EXERCISE

2

Protein Expression, Purification, and Analysis

Background

The plasmid pGEX2–VirD2 (the PCR product *virD2* inserted into pGEX2) will express the fusion protein Gst–VirD2 (Gst = glutathione-*S*-transferase). Transcription of this hybrid gene will come from the *tac* promoter located upstream of *gst*.

The *tac* promoter is a hybrid of the *E. coli trp* and *lac* promoters; it is a very strong promoter regulated in the same manner as the *lac* promoter. The *lacI* gene encodes the Lac repressor, which binds to *lac* promoter/operator regions and inhibits transcription. In *lacI*+ cells, a single copy *lac* (or *tac*) promoter will be repressed by *lacI* unless an inducer, such as lactose or IPTG (isopropylthiogalactoside), is present. In multicopy plasmids (for example, pUC or pGEX), multiple copies of the *tac* promoter titrate all of the Lac repressor made in *lacI*+ strains. To regulate *tac* promoters on these multicopy plasmids, cells must overproduce the LacI protein. Strains carrying the mutant allele $lacI^q$ overexpress LacI at levels sufficient to regulate the *tac* promoter on multicopy plasmids. Full repression of the gene is not necessary, because the Gst–VirD2 fusion protein is not deleterious to *E. coli*.

When expressing foreign proteins in *E. coli*, protein insolubility frequently presents a significant problem. If insolubility problems arise, temperature, inducer concentration, and promoter strength may all be varied to increase the percentage of soluble recombinant protein. Fusion to Gst typically increases solubility.

Protease degradation of foreign protein in wild-type *E. coli* strains can also present problems. Various protease-deficient strains are sometimes used to combat this. For example, *E. coli* strain PR78 is deficient in the *lon* protease. PR78 contains Tn*10* (a transposable element that confers tetracycline resistance) inserted into the *lon* gene (*lon*::Tn*10*) to inactivate its expression. Lon− strains often increase the stability of foreign proteins.

After expression of Gst–VirD2, you will prepare crude cell extracts and purify the fusion protein by glutathione–Sepharose affinity chromatography. The glutathione-*S*-transferase moiety of the fusion protein binds to glutathione. After Gst–VirD2 is mixed with the glutathione–Sepharose beads and permitted to adhere, washing removes most other proteins and cellular debris. We will analyze the crude and purified extracts by SDS-polyacrylamide gel electrophoresis. We will detect the proteins in the gel chemically by the silver stain technique and immunologically by the Western blot technique.

Steps of the experiment are

A. Express and purify fusion protein

B. SDS-polyacrylamide gel electrophoresis

C. Silver stain detection of proteins

D. Western blot (immunoblot) detection of fusion protein

Notes

A. EXPRESSION AND PURIFICATION OF A FUSION PROTEIN

Introduction

You will express Gst–VirD2 in the *E. coli* strain MC1061. To help prevent protein degradation during protein purification, we will include 3 protease inhibitors in our buffer solutions and perform purification steps at 0 to 4°C.

You will extract, purify, and analyze proteins from 3 strains:

1. MC1061 (pGEX2–*virD2*)
2. MC1061 (pGEX2)
3. MC1061

The strain containing pGEX2 will express wild-type Gst protein, which will assure that the glutathione–Sepharose affinity purification works properly. Plasmidless MC1061 will show which chromosomally encoded proteins are not removed by the purification procedure.

Protease degradation can be a significant problem when a protein is expressed in wild-type *E. coli* strains. To examine this, a control group will express our protein in *E. coli* strain PR78, deficient in the Lon protease. Our experience shows that the Gst–VirD2 fusion protein is significantly more stable in Lon− than in Lon+ strains.

The control group will extract, purify, and analyze proteins from these strains:

1. PR78 (pGEX2–*virD2*)
2. PR78 (pGEX2)
3. PR78
4. MC1061 (pGEX2–*virD2*)—compare with PR78

Because MC1061 is not Lon protease deficient, you will see what effect this protease has on yield of recombinant protein.

Safety Precaution

Phenylmethylsulfonyl fluoride (PMSF) is dangerous if inhaled, swallowed, or absorbed through skin. Avoid contact. PMSF is inactivated in aqueous solution. Therefore, prepare a 10× stock solution in isopropanol (7.5 mM = 1.3 mg/mL) and freeze in aliquots. Add one-tenth volume of PMSF stock to PBS just before use. Aqueous solutions of PMSF can be discarded after raising the pH to greater than 8.6 and holding at room temperature for several hours.

Protocol

1. (TAs) Inoculate 2 mL of L broth (+50 μg/mL ampicillin) with 40 μL of fresh overnight culture of MC1061 harboring pGEX2–VirD2 and MC1061 harboring pGEX2. Inoculate 2 mL of LB (no antibiotic) with MC1061. Repeat for PR78. Incubate all cultures at 37°C with aeration for 2.5 to 3 hours until the culture is visibly turbid (Klett = 60). Chill microfuges in the 4°C cooler.
2. **(Students start here)** Add 20 μL of 100 mM IPTG (final concentration 1 mM); continue incubation for 1 to 2 hours.
3. Just before use, prepare a fresh solution of ice-cold PBS + protease inhibitors.
4. Centrifuge cells for 1 minute in microfuge, discard supernatant, and resuspend cells in 300 μL of ice-cold PBS + protease inhibitors. Remove and freeze (at −20°C) two 5-μL samples of intact cells for gels.

5. Lyse the remainder by two 10-second sonicator pulses. From this point, **keep all samples on ice** and process as rapidly as possible.
6. Centrifuge lysed cells for 5 minutes at 4°C in microfuge; retain supernatant.
7. Add 50 μL of glutathione–Sepharose–PBS slurry to supernatant (300 μL); mix gently at room temperature for 2 minutes.
8. Add 1 mL of PBS–protease inhibitors, vortex 30 seconds, centrifuge 5 seconds in microfuge, and remove supernatant.
9. Repeat step 8 three times; allow to stand for 5 minutes between vortex and centrifugation.
10. Remove two 10-μL samples of washed beads (pellet) for analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). To the 10-μL samples of beads, add an equal volume of 2× sample buffer and boil for 1 minute.
11. Microfuge 15 seconds, then hold samples on ice until ready to load the SDS-polyacrylamide gel.

Solutions for Protein Expression and Purification

100 mM IPTG: dissolve in sterile distilled water in a sterile tube; use a sterile spatula; store at −20°C.

Phosphate-buffered saline (PBS):

To make 1 L add	Final concentrations are
8.0 g NaCl	137 mM NaCl
0.2 g KCl	2.7 mM KCl
1.15 g $Na_2HPO_4 \cdot 7\ H_2O$	4.3 mM $Na_2HPO_4 \cdot 7\ H_2O$
0.2 g KH_2PO_4	1.4 mM KH_2PO_4

Protease inhibitor stocks:

7.5 mM PMSF; dissolve in isopropanol
100 mM Phenanthroline; dissolve in methanol
0.5 *M* EDTA, pH 8.0
2-Mercaptoethanol (14.4 *M*)
10 mg/mL Aprotinin

For each 10 mL of PBS add	Final concentrations are
1 mL PMSF	0.75 mM PMSF
500 μL phenanthroline	5 mM phenanthroline
200 μL EDTA	10 mM EDTA
6 μL mercaptoethanol	10 mM mercaptoethanol
2 μL aprotinin	2 μg/mL aprotinin

(A number of other protease inhibitors may also be used.)

Glutathione–Sepharose (agarose) slurry: Wash 1 mL of Pharmacia glutathione–Sepharose or Sigma glutathione agarose slurry 3 times with excess PBS; store as a 50% slurry in PBS at 4°C.

2× Sample buffer:
10 mL of 0.5M Tris, pH8.0
10 mL of 10% SDS
5 mL of 2-mercaptoethanol
10 mL glycerol
65.4 mg EDTA
4.0 mg bromphenol blue
65 mL H_2O

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B. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

Introduction

We use SDS-PAGE to separate proteins primarily according to their size. The anionic detergent SDS (sodium dodecyl sulfate) binds to the proteins, causes the polypeptides to denature, and imparts a large negative charge on the proteins. This SDS charge masks any charge normally present and equalizes the charge along the protein molecules. When an electric current is applied during SDS-PAGE, each protein in a sample migrates toward the anode at a rate inversely proportional to its molecular weight. For highly positively charged proteins, molecular weights calculated from migration during SDS-PAGE may differ significantly from the true molecular weight.

Polyacrylamide gel polymerization is catalyzed by tetramethyl ethylenediamine (TEMED) and ammonium persulfate. Once these reagents are added, the gel will begin to polymerize. Oxygen inhibits polymerization. The butanol overlay protects the gel surface from oxygen. You can tell when the gel is fully polymerized because a well-defined interface will form between the gel and the overlay; this usually takes about 15 minutes.

You will use a discontinuous gel. The purpose of this gel system is to concentrate the protein sample into a small volume, which increases the resolution of the gel. The top component, called the stacking gel, will be 6% acrylamide, pH 6.8. The sample buffer is also pH 6.8. The bottom component, called the resolving gel, is 12% acrylamide, pH 8.8. The sample moves quickly through the stacking gel and forms a tight band as it enters the resolving gel. The resolving gel then separates proteins according to their molecular weights.

Proteins are usually detected after SDS-PAGE by staining with silver or Coomassie blue (R250), or by the West-

ern blot procedure. Proteins may be radiolabeled during expression (usually with ^{35}S-methionine) and detected by autoradiography. Note that all of these detection methods are qualitative; do not use SDS-PAGE to quantify the amount of protein present in a sample.

Safety Precautions

Acrylamide is a neurotoxin; use gloves. Acrylamide is available in solution form; this prevents dust inhalation during handling.

Protocol

1. **Clean** and assemble glass plates and spacers. Clamp in place.
2. Prepare and pour resolving gel

   Combine: 3 mL of the 40% acrylamide stock
   2.5 mL of 1.5 *M* Tris, pH 8.8
   100 μL of 10% SDS
   4.3 mL of water
   in a flask and swirl gently to mix.

   Add: 100 μL of 10% ammonium persulfate
   10 μL of TEMED
   to mixture and swirl gently to mix.

   Pour gel using automatic pipettor (Pipet-Aid) or Pasteur pipet, and overlay with water-saturated *n*-butanol using a Pasteur pipet.
3. After resolving gel has polymerized, remove butanol, wash top surface with distilled water, dry gel surface with a Kimwipe, and place comb in position.
4. Prepare and pour stacking gel

Combine: 0.75 mL of the 40% acrylamide stock
1.25 mL of 0.5 *M* Tris, pH 6.8
50 μL of 10% SDS
2.93 mL of water
in a flask and swirl gently to mix.

Add: 50 μL of 10% ammonium persulfate
5 μL of TEMED
to mixture, swirl gently to mix, and pour gel using automatic pipettor or Pasteur pipet. To avoid catching air bubbles underneath the gel comb, slowly pipet gel solution down the inside edge of the apparatus.

5. After stacking gel has polymerized, pipet water onto top of gel, remove comb, and rinse wells with distilled water. Rinsing removes unpolymerized gel material; failure to rinse leads to uneven sample wells.

6. Remove bottom spacer, place gel in apparatus, add electrophoresis buffer, and remove air pocket underneath gel with a buffer-filled syringe and bent needle.

7. Add 1 volume of 2× sample buffer to each sample (except Sepharose bead samples, which are already prepared) and boil for 1 minute. For molecular weight marker, use 7.5 μL marker with 7.5 μL of sample buffer.

8. Load samples. Secure electrodes and apply 100 V (constant voltage) while samples migrate through stacking gel. When samples reach resolving gel, increase to 200 V. Proteins will migrate toward the positive electrode (anode). Continue electrophoresis until the bromphenol blue dye reaches the bottom of the gel. This will take approximately 20 minutes.

SDS-POLYACRYLAMIDE GELS: LOAD ORDER

A. MC1061

Lane	Plasmid	Strain	Sample	Volume Sample (μL)	Volume 2× SB (μL)
1			molecular weight marker	7.5	7.5
2	none	MC1061	resuspended cells	5	5
3	pGEX2	MC1061	resuspended cells	5	5
4	pGEX2–VirD2	MC1061	resuspended cells	5	5
5			molecular weight marker	7.5	7.5
6	none	MC1061	purified extract	10	10
7	pGEX2	MC1061	purified extract	10	10
8	pGEX2–VirD2	MC1061	purified extract	10	10
9			molecular weight marker	7.5	7.5

B. PR78 (*lon*−) controls

Lane	Plasmid	Strain	Sample	Volume Sample (μL)	Volume 2× SB (μL)
1			molecular weight marker	7.5	7.5
2	none	PR78	resuspended cells	5	5
3	pGEX2	PR78	resuspended cells	5	5
4	pGEX2–VirD2	PR78	resuspended cells	5	5
5	pGEX2–VirD2	MC1061	resuspended cells	5	5
6	none	PR78	purified extract	10	10
7	pGEX2	PR78	purified extract	10	10
8	pGEX2–VirD2	PR78	purified extract	10	10
9	pGEX2–VirD2	MC1061	purified extract	10	10
10			molecular weight marker	7.5	7.5

Solutions for SDS-Polyacrylamide Gel Electrophoresis

Acrylamide stock: use purchased 40% acrylamide, 29:1 acrylamide:bis-acrylamide. Or use 40% acrylamide stock:

Combine 387 g of acrylamide + 13 g bis-acrylamide (bis controls the extent of cross-linking). Bring to 1 L with distilled water.

0.5 *M* Tris, pH 6.8 (use Tris base)

1.5 *M* Tris, pH 8.8 (use Tris base)

10% SDS

TEMED

10% Ammonium persulfate: make fresh and store at 4°C for no longer than 1 week; use distilled water.

TG-SDS running buffer:	Final concentration
57.6 g glycine	380 mM
12.0 g Tris base	50 mM
2.0 g SDS	0.1 % (w/v)

Bring to 2 L with water

2× Sample buffer:

10 mL of 0.5 *M* Tris, pH 6.8
10 mL of 10% SDS
5 mL of 2-mercaptoethanol
10 mL glycerol
65.4 mg EDTA
4.0 mg bromphenol blue
65 mL water

100 mL total volume

Molecular weight markers:

Bio-Rad Low Range Silver Stain SDS-PAGE standards
Bio-Rad Kaleidoscope Prestained Standards (for Western blot)

Notes

C. SILVER STAIN DETECTION OF PROTEINS

Introduction

We will use the ICN Rapid-Ag silver stain kit. If you have questions about the kit, refer to the instructions provided with the kit. Procedures are also available to prepare silver stain reagents from laboratory stocks. To prevent contamination of stock solutions, wear gloves while preparing solutions and use a fresh pipet for each measurement.

Technical Tip

Silver stain will detect fingerprints, glove prints, dust, and anything else that touches your polyacrylamide gel. Handle gels with gloves, and only at the edges.

Procedure

1. Prepare 200 mL of gel fixing solution (40% methanol + 10% glacial acetic acid).
2. **Do not handle gel.** Use a glass plate to support the gel; place both in a tray containing 200 mL of gel fixing solution and gently float the gel free of the glass plate. Soak the gel for at least 30 minutes with gentle agitation (at room temperature). There is no time limit on this step; it can continue overnight.
3. Rinse with 200 mL water, then agitate in 200 mL water for 15 minutes. Aspirate to remove solutions; this reduces stress on the gel.
4. Prepare pretreat solution, silver stain, developer, and stop bath.

5. Remove water, add 200 mL pretreat solution, and agitate for 10 minutes.
6. Remove pretreat solution, add 200 mL silver stain solution, and agitate 30 minutes.
7. Remove silver stain and rinse gel with 200 mL water.
8. Wash 4 times with 200 mL water (each) for 2 minutes per wash.
9. Add 200 mL developer and agitate until bands reach the desired darkness. Stop development by adding 2 mL of glacial acetic acid.
10. Remove developer and soak gel in 200 mL of 1% glacial acetic acid for 5 minutes.
11. Remove dilute glacial acetic acid and soak gel in 200 mL water for 5 minutes.
12. Glycerol fix: soak gels in 20% ethanol, 10% glycerol for 30 minutes before drying. This prevents cracking of the gel.
13. Soak 2 pieces of cellophane in glycerol fix. Lay 1 piece of cellophane on plastic wrap. Place your gel on top. Pour 5 to 10 mL glycerol fix over the gel. With a rolling motion, lay the 2nd piece of cellophane over the gel. Remove bubbles. Lay the gel and cellophane on the plastic square, put the plastic frame on top, and clamp. (The apparatus resembles a picture frame and back, minus the glass; it holds the gel flat.) Dry for 2 days.

Solutions for Silver Stain Detection of Proteins

ICN Rapid-Ag Stain Kit

Gel fixing solution:	80 mL methanol
	20 mL glacial acetic acid
	100 mL water
	200 mL total
Pretreat solution:	
combine (sequentially):	80 mL methanol
	20 mL ethanol
	90 mL water
	10 mL ICN solution #1
swirl to mix	200 mL total
Silver stain:	
combine (sequentially):	10 mL ICN solution #2
	10 mL ICN solution #3
(swirl to dissipate	10 mL ICN solution #4
brown color)	170 mL water
	200 mL total
Developer:	380 mL water
	10 mL ICN #5
	10 mL ICN #6
	400 mL total

Stop bath: 1% glacial acetic acid

20% Ethanol, 10% glycerol

Notes

D. WESTERN BLOT (IMMUNOBLOT) DETECTION OF PROTEINS

Introduction

The Western blot procedure is used to detect a specific protein among a mixture of proteins, or to show that a particular purified (or partially purified) protein is recognized by a specific antiserum. After SDS-PAGE, proteins are transferred by electrophoresis (also called electroblotting) to a nitrocellulose, nylon, or PVDF (polyvinylidene difluoride) membrane. A blocking agent binds nonspecific sites on the membrane. Primary antibodies raised against an antigen (the protein of interest) then bind specifically to protein antigens fixed to the membrane. The primary antibody is bound by a secondary antibody, which may be conjugated to a number of different "reporter" enzymes or molecules such as alkaline phosphatase, peroxidase, or biotin. In our experiment, the secondary antibody is conjugated to alkaline phosphatase; alkaline phosphatase activity is detected by conversion of a colorless substrate, 5-bromo-4-chloro-3-indolyl phosphate (BCIP), into a blue indigo derivative.

Because the strength of the Western blot signal (color reaction) depends on both the affinity of the antibody for the antigen and the concentration of antigen present, the Western blot technique is **not** considered a quantitative measure of antigen concentration. An enzyme-linked immunosorbent assay (ELISA) **can** be used as a quantitative measure of antigen concentration. ELISAs are usually based on the same types of color reactions as immunoblots.

Two different types of antibody preparations are used for immunoblotting: polyclonal and monoclonal antibodies. Polyclonal antibodies are prepared by direct immunization of an animal with the antigen, and consist of the full repertoire of the animal's circulating antibodies. Po-

lyclonal antisera may contain antibodies highly specific for your antigen and others that recognize spurious antigens. Monoclonal antibodies are prepared by repeated direct immunizations of an animal followed by dissection of the animal's spleen (source of antibody-producing cells) and fusion of those spleen cells with a tumor cell line. The resulting monoclonal cell line expresses antibodies to a single antigenic epitope.

Technical Tips

Nitrocellulose membranes are fragile and sensitive to fingerprints. **Pick up the membrane (in a corner) with clean forceps only.**

For experiments that use nylon or nitrocellulose membranes (Westerns, Southerns, Northerns), contaminants on the membrane cause significant background. Follow the times and temperatures for membrane washes in each of these procedures; skimping results in dirty blots.

Protocol

1. After SDS-PAGE, place the unstained gel in 50 mL standard transfer buffer and rock for 10 minutes.
2. Cut the nitrocellulose filter to the size of the gel, and cut 2 pieces of Whatman 3 MM paper to the size (15 × 20 cm) of the Scotchbrite pads supplied with the electroblot apparatus (Mini-Genie Immunoblotters from Idea Scientific). Soak the pads, nitrocellulose, and Whatman 3 MM paper in transfer buffer. Do not touch the gel or any membranes or filters with ungloved hands.
3. Lay down 1 pad and cover with a sheet of Whatman 3 MM. Place the gel on the 3 MM paper and lay the

nitrocellulose membrane over the gel. Lay a piece of Whatman 3 MM paper over the membrane. Use a pipet to roll out air bubbles between the gel and the membrane, and finally cover the entire sandwich with the other Scotchbrite pad.

4. Place the sandwich in the apparatus; it is extremely important that the sandwich is tight. Use additional Scotchbrite pads if necessary. Fill the chamber with transfer buffer, and apply 500 mA of current for approximately 30 minutes (this depends on the size of the protein you wish to transfer).

5. Wash the nitrocellulose membrane with rocking for 5 minutes in 15 mL distilled water (in a small plastic container).

6. Soak the membrane in 15 mL TBS for 10 minutes with rocking.

7. Soak membrane for 1 hour in 15 mL blocking solution (TBS + Tween 20 + gelatin), with rocking.

8. Pour off blocking solution. Wash the membrane in 15 mL TBS for 5 minutes with rocking.

9. Add 15 mL TBS. Add 15 μL of serum containing primary antibody (polyclonal antiserum raised in rabbit against glutathione-*S*-transferase antibody, 1 mg/mL); final concentration is 1 μg/mL in TBS. Mix thoroughly. Incubate, with rocking, at room temperature for 1 hour. This incubation can continue overnight.

10. Pour off buffer. Wash 3 times with 15 mL of TTBS for 5 minutes each with rocking.

11. Pour off buffer and add 15 mL TBS to dish. Now add 15 μL of secondary antibody (antiserum raised in goats against rabbit antibodies; the secondary antibody is conjugated to alkaline phosphatase). Incubate for 30 minutes at room temperature with rocking.

12. Pour off buffer, then wash 3 times in 15 mL TTBS for 5 minutes each.
13. Pour off buffer and replace with 15 mL TBS. Leave in TBS until ready to add substrate.
14. Pour off last wash, then add 10 mL of substrate solution. Incubate until color reaction is sufficient.
15. Rinse blot in distilled water. Dry on Whatman paper.

Solutions for Western Blot

Transfer buffer: 25 mM Tris (3 g/L Tris base)
192 mM glycine (14.4 g/L)
20% methanol (200 mL/L)
0.1% SDS (1 mL/L)

TBS (Tris-buffered saline): 20 mM Tris, pH 7.5
150 mM NaCl

TTBS: TBS + 0.1% Tween 20

Blocking solution: TBS
0.1% Tween 20
1% gelatin

Primary antibody: Upstate Biotechnologies anti-Gst; purified polyclonal IgG antibody raised in rabbits against Gst expressed by pGEX2.

ICN Western Blot Staining Kit: this kit supplies secondary antibody (goat polyclonal antiserum made against rabbit antigens), and color staining reagents (nitro blue tetrazolium and BCIP).

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EXERCISE 2. Study Questions

1. Crude extracts of plasmidless MC1061 contained a number of proteins that reacted with the anti-Gst antibody. Do these bands have anything to do with Gst or the Gst–VirD2 fusion protein? Explain.

2. You saw bands on the Western blot that were plasmid-specific. We suspect that these bands correspond to Gst and Gst–VirD2. Measure the mobility of the molecular weight standards, plot a standard curve on semilog paper, and estimate the molecular weight of each plasmid-specific band. Can you conclude that these antibody-stained bands represent Gst and Gst–VirD2?

3. Do the antibody-stained bands on the Western blot match silver-stained bands on the previous gel?

4. There are 2 faint plasmid-specific bands corresponding to low molecular weight positions on some of the Western blots. How are these formed?

5. Must plasmid-specific bands correspond to proteins encoded by genes on the plasmid? How could you prove that a protein is plasmid encoded, and not simply regulated by a gene on a plasmid?