Protein Production & Assays

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Protocol

Heterologous expression of recombinant proteins in *E. coli*

1 GENERAL INFORMATION

E. coli cells are the most common expression host for protein production, due to their easy handling, short doubling time and costs. Use of a promoter whose activity can be tightly controlled to regulate heterologous biosynthesis is generally recommended, since production of a heterologous protein often impairs the growth of *E. coli* cell cultures. Especially if the foreign protein is cytotoxic, the production of minute quantities can result in a selection against *E. coli* cells harboring the expression plasmid. Promoters that can be induced by the addition of chemicals are a convenient solution to this problem.

The *E. coli* expression vectors pASK-IBAxC/pASG-IBA carry the promoter/operator region from the *tet*A resistance gene, a state-of-the-art solution for such an inducible expression system. It can be fully induced by adding anhydrotetracycline at a concentration that is not antibiotically effective (200 ng/ml). The strength of the *tet*A promoter is comparable with that of the *lac*-UV5 promoter (nearly 25% activity of the T7 promoter). Additionally, the *tet* repressor gene is also encoded on the expression plasmid and constitutively expressed, guaranteeing the repression of the promoter in the absence of the inducer.

The commonly used *lac* promoter is susceptible to catabolite repression (cAMP-level, metabolic state) and chromosomally encoded repressor molecules. In contrast, the *tet*A promoter/operator is not coupled to any cellular regulation mechanisms. Subsequently, there are basically no restrictions in the choice of culture medium or *E. coli* expression strain when using the *tet* system. For example, glucose minimal media and even the bacterial strain XL1-Blue, which carries an episomal copy of the tetracycline resistance gene, can be used for expression.

2 REQUIRED MATERIAL

The *E. coli* expression vectors, pASK-IBAxC and pASG-IBA do not require a specific *E. coli* strain for expression and allow the use of a strain that meets the target protein needs. Such strains can be, for example JM83, WK6, B, BL21, MG1655, W3110, XL1-Blue, BL21-CodonPlus™. For periplasmic secretion, JM83 and W3110 are recommended.

For protein expression in *E. coli*, several buffers and solutions are required (table below). Anhydrotetracycline (25 mg, Cat. No. 2-0401-002; 50 mg, Cat. No. 2-0401-001) and 10x Buffer W (tenfold concentrated stock solution, 100 ml, Cat. No. 2-1003-100) are offered by IBA Lifesciences.

Buffer/Solution	Concentration	Storage and notes
Ampicillin (pASG-IBA)	100 mg/ml in H_2O, sterile filtered	Store in aliquots at -20 °C.
Chloramphenicol (pASK-IBAxC)	30 mg/ml in ethanol	Store at -20 °C.
Anhydrotetracycline	2 mg/ml in Dimethylformamide (DMF)	Store at -20 °C.
LB medium	10 g/l tryptone 5 g/l yeast extract 5 g/l NaCl	Autoclave and store at room temperature.
1x Buffer W	100 mM Tris/HCl, pH 8.0 150 mM NaCl 1 mM EDTA	Store at 4-8 °C.
5x SDS-PAGE sample buffer	0.25 M Tris/HCl, pH 8.0 25% glycerol 7.5% SDS 0.25 mg/ml bromophenol blue 12.5% v/v 2-mercaptoethanol	Store at -20 °C.
1x Buffer P	100 mM Tris/HCl pH 8.0 500 mM sucrose 1 mM EDTA	Used for cell lysis of periplasmic expressed proteins. It is recommended to work with 2 mg/ml polymyxin B sulfate instead of 1 mM EDTA when metalloproteins are isolated.

3 PROTOCOL

3.1 Production of an expression culture and subsequent cell harvest

- The colony should not be older than one week, the use of an overnight colony is recommended. Do not inoculate from glycerol stocks.
- The yield of soluble, functional protein can be substantially increased in most cases by lowering the preculture and expression culture growth temperature to 22-30 °C. Take care that cells do not reach the stationary phase for extended periods prior to inoculating the expression culture.

3.1.1 Inoculate 2 ml of LB medium containing 100 µg/ml ampicillin (pASG-IBA) or 30 µg/ml chloramphenicol (pASK-IBAxC) with a fresh colony harboring the expression plasmid.

3.1.2 Shake overnight at 200 rpm and 37 °C.

3.1.3 Inoculate 100 ml of LB medium containing 100 µg/ml ampicillin or 30 µg/ml chloramphenicol depending on the used plasmid with 1 ml of the preculture to obtain the expression culture.

3.1.4 Shake the expression culture at 37 °C and monitor the optical density at 550 nm (OD_{550}).

3.1.5 Take a 1 ml sample immediately before induction. This sample is the non-induced control. Pellet cells by centrifugation at maximum speed for 30 sec and resuspend them in 80 µl 1x Buffer W. Add 20 µl 5x SDS-PAGE sample buffer and mix. Store the sample at -20 °C until SDS-PAGE analysis. Prior to SDS-PAG analysis, the sample should be incubated in an ultrasonic bath for 15 min to shear the chromosomal DNA into small pieces and be heated to 70 °C for 10 min.

3.1.6 When OD_{550} equals 0.5-0.6, add 10 µl of anhydrotetracycline stock solution.

3.1.7 Shake at 200 rpm. The optimal growth temperature and period is protein dependent and has to be determined. Usually, temperatures between 16-37 °C are recommendable. At 37 °C an incubation period of 3 hours is sufficient, at lower temperatures longer incubation periods are necessary. At 16 °C an overnight incubation is recommended.

3.1.8 Harvest the cells by centrifugation at 4.500 x g and 4 °C for 12 min. Directly proceed with the preparation of the cell lysate or store the pellet at -20 °C.

3.2 Preparation of cell lysate after cytoplasmic expression

3.2.1 Chill 1x Buffer W at 4 °C.

3.2.2 Resuspend the pellet of a 100 ml culture in 1 ml 1x Buffer W.

3.2.3 Take a 10 µl sample for analysis of the total protein content via SDS-PAGE and/or western blot analysis. The 10 µl sample should be thoroughly mixed with 90 µl 1x Buffer W and 25 µl of 5x SDS-PAGE sample buffer. Store it at -20 °C. Prior to SDS-PAG analysis, the sample should be incubated in an ultrasonic bath for 15 min to shear the chromosomal DNA into small pieces and be heated to 70 °C for 10 min.

3.2.4 Sonicate the residual suspension under ice-cooling and with cooling intervals between each pulse. Take care that the suspension does not become warm which may denature proteins or activate proteases. French pressing is possible as well. Lysis should be complete and can be monitored by measuring the optical density at 590 nm [% lysis = $(1 - A_{590,\ sonicated}/A_{590,\ suspension}) \times 100$]. Optional: If the lysate is very viscous, add RNase A (10 µg/ml) and DNase I (5 µg/ml) and incubate on ice for 10-15 min.

3.2.5 Centrifuge the suspension at 13000 rpm for 15 min at 4 °C. Insoluble cell components are sedimented. Please note that if the recombinant protein forms inclusion bodies, it will be present in the sediment.

3.2.6 Carefully transfer the clear supernatant to a clean tube. For analysis of the insoluble part of the expressed protein, dissolve the sediment with 1.25 ml 1x SDS-PAGE sample buffer (= 250 µl 5x SDS-PAGE sample buffer mixed with 1 ml 1x Buffer W). Store the supernatant on ice until protein purification or at -20 °C if the protein purification cannot be performed the same day.

3.3 Preparation of cell lysate after periplasmic expression

3.3.1 Chill 1x Buffer P at 4 °C.

3.3.2 Resuspend the pellet of a 100 ml culture in 1 ml 1x Buffer P and incubate 30 min on ice. These conditions will usually sufficiently permeabilize the outer membrane of *E. coli* to release the soluble periplasmic components and leave the spheroplasts intact. Harsher treatments, e.g., osmotic shock or lysozyme may be used if the periplasmic components are not completely released.

3.3.3 Take 10 µl as a sample for total analysis of the protein content via SDS-PAGE and/or western blot analysis. The sample should be thoroughly mixed with 90 µl 1x Buffer W and 25 µl 5x SDS-PAGE sample buffer. Store it at -20 °C. Prior to SDS-PAG analysis, the sample should be incubated in an ultrasonic bath for 15 min to shear the chromosomal DNA into small pieces and be heated to 70 °C for 10 min.

3.3.4 Remove spheroplasts by centrifugation at 13,000 rpm for 5 min at 4 °C.

3.3.5 Carefully transfer the supernatant in a clean tube. To check whether a part of the expressed protein remained in the cells, resuspend the sedimented spheroplasts with 1 ml 1x Buffer P and add 250 µl 5x SDS-PAGE sample buffer and perform SDS-PAGE, optionally followed by Western blotting. Store the supernatant on ice until protein purification or at -20° C if protein purification cannot be performed on the same day.

4 TROUBLESHOOTING

No or low expression	Check the culture condition (e.g., anhydrotetracycline, antibiotics), the sequence and frame of the target gene and whether the protein is found in the insoluble fraction. Reduction of the temperature during cultivation may solve this problem (e.g., 16 °C, 22 °C, 26 °C, or 30 °C) or test different *E. coli* strains.
Protein is degraded	Use a protease deficient *E. coli* strain. If degradation occurs during cell lysis, add protease inhibitor. If the protein is small (<1 kDa), consider adding a terminal carrier protein. A lower temperature during the induction or secretion of the recombinant protein into the periplasmic space can reduce the problem.
Protein is secreted	Remove all signal sequences from the coding region.
Inclusion bodies are formed/protein is insoluble	Modify growth and induction conditions, e.g., lower the culturing temperature (16 °C, 22 °C, 26 °C, 30 °C) or test different *E. coli* strains.

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strep-tag@iba-lifesciences.com

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IBA Lifesciences GmbH · Rudolf-Wissell-Str. 28 · 37079 Goettingen · Germany · Phone + 49 551 50672-0 · info@iba-lifesciences.com