Although the required techniques are taught, this project allowed the students to work through an entire research project from beginning to end. The goal of this laboratory course is to teach students the connection between experiments and, within the limits of the course, the necessity of successfully completing one experiment before the next experiment could begin. This project was also connected to significant topics in biotechnology research including cell signaling in normal and defective cells.

This research laboratory project has been performed twice. With this experience, it has been determined that maintaining flexibility while completing the required laboratories takes time, forethought and experience. Our classes routinely perform competent cell production, DNA isolation and DNA amplification with few problems. The digestion and ligation reactions used to produce a DNA construct were performed with varying degrees of success. Finally, expression and purification of a protein product were difficult for students unfamiliar with these techniques. However, as is true with research, sometimes making mistakes in the experiments can also teach about the research being performed.

It should be noted that the laboratory was divided into four groups, all of which were performing the same set of experiments. Thus, failure of one group to succeed would not delay the class considerably. Although performing the same experiments by multiple groups does not correlate with standard research practice in biotechnology, some compromises were essential in order to satisfy the necessary requirements of a semester course. A further consideration is the time-frame in which these experiments were performed. Our present schedule of two 4-h meetings per week proved sufficient for most experiments. However, some experiments benefited from work performed outside the lab period.

Although this course covers many of the techniques required in biotechnology, the course is not comprehensive. Experience in tissue culture, DNA sequencing, Southern blots and Northern blots are all important skills. These experiments have not been incorporated into this course either due to time constraints, expense, or the ability for students to learn these techniques in another course offered by the university. However, any or all of these techniques can be incorporated into this research project. For example, a genomic Southern blot of RET, a Northern blot of RET expression in tissue or in expressed cells, expression of RET in mammalian cells and sequencing of the amplified RET gene would all strengthen the scientific study of RET and benefit the course.

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