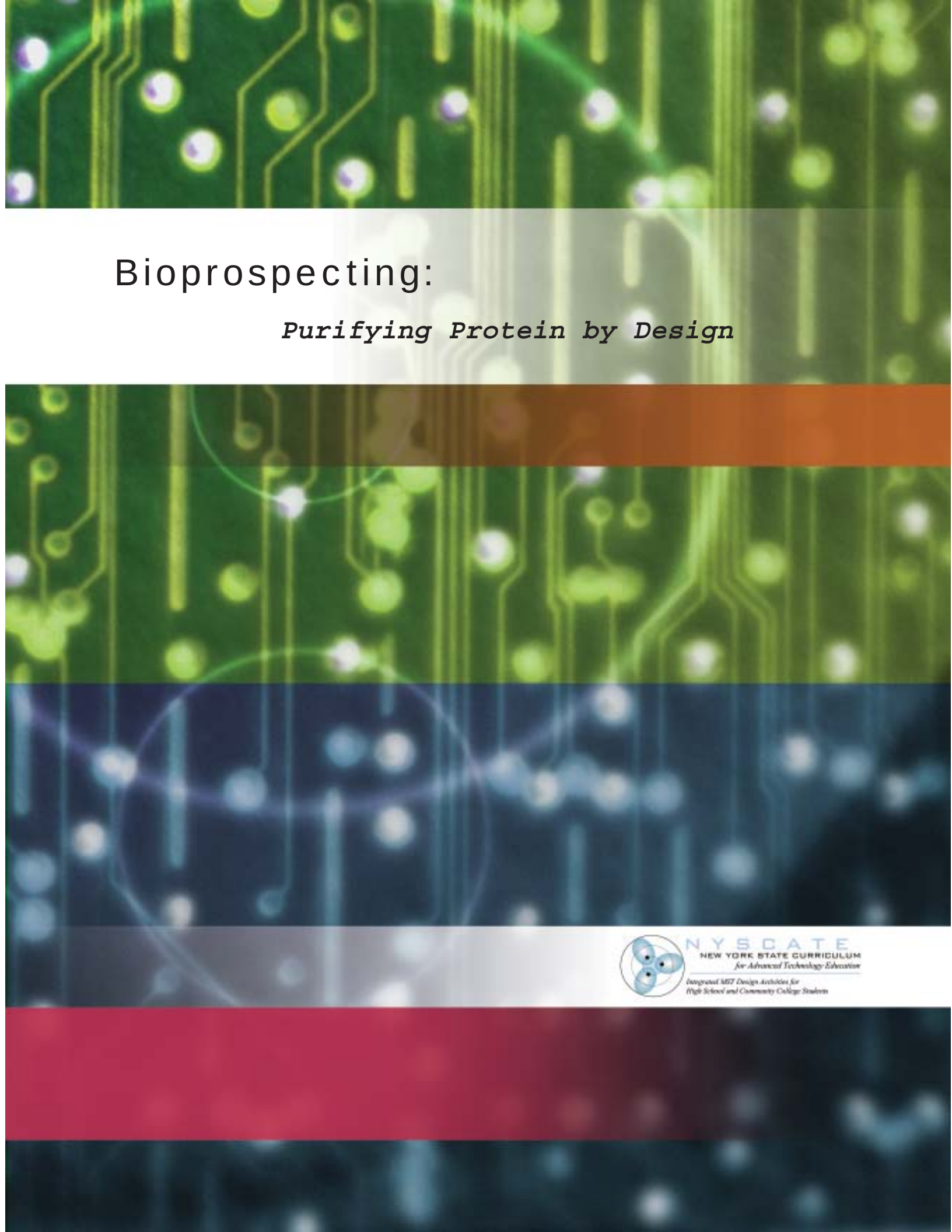
Bioprospecting:

Purifying Protein by Design



NYSCATE
NEW YORK STATE CURRICULUM
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Integrated MST Design Activities for High School and Community College Students

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Bioprospecting: Purifying Protein by Design

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Bioprospecting: Purifying Protein by Design

I. INTRODUCTION AND OVERVIEW

ABSTRACT

This module, one of 13 NYSCATE modules, features the integration of mathematics, science, and technology (MST) through the design of a process to isolate and purify a protein from a mixture of proteins. In response to a challenge, groups of students consider specifications and work within constraints as they design, construct, and test their protocol. They use the SDS-PAGE technique to test the effectiveness of their two-step purification process.

Rather than proceed by trial and error alone, students are expected to design a purification process based on mathematical and scientific principles that they apply consciously. To assist students in using informed design, as opposed to trial and error, this module includes a number of activities that are Knowledge and Skill Builders (KSBs). Topics included in the KSBs are informed design, methods for protein purification, bioinformatics, preparation of solutions and media, cloning, screening of bacterial cultures, chromatography, testing for purity, and bioethics.

GRADE LEVEL

This module is most appropriate at a second-year college level.

TIME ALLOCATION

In a course with three hours of class time and a two-hour lab per week, this module would require five to seven weeks to complete. It is assumed that all classroom and laboratory time will be devoted to this module for that time period. The time may vary depending on how much presentation/review you provide for students on basic protein structure and function. Some classroom time will need to be allocated to prepare students for the laboratory work they will be doing each week.

EXISTING COURSES ENHANCED BY THE MODULE

This module might be used for general biology, biochemistry, genetics, or biotechnology courses, especially at a second-year college level.

CURRICULUM PARTNER

This curriculum is a modified version of the Secrets of the Rainforest Kit, which is produced by Bio-Rad Laboratories as part of their Biotechnology Explorer Series. The Secrets of the Rainforest curriculum was adapted and placed into the context of the scenario in the Design Challenge of this curriculum module. Information on the Explorer Series can be found at www.bio-rad.com.

SOURCES

Biotechnology Explorer, *Secrets of the Rain Forest*. Bio-Rad Laboratories, 1997.

Lyons, Eilene. *Bioinformatics: Basic Blast Search.* St. Louis Community College. Biotech Lab, 2000.

Protein Purification Handbook. Amersham Pharmacia Biotech. 1999. *Polymer Research and Development*. Science in a Technical World. W.H. Freeman and Company. N.Y. 2000.

Seidman, Lisa A. and Moore, Cynthia, J. *Basic Laboratory Methods for Biotechnology: Textbook and Laboratory Reference.* Prentice Hall, 2000.

II. DESIGN CHALLENGE OVERVIEW

SETTING THE CONTEXT FOR STUDENTS

Introduction

At a recent conference sponsored by the pharmaceutical industry, you and your research partners attended a presentation in which you learned of an effective folk remedy that is used for the prevention of fungal diseases in humans. During the presentation you learned that there is evidence that, among the people who take this remedy, there is a lower incidence of breast cancer and heart disease. To explore this further, you have obtained a sample of this folk remedy and determined that it contains the following ingredients:

1. Water
2. Salt (NaCl)
3. A prepared extract from pigeon feathers
4. Muskmelon seeds
5. Southern copperhead snake venom

A chemical analysis of the material reveals that the remedy contains three proteins that may be responsible for the beneficial effects that you learned about at the conference. You and your partners have decided to start a new biotechnology company that will identify the protein that may be responsible for reducing the risk of breast cancer and heart disease. Your company will then clone the gene for this protein into bacteria. By growing these bacteria in nutrient culture, your company expects to produce large quantities of the protein that can be purified and then tested on humans in clinical trials.

Design Challenge

As part of a group, you will design a way to purify a protein in large quantities for use in clinical trials. You will purify this protein from a bacterial culture through a two-step purification process.

Specifications

Purify Green Fluorescent Protein (GFP) from a bacterial culture lysate and use SDS–PAGE technique to test the effectiveness of the purification protocol that you designed.

Constraints

You will be provided with a variety of materials used to purify proteins that will improve your design solution. The materials will come from many of the currently applied methods used in protein purification (ion exchange, affinity chromatography, size exclusion, etc.). The following constraints will be imposed:

- The design will be constrained by the materials supplied for use in protein purification.

- The design must be limited to a two-step chromatographic purification process.

III. GOALS AND LEARNING OUTCOMES

In this module the teacher:

- introduces the students to the biotechnology industry, including DNA recombinant technology and production of pharmaceuticals
- reviews protein structure and function
- helps students understand implementation of the design process and its relationship to the methods of science, including human clinical trials
- provides students with a Design Challenge
- facilitates student acquisition of skills and knowledge needed to design a protein purification protocol
- works with groups as they compose, construct, test, and improve their protein purification protocols
- helps student groups in the preparation of class presentations on the results of their work.

In this module the students:

- work in teams to address the Design Challenge presented in this module
- follow important safety guidelines in the laboratory as they work with the Design Challenge
- maintain a proper laboratory notebook throughout the module, which will include a detailed record of work as it relates to the Design Challenge
- complete all of the necessary Knowledge and Skill Builder (KSB) activities that are associated with the Design Challenge in this module
- participate in classroom discussions throughout the module
- work within the team to produce and deliver a classroom presentation on the results of the design activities.

IV. Timeline Chart

WEEK	FOCUS MODEL COMPONENT (for Teacher)	INFORMED DESIGN LOOP COMPONENT (for Student)	CLASSROOM ACTIVITY	LABORATORY ACTIVITY
1	Focus discussion on problem context Organize for informed design	Clarify design specifications and constraints (Student Handout #1: Overview of the Module and Design Challenge)	Review protein structure and function, biotechnology methods, and recombinant DNA technology. Introduce module. Discuss Design Challenge. Review design process and compare to scientific inquiry.	Review general lab safety and precautions relevant to module. Students complete: KSB 1: The Laboratory Notebook KSB 2: Biotechnology and the Economy KSB 3: Methods for Protein Purification
2***	Coordinate student progress	Conduct research and investigation	Begin: KSB 4: Bioinformatics	Students complete: KSB 4: Bioinformatics KSB 5: Solutions and Media
3	Coordinate student progress	Conduct research and investigation	Discuss concept of fusion proteins. Begin: KSB 6: Cloning KSB 7: Screening KSB 8: Bacterial Concentration	Students complete: KSB 6: Cloning KSB 7: Screening KSB 8: Bacterial Concentration
4	Coordinate student progress	Generate alternative designs Choose and justify optimal design Carry out protocol	Introduce Design Challenge to students. Discuss physical properties of GFP and bacterial lysate. Discuss design of purification protocol.	Students design and carry out protocol to meet Design Challenge.
5	Unite class thinking on accomplishments	Test and evaluate the design solution (protocol)	Discuss principles of electrophoresis and SDS-PAGE specifics.	Complete: KSB 9: SDS-PAGE to Test Purity
6	Sum up progress on learning goals	Share methods and results with other teams	Complete: KSB 10: Clinical Test KSB 11: Bioethics	Complete: KSB 11: Bioethics and Human Testing KSB 12: Classroom Presentations

*** Time commitment for Week 2 varies depending on student involvement in solution preparation—see details for Week 2 in the instructor section below.

V. MATERIALS AND RESOURCES

Materials

The following materials can be obtained from Bio-Rad Laboratories (WWW.BIORAD.COM, 1-800-4-BIORAD):

Cloning and Screening Knowledge and Skill Builders This is an all-inclusive list for the whole module. Individual supply needs are outlined in each section:

Bacterial library — lyophilized	1 vial
LB-agar tablets	5
Petri dishes — 60 mm, sterile	20
Inoculation loops — pack of 10 loops	1 pack
Pipettes — sterile, individually wrapped	10
Ampicillin — lyophilized	1 vial
Arabinose — lyophilized	1 vial
LB-broth tablets (to make 100 mL)	2 tablets
Inoculation loops — pack of 10 loops	2 packs
Pipettes — sterile, individually wrapped	40
Culture tubes — 15 mL, sterile (pack of 25)	1 pack

Design and construction of the protein purification protocol

TE buffer (10 mm Tris, 1 mm EDTA, pH 8.0; sterile)	1 L
Column buffer (10x PBS)	1 L
Tris buffer (50 mm, pH 8.3)	1 L
Equilibration buffer — (2 M $[NH_4]_2SO_4$)	1 bottle
Binding buffer — (4 M $[NH_4]_2SO_4$)	1 bottle
Wash buffer — (1.3 M $[NH_4]_2SO_4$)	1 bottle
Microtubes — 2.0 mL, clear	100
Lysozyme — lyophilized	1 vial
Collection tubes — 5 mL polystyrene	150
PolyPrep sizing columns	8
PolyPrep chromatography columns	8
HIC chromatography column	8
MacroPrep HighQ support for IEX	1 bottle
Column end caps	1 bag
Foam microtube rack	8

Design testing

Mini-PROTEAN 3 Cell electrophoresis unit from Bio-Rad Laboratories
(catalog #165-3301, 165-3302)

PowerPac 300 power supply from Bio-Rad Laboratories
(catalog #165-5050, 165-5051)

Ready Gel precast gels from Bio-Rad (15% Tris HCl, 10 well)
(catalog #161-1103)

10x Tris/Glycine/SDS running buffer (dilute to 1x concentration)
(catalog #161-0732 or see formulation in KSB 9)
Sample buffer (Laemmli Sample Buffer from Bio-Rad Laboratories)

Broad range protein standard (Bio-Rad Laboratories)
pipettes with tips
Coomassie blue gel staining solution
(1 L, Bio-Rad catalog #161-0786)
Visible light box
Polaroid camera and film for photo documentation

Other materials required for the module:
UV safety goggles or glasses
UV lamp — Long Wavelength
Bio-Rad catalog #166-0500
Microwave oven
1 L flasks
250 mL flasks
100 mL and 250 mL graduated cylinders
50, 100, 200 mL volumetric flasks
Distilled water
Thermometers
Centrifuge
Magnetic stir plate
Reagent bottles
Beakers of water for rinsing pipettes
Refrigerator/freezer
Computers with Internet access
Shaking/rocking platform or incubator
Set of pipettes and tips
Gloves

SAFETY CONSIDERATIONS

Review general lab safety and safety issues specifically related to the use of the SDS–PAGE technique. In addition, students will use UV lamps to view Green Fluorescent Protein. Ultraviolet radiation can cause damage to eyes and skin. Short-wave UV is more damaging than long-wave UV light. The Bio-Rad UV lamp recommended for this module is long-wave. NYSCATE recommends the use of UV-rated safety glasses or goggles.

RESOURCES

Computers having Internet access.

VI. PROCEDURAL SUGGESTIONS

PEDAGOGICAL FRAMEWORK REFERENCE

A separate document, the NYSCATE *Pedagogical Framework* (www.nyscate.net), provides an in-depth understanding of the NYSCATE challenge statements, the FOCUS on Informed Design pedagogical model for teachers, student Knowledge and Skill Builders (KSBs), the informed design loop for students, and more.

SUGGESTIONS FOR TEACHERS

The following pages provide suggestions for preparing and presenting the KSBs and Design Challenges. The text boxes that appear in this section represent the first pages only of the relevant student handouts. For the complete handouts, turn to the Student Handout section.

Week 1

Classroom:

In Week 1, provide the students with a brief review of protein structure and function along with the concepts of transcription and translation. You may wish to incorporate *Bioprospecting*: *Purifying Protein by Design* into your unit on proteins. If so, it may require more than one week of classroom time to prepare students for the module. If you have not done so already, you will want to provide students with classroom material that discusses some of the methods used in biotechnology. You will want to give special attention to the principles of recombinant DNA technology and its application in the production of pharmaceuticals. Most general biology textbooks provide an adequate description of these processes.

After students have reviewed proteins and biotechnology methods, distribute Overview of the Module and Design Challenge. Introduce the module, and discuss the scenario that is provided at the beginning of that handout. Encourage students to begin thinking about how drugs are discovered. There are many good examples of materials with benefits unrelated to the purpose for which the materials were designed. One such example is the Avon product Skin So Soft, which now is being used as a mosquito repellent.

Have students suggest ways that the folk remedy might be administered to people who take it (note that this aspect of the role-play is not specifically mentioned in the Design Challenge scenario). Ask students what they think would happen if this remedy were taken internally and how that would affect the therapeutic value of the proteins to the patient (most protein drugs must be administered through IV injection to avoid being digested in the gut).

During the first week be sure to present/review the process of design (see the separate NYSCATE *Pedagogical Framework* document for an elaborated discussion of the design process). Since the students are likely to be familiar with

the formal methods of science, it is a good idea to elicit how the methods of science are similar to the design cycle. For example, testing a hypothesis is very similar to testing a design. Also, once a test is complete, the observations can be used to generate a new hypothesis or a new design (redesign).

Laboratory:

The procedural suggestions given throughout this module assume that laboratory groups consist of three students. If you have not done so already, orient your students to the important safety considerations that must be followed during this module. Review general lab safety and discuss some of the hazards of UV light and the SDS-PAGE technique. In the first week of lab, student groups will need to be at a computer having Internet access. During this time, distribute the following Knowledge and Skill Builders (KSBs) and ask students to work through them:

- KSB 1: The Laboratory Notebook
- KSB 2: Biotechnology and the Economy
- KSB 3: Methods for Protein Purification.

KSB 1: The Laboratory Notebook

A critical skill that students must develop and maintain is proper documentation of laboratory work. Many may be familiar with the concept of the laboratory notebook, but not everyone is familiar with the importance of this form of documentation in the biotechnology workplace. This importance can be summarized with a simple phrase that is part of the Food and Drug Administration (FDA) regulations: “If it isn’t written down, it wasn’t done.” There are documented cases in which a biotechnology company threw an entire batch of pharmaceuticals away because a technician failed to record a simple measurement taken during the process. This type of oversight can cost companies millions of dollars in profits.

Since the students are essentially a small-scale biotechnology company, they must be able to maintain a proper laboratory notebook that contains a chronological log of everything they do in the laboratory. A measure of a good laboratory notebook is that it is complete enough that the person, or someone else, could repeat the work on the basis of the information that is documented. Although this type of document may vary somewhat from company to company, there are some very basic guidelines that students should follow (see KSB 1 in the Student Handout section).

To help students maintain good records, each team, representing a biotechnology company, will have a lab manager for each laboratory session. You may want to rotate the position of lab manager each week. The lab manager’s responsibility is to see that all protocols are followed exactly as written or designed, all tasks for that week are completed, and work is documented completely and accurately. The lab manager will sign off on the laboratory notebooks of the other members of the group at the end of each day.

These components and guidelines are designed to serve as a reference for students when they begin to document their progress for this module. Have students refer to them often during the module. With practice, many of the details regarding documentation will become automatic. To help students become comfortable with this skill, encourage them to use proper documentation while working with the other two KSBs that they will explore in the first week (KSB 2: Biotechnology and the Economy, and KSB 3: Methods for Protein Purification). Direct the students as follows:

- Have students set up their laboratory notebook with the identifiers and page numbers.
- While working through KSB 2 (Biotechnology and the Economy) and KSB 3 (Methods for Protein Purification), have them follow all of the guidelines for preparing laboratory notebooks.

- When students finish each KSB, make sure that their name appears at the top of every page, the lab manager has initialed or signed the pages, and the table of contents has been updated.
- Have students keep detailed notes of all of their work, classroom discussions, etc. that are part of the KSBs for this week. Now is the time to develop good habits so that they become automatic throughout the rest of the module.

KSB 2: Biotechnology and the Economy

In this KSB, each group will form a biotechnology company, as described in the Student Handout section. After students research information on their new biotechnology company and the types of procedures used in protein purification, have the groups report back to the entire class on their findings. Each group should give a report. The report from each group and the class discussion that follows should focus on the following:

- The company name.
- Methods used in protein purification: A class list of methods can be maintained for students to view during discussions. (Information on protein purification is provided in the Additional Support for Teachers section at the end of this teacher section.)
- How protein purification methods address variations in protein structure. (The class should discuss how the different separation methods work at this time.)

During this first lab, students will be setting up their laboratory notebooks, which at the end of the lab period should contain information about their new biotechnology company, a preliminary list of materials, and notes on protein purification. Make sure that you sign off on the lab manager's notebook from each company and that the lab manager in each company signs off on the notebooks of the other two members of the company. You may wish to have the students keep their laboratory notebooks in the lab and not take them home to simulate the policy that biotechnology companies follow.

KSB 3: Methods for Protein Purification

Using the World Wide Web or printed materials, students will explore the factors involved in the purification of proteins. Student groups should consider a variety of different questions in their explorations:

Why do scientists purify proteins?
What physical properties of proteins do the purification methods address?
What are the different types of purification methods?
How do the different purification methods work?

When students have had a chance to explore this topic, have the groups come back together as a class to take part in a class discussion on the topic. Encourage students to take very detailed notes and ask them to be very specific in

their discussions. For example, if students are discussing a purification method, ask them to explain how that method takes advantage of a physical property of the protein they are trying to purify. Each company will also be conducting research on "contract manufacturing." Allow for some time to discuss the role of this type of company in the pharmaceutical industry.

Week 2

Classroom:

At the beginning of each new week, the job of lab manager should rotate to a new member of each group. During Week 2 of this module, distribute KSBs 4 and 5.

Laboratory:

NOTE: The time commitment for laboratory in Week 2 will vary depending on whether or not the students prepare all of the solutions that will be used in the purification that will be run later in the semester.

Refer to KSB 5 in the Student Handout section for detailed information on how these solutions will be prepared. If the students will be preparing all of the "solutions and dilutions" described in this KSB, then an ***extra week*** should be added to the curriculum. The extra class period would be used for instruction on how solutions and dilutions are prepared. The student groups could then work through their calculations for the solutions that need to be made. The laboratory for that week would then be used for preparing the solutions. All of the information for the "Optional Student Activity" can be found in the student handout for KSB 5.

If the instructor is preparing the chromatography solutions, then the laboratory for Week 2 requires only about 1 hour and 30 minutes to prepare the agar and media described in KSB 5. In this case, some of the laboratory time for Week 2 could be allocated to KSB 4: Bioinformatics, which the students are working on in class during this week.

The students should complete KSB 5: Solutions and Media during a laboratory period. The materials that students will help prepare are:

- agar plates
- liquid media.
- chromatography solutions (optional activity — see KSB 5)

Once the class has prepared the materials, each company can collect, label, and store what they will need for the following week.

KSB 4: Bioinformatics

The National Center for Biotechnology Information (NCBI) maintains a very large database of DNA and protein sequences that is constantly being updated.

The NCBI is within the National Library of Medicine (NLM), which is part of the National Institutes of Health (NIH). In addition to the massive amount of human DNA sequence information produced from the Human Genome Project, the NCBI contains DNA and protein sequence information from a variety of different organisms. The NCBI makes this data available over the Internet at no charge for use by scientists to identify DNA sequences and proteins. In addition, the database can be used to look for homology between sequences when similar genes or proteins are found in a variety of different organisms.

The computer program that is used to search the database is called BLAST (Basic Local Alignment Search Tool). There are BLAST programs designed to search both DNA and protein sequences on the NCBI server. The specific program that students will use to explore their proteins is BLASTP (P = protein). In this KSB, students will be introduced to the fundamentals of a basic Internet BLAST search to identify the three proteins isolated from the folk remedy in the scenario. Before exploring the three unknown protein sequences, students will first work through an online tutorial and a couple of practice exercises. Once the students are comfortable performing a BLAST search, they will begin exploring their unknowns. The details are explained in KSB 4 in the Student Handout section.

The answers to the questions in the KSB are as follows:

1. What is the probable identity of the unknown protein?

   The most probable match is pig alpha-amylase.

2. What tissues or organs produce the unknown protein?

   Pancreas and salivary glands

3. Use your mouse to select the first red line in the graphical output and determine how similar that subject protein is to the unknown protein by listing the %identity.

   <u>First red line: Pig alpha amylase; 82%</u>
   pdb|1PPI| Alpha Amylase (Ppa) (E.C.3.2.1.1) Complexed With Acarbose. Length = 496 Score = 352 bits (894), Expect = 1e-96 Identities = 160/195 (82%), Positives = 170/195 (87%), Gaps = 1/195 (0%)

4. Identify the names of the organisms that produce this unknown protein. Only list the animal sources that show a Score (bits) of greater than 250. In order to find the animal source for some of the matches, you may need to use your mouse to select some of the accession number links. Recall that selecting these links will take you to a page that contains more detailed

information for that match. For each animal that you list, report the highest %identity that you can find from the alignments.

Organism	***Score***	***%Identity***
Pig	***352***	***82%***
Human	***309***	***71%***
Chicken	***302***	***67%***
House mouse	***293***	***67%***
Rat	***279***	***63%***
Winter flounder	***253***	***54%***

KSB 5: Solutions and Media

You will need to determine which solutions and media you will prepare and which the students will prepare. For instructions on preparing the solutions and media, see the Student Handout section. Note that there are some scheduling considerations that you should take into account when planning for this KSB:

1. Agar plates should be prepared at least two days before you plan to begin KSB 6: Cloning.

2. Plan to rehydrate the ampicillin and arabinose on the same day that you pour the agar plates.

3. The agar plates can dry out (cure) at room temperature for two days before they need to be refrigerated. This can conveniently be done on a Friday with the covered plates left to cure over the weekend.

4. It is most convenient to prepare the liquid nutrient medium used in KSB 7: Screening on the same days as you prepare the nutrient agar. Each student workstation will require two culture tubes containing 5 mL of liquid nutrient media. Store the culture tubes in a refrigerator until the day of use.

Week 3

Classroom:

Distribute KSBs 6, 7, and 8, which students will be working on this week (approximately 50 minutes each). Before the students begin these KSBs, you should allocate additional time to explore the concept of fusion proteins and the process of bacterial transformation. (Information on fusion proteins and transformation is provided in the Additional Support for Teachers section.) By this time, the students will have discovered that the protein of interest is a disintegrin found in the snake venom of a southern copperhead (background material on disintegrins is provided in Additional Support for Teachers section). In KSB 6: Cloning, students will work with transformed bacteria. Students should understand

that we are simulating the idea that the gene for the disintegrin protein was fused to the gene for GFP and then cut and pasted into a plasmid. Cultures of *E. coli* bacteria were then transformed with this gene construct. Since the process of transformation is not 100% efficient, only some of the bacteria will contain the gene for disintegrin. The bacteria that do contain the gene and are expressing it will turn green under UV light, because GFP will fuse to the disintegrin protein. The goal of Week 3 is to grow bacteria on agar plates (KSB 6: Cloning), identify which bacteria actually contain the disintegrin gene (KSB 7: Screening), and then grow and concentrate a large quantity of bacteria that carry the gene for disintegrin (KSB 8: Bacterial Concentration).

Laboratory:

Week 3 of the module will require that students either come into lab outside of their normal laboratory time or use some class time to complete some laboratory tasks. Because of this necessity for extra time, schedule a combination of classroom and laboratory time in order to complete the KSBs for this week:

- KSB 6: Cloning
- KSB 7: Screening
- KSB 8: Bacterial Concentration.

KSB 6: Cloning

As noted under KSB 5: Solutions and Media, you should have prepared the agar plates at least two days before you plan to begin this KSB. The transformed bacteria are shipped dehydrated and need to be rehydrated prior to use. Because of the ever-present possibility of external contamination, it is important that rehydration of the bacteria be done on the day of this KSB by you or a student. For instructions on these procedures, follow the instructions provided to students. (See Student Handout section.)

KSB 7: Screening — Follow the instructions provided to students. (See Student Handout section.)

KSB 8: Bacterial Concentration

At this point, student groups will have produced two living cultures of bacterial clones. The students will now extract the GFP from the bacterial cells. In this KSB, students will collect a large number of these bacterial cells in a concentration step that is part of a typical purification protocol. Follow the instructions provided to students. (See Student Handout section.)

<u>Week 4</u>

Classroom:

In the beginning of Week 4, you should distribute Design Challenge: Protein Purification and introduce the Design Challenge to the students. The solution that

the students will be working with contains thousands of bacterial proteins from which a Green Fluorescent Protein (GFP) must be separated. The students need to know that this is a simulation in which the GFP is simulating a fusion protein (GFP fused to disintegrin). Although we are simulating the idea that GFP is fused to a disintegrin, in reality there is no disintegrin protein fused to the GSP that students are using. GFP is being used to represent the fusion protein because GFP can be visualized under UV light throughout the entire process. Students must understand this because they will have to explore the physical properties of GFP before they can design a way to purify it from the other proteins found in the bacterial lysate.

Before students begin to design their specific protein purification protocols, it is important that they have an understanding of the process of design and the design cycle in the context of the NYSCATE FOCUS on Informed Design. Information on the components of the design cycle and the FOCUS model can be found in the NYSCATE *Pedagogical Framework*. It is important that students understand that their solutions are not a trial-and-error approach to a problem. The protocol that they design should be informed by the parameters of the purification methods available and the chemical and physical properties of the protein that they will be purifying.

At this point, students will have already explored the principles of protein purification (see Week 1) and, therefore, they will be able to focus their attention on the physical properties of GFP and the proteins found in the bacterial lysate. Refer to the student instructions for the Design Challenge for further details.

Laboratory:

Students will be provided with materials and protocols for performing three different chromatography procedures in an attempt to purify GFP from the bacterial lysate. The three methods include size exclusion chromatography (SEC), ion exchange chromatography (IEX), and hydrophobic interaction chromatography (HIC). Students must design a multistep purification protocol to purify GFP. Knowledge of the parameters of each method, along with the physical and chemical properties of GFP, will inform their design.

Week 5

Classroom:

Distribute KSB 9: SDS–PAGE to Test Purity. The general principles of electrophoresis should be reviewed with special attention given to the use of SDS-PAGE to examine proteins. Native and denaturing conditions should be compared so that students understand the importance of protein structure and shape in relationship to protein function.

Laboratory:

Students will test for the purity of their samples by running SDS-PAGE gels. By comparing samples that were processed with their purification protocol to control samples, students will be able to evaluate the efficacy of their design for purifying GFP. Procedures for performing SDS-PAGE will be reviewed, and then students will use this technique to examine the purity of their samples. Results from this test should be used to compare designs from different groups. Complete the KSBs for this week: KSB 9: SDS-PAGE to Test Purity.

KSB 9: SDS–PAGE to Test Purity

In this KSB, students will analyze the relative purity of their sample, and thus the efficacy of their purification protocol, with SDS-PAGE. The materials and instructions provided are specific to the Bio-Rad Mini-PROTEAN 3 system, but they can be easily modified for any SDS-PAGE system. For specific instructions and list of materials, refer to KSB 9 in the Student Handout section.

Week 6

Classroom:

Classroom time will be devoted to the completion of the last three KSBs. Having students prepare work related to these KSBs outside of class should conserve classroom time. In KSB 10, students will be analyzing data produced during a simulated clinical trial used to test the efficacy of the disintegrin protein on breast cancer. (Information on clinical trials and breast cancer is provided in the Additional Support for Teachers section.) In KSB 11, students will be examining one of several ethics cases that deal with drug testing and clinical trials. In the final KSB (KSB 12: Classroom Presentations) students will produce a presentation to the class that will cover information on their purification protocol design and the analysis of the clinical trial data from KSB 10.

Laboratory:

In the final week of this program, students will be working on KSBs 10–12. Since these KSBs do not require laboratory space, the time allocated to classroom and lab can be combined to accommodate all three. If more time is needed for KSB 9 (SDS-PAGE), the laboratory time for this week could be used here. In this instance, an extra week of classroom time will be needed to accommodate KSB 12 (Classroom Presentations).

KSB 10: Clinical Test

Before the drug can be tested in human subjects, the FDA (Food and Drug Administration) requires that the drug be tested in animals. Although there are many ethical issues related to animal testing, it is important to explore any toxic effects that the drug might have before administering it to human subjects. Although there will be a discussion on the ethics of human testing in the next KSB, this may be a good time to introduce the issues related to the use of animals in scientific research. For the purposes of this KSB, we will assume that the drug was found to be safe in animals and is ready for human testing in what is called a

clinical trial. (Information on clinical trials can be found in the Additional Support for Teachers section.) Refer to KSB 10 in the Student Handout section for details on this activity.

OPTIONAL CLASSROOM EXERCISE

- Building upon the KSB that deals with the breast cancer drug trial (KSB 10: Clinical Test), give the students the responsibility of exploring a different disease through designing a similar experiment for testing the effects of the drug on reducing a person's risk of heart disease.
- Have the students search the literature on heart disease risk and develop similar background knowledge to that which was covered for breast cancer.
- Have the student groups design a clinical trial for testing the effectiveness of the drug on high-risk heart disease patients. Make sure that the students consider their primary end points (what they will measure).

KSB 11: Bioethics and Human Testing

In this KSB, students will explore some very difficult ethical dilemmas that face scientists as they design and conduct clinical trials to test the effectiveness of new therapies. What follows is a series of ethical case studies that are each followed by a challenge to find a specific solution. Student groups should be assigned a case to work with and then they will be asked to report on their solution to the class. This KSB can be done as either an in-class or a homework assignment. In either case, sufficient time should be allowed for the students to conduct necessary background research and to take part in group discussions. After assigning a case to each of the groups, give them a deadline for when they will have to report on their solution to the class. Make sure you allow enough time for classroom discussions after each of the solutions is presented.

KSB 12: Classroom Presentations

In this KSB, student groups will be presenting the work that they have conducted over the course of this module. Since many of the activities that students have taken part in are similar from group to group, the presentations should focus on two areas:

1. The details of the solution to the Design Challenge
2. The conclusions drawn from the data on the clinical trials to determine the effectiveness of the new drug.

The amount of time allocated and the basic format for the presentations are left up to the instructor. You may decide to have students take part in a poster session or you may decide to have the format consist entirely of oral presentations. Although the details regarding the format of the presentation are flexible, you should encourage students to make sure that they cover the following topics:

Details Regarding the Design Challenge

- What factors did you consider in your design?
- Which of these factors did you think was the most important in determining your design?
- What were some of the alternative designs that you considered?
- How effective was your design? (Present data from KSB 9: SDS-PAGE to Test Purity.)
- If your design was not optimal, what factors do you think contributed to this?
- If given a chance (and more capital), would you implement one of your alternatives? Why or why not?

Clinical Trial Data

- Does the drug reduce a person's risk of developing breast cancer? By how much and at what dose?
- Does the drug delay the onset of breast tumors in a high-risk patient? By how much and at what dose?
- Does the drug help to prevent metastatic tumors in patients who develop breast cancer? How well does the drug prevent breast cancer and at what dose is it effective?
- Does the drug help to delay the onset of metastatic tumors in those who develop them? By how much and at what dose?

VII. ADDITIONAL SUPPORT FOR TEACHERS

I. Physical and Chemical Properties of Proteins

The chromatographic techniques available to students for their Design Challenge focus on three very important chemical properties of proteins: molecular weight, pI, and hydrophobicity. It is important that, at a minimum, students understand the theory behind these principles and that they have collected this information for GFP.

Molecular weight

The molecular weights of proteins are often reported in *daltons*. Named after John Dalton, who developed the atomic theory of matter, the dalton is equal to 1.0000 atomic mass units. A *kilodalton* (kd) is a unit of mass equal to 1000 daltons. The mean molecular weight of an amino acid is around 110 daltons. Most natural proteins contain between 50 and 2500 amino acids, and, therefore, the molecular weights of most proteins are between 5500 and 275,000 daltons (5.5 – 275 kd). Size exclusion chromatography (SEC), which is also referred to as gel filtration (GF), takes advantage of the different sizes of proteins to separate them out.

pI

The net charge of a protein varies according to the pH of the environment. The pI of a protein (or isoelectric point) is defined as the pH at which there is a net charge of zero. At its pI, a protein will not migrate in an electric field (i.e., it will not move toward a negative or positive pole) and it will not be attracted to other charged molecules. The pI is an important measure of a protein when considering a protein purification protocol, because net charge is the key factor in ion exchange chromatography (IEX).

Hydrophobicity

Most biomolecules have some degree of hydrophobicity (The state of being “water fearing”). In proteins, the degree of hydrophobicity is determined by the amino acids that are contained in the primary structure of the protein. The incorporation of hydrophobic and hydrophilic amino acids partially determines the tertiary structure of that protein. Hydrophobic amino acids are those that have nonpolar, or uncharged, side chains (*R* groups). These include the various aromatic side chains on tryptophan, phenylalanine, and tyrosine and the large nonpolar groups of leucine, isoleucine, and valine. Hydrophilic amino acids include serine and glutamine, which contain polar groups but have no charge, and glutamate, aspartate, lysine, and arginine, which contain charged side chains.

In the aqueous environment of a protein, most hydrophilic amino acids are found on the protein surface, while the interior tends to be very hydrophobic. This makes sense when you consider an amino acid chain folding up to produce the tertiary structure of the protein. If you have a chain of amino acids, the chain will be much more stable in water if it is folded so that all the hydrophilic amino acids are on the outside and all the hydrophobic amino acids are on the inside. This relationship is not absolute, so it is possible to find hydrophobic amino acids on the protein surface and hydrophilic amino acids in the interior. The relative number of hydrophobic amino acids on the surface of a protein will determine its hydrophobicity and thus its solubility in water.

II. Protein Purification

The purification of proteins is an essential step in the production of biopharmaceuticals. Using recombinant DNA technology, the gene for a specific protein is "cut and pasted" into a host organism. The host is grown under conditions that are optimal for the expression of that gene and the production of the target protein. Once the protein has been produced in large enough quantities, it must then be separated from all of the other biomolecules in the "mix." In this module, the host organism is a strain of *E. coli (Eschericia coli).* When the host bacteria are lysed to release the GFP, a large number of contaminants will also be released from the host itself. The goal of the purification step is to purify the target protein from this mix.

To design an optimal purification scheme, you need to have an understanding of the physical properties of the protein you wish to purify and the molecules that you consider to be contaminants. The following table lists some of the physical properties to be considered and some of the most common purification methods that address those characteristics (Seidman & Moore, 2000; Protein Purification Handbook, 1999):

NET CHARGE	Ion exchange chromatography Electrophoresis Isoelectric focusing
POLARITY	Reverse phase chromatography Hydrophobic interaction chromatography
SIZE	Gel electrophoresis Gel filtration chromatography Dialysis and ultrafiltration Analytical ultracentrifugation
BIOLOGICAL RECOGNITION	Affinity chromatography
THERMAL STABILITY	Differential denaturation

One of the most commonly employed methods in protein purification is chromatography. A variety of different chromatographic methods will be made available in this module as part of the Design Challenge. In KSB 3: Methods for Protein Purification, students will research a variety of methods used in purifying proteins. Encourage them to develop a complete list even though, for the Design Challenge, methods will be confined to chromatography only.

Chromatography is a method involving the separation of molecules on the basis of differences in their structure and/or composition. In its general form, the method involves moving a solution of mixed molecules over a stationary support (substrate). The molecules in the solution will have various interactions with the support, which will lead to the separation of similar molecules. Molecules that have tighter interactions with the support will move more slowly than those molecules that have weaker interactions.

Chromatographic separations can be conducted using a variety of supports. Thin layer chromatography involves an immobilized layer of silica on glass plates. In gas chromatography, the support is a volatile gas, while paper is the support used in paper chromatography. Liquid chromatography may incorporate hydrophobic, insoluble molecules as a support.

In this module, materials will be made available in the Bio-Rad kits for performing four different types of chromatographic separations (ion exchange, hydrophobic interaction, gel filtration, and affinity chromatography). The students will be designing a two-step purification process, using these methods. The following sections provide some background on these four different methods.

Ion Exchange Chromatography

This is perhaps the most frequently used chromatographic technique for protein purification. In its basic form, this method separates molecules on the basis of their charge. The support in this method uses charge to bind and release molecules as they are purified.

The molecular structure of a protein determines its overall charge. Some of the amino acids will have positively charged *R* groups while others will be negatively charged. A comparison of the overall number of positive and negative charges determines the overall charge of the protein. Ion exchange chromatography takes advantage of this protein variability; either the protein interacts with the charge, binding the protein to the support, or the charge causes a release so that the protein moves through the support. The net surface charge of a protein can be altered by changes in pH. At its isoelectric point, the pH where the protein has zero net charge, the protein

will not bind to any support and will move through the column. Above its isoelectric point, a protein will have a net negative charge and will, therefore, bind to an anion exchange support that contains positively charged chromatographic beads.

Elution from the column can be achieved by a change in pH or by the addition of salt. Changes in pH will alter the net charge of a bound protein and will cause it to release from the column as the pH reaches its isoelectric point. Addition of salt will cause the protein to elute because the chloride or sodium ions will compete with the protein for binding sites on the column. By slowly increasing the salt concentration across a gradient, proteins that are bound to the column at different strengths (on the basis of their net charge) will elute.

Most commonly, samples are eluted with salt rather than by changing pH. The protocols and materials for this module require a step-wise elution in which the salt concentration is increased in steps instead of a steady gradient. Because the fractions that are collected will come off of the column in high concentrations of salt, an inter-step conditioning of the sample may be required if the next step in the protocol requires a starting sample with low salt or low ionic strength.

The stationary phase (support), called an ion exchanger, is a solid substance that interacts with the components of the sample. Synthetic materials called resins, which are composed of very small beads that contain polymers with ionizable chemical groups, are commonly used as the stationary phase. In this form of chromatography, solutes (proteins) are continuously being bound and released. When released, the solutes (proteins) move through the resin in the mobile liquid phase. When a protein with a strong charge comes in contact with a support that carries the opposite charge, the protein will bind. The strength of binding is controlled by the size of the net charge on individual components of the sample.

Hydrophobic Interaction Chromatography (HIC)

In this method, the separation takes advantage of the parts of the protein that contain no charge and, therefore, are hydrophobic (water fearing). Most of the hydrophobic portions of a protein are buried deep within the biologically active form of the protein once it has folded. However, there is usually a distribution of hydrophobic amino acid residues on the surface of the molecule. These hydrophobic residues will interact strongly with other hydrophobic molecules (the clustering effect). By providing a hydrophobic support and a very hydrophilic environment, it is possible to bind the protein to the support through the interaction of hydrophobic portions of the surface of the protein.

Within a protein mixture, the most hydrophobic proteins will be adsorbed on the matrix first. The high salt conditions created with the binding buffer provide favorable binding conditions. They increase the exposure of the hydrophobic portions of the proteins, which leads to stronger binding to the hydrophobic chromatography support. After sample loading and binding, a decreasing salt gradient is used to elute the samples from the gel. As the ionic strength of the buffer decreases, the hydrophobic portions of the proteins will be less exposed, and, therefore, they will be bound less tightly to the matrix. As the ionic strength continues to decrease, only the most hydrophobic proteins will remain bound to the matrix and the least hydrophobic proteins will elute from the column first.

GFP has surfaces that are very hydrophobic. In salt water, these parts of the protein tend to stick tightly to other hydrophobic surfaces. Protein mixtures containing GFP can be poured through a column packed full of hydrophobic beads. When the beads are in salt water, hydrophobic proteins—like GFP—passing through the column will stick to the beads, while the other proteins will drip through. When the salt is removed, the shape of the GFP protein changes so that the hydrophobic surfaces are less exposed than before. The result is that GFP no longer sticks to the beads and will drip out the bottom of the column. In this way GFP can be separated from the other bacterial proteins.

Gel Filtration Chromatography

This is the only chromatographic technique that does not involve the binding of proteins to the support. In this method, proteins are separated on the basis of their size and shape. The support for gel filtration is a mass of beads that contain holes (pores) of a specific size. Small molecules tend to diffuse into the interior of the porous particles so that their flow is restricted, while large molecules are unable to enter the pores and tend to flow unhindered. The large molecules will move through the support more quickly and will be followed by the progressively smaller molecules that can fit through the pores in the beads. This movement of the smaller molecules slows down the flow of the particles. (Seidman & Moore, 2000; Protein Purification Handbook, 1999)

III. Fusion Proteins

The use of GFP in fusion proteins has become a powerful tool for cell biologists. In most uses of this technique, a recombinant DNA is constructed with the coding sequence of GFP joined with the coding sequence of a protein under study. This construct is then introduced into cells, which will be expressed to produce a chimeric protein that is a

combination of GFP and the protein of interest. It was discovered that, in many cases, the attachment of GFP to the end of the protein being studied did not affect the normal function of the protein. Then, the expression of the protein is easy to follow because of the amount of green fluorescence produced. In addition, the location of the protein within the cell can be followed over time under a variety of experimental conditions by measuring fluorescence.

When GFP is being used simply to look for the expression of the construct partner, then the coding sequence for GFP may be referred to as a reporter gene. In this way, GFP “reports” when the gene of interest is being expressed because it is fused to the gene that codes for the protein under study. This technique is very powerful when experiments are designed to induce or suppress the expression of a gene.

Another important use of GFP as a reporter gene is when a gene is introduced into a host cell (transfection and transformation). Since this introduction is never 100% efficient, it is important that the cells that have successfully taken up the DNA can be easily identified. In KSB 7: Screening, this technology is being employed to identify colonies of bacteria that will be cultured.

GFP fusion proteins are also valuable in the process of protein purification. In the Design Challenge, students will use chromatography to purify protein. The presence of fluorescence in the column and in the fractions collected from the columns makes it relatively easy to track the movement of the protein as it binds or moves through. This is a very useful tool when trying to optimize a protein purification protocol.

IV. Bacterial Transformation

Genetic transformation literally means "change caused by genes" and it occurs when a bacterial cell’s genotype is altered by the uptake of naked, foreign DNA from the surrounding environment. The ability of bacteria to take up DNA has been a useful tool for many areas of biotechnology.

In a typical transformation protocol, a eukaryotic gene (such as the disintegrin and GFP genes in this module) is cut and pasted into a bacterial plasmid (a circular piece of double-stranded DNA). This plasmid is then introduced into bacteria through the process of transformation. In some instances, the bacteria may need to be made competent (given the ability to take up naked DNA) with a treatment such as a high concentration of calcium chloride followed by a heat shock. This treatment encourages the bacteria to take up the introduced plasmid DNA. The cells are then cultured, and this process leads to the production of cloned copies of bacteria with

the introduced plasmid. In a final step, the plasmid is extracted from the bacterial cells and purified. In some cases, the idea is to have the bacteria express genes on the plasmid, which leads to the production of a protein that is then purified from the culture.

V. Proteins in the Folk Remedy

Keratin

Keratin is a member of the structural class of proteins. Other examples include collagen and elastin, which provide a fibrous framework in animal connective tissues, such as tendons and ligaments. Keratin is a highly fibrous and resistant protein that makes up most of the material in the cells forming the epidermis, hair, nails, scales, feathers, beaks, horns, and hooves of animals. Most animals have two forms of keratin (alpha and beta). In birds, feathers contain almost 100% of the beta form. Beta-keratins do not have a coiled helical shape as alpha-keratins do, but instead have beta sheet structures.

The keratin found in animal epidermis consists of long polypeptide chains of keratin arranged in parallel sheets held together by hydrogen bonding. Of the amino acids in keratin, cystine may account for as much as 24%. The numerous disulfide bonds formed by cystine are responsible for the great stability of keratin; it is completely insoluble in hot or cold water and is not attacked by proteolytic enzymes (the enzymes that cleave to protein molecules).

Chitinase

Most fungi build their cell walls out of a molecule called chitin. This molecule is a strong, but flexible, nitrogen-containing polysaccharide that is similar to the chitin that can be found in arthropod exoskeletons. Chitinases are a class of enzymes that have the ability to break some of the stabilizing bonds in the molecule, which leads to a loss of integrity in the cell walls of fungi. Because these enzymes offer an important protection from fungal diseases, there has been some recent interest in cloning the genes for specific chitinases and introducing them into crop plants.

Disintegrin

Disintegrins are snake venom proteins that inhibit the interaction of adhesion molecules on the surface of cell receptors with the extracellular substrate. Specifically, they prevent the interaction of fibrinogen with the adhesion glycoprotein IIb-IIIa complex. This adhesion protein, which is found on the surface of many cell types, is part of a class of proteins called integrins. Since platelet aggregation is mediated by this interaction, there is

a wealth of evidence that shows that these proteins found in snake venoms have a strong effect on platelet function. In addition, since the migration of cells involves the interaction of integrin receptors with the extracellular matrix, there is some evidence that disintegrins can disrupt cancer cell adhesion and invasion.

Disintegrins are peptides of about 70 amino acid residues that include many cysteines, all of which are involved in disulfide bonds. Disintegrins contain an Arg-Gly-Asp (RGD) sequence. The RGD sequence is a well-known binding site of proteins to integrin receptors. It is the RGD sequence of disintegrins that binds with the integrin receptor and produces the antagonistic effect.

VI. The Role of Arabinose: Gene Regulation — One Gene, One Protein

Regulation of genes allows adaptation to different environments. For example, bacteria can use the sugar arabinose as a source of carbon for respiration. The bacterial enzymes needed to digest arabinose are only made when arabinose is present. In other words, arabinose turns on the genes for these digestive enzymes. When the arabinose is used up, these genes are turned off.

The pGLO plasmid has been engineered to combine these controlling elements with the GFP gene. Therefore, in the presence of arabinose, GFP protein is produced. Cells fluoresce a brilliant green color as they accumulate GFP protein. In the absence of arabinose, the GFP gene is shut off. When the GFP protein is not made, bacterial colonies will appear white with no fluorescence.

Organisms regulate expression of their genes and ultimately the amounts and kinds of proteins present within their cells for a myriad of reasons, including development, cell specialization, and adaptation to the environment. Gene regulation not only allows for adaptation to differing conditions, but also prevents wasteful overproduction of unneeded proteins that would put the organism at a competitive disadvantage. The genes involved in the transport and breakdown (catabolism) of food are good examples of highly regulated genes. For example, the sugar arabinose is both a source of energy and a source of carbon. *E. coli* bacteria produce three enzymes needed to digest arabinose as a food source. The genes that code for these enzymes are not expressed when arabinose is absent, but they are expressed when arabinose is present in their environment.

Regulation of the expression of proteins often occurs at the level of transcription from DNA into RNA. This regulation takes place at a very specific location on the DNA template, called a promoter, where RNA attaches to the DNA and begins transcription of the gene. In bacteria,

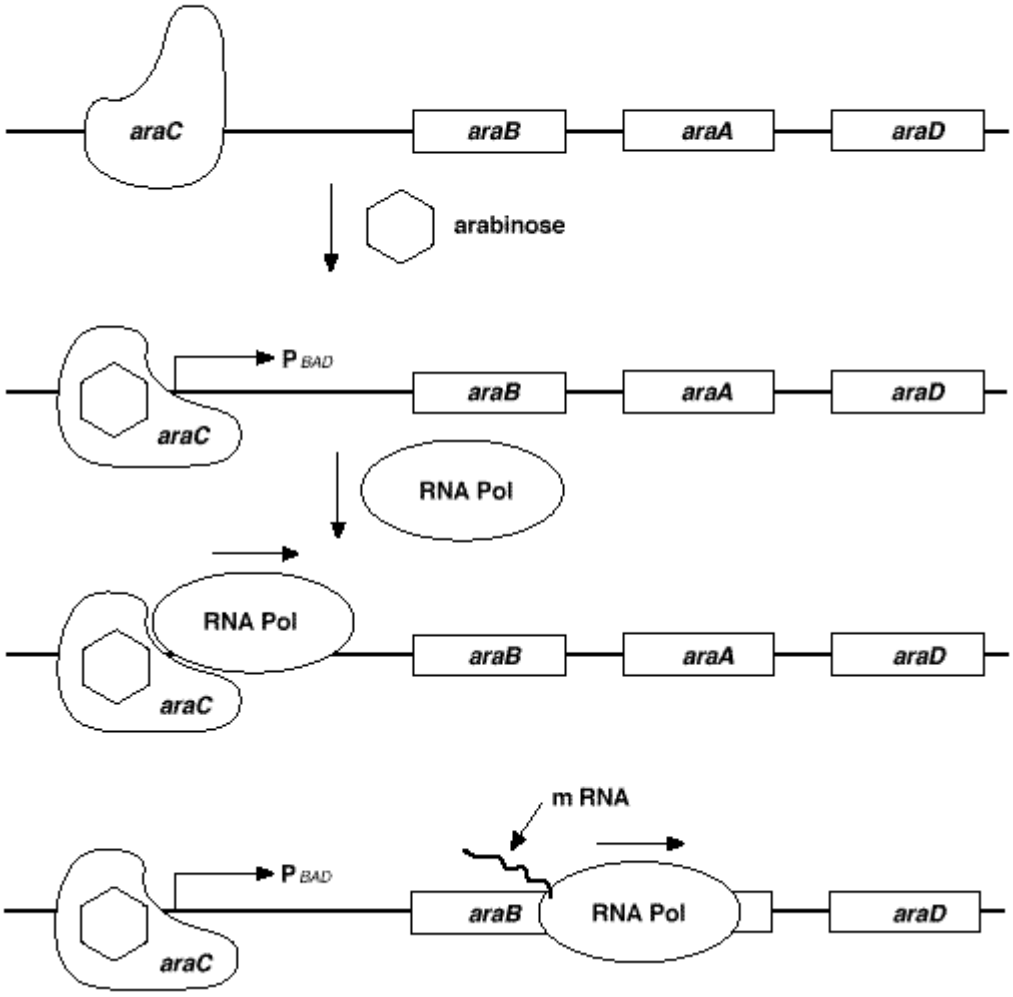
groups of related genes are often clustered together and transcribed into RNA from one promoter. These clusters of genes controlled by a single promoter are called operons.

The three genes (araB, araA, and araD) that code for three digestive enzymes involved in the breakdown of arabinose are clustered together in what is known as the arabinose operon. These three proteins are dependent on initiation of transcription from a single promoter, PBAD. Transcription of these three genes requires the simultaneous presence of the DNA template (promoter and operon), RNA polymerase, a DNA binding protein called araC, and arabinose. AraC binds to the DNA at the binding site for the RNA polymerase (the beginning of the arabinose operon). When arabinose is present in the environment, bacteria take it up. Once inside the bacterial cells, the arabinose interacts directly with araC, which is bound to the DNA. The interaction causes araC to change its shape, which in turn promotes (actually helps) the binding of RNA polymerase, and the three genes B, A, and D are transcribed. Three enzymes are produced; they have the function of breaking down the arabinose, and eventually the arabinose runs out. In the absence of arabinose, the araC returns to its original shape and transcription is shut off.

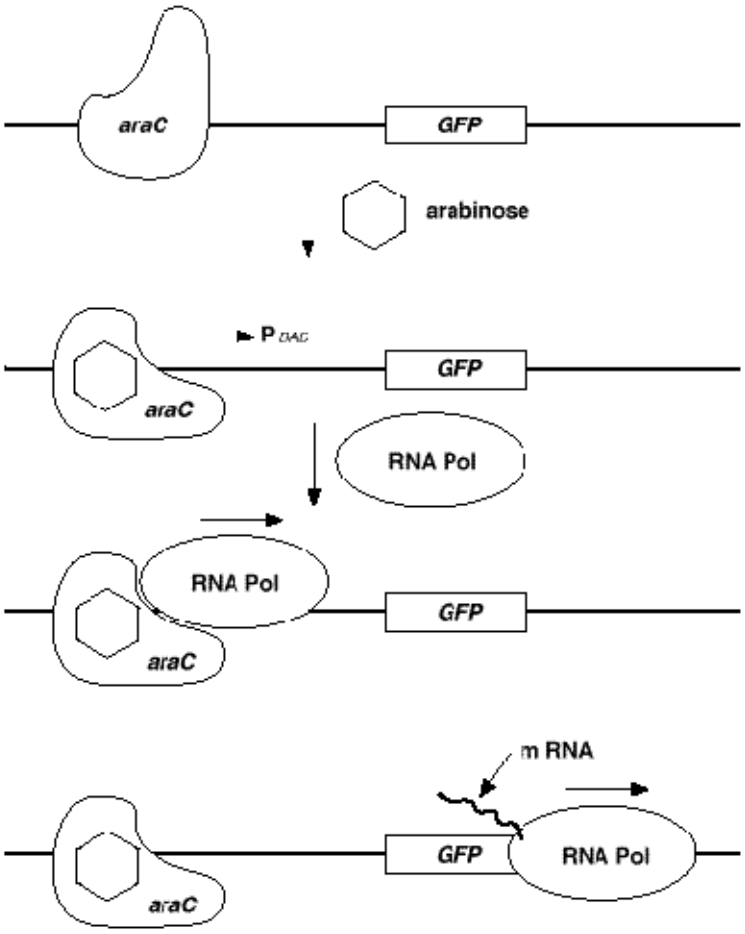
The DNA code of the pGLO plasmid has been engineered to incorporate aspects of the arabinose operon. Both the promoter (PBAD) and the araC gene are present. However, the genes that code for arabinose catabolism, araB, araA, and araD, have been replaced by the single gene that codes for the Green Fluorescent Protein (GFP). Therefore, in the presence of arabinose, araC protein promotes the binding of RNA polymerase and GFP is produced. Cells fluoresce a brilliant green color as they produce more and more protein. In the absence of arabinose, araC no longer facilitates the binding of RNA polymerase and the GFP gene is not transcribed. When the GFP protein is not made, bacteria colonies will appear to have a wild-type (natural) phenotype, namely, white colonies with no fluorescence under UV light.

This is an excellent example of the central dogma of molecular biology in action: DNA > RNA > PROTEIN > TRAIT.

The Arabinose Operon



Expression of Green Fluorescent Protein



VII. Sterile Technique

Contamination

Because contaminating bacteria are ubiquitous and are found on fingertips, bench tops, etc., it is important to minimize contact with contaminating surfaces. When students are working with the inoculation loops and agar plates, you should stress that the round circle at the end of the loop, the tip of the pipette, and the surface of the agar plate should not be touched or placed onto contaminating surfaces.

Working with E. coli

The host organism, an *E. coli* K-12 strain, and the plasmid containing the GFP gene are not pathogenic organisms like the *E. coli* O157:H7 strain that has been in the news. However, handling of the *E. coli* K-12 strains provided by Bio-Rad requires the use of standard microbiological practices. These practices include, but are not limited to, the following:

- Work surfaces are decontaminated with 10% bleach once a day and after any spill of viable material.
- All contaminated liquid or solid wastes are decontaminated before disposal.
- Persons wash their hands:
  (i) after they handle materials involving organisms containing recombinant DNA molecules
  (ii) before exiting the laboratory.
- All procedures are performed carefully to minimize the creation of aerosols.
- Mechanical pipetting devices are used; **mouth pipetting is prohibited.**
- Eating, drinking, smoking, and applying cosmetics are not permitted in the work area.

Decontamination and Disposal

If an autoclave is not available, all solutions and components (loops and pipettes) that have come in contact with bacteria can be placed in a fresh 10% bleach solution for at least 20 minutes to sterilize them. A shallow pan of this solution can be placed at every lab station. Sterilize petri dishes by covering the agar with 10% bleach solution. Let the bleach stand for one hour or more. Once sterilized, the agar plates can be double-bagged and treated as normal trash. Safety glasses or goggles are recommended when using bleach solutions.

VIII. Clinical Trials

Clinical trials are the last stage in a long process of research and development for a new drug. The FDA (Food and Drug Administration) maintains regulations that must be followed before a new drug can be brought to the marketplace. These regulations include the protocols that must be followed in the clinical trial process. A new drug must be tested first in the test tube, then on animal models, and then on patients. Clinical trials involve human subjects and are designed to assess the safety and efficacy of the therapy under highly controlled conditions. Clinical trials usually last two to four years and go through at least three separate phases. After the trials, the Food and Drug Administration must approve the drug for the marketplace; the approval process takes about two years. It is not uncommon for a drug to take 15 years to go “from bench to shelf” (research lab to store shelf).

There are a variety of different types of clinical trials that are recognized by the FDA:

- *Treatment trials* — involve the testing of novel drugs, surgeries, or therapies for human diseases
- *Prevention trials* — test the ability of new therapeutic approaches to reduce the risk of people developing a disease who have never had the disease but may be at a certain level of risk

- *Screening trials* — test new ways to find and diagnose disease
- *Supportive care trials* — test new ways for improving the comfort level and quality of life for people living with cancer.

For most clinical research, there is a series of steps (called phases) that are followed as novel drugs and therapeutic strategies are tested for the ability to prevent or eliminate sickness and disease. Often referred to as Phases I, II, and III, this series of experiments is designed to produce some very important information regarding the safety and effectiveness of a new drug, surgery, or therapy.

- Phase I trials — During this first phase, a small number of patients are enrolled to evaluate how the therapy will be administered and what dose is safe. For example, the drug that the students will be working with is a protein and is being administered through an IV injection. An oral route was not appropriate because the protein drug would be digested before it could enter the bloodstream and have its effect on tumor development.

- Phase II trials — Once Phase I studies have been completed and a safe dosage level is known, Phase II studies can start. More patients are enrolled at this next stage. Phase II studies test the treatment in humans with various forms of a disease (for example, different types of tumors) to determine the effectiveness of the therapy against specific disease types. More information on complications and side effects may also be gained at this stage.

- Phase III trials — Phase III trials usually involve large numbers of patients at multiple sites (offices, clinics, hospitals, etc.). In these trials, a participant will be assigned either to a group that receives standard treatment for the disease (control) or a group that receives the treatment being tested. The goal is to generate large amounts of data to support the claims that are made during Phase II trials regarding the safety and effectiveness of the therapy.

The double-blind randomized placebo-controlled study described in this module involves some very common controls that are found in many clinical studies. The double-blind design means that both the doctors and the patients are unaware of (blind to) which patients are receiving the new therapy and which are receiving a placebo. The placebo is usually something that has no therapeutic value, but appears identical to the drug being tested. Once a patient has been enrolled in the study, a computer randomly assigns the patient to either the experimental or control group. The purpose and importance of these controls should be discussed in class before the students begin KSB 10: Clinical Test, which deals with experimental design and data analysis.

For more information, visit “Cancer Trials: Understanding Trials” at http://mednav10.vh.shore.net/understanding, a service of the National Cancer Institute.

IX. Facts About Breast Cancer

Epidemiology and Risk Factors

The incidence of breast cancer in the United States increased very steadily over the last 60 years. In 1996 and 1997, the American Cancer Society reported over 180,000 new cases of breast cancer each year. In addition, they projected that more than 40,000 of the patients from each of those two years would die from the disease. However, the incidence of breast cancer and mortality associated with the disease has leveled off recently. Much of this stabilization can be attributed to increased screening (such as mammography and self-exams) and better treatment regimens for patients who develop the disease.

There are many risk factors associated with the incidence of breast cancer, including the following:

- Gender — Being female is the main risk factor for developing breast cancer. Although men can be affected, the incidence of breast cancer is 100 times greater in the female population.
- Age — The incidence of breast cancer increases with age and then slows after menopause.
- Long-term estrogen-replacement therapy — Many breast tumors are sensitive to estrogen levels and long-term estrogen therapies can increase the risk of developing breast cancer.
- Alcohol — Alcohol consumption leads to an increased risk of breast cancer.
- Genetics — The genetic component of breast cancer is one of the greatest predictors of risk. For example, it is believed that mutations in the BRCA1 and BRCA2 genes can lead to a 50–80% increase in risk.
- Family history — Having an immediate family member with the disease means a person’s risk is doubled. If two family members are diagnosed with the disease, the risk increases fivefold.

Prevention and Detection

The American Cancer Society recommends that women begin receiving annual mammogram screenings at age 40. If a person is deemed to be at high risk (family history or BRCA1 and BRCA2 mutations), then the screenings may start as early as 25 years of age, or five years earlier than the earliest age at which breast cancer was diagnosed in a family member. In addition, beginning at age 20, women should perform a breast self-examination (BSE) every month. It is also recommended that women have

a clinical breast examination conducted by a health professional every three years between the ages of 20 and 39.

Biology

Many factors are involved in the stimulation or inhibition of tumor development and growth. Specific hormones, such as estrogen, may have a strong positive effect on the growth of a tumor. In addition, breast cancer cells induce the development of new blood vessels (angiogenesis) that are needed to support and nourish the tumor tissue. Many of the therapies currently employed involve blocking the effects of growth hormones, such as estrogen, or preventing the growth of new blood vessels (starving the tumor cells).

One of the unfortunate biological aspects of cancer is the ability of the cancer cells to break off of the primary tumor, enter the blood or lymph system, and travel to a distant site where they attach and establish a new tumor. This process is referred to as metastasis and is a major source of mortality for many forms of cancer. Many instances of early detection of cancer are effective because the tumors are found and treated locally (with drugs or surgery) before they have the chance to spread to vital organs.

There are many different organs/tissues that become the target of metastatic breast cancer cells, including the brain, lungs, and bone. The metastatic breast cancer discussed in this module is not a simple process and involves an interaction among the breast cells, cancer cells, blood vessels, and normal surrounding tissue at both the primary and metastatic site. In order for cancers to metastasize, the cells must be able to invade blood or lymph vessels and then attach to distant tissues, where they begin the new tumor. This often involves adhesion molecules in the membranes of the cancer cells that are a part of a class of molecules called integrins. The disintegrin protein in this module that the students are addressing as a drug candidate binds to these molecules in an antagonistic way. This may account for its effect of preventing tumors from metastasizing once they develop because the cells cannot stick to the distant tissue. As an analogy, imagine a piece of adhesive tape that gets covered with lint. The tape appears to be unchanged, but it has lost its ability to stick to anything.

For more information, visit the American Cancer Society Web site at
http://www.cancer.org

X. References and Resources

GFP

GFP Applications Page
http://pantheon.cis.yale.edu/~wfm5/gfp_gateway.html

The molecular structure of GFP
http://www-bioc.rice.edu/Bioch/Phillips/Papers/gfpbio.html

The structure and function of GFP
http://public-1.cryst.bbk.ac.uk/PPS2/projects/jonda/

The GFP home page
http://www.biochem.mcw.edu/science_ed/Pages/gfp/

Illuminating the Structure of Green Fluorescent Protein
http://www.npaci.edu/online/v4.14/gfp.html

Chalfie, M., et al. (1994). Green Fluorescent Protein as a marker for gene expression. *Science*, 263:802.

Chalfie, M. and Kain, S. (Eds.). (1998). Green Fluorescent Protein: Properties, applications and protocols. New York: John Wiley and Sons, Inc.

Gura, T. (1996). Structure of the gene-tag protein solved. *Science*, 273:1336.

Ormo, M., et al. (1996). Crystal structure of the Aequorea victoria Green Fluorescent Protein. *Science*, 273:1392-1395.

Prasher, D.C. et al. Using GFP to see the light. *Trends in Genetics*, 11(8):320-329.

Yang, T., Moss, L.G., and Phillips, G.N. (1996). The molecular structure of Green Fluorescent Protein. *Nature Biotechnology*, 14:1246.

Chromatography and Protein Purification

An introduction to chromatography
http://www.chem.neu.edu/Courses/1221PAM/chromatog/ppframe.htm

Amersham Pharmacia Biotech Chromatography Page
http://www.chromatography.apbiotech.com/

Protein Purification
http://www.biotech.vt.edu/classes/bion_4784/9-ProteinPurification/ProteinPurification.html

ebioinfogen Protein Purification Protocol page
http://www.ebioinfogen.com/PROTPURI.htm

VIII. STUDENT HANDOUT SECTION

The Student Handout section begins on the next page.

STUDENT HANDOUT #1

Overview of the Bioprospecting Module and Design Challenge

HERE'S WHAT YOU WILL DO

Throughout the seven-week session, you will work in a group to:

- Explore the biotechnology industry and set up your own small-scale company.
- Review the concept of protein structure and function and learn how biotechnological methods are used to research and develop protein drugs.
- Develop an understanding of the design process and how it relates to the methods of science, including human clinical trials.
- Research information on drug development and protein purification.
- Develop important skills related to drug development and protein purification by completing Knowledge and Skill Builder (KSB) activities.
- Explore and discuss a Design Challenge related to protein purification that will be presented to you by the instructor.
- Compose, construct, test, and improve a design that relates to protein purification.
- Prepare a classroom presentation on the results of your work.

PROBLEM CONTEXT

Introduction

At a recent conference sponsored by the pharmaceutical industry, you and your research partners attended a presentation where you learned of an effective folk remedy that is used for the prevention of fungal diseases in humans. During the presentation, you learned that there is evidence that among those who take this remedy, there is a lower incidence of breast cancer and heart disease. To explore this further, you have obtained a sample of this folk remedy and determined that it contains the following ingredients:

1. Water
2. Salt (NaCl)
3. A prepared extract from pigeon feathers
4. Muskmelon seeds
5. Southern copperhead snake venom

A chemical analysis of the material reveals that the remedy contains three proteins that may be responsible for the potential therapeutic effects that you learned about at the conference. You and your partners have decided to start a new biotechnology company to design a way to identify the protein in the remedy that may be responsible for reducing the risk of breast cancer and heart disease. Your company will then clone the gene for this protein into bacteria. By growing these bacteria in nutrient culture, your company hopes to produce large quantities of the protein that can be purified and then used in testing on humans (such tests are referred to as clinical trials).

DESIGN CHALLENGE

As part of a group, you will design a way to purify a protein in large quantities for use in clinical trials (human testing). You will purify this protein from a bacterial culture through a two-step purification process.

SPECIFICATIONS

Purify Green Fluorescent Protein (GFP) from a bacterial culture lysate and use the SDS-PAGE technique to test the effectiveness of the purification protocol design.

CONSTRAINTS

You will be provided with a variety of materials used to purify proteins that will improve your design solution. The materials will come from many of the currently applied chromatographic methods used in protein purification (ion exchange, affinity chromatography, size exclusion, etc.). The following constraints will be imposed:

- The design will be constrained by the materials supplied for use in protein purification.
- The design must be limited to a two-step chromatographic purification process.

STUDENT REQUIREMENTS

In the NYSCATE module *Bioprospecting*: *Purifying Protein by Design*, you will be required to:

- Work in a team to address the Design Challenge presented in this module.
- Follow important safety guidelines in the laboratory as you work with your Design Challenge.
- Maintain a proper laboratory notebook throughout the module; the notebook will include a detailed record of your work as it relates to the Design Challenge.
- Complete all of the necessary Knowledge and Skill Builder (KSB) activities that are associated with the Design Challenge in this module.
- Participate in classroom discussions as you work through the module.
- Work with your group to produce and deliver a classroom presentation on the results of your activities.

STUDENT HANDOUT #2: KSBs 1–3
KSB 1: The Laboratory Notebook

One of the most critical skills that you must develop and maintain is proper documentation of laboratory work. You may be familiar with the concept of the laboratory notebook (Seidman & Moore, 2000; Polymer Research and Development, 2000), but you may not be familiar with the importance of this form of documentation in the biotechnology workplace. This importance can be summarized with a simple phrase that is part of the Food and Drug Administration (FDA) regulations: "If it isn't written down, it wasn't done." There are documented cases in which a biotechnology company threw an entire batch of pharmaceuticals away because a technician failed to record a simple measurement taken during the process. This type of oversight can cost companies millions of dollars in profits.

Since your group is essentially a small-scale biotechnology company, they must be able to maintain a proper laboratory notebook that contains a chronological log of everything that they do in the laboratory. A measure of a good laboratory notebook is that it is complete enough that the person, or someone else, could repeat the work on the basis of the information that is documented. Although this type of document may vary somewhat from company to company, there are some very basic guidelines that you should follow.

Guidelines

- Use only a bound notebook.
- Make sure every page is numbered before you use the notebook.
- Never remove pages from the notebook for any reason.
- Only use black ink. This will ensure that all writing will be visible after photocopying.
- Make sure your handwriting is clear, complete, and legible.
- Make sure you record your observations and data immediately and directly into the notebook and not on a separate sheet of paper. Transferring data from separate pieces of paper, backs of envelopes, paper towels, or your hand is a waste of time and can increase chances for clerical error.
- If you have separate pages (such as instrument printouts) that need to be included in the notebook, never cover information up when the page is added. Never fold a page into your notebook.
- If you have to affix material into your notebook, tape or paste all sides of it to the notebook page. Write "NWUI" (no writing under the insert) on the tape or near the material along with your initials and the date on which the material was added.
- Cross out any errors with a single line so that the original text is still visible and can be read. Add your initials and a date next to the correction.
- Make clear notes of any problems encountered. Do not try to erase or hide mistakes. Unused portions of a laboratory notebook page should be crossed out with a diagonal line so that nothing can be added to the page at a later time.
- Be objective in your documentation. (Avoid personal commentary and notes.)
- Make sure that information on materials and methods is detailed enough that the generated designs and experiments can be repeated using the information in your laboratory notebook (including vendor names, equipment model numbers, etc.).

You should include the following components in your laboratory notebooks.

Key Components

- Identifying Information — The front of the notebook should contain identifying information. This should include your name, company name, project name, date, an identification number for the

notebook (typically number the notebook if it is part of a series of notebooks), and any other important identifiers that may be appropriate.
- Contents — A table of contents with page numbers referenced.
- Page Numbers — A page number should appear on every page. It is best to number all of the pages before using the notebook for the first time.
- Statement of Responsibility — At the top of every page there should be a statement that reads "prepared by" or "recorded by" with your name. At the bottom of every page there should be a statement that reads "witnessed by" or "read and understood by" and the initials or signature of the lab manager (see KSB 2: Biotechnology and the Economy). The lab manager can sign or initial the lab notebooks at the end of each classroom and laboratory session.
- Information Sources — For each project (or KSB) there should be a thorough listing of the results of literature searches or information collected from colleagues.
- Dates, Titles, and Descriptions — For each day's work there should be a date, title, and description of the day's activities and objectives. Each day should begin on a new page with a diagonal line drawn through any unused portion of the previous day's work.
- Rationale — A statement of the rationale for the activities that will be documented should explain the reason for performing a documented task.
- Equations and Calculations — Relevant equations and calculations must be included.
- Equipment and Materials — A complete description of equipment and materials used in all of the work should be included.
- Procedural Details — The steps of all protocols should be documented in the notebook. In a biotechnology company, this may simply be a reference to a standard operating procedure (SOP) that the company keeps on file. Another option is to cut and paste a written protocol into a notebook page to avoid having to rewrite every step.
- Data — Data that is collected should be documented in the notebook. If this involves printouts or other forms of output (such as pictures of electrophoresis gels), they should be clearly labeled and affixed in the notebook according to the directions in the guidelines (see above).
- Observations — As work is being conducted, all relevant observations for a given activity should be documented.
- Summaries — When an activity is completed, a brief written summary should be produced.
- Conclusions and Interpretations — It is appropriate to summarize preliminary interpretations of observations and data in the notebook.

Practice

- Set up your laboratory notebook with the identifiers and page numbers.
- When you finish each KSB, make sure your name appears at the top of every page, your lab manager has initialed or signed the pages, and your table of contents has been updated.

While working through KSB 2: Biotechnology and the Economy, and KSB 3: Methods for Protein Purification, follow all of the guidelines for preparing laboratory notebooks.

Keep detailed notes of all of your work, classroom discussions, etc. that are part of the KSBs for this week. It is important to develop strong habits early on so that they become automatic throughout the rest of the module.

KSB 2: Biotechnology and the Economy

In this section, you will develop a name for your company by researching current biotechnology companies on the Internet. In addition, you will be exploring several aspects of the biotechnology industry that relate to the business of your small start-up company. Depending on your background, you may need some information and practice on how to perform a basic Internet search using a Web browser. Your instructor may provide you with this information or you might consider joining a group that contains at least one experienced computer user.

Although every member of your group should be maintaining a proper laboratory notebook, every week you will have a new lab manager who will sign off on your books after reviewing them for proper format. The members of your group will rotate into this role every week so that everyone has this experience. Below you will find the job description for this responsibility.

Lab Manager — The lab manager's responsibility is to see that all protocols are followed exactly as written or designed and all tasks for that week are completed. If the company decides that it will modify any of the steps of a protocol, the lab manager should discuss this with the company and then have it approved by the instructor. The lab manager should check to be sure that all steps are being followed, all safety considerations are being addressed, and all work is being documented. The lab manager will sign off on the laboratory notebooks of the other members of the group at the end of the day. It is extremely important that the manager ensures proper documentation. The instructor should always sign the lab manager's notebook.

NOTE: As the lab manager, you are responsible for your company's documentation. Every week your instructor may require that you switch responsibilities with other members of the company.

Protocol

Each member of your group is responsible for working on the research questions found below. You should discuss your findings and share information as you are working. This is your chance to practice proper documentation without the pressure of working through complicated laboratory protocols. Your instructor may spend some time discussing how to perform an Internet search before you begin.

As you work, you have your first opportunity to practice the use of the laboratory notebook. During your research, you should follow proper documentation rules:

- Begin with a rationale—a statement of the rationale for the activities that will be documented should explain the reason for performing a documented task.
- As you use information from a resource, document that source (e.g., Web site address).
- Make sure you record your observations and data immediately and directly into the notebook and not on a separate sheet of paper.
- Never fold separate sheets into a page into your notebook.
- When affixing separate sheets, tape each side and write "NWUI" (no writing under the insert) on the tape or near the material along with your initials and the date on which the material was added.
- Cross out any errors with a single line.
- If you leave portions of a page blank, cross out the blank areas with a diagonal line so that nothing can be added to the page at a later time.

Using information obtained from text resources and searches conducted on the Internet, explore answers to the following questions:

1. What is biotechnology? How would you define this type of technology?

2. The company that you have formed can be classified as a biopharmaceutical company. What is the difference between a pharmaceutical and a biopharmaceutical?

3. The development of a drug for the market is a highly regulated activity in this country. The Food and Drug Administration (FDA) develops these regulations. During the development of your drug, you will need to file important applications with the FDA. For each of the following, briefly describe what the application is for, and identify at what stage of development you would file these applications:

    a. IND — Investigational New Drug application

    b. NDA — New Drug Application

4. The large-scale production of biopharmaceuticals can be a very expensive process for a start-up company. In response to this expense, some companies elect to take advantage of "biopharmaceutical contract manufacturing." Summarize answers to the following questions:

    a. What is "contract manufacturing"?

    b. Why would it be beneficial to your start-up company to consider this resource?

    c. Identify some specific contract manufacturers that can handle the production of the type of drug you hope to produce.

Choosing a company name

Before you continue, your group needs to establish a name for your company. Continue to conduct research on companies that produce biopharmaceuticals so that you have a feel for the types of company names that have been used. Come up with a name for your company that you will use throughout the rest of this course. Display the name of this company with the identifying information in your lab notebook.

KSB 3: Methods for Protein Purification

Develop your understanding

Using the World Wide Web or printed materials, explore the factors involved in the purification of proteins. Your company should consider a variety of different questions in your explorations.

Why do scientists purify proteins?

What physical properties of proteins do the purification methods address?

What are the different types of purification methods and what properties of the proteins do they target?

How does each of the different purification methods work?

Activities

After you have had some time to explore this topic, all of the groups will come back together as a class to take part in a class discussion on the topic. Make sure that you take very detailed notes and are very specific in your discussions. For example, if you are discussing a purification method, make sure that you can explain how that method takes advantage of a physical property of the protein you are trying to purify.

When the entire class has completed this KSB, your notebook should contain a record of your research (including references) and the discussions that followed when the class reconvened. Make sure you review those notes with your company before your lab manager signs off at the end of the day.

LAB MANAGER

When Week 1 activities are complete, make sure the lab manager inspects the notebooks of all of the company scientists and identifies any mistakes or errors before signing off on each page. The instructor will then sign off on the notebooks the lab manager has inspected.

STUDENT HANDOUT #3: KSBs 4–5
KSB 4: Bioinformatics

The National Center for Biotechnology Information (NCBI) maintains a very large database of DNA and protein sequences that is constantly being updated. The NCBI is within the National Library of Medicine (NLM), which is part of the National Institutes of Health (NIH). In addition to the massive amount of human DNA sequence information produced from the recently completed Human Genome Project, the NCBI contains DNA and protein sequence information from a variety of different organisms. The NCBI makes this data available over the Internet at no charge for use by scientists to identify DNA sequences and proteins. In addition, the database can be used to look for homology (similarity) between sequences when similar genes or proteins are found in a variety of different organisms.

The computer program that we will use to search the database is called BLAST (Basic Local Alignment Search Tool). There are BLAST programs designed to search both DNA and protein sequences on the NCBI server. The specific program that you will use to explore proteins is BLASTP (P = protein). In this KSB, you will be introduced to the fundamentals of a basic Internet BLAST search to identify the three proteins isolated from the folk remedy in the scenario.

As with any new skill, it is important that you are able to practice before trying to use this tool in your research. Before exploring the three unknown protein sequences, your instructor may spend some time giving you an orientation to the BLAST program and the NCBI Web site. If your instructor does not provide an orientation, then you might find it useful to use the tutorial tools supplied by the NCBI. You may find that an exploration of these tutorials is helpful even if your instructor is providing an orientation. In addition, we will be working through a practice sequence to help develop the skills needed to use this very powerful bioinformatics tool.

Access to the NCBI Server:

1. To begin the exploration of the BLAST program, access the NCBI home page at http://www.ncbi.nlm.nih.gov/
2. From the main NCBI page, select the *BLAST* link that is found in the banner at the top of the page.
3. The main BLAST information page contains links to a variety of topics. If you are ready to perform a BLAST search with a practice protein amino acid sequence, then skip to the section "Performing a BLAST search." If you would like to make use of the BLAST tutorials, then move on to the "BLAST tutorial" section below.

BLAST tutorial

Before beginning any of the tutorials, you should open two different browser windows. In one window you will be able to run through the tutorial, and then as the tutorial gives instructions, you can switch to the other window to see how it will work in the actual BLAST program by running a BLAST search.

1. After accessing the NCBI page and then the main BLAST page, locate the link for BLAST tutorial to the left of the page and select this link.
2. The first tutorial link on the BLAST information page is called *Query Tutorial*. This tutorial is designed for novice users with little or no experience. Select this link to begin the tutorial.

3. The Query Tutorial page is broken into sections and begins with an INTRODUCTION. Under the second section, “Selecting the BLAST Program,” make note of the fact that you will be using the BLASTP program for searching proteins.
4. Under the third section, “Selecting the BLAST Database,” make note of the fact that you will use the *nr* database for proteins. Using this database will give you the most comprehensive search in the BLAST program.
5. At the end of this tutorial, there is a very important discussion regarding the type of information that can be input into the program. The BLAST program accepts three forms of information for searching:
    a. An amino acid sequence in FASTA format (see below)
    b. NCBI accession number (a code assigned to the protein)
    c. A GenBank (GI) Identifier (another database code).
6. In this KSB, you will be entering an amino acid sequence in FASTA format. The table below describes the symbols that are used in this type of format.
7. The tutorial directs you to enter a sequence into the search window in FASTA format. When you are ready to do this, skip to the practice problem in the “Performing a BLAST search” section below

FASTA FORMAT

In this format, sequences are entered using the standard IUB/IUPAC amino acid and nucleic acid codes. These codes are single-letter codes representing the amino acids and nucleotides. The accepted codes for the amino acids are as follows:

AMINO ACID	Standard three-letter code	IUB/IUPAC used in FASTA format
Alanine	Ala	A
Aspartate or Asparagine	Asp or Asn	B
Cystine	Cys	C
Aspartate	Asp	D
Glutamate	Glu	E
Phenylalanine	Phe	F
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Lysine	Lys	K
Leucine	Leu	L
Methionine	Met	M
Asparagine	Asn	N
Proline	Pro	P
Glutamine	Gln	Q
Arginine	Arg	R
Serine	Ser	S
Threonine	Thr	T
Selenocysteine	Sel	U
Valine	Val	V
Tryptophan	Try	W
Tyrosine	Tyr	Y
Glutamate or Glutamine	Glu or Gln	Z
Gap of intermediate length		-
Translation stop code		*
Any amino acid		X

For example, the amino acid sequence

Gln tyr ala pro gln thr gln ser gly arg thr ser ile val his leu

would be represented in FASTA format as

Q Y A P Q T Q S G R T S I V H L

Performing a BLAST search

Practice Problem

1. Before working with the unknowns, practice performing a BLAST search with the sequence found below and then answer some simple questions relating to the output that is generated.
2. Access the BLAST page from the main NCBI page as described in the first section of this KSB.
3. Make sure that you select the correct program and database for performing the search. If you are unsure, then you should return to the "BLAST tutorial" section above. We will be performing a standard protein-protein BLAST [blastp].
4. Assume the following protein sequence was obtained from experimental data and must be identified:

qyapqtqsgrtsivhlfewrwvbialecerylgkgfggvqvsppnenvvvtnpsrpwweryqpvsyklctrsgnenefrbmvtrcnnvgvr
iyvbavinhmcgsgaaagtgttcgsycnpgsrefpavpysawbfnbgkcktasggiesynbpyqvrbcqlvgllblalekbyvrsmiaby
lnklibigvagfr

5. To save time, this sequence can be found on the NYSCATE Web site (www.nyscate.net) and can be cut and pasted into the BLAST search window. Make sure that all carriage returns are removed from the BLAST window after pasting it.
6. After the sequence has been pasted into the search window, click on the BLAST button.
7. After a short wait, you will be shown a format page. From this page, you can select the various ways that your results can be displayed. For now, just leave the defaults as they are and then click on the FORMAT button to go to the results page.

BLAST OUTPUT

The information given in the tutorial on BLAST output is fairly technical and much of the information that is highlighted will not be important for this KSB. For the purposes of this KSB, you will need the following highlights:

- The BLAST output is given in both text and graphical format. The graphical format is presented first. The graphical output is interactive and when the mouse is dragged across any of the graphical bars, it will yield information about that part of the search output.
- In the graphical and text portions of the output, the sequences in the NCBI database that show homology (similarity) to the input sequence are listed from the greatest to the least amount of homology.
- In the text output, the *Score (bits*) and *E value* are statistical measures of how well the input sequence matches the sequences found in the database. A very high Score (bits) and a very low E value correspond to the greatest homology. (NOTE: Very small E values are represented in scientific notation. For example, an E value of "3e-85" would be $3x10^{-85}$.)
- The Score values act as links to take a searcher to the bottom of the page where all of the matches (alignments) are presented. If the mouse is used to select a Score, a graphical representation will appear that shows how your input sequence (query) matches with the sequence found in the database, which is referred to as the subject sequence (sbjct). One of the numbers to look for when viewing the alignments is the "Identities" number that is listed at

the top of each alignment and is represented as a percentage. This number is a measure of the percentage of amino acids in the input sequences that match the subject sequence.

- Each match that is listed in the text output begins with a GenBank Identifier (GI) or an accession number. These numbers act as links. If the mouse is used to select the link, a page that contains more detailed information on that protein will come up. Select some of these links to see what type of information is available. This will be an important task when working with the unknowns.

PRACTICE PROBLEM QUESTIONS

After performing a BLAST search with the practice sequence, answer the following questions as a group. Discuss ways that you can break up the work so that you are able to collect all of the information from the BLAST output. You should use your laboratory notebooks to record your observations and discussion answers.

1. What is the probable identity of the unknown protein?
2. What tissues or organs produce the unknown protein?
3. Use your mouse to select the first red line in the graphical output and determine how similar that subject protein is to the unknown protein by listing the %identity.
4. Identify the names of the organisms that produce this unknown protein. Only list the animal sources that show a Score (bits) of greater than 250. In order to find the animal source for some of the matches, you may need to use your mouse to select some of the accession number links. Selecting these links will take you to a page that contains more detailed information for that match. For each animal that you list, report the highest %identity that you can find from the alignments.

Identifying the unknown proteins in the folk remedy

The scenario for this module describes a folk remedy that contains three proteins that may be responsible for reducing a person's risk of developing breast cancer and heart disease. You will perform a BLAST search on these three proteins to try to determine the source and function of these three proteins. As a start-up company, you have only enough resources to pursue the development and testing of one of the three proteins. The results of the BLAST search and subsequent research will be used to justify the research and development that will follow.

Once the identity of each of the three unknown proteins is discovered, your company will have to do some research on the natural functions of those proteins. Assuming a group of three students, it is possible to assign the research work for one protein to each member in the group. Your instructor will assign the research that follows the BLAST search either as a homework assignment or as an in-class exercise. In either case, the results will become part of a class discussion prior to beginning the activities for Week 3.

1. Access the NCBI server and select the BLAST link. Select the Protein BLAST link to open a search window.
2. The amino acid sequences for each of the three unknowns can be typed into a BLAST search window or cut and pasted from the NYSCATE Web site. If you are cutting and pasting the sequences into the search window, make sure that you remove any carriage returns before performing the search.

   Unknown sequence #1:

mklysllsllflgvlwrsegvasssnddvgrlinvttfnamfkyqkdpqcpsqgfysyqafltaarsfgklgfattgklatrkrellaflaqtsh
qttggwltapdgplfwgychirestedsyckadpkwpcakgqkyygrgpmqlkgnqnygqagkalgldllknpdlvakdpvvsfkt
aiwfwmtaqgikpschdvmvgkwkpteadkaakrvpgygvvsniiggsecgsgantdvadrfgfyvryckmlgvnpgkhldcff
qqpftrm

Unknown sequence #2:
msccnpclpcqpcgptplanscnepcvrqcqsssviiepssvvvilpgpilssfpqntvvgsstsaavgsilscegvpinsggfdlsit
srycgsrcrpc

Unknown sequence #3:

dapanpccdaatckllttgsqcadglccdqckfmkegtvcrrargddlddycngisagcprnpfha

3. Perform a BLAST search on each of the three unknown proteins from the folk remedy.
4. Collect information from the BLAST output and the various links to information from the output page to identify the name of each unknown protein.
5. Conduct library and Internet research to gather information on the three proteins once they are identified. You will also be able to collect information from links on the BLAST output page. Your instructor may have you do this as an in-class activity or as a homework assignment.
6. Answer the questions in the following section:

DISCUSSION QUESTIONS FOR UNKNOWNS

For each of the unknown sequences, answer the following questions:

1. What is the name of the protein?
2. What is the most likely source of this protein? (What is the source organism?)
3. What other organisms make this protein or a protein similar to this one?
4. What function does this protein play in the source organism? (What use does the source organism have for this protein?)
5. What function does this protein play in the folk remedy with which you are working?
6. Is this protein a likely candidate as a cancer or cardiovascular drug? If so, explain why you think this protein is a candidate by describing how the function of the protein might be useful to treat these diseases.

CLASS DISCUSSION:

- Once the BLAST search and research are complete and you have had time to discuss within your company, your class will come together for a large group discussion of the results.
- As a class, you will try to come to a consensus as to the component of the folk remedy that is the most likely candidate responsible for reducing a person's risk of developing breast cancer and heart disease.
- Document all of your observations and notes in the laboratory notebook using proper documentation.

KSB 5: Solutions and Media

Your instructor will determine which solutions you will need to prepare as a company, and which solutions will be provided for you to use during the purification of your target protein. The following solutions will be required:

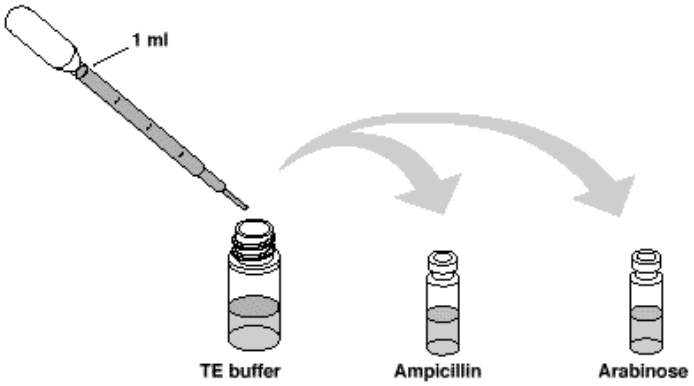
- Ampicillin and arabinose solutions
- LB agar with ampicillin and arabinose
- LB liquid medium with ampicillin and arabinose
- Buffers and salt solutions for chromatography

NOTE: Some of the chromatography buffers are supplied as stock, but many of the buffers required for running ion exchange chromatography and hydrophobic interaction chromatography will need to be prepared. The instructions for preparing these solutions are included in this KSB. Your instructor will determine whether you will be preparing these solutions as an activity or whether they will be supplied to your group.

Prepare Ampicillin and Arabinose Solutions

Ampicillin and arabinose are shipped dry in small vials. After being rehydrated, both are added to the molten agar (before plates are poured) and to the liquid growth media. Ampicillin is an antibiotic that inhibits growth of bacterial contaminants that may be introduced from the environment. Arabinose is a sugar that promotes the overexpression of the Green Fluorescent Protein in cloned cells.

1. Using a sterile pipette, add 3 mL of TE buffer directly to the vial containing the ampicillin.
2. Using another sterile pipette, add 3 mL of TE buffer to rehydrate the arabinose.
3. Mix the vials and gently vortex to aid in rehydration.
4. Put the vials aside and begin preparing the agar solution.



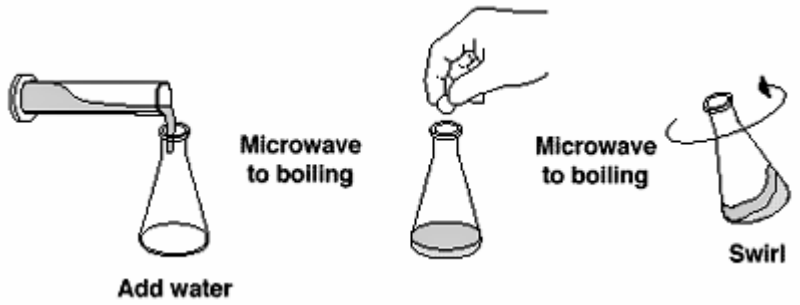
NOTE: Rehydrate ampicillin and arabinose the day you pour agar plates. Arabinose requires 10 minutes to dissolve—be patient.

Prepare LB Agar

NOTE: Agar plates should be prepared at least two days before KSB 6: Cloning begins.

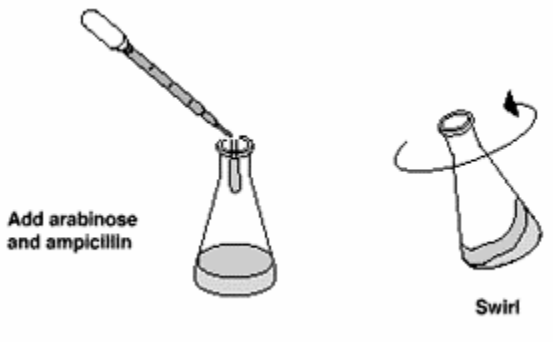
The agar plates can dry out (cure) at room temperature for two days before they need to be refrigerated. This can conveniently be done on a Friday, with the covered plates left out over the weekend to dry adequately. Drying out for two days at room temperature allows the agar to cure sufficiently to readily take up the bacterial library. Plates that are prepared more than two days before KSB 6: Cloning begins should be refrigerated until used. In the suggested timeline of this module, the plates will need to be stored in the refrigerator for one week before being used in the KSB on cloning. If necessary, plates can be poured and stored in the refrigerator for up to three months.

The vial of arabinose contains 600 mg and the vial of ampicillin contains 30 mg. The agar and nutrient media should be prepared to give a final arabinose concentration of 0.2% (2 mg/mL) and an ampicillin concentration of 100 g/mL. Although directions are provided for preparing these solutions, you should calculate the correct volumes and concentrations for preparing these solutions and record them in your notebook.



Prepare the agar by adding 210 mL of distilled water to a 1 liter Erlenmeyer flask and heat solutions and record them in your notebook.

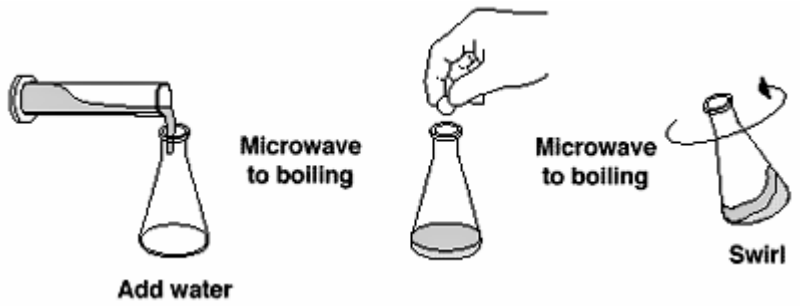
1. Prepare the agar by adding 210 mL of distilled water to a 1 liter Erlenmeyer flask and heat to boiling in a microwave oven.
2. If LB-agar is in tablet form, it is helpful to crush the tablets into a powder form before heating in the flask to help it dissolve.
3. Add 4 LB-agar tablets to the flask and let the tablets soak in the hot water for 20 minutes—this will aid in dissolving and prevent clumping of agar particles.
4. Heat the flask again to boiling in the microwave, then swirl.
5. Allow the flask to cool for 30 seconds before swirling so that the molten agar does not suddenly bump or boil over onto your hand when swirled.
6. Use gloves or a folded paper towel to hold the flask and protect your hand while swirling.
7. Repeat heating and swirling until all of the agar is dissolved and the solution no longer appears cloudy. Taking evaporation into consideration, the volume should now be about 200 mL.
8. Before pouring plates, ampicillin and arabinose must be added to the hot liquid agar. Because excessive heat (>50 °C) will destroy ampicillin and arabinose, allow the agar to cool so that the outside of the flask is just barely comfortable to hold (~50 °C).
9. When the agar has cooled to 50 °C, use a new sterile pipette and add 2.0 mL of the ampicillin solution you prepared to the flask of agar.
10. Using a new pipette, transfer 2.0 mL of the arabinose solution to the 200 mL of cooled liquid agar. Swirl to mix.
11. Remember that the agar will solidify at 27 °C, so after the ampicillin and arabinose are added, you should pour all 16 plates from start to finish without interruption.
12. The remaining 1.0 mL of ampicillin and arabinose will be used for the preparation of the liquid nutrient broth in the next step.



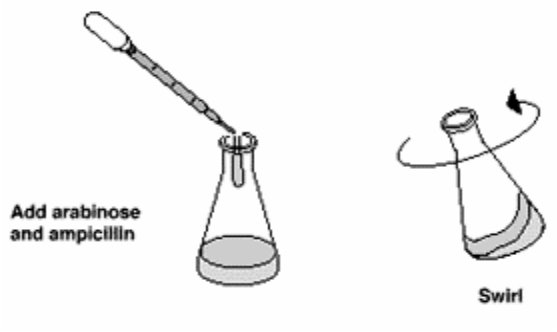
Prepare Liquid LB Medium

NOTE: Observe sterile technique when preparing broth. Liquid nutrient medium is used in KSB 7: Screening; each student workstation will require two culture tubes containing 5 mL of liquid nutrient media. These will be used to grow bacterial cultures. The preparation of the liquid LB medium is placed here because it is most conveniently prepared on the same day as the nutrient agar.

1. To prepare the liquid medium, add 110 mL of distilled water to a 250 mL Erlenmeyer flask and heat to boiling in a microwave.
2. Add two LB tablets to the flask. Let the tablets soak in the hot water for several minutes; this will aid in dissolving.
3. Heat the flask again to boiling in the microwave.
4. Swirl (note safety comment in #5) the flask to dissolve the tablets.



5. Repeat heating and swirling several times until the tablets are dissolved, but be careful to allow the flask to cool a little each time before swirling so that the hot medium does not boil over onto your hand.
6. When the tablets have dissolved, allow the LB to cool so that the outside of the flask is comfortable to hold, or below 50 °C.
7. While the media is cooling, get the ampicillin and arabinose solutions that were prepared in the step above.
8. When the media has cooled, transfer the remaining 1.0 mL of arabinose and ampicillin into the flask.



9. Using a new pipette, have each group aliquot 5 mL of the liquid media into two of the culture tubes. (This can be accomplished by transferring the media in five 1 mL aliquots from a sterile pipette.)

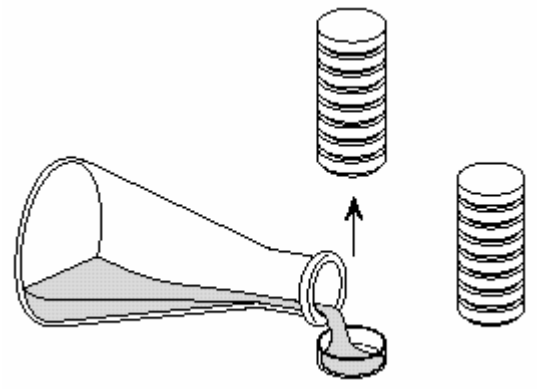
10. Store the culture tubes in a refrigerator until the day of use. Extra culture tubes are provided. Fill these extra tubes with medium and use them as backup cultures for KSB 7: Screening.

Pour Agar Plates

1. Stack the empty plates (with lids on) in piles four to eight high. Starting with the bottom plate, lift the lid (and the upper plates) up and to the side with one hand and pour the agar, using your other hand.

2. Fill the plate about one-third of the way (<12 mL) with agar, replace the lid, and continue up the stack.

3. Pour 16 plates in this fashion, or you may choose to employ your own pouring technique. The agar should solidify within 15-20 minutes.



NOTE: After the plates are poured, do not disturb them until the agar has solidified.

Storing Agar Plates

After the plates have cured for two days at room temperature, they can be stacked (20 high) and the plastic sleeve slipped back over them. The stack should then be inverted and the sleeve taped closed. Plates can be stored upside down in a refrigerator for up to three months.

OPTIONAL STUDENT EXERCISE:

(Your instructor may have prepared these solutions ahead of time and will provide them, or your group may be asked to prepare these as part of your course activities.)

Preparing Buffers and Salt Solutions for Protein Purification

Your workstation	Number
Reagent stock bottles	8–10
Volumetric flasks (50,100, 200 mL)	2
Graduated cylinders (10, 25, 100 mL)	3
Beakers (100, 200, 400 mL)	3
Marking pen	1
Microcentrifuge tubes	4

Shared classroom materials

Magnetic stir plate and magnetic bars
Ph meters
1 M HCl stock with glass pipettes and bulbs
Scale and weight boats/paper
Distilled water
Tris solid
NaCl solid
TE buffer
HIC equilibration buffer stock
HIC binding buffer stock
HIC wash buffer stock

Each of the three different purification techniques provided requires buffers and salt solutions that must be prepared ahead of time so that they will be available during the purification stage of your drug development. Scientists use a fairly standard protocol to prepare these solutions, and biotechnology companies maintain these protocols as a standard that their technicians and scientists can follow to ensure consistency. A standard operating procedure (SOP) is a document that instructs company personnel in how to perform specific tasks relating to their operations. Your instructor may explore this in more detail and may ask your company to develop SOPs for your buffer and solution preparation protocols.

Concentration expressions — Solutions for Ion Exchange Chromatography (IEX)

There are a variety of different ways to express the concentration of a solute in a solution, but one of the more common ways is to use an expression of molarity. Molarity describes the number of moles of a solute dissolved in one liter of a solvent. For any element, a mole is described as 6.02×10^{23} atoms of that element. The atomic mass of that element gives you the weight (in grams) of one mole of that element. When applied to a compound, the weight (in grams) of one mole of that compound can be calculated by adding together the atomic mass of each of the elements in that compound. An example is given below.

1 mole of Na_2SO_4 weighs 142.04 grams:

2 sodium atoms x 22.99g/mole = 45.98g
1 sulfur atom x 32.06g/mole = 32.06g
4 oxygen atoms x 16.00g/mole = 64.00g
TOTAL
142.04g

Since molarity describes the number of moles of solute dissolved in one liter of solvent, one liter of a one molar (1 M) solution of Na_2SO_4 can be prepared by dissolving 142.04 grams into a liter of distilled water. Since many times you would need much smaller volumes of a solution, or at a variety of concentrations, it is important that you can calculate how to prepare a solution that you can use in your laboratory. Very often, you are interested in knowing how much solute is required to prepare a given solution. Examples are given below:

How do I prepare 300 mL of a 0.8 M solution of calcium chloride?

(NOTE: In many cases, you will not have to calculate the molar weight of a solute because it will be given to you as part of the information presented on the stock label.)

Solute required = (grams/mole)(moles/liter)(liters)

One mole of calcium chloride = 111.0g

Solute required = (111)(0.8)(0.3) = 26.64 grams

How do I prepare 500 mL of a 200 mM solution of NaCl?

One mole of sodium chloride = 58.44g

Solute required = (58.44)(0.2)(0.5) = 5.84 grams

To prepare the solution, you would follow these steps:

1. Find the molar weight of the solute (on the label use either FW or MW; FW is the gram Formula Weight and MW is the Molecular Weight).
2. Determine the volume of the solutions that you will want to prepare.
3. Determine the concentration (molarity) that you will need.
4. Calculate the amount of solute needed.
5. Weigh out the amount of solute needed.
6. Measure out a volume of solvent (dH_2O unless otherwise stated) that is less than what the final volume will be (about 80–90% of final volume).
7. Pour solvent into appropriate beaker with a stir bar and a stir plate if needed.
8. Dissolve solute into solvent.
9. Adjust pH (if necessary—see below).
10. Pour solution into appropriate volumetric flask and bring to volume.
11. Store solution in a well-labeled reagent bottle.

Adjusting pH — Use stock solutions of 1 M HCl and NaOH to adjust pH. Using a pH meter and stir plate, slowly drip in appropriate solution and watch pH changes until the desired pH is reached.

Using the information presented above, calculate how you would prepare the following solutions for this purification technique:

100 mM Tris, pH 8.3 stock solution
50 mM Tris, pH 8.3 stock solution
50 mM Tris, pH 8.3, 130 mM NaCl
50 mM Tris, pH 8.3, 200 mM NaCl
50 mM Tris, pH 8.3, 300 mM NaCl
50 mM Tris, pH 8.3, 500 mM NaCl

After checking your calculations (or signing your SOP if you are preparing one of these documents), your instructor will provide you with the materials that you need to prepare these solutions. Make sure you document all of your activity in your lab notebook. The details from the labels of any reagent or stock solution that you use should be documented with your list of materials.

Preparing Dilutions — Solutions for Hydrophobic Interaction Chromatography (HIC)

A very common practice in a laboratory is to prepare a stock solution at a concentration that is greater than what would be used in a laboratory protocol, and then prepare working solutions from that stock as needed. In order to do this, it is necessary that you know how to calculate these dilutions. One of the most common formulas used to prepare a dilution from a concentrated stock is the following:

$$C_1V_1 = C_2V_2$$

Where
C_1 = Initial stock concentration
C_2 = Final dilution concentration
V_1 = Volume of stock required (UNKNOWN)
V_2 = Volume of dilution to be prepared

For example, if I wanted to make 100 mL of a 130 mM NaCl working solution from a stock solution of 200 mM NaCl, I would calculate the following:

$(200\ mM)(V_1) = (130\ mM)(100\ mL)$

$V_1 = (130)(100)/200 = 65\ mL$

To prepare this dilution, I would measure 65 mL of the stock solution into beaker or volumetric flask. I would then measure out and add 35 mL of the solvent that was used to prepare the stock solution (usually distilled water) or bring to volume in a 100 mL volumetric flask with that solvent.

To prepare the dilution, I would do the following:

1. Determine the volume of working solution you want to prepare.
2. Determine the concentration of the working solution you want to prepare.
3. Determine the concentration of the stock solution you are using.
4. Calculate the volume of stock solution needed to prepare dilution.
5. Measure out the required volume of stock solution into an appropriate volumetric flask or beaker.
6. Bring to desired volume of working solution with the solvent that was used to prepare the stock solution (usually distilled water).
7. Store dilution in a well-labeled reagent bottle.

For HIC, we will need to prepare some dilutions of ammonium sulfate. The binding buffer, equilibration buffer, and wash buffer provided for use with HIC can act as your stock solutions. The binding buffer contains 4 M ammonium sulfate. The equilibration buffer contains 2 M ammonium sulfate. The wash buffer contains 1.3 M ammonium sulfate. Using the information presented above, calculate how you would prepare the following solutions for this purification technique:

1.5 M ammonium sulfate solution for equilibration of the HIC column
0.8 M ammonium sulfate solution for a wash of the column
0.4 M ammonium sulfate solution for a wash of the column
0.16 M ammonium sulfate solution for elution of your protein

For the two wash and elution buffers, you will only be needing 250 μL of each of these solutions during your purification, so make sure that you prepare a working volume that is appropriate. Since you will be working in small volumes, you can prepare the volumes using a pipette, and you can store the reagents in microcentrifuge tubes. Consider how you would prepare and store the equilibration buffer. You will require a larger volume of this buffer for HIC (2 mL).

After checking your calculations (or signing your SOP if you are preparing one of these documents), your instructor will provide you with the materials that you need to prepare these solutions. Make sure you document all of your activity in your lab notebook. The details from the labels of any reagent or stock solution that you use should be documented with your list of materials.

STUDENT HANDOUT #4: KSBs 6-8
KSB 6: Cloning

The gene for Green Fluorescent Protein (GFP) was inserted into a DNA plasmid and then placed into *E. coli* bacteria through the process of transformation. In this case, we are simulating the idea that the GFP gene was fused to the disintegrin gene before being cut and pasted into the plasmid. If this fused gene were to be expressed as a fusion protein inside the bacteria, it would be easy to identify, because it would glow green under UV light. Since the process of transformation is not 100% efficient, only some of these bacterial cells now contain the gene for the disintegrin protein. These need to be found and separated from other bacteria.

First streak out or spread a sample of these transformed bacteria onto a petri plate containing a special blend of bacterial food. In a day or two, individual colonies should appear on the plate. This process of separating the bacteria on the surface of the plate so that each individual cell can grow up into a clump of identical cells (a colony) is called cloning. Because all the cells in a single colony are genetically identical, they are called clones.

Follow the procedure outlined below to streak out the bacterial library.

Your workstation	Number
Inoculation loops — Sterile	1
Poured agar plates — Sterile	3
Marking pen	1
Shared classroom materials	
Lyophilized transformed bacteria	1
37 °C incubator	1

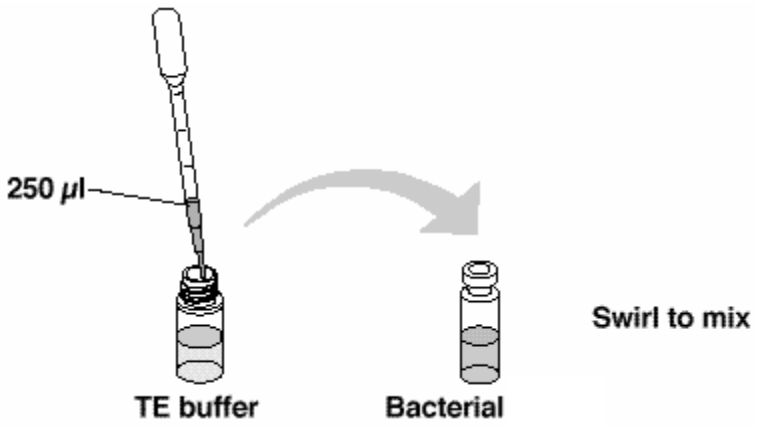
The transformed bacteria are shipped dehydrated and need to be rehydrated prior to use. Because of the ever-present possibility of external contamination, it is important that rehydration of the bacteria be done on the day of this KSB. Since the class will share this solution, your instructor or a student from one of the groups can prepare it.

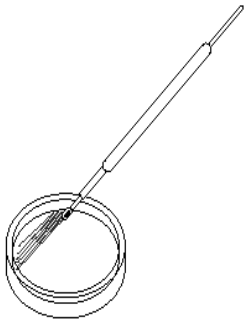
1. Using a new sterile pipette, add 250 µL of TE buffer to the bacterial vial. Swirl gently to resuspend. The bacteria settle quickly; the vial should be thoroughly mixed before you streak your plates.

2. Turn your petri dishes upside down. Using a marker, label your two agar plates with your company name and class section.

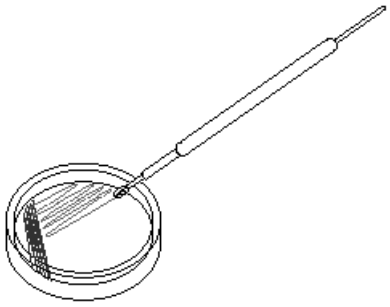
3. Using the rehydrated bacteria, streak two plates. The purpose of streaking is to generate single colonies from a concentrated suspension of bacteria. A minute amount of the bacterial suspension goes a long way. Under favorable conditions, one colony cell multiplies to become millions of genetically identical cells in just 24 hours.
    a. Insert a sterile inoculation loop straight into the vial of the bacteria without tilting the vial. Remove the loop and streak for single colonies as illustrated below. Streaking takes place sequentially in four quadrants on each plate. The first streak is to just spread the cells out a little. Go back and forth with the loop about a dozen times in each of the small areas shown. In subsequent streaks

the cells become more and more dilute, increasing the likelihood of producing colonies from single bacteria.

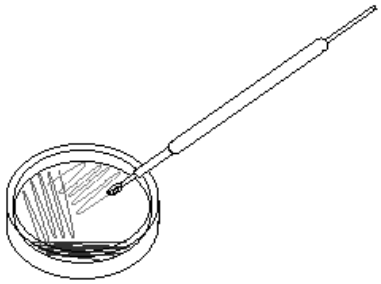




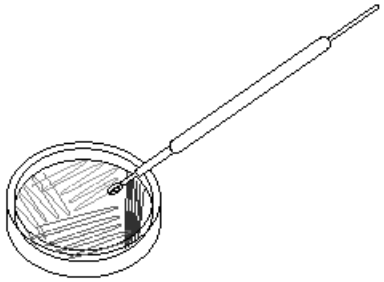
b. For subsequent streaks, the goal is to use as much of the surface area of the plate as possible. Rotate the plate approximately 45° (so that the streaking motion is comfortable for your hand) and start the second streak. Go into the previous streak about two times and then back and forth as shown for a total of about 10 times.



c. Rotate the plate again and repeat streaking.



d. Rotate the plate for the final time and make the final streak. Repeat steps a–c with the remaining agar plate. Use the same inoculation loop for all of your plates. When you are finished with each plate, cover it immediately to avoid contamination.



e. Place the plates upside down inside the incubator overnight at 37 °C. Use within 24–36 hours and do not refrigerate before use.

KSB 7: Screening

Recall that a protein named GFP (Green Fluorescent Protein) is being used to simulate a protein that contains GFP, which is fused to the disintegrin protein that you are looking for in the folk remedy. The method used to identify colonies that contain the disintegrin protein would be to look for the presence of GFP (GFP will be fused to it). Although this is only a simulation, fusion proteins are a good method for identifying colonies that are expressing a target gene that does not produce a protein product that is easy to measure. The bacterial colonies are ready for examination under an ultraviolet (UV) lamp. The GFP protein is easy to find because it "glows" under UV light. If a colony produced from the transformed bacterial culture contains the gene for the protein, it will emit a green fluorescence under the UV lamp.

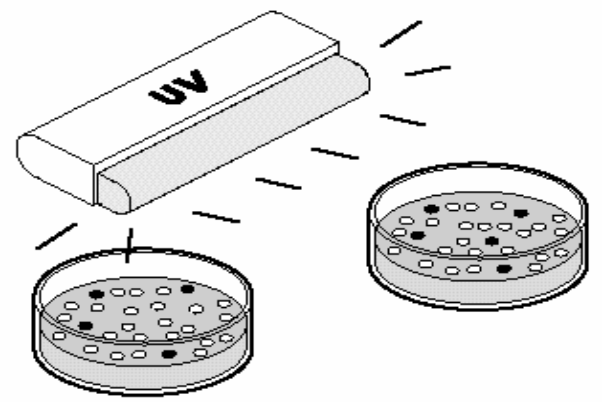
Workstation Checklist

Your workstation	Number
Safety glasses or goggles	3
Streaked bacterial plates	2
Inoculation loops	2
Culture tubes — containing 2 mL growth medium	2
Marking pen	1
Test tube holder	1

Shared classroom materials

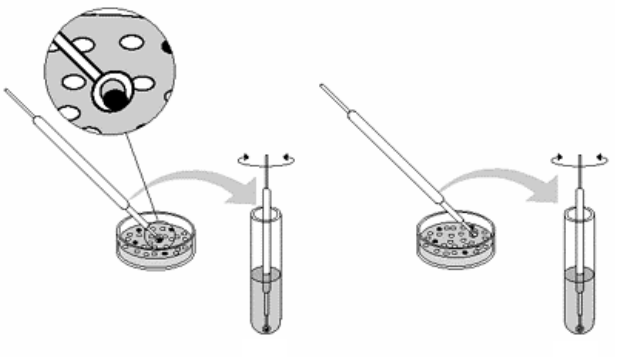
Shaking platform and incubator	1
UV lamp	1 or more

1. Remove your streaked plates from the incubator and examine them.
2. First use normal room lighting, then use UV light in a darkened area of your laboratory.
3. Note your observations.



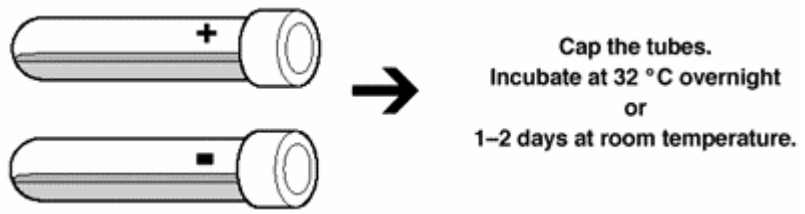
4. WARNING: To prevent damage to your skin or eyes, avoid exposure to UV light. Never look directly into the UV light source.
5. Identify several green colonies that are not touching other colonies on your plates. Examine all of the plates for colonies that will be easy to isolate.

6. Turn the plates over and circle several of these green colonies.
7. If you have not done so already, prepare two culture tubes with 5 mL of nutrient growth media. 100 mL of the growth media was prepared in KSB 5: Solutions and Media.
8. Using a sterile inoculation loop, lightly touch the loop end to a circled single green colony and scoop up the cells without grabbing big chunks of agar.
9. Immerse the loop into one of the culture tubes.
10. Spin the loop between your index finger and thumb to disperse the entire colony.
11. Repeat this process one more time for the first culture tube.



12. Using a new sterile loop, repeat for your other culture tube.
13. Cap your tubes and place them in the shaker or incubator.

Let the tubes incubate for 24 hours at 32 °C or for up to two days at room temperature. If a shaker is not available, lay the tubes down horizontally in the incubator.
If a shaker is available but an incubator is not, tape the tubes to the platform and let them shake for 24 hours at 32 °C or at room temperature for up to 48 hours.



Culture Condition	Days Required
32 °C — shaking	1 day
32 °C — no shaking	1–2 days*
Room temperature — shaking	2 days
Room temperature — no shaking	(not recommended)

*Periodically shake by hand and lay tubes horizontally in incubator.

NOTE: In order to ensure that you will have enough protein for your purification design, it is strongly recommended that culture conditions include both shaking and incubation.

KSB 8: Bacterial Concentration

At this point, you will have produced living cultures of bacterial clones. These clones contain a gene that produces the Green Fluorescent Protein (simulating a fusion protein of disintegrin from the folk remedy fused to GFP). You will now extract the GFP from the bacterial cells. In this KSB, you will collect a large number of these bacterial cells in a concentration step that is part of a typical purification protocol.

Develop your understanding

A good way to concentrate a large number of cells is to place a tube containing the liquid cell culture into a centrifuge and spin it. Where do you expect the cells to collect—in the liquid portion (supernatant) or at the bottom of the tube (pellet)? Why?

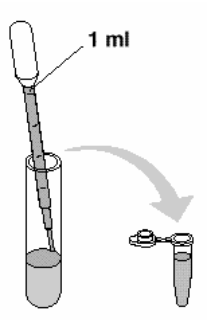
Workstation Checklist

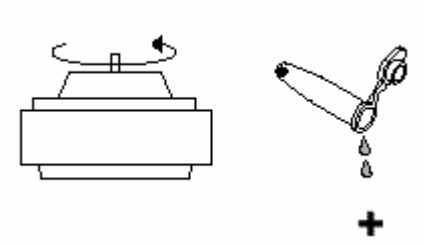
Student workstation	Number
Safety glasses or goggles	3
Microtubes	2
Pipettes	3
Microtube rack	1
Marker	1

Shared classroom materials

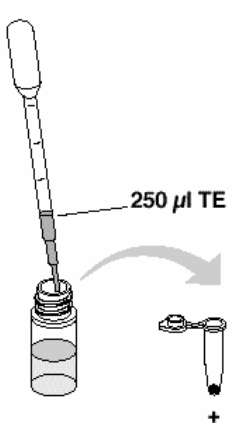
TE buffer	1 vial
Lysozyme (rehydrated in 1 mL TE buffer)	1 vial
Centrifuge	1
UV lamp	1–4

1. Using a marker, label two new microtubes with your name and period.
2. Remove your two liquid cultures from the shaker or incubator and observe them in normal room lighting and then with UV light. Note any color differences that you observe.
3. Using a clean pipette, transfer 2.0 mL of each of the liquid cultures into the 2 mL microtubes and then cap them.
4. Spin the microtubes for 5 minutes in the centrifuge at maximum speed. Be sure to balance the tubes in the machine. If you do not know how to balance the tubes, do not operate the centrifuge. Ask the teacher for instructions.
5. After the bacterial liquid culture has been centrifuged, open the tube and slowly pour off the liquid supernatant above the pellet.

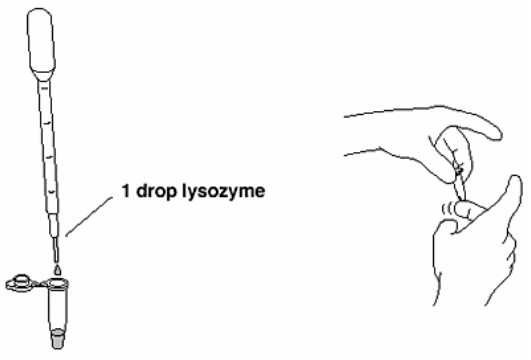




6. After the supernatant has been discarded, there should be a large bacterial pellet remaining in the tubes.
7. Add another 2 mL of each of the liquid cultures to the tubes, cap them and centrifuge them as before.
8. As before, carefully pour off the supernatant and observe the bacterial pellets in the bottom of the tubes.
9. Repeat this procedure until you have processed the entire liquid culture.
10. Observe the pellets under UV light. Note your observations.
11. Using a new pipette, add 250 µL of TE buffer to each tube.
12. Resuspend the bacterial pellets thoroughly by rapidly pipetting up and down several times with the pipette.



13. Using a new pipette, add 1 drop of lysozyme to the resuspended bacterial pellets (lysozyme resuspended in 1 mL of TE buffer).
14. Cap and mix the contents by flicking the tube with your index finger. The lysozyme will start digesting the bacterial cell walls.
15. Observe the tubes under the UV light.
16. Place the microtubes in the –20 °C freezer for at least one hour, or until the next laboratory period. The freezing will cause the bacteria to explode and rupture completely.


1 drop lysozyme

STUDENT HANDOUT #5
Design Challenge: Protein Purification

Materials:
(Bio-Rad Laboratories: www.bio-rad.com)

Size Exclusion Chromatography

Student Workstation	Number
250 µl GFP in TE (bacterial lysate) (or fraction from purification)	1
PolyPrep sizing columns (from Bio-Rad B12, hemoglobin SEC kit (166-008)	1
Column end caps	1
Column buffer (1x PBS)	10 mL
NOTE: Buffer may be kept in a stock solution for the class.	
Collection tubes	12
Test tube rack	1
Lab marker	1
Pipette	1
Beaker of water for rinsing pipettes	1

Ion Exchange Chromatography

Student Workstation	Number
250µl GFP in TE (bacterial lysate) (or fraction from purification)	1
MacroPrep HighQ (Bio-Rad) for IEX	1 mL
PolyPrep chromatography column	1
Column end cap	1
Column top cap	1
50 mM Tris, pH 8.3	10 mL
50 mM Tris, pH 8.3, 130 mM NaCl	10 mL
50 mM Tris, pH 8.3, 200 mM NaCl	10 mL
50 mM Tris, pH 8.3, 300 mM NaCl	10 mL
50 mM Tris, pH 8.3, 500 mM NaCl	10 mL
NOTE: Buffers may be kept in larger volumes as a stock solution for the class.	
Pipettes	5
Collection tubes	6
Test tube rack	1
Lab marker	1

Hydrophobic Interaction Chromatography

Student Workstation	Number
250 µl GFP in TE (bacterial lysate) (or fraction from purification)	1
Collection tubes	6
Pipettes	6
Microtube rack	1
Marker	1
HIC chromatography column w/ resin (from Bio-Rad Rainforest kit)	1
Column end cap	1
Test tube or beaker to collect waste	1

The following buffers in a stock solution will be used during the purification:
Equilibration buffer — A high-salt solution (2 M $[NH_4]_2SO_4$)
Binding buffer — A very high-salt solution (4 M $[NH_4]_2SO_4$)
Wash buffers — medium-salt solutions
- 1.3 M $[NH_4]_2SO_4$
- 0.8 M $[NH_4]_2SO_4$
- 0.4 M $[NH_4]_2SO_4$

Elution buffers – A low-salt solution (0.16 M $[NH_4]_2SO_4$)
TE buffer — A very low-salt solution (10 mM Tris/EDTA)

Introduction:

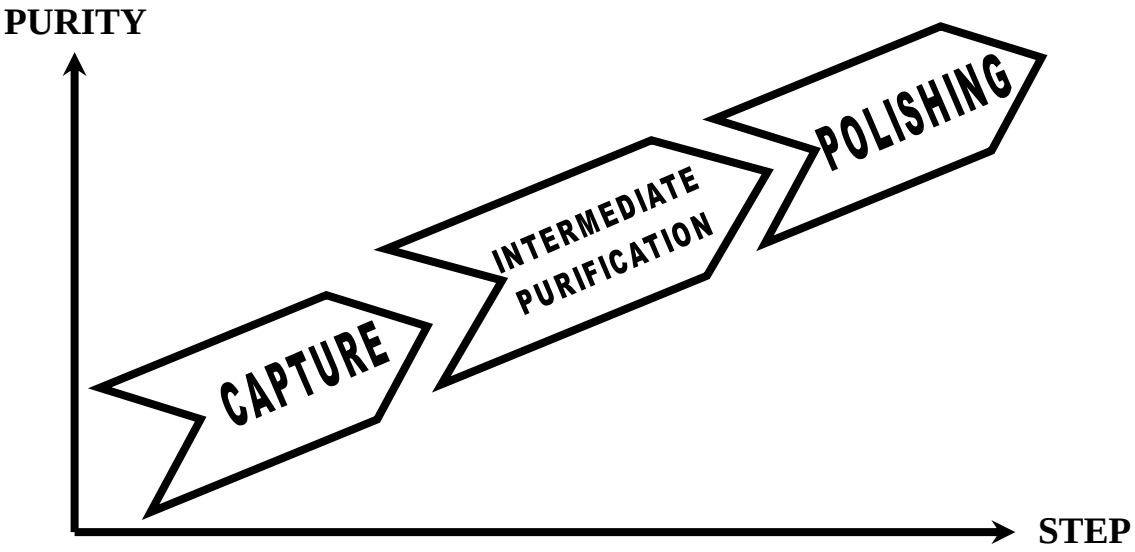
Before you begin to explore your specific protein purification protocols, it is important that you have an understanding of the process of design. Your instructor may review the process of design with the class before you begin. It is important that you understand that your solutions are not a trial-and-error approach to a problem and that the protocol that you design is informed by the parameters of the purification methods available and the chemical and physical properties of the protein that you will be purifying. There are many things that you should consider when attempting to design a purification protocol for your protein. Since the protein has already been extracted from the bacterial culture, you will only have to focus on how you will purify the GFP from this lysate. In addition, since you are limited to three chromatography methods, you will be able to focus your design on one of a limited number of combinations.

In industry, a major part of the design of the purification protocol involves economy. Economic considerations are not simply monetary and include reproducibility, resolution, efficiency, time commitments, personal and practical convenience, and the cost and availability of materials.

Structuring the Purification Protocol: Introduction

The purpose of a protein purification procedure is to isolate one protein from a complex mixture of proteins released into the cell lysate. The process of protein purification can be broken down into three different stages: capture, intermediate purification, and polishing. In the capture stage, the target protein is concentrated from the mixture to isolate it from harmful contaminants that we released when the cells were lysed to release the protein. In the intermediate purification stages the

bulk contaminants are removed. At the polishing stage, any trace impurities will be removed. At each step, the purity of the sample increases.



The tasks that are performed at the various stages of the purification require a variety of different methods and techniques. These techniques can be subdivided into two categories: differential solubility techniques and high-resolution techniques.

Differential solubility techniques are generally applied at the start of a protocol because of their high capacity. The capacity of a technique refers to the sample size (both volume and protein concentration) that can be handled in a single step. Although the differential solubility techniques have a high capacity, they have low resolving power. The resolving power of a technique refers to its ability to separate the target protein from other proteins in the mixture. Of the differential solubility techniques, probably the most widely used is the precipitation of protein with salt (salt fractionation). Ammonium sulfate is the most widely used salt because it is very soluble in water and has a stabilizing effect on the protein. Salt fractionation has low resolving power because a large fraction of the protein in the mixture will precipitate with this technique. Because of its high capacity and low resolving power, the differential solubility techniques are often used in the capture stage of protein purification.

The techniques that are available to you are all high-resolution chromatographic techniques. Only rarely will a single high-resolution technique provide an efficient one-step purification of the target protein. Usually a combination of several techniques, each of which separates the molecules on the basis of a different molecular property, is required to reach an acceptable level of purity. The three properties that will be targeted with the available chromatographic techniques include protein size, charge, and hydrophobicity. In theory, one can picture the proteins in a mixture distributed in a three-dimensional matrix, where each protein in the mixture occupies a specific location within the matrix on the basis of these three properties. Any single chromatographic technique will be unable to target the specific location of a single protein within the matrix. By performing successive steps in a well-structured protocol, it is possible to isolate a single protein from a heterogeneous mixture by combining the specificity and resolving powers of three techniques in a specific order.

Structuring the Purification Protocol: Choosing and Combining Techniques

There are no hard-and-fast rules for ordering the various techniques, but there are many things to consider when structuring the protocol. Before any of the techniques can be explored, it is important that you have as much information about your target protein as you can find. Special attention should be given to molecular size, charge (pI), and hydrophobicity since these are the three characteristics targeted by the techniques. Your instructor may review these properties with the class before you begin.

The following section describes some of the things to consider when you structure your purification schemes:

Capacity
One of the parameters to consider is capacity. Since the starting material contains high volume and high protein concentration, it is important that techniques with high capacities are considered. In addition, it would not be beneficial to begin with techniques that will dramatically increase the sample volume early in the protocol.

Resolving Power
In general, the resolving power becomes more important as the separation progresses. During the later stages of the protocol, the proteins that remain in the mixture will be ones that have co-purified with your target protein due to their chemical and physical similarities. For this reason, very high-resolution techniques are often applied near the end of the purification.

Minimize Inter-Step Conditioning
Since every manipulation of the sample reduces yield, it is important that the amount of conditioning between steps be reduced. For example, it may be important to concentrate the sample, change buffers, or remove salt (desalt) before proceeding to the next step. The number of inter-step manipulations should be kept to a minimum. One way to do this is to combine steps that are compatible with each other.

Compatibility of Steps
The chromatographic process often requires that the sample be modified during the purification process (pH, buffers, salt, dilution, etc.). It is important to select methods that are compatible with each other. If a fraction containing the target protein is collected from one step, it is helpful if the composition of the target fraction is such that it has become appropriate starting material for the next step. In this way, the amount of inter-step conditioning can be kept to a minimum. For example, if one step increases the salt concentration of the sample, it would be helpful if the next step requires a starting sample that is high in salt. An example of this would be a salt fractionation as a capture step followed by HIC as an intermediate purification step (Seidman & Moore, 2000; Protein Purification Handbook, 1999).

Preparing for the Design Challenge

Using library materials and the Internet, describe the physical properties of GFP with special attention given to molecular weight, charge (or pI), and hydrophobicity.

Using library materials and the Internet, collect as much information as you can find on the three different chromatography techniques being used for the purification.

For each of the three chromatographic techniques, describe the following:

- Capacity
- Resolving power
- Compositional changes to target protein fraction as compared to starting material
- Suitability as a capture, intermediate purification, or polishing step
- Compatibility with the other techniques (minimizing inter-step conditioning of the sample).

Describe a purification protocol that would be suitable for purifying GFP. Your design should be informed by what you know about GFP and the compatibility of the three techniques available to you.

Follow the protocols and notes provided to perform your purification.

Preparing the Bacterial Lysate for the Design Challenge

Following KSB 8, you will have a sample of bacteria expressing GFP. The bacteria have been broken open (lysed) because they have been exposed to lysozyme and then frozen. Before beginning any of the purification protocols, the following preparation should be carried out.

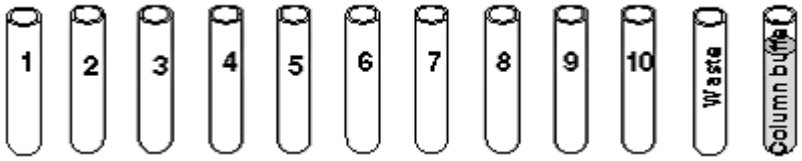
1. Remove the microtube from the freezer and thaw, using hand warmth.
2. Place the tube in the centrifuge and pellet the insoluble bacterial debris by spinning for 10 minutes at maximum speed.
3. After the 10-minute spin, immediately remove your tube from the centrifuge.
4. Examine the tube with the UV light.
5. Using a new pipette, transfer 250 µl of the supernatant into a new microtube.
6. This bacterial lysate solution will be the working sample for the protein purification protocol.
7. Save another 10 µl sample of this original bacterial lysate for analysis with SDS-PAGE.

Ion Exchange Chromatography
(MacroPrep HighQ anion exchange)

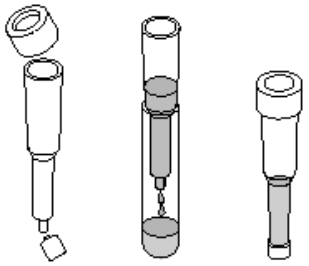
1. Snap off the end of an empty PolyPrep chromatography column. Cap the bottom of the column.
2. Place 6 collection tubes into a test tube rack.
3. Pipette 1 mL of HighQ resin into the empty column and allow the storage solution to drain. The HighQ support from Bio-Rad is a strong anion exchanger $-N^{+}(CH_3)_3$.
4. Equilibrate the column by adding 2 mL 50 mM Tris, pH 8.3.
5. If you have not already done so, a 10 ul sample of the cleared bacterial lysate needs to be saved as an analysis of the load fraction.
6. Load the cleared lysate (or the elution fraction from a previous purification step) and collect the flow-through as a fraction.
7. Carefully add 250 µl of 50 mM Tris, 130 mM NaCl, pH 8.3 buffer to the top of the column and collect the flow-through as the first wash fraction. This is best done by inserting the pipette tip into the column so that it rests just above the surface of the column matrix. Carefully let the buffer run down the side of the tube and onto the top of the bed. (NOTE: The column will work best when the column bed is left undisturbed.)
8. Examine the column under UV light and make note of the location of the GFP on the column
9. Move the column into the next collection tube and carefully add 250 µl of 50 mM Tris 200 mM NaCl, pH 8.3 to the column. Collect the wash fraction.
10. Examine the column under UV light. The GFP will have moved down into the column.
11. Move the column to the next collection tube.
12. In the next step, add 750 µl of 50 mM Tris 300 mM NaCl, pH 8.3. Observe the column under UV light when collecting the flow-through. The first couple of drops will be non-glowing. As soon as you observe the first glowing drop coming off the column, switch the column to a new collection tube. This process will help to concentrate the target protein into a single fraction.
13. Finish with a 250 µl wash of the column with 50 mM Tris 500 mM NaCl, pH 8.3 and collect the flow-through as your final fraction.
14. If you will be following this procedure with another purification protocol, transfer 10 µl of the "glowing fraction" to a clean (and labeled) microcentrifuge tube and refrigerate for later analysis using SDS-PAGE. The remaining "glowing fraction" can be used in the next purification step. The other fractions should be transferred to microcentrifuge tubes and refrigerated for later analysis.
15. If this is the final step in your purification, the fractions contained in all of the tubes can be transferred to microcentrifuge tubes and refrigerated for later analysis.

Size Exclusion Chromatography

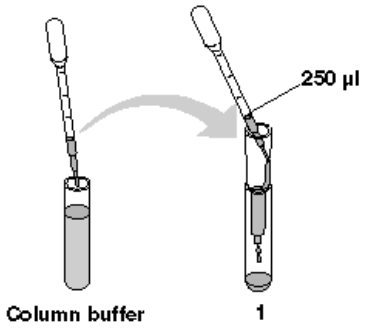
1. Obtain 12 collection tubes and label 10 sequentially from 1 to 10. Label the tubes with your company name. Label the final two tubes "waste" and "column buffer." Using a clean pipette, transfer 4 mL of column buffer into the tube labeled "column buffer" (PBS).



2. Remove the cap and snap off the end of the sizing column. Columns come prepoured with the matrix used for SEC. Allow the entire buffer to drain into the waste tube. Cap the bottom of the column when you see the column buffer drain down to the level of the SEC matrix.

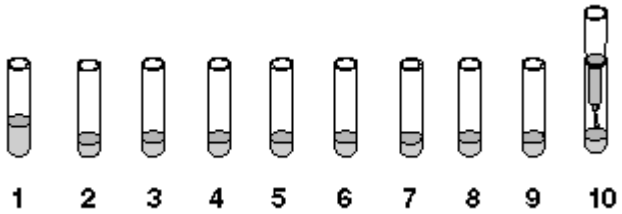


3. Equilibrate the column with 2 mL 1x PBS. When the last of the buffer has reached the surface of the SEC matrix, proceed to the next step below.
4. Load 250 µl of the bacterial lysate (or the elution fraction from a previous purification step). Save 10µl for gel analysis of load fraction.
5. As soon as the drop of protein mix enters the column bed, carefully add 250 µl of column buffer to the top of the column. This is best done by inserting the pipette tip into the column so that it rests just above the surface of the column matrix. Carefully let the buffer run down the side of the tube and onto the top of the bed. (NOTE: The size separation will work best when the column bed is left undisturbed.) Begin to collect drops into tube 1.



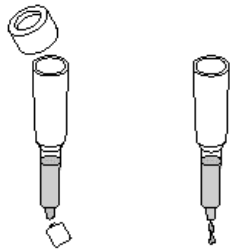
6. Add another 250 µl of column buffer to the top of the column. Add the buffer as before, by placing the pipette just above the top of the column and letting the buffer run down the side of the tube. Continue to collect drops into tube 1.
7. Carefully add 4 mL of PBS to the top of the column, after the lysate has entered, without disturbing the resin bed. At this time the protein mix has entered the column far enough so that slight disturbances to the column bed will not affect the separation. Transfer the column to tube 2 and begin to count the drops that enter into each tube. Collect 5 drops of buffer in tube 2.

8. Continue to collect fractions in your 10 tubes, maybe 5 drops each. Observe the GFP with the UV light to see when and where it comes off the column.
9. When you reach tube 10, collect a total of 10 drops. Cap the column and if necessary, Parafilm® or cover your fractions until the next laboratory period. Store the fractions in the refrigerator. Record your results in your laboratory notebook.
10. If you will be following this procedure with another purification protocol, transfer 10 µl of the "glowing fraction" to a clean (and labeled) microcentrifuge tube and refrigerate for later analysis using SDS-PAGE. The remaining "glowing fraction" can be used in the next purification step. The other fractions should be transferred to microcentrifuge tubes and refrigerated for later analysis.
11. If this is the final step in your purification, the fractions contained in all of the tubes can be transferred to microcentrifuge tubes and refrigerated for later analysis.

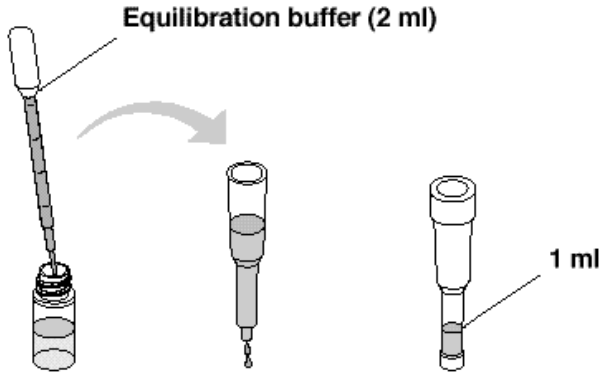


Hydrophobic Interaction Chromatography

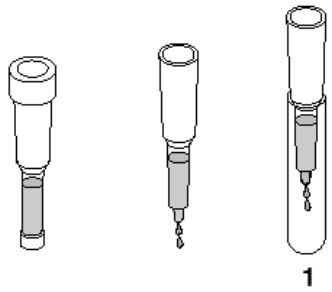
1. Prepare the chromatography column. Remove the cap and snap off the bottom from the prefilled HIC column. Allow all of the liquid buffer to drain from the column (~3–5 minutes). You can discard the solution that drains from the column.



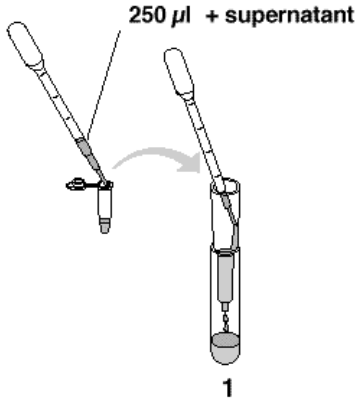
2. Prepare the column by adding 2 mL of equilibration buffer to the top of the column. This is done by adding two 1 mL aliquots with a rinsed pipette. Drain the buffer to the 1 mL mark on the column. The equilibration buffer is a 2 M ammonium sulfate stock solution. If you or your instructor prepared a 1.5 M dilution of this stock in KSB 5: Solutions and Media, then this would be the appropriate buffer to use in this step. You do not need to collect the solution that drains from the column.
3. If you run out of time, you can cap the top and bottom of the column. The column can be stored at room temperature until the next laboratory period. Otherwise, let the buffer drain from the column. You may now proceed to the next step.



4. Using a well-rinsed pipette, transfer 250 µl of binding buffer to the microtube containing the GFP in TE (this is either the cleared lysate, or the elution fraction from a previous purification step). The “binding buffer” provided is a solution of 4 M ammonium sulfate. Very often, a binding buffer will be identical to the equilibration buffer. The transfer of an equal volume of the binding buffer provided to the lysate produces a dilution of 2 M ammonium sulfate. If you used a 1.5 M ammonium sulfate solution to equilibrate the column, then it would be appropriate to adjust this step so that the binding buffer is diluted to a similar concentration (although it is not necessary).
5. Label collection tubes 1–6 and place these tubes in the foam rack or in a rack supplied in your laboratory. When the last of the equilibration buffer has reached the surface of the HIC matrix, place the column in the first collection tube and proceed to the next step below.

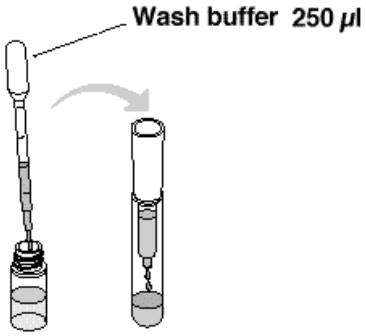


6. Using a new pipette, carefully and gently load your GFP in binding buffer (from step 4) onto the top of the column. Hold the pipette tip against the side of the column wall, just above the upper surface of the matrix, and let the supernatant drip down the side of the column wall. Examine the column, using UV light. Note your observations. After it stops dripping, transfer the column to collection tube 2.



7. Using the rinsed pipette, add 250 µl of each of the following wash buffers. The preparation of these solutions is described in KSB 5: Solutions and Media. For each buffer, move the column into a fresh collection tube. After each addition, examine the column, using UV light. Note your observations.

Tube 2: Wash in 1.3M ammonium sulfate. ("wash buffer" is provided)
Tube 3: Wash in 0.8M ammonium sulfate. – prepared by students OR instructor
Tube 4: Wash in 0.4M ammonium sulfate. – prepared by students OR instructor



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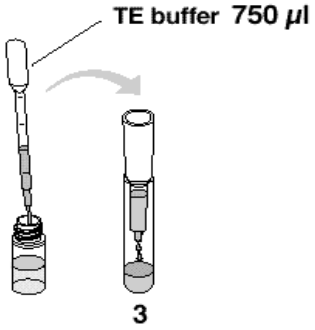
8. After placing the column into tube 5, elute the GFP by adding 250 µl of 0.16 M ammonium sulfate (prepared by students OR instructor in KSB 5: Solutions and Media) and let the entire volume flow into the column. Examine the column, using UV light. Note your observations. Collect the fraction.
9. Finish with a wash of 500 µl of TE buffer. If you noticed GFP still on the column after the previous step (elution), then collect the first few glowing drops in the same tube (tube 5) and then transfer to tube 6 when you notice the first "non-glowing" drop appear.



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10. If you will be following this procedure with another purification protocol, then transfer 10 µl of the "glowing fraction" (tube 5) to a clean (and labeled) microcentrifuge tube and refrigerate for later analysis using SDS-PAGE. The remaining fraction in tube 5 can be used in the next purification step. The other fractions should be saved for later analysis.
11. If this is the final step in your purification, the fractions contained in all of the tubes can be transferred to microcentrifuge tubes and refrigerated for later analysis.

STUDENT HANDOUT #6
KSB 9: SDS–PAGE to Test Purity

In this KSB, you will analyze the relative purity of your sample, and thus the efficacy of your purification protocol, with SDS-PAGE. The materials and instructions provided are specific to the Bio-Rad Mini-PROTEAN 3 system, but they can be easily modified for any SDS-PAGE system.

Polyacrylamide gel electrophoresis separates molecules according to the size and charge of the molecules contained in the mixture. In the SDS-PAGE systems, a solution of sodium dodecyl sulfate (SDS) is added to the samples, which are then heated to denature the proteins. In some cases, a reducing agent such as 2-mercaptoethanol (βME) is added to the sample. The denatured proteins will then separate on the basis of their molecular weights.

In the native PAGE system, the samples are not exposed to denaturing conditions and therefore the proteins retain their biological activity. In contrast to SDS-PAGE, the separation of proteins in a native PAGE system depends on both the size and charge of the molecules in the mix.

The incorporation of a protein standard (protein ladder) helps to identify the molecular weights of the proteins being separated in the SDS-PAGE system. When performing SDS-PAGE, you should understand that the denatured GFP will not fluoresce under UV light because it will lose its biological activity (its shape). This should be discussed with your group so that you can make the connection between native and denaturing conditions.

The use of SDS-PAGE provides a qualitative method for exploring the efficacy of a specific purification protocol. By comparing samples from the target fractions to samples from the load fractions and bacterial lysate, you will have a measure of the purity of your sample. You should be able to identify the GFP band on the basis of its molecular weight by comparing the band to the protein standard. The results from this analysis will be presented as part of the class presentation that your company must give, so it is important to fully document your results in your laboratory notebook.

Materials:

Mini-PROTEAN 3 Cell electrophoresis unit from Bio-Rad Laboratories
PowerPac 300 power supply from Bio-Rad Laboratories
Ready Gel precast gels from Bio-Rad Laboratories (15% Tris HCl, 10 well)
10x Tris/Glycine/SDS running buffer (dilute to 1x concentration)
Sample buffer (SDS reducing buffer)
Broad range protein standard Pipettes with tips
Coomassie blue gel staining solution
Visible light box
Polaroid camera and film for photo documentation

Sample Preparation

1. Label microcentrifuge tubes for the following:
    a. protein standard (ladder)
    b. load fractions that were collected (the 10 µl fractions saved before loading sample into a column)
    c. bacterial lysate (the original 10 µl sample of lysate collected before running the purification protocol)
    d. label tubes for all of the different fractions collected from the chromatography methods that you produced

2. Load 10 µl of each of the above into the labeled microcentrifuge tubes.
3. Add 10 µl reducing buffer (or straight Laemmli buffer) to each of the samples and mix.

    a. NOTE: To prepare a reducing buffer, add 50 µl β-ME to 950 µl of the Laemmli sample buffer prior to use.

4. Pulse sample in a microcentrifuge to return contents to the bottom of the tubes.
5. Heat samples at 95 ^{o}C for 4 minutes.
6. Remove samples from heat and return to room temperature.
7. Pulse samples again to return contents to bottom of tube.

Electrophoresis using Mini-PROTEAN 3 system from Bio-Rad

1. Follow instructions for preparing the Ready Gel precast gel and assembling the Mini-PROTEAN 3 system.
2. Add approximately 125 mL of running buffer to the inner chamber (do not overfill).
3. Add approximately 200 mL of running buffer to the Mini Tank (lower buffer chamber).
4. Load all 20 µl of each of your samples into a lane of the gel (be sure to record the contents of each lane in your lab notebook).
5. Fill any unused lanes with 20 µl of running buffer.
6. Connect gel box to power supply.
7. Run-time on the Mini-PROTEAN 3 is approximately 35 minutes at 200 volts.
8. Run until the bromophenol blue dye front comes to within about 2 cm of the bottom of the gel.
9. Follow the instructions for removing the precast gel from the gel box.
10. If time permits, washing the gel in a bath of dH_2O 3 times for 5 minutes each will help remove residual SDS in the gel and help improve staining.
11. Slide gel into enough Coomassie blue staining solution so that the staining solution covers it well.
12. Shake for 40 minutes to an hour in the stain (maximum intensity will be reached after about an hour).
13. Destain by rinsing the gel extensively in water until the background is clear and the bands are visible (20–30 minutes).
14. Photodocument using a visible light box and camera (or digital documentation system). Document the result in your notebook. If image capture is not possible, produce accurate sketches and descriptions in the laboratory notebook.

STUDENT HANDOUT #7: KSBs 10–12
KSB 10: Clinical Test

A biotechnology company faces a tremendous cost when bringing a drug "from bench to shelf" (from the lab to the market). Conduct some research on the costs associated with this effort. Collect enough information so that you can have a discussion with your company on the answers to the following questions:

- How long does it take a drug to go from bench to shelf?
- How much does the average clinical trial cost?
- What is the total cost to a company to bring a drug from bench to shelf?
- Why is it difficult for companies to develop drugs for diseases that are very rare?

Before the drug can be tested in human subjects, the FDA (Food and Drug Administration) requires that the drug be tested in animals. Although there are many ethical issues related to animal testing, it is important to explore any toxic effects that the drug might have before administering it to human subjects. Although there will be a discussion on the ethics of human testing in the next KSB, your instructor may decide to introduce the issues related to the use of animals in scientific research. For the purposes of this KSB, we will assume that the drug was found to be safe in animals and is ready for human testing in what is called a clinical trial.

In order to establish the effectiveness of the drug in reducing a person's risk for heart disease or breast cancer, you will be working with human subjects who are at a high risk for these diseases. One of the important decisions that must be made is what measurements will be made to determine if your drug is working. In a clinical trial, these are referred to as primary end points. In the following section, you will be presented with a description and the results of an experiment that tests the drug on high-risk breast cancer patients. You will analyze the data to determine the effectiveness of the drug in reducing a person's risk for developing breast tumors.

Breast Cancer Trial

The most important risk factor for breast cancer is a family history of the disease. In the following activity, you will be examining the effect of various concentrations of the drug on human subjects who are known to be at high risk for developing the disease due to a known family history of breast cancer. Before you begin analyzing your data, you should discuss breast cancer in the classroom. In addition, you should have a basic understanding of the clinical trial process and the concept of the double-blind randomized placebo-controlled study in drug testing. The following topics should be discussed:

What are the risk factors for breast cancer?
How is breast cancer normally treated?
WHAT DOES IT MEAN WHEN A CANCER METASTASIZES?
WHAT TISSUES AND ORGANS BECOME INVOLVED IN METASTATIC BREAST CANCER?
What is a clinical trial?
What process is involved in a double-blind randomized placebo-controlled study?
What are the reasons why the experiments are double-blind, and why do they involve placebos?

This Phase III trial was conducted as a double-blind randomized placebo-controlled study. For each level of dosing (10 levels total), 300 high-risk patients were enrolled. Experimental subjects (150 patients) were given a daily dose of 200 to 2000 mg of the drug intravenously (through an IV) for 10 years while control subjects (150 patients) were administered an IV placebo identical in appearance.

The primary end points (measurements) for this experiment are the appearance of a breast tumor and the length of time until the first tumor appears. It has been well established that bone is the most common site of metastases in breast cancer, and therefore, the appearance of new bony metastases and the length of time until the first metastases appear were also measured in subjects who developed breast cancer during the study period. All patients who developed breast tumors in both the control and experimental groups received standard treatment during the study period (surgical, hormonal, and chemotherapy).

The data are summarized below. The total number of patients involved at each level of dosing was 300 with 150 assigned to the experimental group (150 received the drug) and 150 to the control group (150 received the placebo).

- The first column presents the dose of the drug administered (in milligrams).
- The second and third columns present the number of patients who developed breast tumors within the 10-year study period.
- The fourth and fifth columns present the average length of time it took for a tumor to appear in patients who developed them.
- Columns 6 and 7 present the data on metastatic tumors. Metastases were measured only in patients who developed breast tumors. For example, at the 200 milligram dose level, 21 of the 150 patients in the experimental group developed breast tumors. Of those 21 patients, 10 developed a metastatic tumor.
- Columns 8 and 9 present the average length of time it took for a metastatic tumor to appear in patients who developed them. Using the same example presented above, at the 200 milligram level of dosing, 10 patients in the experimental group and 11 patients in the control group developed metastatic tumors. At this level of dosing, it took an average of 0.6 years for those metastatic tumors to show up in both groups after the initial breast tumor was discovered.

DOSE (mg)	TUMORS (no. of patients)		TUMOR APPEARANCE (years)		METASTASES (no. of patients)		METASTATIC TUMOR APPEARANCE (years)	
	E	C	E	C	E	C	E	C
200	21	22	1.1	1.3	10	11	0.6	0.6
400	18	25	1.5	1.9	9	13	0.5	0.7
600	26	13	1.3	1.5	14	7	0.7	0.7
800	13	17	1.9	1.2	6	9	1.6	0.5
1000	17	24	2.8	1.7	5	12	2.9	0.7
1200	24	28	3.3	1.6	5	13	3.7	0.9
1400	28	18	4.2	1.4	2	9	3.6	0.5
1600	11	13	4.3	1.6	0	7	*	0.6
1800	20	19	4.2	1.5	1	10	3.7	0.7
2000	18	19	4.3	1.5	0	9	*	0.6

Note: E = Experimental; C = Control

* None of the patients in the experimental group at the 1600 mg and 2000 mg levels developed metastatic tumors; therefore, the time until appearance of a metastatic tumor is not applicable.

Data analysis

- Begin discussing within your company how you will treat the data. For example, you should think about the types of calculations you will need to make on the raw data.
- Consider the types of graphs you will want to prepare in presenting the data.
- It is a good idea to come back together as a class and discuss the data treatments before you begin your analysis.
- There are several questions you should consider when planning the analysis of your data:

  Does the drug reduce a person's risk of developing breast cancer? By how much and at what dose?

  Does the drug delay the onset of breast tumors in a high-risk patient? By how much and at what dose?

  Does the drug help to prevent metastatic tumors in patients who develop breast cancer? How well does it prevent this and at what dose is it effective?

  Does the drug help to delay the onset of metastatic tumors in those who develop them? By how much and at what dose?

- Once you are comfortable with the data and the type of analysis you will want to perform, prepare the calculations and graphs that will be beneficial in helping you address the study questions.
- Since you will not be performing any statistical analysis on this data, it is important to understand that the conclusions you will draw will be somewhat subjective.
- In the analysis, one group may find that there are significant differences between the experimental and control groups at one dose while another group may decide that the difference is not large enough to be considered significant. For this reason, it is a good idea to discuss the concept of experimental error and statistical significance. One example that is always good to use is the coin flip. If you flip two coins 100 times each and observe tails 57 times on one coin and 52 times on the other, does this make the coins different from each other or can we count on random error to explain the difference?
- Try to draw some conclusions based on your analysis. These conclusions will become an integral part of KSB 12: Classroom Presentations.

OPTIONAL CLASSROOM EXERCISE

- Building upon the KSB that deals with the breast cancer drug trial (KSB 10: Clinical Test), explore a different disease through designing a similar experiment for testing the effects of the drug on reducing a person's risk of heart disease.
- Search the literature on heart disease risk and develop similar background knowledge to that which was covered for breast cancer.
- Design a clinical trial for testing the effectiveness of the drug on high-risk heart disease patients. Make sure that you consider the primary end points (what you will measure).

KSB 11: Bioethics and Human Testing

In this KSB, you will explore some very difficult ethical dilemmas that face scientists as they design and conduct clinical trials to test the effectiveness of new therapies. What follows is a series of ethical case studies that are each followed by a challenge to find a specific solution. Your group will be assigned a case to work with and then you will be asked to report on your solution to the class. Your instructor will determine if this KSB will be an in-class or a homework assignment.

CASE #1

During the Phase III clinical trial to test the effectiveness of the disintegrin drug, doctors are concerned that there may be potentially dangerous side effects associated with the drug. Physical exams of several of the patients have revealed some mysterious bruising that does not seem to resolve (heal). Other patients report unexpected nose bleeding. Since the doctors do not know who is receiving the drug and who is receiving the placebo, it is impossible for them to know if some aspect of the study might be causing these problems. Several doctors have banded together to argue for putting a stop to the clinical trial to look into the problem. Because you belong to a start-up company with limited funds, you very likely will not be able to afford this delay.

Questions to consider:

1. Discuss the possibility that your drug might be directly responsible for the observed side effects. Can you propose a mechanism for this effect?
2. If the side effects are real, are they acceptable risks if the drug is found to be highly effective against breast cancer?
3. Split into two groups and take on one of the following roles:
    a. Group 1 consists of doctors trying to stop the trial. What are your concerns?
    b. Group 2 consists of the investors (partners) in your start-up company. How do you convince the doctors that the trial should continue?
4. Report out to the full class. Come up with a way either to convince the group of doctors that they should continue the trial as it was designed or to convince your partners that they should cease conducting the trials.

CASE #2

In this dilemma, you are trying to recruit a group of doctors into your clinical trial. The doctors are some of the most respected physicians in the field of breast cancer and are very important to your study. They claim that their obligation is to the individual patient that they are treating and that they are committed to acting in every patient's best interest. These doctors have an ethical problem with the placebo-controlled trial because they will unknowingly be giving a potentially beneficial drug to only half of their breast cancer patients. In other words, you are asking them to act in the best interests of only 50% of their patients.

Questions to consider:

1. Discuss within your group the ethical problem the doctors are having. Try to put yourselves in their place as you discuss this issue.
2. Come up with a way to convince these doctors that they should work with your company on this clinical trial. In your solution, make sure you address the specific ethical concerns that the doctors are having.

CASE #3

Consider a case in which a clinical trial is being conducted for a disease for which there is no satisfactory therapy—for example, advanced cancer or acquired immunodeficiency syndrome (AIDS). A new drug that promises more effectiveness is the subject of the study. The control group must be given either an unsatisfactory treatment or a placebo. Your company is trying to recruit patients into the study to test the new drug. Many of the patients that become interested in the study are desperate to try any new potential therapy to treat their disease. In your study, patients will have to halt any of the traditional therapies that they are currently using if they become a part of the control group.

The FDA regulates that in order for a person to become a patient in the study, he or she must sign a consent form that explicitly states the nature of the study and the details of the design (including the potential for being randomly placed in a control group). Your company needs to come up with a consent form that addresses this FDA regulation but does not discourage patients from taking part.

Questions to consider:

1. Design and construct a professional-looking consent form that you feel would encourage patients to take part in your study but would still provide the appropriate details regarding the nature of the study design.
2. Share the consent form with the class during the class discussions. This can be done either by having copies made for the entire class or by projecting the form in the front of the classroom. After presenting the forms, have every student take on the role of a patient and see how many would sign the form to take part.
3. During the class discussions, encourage other students to discuss their considerations when making the decision.

CASE #4

You are the director of an advocacy group for patients who are suffering from a lethal disease for which there is no cure. A local biotechnology company is working with area clinics and hospitals to design a clinical trial for a new treatment for this disease that the company has developed. The physicians are finding that it has become very difficult to recruit patients into the study. Although the drug appears promising, it has some serious side effects that have scared some patients away when they read the consent form. In order to move the development of the drug along, they decide to offer compensation of $1,000.00 to patients who are willing to take part in the study. Your advocacy group strongly discourages the practice of offering compensation to patients for enrolling in clinical trials.

Questions to consider:

1. What are the concerns your advocacy group has regarding monetary compensation for clinical trial participation?
2. Would your group have a different view of this case if the $1,000.00 were offered from the start, and not after the trial was having difficulty finding patients?
3. How could a clinical trial be run so that monetary compensation could be used while taking care of the concerns you identified in question 1?
4. What are other ways that trials could recruit patients into a study with a consent form that scares away potential trial participants?

CASE #5

You are a physician/scientist who is in charge of a clinical trial for the disintegrin drug for treating breast cancer. During the study, a participating physician who is well respected in the field of breast cancer is able to "crack the code" for the drug and the placebo and can now tell which is being administered to the patient she is treating. She does not tell anyone of her new knowledge. During the study, this doctor quickly realizes the power of this new drug for preventing metastatic breast cancer (the type that spreads to other tissues and is often fatal). Out of compassion for her patients, she decides to occasionally administer the drug to patients in the control group when they come into her clinic with very serious breast tumors. Near the end of the study, you learn of this "rogue" doctor and obtain records of all of the patients she has treated. When the study is over, you are left with two apparent facts. One, your drug is a very powerful preventative therapy for metastatic breast cancer, and two, your rogue doctor has sabotaged your study to the point where it will not meet FDA regulations for a clinical trial.

Questions to consider:

1. Describe the ethical issues associated with this case. Are the doctor's actions unethical?
2. Large numbers of doctors at multiple centers are involved in this trial. Is an occasional rogue doctor acceptable in a large trial? Why or why not?
3. Do you feel that it is unethical that the doctor was not giving the drug to all of her patients?
4. Should the FDA be informed of this problem? How will you approach the FDA? In your discussions, keep in mind that the FDA will consider the study flawed and your company likely will not be able to afford another study. This will mean that you will not be able to bring the drug to market.
5. Consider the fate of the doctor in this case. How should she be dealt with?

KSB 12: Classroom Presentations

In this KSB, your group will be presenting the work that you have conducted over the course of this module. Since many of the activities that the various groups have taken part in are similar from group to group, the presentations should focus on two areas:

- **The details of the solution to the Design Challenge including the information that you collected from testing your design using SDS-PAGE analysis.**
- **The conclusions drawn from the data on the clinical trials to determine the effectiveness of the new drug.**

Your instructor will determine the amount of time allocated and the basic format for the presentations. Regardless of the format of the presentation, your group should make sure that you cover the following topics:

Details Regarding the Design Challenge

- What factors did you consider in your design?
- Which of these factors did you think was the most important in determining your design?
- What were some of the alternative designs that you considered?
- How effective was your design? (Present data from KSB 9: SDS-PAGE to Test Purity.)
- If your design was not optimal, what factors do you think contributed to this?
- If given a chance (and more capital), would you implement one of your alternatives? Why or why not?

Clinical Trial Data

- Does the drug reduce a person's risk of developing breast cancer? By how much and at what dose?
- Does the drug delay the onset of breast tumors in a high-risk patient? By how much and at what dose?
- Does the drug help to prevent metastatic tumors in patients who develop breast cancer? How well does the drug prevent breast cancer and at what dose is it effective?
- Does the drug help to delay the onset of metastatic tumors in those who develop them? By how much and at what dose?
- How much time and what financial resources are required to bring a drug to the marketplace?