

Cloning and Expressing the Cat Insulin Gene as a Course-Based Research Project in an Introductory Biology Course

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In this course-based undergraduate research experience (CURE) for an introductory biology course, students learn concepts of gene expression and practice techniques in molecular biology as they examine the research problem of cloning and expressing the cat insulin gene in *E. coli*. Over the course of several weeks, students design a DNA sequence for synthesis that is optimized for expression in *E. coli*, amplify the synthesized DNA, clone PCR products into a vector, screen colonies for inserts, and analyze DNA sequences of clones. Assessment of this experience indicated a positive impact on students.

Keywords: CURE, protein expression, cloning, molecular biology, insulin

Introduction

Recent reports on improving biology education in higher education have advocated for implementing genuine research experiences within courses early in the undergraduate career (American Society for the Advancement of Science, 2011; National Academies of Sciences, Engineering, and Medicine, 2015). In response to these reports, numerous course-based undergraduate research experiences (CUREs) have been developed (Small World Initiative, Prevalence of Antibiotic-resistance in Environment, Authentic Research Experience in Microbiology) and implemented in laboratory components of courses worldwide. CUREs are differentiated from inquiry-based laboratory exercises in that the research problem is of interest to the larger scientific community (Auchincloss, 2014). CUREs have been shown to be beneficial to students as students have the opportunity to be scientists and practice data collection, analysis, and interpretation. CUREs positively affect student attitudes towards science, confidence in science and technical skills, and the ability to think like scientists (Auchincloss, 2014, Bascom-Slack, 2012, Lopatto, 2004, Shortlidge, 2016). Because the research project is integrated into a course, more students are impacted than in usual apprentice-type research opportunities. We sought to develop a CURE to replace our traditional laboratory in our cell structure and function course (a course in the general biology sequence required for first-year biology

majors). Because enrollments in our course include science, engineering, and math majors, we chose to study an applied biological research problem: cloning and expressing the cat insulin gene. This project enabled students to practice techniques encountered in the traditional course but in the context of a research problem. Our specific learning outcomes for this research experience are:

1. Perform technical skills in cellular and molecular biology (aseptic technique, PCR, transformation, gel electrophoresis, NCBI database search)
2. Explain how DNA-encoded information becomes expressed as protein
3. Differentiate between bacterial and eukaryotic gene expression
4. Describe the process of science and its challenges
5. Identify sources for information on experiments and differentiate primary sources of information from other sources.

Background on Insulin

Felis catus, the domestic cat, is currently prescribed a human form of insulin to treat diabetes mellitus. Human insulin differs in primary amino acid sequence, raising the question of specificity. *In vivo*, insulin synthesis involves the processing of a precursor protein: preproinsulin. Preproinsulin contains a signal

sequence that directs the protein to the RER and facilitates the threading of the protein through the ER membranes into the lumen of the ER. The ER signal peptidase cleaves the signal peptide from proinsulin producing an intermediate protein: proinsulin. Proinsulin contains 3 domains: B, C, and A. Endopeptidases in the ER cleave the C domain from the B and A domains. Functional insulin consists of the B and A domains connected through disulfide bridges.

Human insulin was first cloned and expressed in *E. coli* by separately cloning synthetic genes for B and A domains of insulin (Goeddel, 1979). Upon purification of the B and A domains from *E. coli*, the B and A domains were mixed together, and spontaneous di-sulfide bond formation occurred upon oxidation by air. Another approach to insulin expression in *E. coli* involved cloning the entire proinsulin cDNA (Chan, 1981). Functional insulin can be produced by allowing air oxidation and then treatment *in vitro* with endopeptidases to remove the C domain (Heath, 1992).

Expression of a eukaryotic gene in *E. coli* involves a few challenges. First, insulin synthesis involves translation of a precursor by ribosomes of the RER and subsequent processing of the precursor using enzymes found in the ER. Bacteria lack organelles. Second, eukaryotes have introns within their genes that require splicing machinery to remove them during RNA processing. *E. coli* does not have the ability to perform splicing. Third, although the genetic code is universal, distinct species vary in preference for particular codons to use for a given amino acid. Last, translation in bacteria and eukaryotes differs. In eukaryotes, ribosomes bind the 5' end of mRNA and scan to the first start codon to initiate translation. In bacteria, ribosomes bind internally near the initiation site at a sequence known as the Shine-Dalgarno sequence. Because synthesized DNA can be purchased a manufacturer, students can avoid the multiple hurdles to addressing these challenges *in vivo* by carefully designing a DNA sequence to use for cloning into a vector for expression in *E. coli*. Synthesis of a DNA sequence also eliminates the need to use a cat or cat cells in the experiment.

Implementation of Project

We have implemented this research project in the laboratory component of a cell structure and function course, a 100-level course. Our course follows a 10-week schedule with weekly laboratory periods of 150 minutes. In the first 3 weeks, students use the NCBI database and BLAST to find the cat insulin, align the cat insulin gene and proinsulin protein with the human insulin gene and proinsulin, and then annotate the cat gene and the proinsulin protein. After examining each of the challenges

to expressing cat insulin in *E. coli*, students propose an approach for cloning cat insulin (often similar to those seen in the literature), their DNA sequence (or sequences) designs, and DNA primers for PCR amplification for use in cloning a cat insulin-producing gene in *E. coli*. After ordering the student-generated DNA sequences, we take a one-week hiatus from the project to provide ample time for the synthesis of DNA sequences. During this week, we usually revert to a traditional lab to support course concepts. During weeks five through nine, students perform PCR, gel electrophoresis, cloning using a TOPO-TA cloning vector, transformation of plasmid into *E. coli*, colony PCR screening, mini-preparation of plasmid DNA from *E. coli*, restriction mapping of recombinant plasmids, and sequencing analysis. Week 10 is a buffer week in case we need to repeat an aspect of the research. Weekly pre-labs enable students to practice looking for scientific information and using the information to prepare an experimental design.

Students are challenged through this research as they need to learn about the gene expression and the differences in gene expression in eukaryotes versus bacteria prior to the introduction of the content in the lecture portion of the course. Students are assisted in developing an experimental design by examining primary literature on the expression of human insulin in *E. coli* and through discussions with peers and the instructor.

For instructors, the preparation time for the laboratory is comparable to preparing for traditional laboratory exercises as similar molecular techniques are included. For the same reason, the cost of the research project is similar to our traditional laboratory.

Student Outline Cat Insulin Project

Week 1: Introduction

Research Experiences for Undergraduate Biology Education

Recent reports from scientific societies have advocated for renovating biology education. The American Association for the Advancement of Science 2011 report “Vision and Change in Undergraduate Biology Education” advocated for a complete overhaul of biology education by focusing on “core competencies and disciplinary practice” in biology and incorporating authentic research experiences into the curriculum. A more recent report “Integrating Discovery-Based Research into the Undergraduate Curriculum” published by the National Academies of Sciences, Engineering, and Medicine in 2015 touted the numerous benefits of undergraduate research and recommended incorporating true research experiences within courses early in the curriculum. In this course, we are providing you the opportunity to perform research on a project to isolate and clone the gene for cat insulin for its expression in *E. coli*.

Case Study

Fog, a 15-year old male grey tiger-striped cat is brought to the veterinarian by his family. In the past few months his family has noted that Fog has lost considerable weight (despite being hungry all the time) and is having difficulty jumping. Usually, he can jump quite high (from the kitchen island to the top of the refrigerator) but he is unable to summon the strength any more. His family also states that he might be producing more urine than normal but that it is difficult to tell because sometimes the dog uses the cat’s litter box. The veterinarian orders blood tests. Fog’s tests reveal high glucose levels in his blood. The veterinarian diagnoses Fog as having diabetes mellitus and prescribes insulin and a diet of dietetic cat food (high in protein). Fog is fed the dietetic cat food twice a day and 1.25 units of insulin is administered after each feeding. After a month, Fog is gaining weight and his strength is returning. A year later, Fog is normal weight and strength and his diabetes is being managed through food and insulin.

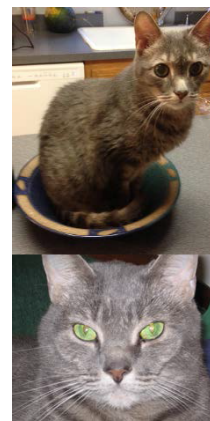


Figure 1. Fog the cat was diagnosed with diabetes.

Diabetes

Diabetes mellitus is a disease resulting from the failure of the body to regulate levels of the sugar glucose found in the blood. Regulation of most of the body’s functions is controlled by hormones synthesized and secreted by organs of the endocrine system. Hormones are cell signaling molecules that are secreted into extracellular fluids and bind to cell receptors to signal cells to elicit a given response. The amount of glucose in the blood is regulated by two hormones produced by the pancreas: insulin and glucagon. These hormones work antagonistically against one another. Insulin signals cells to remove glucose from the blood and store glucose in long chains called glycogen. Glucagon signals cells to break down glycogen and release glucose to the bloodstream. When fasting, the blood glucose level falls below the normal range of 65-135 mg/dL blood (in cats) and the α cells of the pancreas secrete glucagon, promoting glucose stores to be released into the bloodstream. After a meal, blood glucose levels rise above the normal range and the β cells of the pancreas secrete insulin which acts to lower blood glucose levels by directing cells to convert recently absorbed glucose molecules to glycogen stores. In insulin-dependent diabetes mellitus or IDDM (also known as Type I diabetes mellitus), β -cells fail to synthesize and secrete functional insulin. IDDM is both an endocrine disease and an autoimmune disease as β cells are destroyed by the body’s own immune system. In cats, one half to three fourths of diabetic cats have IDDM. Non-insulin-dependent diabetes mellitus or NIDDM (also known as Type II diabetes mellitus) involves cells failing to recognize insulin or becoming resistant to insulin. IDDM can be treated with insulin whereas NIDDM is usually managed through changes in diet and exercise.

Question

In the case study, Fog lost weight and muscle strength. Knowing that he had diabetes, explain why this happened.

Insulin and the Treatment of Diabetes in Cats

Charles Best and Frederick Banting were involved in the discovery of insulin and were the first to use insulin extracted from a dog pancreas to treat a boy with diabetes in 1921. For years, human IDDM was treated using insulin extracted from the pancreas of pigs or cows until the human version was cloned and expressed in *E. coli*. Humulin (human insulin made through recombinant DNA technology) was approved by the FDA for use in 1982. Functional insulin involves two polypeptides A and B. In humans, the two polypeptides are synthesized from one precursor protein, proinsulin, which is cleaved into two separate

polypeptides which are associated with one another through disulfide bridges. The first insulin pharmaceutical developed expressed the A and B chains individually and then united the polypeptides later in pharmaceutical production. In 1986, human proinsulin was cloned and expressed in *E. coli*. The proinsulin is then cleaved into A and B chains by an enzyme. Diabetes in the common house cat (*Felis catus*) is treated using a human form of insulin, protamine zinc insulin (PZI U-40).

Google to find research abstracts on human insulin as a drug before answering the questions.

Questions

What were the advantages of using a “cloned” human insulin versus insulin extracted from another species in treating human diabetes? What advantages would exist for treating cat diabetes with “cloned” cat insulin?

Pre-lab for Week 2

Investigate how human insulin was cloned and expressed. To start, go to chapter 17 of your OpenStax text to learn about recombinant DNA techniques. Then, do some research on the internet. Use at least 3 sources available over the internet. Find at least one peer-reviewed scientific article on the cloning and expression of human insulin. Discuss your findings with at least one other BIO 110 student to brainstorm how to best go about cloning and expressing the gene for cat insulin.

- ❖ Peer-reviewed means that the article comes from a science journal that requires other scientists to review and approve its publication. These articles are considered primary literature as they are the original source of information.

Pre-lab Submission

How would you go about cloning and expressing the genes for cat insulin? Outline the steps required and provide a list of references used in preparing your experimental design. Please submit your pre-lab submission to the Moodle submission box by next lab period at 8am.

Week 2: Finding and Annotating the Cat Insulin Gene

Discussion of Pre-Lab Assignment with Lab Team

In your lab team, take approximately 10 minutes to discuss and compare your experimental designs. Were they similar? In what ways? Did anyone have a unique approach? After this discussion, are there changes you wish to make to your experimental design? In your pre-lab assignment, you were asked to outline the steps necessary to clone the gene and express insulin. What did you indicate as your first step?

Your first step probably was related to identifying the sequence for the cat insulin gene. Let's start with learning how to find a gene.

Using NCBI to Find the Insulin Gene

The National Center for Biotechnology Information (NCBI) (available at www.ncbi.nlm.nih.gov) is a site where researchers can find and access numerous databases and tools for molecular analyses. From the NCBI homepage, you can choose an item from dropdown box to utilize a specific database. Clicking the "Learn" button on the homepage will direct you to resources (tutorials, instructions, FAQs, etc.) to help you use the different databases.

Searching for Scientific Articles Regarding Insulin

If you wanted to search for citations and abstracts from scientific articles related to the biomedical field, you would choose PubMed in the dropdown box. Enter your search terms and hit "search".

Question

Search PubMed for some articles related to cloning insulin or cat insulin. Did you find any that may be helpful for this project? **Copy and paste at least one useful abstract from PubMed into a document for submission.**

Finding the Sequence of the Insulin Gene

The NCBI houses databanks of DNA, RNA, and protein sequences. You can search for sequences by name or use specific molecular data (from sequencing). To find a specific gene, you probably want to use the "gene" database. It focuses on specific genes only. Choose "gene" on the homepage dropdown box and enter your search terms.

Question

What search term did you choose first? Was this helpful in finding the cat insulin gene? Why or why not?

*It is helpful to include the scientific name of the organism you are studying. The scientific name for the common house cat is *Felis catus*.

You may find that your results include many other genes that encode other proteins including the term "insulin" such as the insulin receptor or the insulin-like growth factor.

Click on the cat insulin gene and examine it. You may download the specific sequence of the cat proinsulin gene by hovering over the green box with arrows (which represents the gene). Another box should appear, and you can download the protein sequence to a notepad by clicking on the NP# (for protein sequences) next to the "download" label. **Copy and paste the protein sequence for cat proinsulin onto your document for submission.** For the gene, it is best to open the number beside the FASTA view as it will have your sequence in a format that can be easily compared to other sequences later. **Copy the DNA sequence into your submission document.** Click on the number beside the GenBank view (GenBank was the original database for DNA sequences). A summary describing the sequence is displayed. **Copy the summary to your document.**

Question

Examine the summary, what sort of information can you learn about the sequence?

Return to the homepage and select the "gene" dropdown and search for the gene for human (*Homo sapiens*) insulin.

Copy the protein sequence for human proinsulin to your submission document. Then copy the DNA sequence for human proinsulin to your submission document.

Align the Genes of Human and Cat Proinsulin

To align the genes of human and cat proinsulin, go to the homepage of NCBI. On the right side, there is a list of popular resources. Click on “BLAST”. To compare two nucleotide sequences, click on “Nucleotide BLAST”. Copy your cat proinsulin gene sequence in FASTA form (the form is what showed up when you opened the notepad of the sequence). Just copy the DNA sequence with no identifiers. Click on the box “align two or more sequences”. Another box will appear. Copy the human proinsulin DNA sequence in FASTA form to this box. Click on the box beside the “somewhat similar sequences/blastn”. “BLAST” to align these sequences. The aligned sequences should appear after a short time. Scroll down to see the aligned sequences. **Copy and paste the sequence alignments to your submission document.**

Align the Protein Sequences of Cat and Human Proinsulin

To align the protein sequences of human and cat proinsulin, go to the homepage of NCBI. On the right side, there is a list of popular resources. Click on “BLAST”. To compare two protein sequences, click on “Protein BLAST”. Copy your cat proinsulin protein sequence in FASTA form (the form is what showed up when you opened the notepad of the sequence). Just copy the protein sequence with no identifiers. Click on the box “align two or more sequences”. Another box will appear. Copy the human proinsulin sequence in FASTA form to this box. “BLAST” to align these sequences. The aligned sequences should appear after a short time. Scroll down to see the aligned sequences. **Copy and paste the sequence alignments to your submission document.**

Question

What are the differences between the proteins? How might specific amino acid changes affect protein function?

*Challenges to Expressing Cat Insulin in *E. coli**

Expressing cat insulin in *E. coli* presents many challenges because of the differences in gene expression that exist between Eukarya and Bacteria.

1. Eukaryotes have introns (interfering sequences) within the gene which are removed from a pre-mRNA through a process called splicing; bacteria do not have introns and lack the machinery to perform splicing.
2. Functional insulin requires the processing of proinsulin to proinsulin to insulin. The enzymes required for processing the protein are contained within the lumen of the endoplasmic reticulum, an organelle not possessed by bacteria.
3. Codon bias, the preference of a given species to use a particular codon for a given amino acid, may be different between cats and *E. coli*.
4. Translation mechanisms vary between eukaryotes and bacteria. Ribosomes bind to a 5' cap on eukaryotic mRNA and then scan down to the start site while prokaryotic mRNAs have a Shine-Dalgarno sequence for ribosomal binding located directly at the start site.

Use your text to review gene expression.

Challenge 1: Introns

The central dogma of genetics outlines the expression of proteins. Instructions on how to build the protein is contained in DNA, the genetic material. The gene is the unit of inheritance that encodes a polypeptide or protein. To express a protein, DNA is transcribed to an intermediate RNA molecule. Ribosomes perform “translation” using the RNA molecule as a template for producing the protein. In eukaryotes, intervening sequences called introns exist within the gene. These sequences are removed from pre-mRNA through an RNA processing mechanism called splicing. The resulting molecule, mRNA, contains only the useful parts (called exons). Ribosomes use the mRNA as a template to synthesize protein through a process called translation.

During translation the protein building information is matched via codons. A codon is composed of 3 nucleotides and corresponds to a particular amino acid used in protein synthesis. Ribosomes begin translation at the AUG or start codon on the mRNA and finish at one of 3 stop codons. Transfer RNAs ensure that the correct amino acid is matched according to the proper codon. A codon table can be used to “decode” the amino acid sequence that will be translated from an mRNA.

An open reading frame (ORF) is uninterrupted region with the potential to encode a polypeptide. An ORF begins with a start codon and ends with a stop codon. Examination of ORFs are helpful in identifying genes and introns and exons within genes.

The program GenScan at MIT (<http://genes.mit.edu/GENSCAN.html>) can be used to identify potential ORFs. The ORFs can be compared to the predicted protein sequence for proinsulin so that exons and introns can be identified. Because bacteria do not have introns and therefore any splicing machinery, the introns will have to be removed from the sequence for protein expression in *E. coli*. Go to GenScan and paste your DNA sequence in FASTA form into the box. **Copy the possible exons identified through this program into your document.**

Compare the predicted amino acid sequence for cat proinsulin obtained from NCBI with the ORFs predicted by GenScan.

While comparing, keep in mind that there are 3 possible reading frames (one starting with the first nucleotide, one starting with the second nucleotide and one starting with the third nucleotide).

Examine a codon table in your textbook to help you.

Question

Where is the location of any introns? How many exons are involved? **In your document, annotate from the beginning (from the start codon) to the end of the gene. Identify all exons and introns. Identify the start and stop codon.**

Week 2 Submission

As a team, submit one document including all items indicated with grey shading in this document. Please use the submission document and include item "titles" to keep things easy. Please submit your submission to the Moodle submission box by 8 am of the day of your lab section.

Pre-lab for Week 3

Investigate how the insulin protein is synthesized in the human pancreas. Identify important steps in its synthesis and post-translational modifications to produce a functional insulin protein. Use at least 3 sources available over the internet. Find at least one peer-reviewed article on insulin synthesis.

Pre-lab Submission

As a team, submit a document summarizing the steps involved in the synthesis of FUNCTIONAL insulin in humans. Provide a list of references used in preparing your summary. Please submit your pre-lab submission to the Moodle submission box by 8am of the day of your lab section.

Week 3: Designing a Sequence for the Expression of Cat Insulin in *E. coli*

Challenges to Expressing Cat Insulin in E. coli

Expressing cat insulin in *E. coli* presents many challenges because of the differences in gene expression that exist between Eukarya and Bacteria.

1. Eukaryotes have introns (interfering sequences) within the gene which are removed from a pre-mRNA through a process called splicing; bacteria do not have introns and lack the machinery to perform splicing.
2. Functional insulin requires the processing of preproinsulin to proinsulin to insulin. The enzymes required for processing the protein are contained within the lumen of the endoplasmic reticulum, an organelle not possessed by bacteria.
3. Codon bias, the preference of a given species to use a particular codon for a given amino, may be different between cats and *E. coli*.
4. Translation mechanisms vary between eukaryotes and bacteria. Ribosomes bind to a 5' cap on eukaryotic mRNA and then scan down to the start site while prokaryotic mRNAs have a Shine-Dalgarno sequence for ribosomal binding located directly at the start site.

Last week, we addressed challenge #1 to expressing cat insulin in *E. coli*. This week, we will address the remaining challenges and design the appropriate sequence to synthesize for cat insulin expression.

Challenge 2: Identification of Functional Protein Domains

Human insulin preproinsulin contains a signal peptide sequence at the N' terminus. Translation begins with free ribosomes. As the signal peptide emerges, translation is transferred to the ribosomes on the ER. The signal peptide threads the preproinsulin into the RER. Signal peptidase, an ER enzyme, cleaves the signal peptide from preproinsulin. The remaining protein (proinsulin) is transferred entirely into the ER. Proinsulin contains three domains: the N' B domain, a C' A domain, and a connecting (C) domain in between the B and A chain. Endopeptidases located in the ER cleave the C domain from the A and B domains which remain together through disulfide bonds. Bacteria cannot process the protein in this manner because it lacks an ER.

Question

What approaches may be taken to clone this gene for proper expression in *E. coli*?

The domains of the human insulin have been elucidated. The sequences of the human domains can be compared to the cat sequences to predict domains in the cat proinsulin. Examine figure 1 in Yang's 2010 paper *Solution Structure of Preproinsulin* to learn how preproinsulin is processed to insulin. Use figure 2 in Yang's paper to identify the domains of preproinsulin.

Copy you sequence of the human and cat proinsulin proteins further down on your document. Annotate you sequence with predicted domains signal sequence, the B domain, the C and the A.

Now make a combined annotated sequence with both amino acids of the preproinsulin and the codons in the gene of preproinsulin incorporated. Annotate the domains on the protein sequence and the exons, introns, start, and stop codons on the gene.

Challenge 3: Codon Bias

A species may tend to use a particular codon for an amino acid more frequently than the other codons for that same amino acid. This phenomenon is known as codon bias. Codon bias may affect the productivity of insulin expression because the gene will contain the codons preferred to be used in the cat yet the gene will be expressed in *E. coli*. Using the codon table below, identify what codons need to be changed for effective expression of insulin in *E. coli*. **Copy over your combined protein and gene sequences and replace the codons needed to be replaced because of codon bias.** To track your changes, use another color of ink for your changes. The ratio number indicates the ratio of codon use compared to other codons for the same amino acid. For example, phenylalanine (Phe or F abbreviation) has two codons. The ratio for UUU is 0.51 (meaning it is used 51% of the time) while the ratio for UUC is 0.49 (meaning it is used 49% of the time); codon bias is not really an issue because the two codons are both used frequently in *E. coli*. For the amino acid lysine (Lys or K abbreviation), the ratio for codon AAA is 0.76 (meaning it is used 76% of the time) whereas the ratio for the AAG codon is 0.24 (meaning it is used 24% of the time). In this case, *E. coli* has a definite preference for the AAA codon over the AAG codon.

Google a table of codon usage for *E. coli* to assist you in identifying preferred codons. You may also want to try this website: http://www.bioinformatics.org/sms2/rev_trans.html You can paste in the cat insulin primary amino acid sequence and it will reverse translate (to DNA) using codons optimized for *E. coli*. ☺

Challenge 4: Ensuring Efficient Translation

In eukaryotes, ribosomes recognize a 5' cap (a methylated guanine) on the end of the mRNA. Within the huge complex of RNAs and proteins that make up the eukaryotic ribosomes, a cap-binding protein is found. The cap-binding protein interacts with the 5' cap on the mRNA and the ribosomes scan down the mRNA until it reaches the start codon where it begins translation. In bacteria, ribosomes bind through an rRNA to a specific sequence located just upstream of start site. This sequence is called the Shine-Dalgarno sequence.

Visit the iGEM website (http://parts.igem.org/Ribosome_Binding_Sites/Design) to learn about Shine-Dalgarno sequences that have successfully been used for the expression of proteins in *E. coli*. The iGEM competition is really cool as you engineer microorganisms to perform a specific task. Rose has a team – see Dr. Anthony for more details.

Identify a viable Shine-Dalgarno sequence and copy it to your submission document. Make sure a start codon (ATG in DNA) is included

“Designing” Your Gene to Produce Functional Insulin

To obtain DNA from a cat, you must submit an extensive document for review by an animal care committee (IACUC). In your proposal, you must explain the rationale for the experiments, provide an explanation of the use of the species, and describe the protocol the animal will be subjected to (including pain and management of pain). Once written, the proposal would go to the committee for review and approval. This process may take weeks. An alternative to seeking IACUC approval is to use a cat cell line which may be purchased from ATCC. Cell lines cost \$350-\$400 and it requires a couple weeks to culture so that the cells are growing effectively. Another option is to have the DNA synthesized. This option would have many advantages:

1. We can avoid the long IACUC process, the distress to the cat (what cat likes to have its cheek swabbed?), and potential distress to the cat's owner (who will most likely get scratched).
2. We can avoid the cost of purchasing cat cells and reagents to culture cat cells.
3. We can avoid the time needed to invest in culturing cat cells for DNA extraction.
4. We can alter the sequence on paper (which is much, much easier than doing it in the plasmid) and have the sequence modified to account for codon bias, need for Shine-Dalgarno, encode specific domains.

Brainstorm two different strategies to express insulin in *E. coli*. Remember that functional insulin is composed of the A and B domains connected by disulfide bridges. Would you clone A and B separately? Together? How would you approach this?

Choose a strategy and design a sequence to be synthesized. Copy your design onto your submission document.

Designing Primers to Amplify the Synthesized DNA for Cloning

To clone your DNA sequence, you will need to amplify the DNA. The polymerase chain reaction (PCR) is a method for amplification of DNA. PCR is essentially DNA synthesis in a test tube. In the cell, DNA synthesis involves a DNA template, a DNA polymerase (enzyme to synthesize new DNA), RNA primers to provide free 3' OH groups for addition of nucleotides, nucleotides, and other proteins. DNA is always synthesized in the 5' to 3' direction (remember your dehydration reactions for nucleic acids) off a single-stranded DNA template. To perform DNA synthesis in a test tube, you need a DNA template, a DNA polymerase, nucleotides, and DNA primers. To denature DNA to make single-stranded DNA template, the reaction must be increased to 94°C. Taq DNA polymerase, a special polymerase isolated from the microorganism *Thermus aquaticus* found in hot springs in Yellowstone National Park, is used as this enzyme can withstand temperatures at 94°C.

Amplification through PCR will provide you with a greater yield of DNA to work with and also add 'A' nucleotides that overhang at the ends of the DNA which is important for cloning into a TOPO TA vector, a cloning plasmid for introduction of the gene into *E. coli*. A set of two “primers”, one forward primer and one reverse primer, are required to start the copying of the DNA sequence in PCR amplification. Primers are short pieces of DNA that can anneal (form hydrogen bonds with) to specific sections of DNA template.

Rules for Designing Primers

1. The primer length can be from 18 to 30 nucleotides long. Usually most primers are between 18 and 22 nucleotides.
2. The GC content should be between 40 to 60% of the primer.

3. A G or C is needed at (or near) the 3' end of a primer.
4. Avoid the formation of secondary structures (can any region of bases pair with another region?).
5. Avoid strings of 4 or more nucleotides.
6. Avoid repeated dinucleotides, etc.
7. Examine forward and reverse primers for homology.

Primer Melting Temperature Estimator

$$T_m = 2 (\#A+\#T) + 4 (\#G+\#C) < 14 \text{ bases}$$

$$T_m = 64.9 + 41 * (\#G+\#C - 16.4) / (\#A+\#T + \#G+\#C) \geq 14 \text{ bases}$$

Design a set of forward and reverse primers for your DNA sequence.

Week 3 Submission

As a team, submit one document including all items indicated with grey shading in this document. Please use the submission document and include item "titles" to keep things easy. Please submit your submission to the Moodle submission box by 8 am of the day of your lab section.

Week 4: Waiting for DNA to be Synthesized (do another activity)

Week 5: Amplifying the Synthesized Insulin Gene via PCR

In week 3, we designed a DNA sequence for the expression of cat insulin in *E. coli*. Our design removed an interfering intron, incorporated a Shine-Dalgarno sequence for translation in *E. coli*, made changes in codons to suit *E. coli* codon bias, and included a strategy to meet the challenge of needed protein processing. We also designed forward and reverse primers for use in the polymerase chain reaction. This week, we will amplify the synthesized DNA sequence through PCR.

Polymerase Chain Reaction

The technique known as PCR is useful to amplify small amounts of DNA. The procedure is outlined in figure 2. Essentially, DNA replication is performed in a test tube. A template of DNA that is to be amplified is placed in a tube with specific DNA primers, deoxynucleotide triphosphates (dNTPs), buffer solution, and *Taq* DNA polymerase. The tube is placed in a thermocycler, a machine that rapidly increases and decreases temperatures. Often, a long initial denaturation step is involved (for example, the DNA is heated to 94°C for 2 minutes) prior to beginning the PCR cycle. This step ensures that DNA has melted into single strands. PCR cycles through a denaturation step, an annealing step, and an extension step. The denaturation step involves heating the reaction to 94°C for a short time (30 seconds to 1 minute) to allow separation of the strands. The annealing step cools the reaction to 50-65°C to allow the primers (small oligonucleotides that bind to specific regions of the DNA genome) to anneal. In the extension step, the reaction temperature is raised again to 72°C so the *Taq* polymerase can extend the DNA from the primers to yield a full-length complementary strand of DNA. The denaturing, annealing, and extending steps are repeated for 20-40 cycles to repeat amplification of the DNA. A final extension 5-10 minutes at 72°C is often included.

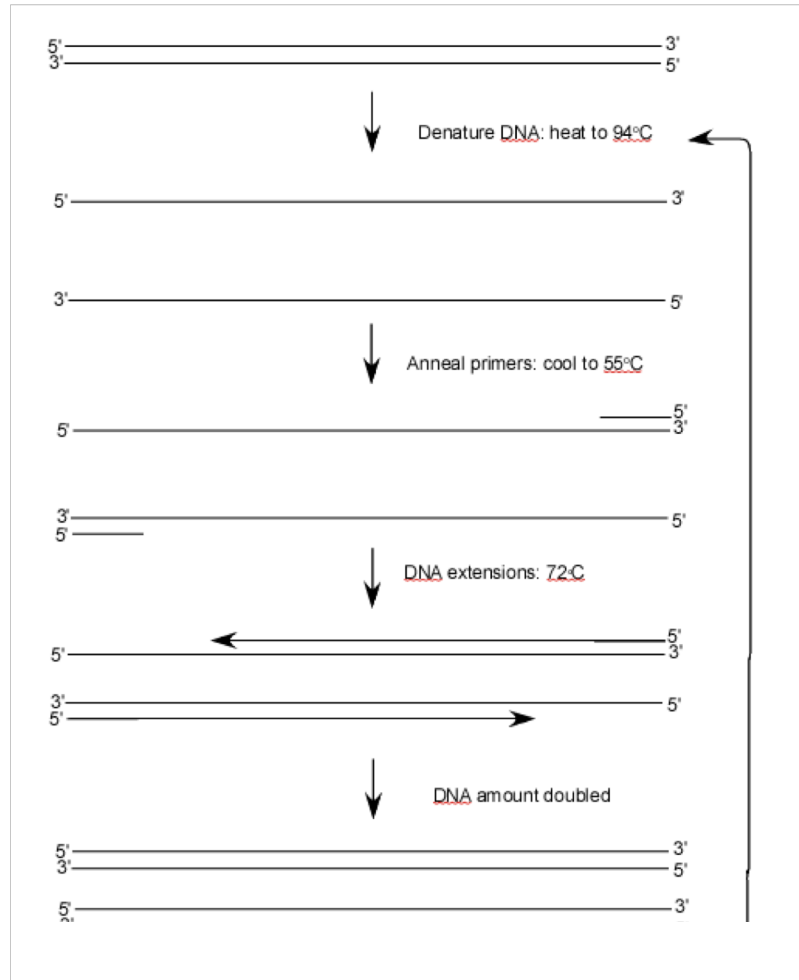


Figure 2. Diagram outlining the steps in the polymerase chain reaction.

PCR Reaction Protocol

Reagents per PCR reaction

- Template DNA (synthesized)
- Sterile molecular grade water
- 10x Taq Buffer
- dNTPs (10mM)
- Forward Primer (10μM)
- Reverse Primer (10μM)
- Taq DNA polymerase (5U/μL)

Equipment

- 0.5 mL PCR tubes
- Pipettes and tips
- Ice bucket with ice
- Thermocycler

PCR Protocol

1. Obtain a 0.5 mL PCR tube.

2. Add the following reagents to the tube:
 - 40.5 μ L of sterile water
 - 5 μ L 10X Taq buffer
 - 1 μ L DNA template
 - 1 μ L dNTPs (10mM)
 - 1 μ L Forward primer (10 μ M)
 - 1 μ L of Reverse primer (10 μ M)
 - 0.5 μ L Taq DNA polymerase
3. Label your tube.
4. Place tubes in thermocycler that has been set for the following parameters:
 - a. Initial denaturing step. Incubate at 94°C for 5 minutes.
 - b. Denature. Incubate at 94°C for 30 seconds.
 - c. Annealing. Incubate at 50-65°C for 30 seconds. *** Use an annealing temperature about 5°C of the melting temperature of primers.
 - d. Extension. Incubate at 72°C for 1½ minutes.
 - e. Repeat steps b-d thirty (30) times.
 - f. Final extension. Incubate at 72°C for five minutes.
5. Store in -20°C freezer if necessary.

Preparing a Gel for the Gel Electrophoresis Protocol

1. Place 50 mL of 1X TBE “running buffer” in the flask with 0.5 g agarose. (This is considered to be a “1% gel”.)
2. Microwave flask until agarose is in solution. MICROWAVE FOR 30 SECONDS AT A TIME. Using folded paper towels, gently swirl the agarose as it approaches boiling temperature. For the gel to properly form, the agarose-TBE solution MUST boil.
3. Allow the gel to cool until it is no longer steaming.
4. Add 5 μ L of gel red dye to the agarose solution before pouring.
5. Orient the gel tray such the rubber gasket is flush with the sides of the gel box.
6. Pour agarose into gel tray. Immediately after pouring, insert the gel “comb” into the molten agarose to form the wells. Allow to sit until the agarose has solidified.
7. Wrap in plastic wrap and refrigerate for next week.

Lab Submission

There will be no lab submission regarding the cat insulin project for this week.

Pre-lab for Week 6

Investigate the use of gel electrophoresis for the separation of DNA molecules. Use at least 3 resources including one from primary literature.

Pre-lab Submission

As a team, submit a document summarizing the how gel electrophoresis works to separate DNA molecules by size and include a list of steps involved in gel electrophoresis. Cite your references. Please submit your pre-lab submission to the Moodle submission box by 8am of the day of your lab section.

Week 6: Examining and Cloning PCR Products

In week 5, we amplified our synthesized DNA sequence through PCR. Today, we will examine our PCR product to see that it amplified correctly using gel electrophoresis. If successful in our amplification, we will clone it into a TA cloning vector and transform the plasmid into *E. coli*.

Gel Electrophoresis

In some cases, large biomolecules must be separated into distinct pieces. There are several separation methods available (HPLC, Column Chromatography, Gel Electrophoresis, Dialysis, etc.). One of the most common methods of separating nucleic acid (DNA or RNA) fragments is gel electrophoresis.

Gel Electrophoresis separates molecules based on size. The driving force used to fractionate the sample is electricity (current). The “matrix” or substance through which the nucleic acid passes can be made from agarose (a carbohydrate similar to gelatin) or polyacrylamide. Polyacrylamide has very different properties and has better resolution in separating substances. Agarose is more versatile, safer to use, and more commonly used for nucleic acid separation (polyacrylamide is more commonly used in DNA sequencing).

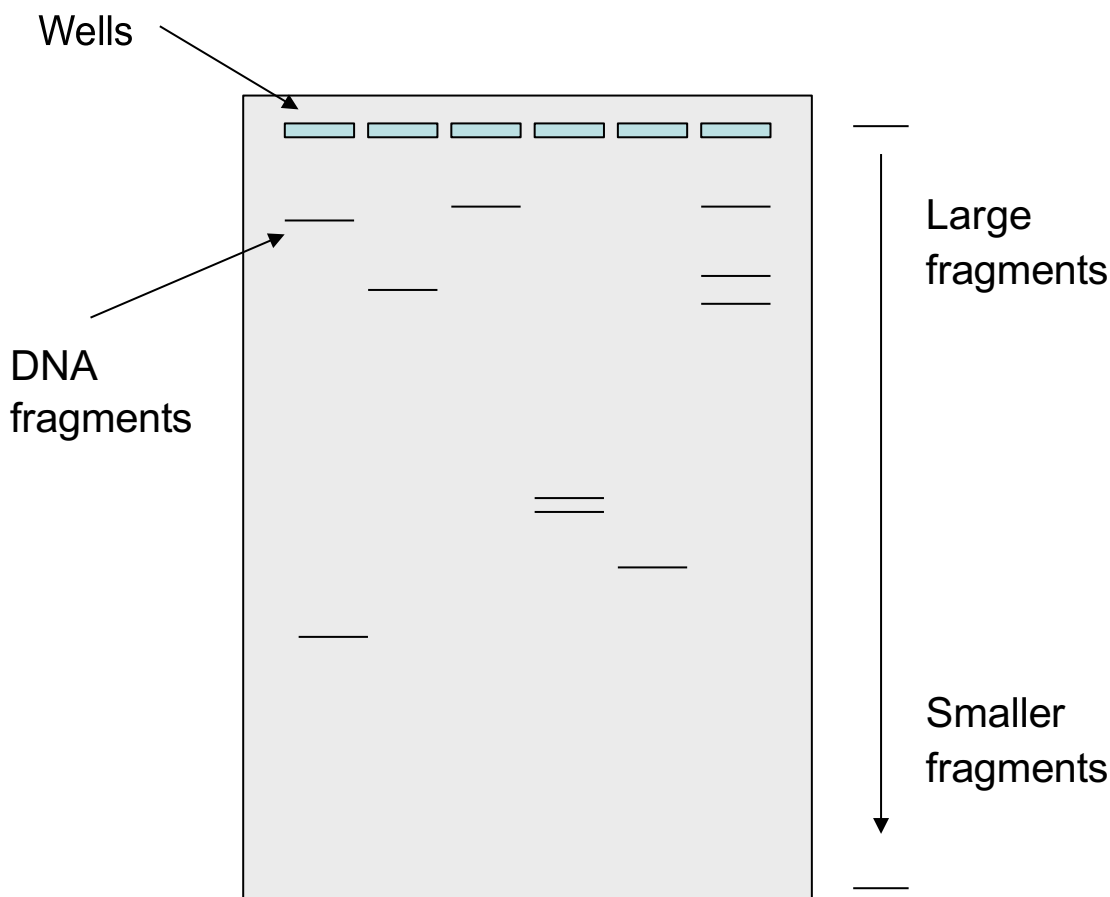


Figure 3. Diagram illustrating gel electrophoresis.

When DNA or RNA fragments pass through the agarose matrix, they must “fit through the openings”. Large pieces move more slowly as they have a more difficult time “fitting” through the matrix, and small pieces move more quickly. Thus, once current has been applied to the gel and samples, large pieces will be closer to the site of origin and small pieces will be near the bottom of the gel. The more agarose in the matrix, the more difficult it is to separate large pieces of nucleic acid. It is, therefore, important to select the agarose percentage that meets your needs (separation of large or small pieces).

In order to get the samples to move through the gel, current is applied. Electrical current is more effective if the gel is submerged in an aqueous solution (thus the term subgel). The aqueous solution contains several ions that enhance the conduction of current. Common chemicals used to establish this ion concentration include Tris, EDTA, and Boric Acid. These substances also act as buffers (maintain the pH so the nucleic acid is not damaged). Therefore, these substances are referred to as the “running buffer”. The running buffer also serves to maintain a cooler temperature (heat will also denature or destroy the samples) when current is applied.

DNA has a slightly negative charge, so when it is loaded into a gel and an electric current is applied, it will travel away from the negative pole (black) toward the positive pole (red). The larger fragments will be “caught” in the gel material sooner than the smaller fragments due to their size. This means that larger fragments are always near the top (nearest the load area or wells) and fragments further away from this area get progressively smaller (Figure 3).

The last major piece to gel electrophoresis is visualization. These molecules are not visible when running through the gel, so a dye is added to the sample that (1) indicates where the leading edge of the sample is, and (2) uniformly coats each piece of DNA with a negative charge. This is important because application of current forces all negatively charged molecules in one direction and positively charged molecules in the opposite direction. Once the pieces are separated, individual sizes must be visualized. There are several staining techniques that can be used to stain the pieces in the gel or transfer the pieces from the gel to a nylon membrane for visualization there.

Cloning into a TA Cloning Vector

Once you have ascertained that you have the desired PCR product, you will set up a ligation reaction to insert your PCR product into a plasmid vector. We will be using the TOPO TA cloning system described below (information from TOPO TA cloning manual from manufacturer). Examine the TOPO TA Cloning Manual to learn how topoisomerase works.

Transformation

In class, we learned about Griffith’s experiment that demonstrated the “transforming principle”. The heat-killed virulent, smooth colonies released DNA which was taken up by the live, avirulent rough colonies. The uptake of foreign DNA by bacteria is called transformation. When bacteria are able to take up DNA, they are said to be “competent”. In nature, specialized competence proteins facilitate DNA uptake. In the laboratory, we can make bacterial cells competent by treating the cells with cold CaCl_2 (or other similar salts). The calcium chloride pokes holes in the cell’s membranes. When the cells are incubated with DNA on ice and then “heat-shocked” at 42°C , the cell holes open up and DNA is taken into the cell. Placing the cells on ice quickly after heat-shock helps retain the DNA in the cell.

Experimental Protocols

Gel Electrophoresis Protocol

1. Obtain gel from last week.
2. Place in gel apparatus so that the wells are closest to the cathode (black end).
3. Pour 1x TBE buffer over the gel until it just covers the top of the gel (~ 300 mL).
4. Allow the gel to rehydrate if necessary.

Loading Samples

1. Using a strip of parafilm, make 3 μL “drops” of loading dye on the parafilm. Add 20 μL of PCR sample to each drop of dye. Mix by pipetting up and down. Obtain a DNA marker with loading dye.
2. Load 20 μL of each sample into a lane on the gel.
3. Obtain a DNA marker with loading dye and load a few μL into a well.

Electrophoresis

1. Attach the leads to the electrodes:
 - a) the **cathode** (*negative* end, black) is nearest to the sample wells in the gel
 - b) the **anode** (*positive* end, red) is at opposite end (Note: this is not a typo. In gel electrophoresis, the cathode is actually the negative electrode, and the anode is the positive electrode. This is a common MCAT question).
2. Turn the power supply on. “Run” the gel at 100V.
*Make sure the DNA is set to run toward the positive end the gel box.
3. Run the gel until the blue dye migrates 2/3 way down the gel.

Staining the Gel

1. Because you added gel red to the gel, you do not need to stain the gel.
2. You can stop your gel at any time and examine results on the UV light box.
3. Return your gel to the apparatus to completely separate products for photographing.

Perform the TOPO cloning reaction and the transformation protocol as instructed in the TOPO TA manual. Maps of the TOPO vectors may be found in the TOPO TA manual.

Lab Submission

As a group, create a figure illustrating the gel electrophoresis of your PCR product. Please submit your submission to the Moodle submission box by 8am of the day of your lab section.

Pre-lab for Week 7

Investigate different methods to screen colonies for the presence of recombinant plasmids.

Pre-lab Submission

As a team, submit a document summarizing how screening works and describe how you will be able to tell if your cloning was successful. Include 3 references with one from primary literature. Please submit your pre-lab submission to the Moodle submission box by 8am of the day of your lab section.

Week 7: Screening for Cloned Inserts

In week 6, we cloned the PCR product into a TOPO TA cloning vector (plasmid pCR2.1-TOPO) and then transformed the plasmid into *E. coli*. Today, we will examine the colonies of *E. coli* and determine whether the insert has made its way into the cloning vector. Blue/white colony screening provides a hint at whether an insert is present. Restriction digestion of the plasmid or PCR can provide more certain corroboration. To be completely confident of your insert, you need to sequence the insert section of the plasmid.

Examination of Colonies

Antibiotics are drugs that kill bacterial cells. Last week, we plated our transformed *E. coli* cells on plates containing the antibiotic kanamycin. The presence of kanamycin in the LB plates inhibits the growth of all *E. coli* cells that have not been successfully transformed with the plasmid (and they outnumber transformed cells by a large margin). Only cells containing the plasmid grow on the plates because the plasmid contains an antibiotic resistance gene that confers kanamycin resistance to cells containing the plasmid. Thus, the antibiotic acts as a “selection” mechanism.

Restriction Digest Analysis

One method to determine whether DNA has been inserted into the plasmid is to perform restriction digests on the plasmid. Restriction enzymes are enzymes made by bacteria that can cleave DNA at specific sites. For example, EcoRI cuts DNA specifically at the site:



Plasmids often have multiple cloning sites or unique restriction sites to cut the plasmid for cloning pieces of DNA. The unique sites can also be used to make a circular plasmid linear; this is important as circular plasmids tend to supercoil therefore create multiple bands of various sizes on a gel. Linear DNA will run as the correct size on the gel.

Examine the plasmid map. Identify two restriction enzymes that you could cut the plasmid with to obtain linear DNA (meaning the enzyme only cut in one location). See the plasmid map from the Week 6 handout. Also, this site may be helpful: Add a gene – pCR2.1 sequence analysis <https://www.addgene.org/vector-database/2285/>

Determine the sizes of DNA fragments if pCR2.1 was cut with the restriction enzyme HindIII. Sizes if cut with restriction enzyme SphI?

*HindIII cuts at 5' A↓AGCTT3' and SphI cuts at 5' GCATG↓C3'

Examine your insert DNA sequence. Identify two restriction enzymes that would cut your insert one time. Do these restriction enzymes also cut the pCR2.1 plasmid? Also, this website might be helpful: Restriction Mapper site: <http://www.restrictionmapper.org/>

Reminder of cat insulin DNA order

```
5'GGCATAAAGGAGGTAAATAATGTTTCGTTAACCAGCACCTGTGCGGTTCTCACCTGGTTGAAGCGCTGTACCT
GGTTTGCGGTGAACGTGGTTTCTTCTACACCCCGAAAGCGCGTCGTGAAGCGGAAGACCTGCAGGGTGAACC
GCCGCTGCCGCCGCTGGGTAAAGACGCGGAACCTGGGTGAAGCGCCGGGTGCGGGTGGTCTGCAGCCGTCTGC
GCTGGAAGCGCCGCTGCAGAAACGTGGTATCGTTGAACAGTGCTGCGCGTCTGTTTGGCTCTCTGTACCAGCTG
GAACACTACTGCAACTAACGC 3'
```

*Remember that restriction enzyme sites can be created at the insert/vector ligation sites.

Preparation of Plasmid DNA (Mini-Preparation)

To digest your plasmid DNA, you will first have to perform a “mini-prep” of your plasmid DNA or obtain a clean preparation of your plasmid DNA. In lab today, we will use the Zyppy Plasmid Miniprep Kit to obtain pure plasmid (see attached protocol). The resulting plasmid DNA is ready for use in a restriction digest (or to be subcloned into another vector).

Colony PCR Screening

Another way to determine whether DNA has been inserted into the plasmid is to perform PCR screening. PCR is performed on DNA from a bacterial colony (or purified plasmid) using general primers that bind to regions on the plasmid on either side of where the insert would be located. We will use M13 forward and reverse primers (with binding sites located on the map to the right).

Calculate the size of the PCR fragment if no insert is obtained:

Calculate the size of the PCR fragment if the cat insulin insert is successful:

Protocols

Mini-Preparation of Plasmid DNA protocol: Zyppy

Follow the Zyppy protocol. See attached document.

Restriction Digest Screening Protocol

Reagents for Restriction Digest

HindIII restriction enzyme
HindIII 10x buffer
Molecular grade water

SphI restriction
enzyme SphI 10x
buffer
DNA template from mini-prep

Equipment

1.5 mL tubes
Pipettes and tips
Ice bucket with ice
Water bath at 37°C

Restriction Digest Protocol

1. For each restriction enzyme, set up a restriction digest in the following manner in a 1.5 mL microfuge tube:
2 μ L 10x buffer (specific to restriction enzyme)
2 μ L DNA
15.5 μ L molecular grade water
0.5 μ L restriction enzyme
2. Incubate your digest for at least 1 hour in a water bath set at the optimal temperature for the enzyme (for both HindIII and PstI, the optimal temperature is 37°C).
3. Store DNA at -20°C until use.
4. Examine products using gel electrophoresis (next week).

Colony PCR Screening Protocol

Reagents per PCR reaction

Colonies
Sterile molecular grade water
10x Taq Buffer
dNTPs (10mM)
M13 Forward Primer (10 μ M)
M13 Reverse Primer (10 μ M)
Taq DNA polymerase (5U/ μ L)

Equipment

0.5 mL PCR tubes
Pipettes and tips
Ice bucket with ice
Thermocycler

PCR Protocol

1. Obtain 4 0.5 mL PCR tubes.
2. Add the following reagents to each of the tubes:

- 40.5 μ L of sterile water
 - 2.5 μ L 10X Taq buffer
 - 0.5 μ L dNTPs (10mM)
 - 0.5 μ L M13 Forward primer (10 μ M)
 - 0.5 μ L M13 Reverse primer (10 μ M)
 - 0.5 μ L Taq DNA polymerase
3. Choose a colony for screening and pick a small amount of the colony from the plate using a toothpick and place it into the tube. *Note: you should do this quickly and scrape the colony along the side of the tube in the liquid. If you leave the toothpick in the liquid too long, it will soak up the liquid.*
 4. Label your tube.
 5. Place tubes in thermocycler that has been set for the following parameters:
 - a. Initial denaturing step. Incubate at 94°C for 5 minutes.
 - b. Denature. Incubate at 94°C for 30 seconds.
 - c. Annealing. Incubate at 50-65°C for 30 seconds. *** Use an annealing temperature about 5°C of the melting temperature of primers.
 - d. Extension. Incubate at 72°C for 1½ minutes.
 - e. Repeat steps b-d thirty (30) times.
 - f. Final extension. Incubate at 72°C for five minutes.
 6. Store in -20°C freezer if necessary.

Lab Submission

No lab submission this week.

Pre-lab for Week 8

Use your handout from week 7 and go to the suggested websites to help you investigate the pCR2.1 vector. If this vector was cut with the restriction enzyme Hind III, how many DNA fragments would you expect and what are their sizes without the cat insulin insert? And with the cat insulin insert? What results would you expect from a digest with Sph I? A double digest with both Hind III and Sph I? What is the expected size of the fragment from amplification of DNA using M13 primers?

Pre-lab Submission

As a team, submit a document summarizing what sizes of DNA fragments you expect to see on the gel if the cat insulin insert was successfully or unsuccessfully cloned into the pCR2.1 plasmid for both the PCR screening and the restriction digest analysis.

Week 8: Analysis of Screenings

In week 7, we examined antibiotic-resistant colonies on plates, prepared plasmid DNA from a colony to digest with restriction enzymes and performed colony PCR on 4 colonies. Today, we will examine our PCR products and restriction enzyme digests to see which colonies contained cat insulin gene inserts.

Gel Electrophoresis Protocol

We will set up two gels today; one gel will be a 0.8% agarose gel for separating the rather large DNA fragments from the restriction digest and a 1.2% gel to separate the rather small fragments amplified from the colony PCR.

1.8% Agarose Gel

1. Place 50 mL of 1X TBE “running buffer” in the flask with 0.4 g agarose. (This is considered to be a “0.8% gel”.)
2. Microwave flask until agarose is in solution. MICROWAVE FOR 30 SECONDS AT A TIME. Using folded paper towels, gently swirl the agarose as it approaches boiling temperature. For the gel to properly form, the agarose-TBE solution MUST boil.
3. Allow the gel to cool until it is no longer steaming.
4. Add 5 μL of gel-red dye to the flask and swirl to mix.
5. Orient the gel tray such the rubber gasket is flush with the sides of the gel box.
6. Pour agarose into gel tray. Immediately after pouring, insert the thicker side of the 12-well gel “comb” into the molten agarose to form the wells. Allow to sit until the agarose has solidified.

1.2% Agarose Gel

1. Place 50 mL of 1X TBE “running buffer” in the flask with 0.6 g agarose. (This is considered to be a “1.2% gel”.)
2. Microwave flask until agarose is in solution. MICROWAVE FOR 30 SECONDS AT A TIME. Using folded paper towels, gently swirl the agarose as it approaches boiling temperature. For the gel to properly form, the agarose-TBE solution MUST boil.
3. Allow the gel to cool until it is no longer steaming.
4. Add 5 μL of gel-red dye to the flask and swirl to mix.
5. Orient the gel tray such the rubber gasket is flush with the sides of the gel box.
6. Pour agarose into gel tray. Immediately after pouring, insert the thicker side of the 12-well gel “comb” into the molten agarose to form the wells. Allow to sit until the agarose has solidified.

Loading Samples

1. When gel is set, remove combs. Pour 1X TBE running buffer over the top. Fill the gel box to an approximately 1-2 mm depth over the gel.
2. Place 6 “dots” (3-5 μL) of loading dye on a strip of parafilm.
3. Add 20 μL of each colony PCR sample to 4 of the dots and mix each sample on the parafilm.
4. Add 20 μL of each of the restriction digests to the other 2 dots and mix on the parafilm.
5. Load the colony PCR samples into wells in a 1.2% agarose gel.
6. Load the restriction enzyme digest samples into wells in a 0.8% agarose gel.
7. Obtain a DNA marker with loading dye.
8. A DNA ladder with loading dye should be included in one well on each gel.

Electrophoresis

1. Attach the leads to the electrodes:
 - a. the **cathode** (*negative* end, black) is nearest to the sample wells in the gel
 - b. the **anode** (*positive* end, red) is at opposite end (N.B.: this is not a typo: in gel electrophoresis, the cathode is actually the negative electrode, and the anode is the positive electrode. This is a common MCAT question.
2. Turn the power supply on. “Run” the gel at 100V. ***Make sure the DNA is set to run toward the positive end the gel box.***
3. Run the gel until the blue dye migrates 2/3 way down the gel.

Visualizing the DNA

1. Visualize the gel on the UV light box.
2. Take a picture of your gel using the photo documentation system.

Reporting Results

1. For each result indicating a positive insert, record the data and submit to your lab instructor.

Lab Submission

As a group, design an experiment to test whether your cat insulin is functional after expression in *E. coli*. Assume that the vector you have the DNA inserted into works well to produce the polypeptide encoded by the DNA we had synthesized and that there were no problems in isolating the protein from cell extracts or treating with endopeptidase. How would you know that your insulin protein was functional? Include references for your ideas. Please submit your submission to the Moodle submission box by 8am of the day of your lab section.

Pre-lab for Week 9

Investigate how DNA sequencing is performed. How is it determined what nucleotide is present at a given site? Use at least 3 references with one from primary literature. Please submit your pre-lab submission to the Moodle submission box by 8 am of your lab section.

Week 9: Analysis of Sequencing Data

In week 8, we identified colonies positive for inserts through PCR screening and restriction enzyme analysis; we then cleaned up our positive PCR products to send them off for sequencing. Today, we will examine our sequences and compare them to the expected sequences.

DNA Sequencing

Recent multidisciplinary advancements in biology, chemistry, physics, and computer science have enabled molecular biologists to perform relatively inexpensive, efficient, and rapid sequencing of nucleic acids. Today, the average molecular biology laboratory is able to determine the sequence of a given strand of DNA (up to several thousand base pairs) in a single afternoon.

The most common method for sequencing nucleic acids involves the use of fluorescent nucleotides and capillary-based electrophoresis. Called “dideoxy sequencing”, this method can reliably sequence up to 1000 base pairs of DNA at a time.

Dideoxy sequencing begins by replicating DNA in a test tube, generating thousands of copies of the DNA molecule to be sequenced. During replication, the DNA is incubated with the necessary enzymes (DNA polymerase) needed to replicate the molecule, regular nucleotides, and modified nucleotides that are fluorescently tagged. Short, single-stranded molecules of DNA called “primers” are also added to direct the start point of replication. During the replication process, incorporation of regular nucleotides enables elongation of the complementary strand to continue; in contrast, incorporation of a modified, fluorescently-tagged nucleotide terminates the elongation process. As the thousands of copies of the DNA molecule to be sequenced are simultaneously replicated, regular nucleotides and modified nucleotides are randomly incorporated into the complementary strands. This process results in complementary strands of varying length since the incorporation of modified nucleotides into the complementary strand terminates the copying process. (Modified nucleotides prevent additional nucleotides from being added to the growing complementary strand.) The complementary strands are then separated using a process analogous to gel electrophoresis (capillary electrophoresis). By reading the fluorescent tags of the modified nucleotides (analyzed by a laser), it is possible to determine the nucleotide sequence of the original.

Viewing Your Sequences

Download FinchTV from Geospiza. Here is the link: <https://digitalworldbiology.com/FinchTV>

1. Click on the the version for windows.
2. A zip file should download. Open the zip file and click in the installer file.
3. Click “run” to install the program.

Reading Your Sequences

To read your sequences, unzip the zip file of sequences and choose the sequence ending in the AB1 format (.AB1). When you click on this file, it should open automatically in FinchTV. A chromatogram with the nucleotide sequence above should appear.

A good sequence should yield a chromatogram that has evenly spaced peaks clustered close together as shown in Fig. 4 below:

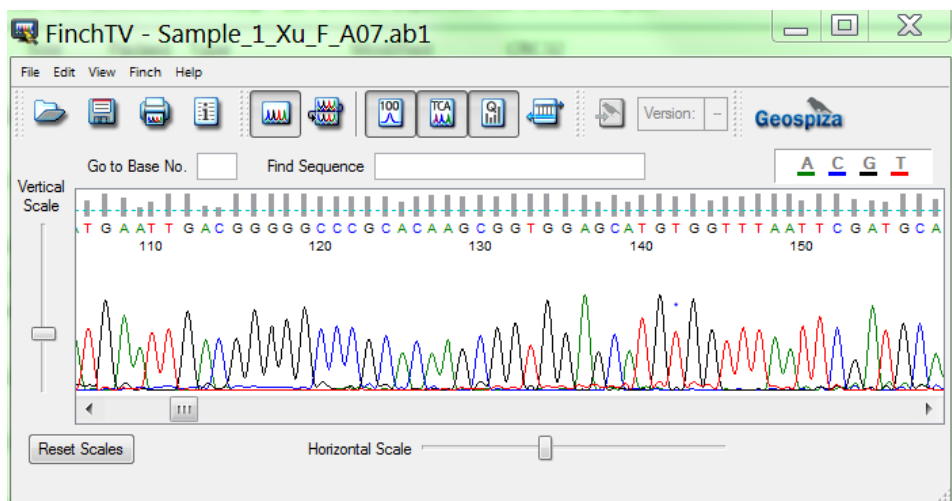


Figure 4. A chromatogram exhibiting distinct peaks representing nucleotides in the DNA sequence.

Ignore parts that have large peaks or peaks that lay over one another (possibly from multiple sequences) such as seen in Fig. 5 below:

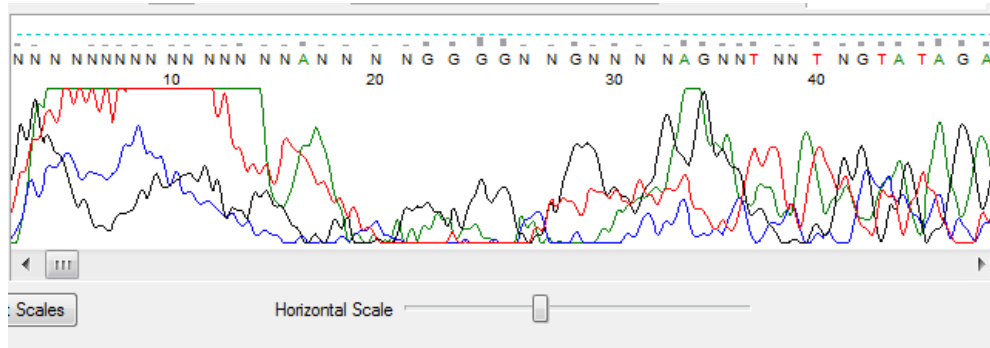


Figure 5. A chromatogram depicting large peaks and unreadable sequence data.

Notice the numerous ‘N’s listed on the above sequence. An ‘N’ means “any” nucleotide and the sequencer was unable to differentiate among the nucleotides at this spot.

Your PCR products have been sequenced twice using M13 forward primer and M13 reverse primer. Please examine both sets of results to see which sequence is cleaner for BLASTING.

Analyzing Your Sequences

To compare your sequence to those in the NCBI databank, FinchTV has a button that does it automatically.

1. Under the Edit dropdown, choose BLAST sequence with the Nucleotide option.
2. A window from NCBI should appear with your sequence uploaded in the FASTA sequence window.
3. Click the box beside “Align two or more sequences”. Another box should appear.
4. Copy and paste the cat insulin synthesized DNA sequence into that box.
5. Click on the “BLAST” button and wait.
6. Results will appear with a box showing lines of alignment of your sequence with the cat insulin synthesis.

Reminder of Cat Insulin DNA Order:

```
5'GGCATAAAGGAGGTAATAATGTTTCGTTAACCAGCACCTGTGCGGTTCTCACCTGGTTGAAGCGCTGTACCT
GGTTTGC GG TGAACGTGGTTTCTTCTACACCCCGAAAGCGCGTTCGTGAAGCGGAAGACCTGCAGGGTGAACCG
CCGCTGCCCGCGCTGGGTAAGACGCGGAACTGGGTGAAGCGCCGGGTGCGGGTGGTCTGCAGCCGTCTGCGC
TGGAAGCGCCGCTGCAGAAACGTGGTATCGTTGAACAGTGCTGCGCGTCTGTTTGTCTCTGTACCAGCTGGAA
CACTACTGCAACTAACGC 3'
```

Sequencing Analysis Questions

1. How well did your sequence align with the synthesized cat insulin gene? Did you have any mismatches?
2. If you had any mismatches, examine your annotated cat insulin sequence submitted in week Predict the effect of any mutation. Identify the type of mutation (silent, nonsense, missense, frameshift).

Lab Submission

As a group, summarize the results of sequencing of your construct (if yours did not work, borrow someone else’s data). A figure containing an alignment of the synthesized DNA sequence we purchased and the DNA sequence of your construct would easily demonstrate the relationship between the two sequences. A sentence title explaining the result and a few sentences of analysis would work. Did the sequence of the construct match the sequence you expected? If not, hypothesize why.

Please submit your document to Moodle submission box by Monday 8 am.

Week 10: Built-in Research Buffer Week

Research can be challenging as many things can go wrong. For that reason, this week is an extra in case we need to repeat something or need more time to analyze our sequences.

Materials

List of Equipment and Supplies

For a Class of 25 Students

The equipment needed to perform this research is listed in Table 1. A list of supplies typically ordered at the beginning of the term and respective suppliers are listed in Table 2. Other supplies needed that are generally on hand for multiple biology laboratories are listed in Table 3. A list of activities and supplies needed by week is provided in Table 5 in the appendix.

Table 1. List of equipment needed.

Thermocycler	Pipettors
Microwave	Incubator
-20°C freezer	Hockey sticks for spread plating
Water bath	Turn tables (nice but not necessary)
Ice buckets	Bunsen burners or alcohol burners
Gel apparatus with combs, casting tray	Autoclave (or plates can be purchased)
Power supply for gel electrophoresis	Erlenmeyer flasks

Table 2. Items ordered at the beginning of term.

Supplies to be ordered	Supplier name or link
TOPO TA Cloning kit	ThermoFisher https://www.thermofisher.com/us/en/home/life-science/cloning/topo/topo-ta-cloning/topo-ta-for-subcloning.html
PCR cleanup kit *	IBI Science https://www.ibisci.com/products/gel-pcr-dna-fragment-extraction-kit-300-preps
Mini-prep kit	Zymo Research https://www.zymoresearch.com/dna/plasmid-dna-purification/zypzy-plasmid-miniprep-kit
TOP 10 or DH5 α competent cells	VWR or Zymo
PCR reagents (Taq polymerase and buffer, dNTPs, molecular grade water)	Currently ordering from IBI, sometimes VWR
Restriction enzymes (Hind III and Sph I)	NEB through VWR

Table 3. Supplies generally on hand for other laboratories.

LB agar media	Agarose	Glo germ + UV light
Kanamycin	10x TBE buffer	Shaving cream
PCR tubes (0.2 mL or 0.5 mL)	Gel loading dye	Gloves (all sizes)
Microfuge tubes (1.5 mL)	1 kb DNA ladder	Biohazard disposal bags
Pipette tips (all sizes)	Matches	Plastic wrap
Sharpies	Gel red dye	Toothpicks
Parafilm		

*Primers and DNA sequences are ordered after students design experiments (after week 3 session). We ordered our primers through Thermo Fisher Scientific and our synthetic DNA from Integrated DNA Technologies.

Notes for the Instructor

A minimum of 8 laboratory periods (150-minute periods) is required for the project. More background information regarding the exercises each week is provided in the student handouts. Specific information needed for preparing for each week's lab is contained in the supply list.

Week 1: Biosafety Training, Pipetting Practice, Introduction to Research Project

Estimated preparation time for lab: 10 minutes

- During the Biosafety training, two active learning exercises are performed to emphasize proper hand-washing technique and care in removal of contaminated gloves. The hand-washing activity involves placing a small amount of liquid glo germ into each student's hand. Students rub their hands to distribute the glo germ evenly then the students wash their hands as usual. Glo germ is visualized under a handheld UV light. The glove removal activity involves the instructor placing a small amount of shaving cream in the gloved hand of a student. Students rub their hands to distribute the shaving cream. Students should take off their gloves without contaminating themselves or their neighbors with shaving cream.
- Pipetting error is a common cause of failure in molecular biology labs. During this week students will pipet predetermined volumes of colored water to compare against instructor standards. Common volumes students will pipet are 1ul, 5ul, 10ul, and 50ul.
- Students are introduced to the general concept of blood glucose regulation by the protein hormone

insulin. Students will investigate and discuss how insulin is normally synthesized and processed in the human pancreas, including secretion and post-translational processing.

- Students are introduced to the concept of recombinant insulin production, particularly the two original approaches: 1) synthesizing the A and B chains separately in *E. coli*, and 2) synthesizing and secreting proinsulin in *E. coli*.

Week 2: NCBI Database – Identifying and Annotating Insulin Gene from Cat; Introns

Estimated preparation time for lab: None

Big picture: Students learn to use NCBI databases and begin comparing human and cat insulin gene sequences.

- In this week the students are charged with finding domestic cat (*Felis catus*) preproinsulin genomic sequence using NCBI Genbank. To find a specific gene, students will try different search terms in the “gene” database. For example, the students might search for “cat insulin”, but will likely narrow the search to “*Felis catus*” and “preproinsulin”.
- This week is a good time to discuss homology of mammalian insulin genes, specifically the differences in primary amino acid sequence between cats and humans. To do this, students will download both the nucleotide and amino acid sequence of cat and human preproinsulin genes and proteins, respectively.
- There are a number of alignment programs freely available on the internet, however NCBI nucleotide BLAST is a useful tool to align two or more sequences. Let the students search for their own favorite program and compare results with other groups.
- Cat and human insulin genomic sequences also include introns that will need to be removed before introducing to *E. coli*. Have the students find intron-predicting websites and analyze both cat and human insulin for introns.

Week 3: Identify Functional Domains; Codon Bias; Translation Issues; Primer Design

Estimated preparation time for lab: None

Big picture: Students determine the sequence of cat proinsulin. Sequence is then synthesized by Integrated DNA Technologies.

- Expression of eukaryotic genes in bacteria face several obstacles. Bacteria will not remove eukaryotic introns or perform eukaryotic-specific post-translational processing (e.g. removal of the eukaryotic leader sequence from preproinsulin and removal of the “C” chain from proinsulin.). Allow the students brainstorm various approaches to overcome these obstacles.

- Provide the students with figures from “Solution structure of proinsulin. 2010. Journal of Biological Chemistry, 285 (11): 7847-51”. The students will compare the cat proinsulin sequence to human insulin to identify putative B, C, and A chains.
- In a Word document, students will now make a combined annotated sequence with both amino acids of the proinsulin and the codons in the gene of proinsulin incorporated. Students should annotate the domains on the protein sequence and the exons, introns, start, and stop codons on the gene.
- An organism’s codon bias—preference of one codon for a particular amino acid—may affect the productivity of insulin expression because the gene will contain the codons preferred to be used in the cat yet the gene will be expressed in *E. coli*. Using a provided table of *E. coli* codon preferences, the students will alter their sequence of cat proinsulin with preferred *E. coli* codons.
- Discuss the importance of a Shine-Dalgarno sequence in bacterial translation. Students should search for a Shine-Dalgarno consensus sequence in *E. coli* and add to the 5’ end of their codon-updated cat proinsulin sequence.
- Introduce students to the concept of PCR focusing on the role of DNA oligonucleotides (primers). Explain the general properties of robust primers (length, GC content, melting temperature, primer dimers, and potential hairpin loops). Students will design their own primers to PCR the modified cat proinsulin gene.
- → Instructor will order the synthesis of modified cat proinsulin and primer sequences.

Week 4: Wait For Synthesis of DNA and Primers Perform Another Activity.

Week 5: PCR (Polymerase Chain Reaction) of Cat Proinsulin Gene

Estimated preparation time for lab: 1 hour

Big picture: Students use PCR to copy the synthesized cat insulin gene.

- In week 3 the students designed a DNA sequence for the expression of cat proinsulin in *E. coli*. Their design removed an intron, incorporated a Shine-Dalgarno sequence for translation in *E. coli*, and made changes in codons to suit *E. coli* codon bias. The students also designed forward and reverse primers for use in the polymerase chain reaction. This week, we will amplify the synthesized DNA sequence through PCR.
- Although the proinsulin gene construct was synthesized, there is not enough DNA for an

entire course to clone and transform into *E. coli*. Therefore, the students will use their self-designed primers to PCR amplify the construct.

- PCR is useful to amplify the small amount of synthesized cat proinsulin DNA. The procedure is outlined in student handouts.
- Students will also prepare gels for gel electrophoresis this week. Gel red dye may be added to the agarose solution prior to pouring. Having this dye in the gel allows for visualization of the DNA as the gels are being run.

Week 6: Gel Electrophoresis and Cloning of PCR Products

Estimated preparation time for lab: 1 hour

Big picture: Students use gel electrophoresis to determine if PCR was successful, and to clone these products into a plasmid for transformation into *E. coli*.

- Students will perform agarose gel electrophoresis of their PCR reaction (week 4). If PCR were successful, the students should observe a ~500 bp band on the gel.
- When preparing the samples for gel electrophoresis, it is important that the students only load a portion (typically 20 μ L or less) of their PCR reaction. The remaining ~30ul of PCR product is saved for later use.
- If a correct size band is present on the gel, proceed to cloning and transformation. This protocol is described in detail in the student handouts. Briefly, students will use ~2 μ L of the remaining PCR product as an “insert” for TOPO-TA cloning into the pCR2.1 cloning vector.
- Following a benchtop incubation, ligated plasmids are transformed into chemically competent *E. coli* using the heat shock method. *E. coli* are then plated on LB-Kan50 (Kanamycin 50ug/mL final concentration) per user manual instructions. Plates are incubated at 37°C overnight.

Week 7: Screening of Colonies for Recombinant Plasmid

Estimated preparation time for lab: 1 hour

Big picture: If transformation is successful, students will screen for colonies that contain recombinant plasmids (i.e. plasmids containing the cat proinsulin gene).

- Since cloning and transformation is not 100% efficient, it is possible that some *E. coli* colonies contain “empty” plasmids, i.e. plasmids lacking the cat insulin gene.
- Students will perform two complementary methods for selecting colonies that contain recombinant plasmids: 1) Colony PCR and 2) Restriction digests.

- Students will select 3 or 4 random colonies off the plate for “colony PCR”. Colony PCR is identical to regular PCR with one major change: the “template” is not purified DNA, but a tiny dab of the colony itself. During the initial denaturation step of PCR, some of the bacteria in the PCR mixture will lyse open releasing the plasmid(s) they contain. These plasmids then serve as template for PCR.
- Emphasize to students that too much of the colony will inhibit the PCR. Have students add a barely visible amount of colony to each PCR reaction.
- The primers used for colony PCR should be the laboratory standard M13 Forward and M13 Reverse primers. These primers are typically shipped along with TOPO-TA cloning kits and are commonly used in colony PCR reactions. These primers bind to sequences that flank the location of an insert (i.e. the cat proinsulin gene).
- Successful colony PCR will result in ~500 bp product in gel electrophoresis. Colonies that resulted in successful PCR should be saved for future analysis, either temporarily at 4°C or preferably -80°C glycerol stock.
- Students will choose a colony and resuspend it in the lysis buffer, plasmids are purified using any number of commercially available “miniprep kits”. See student handout for example kit. These kits allow the student to purify plasmids from up to 5mL of cultured bacteria.
- Students are provided with a restriction map of the pCR2.1 cloning vector (from user’s manual) and asked to perform a virtual restriction digest using a variety of commercially available restriction enzymes. The students can sketch mock gels indicating expected sizes of DNA fragments.
- HindIII and SphI are efficient enzymes for the actual digest. Neither HindIII nor SphI cut within the cat proinsulin sequence, and they share compatible buffers for double digests (cutting with both at the same time). HindIII cuts once in pCR2.1, and SphI cuts twice. When double digested with HindIII and SphI, three fragments of DNA should appear on the gel.
- The relative restriction sites and resulting DNA fragments allow the student to determine whether or not the plasmid contains an extra ~500 bp (corresponding to the cat insulin gene).
- Both colony PCR and restriction digests are used to confirm the presence of the cat proinsulin sequence in the plasmids. While only one approach is typically necessary in the “real world”, having the students complete both complementary approaches teaches two sets of principles: colony PCR and restriction mapping.

- Save PCR products and restriction digests in -20°C freezer for next week.
- Have your students “patch” plate the colonies tested on another LB plate with kanamycin and incubate them at 37°C. The colonies will be saved for later use if they yield positives.

Week 8: Gel Electrophoresis of Colony PCR Products & Restriction Digests

Estimated preparation time for lab: 1 hour

Big picture: Students will examine results from the colony PCR screening and the restriction digests to determine if any are positive for the cat insulin insert.

- At the beginning of the lab, students should discuss the expected sizes of DNA fragments from colony PCR screening and restriction enzyme digests for both empty plasmids and plasmids with the cat insulin insert.
- In order to determine if colony PCR and restriction mapping is successful, students will need to perform gel electrophoresis identical to week 5 (above).
- Students will prepare two gels: one 0.8% gel for examining the restriction digest and one 1.2% gel for examining the colony PCR products.
- Recombinant plasmids identified by either colony PCR or restriction digests should be purified using a PCR clean up kit and sent for sequencing (see student handouts) using plasmid-specific M13F and R primers.

Week 9: Review DNA Sequences

Estimated preparation time for lab: None

Big picture: Students will analyze the raw sequence file of their recombinant plasmids; attempt to align in BLAST.

- Students will learn about dideoxy sequencing and how to analyze and interpret chromatograms.
- If using raw sequence files in ABI file format, the students will need to download a suitable analysis program (*e.g.* FinchTV, see student handout for link).
- Students will attempt to align the sequence with the known cat proinsulin gene to confirm that the construct was correctly synthesized, cloned into pCR2.1, and transformed into *E. coli*.
- Students will attempt to identify any mismatches. As a group, the students will summarize the results of sequencing and generate a figure containing an alignment of the synthesized DNA sequence the class purchased and the DNA sequence of the cloned construct.

Week 10: Extra Week for Problems that May Arise

Sample Results

Results from student experiments are provided for colony PCR screening (Fig. 6), restriction digest screening (Fig. 7), sequencing data (Fig. 8), BLAST analysis of cat insulin recombinant DNA with cat insulin gene (Fig. 9), and alignment of the predicted proinsulin from recombinant DNA with cat proinsulin (Fig. 10).

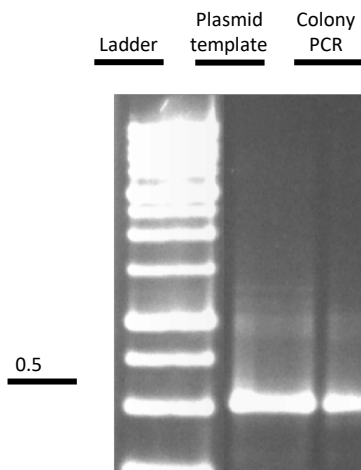


Figure 6. Colony PCR of Kan-resistant colonies confirm presence of recombinant plasmids. Individual colonies were used as templates in colony PCR with plasmid-specific M13 forward and reverse primers. The presence of a ~500 bp product (arrow) confirms presence of an insert indicative of a proinsulin insert. Previously-confirmed plasmids were used as a positive control for PCR.

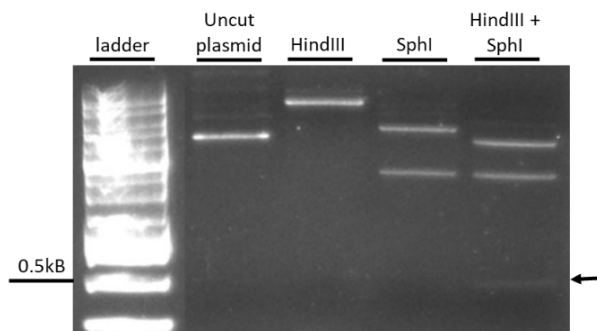


Figure 7. Restriction digests confirm presence of insert in recombinant plasmids. Purified recombinant plasmids isolated from colonies were digested with HindIII and SphI. Double digests (HindIII and SphI) produce three bands, including a ~500 bp band indicative of the pro-insulin insert.

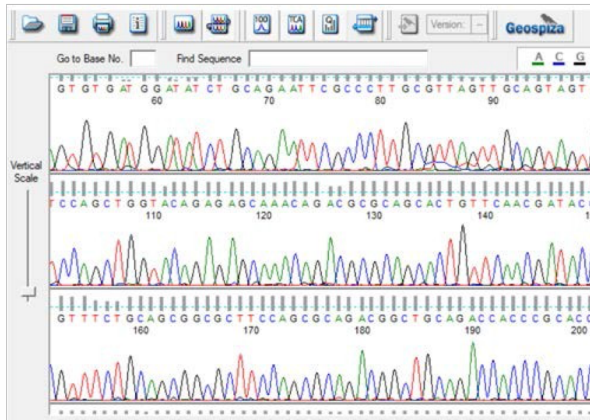


Figure 8. Sequencing of cat proinsulin from a purified plasmid template yields clean chromatograms for student analysis. Purified recombinant plasmids were sequenced using M13 forward and reverse plasmids and analyzed using FinchTV software.

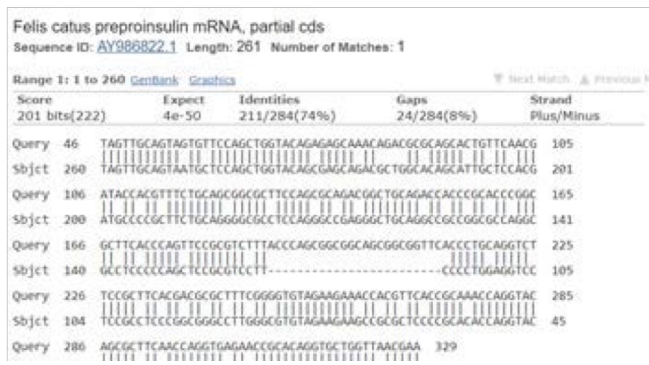


Figure 9. Recombinant cat proinsulin successfully matches with cat preproinsulin in BLAST. NCBI nBLAST using sequences obtained from recombinant plasmids (Query) match 74% with partial coding sequence of cat preproinsulin (Sbjct, AY986822.1). Mismatches indicate *E. coli*-optimized codons. The 24 base pair gap is a partial intron not removed before original DNA synthesis.

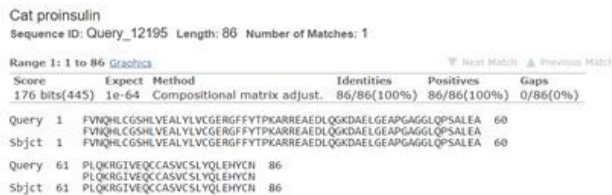


Figure 10. Predicted primary amino acid sequence of codon-optimized cat proinsulin (Query) align 100% with published cat proinsulin (Sbjct).

Evaluation of the Activity

Since its inception, our biology program has incorporated a research experience in the form of a senior thesis research project in which students develop a research proposal, design and perform experiments, analyze results, present their research orally and written as a thesis. Our current departmental goal is to institute real research experiences earlier in the curriculum. The cat insulin project is our first attempt at designing our own research project for implementation in a course laboratory; from our viewpoint, this experience is what a laboratory course should be. Students are introduced to the process of science and how to approach a scientific problem. Students also gain a more realistic view of science and the challenges that occur during the research process. The technical skills that students learned in the traditional laboratory format are still intact, but now these skills have context to their use. The laboratory reinforces lecture content on gene expression.

This experience has been implemented in four laboratory sections of our cell and molecular biology-focused general biology course in the fall, 2016 and another two sections in the fall and winter of 2017-18. Informal feedback from students has been positive. Students from last year's courses continue to ask about the project. Students asking for recommendation letters often provide curriculum vitae or resumes that contain research experience sections highlighting their work on the cat insulin project. Student comments on anonymous course evaluations have also positive (Table 4).

Table 4. Comments from students on instructor course evaluations.

I liked the opportunity to do a real experiment as opposed to a guided experiment in which we all knew exactly what was going to happen. I felt it gave a much more accurate experience of what a lab environment should be.

Keep the cat insulin project.

The lab was very interesting. Sometimes it was a bit confusing, but it was still really cool to "clone" cat-insulin. There were so many different parts to it as well. Furthered the topics discussed in class.

The lab was very interesting and it was a process that showed us the way a real experiment would work and it addressed the things we were learning in class well. The labs we did also helped with learning how to use the lab equipment.

Very interesting lab that usually is not done at the freshman level. I really enjoyed it.

We were able to conduct an actual research project during the course of the quarter.

A formal assessment of this research experience was performed on the winter section of the course. A

survey including statements and a Likert scale for responses (1= strongly disagree, 2= disagree, 3= neutral, 4= agree, 5= strongly agree) was administered to students before and after the research experience. Statements on the survey were from the Colorado Learning Attitudes Survey (Semsar *et al.*, 2011), the Laboratory Course Assessment Survey (Corwin *et al.*, 2015) or statements specific to our course to measure learning outcomes, understanding of the scientific process, and attitudes towards science. Analysis of the pre- and post- survey data indicated that the cat insulin research experience positively impacted students. The research experience helped students practice finding information on experimental methods (Fig. 11), acquire technical skills in molecular biology (Fig. 12), understand the challenges of expressing a mammalian gene in *E. coli* (Fig. 13), learn the process of gene expression (Fig. 14), differentiate gene expression in bacteria and eukaryotes (Fig. 15), practice discussing scientific methods (Fig. 16), assist others in data collection and analysis (Fig. 17), and discuss problems related to research (Fig. 18). Approval to survey and evaluate student responses was obtained from the Rose-Hulman Institutional Review Board Human subject research protocol IR# RHS0306.

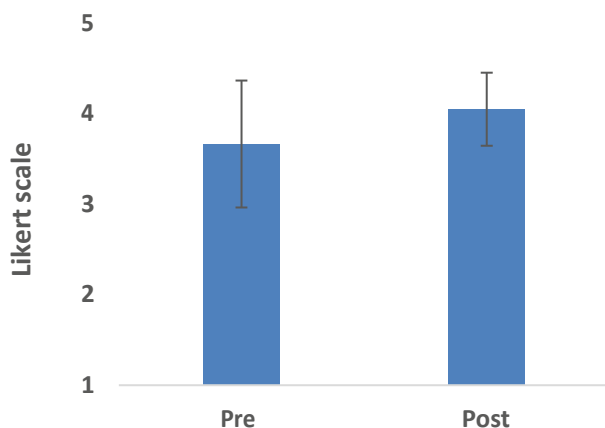


Figure 11. The cat insulin research experience enabled students to practice finding information on experimental methods as indicated by the student responses to the statement “I am able to find information on experimental methods” in the pre- and post-surveys. ($p = 0.02$)

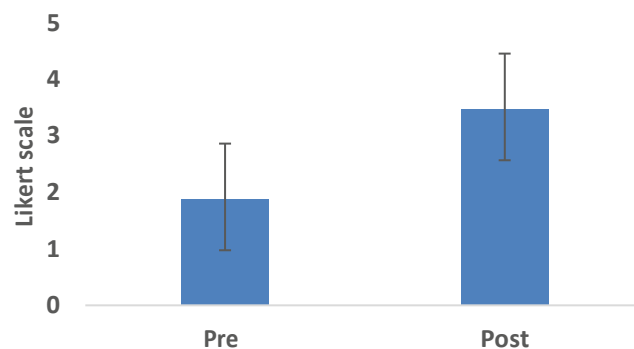


Figure 12. The cat insulin research experience enabled students to learn technical skills in molecular biology as indicated by the student responses to the statement “I have technical skills in molecular biology” in the pre- and post-surveys. ($p < 0.001$)

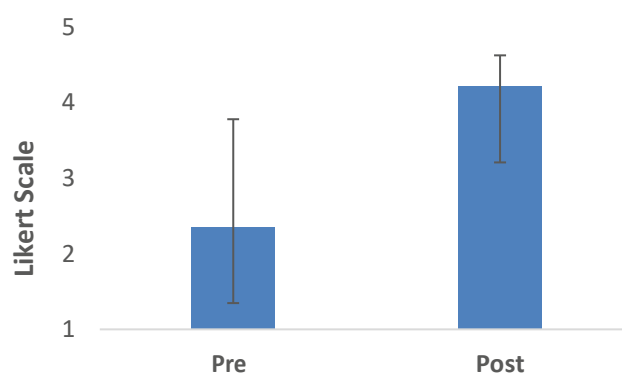


Figure 13. The cat insulin research project helped students understand the challenges of expressing a eukaryotic gene in *E. coli* as indicated by the student responses to the statement “I understand the challenges of expressing a gene from a mammal in *E. coli*.” ($p < 0.001$)

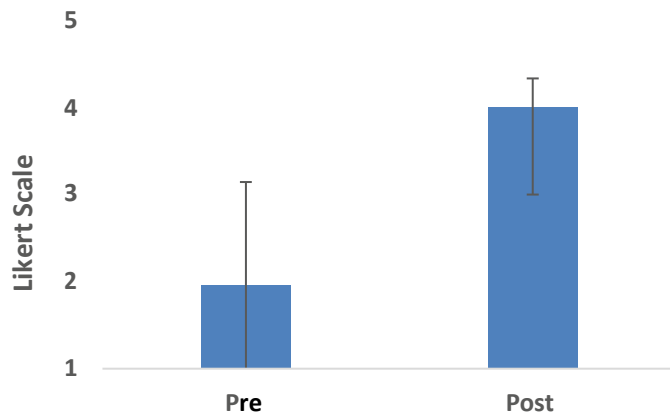


Figure 14. The cat insulin research project helped students learn gene expression as indicated by the student responses to the statement “I understand the process of gene expression.” ($p < 0.001$)

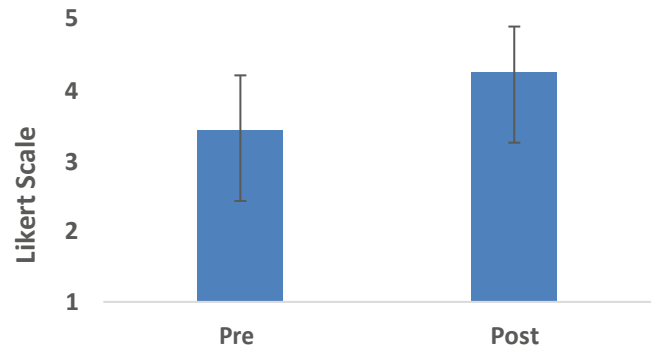


Figure 16. The cat insulin research project encouraged students to share their research with peers and the instructor as indicated by the student responses to the statement “In this course, I was encouraged to discuss elements of my investigation with classmates or instructors.” ($p < 0.001$)

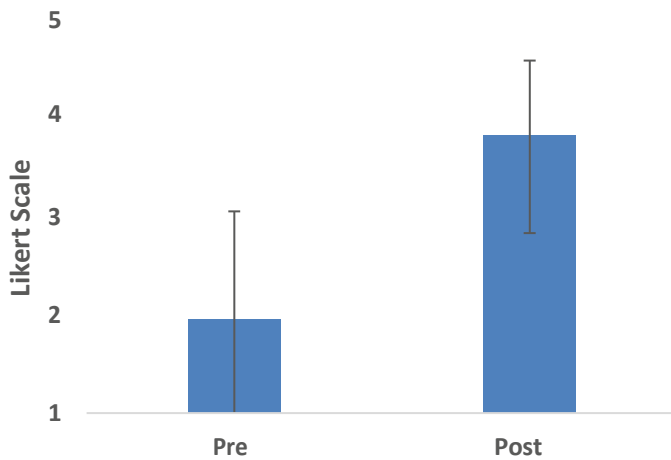


Figure 15. The cat insulin research project helped students differentiate gene expression in eukaryotes and bacteria as indicated by the student responses to the statement “I can discuss the differences between gene expression in bacteria and eukaryotes.” ($p < 0.001$)

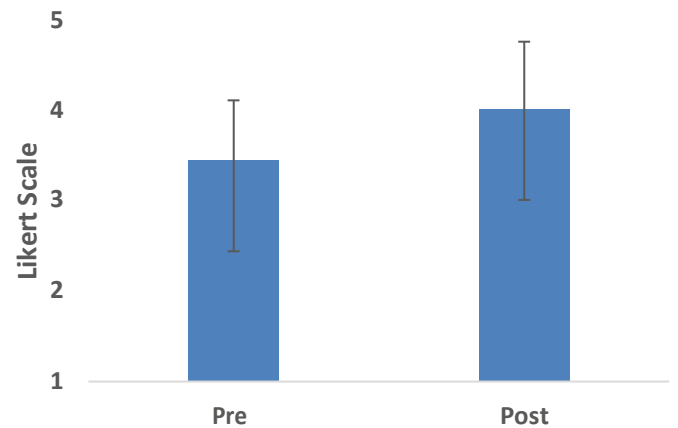


Figure 17. The cat insulin research project encouraged students to help other students collect or analyze data as indicated by the student responses to the statement “In this course, I was encouraged to help other students collect or analyze data.” ($p = 0.006$)

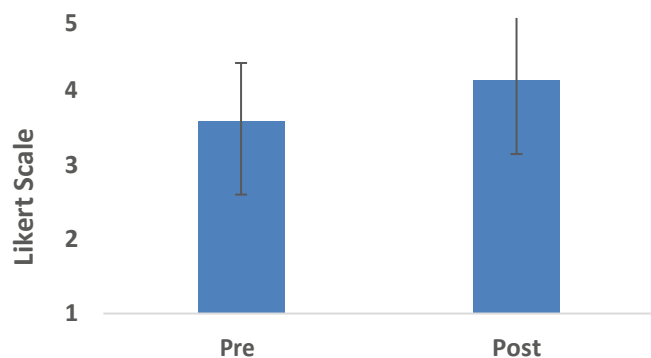


Figure 18. The cat insulin research project encouraged students to discuss problems encountered during their research as indicated by the student responses to the statement “In this course, I was encouraged to share the problems I encountered during my investigation and seek input on how to address them.” (p-0.008)

Cited References

- American Society for the Advancement of Science. 2011. Vision and Change in Undergraduate Biology Education: A Call to Action. AAAS, Washington, DC, 79 pages.
- Auchincloss, LC, SL Laursen, JL Branchaw, K Eagan, M Graham, DI Hanauer, G Lawrie, CM McLinn, N Pelaez, S Rowland, M Towns, NM.Trautmann, P Varma-Nelson, TJ Weston, and EL Dolan. 2014. Assessment of course-based undergraduate research experiences: a meeting report. *CBE-Life Sci Educ*, 13: 29-40.
- Bascom-Slack, CA, AE Arnold, and SA Strobel. 2012. Student-directed discovery of the plant microbiome and its products. *Science*, 338: 485-486.
- Chan, SJ, J Weiss, M Konrad, T White, C Bahl, S-D Yu, D Marks, and DF Steiner. 1981. Biosynthesis and periplasmic segregation of human proinsulin in *Escherichia coli*. *P Natl Acad Sci USA*, 78: 5401-5405.
- Corwin, LA, C Runyon, A Robinson, and EL Dolan. 2015. The laboratory course assessment survey: a toll to measure three dimensions of research-course design. *CBE-Life Sci Educ*, 14: 1-11.
- Goeddel, DV, DG Kleid, F Bolivar, HL Heyneker, DG Yansura, R Crea, T Hirose, A Kraszewski, K

Itakura, AD Riggs. 1979. Expression in *Escherichia coli* of chemically synthesized genes for human insulin. *P Natl Acad Sci USA*, 76: 106-110.

Heath, WF, RM Belagaje, GS Brooke, RE Chance, JA Hoffman, HB Long, SG Reams, C Roundtree, WN Shaw, LJ Sliker, KL Sundell, and RD DiMarchi. 1991. (A-C-B) human proinsulin, a novel insulin agonist and intermediate in the synthesis of biosynthetic human insulin. *J Biol Chem*, 267: 419-425.

Lopatto, D. 2004. Survey of undergraduate research experiences (SURE): first findings. *Cell Biol Educ*, 3: 270-277.

National Academies of Sciences, Engineering, and Medicine. 2015. Integrating Discovery-Based Research into the Undergraduate Curriculum: Report of a Convocation. National Academies Press, Washington, DC, 136 pages.

Semsar, K and JK Knight, G Birol, and MK Smith. 2011. The Colorado learning attitudes about science survey (CLASS) for use in biology. *CBE-Life Sci Educ*, 10: 268-278.

Shortlidge, EE and SE Brownell. 2016. How to assess your CURE: a practical guide for instructors of course-based undergraduate research experiences. *J Microbiol Biol Educ*, 17: 399-408.

Yang, Y, Q Hua, EH Shimizu, MH Choquette, RB Mackin, and MA Weiss. 2010. Solution structure of proinsulin. *J Biol Chem*, 285: 7847-7851.

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About the Authors

Jennifer O'Connor and Peter Coppinger are Associate Professors of Biology at Rose-Hulman Institute of Technology where they are engaged in teaching introductory courses in biology for science and engineering majors. Jennifer teaches upper-level courses in microbiology, immunology, and cancer biology. Peter teaches upper level courses in genetics, plant biology, and applied microbiology.

Appendix: Timetable

Table 5. Weekly list of activities and supplies needed per lab section and student groups.

Week	Activities	Supplies per lab section	Supplies per group of 4 students
1	Biosafety training	Glo germ and UV light Shaving cream Gloves (all sizes)	
	Pipetting practice	pipettors (all volumes) Pipette tips (all sizes) Water (1 mL aliquot) Microfuge tubes Pipetting standards with blue dye (562 μ L, 50 μ L, 10 μ L, 1 μ L) – 1 set with instructor	Set of pipettors Container of each size pipette tip 4 tubes of 1mL water Container of microfuge tubes Disposal bags Sharpies
	Introduction to research project	Handouts	
2	Finding and annotating cat preproinsulin gene	Computers Internet access	
3	Designing a sequence for expression of cat insulin in <i>E. coli</i>	Computers Internet access ***Order synthetic DNA and primers.	
4	Break week – do another activity		
5	Amplification of synthetic DNA	Forward cat insulin primer (10 μ M) Reverse cat insulin primer (10 μ M) Taq polymerase buffer Molecular grade water dNTPs (10mM) Taq polymerase (for instructor only) Synthesized DNA template (10ng/ μ L) Thermocycler Ice buckets and ice 0.5 ml PCR tubes Agarose 1x TBE buffer Gel red dye Erlenmeyer flasks Gel casting apparatus Plastic wrap Sharpies Pipettors Pipette tips (all sizes)	Microfuge tubes containing at least: 40.5 μ L molecular grade water 1 DNA template (10ng/ μ L) 1 μ L forward primer (10 μ M) 1 μ L reverse primer (10 μ M) 5 μ L buffer 1 μ L dNTPs (10 mM) Ice bucket and ice 0.5 g agarose 400 mL 1x TBE buffer 5 μ L gel red dye Erlenmeyer flask Gel casting apparatus with combs Plastic wrap Sharpies Set of pipettors Container of each size pipette tip
6	Gel electrophoresis of PCR products; TOPO TA cloning and transformation	PCR products Gel apparatus Power supplies Loading dye	Team PCR product on ice Ice bucket with ice Team poured gel Power supply

		<p>1 kb DNA marker Ice buckets and ice TOPO reagents Microcentrifuge tubes Sharpies Competent cells SOC media) 300 μL Xgal solution (40mg/mL) 42°C water bath with floaters for tubes LB plates with kanamycin Hockey sticks Turn tables for spreading Petri dishes with alcohol Bunsen burners Matches 37°C incubator Pipettors</p>	<p>Loading dye (7 μL) 1 kb DNA marker (5 μL) TOPO salt solution (> 1 μL) Molecular grade water (>5 μL) TOPO vector (exactly 1 μL) Container of microfuge tubes Sharpie Competent cells (50 μL) SOC media (300 μL) Xgal solution (120 μL) LB plates with kanamycin – 2 plates per group Hockey sticks – 1 per group Turn tables for spreading – 1 per group Petri dishes with alcohol – 1 per group Bunsen burners – 1 per group Matches – 1 per group Set of pipettors Container of each size pipette tip</p>
7	Examination of plates; mini-prep of plasmid DNA; restriction digest; PCR screening	<p>Plates of transformed colonies Sharpies Microcentrifuges Microcentrifuge tubes (1.5 mL) Sterile toothpicks (for picking colonies) Zyppy mini-prep kit HindIII restriction enzyme (added by instructor) HindIII 10x buffer Water bath at 37°C PstI restriction enzyme (added by instructor) PstI buffer Molecular grade water Water bath at 37°C Pipettors Pipette tips (all sizes) M13 forward primer from TOPO kit M13 Reverse primer from TOPO kit 10X Taq polymerase buffer dNTPs (10 mM) Molecular grade water 0.5 PCR tubes 0.5 μL Taq polymerase (for instructor) Thermocycler</p>	<p>Team plates of transformed colonies Sharpie Container of microfuge tubes Sterile toothpicks Aliquots of Zyppy plasmid reagents and column tube HindIII 10x buffer (>2 μL) PstI buffer (>2 μL) Molecular grade water (50 μl) Set of pipettors Container of each size pipette tip 1 μL of M13 Forward primer – from TOPO TA kit 1 μL of M13 Reverse primer – from TOPO TA kit 5 μL 10X Taq polymerase buffer 1 μL dNTPs (10 mM) 41 μL Molecular grade water Container of 0.5 mL PCR tubes</p>
8	Gel electrophoresis of colony PCR and restriction digests	<p>Colony PCR products (-20°C freezer) Restriction enzyme digests (-20°C freezer) Sharpies</p>	<p>Team colony PRC products on ice Team restriction digests on ice Sharpies Strip of parafilm Ice bucket and ice</p>

		Strips of parafilm Ice buckets and ice Gel apparatus and 12-well combs Power supply Loading dye TBE buffer Gel red dye Agarose Erlenmeyer flasks PCR clean-up kit (one per pipettors Pipette tips (all sizes) Microfuge tubes (1.5 mL) *** Send sequences off to be sequenced using M13 forward and reverse primers – we use MC Labs)	Gel apparatus and 12-well comb Power supply Loading dye (35 μ L) 1 kb DNA marker (10 μ L) TBE buffer (500 mLs) Gel red dye (10 μ L) Agarose (1 g for two gels – one 0.8% and one 1.2%) 2 Erlenmeyer flasks Container of microfuge tubes Set of pipettors Container of each size pipette tip
9	Analysis of Sequences	Computers Internet access	
10	Extra week – for when something goes wrong		

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