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HSV-1 Protein Expression Using Recombinant Baculoviruses

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Abstract

The baculovirus expression system is an invaluable method for the expression of Herpes Simplex Virus 1 (HSV-1) proteins. The use of insect cells provides a eukaryotic system for the robust expression of heterologous proteins under control of the baculovirus polyhedrin gene promoter that naturally drives the high expression of the polyhedrin protein. Additionally, insect cells often initiate the necessary posttranslational modifications and/or disulfide-bond formation important for the proper folding of the protein. We and others have successfully expressed and purified several HSV-1 proteins including the polymerase, helicase-primase, single-strand DNA binding protein, and alkaline nuclease. The following protocol is based on 15 years of experience from our laboratory.

Keywords

Baculovirus; HSV protein expression; Protein expression; Viral protein expression; Insect cells; Sf9; Transfer plasmid; pFastBac; Bacmid

1 Introduction

The Baculoviridae family of insect viruses contains a large, double-stranded, circular DNA genome, and these viruses have been utilized extensively for recombinant protein expression in cultured insect cells [1–3]. This system is widely used for the expression of Herpes Simplex Virus 1 (HSV-1) proteins and has many advantages over *Escherichia coli* expression systems that can result in poor protein expression especially for proteins that require posttranslational modification or disulfide-bond formation for proper folding and activity. Examples of HSV-1 proteins that have been successfully expressed and purified in the baculovirus system include the polymerase, helicase-primase, single-strand DNA binding protein, and alkaline nuclease [4–8].

The expression of recombinant protein in the baculovirus system is generally under control of the late polyhedrin gene promoter. Polyhedrin is the most abundantly expressed baculovirus protein and makes up approximately half of the total protein present at the end of the viral life-cycle [9]. Importantly, polyhedrin was found to be nonessential for

production of viral progeny in cultured insect cells making it an ideal target for gene swapping [10]. The most commonly used baculovirus for recombinant protein expression is *Autographa californica* multiple nucleopolyhedrosis virus (AcMNPV) that readily infects *Spodoptera frugiperda* cells (Sf9) and *Yes, correct the species name as suggested* (High Five) insect cells.

We have used the Bac-to-Bac system developed by Life Technologies-Invitrogen to produce recombinant viruses for the expression of HSV-1 proteins. Briefly, a viral gene is cloned into a transfer plasmid that is then used to transform a specially engineered *Escherichia coli* strain, DH10Bac (Life Technologies-Invitrogen). DH10Bac contains the baculovirus bacmid as well as a helper plasmid that expresses the transposase. The expression of transposase from the helper plasmid initiates the transposition of the viral gene from the transfer plasmid into the bacmid. The transfer vector contains two Tn7 coding sequences flanking the cloned viral gene. Transposition occurs between the transfer vector and the bacmid containing both a mini-ATT Tn7 target site (directing the location of the transposed viral gene) and an engineered *LacZ* gene that is replaced by the gene of interest. Positive baculovirus recombinants are identified by the loss of *LacZ* expression on selective LB agar plates. The recombinant baculovirus DNA is purified and used to transfect insect cells to generate a baculovirus stock, which in turn can be used for the expression of the HSV-1 viral protein within insect cells.

2 Materials

1. Transfer plasmid: such as pFastBac (Life Technologies-Invitrogen), engineered to contain the HSV-1 viral gene of interest.
2. DH10Bac competent cells (Life Technologies-Invitrogen), which contain the baculovirus shuttle vector and helper plasmid containing transposition genes, necessary to generate the recombinant bacmid.
3. 42 °C waterbath.
4. S.O.C. broth medium (NEB).
5. Incubator at 37 °C (Fisher Scientific).
6. pFastBac transfer plasmid selection plates and medium: LB agar plates and LB medium containing 100 µg/mL ampicillin.
7. Recombinant bacmid selective LB agar plates: LB agar containing 50 µg/ml kanamycin, 7 µg/ml gentamicin, 10 µg/ml tetracycline, 100 µg/ml Bluo-gal (Life Technologies-Invitrogen), and 40 µg/ml IPTG (isopropyl β-D-1-thiogalactopyranoside) (*see* Note 1).
8. Recombinant bacmid selective LB medium: LB medium containing 50 µg/ml kanamycin, 7 µg/ml gentamicin, and 10 µg/ml tetracycline.

¹Bluo-gal is light sensitive, so plates should be stored in the dark at 4 °C.

9. Cell resuspension buffer: 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100 µg/ml RNaseA.
10. Cell lysis solution: 200 mM NaOH, 1 % SDS.
11. Neutralization buffer: 3.0 M potassium acetate, pH 5.5.
12. TE Buffer: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.
13. 3 M sodium acetate, pH 5.5.
14. 70% and 100% Ethanol.
15. Laminar flow hood.
16. Phase contrast inverted microscope.
17. Hemocytometer.
18. Trypan blue solution, 0.4 % (Sigma).
19. Cellfectin Reagent (Life Technologies-Invitrogen). A newer formulation of this transfection reagent (Cellfectin II Reagent) is now available, and the reader is directed to the manufacturer's instructions for its use as the transfection procedure differs from that described below for the Cellfectin Reagent.
20. Sf-900 II serum-free insect cell culture medium (Life Technologies-Invitrogen).
21. Fetal Bovine Serum (FBS) (Gemini Bio-Products).
22. Insect cells such as Sf9 for the generation of viral stocks and/or protein expression. High Five cells for protein expression.
23. Phosphate-buffered saline (PBS): 137 mM NaCl, 27 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4.
24. Lysis buffer for insect cells: 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 % Triton X-100, protease inhibitors.
25. Protease inhibitor cocktail (Sigma).
26. 1× SDS-PAGE loading buffer: 50 mM Tris-HCl, pH 6.8, 10 % glycerol, 2 % SDS, 100 mM DTT, 0.05 % bromophenol blue.
27. Low temperature incubator at 27 °C (Precision Scientific 815).
28. Orbital shaker at 27 °C, 120 rpm Innova 44 (New Brunswick).
29. Bench top centrifuge, Allegra X-15R (Beckman Coulter).
30. Refrigerated centrifuge, Sorvall RC-5B (Du Pont Instruments).
31. 15 ml polypropylene conical tubes (BD falcon).
32. 50 ml polypropylene conical tubes (BD falcon).
33. 14 ml polypropylene round-bottom tubes (BD falcon).
34. 5 ml polystyrene round-bottom tubes (BD falcon).

35. 6-well tissue culture plates (BD falcon).
36. 60 mm tissue culture plates (BD falcon).
37. Tissue culture flasks: T75 and T165 (Corning).
38. Flasks for cell suspensions: 125, 250, 500, and 1,000 ml (Corning).
39. 15 ml glass dounce tissue grinder set (Wheaton).
40. 50 ml Oakridge tubes.

3 Method

This protocol was adapted from the Life Technologies-Invitrogen Bac-to-Bac instruction manual and begins after the successful cloning of the gene of interest into the multi-cloning site of an appropriate transfer vector. Transfer plasmids containing fusion tags are also available that may simplify subsequent purification steps; however, the addition of a tag could interfere with the function of the protein. Thus, it is important to ensure that the tagged recombinant protein is properly folded and functional.

3.1 Transformation, Transposition, and Selection of Positive Recombinants in DH10Bac Cells

1. Purify the transfer plasmid containing the viral gene of interest using standard methods for plasmid purification. For pFastBac derived transfer plasmids use pFastBac transfer plasmid selection plates and medium.
2. Thaw an aliquot of competent DH10Bac cells (Life Technologies-Invitrogen) on ice. For each transformation use 30 μ l of competent cells (*see* Note 2).
3. Add 40–80 ng of the transfer plasmid from Subheading 3.1 **step 1** to 30 μ l of competent cells in a sterile microcentrifuge tube, tap cells gently to mix and keep mixture on ice for 30 min. Include negative control with no DNA and a positive control plasmid that will not undergo transposition (*see* Note 3).
4. Heat-shock cells for 90 s in a 42 °C waterbath.
5. Place tubes back on ice for 2 min.
6. Add 900 μ l of S.O.C. medium.
7. Shake cells at 37 °C at 220 rpm for 4 h. The cells transformed with the positive (no transposition) control should be shaken for only 1 h to ensure recovery of the transformed cells and avoid the loss of the plasmid.
8. Make tenfold serial dilutions (10^{-1} , 10^{-2} , 10^{-3}) for each transformation using SOC medium. Spread 100 μ l of each dilution onto the recombinant bacmid selective LB agar plates.

²Use sterile techniques for transforming cells with recombinant transfer plasmids. Use autoclaved or sterile microcentrifuge tubes, pipette tips, media, etc.

³Invitrogen supplies positive controls to identify colonies that have successfully undergone transposition in the Bac-to-Bac System.

9. Incubate plates at 37 °C for a minimum of 48 h. Positive recombinants form white colonies (*see* Note 4).
10. Streak the white positive colonies onto bacmid selective LB agar plates to confirm the growth phenotype and to isolate a purified colony (*see* Note 5).

3.2 Extraction and Purification of the Recombinant Bacmid

1. Pick a colony of each isolated recombinant bacterial strain and inoculate 5 ml of recombinant bacmid selective LB medium. Incubate at 37 °C at 200 rpm for 14–16 h.
2. Pellet the 5 ml cell culture in a 15 ml conical tube at $3,000 \times g$ for 5 min at 4 °C in a Sorvall RC-5B refrigerated centrifuge using an SS-34 rotor fitted with tube adapters (*see* Note 6).
3. After the spin, pour off the supernatant (*see* Note 7).
4. Resuspend cell pellet in 300 µl of resuspension buffer. Transfer resuspended cells to a 1.5 ml microcentrifuge tube.
5. Add 300 µl of the cell lysis solution, to the resuspended cells. Invert tube six times to mix well and let sit at room temperature 5 min. In this step and in Subheading 3.2, **step 6**, care should be used to invert the tube gently to avoid shearing of the DNA.
6. Add 420 µl of the neutralization buffer. Gently invert tube six times to mix well and place on ice for 5 min.
7. Centrifuge lysed cells in a microcentrifuge at $15,000 \times g$ for 10 min.
8. Carefully transfer the cleared lysate (supernatant) to a clean 14 ml round-bottom centrifuge tube taking care to avoid the pellet.
9. Add 1/10 volume of 3 M sodium acetate, pH 5.5 to the cleared lysate and gently mix.
10. Add 3 volumes of ice cold 100 % ethanol and gently mix.
11. Place the tube on dry ice or at –80 °C for 15 min to precipitate the DNA.
12. Pellet DNA at $10,000 \times g$ at 4 °C for 1 h in a benchtop centrifuge, Allegra X-15R (Beckman Coulter) using an FX-6100 rotor fitted with 18 mm adapters.
13. Decant the supernatant and wash the remaining DNA pellet with 1 ml 70 % ethanol. Transfer the ethanol containing the washed pellet to a 1.5 ml microcentrifuge tube.

⁴If it is difficult to differentiate the white colonies from blue colonies, store plate in the cold room for an additional 24 h. Negative colonies produce a deeper blue color with increased storage time making it easier to identify positive clones.

⁵Approximately 5–10 % of the plated cells will be positive. Isolate 6–10 colonies for each recombinant virus and verify the presence of the viral gene by colony PCR using primers from within the baculovirus backbone that are designed to amplify the viral gene. Make a glycerol stock of each positive bacterial strain for long-term storage at –80 °C.

⁶Any centrifuge capable of generating a similar g force could be used in this and other steps in this protocol that require centrifugation.

⁷For the best transfection efficiency, prepare the bacmid DNA within 1–3 days before performing the transfection.

14. Spin for 5 min at $15,000 \times g$ in a microcentrifuge to repellet the DNA.
15. Carefully pipette or aspirate the ethanol from the pellet and allow it to air dry.
16. Solubilize the DNA pellet in approximately 75 μ l of TE buffer (*see* Note 8).
17. Measure the concentration of the recombinant DNA and load 1 μ g on a 0.5–0.7 % agarose gel to visualize the DNA prep. Two bands are expected: the slower migrating band corresponds to the recombinant bacmid (>100 kb) and a faster migrating band that corresponds to the helper plasmid (approximately 6 kb).
18. Confirm that the viral gene of interest is present in the bacmid by PCR. Primers are available from Invitrogen; pUC/M13 forward and reverse primers anneal to a sequence in the bacmid and may be used to confirm the presence of the gene of interest. Alternatively, a primer pair containing one of the pUC/M13 primers that anneals to the bacmid and a second primer that anneals within the gene of interest can be used.

3.3 Transfection of Sf9: Production of P1 Viral Stock

Cell culture work should be performed under a laminar flow tissue culture hood using sterile conditions.

1. Seed 60 mm tissue culture plates with 1.2×10^6 Sf9 cells (50–60 % confluent) grown in Sf-900 II Insect Cell Culture medium supplemented with 5 % FBS and incubate at 27 °C for a minimum of 2 h to ensure the cells are well attached to the surface of the plate.
2. Transfect Sf9 cells with 1–2 μ g of freshly prepared recombinant bacmid DNA. For each transfection prepare two 1.5 ml microcentrifuge tubes with 100 μ l of Sf-900 II medium without FBS. Add 1–2 μ g of DNA to one tube and 6–10 μ l of Cellfectin lipid reagent to the second tube. Include a negative control containing only Cellfectin reagent (*see* Note 9).
3. Mix the two tubes and incubate at room temperature for 30–45 min.
4. While the DNA–lipid mixture is incubating (Subheading 3.3, **step 3**), gently wash the 60 mm plates containing the cultured Sf9 insect cells two times with Sf-900 II medium without FBS (*see* Notes 10 and ¹¹).
5. After the incubation of DNA with Cellfectin (Subheading 3.3, **step 3**), add 800 μ l of Sf-900 II serum-free medium to the DNA–lipid mixture. Remove the Sf-900 II medium from the washed cells in **step 4**, and carefully pipette the DNA–lipid mixture over the cells taking care not to dislodge the cells. Gently rock the plates to

⁸The volume of TE buffer used to solubilize the DNA pellet depends on the size of the pellet. In this step, 75 μ l is a suggested volume for solubilizing a DNA pellet of approximately 1–2 mm in diameter. Store the DNA in single use aliquots at –20 °C.

⁹If the concentration of bacmid DNA is less than the helper plasmid (determined in Subheading 3.2) it may be necessary to increase the amount of DNA. Additionally, the volume of the Cellfectin reagent may need to be optimized. Typically 10 μ l works well in our hands. Mix the Cellfectin reagent, by inverting 5–10 times before use.

¹⁰Use caution when washing the cells to avoid releasing them from the surface of the plate. Add the medium to the side of the well and avoid releasing the liquid with too much force.

¹¹To prevent the Sf9 cells from drying out, leave them in the serum-free medium from the second wash until the incubation of the DNA–lipid mixture is complete. Remove the medium before adding the transfection mixture.

ensure the mixture covers all the cells and incubate at 27 °C for 5 h with occasional rocking, every 15–30 min.

6. Remove the transfection medium and add 3 ml of fresh Sf-900 II medium supplemented with 5 % FBS.
7. Return plates to the 27 °C incubator for a minimum of 72 h. After 48 h of incubation, plates can be monitored for morphological changes. Control cells continue to divide and become overly confluent, while transfected cells fail to divide due to senescence and appear larger in size (*see* Note 12).
8. Harvest by scraping the medium and cells into a sterile 15 ml conical tube and centrifuge at $1,000 \times g$ for 5 min at 4 °C. The supernatant is expected to contain recombinant baculovirus (P1 viral stock) and should be stored in a foil covered tube (protected from light) at 4 °C. To confirm the presence of recombinant baculovirus, the cell pellet can be analyzed for expression of recombinant protein by resuspending the cell pellet in 200 µl of 1× SDS-PAGE loading buffer and subjecting a 20 µl aliquot to SDS-PAGE followed by Coomassie staining or Western analysis.

3.4 Infection of Sf9: Virus Amplification to Generate P2 Stock

1. Prepare a 15 ml suspension culture of Sf9 insect cells using a 125 ml flask at a cell concentration of 2×10^6 cells/ml. Infect cells with the recombinant baculovirus by adding 100–500 µl of the P1 stock. Include an uninfected control using the same conditions to compare cell morphology and cell growth as the infection proceeds (*see* Notes 13–15).
2. Incubate each flask at 27 °C and 120 rpm in an orbital shaker.
3. Monitor the changes in the cell morphology beginning at 48 h post infection by collecting a 100 µl aliquot from the infected and uninfected culture flasks. Cells are mixed with an equal volume of trypan blue for observation under an inverted microscope using a hemocytometer. Size changes in the infected cell population can be observed using the hemocytometer grid. When approximately 70–80 % of the cells are dead as determined by trypan blue staining, the virus stock should be harvested.
4. Collect the cells and medium in a 15 ml conical centrifuge tube, pellet cells at $1,000 \times g$ for 5 min at 4 °C and collect the P2 viral stock (supernatant) in a sterile tube. Store the viral stock protected from light at 4 °C (*see* Note 16).

¹²Changes in cell morphology may be difficult to visualize. If so, transfected cells can be analyzed directly to confirm expression of recombinant protein via Coomassie stain or Western blot analysis following SDS-PAGE. An antibody that detects the HSV protein of interest directly or the fusion tag (if present) can be used for Western blot analysis.

¹³Since the virus titer in the P1 stock can be variable and low, it is important to monitor the cells beginning after 48 h to assess how the infection is proceeding. It may be necessary to use a larger inoculum if the cells do not appear to be infected (cell number is similar to the uninfected control and cells do not increase in size). On the other hand, extensive cell lysis would suggest that a smaller volume of the viral stock should be used.

¹⁵This step may also be performed using a monolayer of cells in a 162 cm² tissue culture flask.

¹⁶The cells from this step can be analyzed to confirm expression of recombinant protein via Coomassie stain or Western analysis following SDS-PAGE.

3.5 Determination of Inoculum Volume and Cell Type for Optimal Protein Expression

Although it is possible to determine viral titer of the P2 stock via plaque assay, we and others have found baculovirus plaques somewhat difficult to count, and we have developed an empirical method for optimizing the amount of P2 stock for protein expression.

1. Seed the wells of one six-well plate with 2×10^6 Sf9 cells and the wells of a second six-well plate with 1×10^6 High Five cells in Sf-900 II supplemented with 5 % FBS. Cells should be 70–80 % confluent before infection (*see* Note 17).
2. Prepare a series of twofold dilutions of the P2 stock in serum-free Sf-900 II medium by placing 1 ml of concentrated P2 stock in a sterile tube. The second dilution will contain 500 μ l of P2 stock and an equal volume of media. Continue three more times to make a series of 5 dilutions including the concentrated stock. Include a mock-infected control.
3. Carefully remove the medium from the wells, and add 250 μ l of each dilution to the sidewall of each well to avoid disrupting the cell monolayer. Gently rock the plate from side-to-side to evenly spread the inoculum over the cells.
4. Incubate the plates at 27 °C for 1 h while rocking the plates every 15 min.
5. After 1 h add 2 ml of Sf-900 II medium containing 5 % FBS and incubate the plates at 27 °C for 48 h (*see* Note 18).
6. Scrape the media and cells with a rubber spatula and collect the total contents into 5 ml round-bottom centrifuge tubes. Pellet the cells at $1,000 \times g$ for 5 min at 4 °C.
7. Remove the supernatant and resuspend each pellet in 200 μ l of 1 $\times$ SDS-PAGE loading buffer and subject a 10–20 μ l aliquot to SDS-PAGE followed by Coomassie staining or Western analysis. Identify the dilution of P2 stock capable of producing the largest amount of protein and calculate the volume of P2 stock in that dilution. For instance, in the dilution series from Subheading 3.5, **step 2**, the undiluted stock corresponds to 250 μ l, the first dilution to 125 and so on. This number will be used to calculate a “multiplicity of infection” for large-scale protein production in Subheading 3.6.

3.6 Scaling Up the Infection for Protein Expression

Once the optimal inoculation volume, cell type and infection period have been determined, large-scale protein production can be performed using cells grown in suspension or monolayer culture (*see* Note 19).

1. For protein expression in suspension culture, add 500 ml of cells at 2×10^6 cells/ml (10^9 cells) to a 2 L flask. Infect by adding P2 stock directly to the suspension

¹⁷Some recombinant proteins can be expressed more efficiently in High Five and others in Sf9 cells.

¹⁸The length of infection (48, 60, or 72 h) can also influence protein production and should be optimized for each new recombinant virus.

¹⁹Generally, Sf9 cells can be easily converted from a monolayer to a suspension culture by scraping the monolayer of cells from the surface of the flask in the presence of Sf-900 II medium. The cell solution is repeatedly pipetted into a serological pipette to obtain a homogenous cell suspension. In our hands, High Five cells have been less amenable to conversion to suspension culture; therefore, we generally use monolayer cultures in 162 cm² for protein expression in these cells.

culture using the following calculation: multiply the volume of P2 stock (in milliliters) that generated optimal protein expression by the culture volume, 500. Divide this number by 2 to determine the final inoculation volume. For example, if the first dilution gave maximal expression, convert 125 μ l to 0.125 ml, multiply by 500 and divide by 2. Inoculum will be approximately 31 ml.

2. For protein expression in monolayer cell cultures, multiple 162 cm² flasks are seeded to 80–90 % confluency (approximately 2×10^7 cells per flask). Multiply the volume of P2 stock (in μ l) by 5. For example if the second dilution was optimal, multiply 125 by 5 and add 625 μ l of the P2 stock to 5 ml of Sf-900 II medium. Remove medium from each flask and infect cells with the 5 ml inoculum. Incubate at 27 °C for 1 h while rocking the flasks every 15 min. After 1 h, add 15 ml of Sf-900 II medium containing 5 % FBS.
3. Incubation of the infected suspension (Subheading 3.6, **step 1**) or monolayer (Subheading 3.6, **step 2**) cultures are carried out at 27 °C for varying lengths of time ranging from 46 to 72 h (*see* Note 20).
4. After the incubation period, harvest suspension cells by pelleting the infected cells in 50 ml conical centrifuge tubes at $1,000 \times g$ at 4 °C. Monolayer cultures are harvested by scraping the cells and media into 50 ml conical centrifuge tubes followed by centrifugation at $1,000 \times g$ at 4 °C. Remove the supernatant and resuspend the pellet in 40 ml of cold PBS containing protease inhibitors. Repellet cells at $1,000 \times g$ at 4 °C and pour off the supernatant. Freeze the cell pellet in liquid nitrogen and store at –80 °C until ready for protein purification.

3.7 Protein Purification

Infected cell pellets are lysed for protein purification as outlined below:

1. Resuspend the frozen cell pellet in approximately 20–40 ml of insect cell lysis buffer and keep on ice for 15–30 min to allow the cells to swell (*see* Note 21).
2. Lyse the cells using a 15 ml dounce homogenizer (20–25 times on ice). Alternative methods include sonication and/or freeze/thawing of the cells in liquid nitrogen. Collect the disrupted cells in 50 ml Oakridge tubes.
3. Separate the lysed cells into nuclear and cytoplasmic fractions by centrifugation at $1,000 \times g$ for 10 min at 4 °C. Collect the cytosolic fraction (supernatant) in one tube and resuspend the nuclear fraction (pellet) in an equal volume of PBS containing protease inhibitors and subject these fractions to SDS-PAGE analysis to determine the location of the recombinant protein.

After lysis, subsequent purification steps must be tailored to each protein of interest based on whether the protein is native or tagged and whether it is in the nucleus or cytoplasm. For proteins that localize to the nucleus, the isolated nuclei are treated with high salt to extract

²⁰The length of incubation for optimal expression varies and can range from 46 to 72 h. Incubating longer than 72 h is not recommended due to excessive cell lysis leading to diminished amounts of recovered protein.

²¹Depending on the protein to be purified, it may be necessary to adjust the NaCl or KCl concentration in the lysis buffer. For instance, purification of the major DNA binding protein, ICP8, requires 1.2 M NaCl to facilitate extraction from the nucleus.

the protein. On the other hand, for proteins that localize in the cytosol, the cytoplasmic fraction is used for the subsequent purification steps. Native proteins can be purified using ammonium precipitation followed by ion exchange, affinity, and/or gel-filtration chromatography. Tagged proteins can be utilized to facilitate purification; for example, His-tagged proteins can be directly purified using a Ni²⁺ affinity column although additional purification steps may be necessary. In addition, it is important to collect and analyze a sample from each step of the purification process to learn where the protein may have been lost if the final protein yield is low.

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