Purification and Identification of Recombinant GFP

Laboratory 7 introduces a rapid method to purify recombinant green fluorescent protein (GFP) using hydrophobic interaction chromatography (HIC). The purified protein is then identified using polyacrylamide gel electrophoresis (PAGE). This lab is divided into two parts: Purification of GFP by HIC and PAGE Analysis of Purified GFP.

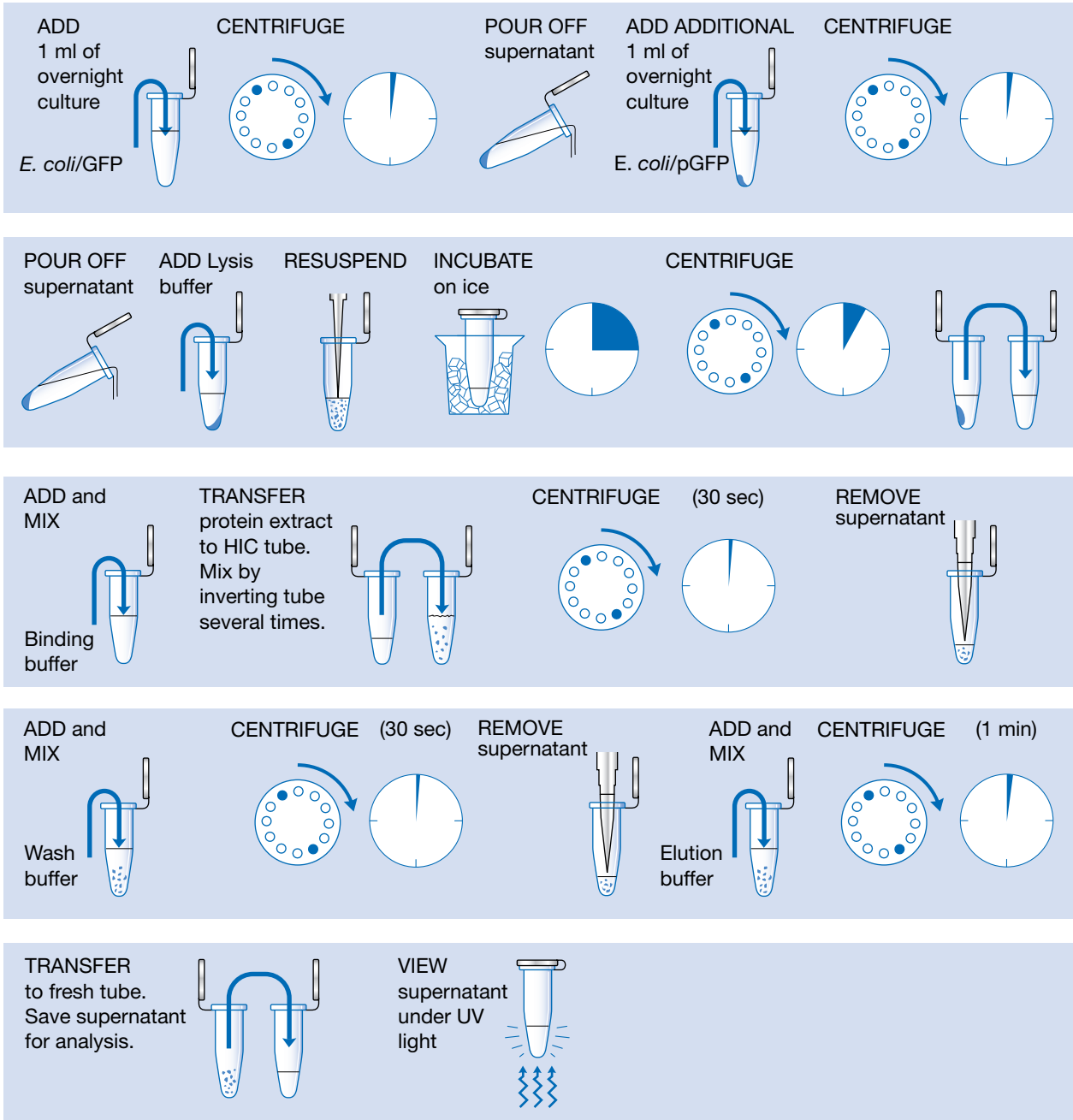
- Part A provides a procedure for purifying GFP. Cells taken from a GFP/ampicillin-resistant colony are grown to stationary phase in suspension culture. The cells from 2 ml of culture are harvested and lysed on ice to liberate GFP and other cellular proteins. During the first step of the purification, the cell lysate is incubated in a high-salt binding buffer. The charged ions in the binding buffer repel ions on the exterior of the GFP molecule, essentially flipping the GFP molecule inside out to reveal the hydrophobic chromophore. The exposed chromophore then binds tightly to the HIC resin, and successive washes in mid-salt elute unbound and weakly interacting proteins. Finally, incubation with low-salt TE buffer restores the normal structure of GFP and releases the protein from the resin. The eluted protein is transferred to a separate tube and its characteristic fluorescence is detected by exposure to long-wavelength UV light ("black light").
- Part B provides a technique whereby samples of the cell lysate, mid-salt wash, purified GFP, and a protein molecular-weight ladder are coelectrophoresed in a polyacrylamide gel and stained with Coomassie Blue. A single band of GFP protein is present in the lane of purified protein and comigrates with a size marker of approximately 27 kD. Bands and smears representing numerous cellular proteins are visible in the lysate and wash lanes, but are absent from the lane containing purified GFP.

Kits based on this laboratory are available from the Carolina Biological Supply Company.

- Catalog no. 21-1082: Module 1 (Green Gene Colony Transformation Kit)
- Catalog no. 21-1070: Module 2 (GFP Purification)
- Catalog no. 21-1071: Module 3 (Electrophoretic Analysis of GFP Purification)

PART A

Purification of GFP by HIC



PRELAB NOTES

GFP Expression in *E. coli*

It is critical that overnight cultures of *E. coli*/GFP be shaken *vigorously* to provide good aeration. Shake the cultures hard enough so that some air bubbles are introduced at the surface of the culture medium. Of course, oxygen is required for *E. coli* reproduction, plasmid amplification, and transcription/translation needed for good expression of GFP. However, oxygen is also critical for the post-translational formation of the GFP chromophore, upon which fluorescence depends. Without proper aeration, GFP-transformed strains of *E. coli* will fail to fluoresce.

The best protein yield is obtained from *fresh* cells grown in overnight suspension culture. Use cells taken directly from overnight culturing of not more than 16 hours; GFP begins to degrade in stationary-phase cells.

Batch Technique for Protein Purification

The best known methods of HIC involve passing a cell lysate through a vertical column packed with resin. However, the passage of lysate and washes through a column is inherently time-consuming. Packing and handling a column can be problematic, since even small air spaces in the resin inhibit the capillary action on which flow-through depends. Fortunately, the strong binding of GFP to hydrophobic resin makes column chromatography unnecessary. Rather, this simplified method uses a "batch technique" in which the entire purification takes place in a 1.5-ml tube. Use only clear tubes, since it is important to remove as little of the HIC resin as possible during the purification process.

HIC Bead Resin

The HIC bead resin consists of 50-μm porous beads that contain methyl groups ($-CH_3$). The methyl groups provide a strong interaction with hydrophobic proteins under high-salt conditions.

SDS-PAGE Molecular-weight Standards

Molecular-weight markers are available from many suppliers and assist in identifying GFP protein. Since GFP protein is about 27 kD, protein markers should range from 20 to 150 kD.

For Further Information

The protocol presented here is based on the following published methods.

Lin F.Y., Chen W.Y., and Hearn M.T. 2001. Microcalorimetric studies on the interaction mechanism between proteins and hydrophobic solid surfaces in hydrophobic interaction chromatography: Effects of salts, hydrophobicity of the solvent, and structure of the protein. *Anal. Chem.* **73:** 3875–3883.

PRELAB PREPARATION

Before performing this Prelab Preparation, please refer to the cautions indicated on the Laboratory Materials list.

1. The day before starting this laboratory, prepare an *E. coli* culture according to the protocol in Laboratory 2B, Overnight Suspension Culture. Inoculate the culture with a cell mass scraped from one colony selected from a +pGREEN plate, from Laboratory 5, Rapid Colony Transformation of *E. coli* with Plasmid DNA. Maintain antibiotic selection with LB broth plus ampicillin. Before incubating the overnight culture, gently swirl the culture tube until the colony is dispersed. Vigorous aeration in a shaking water bath or environmental shaker is essential to GFP fluorescence. Although incubation at 33°C is recommended for best expression, we have found that 37°C also works well.
2. The day before the experiment, equilibrate the HIC bead resin. Add 1000 µl of equilibration buffer to 500 µl of HIC beads in a 1.5-ml tube. Invert the tube several times to mix. Centrifuge at high speed for 1 minute. Remove the buffer layer without disturbing the equilibrated HIC beads.
3. Prepare aliquots for each experiment:

   2 ml of *E. coli*/GFP overnight culture
   500 µl of preequilibrated HIC beads
   600 µl of lysis buffer
   300 µl of binding buffer
   600 µl of wash buffer
   300 µl of elution buffer (TE)
4. Review Part B, PAGE Analysis of Purified GFP.

MATERIALS

REAGENTS

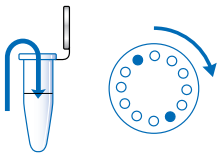
Binding buffer
E. coli/GFP overnight culture
Equilibration buffer
Glucose/Tris▼/EDTA (GTE)
Hydrophobic (HIC) bead resin
Lysis buffer
Tris▼-EDTA (TE) buffer
Wash buffer

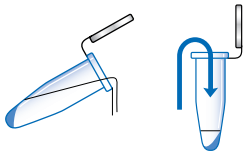
SUPPLIES AND EQUIPMENT

Beaker for waste/used tips
Microfuge
Micropipettor (100–1000 µl) + tips
Permanent marker
Shaking water bath (37°C) or incubator
Test tube rack
Tubes (1.5-ml clear)

▼ See Appendix 4 for Cautions list.

METHODS

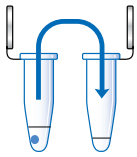




Purification of GFP by HIC

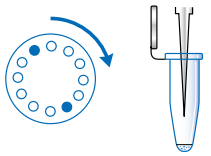
(40 minutes)

1. Shake the culture tube to resuspend *E. coli* cells.
2. Use a micropipettor to transfer 1 ml of the overnight *E. coli*/GFP culture into a 1.5-ml tube.
3. Close cap, and place the tube in a *balanced* configuration in the microfuge rotor. Spin for 1 minute to pellet cells.
4. Carefully pour off the supernatant into a waste beaker for later disinfection. *Do not disturb the green cell pellet.*
5. Repeat Steps 1–4 *in the same 1.5-ml tube* to pellet cells from a second 1-ml sample on top of the first pellet. This will result in a large green cell pellet. Resuspend the pellet in 50 µl of GTE buffer.
6. Add 500 µl of lysis buffer to the tube. Resuspend the pellet by pipetting in and out several times.
7. Incubate the tube for 15 minutes on ice. Incubation on ice helps prevent protein degradation.



Transfer 100 µl of remaining lysate to a new tube and freeze at –20°C for PAGE analysis in Part B.

8. Place the tube in a *balanced* configuration in the microfuge rotor. Spin for 5 minutes to pellet the insoluble cellular debris.
9. Transfer 250 µl of green cell extract (supernatant) into a clean 1.5-ml tube. *Do not disturb the pellet of cellular debris.*
10. Add 250 µl of binding buffer to tube containing 250 µl of green cell extract. Close cap, and mix solutions by rapidly inverting the tube several times.
11. Add 400 µl of the cell extract/binding buffer mixture to tube of hydrophobic bead resin. Close cap, and mix by inverting the tube several times.
12. Microfuge for 30 seconds. Gently remove the supernatant with a micropipettor. *Do not disturb the hydrophobic bead pellet.*
13. Add 400 µl of wash buffer to the hydrophobic bead pellet. Mix by inverting the tube several times. This step unbinds weakly interacting cellular proteins from the resin.



The hydrophobic chromophore of GFP binds to the resin beads.

14. Microfuge for 30 seconds. Gently remove the supernatant with a micropipettor. *Do not disturb the hydrophobic bead pellet.*
15. Elute the recombinant GFP by adding 200 µl of TE buffer to the hydrophobic bead pellet. Mix by inverting the tube several times.

16. Microfuge for 1 minute. The supernatant now contains the purified GFP. Use a micropipettor to transfer the recombinant GFP to a new 1.5-ml Eppendorf tube.
17. After observing under UV light, freeze purified GFP at –20°C for PAGE analysis in Part B. GFP will retain fluorescence while frozen.

RESULTS AND DISCUSSION

HIC is a simple and efficient means of isolating proteins with a hydrophobic domain. You should be familiar with the biochemical interactions taking place during each step of the purification.

- Chloride ions in the binding buffer repel negative charges in the exterior β sheath of the GFP molecule. This repulsion causes the molecule to flip inside out, exposing the nonionic (hydrophobic) chromophore.
- The exposed chromophore binds tightly to nonpolar methyl groups attached to the HIC resin.
- The mid-salt wash maintains GFP in its hydrophobic state, but it elutes unbound or weakly bound proteins in the cell lysate. These remain in solution.
- The low-salt buffer (TE) allows the chromophore to return to its normal position on the inside of the GFP molecule, releasing GFP from the resin.

1. What class of molecules does the lysis buffer interact with to release GFP from *E. coli* cells?
2. What aspect of GFP structure allows it to interact so strongly with the HIC beads?
3. How does the TE buffer release the GFP molecules from the HIC beads in Step 15?
4. HIC chromatography does not yield 100% pure GFP. What other types of cellular proteins would most likely be found in the GFP preparation?

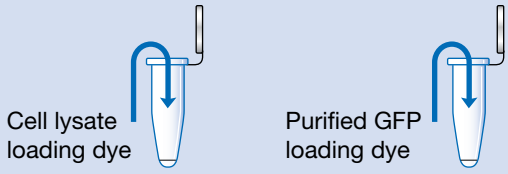
FOR FURTHER RESEARCH

Use a second method of protein chromatography to purify your HIC-purified GFP. Run protein samples from single and double chromatography purifications on SDS-polyacrylamide gel. Analyze the level of background native proteins between single and double purification schemes.

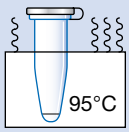
PART B

PAGE Analysis of Purified GFP

ADD

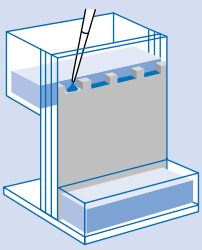


HEAT
tubes

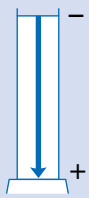


(2 min)

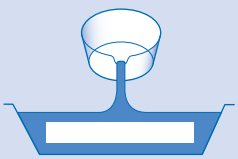
LOAD
gel



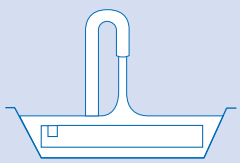
ELECTROPHORESE



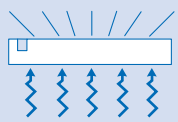
STAIN gel



RINSE gel



VIEW gel



PHOTOGRAPH gel

PRELAB NOTES

Analytical electrophoresis of proteins is usually carried out under denaturing conditions using a polyacrylamide gel. Before loading onto the gel, samples are incubated at 95°C to denature the proteins. The negatively charged detergent SDS, incorporated into the gel and running buffer, maintains the dissociation of the proteins into their polypeptide subunits and prevents protein aggregation.

Use Pre-Cast Polyacrylamide Gels

Acrylamide and bisacrylamide are neurotoxins, and the risk of handling these reagents makes them too hazardous to use in the teaching lab. We strongly recommend using pre-cast polyacrylamide gels, which are available from several supply companies. Although the polymerized polyacrylamide is not believed to be an immediate hazard, wear gloves even when working with pre-cast gels. Because pre-cast gels are relatively expensive, this is a good time to share a single gel among a number of groups.

Coomassie Blue

We recommend a Coomassie Blue stain designed for classroom use.

For Further Information

The protocol presented here is based on the following published methods:

Davis B.J. 1964. Disc electrophoresis. II: Method and application to human serum proteins. *Ann. N.Y. Acad. Sci.* **121:** 404–427.

Hames B.D., ed. 1998. *Gel electrophoresis of proteins: A practical approach,* 3rd edition. Oxford University Press, New York.

Laemmli U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680–685.

Ornstein L. 1964. Disc electrophoresis. I: Background and theory. *Ann. N.Y. Acad. Sci.* **121:** 321–349.

Sharp P.A., Sugden B., and Sambrook J. 1973. Detection of two restriction endonuclease activities in *Haemophilus parainfluenzae* using analytical agarose-ethidium bromide electrophoresis. *Biochemistry* **12:** 3055–3063.

PRELAB PREPARATION

Before performing this Prelab Preparation, please refer to the cautions indicated on the Laboratory Materials list.

1. Prepare aliquots for each experiment:

   5 μg of protein markers (store on ice). Volumes vary depending on supplier.
   40 μl of 2x protein loading dye.

2. Prepare 1x Tris-glycine-SDS buffer for electrophoresis (400–500 ml per gel).
3. Prepare Coomassie Blue protein stain (~100 ml per gel).
4. Adjust water bath to 95°C.

MATERIALS

REAGENTS

Coomassie Blue▼protein stain
HIC cell lysate
HIC-purified GFP
Tris/HCl/SDS polyacrylamide gel (12.5%)▼
2x Protein loading dye
Protein markers
1x Tris/glycine/SDS buffer▼

SUPPLIES AND EQUIPMENT

Beaker for waste/used tips
Electrophoresis chamber
Latex gloves
Micropipettor (0.5–10 µl) + tips
Permanent marker
Plastic wrap (optional)
Power supply
Test tube rack
Tubes (1.5-ml)
White light transilluminator▼ (optional)
Water bath (95°C)

▼ See Appendix 4 for Cautions list.

METHODS

I. Denature Proteins, Load Gel, and Separate by Electrophoresis

(1 hour, 30 minutes)

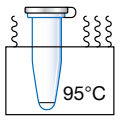
Your instructor may assign you a single sample to prepare for loading into a communal gel.

Refer to Laboratory 3, DNA Restriction and Electrophoresis, for detailed instructions.

1. Use a permanent marker to label two 1.5-ml tubes

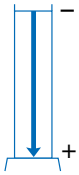
   CL = cell lysate (from Part A, Step 9)
   GFP = purified GFP (from Part A, Step 16)

2. Transfer 5 µl of cell lysate (CL) and 15 µl of purified GFP (GFP) into the appropriate tubes. Obtain a tube containing protein markers (PM). These volumes correspond to approximately 10 µg of total protein.
3. Add 5 µl of 2x protein loading dye to tube CL. Add 15 µl of 2x protein loading dye to tube GFP.
4. Heat the samples (including markers) for 2 minutes at 95°C to denature the proteins.
5. Load pre-cast 12.5% Tris/HCl/SDS polyacrylamide protein gel into a vertical gel chamber, and add 1x Tris-glycine-SDS buffer to both gel buffer chambers.
6. Load entire contents of each sample tube into a separate well in the gel, as shown in diagram on the following page. *Use a fresh tip for each reaction.*



It is often useful to wash the well by repeatedly pipetting running buffer into the wells. This should be done immediately before loading the wells to remove any unpolymerized acrylamide.

7. Close the tank of the electrophoresis unit and connect the electrical leads to a power supply, anode to anode and cathode to cathode. Electrophorese at 175 volts, until the bromophenol blue band has moved to the bottom of the gel. This should take approximately 1 hour.



8. Turn off power supply, disconnect leads from the inputs, and remove top of electrophoresis box.
9. Carefully remove gel cassette from the electrophoresis chamber. Open the cassette with a spatula, and transfer the gel to a staining tray.

CAUTION
Handle carefully. Polyacrylamide gels are easily torn.

STOP Cover electrophoresis tank and save gel until ready to continue. Gel can be stored in a zip-lock plastic bag and refrigerated overnight for viewing/photographing the next day. However, over longer periods of time, the proteins will diffuse through the gel and the bands will become indistinct or disappear entirely.

aining may be performed y an instructor in a con- olled area when students re not present.

II. Stain Gel with Coomassie Blue and View (Photograph) (3+ hours)

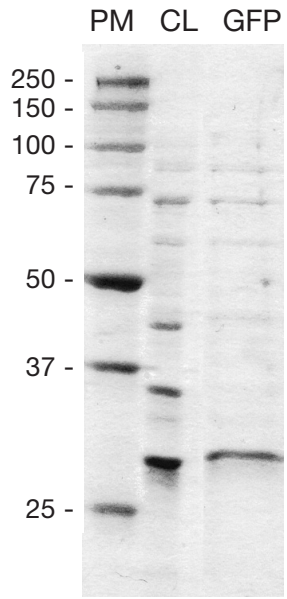
CAUTION
Wear latex gloves when staining, viewing, and photographing gel and during clean up. Confine all staining to a restricted sink area.

1. Flood the gel with Coomassie Blue protein stain, cover the staining tray, and allow the gel to stain from 1 hour to overnight.

2. Wearing gloves, pour off the staining solution, and remove excess stain by

RESULTS AND DISCUSSION

1. Describe the different properties and uses of agarose *versus* polyacrylamide gels.
2. Why are the protein samples incubated at 95°C prior to loading onto the polyacrylamide gel?
3. What is the function of the SDS detergent in the gel and running buffer?
4. How does Coomassie Blue stain the proteins in the gel? Why is it important to destain for a sufficient amount of time?
5. View your stained gel on a light box. Compare your gel with the ideal gel shown below, and try to determine which band represents GFP.
   a. Expose the gel to a UV light. Explain why the GFP band does not fluoresce.
   b. Why might you observe some bacterial proteins in your purified GFP lane?
6. Troubleshooting gels. What effect will be observed in the stained bands of protein in a polyacrylamide gel
   a. if the samples are not incubated at 95°C prior to loading?
   b. if the gel is run at too high or too low a voltage?
   c. if too much protein is loaded?
7. Predict the pattern of protein banding observed following SDS-PAGE of the lysate mixed with binding buffer (Part A, Step 10). How would this pattern differ following incubation with HIC beads (Part A, Step 12)? What protein banding pattern would be observed if the wash buffer (Part A, Step 14) was subjected to SDS-PAGE?



Ideal Gel

FOR FURTHER RESEARCH

Run your purified GFP samples on other types of polyacrylamide gels.

1. Under nondenaturing conditions, native PAGE allows proteins to maintain biological activity, so the GFP band should fluoresce when exposed to UV light. How does the staining pattern differ from SDS-PAGE?
2. Gradient PAGE uses differing gel concentrations along the length of the gel to achieve optimal separation of proteins in a wide range of molecular-weight proteins. How does the staining pattern differ from nongradient PAGE?