Chemical Biology & Biochemistry Laboratory Using

Genetic Code Expansion

Manual

Table of Contents

Background

Proteins play vital roles in most biological processes; these roles include acting as catalysts for physiological reactions, as regulators for those reactions, or as structural framework around which these processes can occur. Proteins' complex organization of diverse functionality in 3D space leads to an astonishing range of function for living organisms. Understanding this intimate relationship between structure and function is the backbone of understanding the natural world and is the key to controlling it. Site-directed mutagenesis (replacing one amino acid codon in a gene with another one) is a common and useful tool for investigating protein function. However, despite their functional complexity, proteins have a relatively simple structural basis: all organisms use the same 20 amino acids to build proteins, all of which have limited chemical functionality (Figure 1). Therefore, in order to expand the range of studies that can be performed on proteins, non-canonical amino acids (ncAAs; also referred to as unnatural amino acids, UAAs) with unlimited chemical functionality are obvious tools for studying these structure-function relationships.

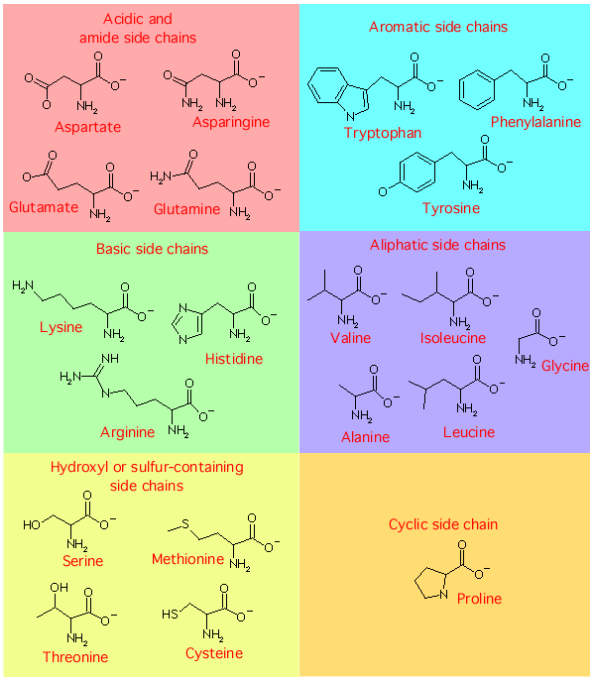


Figure 1. The twenty natural amino acids

Manipulating proteins to permit use of ncAAs involves exploiting the central dogma of molecular biology—the transcription of a DNA sequence into mRNA, and the translation of this mRNA sequence into a foldable, functional peptide chain. Modification

at the DNA level, therefore, will result in a modified protein. The nature of the genetic code, however—with multiple degenerate codons specifying the same amino acid—leaves no "unused" codons for easily adding ncAAs *in vivo*. Therefore, a codon must be "hijacked" so that it can be used to specify the ncAA by replacing a natural codon in a sequence. The stop codon TAG, the least used of the three stop codons, serves this role and can easily replace any existing codon along a sequence via site-directed mutagenesis. In order to preclude this codon from signifying the termination of the peptide chain, it must function as the other "natural" codons do: having an aminoacyl-tRNA synthetase (RS) bind an amino acid to a custom tRNA, which then adds the amino acid to the peptide chain via the ribosome (Figure 2). Since the amino acid is not naturally occurring, no corresponding RS/tRNA pair is naturally present in the cell.

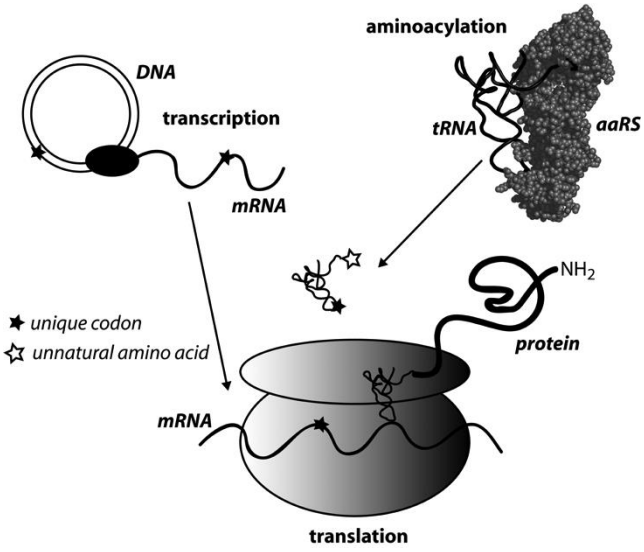


Figure 2. Processes of transcription and translation

To facilitate specific ncAA incorporation, the cell must therefore be provided with a ncAA-RS that aminoacylates the paired tRNA with only the ncAA, but no endogenous amino acids. To do this, first a RS and a tRNA must be found in nature that can be used by (are *orthogonal* to) a host cell. This pair must be able to work both together and with the ribosome of *E. coli*, and the tRNA must be able to site-specifically recognize the TAG codon. A new, non-toxic ncAA can be chosen for the interesting chemical properties that it may introduce into the protein. Then an RS must be selected that is able to bind the

ncAA of interest when aminoacylating the tRNA. Directed evolution of RSs involves rounds of positive and negative selection on a library of RSs (up to 10^8 members), which all share the same overall structure but have a variety of mutations focused around the amino acid binding site. Positive selection rounds involve transforming library members into cells containing a plasmid with the chloramphenicol acetyltransferase gene (CAT), which confers chloramphenicol resistance, containing a TAG codon. The cells are then grown in the presence of both the ncAA and the antibiotic chloramphenicol; the members that can successfully incorporate the ncAA (as well as those that incorporate endogenous amino acids) produce fully functional CAT protein and can survive in the chloramphenicol-containing medium. To exclude the remaining members that used an endogenous amino acid instead of the ncAA, a round of negative selection is then performed. Negative selection rounds involve isolating the RS-containing plasmids from surviving positive selection cells, then transforming the remaining RS members into cells with another plasmid. This plasmid contains the toxic barnase gene with a TAG codon in the middle, which encodes a toxic protein that kills the cell if successfully produced. ncAA is excluded from the media in negative selection rounds, so that RS members that incorporate a natural amino acid in response to the TAG codon do not survive. Several alternating rounds of positive and negative selection are performed until the remaining RSs are those that can efficiently attach a ncAA to a tRNA (and therefore make protein containing a TAG codon), but cannot attach a natural amino acid to that tRNA.

In order for the cell to produce mutant TAG protein and its necessary ncAA-RS/tRNA pair, it must be provided with the genes that encode them. The genes are introduced into *E. coli* cells via the plasmids *pBad* and *pDule* (Figure 3).

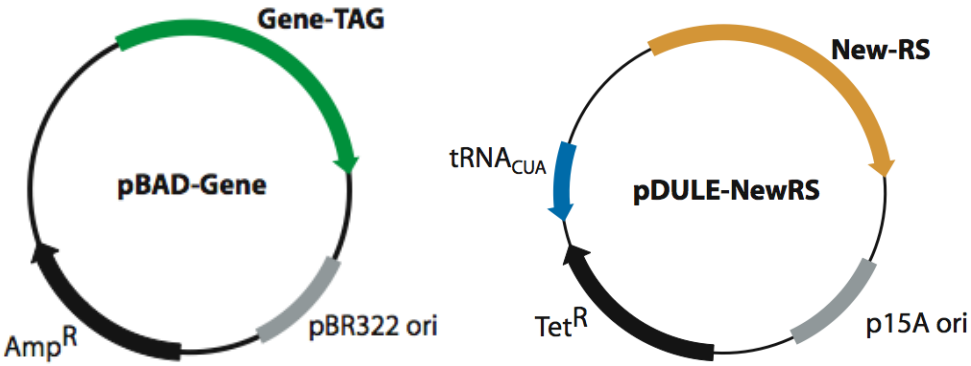


Figure 3. Genes and elements on the pBad and pDule plasmids

The *pBad* plasmid contains the gene (containing a TAG codon) that encodes the protein of interest controlled by an arabinose-induced promoter, an origin of replication, and a gene that encodes β-lactamase (which confers ampicillin resistance). When cloned into the pBad plasmid, codons for 6 histidines are added to either the N- or C-terminus of the gene, which allows for affinity purification of the overexpressed protein. The *pDule* plasmid contains genes that encode the ncAA-RS and the $tRNA_{CUA}$, an origin of replication (must be compatible with the pBAD origin of replication), and a gene that encodes TetA protein (which confers tetracycline resistance). These plasmids must both be transformed into the cell in order for full-length, ncAA-containing protein to be produced. To verify that the cells contain both plasmids before initiating overexpression, the cells are grown in the presence of the antibiotics ampicillin and tetracycline, ensuring that only cells that contain both plasmids will be able to grow in the media.

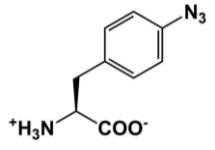
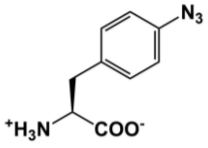
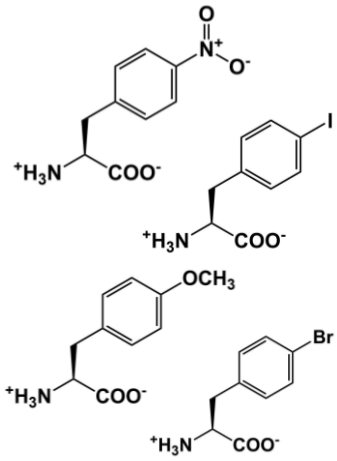
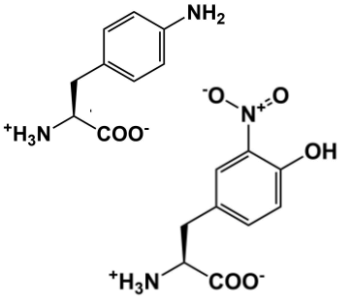
Cells containing the appropriate plasmids can then be induced to overexpress the protein of interest using arabinose autoinduction media. The *pBad* plasmid contains an arabinose promoter system that activates expression of the gene on that plasmid in the presence of arabinose. The autoinduction media is designed to allow cells to reach high density *before* overexpression is induced, so that a larger number of cells are available to

overexpress protein when induction begins. The media autoinduces by using defined sugar concentrations: when the glucose levels begin to decrease due to cellular metabolism and growth, the cells begin to uptake the arabinose that is available. Although they cannot metabolize it for further growth, the arabinose functions as an activator for the promoter on the *pBad* plasmid, thereby inducing protein overexpression. Crude protein gel electrophoresis can then easily verify the success of this process by monitoring the size of protein (full-length vs. truncated) produced in the presence and absence of ncAA. Full-length protein produced in the presence of ncAA, and truncated protein produced in the absence of ncAA, would indicate that the ncAA-RS/$tRNA_{CUA}$ system was able to recognize the TAG codon and incorporate the ncAA, but not any endogenous amino acids.

The quality of a ncAA-protein study is defined by what can be understood about the protein's structure or function, or by the new ability conferred due to the ncAA's presence in the protein. Because ncAAs expand the limited chemical potential of amino acid residues, a wide variety of studies and applications become feasible that explore the sensitive yet resilient nature of proteins when using ncAAs. Depending upon the location and type of amino acid substitution, a wide range of effects on protein stability and/or function may occur: both may be unaffected, the stability may be unaffected but activity destroyed, or the protein may not even fold properly, thereby destroying function. Some ncAAs placed in the enzyme active site have even been shown to *improve* the function of the enzyme by altering the electrostatics of binding or catalysis. If the stability and function of the protein appear largely unperturbed by the addition of the ncAA, then the chemical properties of the ncAA may be utilized to yield relevant information about

the structural states of the protein. Examples of different ncAAs and their applicable studies are outlined in Table 1.

Table I. *Some ncAA families and their applications*

Category of ncAA	**Example Structure**	**Applications**
Photocrosslinking		Provide snapshots of *in vivo* protein interactions
Biorthogonal ligation		Conjugate fluorophores; surface functionalization
Size and polarity probes		Alter packing, sterics, and other interactions to probe structure/function relationships
pH probe		Add, remove, and alter hydrogen bonding interactions to study structure/function relationships

Whether the function of the protein is improved or better elucidated, or the structure and dynamics of the enzyme are better understood, the use of ncAAs has enabled a greater range of studies on protein structure-function relationships.

Deep Thoughts on Deep Thoughts

Throughout this manual, you will find asides, entitled “Deep Thoughts,” on a variety of different topics. These are intended to guide you to develop a deeper understanding of your project and of the techniques you are using as well as to provide some things to consider when designing your experiment, analyzing your results, and carrying out various steps of this laboratory. These suggestions are by no means exhaustive but are intended to help you think critically about what you are doing and guide your design and thought process.

We expect that you have read, considered, and answered these questions on your own before asking the instructor or TA for help.

Week	Overview of Laboratory Responsibilities Tentative Schedule Refer to separate schedule for specific assignment due dates
1	Perform a literature search on enzyme, its genetic sequence, and potential ncAAs and sites of incorporation. • Students' laptops will be needed to perform a literature search on their enzyme, its genetic sequence, and potential ncAAs and sites of incorporation • Download and study crystal structure of enzyme • Investigate the ncAAs and sites that will be studied throughout the term *To prevent bottlenecks using equipment for kinetic assays, three groups in each section have been assigned carbonic anhydrase and three groups catalase.*
2	Express wild-type protein and continue to develop hypothesis and plan experiments. • Mix autoinduction media and obtain *E. coli* cells with appropriate expression plasmids to start overnight protein expression of wild-type protein. • Develop experimental hypothesis based on the ncAAs and sites that your team is selecting to study. • Tell professor by Thursday, Week 2 at 5 pm which protein sites and ncAAs will be expressed so starter cultures can be prepared for class week 3. • Begin a literature search for assay procedures. • Harvest cells 48 hours after induction and store at -80 °C. • Prepare SDS-PAGE solutions. • Analyze wild-type protein production by crude SDS-PAGE.
3	Express ncAA-mutant and wild-type proteins, purify wild-type protein, and continue to develop assay(s). • Mix autoinduction media and obtain *E. coli* cells with appropriate expression plasmids to start overnight protein expression of ncAA-mutant proteins and wild-type protein. • Prepare protein purification buffers. • Understand assay procedure. • Purify wild-type protein from *E. coli* cells. • Make assay/storage buffer(s). • Desalt wild-type protein sample into assay/storage buffer. • Harvest cells 48 hours after induction and store at -80 °C. • Analyze ncAA-mutant protein production by crude SDS-PAGE.

4	Purify ncAA-mutant proteins and preliminary assessment of purified wild-type protein. • Analyze purified wild-type protein by SDS-PAGE. • Determine protein concentration. • Determine if pure protein is active. • Purify ncAA-mutant proteins from cells.
5	Preliminary kinetics assay of wild-type protein and preliminary assessment of purified ncAA-mutant proteins. • Begin preliminary kinetic assay trials with wild-type protein. • Analyze purified ncAA-mutant proteins by SDS-PAGE. • Determine protein concentrations. • Determine if pure protein is active.
6-9	Continue to design and execute studies on pure protein including testing of wild-type and ncAA-mutant proteins with kinetic assays.
10	Lab clean-up and Presentations in lab sections. • Final paper and notebooks due Monday of Finals Week at 4 pm.

Objectives for Week 1

- Laptops will be used for reading and understanding the background information on expressing proteins containing non-canonical amino acids (ncAAs).
- Obtain and discuss with peers information on the genes and sites of ncAA incorporation available this term.
- Determine the amino acid sequence of the proteins and ncAA locations.
- Register to use and install the free educational version of Pymol at http://pymol.org/edu/
- Download a crystal structure of the proteins from the online Protein Data Bank.
- Load and study the protein structure in Pymol (for help getting started see http://www.pymolwiki.org/index.php/Practical_Pymol_for_Beginners as well as the PyMol Tutorial posted on Canvas).
- Consider which location and which ncAAs will be incorporated/studied based on literature searches and the Pymol image. Identify sites that have already been mutated. Finalize selection of which protein you will study this term and begin to develop hypothesis for term-long projects.
- Set up group bench and calibrate and sterilize pipettes.
- Obtain instructions on expressing protein from the plasmid-containing cells.
- Ensure that the necessary media component solutions and sterile equipment are prepared and available for expressions during week 2 (wild-type protein) and week 3 (set of wild-type and ncAA-mutant proteins).

Concepts for Choosing and Expressing Mutants

Your goal is to design a meaningful scientific study surrounding one of the enzymes that has been chosen for you: thermostable human carbonic anhydrase II or *E. coli* catalase HPII. Traditionally, biochemists are able to change any amino acid in a protein to any of the other naturally occurring amino acids by standard mutagenesis methods. This is common to facilitate the understanding of how a protein works (regulation, binding etc.) and to improve its utility for other applications (stability, activity etc.). Here, we will break from tradition and enable you to select from new amino acids structures (non-canonical amino acids) to study proteins in ways that were not possible before. One could put the ncAAs anywhere in the protein via genetic code expansion, but because of time constrains of mutagenesis, we are forced to select some sites for you to explore. The key to this endeavor is to identify what is important about the sites we have selected for you regarding the proteins' stability, activity, regulation, etc. and then select from the new chemical ability of the ncAAs to develop a meaningful scientific study that has never been possible before.

Using the list of available mutation sites and ncAAs provided by the instructor (Appendix 6), choose one or two sites to explore by the incorporation of ncAAs. You will be able to generate two new mutant proteins to study in parallel with the wild-type protein. Searching the literature on your particular enzyme can highlight particular residues in that enzyme that are thought to have implications on the protein's structure or function. Carefully consider the chemical properties (i.e., the wild-type amino acid side chain) that already exist at the site(s), and the new properties that may be introduced by the ncAA. Is the site supposedly involved in the catalysis of the enzyme? Is it responsible for maintaining structural characteristics of the protein, or involved in relaying structural changes to different areas of the protein? These are important aspects to consider when selecting the sites and ncAAs that will be studied for the entire term. Be sure to base your study in the context of the scientific literature.

To facilitate the production and purification of the chosen ncAA-mutant proteins, the genes of interest a thermostable variant of human carbonic anhydrase II (CA) as well as *E. coli* catalase HPII (HPII) were commercially synthesized to optimize their codon usage for expression in *E. coli*. Both genes were cloned into the pBad expression plasmid. The cloning event removes the stop codon and adds a C-terminal histidine-rich affinity tag, allowing easy purification of the protein product. A stop codon (TAG) was then incorporated in place of a codon in the wild-type gene by using mutagenic primers. These two plasmids—one containing the wild-type gene, and the other with the TAG mutation—will allow production of wild-type and ncAA-mutant versions of the protein, respectively. With the set of pure proteins (wild-type and ncAA-mutant), each group will perform comparison studies of the structure and function of the wild-type and ncAA-mutant proteins. Relevant genetic sequences for each protein can be found in the corresponding tables in Appendix 2, and available ncAA structures can be found in Appendix 3. Using these genetic sequences, the predicted molecular weight and protein product sequence can be determined using the provided web resource. This information will be useful in later steps of the purification process.

Deep Thoughts: Amino Acid Structure

Consider the features of the naturally occurring amino acids you will be replacing. Naturally occurring amino acids are generally classified into a number of different categories: hydrophobic, hydrophilic, polar, non-polar, charged (negative or positive), uncharged, acidic, basic, aromatic, aliphatic (certainly the list could go on). Non-canonical or unnatural amino acids can be categorized into even more complex categories and often belong in many different categories at the same time. (For example, 4-bromophenylalanine, an aromatic amino acid which has a halogen substituent, allowing for electrostatic interactions not found in nature). It's important to consider how the features of the naturally occurring amino acid compare to the non-canonical amino acid you will be replacing it with. Are they both hydrophobic? Aromatic? Charged? Aromatic and charged? If it's different, how might these changes affect the structure and/or function of your protein? If it's similar, how will it be able to maintain the structure and/or function of your protein?

Another important feature of each amino acid that is important to consider (again linking to structure-function relationships) is hydrogen bonding. Hydrogen bonds are essential for holding proteins together and you have to consider how your new amino acid will affect hydrogen bonding interactions. Is your naturally occurring amino acid involved in hydrogen bonds? Can your new non-canonical amino acid participate in these same hydrogen bonds? Maybe it can't form any hydrogen bonds or maybe it forms new ones. Just because two things are close to each other does not mean they are going to hydrogen bond! You must consider not only distance and proximity but geometry and the surrounding hydrogen bond environment (other hydrogen bonds, being a donor or an acceptor, etc.). Hydrophobicity also plays into this reasoning. When you're considering improving thermostability like in the case of human carbonic anhydrase, establishing new hydrogen bonds has been shown to be an effective strategy for this. And since we're using non-canonical amino acids, even more bonds are possible!

Necessary Materials (to obtain for use in Week 2)

Equipment

- 250 mL sterile baffled flasks (1 for wild-type)
- Sterile pipette tips
- Sterile bottles and flasks (250 mL)
- Sterile 1.7 mL microcentrifuge tubes
- Sterile 14 mL round-bottom culture tubes for sfGFP positive control expression

Culture Preparation (Media components will be prepared and aliquoted by TAs)

- Sterile H_2O (autoclaved in 250 mL volume)
- Aspartate (5%, pH 7.5; adjust pH with NaOH, autoclave)
- Glycerol (10% vol/vol; autoclave)
- 18 AA mix (25 x) (stored at 4 °C)
- 25 x Mineral salts ("25 x M")
- Arabinose (20% wt/vol, sterile filter)
- $MgSO_4$ (1 M; autoclave)
- Glucose (40% wt/vol; autoclave)
- Trace metals stock solution (5000x)
- Ampicillin (1000x stock) (100 mg/mL in H_2O; sterile filter) (stored at -20 °C)
- Tetracycline (1000x stock) (25 mg/mL in DMF) (stored at -20 °C)
- 8 M NaOH
- Non-canonical amino acids of interest (powder form)

Suggested Resources and Protocols

- *Calibration of micropipettes*: See Appendix 1.
- Pymol http://pymol.org/edu/ and PyMol tutorials http://www.pymolwiki.org/index.php/Practical_Pymol_for_Beginners
- Protein Data Bank http://www.rcsb.org/pdb/home/home.do
- *Crystal structure program Pymol*: Go to the PDB (protein data bank) on the web and find a PDB file of the wild-type proteins human carbonic anhydrase II or *E.coli* catalase HPII. http://www.rcsb.org/pdb/home/home.do
- *Determining protein sequences and calculating protein molecular weights*: http://db.systemsbiology.net:8080/proteomicsToolkit/
- *Media preparation and protein expression*: A basic outline for this procedure is in Week 2. For a more in-depth discussion on the production, monitoring, and purification of ncAA-mutant proteins, see the following article, available on Canvas:
  Hammill, J.T.; Miyake-Stoner, S.; Hazen, J.L.; Jackson, J.C; Mehl, R.A. (2007) Preparation of site-specifically labeled fluorinated proteins for 19F-NMR structural characterization. *Nature Protocols* 2(10), 2601-2607.

Objectives for Week 2

- Obtain *E. coli* cells containing the appropriate expression plasmids of wild-type enzymes.
- Prepare autoinduction media (lab 1).
- Start 40-48 hr expression of wild-type and sfGFP control protein samples.
- Determine which type of polyacrylamide gel to use with for crude protein gel (SDS-PAGE) using information about the predicted protein structure and molecular weight (lab 1).
- Prepare SDS-PAGE buffers.
- Harvest cells and prepare samples for crude protein gel (lab 2).
- Pour and run SDS-PAGE on crude samples (lab 2).
- Begin a literature search for an assay procedure to test the enzyme kinetics. Resources will be provided on Canvas.
- Analysis of protein production by SDS-PAGE
    - Separate proteins on polyacrylamide gel
    - Fix and stain proteins on gel
    - Digitally image gel
- Read about protein purification procedures.
- Prepare purification buffers.
- Refine hypothesis and finalize selection of mutations and ncAAs for term project.

By end of Thursday of Week 2:

- **Notify professor which protein, mutations, and ncAAs will be used.**

Deep Thoughts on Lab Citizenship

As you embark on the experimental component on this course, it is important to consider appropriate lab citizenship. You are working in a lab space shared with many other students- not only your lab group but also the other lab groups in your class AND the lab groups in other sections, some of which are using the same bench space as you. You need to be considerate of all these people as well as any instructors and TAs when working in the lab- that's a lot of people!

A few recommendations:

- **Clean up after yourself**. Clean up your bench space and any common areas of the lab immediately after you use them (this includes the balance area, refilling pipette tip boxes, etc.)
- **Be courteous and respectful**. Be respectful of your group members and other people in lab. Be respectful of people's things- do not use or interfere with someone else's experiments or reagents.
- **Be prepared**. You are expected to show up prepared for that day's lab. Develop a plan ahead of time for what your group wishes to accomplish in lab and how you plan to go about accomplishing it. You have a limited amount of time in lab to work. Coming in with a plan already established is respectful to not only your other group members and the instructors but will also help you work efficiently and effectively.
- **Use lab resources wisely**. The reagents and supplies are expensive and your lab fees only cover a fraction of the costs of running this project-based lab.
- **Label**. All of your supplies should be appropriately labeled with your group names and contents. This not only assists your other group members in locating necessary reagents but also avoids the potential hazards and hassle of dealing with unknown reagents in the case of a spill or cleanup.
- **Be punctual**. Just as you would show up on time for a lecture course, you are expected to show up on time and prepared to work at the start of class.
- **Be safe.** Follow all appropriate safety procedures and ask if you have questions.

Concepts for Protein Expression

The appropriate proteins will be produced by growing cultures of *E. coli* DH10B cells, which have different plasmids in them depending on the ability needed. To ensure that only cells with the appropriate plasmids can grow in the media, they must be grown with antibiotics present. The wild-type protein will be produced from a pBad plasmid (pBad-gene), and cells containing pBad need to be grown in the presence of ampicillin. The ncAA-mutant protein will also be produced from a pBad plasmid, which contains the same gene but with a TAG site at one of the codons (pBad-TAG-gene). Furthermore, in order to produce proteins with non-canonical amino acid, one also needs to add unnatural translational machinery. This unnatural machinery is on the pDule-RS plasmid, and cells containing pDule need to be grown in the presence of tetracycline. In addition, the non-canonical amino acid must be supplied in the media because the *E. coli* cells do not produce the ncAAs naturally. Therefore, while the wild-type protein producing cells only need to be grown in the presence of ampicillin to produce wild-type protein, the ncAA-mutant protein producing cells will need to be grown with ampicillin, tetracycline, and non-canonical amino acid in the media in order to produce ncAA-mutant protein.

As a further control of monitoring protein production, negative control expressions must be run for each type of ncAA used. These expressions ensure that no natural amino acids are being used by the synthetase and inserted into the TAG site. Negative control expressions lack ncAA in the media; therefore, only truncated proteins should be produced by these expressions. As a positive control for your media, you will express 5 mL cultures of a superfolder green fluorescent (sfGFP) protein construct. These cells will contain only the pBad-sfGFP plasmid (amp only) and will turn green if the media was prepared correctly.

After approximately 24-48 hours of expression, the large volume cell cultures should be saturated with cells that have been induced to overexpress the protein of interest. Separating the cells from the media and storing the cell pellets at -80 °C after the expression is complete will allow the protein to be purified from the cells at a later time.

Note: This year we are staggering the expression and purification of wild-type and ncAA-mutant proteins to allow you to spend more time understanding your protein and developing an experimental hypothesis. Teams will just grow cultures of their wild-type protein and a sfGFP control during week 2 and then the entire set of wild-type and ncAA-mutant proteins during week 3. Scale the media components appropriately.

Remember these instructions for volumes to prepare are guidelines—make sure you understand the principles and can adjust how much media you make and the volume of cultures you grow accordingly. Do not prepare more media than you need for that week's expression.

Necessary Materials

Equipment for 50 mL expression cultures

- 250 mL sterile baffled flasks (one for wild-type, two for ncAA-mutant proteins)
- Two sterile culture tubes for negative control expression
- Sterile bottles (150 mL, 250 mL, 500 mL)
- Sterile pipette tips
- Large centrifuge
- Microcentrifuge
- Sterile 1.5 mL microcentrifuge tubes
- Oakridge centrifuge tubes
- Sterile conical 50 mL centrifuge tubes
- Shaker (temperature set to 37 °C)
- Shaker clamps (3 small) for each group—TAs and instructors will provide these

Culture Preparation

- Appropriate starter cell cultures grown in non-inducing media (See Appendix 4)
- Sterile H_2O
- Aspartate (5%, pH 7.5)
- Glycerol (10%)
- 18 AA mix (25 x) (stored at 4 °C)
- 25 x Mineral Salts
- Arabinose (20%) (stored at -20 °C)
- $MgSO_4$ (1 M)
- Glucose (40%)
- Trace metals (5000x)
- Ampicillin (1000x stock) (100 mg/mL in H_2O) (stored at -20 °C)
- Tetracycline (1000x stock) (25 mg/mL in DMF) (stored at -20 °C)
- 8 M NaOH
- Non-canonical amino acids of interest

Suggested Resources and Protocols

- *Media preparation and protein expression*: A basic outline for this procedure can be found below. For a more in-depth discussion on the production, monitoring, and purification of ncAA-mutant proteins, see the following article:
  Hammill, J.T.; Miyake-Stoner, S.; Hazen, J.L.; Jackson, J.C; Mehl, R.A. (2007) Preparation of site-specifically labeled fluorinated proteins for 19F-NMR structural characterization. *Nature Protocols* 2(10), 2601-2607.

- Selection of polyacrylamide gels: The following resource from Bio-Rad contain relevant concepts for protein gel electrophoresis and can be found in the literature resource area on Canvas:

Bio-Rad Mini Protean Tetra Cell handbook (in particular Section 4.2 contains protocols for making stock solutions for discontinuous Laemmli SDS-PAGE gels and buffers).

Deep Thoughts on Sterile Technique

Maintaining sterile technique is a key part of working with *E. coli* and molecular biology in general. You want your desired *E. coli* to grow and nothing else. But germs are everywhere! And what wouldn't want to grow in a nice, warm, aerated nutrient rich broth? In order to prevent other things from growing, we use sterile technique (i.e. killing anything we don't want with heat or ethanol). Practicing good sterile technique will first and foremost help you in the laboratory setting.

But beyond this lab course, sterile technique may prove to be useful to you. Consider any sort of medical practice- whether you are pre-medicine, pre-dental, or someone who will likely visit a doctor at some point in their life- sterile technique limits the spread of pathogens and reduces your chances of developing a harmful disease. Consider carefully how to organize your workspace and tools to maintain sterility.

Deep Thoughts on Exploiting the Central Dogma

The basic details of genetic code expansion are detailed in the Background of this lab manual. The goal of genetic code expansion is to site-specifically incorporate a non-canonical amino acid into your protein of choice. How exactly does this work? How is the genetic code facilitated through tRNA? What is the anticodon on the orthogonal tRNA? Where in the protein is your non-canonical amino acid incorporated?

Genetic code expansion requires at least four different components. What are these components? Where do you need them to be? How are these components made? What would happen if any of these components were missing? For example, what would happen if the non-canonical amino acid were not included in your media? Or if the orthogonal tRNA synthetase were missing or an incompatible orthogonal tRNA synthetase included?

Thinking about these questions can also help you troubleshoot your expression if things do not seem to be going according to plan.

Methods for Protein Expression

Autoinduction Media Preparation: Media should be prepared with the sterile solutions and sterile equipment obtained in Week 1.

ADVICE: All of these steps should be done using sterile technique with a flame on. Both the pipettes and the benchtop should be cleaned with water and ethanol before starting. Make sure to only use sterile tips, flasks, pipettes, water, and media.

Preparation of Autoinduction Media for Expressions (100 mL) Scale to make amount you need for your experiment based on the following amounts per 100 mL. Prepare fresh and do not plan on storing extra AIM.

Aspartate (5%, pH 7.5)	5 mL
Glycerol (10%)	5 mL
25x Mineral Salts	4 mL

Glucose (40%)	0.125 mL
$MgSO_4$ (1 M)	0.2 mL
Arabinose (20%)	0.25 mL
Trace metals (5000x)	20 μL
18 AA mix (25x) stored @ 4°C	4 mL

Add appropriate antibiotics (ampicillin to final concentration of 100 μg/mL and tetracycline to 25 μg/mL).
Add sterile water to a final volume of 100 mL.

Recommended volumes and flasks for the expressions during weeks 2 and 3:

Wild-type protein	75 mL expression in a 250 mL flask
ncAA protein 1	75 mL expression in a 250 mL flask
ncAA protein 1 (negative control -ncAA)	5 mL expression in culture tube
ncAA protein 2	75 mL expression in a 250 mL flask
ncAA protein 2 (negative control -ncAA)	5 mL expression in culture tube
sfGFP wild-type control (-ncAA)	5 mL expression in culture tube

ADVICE (EXAMPLE FOR 250 mL—SCALE ACCORDING TO YOUR NEEDS):
It is convenient to start with a 250 mL bottle of sterile water and remove about 50 mL of it by placing it into another sterile vessel (for later use). Then all of the media components can be added to the 200 mL of sterile water and topped off with sterile water to a final volume of 250 mL. Then add 250 μL of 1000x ampicillin to the media, and then place 75 mL of it in a 250 mL baffled flask for expression of wild-type protein. 175 μL of 1000x tetracycline can then be added to the remaining media (175 mL) and separated into expression flasks. ncAAs will be added later.
Each lab section should use the appropriate code of tape color to label flasks so it is easy to recover them. Remember there are 4 sections, over 80 students and some weeks 80-100 flasks growing.

Addition of Cells from Starter Cultures:
To the prepared flasks (75 mL media/flask), saturated cell cultures in non-inducing media of the appropriate cell line must be added to start the expressions. Dilute cells of appropriate overnight starter culture 1:100-1:200 to each flask containing 75 mL. Incubate these cultures for 0.5 -1 hr before adding the ncAA (this incubation may be shortened for certain ncAAs).

ncAA Preparation and Addition: (Week 3)
The concentration recommended for the ncAA is >1 mM final ncAA concentration in the expression media. The ncAAs may need assistance dissolving before adding to the media, depending on the chemical characteristics of the ncAA. It is recommended to weigh out slightly more than the appropriate amount of ncAA directly into a microcentrifuge tube. Start by adding 0.5 mL of sterile water and mixing – if it dissolves, add it to the appropriate expression flask. If *most* of the ncAA dissolves, first try adding

an additional 0.5 mL of sterile water. If very little is dissolved after the first 1 mL, try adding 1 molar equivalent of NaOH from the 8 M stock solution (add 5 µL 8 N NaOH at a time and do not exceed 20 µL total). Adding an excess of NaOH can damage some of the ncAAs and cause problems with cell growth. Once ncAAs are added to their appropriate flasks, let them incubate while shaking at 250-300 rpm at 37°C for up to 48 hours (this long incubation is designed to meet the schedule of BB 494; typically cultures are grown for 24-40 hours before harvesting).

To obtain a zero time point of protein expression, remove 250 µL of cell culture from the original starter cells in non-inducing medium and centrifuge at 3000 – 5000 rcf for 5-10 min. Discard the supernatant and store the small cell pellet at -20°C in your freezer box (label all samples well, so you and your partners can identify in the future).

Express protein for 24-48 hours. Before harvesting cells by centrifugation, remember to remove 250 µL cells for final time points for a crude gel first. Also, determine the OD_{600} of the cultures. Divide each cell sample into 25 mL aliquots in pre-weighed conical tubes. Spin the cells in 50 mL conical tubes for 10 min at 5000-8000 rcf. in the bench top centrifuge (not the Sorvall). Remove the supernatant by pouring it off, determine the mass of each cell pellet, and store the cell pellets at -80 °C. Again label tubes well with section, group number, date, and sample. Store cell pellets in small Ziploc bags that are well labeled with pen directly on the bag with your lab section, group number, and date. Do not use tape to label tubes or bags—it falls off at -80 °C.

Deep Thoughts on Bacterial Strains and Plasmids

We are providing you with starters grown in non-inducing media with which to inoculate your larger cultures but what exactly are you using? Are you blindly taking what the TA or instructor gives you and following the instructions for expressing protein in this manual or do you know how the system works and what exactly is in that starter culture?

If you are adding antibiotics to your media, why are you doing it? How do you know what the correct antibiotics are? Not all of your expressions use the same antibiotics and why is this? How is resistance to these antibiotics imparted?

There are a huge variety of different bacterial strains we could be using, each with unique features optimized for specific purposes and with advantages and disadvantages. Some are designed to tightly control expression and are used for expressing proteins that may be toxic to a cell. Others lack or contain mutants of certain genes in order to prevent protein degradation, promote disulfide bond formation, or limit production of certain metabolites. What bacterial strain are you using? What are some of its advantages and disadvantage? Why do you think it may have been chosen for these experiments?

Due to the time constraints of this course, the bacteria containing the appropriate plasmids were also provided for you. Just because you did not carry out this part of the experiment yourself doesn't mean you don't have to understand how it was done. How were these plasmids generated? What do each of the plasmids contain? How did these plasmids get into the bacteria where your protein of interest can be overexpressed?

At the very least, you'll want to know and understand: the type of *E.coli* you are using, the plasmids you are using, and what is encoded on each of these plasmids.

Concepts for SDS-PAGE

Before running protein samples using gel electrophoresis, it is useful to know the anticipated position of the bands on the gel by calculating the anticipated size of the protein. Chapter 6 of Voet and Voet outlines basic principles of SDS-PAGE. Knowing the anticipated size of the bands is also important when selecting the concentration of polyacrylamide that the gel should contain, since different levels of polyacrylamide are more efficient at separating different ranges of protein sizes. The sources *Molecular Cloning* (which also discusses the stain used in this process, Coomassie G-250) and *Bio-Rad manual* are also good resources for principles of gel electrophoresis.

Deep Thoughts on Controls

What is the purpose of a control? Generally, a control can be used to verify that your system is behaving how you would expect it to. Additionally, a control can be used to track down what is happening if/when something goes wrong in your experiment and correct it. In this laboratory, you will be required to use controls in at least two contexts: protein expression (for ncAA incorporation) and assays. Are there any other controls you should be incorporating into your experiments?

For your protein expression, you should be setting up both a positive and negative controls. What is each of these controls? What results do you expect to observe from your positive and negative controls? If your controls deviate from this expectation, what is going on? Do you need any purification controls?

For your assay, a control is an important way to verify that the observed activity is coming from your protein and not from another source. How should this control be set up? It's okay if some activity is observed in your control- this is often referred to as "background." In this case, how might you use the results of this control?

Necessary Materials for SDS-PAGE

Equipment

- Glass plates, casting stand, casting clamps, comb
- Gel Rigs (with dams)
- Gel-loading tips
- Bottles for 1X Running Buffer,
- Plastic gel storage containers (one per group)
- pH meters-common stations.

SDS-PAGE-refer to Bio-Rad SDS-PAGE guide for Mini Protean II System discontinuous Laemmli gels

- 30% polyacrylamide solution (**Caution: neurotoxin**—wear gloves, goggles, lab coat) (aliquots provided)
- Temed (purchased as solution)
- Ammonium persulfate-10% wt/vol in water; make fresh daily (1 mL)
- Separation gel buffer (1.5 M Tris pH 8.8) (make 100 mL)
- Stacking gel buffer (0.5 M Tris pH 6.8) (make 100 mL)
- 10% SDS solution (prepared for you)
- 2X SDS load dye, pH 6.8 (contains 125 mM Tris-HCl, 4% SDS, 20% glycerol, 0.02% bromophenol blue, and 5% β-mercaptoethanol) (10 mL)
- 10X Running Buffer, pH 8.3 (contains 250 mM Tris base, 1.92 M Glycine, and 1% SDS in distilled H_2O) (make 500 mL)
- Molecular weight markers (Bio-Rad Precision Plus Dual Color; load 5-7.5 µL in a lane; store aliquot at -20°C)
- Time point samples (spun down cell pellet from 250 µL of each expression)

- Stain /Destain solutions: will be provided for class at a station in the fume hood.
    - Coomassie Brilliant Blue Stain G-250
    - Methanol 40%
    - Acetic Acid 10%

Methods for SDS-PAGE

To prepare the crude extract samples for gel electrophoresis, first obtain the 250 μL cell pellets that were stored at -20 °C. Resuspend the pellets in 50 μL of distilled H_2O—pipet up and down and vortex to get the entire cell pellet resuspended. Then add 50 μL of 2X SDS dye. Make sure that the SDS dye is completely dissolved before adding to the samples, as SDS precipitates out of solution at low temperatures. Heat samples to 100 °C for about 5-10 minutes, vortexing as necessary to ensure complete dissolution. Centrifuge the samples at >10,000 rcf for 5 minutes. After the gel has been prepared as outlined in the Instruction Protocol, 20 μL of the supernatant can be placed in each well of the polyacrylamide gel. Remember to add 5 μL of molecular weight markers to one well.

To run the gel, follow the Bio-Rad Protean II Instruction Protocol. Once the dye front has reached the bottom of the gel, turn off the voltage and remove the gel from the glass plates. Place the gel in Coomassie Brilliant Blue G-250 staining solution in a plastic container and rock for several hours (or follow Advice below). The staining solution can then be poured off and destain poured onto the gel, followed by additional rocking. The destain can be poured off into the designated waste container in the fume hood and replaced several times until the bands can be clearly visualized against the background of the gel.

- **ADVICE**: To speed up the staining process, since the staining steps function on electrostatic attraction and diffusion, the gel (in either stain *or* destain) can be heated in the microwave in the fume hood to boiling (about 20-30 seconds) before placing on the rocker for 10 minutes. Be sure to wear protective eyewear and gloves! Do not leave in destain longer than overnight.

Necessary Materials for Talon-resin Protein Purification

Purification Buffer Preparation and Components

- pH calibration buffers
- 5 N HCl and 8 N NaOH
- Imidazole
- NaCl
- NaH_2PO_4, Na_2HPO_4, H_3PO_4 Na_3PO_4 salts

Suggested Resources and Protocols

- *Use of polyacrylamide gels*: The following resources contain useful information for protein gel electrophoresis theory and practice. See page 15 for locations of these resources:

Bio-Rad Mini Protean Tetra Cell handbook (in particular Section 4.2 contains protocols for making stock solutions for discontinuous Laemmli SDS-PAGE gels and buffers).
Voet and Voet
Sambrook's *Molecular Cloning: A Laboratory Manual*

- *Protein purification buffer preparation*: The manual for "TALON Metal Affinity Resins" is posted on the course Canvas site.

Objectives for Week 3

- Express entire set of wild-type and ncAA-mutant proteins.
- Purify wild-type protein from cells harvested in Week 2.
- Make storage and assay buffers.
- Desalt the wild-type protein sample into the storage buffer.
- Analyze purified protein by SDS-PAGE.
- Determine if protein is active.
- Continue literature search for an appropriate and feasible assay to test enzyme kinetics.
- Obtain chemicals and supplies for assays.

Concepts for Protein Purification and Desalting

The polyhistidine tail on the protein of interest allows it to be purified from other proteins using metal affinity resin. Using the proper amount of resin is important: too much resin may indiscriminately bind to proteins that lack the polyhistidine tail, resulting in impure protein, but too little resin will cause some of the desired protein to be lost as the resin is overloaded, resulting in reduced yield.

The elution buffer used to elute the polyhistidine-tagged protein during the purification procedure (containing sodium phosphate, sodium chloride, and imidazole) is likely not an optimal buffer for your assays. Therefore, the protein must be removed from elution buffer and placed into a storage or assay buffer. The method of purification used to desalt protein samples—gel filtration chromatography—is conceptually important to understand. The principles of it are described in Chapter 5 of Mathews, Van Holde, Appling, and Athony-Cahill or Chapter 6 of Voet and Voet.

Deep Thoughts on Protein Purification

There are many different ways in which proteins can be purified. In this course, we are using one of the simpler and more common ways to purify recombinantly expressed protein- polyhistidine affinity purification. How does this method actually work? What does it rely on in order to work effectively? If you are having problems with your purification, what could be going wrong and how might you troubleshoot?

There are many other purification techniques you could use other than polyhistidine affinity purification which rely on size, charge, or the affinity of other molecules as an alternative purification technique or in addition to polyhistidine affinity purification in order to obtain a purer sample. What are some of these techniques? What would they require in order to effectively purify your protein of interest and what would be the pros and cons of using them to purify your protein of interest?

Deep Thoughts on Buffers

You've spent a lot of time learning about buffers and pH in classes since high school. But do you remember what a buffer is and what the purpose of a buffer is? If you don't, you'll want to brush up on this before continuing.

You've used and made a variety of buffers already but what makes a good buffer? For example, buffers for SDS-PAGE and polyhistidine affinity purification have prescribed recipes optimized for each specific purpose. Now, it's up to you to select and make your own buffers for your experiments. At the very least, you'll need to select a buffer suited for storing your protein in which to desalt. What makes a good buffer in this case? It depends on what qualities you want in your buffer. In this case, you probably want a buffer in which your protein in stable. In order to avoid changing buffers again, you also likely want a buffer that will not interfere with your assay of choice. There may be additional qualities to consider when choosing and making your buffer.

Necessary Materials

Equipment

- Microfluidizer (available for BB494)
- Sonicator with a power source and ear protection (alternate method of lysing)
- Oak Ridge high speed centrifuge tubes
- Sorvall and SS34 rotor
- Poly-prep columns –Bio-Rad (reusable)
- Prepacked PD-10 desalting columns from GE Health Care-reuse these

Protein Purification and Desalting

- BD Talon Metal Affinity Resin (teams will receive aliquot of resin in long-term storage buffer. Resin will need to be equilibrated).

- 5X Talon Equilibration Buffer (250 mM sodium phosphate, 1.5 M NaCl pH 7.0). Important Note: For using the microfluidizer, prepare a 0.5 L stock of 5X. Dilute to 1X as needed each time you purify.
- 1X Talon Equilibration/Wash buffer pH 7.0 (50 mM sodium phosphate, 300 mM NaCl) (also referred to Tractor buffer in Talon manual)
- Talon Elution buffer, pH 7.0 (50 mM sodium phosphate, 300 mM NaCl, 250 mM imidazole) (make 100 mL or less)
- Hen egg white T4 lysozyme if sonicating
- Cell pellets from spun down expressions
- Bradford Protein Assay Reagent and bovine serum albumin protein standard

Suggested Resources and Protocols

- *Protein purification buffer preparation*: The manual for TALON Metal Affinity Resins is posted on Canvas site. Note: Talon manual refers throughout to Tractor Buffer—you will make your own Talon resin Equilibration/Wash and Elution buffers (see list above).

- *Protein Desalting*: The manual for using PD-10 Desalting columns can be downloaded from the manufacturer's website and is also posted on Canvas. http://www6.gelifesciences.com Select a buffer for desalting that is compatible with your assays. Make sure you understand the general concept and use of terms equilibration and elution buffer.

Deep Thoughts on Protein Stability

Protein stability is applicable to this course in multiple ways. First, you'll want to consider the stability of your protein over time in the context of conducting your experiments. Second, stability (over time or perhaps thermostability) may be a feature of your enzyme that you'll want to assess as part of your research. Changes in stability are often of interest and increased thermostability is often a desired outcome when engineering an enzyme.

First, we will focus on stability in terms of maintaining the integrity of your protein. You will be expressing and purifying your native protein as early as Week 3 of the term. Do you expect this purified protein to have the same activity and behave the same once you get to your final assays in Week 9? What can you do to maintain the integrity of your protein over time?

One key factor to consider is how your protein is stored; understanding and choosing conditions under which your protein may be stored without appreciable loss of activity is essential for effectively evaluating your protein. In order to maintain maximum activity and obtain consistent results from lab period to lab period, how should you store your protein? At what temperature? In what buffer? At what concentration? How should your protein be handled when you are using it?

If you are observing loss of activity over time, what could be happening to your protein to reduce its activity? What could you do to mitigate the loss of activity? What could you do to limit the effect of the loss of activity of your protein over time on your results? Aside from measuring reduced activity, how might you detect that the integrity of your protein is being reduced with time?

Additionally, if you want to assess the stability of your protein as an additional feature, how might you go about doing so? What is an appropriate assay and how might you challenge your enzyme?

Methods for Protein Purification and Desalting

Once samples have been bound to the metal affinity resin, the procedures for purification and desalting are clearly outlined in the respective manufacturers' manuals. To prepare the cell pellets for binding to the resin, the cells will be passed through the Microfluidizer in the Mehl Core Lab followed by centrifugation of the lysed cells. Alternatively, cells must be sonicated and centrifuged according to the general procedure provided later in this manual. Note that throughout the purification process, the samples should be kept on ice to prevent less stable proteins from unfolding.

Of the three types of cell pellets being stored at -80 °C, purify at least one pellet of each type. Save the remaining pellets at -80°C. More protein expressions can be done later as needed. Note, however, that at least three days' notice is required to prepare more non-inducing starters.

Microfluidizer Lysis Method

From your 5X stock prepare 1X Equilibration Buffer (50 mM sodium phosphate pH 7.0, 300 mM NaCl)—you will need about 1 L total for resuspending the cells and washing the microfluidizer between samples.

Place the pellets in the tubes on ice and resuspend each completely (no chunks) in 10 mL of 1X Talon Equilibration buffer minus imidazole. Resuspend one pellet of the wild-type and one each of two ncAA-mutant proteins (week 3). Take your resuspended cells on ice, your bottle of 1X Equilibration Buffer, and Oakridge centrifuge tubes to the Core Lab in ALS 2124. Your TA will show you how to use the microfluidizer. We will send groups up one at a time. **To finish lab on time, you must microfluidize in the first hour of lab. Groups will not be allowed to begin microfluidizing after the first hour. You also need to get all the way through to eluting the protein from the resin before lab ends. Work efficiently!**

Once you have recovered your lysed cells, return to the BB Teaching Lab and centrifuge your samples in the Sorvall SS34 rotor for 18,000 rpm for 20 minutes, 4ºC. Be sure to balance the tubes first.

Preparation of TALON Resin and Sample Binding:

While microfluidized cells are centrifuging, prepare and wash the BD TALON metal affinity resin, following the protocol outlined in the Talon manual (specifically, the protocol for a native purification at pH 7.0). This manual also includes the recommended amount of bed volume of resin to use—anticipate that there will be at least 20 mg of protein per 100 mL of culture for wild-type protein (the yield varies depending on how stable each protein is and the efficiency of translational machinery, so each TAG site will be different depending on its location and the ncAA incorporated). Once the resin has been washed and combined with the sample in a 50 mL Falcon tube, bind the sample to the resin for at least 30 minutes (at room temperature or colder) with rocking. Once the sample has been bound, it can either be washed in batch or be applied to the column and washed extensively (2 x 10 mL approximately). The extent of washing can be monitored with Bradford Coomassie stain—if a blue color appears when 500 μL of stain is combined with 20 μL of eluent, then there is likely still non-polyhistidine tagged protein still being washed from the resin. Once no blue color appears, elute the purified protein with 1.5-2 mL of elution buffer. This is a convenient volume to use for the next step, desalting.

- **ADVICE**: Adding the 1.5- 2 mL of elution buffer in smaller portions to the resin tends to improve yield of purified protein.
  Store the eluted protein fractions at 4˚C (**not frozen!!!**) or on ice until you are ready to proceed with the next step.

To desalt the protein sample into the storage buffer, follow the procedure outlined in the PD-10 desalting columns manual. Anticipate that it will take approximately 20

minutes to wash the desalting column with the storage buffer. You may do this during the next lab session.

Sonicating Method to Lyse Cells (alternative method):
Place the pellets on ice and add an appropriate amount of wash buffer. Combine all pellets of the same protein type (i.e., wild-type vs. the two ncAA-mutant pellets) into a single 50 mL conical tube.

- **ADVICE**: Since the resuspended cells will eventually be centrifuged in special high-speed tubes that hold 40 mL, make sure that the total volume used to resuspend all pellets of a protein type does not exceed 40 mL, so that all pellets of a single type can be in the same tube. Use 15 mL lysis buffer/25 mL cell pellet

Once the pellets have been combined, add ~20 mg of lysozyme (not exceeding a final lysozyme concentration of 1 mg/mL) to the resuspended cells. Mix the lysozyme into solution by gentle inversion only—do not shake, foam (i.e., whip air into), or warm the samples, as the protein may be unstable when exposed to air or heat. Any remaining pellet pieces can be resuspended with brief sonication.

To lyse the cells more completely, they can be sonicated. Sonication ruptures the cell walls much the same way that standing next to a concert speaker can rupture eardrums. Therefore, ear protection should be worn throughout this process. Use the highest power setting to sonicate without foaming the sample. Moving the sonicator tip near the surface of the solution will make it foam, and touching the sonicator tip to the plastic tube will prevent it from lysing the cells well. Do not sonicate in glass. Sonicating each sample for three one-minute intervals should be sufficient. Do not let the sonicator warm the sample to above room temperature—if the sample starts to warm, stop sonicating until it cools down.

To remove cell debris, the samples must be centrifuged really fast in special high-speed tubes with caps. After ensuring that the tubes are balanced in the rotor (check the tubes with a balance) and that they are not cracked or structurally compromised, spin them as fast as the centrifuge will allow for 30 minutes.

Objectives for Week 4

- Determine the concentration of the pure, desalted wild-type protein samples.
- Determine if wild-type protein is active.
- Run SDS-PAGE of crude extracts of ncAA-mutant proteins expressed in Week 3.
- Run SDS-PAGE of purified wild-type proteins.
- Purify ncAA-mutant proteins.
- Develop kinetic assay and determine catalytic constants for wild-type and ncAA-mutant enzymes (see below for advice on running assays).

Necessary Materials

Determining Concentration of Protein

- Coomassie (Bradford) Protein Assay Kit, containing
    - Coomassie Protein Assay Reagent
    - Bovine Albumin Standard aliquots (2 mg/mL)

Determine activity and catalytic constants of Carbonic Anhydrase and Catalase

Materials will vary by group depending upon the nature of the assay. Each group is responsible for ensuring that the necessary equipment and materials are available from week to week for executing their assays.

Suggested Resources and Protocols

- *Protein Concentration*: The manual for the Bio-Rad Bradford Assay can be found online. http://www.bio-rad.com/webroot/web/pdf/lsr/literature/4110065A.pdf
- *SDS-PAGE*: Use resources from Week 3.
- *Enzyme Assay*: See Weeks 5-9 for details about assay resources.

Methods for Protein Standard Curves and SDS-PAGE

The method for generating a standard curve and measuring concentrations of samples is clearly outlined in the Bradford Assay Manual. Anticipate a working range of 100-1,500 μg/mL protein. Standard bovine serum albumin samples will be provided.

Caution: The Bradford Coomassie solution is a very strong acid (phosphoric acid)—be very cautious when working with it, and if exposed wash immediately with water. Collect used reagent in appropriate waste container in the fume hood.

When preparing and running the SDS-PAGE gel of the purified protein sample, the only difference lies in sample preparation. Approximately 5-20 μL of desalted protein sample can be mixed with 20 μL of 2x SDS, which can then be heated at 100 °C for 5-10 minutes. Briefly centrifuge the tubes to get the entire sample to the bottom of the tube. 20 μL of this mixture can be loaded into the wells of the gel. A rule of thumb for mini gels is to load about 0.5 microgram protein per expected band, so the gel is not overloaded.

Deep Thoughts on Protein Concentration

There are many ways you can measure protein concentration and, as is often true, each way has its advantages and disadvantages. How are you measuring protein concentration? The reagents for a Bradford Assay are provided, but you should still consider whether this is the best method for measuring the concentration of your protein. In addition to the Bradford Assay, two of the most common ways to determine protein concentration are the BCA Protein Assay and Absorbance at 280 nm using a Nanodrop or Picodrop.

A few things to consider when selecting a method include compatibility with the sample type and buffer components (including added zinc), assay range and required sample volume, speed and convenience for the number of samples to be tested, availability of spectrophotometer or plate reader to measure the absorbance by the assay, utility in measuring whole cell lysate or pure protein, uniformity (how much protein-to-protein variation there is) and, if used, appropriateness of the standard. In your case, you'll also need to consider the effect adding a non-canonical amino acid to your protein might have on your measurements.

The Bradford Assay relies on the proportional binding of Coomassie dye to proteins. Within the linear range of the assay, the more protein present, the more Coomassie binds, and the darker the color of the sample becomes. Coomassie absorbs at 595 nm so using a protein standard, most commonly BSA (bovine serum albumin), you can generate a standard curve and use this to measure the protein concentrations of your samples.

The BCA Protein Assay is also a colorimetric assay used to quantify protein concentration. This method relies on the reduction of copper and subsequent detection of the reduced copper by BCA (bicinchoninic acid). This BCA/copper complex exhibits a strong linear absorbance at 562 nm with increasing protein concentrations. Like with a Bradford assay, a protein standard, most commonly BSA, is used to generate a standard curve with which to determine the protein concentrations of your samples.

A Nanodrop is a common lab spectrophotometer that can be used to determine protein, DNA, and RNA concentration. To measure protein concentration, the Nanodrop uses absorbance at 280 nm, often referred to as A280, and an extinction coefficient to determine concentration of a pure protein sample. What's an extinction coefficient? An extinction coefficient is a constant for a protein reflecting how much UV light it will absorb based on its amino acid composition; aromatic amino acids are primarily responsible for UV-light absorption. The Nanodrop has the advantages that it requires only very small sample sizes (~1-2 µL) and is fast.

Objectives for Weeks 5-9

- Develop kinetic assay and determine catalytic constants for wild-type and ncAA-mutant enzymes (see below for advice on running assays).
- Modify assay as necessary to obtain meaningful, reproducible results. Purify additional protein if necessary.
- Obtain kinetic parameters for wild-type and ncAA-mutant enzymes so that comparisons can be made, both to literature values as well as among the enzymes tested in this experiment.
- Design and execute individual assays for protein structure and function.

- Based on the known structure of the enzyme, rationalize the obtained results and attempt to determine the structure-function relationship of that particular site.
- Design and run experiments to test your hypotheses on structure and function.

- Mass spec analysis of purified proteins to confirm expected composition-if mass spec time is available to lab class.

Concepts for Enzyme Assays

Essentially, an enzyme assay is taking an enzyme and combining it with the starting materials. Therefore, it is necessary to have a way to monitor the disappearance of the starting materials or the formation of the products. If the enzyme does increase the rate of the reaction, then the formation of products will be noticeably faster with the enzyme than without the addition of the enzyme. Because the catalyst being used here comes from a living organism, it is sensitive to salt concentration, pH, and the concentrations of the starting materials and products. Literature on the protein can give useful information on these values.

When beginning the initial crude assays, do not stress out – the likelihood of it looking correct on the first try is extremely slim. There are too many factors that will need to be tweaked to get good data. When starting out, give the assay the best chance of showing catalytic activity, without wasting time being precise with measurements and making stock solutions.

Deep Thoughts on Kinetics and Assays

You will be at least comparing the kinetics of your wild-type protein to ncAA-mutant protein using an assay. But what do these changes in kinetic parameters actually mean and how do you interpret your results? Interpreting your results in a meaningful way may be one of the most challenging parts of your experiments.

First, let's brush up on some basic kinetics values. K_M is the Michaelis constant and is the substrate concentration at 1/2 V_{max}. V_{max} is the maximum velocity of the enzyme. k_{cat} is turnover number and measures the number of substrate molecules turned over per enzyme molecule per second. k_{cat}/K_M is often used as a measure of catalytic efficiency. How are you going to obtain these values (and perhaps others) using your kinetic assay?

Typically we assume enzymes follow Michaelis-Menton kinetics, but is this true for your enzyme? What is the most appropriate model with which to model the kinetics of your enzyme and how has it been modeled in the literature? To assess the kinetics of your proteins, at what time points should you take your measurements and for how long? What other factors do you need to consider in order to conduct an accurate and consistent kinetic assay? For example, how do temperature, pH, and buffer composition affect your assay? What variables should be constant and which variables should change as your carry out your assay? (If your enzyme follows Michaelis-Menton kinetics, enzyme concentration should stay the same and substrate concentration should change.)

Once you have your results, what do you do with them? How might you assess the accuracy and validity of your measurements? Are your results self-consistent and do they make sense? You will also be able to find accepted kinetic parameters for your native protein in literature. What makes for a meaningful comparison between your values and those found in literature? What do changes in the various kinetic parameters mean?

Necessary Materials

- Materials will vary by group depending upon the nature of the assay. Each group is responsible for ensuring that the necessary equipment and materials are available from week to week for executing their assays.

Suggested Resources and Protocols

- *Assay Background Information*: Chapter One of the book *Enzyme Assays* by Eisenthal and Danson covers many of the basics for running assays and performing kinetic measurements. A copy will be in the teaching lab.

Methods for Enzyme Assays

Although the specifics for the assays will vary by protein, it is recommended to start with the most concentrated wild-type protein solution and crudely measured out reagents. Making stock solutions, and the order in which components are mixed together, is of utmost importance. It is prudent to keep separate stock solutions of every component until the behavior of every component is known (Is it temperature sensitive? Precipitate over time? Does it kill protein? Or, only last one class period?). Mixing reagents together in one big stock solution at the beginning of running assays sacrifices much of the control in the experiment.

Here are some tips for finding a reproducible assay method:

- First, how is the reproducibility? Can the assay be repeated three times in a row and yield the same initial reaction velocity? Test this with the crude assay mixture.
- Adding too much enzyme can finish the reaction before mixing is complete (i.e., the zero baseline should change).
- Putting the cuvette in the wrong way or using the wrong type of cuvette can frustrate even the smartest of groups.
- Make sure the pH is correct and that it does not change substantially during the reaction.
- When mixing components together for assays, try not to use volumes less than 10 µl. If need be, make a more dilute stock solution of enzymes and substrates to accomplish this goal.
- Some reagents decompose (over the course of a lab period) if placed in the assay buffer. Instead, make a 100x stock solution of that reagent and keep it in water or storage solvent on ice.
- The enzyme may need to warm up to room temperature before it works well.
- Improper mixing makes the data look groovy and not linear.
- Determine the longevity of the enzyme—does the enzyme die quickly? Will it have the same activity next week?

Note: the following example is not an assay that is appropriate for your enzymes-rather it is just an example of an assay that can be monitored spectrophotometrically!

Here is an example for a 1 mL enzyme assay reaction that needs Zn^{+2} ion. 10x stock solutions of all the components (dissolved in assay buffer) can be made.

Place 780 µl of assay buffer into a cuvette (stored at room temperature)
Add 100 µl of $ZnCl_2$ solution (10x, in assay buffer) (stored at necessary temperature)
Add 100 µl of substrate solution (10x, in assay buffer) (stored at necessary temperature)
Mix

Place the cuvette into the instrument and zero it. It may be useful to run the instrument through a full data collection set to see what happens. Nothing should happen, since the enzyme has not been added yet. If something *is* changing, this is the background reaction and it is important to monitor.

Add 20 µl of the most concentrated wild-type enzyme solution (stored on ice)
Quickly mix by pipetting with a P1000 pipette.
Collect data
Is there activity? Yeah! Keep in mind you will want to run a control reaction without enzyme to make sure that the data looks different.

Now modify the assay so that all the needed kinetic constants for publication can be gathered and the ncAA-mutant enzymes can be tested. Streamlining the assay before taking serious measurements can be useful—aspects such as temperature control, taking data in triplicate for error bars, and other aspects are good to outline before taking a series of measurements for all of the enzymes.

Deep Thoughts on "How do I write a good paper? What do I need for publication?"

By the end of this term, you will hopefully have been exposed to a significant body of literature surrounding your protein of interest. Now, you have to prepare your research in a manuscript. Think back to some of the papers you read. What did you like about these papers? What did you dislike about these papers? Perhaps you liked when the authors told a story that drew you in or when the data was presented in a clear and concise manner. You can use your own experience as a reader to guide your writing.

Aside from creating a manuscript that people will actually enjoy reading, it's also important to consider the utility and reliability of your work. Much like you did at the beginning of this term, when embarking on a new project, pursuing a new area of research, or investigating a new technique, scientists tend to scour the existing literature to find out what has been done and what is already known. As such, it's important that these papers include the information required to repeat the experiments and replicate the results.

Any reader will also want to be able to assess the quality and meaning of your work. For something like a kinetics assay, it's important to carry out multiple trials (ideally, triplicate) and to include error bars. This allows the reader to decide if your results were just a fluke or if they were consistent and meaningful.

Appendix 1: Micropipette Calibration

Before starting any type of lab work, it is important to check the accuracy of the micropipettes that will be used for the duration of the semester. Water provides a simple way to do this, since it has a density of 1 kg/L. Thus, by recording the weights of different volumes of water, it is easy and quick to evaluate the accuracy of the pipette, given the reliability of the balance.

Use the protocol below to fill out the following table for each pipette:

Micropipette # _____ **Micropipette Maximum Volume: _____ µL**

Volume setting: _____ µL (max)	Volume setting: _____ µL (1/4 max)
Mass of distilled H_2O (mg)	Mass of distilled H_2O (mg)
_____	_____
_____	_____
_____	_____
_____	_____
_____	_____

1. Using a balance capable of reading in milligrams or smaller, tare a plastic weighing boat on the pan of the balance.

2. Set the micropipette to its maximum capacity and carefully transfer that volume of distilled water to the weighing boat.

3. Repeat the operation four times recording in the table below the weight of the distilled water transferred each time.

4. Set the micropipette to transfer 20-25% of its maximum volume and repeat step 3.

5. Calculate the average (x-bar) and the standard deviation (σ) for each set of measurements, using the appropriate button on a calculator.

6. Calibrate the 3 micropipettes and fill out a table for each as shown above.

7. Using the parameters that were calculated, assess the accuracy of the pipettes.

Appendix 2: Genetic Sequences

Electronic versions of the sequences are available on Canvas.

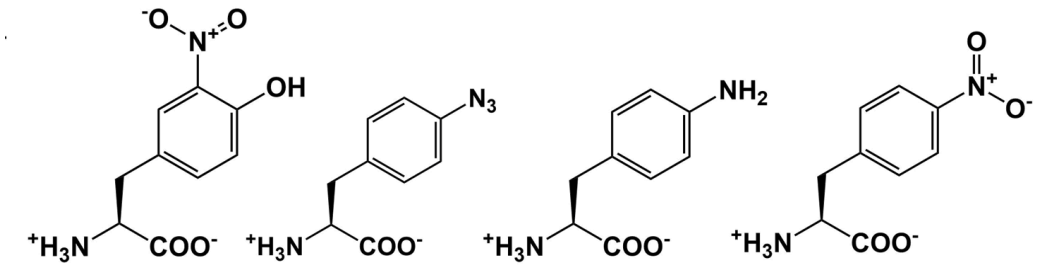
CA	**Human carbonic anhydrase II thermostable variant gene** Gene with available TAG sites (bold and underlined-Phe20, Phe93, Trp97, Lys126, Glu186, and Glu233), 6his-tag (lowercase), and thermostable mutations (lowercase)	ATGGCGCATCATTGGGGTTACGGTAAACACAACGGTCCGGAGCATTG GCACAAAGAT**TTT**CCAATTGCGAAGGGCGAACGTCAAAGCCCGGTT GACATTGATACGCACACGGCAAAGTACGACCCGAGCCTGAAACCGC TGAGCGTTTCCTATGACCAGGCTACGAGCCTGCGTATCCTGAACAAT GGCCACacctTCAACGTGGAGTTTGATGATTCCCAAGATAAGGCGGTT CTGAAAGGTGGTCCGTTGGATGGCACCTACCGCCTGATCCAA**TTT**CA CTTTCAC**TGG**GGTAGCcacGACGGTCAGGGCAGCGAGCATACCGTGG ACAAAAAGAAGTATGCAGCCGAACTGCACCTGGTGCATTGGAACAC G**AAG**TACGGCGACTTCGGTAAAGCGGTCCAGCAACCGGACGGTCTG GCTGTTCTGGGTATTTTCCTGAAGGTCGGCAGCGCGaacCCGGGTCTG CAGAAAGTGGTTGACGTGTTGGACTCTATCAAGACCAAAGGCAAGA GCGCGGACTTCACCAATTTCGATCCGCGTGGTCTGCTGCCG**GAG**AGC CTGGATTACTGGACTTATCCGGGCAGCCTGACCACCCCGCCATTGCT GGAGTGCGTGACCTGGATCGTCTTGAAAGAACCGATCAGCGTTAGCT CTGAACAGGTCagcAAGTTCCGCAAGCTGAATTTCAATGGT**GAG**GGC GAGCCGGAAGAAccgATGGTCGATAATTGGCGTCCTaccCAACCGCTG AAAAACCGCCAGATTAAAGCATCCTTTAAGcatcaccaccaccatcactaa
	Mutations to increase thermostability	A65T, L100H, K153N, L223S, L239P, A247T

HPII	***E.coli* catalase HPII gene** Gene with 6his-tag and available TAG sites (bold and underlined-Phe206, Glu283, Lys348, His392, Tyr415, and Asp568) and 6his-tag with linker (lowercase)	atgggggttctcatcatcatcatcatggtatggctagcatgactggtggacagcaaatgggtcgggatctgta cgacgatgacgataaggatcgatggggatccgagctcgagTCGCAACATAACGAAAAGAAC CCACATCAGCACCAGTCACCACTACACGATTCCAGCGAAGCGAAAC CGGGGATGGACTCACTGGCACCTGAGGACGGCTCTCATCGTCCAGCG GCTGAACCAACACCGCCAGGTGCACAACCTACCGCCCCAGGGAGCC TGAAAGCCCCTGATACGCGTAACGAAAAACTTAATTCTCTGGAAGAC GTACGCAAAGGCAGTGAAAATTATGCGCTGACCACTAATCAGGGCG TGCGCATCGCCGACGATCAAAACTCACTGCGTGCCGGTAGCCGTGGT CCAACGCTGCTGGAAGATTTTATTCTGCGCGAGAAAATCACCCACTT TGACCATGAGCGCATTCCGGAACGTATTGTTCATGCACGCGGATCAG CCGCTCACGGTTATTTCCAGCCATATAAAAGCTTAAGCGATATTACC AAAGCGGATTTCCTCTCAGATCCGAACAAAATCACCCCAGTATTTGT ACGTTTCTCTACCGTTCAGGGTGGTGCTGGCTCTGCTGATACCGTGCG TGATATCCGTGGCTTTGCCACCAAGTTCTATACCGAAGAGGGTATTT TTGACCTCGTTGGCAATAACACGCCAATC**TTC**TTTATCCAGGATGCG CATAAATTCCCCGATTTTGTTCATGCGGTAAAACCAGAACCGCACTG GGCAATTCCACAAGGGCAAAGTGCCCACGATACTTTCTGGGATTATG TTTCTCTGCAACCTGAAACTCTGCACAACGTGATGTGGGCGATGTCG GATCGCGGCATCCCCCGCAGTTACCGCACCATGGAAGGCTTCGGTAT TCACACCTTCCGCCTGATTAATGCC**GAA**GGGAAGGCAACGTTTGTAC GTTTCCACTGGAAACCACTGGCAGGTAAAGCCTCACTCGTTTGGGAT GAAGCACAAAAACTCACCGGACGTGACCCGGACTTCCACCGCCGCG AGTTGTGGGAAGCCATTGAAGCAGGCGATTTTCCGGAATACGAACTG GGCTTCCAGTTGATTCCTGAAGAAGATGAATTC**AAG**TTCGACTTCGA TCTTCTCGATCCAACCAAACTTATCCCGGAAGAACTGGTGCCCGTTC AGCGTGTCGGCAAAATGGTGCTCAATCGCAACCCGGATAACTTCTTT GCTGAAAACGAACAGGCGGCTTTC**CAT**CCTGGGCATATCGTGCCGG

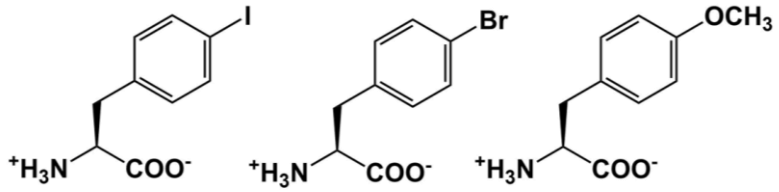
		GACTGGACTTCACCAACGATCCGCTGTTGCAGGGACGTTTGTTCTCC TA**TAC**CGATACACAAATCAGTCGTCTTGGTGGGCCGAATTTCCATGA GATTCCGATTAACCGTCCGACCTGCCCTTACCATAATTTCCAGCGTG ACGGCATGCATCGCATGGGGATCGACACTAACCCGGCGAATTACGA ACCGAACTCGATTAACGATAACTGGCCGCGCGAAACACCGCCGGGG CCGAAACGCGGCGGTTTTGAATCATACCAGGAGCGCGTGGAAGGCA ATAAAGTTCGCGAGCGCAGCCCATCGTTTGGCGAATATTATTCCCAT CCGCGTCTGTTCTGGCTAAGTCAGACGCCATTTGAGCAGCGCCATAT TGTCGATGGTTTCAGTTTTGAGTTAAGCAAAGTCGTTCGTCCGTATAT TCGTGAGCGCGTTGTTGACCAGCTGGCGCATATTGATCTCACTCTGG CCCAGGCGGTGGCGAAAAATCTCGGTATCGAACTGACT**GAC**GACCA GCTGAATATCACCCCACCTCCGGACGTCAACGGTCTGAAAAAGGATC CATCCTTAAGTTTGTACGCCATTCCTGACGGTGATGTGAAAGGTCGC GTGGTAGCGATTTTACTTAATGATGAAGTGAGATCGGCAGACCTTCT GGCCATTCTCAAGGCGCTGAAGGCCAAAGGCGTTCATGCCAAACTGC TCTACTCCCGAATGGGTGAAGTGACTGCGGATGACGGTACGGTGTTG CCTATAGCCGCTACCTTTGCCGGTGCACCTTCGCTGACGGTCGATGC GGTCATTGTCCCTTGCGGCAATATCGCGGATATCGCTGACAACGGCG ATGCCAACTACTACCTGATGGAAGCCTACAAACACCTTAAACCGATT GCGCTGGCGGGTGACGCGCGCAAGTTTAAAGCAACAATCAAGATCG CTGACCAGGGTGAAGAAGGGATTGTGGAAGCTGACAGCGCTGACGG TAGTTTTATGGATGAACTGCTAACGCTGATGGCAGCACACCGCGTGT GGTCACGCATTCCTAAGATTGACAAAATTCCTGCCTGA

Appendix 3: Structures of Available Non-canonical Amino Acids

(Note: Not all structures are available for every site of every enzyme)



3-nitrotyrosine (3-NY) | *p*-azido-L-phenylalanine (pAzF) | *p*-aminophenylalanine (pAF) | *p*-nitrophenylalanine (pNO_2F)



4-iodophenylalanine (pIF) | 4-bromophenylalanine (pBrF) | *o*-methyl-L-tyrosine ($o\text{-}CH_3Y$)

Appendix 4: Non-Inducing and Autoinduction Media

Non-Inducing Media for Starter Cultures

When inoculating auto-induction media with cells, saturated rich media starters (i.e., saturated cells in 2XYT broth) can be used. However, saturated starters grown in non-inducing media can also be used, and also are stable for a much longer period of time when stored at 4 °C (for up to, or even over, a week). To make 1 liter of non-inducing media, follow the directions below.

Non-inducing media for starters (1L)

Aspartate (5%, pH 7.5)	50 mL
25X Mineral salts	40 mL
Glucose (40%)	12.5 mL
$MgSO_4$ (1 M)	2 mL
Trace metals (5000x)	200 µL
18 AA mix (25X) (@ 4°C)	40 mL (for faster starter growth)

Add appropriate antibiotics (ampicillin to final concentration of 100 µg/mL and tetracycline to 25 µg/mL).
Add sterile water to final volume of 1 L.

Approximately 4-5 mL of non-inducing media is sufficient for a starter of a particular cell culture. Inoculate this volume of non-inducing starter with 40-50 µL of saturated 2XYT or other rich media starter.

Autoinduction Media for Expressions

To make 100 mL of autoinduction media (AIM), follow the directions below.

Scale the make amount you need for your experiment based on the following amounts per 100 mL. Prepare fresh and do not plan on storing extra AIM.

Aspartate (5%, pH 7.5)	5 mL
Glycerol (10%)	5 mL
25x Mineral Salts	4 mL
Glucose (40%)	0.125 mL
$MgSO_4$ (1 M)	0.2 mL
Arabinose (20%)	0.25 mL
Trace metals (5000x)	20 µL
18 AA mix (25x) stored @ 4°C	4 mL

Add appropriate antibiotics (ampicillin to final concentration of 100 µg/mL and tetracycline to 25 µg/mL).
Add sterile water to a final volume of 100 mL.

Appendix 5: Master Supply List

General Lab Equipment

- Rocker for gels (on side counter)
- Computers (1 per group)-bring a laptop
- UV/Vis spectrophotometers (in adjoining room)
- Vernier Lab Quest 2 and assorted probes (pH sensor, gas pressure sensor, dissolved oxygen sensor)
- 100 °C Heat block (in fume hood)
- Vortexer
- Carboy containing Millipore water
- Biohazard container for tip disposal-BSL1 waste
- pH meter, standards, 5 N HCl, 8 N NaOH
- Centrifuges (one large and one small)
- Balance
- Spatulas

Minimal-Use Equipment

- Microfluidizer –Mehl Core Lab. Ask TA for assistance.
- High speed centrifuge tubes (40 mL; reusable) (referred to as Oak Ridge tubes)
- SDS-PAGE Gel rigs, glass plates, and combs
- Plastic storage containers (for gels)
- 250 mL baffled flasks with purple membrane caps
- 1 L, 500 mL bottles, 150 mL bottles and flasks
- Erlenmeyer flasks
- Sterile glass culture tubes
- Shaker
- Shaker Clamps

Group

- Sterile water
- Pipette tips: 100-1000 μL, 1-200 μL,
- Falcon tube rack
- 2 group boxes (one for 4 °C and one for -20 °C freezer)
- Microcentrifuge tubes (1.7 mL and 0.65 mL) in jars
- Microcentrifuge tube rack
- Autopipette and charger
- Label tape and marker
- Ice bucket (under table)
- Scissors
- Squirt bottle of Millipore water
- Squirt bottle of 95% ethanol
- Tip disposal vessel

- Liquid disposal vessel
- Set of three micropipettes (20, 200, 1000 µL)
- Pipette stand
- Bunsen burner and tubing
- Striker

Common Drawers, Shelves, and Cabinets or Supply Bench

- 50 ml conical tubes with lids (sterile and nonsterile)
- 15 ml conical tubes with lids (sterile and nonsterile)
- 25 mL serological pipettes
- 10 mL serological pipettes
- 5 mL serological pipettes
- Styrofoam holders
- Razor blades
- Sterile Eppendorf tubes in glass jars
- Paper towels
- Gloves
- Lab Tape

Chemicals (Specialty; suppliers may be substituted)

- Autoinduction media components (adapted from *Nature Protocols*)
- 2X YT Broth (Fisher, BP2467-2)
- Sodium dodecyl sulfate (Fisher, BP166-100)
- Bromophenol blue, sodium salt (Sigma, B-5525)
- β-mercaptoethanol (Sigma, M7154)
- Coomassie brilliant blue G stain (Sigma, B0770)
- Coomassie (Bradford) Protein Assay reagent (Bio-Rad Quick Start)
- Lysozyme (Fisher, BP535-10)
- pH meter buffers (Thomas Scientific, numbers 4120G52, 4120G60, and 4120G68 for pH 4, 7, and 10, respectively)
- Non-canonical amino acids (see Appendix 3 for suppliers)

Prepared Chemicals

- 30% acrylamide solution (37:1) for SDS-PAGE (Bio-Rad; prepared)
- 10X running buffer for SDS-PAGE, pH 8.3 (make 500 mL)
    - 250 mM Tris Base
    - 1.92 M glycine
    - 1% SDS
- 2X SDS load dye for SDS-PAGE, pH 6.8 (make 10 mL)
    - 125 mM Tris HCl
    - 4% SDS
    - 20% glycerol
    - 0.02% bromophenol blue

    - 5% β-mercaptoethanol
- Staining solution for protein gels (TAs will prepare)
    - 250 mL Methanol
    - 200 mL H_2O
    - 50 mL glacial acetic acid
    - 1.25 g Coomassie brilliant blue G stain
- Destaining solution for protein gels (TAs will prepare)
    - 250 mL Methanol
    - 200 mL H_2O
    - 50 mL glacial acetic acid
- 5 M NaCl stock
- 1 M each sodium phosphate monobasic and sodium phosphate dibasic stocks or prepare phosphate stock solution at desired pH
- 5X Equilibration for protein purification, pH 7.0
    - 250 mM sodium phosphate pH 7.0 (refer to chart for mixing of sodium phosphate stocks to achieve 0.1 M pH 7.0 or use stock prepared at desired pH)
    - 1.5 M NaCl
- 1X Equilibration/wash buffer for protein purification, pH 7.0
    - 50 mM sodium phosphate
    - 300 mM NaCl
- 1X elution buffer for protein purification, pH 7.0
    - 50 mM sodium phosphate
    - 300 mM NaCl
    - 250 mM imidazole

Appendix 6: **List of Available Sites, Non-canonical Amino Acids, and Plasmids**

Available Expression Plasmids	Protein Encoded	Antibiotic Resistance	Addgene ID
pBad-CA	CA thermostable wild-type	ampicillin	105665
pBad-CA TAG20	CA thermostable TAG20	ampicillin	105666
pBad-CA TAG93	CA thermostable TAG93	ampicillin	105667
pBad-CA TAG97	CA thermostable TAG97	ampicillin	105668
pBad-CA TAG126	CA thermostable TAG126	ampicillin	105836
pBad-CA TAG186	CA thermostable TAG186	ampicillin	105837
pBad-CA TAG233	CA thermostable TAG233	ampicillin	105838
pBad-HPII	Catalase HPII wild-type	ampicillin	105839
pBad-HPII TAG206	Catalase HPII TAG206	ampicillin	105846
pBad-HPII TAG392	Catalase HPII TAG392	ampicillin	105848
pBad-HPII TAG415	Catalase HPII TAG415	ampicillin	105847
pBad-HPII TAG568	Catalase HPII TAG568	ampicillin	105845
pBad-HPII TAG283	Catalase HPII TAG283	ampicillin	105843
pBad-HPII TAG348	Catalase HPII TAG348	ampicillin	105844
pBad-sfGFP	sfGFP	ampicillin	85482
pBad-sfGFP TAG150	sfGFP TAG150	ampicillin	85483

CA: human carbonic anhydrase II thermostable
HPII: *E. coli* catalase HPII
sfGFP: superfolder green fluorescent protein

Enzyme	TAG Site	ncAAs for Incorporation
CA Human thermostable	F20	3-NY, pAzF, p-NO_2F, pBrF, pAF, pIF, o-CH_3Y
	F93	3-NY, pAzF, p-NO_2F, pBrF, pAF, pIF, o-CH_3Y
	W97	3-NY, pAzF, p-NO_2F, pBrF, pAF, pIF, o-CH_3Y
	K126	3-NY, pAzF, p-NO_2F, pBrF, pAF, pIF, o-CH_3Y
	E186	3-NY, pAzF, p-NO_2F, pBrF, pAF, pIF, o-CH_3Y
	E233	3-NY, pAzF, p-NO_2F, pBrF, pAF, pIF, o-CH_3Y
HPII *E. coli*	F206	3-NY, pAzF, p-NO_2F, pBrF, pAF, pIF, o-CH_3Y
	E283	3-NY, pAzF, p-NO_2F, pBrF, pAF, pIF, o-CH_3Y
	K348	3-NY, pAzF, p-NO_2F, pBrF, pAF, pIF, o-CH_3Y
	H392	3-NY, pAzF, p-NO_2F, pBrF, pAF, pIF, o-CH_3Y
	Y415	3-NY, pAzF, p-NO_2F, pBrF, pAF, pIF, o-CH_3Y
	D568	3-NY, pAzF, p-NO_2F, pBrF, pAF, pIF, o-CH_3Y

GCE Plasmid	Machinery	Compatible ncAAs for Incorporation	Antibiotic Resistance	Addgene ID
pDule-para-aminoPhe	pAF	pAF	tetracycline	85502
pDule-3-nitrotyrosine	NitroY	3-NY	tetracycline	85498
pDule-pCNF	pCNF	pAzF, p-NO_2F, pBrF, pIF, o-CH_3Y	tetracycline	85494

ncAA Abbreviations:

- 3-NY: 3-nitrotyrosine
- pAzF: *para*-azidophenylalanine
- p-NO_2F: *para*-nitrophenylalanine
- pBrF: *para*-bromophenylalanine
- pAF: *para*-aminophenylalanine
- pIF: *para*-iodophenylalanine
- o-CH_3Y: *ortho*-methyltyrosine