

### **I. Pilot protein induction with IPTG**

1. Transform homemade chemically competent C41 bacteria (variant BL21 strain) with the desired pGEX plasmid.
2. At the end of the day, start one 5 ml overnight culture in LB/Amp.
3. Dilute the overnight culture 1:100 by adding 50  $\mu$ l into 5 ml LB/Amp. Start four 5 ml cultures (one each for the IPTG concentrations that will be tested).
4. Place the pilot cultures in a 37°C shaker for 2-3 hours until OD 0.6-0.8 is reached.
  - *Protein induction is optimal when bacteria are in mid-log phase*
  - *Put the tubes in at an angle in the shaker to aerate the cultures better and speed the growth of the bacteria*
5. Add IPTG (1 M stock, Sigma #16758) at the following concentrations to induce protein synthesis: 0.4mM, 1mM, 2mM, 5mM. 1M IPTG stock is stored in the -20°C freezer.
6. Return the cultures to the 37°C shaker for 24 hours, taking samples (30  $\mu$ l culture + 10  $\mu$ l 4X sample buffer) every hour for 5 hours and then one at 24 hours.
  - *For larger proteins that require longer induction times, it may be necessary to take additional time points between 5 and 24 hours to optimize the induction time.*
7. Run the samples on an appropriate percentage SDS-PAGE and stain with Coomassie blue.
  - *For the first pilot, the optimal IPTG concentration and induction time is the condition that leads to the maximum amount of induced protein with the lowest IPTG concentration and the shortest incubation time*

### **II. Large-scale protein induction and pilot purification**

1. From Section I Step #2, add the entire overnight to 500 ml LB/Amp in a 2 L flask. Place the large-scale culture in a 37°C shaker for 2–3 hours until OD 0.6–0.8 is reached.
2. Add IPTG at the optimal concentration determined in Section I and leave in 37°C shaker for the optimal time determined in Section I.
3. Split the 500 ml culture into two 250 ml cultures, spin down (4000 rpm for 10 min) at the end of the induction, and pour off the supernatant. Pellets can be stored at -20°C for about one month.
4. Thaw bacteria pellet from ~250 ml of culture at room temperature for 10 minutes.
5. Resuspend the bacterial pellet in 7.5 ml TNE buffer plus protease inhibitors. Collect the first fraction (F1: 3  $\mu$ l sample + 12  $\mu$ l H<sub>2</sub>O + 5  $\mu$ l 4x sample buffer) to confirm the protein induction in the bacteria. To each 7.5 ml of TNE add 36.5  $\mu$ l 1 M DTT (stored at -20°C) and 40  $\mu$ l 50 mg/ml lysozyme (made in PBS and stored at -20°C).
6. Transfer the bacterial suspension to a polycarbonate centrifuge tube and place on the nutator in the cold room for ~20 min.
7. Add 10 mg deoxycholic acid (sodium salt) as a dry powder. Incubate at room temperature on the nutator for ~15 min.
8. Add 10  $\mu$ l DNase I (Sigma #4527, 5mg/ml stock) and 50  $\mu$ l 2 M MgCl<sub>2</sub>. Incubate at room temperature for 10 min on the nutator.
9. Centrifuge at 9000RPM (~12000g) at 4°C for 15 min. Collect the second fraction (F2: 3  $\mu$ l sample + 12  $\mu$ l H<sub>2</sub>O + 5  $\mu$ l 4x sample buffer) from the supernatant to check whether the induced protein has remained soluble or gone into inclusion bodies.
10. While spinning, prepare the GSH beads. Wash the beads twice with 1 ml TNE lysis buffer. Resuspend the beads in ~500  $\mu$ l TNE lysis buffer and transfer into a 15 ml conical tube (keep on ice).
  - Stock GSH beads are stored in the 4°C fridge as a 1:1 bead slurry (with NETN + 0.02% sodium azide)
  - For each 7.5 ml lysate you need 500  $\mu$ l bead slurry. Aliquot 500  $\mu$ l of bead slurry (use cut pipet tip) into separate microcentrifuge tubes.

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8. Transfer the clarified bacterial lysate into the 15 ml Falcon tube and incubate on the nutator at 4°C for 3.5 hours or overnight.
9. Centrifuge tubes at 1000 rpm for 1 min. Collect the third fraction (F3: 3 µl sample + 12 µl H<sub>2</sub>O + 5 µl 4× sample buffer) from the supernatant to check for the efficiency of GST capture on the GSH beads.
10. Carefully aspirate off supernatant and then wash the beads three times with 10 ml ice-cold PBS + 0.5% Triton and twice with 10 ml ice-cold PBS, spinning at 1000 rpm for 1 min between washes.
11. Resuspend the beads in an equal volume of ice-cold PBS (50:50 slurry) and then collect the fourth fraction with a cut pipet tip (F4: 15 µl sample + 5 µl 4× sample buffer).
  - *The fraction is overloaded compared to the others to check for cleavage products and weak purifications*
12. Run F1-F4 on an appropriate percentage SDS-PAGE and stain with Coomassie blue.
13. For proteins that are retained through F4, beads are now ready for thrombin digest (Section IV) or glutathione elution (Section V). For proteins that are lost at the F2 fraction as inclusion bodies, proceed to Section III.

### III. Troubleshooting insoluble proteins (if needed)

1. Thaw bacteria pellet from ~250 ml of culture at room temperature for 10 minutes.
  - *The second 250 ml pellet from the 500 ml culture can be used for this purpose*
2. Resuspend the bacterial pellet in 7.5 ml TNE buffer plus protease inhibitors. Collect the first fraction (F1: 3 µl sample + 12 µl H<sub>2</sub>O + 5 µl 4× sample buffer) to confirm the protein induction in the bacteria. To each 7.5 ml of TNE add 36.5 µl 1M DTT (stored at -20°C) and 40 µl 50 mg/ml lysozyme (made in PBS and stored at -20°C).
3. Transfer the bacterial suspension to a polycarbonate centrifuge tube and place on the nutator in the cold room for ~20 min.
4. Add 10mg deoxycholic acid (sodium salt) as a dry powder. Incubate at room temperature on the nutator for ~15 min.
5. Add 10 µl DNase I (Sigma #4527, 5mg/ml stock) and 50 µl 2M MgCl<sub>2</sub>. Incubate at room temperature for 10 min on the nutator.
6. Split the bacterial lysate into eight 800 µl aliquots. Supplement each aliquot with one of the following detergent concentrations:
  - 200 µl 10% sarkosyl (2% sarkosyl)
  - 150 µl 10% sarkosyl + 50 µl H<sub>2</sub>O (1.5% sarkosyl)
  - 100 µl 10% sarkosyl + 100 µl H<sub>2</sub>O (1% sarkosyl)
  - 50 µl 10% sarkosyl + 150 µl H<sub>2</sub>O (0.5% sarkosyl)
  - 200 µl 10% Triton X-100 (2% Triton)
  - 100 µl 10% Triton X-100 + 100 µl H<sub>2</sub>O (1% Triton)
  - 50 µl 10% Triton X-100 + 150 µl H<sub>2</sub>O (0.5% Triton)
  - 200 µl H<sub>2</sub>O (no detergent control)
  - *Sarkosyl and Triton can solubilize certain proteins from inclusion bodies, and these concentrations are compatible with glutathione affinity purification*
7. Centrifuge at 9000RPM (~12000g) at 4°C for 15 min. Collect the second fraction from each aliquot (F2: 3 µl sample + 12 µl H<sub>2</sub>O + 5 µl 4× sample buffer) from the supernatant to check whether the induced protein has remained soluble or gone into inclusion bodies.
8. Run F1-F2 on an appropriate percentage SDS-PAGE and stain with Coomassie blue.
  - *The optimal detergent concentration is the lowest that retains the maximum amount of soluble protein in the F2 fraction*

### IV. Pilot and large-scale thrombin digestion

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1. Make 1:25, 1:50, 1:100, and 1:200 serial dilutions of thrombin enzyme (EMD #69671) in Thrombin Dilution/Storage Buffer (this can be stored for several weeks in the  $-20^{\circ}\text{C}$  freezer).
2. Assemble the following five reactions:
  - 5  $\mu\text{l}$  10 $\times$  thrombin buffer
  - 10  $\mu\text{g}$  target protein (estimate 10  $\mu\text{l}$  bead slurry (use cut pipet tip))
  - 1  $\mu\text{l}$  diluted thrombin (one of four dilutions or water negative control)
  - 34  $\mu\text{l}$   $\text{H}_2\text{O}$
  - 50  $\mu\text{l}$  total volume
3. Incubate the reactions at room temperature on the nutator and remove 10  $\mu\text{l}$  samples at 2 hours, 4 hours, 8 hours, and one between 16–24 hours and combine with 10  $\mu\text{l}$  2 $\times$  sample buffer.
  - *For each sample, spin down the reaction and pull the sample from the supernatant, being careful not to pull any beads*
  - *Also take a sample of the beads at the end of the digestion experiment (one for each thrombin dilution) to confirm that the GST fusion protein has been digested completely*
  - *A 0-hour time point from the original bead sample (10  $\mu\text{l}$  bead slurry + 40  $\mu\text{l}$   $\text{H}_2\text{O}$  + 50  $\mu\text{l}$  2 $\times$  sample buffer = 100  $\mu\text{l}$  total, good for five 0-hour time points)*
4. Run the digested samples on an appropriate percentage SDS-PAGE and stain with Coomassie blue.
  - *Remember that the cleaved protein will run 26 kDa lower than the GST-fused protein that was on the beads*
  - *The optimal thrombin condition is the lowest thrombin concentration and shortest incubation time that leads to the maximum release of full-length recombinant protein*
5. For scale-up on the remainder of the GSH beads, assemble the following reaction:
  - 125  $\mu\text{l}$  10 $\times$  thrombin buffer
  - 250  $\mu\text{l}$  beads (use cut pipet tip)
  - X  $\mu\text{l}$  thrombin (from pilot experiment, 1:25 dilution = 2  $\mu\text{l}$ ; 1:50 dilution = 1  $\mu\text{l}$ ;  
1:100 dilution = 0.5  $\mu\text{l}$ ; 1:200 dilution = 0.25  $\mu\text{l}$ )
  - X  $\text{H}_2\text{O}$
  - 1.25 ml total volume
  - *The amount of thrombin must be scaled according to the mass of GST fusion protein*
  - *10  $\mu\text{l}$  1:1 bead slurry  $\rightarrow$  500  $\mu\text{l}$  bead slurry (50-fold increase)*
  - *$\sim$ 40  $\mu\text{l}$  reaction volume  $\rightarrow$  1 ml reaction (25-fold increase)*
  - *Therefore, the concentration of thrombin will be twofold higher in the scaled up digest*
6. After digesting for the optimal time (determined in Step #4), spin down the reaction and pull off the supernatant.
7. Prepare p-aminobenzamidine beads (Sigma #A7155): wash beads (20  $\mu\text{l}$  per 2U thrombin) twice with 10 volumes PBS, then resuspend in an equal volume of PBS and add the entire slurry to the supernatant.
8. Incubate at room temperature on the nutator for 30 min.
9. Spin down the p-aminobenzamidine beads and run the purified protein over a NAP-10 column (Amersham #17-0854-02) for buffer exchange (see separate protocol). Elute into 1 $\times$  PBS, then add glycerol to a final concentration of 10% (v/v) and store working aliquots at  $-80^{\circ}\text{C}$ .
10. Quantify the final protein concentration by SDS-PAGE with a BSA standard.

## V. Glutathione elution

1. Centrifuge tubes at 1000 rpm for 1 min and aspirate the supernatant.
2. Add 1.2 ml glutathione buffer (10 mM Glutathione in 50 mM Tris pH 8.0) + 1.2  $\mu\text{l}$  2 M DTT and incubate for 1 hour at  $4^{\circ}\text{C}$ .
3. Spin at 1000 rpm for 1 minute and transfer the supernatant to a fresh tube (always keeping proteins on ice). Recentrifuge the tubes containing the supernatant to pellet any residual GSH beads.

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4. Take 30  $\mu$ l + 10  $\mu$ l 4 $\times$  sample buffer for SDS-PAGE.
5. Run the remaining protein solution through a NAP-10 column (Amersham #17-0854-02) for buffer exchange (see separate protocol). Elute into 1 $\times$  PBS with 10% glycerol and store working aliquots at –80°C.
6. Quantify the final protein concentration by SDS-PAGE with a BSA standard.

**Buffer recipes**

- **TNE buffer** Prepare fresh  
1.5 ml 1 M Tris, pH 7.4 (50 mM)  
0.9 ml 5 M NaCl (150 mM)  
60  $\mu$ l 0.5 M EDTA (1 mM)  
5  $\mu$ l Leupeptin (10 mg/ml in H<sub>2</sub>O)  
15  $\mu$ l Aprotinin (10 mg/ml in H<sub>2</sub>O)  
30  $\mu$ l Pepstatin (1 mg/ml in MeOH)  
27.5 ml H<sub>2</sub>O  
30 ml total volume (good for 2  $\times$  250 ml cultures)
- **NETN buffer** Store at 4°C  
20 ml 1 M Tris, pH 8.0 (20 mM)  
2 ml 0.5 M EDTA (1 mM)  
5 ml NP-40 substitute (0.5%)  
20 ml 5 M NaCl (100 mM)  
953 ml H<sub>2</sub>O  
1 L total volume

**GSH bead preparation**

1. Rehydrate 10 ml of glutathione agarose beads (Sigma #G4510) in 40 ml H<sub>2</sub>O on a nutator for 1 hr at 4°C.
2. Centrifuge beads at 3000 rpm on a swinging bucket rotor for 5–10 min at 4°C.
3. Aspirate supernatant, wash beads with 40 ml NETN buffer, and repeat Step 2.
4. Repeat Step 3.
5. Resuspend beads in 40 ml NETN buffer + 1% (w/v) BSA and incubate on a nutator for 1 hr at 4°C.
6. Centrifuge beads at 3000 rpm on a swinging bucket rotor for 5–10 min at 4°C.
7. Aspirate supernatant, wash beads with 40 ml NETN buffer, and repeat Step 6.
8. Repeat Step 7 three times (four total washes).
9. Resuspend beads in 10 ml NETN buffer + 0.02% (w/v) sodium azide.
10. Make 0.5 ml aliquots in microcentrifuge tubes and store at 4°C.
  - *Be sure to dispense with a 5- or 10-ml pipette so that the beads do not have time to sediment during aliquotting*