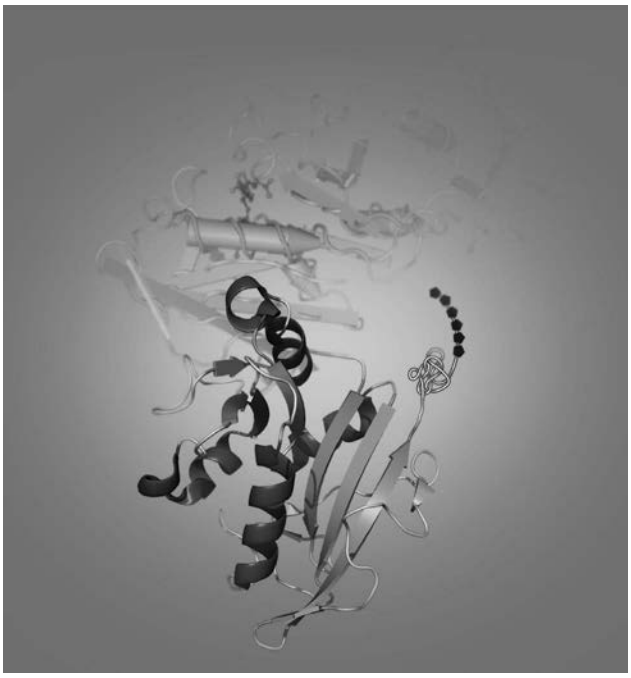

Bio-Rad Explorer™

Protein Expression and Purification Series

Catalog Numbers
1665040EDU Centrifugation Purification Process
1665045EDU Handpacked Purification Process
1665050EDU Prepacked Purification Process
1665070EDU Assessment Module

explorer.bio-rad.com

Please see each individual module for storage conditions.

Please visit explorer.bio-rad.com to access our selection of language translations for Biotechnology Explorer Kit curricula.

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Dear Educator

One of the great promises of the biotechnology industry is the ability to produce biopharmaceuticals to treat human disease. Genentech pioneered the development of recombinant DNA technology to produce products with a practical application. In the mid-1970s, insulin, used to treat diabetics, was extracted from the pancreas glands of swine and cattle that were slaughtered for food. It would take approximately 8,000 pounds of animal pancreas glands to produce one pound of insulin. Rather than extract the protein from animal sources, Genentech engineered bacterial cells to produce human insulin, resulting in the world's first commercial genetically engineered product.

Producing novel proteins in bacteria or other cell types is not simple. Active proteins are often comprised of multiple chains of amino acids with complex folding and strand interactions. Commandeering a particular cell to reproduce the native form presents many challenges. Considerations of cell type, plasmid construction, and purification strategy are all part of the process of developing a recombinant protein.

In the Protein Expression and Purification Series students will explore the process of developing a recombinant protein by inducing *E. coli* to express the protein of interest, dihydrofolate reductase, which is a target for certain cancer treatments. Students will learn how to recover the protein from other cellular components and then purify it away from other proteins in the cell. The dominant method of purifying proteins is chromatography, and this series utilizes one of the most powerful types used today: affinity chromatography. In addition, two options for purification are provided—one on a small scale, using centrifugation, and the other a larger scale, with purification instrumentation. The latter provides a workflow and equipment experience that parallels biomanufacturing processes used today.

Many biology educators have used the pGLO™ system in which a cloned green fluorescent protein (GFP) serves as a model system for transformation and chromatographic separation. The visibility of the protein is a great teaching model and introduction to chromatography. However, in the laboratory and in industrial settings, very few proteins are visible. The Protein Expression and Purification Series is a real-world model system that provides hands-on experience for students to learn the key process in biotechnology—taking recombinant DNA through production to purified protein.

The Bio-Rad Explorer program has a long history of partnering with educators to create laboratory experiences that prepare students for today's careers and providing the understanding that is required for citizens in the rapidly advancing technologies that impact our daily lives. The Protein Expression and Purification Series was developed in response to educators' desire to provide students with authentic laboratory experiences relevant to research and industrial applications. We wish to acknowledge the contribution of several collaborators in the development of the curriculum and assessment utilized in the series: Jim DeKloe from Solano Community College, Kirk Land from University of the Pacific, Joann Lau from Bellarmine University, and Cindy Gay from Steamboat Springs High School. We thank each of these educators for their invaluable guidance and contributions to this series.

We continually strive to improve our curricula and products. We welcome your suggestions and ideas!

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DHFR
Enzymatic
Assay
Module
SDS-PAGE
Electrophoresis
Module
Growth and
Expression
Module
Purification
Module
Option 1
Centrifugation
Purification
Module
Option 3
Prepacked
Cartridge
Purification
Module
Option 2
Handpacked
Column
Purification
Module

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Kit Summary

The Protein Expression and Purification Series is a modular laboratory course designed to serve four to 12 student teams, depending on which purification option is used. The aim of this course is to express and purify His- and GST-tagged dihydrofolate reductase (GST-DHFR-His) and then analyze the isolated purified protein fractions for enzymatic activity.

This project involves growing cultures of GST-DHFR-His, lysing the bacterial cell cultures, separating soluble from insoluble fractions, purifying the GST-DHFR-His via centrifugation or protein purification instrumentation, analyzing protein purity via SDS-PAGE and analyzing protein enzymatic activity. DHFR is an essential protein used in the conversion of dihydrofolate (DHF) into tetrahydrofolate (THF) by the addition of a hydride from NADPH. THF is a methyl (CH_3) group shuttle required for synthesis of essential molecules such as nucleotides and amino acids. DHFR deficiencies are extreme and result in symptoms such as megaloblastic anemia and cerebral folate metabolism disorders. DHFR inhibition or reduction disrupts nucleic acid synthesis and cell growth, resulting in cell death.

This project offers an opportunity to perform true affinity purification with a realistic research workflow in which students can examine the enzymatic activity of the purified protein product. It also introduces students to the world of biomanufacturing where the research process is scaled up and standard operating procedures and work instructions are followed strictly during the production process.

Independent research extensions such as further purification steps, ELISA, Western Blotting, site-directed mutagenesis and many other options are possible once students have purified the protein.

The steps in this project to express and purify GST-DHFR-His (and the corresponding chapter in this manual) are:

1. Understanding recombinant protein expression, DHFR, and protein purification (General Background chapter).
2. Setting up the experiments (Instructor's Advanced Preparation chapters).
3. Growing cultures (Culturing, expression, lysis and SDS-PAGE analysis chapters).
4. Inducing expression of the GST-DHFR-His protein (Culturing, expression, lysis and SDS-PAGE analysis chapters).
5. Lysing the bacterial cells to release the expressed protein (Culturing, expression, lysis and SDS-PAGE analysis chapters).
6. Using SDS-PAGE analysis to verify expression of the protein, identify fractions containing purified protein, and to assess level of purity (Culturing, expression, lysis and SDS-PAGE analysis chapters).
7. Purifing the GST-DHFR-His protein via affinity chromatography followed by desalting of the samples (Purification Protocol chapters).
8. Analyzing the DHFR enzymatic activity (DHFR Enzyme Activity chapter).

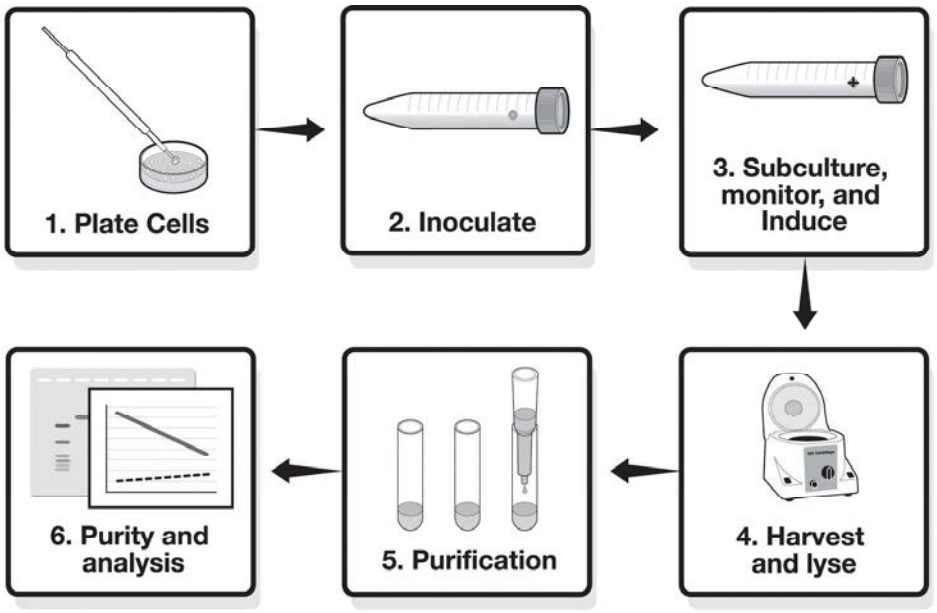
About This Manual

The Protein Expression and Purification Series is accompanied by extensive curricula to enable students to gain a deep understanding about DHFR and the theory behind the techniques used to perform the laboratory series. The manual is provided with the series allowing for the copying and distributing of the entire manual or chosen material to students.

Storage Instructions

See Checklists for Individual Modules on subsequent pages for shipping and storage conditions for each module. Open each module as soon as it arrives and store components in recommended storage conditions.

Protein Expression and Purification Series Overview



Protein Expression and Purification Series Inventory Checklist

This manual provides complete curriculum for all three purification options. The handpacked and prepacked purification processes require chromatography instrumentation.

Centrifugation Purification Process (catalog #1665040EDU)

Kit Components	Quantity	(✔)
Growth and Expression Module	1	❒
SDS-PAGE Electrophoresis Module	1	❒
Centrifugation Purification Module	1	❒
DHFR Enzymatic Assay Module	1	❒

Handpacked Purification Process (catalog #1665045EDU)

Kit Components	Quantity	(✔)
Growth and Expression Module	1	❒
SDS-PAGE Electrophoresis Module	1	❒
Handpacked Column Purification Module	1	❒
DHFR Enzymatic Assay Module	1	❒

Prepacked Purification Process (catalog #1665050EDU)

Kit Components	Quantity	(✔)
Growth and Expression Module	1	❒
SDS-PAGE Electrophoresis Module	1	❒
Prepacked Cartridge Purification Module	1	❒
DHFR Enzymatic Assay Module	1	❒

Checklists for Individual Modules

This section lists the components in each of the Protein Expression and Purification Series individual modules comprising the series. Each module contains sufficient materials to outfit 12 student workstations of one to four students per workstation for centrifugation purification or four student workstations for handpacked column purification or prepacked cartridge purification using chromatography instrumentation. Use the checklist to inventory your supplies before beginning your advanced preparation.

Growth and Expression Module (catalog #1665055EDU)

Kit Components	Quantity	(✔)
EZ Micro™ test tubes (microcentrifuge), 2 ml, natural	500	❒
Petri dishes	20	❒
Inoculation loops, sterile	20	❒
Sterile conical tubes, 50 ml	25	❒
Screwcap microcentrifuge tubes, 1.5 ml	50	❒
Ampicillin, lyophilized	2	❒
LB nutrient agar powder	1	❒
LB broth capsules	12	❒
Lysozyme, lyophilized	1	❒
E. coli strain BL21(DE3) containing pDHFR, lyophilized	1	❒
IPTG, 1 M, 0.1 ml	1	❒
10x PBS, 100 ml	1	❒
Imidazole stock solution, 200 ml	1	❒
20% sterile glucose, 4 ml	1	❒
Sterile water, 2.5 ml	1	❒
Instruction manual	1	❒

Ships at room temperature. Immediately store temperature-sensitive reagent bag at –20°C.

SDS-PAGE Electrophoresis Module (catalog #1665060EDU)

Kit Components	Quantity	(✔)
10x Tris/Glycine/SDS Buffer, 1 L	1	❒
Laemmli sample buffer, 30 ml	1	❒
Bio-Safe™ Coomassie stain, 1 L	1	❒
Precision Plus Protein™ Dual Color standards, 500 µl	1	❒

Ships at room temperature. Precision Plus Protein Dual Color standards should be stored at –20°C. All other reagents should be stored at room temperature.

Centrifugation Purification Module (catalog #1665041EDU)

Kit Components	Quantity	(✔)
Profinity™ IMAC Ni-charged resin, 1.5 ml	1	❒
Micro Bio-Spin™ empty columns	12	❒
Micro Bio-Spin 6, Tris columns	12	❒
Screwcap microcentrifuge tubes, 1.5 ml	50	❒
Spin Column wash tubes	25	❒

Ships at room temperature; store at 4°C.

Handpacked Column Purification Module (catalog #1665046EDU)

Kit Components	Quantity	(✔)
Profinity IMAC Ni-charged resin, 10 ml	1	❒
1.0 cm x 5 cm, 2-pk Glass Econo-Column™ columns	2	❒
Micro Bio-Spin 6, Tris columns	25	❒

Ships at room temperature; store at 4°C.

Prepacked Cartridge Purification Module (catalog #1665051EDU)

Kit Components	Quantity	(✔)
Bio-Scale™ Mini Profinity IMAC Cartridge	4	❒
Micro Bio-Spin 6, Tris columns	25	❒

Ships at room temperature; store at 4°C.

DHFR Enzymatic Assay Module (catalog #1665065EDU)

Kit Components	Quantity	(✔)
Dihydrofolic acid substrate (DHF), 1 mg	1	❒
NADPH, cofactor, 1 mg	1	❒

Ships on blue ice. Immediately store at –20°C.

Assessment Module (catalog #1665070EDU)

This unique assessment guide provides ideas for using formative assessment in your class to guide and increase learning while students perform the lab activities. At the end of the lab series use the summative assessment to evaluate the final learning levels of students. The assessment tool is arranged according to learning levels so that you can choose what best evaluates the comprehension of the students.

Materials Required But Not Supplied

Chemicals	Centrifugation Purification	BioLogic™ LP/BioLogic DuoFlow™ Purification
Dry ice	1 block	1 block
Ethanol	0.5–2 L	0.5–2 L

Plastics and Consumables	Centrifugation Purification	BioLogic LP/BioLogic DuoFlow Purification
100–1,000 µl pipet tips, standard style (catalog #2239350EDU)	12 boxes	4 boxes
2–200 µl pipet tips, standard style (catalog #2239347EDU)	12 boxes	4 boxes
Parafilm sealing film	1	1
UV compatible cuvettes such as trUView™ cuvettes (catalog #1702510EDU)	1 box of 50	1 box of 50
or	or	or
quartz submicrovolume cuvettes (catalog #1702505EDU)	1–4 cuvettes	1–4 cuvettes
1.5 ml standard disposable polystyrene cuvettes (catalog #2239955EDU)	1 box of 100	1 box of 100
or		or
quartz standard cuvettes (catalog #1702502EDU)	1–4 cuvettes	1–4 cuvettes
10 ml syringes	12	16
22 gauge syringe needles	12	4
Fraction collection tubes (catalog #2239751EDU)	N/A	200 tubes

Glassware	Centrifugation Purification	BioLogic LP/BioLogic DuoFlow Purification
500 ml Erlenmeyer flasks	N/A	4
1 L Erlenmeyer flask or autoclavable bottle	2	1
Beakers for dry ice/ethanol bath	1–12	1–4

Temperature Control Equipment and Mixing Devices	Centrifugation Purification	BioLogic LP/BioLogic DuoFlow Purification
Microwave oven	1	1
–20°C freezer	1	1
Incubation oven (catalog #1660501EDU)	1–2*	1
Tube roller (catalog #1660711EDU)	1–2*	N/A
Mini rocker (catalog #1660710EDU)	1**	1
Shaking incubator or shaking water bath	1*	N/A
capable of holding 4 x 500 ml flasks	N/A	1
Dry bath (catalog #1660562EDU) or water bath	1	1

* Initial overnight cell cultures and cell subcultures/induced cells for Centrifugation Purification are prepared in 50 ml sterile conical tubes. Incubation at 37°C with some form of mixing is required. This incubation can be accomplished in either a temperature-controlled shaking incubator, a shaking water bath or using tube rollers in an incubation oven set at 37°C. The Bio-Rad tube roller can hold six 50 ml sterile conical tubes. Therefore, if 12 workstations are being run, two tube rollers, each in their own incubation oven, would be required.

** For the Centrifugation Purification protocol, during the binding of the cell lysate to the Profinity IMAC Ni-charged resin, a tube roller can be used for end over end mixing of the resin and lysate. If a tube roller is not available, the mixing can be accomplished by using a mini rocker. The mini rocker can be further used for rinsing, staining and destaining of SDS-PAGE gels.

Other Equipment

	Centrifugation Purification	BioLogic LP/BioLogic DuoFlow Purification
UV/Vis Spectrophotometer capable of reading to three decimal places (catalog #1702525EDU)	1	1
100–1,000 µl adjustable micropipet (catalog #1660508EDU, 1660553EDU)	12	4
20–200 µl adjustable micropipet (catalog #1660507EDU, 1660552EDU)	12	4
2–20 µl adjustable micropipet (catalog #1660506EDU, 1660551EDU)	12	4
Pipet pump or filler	12	4
10 ml serological pipets	4	12
Power supply (catalog #1645050EDU)	3–12	1–4
Vertical electrophoresis chambers (catalog #1658004EDU, 1658005EDU)	3–12	1–4
Gel documentation system (catalog #1708170EDU, 1708270EDU)	1	1
Microcentrifuge with variable speed setting capable of 16,000 x g (catalog #1660602EDU)	1	1
Centrifuge capable of 16,000 x g with rotors that hold 250 ml centrifuge bottles and 30–50 ml centrifuge tubes	N/A	1
Centrifuge bottles (250 ml) capable of withstanding 4,500 x g	N/A	4
Centrifuge tubes (30–50 ml) capable of withstanding 16,000 x g	N/A	4

Handpacked Column Process Only	Quantity
Flow adaptor, 1.0 cm column ID 1–7 cm functional length (catalog #7380014EDU)	1 per instrument

Equipment Necessary If Using a BioLogic DuoFlow System (catalog# 7600047EDU or 7600037EDU)

	Quantity
Fittings to convert luer to ¼-28 (catalog #7320113EDU)	1 set per instrument
5 ml injection loop (catalog #7500497EDU)	1 per instrument

Miscellaneous

	Centrifugation Purification	BioLogic LP/BioLogic DuoFlow Purification
Marking pens	12	4
Storage boxes for microcentrifuge tubes (catalog #1660482EDU)	3	1
Green racks for microcentrifuge tubes (catalog #1660481EDU)	12	4

Optional Materials	Centrifugation Purification	BioLogic LP/BioLogic DuoFlow Purification
Autoclave	1	1
Vortexer (catalog #1660610EDU)	1	N/A
Imaging system (catalog# 1708270EDU with 1708272EDU)	1	1

Refills Available Separately

Each individual module is available to order as a stand alone. In addition, certain refill items are also available:

Growth and Expression reagent pack, catalog #1665057EDU, includes ampicillin (60 mg), LB agar pouch (20 g), LB capsules (12), lysozyme (25 mg), BL21(DE)3 with pDHFR, 1M IPTG (0.1 ml), sterile water (2.5 ml), 10x PBS (100 ml), imidazole stock solution (200 ml), and 20% sterile glucose solution (4 ml)

LB nutrient agar powder, 20 g, catalog #1660600EDU

LB nutrient agar powder, 500 g, catalog #1660472EDU

Ampicillin, 30 mg, catalog #1660407EDU

10x PBS, 100 ml, catalog #1662403EDU

Sterile water, 500 ml, catalog #1632091EDU

Petri dishes, 60 mm, sterile, 500, catalog #1660470EDU

Inoculation loops, 10 µl, sterile, 80, catalog #1660471EDU

EZ Micro test tube, 2 ml, 500/box, catalog #2239430EDU

1.5 ml conical tubes, with separate O-ring screwcaps, 500, catalog #2240100EDU

1.5 ml conical tubes, with installed O-ring screwcaps, sterilized, 500, catalog #2240110EDU

10x TGS buffer, 1 L, catalog #1610732EDU

Laemmli sample buffer, 30 ml, catalog #1610737EDU

Bio-Safe Coomassie stain, 1 L, catalog #1610786EDU

Precision Plus Protein Dual Color standards, catalog #1610374EDU

Jellyfish foam floating racks, 8 racks, catalog #1660479EDU

Gel staining trays, 4, catalog #1660477EDU

Profinity IMAC Ni-charged resin, 10 ml, catalog #1560131EDU

1.0 cm x 5 cm, 2-pk Glass Econo-Column columns, catalog #7371007EDU

Micro Bio-Spin 6, Tris 25/pkg, catalog # 7326221EDU

Bio-Scale Mini cartridge, IMAC, 5 x 1 ml, catalog #7324610EDU

Flow adaptor, 1.0 cm Column ID, catalog #7380014EDU

Course Objectives

The Protein Expression and Purification Series is appropriate for the laboratory portion of an undergraduate (or early graduate) course in Biotechnology, Biochemistry, Molecular Biology, Cell Biology, Recombinant DNA Techniques, or advanced high school biotechnology related courses. It would also be suitable for students doing independent research. It would be excellent for inclusion in biotechnology degree programs offered by community or technical colleges. The exercise could also prove useful for employers in the biotechnology, pharmaceutical, or industrial sectors as an introduction or refresher to protein purification techniques, and particularly as an introduction to using chromatographic instrumentation.

Due to recent advances in the area of protein technology, the actual laboratory procedures are routine, safe and relatively inexpensive, provided basic laboratory equipment is available. Measures have been taken to ensure the safety of the reagents used. While proper laboratory safety techniques must always be used, the reagents provided are safe to use in the classroom. In order to complete the laboratory project in five to eight sessions, it is assumed that students meet at least once per week in a three-hour laboratory session, and that students can meet during the week to carry out a quick laboratory task or two.

Specific Objectives Met by This Project

1. Students will experience a wide range of laboratory techniques. Some of the techniques implemented in this protein-based project are: cell culturing techniques, protein expression and purification, basic micropipetting, gel electrophoresis, and enzymatic analyses.
2. Students will see that these individual techniques are just steps in a longer investigatory process. Few researchers can complete an entire research project in one or two 3-hour laboratory sessions (the timeframe of most commercially available kits), so this five to eight period project more accurately reflects what goes on in a contemporary molecular biology laboratory.
3. Students will be active participants in the process. There are numerous occasions during the project when students are asked to troubleshoot their results, or to make judgments about what to do next. This exercise does not take a simple “cookbook” approach, but rather it involves more critical thinking.

Note to the Instructor

The amount of advance preparation needed will vary greatly depending on the level of the students and the goals of the instructor. A detailed description of the preparation required for each laboratory stage is provided in two chapters at the beginning of the instruction manual: chapters 3A and 3B. Please note that Chapter 3A pertains to the advanced preparation of the Centrifugation Purification Process whereas Chapter 3B pertains to the advanced preparation required for the Chromatography Instrumentation Purification Processes.

Standard techniques: Basic standard laboratory techniques for preparing, loading and running SDS-PAGE gels; using a SmartSpec™ Plus spectrophotometer; determining a centrifuge's RPM; setting up BioLogic LP or BioLogic DuoFlow instrumentation and fraction collectors are included in the appendices for students or instructors to perform prior to or as part of the laboratory.

Aliquoting reagents: Depending on the level and number of students, the instructor may prefer to prepare aliquots of reagents for student teams or to have students take required reagents from a common stock. A list of requirements for each student workstation is provided at the start of each section. Additional requirements and information for the instructor at each stage are within individual modules later in this manual.

Reagents used multiple times: Some of the reagents supplied are used in multiple places in the course. For example, the 10x PBS provided in the Growth and Expression module is used in both Chapters 4 and 7 or 8 for purification, and also in Chapter 9 for analysis of DHFR Enzyme Activity. Therefore it is important that reagents not be discarded following use at a particular stage.

Protein expression: The protein expression portion of this laboratory is probably the most difficult part of this research workflow with respect to timing and aligning with class periods. Great care has been taken to make the protocols as flexible as possible; however, live organisms such as bacteria and their growing cycles can only be managed to some degree. Due to certain class schedules it may be necessary for students to come in and perform some tasks outside of formal class time, or it may be advantageous for the instructor to perform certain tasks to keep the growth and expression activities in progress. These particular steps are highlighted in Chapters 3 through 5. Please review these sections carefully to obtain the best possible results for your class.

DHFR enzyme activity: It is important to note that the NADPH cofactor and DHF substrate are labile and only function three to four hours after being reconstituted. Be sure to prepare these reagents just prior to performing the enzymatic assay. The reconstituted NADPH and DHF reagents are NOT viable after three to four hours and freezing the solutions will not extend their functionality. If multiple classes will be performing the assay and are scheduled more than three to four hours apart, it will be necessary to purchase an additional DHFR Enzymatic Assay Module.

Helpful hints and tips: Please refer to the helpful hints and tips guide located in Appendix A for more useful checks and suggestions on how to ensure the most successful experience with this laboratory series.

Skills Students Need to Perform This Laboratory

The Protein Expression and Purification Series assumes students and instructors have basic molecular biology and microbiology laboratory skills such as loading and running SDS-PAGE gels, micropipetting with care and accuracy, pouring and streaking agar plates, calculating molarity, etc. Brief protocols for basic skills are included in the instructor's advanced preparation section as well as in appendices as reminders, rather than as thorough lessons. Also, this is not an ideal laboratory to introduce basic skills to students. The Bio-Rad Explorer program has a full range of kits to help teach basic skills in individual laboratories prior to introducing students to how these separate skills connect into a single workflow, as is done in this laboratory course.

Setting Class Expectations for Success

Before beginning the Protein Expression and Purification Series it is important for the instructor to help the students understand what the curriculum involves and set expectations for the type of results they may experience along the way. This curriculum was not created to be a demonstration laboratory with guaranteed results of the sort to which some students may be accustomed. Great attention has been given to the design of the experiments with respect to the creation of robust protocols to try to ensure success.* Some ways to increase successful outcomes are to have students practice molecular biology techniques for a couple of weeks prior to embarking on this project. If using chromatography instrumentation for the purification steps it is highly recommended that a trial run is performed with the appropriate BioLogic Starter Kit (catalog #7318350EDU or #7600135EDU) ahead of time to ensure the equipment is properly plumbed and functioning as desired prior to the students performing their experiments.** Ultimately, this series is designed to provide a safe starting place for students to delve into the real world of research and to see how exciting discovering the world of protein purification can be.

* A class can expect a to get a range of results across student groups, which is typical of research. Appendix B shows examples of typical results.

**Appendix A proides a list of tips and helpful hints to help ensure the best possible results.

Safety Considerations

Some countries outside the U.S. may require a special license to use this kit. Please refer to your country's legislative authorities for proper guidelines.

The *Escherichia coli* bacteria BL21(DE3) strain contained in this kit is not a pathogenic organism like the *E. coli* strain O157 H7 that has sometimes been implicated in food poisoning. BL21(DE3) has been genetically modified to prevent its growth unless grown on an enriched medium. However, handling of the *E. coli* BL21(DE3) strain requires the use of standard Microbiological Practices. These practices include, but are not limited to, the following. Work surfaces are decontaminated once a day and after any spill of viable material. All contaminated liquid or solid wastes are decontaminated before disposal. All persons must wash their hands: (i) after they handle material containing bacteria, and (ii) before exiting the laboratory. All procedures are performed carefully to minimize the creation of aerosols. Mechanical pipeting devices are used, mouth pipetting is prohibited; eating, drinking, smoking, and applying cosmetics are not permitted in the work area; wearing protective eyewear and gloves is strongly recommended.

If an autoclave is not available, all solutions and components (loops and pipets) that have come in contact with bacteria can be placed in a fresh 10% bleach solution for at least 20 minutes for sterilization. A shallow pan of this solution should be placed at every lab station. No matter what you choose, all used loops and pipets should be collected for sterilization. Sterilize petri dishes by covering the agar with 10% bleach solution. Let the plate stand for 1 hour or more, and then pour excess plate liquid down the drain. Once sterilized, the agar plates can be double bagged and treated as normal trash. Safety glasses are recommended when using bleach solutions.

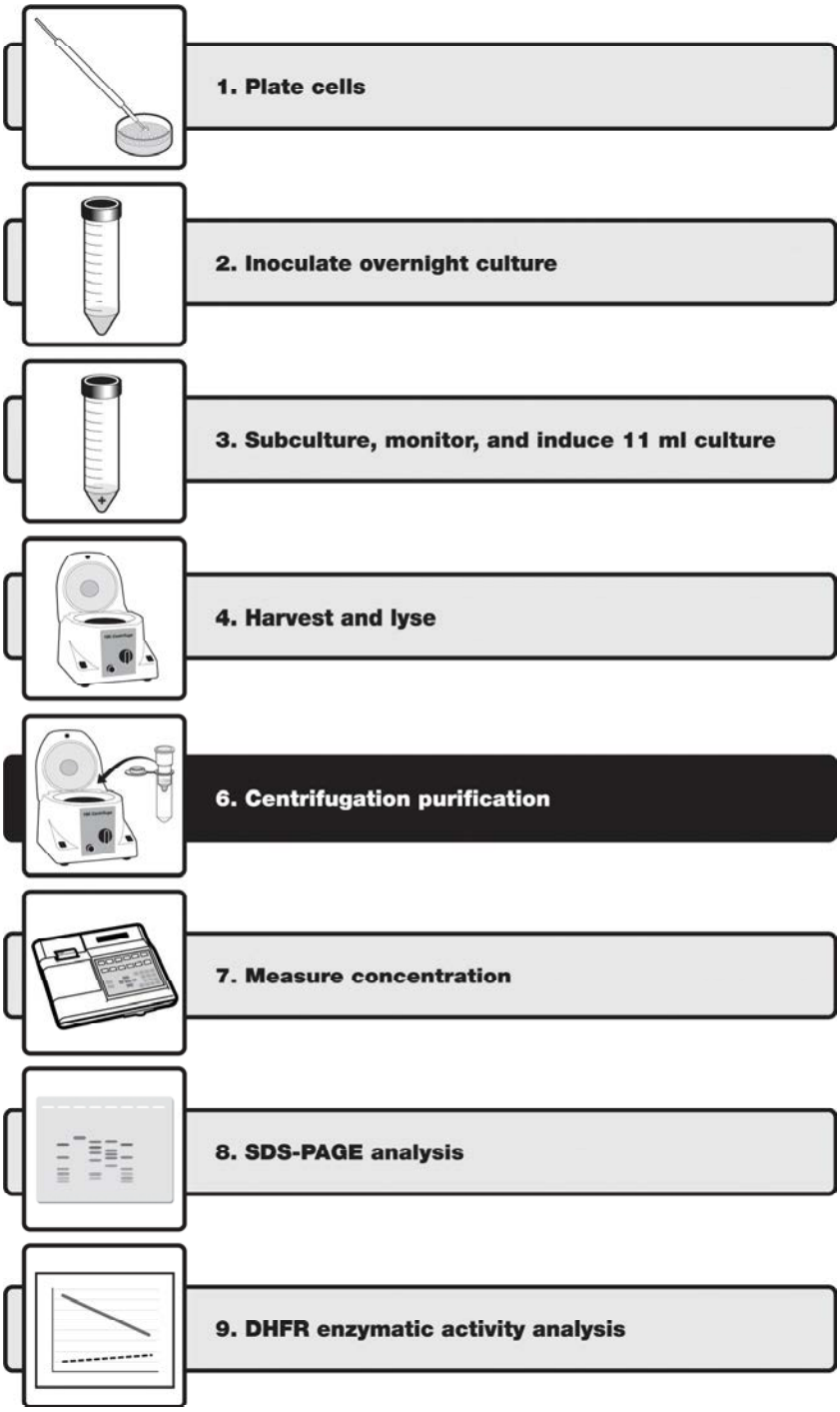
Ampicillin may cause allergic reactions or irritation to the eyes, respiratory system, and skin. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. Wear suitable protective clothing. Ampicillin is a member of the penicillin family of antibiotics. Those with allergies to penicillin or to any other member of the penicillin family of antibiotics should avoid contact with ampicillin.

Please obtain the Material Safety Data Sheets (MSDS) available from Bio-Rad by calling 800-424-6723 in the United States, or see www.bio-rad.com for further information on reagents in this kit. Please consult your local environmental health and safety regulations for proper disposal.

Timelines for the Laboratory Course

The timeline will depend greatly on the level of the students, which purification method is used, the length of class periods, and whether other techniques and analyses are performed in addition to the basic protocol. To assist in planning for the laboratory course, the following pages provide a workflow, timeline, and helpful hints guide for each purification process. The centrifugation purification process begins below (pg 18–24) with the chromatography instrumentation purification process following (pg 25–32).

Centrifugation Purification Workflow



Centrifugation Purification Laboratory Timeline

Note: Tasks that are shaded in grey are preparatory tasks for later stages and may be conducted when spare time is available.

Centrifugation Purification				
Lab Session	Chapter	Task	Estimated Duration	Module Containing Materials
0	3A: Advanced Preparation for Centrifugation Purifiation Protocols	Pour LB/amp plates	30 min	Growth and Expression
		Prepare LB and LB/amp broth	30 min	Growth and Expression
		Prepare IPTG	5 min	Growth and Expression
1	4: Culturing, Expression, Lysis and SDS-PAGE Analysis for 11 ml Cultures and Purification Protocol for Centrifugation Process	Plating *E. coli*	15 min	Growth and Expression
		Grow *E. coli* plates overnight at 37°C	16+ hr*	Growth and Expression
2	4: Culturing, Expression, Lysis and SDS-PAGE Analysis for 11 ml Cultures and Purification Protocol for Centrifugation Process	Prepare overnight culture	15 min	N/A
		Grow overnight culture at 37°C	12+ hr*	N/A
3	4: Culturing, Expression, Lysis and SDS-PAGE Analysis for 11 ml Cultures and Purification Protocol for Centrifugation Process	Measure OD600 of overnight culture	30–45 min	N/A
		Prepare uninduced SDS-PAGE samples	15 min	SDS-PAGE Electrophoresis
		Prepare subcultures	15–30 min	Growth and Expression
		Grow subculture	1 hr	N/A
		Measure OD600 of subculture and induce with IPTG	30–45 min	Growth and Expression
		Culture induced cells at 37°C	4–24 hr*	N/A
Centrifugation Purification continued				

*Time indicated for these tasks is not hands-on time. It is the time needed for reactions to run, bacteria to grow, or time to incubate.

Lab Session	Chapter	Task	Estimated Duration	Module Containing Materials
		Prepare equilibration buffer	5 min	Growth and Expression
		Prepare lysozyme	5 min	Growth and Expression
		Prepare lysis buffers	5 min	Growth and Expression
4	4: Culturing, Expression, Lysis and SDS-PAGE Analysis for 11 ml Cultures and Purification Protocol for Centrifugation Process	Prepare induced SDS-PAGE samples	15 min	SDS-PAGE Electrophoresis
		Pellet cells	30 min–1 hr	Growth and Expression
		Optional stopping point: Store pellet at –20°C to –80°C indefinitely		
		The remaining steps depend on the method chosen for performing lysis procedure:		
		Option 1: Dry ice/ethanol lysis *(recommended method)*		
		Resuspend cell pellet in lysis buffers	10 min	Growth and Expression
		Perform dry ice/ethanol lysis	30–45 min	Growth and Expression
		Optional stopping point: Store lysate at –20°C to –80°C indefinitely		
		Option 2: –70°C to –80°C lysis		
		Resuspend cell pellet in lysis buffers	10 min	Growth and Expression
		Place resuspended cells at –70°C to –80°C	16+ hr*	N/A
		Option 3: –20°C lysis		
		Resuspend cell pellet in lysis buffers	10 min	Growth and Expression
Centrifugation Purification continued				

*Time indicated for these tasks is not hands-on time. It is the time needed for reactions to run, bacteria to grow, or time to incubate.

Lab Session	Chapter	Task	Estimated Duration	Module Containing Materials
		Place resuspended cells at –20°C	16+ hr*	N/A
		Thaw completely	30 min	N/A
		Place resuspended cells at –20° C	16+ hr*	N/A
5	4: Culturing, Expression, Lysis and SDS-PAGE Analysis for 11 ml Cultures and Purification Protocol for Centrifugation Process	Thaw lysate completely	30 min	N/A
		Separate soluble vs insoluble fractions via centrifugation	20 min	N/A
		Decant soluble fraction	10 min	Growth and Expression
		Resuspend insoluble fraction in lysis buffer	15 min	Growth and Expression
		Prepare soluble and insoluble SDS-PAGE samples	15 min	SDS-PAGE Electrophoresis
		Optional stopping point: Store fractions at –20°C indefinitely		
6	4: Culturing, Expression, Lysis and SDS-PAGE Analysis for 11 ml Cultures and Purification Protocol for Centrifugation Process	Prepare Micro-BioSpin column with Profinity IMAC Ni-charged resin	15–30 min	Growth and Expression and Centrifugation Purification
		Bind GST-DHFR-His soluble fraction to IMAC Ni-charged resin	20–30 min	N/A
		Collect flowthrough, wash, and eluate fractions	15–30 min	Growth and Expression and Centrifugation Purification
		Prepare flowthrough, wash and eluate SDS-PAGE samples	15 min	SDS-PAGE Electrophoresis
Centrifugation Purification continued				

*Time indicated for these tasks is not hands-on time. It is the time needed for reactions to run, bacteria to grow, or time to incubate.

Lab Session	Chapter	Task	Estimated Duration	Module Containing Materials
		Optional stopping point: Store fractions at 4°C and SDS-PAGE samples at –20°C indefinitely		
		Prepare desalting columns and desalt eluate fraction	30 min	Centrifugation Purification
		Prepare desalted eluate SDS-PAGE sample	15 min	SDS-PAGE Electrophoresis
		Optional stopping point: Store fractions at 4°C and SDS-PAGE samples at –20°C indefinitely		
7	4 and 8: Culturing, Expression, Lysis and SDS-PAGE Analysis for 11 ml Cultures and Purification Protocol for Centrifugation Process; and DHFR Analysis	Reheat SDS-PAGE samples	15–30 min	N/A
		Electrophorese SDS-PAGE samples	0.5–1 hr	SDS-PAGE Electrophoresis
		Rinse and stain gels	1.25+ hr*	SDS-PAGE Electrophoresis
		Destain gels	8+ hr*	N/A
		Measure and calculate desalted eluate concentration	30 min	N/A
		Prepare enzymatic assay reagents *(Must be prepared within 3–4 hrs of use)*	15 min	DHFR Enzymatic Assay
		Perform enzymatic analysis	2 hr	Growth and Expression and DHFR Enzymatic Assay

*Time indicated for these tasks is not hands-on time. It is the time needed for reactions to run, bacteria to grow, or time to incubate.

Centrifugation Purification Helpful Hints Checklist

Overnight Cultures

- 37°C incubation temperature of cultures is required.
- If using a shaking incubator or shaking water bath, a rotational speed of 250–275 rpm is required.
- If using a tube roller in an incubator, the tube roller should be in end over end mixing mode.

Subcultures

- Ensure the LB/amp medium is prewarmed to 37°C.
- 37°C incubation temperature of cultures is required.
- If using a shaking incubator or shaking water bath, a rotational speed of 250–275 rpm is requried.
- If using a tube roller in an incubator, the tube roller should be in end over end mixing mode.

Induction

- Determine timeline for induction. Four hour induction provides optimal results.
- 37°C incubation temperature of cultures is required.
- If using a shaking incubator or shaking water bath, a rotational speed of 250–275 rpm is required.
- If using a tube roller in an incubator, the tube roller should be in end over end mixing mode.

Cell Lysis

- Prepare lysozyme fresh (within 24 hours of use) for best results.
- Make sure lysozyme was made in 2x PBS.
- Make sure the cell pellets are completely resuspended in lysis buffer 1.

Separating Soluble and Insoluble Fractions

- The centrifuge must be able to generate 16,000 x g of force (RCF). RCF is not equal to RPM. Make sure that the correct conversion from RCF to RPM is calculated. For more information on this conversion please refer to Appendix C.

Centrifugation Affinity Purification

- The microcentrifuge must be able to generate 1,000 x g of force (RCF). RCF is not equal to RPM. Make sure that the correct conversion from RCF to RPM is calculated. For more information on this conversion please refer to Appendix C.
- Do not use a mini-centrifuge for this procedure because they generally generate 2,000 x g (RCF), which is too much force for the affinity Ni-IMAC resin to withstand.
- Ensure the Ni-IMAC resin is fully resuspended before preparing columns.
- Ensure the sample binds to the resin for 20 minutes with rocking.

Size Exclusion Purification (Desalting)

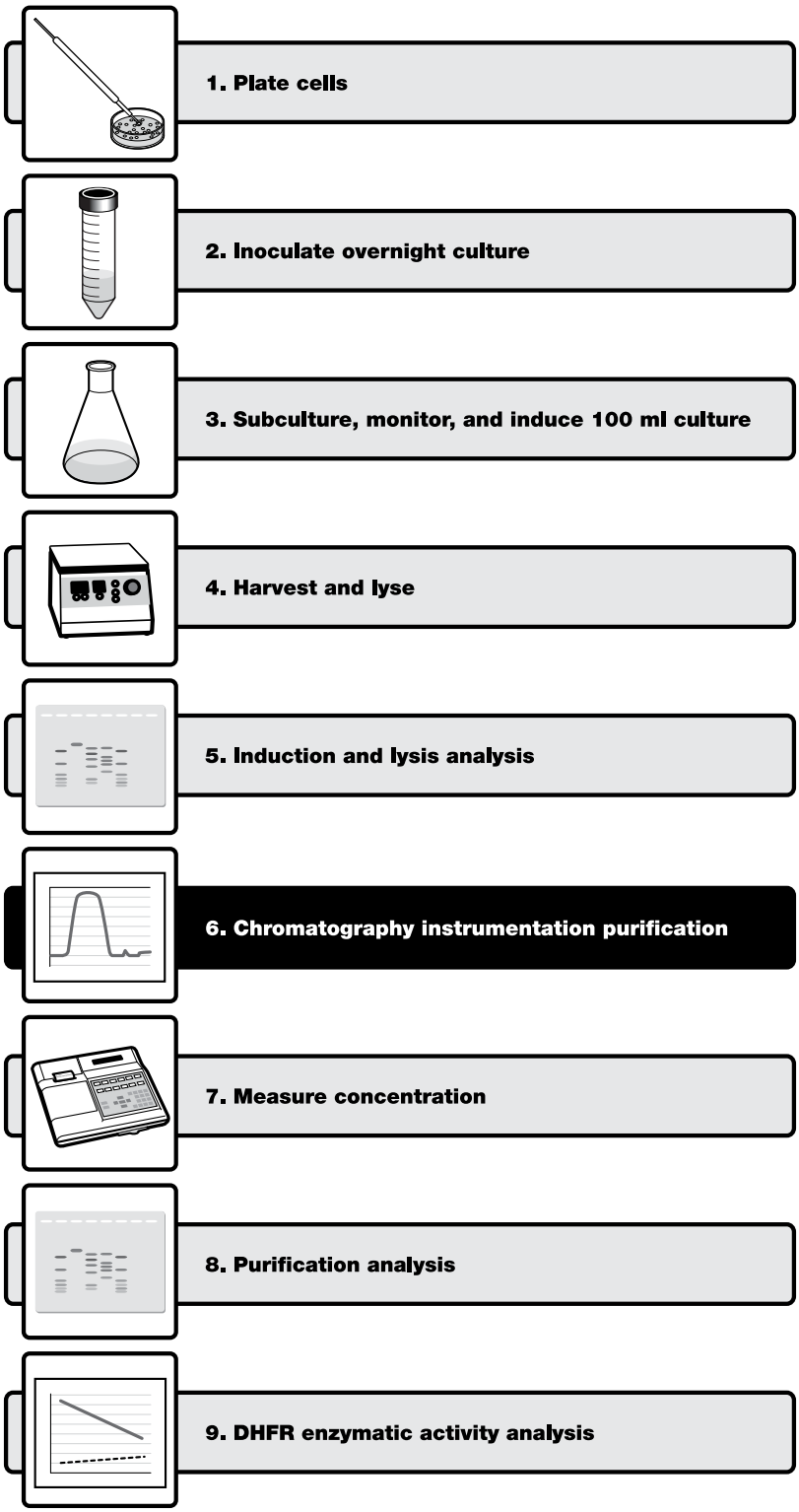
- The microcentrifuge must be able to generate 1,000 x g of force (RCF). RCF is not equal to RPM. Make sure that the correct conversion from RCF to RPM is calculated. For more information on this conversion please refer to Appendix C.
- Do not use a mini-centrifuge for this procedure because they generally generate 2,000 x g (RCF), which is too much force for the Bio-Gel P6 desalting resin to withstand.
- Removal of the storage buffer from the desalting columns is important. Make sure to let the resin drain via gravity to remove the first portion of the storage buffer.
- Make sure to use the 2 ml microcentrifuge tubes during the desalting process. 1.5 ml microcentrifuge tubes are not large enough to allow the buffer to flow from the column into the collection tube.
- Verify that the desalted sample volume is not >150 µl (assuming two desalting runs using the same column). If the volume is >150 µl, then the column storage buffer was likely not removed properly.

DHFR Enzymatic Assay

- Ensure the DHF is reconstituted in 10x PBS.
- Ensure the NADPH is reconstituted in 1x PBS.
- Ensure the DHF and NADPH are used within three to four hours. The reconstituted DHF and NADPH cannot be frozen to retain or extend the activity and shelf life.
- The enzymatic reaction occurs very quickly so make sure to add the DHF to the NADPH solution and then place it immediately in the spectrophotometer. It is best to do this step next to the spectrophotometer to ensure no data points are missed.
- Make sure the spectrophotometer is turned on at least 30 minutes ahead of time to allow the lamp to warm up.
- If using trUView cuvettes, make sure the frosted side is facing the beam path. The frosted side of the trUView cuvettes contains a tiny clear window through which the UV reading is taken.

Chromatography Instrumentation Purification Workflow

The timeline will depend greatly on the level of the students, the length of class periods, and whether other techniques and analyses are performed in addition to the basic protocol.



Chromatography Instrumentation Purification Laboratory Timeline

Note: Tasks that are shaded in grey are preparatory tasks for later stages and may be conducted when spare time is available.

Chromatography Instrumentation Purification				
Lab Session	Chapter	Task	Estimated Duration	Module Containing Materials
0	3B: Advance Preparation—Instrumentation Protocols	Pour LB/amp plates	30 min	Growth and Expression
		Prepare LB and LB/amp broth	30 min	Growth and Expression
		Prepare instrumentation	2+ hr*	N/A
		Optional: Run BioLogic starter kit	2+ hr*	N/A
1	5: Culturing, Expression, Lysis and SDS-PAGE Anlaysis for 100 ml Cultures	Plate *E. coli*	15 min	Growth and Expression
		Grow *E. coli* plates overnight at 37°C	16+ hr*	Growth and Expression
2	5: Culturing, Expression, Lysis and SDS-PAGE Anlaysis for 100 ml Cultures	Prepare overnight culture	15 min	N/A
		Grow overnight culture at 37°C	12+ hr*	N/A
3	5: Culturing, Expression, Lysis and SDS-PAGE Anlaysis for 100 ml Cultures	Measure OD600 of overnight culture	30–45 min	N/A
		Prepare uninduced SDS-PAGE samples	15 min	SDS-PAGE Electrophoresis
		Prepare subcultures	15–30 min	Growth and Expression
		Grow subcultures	1 hr	N/A
		Measure OD600 of subculture and induce with IPTG	30–45 min	Growth and Expression
		Culture of induced cells at 37°C	4–24 hr*	N/A

*Time indicated for these tasks is not hands-on time. It is the time needed for reactions to run, bacteria to grow, or time to incubate.

Chromatography Instrumentation Purification continued				
Lab Session	Chapter	Task	Estimated Duration	Module Containing Materials
		Prepare equilibration buffer	5 min	Growth and Expression
		Prepare lysozyme	5 min	Growth and Expression
		Prepare lysis buffer	5 min	Growth and Expression
4	5: Culturing, Expression, Lysis and SDS-PAGE Analysis for 100 ml Cultures	Prepare induced SDS-PAGE samples	15 min	SDS-PAGE Electrophoresis
		Pellet cells	0.5–1 hr	Growth and Expression
		Optional stopping point: Store pellet at –20°C to –80°C indefinitely		
		The remaining steps depend on method chosen for performing lysis procedure:		
		Option 1: Dry ice/ Ethanol lysis *(recommended method)*		
		Resuspend cell pellet in equilibration and lysis buffer	10 min	Growth and Expression
		Perform dry ice/ethanol lysis	30–45 min	Growth and Expression
		Optional stopping point: Store lysate at –20°C to –80 °C indefinitely		
		Option 2: –70°C to –80°C lysis		
		Resuspend cell pellet in equilibration and lysis buffer	10 min	Growth and Expression
		Place resuspended cells at –70°C to –80°C	16+ hr*	N/A
		Option 3: –20°C lysis		
		Resuspend cell pellet in equilibration and lysis buffer	10 min	Growth and Expression
Chromatography Instrumentation Purification continued				

*Time indicated for these tasks is not hands-on time. It is the time needed for reactions to run, bacteria to grow, or time to incubate.

Chromatography Instrumentation Purification continued				
Lab Session	Chapter	Task	Estimated Duration	Module Containing Materials
		Place resuspended cells at –20°C	16+ hr*	N/A
		Thaw completely	30 min	N/A
		Place resuspended cells at –20°C	16+ hr*	N/A
5	5: Culturing, Expression, Lysis and SDS-PAGE Analysis for 100 ml Cultures	Thaw lysate completely	30 min	N/A
		Separate soluble vs insoluble fractions via centrifugation	20 min	N/A
		Decant soluble fraction	10 min	Growth and Expression
		Resuspend insoluble fraction in equilibration buffer	15 min	Growth and Expression
		Prepare soluble and insoluble SDS-PAGE samples	15 min	SDS-PAGE Electrophoresis
		Optional stopping point: Store fractions at –20°C indefinitely		
		Reheat uninduced, induced, soluble, and insoluble SDS-PAGE samples	10 min	N/A
		Electrophorese SDS-PAGE samples	0.5–1 hr	SDS-PAGE Electrophoresis
		Rinse and stain gels	1.25+ hr*	SDS-PAGE Electrophoresis
		Destain gels	8+ hr*	N/A
6	5: Culturing, Expression, Lysis and SDS-PAGE Analysis for 100 ml Cultures	Optional: Prepare handpacked Profinity IMAC Ni-charged columns	0.5–1 hr	Hand Packed Column Purification Module

*Time indicated for these tasks is not hands-on time. It is the time needed for reactions to run, bacteria to grow, or time to incubate.

Chromatography Instrumentation Purification continued				
Lab Session	Chapter	Task	Estimated Duration	Module Containing Materials
	6: Purification Protocol for BioLogic LP System or 7: Purification Protocol using BioLogic DuoFlow System (Depending on instrument used)	Perform chromatographic separation using chromatography instrumentation	3–4 hr	Growth and Expression and Hand-Packed Column Purification Module or Prepacked Cartridge Purification Module
		Optional stopping point: Store all fractions at 4°C		
7	6: Purification Protocol for BioLogic LP System or 7: Purification Protocol using BioLogic DuoFlow System (Depending on instrument used)	Study chromatogram and determine correct fractions for further analysis	30 min	N/A
		Prepare flowthrough, wash, and 3 eluate fractions SDS-PAGE samples	15 min	SDS-PAGE Electrophoresis
		Prepare desalting columns and desalt eluate fraction	30 min	Hand Packed Column Purification Module or Prepacked Cartridge Purification Module
		Prepare desalted eluates SDS-PAGE samples	15 min	SDS-PAGE Electrophoresis
		Optional stopping point: Store fractions at 4°C and SDS-PAGE samples at –20°C (reheat samples prior to electrophoresis if stored as noted)		
		Electrophorese SDS-PAGE samples	0.5–1 hr	SDS-PAGE Electrophoresis
		Rinse and stain gels	1.25+ hr*	SDS-PAGE Electrophoresis
		Destain gels	8+ hr*	N/A

*Time indicated for these tasks is not hands-on time. It is the time needed for reactions to run, bacteria to grow, or time to incubate.

Chromatography Instrumentation Purification continued				
Lab Session	Chapter	Task	Estimated Duration	Module Containing Materials
		Meaure and calculate desalted eluate concentration	30 min	N/A
		Prepare enzymatic assay reagents *(Must be prepared within 3–4 hrs of use)*	15 min	DHFR Enzymatic Assay
		Perform enzymatic analysis	2 hr	Growth and Expression and DHFR Enzymatic Assay

*Time indicated for these tasks is not hands-on time. It is the time needed for reactions to run, bacteria to grow, or time to incubate.

Chromatography Instrumentation Purification Helpful Hints Checklist

Overnight Cultures

- 37°C incubation temperature of cultures is required.
- If using a shaking incubator or shaking water bath, a rotational speed of 250–275 rpm is required.
- If using a tube roller in an incubator, the tube roller should be in end over end mixing mode.

Subcultures

- Ensure the LB/amp medium is prewarmed to 37°C.
- 37°C incubation temperature of cultures is required.
- If using a shaking incubator or shaking water bath, a rotational speed of 250–275 rpm is required.
- If using a tube roller in an incubator, the tube roller should be in end over end mixing mode.

Induction

- Determine timeline for induction. Four hour induction provides optimal reqults.
- 37°C incubation temperature of cultures is required.
- If using a shaking incubator or shaking water bath a rotational speed of 250–275 rpm is required.
- If using a tube roller in an incubator, the tube roller should be in end over end mixing mode.

Cell Lysis

- Prepare lysozyme fresh (within 24 hours of use) for best results.
- Make sure lysozyme was made in 2x PBS.
- Make sure the cell pellets are completely resuspended in lysis buffer.

Separating Soluble and Insoluble Fractions

- The centrifuge must be able to generate 16,000 x g of force (RCF). RCF is not equal to RPM. Make sure that the correct conversion from RCF to RPM is calculated. For more information on this conversion please refer to Appendix C.

Chromatography Instrumentation Affinity Purification

BioLogic LP System

- Run through the instrument's starter kit prior to performing the Protein Expression and Purification Series to ensure that both the wiring and tubing were installed correctly.
- Remember to calculate the dead volume (typically 2–4 ml) when determining which fractions contain the eluted GST-DHFR-His.

BioLogic DuoFlow System

- Run through the instrument's starter kit prior to performing the Protein Expression and Purification Series to ensure that both the wiring and tubing were installed correctly.

Size Exclusion Purification (Desalting)

- The microcentrifuge must be able to generate 1,000 x g of force (RCF). RCF is not equal to RPM. Make sure that the correct conversion from RCF to RPM is calculated. For more information on this conversion please refer to Appendix C..
- Do not use a mini-centrifuge for this procedure because they generally generate 2,000 x g (RCF), which is too much force for the Bio-Gel P6 desalting resin to withstand.
- Removal of the storage buffer from the desalting columns is important. Make sure to let the resin drain via gravity to remove the first portion of the storage buffer.
- Make sure to use the 2 ml microcentrifuge tubes during the desalting process. 1.5 ml micro-centrifuge tubes are not large enough to allow the buffer to flow from the column into the collection tube.

- Verify that the desalted sample volume is not >150 µl (assuming two desalting runs using the same column). If the volume is >150 µl, then the column storage buffer was likely not removed properly.

DHFR Enzymatic Assay

- Ensure the DHF is reconstituted in 10x PBS.
- Ensure the NADPH is reconstituted in 1x PBS.
- Ensure the DHF and NADPH are used within three to four hours. The reconstituted DHF and NADPH cannot be frozen to retain or extend the activity and shelf life.
- The enzymatic reaction occurs very quickly so make sure to add the DHF to the NADPH solution and then place it immediately in the spectrophotometer. It is best to do this step next to the spectrophotometer to ensure no data points are missed.
- Make sure the spectrophotometer is turned on at least 30 minutes ahead of time to allow the lamp to warm up.
- If using trUView cuvettes, make sure the frosted side is facing the beam path. The frosted side of the trUView cuvettes contains a tiny clear window through which the UV reading is taken.

Chapter 1: Recombinant Protein Expression and Purification

Why Produce Proteins Recombinantly?

To be used for research, industrial or pharmaceutical purposes, proteins need to be purified in large quantities. Some proteins, like casein, which makes up 20% of the protein content in milk, can easily be extracted from a readily available source in large quantities. However, most proteins are not naturally produced in a form and in amounts that allow easy purification. The techniques of genetic engineering overcome the limitations of naturally produced proteins by making cells synthesize specific proteins in amounts which can be purified for use in fundamental research or for industrial and therapeutic applications.

Biogen, one of the first companies to develop recombinant proteins, is using genetic engineering to produce human interferon beta-1a in Chinese hamster ovary (CHO) cells and is sold under the tradename Avonex. A similar form of recombinant human interferon, interferon beta-1b, is expressed in *E. coli* and sold by Bayer under the drug name of Betaseron. (An interferon is an immune protein produced in response to a virus, bacteria, parasite, or tumor cell.) Both recombinant human interferon beta-1a and 1b have been developed, tested, and brought to the market to help slow the progression of multiple sclerosis. Without recombinant production of these proteins in CHO cells or in *E. coli*, there would not be an easy way to obtain this protein for therapeutic usage.

Table 1.1 Human proteins produced by genetic engineering. Human proteins produced via genetic engineering and the disease or disorder they are used to treat.

Protein	Used in the treatment of
Insulin	Diabetes
Somatostatin	Growth disorders
Somatrotropin	Growth disorders
Factor VIII	Hemophilia
Factor IX	Christmas disease
Interferon-alpha	Leukemia and other cancers, MS
Interferon-beta	Cancer, AIDS, MS
Interferon-gamma	Cancers, rheumatoid arthritis
Interleukins	Cancers, immune disorders
Granulocyte colony stimulating factor	Cancers
Tumor necrosis factor	Cancers
Epidermal growth factor	Ulcers
Fibroblast growth factor	Ulcers
Erythropoietin	Anemia
Tissue plasminogen activator	Heart attack
Superoxide dismutase	Free radical damage in kidney transplants
Lung surfactant protein	Respiratory distress
alpha 1-antitrypsin	Emphysema
Serum albumin	Used as a plasma supplement
Relaxin	Used to aid childbirth

Choice of Cell Type

The biotechnology industry uses several cell types, both prokaryotic (bacteria) and eukaryotic (animal, fungi, plant), to synthesize recombinant proteins. The choice of the host cell depends on the protein expressed.

Bacteria can express large amounts of recombinant protein, but the expressed proteins sometimes do not fold properly. In addition, bacterial cells cannot carry out the post-translation modifications that are characteristic of some of the proteins made by eukaryotic cells. The most important post-translation modification is glycosylation, the covalent addition of sugar residues to the amino acid residues making up the protein. Glycosylation can change the structure and thus affect the activity of a protein. Many mammalian blood proteins are glycosylated, and the addition of these sugars often changes the rate of turnover (half-life) of the protein in the blood, because proteins that are misfolded will be quickly degraded. If glycosylation is important for the function of the protein, mammalian cells are the cell type of choice, but these cells produce less protein and are more expensive to grow.

In the early days of the biotechnology industry *Escherichia coli* (*E. coli*) was the bacterial host of choice. This species had been used as the primary experimental system to study bacterial genetics for decades. More was known about the molecular biology of *E. coli* than any other species, and many genetic variants were available. In addition *E. coli* grows quickly, can reach high cell concentrations, and can produce large quantities of a single protein. It is also relatively inexpensive to grow. Today *E. coli* remains the bacterial system of choice, and many companies produce recombinant proteins using this bacterial species. Insulin, the first protein produced by genetic engineering, was produced in *E. coli.* Blockbuster products like human growth hormone and granulocyte colony stimulating factor (which increases white cell production in cancer chemotherapy patients) are also produced using this bacterial species. In general, if a protein's properties allow it to be produced in bacteria, then *E. coli* is the system of choice.

For recombinant protein expression in lower eukaryotic cells, two yeast species are commonly used: *Saccharomyces cerevisiae (S. cerevisiae)* and *Pichia pastoris (P. pastoris)*. *S. cerevisiae* is the yeast species used to make bread, wine and beer. Baker's yeast is used in research laboratories as a model system to study the genetics of eukaryotic cells. *P. pastoris* is a yeast species initially discovered by the petroleum industry. It divides rapidly, grows to a very high cell density, and can produce large quantities of a single protein. In addition, it can be genetically engineered to secrete the protein into the surrounding medium to allow easier recovery. Both species can glycosylate proteins, although the glycosylation patterns may differ from mammalian patterns. The sugars that are added to the protein and their position on the amino acid chain may differ between yeast and mammalian cells. Despite these advantages, relatively few biotech companies use yeast as a production system. The exceptions are the vaccine that immunizes against hepatitis B virus and the vaccine Guardasil that immunizes against HPV, the human papillomavirus.

If a protein has a very large and complex structure, or if that protein requires glycosylation to be active, then the protein must be produced in a mammalian cell line. Chinese hamster ovary cells (CHO) is the cell line that is almost always used. CHO cells bear relatively little resemblance to the cells of the hamster from which they were derived in the 1950s; they have adapted to growth in cell culture medium. Cell lines are established when cells from a multicellular organism are separated from one another by a protein-digesting enzyme and grown as if they are really a unicellular organism. The cells require a rich medium that provides them with all of the amino acids, vitamins, and growth factors that they need to grow. This complexity means that mammalian growth medium is many times more expensive than the media used to grow either bacterial or yeast cells. The CHO cell lines can be adapted for growth in suspension culture. The CHO cell lines are most often engineered to synthesize the protein of interest on the ribosomes attached to the rough endoplasmic reticulum, to package and glycosylate the protein in the Golgi apparatus, and to eventually secrete the protein into the extracellular medium where it is easier to purify.

CHO cells can glycosylate proteins with a mammalian glycosylation pattern. If glycosylation is important to the function of the protein, CHO cells should be used. Because CHO cells divide slowly, the production

runs are much longer than with *E. coli* (on the order of weeks rather than days). All equipment and all growth media must be scrupulously sterilized. A single contaminating bacterial cell will overgrow the culture and will lead to that batch being discarded. Although the growth of CHO cells takes longer, uses expensive media, and presents a greater risk of contamination, the isolation of the proteins that these cells produce is usually easier than in bacterial or yeast cells.

Interferon beta provides a good example of how the end product influences the choice of expression system for recombinant proteins. Avonex, the human interferon beta-1a form produced in CHO cells, is glycosylated; while Betaseron, the human interferon beta-1b form produced in *E. coli*, is not glycosylated. Since glycosylation is important for interferon beta-1a function, it is produced in CHO cells.

Table 1.2. Advantages and disadvantages of using bacteria, yeast and mammalian cells to produce recombinant proteins.

Parameter	Bacteria	Yeast	Mammalian
Contamination risk	Low	Low	High
Cost of growth medium	Low	Low	High
Product titer (concentration)	High	High	Low
Folding	Sometimes	Probably	Yes
Glycosylation	No	Yes, but different pattern	Full
Relative ease to grow	Easy	Easy	Difficult
Relative ease of recovery	Difficult	Easy	Easy
Deposition of product	Intracellular	Intracellular or extracellular	Extracellular
Product	Intracellular	Often secreted into media	Secreted

Table 1.3. Examples of pharmaceutical products and the cell line used to produced them.

Product	Cell Line
Insulin	*Escherichia coli*
Human growth hormone	*Escherichia coli*
Granulocyte colony stimulating factor	*Escherichia coli*
Tissue plaminogen activator	CHO cells
Pulmozyme (DNase) cystic fibrosis	CHO cells
Erythropoietin induces red blood cell production	CHO cells
Hepatitus B virus vaccine	Yeast
Human papillomavirus vaccine	Yeast
Rituxan rheumatoid arthritis, non-hodgkins lymphoma, leukemia	CHO cells
Herceptin breast cancer	CHO cells

Choice of Plasmid

Once the cell type has been chosen, the plasmid or vector to express the protein needs to be selected. Different plasmids are used for expression of proteins in bacteria, yeast and higher eukaryotic cells. Some features that need to be considered in the plasmid include: selection system (such as antibiotic resistance), the promoter, the copy number of the plasmid, presence of signal peptide sequence to excrete the expressed protein out of the cell, presence of sequence coding for a protein purification tag or DNA coding for fusion protein partners.

Antibiotic resistance is a common component of both prokaryotic and eukaryotic vector systems. The presence of a gene for antibiotic resistance allows for selective retention of the plasmid and suppression of growth of any cells that do not contain the plasmid. However, to ensure safety for expression systems being used for vaccine and therapeutic protein production, other selection systems can be used such as the expression of a required metabolic enzyme that has otherwise been deleted from the host organism.

The promoter controls the level of gene expression. It can be either constitutive, meaning that it is always active and there is no control over when the protein of interest is expressed, or inducible, meaning that its activity can be triggered by external factors. Examples of inducible promoters are the heat shock promoters, which are activated by a change in temperature. These promoters are derived from naturally occurring sequences in organisms that need to express a different protein when they are in a warm environment versus a cold one. Other inducible promoters are activated by the addition of a chemical such as lactose or its analog IPTG in the case for the LacZ promoter in *E. coli*. The T7 promoter is an example of a chemically induced promoter system. For industrial applications, genes for the protein of interest tend to be under inducible control.

The copy number of a plasmid depends on the origin of replication present in the plasmid. The origin of replication determines the level of control of replication of the plasmid, and if the plasmid is under a relaxed control more copies can be made. The size of the plasmid and size of the insert also affect the number of copies. If there are more copies of the plasmid in cells it is possible for them to produce more protein than cells that have fewer copies of the plasmid. Plasmids used for cloning such as pUC tend to be higher copy number while plasmids used for protein expression tend to be larger and have lower copy number.

To facilitate the purification of the expressed protein, DNA sequences coding for a signal (amino acid sequence) that targets the protein of interest to be secreted into the periplasmic region of *E. coli* or into the extracellular medium for eukaryotic cells, can be fused to the gene of interest. Other tags that are commonly added are fusion proteins to increase the solubility of the expressed protein (such as glutathione-S-transferase, GST) or affinity tags (polyhistidine or GST tags) that can be used to selectively purify the recombinant protein.

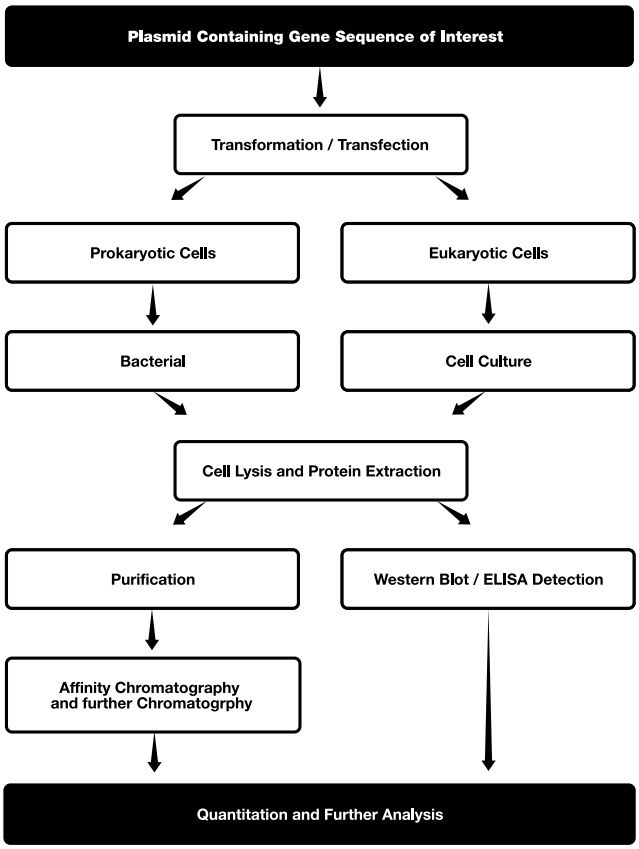


Figure 1.1. Gene Design For Recombinant Protein Production.

Choice of Cell Expression System

Once the cell expression system has been determined for recombinant protein production, the gene construct to express the recombinant protein needs to be designed. This could be as simple as taking the coding sequence of the gene of interest as it exists in the parent organism and inserting this sequence into an expression plasmid for the cell system being used. However, this usually does not produce optimal levels of recombinant protein when the gene is expressed in a heterologous system (a cell or organism different from the one where the gene is naturally found) because the preferred codon usage for various species differs. For example, the codon GGA for glycine can be found at a frequency of 16.4 times per 1000 codons in human genes while it is only used 9.5 times per 1000 codons in *E. coli* genes. Therefore, there is a chance that leaving this codon in the recombinant gene might lead to lower levels of recombinant expression due to a scarcity of tRNA molecules for GGA.

A second consideration for recombinant gene design is whether or not the protein of interest is expressed in a soluble or in an insoluble form. If a protein is expressed in an insoluble form, it can be easier to initially separate it from components, such as nucleic acids, phospholipids and soluble proteins, by centrifugation. An insoluble protein is also relatively protected from the action of proteolytic proteins that are present in the host cell that can be released upon lysis of the cell. However, if a fully functional recombinant protein is desired, it is necessary to refold the insoluble protein to its native conformation, which many times proves extremely problematic especially if the fully refolded protein has disulfide bonds and multiple subunits.

The ability to express a recombinant protein in the soluble form is partially dependent on the protein being expressed as well as the rate of expression of the recombinant protein. If the protein is expressed at an extremely high rate, it could overwhelm the native proteins involved in folding proteins (such as chaperonins) in the cell host. The rate of expression can be controlled by the promoter system involved such as the T7 polymerase system used commonly in *E. coli*.

A final consideration for recombinant protein gene design is how the recombinant protein will be purified from the other host cell components. Some recombinant proteins, such as antibodies, have a specific antigen against which they were raised and hence can be purified by binding to that molecule (affinity chromatography).* Other recombinant proteins have a very large positive or negative charge associated with them and can be purified by binding to charged resins (ion exchange chromatography). Some proteins do not have any strong distinguishing property, and an affinity tag, such as GST or a histidine tag can be added as a DNA sequence to the gene of interest at either the 5' or 3' end of the recombinant gene. The tag attached to the protein enables the specific purification of the recombinant protein using affinity chromatography methods.

*Antibodies can also be purified by binding to Protein A, a surface protein of *Staphylococcus aureus* which has high affinity for immunoglobulins. Protein A is commonly used for the first step in the purification of antibodies in industrial applications.

DHFR—Our Protein of Interest

The Protein Expression and Purification Series focuses on the protein dihydrofolate reductase (DHFR), which is essential for proper cell function and illustrates the importance of basic oxidation–reduction enzymatic reactions.

What is DHFR?

Dihydrofolate reductase, DHFR, is an enzyme that converts dihydrofolate, a folic acid derivative, into tetrahydrofolate (THF) by the addition of a hydride from NADPH. Tetrahydrofolate is a methyl group shuttle required for the synthesis of purines, thymidylic acid, and amino acids, all essential for nucleic acids. DHFR is ubiquitous in prokaryotic and eukaryotic cells, and is found on chromosome 5 in humans. Deficiency in DHFR has been linked to megaloblastic anemia, an anemia disorder with larger-than-normal red blood cells, as well as cerebral folate metabolism disorders. Both are treatable with folic acid and/or Vitamin B_{12}, depending on symptoms. Refer to Bio-Rad's Size Exclusion Chromatography kit (catalog# 166-0008EDU) for an introductory exercise separating Vitamin B_{12} from hemoglobin.

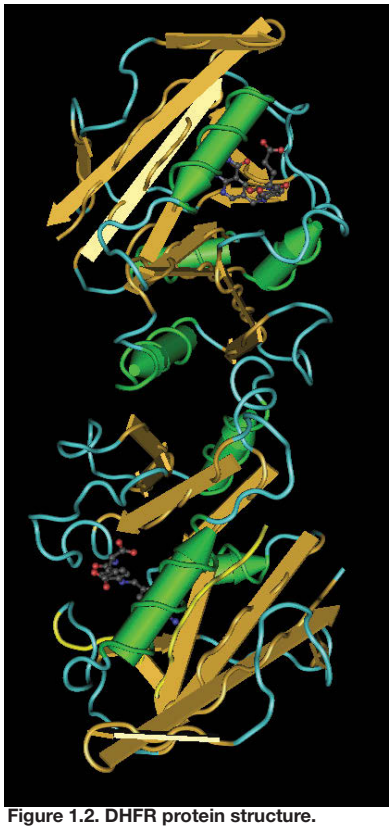


Figure 1.2. DHFR protein structure.

Being able to control DHFR makes it a powerful tool not only for research and gene manipulation but also for medical treatments for cancer and malaria. When DHFR is inhibited or reduced, it

leads to a shortage of thymidylates, interfering with nucleic acid synthesis. A lack of nucleic acid synthesis thus interferes with cell growth, proliferation, and ultimately causes cell death.

DHFR Cancer Connection

Cancer occurs when a particular cell loses the ability to control its division. These dividing cells spread, displace normal cells, disrupt the architecture of tissues, and use up the nutrients required by normal cells. Surgery can remove most of the cells in a solid tumor, but malignant cancers send out colonizing cells called metastases that use the blood and lymph systems to spread far from the tumor and non-solid tumors, such as leukemia or lymphoma, are not confined to one specific area. Clinicians use radiation or chemotherapy to kill these cells.

Chemotherapy drugs target cancer cells by disrupting the functions of actively dividing cells. This strategy exploits the fact that most of the cells in an adult are not dividing. Therefore, chemotherapy damages the rapidly dividing cancer cells by disrupting structures required for mitosis, like spindle fibers, or by disrupting the production of nucleotides required for DNA replication. One of the first chemotherapeutic agents was methotrexate, a folic acid analog that interferes with folic acid metabolism. Treatment with methotrexate limits the ability of dividing cells to make nucleotides by competitively inhibiting the enzyme dihydrofolate reductase (DHFR). When the enzyme DHFR is inhibited, cancer cells cannot divide and spread.

Occasionally after repeated treatments with methotrexate, a patient's cancer will develop a methotrexate resistance and will stop responding to the drug. Some of these resistant cells when examined show that the resistance was due to an increased copy number of DHFR genes. This gene amplification leads to increased levels of DHFR protein in the cell and therefore an increased ability to catalyze its reaction and produce nucleotides, even in the presence of methotrexate.

DHFR and Malaria

DHFR is also integral to parasite cell metabolism. Malaria is caused by the parasite Plasmodium falciparum, which is transmitted to humans by mosquitoes. Once in the human bloodstream, the parasite multiplies, eventually causing headaches, fever, coma, and ultimately, death if untreated.

Like in humans, interrupting the DHFR pathway in Plasmodium leads to reduced DNA synthesis: blocking DHFR successfully blocks Plasmodium falciparum multiplication. There are many drugs to treat malaria, but drug-resistant strains are becoming more and more common. Strains resistant to the once effective DHFR inhibitors pyrimethamine, sulphadoxine, and methotrexate are appearing.

Having a system to produce recombinant DHFR to study its enzymatic activity and develop inhibitors for chemotherapy or antimalarial drugs could be a powerful tool in developing new therapeutics. Scientists continue to search for effective drugs to stop the spread of malaria and cure those infected with malaria. (See Appendix J for a link to the World Health Organization's site on malaria.)

Use of DHFR in Biomanufacturing

The regulation of the DHFR gene amplification phenomenon described above is used to produce genetically engineered CHO cells to biomanufacture particular therapeutic proteins. Cells containing the gene of interest and DHFR are treated with methotrexate leading to the amplification of the DHFR gene; since the gene of interest lies next to the DHFR gene, the transgene is amplified too. This increases the amount of protein produced by the cells. Individual clones are separated and independently tested for their ability to produce protein and to divide. A particular clone that produces a large amount of protein and that retains its ability to divide quickly, will become the master cell bank from which all subsequent cells for production runs will be pulled.

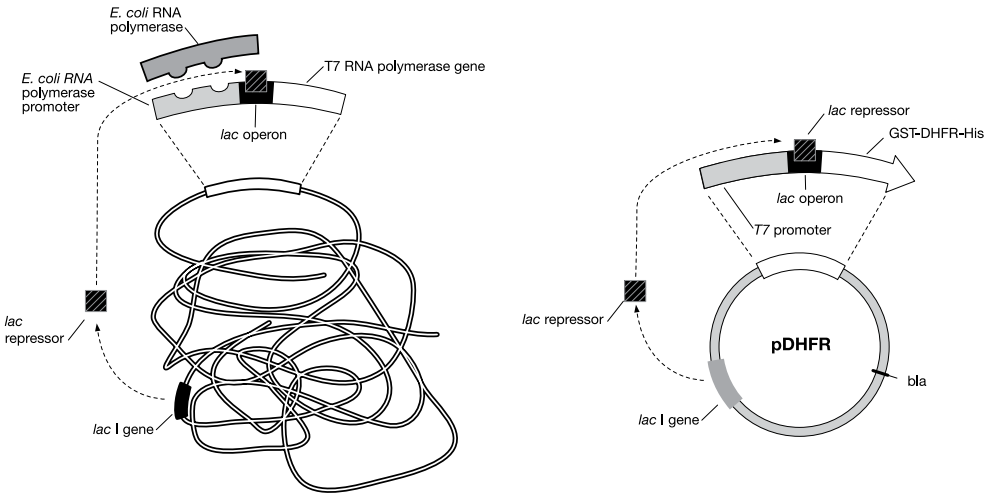
(For more information on biomanufacturing see Appendix G.)

Recombinant Production of DHFR Cell Selection

In this series of laboratory exercises, you will express the human DHFR gene in *E. coli*, a recombinant protein host system that is used extensively for research and industrial purposes. The bacteria have a quick doubling time (20 minutes) and are easy and inexpensive to culture, induce, and lyse to release cell contents. Also, posttranslational modifications such as phosphorylation and glycosylation are not required for the human DHFR to function properly, also making *E. coli* a good selection.

Plasmid Selection

A high level of expression with tight regulation is desired so the pET21a plasmid system will be used. This plasmid contains the constitutively expressed β-lactamase gene that confers resistance to ampicillin. The pET21a vector used contains the T7 promoter. This vector is used with a specific type of *E. coli*, the BL21(DE3) strain. This strain has been engineered to contain the T7 RNA polymerase gene (a gene that is not endogenous to bacteria) placed under the control of the inducible lac promoter. Addition of lactose or its analog IPTG to the growth medium, will induce the expression of the T7 RNA polymerase and, thus, the expression of any gene placed downstream of the T7 promoter. In the absence of the inducer, there is no expression of the gene.



Figures 1.3.a (above) and 1.3.b (below). Protein expression control using the pDHFR plasmid in a BL21(DE3) *E. coli*.

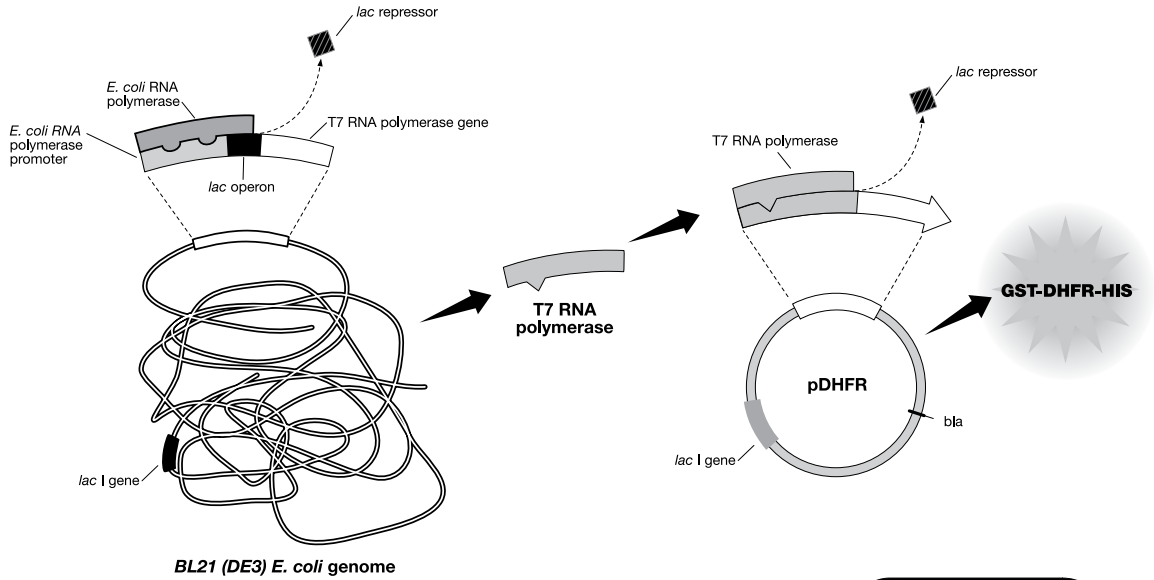


Figure 1.3a shows the use of the pDHFR plasmid in BL21(DE3) *E. coli* having tightly controlled expression of GST-DHFR-His. Both the BL21(DE3) *E. coli* genomic DNA and the pDHFR plasmid contain the lac I gene which codes for the lac repressor protein. Both the BL21(DE3) *E. coli* genomic DNA and the pDHFR plasmid also have recombinant genes that are inserted after a lac operon site. In the BL21(DE3) *E. coli* genomic DNA, the inserted gene codes for bacteriophage T7 RNA polymerase. In the pDHFR plasmid, the inserted gene codes for the fusion protein GST-DHFR-His. Before the lac operon, the BL21(DE3) *E. coli* genomic DNA has a native *E. coli* RNA polymerase promoter site. This is different than on the pDHFR plasmid where before the lac operon, there is a bacteriophage T7 RNA polymerase promoter site. The lac repressor protein is constitutively expressed, and when there is no lactose or its analog IPTG present, the lac repressor protein binds to the lac operon on both the genomic and plasmid DNA, preventing binding of the appropriate RNA polymerase and transcription of genes downstream from the lac operons, T7 RNA polymerase and GST-DHFR-His, respectively.

Figure 1.3b shows that once lactose or its analog IPTG has been added, the lac repressor protein detaches from the lac operon on both the genomic DNA and the pDHFR plasmid. This allows binding of the constitutively expressed *E. coli* RNA polymerase to the *E. coli* RNA polymerase promoter site on the genomic DNA and starts the transcription of the bacteriophage T7 RNA polymerase gene. Once the bacteriophage T7 RNA polymerase gene has been transcribed and translated into protein, the bacteriophage T7 RNA polymerase binds to the promoter region on the pDHFR gene. Once bound, the bacteriophage T7 RNA polymerase transcribes the recombinant gene GST-DHFR-His coded for on the pDHFR plasmid.

Gene Design

When expressed in *E. coli* using the pET21a system, the human DHFR is insoluble. To increase its solubility, a GST tag was added to its N-terminus. A six histidine sequence (polyhistidine tag) was added to the C-terminus of DHFR to allow for easy purification by metal affinity chromatography. The resulting plasmid codes for a GST-DHFR-His fusion protein that can be expressed in *E. coli* BL21(DE3).

Culturing *E. coli* for Protein Expression

The starting point in the Protein Expression and Purification Series is lyophylized BL21(DE3) *E. coli* containing pDHFR which will be rehydrated and plated to generate individual colonies. An initial culture is grown to saturation from a single bacterial colony picked from a Petri dish. This culture is used to initiate a larger culture that is grown to mid-log phase at which stage expression of the recombinant protein is induced by addition of IPTG to the medium. Determining when cells have reached their mid-log phase of growth is accomplished by measuring the absorbance of the culture at 600 nm. An OD600 of 0.6–1.0 is the typical target for induction. At this point the cells are dividing rapidly, and production of the protein will be optimal.

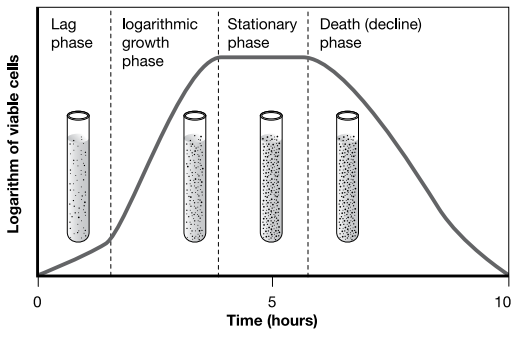


Figure 1.4. Depiction of cell growth phase in relation to overall culture viability.

For the GST-DHFR-His construct in the BL21(DE3) *E. coli* an overnight culture containing 1% glucose is grown from a single colony on an LB/amp plate. The 1% glucose is added to ensure that the lac operon remains repressed and no T7 RNA polymerase or GST-DHFR-His is expressed. Leaky expression (not actively induced) of recombinant proteins is generally undesirable because expression of the recombinant protein may be toxic and prevent bacterial growth. The overnight culture (late log/stationary phase) is diluted in fresh LB medium and allowed to grow until mid-late log phase.

At mid-log phase, expression of GST-DHFR-HIS is induced by addition of IPTG. Cells will use their internal

biochemistry to produce the recombinant protein and will not divide as quickly or at all. After induction has been completed, cells are recovered by centrifugation, and protein is extracted from the cell pellet.

Introduction to Protein Purification

Protein purification is an important step in biotechnology workflows. It is the isolation of the protein of interest so that it may be used in subsequent research, for diagnostic tests, or for pharmaceutical production. The purity needed depends on its end use. For proteins used in research 90–95% purity may be sufficient but for proteins used for pharmaceutical applications, much higher purity levels (up to 99.99%) must be reached. How purification is done will depend on the type of protein engineered, the volume of protein to be purified, the degree of purity required, and the availability of special laboratory equipment.

The first step in the purification workflow is to extract the protein from the cells by lysing, or breaking them open. Techniques used for lysing cells depend on what type of cell—bacterial, plant, or mammalian—was used to produce the protein as well as where the protein is produced in the cell. Freeze-thaw cycles, enzymatic digestion, chemical breakdown, and mechanical disruption methods such as sonication or grinding (mortar and pestle) are some of the cell lysis techniques available. In this laboratory the *E. coli* cells are lysed in a buffer containing lysozyme, an enzyme which digests the cell wall, along with freeze-thaw cycles. Once the cells are lysed, the soluble and insoluble components are separated by centrifugation. The insoluble components form a pellet at the bottom of the tube while soluble components will remain in the aqueous phase. (Not only proteins are found in the cell lysate, but also nucleic acids, sugars, phospholipids and other cell components.) When present in high concentration, nucleic acids (mostly genomic DNA) render the aqueous phase viscous making it difficult to isolate the recombinant protein. To reduce viscosity the genomic DNA can be broken down using sonication (ultrasound), a French Press that shears the DNA by pushing the cell lysate through a narrow opening at very high pressure or shearing the lysate using a narrow gauge syringe needle. These treatments break the genomic DNA down into small fragments and reduce viscosity. The DNA can also be degraded enzymatically by addition of a DNase. A too viscous soluble fraction of protein can be problematic since it may clog the resin and the frit (bed support) of the chromatography column during the subsequent protein purification step.

Isolating the Recombinant Protein of Interest

Proteins are usually purified by chromatography. There are a variety of chromatographic methods to choose from; the method used will depend on the protein's physicochemical properties. In the following sections some of the common chromatographic techniques available are described.

Introduction to Protein Chromatography

Chromatography is commonly used in biotechnology for separating biological molecules, such as proteins, from complex mixtures. Chromatography consists of a mobile phase (solvent and the molecules to be separated) and a stationary phase, such as paper (in paper chromatography) or glass, resin, or ceramic beads (in column chromatography), through which the mobile phase travels. The stationary phase is typically packed in a cylinder known as a column. Molecules travel through the stationary phase at different rates or bind to the solid phase based on their physicochemical properties.

The liquid used to dissolve the biomolecules to make the mobile phase is usually called a buffer. In column chromatography the mixture of biomolecules dissolved in the buffer is called the sample. The sample is allowed to flow through the column bed, and the biomolecules within the buffer enter the top of the column bed, filter through and around the beads, and ultimately pass through a small opening at the bottom of the column. For this process to be completed additional buffer is placed on the column bed after the sample has entered the bed. The mobile phase liquid is collected, as drops, into collection tubes that are sequentially ordered. A set number of drops, known as a fraction, is collected into each tube. Fractions are collected so that they may later be analyzed to see which one or ones contain the protein or proteins of interest.

Chromatography Techniques

There are many ways to perform liquid column chromatography. The choice of chromatography media and buffers depends on the properties of the protein of interest to be purified.

Hydrophobic Interaction Chromatography (HIC) separates molecules based on their hydrophobicity. Hydrophobic (water-fearing) substances do not mix well with water. Exposing a hydrophobic protein to a high salt buffer causes the three-dimensional structure of the protein to change so that the hydrophobic regions of the protein are more exposed on the surface of the protein and the hydrophilic (water-loving) regions are more shielded. The sample in high salt buffer is then loaded onto a chromatography column packed with hydrophobic interaction beads. The hydrophobic proteins in the sample will stick to the beads in the column. The more hydrophobic the proteins are, the more tightly they will stick. When the salt is removed by flowing in a low salt buffer through the column, the three-dimensional structure of the protein changes again so that the hydrophobic regions of the protein now move to the interior of the protein, and the hydrophilic regions move to the exterior. The result is that the hydrophobic proteins no longer stick to the beads and elute (wash out) from the column, separated from the other proteins.

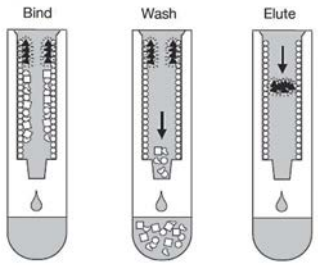


Figure 1.5. Depiction of HIC separation of molecules based on hydrophobicity.

Size Exclusion Chromatography (SEC), also known as gel filtration or desalting chromatography, separates molecules based on their shape and size. The solid phase is made of gel beads that have pores of varying size (think of them like wiffle balls). Larger molecules cannot enter the pores and are excluded, so they merely flow between the beads and are eluted first. Smaller molecules can enter the pores and therefore will take longer to flow down the column. Typically, size exclusion columns are tall, narrow columns so that there is a long path for the molecules to flow through or to be retained by the pores and better separated from each other. Size exclusion chromatography can also be used to exchange the buffer that the molecule of interest is currently in for another buffer.

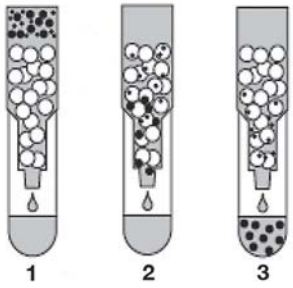


Figure 1.6. SEC separation of molecules based on size. 1) A mixture of large and small proteins is applied to a column of porous beads. 2) As the buffer flows down the column, the small proteins penetrate into the beads and are slowed. 3) The larger protein molecules emerge from the column first.

Ion Exchange Chromatography (IEX) beads have either a positive (cation) or negative (anion) charge. During ion exchange chromatography, the protein of interest binds to the oppositely charged beads. If the charge of the beads is positive, it will bind negatively charged, or anionic, molecules. This technique is called anion exchange (AEX) chromatography. If the beads are negatively charged, they bind positively charged, or cationic, molecules, called cation exchange (CEX). A scientist picks the resin based on the charge of the protein of interest. After contaminants are separated from the protein of interest, a high salt buffer is used to disrupt the interaction and to elute the protein of interest from the column.

Resin Type	Anion Exchanger	Cation Exchanger
Net Charge of Molecule of Interest	-	+
Charge of Resin	+	-

For example, in anion exchange chromatography, the resin beads have a molecule with a positive charge covalently attached to the resin. The molecules to be separated flow across the resin beads and any positively charged molecules are repelled and do not stick to the column, exiting the column with the flow of the buffer. Negatively charged molecules will bind, or adsorb, to the column. The column is then washed with buffer of increasing salt concentration, and those molecules that are more tightly bound will elute. Cation exchange chromatography works in a comparable way except that the resin beads have molecules with negative charges covalently bound to them.

In ion exchange chromatography knowing the isoelectric point (pI) of the protein allows researchers to manipulate the charge of the protein. The pI of the protein is where the protein has equal positive and negative charges. A buffer with pH higher than the pI will give the protein a negative charge; a buffer with pH below the pI of the protein will have a more positive charge. This change of charge depending on buffer pH can be used to elute protein from ion exchange columns by changing buffer pH.

Mixed Mode, or Multimodal, Chromatography resins combine more than one type of chromatography technique, such as having both anion and cation exchange properties on the same bead. Based upon the properties of the molecule of interest and the buffers used, this can be a very selective chromatography method.

In **Affinity Chromatography (AC)**, a ligand with specific affinity for the molecule to be isolated is covalently attached to the beads. A mixture of proteins is added to the column and everything passes through except the protein of interest which binds to the ligand and is retained on the solid support. The desired molecule is primarily eluted by adding a molecule that competes for the ligand.
The affinity chromatography methodology depends on the presence of a specific tag on the recombinant protein such as a polyhistidine (His) affinity tag; a fusion protein partner, such as maltose binding protein or glutathione-s-transferase (GST-tag), or an antibody-antigen interaction such as protein A or protein G and different classes of antibodies.

Polyhistidine-tagged proteins bind to nickel groups that have been attached to the resin, known as immobilized metal affinity chromatography (IMAC). The histidine groups of the polyhistidine-tag bind to the Ni^{++} groups on the resin. The protein of interest can be eluted by the addition of imidazole which competes for nickel binding sites. A second example is using a fusion partner such as GST. This protein will bind to a resin bead coated with glutathione. In order to elute the recombinant protein fused with GST, glutathione is added to the mobile phase and competes with the binding site on the GST and the fusion protein elutes.

Figure 1.7. Depiction of the interaction of the Ni^{++} groups on the resin and the polyhistidine tag.

In this laboratory purification affinity polyhistidine tag chromatography (more specifically, immobilized metal affinity chromatography (IMAC)), will be used to purify DHFR. The recombinant DHFR in this laboratory also has a GST-tag, but in this instance the tag was added to make the protein more soluble and increase the overall molecular weight of the fusion protein. It would be possible to use the GST-tag in a second round of purification. In research and industrial purification of proteins more than one chromatographic method is needed to reach the level of purity desired.

Chromatography Methods

Once the chromatographic purification strategy is chosen for the target protein, a decision needs to be made as to how the chromatographic separation will be performed.

Batch Purification

The simplest way to perform chromatography is in a beaker. To do this, the chromatography resin of choice is resuspended in buffer in a beaker. The sample is added. The beaker is either gently swirled to mix the sample and resin, or a stir bar and stir plate may be used, being careful not to damage the resin. Next, the resin is allowed to settle, the buffer is decanted and wash buffer is added to the beaker. Again, the resin is mixed, and the buffer is decanted. Finally, an elution buffer is added and the decanted buffer is saved since that is where the protein of interest should be. This method may work well for purifying crude extracts where quantity, and perhaps quality, of protein is not a concern.

Gravity Chromatography

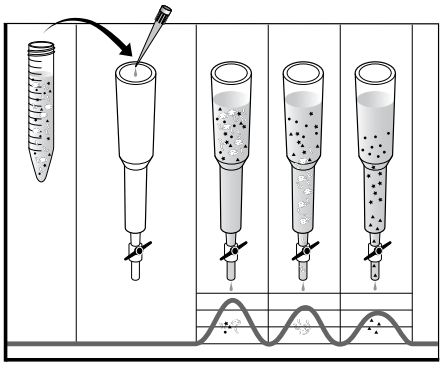


Figure 1.8. Gravity column set-up

The most common way to purify protein is by column chromatography using gravity flow. In gravity chromatography the resin is resuspended in buffer and poured into a column. The column is placed upright in a stand and the resin is allowed to settle into what is known as the resin bed. Once the resin has settled, the sample is loaded onto the column. As the sample flows through the column, buffer is added to the column so that the top of the resin bed stays wet and there is a pressure head to continuously push the sample and buffers through the resin. The buffer and sample rely on gravity to move through the column. During the chromatography process fractions are collected. Samples from these fractions are tested for purity and for the presence of the protein of interest.

The main advantage of gravity chromatography is that it is an inexpensive method of purification. A glass or plastic column, buffers, sample, a column holder, and test tubes to collect the fractions are all that is needed. Columns, and sometimes resin, can be cleaned and reused multiple times. The expense of this method of chromatography is time. A person must monitor the column so that it does not go dry, ensure the correct buffers are used when they should be used, and collect fractions. Gravity chromatography is typically used with larger diameter chromatography beads and softer resins. There needs to be minimal resistance to flow due to gravity or this will not work. (Think of trying to flow water through a small diameter of sand versus around pebbles or rocks.)

Spin Column Chromatography

Figure 1.9. Spin column.

A variation on gravity chromatography is the use of a spin column. Spin columns are typically small (3–5 cm in length) plastic chromatography columns that fit in a standard microcentrifuge. They come empty or in many cases prepacked with resin. The sample volume that can be applied to the column is limited by the small size of the column and by how much protein can bind to the amount of resin in the tube; the advantage, however, is that this is a quick way to perform chromatography. Simply load the resin or add buffer to a prepacked column, spin in the centrifuge, add sample in buffer, spin again to bind the protein, add elution buffer, and spin again. The gravitational force from the spin "pulls" the buffer and sample through the column. The spin column fits in a centrifuge tube, and after each spin the column is moved to a fresh tube so at the final spin the protein of interest will be in the elution tube. Each resin or prepacked column has a spin protocol to follow on how to use it in a spin column and how fast to spin it. (See Appendix C for more information on centrifuge spin speed.)

The advantage of the spin column is that it is quick and relatively inexpensive in terms of columns, resin, and time. Many spin columns come prepacked with popular resins. Most spin columns are single use. They require a centrifuge and sometimes require a certain rotor (the inside of the centrifuge, where the tubes are placed), that will depend on the length of the spin column and type of chromatography resin used.

Prepacked Chromatography Cartridges

Figure 1.10. Prepacked chromatography cartridge.

Prepacked cartridges are a good choice for chromatographic needs when sample size is too large for a spin column, but a short purification time is desired. Cartridges are convenient as they are prepacked with resin, consistent in quality of resin bed from column to column, and disposable. Most cartridges have Luer-Lok or similar screw-like connections that allow them to be used with a syringe with a Luer-Lok end, a mechanical pump or chromatography system.

The simplest way to use a prepacked cartridge is with a Luer-Lok syringe. The syringe is used to deliver the buffers and sample in sequence and collect the eluate or fractions in test tubes. For more complex purifications or for a hands-off approach, a pump can be used to deliver the sample and buffers through the columns. Some monitoring of the pump may be needed depending on the programming capabilities. The cartridge can also be connected to a fraction collection system or apparatus.

Chromatography Systems

Most large-scale purifications, when the sample volume is milliliters to liters and columns range in size from smaller than a tube of toothpaste to 100 L and larger, are performed using a chromatography system. The system will allow for multiple buffers and columns to be used. Systems can range from low pressure to high pressure, and basic to complex. As the complexity of the chromatography equipment rises, so does the price. The decision of which system to use depends on the purification scheme.

Figure 1.11. Bio-Rad BioLogic System.

Protein Expression and Purification Series Laboratory Background

Chromatographic purification of GST-DHFR-His

The purification of the GST-DHFR-His is accomplished in two steps: First the protein will be purified using IMAC. As previously described, the IMAC resin selectively binds polyhistidine-tagged proteins. All other biomolecules will flow through the column. The column is first equilibrated in 20 mM sodium phosphate, 300 mM NaCl and 5 mM imidazole. The 300 mM NaCl prevents the non-specific binding of charged molecules in the *E. coli* lysate soluble fraction from binding to the column. Since imidazole has a similar structure to histidine (see figure 1.12), the 5 mM imidazole prevents non-specific binding of any *E. coli* proteins which contain multiple histidine residues, but the imidazole is not at a high enough level to prevent the binding of the polyhistidine tag on the GST-DHFR-His to the Ni-IMAC beads. After the lysate is added and the GST-DHFR-His binds to the resin and the majority of *E. coli* proteins flow through without binding, the column is washed. The wash buffer contains the same 20 mM sodium phosphate for buffering and 300 mM NaCl to prevent non-specific ionic binding, but also contains a higher level of imidazole. This 10 mM imidazole is slightly more stringent and will wash away many of the *E. coli* proteins that were able to bind under the 5 mM imidazole condition. However, 10 mM imidazole is not stringent enough to effectively compete with the binding of the polyhistidine tag so the GST-DHFR-His remains bound to the column beads during the wash step. GST-DHFR-His is eluted from the column with a buffer containing 250 mM imidazole. At 250 mM, the imidazole is at a much higher concentration than the GST-DHFR-His effectively competing with the polyhistidine tag for binding to the Ni^{++} groups of the resin. There are a lot more molecules of imidazole present than GST-DHFR-His so the GST-DHFR-His is knocked off the Ni^{++} binding sites and elutes from the column.

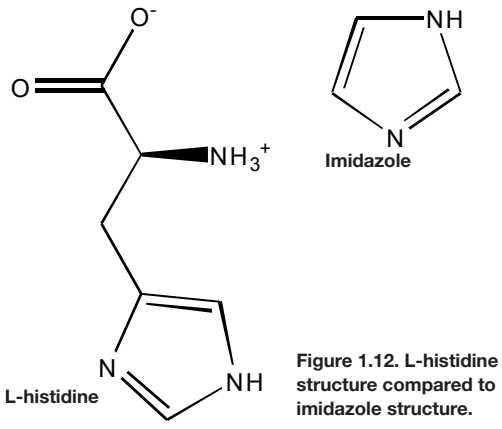


Figure 1.12. L-histidine structure compared to imidazole structure.

The second step in the purification of GST-DHFR-His is removing excess salt and imidazole from the sample. If the sample is not desalted, during subsequent polyacrylamide gel analysis, sample bands may be fuzzy, broad, skewed, or otherwise distorted. The desalting also removes the imidazole, which interferes with measuring the absorbance at 280 nm, used to estimate protein quantity.

Desalting the sample is performed using a size exclusion gel prepacked in a spin column. The gel, Bio-Gel P-6, has a fractionation range of 1,000 to 6,000 Da. This means that the pores of the gel are large enough to allow molecules in the 1 to 6 kD range, like salts and small proteins, to enter, but larger molecules, such as GST-DHFR-His, with a molecular weight of 52 kD, will be excluded and flow through the column. In essence, the salts (imidazole, NaCl and phosphate buffer) are trapped in the gel while the protein of interest comes out "clean" in a Tris buffer.

Methods To Quantify Protein Concentration and Check Protein Purity

Once the protein of interest (GST-DHFR-His) is purified it is necessary to check its purity and determine the quantity of protein purified. There are multiple ways to perform these tasks.

Absorbance at 280 nm

The aromatic amino acids (tryptophan, and to a lesser degree tyrosine and phenylalanine) in proteins absorb at 280 nm. If the extinction coefficient (a parameter that helps define how well a substance absorbs light at a specific wavelength at a particular concentration) is known, the amount of protein present can be calculated using Beer's Law (absorbance = εcL) where ε is the extinction coefficient, c is concentration and L is the pathlength of light. If the extinction coefficient is not known, there are computer programs, such as that from Expasy (see Appendix J), that can calculate an approximate extinction coefficient from empirical relationships.

It should be noted that other molecules such as imidazole absorb at 280 nm and can interfere with calculations. The buffer in which the sample is measured is critical for A280 measurements. This is one of the reasons why the second purification step in this series is performed. It is a desalting step to remove the imidazole from the GST-DHFR-His so that the A280 measurement can be taken.

Colorimetric Protein Assays

There are multiple colorimetric protein assays that have been developed to determine protein concentrations. The first—the Bradford protein assay—is based on a shift in the maximum absorbance of a colored dye, Coomassie Brilliant Blue G-250. The dye interacts mainly with basic amino acid groups (arginine and lysine) as well as aromatic amino acids (phenylalanine, tyrosine and tryptophan). A second method—the Lowry method—is based on reaction of the protein with an alkaline copper tartrate solution and Folin reagent. In the case of the Lowry method, color is mainly due to the presence of tyrosine and tryptophan as well as cysteine and histidine. Each of these assays has its advantages and disadvantages, namely the compatibility of the assay with reagents in the buffer as well as sensitivity.

SDS-PAGE Analysis

Another method of determining which fractions contain the purified protein as well as the progress of the protein purification procedure, is to analyze samples by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). This method is not quantitative unless a protein standard of known concentration is run in one of the lanes and quantitative software is used to examine the gel image. It will, however, allow the determination of which fractions contain the protein of interest if the molecular mass is known and at what relative concentration. It will also allow the assessment of the purity of the protein fraction. If the protein is pure, a single band should be visible on the gel, even when large quantities of the samples are loaded.

Protein Structures and Basic Properties

In contrast to DNA, which is quantified in terms of its length (i.e., the number of base pairs), proteins are quantified in terms of their molecular weights relative to a hydrogen atom, in Daltons. This is because DNA is composed of only 4 nucleotides, which are in roughly equal proportions and about the same molecular weight. Proteins on the other hand are composed of 20 amino acids with molecular weights from 89 to

204 Daltons (the average is 110). They vary considerably in amino acid composition. One Dalton equals the mass of a hydrogen atom, which is 1.66 x 10^{-24} grams. Most proteins have masses on the order of thousands of Daltons, so the term kilodalton (kD) is used for protein molecular masses. Proteins range in size from several kilodaltons to thousands of kilodaltons but most fall between the range of 10 kD and 220 kD. DHFR-GST-His has a primary structure of 410 amino acids, a total molecular weight of 52,000 daltons, or 52 kD.

Using Gel Electrophoresis to Separate and Identify Proteins

A protein's electrical charge and its mass affect its mobility through a gel during electrophoresis. The ratio of charge to mass is called charge density. Since every protein is made of a unique combination of amino acids, the net charge of each protein may be different. The electric charge of proteins must be removed as a factor affecting migration in order for polyacrylamide electrophoresis to be effective as a method of protein molecular weight determination. The intrinsic charges of proteins are masked by placing a strongly anionic (negatively charged) detergent, sodium dodecyl sulfate (SDS), in both the sample buffer and the gel running buffer. SDS binds to and coats the proteins and also keeps them denatured as relatively linear chains. In this form, proteins migrate in a polyacrylamide gel as if they have equivalent negative charge densities, and mass becomes the main variable affecting the migration rate of each protein. (**Note:** Posttranslational modifications such as glycosylation can also affect protein migration).

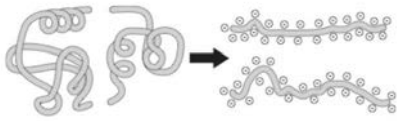


Fig. 1.13. The combination of heat and the detergent SDS denatures proteins for SDS-PAGE analysis.

Aside from obscuring protein charge with SDS, to effectively determine the molecular weight, the secondary (2°), tertiary (3°), and quaternary (4°) structures of the protein complexes within a protein extract are also disrupted prior to electrophoresis. This process of structural disruption is called **denaturation**. A reducing agent, such as β-mercaptoethanol (BME) or dithiothreitol (DTT), is sometimes added to samples to ensure complete breakage of disulfide bonds. (In the case of GST-DHFR-His, no reducing agent is needed as there are no disulfide bonds in the protein structure.) Three factors—heat, ionic detergent, and reducing agent—completely disrupt the 2°, 3°, and 4° structures of proteins and protein complexes, resulting in linear chains of amino acids. The denatured amino acid chains move through the gel at rates proportional to their molecular masses.

Performing Electrophoresis

In this lab, the induction of expression of GST-DHFR-His, solubility of the expressed GST-DHFR-His, and success of purification will be analyzed by SDS-PAGE. To do this, a portion of the fractions will be run on an SDS-PAGE gel against a protein standard of known molecular weight. The samples of the fractions have Laemmli sample buffer added to them and then will be loaded into the wells of a polyacrylamide gel. Once the gel is placed in the electrophoresis cell, the lower and upper chambers are filled with running buffer. The lower part of the cell contains the anode, or positive pole, and the top one contains the cathode, or negative pole. Once assembled, the electrophoresis cell is connected to a power supply. The proteins, now negatively charged due to the presence of SDS, will flow with the electric field toward the anode. When the dye front (from the sample loading dye) reaches the end of the gel, electrophoresis is stopped. The gel will be stained with the Coomassie blue dye. The stained gel will allow students to check the purity of their fractions and quality and relative quantity of the recombinant protein produced.

DHFR Enzyme Activity

Dihydrofolate reductase is a critical enzyme necessary for the conversion of dihydrofolate (DHF) to tetrahydrofolate (THF). This reaction also requires the presence of the cofactor NADPH (nicotinamide adenine dinucleotide phosphate). A **cofactor** is a molecule that is required to be bound to the enzyme in order for the reaction to occur. NADPH is a cofactor that carries electrons from one reaction to another for use in metabolic enzymatic reactions.

The conversion of DHF to THF by dihydrofolate reductase in the presence of NADPH is an example of a classical oxidation-reduction reaction. Oxidation is defined as the loss of at least one electron, while reduction is the gain of that electron. In the case of organic compounds, oxidation is the loss of a hydride ion (H^-), or a negatively charged hydrogen ion that consists of two electrons and one proton. The NADPH donates a hydride ion to DHF, and when this hydride ion reacts with a specific bond of DHF, it removes the existing double bond and is covalently bound to the carbon group producing THF. Dihydrofolate reductase catalyzes the reactions shown in Figure 1.14. As shown, the NADPH loses a hydride ion that is transferred to DHF converting it to THF.

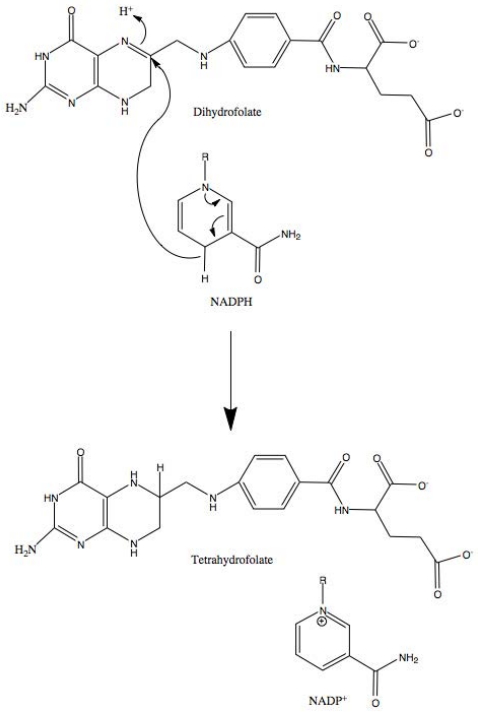


Figure 1.14. The conversion of DHF to THF. NADPH transfers a hydride ion to DHF, converting it to THF.

To measure the enzymatic activity of dihydrofolate reductase, a methodology needs to be developed to track the disappearance of NADPH or DHF or conversely to measure the appearance of $NADP^+$ or THF. For every molecule of NADPH that is converted to $NADP^+$ by the enzyme dihydrofolate reductase, one molecule of THF is produced from the substrate DHF. Unfortunately, none of these four compounds are colored so there is no way to directly visualize the reaction. However, it is possible to measure the rate of this reaction using a spectrophotometer than can detect light in the UV range. NADPH absorbs light in the UV range at 340 nm. However, when NADPH is converted to $NADP^+$, it no longer absorbs at 340 nm. Therefore, by measuring the decrease of absorbance at 340 nm of a reaction containing a known quantity of DHF, NADPH and dihydrofolate reductase, the reaction rate that NADPH is converted to $NADP^+$ can be calculated.

In this laboratory series, students will recombinantly make and purify human dihydrofolate reductase as a fusion construct with GST and a histidine tag (GST-DHFR-His). The concentration of the purified GST-DHFR-His will be calculated using the proteins' intrinsic absorbance of UV light at 280 nm. This known concentration of GST-DHFR-His will then be first combined with a known quantity of NADPH. Since no DHF substrate is present, the NADPH should not be reduced and the absorbance at 340 nm over time should be constant. Once the substrate DHF is added to the solution containing the purified GST-DHFR-His and NADPH, the absorbance at 340 nm should decrease over time as the reaction occurs converting NADPH to $NADP^+$ and DHF to THF.

Chapter 2: Focus Questions

Focus Questions for DHFR Cancer Connection

1. What is DHFR? Why is it important?

2. What role does DHFR play in cancer?

3. How does methotrexate interfere with DHFR?

4. What reaction does DHFR catalyze? What is the cofactor that is necessary for this reaction to occur?

5. Name the primary inhibitor of DHFR. How does this inhibitor affect its action?

Focus Questions for Protein Expression

1. What is a recombinant protein, and why would it be used instead of a native protein?

2. Name at least one pro and one con of using eukaryotic cells to produce a recombinant protein. When would you choose to use eukaryotic cells to produce a recombinant protein?

3. Name at least one pro and one con of using prokaryotic cells to produce a recombinant protein. When would you choose to use prokaryotic cells to produce a recombinant protein?

4. What are three considerations for recombinant protein gene design?

5. What is the log phase of a cell culture, and why is it important to recombinant protein production?

6. Why are subcultures prepared when trying to produce recombinant proteins?

7. What is the purpose of adding glucose to culture media for lac operon systems?

Focus Questions for Protein Purification Introduction

1. What is protein purification?

2. Describe lysis and why it is a necessary part of the protein purification process. Name a lysis technique.

3. Why is it important to remove DNA from the sample?

4. What enzyme is used to break down the DNA in a lysate but leaves the protein intact?

5. What is a common method of capture, or isolation, of protein?

Focus Questions for Introduction to Chromatography

1. What is the purpose of chromatography?

2. What is the mobile phase in chromatography?

3. What is the stationary phase in chromatography? Give three examples of stationary phase.

4. Name four types of liquid chromatography techniques.

5. What is an anion and what is a cation? When would you use anion exchange chromatography versus cation exchange chromatography?

6. What is an affinity chromatography tag? Name two tags.

7. Does adding an affinity tag to the protein change the protein? Explain your answer.

Focus Questions for Centrifugation and Chromatography Instrumentation Purification

1. What is gravity chromatography? Name an advantage and disadvantage of this system.

2. Is centrifugation/spin chromatography the same as gravity chromatography? Explain your answer.

3. What are the advantages of spin chromatography? When would you choose to use centrifugation/spin chromatography and why?

4. What are the advantages of using prepacked cartridges? When would you choose to use prepacked cartridges and why?

5. What is a fraction in chromatography?

Focus Questions for SDS-PAGE Electrophoresis

1. What are four ways to quantify the amount of protein in a sample?

2. What does primary (1°), secondary (2°), tertiary (3°), and quaternary (4°) protein structure refer to?

3. Describe SDS-PAGE analysis. Why would you use SDS-PAGE to analyze your samples?

4. What are BME and DTT and what do they do to a protein?

5. Describe the components and function of each component in Laemmli sample buffer.

Focus Questions for DHFR Enzyme Activity

1. Which cofactor is required in the conversion of dihydrofolate to tetrahydrofolate?

2. What is the purpose of the cofactor?

3. Define oxidation. Define reduction.

4. In the conversion of DHF to THF, which compound donates the hydride ion to DHF?

5. What information does the DHFR enzyme activity provide in the context of recombinant protein expression and purification?

Chapter 3A: Advance Preparation for Centrifugation Purification Protocols

Advance Preparation for Cell Culture and Induction Portions of the Protein Expression and Purification Series

In the Cell Culture activities of the Protein Expression and Purification Series, students will grow a small culture of BL21(DE3) *E. coli* containing pDHFR starting from a single colony from a streaked plate. The initial culture will be subcultured to log phase and then induced to express GST-DHFR-His.

Cell Culture Laboratory Checklist

Components from Protein Expression and Purification Series	Where Provided	(✔)
Petri dishes	Growth and Expression Module	❒
LB nutrient agar	Growth and Expression Module	❒
Ampicillin	Growth and Expression Module	❒
LB broth capsules	Growth and Expression Module	❒
E. coli BL21(DE3) with pDHFR, lyophilized	Growth and Expression Module	❒
Sterile 50 ml conical tubes	Growth and Expression Module	❒
Sterile inoculating loops	Growth and Expression Module	❒
IPTG	Growth and Expression Module	❒
Sterile water	Growth and Expression Module	❒
20% sterile glucose	Growth and Expression Module	❒
Microcentrifuge tubes, 2 ml	Growth and Expression Module	❒
Laemmli sample buffer	SDS-PAGE Electrophoresis Module	❒
Screwcap microcentrifuge tubes, 1.5 ml	Growth and Expression Module	❒

Required Accessories (Not Provided)	Quantity	(✔)
Incubator set to 37°C	1	❒
Autoclave, microwave or hot plate and stir bar	1	❒
Erlenmeyer flask, 500 ml or larger	1	❒
Tube roller, shaking water bath or shaking incubator set to 37°C	1	❒
Microcentrifuge with variable speed setting ≥16,000 x g	1	❒
2–20 µl adjustable-volume micropipets and tips	12	❒
20–200 µl adjustable-volume micropipets and tips	12	❒
100–1,000 µl adjustable-volume micropipets and tips	12	❒
Pipet controller or bulb	12	❒
10 ml sterile serological pipets	5–10	❒
Marking pens	12	❒
Spectrophotometer	1	❒
Semi-microvolume cuvettes	48	❒
Water bath or heat block set to 95°C	1	❒

Tasks to Perform Prior to the Lab

Note: Several reagents are used in different parts of the advance preparation as well as for different laboratory activities. **Do not discard excess reagents used in preparation and store them at the appropriate conditions.**

Streaking starter plates to produce single bacterial colonies on agar plates

1. **Preparation of ampicillin:** With a sterile pipet, add 600 µl of sterile water to each vial containing 30 mg ampicillin to rehydrate the antibiotic, making a 50 mg/ml (500x) solution.

   Note: Ampicillin is used to make LB/amp agar plates as well as LB/amp broth, so do not discard any unused ampicillin solution. The rehydrated ampicillin should be stored at –20°C and is good for one year.

2. **Preparation of LB/amp agar plates:** At least **two days** prior to cell culturing, prepare one LB/amp agar plate per team to be used to inoculate *E. coli* starter cultures.

   Note: Only prepare 200 ml of molten LB agar reagent (enough for 20 plates) to which 200 µl of 50 mg/ml ampicillin will be added to make LB/amp agar plates. Retain excess 50 mg/ml ampicillin at –20°C for use in preparation of LB/amp broth.

   This protocol is used to prepare solid LB/amp agar media to prepare plates for the growth of starter colonies of BL21(DE3) *E. coli* containing the pDHFR plasmid. Each student group will require one LB/amp agar plate. Agar plates should be prepared at least two days before required, left out at room temperature for two days and then refrigerated until they are to be used. Two days on the bench allows the agar to dry out and more readily take up the rehydrated *E. coli* solution.

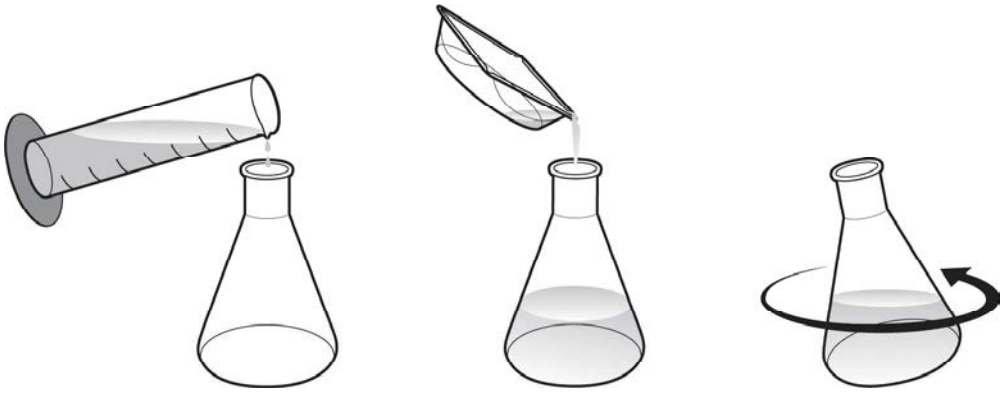


Figure 3A.1. Preparation of LB/amp agar.

A. To prepare 20 LB/amp plates, prepare 200 ml of LB agar. Add 8 g of LB nutrient agar to 200 ml of distilled water in a 500 ml or larger Erlenmeyer flask and cover the flask opening. Swirl the flask to dissolve the agar, or add a magnetic stir bar to the flask and stir on a stir plate. A stir bar will also aid in mixing the solution once sterilization is complete. Autoclave the LB agar on the wet cycle for 30 minutes. Once the autoclave cycle is complete, check the solution to ensure that the agar is completely dissolved.

Note: Be sure to wear appropriate protective equipment and be careful to allow the flask to cool slightly before swirling so that the hot medium does not boil over onto your hand.

If no autoclave is available, heat the agar solution to boiling in a microwave or on a hot plate. Repeat the heating and swirling process about three times until all the agar is dissolved (no more clear specks are noticeable). Use a lower power setting on the microwave to reduce evaporation and boilover. Allow the LB agar to cool so that the outside of the flask is just comfortable to hold (around 55°C). A water

bath set at 55°C is useful for this step. Be careful not to let the agar cool so much that it begins to solidify. Solidified agar can be reheated to melt if antibiotics have not been added.

B. While the agar is cooling, label the plates **LB/amp**. Label the outside of the lower plate rather than the lid of the plates that are to be prepared.

C. Ensure LB agar is cooled to 55°C before adding ampicillin since excessive heat will degrade the ampicillin. Add 200 µl of 50 mg/ml ampicillin to the molten LB agar. The final concentration of the ampicillin in the LB/amp agar plates will be 50 µg/ml. Swirl or use the stir bar and a stir plate to mix the ampicillin into the agar taking care not to introduce bubbles.

D. Pour the LB/amp agar plates. One LB/amp plate is required for each team.

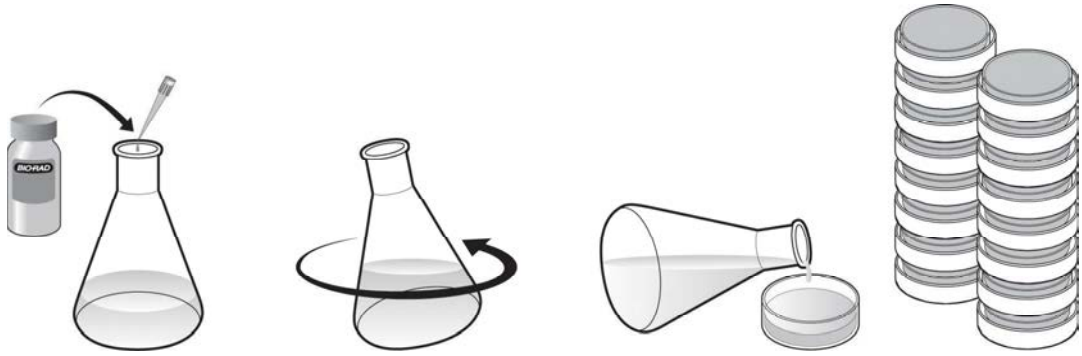


Figure 3A.2. Preparation of LB/amp agar plates.

E. After the plates have dried at room temperature for two days they should be stored at 4°C enclosed in plastic bags or plastic wrap to prevent them drying out. Plates are good for two months. Pour excess agar in the garbage, not in the sink. Wipe any agar drips off the sides of the plates.

3. **Prepare LB broth:** At least **two days** prior to cell culturing, prepare 250 ml of LB broth. To a 1 Liter Erlenmeyer flask, add 250 ml of distilled water and 5 capsules of LB broth. Heat the solution until the capsules dissolve. Autoclave on wet cycle for 30 minutes. Allow the broth to cool to room temperature before use.

   Note: LB broth without ampicillin is used to rehydrate the BL21(DE3) with pDHFR bacteria.

   If an autoclave is not available, LB broth can be filter sterilized through a 0.2 µm filter into a sterile container, or it can be sterilized in the microwave by heating to boiling at least three times. (**Note:** Reduce evaporation by using a lower power setting on the microwave so the solution simmers rather than boils.) Storage of LB broth at 4°C is recommended and can be stored for up to one year. LB broth can also be stored at room temperature if desired; however, this is not recommended if the microwave or filter sterilization methods have been used for sterilization, or if the bottle has been opened after sterilization.

4. **Rehydrate bacteria:** Using a sterile pipet, rehydrate the lyophilized *E. coli* BL21(DE3) containing pDHFR by adding 250 µl of LB broth directly to the vial. Recap the vial and allow the cell suspension to stand at room temperature for 5 minutes. Store the rehydrated bacteria at 4°C until used (within 24 hours for best results).

5. Set up student workstations according to the student workstation list on page 60.

Overnight Cell Culture

1. **Prepare LB/amp broth**: Ensure LB broth is cooled to room temperature before adding ampicillin; excessive heat will degrade the antibiotic. Add 500 µl of 50 mg/ml ampicillin to the remaining ~250 ml of LB broth to produce LB/amp broth. Swirl to mix. (250 µl of LB broth should have been used to rehydrate the bacteria.)
   *LB broth containing antibiotics should be stored at 4°C and is good for one month.

2. **Dilute IPTG:** Add 900 µl of sterile water to the 100 µl of 1M IPTG to produce a 100 mM IPTG solution. Store at –20°C until used. Diluted IPTG is good for one month at –20°C.

3. **Dispense LB/amp broth:** Dispense 3 ml of LB/amp broth into one 50 ml sterile conical tube per workstation. Dispense 11 ml of LB/amp broth into one 50 ml sterile conical tube per workstation. Finally, dispense 2 ml of LB/amp broth into one 2 ml microcentrifuge tube per workstation. Store at 4°C until ready for use.

4. Aliquot 300 µl of 20% sterile glucose into a 2 ml microcentrifuge tube for each workstation.

5. Set up student workstations according to the student workstation list on page 60.

Subculture and Induction

1. Refer to pages 18-22 to help determine the timeline for induction that the class will use. A four hour induction provides optimal results.

2. Make sure that the instrumentation for cell culturing (incubator with tube roller, shaking water bath or incubator shaker) is at 37°C.

3. **Warm up 50 ml conical tubes containing 11 ml LB/amp broth**. At least one hour before the laboratory period, place the 50 ml conical tubes containing 11 ml LB/amp broth in a 37°C incubator with tube roller, shaking waterbath, or incubator shaker and warm the medium to 37°C.

4. If using a tube roller in a 37°C incubator, set up the tube roller for end over end mixing. If you are using a shaking water bath or incubator shaker, set the shaking speed to 250–275 rpm. Once the cells are added, it is essential that the cells remain suspended and oxygenated for proper growth. There should be some foaming when the cells are shaken.

5. **Turn on the spectrophotometer and allow it to warm up for at least 30 minutes.** If you are using the Bio-Rad SmartSpec™ Plus spectrophotometer, step-by-step instructions are available in Appendix D for setting up the instrument to measure absorbance at 600 nm. If using another manufacturer's spectrophotometer, please consult the instruction manual for that instrument for setting it up to measure absorbance at 600 nm.

6. Aliquot 25 µl of 100 mM IPTG into a 2 ml microcentrifuge tube for each workstation.

7. Aliquot 1 ml of Laemmli sample buffer into a 2 ml microcentrifuge tube for each workstation.
   Note: The set up as described involves distributing 1 ml of Laemmli sample buffer at the Subculture and Induction stage for each student workstation. This sample buffer will be used throughout the protocols and should be saved by the students. Alternatively, aliquots can be distributed each time the Laemmli sample buffer will be used.

8. Set up student workstations according to the student workstation list on pages 60-61.

Student Workstations

The following workstation setups are for the initial activities of 1) streaking a starter plate to produce single bacterial colonies on LB/amp agar plates, 2) preparing overnight cell cultures, and 3) subculturing and induction of cells and preparing SDS-PAGE samples for later analysis.

1. Streak starter plates to produce single bacterial colonies on agar plates

Each student team requires the following items to streak a starter plate to produce single bacterial colonies:

Material Needed for Each Workstation	Quantity
LB/amp plate	1
Sterile inoculating loop	1
2–20 µl adjustable-volume micropipet and tips	1
Marking pen	1

Common Workstation	Quantity
Rehydrated BL21(DE3) *E. coli* containing pDHFR	1
Incubator set to 37°C	1

2. Overnight cell culture

Each student team requires the following items to prepare an overnight culture:

Material Needed for Each Workstation	Quantity
LB/amp plate streaked with BL21(DE3) containing pDHFR	1
50 ml conical tube containing 3 ml sterile LB/amp broth	1
20% sterile glucose	300 µl
20–200 µl adjustable-volume micropipet and tips	1
Marking pen	1

Common Workstation	Quantity
Tube roller in a 37°C incubator, shaking water bath or shaking incubator set to 37°C	1

3. Subculture and Induction

Each student team requires the following items to prepare a sample of uninduced cells for SDS-PAGE analysis, to subculture the cells, and to induce the cells:

Material Needed for Each Workstation	Quantity
50 ml conical tube containing 3 ml overnight culture	1
50 ml sterile conical tube containing 11 ml sterile LB/amp broth warmed to 37°C	1
LB/amp broth in two 2 ml microcentrifuge tubes for spectroscopy	4 ml
Screwcap microcentrifuge tube, 1.5 ml	1
100 mM IPTG	25 µl
Laemmli sample buffer	1 ml
20–200 µl adjustable-volume micropipet and tips	1
100–1,000 µl adjustable-volume micropipet and tips	1
Semi-microvolume cuvettes	4
Parafilm squares	2
Marking pen	1

Common Workstation	Quantity
Tube roller in a 37°C incubator; shaking water bath or shaking incubator set to 37°C	1
Water bath or dry bath set to 95°C	1
Microcentrifuge with variable speed setting ≥16,000 x g	1
Spectrophotometer	1–2

Advance Preparation for Cell Lysis Laboratory

In this Cell Lysis activity, cells that have been expressing GST-DHFR-His will be pelleted and a lysis solution will be added. Lysis will be accomplished by multiple freeze-thaw steps using a dry ice/ethanol bath. The lysate will then be centrifuged to separate the soluble components from insoluble components. SDS-PAGE samples will be prepared for the insoluble and soluble cell lysate fractions.

Cell Lysis Laboratory Checklist

Components from Protein Expression and Purification Series	Where Provided	(✔)
Lysozyme	Growth and Expression Module	❒
Sterile water	Growth and Expression Module	❒
Imidazole stock solution	Growth and Expression Module	❒
10x PBS	Growth and Expression Module	❒
Laemmli sample buffer	SDS-PAGE Electrophoresis Module	❒
Microcentrifuge tubes, 2 ml	Growth and Expression Module	❒
Screwcap microcentrifuge tubes, 1.5 ml	Growth and Expression Module	❒

Required Accessories (Not Provided)	Quantity	(✔)
Dry ice	1 block	❒
Ethanol	0.5–2 L	❒
Microcentrifuge with variable speed setting ≥16,000 x g	1	❒
20–200 µl adjustable-volume micropipets and tips	12	❒
100–1,000 µl adjustable-volume micropipets and tips	12	❒
Marking pens	12	❒
10 ml syringes	12	❒
22 gauge syringe needles	12	❒
Water bath or dry bath set to 95°C	1	❒

Tasks to Perform Prior to the Lab

Collecting cell pellet and lysing cells

1. **Prepare 2x PBS:** To prepare 20 ml of 2x PBS, combine 4 ml of 10x PBS and 16 ml of distilled water and mix thoroughly. 2x PBS can be stored at room temperature and is good for one year. The 2x PBS is 20 mM sodium phosphate and 300 mM NaCl.

2. **Prepare lysozyme:** No more than one day in advance, rehydrate lysozyme by adding 500 µl of sterile water to the 25 mg of lyophilized lysozyme to generate a 50 mg/ml solution. Mix gently to aid in the resuspension. Keep the vial of lysozyme on ice or in a refrigerator until use. Once resuspended, it should be used within 24 hours.

3. **Preparation of Lysis buffer 1:** To prepare 20 ml of Lysis buffer 1, combine 20 ml of 2x PBS with 200 µl 50 mg/ml lysozyme solution. Store the Lysis buffer 1 at 4°C until ready to use. Once made, it should be used within 24 hours. The Lysis buffer 1 is 20 mM sodium phosphate, 300 mM NaCl and 0.5 mg/ml lysozyme.

   Aliquot 500 µl of Lysis buffer 1 into a 2 ml microcentrifuge tube for each workstation.

4. **Prepare Lysis buffer 2/Wash buffer:** To prepare 50 ml of Lysis buffer 2/Wash buffer, combine 38 ml of distilled water, 10 ml of 10x PBS and 2 ml of Imidazole stock solution and mix thoroughly. Lysis buffer 2/Wash buffer should be stored at 4°C and is good for one month. The Lysis buffer 2/Wash buffer is 20 mM sodium phosphate, 300 mM NaCl and 10 mM imidazole.

   Aliquot 500 µl of Lysis buffer 2/Wash buffer into a 2 ml microcentrifuge tube for each workstation.

5. **Prepare a dry ice/ethanol bath:** Right before the lab period, add ethanol to a beaker. Add dry ice to the beaker until it no longer melts completely and pieces can still be seen in the solution.

6. Set up student workstations according to the student workstation list shown below.

Separating soluble from insoluble induced cell fractions and prepare SDS-PAGE samples

1. **Thaw lysates:** If the cell lysates have been stored in a –20°C freezer, take them out at least one hour before the laboratory period and allow the lysates to thaw at room temperature. If the lysates do not thaw within one hour, they can be briefly warmed to 37°C to thaw completely.

2. Set up student workstations according to the student workstation list shown below.

Student Workstations

Each student team requires the following items to 1) collect their cell pellet, 2) lyse their cells, 3) separate the soluble from the insoluble lysate fraction, and 4) prepare samples for SDS-PAGE analysis:

1. Collecting cell pellet and lysing cells

Material Needed for Each Workstation	Quantity
Induced cell culture	1 ml
Lysis buffer 1	500 µl
Lysis buffer 2/Wash buffer	500 µl
Microcentrifuge tubes, 2 ml	3
Screwcap microcentrifuge tube, 1.5 ml	1
Laemmli sample buffer (leftover from previous activity)	1 ml
20–200 µl adjustable-volume micropipet and tips	1
100–1,000 µl adjustable-volume micropipet and tips	1
Marking pen	1

Common Workstation	Quantity
Water bath or dry bath set to 95°C	1
Microcentrifuge with variable speed setting ≥16,000 x g	1
Dry ice/ethanol bath	1
Vortexer	1

2. Separating the soluble from the insoluble induced cell fractions and prepare SDS-PAGE samples

Each student team requires the following items to separate the soluble from the insoluble lysate fractions and prepare soluble and insoluble fraction samples for SDS-PAGE analysis:

Material Needed for Each Workstation	Quantity
Thawed cell lysate	1 ml
Lysis buffer 2/Wash buffer	1 ml
Screwcap microcentrifuge tubes, 1.5 ml	2
Microcentrifuge tubes, 2 ml	3
Laemmli sample buffer (left over from previous activity)	1 ml
20–200 µl adjustable-volume micropipet and tips	1
10 ml syringe	1
22 gauge syringe needle	1
Marking pen	1

Common Workstation	Quantity
Water bath or dry bath set to 95°C	1
Microcentrifuge with variable speed setting ≥16,000 x g	1

Advance Preparation for Chromatography Laboratory

In this Chromatography activity, affinity chromatography will be used to isolate the GST-DHFR-His protein. The eluted GST-DHFR-His protein will subsequently be exchanged into a buffer with no imidazole using size exclusion/desalting chromatography in preparation for SDS-PAGE analysis, quantitation, and enzymatic activity analysis.

Protein Purification by Centrifugation Laboratory Checklist

Components from Protein Expression and Purification Series	Where Provided	(✔)
Imidazole stock solution	Growth and Expression Module	❒
10x PBS	Growth and Expression Module	❒
Microcentrifuge tubes, 2 ml	Growth and Expression Module	❒
Screwcap microcentrifuge tubes, 1.5 ml	SDS-PAGE Electrophoresis Module	❒
Profinity IMAC Ni-charged resin	Centrifugation Purification Module	❒
Empty Micro Bio-Spin columns	Centrifugation Purification Module	❒
Desalting columns	Centrifugation Purification Module	❒
Microcentrifuge collection tubes	Centrifugation Purification Module	❒
Lysis buffer 2/Wash buffer	Prepared for Cell Lysis Laboratory	❒

Required Accessories (Not Provided)	Quantity	(✔)
Microcentrifuge with variable speed setting ≥16,000 x g	1	❒
Distilled water	20 ml	❒
2–20 µl adjustable-volume micropipets and tips	12	❒
20–200 µl adjustable-volume micropipets and tips	12	❒
100–1,000 µl adjustable-volume micropipets and tips	12	❒
Tube roller or rocker platform	1	❒
Marking pens	12	❒
UV spectrophotometer	1	❒
trUView cuvettes (or UV compatible cuvettes)	12	❒

Tasks to Perform Prior to the Lab

1. **Equilibration buffer:** To prepare 20 ml of Equilibration buffer, combine 15.6 ml of distilled water, 4 ml of 10x PBS, and 0.4 ml of imidazole stock solution, and mix thoroughly. Equilibration buffer should be stored at 4°C and is good for one month. The Equilibration buffer is 20 mM sodium phosphate, 300 mM NaCl, and 5 mM imidazole.

   Aliquot 500 µl of Equilibration buffer into a 2 ml microcentrifuge tube for each workstation.

2. **Lysis buffer 2/Wash buffer:** Prepared for Cell Lysis laboratory activities. Aliquot 600 µl of Lysis buffer 2/Wash buffer into a 2 ml microcentrifuge tube for each workstation.

3. **Elution buffer:** The imidazole stock solution will be used as the Elution buffer. Elution buffer should be stored at 4°C and is good until the labeled expiration date. Elution buffer is 20 mM sodium phosphate, 300 mM NaCl, 250 mM imidazole, pH 8.0.

   Aliquot 400 µl of Elution buffer into a 2 ml microcentrifuge tube for each workstation.

4. Aliquot 250 µl of Profinity IMAC Ni-charged resin slurry into a 2 ml microcentrifuge tube for each workstation.

5. Aliquot 200 µl of sterile water into a 2 ml microcentrifuge tube for each workstation.

6. Set up student workstations according to the student workstation list below.

   Note: The Profinity IMAC Ni-charged resin is supplied as a 50% slurry in a 20% ethanol solution. Make sure to thoroughly resuspend the resin by vortexing and aliquot 250 µl of the resuspended slurry into microcentrifuge tubes for the student workstations. This is equivalent to 125 µl of resin per student workstation, and the students will only use 100 µl of resin.

Student Workstations

Each student team requires the following items to purify their GST-DHFR-His samples using affinity chromatography followed by size exclusion chromatography (desalting).

Material Needed for Each Workstation	Quantity
Soluble lysate fraction	600–750 µl
Profinity IMAC Ni-charged resin slurry	250 µl slurry
Distilled water	200 µl
Equilibration buffer	500 µl
Lysis buffer 2/Wash buffer	600µl
Elution buffer	400 µl
Empty Micro Bio-Spin column with cap and yellow tip closure	1
Desalting column, with green cap*	1
Microcentrifuge tubes, 2 ml	6
Screwcap microcentrifuge tubes, 1.5 ml	4
Laemmli sample buffer (leftover from previous activity)	1 ml
2–20 µl adjustable-volume micropipet and tips	1
20–200 µl adjustable-volume micropipet and tips	1
100–1,000 µl adjustable-volume micropipet and tips	1
Marking pen	1

***Note:** to avoid confustion between the desalting column and the empty column, you may choose to wait to provide the desalting column until after the affinity purification step has been completed.

Common Workstation	Quantity
Tube roller or mini rocker	1
Water bath or dry bath set to 95°C	1
Microcentrifuge with variable speed setting ≥16,000 x g	1

Advance Preparation for Quantitation of Protein in Desalted Fraction

In this laboratory activity, each student team will quantitate the amount of purified, desalted GST-DHFR-His they have via spectroscopy.

Tasks to Perform Prior to the Lab

1. Turn on the spectrophotometer and allow it to warm up for at least 30 minutes. If using the Bio-Rad SmartSpec Plus spectrophotometer, step-by-step instructions are available in Appendix D for setting up the instrument and measuring the absorbance of your samples at 280 mn. If using another manufacturer's spectrophotometer, please consult the instruction manual for that instrument for setting up to measure absorbance at 280 nm.

   Note: UV compatible cuvettes (trUView or quartz) capable of reading 50–100 µl samples must be used for this step.

Student Workstations

Each student team requires the following items to quantitate the amount of purified, desalted eluate (GST-DHFR-His) they have via spectroscopy:

Material Needed for Each Workstation	Quantity
Desalted eluate sample	150 µl
20–200 µl adjustable-volume micropipet and tips	1
trUView disposable cuvette (or similar UV compatible cuvettes)	1
Marking pen	1

Common Workstation	Quantity
UV spectrophotometer	1–2

Advance Preparation for SDS-PAGE Electrophoresis Activities

In this SDS-PAGE Electrophoresis Laboratory, the uninduced cells, induced cells, soluble fraction of the induced cell lysate, insoluble fraction of the induced cell lysate, flowthrough, wash, eluate and desalted eluate fractions from the chromatographic separations will be separated and visualized using SDS-PAGE.

SDS-PAGE Electrophoresis Laboratory Checklist

Components from Protein Expression and Purification Series	Where Provided	(✔)
10x Tris/Glycine/SDS buffer (TGS)	SDS-PAGE Electrophoresis Module	❒
Precision Plus Protein Dual Color standards	SDS-PAGE Electrophoresis Module	❒
Bio-Safe Coomassie stain	SDS-PAGE Electrophoresis Module	❒

Required Accessories (Not Provided)	Quantity	(✔)
Microcentrifuge with variable speed setting ≥16,000 x g	1	❒
2–20 µl adjustable-volume micropipets and tips	12	❒
20–200 µl adjustable-volume micropipets and tips	12	❒
Water bath or dry bath set to 95°C	1	❒
Marking pens	12	❒
Vertical electrophoresis chambers and power supplies	3–12	❒
4–20% Mini-PROTEAN® TGX™ precast gel	12	❒
Gel staining trays	12	

❒

Tasks to Perform Prior to the Lab

1. **Tris/Glycine/SDS (TGS) running buffer:** One Mini-PROTEAN Tetra cell with two gels requires 700 ml of 1x TGS running buffer. One Mini-PROTEAN Tetra cell using the companion running module to run four gels requires 1.1 L of 1x TGS running buffer. To make 10L of 1x TGS running buffer, mix 1 L of 10x TGS with 9 L of distilled water. Store at room temperature.

   Please refer to Appendix E for more details on using the Mini-PROTEAN Tetra cell. If using other vertical gel boxes please refer to the instruction manual for that instrument for details on setting up the apparatus.

   Tip: You may want to prepare 1–2 L of extra 1x TGS buffer in case your gel boxes leak after assembly. If you do have a leak, the outer chamber of the gel box can be filled to just above the inner small plates to equalize the buffer levels in both reservoirs. This requires approximately 1,200 ml of 1x TGS buffer per gel box and is a more convenient fix than reassembling the apparatus mid-lesson.

2. Aliquot 15 µl of Precision Plus Protein Dual Color standards into a 2 ml microcentrifuge tube for each workstation.

3. Set up student workstations according to the student workstation list on page 69.

Student Workstations

Each student team requires the following items to analyze their samples via SDS-PAGE:

Material Needed for Each Workstation	Quantity
Uninduced PAGE sample	100 µl
Induced PAGE sample	100 µl
Insoluble PAGE sample	100 µl
Soluble PAGE sample	100 µl
Flowthrough PAGE sample	100 µl
Wash PAGE sample	100 µl
Eluate PAGE sample	50 µl
Desalted Eluate PAGE sample	50 µl
Precision Plus Protein Dual Color standards	15 µl
4–20% Mini-PROTEAN TGX precast gel	1
Vertical electrophoresis chamber (per 1–4 gels)	1
1x Tris/Glycine/SDS (TGS) running buffer (per electrophoresis chamber)	700–1,200 ml
Power supply (200 V constant) to be shared between workstations	1
Buffer dam (only required if running 1 gel/box)	1
2–20 µl adjustable-volume micropipet and tips	1
Staining tray	1
Bio-Safe Coomassie stain (per gel)	50 ml
Marking pen	1

Common Workstation	Quantity
Water bath or dry bath set to 95°C	1
Microcentrifuge with variable speed setting ≥16,000 x g	1
Water for gel destaining (tap water is fine)	1 L

Advanced Preparation for the DHFR Enzymatic Assay

In the DHFR enzymatic assay, the enzymatic activity of the purified, desalted GST-DHFR-His is measured spectrophotometerically by detecting the decrease in absorbance at 340 nm over time of the NADPH cofactor as it is converted to $NADP^+$.

DHFR Enzymatic Reaction Assay Checklist

Components from Protein Expression and Purification Series	Where Provided	(✔)
10x PBS	Growth and Expression Module	❒
NADPH cofactor	DHFR Enzymatic Assay Module	❒
DHF substrate	DHFR Enzymatic Assay Module	❒

Required Accessories (Not Provided)	Quantity	(✔)
2–20 µl adjustable-volume micropipets and tips	12	❒
100–1,000 µl adjustable-volume micropipets and tips	12	❒
UV spectrophotometer capable of three decimal place accuracy	1–2	❒
trUView disposable cuvettes (or UV compatible cuvettes)	12	❒
Parafilm	12 squares	
❒		

Tasks to Perform Prior to the Lab

1. Prepare 15 ml of 1x PBS by combining 13.5 ml of distilled water with 1.5 ml of 10x PBS and mix thoroughly.

2. Immediately before the laboratory exercise begins, reconstitute the 1 mg of NADPH by adding 120 µl of **1x PBS** to the vial of NADPH and invert or vortex to mix and ensure complete dissolution. The concentration of the dissolved NADPH is 10 mM. Store the NADPH on ice until ready to use. Once reconstituted, the NADPH solution is only stable for 3–4 hours. The NADPH solution cannot be frozen to retain or extend the activity and shelf life.

   Aliquot 8 µl of NADPH cofactor into a 2 ml microcentrifuge tube for each workstation.

3. Immediately before the laboratory exercise begins, reconstitute the 1 mg of DHF by adding 226 µl of **10x PBS** to the vial of DHF and invert or vortex to mix and ensure complete dissolution. The concentration of the dissolved DHF is 10 mM. Store the DHF on ice until ready to use. Once reconstituted, the DHF solution is only stable for 3–4 hours. The DHF solution cannot be frozen to retain or extend the activity and shelf life.

   Aliquot 10 µl DHF substrate into a 2 ml microcentrifuge tube for each workstation.

   Note: Make sure and use **10x PBS** to reconstitute the 1 mg of DHF. The high salt in 10x PBS helps dissolve the DHF.

4. Turn on the spectrophotometer at least 30 minutes before the lab period to allow the lamp to warm up. If using the Bio-Rad SmartSpec Plus spectrophotometer, step-by-step instructions are available in Appendix D for setting up the instrument in kinetics mode to read at 340 nm. If using another manufacturer's spectrophotometer consult the instruction manual that came with the instrument to determine if kinetics mode can be programmed and how to set the wavelength to 340 nm. If there is no kinetics mode, readings at 340 nm can be taken manually every 15 seconds for 150 seconds and recorded.

   Note: UV compatible cuvettes (trUView or quartz) that can read 1 ml samples must be used for this activity.

5. Aliquot 1 ml of **1x PBS** into a 2 ml microcentrifuge tube for each workstation.

6. Set up student workstations according to the student workstation list below.

Student Workstations

Each student team requires the following items to analyze enzymatic activity of their purified, desalted GST-DHFR-His protein sample:

Material Needed for Each Workstation	Quantity
Purified, desalted eluate protein sample	15 µl
2–20 µl adjustable-volume micropipets and tips	1
100–1,000 µl adjustable-volume micropipets and tips	1
1x PBS	1 ml
Marking pen	1
NADPH cofactor	8 µl
DHF substrate	10 µl
trUView disposable cuvettes (or UV compatible cuvettes)	1
Parafilm	1 square

Common Workstation	Quantity
UV spectrophotometer capable of three decimal place accuracy	1–2

Chapter 3B: Advance Preparation for Chromatography Instrumentation Protocols

Advance Preparation for Cell Culture and Induction Portions of the Protein Expression and Purification Series

In the Cell Culture activities of the Protein Expression and Purification Series, students will grow a small culture of BL21(DE3) *E. coli* containing pDHFR starting from a single colony from a streaked plate. The initial culture will be subcultured to log phase and then induced to express GST-DHFR-His.

Cell Culture Laboratory Checklist

Components from Protein Expression and Purification Series	Where Provided	(✔)
Petri dishes	Growth and Expression Module	❒
LB nutrient agar	Growth and Expression Module	❒
Ampicillin	Growth and Expression Module	❒
LB broth capsules	Growth and Expression Module	❒
E. coli BL21(DE3) w/pDHFR, lyophilized	Growth and Expression Module	❒
Sterile 50 ml conical tubes	Growth and Expression Module	❒
Sterile inoculating loops	Growth and Expression Module	❒
IPTG	Growth and Expression Module	❒
Sterile water	Growth and Expression Module	❒
20% sterile glucose	Growth and Expression Module	❒
Microcentrifuge tubes, 2 ml	Growth and Expression Module	❒
Laemmli sample buffer	SDS-PAGE Electrophoresis Module	❒
Screwcap microcentrifuge tubes, 1.5 ml	Growth and Expression Module	❒

Required Accessories (Not Provided)	Quantity	(✔)
Incubator set to 37°C	1	❒
Autoclave, microwave, or hot plate and stir bar	1	❒
500 ml Erlenmeyer flasks	5	❒
1 L autoclavable bottle or Erlenmeyer flask	1	❒
Shaking water bath or shaking incubator set to 37°C	1	❒
Microcentrifuge with variable speed setting ≥16,000 x g	1	❒
Benchtop or floor centrifuge with rotors for 250 ml centrifuge bottles and 30–50 ml centrifuge tubes	1	❒
250 ml centrifuge bottles capable of withstanding 4,500 x g	4	❒
30–50 ml centrifuge tubes capable of withstanding 16,000 x g	4	❒
2–20 µl adjustable-volume micropipets and tips	4	❒
20–200 µl adjustable-volume micropipets and tips	4	❒
100–1,000 µl adjustable-volume micropipets and tips	4	❒
Pipet controller or bulb	4	❒
10 ml sterile serological pipets	12	❒
Marking pens	4	❒
Spectrophotometer	1	❒
Semi-microvolume cuvettes	16	❒
Water bath or dry bath set to 95°C	1	❒

Tasks to Perform Prior to the Lab

Note: Several reagents are used in different parts of the advance preparation as well as for different laboratory activities. **Do not discard excess reagents used in preparation and store them at the appropriate conditions.**

Streaking starter plates to produce single bacterial colonies on agar plates

1. **Preparation of ampicillin:** With a sterile pipet, add 600 µl of sterile water to each vial containing 30 mg ampicillin to rehydrate the antibiotic, making a 50 mg/ml (500x) solution.

   Note: Ampicillin is used to make LB/amp agar plates as well as LB/amp broth, so do not discard any unused ampicillin solution. The rehydrated ampicillin should be stored at –20°C and is good for one year.

2. **Preparation of LB/amp agar plates:** At least **two days** prior to cell culturing, prepare one LB/amp agar plate per team to be used to inoculate *E. coli* starter cultures.

   Note: Only prepare 200 ml of molten LB agar reagent (enough for 20 plates) to which 200 µl of 50 mg/ml ampicillin will be added to make LB/amp agar plates. Retain excess 50 mg/ml ampicillin at –20°C for use in preparation of LB/amp broth.

This protocol is used to prepare solid LB/amp agar media to prepare plates for the growth of starter colonies of BL21(DE3) *E. coli* containing the pDHFR plasmid. Each student group will require one LB/amp agar plate. Agar plates should be prepared at least two days before required, left out at room temperature for two days and then refrigerated until they are to be used. Two days on the bench allows the agar to dry out and more readily take up the rehydrated *E. coli* solution.

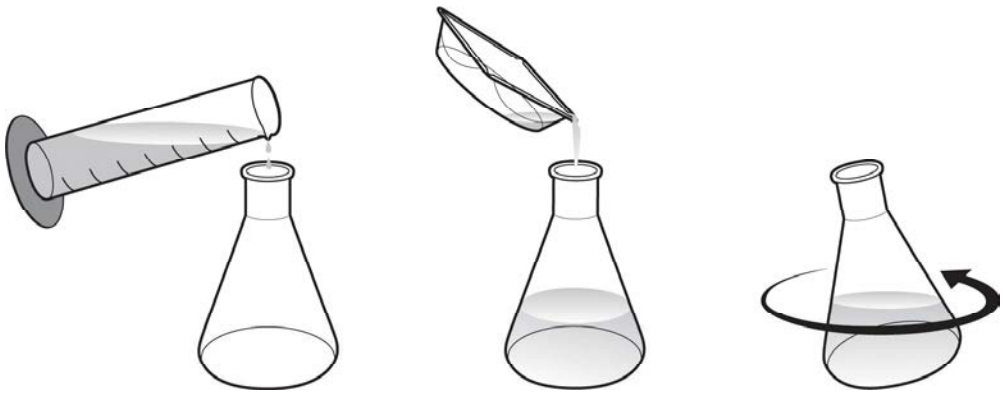


Figure 3B.1. Preparation of LB/amp agar.

A. To prepare 20 LB/amp plates, prepare 200 ml of LB agar. Add 8 g of LB nutrient agar to 200 ml of distilled water in a 500 ml or larger Erlenmeyer flask and cover the flask opening. Swirl the flask to dissolve the agar, or add a magnetic stir bar to the flask and stir on a stir plate. A stir bar will also aid in mixing the solution once sterilization is complete. Autoclave the LB agar on the wet cycle for 30 minutes. Once the autoclave cycle is complete, check the solution to ensure that the agar is completely dissolved.

Note: Be sure to wear appropriate protective equipment and be careful to allow the flask to cool slightly before swirling process so that the hot medium does not boil over onto your hand.

If no autoclave is available, heat the agar solution to boiling in a microwave or on a hot plate. Repeat the heating and swirling about three times until all the agar is dissolved (no more clear specks are noticeable). Use a lower power setting on the microwave to reduce evaporation and boilover.

Allow the LB agar to cool so that the outside of the flask is just comfortable to hold (around 55°C). A water bath set at 55°C is useful for this step. Be careful not to let the agar cool so much that it begins to solidify. Solidified agar can be reheated to melt if antibiotics have not been added.

B. While the agar is cooling, label the plates **LB/amp**. Label the outside of the lower plate rather than the lid of the plates that are to be prepared.

C. Ensure LB agar is cooled to 55°C before adding ampicillin since excessive heat will degrade the ampicillin. Add 200 µl of 50 mg/ml ampicillin to the molten LB agar. The final concentration of the ampicillin in the LB/amp agar plates will be 50 µg/ml. Swirl or use the stir bar and a stir plate to mix the ampicillin into the agar taking care not to introduce bubbles.

D. Pour the LB/amp agar plates. One LB/amp plate is required for each team.

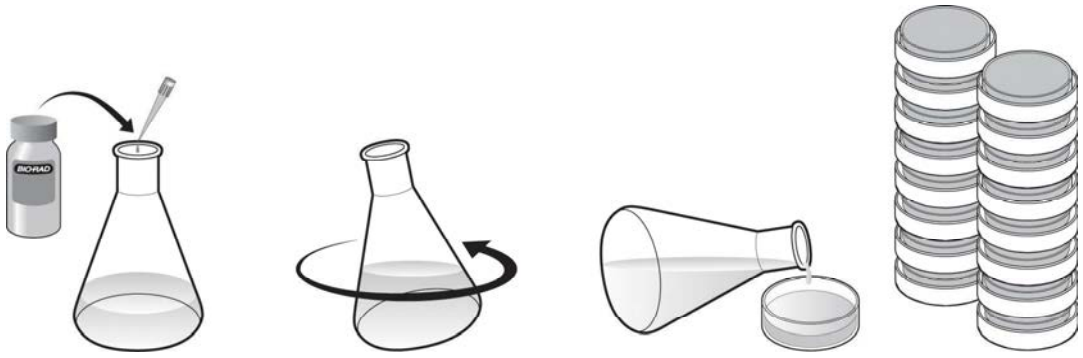


Figure 3B.2. Preparation of LB/amp agar plates.

E. After the plates have dried at room temperature for two days they should be stored at 4°C enclosed in plastic bags or plastic wrap to prevent the plates from drying out. Plates are good for two months. Pour excess agar in the garbage, not in the sink. Wipe any agar drips off the sides of the plates.

3. **Prepare LB broth:** At least **two days** prior to cell culturing, prepare four Erlenmeyer flasks with 100 ml of LB broth: to each of four 500 ml Erlenmeyer flasks, add 100 ml of distilled water and 2 capsules of LB broth. Prepare one Erlenmeyer flask with 200 ml of LB broth: to a 1 L Erlenmeyer flask, add 200 ml of distilled water and 4 capsules of LB broth. Heat the solutions until the capsules dissolve. Autoclave all five flasks on wet cycle for 30 minutes. Allow the broth to cool to room temperature before use.

   Note: LB broth without ampicillin is used to rehydrate the BL21(DE3) with pDHFR bacteria.

   If an autoclave is not available, LB broth can be filter sterilized through a 0.2 µm filter into a sterile container, or it can be sterilized in the microwave by heating to boiling at least three times. (**Note:** reduce evaporation by using a lower power setting on the microwave so the solution simmers rather than boils.) Storage of LB broth at 4°C is recommended and can be stored for up to one year. LB broth can also be stored at room temperature if desired; however, this is not recommended if the microwave or filter sterilization methods have been used for sterilization, or if the bottle has been opened after sterilization.

4. **Rehydrate bacteria:** Using a sterile pipet, rehydrate the lyophilized *E. coli* BL21(DE3) containing pDHFR by adding 250 µl of LB broth directly to the vial. Recap the vial and allow the cell suspension to stand at room temperature for 5 minutes. Store the rehydrated bacteria at 4°C until used (within 24 hours for best results).

5. Set up student workstations according to the student workstation list on page 76.

Overnight Cell Culture

1. **Prepare LB/amp broth:** Ensure LB broth is cooled to room temperature before adding ampicillin; excessive heat will degrade the antibiotic. Add 100 µl of 50 mg/ml ampicillin to each of the four Erlenmeyer flasks containing 100 ml of LB broth. Swirl to mix. Add 200 µl of 50 mg/ml ampicillin to the 200 ml of LB broth in the 1 L Erlenmeyer flask and swirl to mix. The final concentration of ampicillin in the LB broth in all bottles and flasks will be 50 µg/ml.
   * LB broth containing antibiotics should be stored at 4°C and is good for one month.

2. **Dispense LB/amp broth into sterile 50 ml conical tubes:** Using the LB/amp broth from the 1 L Erlenmeyer flask, dispense 15 ml of LB/amp broth into one 50 ml sterile conical tube per workstation. Dispense 2 ml of LB/amp broth into 2 ml microcentrifuge tubes, one per workstation. Store at 4°C until ready for use.

3. Aliquot 1 ml of 20% sterile glucose into a 2 ml microcentrifuge tube for each workstation.

4. Set up student workstations according to the student workstation list on page 76.

Subculture and Induction

1. Refer to pages 26-30 to help determine the timeline for induction that the class will use. A four hour induction provides optimal results.

2. Make sure that the incubator shaker or shaking water bath is set to 37°C.

3. **Warm up Erlenmeyer flasks containing 100 ml LB/amp broth:** At least one hour before the laboratory period, place the four 500 ml Erlenmeyer flasks in a 37°C incubator shaker, or shaking water bath and warm the medium to 37°C.

4. Set the shaking speed of the shaking water bath or incubator shaker to 250–275 rpm. Once the cells are added, it is essential that the cells remain suspended and oxygenated for proper growth. There should be some foaming when the cells are shaken.

5. **Turn on the spectrophotometer and allow it to warm up for at least 30 minutes**. If using the Bio-Rad SmartSpec Plus spectrophotometer, step-by-step instructions are available in Appendix D for setting up the instrument for reading absorbance at 600 nm. If using another manufacturer's spectrophotometer, please consult the instruction manual for that instrument for setting it up to measure absorbance at 600 nm.

6. Aliquot 25 ml of 1 M IPTG into a 2 ml microcentrifuge tube for each workstation.

7. Aliquot 1 ml of Laemmli sample buffer into a 2 ml microcentrifuge tube for each workstation.

   Note: The set up as described involves distributing 1 ml of Laemmli sample buffer at the subculture and induction stage for each student workstation. This sample buffer will be used throughout the protocols and should be saved by the students. Alternatively, aliquots can be distributed each time the Laemmli sample buffer will be used.

8. Set up student workstations according to the student workstation list on page 77.

Student Workstations

The following workstation setups are for the initial activities of 1) streaking a starter plate to produce single bacterial colonies on LB/amp agar plates, 2) preparing overnight cell cultures, 3) subculturing and induction of cells, and 4) preparing SDS-PAGE samples for later analysis.

1. Streak starter plates to produce single bacterial colonies on agar plates

Each student team requires the following items to streak a starter plate to produce single bacterial colonies:

Material Needed for Each Workstation	Quantity
LB/amp plate	1
Sterile inoculating loop	1
2–20 µl adjustable-volume micropipet and tips	1
Marking pen	1

Common Workstation	Quantity
Rehydrated BL21(DE3) *E. coli* containing pDHFR	1
Incubator set to 37°C	1

2. Overnight cell culture

Each student team requires the following items to prepare an overnight culture:

Material Needed for Each Workstation	Quantity
LB/amp plate streaked with BL21(DE3) containing pDHFR	1
50 ml conical tube containing 15 ml sterile LB/amp broth	1
Sterile inoculating loop	1
20% sterile glucose	1 ml
100–1,000 µl adjustable-volume micropipet and tips	1
Marking pen	1

Common Workstation	Quantity
Tube roller in a 37°C incubator, shaking water bath or shaking incubator set to 37°C	1

3. Subculture and induction

Each student team requires the following items to prepare a sample of uninduced cells for SDS-PAGE analysis, to subculture the cells and to induce the cells:

Material Needed for Each Workstation	Quantity
50 ml conical tube containing 15 ml overnight culture	1
500 ml Erlenmeyer flask containing 100 ml sterile LB/amp broth warmed to 37°C	1
LB/amp broth in two 2 ml microcentrifuge tubes for spectroscopy	4 ml
Screwcap microcentrifuge tube, 1.5 ml	1
1 M IPTG	25 µl
Laemmli sample buffer	1 ml
20–200 µl adjustable-volume micropipet and tips	1
100–1,000 µl adjustable-volume micropipet and tips	1
Pipet pump or bulb	1
10 ml sterile serological pipets	2
Semi-microvolume cuvettes	4
Parafilm squares	4
Marking pen	1

Common Workstation	Quantity
Shaking water bath or shaking incubator set to 37°C	1
Water bath or dry bath set to 95°C	1
Microcentrifuge with variable speed setting ≥16,000 x g	1
Spectrophotometer	1–2

Advance Preparation for Cell Lysis Laboratory

In this Cell Lysis activity, cells that have been expressing GST-DHFR-His will be pelleted and a lysis solution will be added. Lysis will be accomplished by multiple freeze-thaw steps using a dry ice/ethanol bath. The lysate will then be centrifuged to separate the soluble components from insoluble components. SDS-PAGE samples will be prepared for the insoluble and soluble cell lysate fractions.

Cell Lysis Activity Checklist

Components from Protein Expression and Purification Series	Where Provided	(✔)
Lysozyme	Growth and Expression Module	❒
Sterile water	Growth and Expression Module	❒
Imidazole stock solution	Growth and Expression Module	❒
10x PBS	Growth and Expression Module	❒
Laemmli sample buffer	SDS-PAGE Electrophoresis Module	❒
50 ml sterile centrifuge tubes	Growth and Expression Module	❒
Microcentrifuge tubes, 2 ml	Growth and Expression Module	❒
Screwcap microcentrifuge tubes, 1.5 ml	Growth and Expression Module	❒

Required Accessories (Not Provided)	Quantity	(✔)
Dry ice	1 block	❒
Ethanol	0.5–2 L	❒
Microcentrifuge with variable speed setting ≥16,000 x g	1	❒
Benchtop or floor centrifuge with rotors for 250 ml centrifuge bottles and 30–50 ml centrifuge tubes	1	❒
250 ml centrifuge bottles capable of withstanding 4,500 x g	4	❒
30–50 ml centrifuge tubes capable of withstanding 16,000 x g	4	❒
20–200 µl adjustable-volume micropipets and tips	4	❒
100–1,000 µl adjustable-volume micropipets and tips	4	❒
Pipet controller or bulb	1	❒
10 ml sterile serological pipets	8	❒
Marking pens	4	❒
10 ml syringes	4	❒
22 gauge syringe needles	4	❒
Water bath or dry bath set to 95°C	1	❒

Tasks to Perform Prior to the Lab

Collecting cell pellet and lysing cells

1. **Prepare 2x PBS:** To prepare 50 ml of 2x PBS, combine 40 ml of distilled water and 10 ml of 10x PBS and mix thoroughly. 2x PBS can be stored at room temperature and is good for one year. The 2x PBS is 20 mM sodium phosphate and 300 mM NaCl.

2. **Prepare Lysozyme:** No more than one day ahead, rehydrate lysozyme by adding 500 µl of sterile water to the 25 mg of lyophilized lysozyme to generate a 50 mg/ml solution. Mix gently to aid in the resuspension. Keep the vial of lysozyme on ice or in a refrigerator until ready to use. Once resuspended, it should be used within 24 hours.

3. **Preparation of Lysis buffer:** To prepare 50 ml of Lysis buffer, combine 50 ml of 2x PBS with 500 µl of 50 mg/ml lysozyme solution. Store the Lysis buffer at 4°C until ready to use. Once made, it should be used within 24 hours. The Lysis buffer is 20 mM sodium phosphate, 300 mM NaCl and 0.5 mg/ml lysozyme.

Aliquot 5 ml of Lysis buffer for each workstation.

4. **Prepare Equilibration buffer:** To prepare 50 ml of Equilibration buffer, combine 38 ml of distilled water, 10 ml of 10x PBS and 2 ml of imidazole stock solution and mix thoroughly. Equilibration buffer should be stored at 4°C and is good for one month. The Equilibration buffer is 20 mM sodium phosphate, 300 mM NaCl and10 mM imidazole.

   Aliquot 5 ml of Equilibration buffer for each workstation.

5. **Prepare a dry ice/ethanol bath:** Right before the laboratory period, add ethanol to a beaker. Add dry ice to the beaker until it no longer melts completely and large pieces can still be seen in the solution.

6. **Centrifuge setup:** Set up your centrifuge with the rotor for use with 250 ml bottles. Use the equation in Appendix C or the chart that came with your centrifuge to determine the speed (RPM) that is required to generate 4,500 x g of force for that rotor. Set the speed dial.

7. Set up student workstations according to the student workstation list shown on the following page.

Separating soluble from insoluble induced cell fractions and prepare SDS-PAGE Samples

1. **Thaw lysates:** If the cell lysates have been stored in a –20°C freezer, take them out at least one hour before the laboratory period and allow the lysates to thaw at room temperature. If the lysates do not thaw within one hour, they can be briefly warmed to 37°C to completely thaw.

2. **Centrifuge setup:** Set up your centrifuge with the rotor for use with 30–50 ml centrifuge tubes. Use the equation in Appendix C or the chart that came with your centrifuge to determine the speed (RPM) that is required to generate 16,000 x g of force for that rotor. Set the speed dial.

3. Set up student workstations according to the student workstation list shown on the following page.

Student Workstations

Each student team requires the following items for 1) collecting their cell pellet, 2) lysing their cells, 3) separating the soluble from the insoluble lysate fraction, and 4) preparing samples for SDS-PAGE analysis:

1. Collecting cell pellet and lysing cells

Material Needed for Each Workstation	Quantity
Induced cell culture	100 ml
Lysis buffer	5 ml
Equilibration buffer	5 ml
250 ml centrifuge bottles capable of withstanding 4,500 x g	1
50 ml sterile conical tube	1
Screwcap microcentrifuge tube, 1.5 ml	1
Laemmli sample buffer	1 ml
20–200 µl adjustable-volume micropipet and tips	1
100–1,000 µl adjustable-volume micropipet and tips	1
Marking pen	1

Common Workstation	Quantity
Water bath or dry bath set to 95°C	1
Microcentrifuge with variable speed setting ≥16,000 x g	1
Benchtop or floor centrifuge with rotor for 250 ml centrifuge bottles	1
Dry ice/ethanol bath	1
Vortexer	1

2. Separating soluble from insoluble induced cell fractions and prepare SDS-PAGE samples

Each student team requires the following items to separate soluble from insoluble lysate fractions and prepare soluble and insoluble fraction samples for SDS-PAGE analysis:

Material Needed for Each Workstation	Quantity
Thawed cell lysate	10 ml
50 ml sterile conical tube	1
30–50 ml centrifuge tube capable of withstanding 16,000 x g	1
Equilibration buffer	5 ml
2x PBS	5 ml
Screwcap microcentrifuge tubes, 1.5 ml	2
Laemmli sample buffer (left over from previous activity)	1 ml
20–200 µl adjustable-volume micropipet and tips	1
Pipet pump or bulb	1
10 ml serological pipet	4
10 ml syringe	1
22 gauge syringe needle	1
Marking pen	1

Common Workstation	Quantity
Water bath or dry bath set to 95°C	1
Microcentrifuge with variable speed setting ≥16,000 x g	1
Benchtop or floor centrifuge with rotor for 30–50 ml centrifuge tubes	1

Advance Preparation for SDS-PAGE Electrophoresis Activities
(Analysis of Induction and Analysis of Purification)

There are two stages at which students will run an SDS-PAGE gel to analyze data. The first stage is after the separation of the soluble and insoluble lysate fractions. The uninduced, induced, soluble, and insoluble cell fraction SDS-PAGE samples will be run on this gel with the purpose of determining that induction and expression of GST-DHFR-His occurred. It will also be determined if the expressed GST-DHFR-His is primarily in the soluble or insoluble fraction. The second SDS-PAGE gel that will be run will be used to examine the success of the purification protocols and will examine the soluble fraction, the flowthrough of the column fraction, the column wash fraction and several eluted protein fractions before and after desalting. The preparation is the same for both of these laboratories, with just the samples being run differing.

SDS-PAGE Electrophoresis Laboratory Checklist

Components from Protein Expression and Purification Series	Where Provided	(✔)
10x Tris/Glycine/SDS buffer (TGS)	SDS-PAGE Electrophoresis Module	❒
Precision Plus Protein Dual Color standards	SDS-PAGE Electrophoresis Module	❒
Bio-Safe Coomassie stain	SDS-PAGE Electrophoresis Module	❒

Required Accessories (Not Provided)	Quantity	(✔)
Microcentrifuge with variable speed setting ≥16,000 x g	1	❒
2–20 µl adjustable-volume micropipets and tips	4	❒
20–200 µl adjustable-volume micropipets and tips	4	❒
Water bath or dry bath set to 95°C	1	❒
Marking pens	4	❒
Vertical electrophoresis chambers and power supplies	1–4	❒
4–20% Mini-PROTEAN TGX precast gel	8	❒
Gel staining trays	4	❒

Tasks to Perform Prior to the Lab

1. **Tris/Glycine/SDS (TGS) running buffer:** One Mini-PROTEAN Tetra cell with 2 gels requires 700 ml of 1x TGS running buffer. One Mini-PROTEAN Tetra cell using the companion running module to run four gels requires 1.1 L of 1x TGS running buffer. To make 3 L of 1x TGS running buffer, mix 300 ml of 10x TGS with 2,700 ml of distilled water. Store at room temperature.

   If using other vertical gel boxes please refer to the instuction manual for that instrument for details on setting up the apparatus. Please refer to Appendix E for more details on using the Mini-PROTEAN Tetra cell.

   Tip: You may want to prepare 1–2 L of extra 1x TGS buffer in case your gel boxes leak after assembly. If you have a leak, the outer chamber of the gel box can be filled to just above the inner small plates to equalize the buffer levels in both reservoirs. This requires approximately 1,200 ml of 1x TGS buffer per gel box and is a more convenient fix than reassembling the apparatus mid-lesson.

2. Aliquot 15 µl of Precision Plus Protein Dual Color standards into each of two 2 ml microcentrifuge tubes (one for each gel) for each workstation.

3. Set up student workstations according to the student workstation list on pages 82-83.

Student Workstations

1. SDS-PAGE electrophoresis analysis of induction

Each student team requires the following items to analyze their induction samples via SDS-PAGE:

Material Needed for Each Workstation	Quantity
Uninduced PAGE sample	100 µl
Induced PAGE sample	100 µl
Insoluble PAGE sample	100 µl
Soluble PAGE sample	100 µl
Precision Plus Protein Dual Color standards	15 µl
4–20% Mini-PROTEAN TGX precast gel	1
Vertical electrophoresis chamber (per 1–4 gels)	1
1x Tris/Glycine/SDS (TGS) running buffer (per electrophoresis chamber)	700–1,200 ml
Power supply (200 V constant) to be shared between workstations	1
Buffer dam (only required if running 1 gel/box)	1
2–20 µl adjustable-volume micropipet and tips	1
Staining tray	1
Bio-Safe Coomassie stain (per gel)	50 ml
Marking pen	1

Common Workstation	Quantity
Water bath or dry bath set to 95°C	1
Microcentrifuge with variable speed setting ≥16,000 x g	1
Water for gel destaining (tap water is fine)	1 L

2. SDS-PAGE electrophoresis analysis of purification

Student Workstations

Each student team requires the following items to analyze their purification samples via SDS-PAGE:

Material Needed for Each Workstation	Quantity
Soluble PAGE sample	100 µl
Flowthrough PAGE sample	100 µl
Column Wash PAGE sample	100 µl
Fraction A PAGE sample	100 µl
Desalted fraction A PAGE sample	50 µl
Fraction B PAGE sample	100 µl
Desalted fraction B PAGE sample	50 µl
Fraction C PAGE sample	100 µl
Desalted fraction C PAGE sample	50 µl
Precision Plus Protein Dual Color standards	15 µl
4–20% Mini-PROTEAN TGX precast gel	1
Vertical electrophoresis chamber (per 1-4 gels)	1
1x Tris/Glycine/SDS (TGS) running buffer (per electrophoresis chamber)	700–1,200 ml
Power supply (200 V constant) to be shared between workstations	1
Buffer dam (only required if running 1 gel/box)	1
2–20 µl adjustable-volume micropipet and tips	1
Staining tray	1
Bio-Safe Coomassie stain (per gel)	50 ml
Marking pen	1

Common Workstation	Quantity
Water bath or heat block set to 95°C	1
Microcentrifuge with variable speed setting ≥16,000 x g	1
Water for gel washing and destaining (tap water is fine)	1 L

Advance Preparation for Chromatography Laboratory

In this Chromatography activity, affinity chromatography will be used to isolate the GST-DHFR-His protein. The eluted GST-DHFR-His protein will subsequently be exchanged into a buffer with no imidazole using size exclusion/desalting chromatography in preparation for SDS-PAGE analysis, quantitation and enzymatic activity analysis.

Please note that there are several steps that can be performed as advanced preparation by the instructor or can be performed by the students as a learning experience in familiarizing them with the chromatography instrumentation. This includes calibrating the instrument and installing (or building) a sample loop. Full instructions on how to perform these tasks are available in the appropriate instruction manual for the instrument.

Chromatography Laboratory Checklist

Components from Protein Expression and Purification Series	Where Provided	(✔)
Imidazole stock solution	Growth and Expression Module	❒
10x PBS	Growth and Expression Module	❒
2 ml microcentrifuge tubes	Growth and Expression Module	❒
Laemmli sample buffer	SDS-PAGE Electrophoresis Module	❒
Handpacked IMAC column	Handpacked Column Purification Module	❒
Bio-Scale Profinity IMAC cartridge	Prepacked Cartridge Purification Module	❒
Micro Bio-Spin 6, Tris columns (desalting columns)	Handpacked Column Purification Module Prepacked Cartridge Purification Module	❒

❒

Required Accessories (Not Provided)	Quantity	(✔)
Microcentrifuge with variable speed setting ≥16,000 x g	1	❒
2–20 µl adjustable-volume micropipets and tips	4	❒
20–200 µl adjustable-volume micropipets and tips	4	❒
Chromatography instrument	1–4	❒
Fraction collector	1–4	❒
Fraction collection tubes	120	❒
10 ml syringe	1–4	❒
Injection needle (DuoFlow only)	1–4	❒
Data collection system (software, chart recorder, etc.)	1–4	❒
Marking pens	12	❒
UV spectrophotometer	1	❒
trUView cuvettes (or UV compatible cuvettes)	12	❒
Flow adaptors for 1 cm ID columns (handpacked columns only)	1–4	❒

Tasks to Perform Prior to the Lab

Affinity purification of GST-DHFR-His

1. **Buffer A (280 ml):** Combine 224 ml of distilled water with 56 ml of 10x PBS and mix well. Buffer A should be stored at 4°C and is good for one month. Buffer A is 20 mM sodium phosphate and 300 mM NaCl, pH 7.2.

2. **Buffer B:** Buffer B is the imidazole stock solution. Buffer B is 20 mM sodium phosphate, 300 mM NaCl, and 250 mM imidazole, pH 8.0.

3. **BioLogic™ LP system ONLY:**
Note: The following instructions assume a basic working knowledge of the BioLogic LP system. If the instrument has not been run before, it is strongly suggested that a Starter kit (catalog #731-8350EDU) is run to familiarize yourself with the instrument components, nomenclature, and programming. Please see Appendi**x** F **f**or diagrams of the plumbing and wiring for a Biologic LP system with either a 2110 or BioFrac™ fraction collector.

   a. Ensure that the plumbing and wiring for your system is comparable to that in Appendix F.

   b. Adjusting the platen: Follow the instructions of Section 4.2 of the BioLogic LP system instruction manual to ensure that the platen is adjusted correctly for the size of tubing (1.6 mm) that is installed on the instrument.

   c. Preparing a 5 ml sample loop: Follow the instructions of Section 4.3, step 6 of the BioLogic LP system instruction manual to cut a piece of tubing for a 5 ml injection loop and attach the appropriate luer fittings and connect the sample loop to the MV-6 sample valve.

   d. Turn the MV-6 sample valve counterclockwise (left) as far as it will go. Fill a syringe with 10 ml of high quality water and attach it to the needle port on the MV-6 sample valve. Push the syringe plunger and watch the water flow. It should flow into the sample loop and then out the waste tube. If this is not the case, remove the syringe, turn the MV-6 valve counterclockwise (left) as far as it will go and replumb the valve as shown in Appendix F.

   e. Calibrate the pump: Follow the instructions of Section 6.2–Pump Calibration of the BioLogic LP system instruction manual to ensure proper flow from the pump.

   f. Checking UV monitor filter: Look on the bottom of the UV optics module—the rectangular filter holder has 254 and 280 markings. The 280 marking should be opposite the arrow, indicating that the UV monitor is set for 280 nm. If this is not the case, loosen the thumbscrew, remove and rotate the filter holder, and reinstall.

   g. Check communication with fraction collector: Turn on the fraction collector (and BioLogic system workstation if you have not already done so). On the BioLogic system keypad, select **manual** from the mode keys. From the instrument keys press **Collector**. Press the **Model** softkey.

      If using a Model 2110 fraction collector, press the **2110** softkey. Press the **Advance** softkey. The drophead on the Model 2110 fraction collector should advance one tube. If not, go to Appendix F and check the wiring and plumbing of the SV3 valve.

      If you are using a BioFrac fraction collector, choose **BioFrac** (or 2128 on older models) softkey. When asked if System Cable 3 has been installed, press the **Yes** softkey. Press the **Engage** softkey on the BioFrac fraction collector to home the drophead. On the BioLogic LP system, press the **Advance** softkey. The BioFrac fraction collector should advance one tube. If not, go to Appendix F and check the wiring of System Cable 3 and the diverter valve.

Optional: Depending on teaching objectives, it is possible to program the instruments with the DHFR purification protocol beforehand, rather than have students perform this task.

1. **BioLogic DuoFlow system ONLY:**
   Note: The following instructions assume a basic working knowledge of use of the BioLogic DuoFlow system. If the instrument has not been run before, it is strongly suggested that a Starter kit (catalog #760-0135EDU) is run to familiarize yourself with the instrument components, nomenclature, and programming.

   a. Install a 5 ml sample loop on each instrument. Remove the 50 µl sample loop that was installed with the instrument and store in a safe place. Replace this sample loop with a 5 ml sample loop by attaching the fittings into port 6 and port 3 of the AVR7-3 injection valve. Please reference your DuoFlow system instruction manual (Section 4.2—Plumbing a DuoFlow System; Section 6—Plumbing the AVR7-3 Inject Valve) for more information.

   b. Injection port needle: It is critical that students use only the injection port needle that was supplied with the DuoFlow system instrument. This needle has a flat bottom (in contrast to the sharp, beveled edge on a traditional syringe needle). The injection port on the instrument has a filter that will be damaged if a syringe needle is used.

   c. Install column: Using fittings to convert the columns fittings from luer-style to ¼-28 style, attach the column after the AVR7-3 valve and before the UV detector. Make sure that the arrow on the column (if using a Bio-Scale mini cartridge) points in the correct direction of flow.

   d. Check flow rates of Pump A and Pump B: In manual mode, set the flow rate to 2.00 ml/min and place the pump inlet tubing for both Pump A and Pump B in high quality water. Set the AVR7-3 to Load. Place the waste outlet tubing coming from the SV3-2 valve for the fraction collector into a graduated cylinder. Choose 100% A and start the pump. Allow Pump A to run for 5 minutes. Check the volume of water collected in the graduated cylinder. It should be close to 10 ml. Repeat for Pump B. If the volume is incorrect for either pump, purge the system to make sure there are no air bubbles present in the lines.

   Optional: Depending on teaching objectives, it is possible to program all of the instruments with the DHFR purification protocol beforehand, rather than have students perform this task.

5. Set up student workstations according to the student workstation list on pages 87-88.

Quantitation of protein in desalted fractions

1. Turn on the spectrophotometer and allow it to warm up for at least 30 minutes. If using the Bio-Rad SmartSpec Plus spectrophotometer, step-by-step instructions are available in Appendix D for setting up your instrument and measuring the absorbance at 280 nm. If you are using another manufacturer's spectrophotometer, please consult the instruction manual for that instrument for setting it up to read absorbance at 280 nm.

   Note: UV compatible cuvettes (trUView or quartz) capable of reading 50–100 µl samples must be used for this step.

2. Set up student workstations according to the student workstation list on pages 87-88.

Tasks to Perform After the Lab

In order to ensure excellent, long-term performance of the BioLogic LP system or DuoFlow instrument, it is important to wash all tubing and components with high-quality water for short-term storage and with a 20% ethanol solution in water for long-term storage. A water wash will remove all salts that can precipitate and clog tubing and flow paths. If instruments are used regularly (at least once or twice a month) this wash should be sufficient. If instruments are used infrequently, washing with water followed by a 20% ethanol wash will wash out salts as well as provide some level of antibacterial activity with the ethanol. Make sure to wash out all tubing and all valve positions.

Student Workstations

1. Advance preparation for handpacking a glass econo-column column with a flow adaptor

If students are handpacking their own columns, the following workstations will need to be set up. Otherwise, if they are using prepacked cartridges this section can be skipped and proceed to section 2 below.

Each student team requires the following items to handpack their own column:

Material Needed for Each Workstation	Quantity
Glass Econo-Column column, 1 cm x 5 cm	1
Profinity IMAC Ni-charged resin slurry (50% slurry)	2 ml slurry
Flow adaptor for 1 cm ID columns	1
2-way stopcock	1
10 ml syringe	1
Beaker filled with 200 ml of distilled water	1
50 ml beaker for waste	1
100-1,000 µl adjustable-volume micropipet and tips	1
Marking pen	1

2. Affinity purification of GST-DHFR-His protein

Each student team requires the following items to purify their GST-DHFR-His samples using affinity chromatography:

Material Needed for Each Workstation	Quantity
Soluble lysate fraction	5–7.50 ml
BioScale Mini Profinity Ni-IMAC cartridge	1
A glass Econo-Column column handpacked with 1 ml of Profinity IMAC Ni-Charged resin	1
10 ml syringe	1
Distilled water	500 ml
Buffer A	70 ml
Buffer B	40 ml
Collection tubes for fraction collector	30
Marking pen	1

Common Workstation	Quantity
BioLogic system instrumentation with sample loop installed	1–4
Injection needle (DUOFLOW System ONLY)	1–4

3. Removing imidazole (desalting) from the purified GST-DHFR-His and preparing samples for SDS-PAGE analysis

Each student team requires the following items to desalt three of their fractions in which they think their GST-DHFR-His samples reside and to prepare samples for SDS-PAGE analysis:

Material Needed for Each Workstation	Quantity
Chromatogram from GST-DHFR-His purification	1
Fractions from GST-DHFR-His purification	varies
Desalting column	3
Screwcap microcentrifuge tubes, 1.5 ml	6
Laemmli buffer (left over from previous activity)	1 ml
Microcentrifuge tubes, 2 ml	6–12
20–200 µl adjustable-volume micropipet and tips	1
Marking pen	1

Common Workstation	Quantity
Microcentrifuge with variable speed setting ≥16,000 x g	1
Dry bath or water bath set at 95°C	1

4. Quantitation of protein in desalted fractions

Each student team requires the following items to quantitate the amount of purified, desalted GST-DHFR-His they have via spectroscopy:

Material Needed for Each Workstation	Quantity
Desalted fractions	3
20–200 µl adjustable-volume micropipet and tips	1
trUView disposable cuvettes (or UV compatible cuvettes)	3
Marking pen	1

Common Workstation	Quantity
UV spectrophotometer	1–2

Advance Preparation for the DHFR Enzymatic Assay

In the DHFR enzymatic assay, the enzymatic activity of the purified, desalted GST-DHFR-His is measured spectrophotometrically by detecting the decrease in absorbance at 340 nm over time of the NADPH cofactor as it is converted to $NADP^+$.

Note: The following quantities are based on assaying the desalted GST-DHFR-His fraction with the highest concentration and darkest band on the SDS-PAGE gel that also appears to be the most pure fraction from the SDS-PAGE analysis. There are enough reagents (1x PBS, NADPH and DHF) for each of the four student workstations to perform three enzymatic reactions for each station, one for each of three desalted fractions. The quantities listed below for the student workstations would need to be adjusted accordingly.

DHFR Enzymatic Reaction Assay Checklist

Components from Protein Expression and Purification Series	Where Provided	(✔)
10x PBS	Growth and Expression Module	❒
NADPH cofactor	DHFR Enzymatic Assay Module	❒
DHF substrate	DHFR Enzymatic Assay Module	❒

Required Accessories (Not Provided)	Quantity	(✔)
2–20 µl adjustable-volume micropipets and tips	4	❒
100–1,000 µl adjustable-volume micropipets and tips	4	❒
UV Spectrophotometer capable of three decimal place accuracy	1–2	❒
trUView disposable cuvettes (or UV compatible cuvettes)	4–12	❒
Parafilm	4 squares	❒

Tasks to Perform Prior to the Lab

1. Prepare 15 ml of 1x PBS by combining 13.5 ml of distilled water with 1.5 ml of 10x PBS and mix thoroughly.

2. Immediately before the laboratory exercise is to be run, reconstitute the 1 mg of NADPH by adding 120 µl of **1x PBS** to the vial of NADPH and invert or vortex to mix and ensure complete dissolution. The concentration of the dissolved NADPH is 10 mM. Store the NADPH on ice until ready to use. Once reconstituted, the NADPH solution is only stable for 3–4 hours. The NADPH solution cannot be frozen to retain or extend the activity and shelf life.

   Aliquot 8 µl of NADPH cofactor into a 2 ml microcentrifuge tube for each workstation.

3. Immediately before the laboratory exercise is to be run, reconstitute the 1 mg of DHF by adding 226 µl of **10x PBS** to the vial of DHF and invert or vortex to mix and ensure complete dissolution. The concentration of the dissolved DHF is 10 mM. Store the DHF on ice until ready to use. Once reconstituted, the DHF solution is only stable for 3–4 hours. The DHF solution cannot be frozen to retain or extend the activity and shelf-life.

   Note: Make sure to use 10X PBS to reconstitute the 1 mg of DHF. The high salt in 10x PBS helps dissolve the DHF.

   Aliquot 10 µl DHF substrate into a 2 ml microcentrifuge tube for each workstation.

4. Turn on the spectrophotometer at least 30 minutes before the lab to allow the lamp to warm up. If using the Bio-Rad SmartSpec Plus spectrophotometer, step-by-step instructions are available in Appendix D for setting up the instrument in kinetics mode to read at 340 nm. If using another manufacturer's spectrophotometer, consult the instruction manual for that instrument to determine if

kinetics mode can be programmed and how to set the wavelength at 340 nm. If there is no kinetics mode, readings at 340 nm can be taken manually every 15 seconds for 150 seconds and written down.

Note: UV compatible cuvettes (trUView or quartz) that can read 1 ml samples must be used for this activity.

5. Aliquot 1 ml of **1x PBS** into a 2 ml microcentrifuge tube for each workstation.

6. Set up student workstations according to the student workstation list below.

Student Workstations

Each student team requires the following items to analyze enzymatic activity of their purified, desalted GST-DHFR-His protein sample:

Material Needed for Each Workstation	Quantity
Purified, desalted eluate protein sample	15 µl
2–20 µl adjustable-volume micropipet and tips	1
100–1,000 adjustable-volume micropipet and tips	1
1x PBS	1 ml
Marking pen	1
NADPH cofactor	8 µl
DHF substrate	10 µl
trUView disposable cuvettes (or UV compatible cuvettes)	1–3
Parafilm	1 square

Common Workstation	Quantity
UV spectrophotometer capable of three decimal place accuracy	1–2

Chapter 4: Culturing, Expression, Lysis and SDS-PAGE Analysis for 11 ml Cultures for Centrifugation Purification Process

Cell Culture and Induction

Student Workstations

Each student team requires the following items to streak a starter plate to produce single bacterial colonies:

Material Needed for Each Workstation	Quantity
LB/amp plate	1
Sterile inoculating loop	1
2–20 µl adjustable-volume micropipet and tips	1
Marking pen	1

Common Workstation	Quantity
Rehydrated BL21(DE3) *E. coli* containing pDHFR	1
Incubator set to 37°C	1

Each lab team will streak their own starter plate as a source of cells for culture for protein production. LB/amp plates should be streaked for single colonies and incubated at 37°C for 16–24 hours before the initial culture activity is planned.

Student Protocol: Streaking starter plates to produce single bacterial colonies on agar plates

Using the rehydrated BL21(DE3) *E. coli* containing pDHFR at the Common Workstation to streak one plate. 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 cell multiplies to become millions of genetically identical cells in just 24 hours. There are millions of individual bacteria in a single 1 mm bacterial colony.

1. Pipet 10 µl of reconstituted *E. coli* using a sterile pipet tip onto an LB/amp plate. Use a sterile loop to perform the streaking. Streaking takes place sequentially in four quadrants. The first streak is to just spread the cells out a little. Go back and forth with the loop about a dozen times in one quadrant as shown in Figure **4.1a**. In subsequent quadrants the cells become more and more dilute, increasing the likelihood of producing single colonies.

2. For subsequent streaks, the goal is to use as much of the surface area of the plate as possible. Rotate the plate approximately 45 degrees (so that the streaking motion is comfortable for your hand) and start the second streak. Do not dip into the rehydrated bacteria a second time. Go into the previous streak about two times and then back and forth as shown in Figure **4.1b** for a total of about 10 times.

3. Rotate the plate again and repeat streaking (Figure **4.1c**).

4. Rotate the plate for the final time and make the final streak (Figure **4.1d**). When you are finished streaking the plate, cover it immediately to avoid contamination. Label the plate with your initials.

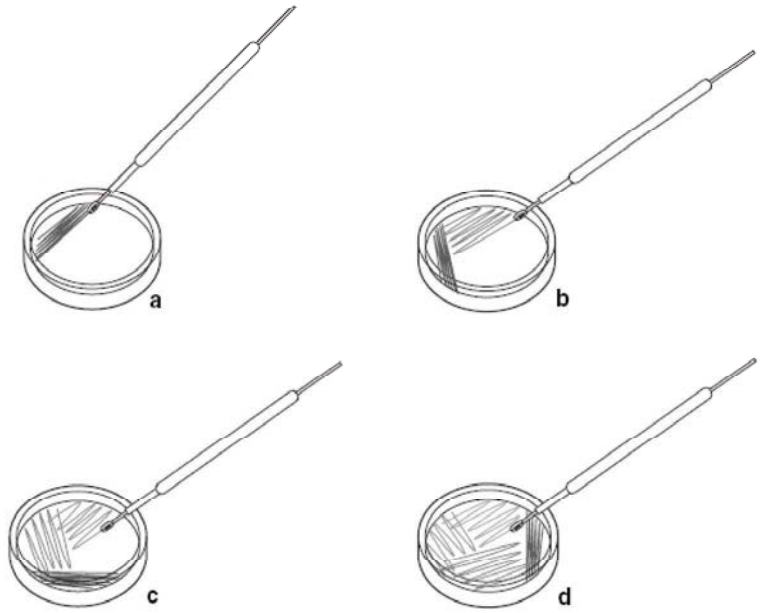


Figure 4.1. Streaking a plate to generate single colonies.

1. Place the plate upside down inside the incubator overnight at 37°C. Once colonies have grown to 1 mm in diameter, they may be stored in a sealed bag upside down at 4ºC until ready to use for up to one month.

6. *E. coli* forms off-white colonies that are uniformly circular with smooth edges. Avoid using plates with contaminant colonies.

Overnight Cell Culture

Student Workstations

Each student team requires the following items to prepare an overnight cell culture:

Material Needed for Each Workstation	Quantity
LB/amp plate streaked with BL21(DE3) containing pDHFR	1
50 ml conical tube containing 3 ml sterile LB/amp broth	1
20% sterile glucose	300 µl
20–200 µl adjustable-volume micropipet and tips	1
Marking pen	1

Common Workstation	Quantity
Tube roller in 37°C incubator, shaking water bath or shaking incubator at 37°C	1

Overnight Culture

1. Label the 50 ml conical tube containing 3 ml of sterile LB/amp broth with your initials.

2. Add 150 µl of 20% sterile glucose to the 3 ml of sterile LB/amp broth to make a final 1% glucose in LB/amp broth solution.

3. Pick a single colony from the starter plate using a micropipettor with a sterile pipet tip and inoculate the 3 ml of 1% glucose, LB/amp broth by twirling the pipet tip vigorously in the broth to dislodge the cells. Recap the tube. Incubate/shake the culture at 37°C for 12–18 hours.

   Note: If using a tube roller in an incubator, make sure to close the cap tightly on the 50 ml conical tube to avoid leakage.

Subculture and Induction

Student Workstations

Each student team requires the following items to prepare a sample of uninduced cells for SDS-PAGE analysis, to subculture the cells, and to induce the cells:

Material Needed for Each Workstation	Quantity
50 ml conical tube containing 3 ml overnight culture	1
50 ml sterile conical tube containing 11 ml sterile LB/amp broth warmed to 37°C	1
LB/amp broth in two 2 ml microcentrifuge tubes for spectroscopy	4 ml
Screwcap microcentrifuge tube, 1.5 ml	1
100 mM IPTG	25 µl
Laemmli sample buffer	1 ml
20–200 µl adjustable-volume micropipet and tips	1
100–1,000 µl adjustable-volume micropipet and tips	1
Semi-microvolume cuvettes	4
Parafilm squares	2
Marking pen	1

Common Workstation	Quantity
Tube roller in a 37°C incubator, shaking water bath or shaking incubator set to 37°C	1
Water bath or dry bath set to 95°C	1
Microcentrifuge with variable speed setting ≥16,000 x g	1
Spectrophotometer	1–2

Prepare uninduced SDS-PAGE sample

1. Label a 1.5 ml screwcap microcentrifuge tube "**Uninduced PAGE**" with your initials.

2. Aliquot 100 µl of the overnight culture into the tube.

3. Centrifuge the tube at 16,000 x g for 2 minutes.

4. Use a pipet to gently remove and discard the supernatant without disturbing the pellet.

5. Add 100 µl Laemmli sample buffer to the pellet and fully resuspend the pellet by pipeting up and down or vortexing.

   Note: It is critical to use only clean pipet tips when aliquoting Laemmli sample buffer or all future samples can become contaminated.

6. Heat the tube at 95°C for 5 minutes.

7. Store the sample at –20°C until ready to analyze via SDS-PAGE analysis.

8. Write your initials on your tube of Laemmli sample buffer and save this tube. This buffer will be used throughout the experiment.

Measure cell density of overnight culture

1. Prepare a 1:10 dilution of your overnight culture by combining 900 µl of sterile LB/amp broth with 100 µl of overnight culture in a clean semi-microvolume cuvette.

2. Cover the cuvette with parafilm and gently invert the cuvette several times to mix the diluted overnight culture.

3. If not already set up, set a spectrophotometer to read absorbance at 600 nm and then blank the instrument using 1 ml LB/amp broth in a cuvette. (**Note:** Save this cuvette containing LB/amp broth to use as a blank for subsequent readings.) If using a Bio-Rad SmartSpec Plus spectrophotometer, see Appendix D for instructions on how to use the instrument.

4. Measure the absorbance at 600 nm (OD600) of the 1:10 dilution of overnight culture cells.

   OD600 of 1:10 dilution of overnight culture: _____

   OD600 of overnight culture = OD600 of 1:10 dilution x 10: _____

Prepare a subculture with a starting OD600 of 0.3

Prepare an 11 ml culture with an OD600 of 0.3 by combining the correct volumes of overnight culture and sterile LB/amp broth that has been warmed to 37°C.

$$\underline{\mathbf{X}} \text{ ml of overnight culture} = \frac{11 \text{ ml} \times 0.3}{\text{OD600 of overnight culture}}$$

1. Calculate the amount of overnight culture that you will need to prepare a subculture with an OD600 of 0.3 and record the value below.

   _____ ml of overnight culture

2. Retrieve your 50 ml conical tube containing 11 ml of LB/amp broth that has been warming to 37°C and label it with your initials.

3. Remove and discard a volume of LB/amp broth, equivalent to the volume you calculated in step 1, from the 50 ml conical tube of overnight culture using a micropipetor and sterile tip.

4. Gently swirl your overnight culture to resuspend all the cells, then add the volume you calculated of overnight culture in step 1 to the remaining LB/amp broth in the 50 ml conical tube using a micropipetor and sterile tips.

5. Screw the cap of the 50 ml conical tube on tightly and grow the 11 ml culture at 37ºC, with shaking or rotating for 1 hour.

6. Record the time that you start your subculture below.

   Subculture Start Time: _____

7. Record the time you stop your subculture below.

   Subculture End Time: _____

Measure OD600 of subculture and induce GST-DHFR-His expression

1. After one hour of incubation measure the OD600 of your subculture.

2. Blank a spectrophotometer at 600 nm with 1 ml LB/amp broth.

3. Prepare a 1:3 dilution of your subculture by combining 600 µl of sterile LB/amp broth with 300 µl of subculture in a clean semi-microvolume cuvette. Cover the cuvette with parafilm and invert gently several times to mix well.

4. Measure the OD600 of your 1:3 diluted subcultured cells and record the results below. Next calculate the OD600 of the subcultured cells and record the value.

   OD600 of 1:3 dilution of subcultured cells: _____

   OD600 of subcultured cells = OD600 of 1:3 dilution x 3: _____

5. Add 25 µl of 100 mM IPTG to the subculture in the 50 ml conical tube and continue incubating and shaking the culture at 37ºC for 4–24 hours. During this time period, most of the resources of the *E. coli* will go toward producing the recombinant GST-DHFR-His protein rather than cell division, though cell division does still occur.

6. Record the time that you start and end your induction below.

   Induction Start Time: _____

   Induction End Time: _____

Collecting cell pellet and lysing cells

Student Workstations

Each student workstation requires the following items to isolate its cell pellet, to prepare an induced cell sample for SDS-PAGE analysis, and to lyse its cells:

Material Needed for Each Workstation	Quantity
Induced cell culture	1 ml
Lysis buffer 1	500 µl
Lysis buffer 2	500 µl
Microcentrifuge tubes, 2 ml	3
Screwcap microcentrifuge tube, 1.5 ml	1
Laemmli sample buffer (leftover from previous activity)	1 ml
20–200 µl adjustable-volume micropipet and tips	1
100–1,000 µl adjustable-volume micropipet and tips	1
Marking pen	1

Common Workstation	Quantity
Water bath or dry bath set to 95°C	1
Microcentrifuge with variable speed setting ≥16,000 x g	1
Vortexer	1
Dry ice/ethanol bath	1

Prepare Induced SDS-PAGE Sample

1. Label a 1.5 ml screwcap microcentrifuge tube "**Induced PAGE**" with your initials.

2. Aliquot 100 µl of the induced culture into the tube.

3. Centrifuge the tube at 16,000 x g for 2 minutes.

4. Use a pipet to gently remove and discard the supernatant without disturbing the pellet.

5. Add 100 µl Laemmli sample buffer to the pellet and fully resuspend the pellet by pipeting up and down or vortexing.

6. Heat the tube at 95°C for 5 minutes.

7. Store the sample at –20°C until ready to analyze via SDS-PAGE.

Pelleting induced cells

1. Label two 2 ml microcentrifuge tubes with "**Induced Cell Pellet**" with your initials. Pellet the induced cells by aliquoting 2 ml of induced cell culture into each of the two labeled 2 ml microcentrifuge tubes. Centrifuge the two 2 ml microcentrifuge tubes at 16,000 x g for 2 minutes. Use a pipet to gently remove and discard the supernatant from both tubes without disturbing the pellets.

2. Aliquot 2 more ml of induced overnight culture into each of the two microcentrifuge tubes labeled "**Induced Cell Pellet**" and centrifuge at 16,000 x g for 2 minutes. Discard the supernatant. Repeat until all of the culture has been centrifuged and each of the two tubes contains a cell pellet. Store the cell pellet at –20°C or continue to lyse the induced cells.

Lysing induced cells

1. Add 250 µl Lysis buffer 1 to each of the two **Induced Cell Pellet** tubes. Resuspend the cell pellets thoroughly by pipeting or vortexing and make sure that there are no cell clumps remaining in both of the tubes.

   Note: As lysis occurs, the solution will get more viscous. Vigorous vortexing and pipetting might be necessary to resuspend the pellet.

2. Label a clean 2 ml microcentrifuge tube "**Lysed Cells**" with your initials. Combine the two 250 µl lysed cell fractions into the labeled microcentrifuge tube for a total of 500 µl of lysed cell solution.

   Note: Use lab tape for the labeling of the 2 ml microcentrifuge tube since the ethanol in the dry ice/ethanol bath will remove the markings from the tube, but not from the tape.

3. Place the tube of lysed cells in the dry ice/ethanol bath for at least five minutes, until the solution is completely frozen. Be careful not to allow the dry ice/ethanol to come in contact with your skin! After five minutes, remove the tube from the dry ice/ethanol bath and thaw completely. It is acceptable to thaw in a 37°C waterbath. Do not thaw by hand to avoid freezerburn to your hand.

   Note: Be careful not to allow the dry ice/ethanol to come in contact with your skin.

4. Repeat two more freeze-thaw steps for a total of three freeze-thaw cycles.

5. After the final freeze-thaw step, add 500 µl Lysis buffer 2 and thoroughly mix by pipetting or vortexing.

6. The lysed cells can be stored at –20°C until they are ready to be purified.

Note: Another method that can be used for efficient lysis is freezing the cells in a –70°C freezer. The cells can be frozen at –70°C overnight and then thawed completely when separation of the soluble from the insoluble fraction is to be performed. If neither dry ice/ethanol or a –70°C freezer is availalble, it is possible to perform two freeze/thaw cycles using a –20°C freezer. Samples can be placed at –20°C overnight (check that samples are completely frozen), thawed the next day and then placed at –20°C overnight again before purification.

Separating Soluble from Insoluble Induced Cell Fractions and Preparing SDS-PAGE Samples

Student Workstations

Each student team requires the following items to separate soluble from insoluble lysate fractions and prepare soluble and insoluble fraction samples for SDS-PAGE analysis:

Material Needed for Each Workstation	Quantity
Thawed cell lysate	1 ml
Lysis buffer 2	1 ml
Screwcap microcentrifuge tube, 1.5 ml	2
Microcentrifuge tubes, 2 ml	3
Laemmli sample buffer (left over from the previous activity)	1 ml
20–200 µl adjustable-volume micropipet and tips	1
100–1,000 µl adjustable-volume micropipet and tips	1
10 ml syringe	1
22 gauge syringe needle	1
Marking pen	1

Common Workstation	Quantity
Water bath or dry bath set to 95°C	1
Microcentrifuge with variable speed setting ≥16,000 x g	1

Separate soluble from insoluble cell lysate components

1. Make sure that your lysate is balanced with either another student's lysate fraction or with a microcentrifuge tube filled with water.

2. Separate the insoluble fraction of the lysed cells from the soluble fraction by centrifugation at 16,000 x g for 20 minutes.

3. Label a clean 2 ml microcentrifuge tube "**Soluble fraction**" with your initials. Gently pour the supernatant from the "**Lysed Cells**" tube into the "**Soluble fraction**" tube being very careful not to decant any of the insoluble fraction (the opaque, viscous blob that is mainly composed of genomic DNA) into your soluble fraction.

   Note: The insoluble fraction does not necessarily adhere to the tube so extreme care is needed when decanting.

4. Relabel the "**Lysed Cells**" microcentrifuge tube containing the remaining insoluble fraction "**Insoluble fraction**."

5. Add 1 ml of Lysis buffer 2 to the "**Insoluble fraction**" tube and resuspend the pellet by shearing with a syringe needle.

   Note: The insoluble fraction contains a large quantity of genomic DNA that can be quite viscous, making resuspension difficult. Using a 22 gauge needle, the insoluble fraction can be pulled up into a 3 ml syringe and expelled from the syringe into a clean tube multiple times to decrease the viscosity of the DNA. Ensure that the needle is disposed of properly in a sharps container.

6. Remove 50 µl of "**Soluble fraction**" and place it in a clean 1.5 ml screwcap microcentrifuge tube labeled "**Soluble PAGE**" with your initials. Add 50 µl Laemmli sample buffer and mix thoroughly.

1. Remove 50 µl of "**Insoluble fraction**" and place it in a clean 1.5 ml screwcap microcentrifuge tube labeled "**Insoluble PAGE**" with your initals. Add 50 µl Laemmli sample buffer and mix thoroughly.

2. Heat the "**Soluble PAGE**" and "**Insoluble PAGE**" samples at 95°C for five minutes.

   Note: **Make sure that you are heating your blue SDS-PAGE samples and not your actual soluble and insoluble fractions!**

3. Store the SDS-PAGE samples "**Soluble PAGE**" and "**Insoluble PAGE**" at –20°C until ready to analyze via SDS-PAGE.

4. Store the soluble fraction and Insoluble fraction at –20°C until ready to purify the soluble fraction.

Affinity and Size Exclusion (Desalting) Purification of GST-DHFR-His

Student Workstations

Each student team requires the following items to purify their GST-DHFR-His samples using affinity chromatography followed by size exclusion chromatography (desalting):

Material Needed for Each Workstation	Quantity
Soluble lysate fraction	600–750 µl
Profinity IMAC Ni-charged resin slurry	250 µl slurry
Distilled water	200 µl
Equilibration buffer	500 µl
Wash buffer	600 µl
Elution buffer	400 µl
Empty Micro Bio-Spin column with cap and yellow tip closure	1
Desalting column	1
Microcentrifuge tubes, 2 ml	6
Screwcap microcentrifuge tubes, 1.5 ml	4
Laemmli sample buffer (leftover from previous activity)	1 ml
2–20 µl adjustable-volume micropipet and tips	1
20–200 µl adjustable-volume micropipet and tips	1
100–1,000 µl adjustable-volume micropipet and tips	1
Marking pen	1

Common Workstation	Quantity
Tube roller or rocking platform	1
Water bath or dry bath set to 95°C	1
Microcentrifuge with variable speed setting ≥ 16,000 x g	1

Pouring, washing, and equilibrating column

1. Snap off the bottom tab of the empty Micro Bio-Spin column, label the column with your initials and place the column in a clean 2 ml microcentrifuge tube.

2. Thoroughly resuspend the resin and pipet 200 µl of the slurry into the column. Centrifuge for 2 minutes at 1,000 x g to remove the packing. (See Appendix C—Instructions on how to calculate the speed that needs to be set to generate a relative centrifugal force, RCF, of 1,000 x g.) Discard the buffer that has collected in the 2 ml microcentrifuge tube. Place the column back into the 2 ml microcentrifuge tube.

   Note: The resin is provided as a 50% slurry in 20% ethanol. It is very important to thoroughly resuspend the resin before pipeting, or the incorrect quantity of resin will be aliquoted.

3. Wash the column by gently adding 200 µl of distilled water to the top of the column taking care not to disturb the resin. Centrifuge for 2 minutes at 1,000 x g to remove the water. Discard the water that was collected in the microcentrifuge tube and place the column back into the 2 ml microcentrifuge tube.

4. Equilibrate the column by gently adding 500 µl of Equilibration buffer and centrifuge for 2 minutes at 1,000 x g to remove the Equilibration buffer. Discard the buffer and the 2 ml microcentrifuge tube. **Do not discard the column!**

Sample binding to the column

1. Attach a yellow tip closure to the bottom of the column.
2. Add up to 600 µl of soluble fraction of the lysate to the column and attach the clear top cap onto the column.
3. Gently mix the column for 20 minutes at room temperature on a tube rotator or a mini rocker. (A uniform slurry should be formed.)
4. Label a 2 ml microcentrifuge tube "**Flowthrough**."
5. Carefully twist and remove the yellow tip closure and place the column into the labeled 2 ml microcentrifuge tube.
6. Remove the clear top cap from the column and centrifuge for 2 minutes at 1,000 x g to collect the flowthrough fraction.
7. Cap and save the flowthrough fraction. Keep the column for the following steps.

Washing the column

1. Label a 2 ml microcentrifuge tube "**Wash fraction**."
2. Place the column into the labeled 2 ml microcentrifuge tube.
3. Gently add 600 µl of wash buffer to the column and centrifuge for 2 minutes at 1,000 x g to collect the wash fraction.
4. Cap and save the wash fraction. Keep the column for the following steps.

Elution of GST-DHFR-His protein from the column

1. Label a 2 ml microcentrifuge tube "**Eluate**."
2. Place the column into the labeled 2 ml microcentrifuge tube.
3. Gently add 400 µl of Elution buffer to the column and centrifuge for 2 minutes at 1,000 x g to collect the eluate fraction.
4. Cap and save the eluate fraction. Discard the column.

Desalting of the GST-DHFR-His protein (eluate fraction) to remove imidazole

1. Invert the desalting column (with a green cap) sharply several times to resuspend the settled gel and to remove any bubbles. The resin should settle into the column and little to no resin should remain in the green cap.
2. Snap off the bottom tab of the column and place the column into a clean 2 ml microcentrifuge tube.
3. Remove the green top cap. If the column does not begin to flow, push the cap back on the column and then remove it again to start the flow.

1. Allow the excess packing buffer to drain by gravity to the top of the gel bed (about 2 minutes), then place the column into a clean 2 ml tube.

2. Centrifuge for 2 minutes in a microcentrifuge at 1,000 x g (see Appendix C for more information about setting centrifuge speed) to remove the remaining packing buffer. Discard the buffer and the microcentrifuge tube. Keep the column for the following steps.

3. Label a clean 2 ml microcentrifuge tube "**Desalted eluate**" with your initials and place the column into the 2 ml microcentrifuge tube. Carefully apply 75 µl of "**Eluate**" fraction directly to the center of the column. Be careful not to touch the resin with the pipet tip.

4. After loading the sample, centrifuge the column for 4 minutes at 1,000 x g.

5. Carefully apply another 75 µl of "**Eluate**" fraction directly to the center of the column, again being careful not to touch the resin with the pipet tip.

6. After loading the sample, centrifuge the column for 4 minutes at 1,000 x g.

7. You should now have approximately 150 µl of desalted eluate in the labeled tube.

8. Discard the column.

Prepare SDS-PAGE samples

1. In a clean 1.5 ml screwcap microcentrifuge tube, combine 50 µl of Laemmli sample buffer with 50 µl of flowthrough and label the tube "**Flowthrough PAGE"** with your initials.

2. In a clean 1.5 ml screwcap microcentrifuge tube, combine 50 µl of Laemmli sample buffer with 50 µl of wash fraction and label the tube "**Wash PAGE"** with your initials.

3. In a clean 1.5 ml screwcap microcentrifuge tube, combine 25 µl of Laemmli sample buffer with 25 µl of Eluate and label the tube **"Eluate PAGE"** with your initials.

4. In a clean 1.5 ml screwcap microcentrifuge tube, combine 25 µl of Laemmli sample buffer with 25 µl of desalted eluate and label the tube "**Desalted Eluate PAGE"** with your initials.

5. Heat the Soluble PAGE, Flowthrough PAGE, Wash PAGE, Eluate PAGE and Desalted Eluate PAGE samples at 95°C for 5 minutes.

6. All SDS-PAGE samples can be stored at –20°C until SDS-PAGE analysis is to be performed.

Storage of chromatography fractions

The flowthrough, wash, eluate and desalted eluate fractions should all be stored at 4°C until the DHFR Enzymatic Assay is to be performed. Do not freeze the samples.

Quantitation of Protein in Desalted Fraction

Student Workstations

Each student team requires the following items to quantitate the amount of purified, desalted eluate (GST-DHFR-His) they have via spectroscopy:

Material Needed for Each Workstation	Quantity
Desalted eluate sample	150 µl
20–200 µl adjustable-volume micropipet and tips	1
trUView disposable cuvette (or similar UV compatible cuvettes)	1
Marking pen	1

Common Workstation	Quantity
UV spectrophotometer	1–2

Protocol: Quantitation of Protein in Desalted Fraction

1. Make sure that your cuvettes are completely clean and dry before usage.

2. Set your spectrophotometer to read at 280 nm

   Note: If using a Bio-Rad SmartSpec Plus spectrophotometer, please see Appendix D for setup instructions.

3. Blank your spectrophotometer with 100 µl distilled water.

4. Pipet 100 µl of your desalted eluate sample into a clean cuvette.

5. Measure the absorbance at 280 nm of your desalted eluate sample.

   A_{280} Desalted eluate sample: _____

6. Pipet the sample from the cuvette back into your tube of desalted eluate sample. Make sure that you have at least 15 µl of desalted eluate sample to run your enzyme assay.

7. Calculate the concentration of GST-DHFR-His in your desalted eluate fraction as follows:

   A. The extinction coefficient (ε) of the entire GST-DHFR-His construct is theoretically calculated to be 75,540 M^{-1} cm^{-1}.

   Knowing that Absorbance = ε x C x L,

   where ε is 75,540 M^{-1} cm^{-1}
   L is the pathlength of the cuvette in cm (usually 1)
   and the absorbance at 280 nm is being measured

   The concentration of GST-DHFR-His (M) = Absorbance/75,540

   Concentration of GST-DHFR-His = _____ M

B. Convert from molarity to mg/ml for the amount of GST-DHFR-His in your purified desalted eluate fraction knowing that the molecular mass of GST-DHFR-His is 50,361 g/mol.

Concentration GST-DHFR-His (mg/ml) = Concentration GST-DHFR-His (M) x 50,361 g/mol

Concentration of GST-DHFR-His = _____mg/ml

This is the concentration of GST-DHFR-His that you produced and purified and is contained in your 150 µl desalted eluate fraction.

SDS-PAGE Electrophoresis to Check Expression and Purity

Student Workstations

Each student team requires the following items to analyze their samples via SDS-PAGE:

Material Needed for Each Workstation	Quantity
Uninduced PAGE sample	100 µl
Induced PAGE sample	100 µl
Insoluble fraction PAGE sample	100 µl
Soluble PAGE sample	100 µl
Flowthrough PAGE sample	100 µl
Wash PAGE sample	100 µl
Eluate PAGE sample	50 µl
Desalted eluate PAGE sample	50 µl
Precision Plus Protein Dual Color standards	15 µl
4–20% Mini-PROTEAN TGX precast gel	1
Vertical electrophoresis chamber (per 1–4 gels)	1
1x Tris/Glycine/SDS (TGS) running buffer (per electrophoresis chamber)	700–1,200 ml
Power supply (200 V constant) to be shared between workstations	1
Buffer dam (only required if running 1 gel/box)	1
2–20 µl adjustable-volume micropipet and tips	1
Staining tray	1
Bio-Safe Coomassie stain (per gel)	50 ml
Marking pen	1

Common Workstation	Quantity
Water bath or dry bath set to 95°C	1
Microcentrifuge with variable speed setting ≥16,000 x g	1
Water for gel destaining (tap water is fine)	1 L

Load, Run, Stain, and Destain the Gel

1. **Prepare samples:** Reheat SDS-PAGE samples at 95°C for 2 minutes to redissolve any precipitated detergent and then centrifuge the samples for 2 minutes at 16,000 x g.

2. **Assemble gel boxes:** If using Bio-Rad Mini-PROTEAN Tetra gel boxes and TGX precast gels, see Appendix **E** for information on how to assemble gel boxes and prepare gels.

3. **Load the gel:** Using a fresh tip each time, load the following volumes of each sample into the appropriate well of your gel.

Well	Volume	Sample
1	10 µl	Precision Plus Protein Dual Color standard
2	7.5 µl	Uniduced PAGE
3	15 µl	Induced PAGE
4	10 µl	Insoluble PAGE
5	10 µl	Soluble PAGE
6	10 µl	Flowthrough PAGE
7	20 µl	Wash PAGE
8	20 µl	Eluate PAGE
9	20 µl	Desalted eluate PAGE
10	10 µl	Laemmli buffer

1. Run the gel at 200 V for 30 minutes. (If using a Bio-Rad Mini-PROTEAN TGX precast gel, the gel can be run at 300 V for 15 minutes.)

2. After the run is complete, remove the gel from the cassette and place it in the gel staining tray.

3. Wash the gel three times with 50 ml of distilled water for five minutes each rinse. Discard all the rinse water.

   Note: Make sure that all of the wash water has been removed since excess water diluting the gel stain will interfere with staining efficiency.

4. Stain the gel with 50 ml of Bio-Safe Coomassie stain for one hour.

5. After one hour discard the Bio-Safe Coomassie stain and add 100 ml of distilled water and destain the gel overnight.

6. Image the gel if you have an imaging system, such as the Bio-Rad Gel Doc™ EZ system, or dry the gel if you have a cellophane drying system.

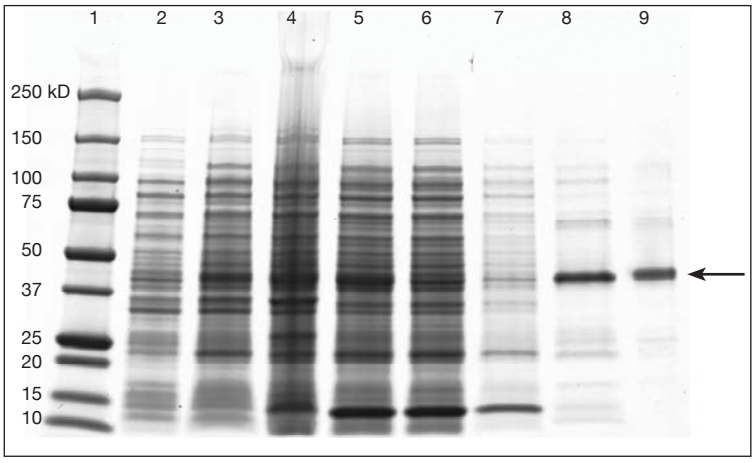


Figure 4.2. Centrifugation purification process results analyzed by SDS-PAGE.

Lane 1. Precision Plus Dual Color standards.
Lane 2. Uninduced cells. There is no strong band at approximately 43 kDa so no GST-DHFR-His has been expressed
Lane 3. Induced cells. A strong band is present at approximately 43 kDa that was not present in the uninduced cell sample (lane 2) and is GST-DHFR-His
Lane 4. Insoluble fraction of *E. coli* cell lysate. A band is present that is the same size as the induced band in lane 3 and this represents the GST-DHFR-His that aggregated or was not folded properly and hence is not soluble and spins down with the cellular debris including cell walls, cell membranes, and the *E. coli* proteins which are not soluble in the 20 mM sodium phosphate, 300 mM NaCl, with 5 mM imidazole buffer
Lane 5. Soluble fraction of *E. coli* cell lysate. A band is present that is the same size as the induced band in lane 3 and this represents the GST-DHFR-His which is soluble. The large band at approximately 12 kDa is the lysozyme, which is also soluble, that was used to lyse the cells open.
Lane 6. Flowthrough fraction which did not bind to the Ni-IMAC resin. This is the fraction of proteins from the soluble fraction that did not bind to the Ni-IMAC resin. The soluble fraction was suspended in 20 mM sodium phosphate buffer, 300 mM NaCl and 5 mM imidazole. The high salt helped prevent *E. coli* proteins and the lysozyme from non-specifically sticking to the Ni-IMAC beads. The 5 mM imidazole helped prevent *E. coli* proteins with multiple histidine groups from binding to the Ni-IMAC beads. There is a decrease in the amount of GST-DHFR-His in the flowthrough fraction versus the soluble fraction (lane 5) and this is representative of the GST-DHFR-His binding to the Ni-IMAC resin.
Lane 7. Wash fraction. This fraction contains proteins that were washed off the Ni-IMAC beads when a wash buffer that has a slightly higher imidazole level (10 mM) was added to wash off more non-specifically bound proteins. No GST-DHFR-His should wash off in this fraction since 10 mM imidazole is not enough to compete with the 6 histidine tag of GST-DHFR-His bound to the Ni-IMAC resin.
Lane 8. Eluate fraction of GST-DHFR-His. This fraction contains the GST-DHFR-His. The elution buffer has 250 mM imidazole in it and this level of imidazole competes with the six histines of GST-DHFR-His and knocks them off the Ni sites and hence the GST-DHFR-His elutes or comes off the resin to be collected. This fraction is predominantly GST-DHFR-His protein relative to the unpurified soluble fraction in lane 5 that contains many other proteins.
Lane 9. Desalted GST-DHFR-His. This fraction contains the purified GST-DHFR-His but has had the 250 mM imidazole removed. Also, since the desalting column removes smaller molecular weight compounds, some of the smaller molecular weight impurities found in lane 8 are not present in the desalted fraction.

Centrifugation Purification Workflow

Cell Culture and Induction

Streaking starter plates to produce single bacterial colonies on agar plates. Pipet 10 µl BL21(DE3) *E. coli* containing pDHFR onto LB/Amp plate; streak out using sterile inoculation loop. Incubate overnight at 37°C.

Overnight culture. Label the 50 ml conical containing 3 ml of sterile LB/Amp broth with your initials. Add 150 µl 20% glucose and single *E. coli* colony to 3 ml of LB/amp liquid media. Incubate overnight at 37°C in shaking incubator or incubator/tube roller.

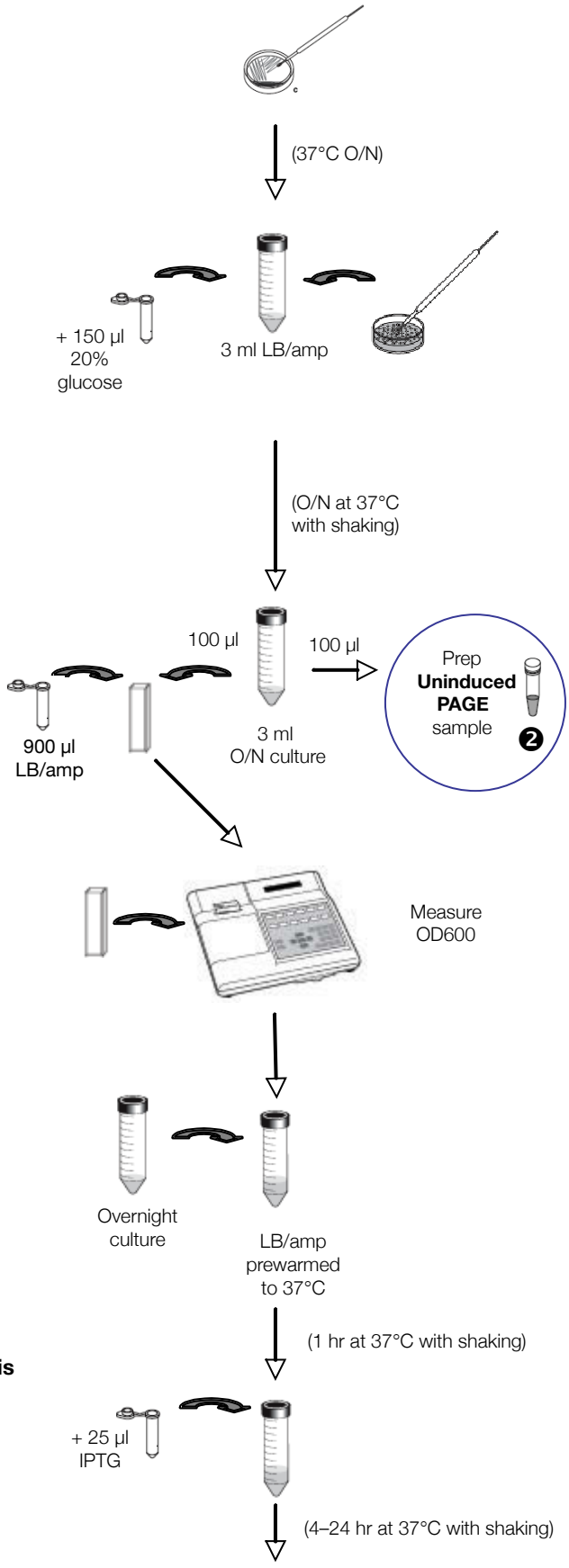
Prepare uninduced SDS-PAGE sample. Label 1.5 ml screwcap microcentrifuge tube **Uninduced PAGE** with your initials. Take 100 µl aliquot from overnight culture and prepare for SDS-PAGE per manual instructions.

Measure cell density of overnight culture. Add 900 µl LB/amp to cuvette; take 100 µl aliquot from overnight culture and add to same cuvette. Using a spectrophotometer, measure absorbance at 600 nm (OD600).

Prepare a subculture with a starting OD600 of 0.3
Determine amount to add to LB/amp for subculture with a starting OD600 of 0.3. Remove LB/amp equivalent volume of calculated cells to be added from prewarmed 50 ml tube containing LB/amp. Add calculated amount of cells from overnight culture to prewarmed 50 ml tube.

Measure OD600 of subculture and induce GST-DHFR-His expression. Incubate subculture for one hour at 37°C with shaking. Measure OD600 and record.

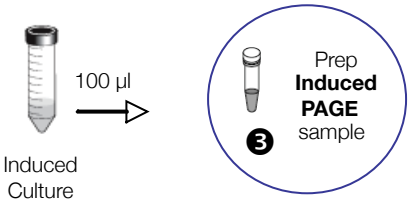
Induce expression by adding 25 µl 100 mM IPTG to culture and incubate 4–24 hours at 37°C with shaking.



Centrifugation Purification Workflow

Collecting cell pellet and lysing cells

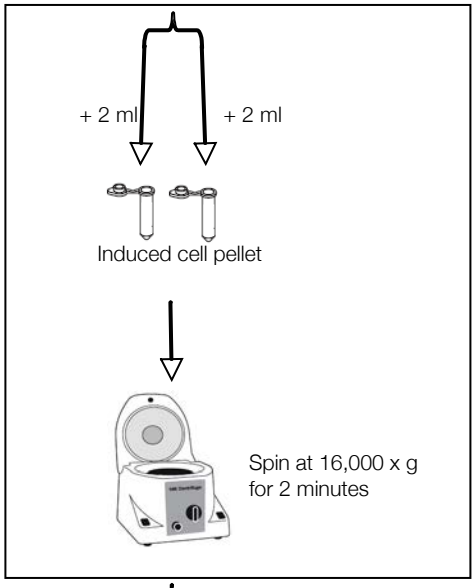
Prepare Induced SDS-PAGE sample. After induction is completed, take 100 µl aliquot from induced culture and prepare for SDS-PAGE per manual instructions.



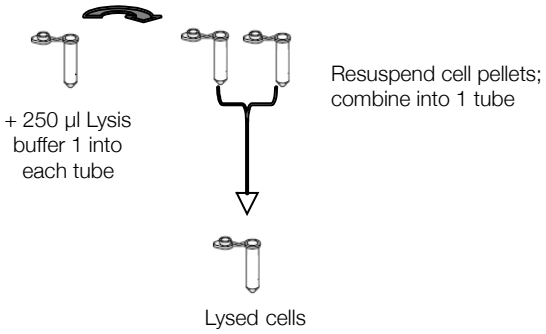
Pelleting induced cells. Label two 2 ml microcentrifuge tubes with **Induced cell pellet** and your team's initials. Add 2 ml of induced culture into each of two 2 ml tubes.

Centrifuge for two minutes at 16,000 x g.

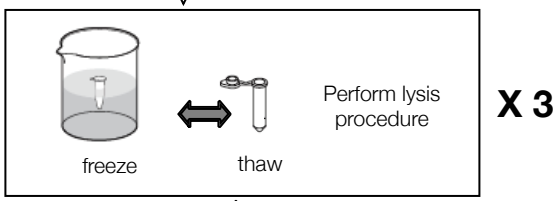
Carefully decant and discard the supernatant Repeat the addition of 2 ml induced culture to each tube; Centrifuge; decant; repeat once more until all cells are pelleted (three times total).



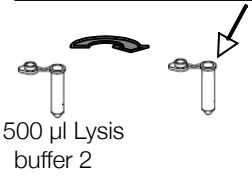
Lysing induced cells. Add 250 µl Lysis buffer 1 to each of the two tubes containing a cell pellet. Using a vortexer or pipet, fully resuspend the pellets. Label a clean 2 ml microcentrifuge tube **Lysed cells** with your initials. Combine resuspended, lysed pellets into the lysed cells tube.



Place lysed cells into dry ice/ethanol bath for five minutes. Remove and thaw completely. Repeat two more times.



Add 500 µl Lysis buffer 2 to tube.



Centrifugation Purification Workflow

Separating Soluble from Insoluble Induced Cell Fractions and Preparation of SDS-PAGE Samples

Separate soluble from insoluble cell lysate components. Centrifuge for 20 minutes at 16,000 x g (thaw first if necessary).

Label 2 ml microcentrifuge tube **Soluble fraction** with your initials.

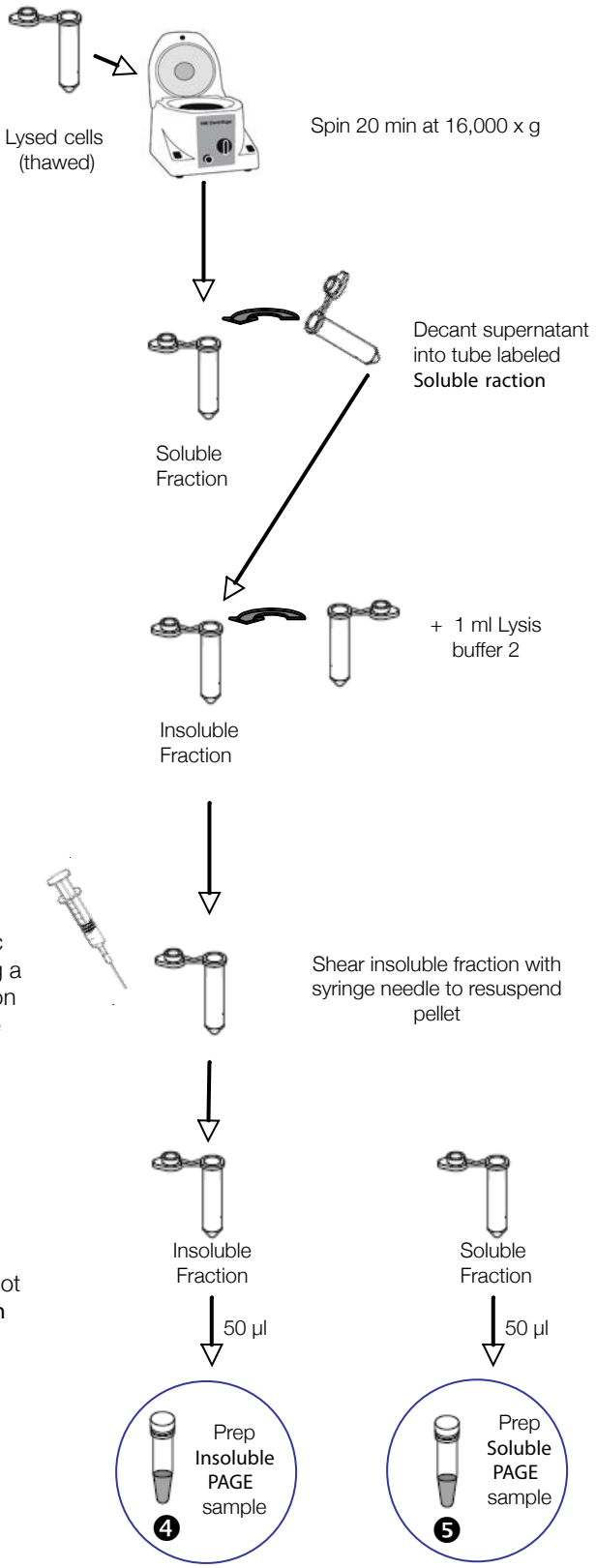
The supernatant contains the soluble fraction, and the pellet contains the insoluble fraction. Gently pour the supernatant into the tube labeled soluble fraction. Relabel the Lysed cells tube **Insoluble fraction.**

Add 1 ml Lysis buffer 2 to the insoluble fraction tube.

The insoluble pellet contains a large quantity of genomic DNA, making it viscous and difficult to resuspend. Using a syringe with 22 gauge needle, repeatedly pull the solution through the syringe to shear the pellet and decrease the viscosity.

Label two clean 1.5 ml screwcap microcentrifuge tubes **Soluble PAGE** and **Insoluble PAGE.** Take a 50 µl aliquot from each fraction Soluble fraction and Insoluble fraction and prepare for SDS-PAGE per manual instructions

Store the SDS-PAGE samples, Soluble and Insoluble fractions at –20°C until ready for further steps.



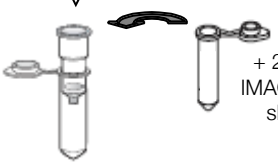
Centrifugation Purification Workflow

Affinity and Size Exclusion (Desalting) Purification of GST-DHFR-His

Pouring, washing and equilibrating column. Snap off bottom tab of empty Micro Bio-Spin column, label the column with your initials, remove cap and place in 2 ml microcentrifuge tube.

Snap off bottom tab and remove cap

Add 200 µl of IMAC resin slurry to empty column.

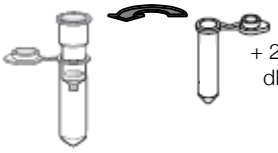


+ 200 µl IMAC resin slurry

Centrifuge for two minutes at 1,000 x g. After spin, discard buffer that has collected in the 2 ml microcentrifuge tube.

Spin 2 min at 1,000 x g

Add 200 µl of distilled water to column.



+ 200 µl dH_2O

Centrifuge for two minutes at 1,000 x g. After spin, discard water from 2 ml microcentrifuge tube.

Spin 2 min at 1,000 x g

Add 500 µl of Equilibration buffer to column.

+ 500 µl Equilibration buffer

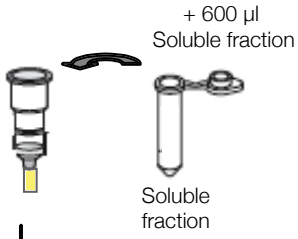
Centrifuge for two minutes at 1,000 x g. After spin, discard Equilibration buffer and 2 ml microcentrifuge tube. The column is now ready to use.

Spin 2 min at 1,000 x g

Centrifugation Purification Workflow

Affinity and Size Exclusion (Desalting) Purification of GST-DHFR-His (continued)

Sample binding to column. Place yellow tip closure on bottom of column. Add 600 µl (max) Soluble fraction to column; Put on clear top cap.



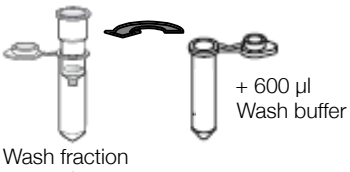
Gently mix for 20 minutes.

Label a 2 ml microcentrifuge tube **Flowthrough.** Remove yellow tip closure and place in 2ml microcentrifuge tube. Remove clear top cap. Centrifuge for two minutes at 1,000 x g.

Spin 2 min at 1,000 x g

Set aside Flowthrough.

Washing the column. Label a 2 ml microcentrifuge **Wash fraction**. Place column in tube. Add 600µ l Wash buffer to column.



Centrifuge for two minutes at 1,000 x g.
Set aside Wash fraction.

Spin 2 min at 1,000 x g

Centrifugation Purification Workflow

Affinity and Size Exclusion (Desalting) Purification of GST-DHFR-His (continued)

Elution of GST-DHFR-His protein from the column. Label a 2 ml microcentrifuge tube **Eluate**. Place column in tube. Add 400 µl Elution buffer to column.

Eluate

Spin 2 min at 1,000 x g

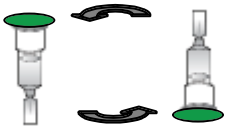
Centrifuge for two minutes at 1,000 x g.

Set aside Eluate.

Centrifugation Purification Workflow

Affinity and Size Exclusion (Desalting) Purification of GST-DHFR-His (continued)

Desalting of the GST-DHFR-His protein (Eluate fraction) to remove imidazole. Prepare desalting column (with green cap) by inverting sharply several times to resuspend gel.



Snap off bottom tab and place in 2 ml microcentrifuge tube. Remove green top cap. If the column does not begin to flow, push the cap back on the column and then remove it again to start the flow.

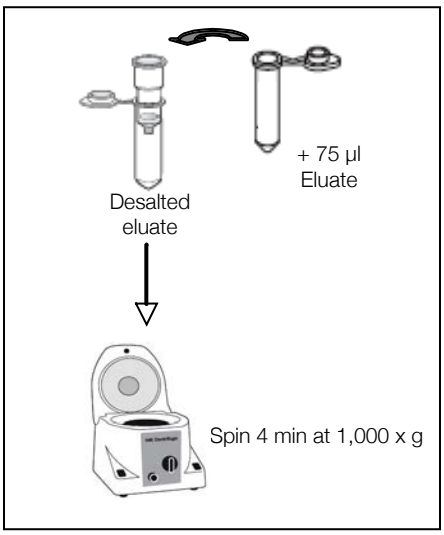
Allow excess packing buffer to drain by gravity to top of resin bed, then place column in clean 2 ml tube.

Centrifuge for two minutes at 1,000 x g. Discard remaining packing buffer and collection tube.

Spin 2 min at 1,000 x g

Label new 2 ml tube **Desalted Eluate**. Carefully apply 75 µl of Eluate fraction directly to the center of column. Be careful not to touch resin with pipet tip.

Centrifuge for four minutes at 1,000 x g. Repeat addition of 75 µl of Eluate fraction to column and centrifugation.

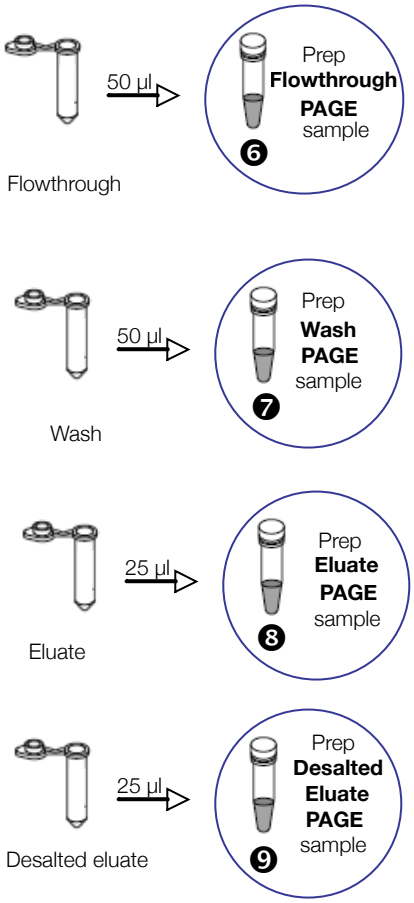


X 2

After second spin, discard column. You should now have ~150 µl of Desalted eluate (GST-DHFR-His).

Centrifugation Purification Workflow

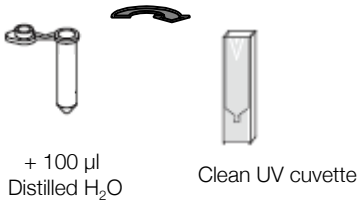
Prepare SDS-PAGE samples. Add equal volume of Laemmli buffer to each sample aliquot per manual instructions. Mix well. Heat all samples at 95°C for five minutes.



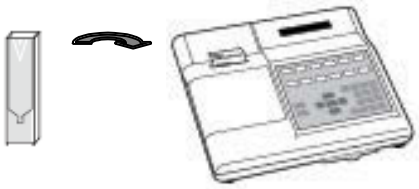
Centrifugation Purification Workflow

Quantitation of Protein in Desalted Fraction

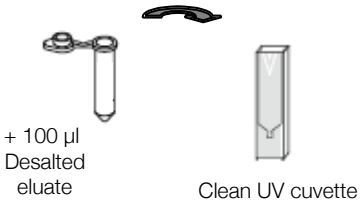
Turn on spectrophotometer and set absorbance to 280 nm. Add 100 µl distilled water to clean UV compatible cuvette.



Blank spectrophotometer with distilled water.



Pipet 100 µl of your **Desalted eluate** sample (GST-DHFR-His) into clean UV compatible cuvette.



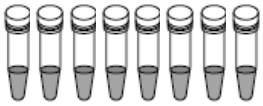
Measure absorbance of sample at 280 nm. Return sample to 2 ml tube.

Calculate concentration of GST-DHFR-His in your Desalted eluate fraction as described in instruction manual and record data.

Centrifugation Purification Workflow

SDS-PAGE Electrophoresis to Check Expression and Purity

Load, run, stain and destain the gel. Reheat SDS-PAGE samples at 95°C for two minutes. Centrifuge samples for two minutes at 16,000 x g.



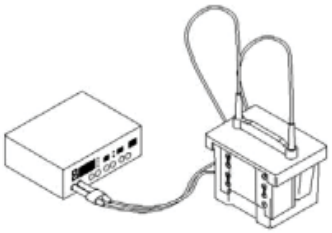
Heat 2 min at 95°C

Spin 2 min at 16,000 x g

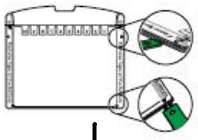
Using a fresh tip each time, load the following volumes of each sample into the appropriate well of your gel

Lane	Volume	Sample
1	10 µl	Precision Plus Dual Color standard
2	7.5 µl	Uninduced PAGE
3	15 µl	Induced PAGE
4	10 µl	Insoluble PAGE
5	10 µl	Soluble PAGE
6	10 µl	Flowthrough PAGE
7	20 µl	Wash PAGE
8	20 µl	Eluate PAGE
9	20 µl	Desalted eluate PAGE
10	10 µl	Laemmli buffer

Run the gel at 200 V for 30 minutes. (If using Mini-PROTEAN TGX precast gel, the gel can be run at 300 V for 15 minutes.)



After the run is complete, remove the gel from the cassette and place it in the gel staining tray.



Wash the gel three times with 50 ml of distilled water for five minutes each rinse. Discard all the rinse water.

Stain the gel with 50 ml of Bio-Safe Coomassie stain for one hour.

After one hour, discard the Bio-Safe Coomassie stain, add 100 ml of distilled water, and destain the gel overnight.

Image the gel if you have an imaging system or dry the gel if you have a cellophane drying system.

Chapter 5: Culturing, Expression, Lysis and SDS-PAGE Analysis for 100 ml Cultures for Chromatography Instrumentation Purification Process

Cell Culture and Induction

Student Workstations

Each student team requires the following items to streak a starter plate to produce single bacterial colonies:

Material Needed for Each Workstation	Quantity
LB/amp plate	1
Sterile inoculating loop	1
2–20 µl adjustable-volume micropipet and tips	1
Marking pen	1

Common Workstation	Quantity
Rehydrated BL21(DE3) *E. coli* containing pDHFR	1
Incubator set to 37°C	1

Each lab team will streak their own starter plate as a source of cells for culture for protein production. LB/amp plates should be streaked for single colonies and incubated at 37°C for 16–24 hours before the initial culture activity is planned.

Student Protocol: Streaking starter plates to produce single bacterial colonies

Using the rehydrated BL21(DE3) *E. coli* containing pHDFR plasmid at the common workstation to streak one plate. 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 cell multiplies to become millions of genetically identical cells in just 24 hours. There are millions of individual bacteria in a single 1 mm bacterial colony.

1. Pipet 10 µl of reconstituted *E. coli* using a sterile pipet tip onto an LB/amp plate. Use a sterile loop to perform the streaking. Streaking takes place sequentially in four quadrants. The first streak is to just spread the cells out a little. Go back and forth with the loop about a dozen times in one quadrant (Figure **5.1a**). In subsequent quadrants the cells become more and more dilute, increasing the likelihood of producing single colonies.

2. For subsequent streaks the goal is to use as much of the surface area of the plate as possible. Rotate the plate approximately 45 degrees (so that the streaking motion is comfortable for your hand) and start the second streak. Do not dip into the rehydrated bacteria a second time. Go into the previous streak about two times and then back and forth (Figure **5.1b**) for a total of about 10 times.

3. Rotate the plate again and repeat streaking (Figure **5.1c**).

4. Rotate the plate for the final time and make the final streak (Figure **5.1d**). When you are finished streaking the plate, cover it immediately to avoid contamination. Label your plate with your initials.

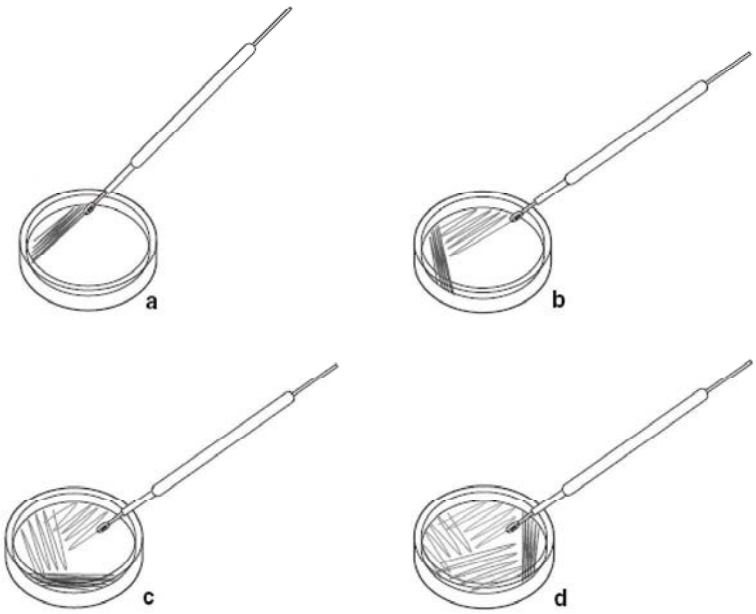


Figure 5.1. Streaking a plate to generate single colonies.

5. Place the plate upside down inside the incubator overnight at 37°C. Once colonies have grown to 1 mm in diameter they may be stored in a sealed bag upside down at 4ºC until ready to use for up to one month.

6. *E. coli* forms off-white colonies that are uniformly circular with smooth edges. Avoid using plates with contaminant colonies.

Overnight Cell Culture

Student Workstations

Each student team requires the following items to prepare an overnight cell culture:

Material Needed for Each Workstation	Quantity
LB/amp plate streaked with BL21(DE3) containing pDHFR	1
50 ml conical tube containing 15 ml sterile LB/amp broth	1
Sterile inoculating loop	1
20% sterile glucose	1 ml
100–1,000 µl adjustable-volume micropipet and tips	1
Marking pen	1

Common Workstation	Quantity
Tube roller in a 37° C incubator, shaking water bath or shaking incubator set to 37°C	1

Overnight Culture

1. Label the 50 ml conical tube containing 15 ml sterile LB/amp broth with your team's initials.

2. Add 750 µl of 20% sterile glucose to 15 ml of sterile LB/amp broth to make a final 1% glucose LB/amp broth solution.

3. Pick a single colony from the starter plate using a sterile loop and inoculate the 15 ml of 1% glucose, LB/amp broth by twirling the loop vigorously in the broth to dislodge the cells. Recap the tube. Incubate/shale the culture at 37°C for 12–18 hours.

   Note: If using a tube roller in an incubator, make sure to close the cap tightly on the 50 ml conical tube to avoid leakage.

Subculture and Induction

Student Workstations

Each student team requires the following items to prepare a sample of uninduced cells for SDS-PAGE analysis, to subculture the cells, and to induce the cells:

Material Needed for Each Workstation	Quantity
50 ml conical tube containing 15 ml overnight culture	1
500 ml Erlenmeyer flask containing 100 ml sterile LB/amp broth warmed to 37°C	1
LB/amp broth in two 2 ml microcentrifuge tubes for spectroscopy	4 ml
Screwcap microcentrifuge tube, 1.5 ml	1
1 M IPTG	25 µl
Laemmli sample buffer	1 ml
20–200 µl adjustable-volume micropipet and tips	1
100–1,000 µl adjustable-volume micropipet and tips	1
Pipet pump or bulb	1
10 ml sterile serological pipets	2
Semi-microvolume cuvettes	4
Parafilm squares	4
Marking pen	1

Common Workstation	Quantity
Shaking water bath or shaking incubator set to 37°C	1
Water bath or dry bath set to 95°C	1
Microcentrifuge with variable speed setting ≥16,000 x g	1
Spectrophotometer	1–2

Prepare uninduced SDS-PAGE sample

1. Label a 1.5 ml screwcap microcentrifuge tube "**Uninduced PAGE**" with your initials.

2. Aliquot 100 µl of the overnight culture into the tube.

3. Centrifuge the tube at 16,000 x g for 2 minutes.

4. Use a pipet to gently remove and discard the supernatant without disturbing the pellet.

5. Add 100 µl Laemmli sample buffer to the pellet and fully resuspend the pellet by pipeting up and down or vortexing.

   Note: It is critical to use only clean pipet tips when aliquoting Laemmli sample buffer or all future samples could become contaminated.

6. Heat the tube at 95°C for five minutes.

7. Store the sample at –20°C until ready to analyze via SDS-PAGE analysis.

8. Write your team's initials on your tube of Laemmli sample buffer and save this tube. This buffer will be used throughout the experiment.

Measure cell density of overnight culture

1. Prepare a 1:10 dilution of your overnight culture by combining 900 µl of sterile LB/amp broth with 100 µl of overnight culture in a clean semi-microvolume cuvette.

2. Cover the cuvette with parafilm and gently invert the tube several times to mix the diluted overnight culture.

3. If not already set up, set a spectrophotometer to read absorbance at 600 nm and then blank the instrument using 1 ml LB/amp broth in a cuvette. If using a Bio-Rad SmartSpec Plus spectrophotometer, see Appendix D for instructions on how to use the instrument.

4. Measure the absorbance at 600 nm (OD600) of the 1:10 dilution of overnight culture cells.

   OD600 of 1:10 dilution of overnight culture: _____

   OD600 of overnight culture = OD600 of 1:10 dilution x 10: _____

Prepare a subculture with a starting OD600 of 0.3

Prepare a 100 ml culture with an OD600 of 0.3 by combining the correct volumes of overnight culture and sterile LB/amp broth that has been warmed to 37°C.

$$\mathbf{X} \text{ ml of overnight culture} = \frac{100 \text{ ml} \times 0.3}{\text{OD600 of overnight culture}}$$

1. Calculate the amount of overnight culture that you will need to prepare a subculture with an OD600 of 0.3 and record the value below.

   _____ ml of overnight culture

2. Retrieve your Erlenmeyer flask containing 100 ml of LB/amp broth that has been warming to 37°C and label it with your team's initials.

3. Remove and discard a volume of LB/amp broth, equivalent to the volume you calculated in step 1, from the 100 ml flask that you would need of overnight culture using a sterile 10 ml serological pipet.

4. Gently swirl your overnight culture to resuspend all of the cells and then add the volume you calculated of overnight culture in step 1 to the remaining LB/amp in the Erlenmeyer flask using a sterile 10 ml serological pipet.

5. Cover the flask with foil and place the 100 ml culture in an incubator shaker at 37ºC with shaking at 250–275 rpm for one hour.

6. Record the time that you start your subculture below.

   Subculture Start Time: _____

7. Record the time you stop your subculture below.

   Subculture End Time: _____

Measure OD600 of subculture and induce GST-DHFR-His expression

1. After one hour of incubation measure the OD600 of your subculture.

2. Blank a spectrophotometer at 600 nm with 1 ml LB/amp broth.

3. Add 1 ml of subculture to a semi-microvolume cuvette and measure the OD600 of the undiluted subculture and record below.

   OD600 of subculture = _____

4. Add 25 µl of 1 M IPTG to the subculture in the 500 ml Erlenmeyer flask and continue incubating and shaking the culture at 37ºC for 4–24 hours. During this time period, most of the resources of the *E. coli* will go toward producing the recombinant GST-DHFR-His protein rather than cell division, though cell division does still occur.

5. Record the time that you start and end your induction below.

   Induction Start Time: _____

   Induction End Time: _____

Collecting cell pellet and lysing cells

Student Workstations

Each student team requires the following items to isolate their cell pellet, to prepare an induced cell sample for SDS-PAGE analysis, and to lyse their cells:

Material Needed for Each Workstation	Quantity
Induced cell culture	100 ml
Lysis buffer	5 ml
Equilibration buffer	5 ml
250 ml centrifuge bottles capable of withstanding 4,500 x g	1
50 ml sterile conical tube	1
Screwcap microcentrifuge tube, 1.5 ml	1
Laemmli sample buffer (left over from previous activity)	1 ml
20–200 µl adjustable-volume micropipet and tips	1
100–1,000 µl adjustable-volume micropipet and tips	1
Marking pen	1

Common Workstation	Quantity
Water bath or dry bath set to 95°C	1
Microcentrifuge with variable speed setting ≥16,000 x g	1
Benchtop or floor centrifuge with rotor for 250 ml centrifuge bottles	1
Vortexer	1
Dry ice/ethanol bath	1

Prepare induced SDS-PAGE sample

1. Label a 1.5 ml screwcap microcentrifuge tube "**Induced PAGE**" with your initials.
2. Aliquot 100 µl of the induced culture into the tube.
3. Centrifuge the tube at 16,000 x g for 2 minutes.
4. Use a pipet to gently remove and discard the supernatant without disturbing the pellet.
5. Add 100 µl Laemmli sample buffer to the pellet and fully resuspend the pellet by pipeting up and down or vortexing.
6. Heat the tube at 95°C for five minutes.
7. Store the sample at –20°C until ready to analyze via SDS-PAGE.

Pelleting induced cells

1. Place the 100 ml of induced culture in a 250 ml centrifuge bottle that will fit into your benchtop or floor model centrifuge.
2. Make sure that your vessel is balanced with either another student's bottle or with a bottle filled with water. If you are balancing your bottle with another student's bottle, sterile water can be added to achieve balanced bottles.

3. Centrifuge the cell pellet at 4,500 x g for 10 minutes.

4. When the centrifugation is done the supernatant should look transparent. If it is not transparent centrifuge the bottle for another 10 minutes. Carefully decant or pipet the supernatant making sure not to disturb the pellet and to dispose of the supernatant properly. Store the cell pellet at –20°C or continue on to lysing induced cells.

Lysing induced cells

1. Add 5 ml of lysis buffer to the cell pellet and using a pipet or a vortexer fully resuspend the cells in the lysis buffer. Make sure that there are no cell clumps remaining in the vessel. Pour the resuspended cells in lysis buffer into a 50 ml conical tube and label it "**Lysed Cells**" with your initials.

   Notes: As lysis occurs, the solution will get more viscous. Vigorous vortexing and pipeting might be necessary to resuspend the pellet.

   Use lab tap for the labeling of the 50 ml conical tube since ethanol in the dry ice/ethanol bath will remove the markings from the tube, but not from tape.

2. Place the tube of lysed cells in the dry ice/ethanol bath for five minutes. After five minutes remove the tube from the dry ice/ethanol bath and thaw completely. It is acceptable to thaw in a 37°C waterbath. Do not thaw by hand to avoid freezer burn to your hand.

   Note: Be careful not to allow the dry ice/ethanol to come in contact with your skin.

3. Repeat two more freeze–thaw steps for a total of three freeze–thaw cycles.

4. After the final thaw step add 5 ml Equilibration buffer and thoroughly mix by pipetting or vortexing.

5. The lysed cells can be stored at –20°C until they are ready to be purified.

Note: Another method that can be used for efficient lysis is freezing the cells in a –70°C freezer. The cells can be frozen at –70°C overnight and then thawed completely when separation of the soluble from the insoluble fraction is to be performed. If neither dry ice/ethanol or a –70°C freezer is availalble, it is possible to perform two freeze/thaw cycles using a –20°C freezer. Samples can be placed at –20°C overnight (check that samples are completely frozen), thawed the next day and then placed at –20°C overnight again before purification.

Separating Soluble from Insoluble Induced Cell Fractions and Preparing SDS-PAGE Samples

Student Workstations

Each student team requires the following items to separate soluble from insoluble lysate fractions and prepare soluble and insoluble fraction samples for SDS-PAGE analysis:

Material Needed for Each Workstation	Quantity
Thawed cell lysate	10 ml
50 ml sterile conical tube	1
30–50 ml centrifuge tube	1
Equilibration buffer	5 ml
2x PBS	5 ml
Screwcap microcentrifuge tubes, 1.5 ml	2
Laemmli sample buffer (left over from previous activity)	1 ml
20–200 µl adjustable-volume micropipet and tips	1
Pipet controller or bulb	1
10 ml serological pipet	4
10 ml syringe	1
22 gauge syringe needle	1
Marking pen	1

Common Workstation	Quantity
Water bath or dry bath set to 95°C	1
Microcentrifuge with variable speed setting ≥16,000 x g	1
Benchtop or floor centrifuge with rotor for 30–50 ml centrifuge tubes	1

Separate soluble from insoluble cell lysate components

1. Transfer the thawed cell lysate to the 30–50 ml centrifuge tubes that will fit into your model of centrifuge.

2. Make sure that your lysate is balanced with either another student's lysate fraction or with a centrifuge tube filled with water.

3. Separate the insoluble fraction of the lysed cells from the soluble fraction by centrifugation at 16,000 x g for 20 minutes.

4. Label a clean 50 ml sterile conical tube "**Soluble fraction**" with your initials. Gently pour the supernatant from the "**Lysed Cells**" tube into the "**Soluble fraction**" tube being very careful not to decant any of the insoluble fraction (the opaque, viscous blob that is mainly composed of genomic DNA) into your soluble fraction tube.

   Note: The insoluble fraction does not necessarily adhere to the tube, so extreme care is needed when decanting.

5. Relabel the "**Lysed Cells**" tube containing the remaining insoluble fraction **"Insoluble fraction**."

6. Add 5 ml of Equilibration buffer and 5 ml of 2x PBS to the insoluble fraction and resuspend the pellet by shearing with a syringe needle.

Note: The insoluble fraction contains a large quantity of genomic DNA that can be quite viscous, making resuspension difficult. Using a 22 gauge needle, the insoluble fraction can be pulled into a 10 ml syringe and expelled from the syringe into a clean tube multiple times to decrease the viscosity of the DNA. Ensure that the needle is disposed of properly in a sharps container.

7. Remove 50 µl of "**Soluble fraction**" and place it in a clean 1.5 ml screwcap microcentrifuge tube labeled "**Soluble PAGE**" with your initials. Add 50 µl Laemmli sample buffer and mix thoroughly.

8. Remove 50 µl of "**Insoluble fraction**" and place it in a clean 1.5 ml screwcap microcentrifuge tube labeled "**Insoluble PAGE**" and your team's initials. Add 50 µl Laemmli sample buffer and mix thoroughly.

9. Heat the "**Soluble PAGE**" and "**Insoluble PAGE**" samples at 95°C for five minutes.

   Note: Make sure that you are heating your blue SDS-PAGE samples and not your actual soluble and insoluble fractions!

10. Store the SDS-PAGE samples "**Soluble PAGE**" and "**Insoluble PAGE**" at –20°C until ready to analyze via SDS-PAGE.

11. Store the soluble fraction and insoluble fractions at –20°C until ready to purify the soluble fraction.

SDS-PAGE Electrophoresis Analysis of Induction

Student Workstations

Each student team requires the following items to analyze their induction samples via SDS-PAGE:

Material Needed for Each Workstation	Quantity
Uninduced PAGE sample	100 µl
Induced PAGE sample	100 µl
Insoluble PAGE sample	100 µl
Soluble PAGE sample	100 µl
Precision Plus Protein Dual Color standards	15 µl
4–20% Mini-PROTEAN TGX precast gel	1
Vertical electrophoresis chamber (per 1–4 gels)	1
1x Tris/Glycine/SDS (TGS) running buffer (per electrophoresis chamber)	700–1,200 ml
Power supply (200 V constant) to be shared between workstations	1
Buffer dam (only required if running 1 gel per box)	1
2–20 µl adjustable-volume micropipet and tips	1
Staining tray	1
Bio-Safe Coomassie stain (per gel)	50 ml
Marking pen	1

Common Workstation	Quantity
Water bath or dry bath set to 95°C	1
Microcentrifuge with variable speed setting ≥16,000 x g	1
Water for gel destaining (tap water is fine)	1 L

Load, Run, Stain and Destain the Gel

1. **Prepare samples**: Reheat SDS-PAGE samples at 95°C for two minutes to redissolve any precipitated detergent and then centrifuge the samples for two minutes at 16,000 x g.

2. **Assemble gel boxes**: If using Bio-Rad Mini-PROTEAN Tetra gel boxes and TGX precast gels, see Appendix E for more information on how to prepare gels and assemble gel boxes.

3. **Load the gel**: Using a fresh tip each time, load the following volumes of each sample into the appropriate well of your gel:

Well	Volume	Sample
1	10 µl	Precision Plus Protein Dual Color standard
2	7.5 µl	Uninduced PAGE
3	15 µl	Induced PAGE
4	10 µl	Insoluble PAGE
5	10 µl	Soluble PAGE
6–10	Empty (or a second lab group can use these wells to load their samples)	

4. Run the gel at 200 V for 30 minutes. (If using a Bio-Rad Mini-PROTEAN TGX precast gel, the gel can be run at 300 V for 15 minutes.)

5. After the run is complete, remove the gel from the cassette and place in the gel staining tray.

6. Wash the gel three times with at least 50 ml of distilled water for five minutes each rinse. Discard all the rinse water.

   Note: Make sure that all of the wash water has been removed since excess water diluting the gel stain will interfere with staining efficiency.

7. Stain the gel with 50 ml of BioSafe Coomassie stain for one hour.

8. After one hour discard the Bio-Safe Coomassie stain and add 100 ml of distilled water and destain the gel overnight.

9. Image the gel if you have an imaging system, such as the Bio-Rad Gel Doc™ EZ system, or dry the gel if you have a cellophane drying system.

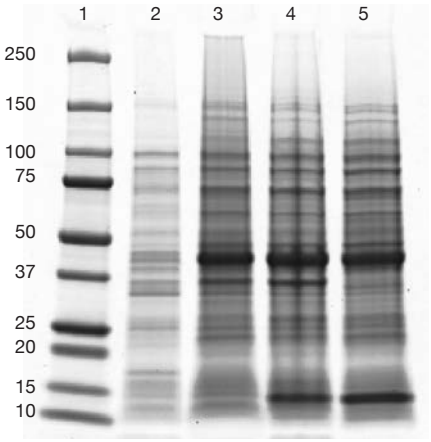


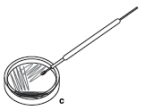
Figure 5.2. Results of uninduced, induced, insoluble, and soluble fractions analyzed with SDS-PAGE.

Lane 1. Precision Plus Dual Color standards.
Lane 2. Uninduced cells. There is no strong band at approximately 43 kDa so no GST-DHFR-His has been expressed.
Lane 3. Induced cells. A strong band is present at approximately 43 kDa that was not present in the uninduced cell sample (lane 2) and is GST-DHFR-His.
Lane 4. Insoluble fraction of *E. coli* cell lysate. A band is present that is the same size as the induced band in lane 3 and this represents the GST-DHFR-His that aggregated or was not folded properly and hence is not soluble and spins down with the cellular debris including cell walls, cell membranes, and the *E. coli* proteins which are not soluble in the 20 mM sodium phosphate, 300 mM NaCl, with 5 mM imidazole buffer
Lane 5. Soluble fraction of *E. coli* cell lysate.

Chromatography Instrumentation Purification Workflow

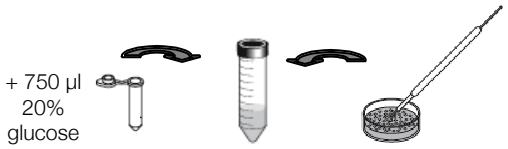
Cell Culture and Induction

Streaking starter plates to produce single bacterial colonies on agar plates. Pipet 10 µl BL21(DE3) *E. coli* containing pDHFR onto LB/amp plate; streak out using sterile inoculation loop. Incubate overnight at 37°C.



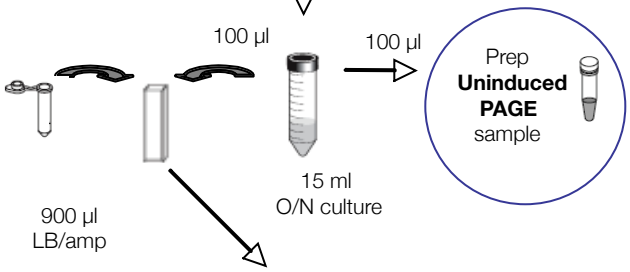
(37°C O/N)

Overnight culture. Label the 50 ml conical containing 15 ml of sterile LB/Amp broth with your initials. Add 750 µl 20% glucose and single *E. coli* colony to 15 ml of LB/amp liquid media. Incubate overnight at 37°C in shaking incubator or incubator/tube roller.



(O/N at 37°C
with shaking)

Prepare uninduced SDS-PAGE sample. Label 1.5 ml screwcap microcentrifuge tube **Uninduced PAGE** with your initials. Take 100 µl aliquot from overnight culture and prepare for SDS-PAGE per instruction manual.



Measure cell density of overnight culture. Add 900 µl LB/amp to cuvette; take 100 µl aliquot from overnight culture and add to same cuvette. Using a spectrophotometer, measure absorbance at 600 nm (OD600).

Measure
OD600

Prepare a subculture with a starting OD600 of 0.3. Determine amount to add to LB/amp for subculture with a starting OD600 of 0.3. Remove LB/amp equivalent volume of calculated cells to be added from prewarmed 500 ml flask containing LB/amp. Add calculated amount of cells from overnight culture to prewarmed 500 ml flask.

(1 hr at 37°C with shaking)

Measure OD600 of subculture and induce GST-DHFR-His expression. Incubate subculture for one hour at 37°C with shaking at 250–275 rpm. Measure OD600 and record.

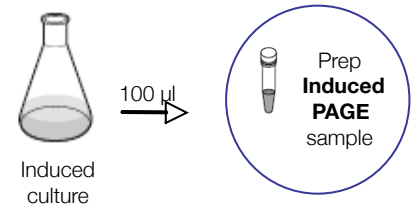
Induce expression by adding 25 µl 1M IPTG to culture and incubate 4–24 hours at 37°C with shaking at 250–275 rpm.

(4–24 hr at 37°C with shaking)

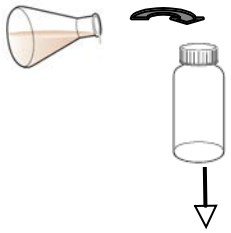
Chromatography Instrumentation Purification Workflow

Collecting cell pellet and lysing cells

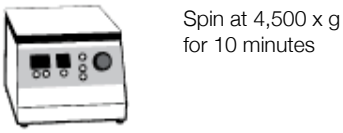
Prepare induced SDS-PAGE sample. After induction is completed, take 100 µl aliquot from induced culture and prepare for SDS-PAGE (label tube **Induced PAGE**).



Pelletinginduced cells. Pour the 100 ml induced culture into a 250 ml centrifuge bottle.



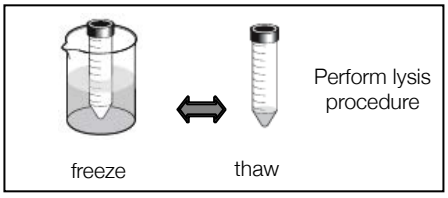
Balance centrifuge and centrifuge for 10 minutes at 4,500 x g to pellet the cells.



Carefully decant and discard the supernatant.

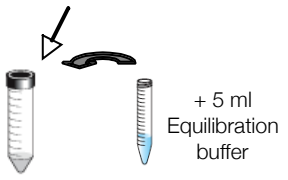
Lysing induced cells. Add 5 ml Lysis buffer to cell pellet. Using a vortexer or pipet, fully resuspend the pellet. Pour the resuspended cells in lysis buffer into a 50 ml conical tube and label **Lysedcells.**

Place Lysed cells into dry ice/ethanol bath for five minutes. Remove and thaw completely. Repeat two more times.



X 3

After the final thaw step, add 5 ml Equilibration buffer and thoroughly mix by pipetting or vortexing. Lysed cells can be stored in the –20°C freezer until ready to be purified.

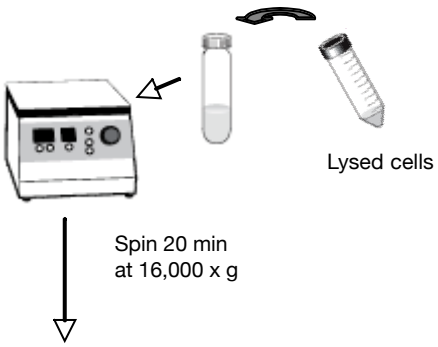


Chromatography Instrumentation Purification Workflow

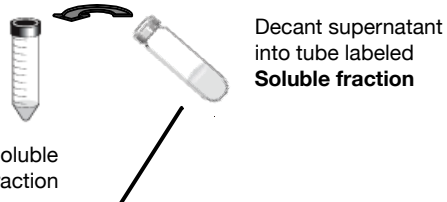
Separating Soluble from Insoluble Induced Cell Fractions and Preparation of SDS-PAGE Samples

Separate soluble from insoluble cell lysate components. Transfer thawed, lysed cells to a 30–50 ml centrifuge tube. Balance centrifuge and centrifuge for 20 minutes at 16,000 x g.

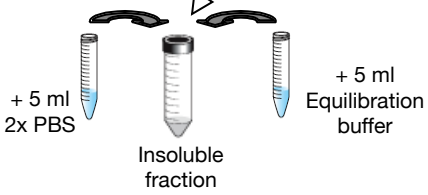
Label 50 ml sterile conical tube **Soluble fraction** with your initials.



The supernatant contains the soluble fraction, and the pellet contains the insoluble fraction. Gently pour the supernatant into tube labeled Soluble fraction. Relabel the Lysed Cells tube **Insoluble fraction**

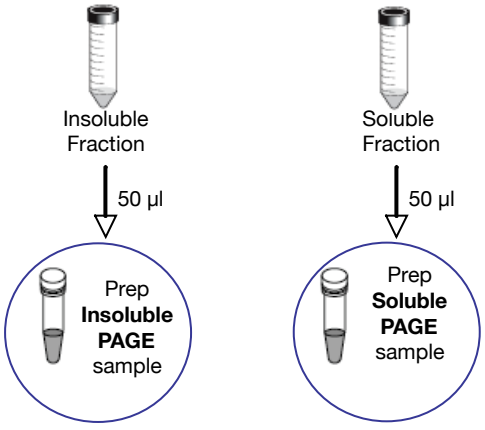


Add 5 ml Equilibration buffer and 5 ml 2x PBS to the Insouble fraction.



The insoluble pellet contains a large quantity of genomic DNA, making it viscous and difficult to resuspend. Using a syringe with 22 gauge needle, repeatedly pull the solution through the syringe to shear the pellet and decrease the viscosity.

Label two clean 1.5 ml screwcap microcentrifuge tubes **Soluble PAGE** and **Insoluble PAGE.** Take a 50 µl aliquot from each fraction **Soluble fraction** and Insoluble fraction and prepare for SDS-PAGE per instruction manual.



Store the SDS-PAGE samples, Soluble and Insoluble fractions at –20°C until ready for further steps.

Chromatography Instrumentation Purification Workflow

SDS-PAGE Electrophoresis Analysis of Induction

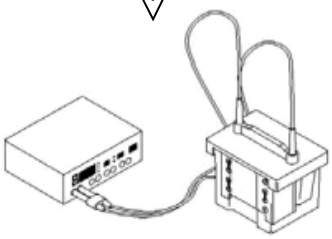
Load, run, stain and destain the gel. Reheat SDS-PAGE samples at 95°C for two minutes. Centrifuge samples for two minutes at 16,000 x g.

Heat 2 min
at 95°C

Spin 2 min
at 16,000 x g

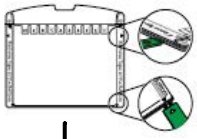
Using a fresh tip each time, load the following volumes of each sample into the appropriate well of your gel

Lane	Volume	Sample
1	10 µl	Precision Plus Dual Color standard
2	7.5 µl	Uninduced PAGE
3	15 µl	Induced PAGE
4	10 µl	Insoluble PAGE
5	10 µl	Soluble PAGE
6 - 10	Empty (or a second lab group can use these wells to load their samples)	



Run the gel at 200 V for 30 minutes. (If using Mini-PROTEAN TGX precast gel, the gel can be run at 300 V for 15 minutes.)

After the run is complete, remove the gel from the cassette and place in the gel staining tray.



Wash the gel three times with 50 ml of distilled water for five minutes each rinse. Discard all the rinse water.

Stain the gel with 50 ml of Bio-Safe Coomassie stain for one hour.

After one hour, discard the Bio-Safe Coomassie stain, add 100 ml of distilled water, and destain the gel overnight.

Image the gel if you have an imaging system or dry the gel if you have a cellophane drying system.

CHAPTER 6: Handpacking a Glass Econo-Column Column Attached to a Flow Adaptor

Student Workstations

If students are handpacking their own columns they will need to use the following protocol. Otherwise, if they are using prepacked cartridges, this section can be skipped.

Material Needed for Each Workstation	Quantity
Glass Econo-Column column 1 cm x 5 cm	1
Profinity IMAC Ni-charged resin slurry (50% slurry)	2 ml slurry
Flow adaptor for 1 cm ID columns*	1
Two-way stopcock	1
10 ml syringe	1
Beaker with distilled water	1
Beaker for waste	1
100–1,000 µl adjustable-volume micropipet and tips	1
Marking pen	1

*__Note__: For complete instructions on using the flow adaptor, please see the instruction manual included with the product.

Introduction

The Econo-Column column flow adaptor provides a number of advantages in column chromatography. Specifically, for purification of GST-DHFR-His it can be used for:

- Sample application with minimal dilution
- More efficient elution of GST-DHFR-His, because the 250 mM imidazole stock solution is not diluted when applied to the column

The Econo-Column column flow adaptors are shipped preassembled and ready to use. Figure 6.1 shows the parts of the assembled flow adaptor.

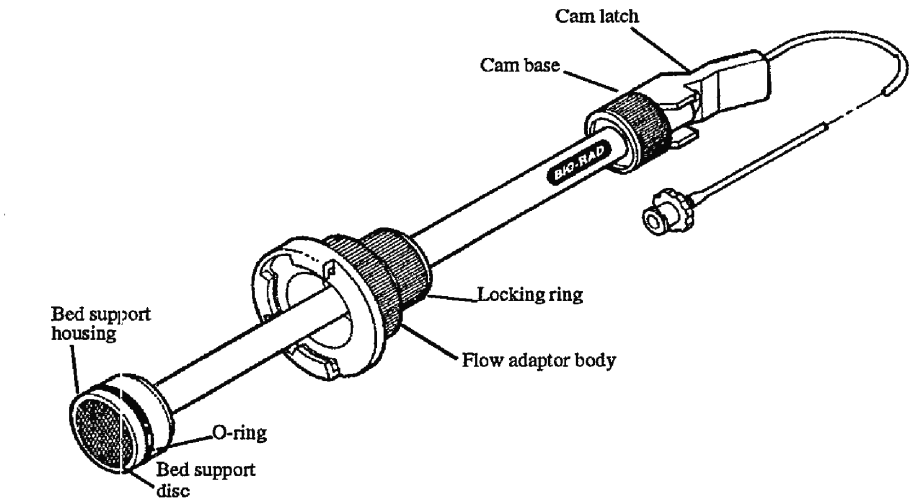


Figure 6.1. Econo-Column column flow adaptor.

Flow Adaptor Description

The cam latch can be adjusted to three positions allowing the flow adaptor to be situated into a glass column, and the O-ring to be tightened to seal the flow adaptor securely into the column so that there is no movement once the flow adaptor has been set up (Figure 6.2).

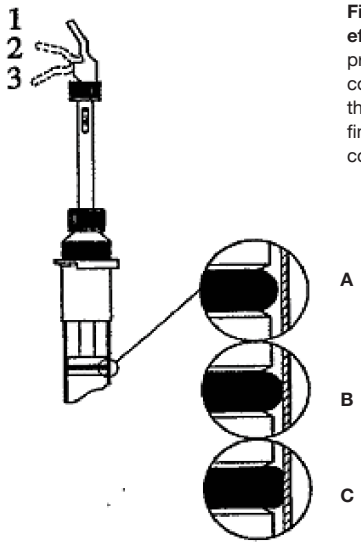


Figure 6.2. The three positions of the cam latch and the corresponding effects on the O-ring. When the cam latch is in position 1, there is little pressure on the O-ring (A), and it should slide with ease through the glass column. When the cam latch is in position 2, the O-ring seals against the glass column wall (B) with a small amount of movement possible for final height adjustments. When the cam latch is in position 3, the O-ring completely seals the flow adaptor into position (C).

Fitting the Flow Adaptor to the Glass Econo-Column column

Due to normal tolerances in glass diameters, each flow adaptor should be adjusted to fit to individual columns. This will ensure optimal performance.

1. Pull the white flow adaptor body all the way up into the collar of the black flow adaptor body (See Figure 6.3).

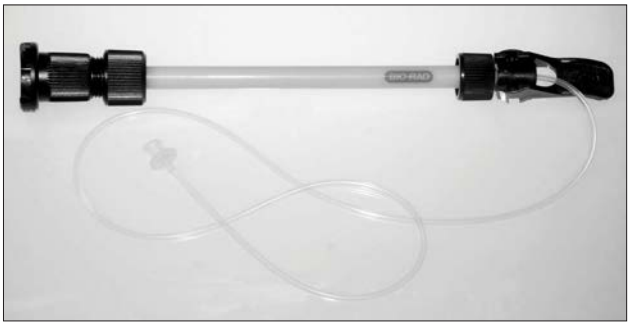


Figure 6.3. Preparing the flow adaptor to be fit into a glass Econo-Column column.

2. Attach the flow adaptor body to the column by sliding the flow adaptor body onto the yellow flange on the top of the glass column (Figure 6.4).

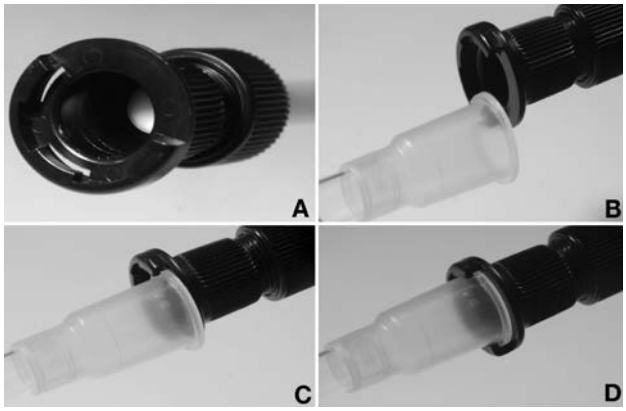


Figure 6.4. Attaching the flow adaptor to the glass Econo-Column column. A) Look for the groove on the end of the black flow adaptor body. B) Line up the groove on the black flow adaptor body with the top of the yellow flange on the column. C) Gently slide the flow adaptor onto the glass column. D) The flow adaptor should snap into place when completely positioned on the top of the column.

3. Make sure the cam latch is in position 1 and slide the white flow adaptor into the column until the black O-ring is visible through the glass (Figure 6.5).

   Note: If the flow adaptor will not slide easily into the glass column do not force it! Continue to Step 4 to adjust the tension on the O-ring.

4. Adjust the tension on the O-ring if the flow adaptor will not slide into the column.

   Note: Proceed to Step 5 if your flow adaptor was able to slide into the column (similar to Figure 6.5).

   If the flow adaptor does not slide easily into the glass column, then the O-ring needs to be adjusted. Holding the cam base, slowly turn the cam latch counterclockwise until the flow adaptor can be easily slid into the column (Figure 6.6).

5. Adjust the final tension on the O-ring by holding the cam base and slowly turning the cam latch clockwise until the O-ring begins to engage the glass, then turn it clockwise ¼ turn (Figure 6.7).

Figure 6.5. Adjusting the O-ring. Cam latch in position 1. White flow adaptor in the glass column.

Figure 6.6. Adjusting the tension on the O-ring if the flow adaptor will not slide into the column. The flow adaptor cannot slide beyond the yellow plastic flange due to the O-ring not fitting. While holding the cam base with one hand, the cam latch is turned counterclockwise to loosen the O-ring. (Figure 6.7) White flow adaptor in the column. (Figure 6.5)

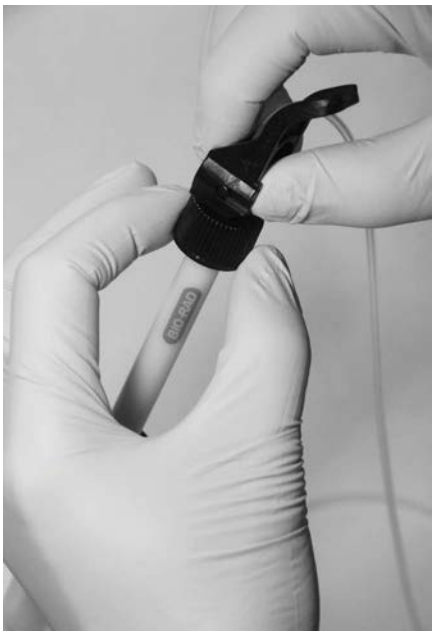


Figure 6.7. Final adjustment of the tension on the O-ring. While holding the cam base with one hand, the cam latch is turned clockwise to tighten the o-ring and turned counterclockwise ¼ turn to loosen the O-ring enough to remove the flow adaptor to add resin to the column.

6. Remove the flow adaptor from the column. Place the two-way stopcock on the luer fitting at the bottom of the column and close the stopcock. This is done by turning the white arm perpendicular to the flow path (Figure 6.8). Your column is now ready to pack.

Figure 6.8. Two-way stop cock. (A) Closed configuration (B) Open configuration.

Packing a 1 ml Profinity IMAC Ni-Charged Resin Column and Inserting the Flow Adaptor

Packing the Resin Bed

1. Fully resuspend the Profinity IMAC Ni-charged resin and then carefully pipet 2 ml of the resin slurry into the column. Stand the column upright and allow the resin to settle onto the bed support of the column (Figure 6.9, left picture).

2. Gently pipet 2 ml of distilled water into the column and allow any disturbed resin to settle (Figure 6.9, right picture).

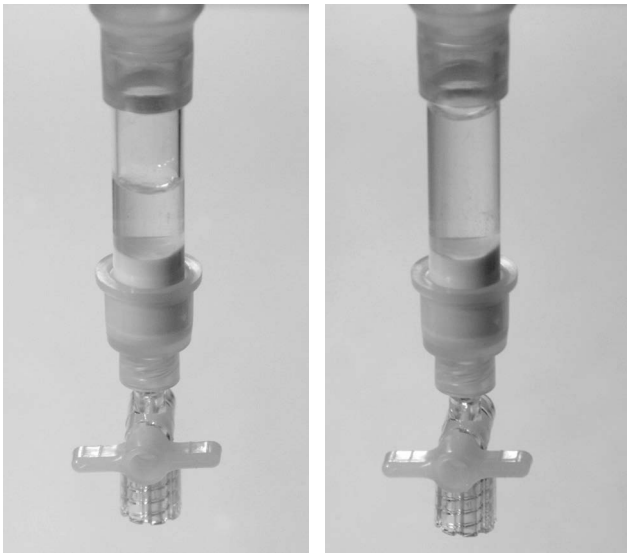


Figure 6.9. Packing the column with resin. 2 ml of 50% slurry were dispensed into the column. After the resin has settled, there is 1 ml of resin and 1 ml of packing buffer above the resin. Note that the stopcock is in the closed position. (Left picture)
2 ml of water are added to the column and the resin is allowed to settle again before the flow adaptor is inserted. (Right picture)

Inserting the Flow Adaptor into the Packed Column

1. Priming (filling with water) the flow adaptor tubing

   A. Fill a 10 ml syringe with distilled water.

   B. Attach the syringe to the female luer lock fitting at the top of the flexible tubing on the flow adaptor (Figure 6.10)

   C. With the bottom of the flow adaptor over the sink or a beaker, gently push 5 ml of water out of the syringe and through the flow adaptor until water is flowing out of the bottom of the flow adaptor.

   D. Leave the syringe attached to the flow adaptor tubing.

Figure 6.10. Priming the flow adaptor. Priming the flow adaptor involves filling the tubing of the flow adaptor with water and removing all air bubbles from the flow path.

2. Attaching the flow adaptor to the column.

A. Pull the flow adaptor bed support all the way up into the flow adaptor body (see Figure 6.3).

B. Attach the flow adaptor body to the top of the column by gently sliding the flow adaptor body over the yellow flange of the column top. Be careful since the glass column can break (see Figure 6.4).

C. Place the flow adaptor over a beaker or the sink and open the two way stopcock on the bottom of the column (Figure 6.11).

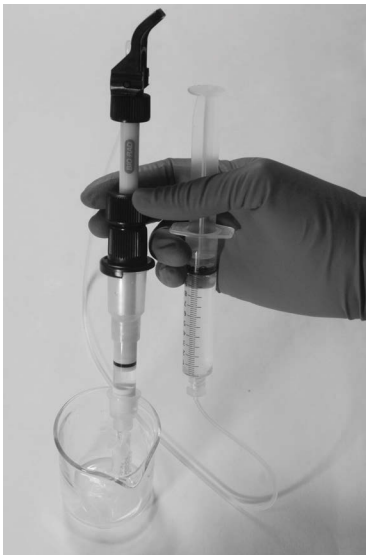


Figure 6.11. Inserting the flow adaptor into the column packed with resin. The cam latch is in position 1 and the flow adaptor has been inserted into the column to the top of the water above the resin. The two-way stopcock at the bottom of the column is open to allow buffer to drain out as the flow adaptor is lowered. The syringe is still attached to the tubing since this prevents water from flowing back up into the syringe at this time.

Figure 6.12. Adjusting the flow adaptor height to the resin bed. The cam lock is moved to position 2. The syringe is removed from the tubing and the tubing is placed into the beaker. The two-way stopcock is moved to the closed position. The flow adaptor has been fully lowered to just above the resin bed. The lowering of the flow adaptor body should force the excess buffer out of the flexible tubing into the beaker.

Figure 6.13. Closing the cam latch, position 3.

D. Make sure the cam lock is in position 1. Carefully insert the flow adaptor into the column until it touches the top of the water above the settled resin bed.

F. Move the cam latch to position 2 (Figure 6.12). Remove the syringe from the flexible tubing at the top of the flow adaptor. Close the two-way stopcock.

G. Slowly lower the flow adaptor until it touches the top of the packed gel bed. The O-ring seal should be tight enough so that water should now be flowing out of the top of the flexible tubing. Make sure the tubing is over a beaker or paper towel, not your lap!

H. Move the cam latch to position 3 (Figure 6.13).

I. Secure the flow adaptor by turning the black locking ring clockwise.

J. Your column is now ready to be washed to remove the remaining storage buffer and to be used for purifying proteins.

Chapter 7: Purification Protocol for BioLogic LP System

Affinity Purification of GST-DHFR-His Using a BioLogic LP System

Student Workstations

Each student team requires the following items to purify their GST-DHFR-His samples using affinity chromatography:

Material Needed for Each Workstation	Quantity
Soluble lysate fraction	5–7.5 ml
Either a BioScale Mini Profinity Ni-IMAC Cartridge	1
or	or
An Econo-Column Glass column handpacked with 1 ml of Profinity IMAC Ni-Charged resin	1
10 ml syringe	1
Distilled water	500 ml
Buffer A	70 ml
Buffer B	40 ml
Collection tubes for fraction collector	32
Marking pen	1

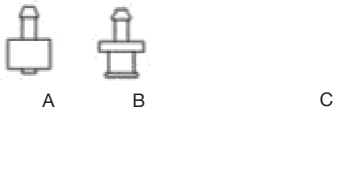
Common Workstation	Quantity
BioLogic LP system instrumentation, with sample loop installed	1–4

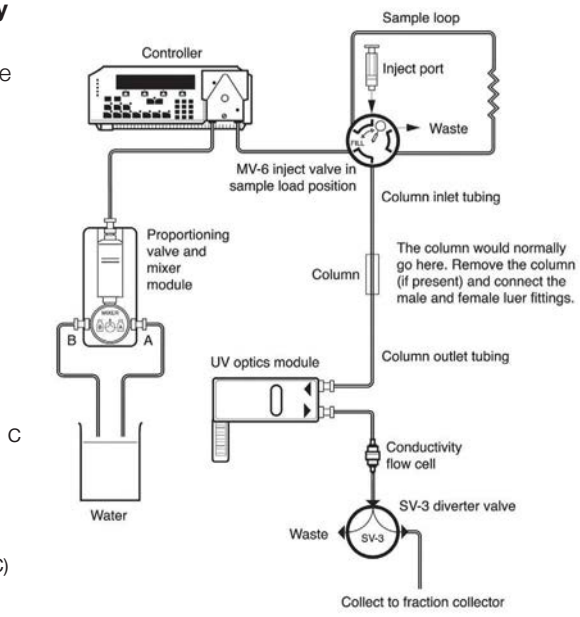
Preparing the Biologic LP for Use

Starting up the BioLogic LP system

Before a purification method can be run, you must take proper steps to evacuate all air from the system and fill up the instrument lines with water to purge any storage buffer that might be in the tubing.

1. Make sure that there are **no chromatography columns connected** to the system. The column inlet tubing should be connected to the column outlet tubing (Figure 7.1).





C

Figure 7.1. System connections without a column in place. (A) Male luer fitting with barb (B) Female luer fitting with barb (C) Flow path of the BioLogic LP system.

1. Verify that the buffer inlet lines for Buffer A and Buffer B are submerged in a container of 500 ml of high-quality water (Figure 7.2). Make sure that the tubing goes all the way to the bottom of the container since it is important to not pull air into the tubing.

2. Make sure that the waste line flows into a large beaker or bottle.

3. Turn on the BioLogic LP system controller using the power switch on the front of the instrument.

4. Verify that the arrow on the left side of line 2 of the LCD points to the right (Figure 7.3). This arrow indicates the direction of flow. If the arrow points to the left press the **Flow** softkey followed by the **Forward** softkey to change the direction of flow. Press the **OK** softkey to confirm the change.

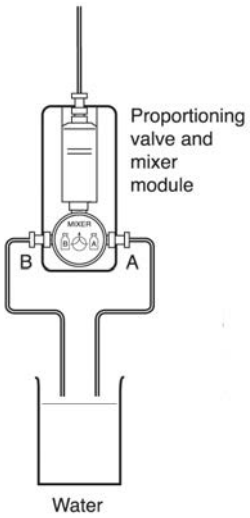


Figure 7.2. Inlet tubing for the mixer, immersed in water.

6. **If you have DataView software (otherwise skip to Purging air from the system by flushing with water, below):** Launch your LP DataView software and make sure that the BioLogic LP system is properly communicating with the software by the presence of a message "**Receive**" with a green dot next to it (immediately to the left of the Bio-Rad logo.)

7. Press **Record** in the LP DataView software to begin recording the UV and conductivity data.

Purging air from the system by flushing with water

Initially you will be running water through the system. The water will be run through all the tubing starting at both the A inlet and B inlet to the mixer/proportioning valve (Figure 7.2) and from there through the UV detector, the conductivity detector and then to waste. This is called purging the system since buffer or storage solution in the system will be flushed out along with any air bubbles. First, you will draw water up only through the A inlet tubing, and then you will learn how to change which inlet the pump draws fluid from by changing to the B inlet. At the end of the purging phase, the entire fluidics system will be filled with water and there should be no air bubbles in any of the lines.

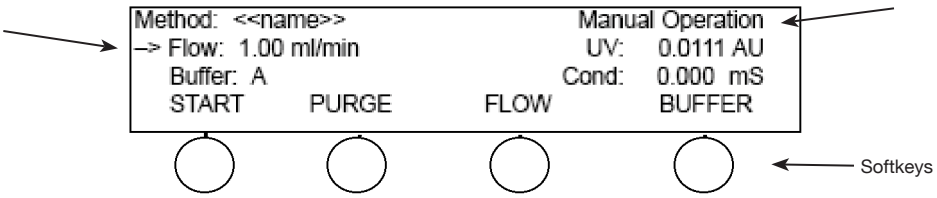


Figure 7.3. When you turn on the BioLogic LP system controller, the system automatically shows Manual mode for pump operation.

1. **Making sure the injection valve is in load position:** Turn the MV-6 injection valve fully counterclockwise (as far left as it will go) (Figure 7.4).

2. **Select Buffer A Inlet:** Press the **BUFFER** softkey, select **A** by pressing the **Next or Previous** keypad buttons (if necessary)**,** then press the **OK** softkey.

3. Press the **PURGE** softkey to run the pump at its maximum flow rate. Water will now flow through the system at a flow rate of 6.5 ml/min. Allow the flow to continue for five minutes.

4. **Change to Buffer B:** While the pump is still running, press the **BUFFER** softkey, select **B** by pressing the **Next** instrument key**,** then press the **OK** softkey. You should hear a soft click as the proportioning valve moves. Visually inspect all tubing and make sure that there are no air bubbles anywhere in the system.

5. You will continue to have water flow through the system while you set the UV monitor range, zero the UV monitor and set the conductivity range. Make sure that you have plenty of distilled water remaining in your container and that the inlet tubing is near the bottom of the container.

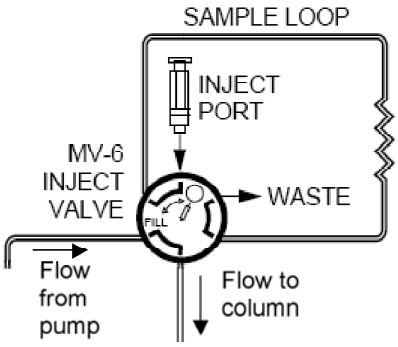


Figure 7.4. MV-6 valve in sample load position. The MV-6 valve has two positions. When it is turned all the way to the left (counterclockwise), the valve is in the LOAD position. In this position, a sample can be loaded into the sample loop. Fluid from the pump does not go through the sample loop but instead flows directly onto the column.

Set the UV Monitor range, and zero the UV monitor

1. Press the **UV** instrument key, then press the **SET RANGE** softkey.

2. Using the **INCREASE** and **DECREAS**E softkeys, set UV monitor range to 1.00 AUFS. AUFS stands for absorbance units full scale and 1.00 is the maximum absorbance that is expected for this run.

3. Press the **OK** softkey.

4. Press the **ZERO** softkey—the system will set the UV reading to zero.

5. Press the **Pump** instrument key.

Set the conductivity monitor minimum and maximum range

1. Press the **Cond** instrument key.

2. Press the **MIN/MAX** softkey.

3. In the Min field, enter 0 mS using the number keypad.

4. Press the **SETMAX** softkey.

5. Enter the conductivity of 90 mS using the number keypad.

6. Press **OK.**

7. Press the **Pump** instrument key.

Finish purging the BioLogic LP system

1. While the purging is occurring, look at the BioLogic LP system front panel (or DataView software if you have it) and see what the absorbance and conductivity readings are. If the readings are not steady (changing more than 0.05 AU and 0.1 mS units), follow the fluid flow path from the inlet tubing, through the mixer, into the UV detector, and into the conductivity meter and look for bubbles.

2. Continue running water through the lines until the absorbance and conductivity values are steady (changing less than 0.05 AU and 0.1 mS units).

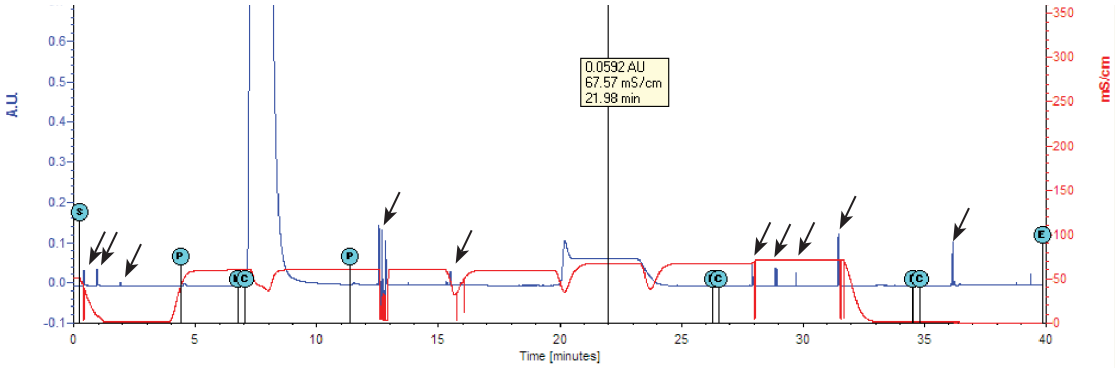


Figure 7.5. DataView software showing air bubbles in the lines during a purification run. The arrows point to times where air bubbles went through the detectors resulting in spikes in the signals.

1. Record the absorbance and conductivity values.

   Absorbance _____ AU Conductivity _____mS

   Since just water is running through the system and you have zeroed the absorbance value, the absorbance should be around 0.000 AU, and the conductivity around 0–3.0 mS (dependent on the level of deionization of the water).

2. Press the **STOP** softkey to stop the pump.

Equilibrating the column and priming the tubing with buffers A and B

The tubing is currently filled with water and needs to be primed with buffers A and B. Also, the column to be used needs to be washed and equilibrated with the same buffer as that used to generate the soluble lysate fraction. First, Buffer B (the buffer that will be used for eluting your GST-DHFR-His) will be run through the system and the column to wash off any residual proteins on the column. Second, an equilibration buffer that is a combination of Buffer A and Buffer B will be run through the system and column to wash away all the Buffer B on the column (so that the GST-DHFR-His will bind to the resin) and to equilibrate the column for usage.

Installing the prepacked column—Bio-Scale Mini Profinity IMAC Cartridge

(If you are using a handpacked column, go to Chapter 6: Handpacking a Glass Econo-Column Column attached to a Flow Adaptor, page 106.)

1. Separate the luer fittings that connect the column inlet and column outlet tubing (Figure 7.1 and Figure 7.6 A).

2. Connect the female luer fitting from the column outlet tubing to the bottom, male luer fitting of the column outlet (Figure 7.1 and Figure 7.6B).

3. Using a pipet, dribble high-quality water into the inlet luer fitting of the Bio-Scale Mini column (Figure 7.6C). Filling the luer fitting with water prevents the air that is in this fitting from being pushed into the column bed.

4. Connect the male luer fitting from the column inlet tubing into the top of the column (Figure 7.6D).

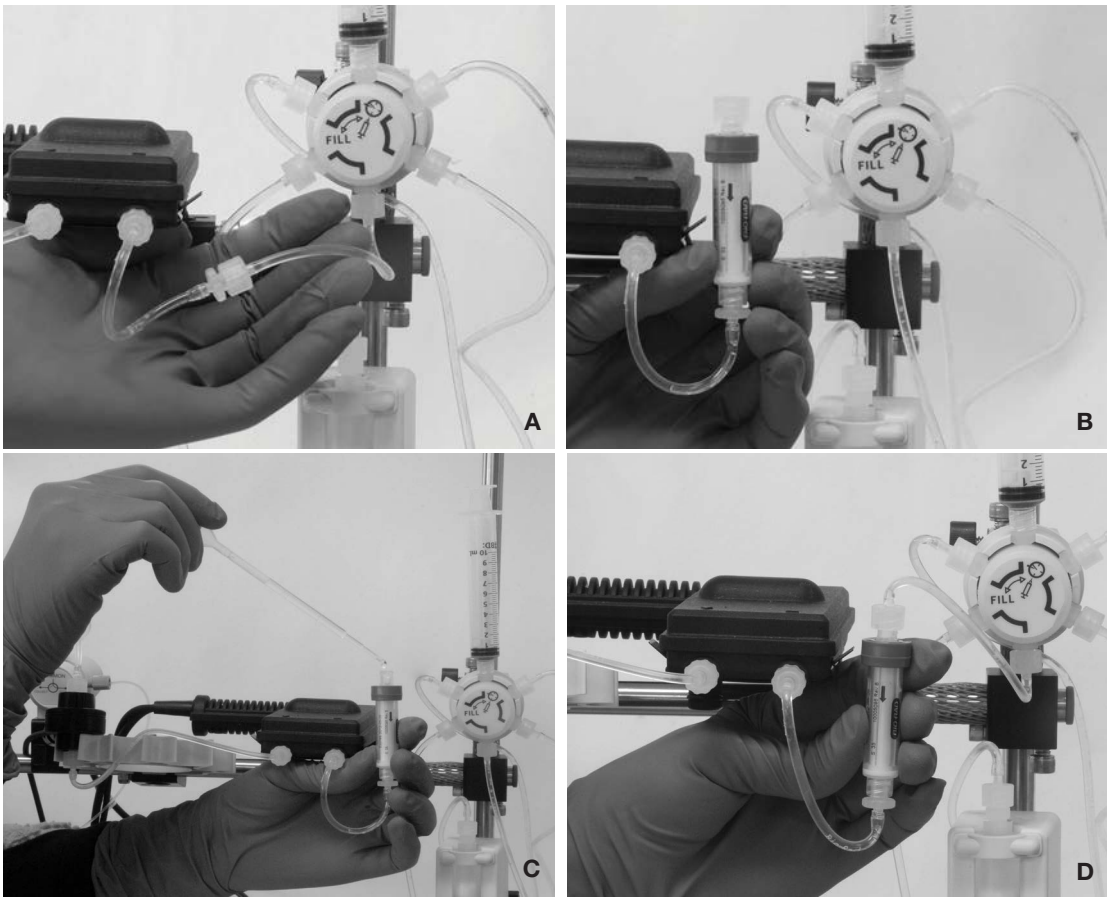


Figure 7.6. Installing the column. (A) Disconnect the column inlet and outlet tubing fittings. The column inlet tubing connects the MV-6 injection valve to the column. The column outlet tubing connects the outflow of the column to the UV detector. (B) Attach the bottom of the column (note direction of the arrow on the column) to the female luer fitting on the column outlet tubing. (C) Fill the female luer fitting at the top of the column with water using a pipet. If the luer fitting is not filled with water, an air bubble will be pushed through the column that can disturb the resin bed packing, which can disrupt purification. (D) Attach the male luer fitting on the column inlet tubing to the female luer fitting on the top of the column

Installing the Column—Handpacked Column with a Flow Adaptor

1. Fill a 10 ml syringe with 10 ml of high quality-water and attach the syringe to the inlet luer fitting on the tubing on the top of the flow adaptor (Figure 7.7A).

2. Remove the two-way stopcock from the outlet luer fitting of the column.

3. With the outlet of the column over a waste beaker, gently push the syringe plunger and flow 5 ml of water through the column (Figure 7.7A).

4. Separate the luer fittings that connect the column inlet and column outlet tubing of the BioLogic LP system (Figure 7.1 and Figure 7.7B).

5. Attach the column outlet tubing of the BioLogic LP system to the male luer fitting on the bottom of the column and press the syringe plunger to flow 3 more ml of water through the column and BioLogic LP system tubing to make sure that all air bubbles are removed from the column (Figure 7.7C)

6. Remove the syringe and attach the column inlet tubing of the BioLogic LP system to the female luer lock fitting at the end of the flow adaptor tubing (Figure 7.7D).

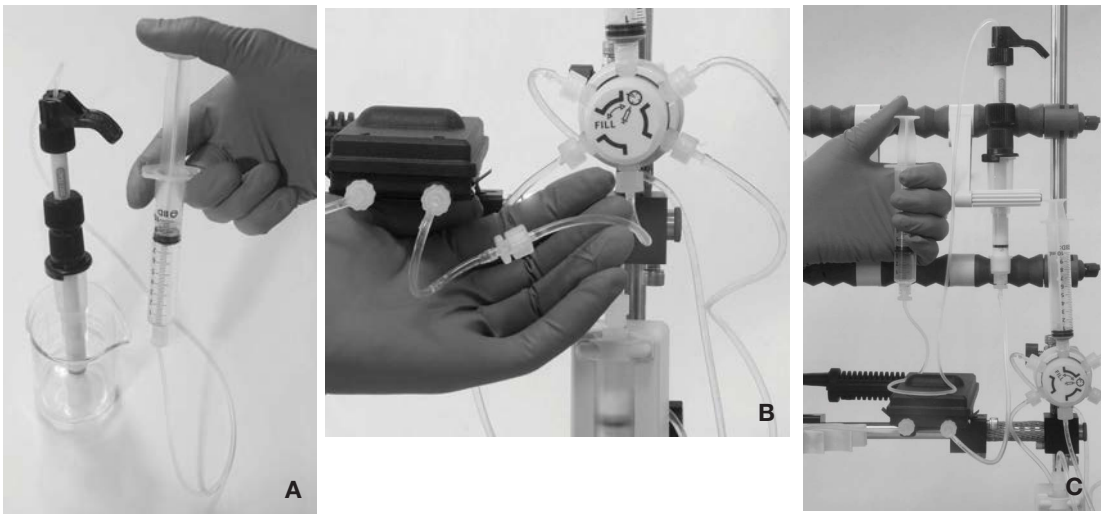


Figure 7.7. Installing a handpacked column with a flow adaptor. A) A syringe filled with 10 ml of water is connected to the tubing on the flow adaptor via the luer lock fittings. 5 ml of water is flowed gently over the column into a waste beaker. This serves to fill up the tubing with water and provide a first rinse of the resin with water to remove storage buffer. B) The column inlet and column outlet tubing of the BioLogic LP system are separated for installation of the column. C) The column outlet tubing of the BioLogic LP system is attached to the outlet luer lock fitting on the column. 3 ml more water is gently pushed through the column using the syringe still attached to the flow adaptor tubing. This water serves to further rinse the resin as well as to purge air bubbles out of the column oulet tubing. D) The syringe is removed from the flow adaptor tubing and the column inlet tubing that comes from the MV-6 injection valve is connected to the column putting the column inline for flow of buffers from the BioLogic LP system.

Priming with buffer A and buffer B

1. Using the system's manual mode operation of the pump, set a flow rate that is safe for the column, which in this case is 2 ml/min. To do this follow the steps below:

    a. From the front panel of the BioLogic LP system, press the **Manual** mode key, followed by the **Pump** instrument key. The yellow light above each key should be on.

    b. Using the softkeys below the front panel's display, press the **FLOW** softkey.

    c. Using the front panel's keypad, enter a flow rate of 2 ml/min, then press the **OK** softkey to accept.

    d. Using the softkeys below the front panel's display, press the **BUFFER** softkey.

    e. Using the **Previous** and **Next** keys, toggle until **B** is bracketed and then press the **OK** softkey to select Buffer B.

2. Place the inlet tubing for Buffer A in the bottle of Buffer A and the inlet tubing for Buffer B in the bottle of Buffer B. Make sure the tubing goes to the bottom of the bottles.

3. Press the **START** softkey to begin flow and to purge air that may have entered the system when the column was connected. Prime the Buffer B inlet tubing with Buffer B and do a final wash of the column with Buffer B. Allow the system to run for seven minutes (14 ml of Buffer B) then press the **STOP** softkey.

4. Look at the front panel of the BioLogic LP system and record the final conductivity and absorbance values after pure Buffer B has been run through the system.

   Absorbance _____ AU

   Conductivity _____mS

   Buffer B has been running through the system, and it contains high salt and imidazole levels which contribute to a higher conductivity reading. The conductivity readings after equilibrating with Buffer B should be approximately 35–55 mS. Imidazole also absorbs at 280 nm so there should be some absorbance due to the 250 mM imidazole in Buffer B. The absorbance should be approximately 0.00–0.15 AU.

   Now the column will be primed with Equilibration buffer (98% Buffer A, 2% Buffer B = 20 mM Phosphate buffer, 300 mM NaCl, 5 mM Imidazole) in preparation for binding of the GST-DHFR-His.

5. Press the **BUFFER** softkey and then press the **MIX** softkey.
   Note: MIX is a mixture of Buffer A and Buffer B.

6. Using the number keypad, enter **2% B** then press the **OK** softkey.

7. Press the **START** softkey to begin flow. Allow the system to run for seven minutes (14 ml of Equilibration Buffer). You will hear a clicking sound which is the proportioning valve/mixer pulling from both beakers of buffer.

8. After seven minutes, press the **STOP** softkey to stop the flow.

9. Note the final conductivity and absorbance values after 2% Buffer B has been run through the system.

   Absorbance _____ AU Conductivity _____mS

Only 2% Buffer B has been running through the system and it contains high salt but low imidazole levels. The high salt contributes to a higher conductivity reading (expected range of 35-55 mS). However, in comparison to 100% Buffer B, only 2% Buffer B should have little to no absorbance at 280 nm (in the range of 0.000–0.05 AU).

Select your Fraction Collector

BioFrac fraction collector only—fraction collector setup

Note: If you are using a Model 2110 fraction collector, proceed to the Model 2110 Fraction Collector setup on the next page.

1. Turn the BioFrac fraction collector's power switch On. The switch is in the back right hand side of the instrument.

2. On the BioFrac fraction collector front panel, using the **Cursor Control Arrow** keys, move the flashing cursor until it is at **Mode:** options. Press the **ENTER** key to see the options. Use the **Cursor Control Arrow** keys to select LP/Econo and then press the **ENTER** key again.

1. Make sure that the rack that is listed on the Biofrac fraction collector's front panel is the one being used. The standard rack is the F1 rack, which is 6 x 15 tube spaces. Otherwise, press the **Rack** softkey and use the **Cursor Control Arrow** keys to toggle to the **Rack:** options. Choose the correct rack option and then use the **Cursor Control Arrow** keys to choose Divert between tubes: On and Collection pattern: Serpentine if not already the defaults. Press the **Done** softkey to exit rack selection.

2. Use the **Cursor Control Arrow** keys to select the Multirun: Overlay option and then press the **ENTER** key.

3. All other settings can be left as the defaults.

4. Press the **ENGAGE** softkey to move the drop collector to the first tube and allow communication between the BioLogic LP system and the BioFrac fraction collector (Figure 7.8).

   Note: If the drop former does not move to the postion above tube 1, consult your instructor.

5. On the BioLogic LP system, press the **Collector** instrument key. Then press the **MODEL** softkey.

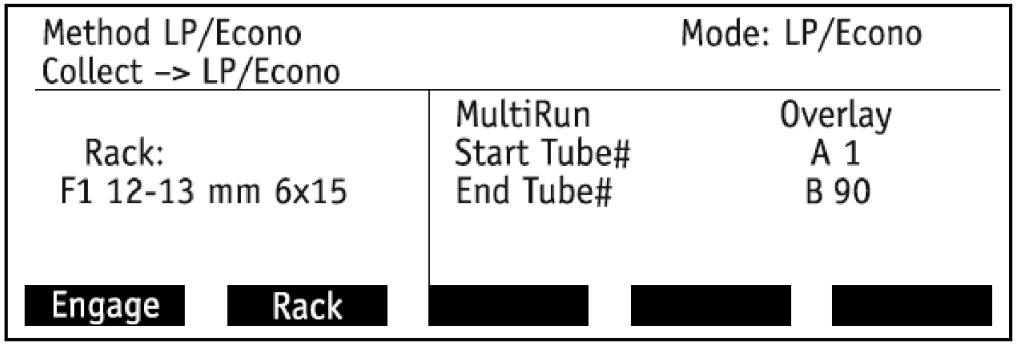


Figure 7.8. Faceplate of the BioFrac Fraction Collector.

Select the **BIOFRAC** softkey (on older models this would be the **2128** softkey).

6. The question "External diverter cable connected" will appear on the screen. Press **YES**.

Model 2110 fraction collector

1. Turn on the Model 2110 fraction collector.

2. On the BioLogic LP system, press the **Collector** instrument key. Then press the **MODEL** softkey. Select the 2110 softkey.

Clean Out the Injection Loop

1. Turn the MV-6 injection valve fully counterclockwise (as far left as it will go) (Figure 7.9).

2. Fill a 10 ml syringe with 10 ml of clean water and screw it into the MV-6 injection port.

3. Make sure that the waste tubing is flowing into a waste collection beaker.

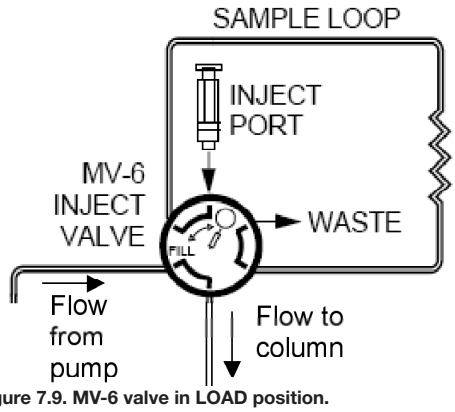


Figure 7.9. MV-6 valve in LOAD position.

4. Slowly push the syringe to flow water through the injection loop. You should see the loop fill and then flow out through waste into the waste beaker.

5. Leave the syringe attached to the injection port.

Program the Pump Method

At this point, you should have manually purged the tubing of all air, primed all the tubing first with Buffer B and secondly with 98% Buffer A + 2% Buffer B. The column should be inline and have been equilibrated with 98% Buffer A + 2% Buffer B. If you are using a BioFrac fraction collector or Model 2110 fraction collector, the BioLogic LP system is programmed to recognize that it is there, and the fraction collector has been programmed to be enslaved by the BioLogic LP system. The UV Flowcell has been zeroed and the conductivity monitor has been set to read within the range of conductivities expected from the lysate and solutions. Now, you will program a method that will be used to purify your protein and collect fractions.

You will be purifying the GST-DHFR-His protein from all the other soluble *E. coli* proteins in your soluble fraction of lysate using an IMAC Ni-charged column. The soluble fraction will flow over the IMAC Ni-charged resin in a solution of 2% Buffer B* (20 mM Phosphate, 300 mM NaCl, 5 mM imidazole) that is the same composition as what the lysis buffer contained (minus lysozyme). The GST-DHFR-His should adhere to the column through the polyhistidine-tag as might other proteins that might contain multiple histidines. All other proteins will not bind to the column and will be part of the FLOWTHROUGH fractions. The column will then be washed with a 4% Buffer B wash solution (20 mM Phosphate, 300 mM NaCl, 10 mM imidazole) and finally the GST-DHFR-His will be eluted in 100% Buffer B (20 mM Phosphate, 300 mM NaCl, 250 mM imidazole). After the elution, the column will be washed with more 100% Buffer B and finally requilibrated into 0% Buffer B (20 mM Phosphate, 300 mM NaCl) so it is ready to be reused.

*In chromatography, usually buffer B contains the component that will elute the proteins of interest be it higher salt, imidazole, different pH, etc. For nomenclature, solutions are referred to as percentage of B in the mix. Thus, the Equilibration solution is referred to as 2% Buffer B meaning that there is 98% Buffer A + 2% Buffer B.

Press the **Program** mode key, and select the **NEW METHOD** softkey.

1. Select **VOLUME** programming mode.

2. Enter the pump program:

a. Press the **ADD** softkey

b. Select the **MIX** softkey, then enter **2% B** and press the **OK** softkey.

c. Enter a step length of **7 ml**, then press the **OK** softkey.

d. Enter flow rate of **1 ml/minute**, then press the **OK** softkey.

e. You have now entered the first step of the method (2% Buffer B, step length 7 ml, flow rate 1 ml/minute). Enter the remaining steps by repeating steps 2–4 with the appropriate information.

Step 2. 4% Buffer B, step length 10 ml, flow rate 2.0 ml/minute.

Step 3. 100% Buffer B, step length 8 ml, flow rate 2.0 ml/minute.

Step 4. 100% Buffer A, step length 15 ml, flow rate 2.0 ml/minute.

f. After entering Step 4, press the **OK** softkey.

3. Enter the alarm program:

The alarm is programmed to sound after 7 ml (once the lysate has been fully injected onto the column plus a bit of extra buffer) to remind you to turn the MV-6 valve back to the load position. If left in the

injection position, the buffers will flow through the loop first instead of going directly to the column and this leads to 5 ml of delay (at 2 ml/min this is equivalent to 2.5 minutes) before buffer changes are seen on the column.

a. Press the **Alarm** instrument key.
b. Press the **ADD** softkey.
c. Enter a volume of 7 ml for Alarm 1 and check that Hold Methods is set to NO.
d. Press the **OK** softkey twice.

4. Enter the fraction collector program:

a. Press the **Frac Coll** softkey.
b. Select the **WINDOWS** softkey.
c. Select the softkey for **ADD.**
d. Enter a Start time of 0 ml then press the **NEXT** key.
e. Enter an End time of 17 ml then press the **OK** softkey.
f. Enter a Fraction Size of 4 ml and then press the **OK** softkey.
g. Add a second fraction collection window by selecting the softkey for **ADD.**
h. The second window will collect from 17 ml to 30 ml, with a fraction size of 1 ml.
i. Set a third window from 30 ml to 40 ml, a fraction size of 2 ml.
j. Press the **OK** softkey to accept the fraction collection windows.
k. Press the **DONE** softkey.

5. Save the program with the name DHFR:

a. Press the **SAVE** softkey.
b. Press the **A-J** softkey and then press the 4 number key.
c. Press the **A-J** softkey and then press the 8 number key.
d. Press the **A-J** softkey and then press the 6 number key.
e. Press the **K-T** softkey and then press the 8 number key to name your program “DHFR.”
f. Press the **DONE** softkey to finish.

The programmed method can now be viewed by pressing the **VIEW METHOD** softkey while in the Program mode. Select your program, and it should look as shown in Table 7.1. Use the **Next** and **Previous** instrument keys to scroll through the program.

List:	Volume (ml)	Buffer	ml/min
	0.00 Coll: 4.00 ml		
01	0.00 to 7.00	Mix 2% B	1.00
	7.00 Alarm		
02	7.00 to 17.00	Mix 4% B	2.00
	17.00 Divert to waste		
	17.00 Coll: 1.00 ml		
03	17.00 to 25.00	Buffer B	2.00
04	25.00 to 40.00	Buffer A	2.00
	30.00 Divert to waste		
	30.00 Coll: 2.00 ml		
	40.00 Divert to waste		
	<End of method>		

Table 7.1. Example of programmed method in VIEW METHOD.

Final Check Before Starting Method

1. Check for leaks or air in the lines.

2. Check to make sure that your buffer inlet lines are at the bottom of the beakers containing Buffer A and Buffer B.

3. Make sure that you have at least 50 ml of Buffer A and 20 ml of Buffer B.

4. Place collection tubes in the fraction collector rack in the first two columns (30 tubes total) for the BioFrac fraction collector or the first 30 spaces for the Model 2110 fraction collector.

5. If you are using LP DataView software, press **STOP** on the software menu. Press **CLEAR** to clear all previous information. A menu box will appear. Choose "NO." Press **RECORD** to record your purification run.

Load Sample Loop with GST-DHFR-His Soluble Fraction

1. Make sure the MV-6 injector valve knob counter is turned counterclockwise (left) as far as it will go (Figure 7.10).

2. Draw 5 ml of the soluble fraction of GST-DHFR-His into the 10 ml syringe. Avoid pulling in any air bubbles. If you have more than 5 ml of soluble fraction, you can draw the entire fraction into the syringe.

3. Make sure to have the waste port tubing of the MV-6 injector valve over a waste beaker.

4. Insert the syringe in the top port of the MV-6 injector valve and fill the sample loop. The excess water that was in the sample loop from the cleaning of the loop will be pushed out of the waste port.

   Note: Avoid pushing air bubbles into the sample loop since these will flow into the column and through the detectors.

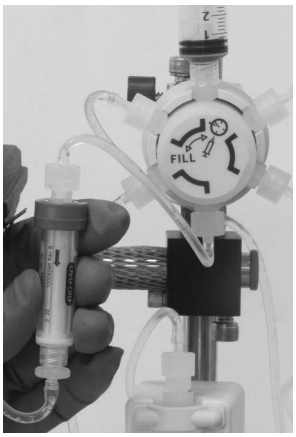


Figure 7.10. MV-6 in Sample Load position, turned as far left as possible.

5. Leave the syringe in the port when you have injected the sample; this prevents the sample from siphoning out of the loop.

Start Method

1. Press the **Run** mode key.

2. **Immediately** turn the injector valve knob clockwise (to the right) as far as it will go (Figure 7.11).

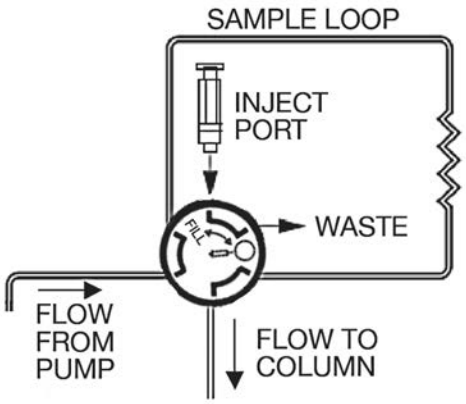


Figure 7.11. MV-6 injection port in the INJECT position. The inject position (turned fully clockwise) opens up the fluid pathway so that the buffer coming from the pump will push the sample out of the sample loop and onto the column.

3. After seven minutes, the alarm will sound. Turn the MV-6 injection valve knob counterclockwise (to the left) as far as it will go. Press the **Alarm** instrument key to shut off the alarm (Figure 7.12).

   As the run progresses, you can monitor your run by either watching the live values of the UV and conductivity detectors on the BioLogic LP system or by watching the data collection if you have DataView software (Figure 7.13). As the sample is loaded on the column, there should be an increase in the absorbance as the non-binding proteins flow through and do not bind. After all the lysate has been loaded, there should be a drop in absorbance as the wash buffer flows through. Once the system switches to 100% Buffer B to elute the GST-DHFR-His, the absorbance should increase again. Since the 100% Buffer B contains 250 mM imidazole, the absorbance will not drop to zero, even once the GST-DHFR-His has eluted. However, once 0% Buffer B is run across the column in a final wash, the absorbance should return to close to zero.

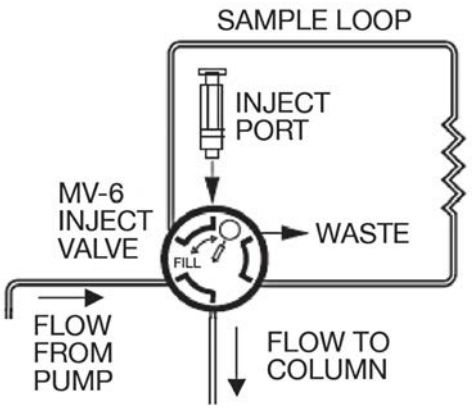


Figure 7.12. Returning MV-6 valve to LOAD position after seven minutes. At this point, all of the sample in the sample loop will have been loaded onto the column. By changing to the load position, the flow from the pump will go directly to the column and will bypass the extra 5 ml of tubing pathway of the sample loop, which at this point is only filled with buffer.

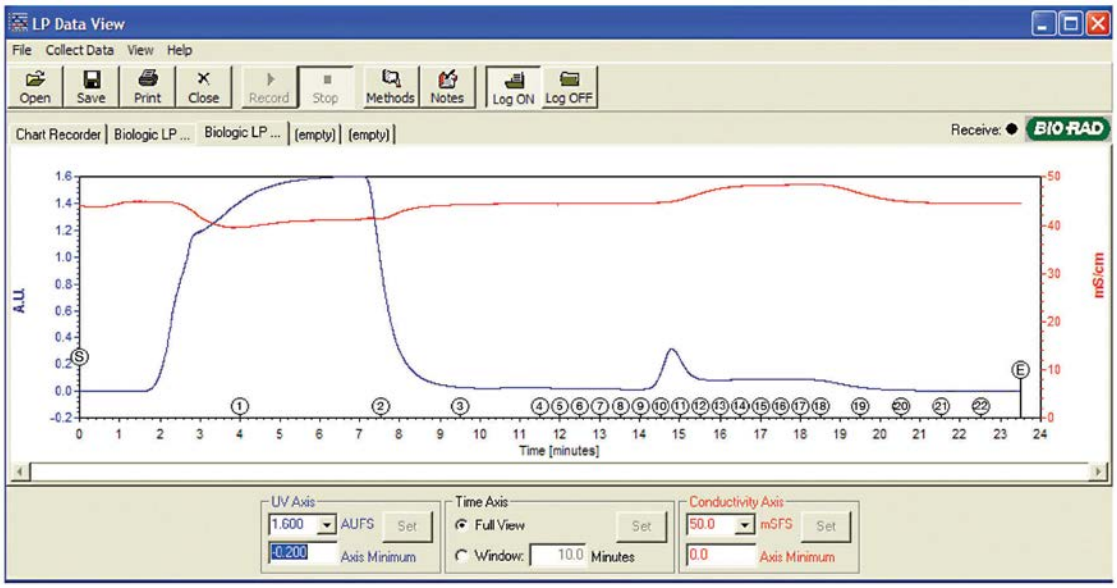


Figure 7.13. Example of DataView chromatogram. The S icon denotes the start of the run. The numbered circles denote fractions collected. The top line is the conductivity recording. Initially, the buffer is 2% Buffer B and contains 300 mM NaCl along with 5 mM imidazole. When the protein that does not bind flows past the conductivity meter, the conductivity decreases since the proteins can buffer the charges of the salts. The conductivity then increases slightly with the wash (that contains 10 mM imidazole and much fewer proteins.) The conductivity then increases once 250 mM imidazole is used and finally drops once there is just Buffer A. The UV absorbance shows a large peak for the proteins that do not bind to the column and then a smaller peak to denote the eluted GST-DHFR-His. After the elution peak is a plateau that is the absorbance of the 250 mM imidazole buffer. Finally the absorbance drops to baseline when just phosphate buffer with 300 mM NaCl is running.

4. When the run has completed, make sure that the pump has stopped.

5. If you used DataView software, save your run data.

6. If you used a BioFrac fraction collector, on its faceplate, press the Stop softkey to rehome the drop collector and confirm the end of the run by pressing “Yes.”

7. Label your fractions numerically (1, 2, 3, etc.) making sure to follow the serpentine path that the fraction collector used.

8. Cover your fractions with Parafilm and store them at 4°C (for up to one week) until you are ready to desalt and analyze them via SDS-PAGE and enzyme activity. Do not freeze your fractions.

9. Remove your column and connect the column inlet and column outlet tubing. Cap your column and store it at 4°C.

Cleaning the BioLogic LP System After Use

In order to keep any instrument in good running form, it is critical to perform certain tasks to make sure that the instrument is clean and ready for use by the next person. For the BioLogic LP system it is important to remove all salt-containing solutions from the lines as well as remove all bacterial lysate components. The salts can precipitate out over time clogging fittings, tubing and flow cells. Bacterial lysates can degrade, resulting in growth of molds and mildews that also will clog and contaminate fittings, tubing and flow cells.

1. Remove your column and connect the column inlet and outlet tubing, if you have not already done so.

2. Place the buffer A and buffer B inlet lines into a beaker containing at least 200 ml of high-quality water.

3. Turn the MV-6 sample injector valve all the way counterclockwise.

4. In manual mode, set the buffer composition to a mix of 50% Buffer A and 50% Buffer B.

5. Set the flow rate to 5 ml/min.

6. Make sure that the waste beaker is empty and that the waste tubing flows into the waste beaker.

7. Press the **Start** softkey and allow the flow to continue for five minutes.

8. After 5 minutes, turn the MV-6 sample injector valve all the way clockwise to allow flow through the sample loop.

9. Continue flow for another five minutes.

10. Stop the pump.

11. If requested by your instructor, repeat steps 3–10 using 20% ethanol in place of high-quality water.

12. Your instrument is now ready for storage or use by another student group.

Removing Imidazole (Desalting) from the Purified GST-DHFR-His and Preparing Samples for SDS-PAGE Analysis

Student Workstations

Each student team requires the following items to desalt three of their fractions in which they think their GST-DHFR-His samples reside, and to prepare SDS-PAGE samples:

Material Needed for Each Workstation	Quantity
Chromatogram from GST-DHFR-His purification	1
Fractions from GST-DHFR-His purification	varies
Desalting column	3
Screwcap microcentrifuge tubes, 1.5 ml	6
Laemmli buffer (left over from previous activity)	1 ml
Microcentrifuge tubes, 2 ml	6–12
20–200 µl adjustable-volume micropipet and tips	1
Marking pen	1

Common Workstation	Quantity
Microcentrifuge with variable speed setting ≥16,000 x g	1
Dry bath or water bath set at 95°C	1

At this point, there might be several fractions that contain the eluted GST-DHFR-His protein. It is important to determine which fractions the purified GST-DHFR-His is present in and which fraction(s) have the highest concentration of GST-DHFR-His. It might be assumed that the fractions marked by the LP DataView software on the chromatogram where the peak eluted are the specific fraction tubes where the protein can be found. However, there is a time delay from when the absorbance of the fraction is measured on the UV detector to when it flows through the tubing past the conductivity meter and out into the fraction collector. This is called a delay time (or volume). For example, in Figures 7.14 and 7.15, according to the chromatogram, the protein should have eluted in fractions 10–11. However, again, this would be assuming that there is no delay between when the protein is detected in the UV detector and when it drops into a fraction collector tube.

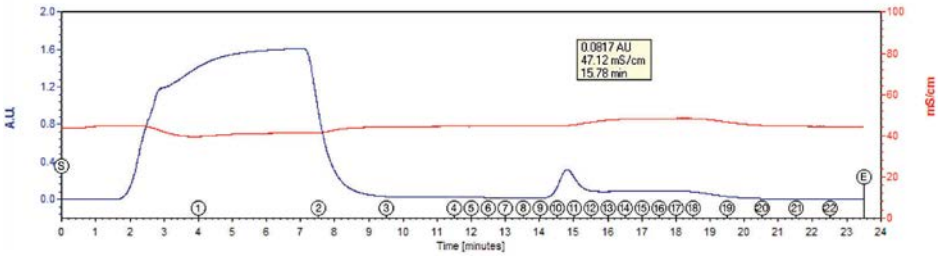


Figure 7.14 Example chromatogram of a run.

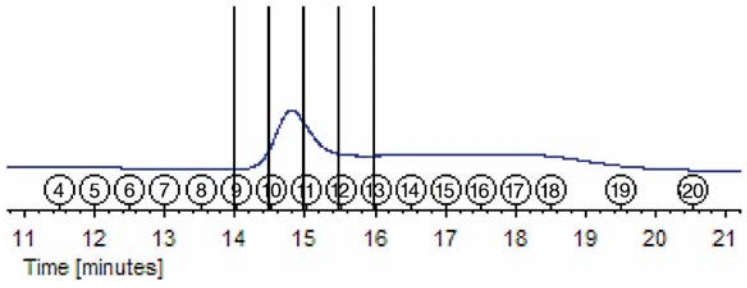


Figure 7.15. Magnified region where the GST-DHFR-His eluted.

The delay time (volume) can be calculated by measuring the length of tubing present between the detector and the fraction collector as well as knowing the volume of all fittings in between the UV monitor and the fraction collector. It can also be measured experimentally

ahead of time by attaching a syringe to the fitting at the inlet of the UV detector when the tubing is filled with fluid and pushing all this fluid out through the fraction collector drop former and measuring the volume.

Using the calculation method, a loose estimate can be determined by calculating the delay volume of the tubing present.

The silicone tubing has an inner diameter (ID) of 0.16 cm.

The volume of fluid in a specific length (L) of tubing would be ϖ x $(ID/2)^2$ x L.

Therefore, as an example, assuming that 1.6 mm ID tubing is in place and that there are 30 cm of tubing between the UV detector and the fraction collector, the delay volume would be at a minimum:

ϖ x $(0.16\ cm/2)^2$ x 30cm = 0.6 cm^3 or 0.6 ml.

As can be seen from the calculation, this is a volume equivalent to about half of the eluted fraction volume size. However, there are also delay volumes as part of the UV flow cell, conductivity flow cell and fittings. Since 1 ml fractions are being collected for the eluate, it is important not to discard any fractions until all analyses are done! In the case of the example shown above, the majority of the GST-DHFR-His has eluted in fractions 11, 12 and 13. The highest concentration was in fraction 12, which would represent a delay volume of approximately 2 ml.

Two other fractions will also be examined. 1) The flowthrough fraction that contains all proteins that did not bind to the column when the column was initially loaded with the soluble fraction, and 2) the wash fraction that contains any proteins that were washed off when the imidizole level was increased to 4% (or 10 mM imidazole). Again, these fractions can be determined by looking at the chromatogram from the BioLogic LP system run. For example, in Figure 7.14, if delay volume is considered, the majority of the flowthrough fraction should be in fraction 2. The majority of the wash fractions would be in fractions 5-8 in the example chromatogram in Figures 7.14 and 7.15.

Protocol

Choosing your flowthrough, wash, and three eluted GST-DHFR-His fractions

Examine your chromatogram and determine which fractions are most likely to contain your flowthrough and wash fractions, and three fractions which might contain your eluted GST-DHFR-His. Remember that the delay volume is probably between 1–3 ml depending on the tubing length of your instrument. Record the five fraction numbers below.

Flowthrough fraction : _____

Wash fraction: _____

First eluted GST-DHFR-His fraction: _____

Second eluted GST-DHFR-His fraction: _____

Third eluted GST-DHFR-His fraction: _____

Preparing SDS-PAGE samples of flowthrough, wash and eluted GST-DHFR-His fractions

1. In a clean 1.5 ml screwcap microcentrifuge tube, combine 50 µl of Laemmli sample buffer with 50 µl of your flowthrough fraction and label the tube "**Flowthrough PAGE"** with your initials.

2. In a clean 1.5 ml screwcap microcentrifuge tube, combine 50 µl of Laemmli sample buffer with 50 µl of your wash fraction and label the tube "**Wash PAGE"** with your initials.
3. In a clean 1.5 ml screwcap microcentrifuge tube, combine 50 µl of Laemmli sample buffer with 50 µl of your first eluted GST-DHFR-His fraction and label the tube "**Fraction A PAGE"** with your initials.

4. In a clean 1.5 ml screwcap microcentrifuge tube, combine 50 µl of Laemmli sample buffer with 50 µl of your second eluted GST-DHFR-His fraction and label the tube "**Fraction B PAGE"** with your initials.

5. In a clean 1.5 ml screwcap microcentrifuge tube, combine 50 µl of Laemmli sample buffer with 50 µl of your third eluted GST-DHFR-His fraction and label the tube "**Fraction C PAGE"** with your initials.

6. Heat the five SDS-PAGE samples at 95°C for five minutes.

7. Store the SDS-PAGE samples at –20°C until they are ready to analyze via SDS-PAGE.

Desalting your three eluted GST-DHFR-His fractions

1. Label three Micro Bio-Spin desalting columns A, B, and C with your initials. These fractions will correspond to the three fractions you chose to be the eluted GST-DHFR-His fractions from your chromatogram:

   Fraction A = First eluted GST-DHFR-His fraction
   Fraction B = Second eluted GST-DHFR-His fraction
   Fraction C = Third eluted GST-DHFR-His fraction

2. Invert three desalting columns (with green caps) sharply several times to resuspend the settled gel and remove any bubbles. The resin should settle into the column, and little to no resin should remain in the green cap.

3. Snap off the bottom tab of the column and place the column into a clean 2 ml microcentrifuge tube.

4. Remove the columns' green caps. If the columns do not begin to flow, push the caps back on the columns and then remove them again to start the flow.

5. Allow the excess packing buffer to drain by gravity to the top of the gel bed (about two minutes), then place the three columns into three clean 2 ml microcentrifuge tubes.

6. Centrifuge the three columns for two minutes in a microcentrifuge at 1,000 x g (See Appendix **C** for more information on setting centrifugation speeds) to remove the remaining packing buffer. Discard the buffer and the three microcentrifuge tubes. Keep the three columns for the following steps, being careful not to disturb the resin.

7. Label three clean 2 ml microcentrifuge tubes "**Desalted A,**" "**Desalted B**" and "**Desalted C**" and each with your initials, and place the columns into the corresponding 2 ml micocentrifuge tubes. Carefully apply 75 µl of each of your eluted fractions chosen in step 1 directly to the center of the column. Be careful not to touch the resin with the pipet tip.

8. After loading the samples, centrifuge the column for four minutes at 1,000 x g.

9. Carefully apply another 75 µl of each of your eluted fractions chosen in step 1 to the correspondingly labeled columns and 2 ml microcentrifuge tubes and centrifuge for four minutes at 1,000 x g to produce a final volume of 150 µl of desalted GST-DHFR-His for each elution fraction you chose.

10. Discard the columns.

Preparing SDS-PAGE samples of desalted GST-DHFR-His fractions

1. In a clean 1.5 ml screwcap microcentrifuge tube, combine 25 µl of Laemmli sample buffer with 25 µl of your desalted GST-DHFR-His fraction A and label the tube "**Desalted A PAGE**" with your initials.

2. In a clean 1.5 ml screwcap microcentrifuge tube, combine 25 µl of Laemmli sample buffer with 25 µl of your desalted GST-DHFR-His fraction B and label the tube "**Desalted B PAGE**" with your initials.

3. In a clean 1.5 ml screwcap microcentrifuge tube, combine 25 µl of Laemmli sample buffer with 25 µl of your Desalted GST-DHFR-His fraction C and label the tube "**Desalted C PAGE** with your initials.

4. Heat the three SDS-PAGE samples at 95°C for five minutes.

5. Store the SDS-PAGE samples at –20°C until ready to analyze via SDS-PAGE.

6. Cover with parafilm and store the **Desalted A**, **B** and **C** fractions at **4°C** until ready to analyze via spectroscopy, SDS-PAGE, and enzymatic activity assay. Do not freeze your fractions.

Quantitation of Protein in Desalted Fractions

Student Workstations

Each student team requires the following items to quantitate the amount of purified, desalted eluate (GST-DHFR-His) it has via spectroscopy:

Material Needed for Each Workstation	Quantity
Desalted fractions (150 µl each)	3
20–200 µl adjustable-volume micropipet and tips	1
trUView disposable cuvettes (or UV compatible cuvettes)	3
Marking pen	1

Common Workstation	Quantity
UV spectrophotometer	1–2

Protocol: Quantitation of Protein in Desalted Fractions

1. Make sure that your cuvettes are completely clean and dry before use.

2. Set your spectrophotometer to read at 280 nm.
   Note: If using a Bio-Rad SmartSpec Plus spectrophotometer, please see Appendix **D** for setup instructions.

3. Blank your spectrophotometer with 100 µl distilled water.

4. Pipet 100 µl of your desalted fractions A, B, and C each into its own clean cuvette.

5. Measure the absorbance at 280 nm of all your desalted fractions.

   A_{280} Desalted fraction A _____

   A_{280} Desalted fraction B _____

   A_{280} Desalted fraction C _____

6. Pipet each desalted fraction back into the tube containing that fraction after measuring its absorbance.

7. Make sure that you have at least 15 µl of desalted GST-DHFR-His to run your enzyme assay.

8. Calculate the concentration of GST-DHFR-His in your desalted fractions as follows:

   A: The extinction coefficient (ε) of the entire GST-DHFR-His construct is theoretically calculated to be 75,540 M^{-1} cm^{-1}.

   Knowing that absorbance = ε x C x L,

   where ε is 75,540 M^{-1} cm^{-1}

   L is the pathlength of the cuvette in cm (usually 1),
   and the absorbance at 280 nm is being measured

The concentration of GST-DHFR-His (M) = Absorbance/75,540

Concentration of GST-DHFR-His (Desalted fraction A) = _____ M

Concentration of GST-DHFR-His (Desalted fraction B) = _____ M

Concentration of GST-DHFR-His (Desalted fraction C) = _____ M

B: Convert from molarity to mg/ml for the amount of GST-DHFR-His in your purified desalted fractions knowing that the molecular mass of GST-DHFR-His is 50,361 g/mol.

Concentration GST-DHFR-His (mg/ml) = Concentration GST-DHFR-His (M) x 50,361 g/mol

Concentration of GST-DHFR-His (Desalted fraction A) = _____mg/ml

Concentration of GST-DHFR-His (Desalted fraction B) = _____mg/ml

Concentration of GST-DHFR-His (Desalted fraction C) = _____mg/ml

These are the concentrations of GST-DHFR-His that you produced and purified and are contained in your 150 µl desalted eluate fractions.

SDS-PAGE Electrophoresis Analysis of Purified Fractions

Student Workstations

Each student team requires the following items to analyze their purification samples via SDS-PAGE:

Material Needed for Each Workstation	Quantity
Soluble PAGE sample	100 µl
Flowthrough PAGE sample	100 µl
Wash PAGE sample	100 µl
Fraction A PAGE sample	100 µl
Desalted A PAGE sample	50 µl
Fraction B PAGE sample	100 µl
Desalted B PAGE sample	50 µl
Fraction C PAGE sample	100 µ
Desalted C PAGE sample	50 µl
Precision Plus Protein Dual Color standards	15 µl
4–20% Mini-PROTEAN TGX precast gel	1
Vertical electrophoresis chamber (per 1–4 gels)	1
1x Tris/Glycine/SDS (TGS) running buffer (per electrophoresis chamber)	700–1,200 ml
Power supply (200 V constant) to be shared between workstations	1
Buffer dam (only required if running 1 gel/box)	1
2–20 µl adjustable-volume micropipet and tips	1
Staining tray	1
Bio-Safe Coomassie stain (per gel)	50 ml
Marking pen	1

Common Workstation	Quantity
Water bath or heat block set to 95°C	1
Microcentrifuge with variable speed setting ≥16,000 x g	1
Water for gel washing and destaining (tap water is fine)	1 L

Load, Run, Stain and Destain the Gel

1. **Prepare samples**: If samples have been stored at –20°C, reheat SDS-PAGE samples at 95°C for two minutes to redissolve any precipitated detergent and then centrifuge the samples for two minutes at 16,000 x g.

2. **Assemble gel boxes**: If using Bio-Rad Mini-PROTEAN Tetra gel boxes and TGX precast gels, see appendix **E** for more information on how to prepare gels and assemble gel boxes.

3. **Load the gel**: Using a fresh tip each time, load the following volumes of each sample into the appropriate well of your gel:

Well	Volume	Sample
1	10 µl	Precision Plus Protein Dual Color standard
2	10 µl	Soluble PAGE
3	10 µl	Flowthrough PAGE
4	20 µl	Wash PAGE
5	20 µl	Fraction A PAGE
6	20 µl	Desalted A PAGE
7	20 µl	Fraction B PAGE
8	20 µl	Desalted B PAGE
9	20 µl	Fraction C PAGE
10	20 µl	Desalted C PAGE

4. Run the gel at 200 V for 30 minutes. (If using a Bio-Rad Mini-PROTEAN TGX precast gel, the gel can be run at 300 V for 15 minutes.)

5. After the run is complete, remove the gel from the cassette and place it in the gel staining tray.

6. Wash the gel three times with 50 ml of distilled water for five minutes each rinse. Discard all the rinse water.

   Note: Make sure that all of the wash water has been removed since excess water diluting the gel stain will interfere with staining efficiency.

7. Stain the gel with 50 ml of Bio-Safe Coomassie stain for one hour.

8. After one hour discard the Bio-Safe Coomassie stain and add 100 ml of distilled water and destain the gel overnight.

9. Image the gel if you have an imaging system or dry the gel if you have a cellophane drying system.

10. Pick the desalted GST-DHFR-His fraction that has the highest concentration (darkest band) but also looks the most pure on the SDS-PAGE gel to analyze enzymatic activity. The most pure sample would show a dark and obvious band for GST-DHFR-His, and few or faint bands at other molecular weights in the lane on the SDS-PAGE gel.

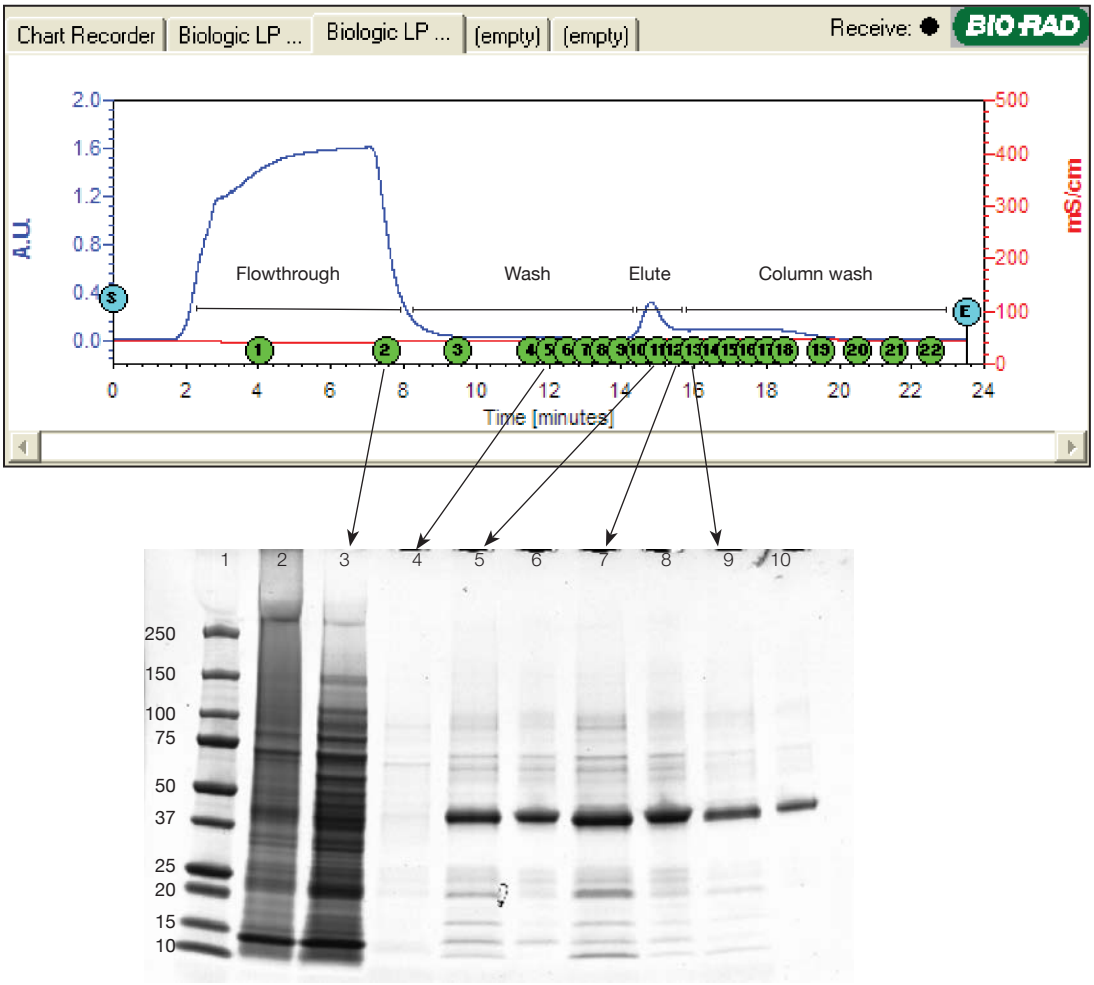


Figure 7.16. Example of fractions selected from the chromatogram and the corresponding SDS-PAGE results.

Lane 1. Precision Plus Dual Color standards
Lane 2. Soluble fraction of *E. coli* cell lysate.. A band is present that is the GST-DHFR-His which is soluble. The large band at approximately 12 kDa is the lysozyme, which is also soluble, that was used to lyse the cells open.
Lane 3. Flowthrough fraction that did not bind to the Ni-IMAC resin. This is the fraction of proteins from the soluble fraction that did not bind to the Ni-IMAC resin. The soluble fraction was suspended in 20 mM sodium phosphate buffer, 300 mM NaCl and 5 mM imidazole. The high salt helped prevent E. coli proteins and the lysozyme from non-specifically sticking to the Ni-IMAC beads. The 5 mM imidazole helped prevent E. coli proteins with multiple histidine groups from binding to the Ni-IMAC beads. There is a decrease in the amount of GST-DHFR-His in the flowthrough fraction versus the soluble fraction (lane 2) and this is representative of the GST-DHFR-His binding to the Ni-IMAC resin.
Lane 4. Wash fraction. This fraction contains proteins that were washed off the Ni-IMAC beads when a wash buffer that has a slightly higher imidazole level (10 mM) was added to wash off more non-specifically bound proteins. No GST-DHFR-His should wash off in this fraction since 10 mM imidazole is not enough to compete with the 6 histidine tag of GST-DHFR-His bound to the Ni-IMAC resin.
Lanes 5, 7, and 9. Eluate fraction of GST-DHFR-His. These fractions contain the GST-DHFR-His. The elution buffer has 250 mM imidazole in it and this level of imidazole competes with the six histines of GST-DHFR-His and knocks them off the Ni sites and hence the GST-DHFR-His elutes or comes off the resin to be collected. These fractions are predominantly GST-DHFR-His protein relative to the unpurified soluble fraction in lane 2 that contains many other proteins.
Lane 6, 8, and 10. Desalted GST-DHFR-His. These fractions contain the purified GST-DHFR-His but have had the 250 mM imidazole removed. Also, since the desalting column removes smaller molecular weight compounds, some of the smaller molecular weight impurities found in lanes 5,7 and 9 are not present in the desalted fractions.

Purification Workflow for BioLogic LP System

Affinity Purification of GST-DHFR-His Protein

Follow the preceding protocols for your instrument and either handpacked columns or prepacked cartridge purificaton.

BioLogic LP System

Post-Purification: Choosing your flowthrough, wash, and three eluted GST-DHFR-His fractions

Examine your chromatogram and determine the fractions that you feel are most likely to contain your flowthrough and wash, and three fractions which might contain your eluted GST-DHFR-His. Remember that you need to calculate the delay volume to help determine which fractions contain your samples. Record the five fraction numbers you will examine at right.

Flowthrough fraction: _____

Wash fraction: _____

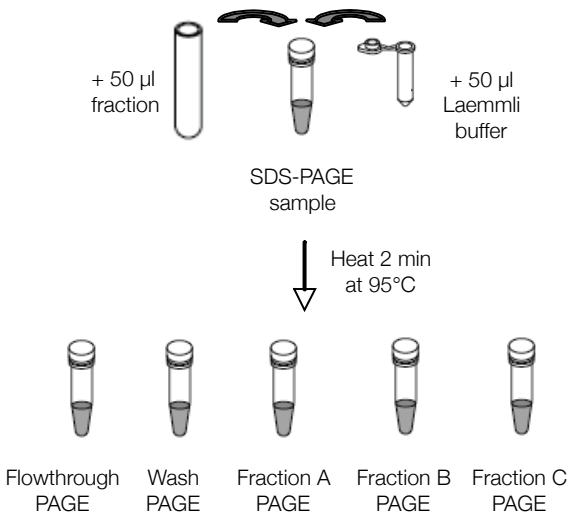
First eluted GST-DHFR-His fraction: _____

Second eluted GST-DHFR-His fraction: _____

Third eluted GST-DHFR-His fraction: _____

Preparing SDS-PAGE samples of flowthrough, wash, and eluted GST-DHFR-His fractions

Label 1.5 ml screwcap mircrocentrifuge tubes according to the fraction as indicated on right. Add 50 µl of fraction to 50 µl Laemmli buffer into corresponding 2 ml screwcap tube. Mix well. Heat all samples at 95°C for five minutes.



Purification Workflow for BioLogic LP System

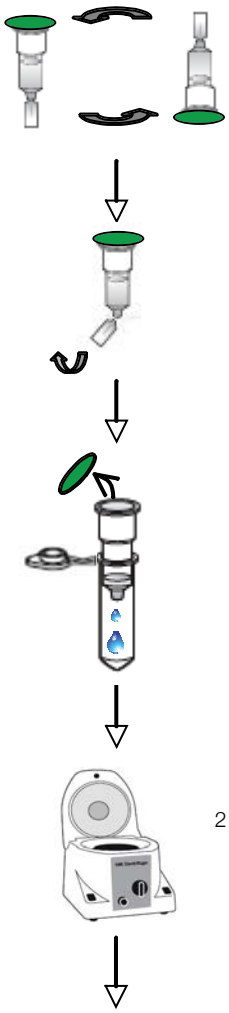
Desalting your three eluted GST-DHFR-His fractions

Label three Micro Bio-Spin desalting columns A, B, and C. These fractions correspond to the three fractions you chose from your chromatogram.

Prepare desalting columns by inverting sharply several times to resuspend gel.

Snap off bottom tabs and place each column into a 2 ml microcentrifuge tube. Remove green top cap. If the column does not begin to flow, push the cap back on the column and then remove it again to start the flow.

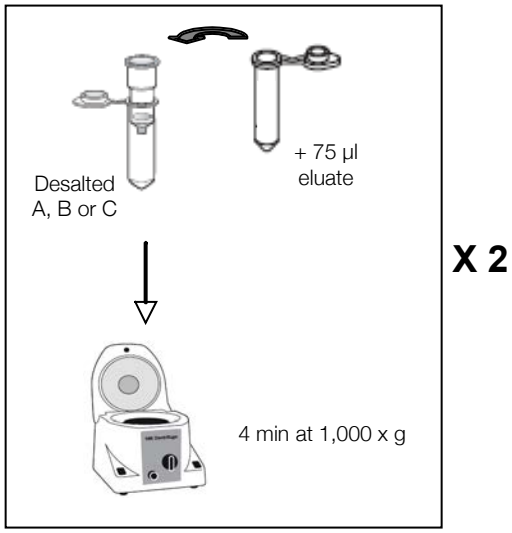
Allow excess packing buffer to drain by gravity to top of resin bed. After draining, place columns in clean 2 ml tubes.



Centrifuge for 2 minutes at 1,000 x g. Discard remaining packing buffer and collection tubes.

Label three clean 2 ml microcentrifuge tubes **Desalted A, Desalted B, Desalted C.** Carefully apply 75 µl of each of your eluted fractions directly to the center of the corresponding column. Be careful not to touch resin with pipet tip.

Centrifuge for four minutes at 1,000 x g. Carefully apply another 75 µl of each of your eluted fractions to the corresponding column and centrifuge again.

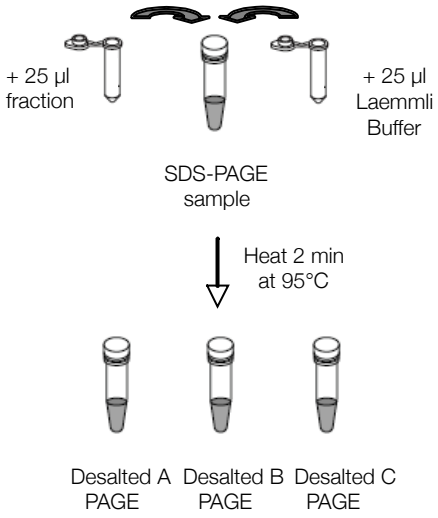


After second spin, discard columns. You will have ~150 µl of Desalted GST-DHFR-His for each elution fraction.

Purification Workflow for BioLogic LP System

Preparing SDS-PAGE Samples of Desalted GST-DHFR-His Fractions

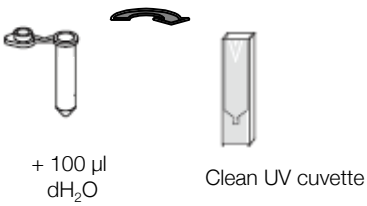
Prepare SDS-PAGE samples. Add 25 µl of each fraction to 25 µl Laemmli buffer in 2 ml screwcap tube. Mix well. Heat all samples at 95°C for five minutes.



Purification Workflow for BioLogic LP System

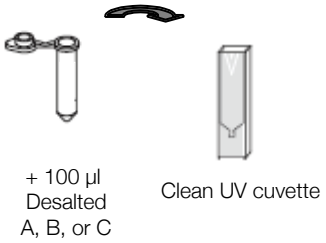
Quantitation of Protein in Desalted Fractions

Turn on spectrophotometer and set absorbance to 280 nm. Add 100 µl distilled water to clean UV compatible cuvette.



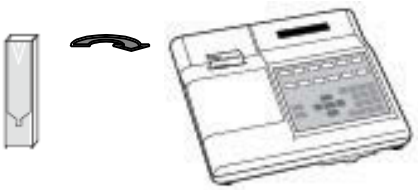
Blank spectrophotometer with distilled water.

Add 100 µl Desalted fractions A, B, and C each into its own clean UV compatible cuvette.



Measure absorbance of each fraction at 280nm. Return fraction to 2 ml tube.

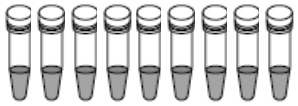
Calculate concentration of GST-DHFR-His in each desalted fraction as described in instruction manual and record data.



Purification Workflow for BioLogic LP System

SDS-PAGE Analysis of Purified Fractions

Load, run, stain and destain the gel. Reheat SDS-PAGE samples at 95°C for two minutes. Centrifuge samples for two minutes at 16,000 x g.

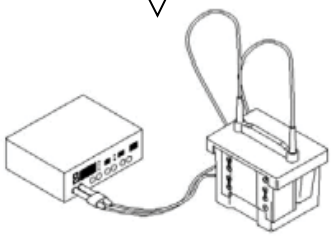


Heat 2 min at 95°C

Spin 2 min at 16,000 x g

Using a fresh tip each time, load the following volumes of each sample into the appropriate well of your gel

Lane	Volume	Sample
1	10 µl	Precision Plus Dual Color standard
2	10 µl	Soluble PAGE
3	10 µl	Flowthrough PAGE
4	20 µl	Wash PAGE
5	20 µl	Fraction A PAGE
6	20 µl	Desalted A PAGE
7	20 µl	Fraction B PAGE
8	20 µl	Desalted B PAGE
9	20 µl	Fraction C PAGE
10	20 µl	Desalted C PAGE



Run the gel at 200 V for 30 minutes. (If using Mini-PROTEAN TGX precast gel, the gel can be run at 300 V for 15 minutes.)

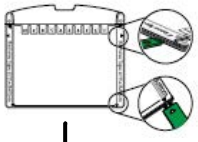
After the run is complete, remove the gel from the cassette and place in the gel staining tray.

Wash the gel three times with 50 ml of distilled water for five minutes each rinse. Discard all the rinse water.

Stain the gel with 50 ml of Bio-Safe Coomassie stain for one hour.

After one hour, discard the Bio-Safe Coomassie stain, add 100 ml of distilled water, and destain the gel overnight.

Image the gel if you have an imaging system or dry the gel if you have a cellophane drying system.



Chapter 8: Purification Protocol for BioLogic DuoFlow System

Affinity Purification of GST-DHFR-His Using a BioLogic DuoFlow System

Student Workstations

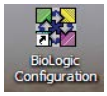
Each student team requires the following items to purify their GST-DHFR-His samples using affinity chromatography:

Material Needed for Each Workstation	Quantity
Soluble lysate fraction	5–7.5 ml
10 ml syringe	1
Distilled water	500 ml
Buffer A	70 ml
Buffer B	40 ml
Collection tubes for fraction collector	32
Marking pen	1

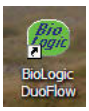
Common Workstation	Quantity
BioLogic DuoFlow chromatography instrument with 5 ml sample loop and column installed: Either a BioScale Mini Profinity Ni-IMAC Cartridge or A glass Econo-Column column handpacked with 1 ml of Profinity IMAC Ni-charged resin	1–4
BioLogic DuoFlow injection needle	1–4

Preparing the DuoFlow system for Use

1. Turn on the power switches for all hardware (e.g. Maximizer, if you have one), Workstation, and BioFrac fraction collector. Turn on the power for the Controller (computer) once all the other hardware has been turned on.

2. Double click on the BioLogic Configuration icon. 

3. Make sure that the configuration is set up for the system that you have installed (Figure 8.1), then click "OK" to close out the Configuration Utility.

4. Double click on the BioLogic DuoFlow application icon to launch the software. 

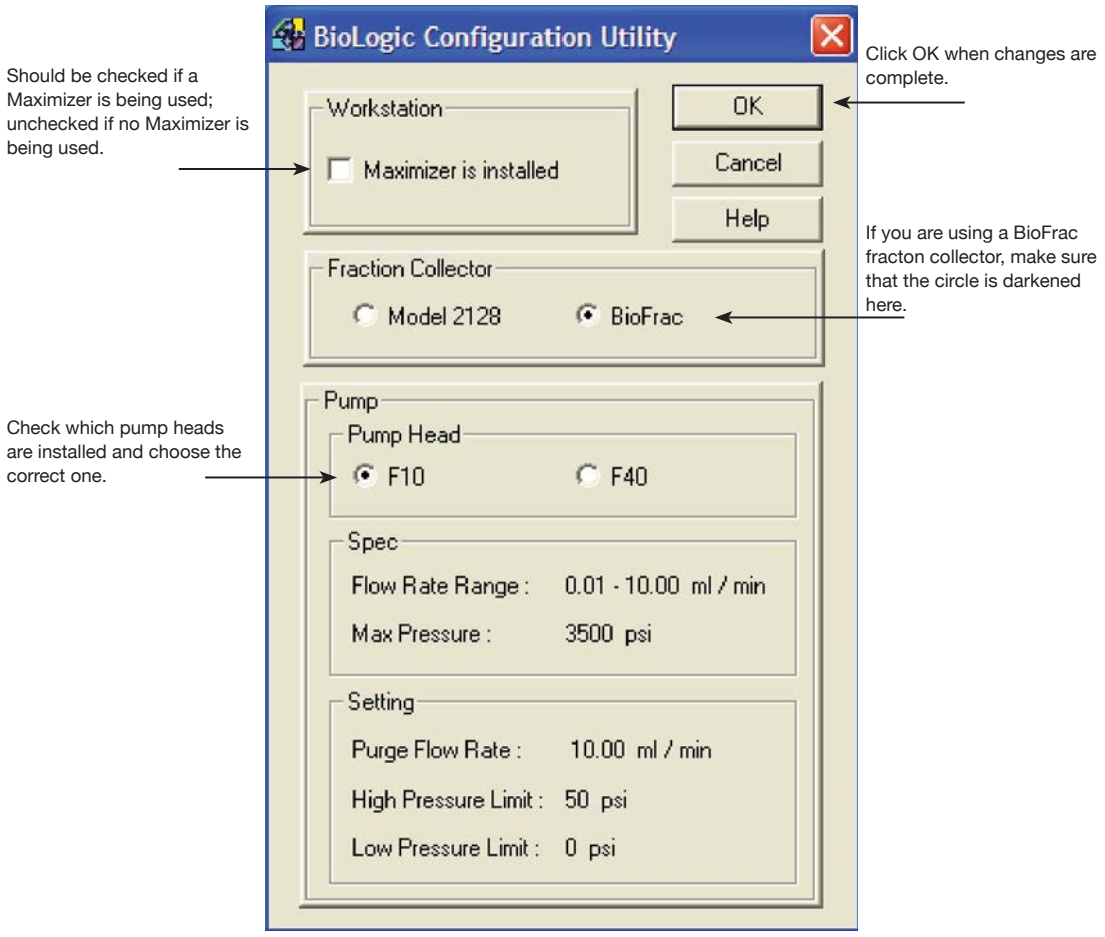


Figure 8.1. BioLogic System Configuration Utility. This utility is used to communicate the configuration of the hardware installed with the BioLogic DuoFlow Controller software. If a Maximizer is installed, the box should be checked. Next, the type of fraction collector should be checked. Finally, the pump head size should be highlighted—F10 for the pump heads that can produce 10 ml/min maximum flow and F40 for the pump heads that can produce 40 ml/min maximum flow.

Section 1. DuoFlow System Preparation

When the DuoFlow system is turned on, the Manual screen is displayed (Figure 8.2). This screen displays instrument control panels that provide direct control of the pumps, valves, fraction collector, and UV detector. Only those valves and accessories connected to the system will be displayed.

1.1 Prime the workstation pumps

Priming the workstation pumps involves filling the buffer inlet tubing with water. If there is air or air bubbles in the inlet lines, the pumps cannot pull fluid efficiently. Priming the pumps also serves as a first rinse of the inlet lines to remove any storage buffers that might be present.

1. Make sure that the waste lines of the AVR7-3 and the SV3-2 valve for the BioFrac fraction collector are flowing into a waste container.

2. Immerse the workstation pump A and B inlet lines in a bottle containing 500 ml of HPLC grade (filtered, degassed) or other high-quality water.

3. Connect a clean 10 ml syringe to the priming port of pump A (Figure 8.3).

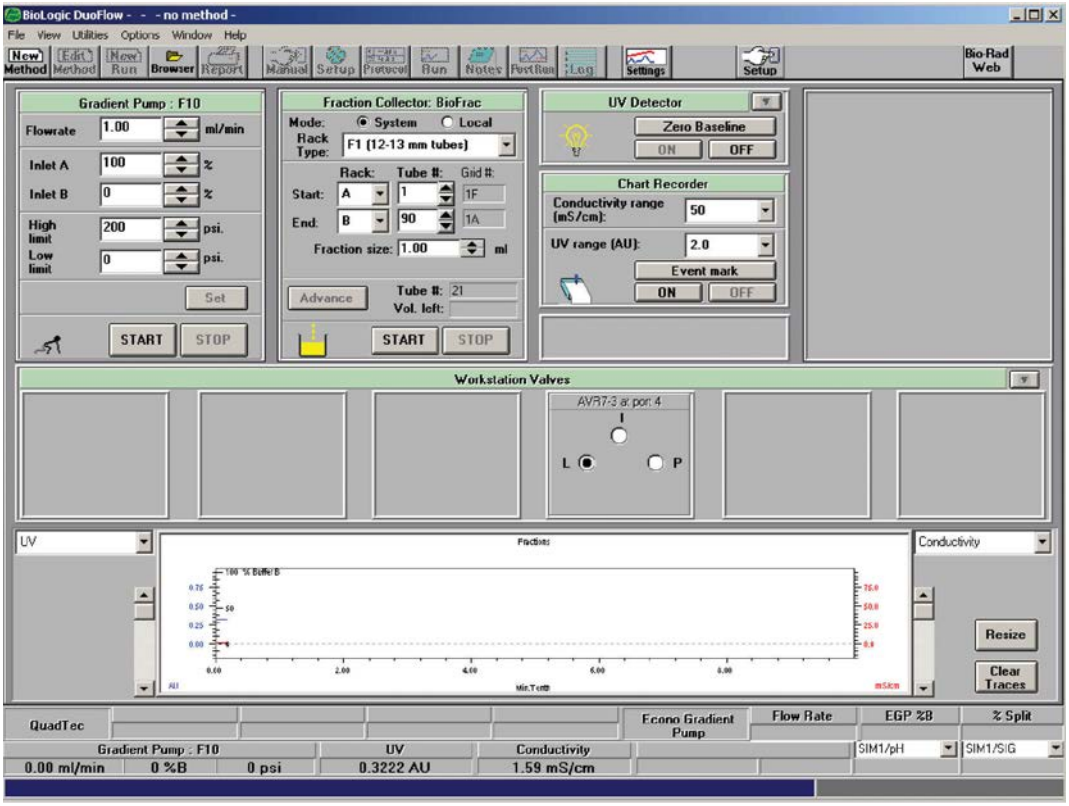


Figure 8.2. Manual control screen with UV detector. This screen allows manual control of the gradient pump (flow rate, % Buffer A/B, high and low pressure limit for the pump), control of the Fraction collector, the UV detector, and the workstation valves. On the bottom of the screen is a chromatogram that can display all variables that are being monitored such as UV, conductivity, pressure, and % Buffer B. The real time display of the gradient pump flow rate, buffer composition, pressure reading, UV reading and conductivity reading are displayed on the bottom line of the manual control page.

1. Turn the priming port counterclockwise one full turn to open the seal. Gently withdraw the syringe plunger to draw 10 ml of water into the pump head and tubing.

2. Look at the inlet tubing that goes from the beaker of water to Pump A. If there are any air bubbles in the tubing, tighten the priming port by turning it clockwise, empty the syringe, and repeat steps 3–5.

3. Tighten the priming port by turning it clockwise and then remove the syringe.

4. Repeat this priming procedure for pump B.

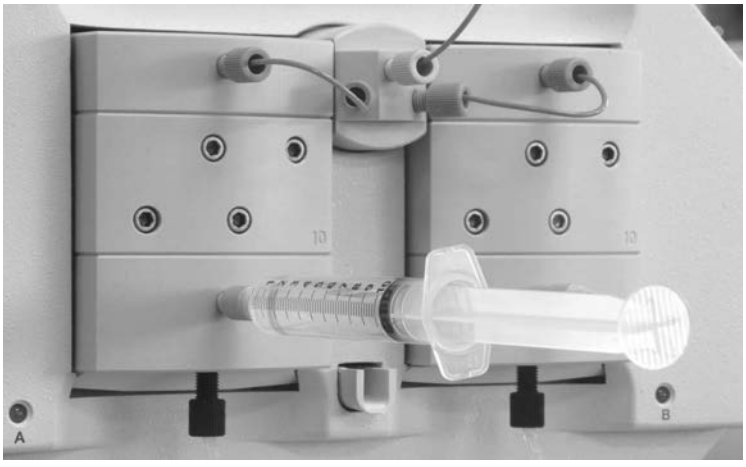


Figure 8.3. Priming the workstation pumps. A complete pump head on the DuoFlow system is shown here. Priming pump: A syringe is used to draw fluid into the inlet tubing for Pump A and Pump B. The pumps cannot pull fluid into the lines if there is air in the lines. Priming also serves to remove any air bubbles from the lines that could damage columns further downstream.

1.2 Move the AVR7-3 inject valve to the purge position

Prior to purging the pumps at 10 ml/min it is essential to place the AVR7-3 valve in the purge position. This directs the flow to waste and not to the column and detectors.

1. To change the position of the AVR7-3 inject valve select “P” from the manual screen valve control panel for the AVR7-3 valve (Figure 8.4B).

2. Look on the AVR7-3 valve itself. The “P” should be showing on the valve between ports 1 and 2 (Figure 8.4B).

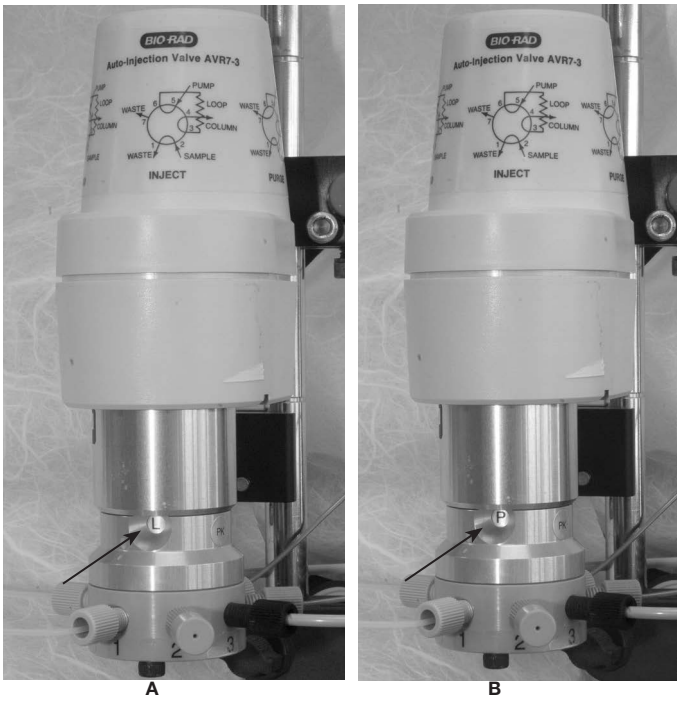


Figure 8.4. Workstation valve manual controls. A) The default setting upon startup of the BioLogic DuoFlow system for the AVR7-3 valve is L (Load). This valve gets its name from the fact that there are seven ports in total and of those, three are inlet ports. One of the three settings that the AVR7-3 valve can be set for is L (Load) in which a sample can be loaded into the sample loop with any excess solution from the sample loop being pushed to waste. At the load setting, the pump pushes fluid into the AVR7-3 valve and out a second port without pushing any of the sample in the sample loop further along the fluid pathway onto the column. The I (Inject) position is where the valve moves so that now the fluid pushed through the pumps pushes the sample out of the sample loop and injects it onto the column.
B) The P (Purge) setting allows fluid from the pumps to be pushed into the AVR7-3 valve and then out a waste port. In purge mode, the fluid from the pumps does not push the sample in the sample loop onto the column and in fact, does not even continue to flow buffers from the pumps any further along the fluid pathway. The purge setting is used to remove or exchange buffers in the tubing bypassing the column at a high flow rate that the column potentially cannot withstand. The AVR7-3 is in purge mode as shown by the “P” on the valve.

1.3 Purge the workstation pumps

Purging of the workstation pumps flushes out any storage buffer or remaining solutions that are in the tubing between the buffer inlets and the AVR7-3 valve. This serves to clean out the tubing and also replace whatever buffer was in the tubing with water.

1. Press the Purge button A on the front of the workstation (Figure 8.5) and allow it to run for two minutes. The green light next to pump A should be flashing green.

2. Press the Purge button A on the front of the workstation a second time to stop pump A.

3. Press the Purge button B to start this pump and allow it to run for two minutes. The green light next to pump B should be flashing green.

4. Press the Purge button B again to stop the pump.

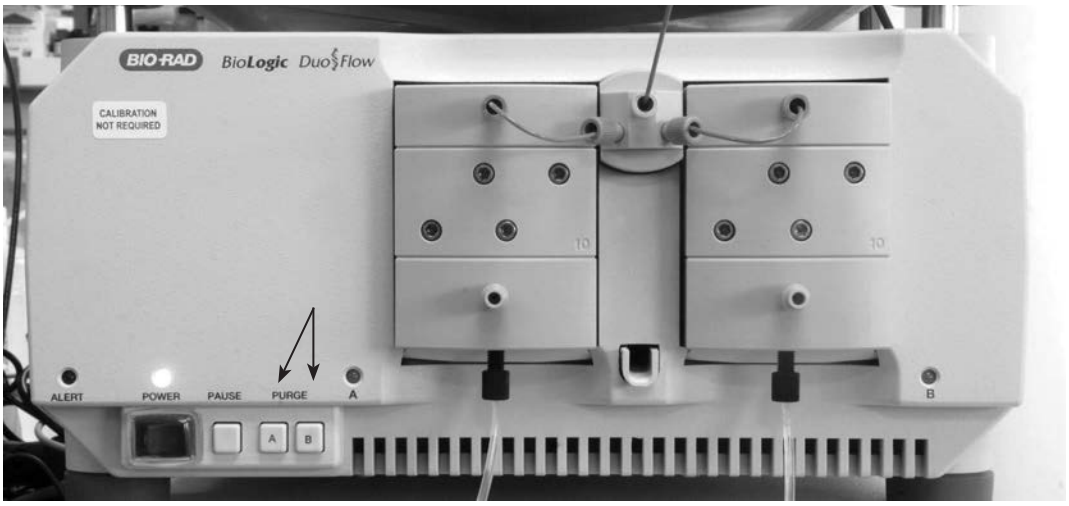


Figure 8.5. The purge buttons for Pump A and Pump B on the DuoFlow workstation. When the purge buttons on the workstation front are pushed, the workstation pumps will run at a default flow rate of 10 ml/min and the indicator light will flash green. Many columns cannot withstand the pressure that this would generate and therefore, it is critical that the AVR7-3 valve has been set to the P (Purge) setting to ensure that the fluid flow will exit to waste and not flow onto the column at this flow rate.

1.4 Manual Control of the Workstation Pumps

The workstation pump parameters are set from the Manual screen either by clicking in the appropriate field and entering a value from the keyboard or by using the arrows (Figure 8.6).

1. Set the flow rate to 1.00 ml/min by typing 1.00 into the Flowrate box or using the up/down arrows.
2. Set the Inlet A composition to 100%.
3. Set the high pressure limit to 200 psi and the low pressure limit to 0 psi.
4. To start the pump, click the Start button. The green light for Pump A should be lit.
5. Allow Pump A to run for three minutes.
6. With Pump A running, change the Inlet B value to 100% (notice that Inlet A automatically changes to 0%) and press the Set button to initiate the change. The green light for Pump A should go off and the green light for Pump B should turn on.
7. Allow Pump B to run for three minutes and then press Stop to stop the pump.

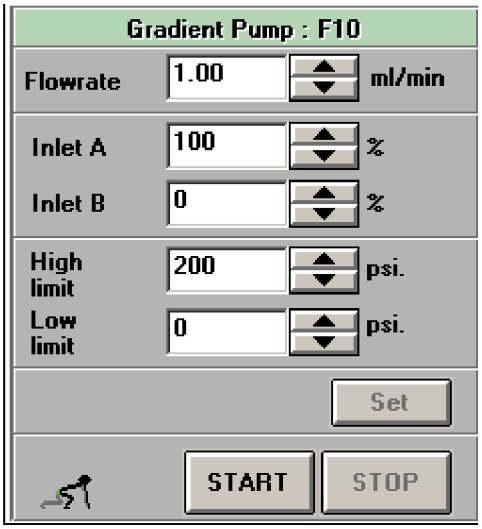


Figure 8.6. Manual pump control. The flow rate for an F10 pump head system can be set between 0.01 to 10 ml/min. The maximum flow rate is determined by the type of column that is being used. Some resins cannot withstand the pressure that builds from a high flowrate and will be crushed (such as soft agarose-based beads). Other resins can withstand much higher pressures/flow rates without being damaged. The Inlet A and Inlet B settings determine how much buffer is pulled from each port. Typical chromatography nomenclature refers to the percentage of buffer B that is being used. So if a 95% Buffer A and 5% Buffer B mixture was required, this would be referred to as 5% Buffer B. The High and Low limits are pressure limits that can be set to have the instrument turn off if they are exceeded or drop below the given values, respectively. These are used to protect the column from overpressuring and to stop the run if the pressure drops to zero, which is usually indicative of running out of buffer or having a large air bubble in the tubing.

1.5 Flush the system through with water to the fraction collector

Up to this point, the inlet tubing for Pump A and Pump B, as well as all the tubing up to the AVR7-3 valve, have been flushed with water. Now it is time to flush the entire system beyond the AVR7-3 valve (the column, UV and conductivity detectors and fraction collector and all associated tubing) with water as well.

1. On the Manual screen, move the AVR7-3 valve back to position L (Load) by clicking "L" (Figure 8.4A). Verify that L is now displayed on the AVR7-3 valve.

2. From the gradient pump control panel on the Manual screen, set the pump flow rate to 2.0 ml/min, set the composition to 50% Buffer B and start the pump (Figure 8.6). Make sure that you have at least 200 ml of water remaining in your beaker and that both the inlet lines are at the bottom of the beaker.

3. In the Fraction Collector window on the Manual screen ensure that the System button is selected (Figure 8.7).

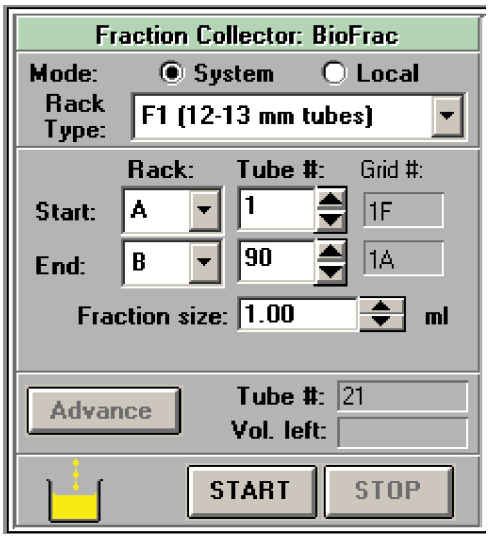


Figure 8.7. Manual DuoFlow system operation fraction collector control. The BioFrac fraction collector has two operating modes: System—controlled by the DuoFlow system and Local—controlled from its own faceplate in stand-alone mode. When in System mode, the fraction collector control panel will show fields for Rack type, Start tube, End tube, Fraction size, Tube number, Volume left, as well as a toggle button for Start and Stop, and a button for Advance.

1.6 Turn on the UV lamp

Check that the lamp is on by looking at the UV Detector panel (Figure 8.8); if it is not, press the ON button. The light bulb icon should be yellow when the UV lamp is on.

Figure 8.8. Manual control of the UV detector. The UV lamp automatically turns on when you turn on power to the workstation. The UV lamp can be turned on and off by clicking the On and Off buttons from the UV detector control panel on the Manual screen.

1.7 Manual screen chromatogram window

A feature of the Manual screen is its ability to display up to eight traces on a chromatogram—including UV/Vis, pH, conductivity, % Buffer B and pressure traces—over a 10-minute interval. This is useful during column equilibration. The chromatogram window is displayed at the bottom of the screen under the valve control panel (Figure 8.9).

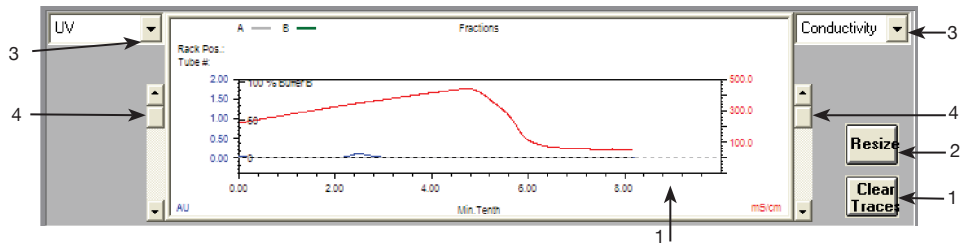


Figure 8.9. Manual screen chromatogram. Features of the chromatogram window include: 1. The time axis is reset automatically at the end of 10 minutes or reset manually by clicking the Clear Traces button. 2. The chromatogram window can be enlarged by pressing the Resize button. 3. Which chromatogram trace is to be displayed can be selected by using the drop-down menus on the upper right and left of the display 4. The Y-axis scale can be changed using the scroll bars on the right or left of the display 5. The maximum and minimum axis settings can be changed by pressing Settings on the Manual screen toolbar (see Figure 8.2).

1. Press the Settings button on the top menu bar. 

2. Select the parameters that you want to be able to track on the Manual screen chromatogram by scrolling down the Trace Device options, selecting an option, and then clicking on the Visible box (Figure 8.10). If there is an "X" in the visible box then that trace can be displayed.

3. Select UV, Conductivity, GP-Pressure and % Buffer B to be visible, and then click OK.

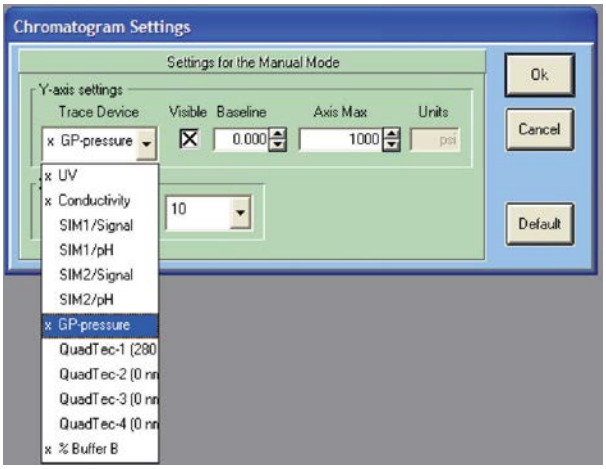


Figure 8.10. Controlling the chromatogram settings. The traces that will be possible to scroll through on the Manual screen chromatogram are selected to be visible under the Settings option. The maximum value for the axis can also be set here or by using the scroll bars on the actual chromatogram (Figure 8.9).

1.8 Status bar

At the bottom of the Manual screen is a status bar that is continually updated with system parameters (Figure 8.11A).

1. Allow the pumps to run for three more minutes.

2. Press the Zero Baseline button on the UV control (Figure 8.8).

3. Press the Clear Traces button (Figure 8.9) to clear the Manual screen chromatogram.

4. At this point, you have primed the entire system and rinsed it out with water. Record the UV and conductivity values from the status bar after the water wash.

   UV _____ Conductivity _____ mS/cm

   Ideally, the UV should be close to zero since high-quality water has minimal to no absorbance at 280 nm. The conductivity should also be close to zero. The conductivity depends on how many ionic species are present. HPLC grade water such as nanopure water, distilled or deionized water should have a conductivity of less than 3 mS/cm.

5. If the UV value and conductivity values are higher than 0.1, continue running high-quality water through the system for another five minutes

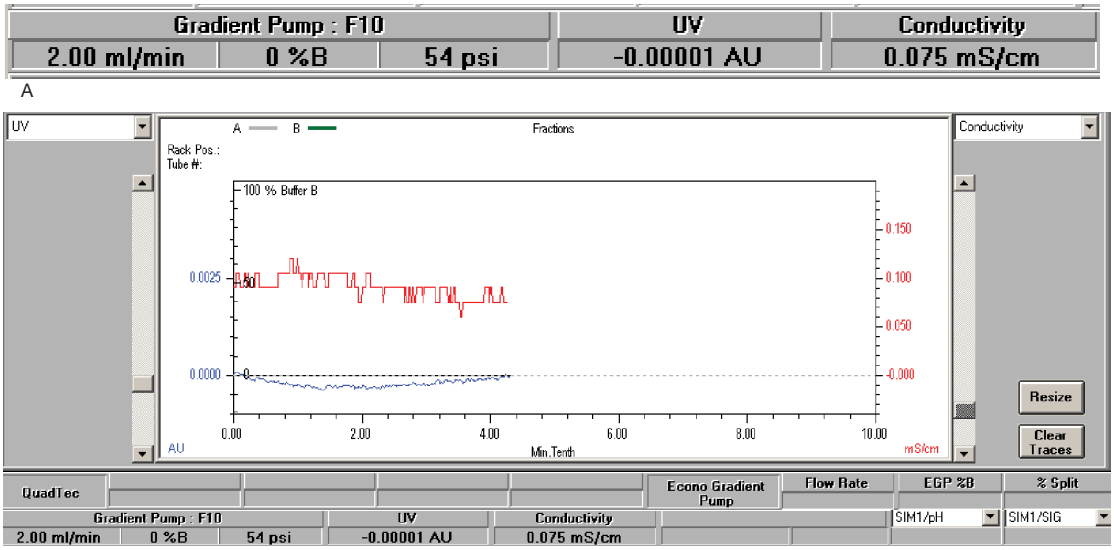


Figure 8.11. Manual screen chromatogram and status bar after five minutes of water washing at 2.00 ml/min. A) This is a magnified view of the status bar. The flow rate is displayed, followed by the % B and the pressure. The UV and conductivity readings are close to zero which means that the system is filled with water and has been cleaned. B) The Manual screen chromatogram shows the UV and conductivity readings for the five minutes of water cleaning time. Note that the Y-axes have been greatly reduced with the AU (UV readings), ranging from -0.005 to 0.0025, and the conductivity readings ranging from 0.000-0.150 mS/cm. Within the accuracy of the detectors, there is little to no change in the conductivity or UV readings for the five minutes, and the values are close to zero.

Section 2. Affinity Chromatography using Native IMAC Protocol to Purify GST-DHFR-His

Manually preparing the DuoFlow system and IMAC Ni-charged column

1. Prime the Pump Inlet tubing with Buffer A and Buffer B

The inlet tubing is currently filled with water. At this point, the inlet tubing will be primed with Buffer A and Buffer B in preparation for cleaning and equilibrating the column.

1. Ensure the pumps are stopped and the AVR7-3 inject valve is in the Load (L) position on the Manual screen.

2. Place the tubing for Pump A into the container of Buffer A. Make sure that the tubing sits near the bottom of the container. Tape the tubing down if necessary.

3. Place the tubing for Pump B into the container of Buffer B. Make sure that the tubing sits near the bottom of the container. Tape the tubing down if necessary.

4. Attach the 10 ml syringe to the priming port for Pump A, turn the priming port counterclockwise to open, and pull 1–2 ml of Buffer A into the syringe to remove any air bubbles introduced into the Inlet line. Turn the priming port clockwise to close and remove the syringe. Expel the buffer in the syringe into the waste beaker.

5. Repeat step 4 for priming port B.

2. Cleaning and equilibrating the IMAC Ni-charged column in Manual mode

At this point, the lines throughout the entire system will first be filled with Buffer B (20 mM sodium phosphate buffer, 300 mM NaCl, 250 mM imidazole, pH 8) to wash any remaining contaminants off of the IMAC column. Then the lines will be filled with 2% Buffer B (20 mM sodium phosphate buffer, 300 mM NaCl, 5 mM imidazole) to flush the 100% Buffer B solution out of the system and replace it with 2% Buffer B (the same composition buffer that the soluble fraction is in). The column will be equilibrated at this point and ready for use.

1. Set the AVR7-3 inject valve to position L (Load).

2. Set the Gradient Pump Flowrate to 2.00 ml/min, Inlet B to 100%, High limit to 200 psi and Low limit to 0 psi, then press the Start button (see Figure 8.12). Allow the wash to proceed for five minutes. While the pump is running, continue to steps 4–6.

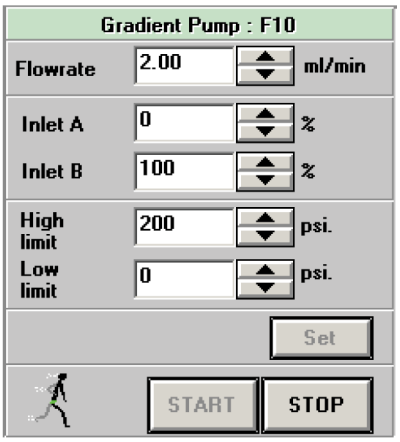


Figure 8.12. Manual wash. The column and tubing are first washed with 100% Buffer B at 2.00 ml/min for five minutes. In chromatography nomenclature, this is a 10 CV (column volume) wash since 10 ml of buffer are flowed over a 1 ml column. This step washed off any storage buffer remaining on the column and any residue from previous purifications (if present).

3. While the pump is running, look at the GP-Pressure curve (Figure 8.13) and determine if the pressure is even.

4. While there might be small oscillations, there should be no large variances. **If there are large fluctuations** (such as going from 50 psi to 18 psi and back), this means there are air bubbles in the lines and you will need to stop. Stop the pump. Prime Inlet line B again, following the protocols in Step 1 and then start Pump B again.

5. Once there are no air bubbles in the line (no pressure fluctuations), record the UV absorbance and conductivity values from the bottom of the Manual screen and then stop the pump.

   UV _____ Conductivity _____

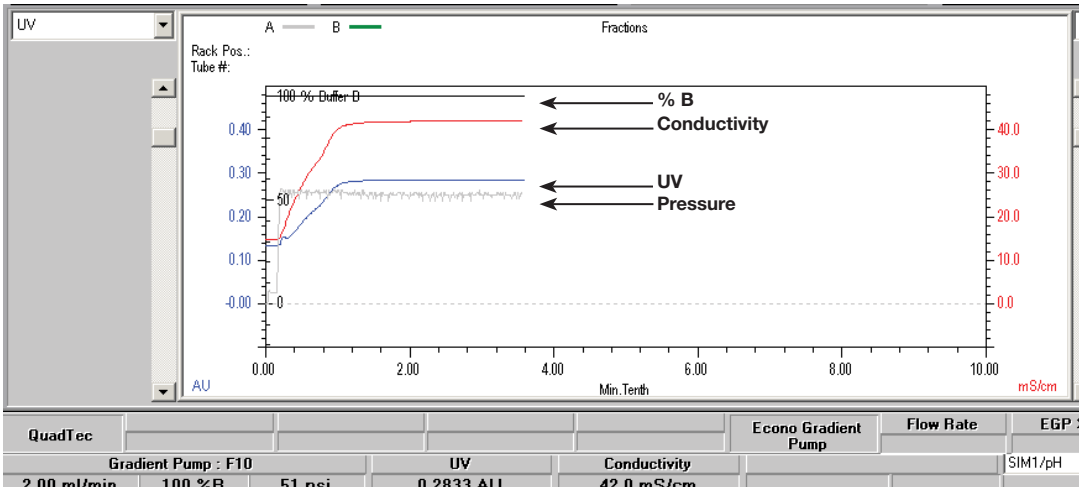


Figure 8.13. Manual wash with 100% B. Imidazole absorbs at 280 nm so you should see a UV value less than 0.5. Buffer B contains a high level of salt (300 mM NaCl and 20 mM sodium phosphate) that contributes to the main portion of the conductivity value. Imidazole contributes some conductivity, but is not a strong ionic species and hence contributes much less to the overall conductivity. The conductivity range expected after the 100% Buffer B wash is between 35–55 mS/cm. The chromatogram shows equilibration of the UV trace, conductivity trace, and pressure reading with the curves that go flat (note the Y-axes have been expanded for easier viewing).

6. Set the buffer composition to 98% Inlet A and 2% Inlet B. Leave the flow rate at 2.00 ml/min and the high and low pressure limits at 200 psi and 0 psi respectively.

7. Press the Start button and equilibrate the system for five minutes (or 10 CV).

8. While the pump is running, look at the GP-Pressure curve (Figure 8.14) and determine if the pressure is even.

9. While there might be small oscillations, there should be no large ones. **If there are large fluctuations** (such as going from 50 psi to 18 psi and back), this means there are air bubbles in the lines and you will need to stop the pumps. Prime Inlet line A again following the protocols in Step 1 and then start the pumps again.

10. After five minutes of equilibration with 2% Buffer B, record the UV and Conductivity values and then stop the pump.

    UV _____ Conductivity _____

1. If your UV values are higher than 0.05, continue flowing 2% Buffer B at 2.00 ml/min over the system for another five minutes.

2. At this point, the column should be equilibrated: no air bubbles should be in any of the lines, and the system should be ready for use once you have programmed your method.

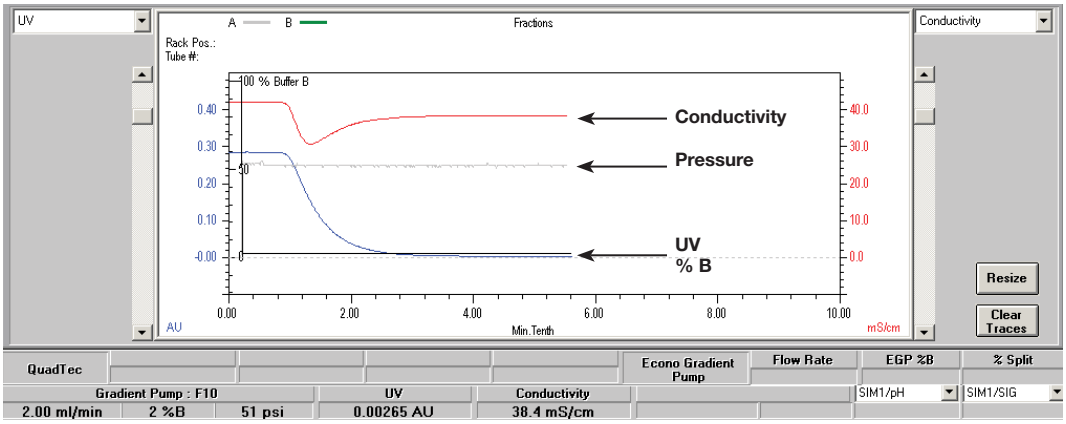


Figure 8.14. Sample of a trace after equilibration with 2% Buffer B. A 2% Buffer B solution contains 20 mM sodium phosphate, 300 mM NaCl, and 5 mM imidazole. Therefore, the UV absorbance at 280 nm should be much lower than the 100% B solution that contained 250 mM imidazole and should be within the range of 0.0–0.008. The conductivity value should not decrease significantly since the sodium phosphate and NaCl are the main contributors to conductivity and should be within the range of 30–55 mS/cm. It is critical that the column is equilibrated in 2% Buffer B since higher compositions of imidazole will prevent GST-DHFR-His from binding to the column.

Creating a New Method for Purifying GST-DHFR-His

There is a hierarchy for how files are written and stored using the DuoFlow system software. The top level of storage is under the designation of a user name. The next level down are Projects. A Project might contain a series of different Methods attempted to try and purify a certain protein. The next level down is the Protocol. This is the actual information sent to the instrument to let it know which buffers to use, what the flow rates should be, when to change buffers, what fractions to collect, etc. Each Method contains information for only one Protocol (and the associated Setup that includes information on which components are being used such as valves, fraction collector, pH meter, etc.). Finally, the most specific designation are the Runs. These are the actual data that are received from the detectors (UV/Vis, Conductivity, Fraction collector, pH meter, etc.) each time a sample is injected to be purified.

User 1➜Project 1 DHFR purification	➜Method 1 IMAC Step	➜Protocol 1 IMAC Step	➜Run 1
	➜Method 2 IMAC Gradient	➜Protocol 2 IMAC Gradient	➜Run 1
			➜Run 2
➜Project 2 GFP purification	➜Method 1 HIC Step	➜Protocol 1 HIC Step	➜Run 1
			➜Run 2
			➜Run 3

Figure 8.15. Hierarchy of data storage for DuoFlow system software.

1. Creating a User Name, Project Name and Method Name

In the Manual screen, select the Browser icon from the tool bar. In the Browser screen you will enter a user name for your method according to the following steps.

1. Select the Browser icon from the tool bar menu. 

2. Click on the yellow New icon on the upper-left side of the Browser screen (Figure 8.15).

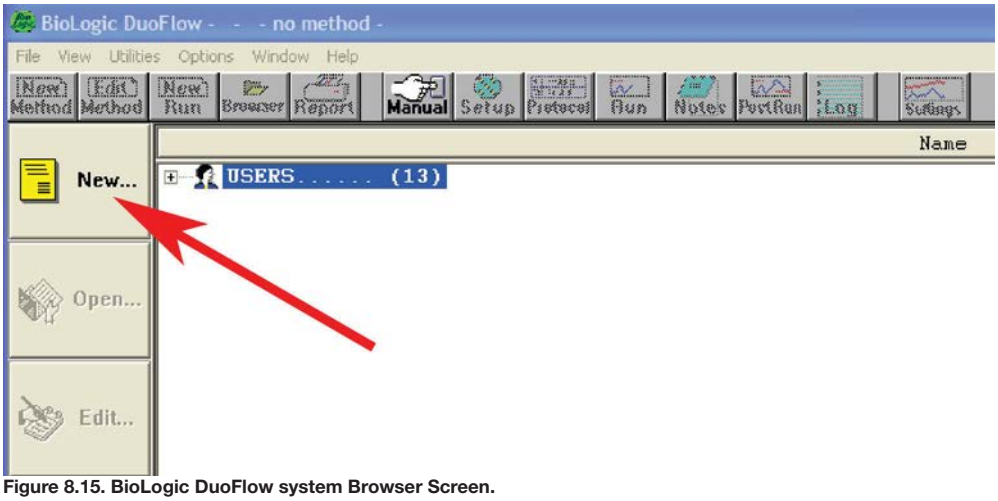


Figure 8.15. BioLogic DuoFlow system Browser Screen.

3. Enter your name in the dialog box and then click OK (Figure 8.16).

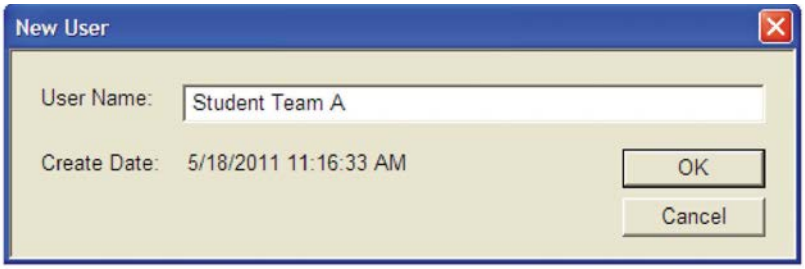


Figure 8.16. BioLogic DuoFlow system New User dialog box.

4. Click on the Project folder icon that is displayed below your name until the line is highlighted in blue (Figure 8.17).

5. Select the yellow New icon again and choose New Method from the options displayed (Figure 8.18). Enter your method name (or use default Method 1).

6. Click OK to proceed to the instrument/devices Setup screen (Figure 8.19).

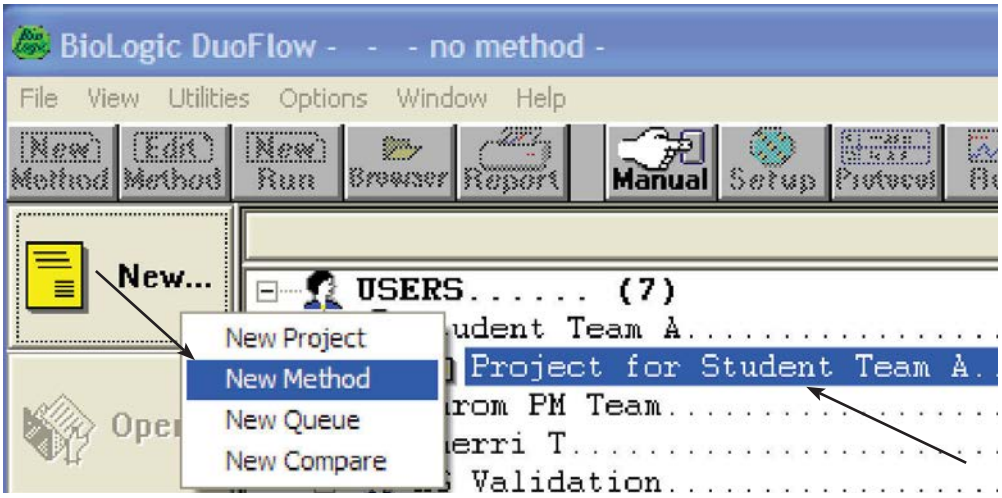


Figure 8.17. BioLogic DuoFlow system New Method creation.

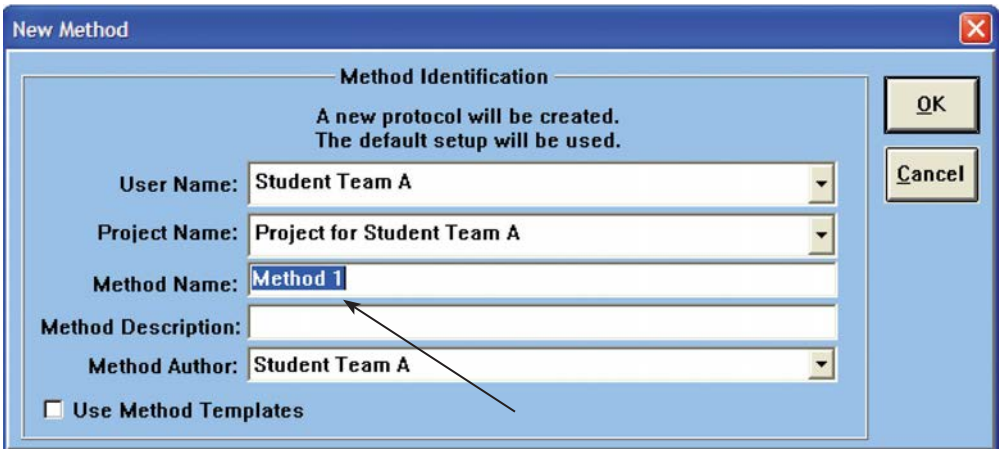


Figure 8.18. BioLogic DuoFlow system New Method dialog box.

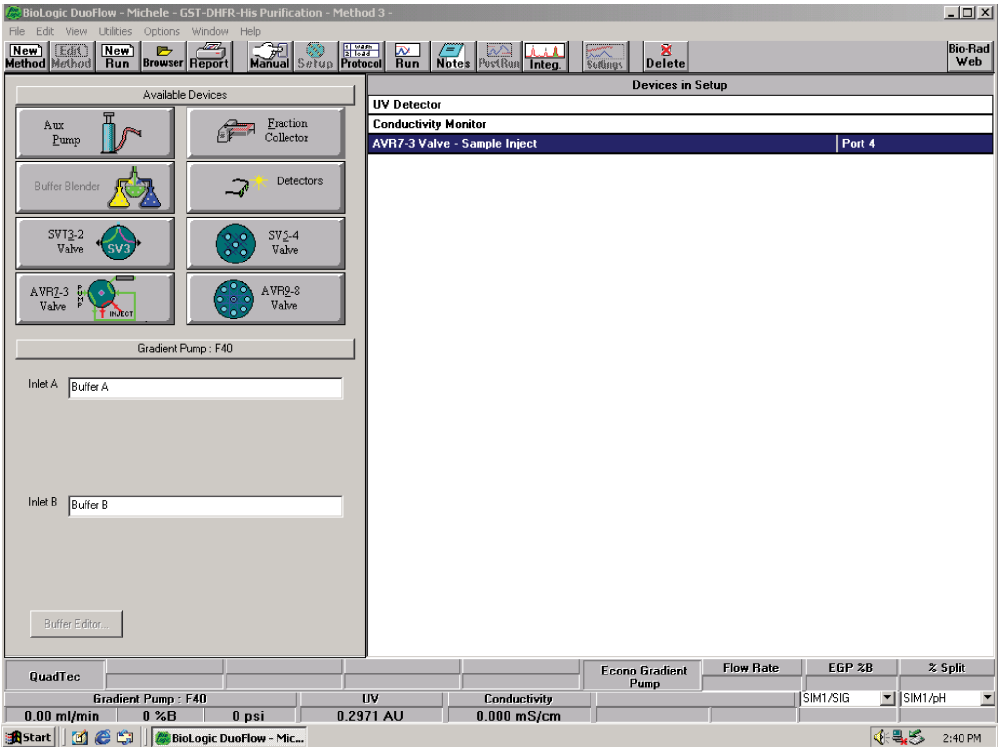


Figure 8.19. Setup editor. In the Setup screen you will select the instruments and devices to be used for the GST-DHFR-His purification method. The icons grouped on the left side of the screen show all the instruments and devices that can be connected to the BioLogic DuoFlow system. The list of devices in the right box (Devices in Setup) identifies those devices selected for use with a specific method. The initial default Devices in Setup are a UV detector, conductivity monitor, and an AVR7-3 inject valve. These come standard with the BioLogic DuoFlow system.

2. Program the Instrument Setup

1. Click on the Fraction Collector button in the Available Devices box (Figure 8.20). A dialog box will appear asking you to choose the type of collector (e.g., a generic collector, a Model 2110, or a BioFrac fraction collector).

   Note: The following instructions pertain to programming the BioFrac fraction collector. If you have a different fraction collector, please consult that instrument's instruction manual.

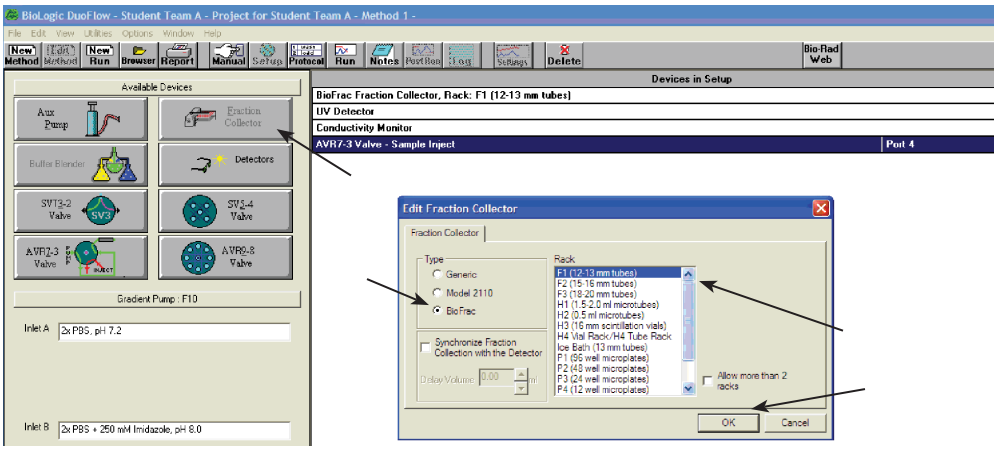


Figure 8.20. Selecting the fraction collector in the device set up window.

2. Click on BioFrac and click the OK button. You will now see BioFrac fraction collector in the Devices in Setup box. The F1 Rack (12–13 mm tubes) is automatically selected. Click OK to choose.

3. In the Gradient Pump section of the setup screen enter your buffer names. In the Buffer A field, type in 2x PBS, pH 7.2. In the Buffer B field, type in 2x PBS + 250 mM Imidazole, pH 8.0.

4. You are now ready to program the separation steps for your method. To program your method, press the Protocol icon on the tool bar. 

3. Program the Method Protocol

From the Options pull-down menu, ensure that Use Volume (ml) is selected so that the programming base is Volume (Figure 8.21).

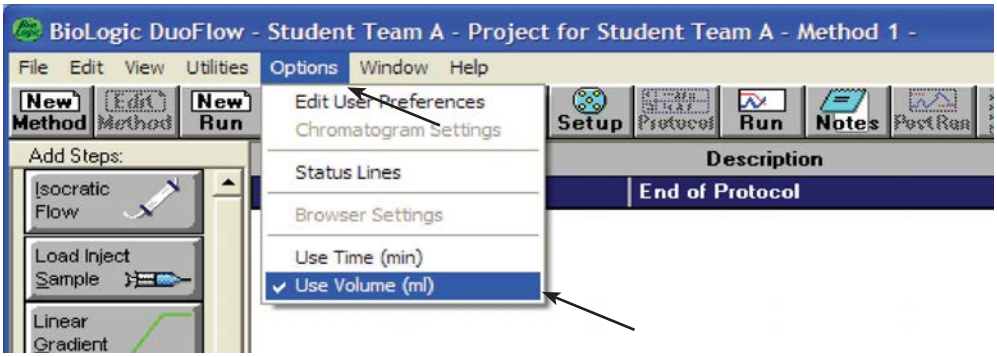


Figure 8.21. BioLogic DuoFlow system setting base to Volume.

Programming the fraction collector (BioFrac fraction collector)

1. From the left side of the screen, press the fraction collection icon (Figure 8.22).

2. In the pop-up window that appears, choose Collection Windows.

3. Make sure the correct rack type is displayed (F1 12-13 mm tubes).

4. Click on the tab Collection/Windows.

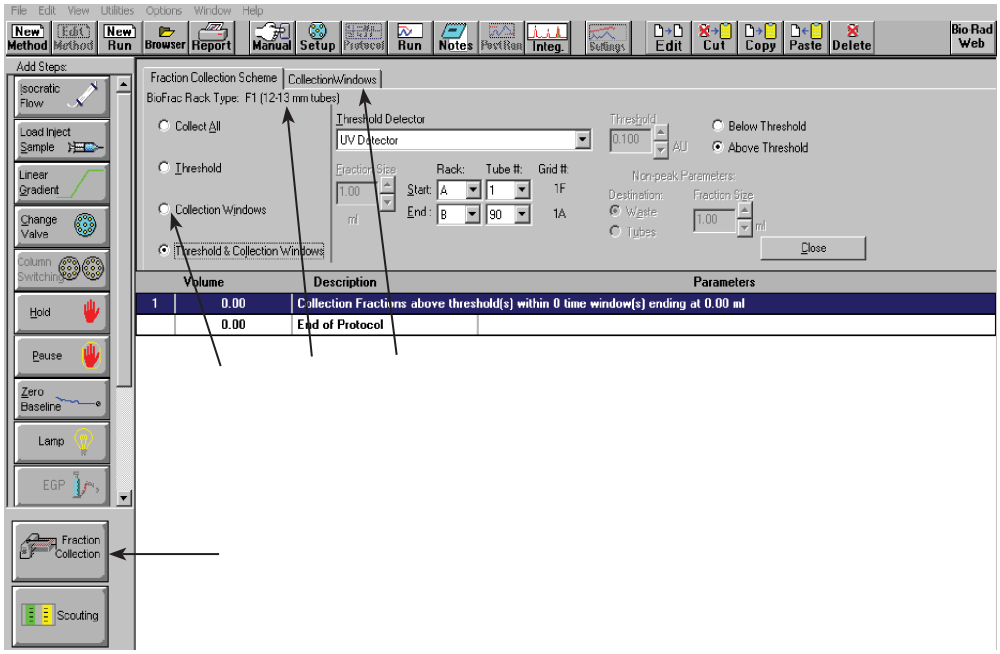


Figure 8.22. Fraction Collection Scheme programming. This window allows programming of the BioFrac fraction collector. The options are: Collect All, where all fractions are collected and each has the same volume; Threshold, where fractions are collected if the absorbance is greater than a set value; Collection Windows, where fractions of differing volume are collected in different time frames (windows); and Threshhold & Collection Windows, where fractions collected during certain time frames have an absorbance greater than a set value. Collection Windows will be used for the GST-DHFR-His protocol. Larger volume fractions will be collected for proteins that do not bind to the column (flowthrough fraction) and are washed off the column (wash fractions) while smaller fractions will be collected when the GST-DHFR-His elutes. This allows for more concentrated GST-DHFR-His samples.

3. Program the first window by typing 6.00 into the Start (ml) box, 12.00 into the End (ml) box and 4.00 into the Frac. Size (ml) box. Press the Save Window button.

Table 8.1 Programming BioFrac fraction collector collection windows.

Step Number	Start (ml)	End (ml)	Frac. Size (ml)	Product being collected
1.	6.00	12.00	4.00	Flowthrough
2.	12.00	23.00	4.00	Wash
3.	23.00	37.00	1.00	Eluted GST-DHFR-His

4. Press the Add Window button. Enter 12.00 for the Start (ml), 23.00 for the End (ml) and 4.00 for Frac. Size (ml). Press the Save Window button.

5. Press the Add Window button. Enter 23.00 for the Start (ml), 37.00 for End (ml) and 1.00 for Frac. Size (ml). Press the Save Window button, then Close.

Programming the pump and UV lamp steps of the protocol

1. Program the remaining steps using the Add Step icons on the left side of the screen. Note that Step 1 has already been added to your protocol since you already programmed the fraction collector. Table 8.2 on page 182 includes all of the remaining steps that need to be programmed. Examples of how to program Steps 2 and 3 are outlined here.

   Note: The software adds a step above whatever line is shaded in blue. For example, in Figure 8.23, if a step were to be added with the Collection Fractions step highlighted, the step would be added before this step. To add a step after Collection Fractions, have the End of Protocol step highlighted.

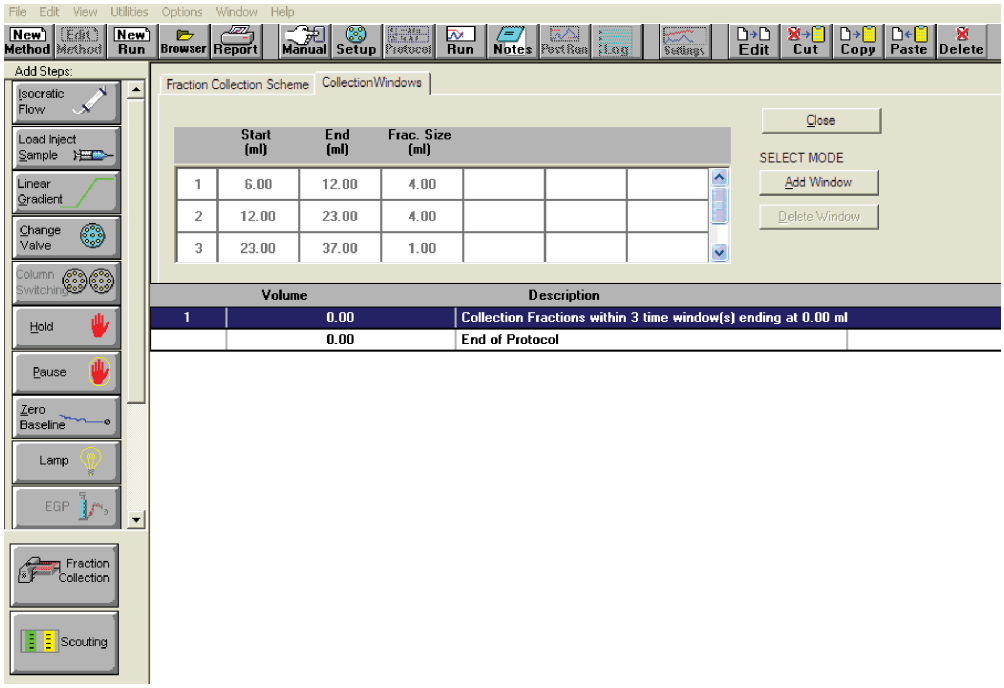


Figure 8.23. Programming the collection windows. Screenshot of the programmed windows.

2. Step 2: Turn on the lamp by clicking on the Lamp button on the left side of the screen and then select the “on”option and click OK (Figure 8.24).

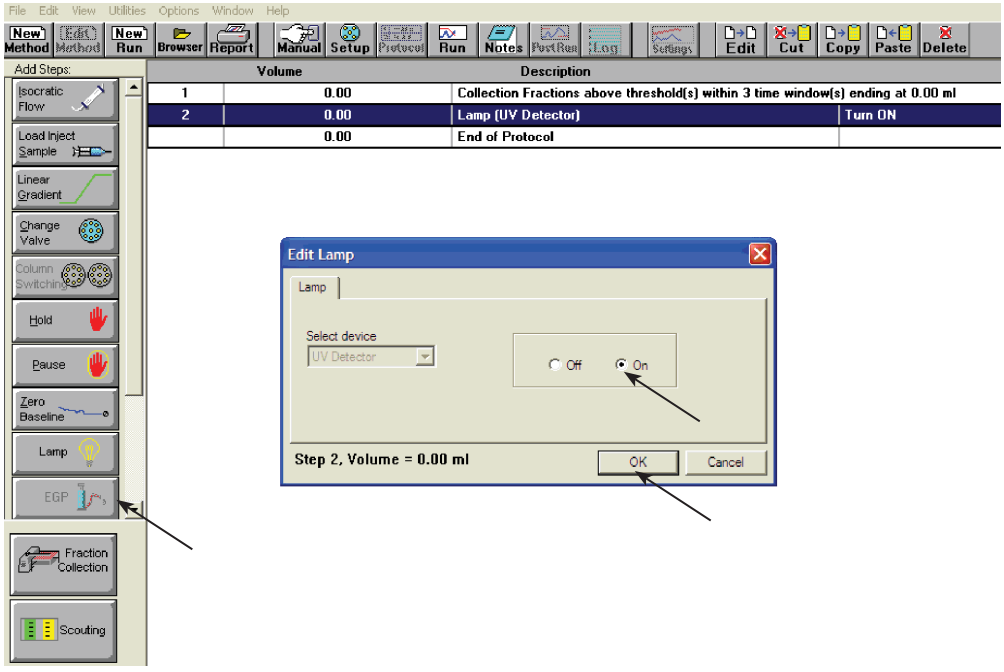


Figure 8.24. Programming Step 2: Lamp (UV Detector) Turn On.

1. Choose Isocratic Flow from the left-side menu. Change the composition of the 2x PBS (Buffer A) to 98%. The % Composition for Buffer B will automatically change to 2%. Change the volume to 5.00 ml and the flow rate to 2.00 ml/min, and then press OK (Figure 8.25).

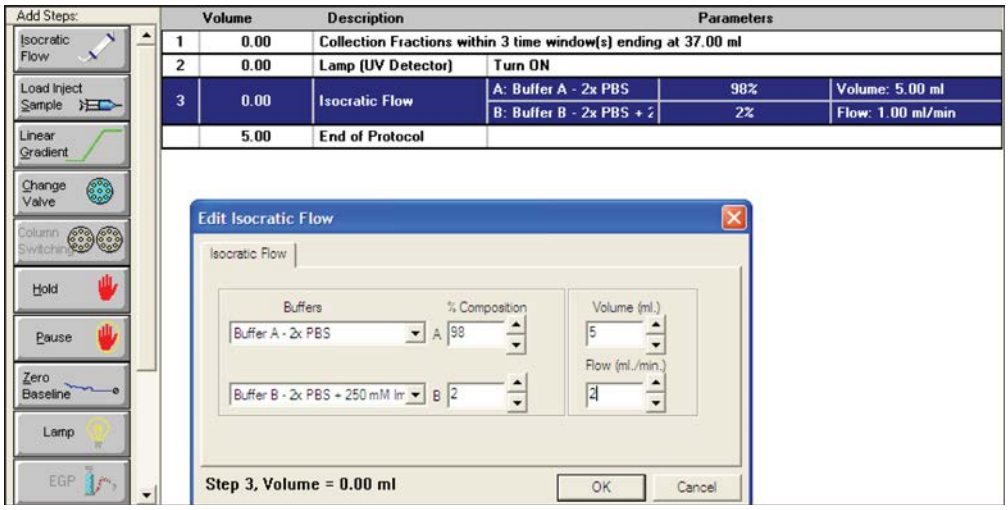


Figure 8.25. Programming Step 3: Equilibrating the column with 2x PBS + 5 mM imidazole for 5 CV at 2 ml/min.

2. Continue programming steps 4–9 as defined in Table 8.2 below. Your final protocol should look like Figure 8.26.

Table 8.2. Programming the Protocol

Step Number	Start (ml)	Purpose	Programming
1	0.00	Programming fraction collector	Collect 4.00 ml fractions during the sample injection and wash steps and then 1.00 ml fraction during the elution step
2	0.00	Turn the lamp on (if not already on)	Lamp Choose on
3	0.00	Equilibrate column	Isocratic flow with 98% 2x PBS and 2% 2x PBS, 250 mM Imidazole for 5 ml at 2.00 ml/min
4	5.00	Zero baseline	Zero baseline to set UV baseline to 0.0
5	5.00	Inject sample onto the column	Load Inject sample, static loop: Inject 6.0 ml* sample at 1.00 ml/min. The injection occurs using 98% 2x PBS and 2% 2x PBS, 250 mM Imidazole
6	11.00	Wash the column	Isocratic flow with 96% 2x PBS and 4% 2x PBS + 250 mM Imidazole for 11 ml at 2.00 ml/min
7	22.00	Elute GST-DHFR-His	Isocratic flow with 0% 2x PBS and 100% 2x PBS, 250 mM Imidazole, for 8 ml at 2.00 ml/min
8	30.00	Final column wash	Isocratic flow with 100% 2x PBS and 0% 2x PBS, 250 mM Imidazole for 15 ml at 2.00 ml/min
9	45.00	Turn off lamp	Lamp Choose off
	45.00	End of protocol	

*A slightly larger volume (6 ml) than the sample loop (5 ml) is used to ensure all the sample is fully pushed onto the column.

Figure 8.26. Protocol for purifying GST-DHFR-His.

	Volume	Description	Parameters	
1	0.00	Collection Fractions within 3 time window(s) ending at 37.00 ml		
2	0.00	Lamp (UV Detector)	Turn ON	
3	0.00	Isocratic Flow	A: 2x PBS, pH 7.2	98% Volume: 5.00 ml
			B: 2x PBS, 250 mM Imidazole, pH 8.0	2% Flow: 2.00 ml/min
4	5.00	Zero Baseline	UV Detector	
5	5.00	Load/Inject Sample	Sample	Volume: 6.00 ml
			Static Loop	Auto Inject Valve Flow: 1.00 ml/min
6	11.00	Isocratic Flow	A: 2x PBS, pH 7.2	96% Volume: 11.00 ml
			B: 2x PBS, 250 mM Imidazole, pH 8.0	4% Flow: 2.00 ml/min
7	22.00	Isocratic Flow	A: 2x PBS, pH 7.2	0% Volume: 8.00 ml
			B: 2x PBS, 250 mM Imidazole, pH 8.0	100% Flow: 2.00 ml/min
8	30.00	Isocratic Flow	A: 2x PBS, pH 7.2	100% Volume: 15.00 ml
			B: 2x PBS, 250 mM Imidazole, pH 8.0	0% Flow: 2.00 ml/min
9	45.00	Lamp (UV Detector)	Turn OFF	
	45.00	End of Protocol		

1. When you have finished programming the method, press the toolbar Run button. 

A dialog box will ask you to name the run. Accept the default Run 1 and click OK. You will now see the Run screen (Figure 8.27).

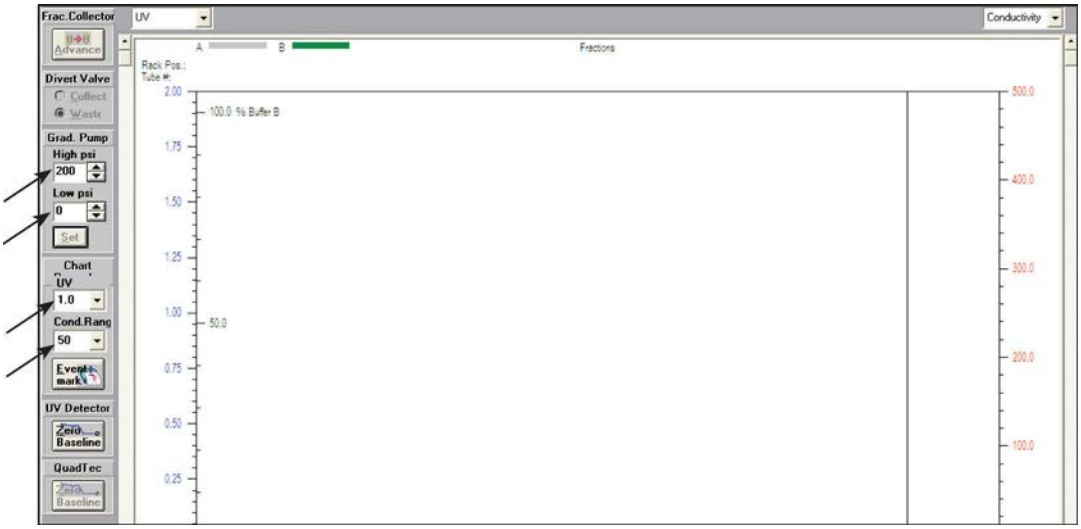


Figure 8.27. Run Screen. The toolbar buttons on the left side of the screen enable you to check that the screen display ranges for UV and conductivity are correctly set and that the gradient pump pressure limits are appropriate.

The Run Screen

1. Make sure that the high psi value is set to 200 and the low psi value is set to 0.

2. Scale the on-screen chromatogram trace display axes by using the scroll bars located on the left axis to have a maximum value of 2.0 and the right axis of the chromatogram window to have a maximum value of 50 mS/cm.

Start the Run

1. Place tubes in the first two columns of the BioFrac fraction collector rack (approximately 30). The drophead will automatically move to tube 1 when the run is started.

2. Ensure that the AVR7-3 valve is in the LOAD position (L). If it is not, return to the Manual screen by clicking the toolbar Manual button and clicking on valve position L.

3. Ensure that the 5 ml sample loop is connected to ports 3 and 6 of the inject valve.

4. Fill a clean 10 ml syringe using the needle provided with 5–6 ml of your soluble fraction. Be careful to avoid getting any air bubbles in the syringe.

5. Insert the syringe needle into the injection port (port 2) on the AVR7-3 valve and completely fill the loop with the Soluble Fraction of GST-DHFR-His. **Do not remove the syringe from the injection port after filling the loop or the sample will siphon to waste.**

6. To launch the run, click on the green Start toolbar button. 

7. When the run is finished, the pumps automatically stop and a “Run Finished” message appears in the bottom right of the status bar.

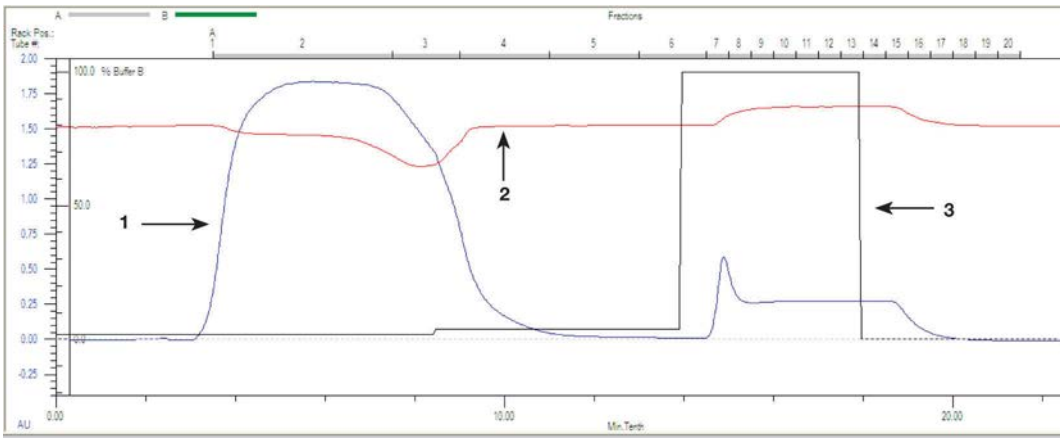


Figure 8.28. Typical trace of purification of GST-DHFR-His. The left axis has been set to a maximum value of 2.00 AU and the right axis is set to a maximum value of 50 mS/cm. Trace 1 is the UV trace. It increases after the sample has been loaded, and all proteins that do not bind (the flowthrough) flow past the column and through the detectors. The UV curve then decreases to baseline (close to 0.0) as the column is washed, and all non-binding proteins are washed off. The % Buffer B can be seen by looking at curve 3. Once % Buffer B goes to 100%, the UV curve increases again as the GST-DHFR-His elutes. The UV curve then decreases as all of the GST-DHFR-His washes off but does not drop to baseline due to the 250 mM imidazole in 100% Buffer B absorbing at 280 nm. Finally, when % Buffer B drops to zero, the UV curve drops back to baseline values. Curve 2 is the conductivity plot. The conductivity drops slightly when the flowthrough passes through the conductivity detector. Once all the non-binding protein passes, the conductivity goes back to its initial set point. Once % Buffer B increases to 100%, the conductivity increases slightly due to the presence of 250 mM imidazole and then drops back to its baseline when % Buffer B goes to 0%.

8. Figure 8.28 shows a typical chromatogram for this separation.

9. Label your fractions numerically (1, 2, 3, etc.) making sure to follow the serpentine path that the fraction collector used.

   Note: The BioFrac fraction collector automatically skips tube 1. Tube 2 will be the first one with liquid in it.

10. Cover your fractions with Parafilm and store at 4°C until you are ready to desalt and analyze them via SDS-PAGE and enzyme activity.

Cleaning the BioLogic DuoFlow System After Use

In order to keep any instrument in good running form, it is critical to perform certain tasks to make sure that the instrument is clean and ready for use by the next person. For the BioLogic DuoFlow system, it is important to remove all salt-containing solutions from the lines and all bacterial lysate components. The salts can precipitate out over time clogging fittings, tubing, and flow cells. Bacterial lysates can degrade resulting in growth of molds and mildews that also will contaminate and clog fittings, tubing, and flow cells.

1. Remove your column but leave the red adaptor fittings attached to the instrument fittings.

2. Place the luer lock closure column fittings on the inlet and outlet of the column to keep the column from drying out when stored.

3. Connect the column inlet and outlet tubing by connecting the two red fittings adaptors still connected to the tubing.

4. Place the buffer A and buffer B inlet lines into a beaker containing 200 ml of high-quality water.

5. In manual mode, make sure that the AVR7-3 valve is in the Load (L) position.

1. In manual mode, set the buffer composition to a mix of 50% Buffer A and 50% Buffer B.
2. Set the flow rate to 5 ml/min.
3. Make sure that the waste beaker is empty and that the waste tubing flows into the waste beaker.
4. In manual mode, press the Start button and allow the flow to continue for five minutes.
5. After five minutes, change the AVR7-3 valve to the Inject (I) position to allow flow through the sample loop.
6. Continue flow for another five minutes.
7. Stop the pump.
8. If requested by your instructor, repeat steps 3–11 using 20% ethanol in place of high-quality water.
9. Your instrument is now ready for storage or use by another student group.

Removing Imidazole (Desalting) from the Purified GST-DHFR-His and Preparing Samples for SDS-PAGE Analysis

Student Workstations

Each student team requires the following items to desalt three of their fractions in which they think their GST-DHFR-His samples reside and to prepare SDS-PAGE samples:

Material Needed for Each Workstation	Quantity
Chromatogram from GST-DHFR-His purification	1
Fractions from GST-DHFR-His purification	varies
Desalting column	3
Screwcap microcentrifuge tubes, 1.5 ml	6
Laemmli buffer (left over from previous activity)	1 ml
Microcentrifuge tubes, 2 ml	6–12
20–200 µl adjustable-volume micropipet and tips	1
Marking pen	1

Common Workstation	Quantity
Microcentrifuge with variable speed setting ≥16,000 x g	1
Dry bath or water bath set at 95°C	1

At this point, there might be several fractions that contain the eluted GST-DHFR-His protein. It is important to determine which fractions the purified GST-DHFR-His is present in and which fraction(s) have the highest concentration of GST-DHFR-His. It might be assumed that the fractions marked by the DuoFlow software on the chromatogram where the peak eluted are the specific fraction tubes where the protein can be found. However, it should be noted, that there is a time delay from when the absorbance of the fraction is measured on the UV detector to when it flows through the tubing past the conductivity meter and out into the fraction collector. This is called a delay time (or volume). For example, in Figure 8.29, according to the chromatogram, the protein should have eluted in fractions 7–8. Again, this would be assuming that there is no delay between when the protein is detected in the UV detector and when it drops into a fraction collector tube.

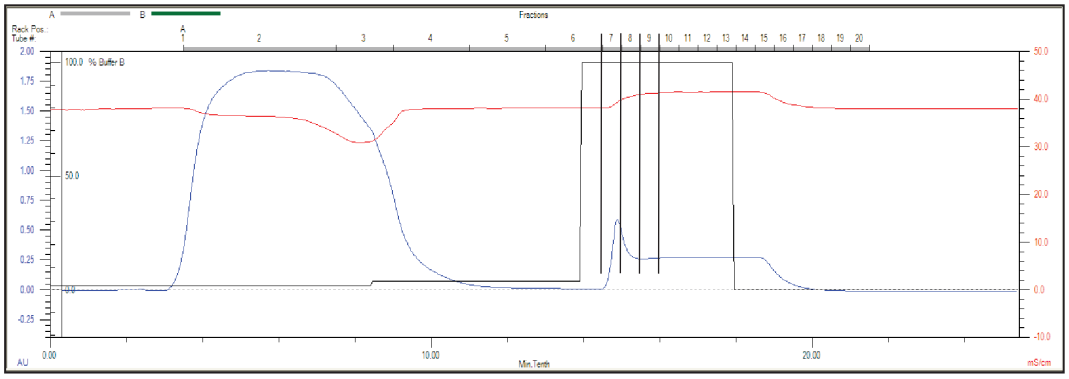


Figure 8.29. Chromatogram of a run. If there was no delay volume between the UV detector and fraction collector, the GST-DHFR-His would be assumed to be in fractions 7, 8, and 9. However, there is a finite delay time between detection and collecting that can be calculated or measured.

The delay time (volume) can be calculated by measuring the length of tubing present between the detector and the fraction collector as well as knowing the volume of all fittings in between the UV monitor and the fraction collector. It can also be measured experimentally ahead of time by attaching a syringe to the fitting

at the inlet of the UV detector when all the tubing is filled with fluid, disconnecting the tubing where it attaches to the fraction collector, pushing all this fluid out, and measuring the volume.

Using the calculation method, a loose estimate can be determined by calculating the delay volume of the tubing present.

The orange PEEK tubing has an inner diameter (ID) of 0.0508 cm.

The green PEEK tubing has an inner diameter (ID) of 0.0763 cm.

The volume of fluid in a specific length (L) of tubing would be ϖ x $(ID/2)^2$ x L.

Therefore, as an example, assuming that green PEEK tubing is in place and that there are 30 cm of tubing between the UV detector and the fraction collector, the delay volume would be at a minimum:

ϖ x $(0.0763\ cm/2)^2$ x 30 cm = 0.137 cm^3 or 0.137 ml

As can be seen from the calculation, this is not a large volume. However, there are much larger delay volumes as part of the UV flow cell, conductivity flow cell and fittings. Since 1 ml fractions are being collected for the eluate, it can be assumed that the majority of the GST-DHFR-His will elute in fractions 7 and 8 as shown in Figure 8.29. However, there might actually be some GST-DHFR-His in fraction 9 as well. **Therefore, it is important not to discard any fractions until all analyses are done!** In the case of the example shown above, the majority of GST-DHFR-His eluted in fractions 7 and 8 with some in 9.

Two other fractions will also be examined. The flowthrough fraction that contains all proteins that did not bind to the column when the column was initially loaded with the soluble fraction, and the wash fraction that contains any proteins that were washed off when the imidizole level was increased to 4% (or 10 mM imidazole). Again, these fractions can be determined by looking at the chromatogram from the BioLogic DuoFlow system run. For example, if delay volume is considered, the majority of the flowthrough fraction should be in fraction 2. The majority of the wash fractions would be in fractions 5–6 in the example chromatogram in Figure 8.29.

Protocol

Choosing your flowthrough fraction, wash fraction, and three eluted GST-DHFR-His fractions

Examine your chromatogram and determine the fractions that you feel are most likely to contain your flowthrough and wash, and the three fractions that might contain your eluted GST-DHFR-His. Remember that the delay volume is probably between 0.5–1.0 ml depending on the tubing length of your instrument. Record the fraction numbers you will examine below.

Flowthrough fraction : _____

Wash fraction: _____

First eluted GST-DHFR-His fraction: _____

Second eluted GST-DHFR-His fraction: _____

Third eluted GST-DHFR-His fraction: _____

Preparing SDS-PAGE samples of flowthrough, wash and eluted GST-DHFR-His fractions

1. In a clean 1.5 ml screwcap microcentrifuge tube, combine 50 µl of Laemmli sample buffer with 50 µl of your Flowthrough fraction and label the tube "**Flowthrough PAGE**" with your initials.

2. In a clean 1.5 ml screwcap microcentrifuge tube, combine 50 µl of Laemmli sample buffer with 50 µl of your Wash fraction and label the tube "**Wash PAGE**" with your initials.

3. In a clean 1.5 ml screwcap microcentrifuge tube, combine 50 µl of Laemmli sample buffer with 50 µl of your first eluted GST-DHFR-His fraction and label the tube "**Fraction A PAGE**" with your initials.

4. In a clean 1.5 ml screwcap microcentrifuge tube, combine 50 µl of Laemmli sample buffer with 50 µl of your second eluted GST-DHFR-His fraction and label the tube "**Fraction B PAGE**" with your initials.

5. In a clean 1.5 ml screwcap microcentrifuge tube, combine 50 µl of Laemmli sample buffer with 50 µl of your third eluted GST-DHFR-His fraction and label the tube "**Fraction C PAGE**" with your initials.

6. Heat the five SDS-PAGE samples at 95°C for five minutes.

7. Store the SDS-PAGE samples at —20°C until ready to analyze via SDS-PAGE.

Desalting your three eluted GST-DHFR-His fractions

1. Label three Micro Bio-Spin desalting columns A, B, and C. These fractions will correspond to the three fractions you chose to be the eluted GST-DHFR-His fractions from your chromatogram.

   Fraction A = First eluted GST-DHFR-His fraction
   Fraction B = Second eluted GST-DHFR-His fraction
   Fraction C = Third eluted GST-DHFR-His fraction

2. Invert three desalting columns (with green caps) sharply several times to resuspend the settled gel and remove any bubbles. The resin should settle into the column, and little to no resin should remain in the green cap.

3. Snap off the bottom tab of the column and place the column into a clean 2 ml microcentrifuge tube.

4. Remove the columns' top green caps. If the columns do not begin to flow, push the caps back on the columns and then remove them again to start the flow.

5. Allow the excess packing buffer to drain by gravity to the top of the gel bed (about two minutes), the place the three columns into three clean 2 ml microcentrifuge tubes.

6. Centrifuge the three columns for two minutes in a microcentrifuge at 1,000 x g to remove the remaining packing buffer (see Appendix C for more information on setting centrifuge speeds). Discard the buffer and the three microcentrifuge tubes. Keep the three columns for the following steps, being careful not to disturb the resin.

7. Label three clean 2 ml microcentrifuge tubes "**Desalted A**," "**Desalted B**" and "**Desalted C**" with your initials and place the columns into the appropriate 2 ml microcentrifuge tubes. Carefully apply 75 µl of each of your eluted fractions chosen in step 1 directly to the center of the column. Be careful not to touch the resin with the pipet tip.

8. After loading the samples, centrifuge the column for four minutes at 1,000 x g.

1. Carefully apply another 75 µl of each of your eluted fractions chosen in step 1 to the corresponding labeled column in the corresponding labeled 2 ml microcentrifuge tubes and centrifuge for four minutes at 1,000 x g to produce a final volume of 150 µl of desalted GST-DHFR-His for each elution fraction you chose.

2. Discard the columns.

Preparing SDS-PAGE samples of desalted GST-DHFR-His fractions

1. In a clean 1.5 ml screwcap microcentrifuge tube, combine 25 µl of Laemmli sample buffer with 25 µl of your Desalted GST-DHFR-His fraction A and label the tube "**Desalted A PAGE**" with your initials.

2. In a clean 1.5 ml screwcap microcentrifuge tube, combine 25 µl of Laemmli sample buffer with 25 µl of your Desalted GST-DHFR-His fraction B and label the tube "**Desalted B PAGE**" with your initials.

3. In a clean 1.5 ml screwcap microcentrifuge tube, combine 25 µl of Laemmli sample buffer with 25 µl of your Desalted GST-DHFR-His fraction C and label the tube "**Desalted C PAGE**" with your initials.

4. Heat the three SDS-PAGE samples at 95°C for five minutes.

5. Store the SDS-PAGE samples at –20°C until ready to analyze via SDS-PAGE.

6. Cover with Parafilm and store the **Desalted A**, **B** and **C** fractions at **4°C** until ready to analyze via spectroscopy, SDS-PAGE, and enzymatic activity assay. Do not freeze the desalted fractions.

Quantitation of Protein in Desalted Fractions

Student Workstations

Each student team requires the following items to quantitate the amount of purified, desalted eluate (GST-DHFR-His) they have via spectroscopy:

Material Needed for Each Workstation	Quantity
Desalted fractions (150 µl each)	3
20–200 µl adjustable-volume micropipet and tips	1
trUView disposable cuvettes (or UV compatible cuvettes)	3
Marking pen	1

Common Workstation	Quantity
UV spectrophotometer	1–2

Protocol: Quantitation of Protein in Desalted Fractions

1. Make sure that your cuvettes are completely clean and dry before use.

2. Set your spectrophotometer to read at 280 nm.
   Note: If using a Bio-Rad SmartSpec Plus spectrophotometer, see Appendix **D** for setup instructions.

3. Blank your spectrophotometer with 100 µl distilled water.

4. Pipet 100 µl of your desalted fractions A, B, and C each into its own clean cuvette.

5. Measure the absorbance at 280 nm of all of your desalted fractions.

   A_{280} Desalted fraction A _____

   A_{280} Desalted fraction B _____

   A_{280} Desalted fraction C _____

6. Pipet each desalted fraction back into the tube containing that fraction after measuring its absorbance.

7. Make sure that you have at least 15 µl of desalted GST-DHFR-His to run your enzyme assay.

8. Calculate the concentration of GST-DHFR-His in your desalted fractions as follows:

   A: The extinction coefficient (ε) of the entire GST-DHFR-His construct is theoretically calculated to be 75,540 M^{-1} cm^{-1}.

   Knowing that Absorbance = ε x C x L,

   where ε is 75,540 M^{-1} cm^{-1}

   L is the pathlength of the cuvette in cm (usually 1)
   and the absorbance at 280 nm is being measured

The concentration of GST-DHFR-His (M) = Absorbance/75,540

Concentration of GST-DHFR-His (Desalted fraction A) = _____ M

Concentration of GST-DHFR-His (Desalted fraction B) = _____ M

Concentration of GST-DHFR-His (Desalted fraction C) = _____ M

B: Convert from molarity to mg/ml for the amount of GST-DHFR-His in your purified desalted fractions knowing that the molecular mass of GST-DHFR-His is 50,361 g/mol.

Concentration GST-DHFR-His (mg/ml) = Concentration GST-DHFR-His (M) x 50,361 g/mol

Concentration of GST-DHFR-His (Desalted fraction A) = _____mg/ml

Concentration of GST-DHFR-His (Desalted fraction B) = _____mg/ml

Concentration of GST-DHFR-His (Desalted fraction C) = _____mg/ml

These are the concentrations of GST-DHFR-His that you produced and purified and are contained in your 150 µl desalted fractions.

SDS-PAGE Electrophoresis Analysis of Purified Fractions

Student Workstations

Each student team requires the following items to analyze their purification samples via SDS-PAGE:

Material Needed for Each Workstation	Quantity
Soluble PAGE sample	100 µl
Flowthrough PAGE sample	100 µl
Wash PAGE sample	100 µl
Fraction A PAGE sample	100 µl
Desalted A PAGE sample	50 µl
Fraction B PAGE sample	100 µl
Desalted B PAGE sample	50 µl
Fraction C PAGE sample	100 µl
Desalted C PAGE sample	50 µl
Precision Plus Protein Dual Color standards	15 µl
4–20% Mini-PROTEAN TGX precast gel	1
Vertical electrophoresis chamber (per 1–4 gels)	1
1x Tris/Glycine/SDS (TGS) running buffer (per electrophoresis chamber)	700–1,200 ml
Power supply (200 V constant) to be shared between workstations	1
Buffer dam (only required if running 1 gel/box)	1
2–20 µl adjustable-volume micropipet and tips	1
Staining tray	1
Bio-Safe Coomassie stain (per gel)	50 ml
Marking pen	1

Common Workstation	Quantity
Water bath or heat block set to 95°C	1
Microcentrifuge with variable speed setting ≥16,000 x g	1
Water for gel washing and destaining (tap water is fine)	1 L

Load, Run, Stain and Destain the Gel

1. **Prepare samples**: If samples have been stored at –20°C, reheat SDS-PAGE samples at 95°C for two minutes to redissolve any precipitated detergent and then centrifuge the samples for two minutes at 16,000 x g.

2. **Assemble gel boxes**: If using Bio-Rad Mini-PROTEAN Tetra gel boxes and TGX precast gels, see appendix **E** for more information on how to prepare gels and assemble gel boxes.

3. **Load the gel**: Using a fresh tip each time, load the following volumes of each sample into the appropriate well of your gel:

Well	Volume	Sample
1	10 µl	Precision Plus Protein Dual Color standard
2	10 µl	Soluble PAGE
3	10 µl	Flowthrough PAGE
4	20 µl	Wash PAGE
5	20 µl	Fraction A PAGE
6	20 µl	Desalted A PAGE
7	20 µl	Fraction B PAGE
8	20 µl	Desalted B PAGE
9	20 µl	Fraction C PAGE
10	20 µl	Desalted C PAGE

4. Run the gel at 200 V for 30 minutes. (If using a Bio-Rad Mini-PROTEAN TGX precast gel, the gel can be run at 300 V for 15 minutes.)

5. After the run is complete, remove the gel from the cassette and place in the gel staining tray.

6. Wash the gel three times with 50 ml of distilled water for five minutes each rinse. Discard all the rinse water.

   Note: Make sure that all of the wash water has been removed because excess water diluting the gel stain will interfere with staining efficiency.

7. Stain the gel with 50 ml of Bio-Safe Coomassie stain for one hour.

8. After one hour discard the Bio-Safe Coomassie stain and add 100 ml of distilled water and destain the gel overnight.

9. Image the gel if you have an imaging system or dry the gel if you have a cellophane drying system.

10. Pick the desalted GST-DHFR-His fraction that has the highest concentration (darkest band) but also looks the most pure on the SDS-PAGE gel to analyze enzymatic activity (Figure 8.30). The most pure sample would show an obvious dark band for GST-DHFR-His and few or faint bands at other molecular weights in the lane on the SDS-PAGE gel.

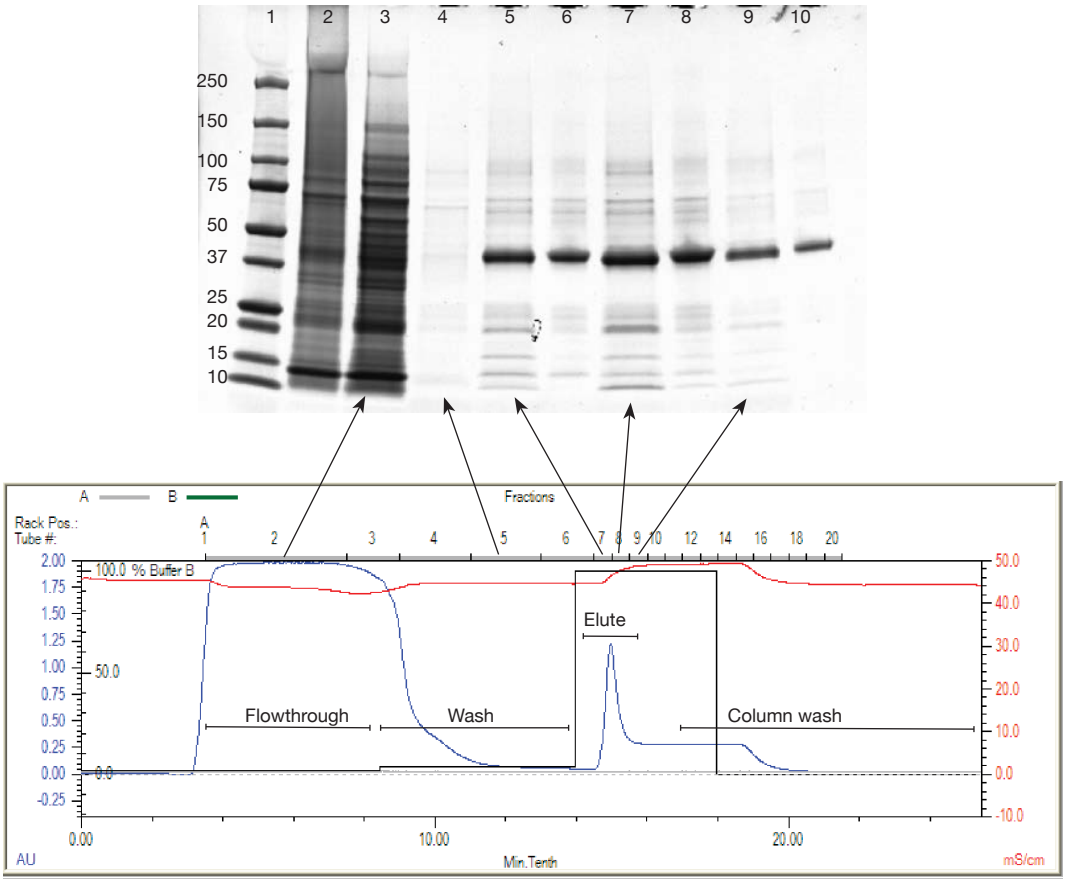


Figure 8.30. Example of fractions selected from the chromatogram and the corresponding SDS-PAGE results.

Lane 1. Precision Plus Dual Color Standards.
Lane 2. Soluble fraction of E. coli cell lysate. A band is present that is the GST-DHFR-His which is soluble. The large band at approximately 12 kDa is the lysozyme, which is also soluble, that was used to lyse the cells open.
Lane 3. Flowthrough fraction which did not bind to the Ni-IMAC resin. This is the fraction of proteins from the soluble fraction that did not bind to the Ni-IMAC resin. The soluble fraction was suspended in 20 mM sodium phosphate buffer, 300 mM NaCl and 5 mM imidazole. The high salt helped prevent E. coli proteins and the lysozyme from non-specifically sticking to the Ni-IMAC beads. The 5 mM imidazole helped prevent E. coli proteins with multiple histidine groups from binding to the Ni-IMAC beads. There is a decrease in the amount of GST-DHFR-His in the flowthrough fraction versus the soluble fraction (lane 2) and this is representative of the GST-DHFR-His binding to the Ni-IMAC resin.
Lane 4. Wash fraction. This fraction contains proteins that were washed off the Ni-IMAC beads when a wash buffer that has a slightly higher imidazole level (10 mM) was added to wash off more non-specifically bound proteins. No GST-DHFR-His should wash off in this fraction since 10 mM imidazole is not enough to compete with the 6 histidine tag of GST-DHFR-His bound to the Ni-IMAC resin.
Lanes 5, 7,and 9. Eluate fraction of GST-DHFR-His. These fractions contain the GST-DHFR-His. The elution buffer has 250 mM imidazole in it and this level of imidazole competes with the six histines of GST-DHFR-His and knocks them off the Ni sites and hence the GST-DHFR-His elutes or comes off the resin to be collected. These fractions are predominantly GST-DHFR-His protein relative to the unpurified soluble fraction in lane 2 that contains many other proteins.
Lane 6, 8, and 10. Desalted GST-DHFR-His. These fractions contain the purified GST-DHFR-His but have had the 250 mM imidazole removed. Also, since the desalting column removes smaller molecular weight compounds, some of the smaller molecular weight impurities found in lanes 5,7 and 9 are not present in the desalted fractions.

Purification Workflow for BioLogic DuoFlow System

Affinity Purification of GST-DHFR-His Protein

Follow the preceding protocols for your instrument and either handpacked columns or prepacked cartridge purificaton.

BioLogic DuoFlow System

Post-purification: Choosing your flowthrough, wash, and three eluted GST-DHFR-His fractions

Examine your chromatogram and determine the fractions that you feel are most likely to contain your flowthrough and wash, and three fractions which might contain your eluted GST-DHFR-His. Remember that you need to calculate the delay volume to help determine which fractions contain your samples. Record the five fraction numbers you will examine at right.

Flowthrough fraction: _____

Wash fraction: _____

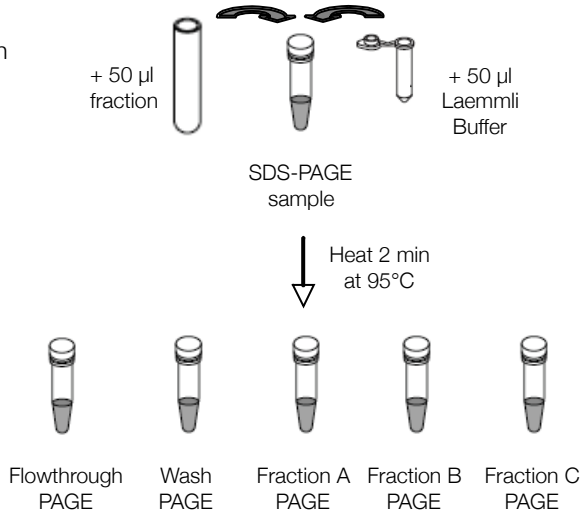
First eluted GST-DHFR-His fraction: _____

Second eluted GST-DHFR-His fraction: _____

Third eluted GST-DHFR-His fraction: _____

Preparing SDS-PAGE samples of flowthrough, wash and eluted GST-DHFR-His fractions

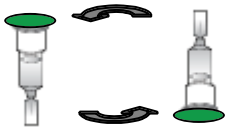
Label 1.5 ml screwcap microcentrifuge tubes according to the fraction as indicated on right. Add 50 µl of fraction to 50 µl Laemmli buffer into corresponding 2 ml screwcap tube. Mix well. Heat all samples at 95°C for five minutes.



Purification Workflow for BioLogic DuoFlow System

Desalting your three eluted GST-DHFR-His fractions

Label three Micro Bio-Spin desalting columns A, B, and C. These fractions correspond to the three fractions you chose from your chromatogram.



Prepare desalting columns by inverting sharply several times to resuspend gel.

Snap off bottom tabs and place each column into a 2 ml microcentrifuge tube. Remove green top cap. If the column does not begin to flow, push the cap back on the column and then remove it again to start the flow.

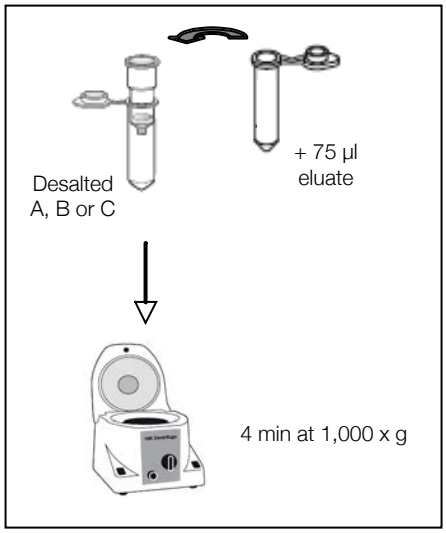
Allow excess packing buffer to drain by gravity to top of resin bed. After draining, place columns in clean 2 ml tubes.

2 min at 1,000 x g

Centrifuge for two minutes at 1,000 x g. Discard remaining packing buffer and collection tubes.

Label three clean 2 ml microcentrifuge tubes **Desalted A**, **Desalted B**, **Desalted C**. Carefully apply 75 µl of each of your eluted fractions directly to the center of the corresponding column. Be careful not to touch resin with pipet tip.

Centrifuge for four minutew at 1,000 x g. Carefully apply another 75 µl of each of your eluted fractions to the corresponding column and centrifuge again.



X 2

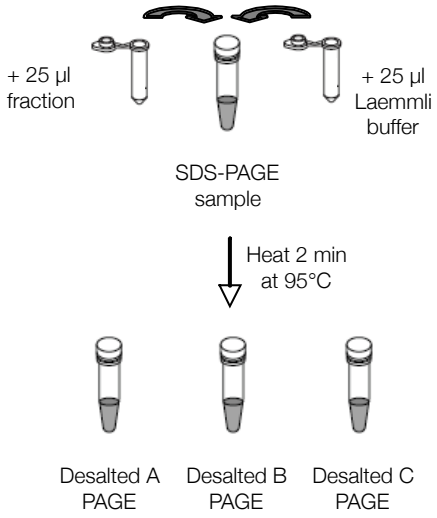
After second spin, discard columns. You will have ~150ul of desalted GST-DHFR-His for each elution fraction.

BIO-RAD

Purification Workflow for BioLogic DuoFlow System

Preparing SDS-PAGE Samples of Desalted GST-DHFR-His Fractions

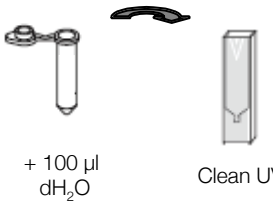
Prepare SDS-PAGE samples. Add 25 µl of each fraction to 25 µl Laemmli buffer in 2 ml screwcap tube. Mix well. Heat all samples at 95°C for five minutes.



Purification Workflow for BioLogic DuoFlow System

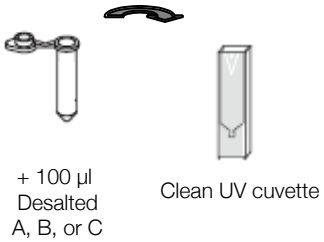
Quantitation of Protein in Desalted Fractions

Turn on spectrophotometer and set absorbance to 280 nm. Add 100 µl distilled water to clean UV compatible cuvette.



Blank spectrophotometer with distilled water.

Add 100 µl Desalted fractions A, B, and C each into its own clean UV compatible cuvette.



Measure absorbance of each fraction at 280nm. Return fraction to 2 ml tube.

Calculate concentration of GST-DHFR-His in each desalted fraction as described in instruction manual and record data.

Purification Workflow for BioLogic DuoFlow System

SDS-PAGE Analysis of Purified Fractions

Load, run, stain and destain the gel. Reheat SDS-PAGE samples at 95°C for two minutes. Centrifuge samples for two minutes at 16,000 x g.

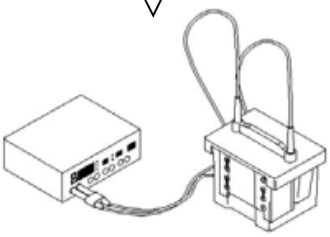
Heat 2 min at 95°C

Spin 2 min at 16,000 x g

Using a fresh tip each time, load the following volumes of each sample into the appropriate well of your gel

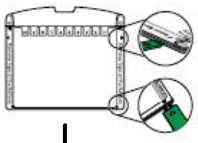
Lane	Volume	Sample
1	10 µl	Precision Plus Dual Color standard
2	10 µl	Soluble PAGE
3	10 µl	Flowthrough PAGE
4	20 µl	Wash PAGE
5	20 µl	Fraction A PAGE
6	20 µl	Desalted A PAGE
7	20 µl	Fraction B PAGE
8	20 µl	Desalted B PAGE
9	20 µl	Fraction C PAGE
10	20 µl	Desalted C PAGE

Run the gel at 200 V for 30 minutes. (If using Mini-PROTEAN TGX precast gel, the gel can be run at 300 V for 15 minutes.)



After the run is complete, remove the gel from the cassette and place in the gel staining tray.

Wash the gel three times with 50 ml of distilled water for five minutes each rinse. Discard all the rinse water.



Stain the gel with 50 ml of Bio-Safe Coomassie stain for one hour.

After one hour, discard the Bio-Safe Coomassie stain, add 100 ml of distilled water, and destain the gel overnight.

Image the gel if you have an imaging system or dry the gel if you have a cellophane drying system.

Chapter 9 : DHFR Enzymatic Activity Assay Student Protocol

Student Workstations

Each student team requires the following items to analyze enzymatic activity of their purified, desalted GST-DHFR-His protein sample:

Material Needed for Each Workstation	Quantity
Gel of purified fractions	1
Purified, desalted eluate (GST-DHFR-His) protein sample*	15 µl
2–20 µl adjustable-volume micropipets and tips	1
100–1,000 µl adjustable-volume micropipets and tips	1
1x PBS	1 ml
Marking pen	1
NADPH cofactor	8 µl
DHF substrate	10 µl
trUView disposable cuvettes (or UV compatible cuvettes)	1
Parafilm	1 square

*If you used a Biologic LP system or Biologic DuoFlow system for purification protocols, you will have three desalted GST-DHFR-His fractions. You can perform the following enzyme reactions either on one fraction with the highest concentration or highest purity (as determined by your SDS-PAGE gel) of GST-DHFR-His, or you can perform the following protocols on all three desalted fractions. Consult your teacher on which protocol you will be using.

Common Workstation	Quantity
UV spectrophotometer capable of three decimal place accuracy	1–2

Dihydrofolate Reductase (DHFR) catalyzes the reversible NADPH-dependent reduction of dihydrofolic acid to tetrahydrofolic acid. The progress of the reaction is monitored spectroscopically by following the decrease in NADPH absorbance at 340 nm.

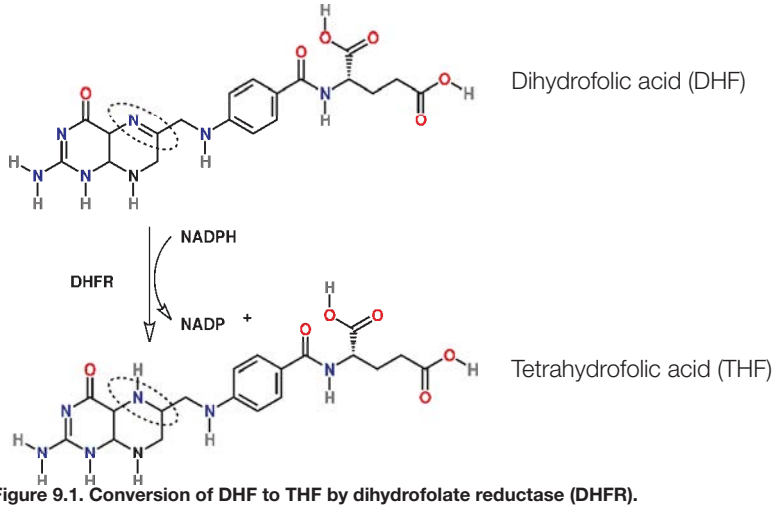


Figure 9.1. Conversion of DHF to THF by dihydrofolate reductase (DHFR).

1. Determining which fraction to test for enzyme activity

In the previous experiment, you determined the protein concentration of your purified GST-DHFR-His fraction(s). Record those concentrations below.

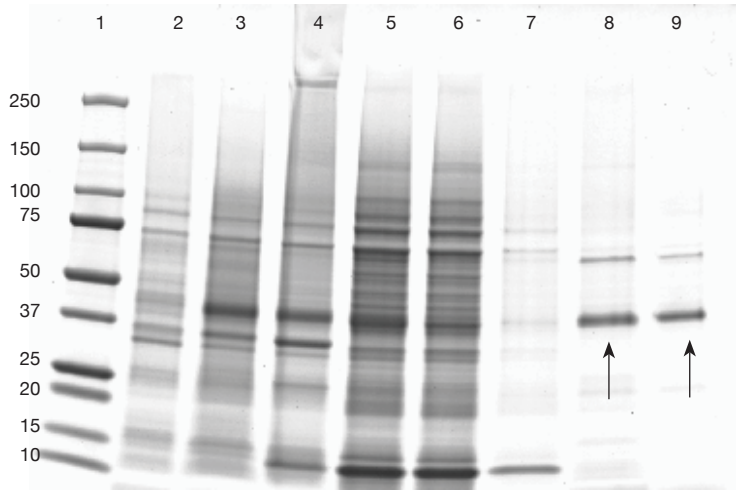
Note: Centrifugation purification will have one desalted fraction while instrumentation-based purification will have three fractions.

Concentration (mg/ml) of GST-DHFR-His (Desalted fraction A): _____

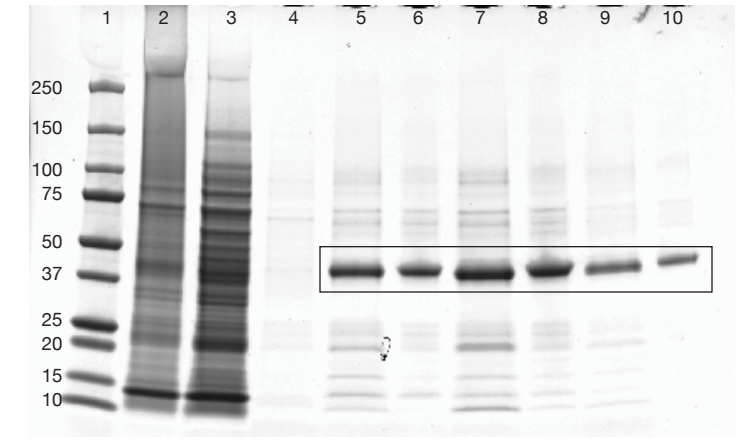
Concentration (mg/ml) of GST-DHFR-His (Desalted fraction B): _____

Concentration (mg/ml) of GST-DHFR-His (Desalted fraction C): _____

Now look at your SDS-PAGE gel from your purification. Sample gels from purification using the centrifugation protocol and instrumentation-based purification are shown below in Figure 9.2.



(A) Centrifugation purification experiment results.
Lane 1 - Precision Plus Protein Dual Color standard
Lane 2 - Uninduced PAGE
Lane 3 - Induced PAGE
Lane 4 - Insoluble PAGE
Lane 5 - Soluble PAGE
Lane 6 - Flowthrough PAGE
Lane 7 - Wash PAGE
Lane 8 - Eluate PAGE
Lane 9 - Desalted eluate PAGE



(B) Chromatography instrument purification experiment results.
Lane 1 - Precision Plus Protein Dual Color standard
Lane 2 - Soluble PAGE
Lane 3 - Flowthrough PAGE
Lane 4 - Wash PAGE
Lane 5 - Fraction A PAGE
Lane 6 - Desalted A PAGE
Lane 7 - Fraction B PAGE
Lane 8 - Desalted B PAGE
Lane 9 - Fraction C PAGE
Lane 10 - Desalted C PAGE

Figure 9.2. Results of purification for GST-DHFR-His from A) centrifugation purification experiment and B) chromatography instrumentation purification experiment.

Centrifugation purification only: choosing a fraction to measure the enzymatic activity

Ideally, you will see a strong band of purified GST-DHFR-His (lane 8) and purified, desalted GST-DHFR-His (lane 9) on your SDS-PAGE gel similar to Figure 9.2A and have a concentration of GST-DHFR-His greater than 0.1 mg/ml.

If you do not see a band in lane 9 (desalted GST-DHFR-His) or your measured concentration of desalted GST-DHFR-His is lower than 0.1 mg/ml, it is possible to perform the enzyme assay on a less pure fraction that shows a dark band on your gel; however, you will not be able to determine any relationship between the concentration of the GST-DHFR-His in that fraction and the enzymatic activity.

1. Look at your gel and concentration of GST-DHFR-His from your data. If you have a strong band in lane 9, then choose your desalted, GST-DHFR-His fraction for testing using the enzyme assay.

2. If you do not see any band or it is very faint in lane 9 of your gel but you do see a strong band in lane 8, then choose your eluted GST-DHFR-His fraction for testing using the enzyme assay.

3. If you do not see any bands in your purified fractions (lane 8 or 9), then speak with your instructor to see if another student group has a strong band so that you can test its sample.

Chromatography instrumentation purification only: choosing a fraction to measure the enzymatic activity

Ideally, you will see at least one strong band of purified GST-DHFR-His in one or more of lanes 5, 7, and 9 and at least one strong band of purified, desalted GST-DHFR-His in one or more of lanes 6, 8 and 10 on your SDS-PAGE gel similar to Figure 9.2B. You should have a concentration of GST-DHFR-His greater than 0.1 mg/ml for one or more of the desalted GST-DHFR-His fractions A, B, or C.

If you do not see any bands in lanes 5, 7, or 9 but do see a peak elute on your chromatogram, it is possible that you chose the incorrect fractions to desalt. More fractions can be desalted and the concentration can be calculated based on the absorbance at 280 nm at the discretion of your instructor.

If you do not see a band in lanes 6, 8, or 10 (desalted GST-DHFR-His), or your measured concentration of desalted GST-DHFR-His is lower than 0.1 mg/ml, it is possible to perform the enzyme assay on a less pure fraction that shows a dark band on your gel. You just will not be able to determine any relationship between the concentration of the GST-DHFR-His in that fraction and the enzymatic activity.

1. Look at your gel and concentration of GST-DHFR-His from your data for your three fractions. If you have a strong band in lane 6, 8 and 10, then choose a desalted GST-DHFR-His fraction that has a strong band but that also looks the most pure (no extra bands).

2. If you do not see any band or it is very faint in lanes 6, 8, and 10 of your gel but you do see a strong band in lane 5, 7 or 9, then choose either Fraction A, B, or C for testing using the enzyme assay.

3. If you do not see any bands in your purified fractions (lanes 5-10), then speak with your instructor and see if another student group has a strong band and you can test its sample.

2. Setting up the spectrophotometer to read at 340 nm in kinetics mode

a. If you are using a Bio-Rad SmartSpec Plus spectrophotometer, please see Appendix D for instructions on how to set up and use your spectrophotometer in kinetics mode.

b. If you are using a different spectrophotometer, consult your instructor to determine if there is a kinetics mode and how to program your instrument to read at 340 nm every 15 seconds for 150 seconds.

3. Blanking the instrument at 340 nm using 1x PBS

a. If you have a spectrophotometer that can be programmed to take multiple readings at a specified time interval (kinetics mode), program the instrument to take readings at 340 nm every 15 seconds for 150 seconds.

b. If you do not have a kinetics mode on your spectrophotometer, set the instrument to read at 340 nm and have a stopwatch handy to measure time intervals.

c. Fill a UV compatible cuvette with 985 µl of 1x PBS.

d. Insert the cuvette into your spectrophotometer and press the key that will blank the instrument at 340 nm so that 1x PBS gives a reading of 0.000 at 340 nm.

e. Take your cuvette out of the spectrophotometer and save it and the 985 µl of 1x PBS for step 4.

4. Running the no substrate control reaction

You will now add your enzyme sample of GST-DHFR-His with DHFR's cofactor, NADPH, to the 1x PBS. Since there is no substrate (DHF) being added to this control reaction, the absorbance at 340 nm contributed by the NADPH should not change since it is not being converted to NADP+.

a. Add 6 µl of 10 mM NADPH to the cuvette already containing 985 µl of 1x PBS.

b. Add 15 µl of your chosen GST-DHFR-His sample to the cuvette.

c. Cover the cuvette with parafilm and invert 10 times to fully mix the GST-DHFR-His and NADPH into the 1x PBS.

d. Place the cuvette into your spectrophotometer and measure the absorbance at 340 nm every 15 seconds for 150 seconds (either manually while timing with a stopwatch or using the kinetics mode if your instrument has one).

Note: If you do not have a printer on your spectrophotometer, record the absorbance values you measure every 15 seconds in your notebook.

e. If you have a printer on your spectrophotometer, after the 150 seconds of readings have been completed, print out your data and label it "No Substrate Control Reaction."

f. Remove your cuvette from the spectrophotometer and save it with all of its contents to be used for running the enzymatic reaction.

5. Running the enzymatic reaction with GST-DHFR-His, NADPH (cofactor) and DHF (substrate)

Note: The enzyme reaction should be prepared while standing at the spectrophotometer. The reaction occurs extremely quickly (within seconds) and it is necessary to mix very quickly, place the cuvette in the spectrophotometer, and begin taking readings as quickly as possible after you have added the DHF substrate.

a. Make sure that your spectrophotometer is still programmed to take readings at 340 nm (in kinetics mode if available).

b. Add 5 µl of 10 mM DHF to the cuvette that already contains 1x PBS, your GST-DHFR-His sample and NADPH.

c. Cover the cuvette with parafilm and invert quickly five times.

d. Place the cuvette in the spectrophotometer and measure the absorbance at 340 nm every 15 seconds for 150 seconds (either manually while timing with a stopwatch or using the kinetics mode if your instrument has one).

Note: If you do not have a printer on your spectrophotometer, record the absorbance values you measure every 15 seconds in your notebook.

e. If you have a printer on your spectrophotometer, after the 150 seconds of readings have been completed, print out your data and label it "GST-DHFR-His Enzyme Reaction."

6. Activity calculation

As the NADPH cofactor is oxidized to $NADP^+$, its absorbance at 340 nm decreases. Therefore, by measuring the decrease in absorbance of a blank (NADPH, GST-DHFR-His, but no DHF substrate) and also the decrease in absorbance of the enzyme reaction (NADPH, GST-DHFR-His, and DHF), the conversion of NADPH to $NADP^+$ that can be attributed to the enzymatic activity of DHFR can be calculated.

a. Look at your data from the "No Substrate Control Reaction." Plot the data with the x-axis as Time in seconds and the y-axis as Absorbance at 340 nm. Example data is pictured below in Figure 9.3.

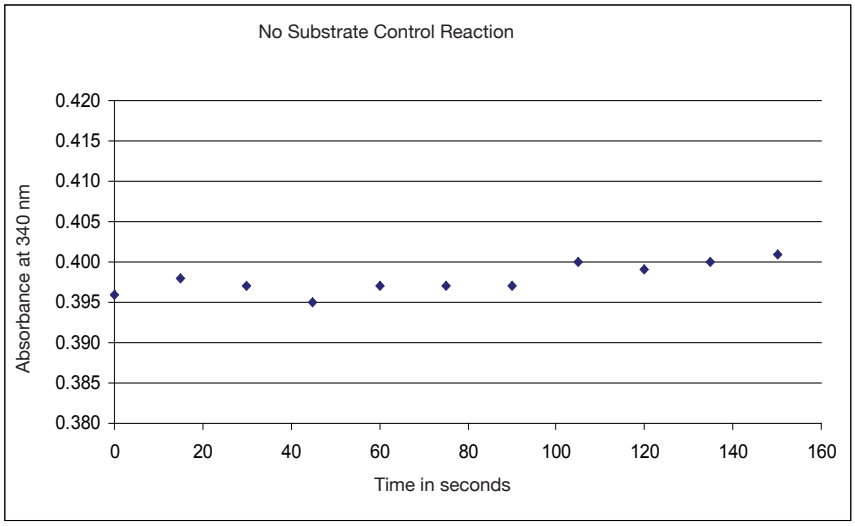


Figure 9.3. No substrate control reaction. When no substrate is present, the absorbance of the NADPH at 340 nm should remain fairly constant since it is not being converted to $NADP^+$. Some small fluctuations in the absorbance can be due to accuracy of the instrument in reading out to the final decimal place, incomplete mixing, or slight degradation of the NADPH in solution. From a regression analysis of the above data, the change in absorbance at 340 nm/minute is 3.33×10^{-5}/second or 0.002/min.

b. Calculate the slope of the line that best fits the No Substrate Control Data. This may be done by drawing a line that best fits all of the data points and determining the change in absorbance at 340 nm over the 150 seconds, or by using a regression computer program or calculator function and determining the slope of the line generated by the data. Record the slope of the No Substrate Control Data below.

Slope of Control Data: _____Change in Absorbance at 340 nm/second

c. Convert this slope from change in absorbance at 340 nm/second to change in absorbance at 340 nm/minute by multiplying by 60.

Slope of Control Data x 60 = _____ Change in Absorbance at 340 nm/minute

This will now be referred to as **ΔOD, control**.

d. Look at your data from the "GST-DHFR-His Enzyme Reaction." Plot the data with the x-axis as Time in seconds and the y-axis as Absorbance at 340 nm. Example data is pictured in Figure 9.4.

Note: Ideally, the enzyme reaction data should be linear over the entire range of 150 seconds. As the reaction is set up, it is limited by the amount of DHF. Therefore, if there is a lot more enzyme than substrate, the substrate will be used up before the 150 seconds is over, and the absorbance will not change appreciably for the remainder of the time course (Figure 9.5). If this is the case, calculate the rate only for data before it flatlines (at 95 seconds in figure 9.5 below). If there are enough reagents available, you can also try diluting your GST-DHFR-His sample in 1x PBS and rerunning the entire enzyme reaction.

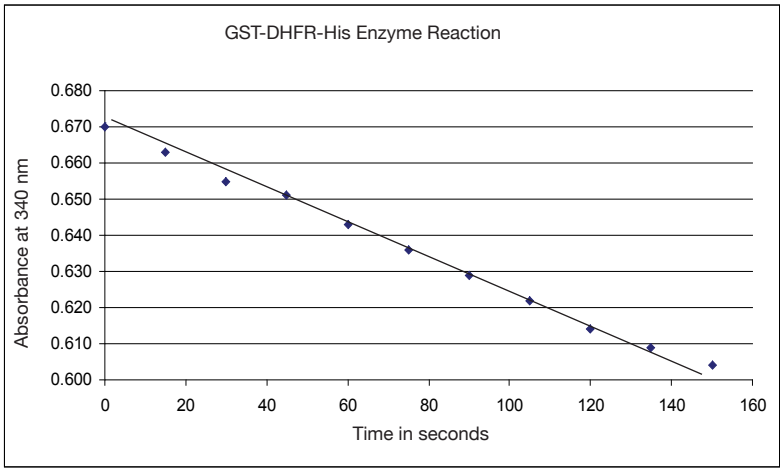


Figure 9.4. GST-DHFR-His enzyme reaction. When substrate, enzyme and cofactor are present, the absorbance of the NADPH at 340 nm should decrease if the enzyme is active since the NADPH is being converted to NADP+. From a regression analysis of the above data, the change in absorbance at 340 nm/minute is -4.5x10^{-4}/second or -0.027/min.

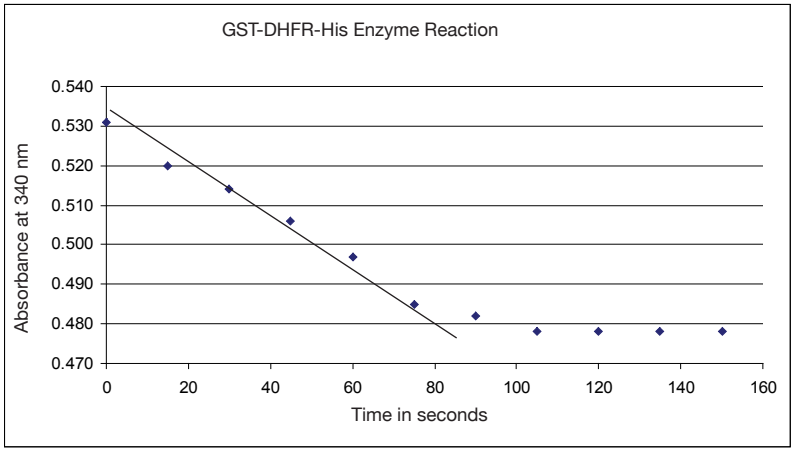


Figure 9.5. A reaction with too much GST-DHFR-His enzyme. This reaction flatlines, or runs out of DHF, by 95 seconds. After that, the absorbance does not change. The timeframe after 95 seconds is analogous to the No DHF Substrate Control reaction since in that reaction, no DHF was added; while in the above reaction, the GST-DHFR-His converted all the DHF present to THF so there was no more need to convert the NADPH to NADP$^+$ and the absorbance values level off. To calculate the rate of the enzyme reaction for this data, the slope of only the data from 0-75 seconds should be used or the GST-DHFR-His should be diluted and the reaction run again if there are enough reagents available.

e. Calculate the slope of the line that best fits the GST-DHFR-His Enzyme Reaction data. This may be done by drawing a line that best fits all of the data points being used and determining the change in absorbance at 340 nm over this timeframe or by using a regression computer program or calculator function and determining the slope of the line generated by the data. Record the slope of the Enzyme reaction below.

Slope of Enzyme reaction data: _____Change in Absorbance at 340 nm/second

f. Convert this slope from change in absorbance at 340 nm/second to change in absorbance at 340 nm/minute by multiplying by 60.

Slope of Enzyme reaction data x 60 = _____ Change in Absorbance at 340 nm/minute

This will now be referred to as **ΔOD, reaction**.

g. Determine the decrease in absorbance at 340 nm due to enzyme reaction. The amount of $NADP^+$ produced due to the DHFR in the GST-DHFR-His converting the NADPH to $NADP^+$ so that DHF can be converted to THF can be calculated by subtracting the absolute value of the change in absorbance with no substrate present to the absolute value of the change in absorbance with substrate present.

Change in absorbance at 340 nm due to enzyme reaction = ΔOD

OD = | OD, reaction| - |ΔOD, control|

Example: From the data shown in the graphs above,
ΔOD, control = 0.002/min so |ΔOD, control| also = 0.002/min
ΔOD, reaction = -0.027/min so |ΔOD, reaction| = 0.027/min

Therefore ΔOD = |ΔOD, reaction| - |ΔOD, control| = 0.027/min – 0.002/min = 0.025/min.

Calculate the ΔOD and record it below:

ΔOD (min^{-1}) = _____

h. Calculate the activity of the purified GST-DHFR-His. The activity of the GST-DHFR-His will be calculated in terms of how many µmol/min of NADPH it can convert to $NADP^+$ per ml of reaction volume. This can be determined from a form of Beer's Law that relates absorbance values to concentration values. In this case, the change in absorbance can be related to the change in concentration of NADPH.

Beer's Law Absorbance (A) = ε x C x l, or for this case
ΔOD = ε x (change in C) x l

where ΔOD = |ΔOD, reaction| - |ΔOD, control|, which you calculated above
Change in C = ΔC = The change in concentration of NADPH over the reaction time course
ε (extinction coefficient) = 6220 M^{-1} cm^{-1} for NADPH
l (length) is the pathlength of the cuvette (usually 1 cm for most cuvettes)

$$\text{Solving for } \Delta C \text{ (mol/liter/min)} = \frac{\Delta OD}{\varepsilon \times l}$$

Example: For the data from the graphs and calculations above:

$$\text{Solving for } \Delta C \text{ (mol/liter/min)} = \frac{0.025/mi}{6220\ M^{-1}\ cm^{-1} \times 1\ cm} = 4 \times 10^{-6} \text{ mol/liter/min}$$

Calculate how many moles of NADPH your GST-DHFR-His sample can break down per minute and record below.

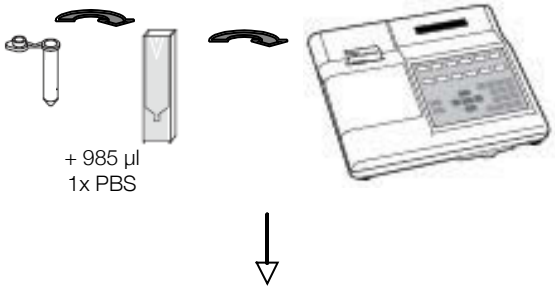
$$\Delta C \text{ (mol/liter/min)} = \frac{\Delta OD\ (min^{-1})}{6220\ M^{-1}\ cm^{-1} \times 1\ cm} = \_\_\_\_\_ \text{(mol/liter/min)}$$

DHFR Enzymatic Activity Assay

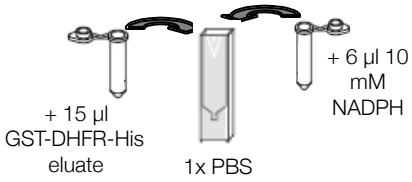
Determining which fraction to test for enzyme activity.
Review your SDS-PAGE gel from your purification to determine which fraction(s) to test. Note that centrifugation purification will have one desalted fraction while instrumentation-based purification will have three fractions.

Set up spectrophotometer for kinetics measurements at 340 nm.

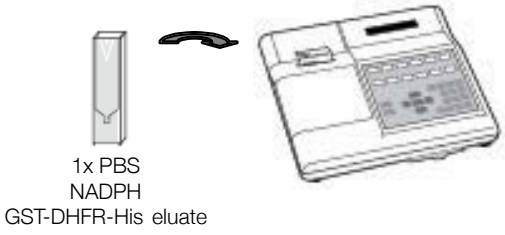
Blanking the instrument. Add 985 µl 1x PBS to cuvette; place in instrument, read as blank. Save cuvette with PBS.



Running the no substrate control reaction.
Add 6 µl of 10 mM NADPH to cuvette containing 985 µl 1x PBS. Add 15 µl of purified, GST-DHFR-His eluate to cuvette. Cover cuvette with parafilm and invert 10 times to mix

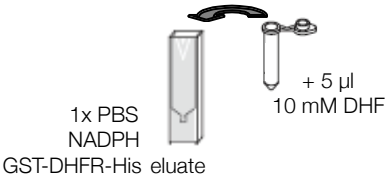


Place cuvette in spectrophotometer and begin kinetics run. As run is proceeding, record absorbance value every 15 seconds for 150 seconds. Remove and save cuvette from the spectrophotometer.

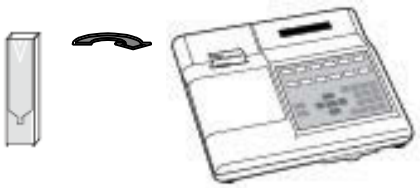


Running the enzymatic reaction with the GST-DHFR-His, NADPH (cofactor) and DHF (substrate).
Note: The enzyme reaction should be prepared while standing at the spectrophotometer. The reaction occurs extremely quickly and it is necessary to place the cuvette in the spectrophotometer and start the readings as quickly as possible once the DHF has been added.

Add 5 µl of 10 mM DHF to the cuvette already containing 1x PBS, your GST-DHFR-His sample and NADPH. Quickly cover the cuvette with parafilm and invert five times.



Place the cuvette in the spectrophotometer and begin kinetics run. As run is proceeding, record absorbance value every 15 seconds for 150 seconds. Remove cuvette from the spectrophotometer.



Calculate the activity following the instructions in the manual.

Appendix A: Tips and Helpful Hints

How to get the best protein expression and purification results

1. Culture, subculture, and induction

The time frames listed in the instruction manual are designed to have as much flexibility as possible to allow for classroom time periods. However, when possible, the following guidelines will help you achieve the best results for cell growth and protein expression.

- Initial overnight cultures should be between 14-20 hours rather than 24 hours.
- Induction levels are the highest when induction occurs for four hours.

The figure below shows a comparison of cells induced for four hours versus 24 hours, demonstrating the clearer induction of the cells induced for four hours.

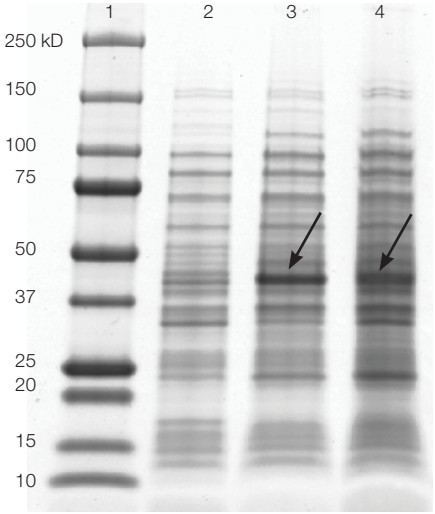


Figure 1. Gel comparing four-hour induction to 24-hour induction. The four-hour induction lane has a much more defined band of GST-DHFR-His (lane 3, arrow) relative to all of the background *E. coli* proteins when compared to 24 hours of induction (lane 4, arrow).

Lane	Sample
1	Precision Plus Protein Dual Color standards
2	Uniduced cells
3	Cells induced 4 hours
4	Cells induced 24 hours

2. Stability of induced protein

While the BL21(DE3) *E. coli* are deficient in some proteases, extra protease inhibitors are not being added to the cell lysate due to chemical safety.

- After induction of the BL21(DE3) cell culture, the best stopping point for protein stability is after pelleting the cells and removing the supernatant to form what is called a cell paste.
- This cell paste is highly stable when stored at –20°C or –70°C.

3. Lysis of cells

The most efficient lysis of cells requires the cells to be completely resuspended followed by complete freezing and thawing of the resuspended cells.

- Completely resuspend the cell paste in the lysis buffer containing lysozyme. This may require vortexing and vigorous pipeting.
- An ethanol/ice bath is the preferred method of freezing.
- Thaw frozen lysed cells completely before beginning each subsequent freeze-thaw cycle.
- If an ethanol/dry ice bath cannot be used, the freeze-thaw can be accomplished in a –70°C freezer overnight or by two cycles of overnight freezing followed by thawing using a –20°C freezer. A –20°C

freezer is the least preferred method of lysing cells. When using the –20°C freezer method, it is imperative that the cell pellets are completely frozen to achieve lysis of the cells. If the resuspended cell paste is not completely frozen, adequate lysis will not occur.

4. Separation of soluble and insoluble fractions

A centrifuge capable of exerting 16,000 x g of force is necessary to separate the soluble and insoluble fractions. Since no DNase is used in this protocol, the insoluble fraction and genomic DNA tend to form a viscous blob rather than a defined pellet. Care is needed to not transfer the genomic DNA into the soluble fraction when separating the soluble and insoluble fractions.

- Spin the lysate at 16,000 x g for 20 minutes. For a Bio-Rad Model 16K microcentrifuge, this is comparable to 14,000 rpm. For different instruments, please refer to the instrument's instruction manual and refer to Appendix **C** for help in determining the appropriate speed to use.
- Extreme care is needed to not decant the insoluble fraction. If it is decanted into the soluble fraction, carefully try to separate the fractions again. If this is not possible the lysate can be centrifuged at 16,000 x g for another 20 minutes to re-separate the fractions.
- If genomic DNA carries over to the soluble fraction and is too viscous, the DNA can be sheared with a 22 gauge needle to break it up for easier handling in subsequent steps.

The figure below shows the insoluble and soluble fractions and the relative amounts of GST-DHFR-His found in each fraction.

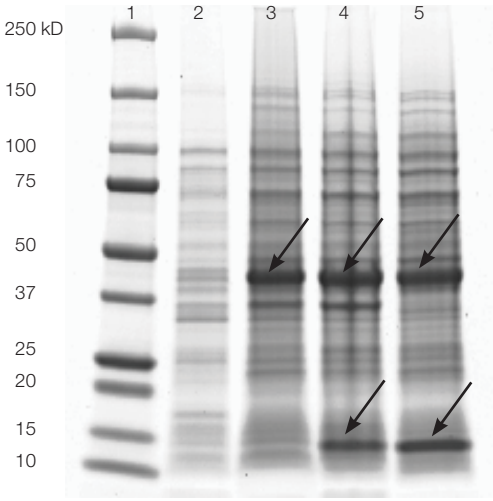


Figure 2. Gel analysis of recombinant GST-DHFR-His expression and location of GST-DHFR-His after lysis. Lane 3 shows a strong band of induced GST-DHFR-His expression (at arrow). Lanes 4 and 5 show comparable levels of GST-DHFR-His in the soluble and insoluble fractions. Lanes 4 and 5 also have a prominent lower band near 14 kDa. This is the band for lysozyme (which is a good way of verifying that students added lysozyme to their samples).

Lane	Sample
1	Precision Plus Protein Dual Color standards
2	Uniduced cells
3	Cells induced 4 hours
4	Insoluble fraction
5	Soluble fraction

5. Affinity purification—Centrifugation methodology

A variable speed microcentrifuge is required for the affinity purification. It is critical to set the variable speed microcentrifuge so that it generates 1,000 x g of force. During the affinity purification, it is important to perform each step in the correct order.

- For the Bio-Rad Model 16K microcentrifuge, 1,000 x g is equivalent to a speed of 3,500 rpm. A Bio-Rad mini-centrifuge should NOT be used since these produce 2,000 x g of centrifugal force which is too much for the Ni-IMAC resin to withstand.
- Make sure to mix the slurry of Ni-IMAC resin fully to resuspend the chromatography beads.
- It is extremely important to make sure that once the column is capped on both ends, that the resin and soluble fraction are thoroughly mixed before putting the column on a tube roller or rocking platform.

6. Affinity purification—Instrumentation protocols

It is highly recommended that the starter kit (BioLogic LP Starter Kit catalog #731-8350EDU or BioLogic DuoFlow Starter Kit catalog #760-0135EDU) be run prior to performing the Protein Expression and Purification Series with your students. This will ensure the equipment is set up with the proper plumbing and electrical connections.

7. Size exclusion purification—Desalting columns

A variable speed microcentrifuge is required for the desalting purification step. It is critical to set the variable speed microcentrifuge so that it generates 1,000 x g of force.

- For the Bio-Rad Model 16K microcentrifuge, 1,000 x g is equivalent to a speed of 3,500 rpm. A Bio-Rad mini-centrifuge should NOT be used since these produce 2,000 x g of centrifugal force which is too much for the Bio-Gel® P–6 desalting resin to withstand.

8. DHFR enzymatic assay

The DHFR enzymatic reaction occurs very quickly. Therefore, it is important to be familiar with the steps involved and to perform them quickly. A very minor change in the absorbance occurs at 340 nm with the conversion of NADPH to NADP+. This change is on the order of 0.05–0.08 OD in 150 seconds.

Please note:

- The DHF needs to be reconstituted in 10X PBS.
- The NADPH needs to be reconstituted in 1X PBS.
- The DHF and NADPH are NOT stable once reconstituted. They should be stored on ice and are only stable for three to four hours. The DHF and NADPH solutions cannot be frozen to retain or extend the activity and shelf life. The DHFR Enzymatic Assay Module can be purchased separately if more reactions are desired or if classes performing the assay are more than three to four hours apart.
- Time is of utmost importance once the DHF is added to the 1x PBS, the GST-DHFR-His, and NADPH solution. Therefore, prior to the addition of the DHF to the cuvette containing the 1x PBS, the GST-DHRF-His sample and the NADPH, the spectrophotometer should be set up and ready to read. The DHF should be added while standing next to the instrument, mixed fast, placed in the instrument, and read immediately to ensure that an accurate measurement of the enzyme activity is captured.
- If using trUView cuvettes please note that the frosted side contains the small clear window through which the UV reading is taken.

Appendix B: Results Analysis

Centrifugation Purification Results Analysis

The gel image below shows how typical results using the Centrifugation Purification Process should appear. Listed below are common steps that can help you analyze your results.

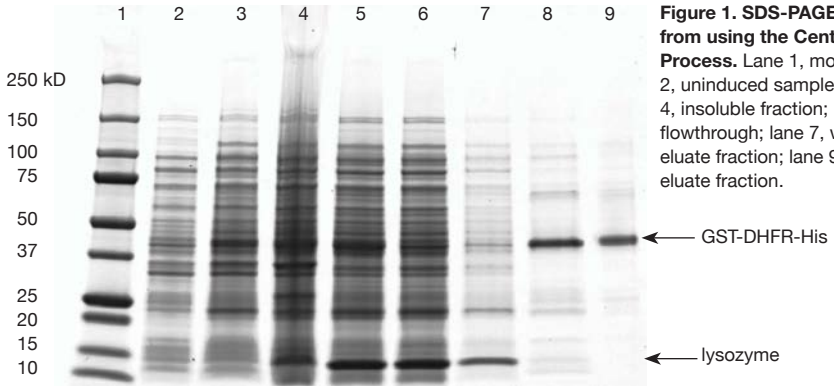


Figure 1. SDS-PAGE gel showing typical results from using the Centrifugation Purification Process. Lane 1, molecular weight markers; lane 2, uninduced sample; lane 3, induced sample; lane 4, insoluble fraction; lane 5, soluble fraction; lane 6, flowthrough; lane 7, wash; lane 8, GST-DHFR-His eluate fraction; lane 9, desalted GST-DHFR-His eluate fraction.

Step 1: Check that induction occurred.

The GST-DHFR-His protein runs on SDS-PAGE gels at an apparent MW of approximately 43 kD. (**Note:** the protein runs at this weight despite the actual protein molecular weight of 52 kD.)

Results: An obvious band should appear in lane 3, the induced sample, between the 37 kD and 50 kD molecular weight marker if the GST-DHFR-His has been strongly induced. This band is not present in lane 2, the uninduced sample.

Step 2: Check that lysis worked.

Lanes 4 and 5 represent the insoluble and soluble fractions, respectively.

Results: If the GST-DHFR-His was strongly induced and the cells lysed, a band of approximately 43 kD should be present in lane 5. It should be the same size as the band that occurred in lane 3 (falls between the 37kD and 50 kD molecular weight markers). Please note that the large band that is apparent at approximately 12 kD in lanes 4, 5, 6 and 7 is the lysozyme used to lyse the cells and not GST-DHFR-His. GST-DHFR-His is expressed in both an insoluble and soluble form. It is likely that there will be strong bands in both lanes 4 and 5 around 43 kD, representing both insoluble and soluble GST-DHFR-His respectively. As long as there is a strong band in lane 5, lysis has occurred, and soluble GST-DHFR-His has been released.

Step 3: Check that the binding of the GST-DHFR-His occurred.

Lane 6 is the flowthrough of proteins that did not bind to the Ni-IMAC resin.

Results: There should be little to no 43 kD band visible in lane 6, the flowthrough, relative to lane 5, the soluble fraction, if the GST-DHFR-His bound well to the column.

Step 4: Check that the GST-DHFR-His eluted from the column when elution buffer was added.

Lane 8 is the GST-DHFR-His eluate fraction.

Results: There should be a band that accounts for the majority of the protein in lane 8 of approximately 43 kD (falls between the 37 kD and 50 kD molecular weight markers). The purity and darkness of the GST-DHFR-His band will depend on the specific culture and induction times used, but the main peak seen in lane 8 should be the GST-DHFR-His.

Step 5: Check that desalting of GST-DHFR-His was successful.

Lane 9 is the desalted GST-DHFR-His eluate fraction.

Results: There should be a band of approximately 43 kD that accounts for the majority of the protein in lane 9 that is of comparable size to the main band in lane 8 and about the same darkness. The absorbance value of this fraction at 280 nm should give a significant value above the blank absorbance.

Step 6: Check that the purified GST-DHFR-His has enzymatic activity.

Results: The "No Substrate Control Reaction" should show a fairly flat line with an absorbance around 0.2–0.4 (Figure 2, below). The enzyme reaction should show a decrease in absorbance at 340 nm over the 150 seconds of reading time (Figure 3, below). The calculated reaction rate will vary depending on the amount of GST-DHFR-His purified.

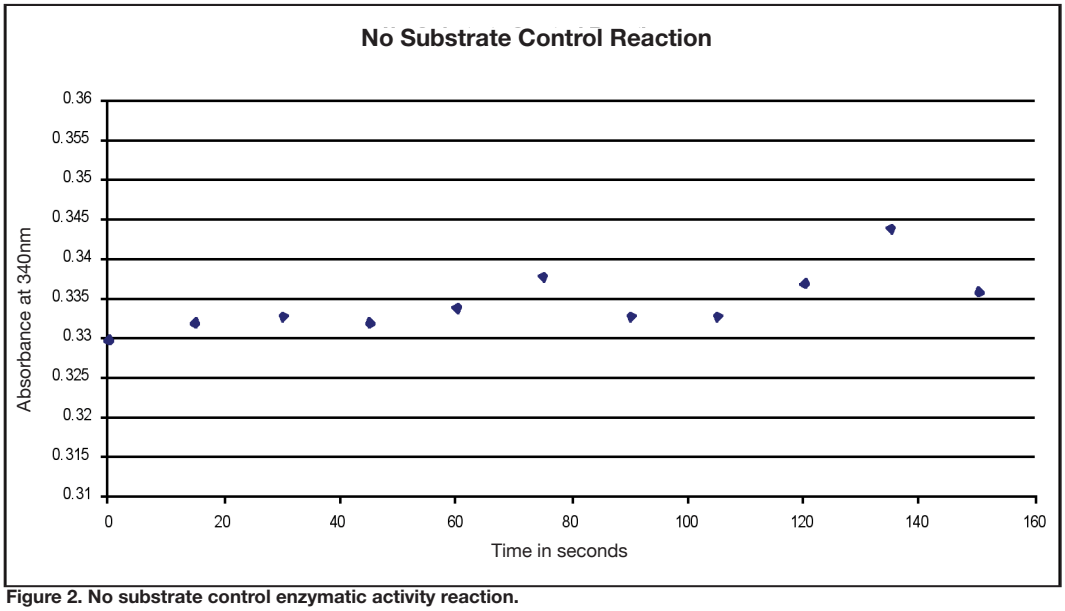


Figure 2. No substrate control enzymatic activity reaction.

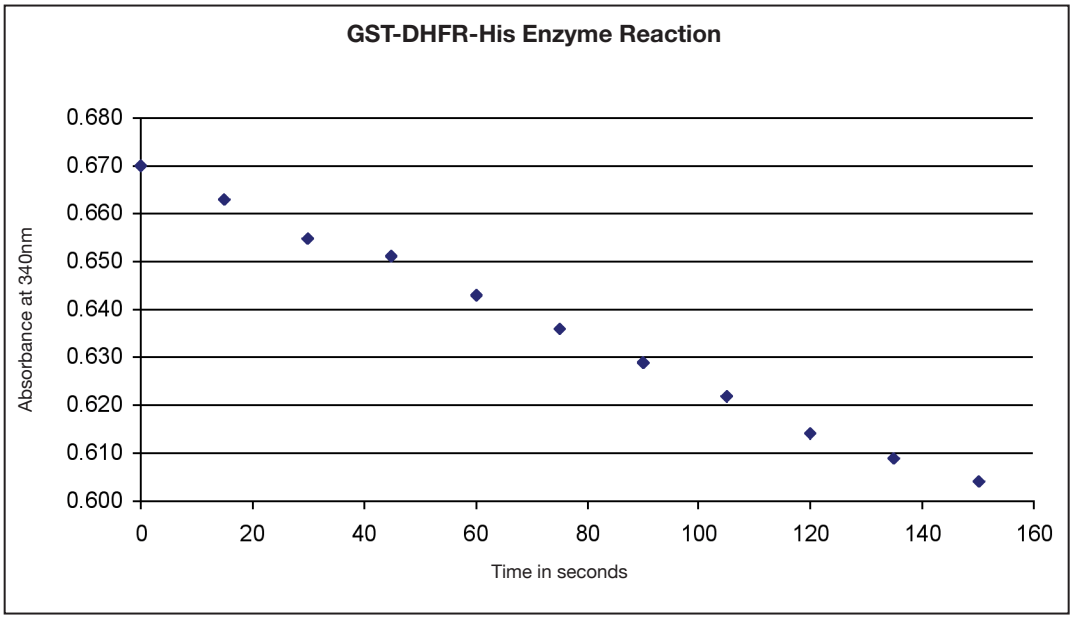


Figure 3. GST-DHFR-His enzyme activity reaction.

BioLogic LP System and BioLogic Duoflow System Purification Results Analysis

The gel image below in Figure 4 shows how typical results using the Chromatography Instrumentation Purification Process should appear. Listed below are common steps that can help you analyze your results.

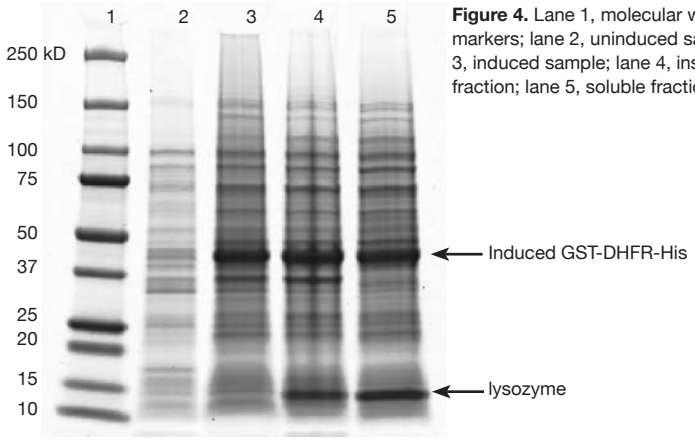


Figure 4. Lane 1, molecular weight markers; lane 2, uninduced sample; lane 3, induced sample; lane 4, insoluble fraction; lane 5, soluble fraction.

Step 1: Check that induction occurred.

The GST-DHFR-His protein runs on SDS-PAGE gels at an apparent MW of approximately 43 kD. (Note that the protein runs at this weight despite the actual protein MW of approximately 52 kD.)

Results: An obvious band should appear in lane 3 that is not present in lane 2 between the 37kD and 52 kD molecular weight markers if the GST-DHFR-His has been strongly induced.

Step 2: Check that lysis worked.

Lanes 4 and 5 represent the insoluble and soluble fractions, respectively.

Results: If the GST-DHFR-His was strongly induced and the cells were lysed, a band should be present in lane 5 that is approximately 43 kD. It should be the same size as the band that occurred in lane 3 (falls between the 37kD and 50 kD molecular weight markers). Please note that the large band that is apparent at approximately 12 kD in lanes 4 and 5 is the lysozyme used to lyse the cells and not GST-DHFR-His. GST-DHFR-His is expressed in both a soluble and insoluble form. It is likely that there will be strong bands in both lanes 4 and 5 at around 43 kD, representing both insoluble and soluble GST-DHFR-His respectively. As long as there is a strong band in lane 5, lysis has occurred, and soluble GST-DHFR-His has been released.

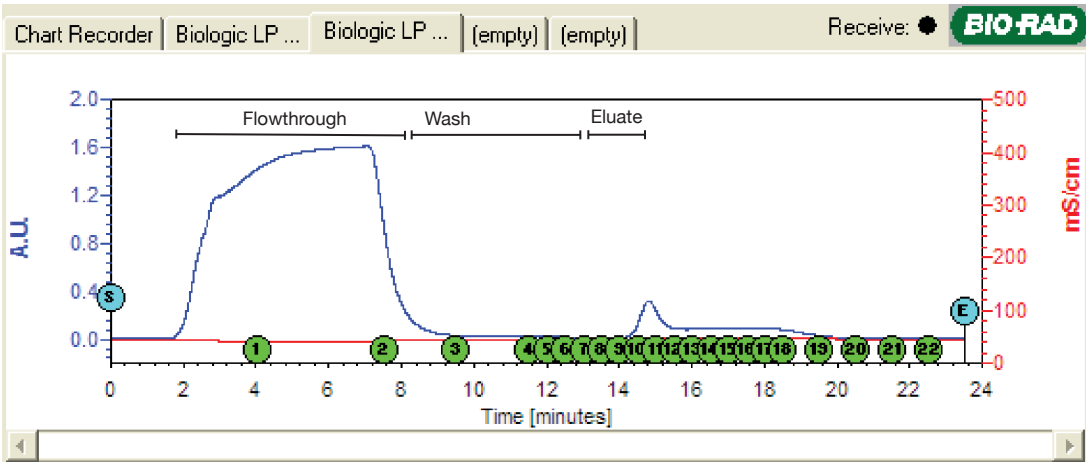


Figure 5. BioLogic LP Chromatogram showing a large peak of flowthrough (between 2–8 minutes in the chromatogram above) and the smaller peak of purified GST-DHFR-His eluate (between 14–16 minutes in the chromatogram above).

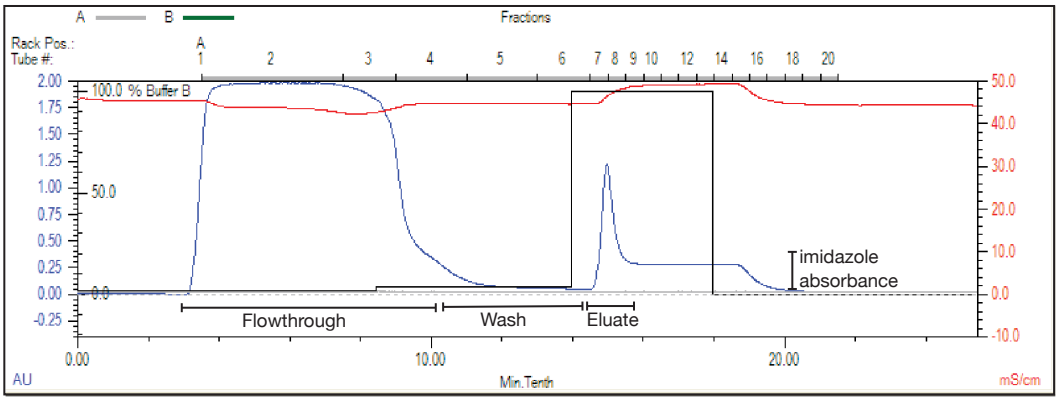


Figure 6. BioLogic DuoFlow system chromatogram showing a large peak of flowthrough (between 2–10 minutes) and the smaller peak of purified GST-DHFR-His eluate (between 14–15 minute). The elevated baseline absorbance between 15–20 minutes represents the increased absorbance from the imidazole in the elution buffer.

Step 3: Check that purification worked by analysis of the chromatogram.

Results: There should be a large peak around 2–8 minutes with a high absorbance at 280 nm that is from the flowthrough of proteins that did not bind to the Ni-IMAC column. There should also be a defined peak around 14–16 minutes that contains the GST-DHFR-His that was eluted with the addition of 250 mM imidazole from the Ni-IMAC column.

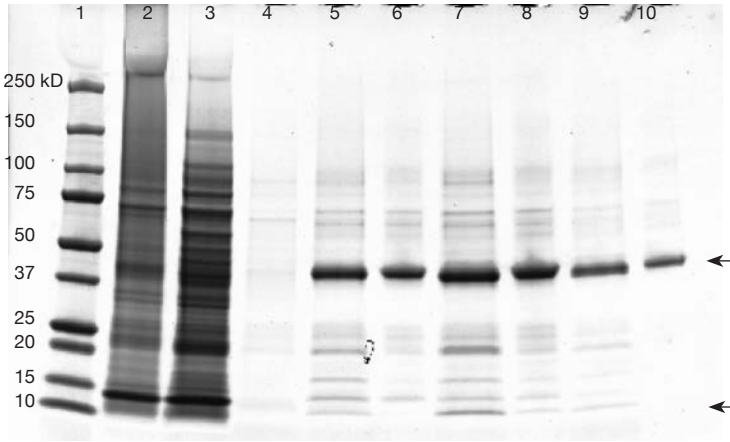


Figure 7. Lane 1, molecular weight markers; lane 2, soluble fraction; lane 3, flowthrough; lane 4, wash; lane 5, fraction A; lane 6, desalted fraction A; lane 7, fraction B; lane 8, desalted fraction B; lane 9, fraction C; lane 10, desalted fraction C.

Step 4: Check that the GST-DHFR-His is present in the fractions chosen after determining delay volume.

There should be a band of approximately 43 kD that accounts for the majority of the protein in lane(s) 5, 7, and/or 9 at approximately 43 kD (between the 37 kD and 50 kD molecular weight markers). The purity and darkness of the GST-DHFR-His band(s) will depend on the specific culture and induction times used.

Step 5: Check that desalting of GST-DHFR-His was successful.

There should be a band of approximately 43 kD that accounts for the majority of the protein in lane(s) 6, 8 and/or 10 that are of comparable size to the main band(s) in lanes 5, 7, and/or 9 and about the same darkness. The absorbance value of this fraction at 280 nm should give a significant value above the blank absorbance.

Step 6: Check that the purified GST-DHFR-His has enzymatic activity.

Results: The "No Substrate Control Reaction" should show a fairly flat line with an absorbance around 0.2–0.4 (Fgure 8). The enzyme reaction should show a decrease in absorbance at 340 nm over the 150 seconds of reading time (Figure 9). The calculated reaction rate will vary depending on the amount of GST-DHFR-His purified.

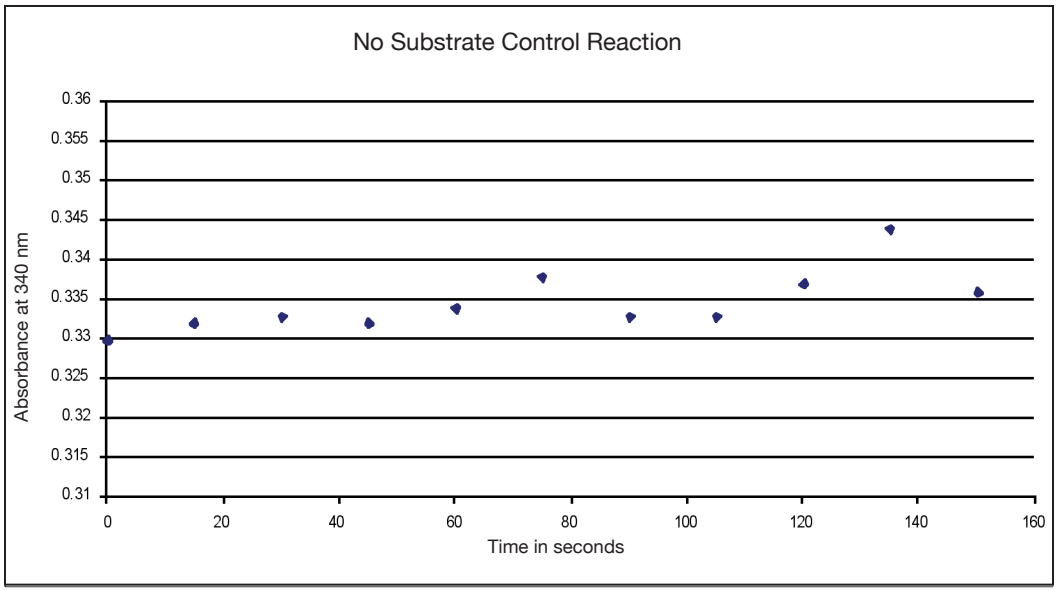


Figure 8. No substrate control enzymatic activity reaction.

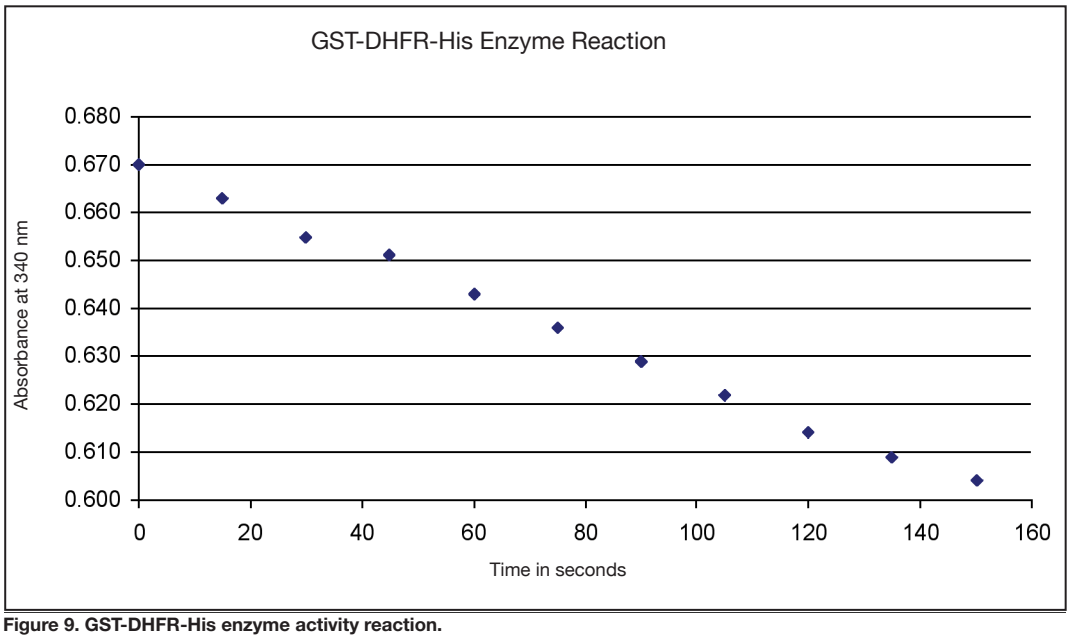


Figure 9. GST-DHFR-His enzyme activity reaction.

Appendix C: Microcentrifuge RCF to RPM Conversion

These directions demonstrate how to calculate the RPM needed to generate a specific amount of force (RCF). It is recommended to first check the instrument's instruction manual since many manuals provide a table showing the instrument's RPM to RCF relationship (similar to Table 1 below).

Calculating the RPM Needed to Generate a Specific Amount of Force

There is a general relationship between RCF (relative centrifugal force) in terms of g and RPM (rotations per minute) of centrifuges:

$$RCF\ (g) = (1.12 \times 10^{-5}) \times (rpm)^2 \times r$$

where r is the radius in centimeters measured from the center of the rotor to the middle of the spin column. This formula can be rearranged to calculate RPM in terms of RCF:

$$RPM = \left(\frac{RCF}{1.12 \times 10^{-5} \times r} \right)^{1/2}$$

This formula can be applied to any centrifuge to calculate the RPM setting to use to achieve a specific RPM.

Bio-Rad Model 16K Microcentrifuge

The Bio-Rad Model 16K microcentrifuge rotor for 1.5 and 2.0 ml microcentrifuge tubes has a radius of 7.3 cm. Therefore, to generate 1,000 x g of force, it would need to spin at a speed of

$$RPM = \left(\frac{1{,}000}{1.12 \times 10^{-5} \times 7.3} \right)^{1/2} = 3{,}497$$

The following table gives the relationship of RPM to RCF for the Bio-Rad Model 16K microcentrifuge:

RPM	RCF
2,500	500
3,500	1,000
5,000	2,000
6,000	3,000
7,000	4,000
7,800	5,000
8,600	6,000
9,300	7,000
9,900	8,000
10,500	9,000
11,000	10,000
11,600	11,000
12,100	12,000
12,600	13,000
13,100	14,000
13,500	15,000
14,000	16,000

Table 1. Bio-Rad Model 16K microcentrifuge RPM to RCF relationship.

Appendix D: Using the Bio-Rad SmartSpec Plus Spectrophotometer for Absorbance Measurements

These directions demonstrate how to use your Bio-Rad SmartSpec Plus spectrophotometer to measure absorbance at 600 nm to determine cell density, to measure absorbance at 280 nm to allow determination of protein concentration, and to run a kinetics experiment measuring absorbance at 340 nm over a 150 second time period. The instructions are laid out such that the tasks are in the numbered steps, and what appears on the screen of the Bio-Rad SmartSpec Plus instrument are below the instructions.

Measuring Absorbance at 600 nm to Measure Cell Density

1. Turn on the Bio-Rad SmartSpec Plus spectrophotometer and press the **OD600** button to measure the absorbance at a wavelength of 600 nm.

A600 1.0 = 5.00e008cell/ml Is this factor OK? YES

2. Press **Enter** to accept that an absorbance of 1.0 is equivalent to 5.00×10^8 cells/ml.

Ready to read absorbance <=Exit Assay >=Options

3. Place the cuvette containing LB/amp broth (the blank) into the chamber. Close the sample compartment door, and press **Read Blank**.

A600= 0.000 >=continue

4. Press ▷ to accept the blank reading.

Ready to read absorbance <=Exit Assay >=Options

5. Remove the cuvette containing the LB/amp broth from the chamber. Place your sample containing cells into the chamber, close the sample compartment door, and press **Read Sample**.

A600= 0.353 Samp #1 Conc= 1.77e+008 cell/ml

Example reading

6. Either press **Print** to print your data or manually record your absorbance value.

7. Remove your cuvette containing your sample from the chamber and dispose of the sample and cuvette properly.

Measuring Absorbance at 280 nm for Calculation of Protein Concentration

1. Turn on the SmartSpec Plus spectrophotometer and press **λ**.

Enter number (1-3) of wavelengths to be read: 1

2. Press the "1" button on the number keypad and then press **Enter**.

Enter wavelength: 1 nm

3. Enter "280" using the number keypad and then press **Enter**.

Do you want to substract background reading? NO

4. Press **Enter** to select NO.

Ready to read absorbance <=Exit Assay >=Options

5. Place the cuvette containing water (the blank) into the chamber. Close the sample compartment door, and press **Read Blank**.

A280= 0.000 >=continue

6. Press ▷ to accept the blank reading.

Ready to read absorbance <=Exit Assay >=Options

7. Remove the cuvette containing water from the chamber. Place your sample containing your desalted, purified GST-DHFR-His sample into the chamber, close the sample compartment door and press **Read Sample**.

A280= 0.126 Samp#1

Example reading

8. Either press **Print** to print your data or record your absorbance value.

9. Remove your cuvette from the chamber and recover your desalted, purified GST-DHFR-His sample.

Measuring Change in Absorbance at 340 nm for Enzyme Activity Calculation

1. Turn on the SmartSpec Plus spectrophotometer and press **Kinetics**.

Enter reading wavelength f or kinetics assay: nm

2. Enter “340” using the numeric keypad and then press **Enter**.

Enter duration of timed assay: seconds

3. Enter “150” using the numeric keypad and then press **Enter**.

Enter interval of data collection: sec

4. Enter “15” using the numeric keypad and then press **Enter**.

Do you want to substract background reading? NO

5. Press **Enter** to select NO.

Ready to read absorbance <=Exit Assay >=Options

6. Place the cuvette containing 1x PBS (the blank) into the chamber. Close the sample compartment door, and press **Read Blank**.

A340= 0.000 >=continue

7. Press ▷ to accept the blank reading.

Ready to read absorbance < =Exit Assay >=Options

8. Remove the cuvette containing 1x PBS from the chamber. Prepare your “No Substrate Control Reaction Sample” in the cuvette and place the cuvette into the chamber, close the sample compartment door and press **Read Sample**.

   Note: If you do not have printer paper in your spectrophotometer, manually record each data point for all time points as they appear on the screen display.

Display Absorbance: 150 A340=0.342 45

Example data for the 45 second time point

9. When the run has completed, the following will be displayed:

```
Press <Print> for report
   <Cancel>=continue
```

10. Press **Print** to print your data (if you have paper) or press **Cancel** if you hand-recorded your data.

```
Ready to read absorbance
<=Exit Assay   >=Options
```

11. Remove the cuvette containing your GST-DHFR-His sample and NADPH in 1x PBS from the chamber. Add the DHF into the cuvette, quickly mix, and immediately place the cuvette into the chamber. Close the sample compartment door and press **Read Sample**.

    Note: If you do not have printer paper in your spectrophotometer, manually record each data point for all time points as they appear on the screen display.

```
Display Absorbance: 150
A340=0.623          75
```

Example data for the 75 second time point

12. When the run has completed, the following will be displayed:

```
Press <Print> for report
   <Cancel>=continue
```

13. Press **Print** to print your data (if you have paper) or press **Cancel** if you hand-recorded your data.

14. Remove the cuvette from the instrument and dispose of the cuvette and the sample properly.

Appendix E : Setting up a Mini-PROTEAN Tetra Gel Box using Mini-PROTEAN TGX Gels

Mini-PROTEAN TGX Gel Set Up

1. **Remove comb**: Position both thumbs on the ridges of the comb. Remove the comb by pushing upward in one smooth continuous motion (Figure 1).

2. **Remove tape**: Pull the green tape gently to remove from the bottom of the cassette (Figure 1).

3. **Rinse wells**: Use a syringe wash bottle or a disposable transfer pipette to rinse the wells with running buffer. Straighten the sides of the wells, if necessary.

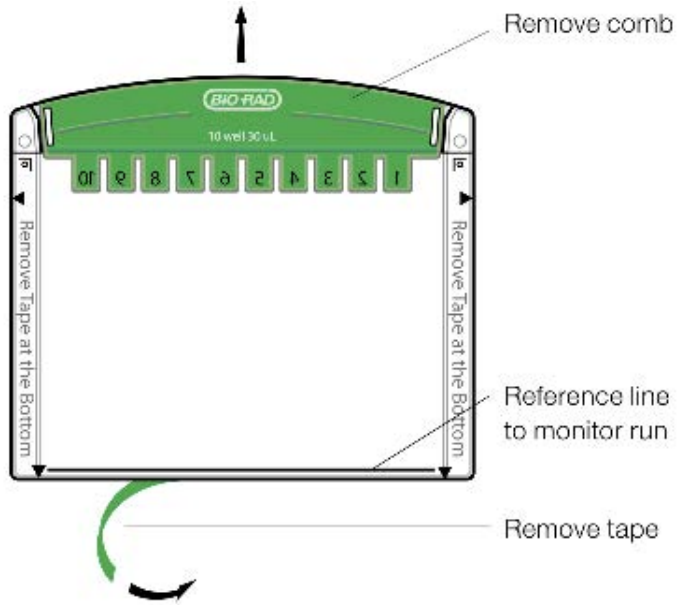


Figure 1. Mini-PROTEAN TGX gel.

Assemble the Mini-PROTEAN Tetra Cell

Note: When running two gels only, use the Electrode Assembly (the one with the banana plugs), NOT the Companion Running Module (the one without the banana plugs). When running four gels, both the Electrode Assembly and the Companion Running Module must be used, for a total of four gels (two gels per assembly).

1. **Set the clamping frame to the open position on a clean flat surface** (see Figure 2A).

2. **Place the first gel sandwich or gel cassette (with the short plate facing inward) onto the gel supports**. Gel supports are molded into the bottom of the clamping frame assembly; there are two supports in each side of the assembly. Note that the gel will now rest at a 30° angle, tilting away from the center of the clamping frame.

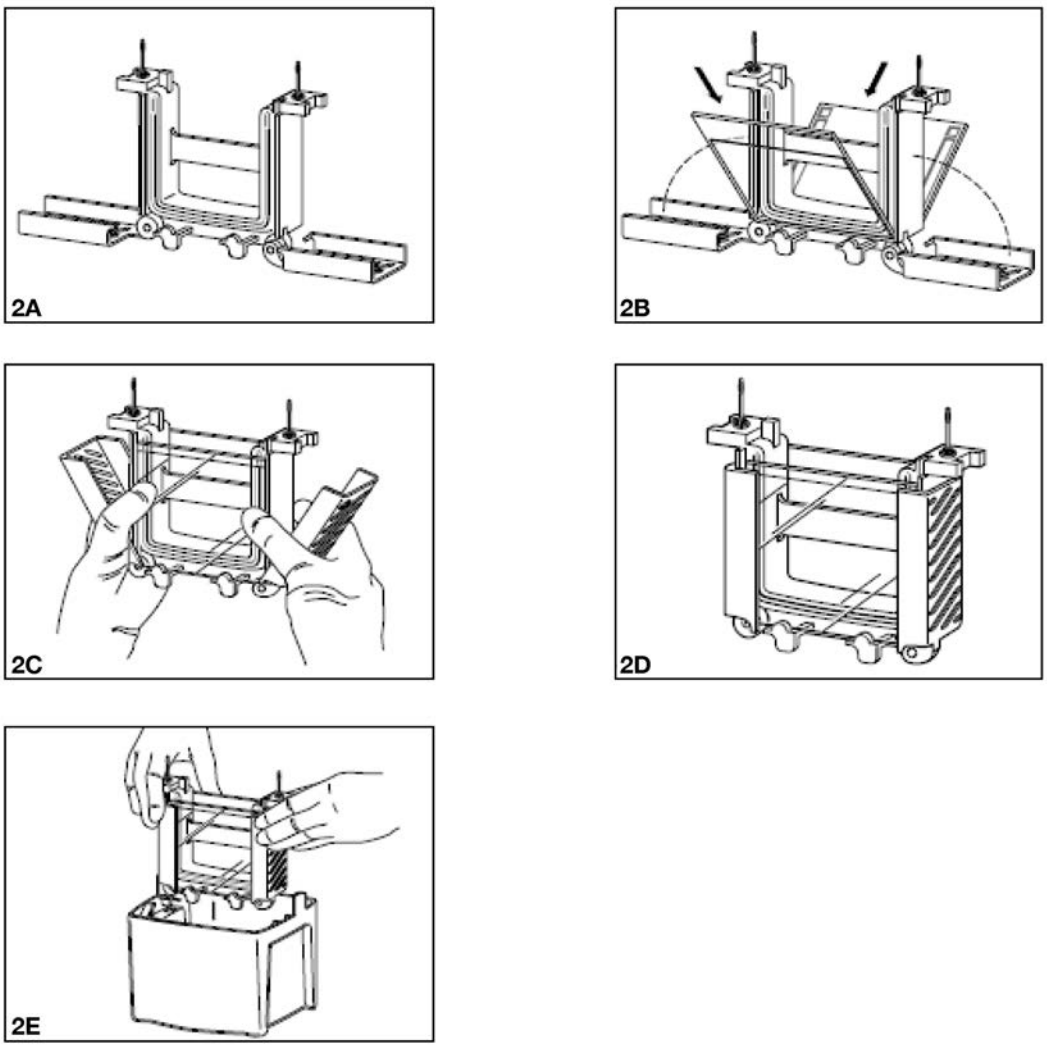


Fig. 2. Assembling the Mini-PROTEAN Tetra Cell Electrophoresis Module.

Please use caution when placing the first gel, making sure that the clamping frame remains balanced and does not tip over. Now, place the second gel on the other side of the clamping frame, again by resting the gel onto the supports. At this point there will be two gels resting at an angle, one on either side of the clamping frame, tilting away from the center of the frame (see Figure 2B).

Note: It is critical that gel cassettes are placed into the clamping frame with the short plate facing inward. Also, the clamping frame requires two gels to create a functioning assembly. If an odd number of gels (one or three) is being run, you must use the buffer dam (see Figure 2B).

1. Using one hand, gently pull both gels toward each other, making sure that they rest firmly and squarely against the green gaskets that are built into the clamping frame. Make certain that the short plates sit just below the notch at the top of the green gasket.

2. While gently squeezing the gel sandwiches or cassettes against the green gaskets with one hand (keeping constant pressure and both gels firmly held in place), slide the green arms of the clamping frame over the gels, locking them into place. Alternatively, you may choose to pick up the entire assembly with both hands, making sure that the gels do not shift, and simultaneously slide both arms of the clamping frame into place (see Figure 2C).

3. The arms of the clamping frame push the short plates of each gel cassette up against the notch in the green gasket, creating a leak-proof seal (check again to make certain that the short plates sit just below the notch at the top of the green gasket). At this point, the sample wells can be washed-out with running buffer (Figure 2D).

   Note: If running more than two gels, repeat steps 1A–D with the Companion Running Module.

   Important Note: Do not attempt to lock the green arms of the clamping frame, without first ensuring that the gel cassettes are perfectly aligned and stabilized against the notches on the green gaskets of the module. To prevent the gels from shifting during the locking step, firmly and evenly grip them in place against the core of the module with one hand.

4. Run the gel(s): Prepare, load and electrophorese the samples according to the instructions in Chapters 4 or 5, depending on whether you are performing the centrifugation process or chromatography instrumentation process for purification. At the completion of the run, disconnect the cell and remove the cassette.

   CAUTION: When running one or two gels only, DO NOT place the Companion Running Module in the tank. Doing so will cause excessive heat generation and prevent electrophoretic separation.

Removing the Gel from the Cassette after the Gel has Been Run

1. **Open the cassette**: Align the arrow on the opening lever with the arrows marked on the cassette (see Figure 3). Insert the lever between the cassette plates at all four locations and apply downward pressure to break each seal. Do not twist the lever. Gently pull apart the two plates beginning from the top of the cassette.

2. **Remove Gel**: Gently remove the gel from the cassette.

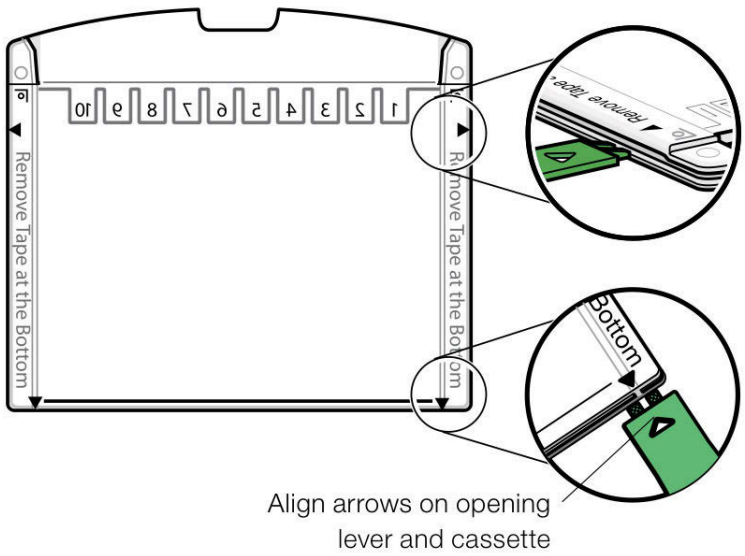


Figure 3. Opening the gel cassette.

Appendix F: Setting-Up, Plumbing, and Wiring BioLogic LP System and Fraction Collectors

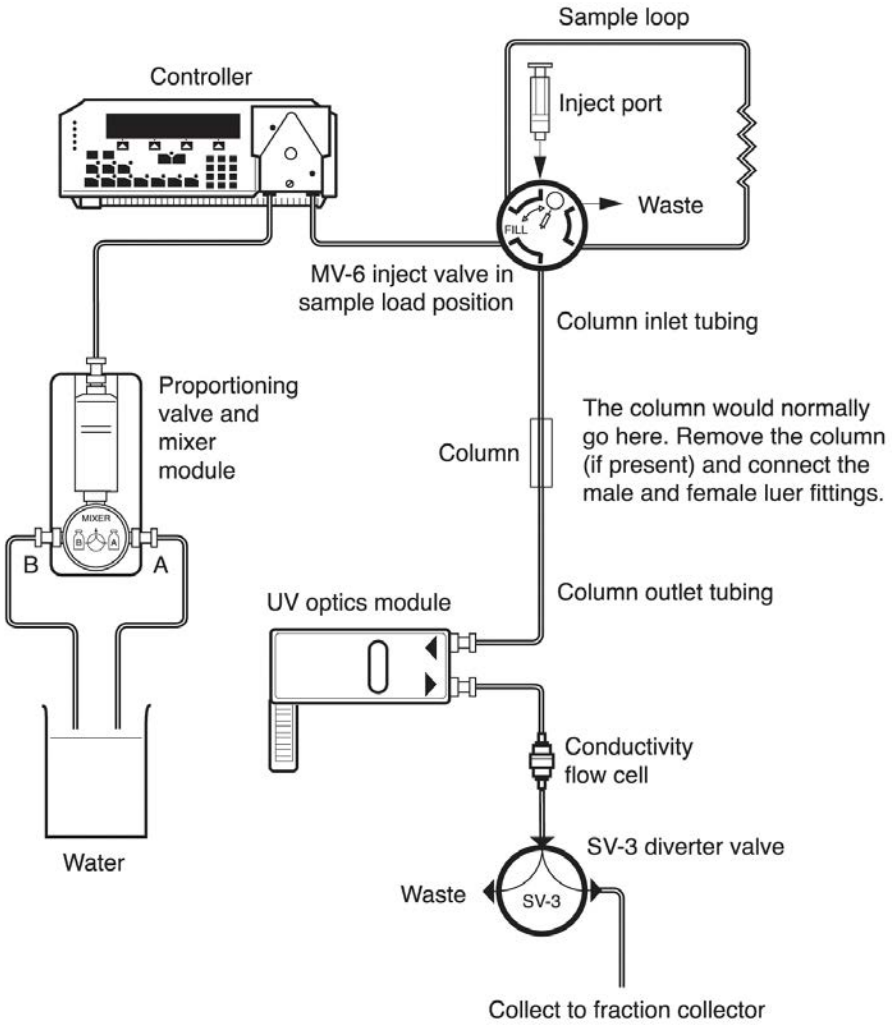


Figure 1. BioLogic LP system plumbing setup.

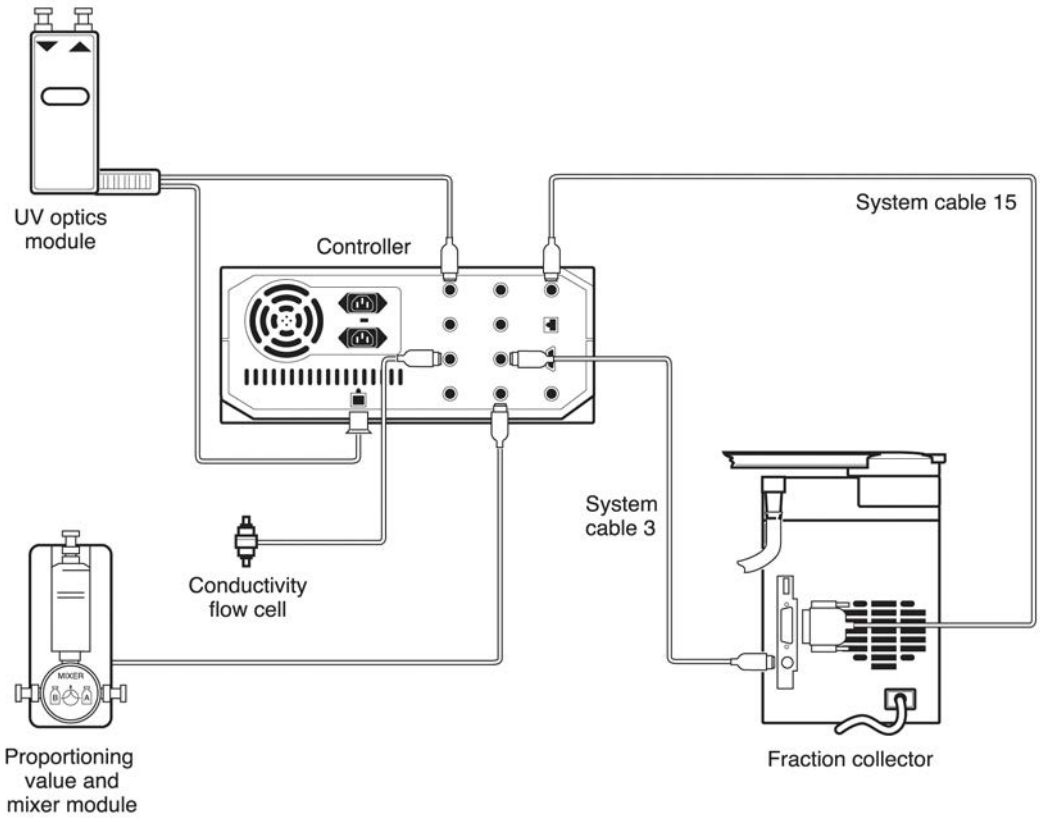


Figure 2.. BioLogic LP system with BioFrac fraction collector cabling.

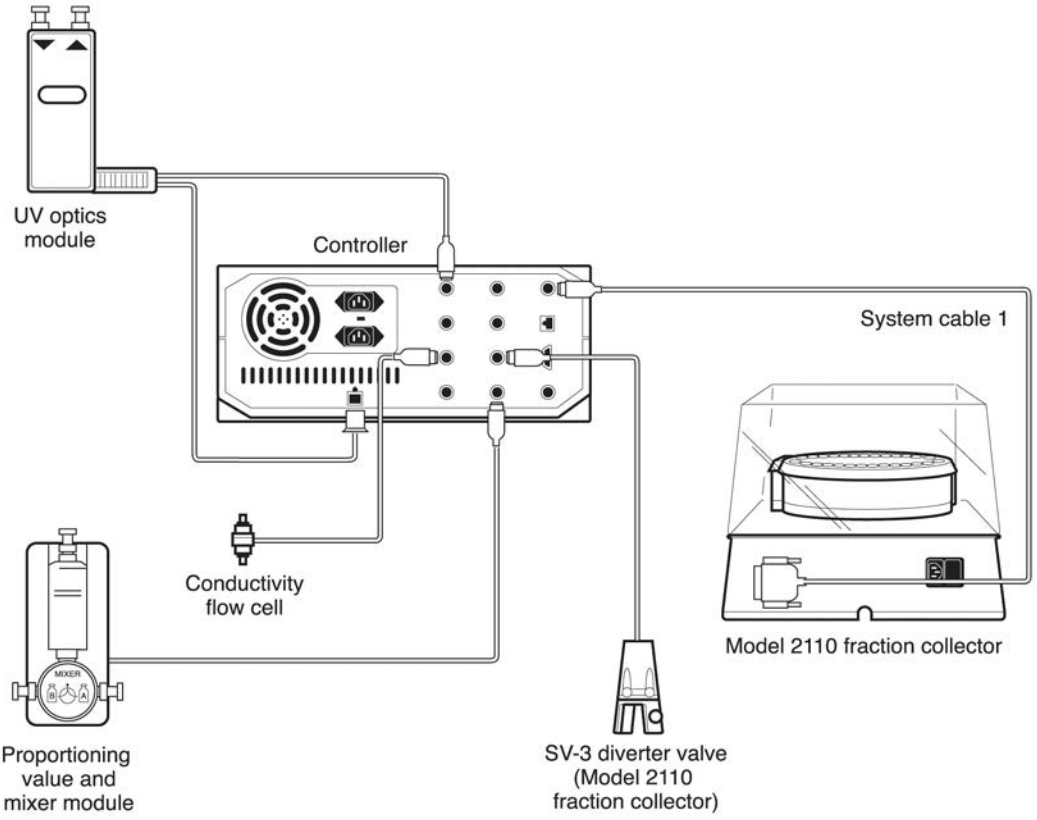


Figure 3. BioLogic LP system with Model 2110 fraction collector cabling.

Appendix G: Biomanufacturing—Protein Pharmaceutical Production Using Genetically Engineered Organisms

Introduction

Industrial biotechnology usually refers to the production of protein pharmaceuticals by living organisms whose genes have been altered in the laboratory. The Research and Development teams of a biotechnology company construct recombinant DNA molecules in their laboratories, introduce this DNA into an appropriate cell type, induce that cell to produce the valuable protein product, and then develop a process where the protein of interest can be purified by separating it from other cellular components and other proteins. The manufacturing team then takes this cell and uses it to produce that pharmaceutical protein on a large scale.

The genetic engineering revolution started in academic laboratories in the mid-1970s, produced major business enterprises in the 1980s, and matured into a multibillion dollar industry in the 1990s. In the 21st century this mode of manufacturing likely will emerge as a dominant industry as a way to manufacture many more pharmaceuticals, as well as to produce biofuels, textiles, and a variety of consumer products.

This process bears some similarities to ancient alchemy. Alchemists tried to turn base metals like lead into gold. Modern biotechnology companies convert the components of the cell growth media into pharmaceutically active proteins that are more valuable than gold. The standard laboratory bacterium, *Escherichia coli*, can take the relatively inexpensive components of a simple growth medium, like simple sugars and salts, and convert them into a product worth many times its weight in gold.

Glucose + Air + Salts ----> Pharmaceutical Proteins

Recombinant Protein	Price per gram
Bovine Growth Hormone (BST)	$ 14
Gold	44
Insulin	60
Growth Hormone	227,000
Granulocyte Colony Stimulating Factor	1,357,000
Note: Prices in 2011 dollars	

These proteins are worth many times their weight in gold, and they often treat diseases for which there was no treatment before the large-scale production of the protein had been developed. The first protein produced was insulin to treat diabetes. This bacterial production of insulin replaced pig insulin that had been isolated from pancreas' collected from slaughterhouses. Next, recombinant human growth hormone, to treat Turner syndrome and other diseases that produced short stature, replaced growth hormone isolated from the pituitary glands of cadavers. Tissue plasminogen activator, an enzyme that triggers the digestion of blood clots, became the standard treatment to remove the blood clots in coronary arteries that caused heart attacks and the blood clots in brain arteries that caused strokes. The hormone erythropoietin raised the level of red blood cells to treat the anemia that accompanies cancer chemotherapy; this protein is also given to patients on dialysis to treat the anemia associated with kidney failure. Biotechnology companies have developed antibodies that specifically recognize proteins on the surface of cancer cells. These antibodies have been combined with traditional chemotherapy to dramatically increase the treatment success rates of some of the most aggressive forms of cancer.

Overview—The Biomanufacturing Process

Biomanufacturing is used to describe the method of producing large quantities of pharmaceutical products from scalable, validated processes. The manufacturing facilities that grow these cells and then purify

the proteins that they produce cost hundreds of millions of dollars to build. These plants manufacture pharmaceuticals under strict government regulations. Every procedure must be validated; that is, proven to be mistake-proof, and every piece of equipment must be proven to perform its function. All employees are specifically trained and must gown-up before entering manufacturing suites. The manufacturing plants are designed for easy cleaning, for a logical personnel flow, and for the airflow to be filtered ensuring it is free of any microbes that might contaminate the product. Each room is continually monitored to make sure that there is the lowest possible level of microbial contamination in the air, on the countertops, and on the floors. Every process follows a standard operating procedure (SOP), and personnel fill out paperwork called a production batch record in which they record every action. Periodically the government inspects the plant and checks these records to make sure that quality standards are strictly followed.

Genetically engineered cells are grown under strictly controlled conditions in very large tanks called bioreactors. After the cells reach their maximum density in the bioreactor, they are separated from the growth medium, then lysed to release the protein that they have produced. Recovery techniques separate cellular proteins away from other cellular components, like DNA and membranes, and then the protein of interest is purified away from other cellular proteins. The Food and Drug Administration (FDA) requires that the final protein injected into a patient be 99.99% pure.

Since the function of a protein depends upon its three-dimensional shape, all production, purification, and delivery techniques must be designed to have the protein maintain this structure. Therefore, proteins are kept away from temperature extremes, pH extremes, organic chemicals, and extreme agitation that might denature (unfold) the protein and render it useless/ineffective. At each stage in the production process the protein must be surrounded by a solution of buffer molecules that moderate pH changes. It is also this consideration of preventing denaturation that dictates whether protein pharmaceuticals will be delivered directly into the bloodstream (parenteral) rather than delivered in a pill form. (If a protein were taken as a pill, the protein would denature the moment that it reached the acidic environment of the stomach.) Therefore, the final preparation of the protein must be placed in a solution that readies it for injection directly into the bloodstream.

Upstream Process—The Growth of Genetically Engineered Cells

The Research and Development team generates a large quantity of the genetically engineered cells and freezes these cells in liquid nitrogen to form a master cell bank; this cell bank will provide the product-producing cells for the entire patent life of the product—20 years. A sample of cells is taken from the master cell bank and used to produce a working cell bank that will provide the cells for a production run, which is called a campaign). These cells are initially grown in a small flask containing a growth medium matching the requirements of the cell. Their cell division causes the culture to increase in concentration, but before the cells reach stationary phase (get too crowded and stop dividing), they will be transferred to a bioreactor and diluted with new media. This bioreactor will stir and aerate (add sterile air to) the cells to make sure that the culture remains aerobic at all times. The goals for the cells are to always generate their energy by cellular respiration and to prevent them from encountering anaerobic conditions that would cause them to supplement ATP production with fermentation. Probes inside the bioreactor monitor the temperature, pH, and dissolved oxygen concentration of the solution. If any of these parameters change, a controller initiates an action to push the altered parameter back to its set point. If the temperature of the medium goes up, cooling water removes heat as it floods through the jacket surrounding the bioreactor. If the pH becomes acid, then a pump turns on to inject base into the medium until the medium is neutralized. If the dissolved oxygen drops, the bioreactor increases the sparge rate of air being pumped in or can supplement the air supply with pure oxygen. This monitoring and reacting to changes maintains the cells in their optimum growth conditions and at their maximum cell division rates.

As the cells divide they are systematically transferred to larger and larger volume bioreactors. The largest bioreactors in a major biotechnology company may be three stories tall. The gene coding for the recombinant protein of interest is always cloned behind an inducible promoter that allows the gene to be turned off for the entire growth phase of production. During this phase the cells devote their energy to

making more cells. It isn't until after the maximum cell concentration is reached in the final bioreactor that the gene is induced, and the cells begin to produce the pharmaceutical protein product. The cells then produce a large concentration (also known as titer) of protein. An optimized process will produce tens of kilograms of protein during a single production run. The cells and their surrounding medium then go to the recovery and purification process.

Downstream Process—The Recovery and Purification of the Protein

Once the cells have produced the protein, the protein must be purified to the 99.99% purity standard required by the FDA. In recovery, the cells are separated from their growth medium by centrifugation or filtration. Typically in *E. coli* the protein is produced as intracellular, therefore the cells are retained and the medium is discarded. If the production cells have been genetically engineered to secrete the proteins into the medium (typical for mammalian cells), then the cell culture fluid is retained and the cells are discarded.

In *E. coli*, when pressure is used to break the cells open to release cellular proteins, the pharmaceutical protein might be 20% of the protein quantity released. In mammalian cell culture (most typically CHO cells), the cells typically are engineered to secrete the protein, and the harvested cell culture fluid comes directly from the centrifuge or filter that removed the cells to be subjected to multiple rounds of chromatography.

The bioseparation technique, chromatography, exploits the unique properties of the pharmaceutical protein to separate it from other proteins in the solution. Different types of chromatography separate proteins according to different properties:

- Size Exclusion (gel filtration or desalting) Chromatography separates proteins according to their size
- Ion Exchange Chromatography separates proteins according to their charge
- Hydrophobic Interaction Chromatography separates proteins according to their hydrophobicity
- Affinity Chromatography separates proteins according to some specific property that they possess
- Mixed Modal Chromatography can be manipulated to separate proteins according to two properties, such as charge and metal affinity

During chromatography purification, the protein containing solution is passed through a column that contains a resin that binds protein of a particular characteristic; for example, an anion exchange chromatography column employs positively charged resin to bind negatively charged proteins and allows neutrally charged and positively charged proteins to pass through. If the protein is an antibody, purification uses a resin coupled with a protein that specifically binds antibodies, such as protein A. The protein will typically be subjected to three, sometimes up to five, different chromatography steps. Contaminating proteins are discarded at each step until the protein is pure enough to place into the final vial.

All of these chromatography steps are carried out at a very large scale. The chromatography columns can be five meters in diameter and can contain several million dollars of chromatography resin. At this scale, it may take thousands of liters of chromatography buffers to wash the proteins during the chromatography processes

After chromatography, the purified protein is often subjected to ultrafiltration. In ultrafiltration the protein-containing solution is passed through a filter of a defined pore size. This step assures sterility and is used to place the protein into its final solution in a process called formulation.

Formulation, Fill, and Finish

Once the protein has been isolated to 99.99% purity, it must be packaged in such a way that prepares it for injection into the patient. Again, all protein pharmaceuticals currently are injected; the ability of stomach acid to denature proteins renders them useless in pill form. To be injected, the proteins must be placed in a solution with the right components; the design of this solution that delivers the drug is called formulation.

Proteins must be surrounded by the same salt concentration as the blood. (If the protein is delivered in pure water, the water would cause the patient's red blood cells to explode.) In addition, the protein is placed in a solution that contains added molecules that buffer the pH, prevents aggregation (proteins sticking together), and helps protect the protein from denaturation.

Once formulation is completed, an automated system dispenses the protein into sterile vials. This process occurs in a specialized facility and is carried out by gowned personnel with specialized training. The sterilized filling room has air that has been filtered to a high degree to remove contaminating microbes. The personnel gowns-up in a manner leaving no skin exposed to avoid shedding bacteria from their bodies. They learn behaviors that help prevent bacteria from inadvertently being placed in the vials. The protein solution within the vials must be absolutely sterile, since it will be injected directly into the bloodstream.

A precisely measured amount of the protein solution is aliquoted into the sterile vial. The filling machine then covers the vial with a sterilized cap and a closure around that cap.

Many proteins are lyophilized, or freeze-dried, into a powder. Lyophilized proteins are more stable than liquid formulations. Lyophilized proteins have the disadvantage that the physician must carry out the extra step of reconstituting the drug by injecting sterile water into the vial and mixing before withdrawing it to inject into the patient.

Labeling and Packaging

An automated process applies labels to the drug-containing vials. Because a mislabeled drug would represent a tremendous danger, the labeling process is carefully controlled. (Mislabeled drugs are one of the top reasons for the recall of a drug.) Each vial is hand-inspected before being placed in a box. The paper insert with the detailed description of the drug's characteristics must be packaged with the drug. Manufacturers' carefully track the destination of each vial in case of a drug recall.

Regulation

In the United States, the Food and Drug Administration, an agency of the Department of Health and Human Services, which is part of the executive branch of the Federal government, regulates the manufacturing of pharmaceuticals. Some would argue that pharmaceutical manufacturing is so carefully monitored that the only industry with greater oversight might be the production of nuclear energy. A pharmaceutical manufacturing company must prove the purity, safety, potency, identity, dosage, and efficacy (effectiveness) of each batch of drug. Each drug production process is examined closely for its ability to produce a consistent product. The stability of each drug must be determined to give it an expiration date.

The law requires that a Quality Assurance unit exist independent of the manufacturing department. This unit tests raw materials, in-process samples, environmental monitoring, utilities, and the final product.

Federal law requires that each pharmaceutical be manufactured under current Good Manufacturing Practices (GMPs). These defined sets of standards and procedures assure that the manufacturing plant follows each production process in exactly the same way for every batch.

To market a drug, a pharmaceutical company must take that drug through clinical trials. The drug is first tested in several model animals. If the pre-clinical animal studies indicate that the drug appears safe, then the drug goes to human clinical trials. These tests involve hundreds of physicians treating thousands of patients. Clinical trials take seven to ten years and cost the company hundreds of millions of dollars. In the end, the FDA must be convinced that the drug is both safe and effective before they issue a formal approval that allows the company to sell the drug in the United States.

Current Good Manufacturing Practice (cGMP) dictates every element of the manufacturing process. The regulations that communicate the provisions of cGMP are found in the Code of Federal Regulations, which containd the following parts:

General Provisions
Organization and Personnel
Buildings and Facilities
Equipment
Control of Containers and Closures
Process and Production Controls
Packaging and Labeling Controls
Holding and Distribution
Laboratory Controls
Records and Reports
Returned and Salvaged Drug Products

The manufacturing buildings must be built in a specific way; their design separates manufacturing from other parts of the biotech company. The regulations, and thus building design, ensure that all personnel gown up and these personnel flow through an airlock before entering the manufacturing areas. The air must be filtered to reduce bacteria, and all surfaces are cleaned regularly. The FDA inspects and approves the facilities before any manufacturing can begin, a process that can take up to four years.

When technicians grow cells and purify proteins, each part of the process follows a Standard Operating Procedure (SOP). Each procedure is carefully designed and validated, that is, proven to perform the function that it claims to perform. During the carrying out of each procedure, a technician will keep strict records by filling out a production batch record. The paperwork may be checked by an FDA official during a formal inspection of the plant, which happens at least every two years.

The Quality Assurance (QA) department checks the product at every stage of production. According to law, it must operate independently of manufacturing and serve as an independent entity to assure that the manufacturing department is following cGMP regulations to the letter. QA operates several Quality Control laboratories to check raw materials coming into the plant, sample and test the product at every stage of the process, and extensively test the final product for purity, identity, potency, consistency, sterility, and stability. The QA department monitors the quality of the air in every room and checks for the presence of bacteria on every surface. Any violation of the provisions of cGMP will render the drug “adulterated” and will prevent the product from being released for consumption. These very strict provisions assure a safe and effective product for the consumer.

Appendix H: Instructor's Answer Guide for Focus Questions

Focus Questions: DHFR Cancer Connection

1. What is DHFR? Why is it important?

DHFR, or dihydrofolate reductase, is an enzyme that converts dihydrofolate into tetrahydrofolate. DHFR is an essential enzyme in the synthesis of purines, thymidylic acid, and aminio acids.

2. What role does DHFR play in cancer?

Inhibited DHFR leads to a shortage of thymidylates, which interferes with nucleic acid synthesis, leading to a lack of cell growth and proliferation. Because cancer involves uncontrolled cell growth and proliferation, a lack of DHFR activity slows down or stops cell growth and proliferation.

3. How does methotrexate interfere with DHFR?

Methotrexate is a folic acid mimic that interferes in folic acid metabolism. Without folic acid, DHFR cannot catalyze the reaction to create thymidylates necessary for DNA synthesis.

Methotrexate is a competitive inhibitor of DHFR. It can bind to the DHFR and block the binding of DHF.

4. What reaction does DHFR catalyze? What is the cofactor that is necessary for this reaction to occur?

DHFR catalyzes the conversion of DHF to THF. It reduces a double bond. NADPH is the cofactor that is required for this reaction to occur.

5. Name an inhibitor of DHFR. How does this inhibitor affect its action?

Methotrexate. Methotrexate has a structure similar to dihydrofolate (DHF). It acts as a competitive inhibitor since it can bind to the active site and prevent binding of DHF. If DHF cannot be bound in the active site by DHFR then it cannot be converted to THF.

Focus Questions: Protein Expression

1. **What is a recombinant protein, and why would it be used instead of a native protein?**

   A recombinant protein is a protein produced from genetically engineered, or recombinant, DNA. The DNA sequence that codes for a particular protein is inserted into a plasmid, which is inserted into a cell, which in turn produces the protein of interest. A protein would be produced recombinantly if it could not be purified from natural sources in large enough quantities to be useful for research or pharmaceutical use.

2. **Name at least one pro and one con of using eukaryotic cells to produce a recombinant protein. When would you choose to use eukaryotic cells to produce a recombinant protein?**

   Pros: Eukaryotic cells perform post-translational modifications such as glycosylation, can refold complicated proteins, and can secrete recombinant proteins into the medium for easier purifications.

   Cons: Eukaryotic cells are slow to grow and can be expensive to grow and maintain.

   Eukaryotic cells would typically be chosen for large or complicated proteins, and when post-translational modifications are needed for proper protein function.

3. **Name at least one pro and one con of using prokaryotic cells to a produce recombinant protein. When would you choose to use prokaryotic cells to a produce recombinant protein?**

   Pros: Prokaryotic cells are fast growing and inexpensive to culture and grow. They can produce large quantities of recombinant protein relative to their total protein content.

   Cons: Prokaryotic cells do not perform post-translational modifications and many times express recombinant proteins in an insoluble form. This can make purification of a refolded active form difficult.

   Prokaryotic cells would typically be chosen when the recombinant protein does not require post-translational modifications and when it can be expressed solubly or easily refolded.

4. **What are three considerations for recombinant protein gene design?**

   Three considerations for recombinant protein gene design are: 1, coding efficiency of the gene; 2, the rate at which the protein will be expressed; 3, how the recombinant protein will be purified away from host cell proteins.

5. **What is the log phase of a cell culture, and why is it important to recombinant protein production?** During log phase the bacteria are actively growing and dividing, producing more cells. For recombinant protein production, it is important that the cells are healthy and actively growing in order to use their cellular machinery to produce protein.

6. **Why are subcultures prepared when trying to produce recombinant proteins?**

   It is hard to predict exactly when cells will be in log phase when grown from a single colony so an initial culture is prepared and used to start a subculture.

7. **What is the purpose of adding glucose to culture media for lac operon systems?**

   The lac operon is repressed when no lactose is present and if glucose levels are high. LB medium is made with casein hydrolysate and yeast extract both which may have endogenous lactose. The addition of glucose ensures the repression of gene expression so that the recombinant protein production is tighly controlled.

Focus Questions: Protein Purification Introduction

1. **What is protein purification?**

   Protein purification is the isolation of a specific protein from other molecules. The level of purity of the isolated protein is dictated by the subsequent use of the protein.

2. **Describe lysis and why it is a necessary part of the protein purification process. Name a lysis technique.**

   Lysis is the breaking open of the cell to release the components in the cytosol. For proteins that are not secreted into the culture medium, cells must be broken open to access the recombinant protein in the cytosolic space in order to begin to isolate that protein. Lysis techniques include freeze-thaw cycles, enzymatic lysis using lysozyme, and physical methods such as sonication and grinding.

3. **Why is it important to remove DNA from the sample?**

   It is important to remove genomic DNA from the sample because genomic DNA from lysed cells is highly viscous. If high-viscosity genomic DNA is flowed over a chromatography column during protein purification steps, it may clog the frit (bed support) that holds the chromatography resin in the column.

4. **What enzyme is used to break down the DNA in a lysate but leaves the protein intact?**

   DNase.

5. **What is a common method of capture, or isolation, of protein?**

   Chromatography.

Focus Questions: Introduction to Chromatography

1. **What is the purpose of chromatography?**

   Chromatography separates biological molecules from complex mixtures using a specific property of the desired molecule such as its charge, hydrophobicity, size, or specific ability to bind or interact with other molecules.

2. **What is the mobile phase in chromatography?**

   In chromatography, the mobile phase is the buffer or solvent containing the molecules to be separated.

3. **What is the stationary phase in chromatography? Give three examples of stationary phase.**

   Stationary phase refers to the medium through which the mobile phase travels. Paper, glass beads, and resin beads are all examples of stationary phase.

4. **Name four types of liquid chromatography techniques**.

   Hydrophobic Interaction (HIC), size exclusion (SEC), ion exchange (IEX), mixed mode, and affinity (AC).

5. **What is an anion and what is a cation? When would you use anion exchange chromatography versus cation exchange chromatography?**

   An anion is a negatively charged ion or molecule. Anion exchange chromatography utilizes a positively charged resin that can be used to bind negatively charged molecules. If the molecule of interest to be captured and purified is negatively charged, then anion exchange chromatography is used.
   A cation is a positively charged ion or molecule. Cation exchange chromatography utilizes a negatively charged resin that can be used to bind positively charged molecules. If the molecule of interest to be captured and purified is positively charged, then cation exchange chromatography is used.

6. **What is an affinity chromatography tag? Name two tags.**

   An affinity chromatography tag is an amino acid sequence added to the recombinant protein that allows for that protein tag to be recognized and bound by an affinity chromatography method. Two types of tags are polyhistidine-tag and GST-tag.

7. **Does adding an affinity tag to a protein change the protein? Explain your answer.**

   Yes, it does. It is the addition of amino acids and this may or may not interfere with the protein's ability to fold correctly and/or function properly.

Focus Questions: Centrifugaton and Chromatography Instrumentation Purification

1. **What is gravity chromatography? Name an advantage and disadvantage of this system.**

   Gravity chromatography uses gravity (via nature or centrifugation) to pull the liquid phase through the solid phase. Advantages of gravity chromatography are that it's inexpensive and does not require any special laboratory equipment. Disadvantages of gravity chromatography are time and the requirement that someone "baby-sit" the experiment.

2. **Is centrifugation/spin chromatography the same as gravity chromatography? Explain your answer.**

   Centrifugation/spin chromatography and gravity chromatography both use gravitational force to push fluids through chromatography columns. Gravity chromatography is limited to 1 x g of force. Centrifugation/spin chromatography controls the g-force by the speed of the centrifuge. Higher speeds exert more g-force and can increase the flow rate. One difference between the two methods is that centrifugation/spin chromatography requires a centrifuge and columns that fit into that centrifuge. As a result, gravity chromatography columns can have a larger variety of lengths and diameters than centrifugation/spin chromatography columns.

3. **What are the advantages of spin chromatography? When would you choose to use centrifugation/spin chromatography and why?**

   Spin chromatography is quick, and the spin columns fit in a standard bench-top centrifuge and are available both empty and prepacked. If the size of column volume and resin can be accommodated, a centrifuge can generate several thousand g-force. This increased force applied to the mobile phase will pull it through the resin more quickly than 1 x g of force for a gravity column. If the resin packed in a chromatography column can withstand higher forces without being crushed, a spin column can be faster than a gravity column.

4. **What are the advantages of using prepacked cartridges? When would you choose to use prepacked cartridges and why?**

   Prepacked cartridges are manufactured to have evenly packed resin beds with no air pockets or channels. There is a lot of uniformity of performance between cartridges. Large scale chromatography columns used in the production of biopharmaceuticals are always handpacked due to the column size and amount of resin used. Prepacked cartidges are typically only used for bench-scale purifications.

5. **What is a fraction in chromatography?**

   A fraction is a sample of known volume of eluate from a chromatography column.

Focus Questions: SDS-PAGE Electrophoresis

1. **What are four ways to quantify the amount of protein in a sample?**

   1, Absorbance at 280 nm using a spectrophotometer; 2, Bradford or Lowry protein assay; 3, ELISA; 4, SDS-PAGE or polyacrylamide gel analysis.

2. **What does primary (1°), secondary (2°), tertiary (3°), and quaternary (4°) protein structure refer to?**

   Primary protein structure refers to the amino acid sequence of the protein. Secondary protein structure refers to the hydrogen bonds that link the amino acids and cause pleating or spiraling. Tertiary structure refers to the 3-D structure of the protein as it is folded up. Quaternary structure refers to the protein complex—more than one amino acid chain, or subunit, joined to make a functional protein.

3. **Describe SDS-PAGE analysis. Why would you use SDS-PAGE to analyze your samples?**

   Sodium dodecyl sulfate polyacrylamide gel electrophoresis is a technique used to separate a mixture of proteins based solely on protein size with the use of a gel medium and an electric field. The relative quantities of different proteins as well as the sizes of those proteins can be visualized using SDS-PAGE. Therefore, for protein expression and purification, because the size of the recombinant protein produced is known, SDS-PAGE can be used to visualize if the protein is expressed, soluble or insoluble, and how well it was purified.

4. **What are BME and DTT and what do they do to a protein?**

   BME, -mercaptoethanol, and DTT, dithiothreitol, are reducing agents that disrupt a protein's disulfide bonds that help stabilize the structure of some proteins.

5. **Describe the components and function of each component of Laemmli sample buffer.**

   Laemmli sample buffer is a commonly used SDS-PAGE sample buffer. It consists of Tris to maintain pH conducive to electrophoresis, glycerol to weigh the sample, SDS to equalize protein charge, potentially a reducing agent to break protein disulfide bonds, and bromophenol blue, which gives the sample color.

Focus Questions: DHFR Enzyme Activity

1. **Which cofactor is required in the conversion of dihydrofolate to tetrahydrofolate?**

   NADPH (nicotinamide adenine dinucleotide phosphate) is required as a cofactor.

2. **What is the purpose of the cofactor?**

   A cofactor is necessary for an enzyme to be fully functional and capable of converting its substrate into the product. In the case of DHFR, NADPH is the cofactor, and it donates a hydride (H^-) group to DHF.

3. **Define oxidation. Define reduction.**

   Oxidation is a reaction in which a molecule or atom loses an electron. Reduction is a reaction in which a molecule or atom gains an electron.

4. **In the conversion of DHF to THF, which compound donates the hydride ion to DHF?**

   NADPH donates the hydride ion to DHF. NAPDH, therefore, undergoes oxidation to $NADP^+$. DHF gains the hydride ion from NADPH and therefore undergoes reduction in converting to THF.

5. **What information does the DHFR enzyme activity provide in the context of recombinant protein expression and purification?**

   The enzyme activity correlates with the functionality of the protein. The protein can be purified and identified, but may have lost its functionality so the enzymatic activity measures and indicates its ability to function properly.

Appendix I: Glossary

Absorbance – The amount of ultraviolet light that is not transmitted (absorbed) through a sample. 280 nm is the ultraviolet wavelength absorbed by the tryptophan, tyrosine, and phenylalanine groups in a protein in solution. The relationship between absorbance of the ultraviolet light and protein concentration is linear.

Affinity chromatography – A chromatography method of separating molecules based on a highly specific biologic interaction such as that between an antigen and antibody, enzyme and substrate, or receptor and ligand.

Anion – A negatively charged ion or biomolecule.

Anode – Positive electrode; attracts negative ions.

Anion exchange chromatography – A chromatography method where a positively charged chromatography resin binds negatively charged molecules, or anions.

Aromatic amino acid groups – Amino acid groups that contain an aromatic ring in the R-group are nonpolar and absorb untraviolet light at 280 nm. Aromatic amino acids are tyrosine (Y, Tyr), tryptophan (W, Trp), and Phenylalanine (F, Phe).

β-mercaptoethanol – (BME) is a reducing agent used to break the diusulfide bonds of proteins, thus disrupting the tertiary and quaternary structure of the protein. It helps to linearize the protein in prepartion for electrophoresis.

Bradford Protein Assay – A test used to measure protein concentration in a sample. The assay relies on the shift in absorbance of Coomassie Brilliant Blue G-250 dye. The dye reacts with mainly basic amino acid and aromatic amino acid groups.

Buffer – The liquid that is used to dissolve the biomolecules that will be applied to the chromatography column.

Cathode – Negative electrode; attracts positive ions.

Cation – A positively charged ion or biomolecule.

Cation exchange chromatography – A chromatography method where negatively charged chromatography resin binds positively charged molecules, or cations.

Cell lysate – All the components, soluble and insoluble, of a cell that have been broken open.

Centrifugation – Spinning a mixture at very high speed to separate heavy and light particles. In protein expression and purification, centrifugation results in a “pellet” found at the bottom of the tube, and a liquid “supernatant” that resides above the pellet.

Charge density – The protein’s ratio of charge to mass.

Chromatogram – A visual output of the chromatographic separation. Peaks on the chromatogram indicate when samples are eluting from the column.

Chromatography – A process for separating complex mixtures of proteins or other molecules. In the case of column liquid chromatography, separation is accomplished by passing a liquid mixture over a column containing a solid matrix. The properties of the matrix can be tailored to allow the selective separation of one kind of molecule from another. Properties include hydrophobicity, molecular size, and charge.

Column – A plastic or glass cylinder that is densely filled ("packed") with small, porous spheres or beads.

Column bed – The volume of beads packed within the chromatography column.

Column volume – (CV), the volume of buffer equal to the volume of resin packed in a chromatography column.

Dalton – One Dalton equals the mass of a hydrogen atom, which is 1.66×10^{-24} grams. A DNA kilobase pair has a mass of approximately 660 kD.

Decant – Gently removing liquid or buffer from a column or other vessel so as not to disturb the resin or sediment bed.

ΔOD/min – Change in optical density, or measured absorbance, per minute. For this series, it is used in calculating the activity of an enzyme.

Denaturation – Process of disrupting a protein's structure.

DHF – Dihydrofolate or dihydrofolic acid.

DHFR – Dihydrofolate reductase.

Disulfide bond – S—S (sulfide—sulfide) bond between amino acids in a polypeptide chain; contributes to tertiary and quaternary structure of proteins.

Dithiothreitol – (DTT) is a reducing agent used to break the disulfide bonds of proteins, thus disrupting the tertiary and quaternary structure of the protein. It linearizes and prepares the protein for electrophoresis.

DNase – Enzyme that specifically breaks down DNA.

Electrophoresis – Means "to carry with electricity." It is the migration of charged molecules in an electric field toward the electrode with the opposite charge.

Eluate – The solution of buffer and biomolecules from elution.

Elute – The removal of a bound molecule from a chromatography resin.

Elution buffer – The chromatography buffer containing chemicals used for the removal of a bound molecule from a chromatography resin.

Exclusion limit – The upper size limit for molecules that cannot penetrate the pores of the porous beads. See *Fractionation range*.

Fraction – A tube that contains material that has flowed through the chromatography column. Multiple tubes or fractions are collected during each chromatography run.

Fractionation range – For size exclusion chromatography gels, the fractionation range is the range of molecular weights that will enter the gel. A fractionation range of 1,000–6,000 kD will have pores large enough for molecules in that size range to pass through. Molecules larger than 6,000 kD will be excluded from entering the gel. The fractionation range is sometimes referred to as the "exclusion limit."

Frit – Bed support of the chromatography column.

Gel electrophoresis – Technique used to separate, or sieve, molecules that carry electric charges. The molecules separate from each other according to the different rates at which they migrate through an electric field set up in a gel that is soaked in a chemical solution.

Glycoslyation – An enzymatic process that adds glycans, or sugars, to a protein or other organic molecule. Glycosylation is known to aid in protein folding.

GST-tag – Glutathione-s-transferase, an enzyme that binds to the substrate glutathione, is a small amino acid sequence (27 kD mw) that is added to the sequence of a recombinant protein. Glutathione is bound to chromatography resin and thus used to purify proteins with the GST-tag. GST is also added to recombinant proteins to aid in solubility.

His-tag – A series of histidine residues (usually 6) fused to a protein that aids protein purification because of its strong binding to nickel (IMAC) columns. Also known as a "polyhistidine tag."

Hydrophilic – A molecule that has a strong affinity for water, "water loving."

Hydrophobic – A molecule that has a strong dislike for or is insoluble in water, "water fearing."

Hydrophobic interaction chromatography – A chromatography method that separates molecules based on their level of hydrophobicity.

IMAC – Immobilized Metal Affinity Chromatography; a chromatography method where the affinity of histidines to metals, such as nickel, is used to purify proteins tagged with polyhistidine sequences.

Inclusion body – Aggregated and precipitated expressed proteins found inside bacteria induced to make high levels of recombinant protein.

Insoluble – The parts of the cell that are not dissolved in water or buffer.

Ion exchange chromatography – A chromatography method where the charge of the molecule is exploited to bind to oppositely charged chromatography media.

Isoelectric point – (pI) The pH at which a molecule has a net charge of 0.

Laemmli sample buffer – The first, and most common, sample buffer used for protein electrophoresis. First described in 1970, this buffer consists of 62.5 mM Tris buffer to maintain pH conducive to electrophoresis; 10% glycerol to increase density of the protein so that it stays sunk in the gel well, 2% SDS to equalize the protein charge; 5% DTT (or BME) can be added to reduce disulfide bonds in the protein; and 0.01% bromophenol blue, which gives the sample color.

Ligand – A molecule, such as an antibody, enzyme, or protein tag, with specific affinity for another molecule.

Loading buffer – (Equilibration buffer) The buffer used to add sample to a chromatography column. The loading buffer is formulated to exploit properties of the biomolecule of interest for the particular chromatography resin and allows the biomolecule to bind to the resin.

Lowry Protein Assay – An absorbance test used to measure the protein concentration in a sample. The assay relies on the reaction of protein with alkaline copper tartrate and Folin and the change in color of the sample.

Luer-Lok – A standardized system of low-pressure fluid fittings used for making leak-free connections between a male-taper fitting and its mating female part on medical and laboratory instruments.

Lyse – To break open a cell.

Mixed-mode chromatography – A method of chromatography in which the resin used utilizes multiple chromatography techniques, such as a combination of anion and cation exchange properties, to resolve a mixture of biomolecules.

Mobile phase – The liquid, solvent, buffer, or sample, that moves through the stationary phase or chromatography resin.

Pellet – The insoluble components of a lysed cell that settle in the bottom of the centrifuge tube during centrifugation of the cell lysate.

Polyhistidine tag – A series of histidine residues (usually 6) fused to a protein that aids protein purification because of its strong binding to nickel (IMAC) columns. Also known as a "His-tag."

Prepacked cartridge – A chromatography column that is prepacked with chromatography resin. The cartridge is capped, keeping the resin contained. The cartridge has a quick-connect fitting on its bottom and top allowing for easy connection to a syringe or chromatography pump or system.

Protein assay – A test using the shift in absorbance of colorimetric dye to determine protein concentration. A spectrophotometer is required to perform the assay. Two popular protein assay methods are the Bradford Protein Assay and the Lowry Method. Which method to use is based on compatibility of the method with reagents in the sample buffer, as well as sensitivity.

Resin bed – The settled, packed chromatography resin in a column.

Sample – A mixture of biomolecules that is dissolved in a buffer and which is applied to a chromatography column.

SDS-PAGE – Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis is a technique of separating a mixture of proteins based solely on its size with the use of a gel medium and an electric field.

Size exclusion chromatography – Also known as gel filtration chromatography, desalting chromatography, and buffer exchange chromatography, size exclusion chromatography uses beads containing tiny holes, or pores, to separate a mixture of molecules based on its size. The larger molecules, which cannot fit through the holes, pass quickly around the beads whereas smaller molecules enter the holes and pass through the column more slowly.

Sodium Dodecyl Sulfate – Also known as SDS, it is a strongly anionic detergent used to coat proteins and give them an overall negative charge.

Soluble – The parts of the cell that are easily dissolved in water or buffer.

Spin column – A small chromatography column that fits into a standard bench-top centrifuge and allows for quick purification of biomolecules. The column may be prepacked or be empty, allowing the user to choose the resin.

Stationary phase – In chromatography, the stationary phase is the chromatography resin that is used to bind the molecule of interest or separate the sample mixture.

Tertiary structure – 3-dimensional shape of a folded polypeptide, maintained by disulfide bonds, electrostatic interactions, and hydrophobic effects.

Appendix J: References, Legal Notices, and Trademarks

Biotechnology Institute Biotech Timeline /www.biotechinstitute.org/teachers/resources/timeline

Drug Development Timeline www.wellcome.ac.uk/bigpicture/drug/runflash2.html

www.malariajournal.com/content/6/1/108

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beta.docguide.com/dihydrofolate-reductase-deficiency-due-homozygous-dhfr-mutation-causes-megaloblastic-anemia-and-cere?tsid=5

The American biotechnology industry has surpassed pharmaceutical companies for the third straight year as the primary source of new medicines, and biotech revenue jumped nearly 16 percent to a record $50.7 billion in 2005. Ernst & Young LLP, 2006.

The biocompatible materials market is estimated to reach nearly $11.9 billion by 2008. Biomaterials are used in every medical device meant for body contact, form orthopedic implants and bone grafts to coronary stents and soluble sutures. Biocompatible Materials for the Human Body, 2003.

The biotech industry has mushroomed since 1992, with U.S. healthcare biotech revenues increasing from $8 billion in 1993 to $39 billion in 2003. Biotechnology Industry Organization (BIO), 2006.

Biotechnology is one of the most research-intensive industries in the world. The U.S. biotech industry spent $17.9 billion on research and development in 2003. Biotechnology Industry Organization (BIO), 2006.

Resources

chemtools.chem.soton.ac.uk/projects/emalaria/index.php?page=1 (This site may be of interest to teachers, although the project is currently on hold.)

DHFR the Movie, from Kraut Research Group at University of California at San Diego: chem-faculty.ucsd.edu/kraut/dhfr.html

Expasy Protein Parameters tool: expasy.org/tools/protparam.html

(Genscript tools; www.genscript.com/cgi-bin/tools/codon_freq_table).

Add a table of the codon sites here or in Appendix G References? A
nother codon usage table: www.kazusa.or.jp/codon/ (This site is referenced a lot in my search for a non-commercial site. It is commercial, but the links to it, I think speak for it). Another useful site molbiol-tools.ca/Translation.htm (This is from Dr Kropinski at University of Guelph)

Visit the World Health Organization's website for more information on malaria: www.who.int/topics/malaria/en.

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