Edvo-Kit # 305

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Fermentation and Bioprocessing of Chromogenic Proteins

Experiment Objective:

Bioprocessing is the production and isolation of desired products from living cells. In this introduction to bioprocessing, students will use small-scale fermenters to produce chromogenic proteins using *Escherichia coli*. Protein extracts will then be separated using column chromatography to analyze the success of the fermentation process. Finally, the protein solutions will be examined by SDS polyacrylamide gel electrophoresis to determine the purity of the chromogenic proteins.

See page 3 for storage instructions.

Version 305.220222

EDVOTEK®

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Safety Data Sheets can be found on our website: www.edvotek.com/safety-data-sheets

Experiment Components

Component		Storage	Check (✓)
A	BactoBeads™ transformed with Purple plasmid	4°C, with desiccant	❑
B	BactoBeads™ transformed with Pink plasmid	4°C, with desiccant	❑
C	LB Growth Media concentrate	4°C	❑
D	Ampicillin	4°C	❑
E	IPTG	4°C	❑
F	Protein Extraction Buffer	4°C	❑
G	Wash Buffer (10x)	4°C	❑
H	Elution Buffer	4°C	❑
I	Dry Ion Exchange Matrix	Room Temp.	❑
J	Standard Protein Markers	-20°C	❑
K	50% Glycerol Solution	-20°C	❑
L	Protein Denaturing Solution	-20°C	❑

Experiment #305 contains materials for up to 5 lab groups.

All remaining components can be stored at room temperature.

- Sterile plastic transfer pipets ❑
- Plastic transfer pipets ❑
- 1.5 mL Snap-top microcentrifuge tubes ❑
- 2.0 mL Snap-top microcentrifuge tubes ❑
- 2.0 mL Screw-top microcentrifuge tubes ❑
- pH paper ❑
- 50 mL centrifuge tubes ❑
- 15 mL centrifuge tubes ❑
- Chromatography Columns ❑
- Tris-Glycine-SDS Electrophoresis Buffer (10x) ❑
- FlashBlue™ Protein Stain Powder ❑
- Practice Gel Loading solution ❑

All experiment components are intended for educational research only. They are not to be used for diagnostic or drug purposes, nor administered to or consumed by humans or animals. None of the experiment components are derived from human sources.

Experiment Requirements *(NOT included in this experiment)*

- Automatic micropipettes (5-50 µL, 20-200 µL recommended)
- Centrifuge (Maximum speed should be 10,000 x G or greater)
- Vertical Gel Electrophoresis Apparatus and D.C. Power supply
- Waste container
- Ring stands and column clamps
- Stir plate and stir bars
- Thermometers
- Graduated cylinders
- Erlenmeyer Flasks (two 250 mL flasks and five 500 mL flasks are recommended)
- 70% Ethanol
- Distilled water
- Spectrophotometer and Cuvettes
- Aluminum Foil
- Vortex
- Laboratory Markers
- Waterbath
- 3 Polyacrylamide gels
- White Vinegar
- Ethanol (95% or higher)
- 750 mL or 1 L flask or beaker
- Small plastic tray or large weigh boat
- Plastic wrap
- Microwave (Optional)
- White light box (recommended)
- Rocking platform (recommended)
- Air pump and flexible plastic tubing (Optional)
- Shaker incubator (Optional)
- Autoclave and autoclave tape or Oven (Optional)

Background Information

For over 6000 years, the process of fermentation has been used for food preservation. However, it was not until the 1850s that microbiologists, including Louis Pasteur, demonstrated that microorganisms were the agents responsible for fermentation. Researchers have since learned that fermentation is the result of these microorganisms breaking the chemical bonds in sugar and starch molecules to create energy. The byproducts of this process (e.g., lactic acid, ethanol and acetic acid) produce staple foods including yogurt, sauerkraut and wine.

Current technologies have extended the utility of fermentation, which can now be exploited to manufacture products as diverse as biofuels, biopharmaceuticals and fine chemicals. Today, studies into the fermentation process continue to yield new and exciting advances. For example, microbial geneticists have identified new strains of microorganisms that grow faster and generate a wide variety of vitamins or antibiotics. Genetic engineering and recombinant DNA have allowed scientists to produce large amounts of important proteins, converting cells into living factories. Insulin, which is a hormone used to control diabetes, was the first medication for human use that was produced by genetic engineering. Recombinant medicines, such as antibiotics, interferon and blood clotting factor VIII, have helped save millions of lives and improved the quality of life for millions more.

Today, commercially relevant fermentation products generally fall into one of four groups:

1. Metabolites naturally produced by the microbial cells:
    a. Primary metabolites that are produced during the normal growth, development, or reproduction of an organism: e.g., ethanol, citric acid, lysine, vitamins, polysaccharides.
    b. Secondary metabolites that are produced by an organism, but are not necessary for its normal growth, development, or reproduction: e.g., antibiotic production.
    c. Enzymes naturally produced by the microbial cells: e.g., amylase, protease, pectinase, cellulase, lipase, lactase, streptokinase.
2. Recombinant protein expressed by microbial cells: e.g., insulin, interferon, clotting factor VIII, the Hepatitis B vaccine.
3. Chemical compounds modified by microbes (bioconversion): e.g., steroid biotransformation.
4. The microbial cells themselves: e.g., whole cell yeast extracts, baker's yeast, *Lactobacillus*, *E.coli*.

The demand for these products has encouraged the development of novel technologies for genetic engineering, fermentation, and biomolecule purification.

UNDERSTANDING MICROBIAL GROWTH

Fermentation requires growth conditions that provide cells with oxygen, water, essential minerals and sources of carbon and nitrogen. Because each organism has different physical and chemical requirements for growth, the formulation can vary greatly depending upon the organism and the process. In a natural fermentation, the growth conditions are provided by the food source being fermented. Conversely, scientists can carefully manufacture the growth media to optimize conditions and maximize the yield in a bioprocessing experiment.

Microbial growth does not occur immediately upon inoculation of the selected nutrient medium. A post-inoculation period, called the lag phase, allows the cells to adapt to the new environment by synthesizing factors necessary for growth and cell division. Once acclimated to the growing conditions, the microbes enter log phase, time during which cells grow and division occurs at an exponential rate. This is the optimal stage for bioprocessing applications, as the biological machinery within the cells is primed for rapid growth and protein expression. Eventually the rate of growth within a culture slows due to decreased nutrient availability, and an increased concentration of toxic compounds causes some cells to die. When the rate of cell death equals the rate of cell growth, the culture has entered what is referred to as stationary phase. The

culture will persist in stationary phase until the nutrients are exhausted or until the toxins in the culture result in cell lysis. At this point, the cells enter the death phase and die at an exponential rate (Figure 1).

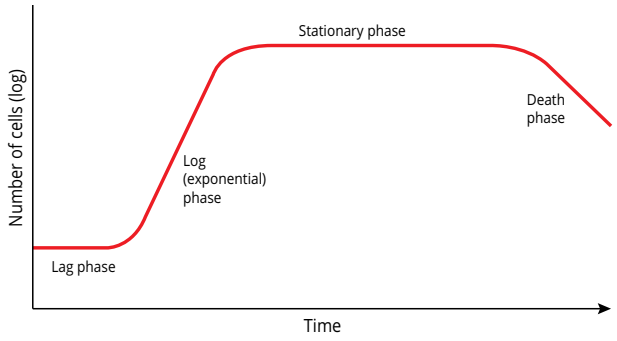


Figure 1: Microbial Growth Curve.

FERMENTATION VESSELS (BIOREACTORS OR FERMENTORS)

In practice, fermentation requires the careful selection of culture conditions to keep cells in a favorable state that allows for the production of the desired product. Cells are grown in a piece of equipment known as a fermentor (or bioreactor), which is fitted with sensors that continuously monitor the environmental conditions during the fermentation process (Figure 2). This information is used to optimize culture conditions. Some of the factors that fermentors can control include temperature, oxygen levels, pH, antifoaming agents, and the rate of mixing.

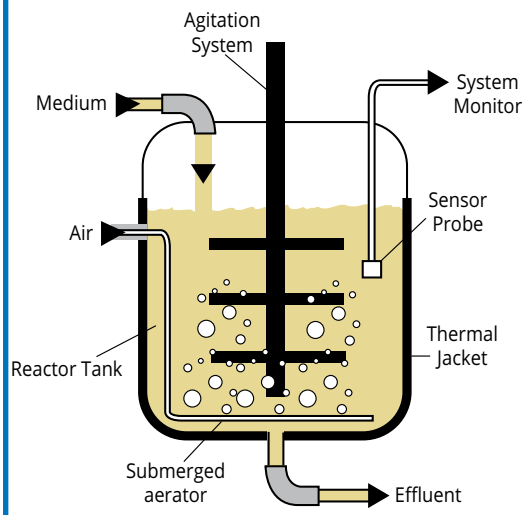


Figure 2: Example of Bioreactor.

Fermentors can be used to grow cultures on vastly different scales. While small cultures (1-10 liters) can be grown, fermentors are especially useful for very large culture volumes (> 1,000 liters). However, a large-scale fermentation reaction cannot be started in such a large volume. Instead, a very small "stock" culture (5-10 mL) of cells is grown, which is then used to inoculate a somewhat greater volume (200 to 1,000 mL) of fresh medium. When these cultures reach log phase growth, they are, in turn, used to inoculate an even larger volume (10-100 liters) in a seed fermentor. As its name suggests, the seed culture is then used to "seed"—or serve as the initial source of cells for—the final culture, grown in a production fermentor (1,000 to 100,000 liters).

There are three main types of fermentation systems: batch, fed-batch or continuous (Figure 3). In batch fermentation, the most basic method, the sterile growth medium is inoculated and fermentation proceeds without any addition or removal of medium. Unfortunately, batch fermentation can lead to the build up of toxins and depletion of nutrients, which can slow culture growth. To counteract nutrient depletion, fed-batch fermentation relies on the addition of fresh growth medium at different times; however, no growth medium is removed until the end of the process. During continuous fermentation, fresh growth medium is added while the used culture is removed. This replenishment of nutrients ensures that the culture remains in log phase, allowing for maximal product production.

Once fermentation is completed, the desired biomolecules must be harvested from the culture. This practice is known as bioprocessing. Sometimes, the product molecule can be secreted directly into the medium by the cells. However, if the molecule is retained intracellularly, the cells themselves must be "disrupted", or ruptured, to liberate the molecule of interest for recovery. Once the product is available in the medium, it can be easily separated from the cells or their debris by centrifugation or filtration. When purified, the product can finally be utilized for commercial and/or industrial purposes (summarized in Figure 4).

USING REPORTER PROTEINS IN BIOTECHNOLOGY

Fluorescent reporter proteins have become an essential tool in cell and molecular biology. The best know fluorescent protein, Green Fluorescent Protein (or GFP), possesses the ability to absorb blue light and emit green light in response with-

out the need for any additional special substrates, gene products, or cofactors. Fluorescent proteins have become an essential tool in cell and molecular biology. Using DNA cloning strategies, proteins can be "tagged" with fluorescent proteins and then expressed in cells. These tags simplify purification because fluorescently labeled proteins can be tracked using UV light.

One of the most useful applications of fluorescent proteins is as a visualization tool during fluorescent microscopy studies. Using genetic engineering techniques, scientists have introduced the DNA sequence for GFP into other organisms, including *E.coli* and the nematode *Caenorhabditis elegans*. Recently, synthetic biologists have engineered a variety of proteins to be used in place of GFP. First, scientists searched a DNA sequence database to identify genes that were predicted to produce colored proteins. Fragments of these genes were linked together to create small chimeric proteins (about 27 kilodaltons in mass). These novel genes were cloned into a plasmid and transformed into *E.coli*. Interestingly, in addition to a variety of fluorescent proteins the scientists also discovered several genes that produced highly pigmented cells. These colorful, chromogenic proteins were visible to the naked eye, meaning that a UV light source or fluorescent microscope was not necessary for visualization. Chromogenic proteins are already being used in biotechnology as controls for protein expression and as visual markers for protein purification.

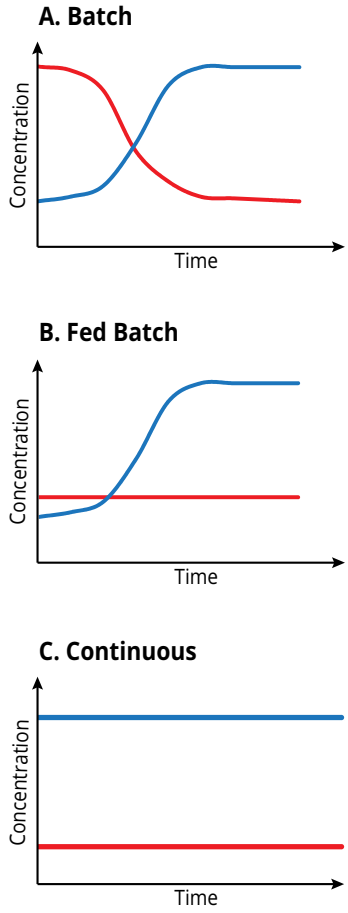


Figure 3: Schematic representation of cell concentration (blue) and reporter protein concentration (red) A) Batch, B) Fed-Batch and C) Continuous.

RECOMBINANT PROTEIN EXPRESSION IN MICROBIAL CELLS

The manufacture of protein products in microbes is an extremely important application of genetic engineering. Using recombinant DNA technology, scientists copy specific genes and insert them into a plasmid, which is a small, extrachromosomal piece of DNA that is propagated by the bacteria. The gene can then be transcribed by RNA polymerase and translated into protein, after which it is harvested from the cells.

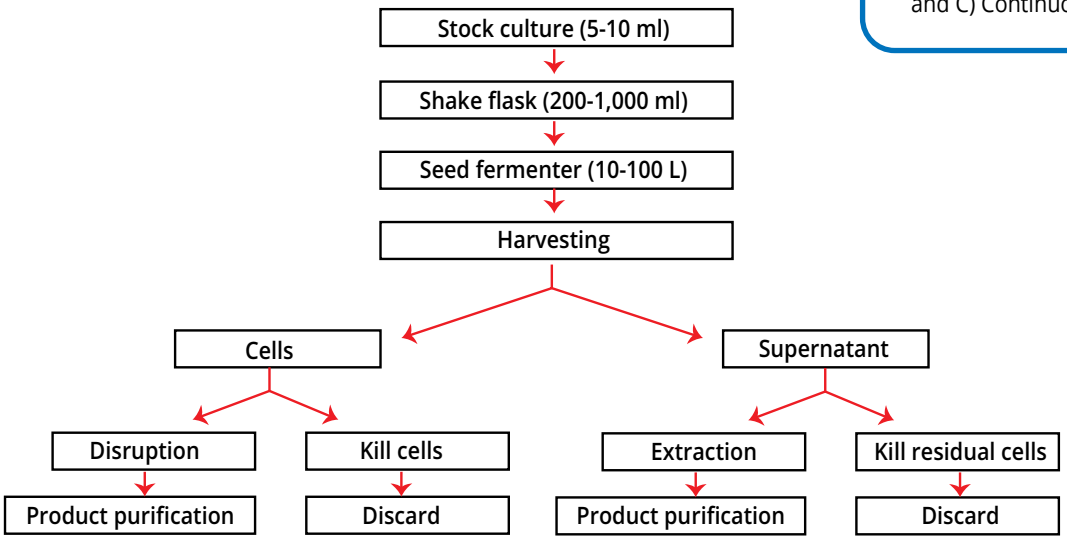


Figure 4: Schematic for large-scale fermentation process.

Many times, expression of our gene of interest is under the control of an inducible promoter. These promoters are only active in the presence of a particular molecule, like arabinose, tetracycline, or isopropyl-β-D-thiogalactopyranoside (IPTG). In this experiment, the host bacterial strain used for protein expression has been genetically engineered to contain the gene for a special RNA polymerase (T7), which is under control of the *lac* promoter. Under normal circumstances, a protein called *lac* repressor binds to the *lac* promoter and blocks transcription of the chromogenic protein. Lac repressor is inactivated in the presence of IPTG, which allows for the expression of T7 polymerase. T7 RNA polymerase then recognizes the T7 promoter on the plasmid, selectively transcribing large quantities of pChromoPink or pChromoPurple mRNA. The mRNA is then translated to produce the Chromogenic Pink and Purple proteins (Figure 5).

ION EXCHANGE CHROMATOGRAPHY

After successfully expressing the protein the final step of most bioprocessing experiments involves purification. In this experiment the chromogenic proteins will be purified using Ion Exchange Chromatography. This process uses a permanently charged matrix to weakly bind the target molecules. Most biological compounds are positively or negatively charged when exposed to a pH in the range of 2-10. The crude cellular lysate is passed over the matrix in a column, allowing any properly charged molecules to bind. Proteins and other molecules that do not bind are then washed away. Finally, the remaining proteins are eluted with an ionic buffer that removes the charged molecules from the matrix.

In this experiment students will explore fermentation and bioprocessing of Chromogenic Pink and Purple proteins. The chromogenic proteins will be expressed by growing transformed *E.coli* in a small-scale fermentor. Protein production will be induced using IPTG, and culture conditions will be monitored to optimize protein production. Cells will be harvested from the fermentor and chromogenic proteins will be isolated and purified using ion-exchange chromatography. Finally, SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) will be performed to determine the purity of the protein purification.

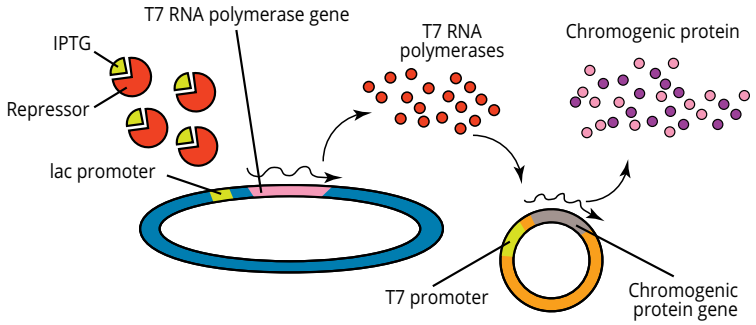


Figure 5: Model of the activation of an inducible promoter.

Experiment Overview

EXPERIMENT OBJECTIVE:

Bioprocessing is the production and isolation of desired products from living cells. In this introduction to bioprocessing, students will use small-scale fermenters to produce chromogenic proteins using *Escherichia coli*. Protein extracts will then be separated using column chromatography to analyze the success of the fermentation process. Finally, the protein solutions will be examined by SDS polyacrylamide gel electrophoresis to determine the purity of the chromogenic proteins.

LABORATORY NOTEBOOKS:

Scientists document everything that happens during an experiment, including experimental conditions, thoughts and observations while conducting the experiment, and, of course, any data collected. Today, you'll be documenting your experiment in a laboratory notebook or on a separate worksheet.

Before starting the Experiment:

- Carefully read the introduction and the protocol. Use this information to form a hypothesis for this experiment.
- Predict the results of your experiment.

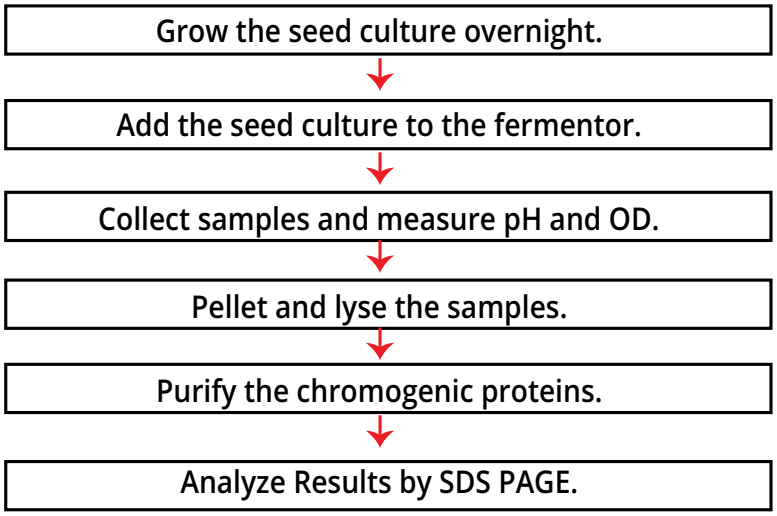
During the Experiment:

- Record your observations.

After the Experiment:

- Interpret the results – does your data support or contradict your hypothesis?
- If you repeated this experiment, what would you change? Revise your hypothesis to reflect this change.

EXPERIMENTAL FLOWCHART:



Laboratory Safety

IMPORTANT -- READ ME!

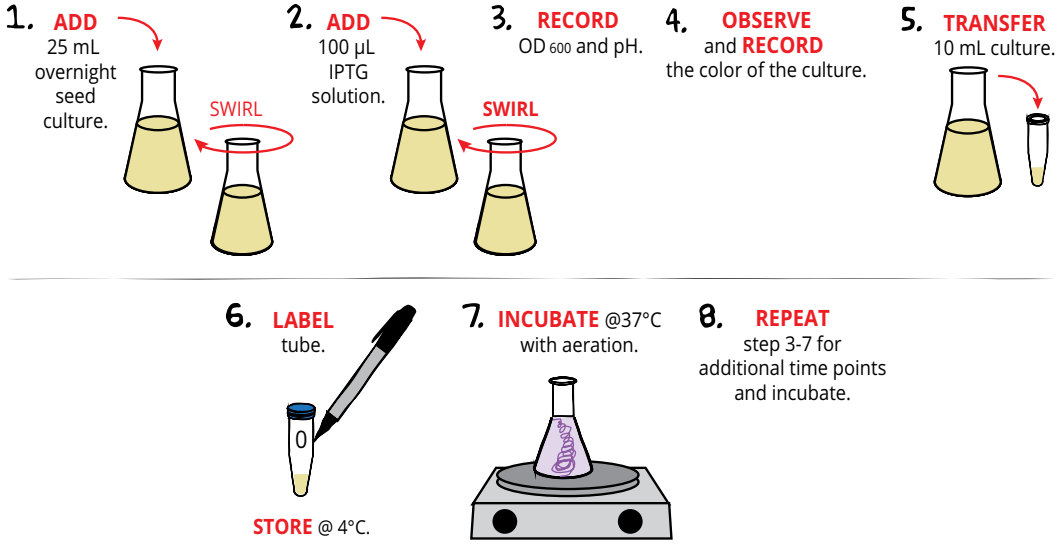
This experiment contains antibiotics that are used to keep cultures free of contamination. Students who have allergies to antibiotics, including AMPICILLIN, should not participate in this experiment.

Although the bacteria used in this experiment are not considered pathogenic, it is good practice to follow simple safety guidelines in handling and disposal of materials contaminated with bacteria.

Wear gloves and safety goggles

1. Wear gloves and goggles while working in the laboratory.
2. Exercise extreme caution when working in the laboratory – equipment used for heating and melting reagents can be dangerous if used incorrectly.
3. Do not mouth pipet reagents - use pipet pumps or bulbs.
4. The *E.coli* bacteria used in this experiment is not considered pathogenic. Although it is rarely associated with any illness in healthy individuals, it is good practice to follow simple safety guidelines in handling and disposal of materials contaminated with bacteria.
5. Properly dispose materials after completing the experiment:
   a. Wipe down the lab bench with a 10% bleach solution or a laboratory disinfectant.
   b. All materials, including pipets, transfer pipets, loops and tubes, that come in contact with bacteria should be disinfected before disposal in the garbage. Disinfect materials as soon as possible after use in one of the following ways:
      - Autoclave at 121°C for 20 minutes.
        Collect all contaminated materials in an autoclavable, disposable bag. Seal the bag and place it in a metal tray to prevent any possibility of liquid medium or agar from spilling into the sterilizer chamber.
      - Soak in 10% bleach solution overnight.
        Immerse open tubes and other contaminated materials into a tub containing a 10% bleach solution. Soak the materials overnight and then discard. Wear gloves and goggles when working with bleach.
6. Always wash hands thoroughly with soap and water at the end of each laboratory period.
7. If you are unsure of something, ASK YOUR INSTRUCTOR!

Module I: Production of Chromogenic Proteins in the Fermentor



Wear gloves and safety goggles

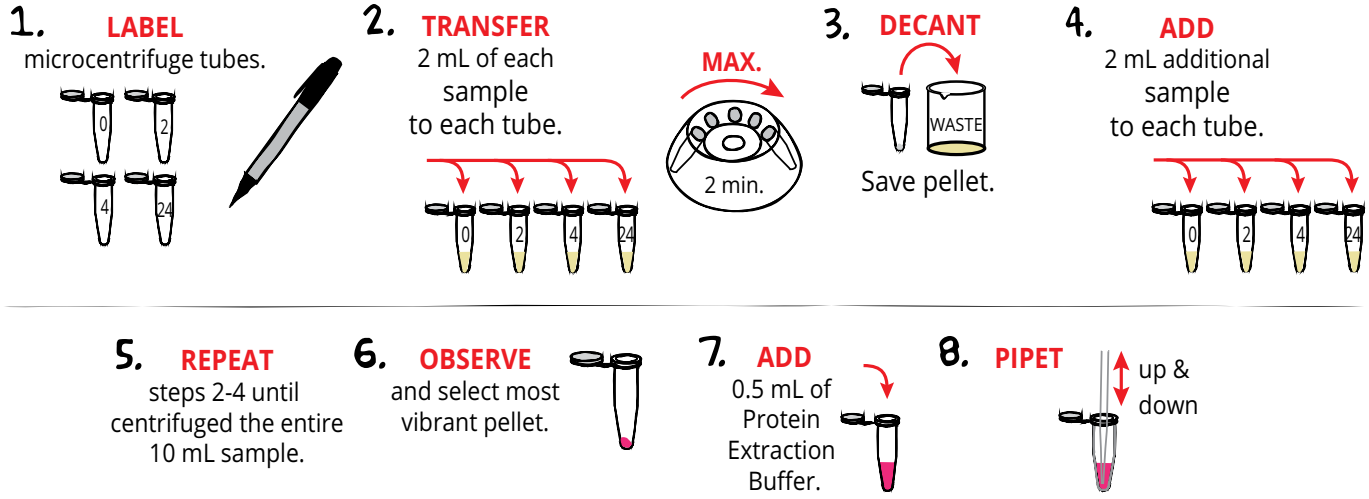
1. **INOCULATE** the media in your flask by adding 25 mL of the overnight seed culture. **SWIRL** to mix.
2. **ADD** 100 µL IPTG solution to the flask. **SWIRL** to mix.
3. Using the spectrophotometer and pH paper **RECORD** the initial optical density at 600 nm (OD_{600}) and the pH value in Table 1. ***NOTE: In a typical culture the starting OD_{600} and pH should measure around 0.2 and 7.0 respectively.***
4. **OBSERVE** the color of the culture. **RECORD** the observations in Table 1.
5. Using a sterile transfer pipet, **TRANSFER** 10 mL of the culture into a 15 mL centrifuge tube.
6. **LABEL** the tube with your initials and the time that the sample was taken. **STORE** the tube at 4°C for later analysis.
7. **INCUBATE** the culture at 37°C with aeration. (i.e.: Stir bar, shaker, air pump)
8. **REPEAT** steps 3 to 7 for any additional time points. We recommend collecting additional measurements and samples at 2 hours, 4 hours, and 24 hours after inoculation.

For cell growth analysis, use leftover LB+AMP as a blank for OD_{600} absorbance measurements. Once you have recorded your measurements, the sample should be safely discarded.

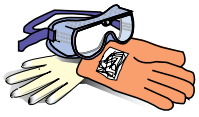
TABLE 1: Monitoring the chromogenic protein fermentation.

Time of Measurement	OD (A600)	pH	Color

Module II: Isolation of Protein

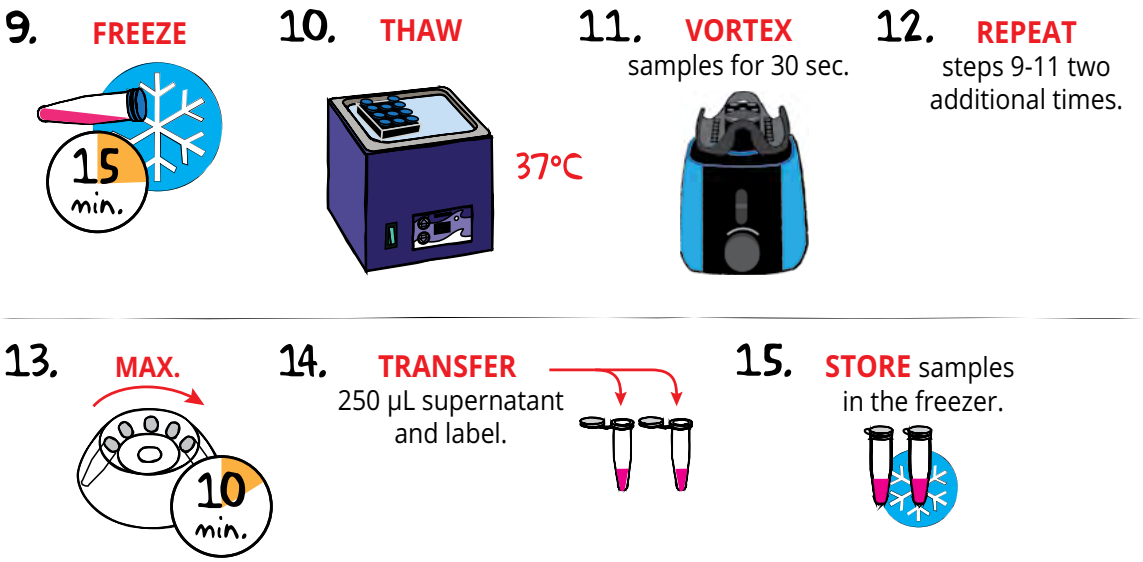


1. **GATHER** culture samples collected in Module I and **LABEL** one 2 mL snap-top microcentrifuge tube for each time point.
2. **TRANSFER** 2 mL of each sample into the appropriate tubes and **CENTRIFUGE** for 2 minutes at max speed.
3. Carefully **DECANT** and discard the supernatant and save the pellet.
4. **ADD** 2 mL of additional sample to each tube.
5. **REPEAT** steps 2-4 until you have centrifuged the entire 10 mL sample.
6. **OBSERVE** each tube and select the tube containing the most vibrantly colored pellet. *This time-point should contain the highest concentration of your chromogenic protein.*
7. **ADD** 0.5 mL of Protein Extraction Buffer to the tube chosen in step 6.
8. **PIPET** up and down or vortex the tube to ensure the pellet is fully resuspended.



Wear gloves and safety goggles

Module II: Isolation of Protein, continued



9. **PLACE** your microcentrifuge tube containing the cells in the -20°C freezer for 15 minutes, or until frozen. **LAY** the tube on its side to ensure rapid freezing.

10. Once the cells are completely frozen, **THAW** the cells by placing the tube in a 37°C waterbath.

11. **VORTEX** the samples vigorously for 30 seconds.

12. **REPEAT** steps 9 – 11 two additional times to fully lyse the cells.

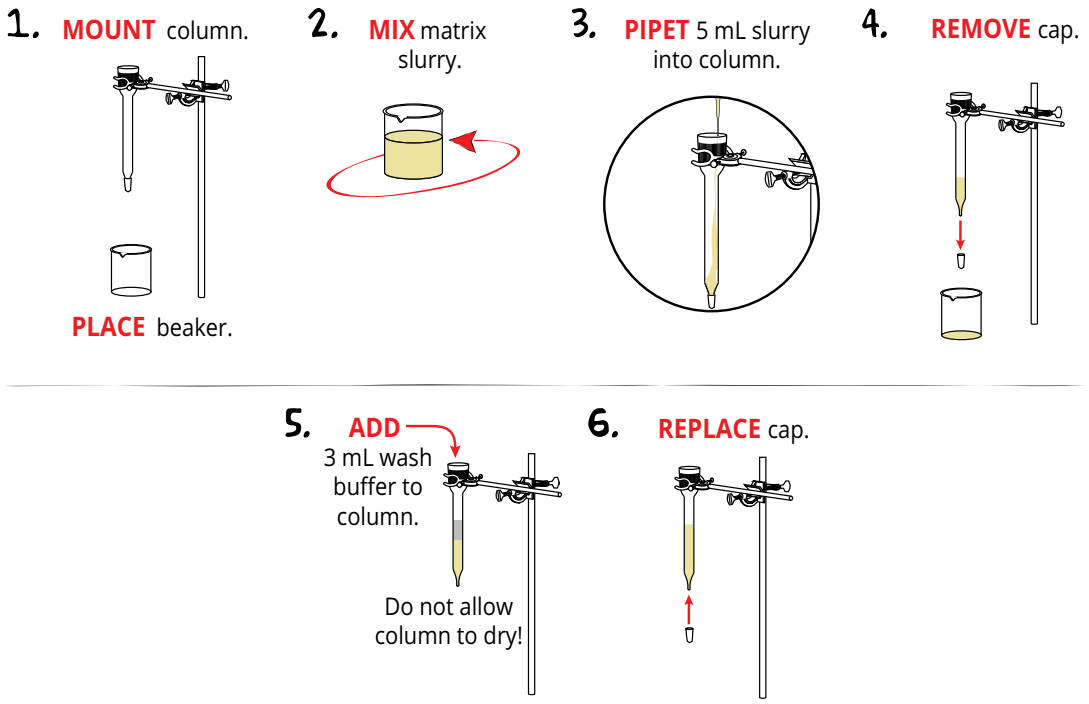
13. **CENTRIFUGE** the tube in a microcentrifuge for 10 minutes at maximum speed.
NOTE: After centrifugation, the supernatant should contain the protein. If the supernatant is not colorful, repeat Steps 9-13 to freeze, thaw, and centrifuge until the supernatant is brightly colored with the chromogenic protein. It is okay for some color to remain in the pellet.

14. **TRANSFER** 250 µl of supernatant into two clean snap-top tubes and label each as "Protein extract" and your group ID.

15. **STORE** the extract in the freezer for the purification in Module III and Module IV.


Wear gloves and safety goggles

Module III: Purification of Protein by Column Chromatography



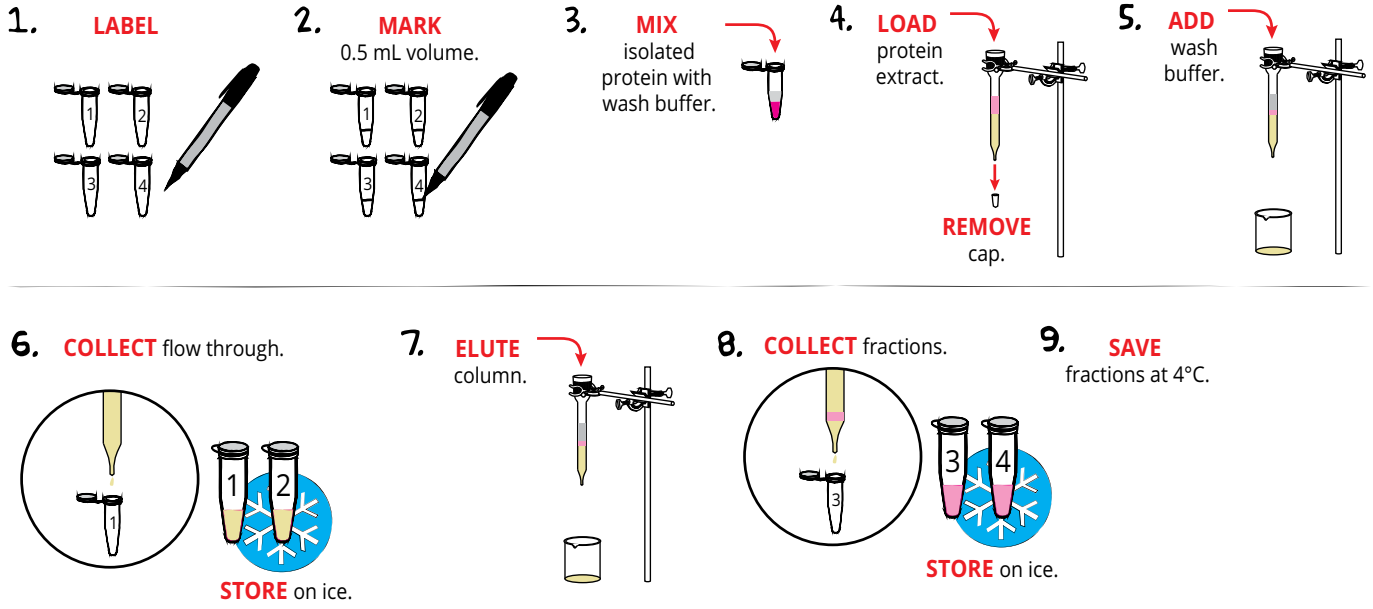
PACKING AND EQUILIBRATING THE COLUMN

1. **MOUNT** the chromatography column vertically on a ring stand. Make sure the column is straight. **PLACE** an empty beaker under the column to collect wash buffer.
2. **MIX** the Ion Exchange Matrix slurry thoroughly by swirling or gently stirring.
3. Carefully **PIPET** 5 mL of the mixed slurry into the column by letting it stream down the inside walls of the column.
   NOTE: If the flow is stopped by an air pocket, stop adding the slurry and firmly tap the column until the air is removed and the slurry continues to flow down the side of the column.
4. **REMOVE** the cap from the bottom of the column and allow the matrix to pack into the column.
5. **ADD** 3 mL of wash buffer to the column and allow the buffer to drain until just above the matrix. ***Do not allow the column to dry!***
6. **REPLACE** the cap and make sure it does not drip.

OPTIONAL STOPPING POINT

The prepared column can be stored at 4°C until needed. Ensure the cap is tight and carefully seal the top to prevent the matrix from drying.

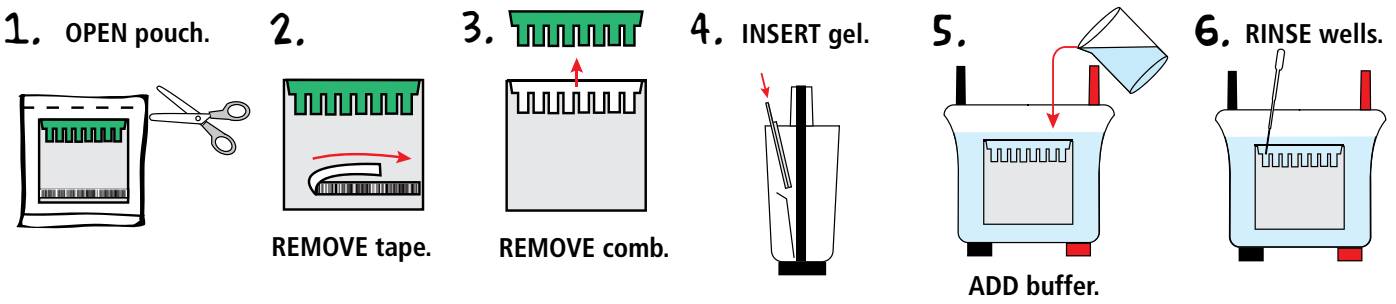
Module III: Purification of Protein by Column Chromatography, continued



COLLECTING COLUMN FRACTIONS OF PROTEIN

1. **LABEL** four snap-top microcentrifuge tubes 1-4.
2. **MARK** each tube with a permanent marker at the 0.5 mL volume.
3. **MIX** 0.25 mL of the protein isolated in Module II with an equal volume of wash buffer.
4. Slowly **LOAD** the column with 0.5 mL of the protein extract. Remove the cap to **ALLOW** the extract to completely enter the column.
5. **ADD** 1 mL of wash buffer to remove protein that is in the flow through.
6. **COLLECT** 0.5 mL of flow through into tube #1 and store on ice. Repeat with tube #2.
7. Sequentially **ELUTE** the column with 2 mL of elution buffer. When the color protein band almost reaches the bottom of the column (near the frit), start collecting the fractions in the microcentrifuges tubes.
8. **COLLECT** 0.5 mL of protein elution into tube #3 and store on ice. Repeat with tube #4.
9. **SAVE** the fractions at 4°C for further analysis.

Module IV - SDS-PAGE Gel Electrophoresis



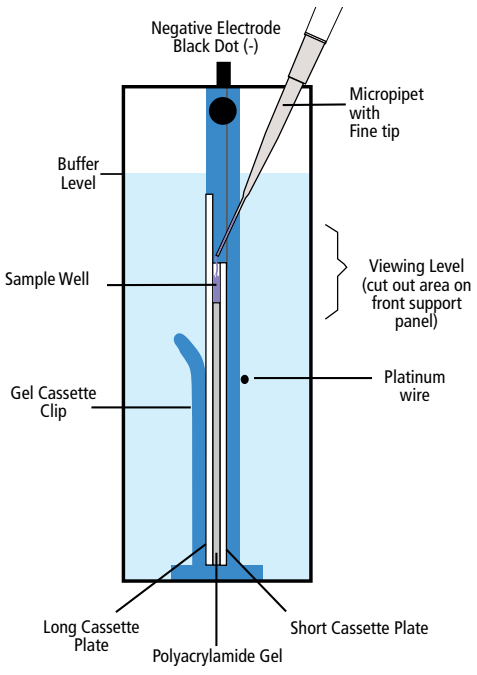
PREPARING THE POLYACRYLAMIDE GEL AND CHAMBER

Wear gloves and safety goggles

NOTE: Although precast polyacrylamide gels and protein chambers will vary slightly in design, the procedure for their use will be similar.

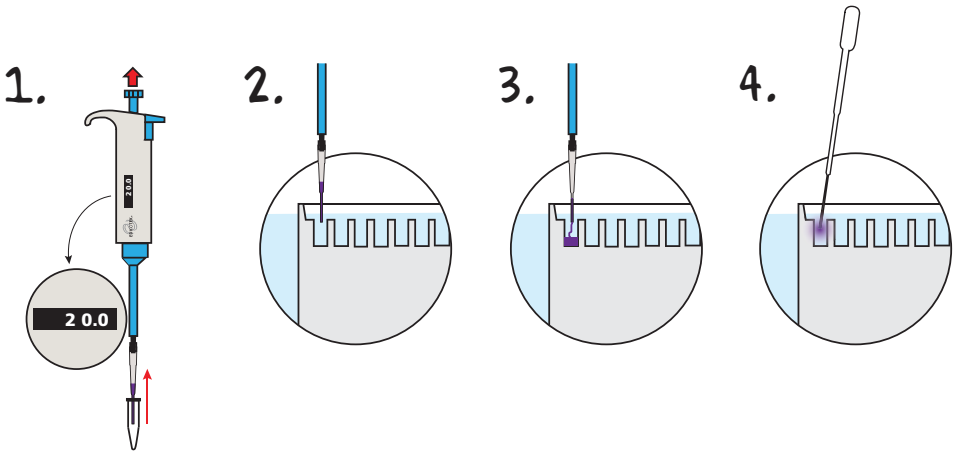
1. **OPEN** the pouch containing the gel cassette. Remove the cassette and place on bench with the shorter front plate facing up.
2. Gels may feature a sticker or tape at the bottom of the front plate. **REMOVE** the tape (if present) to expose the bottom of the gel.
3. Carefully **REMOVE** the comb by gently pulling upwards. Pull the comb straight up to prevent damage to the wells of the gel.
4. **INSERT** the gel into the electrophoresis chamber. Orient the gel according to the manufacturer's instructions. ***NOTE: For EDVOTEK® vertical electrophoresis chambers, the short plate should face the middle of the apparatus.***
5. **ADD** diluted electrophoresis buffer to the chamber. The buffer should cover the top of the shorter plate.
6. **RINSE** each well by squirting electrophoresis buffer into the wells using a transfer pipet. Using the transfer pipet, carefully straighten any wells which may have been distorted during comb removal or rinsing.

The gel is now ready for practice gel loading.



A polyacrylamide gel cassette in the EDVOTEK® Vertical Electrophoresis Apparatus, Model #MV10.

Module IV - SDS-PAGE Gel Electrophoresis, continued



PRACTICE GEL LOADING


Wear gloves and safety goggles

NOTE: EDVOTEK® Cat. #638, Fine Tip Micropipette Tips are recommended for loading samples into polyacrylamide gels. A regular microtip may damage the cassette and result in the loss of protein samples.

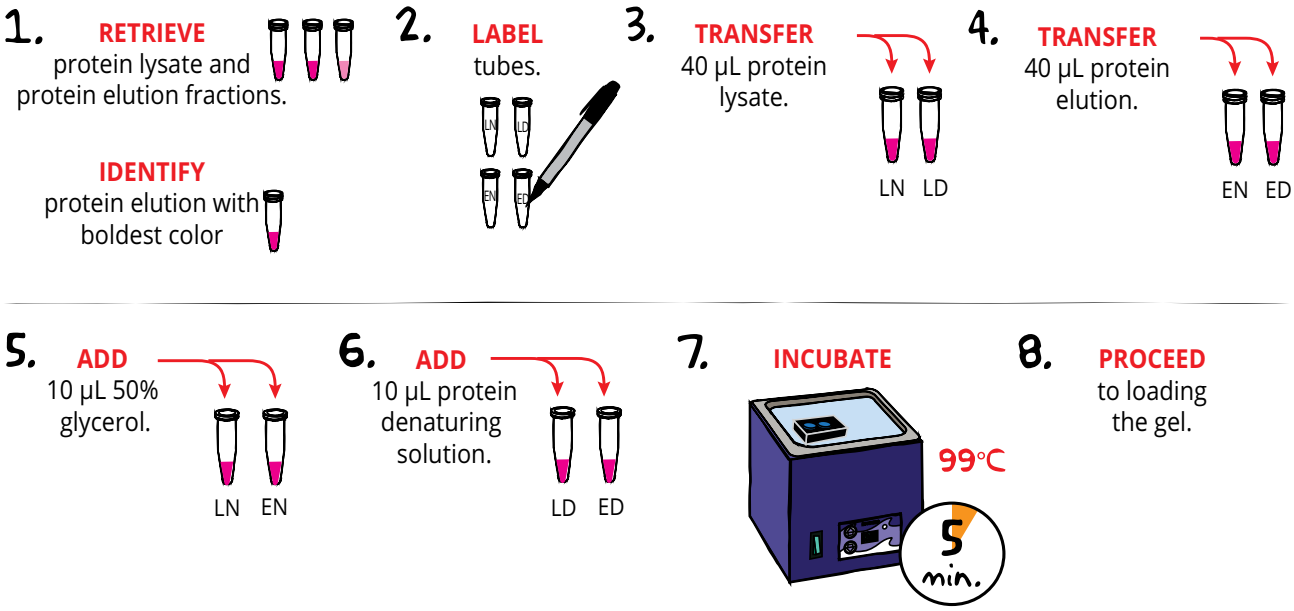
1. **PLACE** a fresh tip on the micropipette. **REMOVE** 20 µL of practice gel loading solution.

2. **PLACE** the lower portion of the pipette tip below the surface of the electrode buffer, directly over a sample well. The tip should be at an angle pointed towards the well. The tip should be partially against the back plate of the gel cassette, but the tip opening should be over the sample well. **Do not try to jam the pipette tip in between the plates of the gel cassette.**

3. **EJECT** all the sample by steadily pressing down on the plunger of the automatic pipette. Do not release the plunger before all the sample is ejected. Premature release of the plunger will cause buffer to mix with sample in the micropipette tip. Release the pipette plunger after the sample has been delivered and the pipette tip is out of the buffer.

4. **REMOVE** the practice gel loading solution from the sample wells. **FILL** a transfer pipet with buffer and **SQUIRT** a stream into the sample wells. This will displace the practice gel loading solution, which will be diluted into the buffer and will not interfere with the experiment. ***NOTE: Practice gel loading solution must be removed from the sample wells prior to sample loading.***

Module IV - SDS-PAGE Gel Electrophoresis, continued



SAMPLE PREPARATION FOR DENATURING SDS-GEL ELECTROPHORESIS

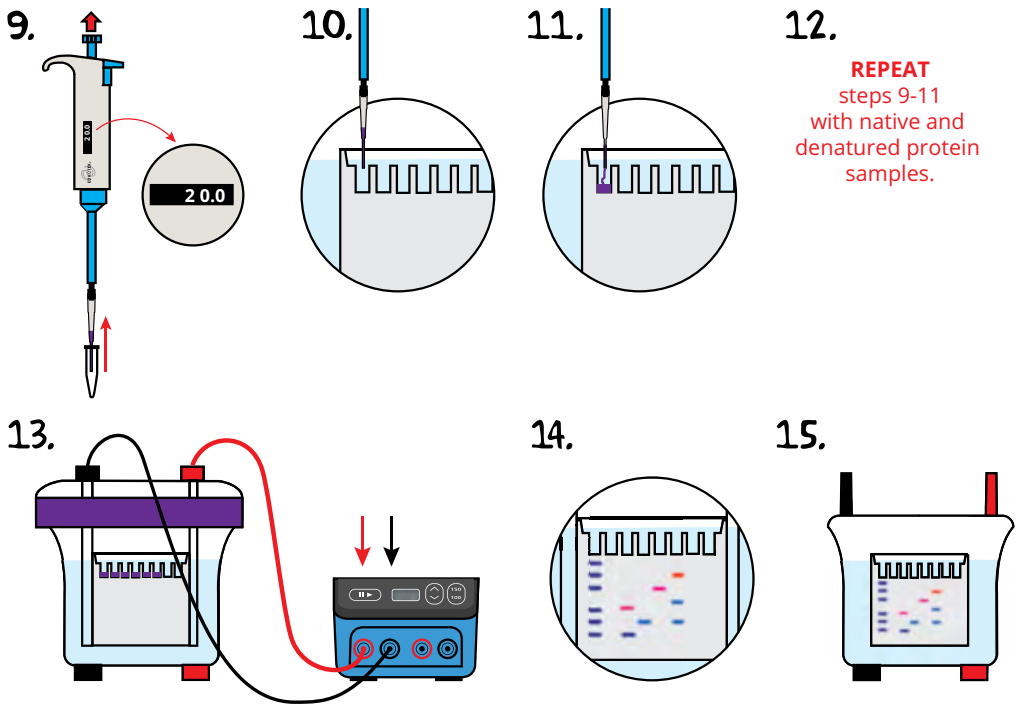

Wear gloves and safety goggles

1. **RETRIEVE** the protein lysate saved at 4°C from Module II as well as the protein elution fractions from Module III. **IDENTIFY** the protein elution tube with the boldest color, this sample will be used for protein analysis.
2. **LABEL** four clean screw-top microcentrifuge tubes "LN", "LD", "EN", and "ED".
3. **TRANSFER** 40 µL of protein lysate to the "LN" and "LD" tubes.
4. **TRANSFER** 40 µL of protein elution to the "EN" and "ED" tubes.
5. **ADD** 10 µL of 50% glycerol to the "LN" and "EN" tubes, then **MIX** each tube and set aside. These will be your native protein samples.
6. **ADD** 10 µL protein denaturing solution to the "LD" and "ED" tubes, then **MIX** each tube and set aside. These will be your denatured protein samples.
7. **INCUBATE** the "LD" and "ED" tubes in a 99°C water bath for 5 minutes.
   NOTE: Denaturing the proteins may remove their color.
8. Immediately **PROCEED** to loading the gel.

TABLE 2: Summary of Protein Sample Preparation

Sample ID	Tube Label	Protein Solution	50% Glycerol	Denaturing Solution
Protein Lysate	LN	40 µL	10 µL	---
	LD	40 µL	---	10 µL
Protein Elution	EN	40 µL	10 µL	---
	ED	40 µL	---	10 µL

Module IV - SDS-PAGE Gel Electrophoresis, continued



9. Using a fresh pipet tip, **MEASURE** 20 µl of the Standard Protein Marker.
10. **PLACE** the pipet tip under the buffer and directly above the sample well, resting gently against the back plate of the gel cassette.
11. Slowly **DISPENSE** the sample by depressing the plunger.
12. **REPEAT** steps 10-12 with the native and denatured protein samples, changing the tip between each new sample. See Table 3.
13. Once all samples have been loaded, carefully **PLACE** the cover onto the electrode terminals and **CONNECT** the electrical leads to the power supply.
14. **SET** the voltage of the power supply and **PERFORM** electrophoresis (see Table A for time and voltage guidelines). Allow the proteins to separate on the gel for the recommended length of time, or until the tracking dye reaches the bottom of the gel.
15. **TURN OFF** the power supply and carefully **REMOVE** the lid. The gel can now be removed from the chamber and stained.

NOTE: Although the native proteins are often visible immediately after concluding the electrophoresis the denature proteins are often invisible on the gel. In order to visualize the proteins, it is necessary to stain with FlashBlue™ Protein stain (see page 20).

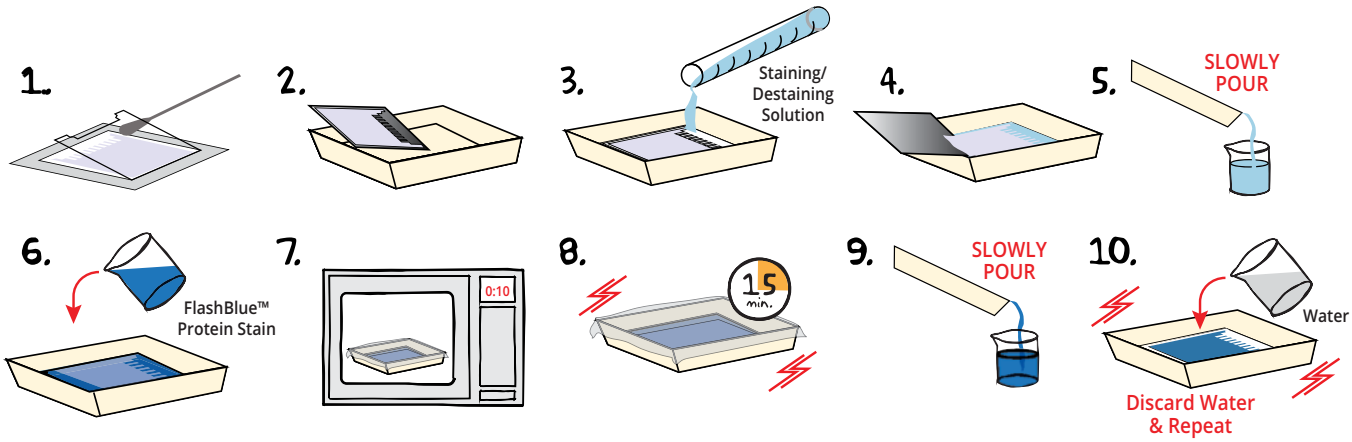
TABLE 3: Gel Loading

Pink Student Group Example Gel	
Lane 1	20 µL of Standard Protein Markers
Lane 2	20 µL of Pink lysate native
Lane 3	20 µL of Pink lysate denatured
Lane 4	20 µL of Pink elution native
Lane 5	20 µL of Pink elution denatured
Purple Student Group Example Gel	
Lane 1	20 µL of Standard Protein Markers
Lane 2	20 µL of Purple lysate native
Lane 3	20 µL of Purple lysate denatured
Lane 4	20 µL of Purple elution native
Lane 5	20 µL of Purple elution denatured

Table A Time and Voltage Guidelines

	Recommended Time	
Volts	Minimum	Optimal
100	80 min.	95 min.
125	60 min.	75 min.
150	50 min.	60 min.

Module V: Gel Staining with FlashBlue™ Protein Stain



1. After electrophoresis, **LAY** the cassette down and **REMOVE** the front plate by placing a thin spatula or screwdriver at the side edge and gently lift it away from the larger back plate. In most cases, the gel will stay on the back plate. If it partially pulls away with the front plate, let it fall onto the back plate. ***Handle very carefully as the thin gels are extremely fragile.***
2. **TRANSFER** the gel on the back plate to a clean tray.
3. **ADD** a sufficient volume (approximately 50-75 mL) of the staining/destaining solution into the tray to **COVER** the gel and back plate.
4. Carefully **REMOVE** the back plate from the tray, leaving just the gel in the tray containing the staining/destaining solution. Bands may be easier to see once the cassette is removed. **OBSERVE** the gel and take a photo/sketch the banding pattern in your notebook before continuing. ***NOTE: If the gel sticks to the plate, gently nudge the gel off the plate using two GLOVED fingers.***
5. **DISCARD** the staining/destaining solution. ***Pour slowly to keep the gel in the container.***
6. **ADD** 30 mL of prepared FlashBlue™ Protein Stain.
7. (OPTIONAL) **COVER** the container with plastic wrap and **MICROWAVE** for 10 seconds to gently heat the solution.
8. **INCUBATE** for 15 minutes at room temperature, **SHAKING** occasionally.
9. **DISCARD** the FlashBlue™ Protein Stain solution. ***Pour slowly to keep gel intact and in container.***
10. **WASH** the gel by partially filling container with water and gently rocking back and forth several times. **DISCARD** the used water and **REPEAT** with fresh water.

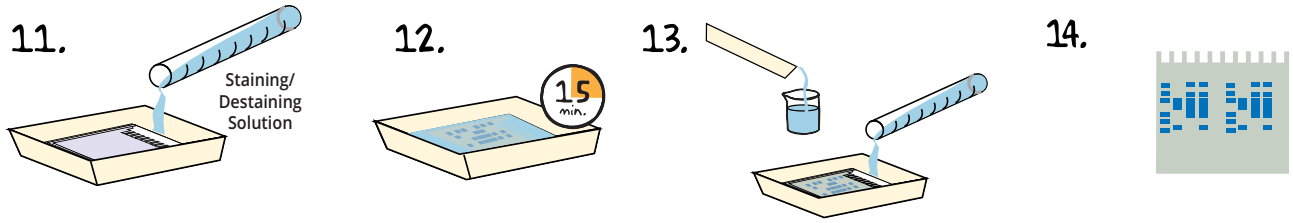
WEAR GLOVES AND SAFETY GOGGLES

Gloves must be worn during this procedure. Avoid touching the gel without gloves.

Polyacrylamide gels are very thin and fragile. Use care in handling to avoid tearing the gel.

continued

Module V: Gel Staining with FlashBlue™ Protein Stain, continued



11. **ADD** 30 mL of staining/destaining solution to the gel.

12. **INCUBATE** for 15 minutes at room temperature. **EXAMINE** the gel.

13. (OPTIONAL) **DISCARD** the used staining/destaining solution and **ADD** an additional 30 mL of staining/destaining solution. **INCUBATE** for 15-60 minutes at room temperature until the appearance and contrast of the protein bands against the background improves.

14. After staining, protein bands will appear medium to dark blue against a light background. A white light box can be used to better visualize the protein bands.

STORING THE GEL

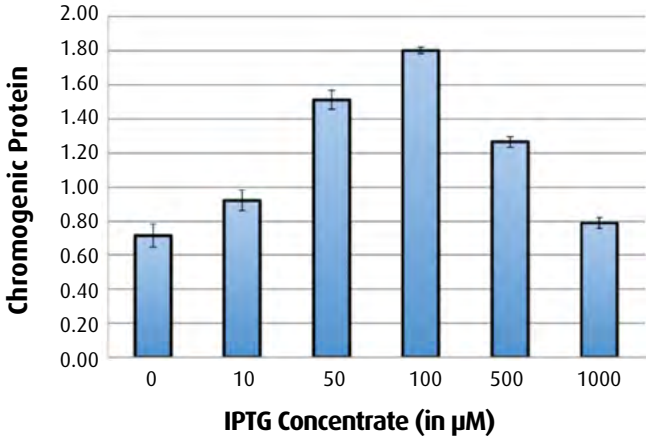
- Gel may be left in deionized water for several hours with no loss in sensitivity and band intensity. This step should be performed once a desired background and stained protein bands are obtained. Pour off the destaining solution from Step 12 (or 13) and add a sufficient amount of deionized water to cover the gel.

- For permanent storage, the gel can be dried between two sheets of cellophane (saran wrap) stretched in an embroidery hoop. Air dry the gel for several days until the gel is paper thin. Cut the "extra" saran wrap surrounding the dried gel. Place the dried gel overnight between two heavy books to avoid curling. Tape it into a laboratory book.

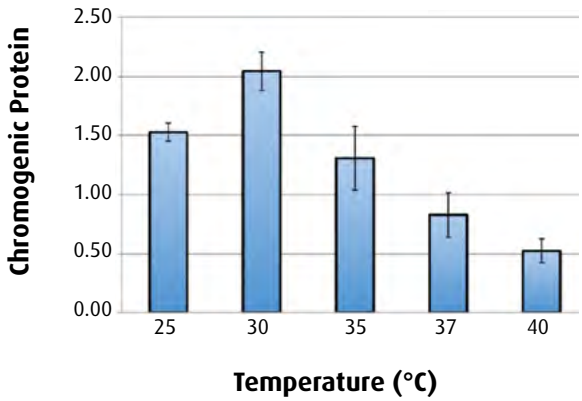
Study Questions

1. Compare and contrast the three types of fermentation. What type of fermentation was performed in this experiment?
2. At which step in the experiment do the cells start producing the chromogenic protein? Why?
3. Why might the pH of the growth medium change during fermentation?
4. What are the most common commercially available fermentation products?
5. What kind of product are the chromogenic proteins? Are they intra- or extracellular?
6. Upstream bioprocessing involves optimizing the microbial growth conditions in order to produce the maximum amount of product. As a Bioprocess Engineer, you have decided to change the temperature and the IPTG concentration of your fermentation process in order to increase the protein yield. You performed the following experiments and collected the following data. Which conditions would you use?

CHROMOGENIC PROTEIN					
Concentration IPTG (uM)	Trial 1	Trial 2	Trial 3	Average	ST DEV
0	0.79	0.69	0.66	0.71	0.068
10	0.95	0.85	0.92	0.92	0.061
50	1.50	1.46	1.57	1.51	0.056
100	1.80	1.82	1.78	1.78	0.020
500	1.24	1.25	1.30	1.26	0.032
1000	0.75	0.80	0.81	0.79	0.032



CHROMOGENIC PROTEIN					
Temperature (°C)	Trial 1	Trial 2	Trial 3	Average	ST DEV
25	1.51	1.46	1.61	1.53	0.076
30	2.01	1.90	2.22	2.04	0.163
35	1.37	1.01	1.54	1.31	0.271
37	0.99	0.62	0.87	0.83	0.189
40	0.63	0.51	0.43	0.52	0.101



Instructor's Guide

IMPORTANT - READ ME!!

This experiment contains antibiotics that are used to keep cultures free of contamination. Students who have allergies to antibiotics, including AMPICILLIN, should not participate in this experiment.

ORGANIZATION AND IMPLEMENTING THE EXPERIMENT

Prior to starting this experiment, carefully check the list of Components and Requirements on pages 3 and 4 to ensure that you have all the necessary components and equipment.

The guidelines that are presented in this manual are based on five laboratory groups. The experiment is divided into four modules and should take approximately one week to perform. The following are implementation guidelines, which can be adapted to fit your specific set of circumstances.

APPROXIMATE TIME REQUIREMENTS

Preparation For:	What to do:	When:	Time Required:
Module I: Production of Chromogenic Proteins	Prepare and autoclave LB Growth Medium	Up to two days before performing the experiment.	2 hours
	Prepare overnight seed culture	One day before performing the experiment.	1 hour
	Prepare the shaker incubator and spectrophotometer	One hour before performing the experiment.	10 min.
	Prepare and aliquot reagents and gather materials for students	Anytime before performing the experiment.	20 min.
Module II: Isolation of Proteins	Aliquot protein extraction buffer and gather materials	Anytime before performing the experiment.	20 min.
	Prepare the waterbath, freezer, centrifuge, & vortex	One hour before performing the experiment.	10 min.
Module III: Purification of Protein by Column Chromatography	Prepare and aliquot reagents	One hour before performing the experiment.	10 min.
	Prepare ion exchange matrix	One hour before performing the experiment.	30 min.
Module IV – SDS-PAGE Gel Electrophoresis	Prepare SDS-PAGE buffer	Up to one day before performing the experiment.	10 min.
	Prepare and aliquot reagents and equipment	Anytime before performing the experiment.	30 min.
	Prepare waterbath at 99° C	Anytime before performing the experiment.	5 min.
Module V: Gel Staining with FlashBlue™ Protein Stain	Prepare Gel Staining and Destaining solutions	Anytime before performing the experiment.	10 min.

Pre-Lab Preparations - Module I

PREPARATION OF THE FERMENTORS

Each group will maintain one flask fermentor. We recommend using 500 mL flasks with 250 mL of media, although smaller flasks and volumes can be used. Always ensure that the flask contains appropriate head space; we recommend filling flasks to no more than 50% capacity.

FOR MODULE I,
Each group should receive:

- 1 Erlenmeyer flask (500 mL)
- 4 Sterile transfer pipets
- 4 Centrifuge tubes (15 mL)
- 1 Tube of IPTG

STERILIZATION OF LAB MATERIAL

Successful fermentation depends heavily on keeping the bacteria cultures free from contamination by microorganisms such as yeast, fungi, and viruses. All materials that come into contact with the flask fermentors must be sterile, and manipulations must not allow any direct link between the cultures and the non-sterile surroundings.

We recommend you sterilize: One 500 mL graduated cylinder, one 100 mL graduated cylinder, five 500 mL flasks, two 250 mL flasks, and seven magnetic stir bars per class.

To prevent contamination, the flasks, graduated cylinders, and stir bars used for this experiment must be sterilized. Many different techniques can be utilized to sterilize the equipment, please check with manufactures to ensure heat or chemical resistance before selecting the method.

Autoclave: Cover the openings of the equipment with aluminum foil. Autoclave at 121°C for 15 minutes.
NOTE: Autoclave indicator tape should be used to ensure that proper temperatures have been achieved.

Dry Heat (Baking): Place components into a preheated oven at 170°C and bake for 60 minutes. Carefully remove the equipment and cover any openings with aluminum foil while still hot.

Cleaning with alcohol: Rinse with 70% Ethanol, ensuring coverage of all surfaces. Allow equipment to air dry before covering any openings with aluminum foil.

PREPARATION OF LB GROWTH MEDIA

The LB growth media can be prepared up to 48 hours before beginning the experiment. The LB Growth Media concentrate (Component C) provided in this kit is sterile. Distilled water can be purchased or tap water can be briefly boiled to sterilize prior to preparing the final media.

1. Follow the table below to **PREPARE** the media you need for the experiment. ***NOTE: We recommend 5 groups with 250 mL cultures each, but smaller fermentors can be used if necessary. Media can be mixed in individual volumes or as one large volume and then aliquoted.***
2. **DISPENSE** 250 mL of media into 5 sterile flasks. Retain the remaining media to prepare seed cultures and to blank the spectrophotometers.
3. **ADD** 0.6 mL of sterile water to the tube of Ampicillin (Component D). Invert to mix.
4. ADD 0.1 mL of the Ampicillin solution to each 250 mL flask of media. Swirl to mix. Store the remaining Ampicillin at 4°C for preparation of seed cultures.

Final Volume	Distilled Water	LB Growth Media Concentrate (Component C)
375 mL	262.5 mL	112.5 mL
750 mL	525 mL	225 mL
1500 mL	1050 mL	450 mL

Pre-Lab Preparations - Module I

PREPARATION OF THE SEED CULTURE

This kit includes bacteria expressing Pink or Purple chromogenic proteins. The two proteins will not separate during the chromatography experiment so it is important that each group select one option in advance.

1. **ALIQUOT** 125 mL of LB Growth Media into two sterile 250 mL Erlenmeyer flasks. **STORE** remaining media at 4°C for Module IV.
2. **ADD** 50 µL of Ampicillin to the Media in each flask.
3. **LABEL** each flask as either "Purple Chromogenic Protein Seed Culture" or "Pink Chromogenic Protein Seed Culture".
4. **ADD** the entire contents of BactoBeads™ transformed with purple or pink plasmid vials to the appropriate flask. Gently swirl to **MIX**, ensuring that the beads are completely dissolved. **COVER** with foil to prevent contamination.
5. **INCUBATE** the flasks overnight at 37°C in a shaking incubator. ***NOTE: If a shaker incubator is unavailable we recommend stirring or shaking the culture at room temperature.***
6. **ALIQUOT** 25 mL of the seed culture into 50 mL conical tubes for each student group.

PREPARATION FOR PRODUCTION OF CHROMOGENIC PROTEINS IN THE FERMENTOR

1. **PREPARE** any option equipment that will be used to aerate fermentors. This includes air pumps, stir plates, and shaker incubators.
2. **GATHER** the spectrophotometer, cuvettes and pH paper for the class.
5. **ADD** 0.6 mL of sterile water to the tube of IPTG (Component E). Invert to mix.
6. **ALIQUOT** 110 µL IPTG into a snap-top microcentrifuge tube for each group. Store the aliquots at -20°C until needed.

Pre-Lab Preparations - Module II

FOR MODULE II,
Each group should receive:
- 6 snap-top microcentrifuge tubes (2 mL)
- 4 Transfer pipets
- 1 tube of Protein Extraction Buffer

PREPARATION FOR ISOLATION OF PROTEIN

1. **ALIQUOT** 2.5 mL of Protein Extraction Buffer (Component F) into a 15 mL conical for each group.
2. **PREPARE** a centrifuge, freezer, and vortex.
3. **PREPARE** a waterbath at 37°C.

Pre-Lab Preparations - Module III

PURIFICATION OF PROTEIN BY COLUMN CHROMATOGRAPHY

Packing the column during Module III should take students between 15 and 30 min. To save time, columns can be prepared by students up to one day ahead of time during incubation steps of Module II or as a separate activity. Packed columns should be capped and stored with 1x wash buffer at 4°C until needed. ***Do not let the matrix dry!***

FOR MODULE III
Each group should receive:
- 1 Waste container
- 4 Snap-top microcentrifuge tubes
- 2 Transfer pipets
- 1 Chromatography Column
- Ring stand
- Column clamps
- Wash buffer
- Ion Exchange Matrix slurry
- Elution buffer

1. **LABEL** five beakers or flasks as "Wash Buffer".
2. **DILUTE** the concentrated Wash buffer (Component G) by adding 10 mL of buffer to 90 mL of distilled water to make a 1x solution.
3. **ALIQUOT** 5 mL of Wash buffer to each of the beakers or flasks. Save the remaining buffer for preparing the ion exchange matrix.
4. **LABEL** five 2 mL snap-top microcentrifuge tubes "Elution Buffer".
5. **ALIQUOT** 2 mL of elution buffer (Component H) into each of the microcentrifuge tubes.

PREPARATION OF ION EXCHANGE MATRIX (SLURRY)

1. **ADD** the entire contents the Dry Ion Exchange Matrix (Component I) to a 100 mL beaker.
2. **ADD** 30 mL of the wash buffer 1X to the beaker containing the ion exchange matrix. Stir occasionally for 15 minutes. Use a spoon or spatula to break apart any hard clumps.
3. **LABEL** five 15 mL conical tubes as "Ion Exchange Matrix".
4. **POUR** 5 mL of the matrix into each tube, mixing between pouring each aliquot.

Pre-Lab Preparations - Module IV

Most precast polyacrylamide gels have 10-12 wells. We suggest TWO students groups share a gel as shown in the results section (page 28). By sharing, the lab only requires three PAGE gels. Additional gels/units will require ordering additional electrophoresis buffer and stain.

FOR MODULE IV,
Two groups will SHARE:
- 4 Screw-top microcentrifuge tubes
- 1 Tube of Standard Protein Markers
- 1 Tube of Practice Gel Loading Solution
- 1 Tube of 50% glycerol
- 1 Tube of Protein Denaturing Solution
- SDS-PAGE gel

SDS ELECTROPHORESIS BUFFER

Tris-Glycine-SDS buffer is supplied as a 10x concentrate and must be diluted before use. To dilute, add 1 part buffer concentrate to 9 parts distilled water. Approximate volumes of 1x electrophoresis buffer for EDVOTEK vertical electrophoresis units are listed below in Table B. For other units please refer to the manufacturer's instructions.

1. **ADD** 160 µl of distilled or deionized water to the tube of Standard Protein Markers (Component J) and allow the sample to hydrate for several minutes. Vortex or flick tube vigorously to mix.
2. **ALIQUOT** 25 µL of resuspended Standard Protein Markers into 1.5 mL snap-top microcentrifuge tubes for each group. Aliquots may be kept at room temperature for immediate use or frozen until needed.
3. **ALIQUOT** 25 µL of 50% glycerol (Component K) into a 1.5 mL snap-top microcentrifuge for each group.
4. **ALIQUOT** 25 µL of Protein Denaturing Solution (Component L) into a 1.5 mL snap-top microcentrifuge for each group.
5. **PREPARE** a 99°C water bath for the class.
NOTE: Multiple groups can share a single SDS-PAGE gel.

Table B — Tris-Glycine-SDS Electrophoresis (Chamber) Buffer

EDVOTEK Model #	Total Volume Required	Dilution: 10x Conc. Buffer	+ Distilled Water
MV10	580 ml	58 ml	522 ml
MV20	950 ml	95 ml	855 ml

Pre-Lab Preparations - Module IV, continued

ALIQUOT SAMPLE PREPARATION SOLUTIONS

1. ALIQUOT 50 mL of protein denaturation solution for each group.
2. ALIQUOT 50 mL of glycerol for each group. Alternatively, because this is a highly viscous solution, it may be easier for the class to share the original tube.

Pre-Lab Preparations - Module V

PREPARATION FOR STAINING GELS

1. Prepare a stock solution of white vinegar and ethanol* by combining 400 mL white vinegar with 200 mL ethanol. Gently mix. Label as "Staining/Destaining Solution".
2. Add 130 mL of the Staining/Destaining Solution to the bottle of FlashBlue™ Protein Stain. Shake briefly to mix.
3. Store both solutions at room temperature until needed.
4. TWO student groups will share: 30 mL FlashBlue™ Protein Stain, 140 mL Staining/Destaining Solution, water, a staining tray, and plastic wrap.

FOR MODULE V,
Two groups will SHARE:
- FlashBlue™ Protein Stain
- Staining/Destaining Solution
- Water
- Staining Tray

**White vinegar, sometimes called distilled or spirit vinegar, is an easy to find cooking and cleaning vinegar with an acetic acid concentration between 5-8% and a pH ~2.6. Ethanol is a common lab supply which is available at various concentrations. Our FlashBlue™ Protein Stain has been designed to work with a wide range of white vinegars. However, we do recommend using 95% Ethanol or higher.*

Experiment Results and Analysis

Below are results from an experiment comparing the fermentation of Purple and Pink Chromogenic Proteins grown at 37°C with shaking for aeration. Your results may vary depending on a number of factors, including the length of incubation, temperature, and accuracy of pipetting.

MODULE I

Flask #1 - Purple Chromogenic Protein

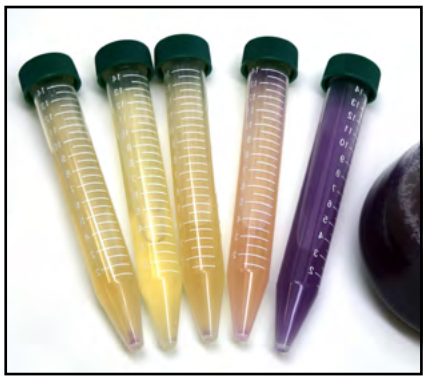
Time of Measurement	OD (A_{600})	pH	Color
10:00 AM	0.184	7.0	Tan
12:00PM	0.788	7.0	Tan
2:00 PM	1.381	7.0	Chestnut
10:00 AM	2.423	8.0	Purple

Flask #2 - Pink Chromogenic Protein

Time of Measurement	OD (A_{600})	pH	Color
10:00 AM	0.164	7.0	Tan
12:00PM	0.889	7.0	Tan
2:00 PM	1.487	7.0	Tan
10:00 AM	2.571	8.0	Pink

Pink and purple protein cultures after 24 hours.

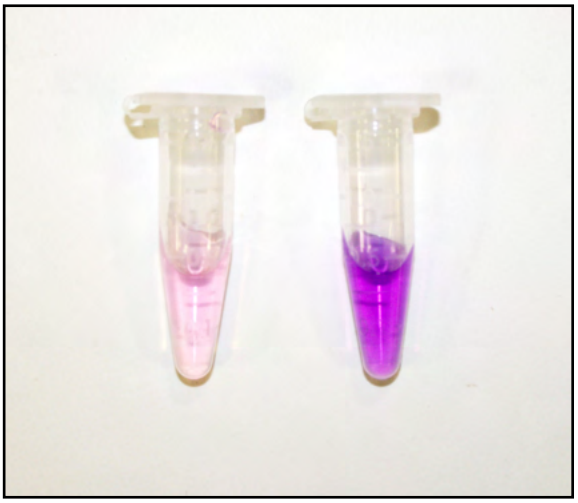
Time-course of purple protein fermentation



Experiment Results and Analysis, continued

MODULE II

Representative image of Pink and Purple protein extracts.

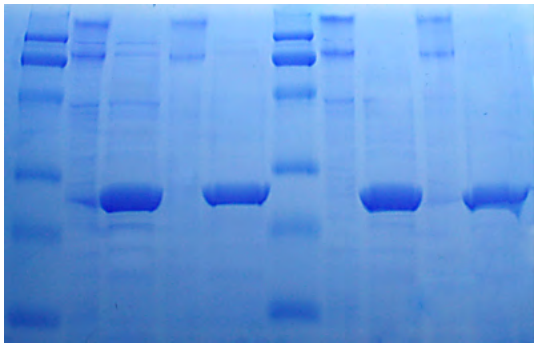


MODULE III

Representative image of Purple protein elution.

MODULE IV

Expected results for stained SDS-PAGE gel.



Pink Student Group Example Gel	
Lane 1	Standard Protein Markers
Lane 2	Pink lysate native
Lane 3	Pink lysate denatured
Lane 4	Pink elution native
Lane 5	Pink elution denatured
Purple Student Group Example Gel	
Lane 1	Standard Protein Markers
Lane 2	Purple lysate native
Lane 3	Purple lysate denatured
Lane 4	Purple elution native
Lane 5	Purple elution denatured

Please refer to the kit insert for the Answers to Study Questions