

Antibody production

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1. Materials

1.1 2X SDS-PAGE Buffer

0.09 M TrisCl, pH6.8

20% glycerol

2% SDS

0.02% bromophenol blue

0.1 M DTT

1.2. Lysis Buffer

50 mM potassium phosphate, pH 7.8

400 mM NaCl

100 mM KCl

10% glycerol

0.5% Triton X-100

10 mM imidazole

1.3. Buffer B

100 mM NaH₃PO₄

10 mM TrisCl

8 M urea

Adjust pH to 8.0 using NaOH

1.4. Buffer C

100 mM NaH₃PO₄

10 mM TrisCl

8 M urea

Adjust pH to 6.3 using HCl

1.5. Buffer D

100 mM NaH₃PO₄

10 mM TrisCl

8 M urea

Adjust pH to 5.9 using HCl

1.6. Buffer E

100 mM NaH₃PO₄

10 mM TrisCl

8 M urea

Adjust pH to 4.5 using HCl

2. Methods

A. Protein purification (for insoluble protein):

1. Clone the genes of interest into pENTR (Invitrogen).
2. Mobilize the gene of interest into pDEST17 (Invitrogen) through L/R reaction.
3. Confirm the correct clones and transform them into BL21-AI competent cells (Invitrogen).
4. Test protein expression conditions:
 - 4.1. Grow 5ml LB medium containing 50 µg/ml carbenicillin to OD₆₀₀ 0.6-1.0.
 - 4.2. Use this culture to inoculate a fresh LB culture to an OD₆₀₀ of 0.05-0.1.
 - 4.3. Grow the culture until OD₆₀₀ is about 0.4.
 - 4.4. Split the culture into two. Add L-arabinose to a final concentration of 0.2% to one of the cultures.
 - 4.5. Remove 500 µl aliquot from each culture at 0, 1, 2, 3 and 4 hours, centrifuge at maximum speed for 30 seconds and freeze the cell pellets at -20°C.
 - 4.6. Resuspend each pellet in 80 µl 1X SDS-PAGE sample buffer.
 - 4.7. Boil for 5 minutes and centrifuge briefly.
 - 4.8. Run 5-10 µl of each sample on an SDS-PAGE gel and choose the best induction condition.

5. Test if the protein of interest is soluble or insoluble:
 - 5.1. Thaw and resuspend each pellet in 500 μ l of Lysis Buffer
 - 5.2. Freeze samples in liquid nitrogen and thaw at 42°C. Repeat 2 to 3 times.
 - 5.3. Centrifuge samples at maximum speed for 1 minute to separate pellet and supernatant.
 - 5.4. Mix equivalent amounts of supernatant and 2X SDS-PAGE sample buffer and boil for 5 minutes
 - 5.5. Add 500 μ l of 1X SDS-PAGE sample buffer to the pellets and boil 5 minutes.
 - 5.6. Run 10 μ l of the supernatant samples and 5 μ l of the pellet samples on SDS-PAGE.
6. Scaled-up expression:
 - 6.1. Inoculate 200 ml LB and grow it to an OD₆₀₀ of 0.4.
 - 6.2. Induce protein expression with L-arabinose according to the optimal induction condition tested.
 - 6.3. Harvest cells by centrifugation at 4,000 xg for 20 minutes
 - 6.4. Freeze pellets at -20°C.
 - 6.5. Thaw pellets on ice for 15 minutes and resuspend in Buffer B at 5 ml per gram wet weight.
 - 6.6. Stir cells for 15-60 minutes at room temperature.
 - 6.7. Centrifuge lysate at 10,000 xg for 30 minutes at room temperature.
 - 6.8. Add 1ml of the 50% Ni-NTA slurry (Qiagen) to 4 ml supernatant and mix by rocking at room temperature for 60 minutes.
 - 6.9. Load lysate-resin mixture into a column.
 - 6.10. Collect flow-through.
 - 6.11. Wash column twice with 4 ml Buffer C.
 - 6.12. Elute 4 times with 0.5 ml Buffer D, followed by 4 times with 0.5 ml Buffer E.
 - 6.13. Analyze collected fractions by SDS-PAGE.
 - 6.14. Measure the protein concentration of the most concentrated fraction.
 - 6.15. Run purified protein on a SDS-PAGE gel, stain with Coomassie blue and send the gel slice containing 500 μ g protein for antibody production.

B. Protein Purification (for soluble protein):

1. A 3 ml culture of LB with 2% glucose and appropriate antibiotics is started with a single colony of bacteria (BL21 DE3 Plys) carrying polyHis- protein plasmids. Culture is grown overnight at 37 °C.
2. One liter of LB media carrying antibiotics is inoculated with the 3ml O/N grown culture, shaking for 3-6 hours at 37°C until OD₆₀₀ is about 0.5.
3. IPTG is added to the culture to a 1 mM final concentration, and incubated at 37 °C for 2 hours.
4. Bacteria are harvested by centrifugation at 5000rpm and stored at -70°C until used.
5. Bacterial pellets are resuspended in sonication buffer (50 mM PiNa, pH 8.0; 300 mM NaCl; 100 µg/ml of PMSF) using about 10 ml of buffer per 500 ml of original culture. Samples are sonicated 3 times for 30 seconds using the small tip of the sonicator at the maximal allowed power, always keeping the tubes in ice.
6. Samples are centrifuged at 12,000rpm for 10 minutes, and the supernatant is filtered through Miracloth into a clean tube.
7. RNase A and DNase I are added to a final concentration of 1 µM.
8. The Ni²⁺ resin is washed twice in sonication buffer and resuspended to give a 50% slurry. 0.5 ml of slurry is added per liter of original culture.
9. The sample is incubated with the resin for 1-2 hours at 4 degree in a rocker.
10. A column is loaded with the sample, washed six times with 5 ml of sonication buffer per liter of culture, and three times with 5 ml of wash buffer per liter of culture (50 mM PiNa, pH8.0; 300 mM NaCl; 1% Tween-20; 10% glycerol; 5 mM beta-MeOH; 10 mM EDTA).
11. All the washes are collected for SDS-PAGE analysis.
12. The protein is eluted with 5 consecutive additions of 5 ml of 50 mM imidazol in wash buffer. Each fraction is collected separately.
13. Samples from the original culture, the flow-through of the column, washes and elutions are tested by SDS-PAGE.

14. Run 500 μg purified protein on a SDS-PAGE and cut the gel slice for generating antibody.

Antibody production (Cocalico Biologicals, Inc):

500 μg protein in the gel slice was sent to Cocalico Biologicals, Inc. for generating polyclonal antibodies using rabbit.

The standard protocol is as follow:

Day 0 - Prebleed/Initial Inoculation

Day 14 - Boost

Day 21 - Boost

Day 35 - Test Bleed

Day 49 - Boost

Day 56 - Test Bleed

The last bleed will be collected from the rabbit and the rabbit will be killed after the antibody is tested.

Antibodies generated for 2010 project: AtMYB88, AP1, CAL, LFY, FIL, PRS and PAN