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.

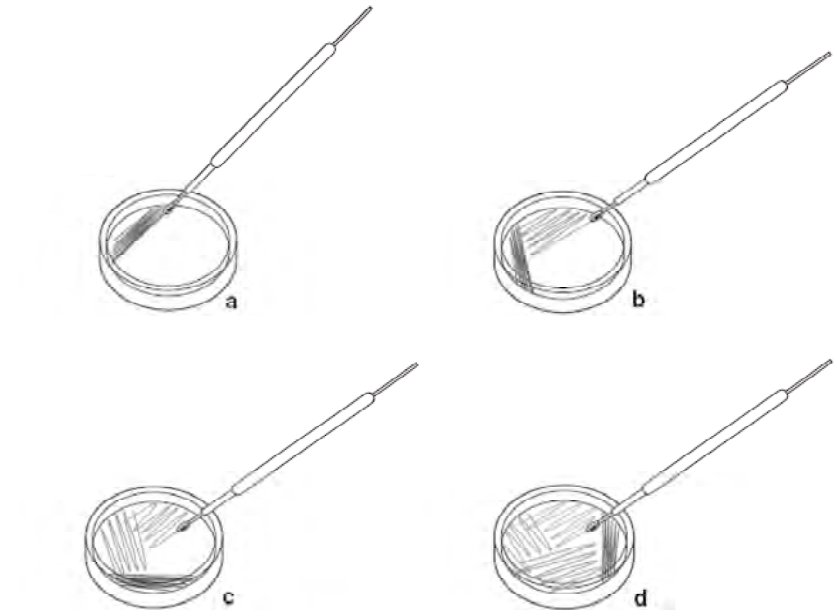


Figure 4.1. Streaking a plate to generate single colonies.

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 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.

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

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.

7. 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.

8. 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!**

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

10. 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.

4. 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.

5. 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.

6. 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.

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

8. 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.

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

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

11. 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

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

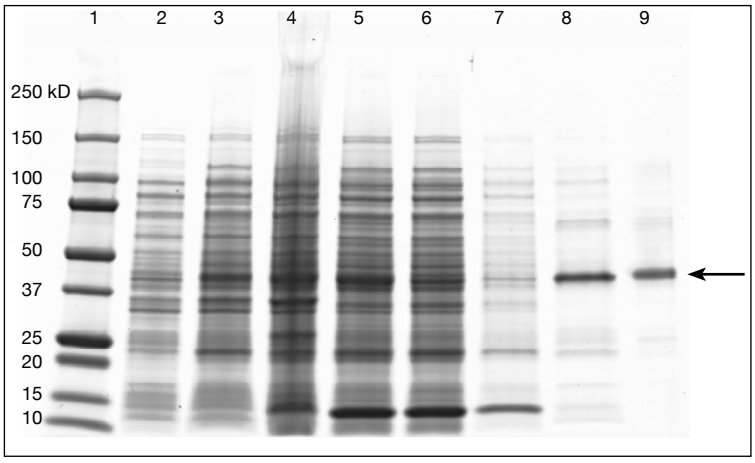


Figure 4.2. Centrifugation purification process results analyzed by SDS-PAGE.

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

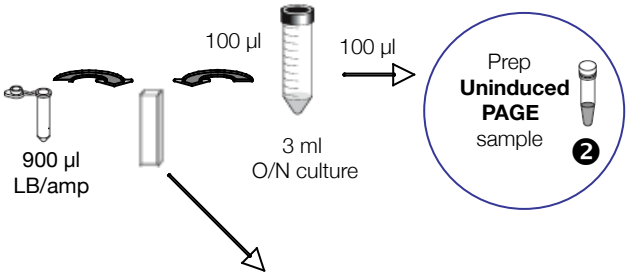
Streaking starter plates to produce single bacterial colonies on agar plates. Pipet 10 µl BL21(DE3) *E. coli* containing pDHFR onto LB/Amp plate; streak out using sterile inoculation loop. Incubate overnight at 37°C.

(37°C O/N)

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.

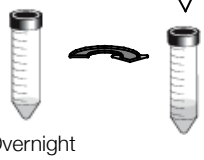
(O/N at 37°C with shaking)

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 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.



(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. Measure OD600 and record.

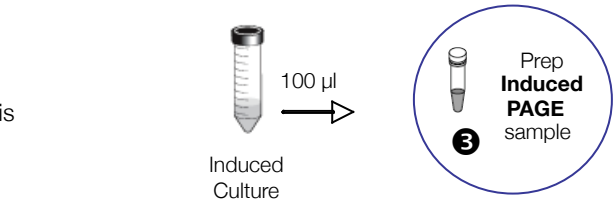
Induce expression by adding 25 µl 100 mM IPTG to culture and incubate 4–24 hours at 37°C with shaking.

(4–24 hr at 37°C with shaking)

Centrifugation Purification Workflow

Collecting cell pellet and lysing cells

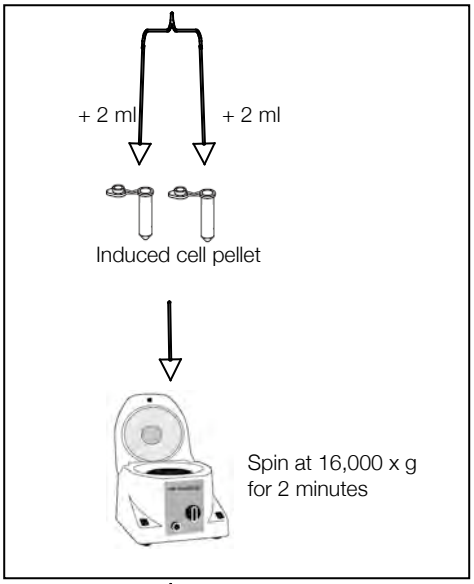
Prepare Induced SDS-PAGE sample. After induction is completed, take 100 µl aliquot from induced culture and prepare for SDS-PAGE per manual instructions.



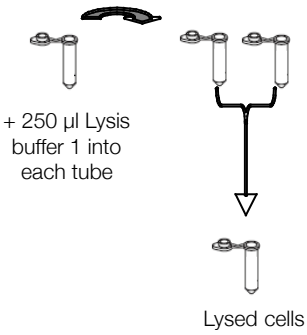
Pelleting induced cells. Label two 2 ml microcentrifuge tubes with **Induced cell pellet** and your team's initials. Add 2 ml of induced culture into each of two 2 ml tubes.

Centrifuge for two minutes at 16,000 x g.

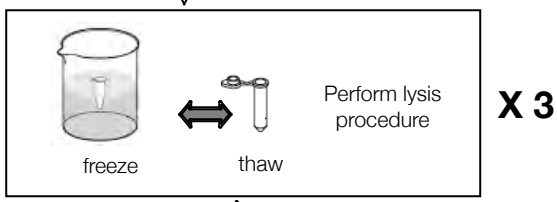
Carefully decant and discard the supernatant Repeat the addition of 2 ml induced culture to each tube; Centrifuge; decant; repeat once more until all cells are pelleted (three times total).



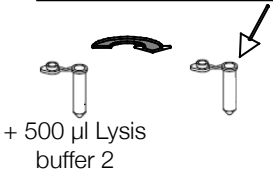
Lysing induced cells. Add 250 µl Lysis buffer 1 to each of the two tubes containing a cell pellet. Using a vortexer or pipet, fully resuspend the pellets. Label a clean 2 ml microcentrifuge tube **Lysed cells** with your initials. Combine resuspended, lysed pellets into the lysed cells tube.



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.

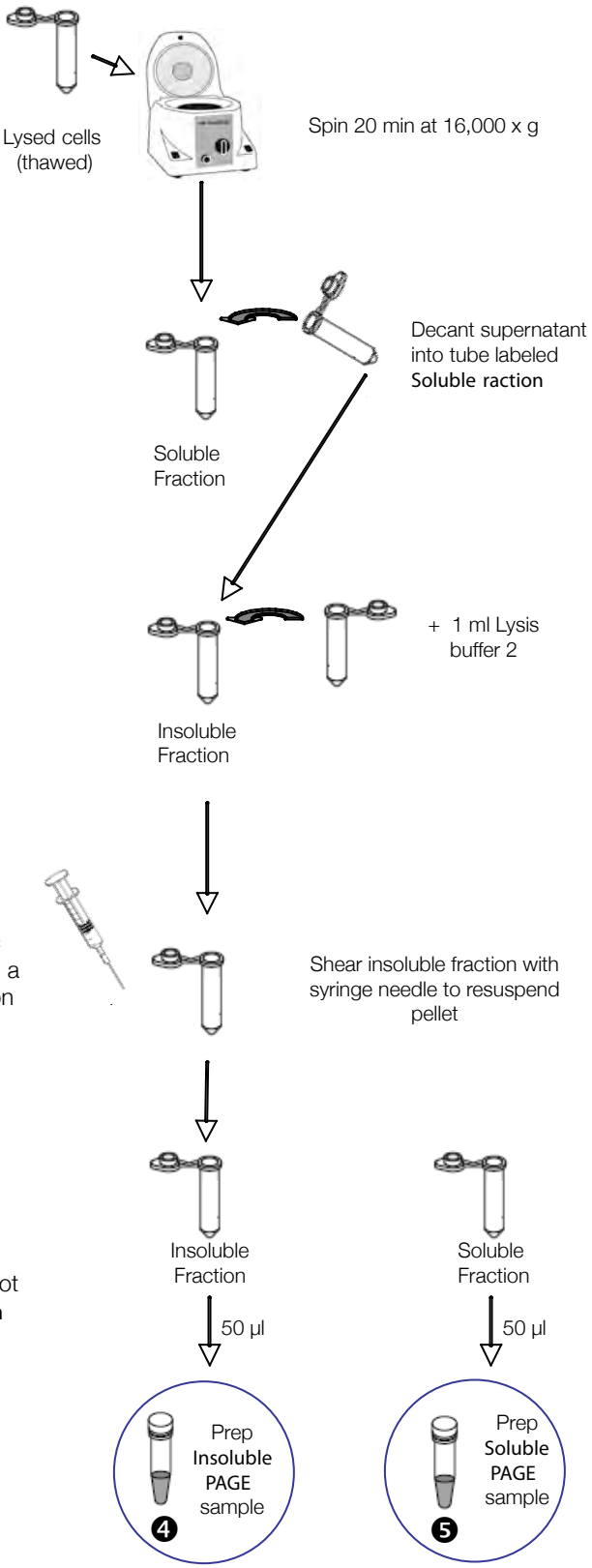
The supernatant contains the soluble fraction, and the pellet contains the insoluble fraction. Gently pour the supernatant into the tube labeled soluble fraction. Relabel the Lysed cells tube **Insoluble fraction.**

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.



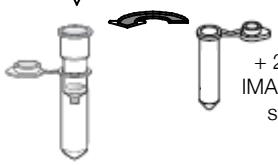
Centrifugation Purification Workflow

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.

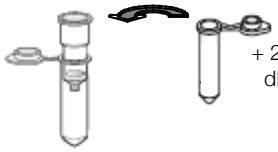


+ 200 µl IMAC resin slurry

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

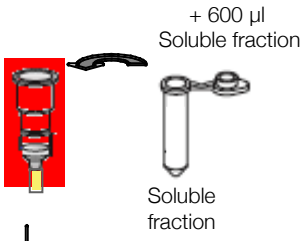
Centrifuge for two minutes at 1,000 x g. After spin, discard Equilibration buffer and 2 ml microcentrifuge tube. The column is now ready to use.

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.

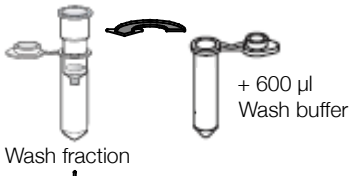


Gently mix for 20 minutes.

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.

Set aside Flowthrough.

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

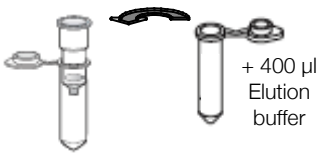


Centrifuge for two minutes at 1,000 x g.
Set aside Wash fraction.

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

Centrifuge for two minutes at 1,000 x g.

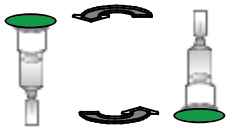
Spin 2 min at 1,000 x g

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.



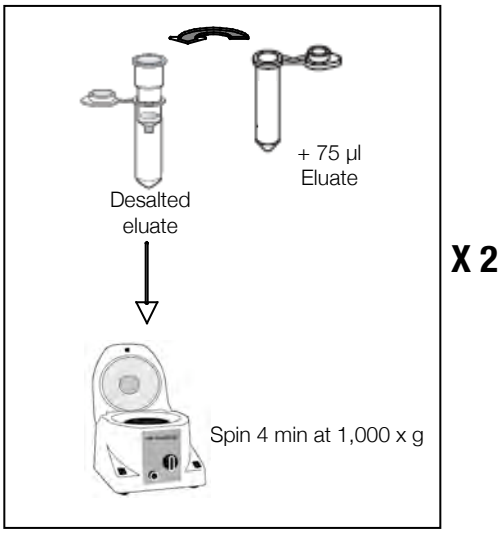
Snap off bottom tab and place in 2 ml microcentrifuge tube. Remove green top cap. If the column does not begin to flow, push the cap back on the column and then remove it again to start the flow.

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.

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.

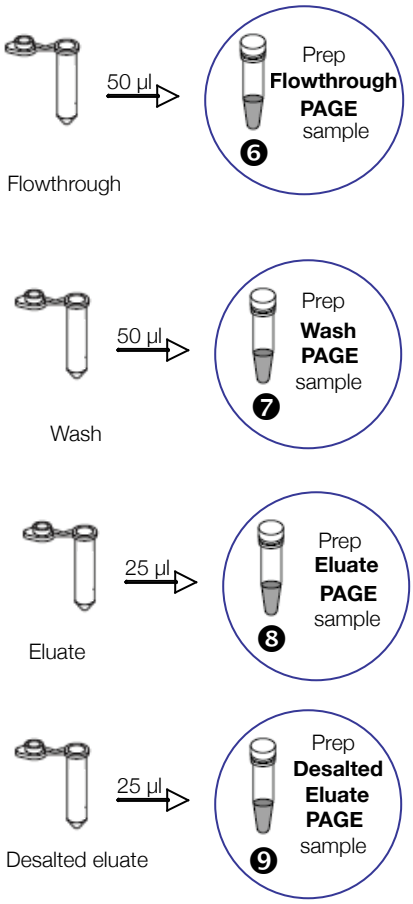


X 2

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

Centrifugation Purification Workflow

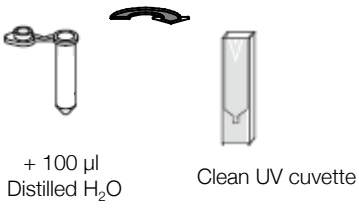
Prepare SDS-PAGE samples. Add equal volume of Laemmli buffer to each sample aliquot per manual instructions. Mix well. Heat all samples at 95°C for five minutes.



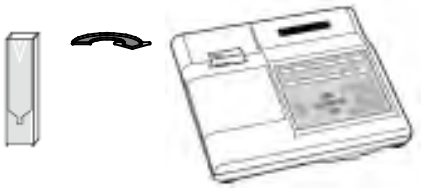
Centrifugation Purification Workflow

Quantitation of Protein in Desalted Fraction

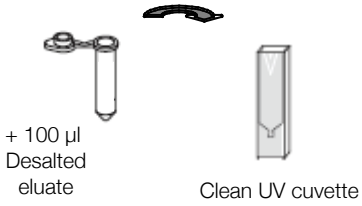
Turn on spectrophotometer and set absorbance to 280 nm. Add 100 µl distilled water to clean UV compatible cuvette.



Blank spectrophotometer with distilled water.

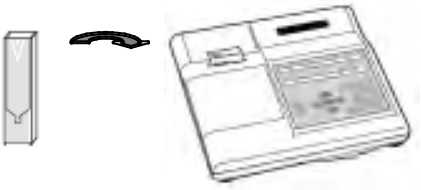


Pipet 100 µl of your **Desalted eluate** sample (GST-DHFR-His) into clean UV compatible cuvette.



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

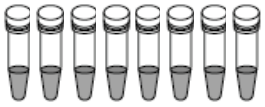
Calculate concentration of GST-DHFR-His in your Desalted eluate fraction as described in instruction manual and record data.



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.

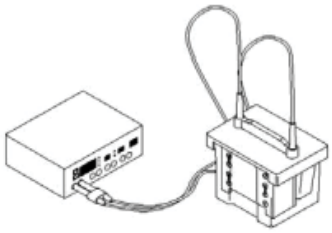


Heat 2 min at 95°C

Spin 2 min at 16,000 x g

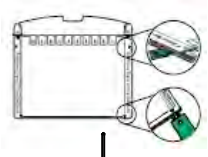
Using a fresh tip each time, load the following volumes of each sample into the appropriate well of your gel

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 9 : DHFR Enzymatic Activity Assay Student Protocol

Student Workstations

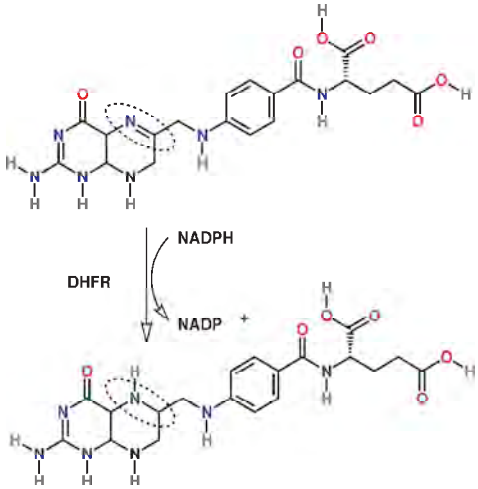
Each student team requires the following items to analyze enzymatic activity of their purified, desalted GST-DHFR-His protein sample:

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.



Dihydrofolic acid (DHF)

Tetrahydrofolic acid (THF)

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.

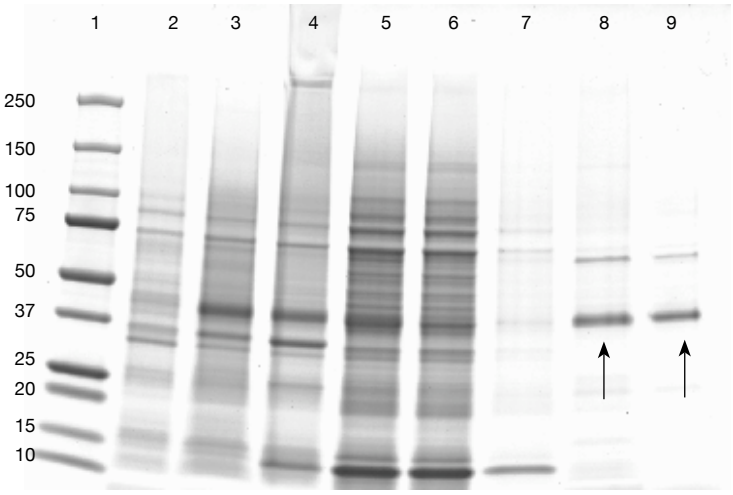
Note: Centrifugation purification will have one desalted fraction while instrumentation-based purification will have three fractions.

Concentration (mg/ml) of GST-DHFR-His (Desalted fraction A): _____

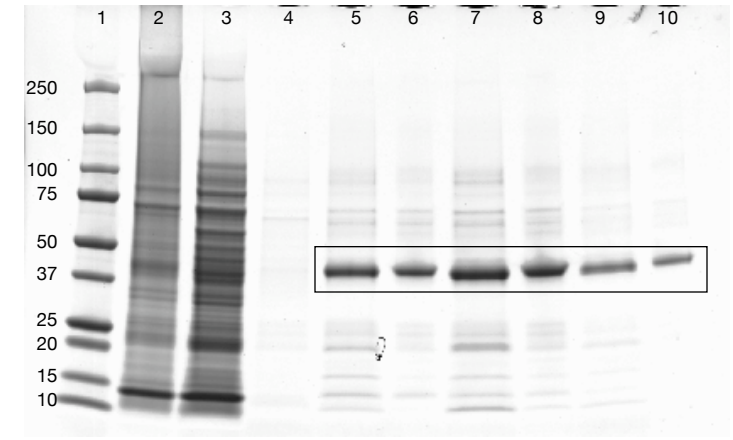
Concentration (mg/ml) of GST-DHFR-His (Desalted fraction B): _____

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.

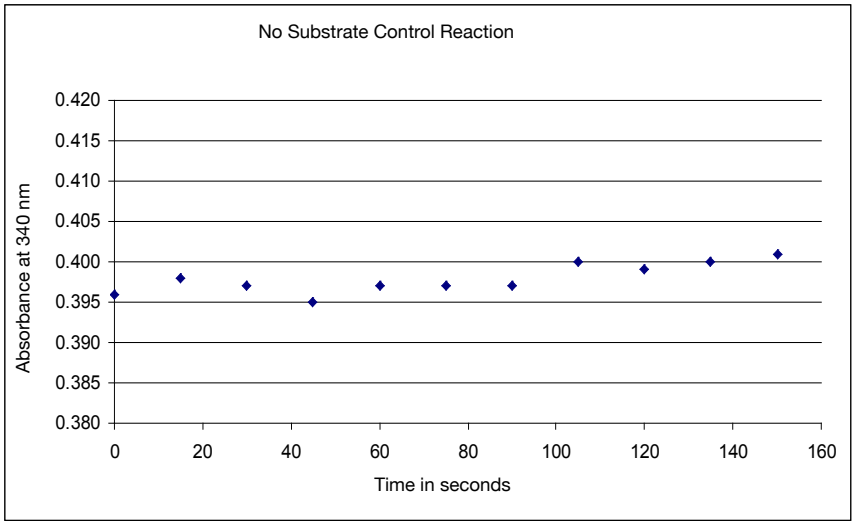


Figure 9.3. No substrate control reaction. When no substrate is present, the absorbance of the NADPH at 340 nm should remain fairly constant since it is not being converted to $NADP^+$. Some small fluctuations in the absorbance can be due to accuracy of the instrument in reading out to the final decimal 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.

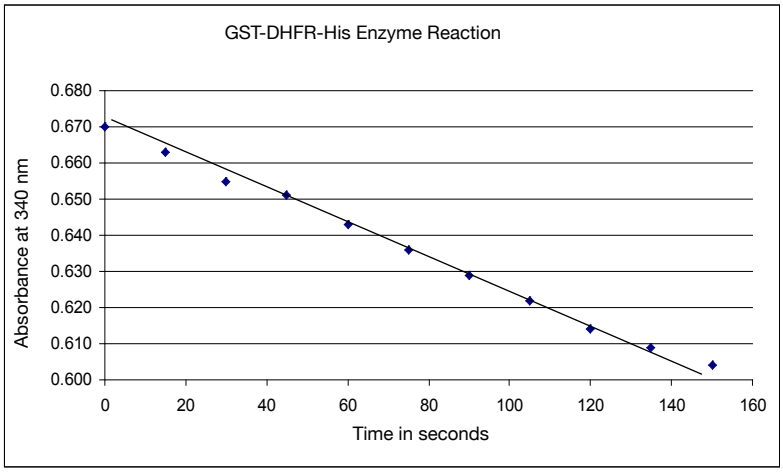


Figure 9.4. GST-DHFR-His enzyme reaction. When substrate, enzyme and cofactor are present, the absorbance of the NADPH at 340 nm should decrease if the enzyme is active since the NADPH is being converted to NADP+. From a regression analysis of the above data, the change in absorbance at 340 nm/minute is -4.5x10^{-4}/second or -0.027/min.

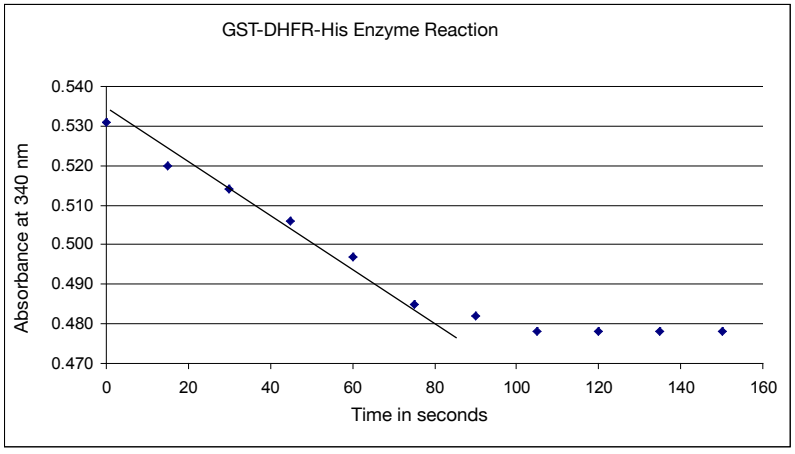


Figure 9.5. A reaction with too much GST-DHFR-His enzyme. This reaction flatlines, or runs out of DHF, by 95 seconds. After that, the absorbance does not change. The timeframe after 95 seconds is analogous to the No DHF Substrate Control reaction since in that reaction, no DHF was added; while in the 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

ΔOD = |ΔOD, 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:

$$\text{Solving for } \Delta C \text{ (mol/liter/min)} = \frac{0.025/\text{mi}}{6220\ M^{-1}\ cm^{-1} \times 1\ cm} = 4 \times 10^{-6} \text{ mol/liter/min}$$

Calculate how many moles of NADPH your GST-DHFR-His sample can break down per minute and record below.

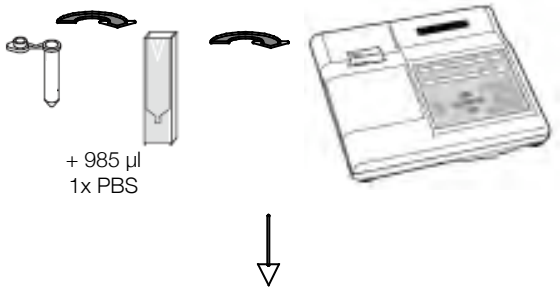
$$\Delta C \text{ (mol/liter/min)} = \frac{\Delta OD\ (min^{-1})}{6220\ M^{-1}\ cm^{-1} \times 1\ cm} = \_\_\_\_\_ \text{(mol/liter/min)}$$

DHFR Enzymatic Activity Assay

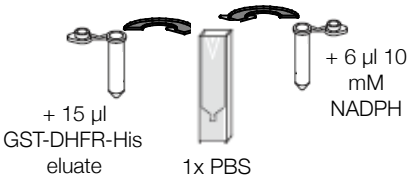
Determining which fraction to test for enzyme activity.
Review your SDS-PAGE gel from your purification to determine which fraction(s) to test. Note that centrifugation purification will have one desalted fraction while instrumentation-based purification will have three fractions.

Set up spectrophotometer for kinetics measurements at 340 nm.

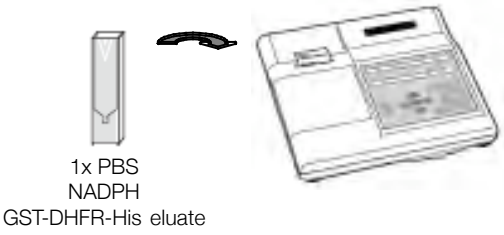
Blanking the instrument. Add 985 µl 1x PBS to cuvette; place in instrument, read as blank. Save cuvette with PBS.



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

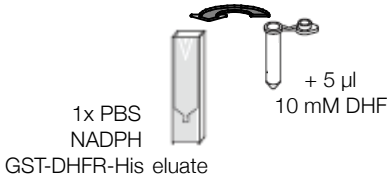


Place cuvette in spectrophotometer and begin kinetics run. As run is proceeding, record absorbance value every 15 seconds for 150 seconds. Remove and save cuvette from the spectrophotometer.



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 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 for 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.

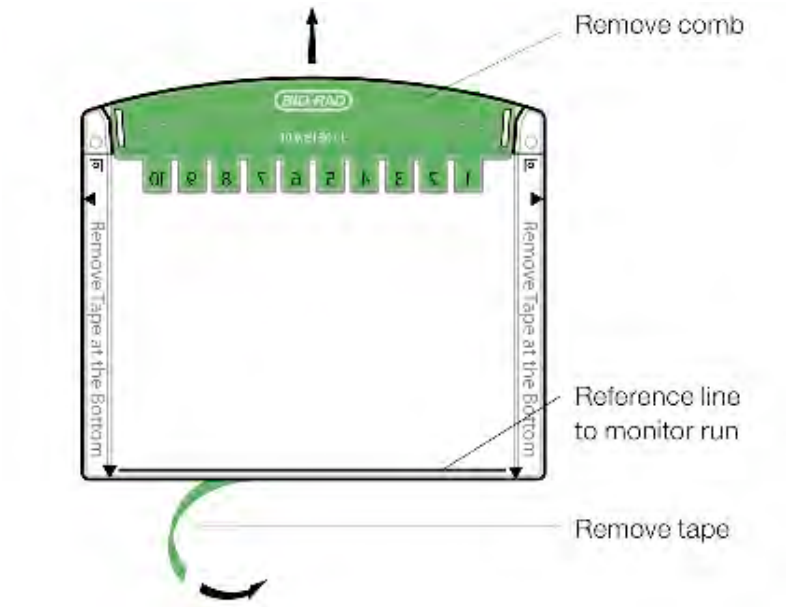


Figure 1. Mini-PROTEAN TGX gel.

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.

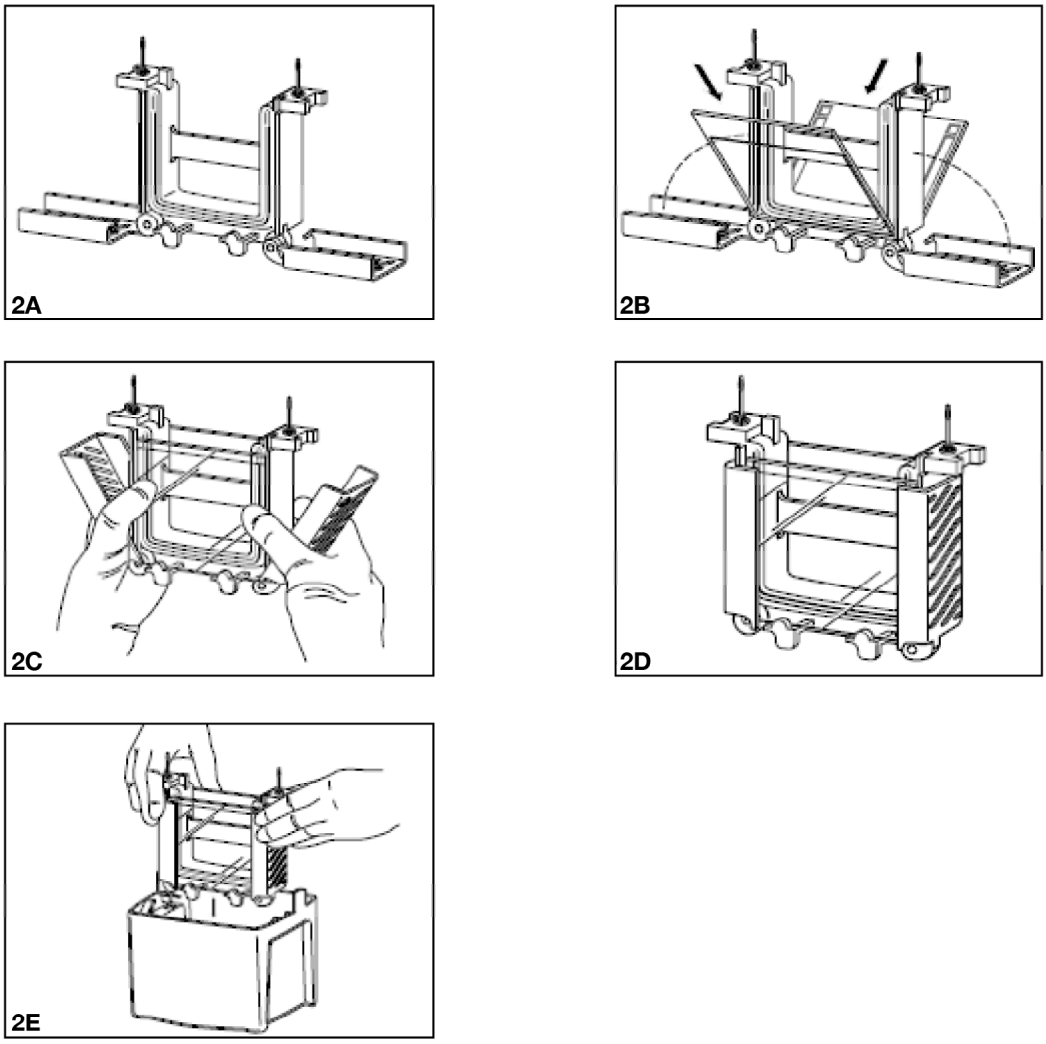


Fig. 2. Assembling the Mini-PROTEAN Tetra Cell Electrophoresis Module.

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).

3. 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.

4. 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).

5. 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.

6. 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.

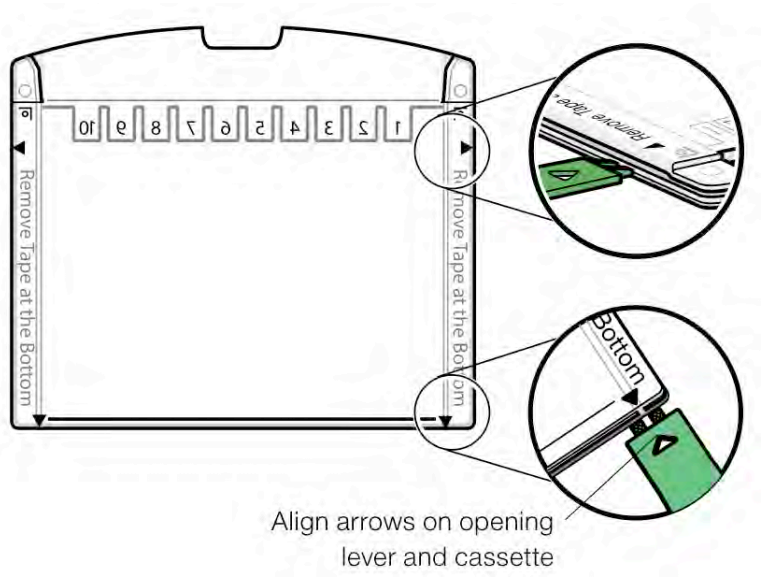


Figure 3. Opening the gel cassette.

Appendix 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.