

Antibody Engineering

Methods and Protocols

Edited by

Benny K. C. Lo

*MRC Laboratory of Molecular Biology
University of Cambridge, Cambridge, UK*

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Expression and Isolation of Recombinant Antibody Fragments in *E. coli*

Keith A. Charlton

1. Introduction

Central to the rapid growth in the study and application of recombinant antibodies in recent years is the ability to reliably and cost-effectively produce large quantities of functional protein. In contrast to glycosylated whole monoclonal antibodies (MAbs), functional derivatives (antibody fragments) carrying antigen (Ag)-binding activities, and comprising at minimum the antibody variable heavy- and light-chain domains, can be readily produced in bacterial cells (1). The range of antibody (Ab) fragments that can be produced in this way includes F(ab)₂ and Fab', which can also be generated by proteolytic digestion of MAb, and smaller variants such as Fv, scFv, and scAb (single-chain antibody). Even single domains that comprise only the heavy-chain or the light-chain variable region can display Ag-binding activity, although these are not discussed specifically in this chapter. The smallest derivatives that retain the Ag-binding specificity of parental MAbs are Fv fragments. However, as the two domains are held together only by non-covalent hydrophobic interactions, they are prone to dissociate. This can be overcome by chemical crosslinking or the introduction of inter-chain disulfide bonds, or more often by the inclusion of a short peptide linker into expression constructs to produce scFv. Further variants include the addition of a range of suitable (C-terminal) tags for the purification or detection of expressed protein, such as a human Ck domain (scAb) (2), c-myc, or hexahistidine.

Ab fragments have a number advantages over MAbs in clinical applications related to their small size. In particular, scFvs, at approx 1/6 the size of whole antibodies, are less immunogenic (when derived from nonhuman origins), have the potential for greater tissue penetration, and are more rapidly cleared from nonspecific tissues. They afford additional opportunities such as the covalent

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attachment of drugs or radioactive isotopes for targeted therapy or imaging and can be engineered to exhibit dual specificities (3) (see Chapter 13). In most applications, for reasons of ease of transformation and manipulation, *E. coli* is the expression host of choice for laboratory research. Although other systems may produce higher yields of some antibodies, as discussed in Chapters 15–17, bacteria are unrivaled for the very rapid production and purification of protein for subsequent analysis (4). The majority of expression vectors currently used are based on the pUC family (5). These include the *ColE1* replication origin to maintain high copy number under optimal growth conditions, such as 37°C. However, copy number can be reduced dramatically when cells are grown at suboptimal temperatures. Essential to efficient high expression of antibody fragments is tight control over transcription. The *lac* (and hybrid *tac*) promoter system is very popular because it is readily induced with isopropyl-1- β -D-thiogalactopyranoside (IPTG) and is repressed by glucose. The latter is of particular importance when expressing proteins that are toxic to the host such as exported antibody fragments, as excessive basal transcription can slow or arrest bacterial growth. Under these conditions, plasmids are not efficiently replicated, and can easily be lost. An in-depth discussion of the various vector systems available is beyond the scope of this chapter, and it will be assumed that readers have access to suitable expression vectors.

There are three locations from which bacterially expressed proteins can be isolated (6)—the cytoplasm, the periplasm, and the culture supernatant (Fig. 1). *E. coli* usually secretes very few proteins into the extracellular medium. Recombinant proteins exported to the medium are least likely to be degraded by bacterial proteases, and purification is simplified by the lack of contaminating proteins. Conversely, the levels of protein found in culture supernatants are generally very low, and the volumes of material to be processed are very high. Antibody fragments directed to the periplasm are often found in culture supernatants, a process often referred to as “secretion into the medium.” More accurately, the observations are usually the result of leakiness of the outer membrane under expression conditions, possibly because of the accumulation of insoluble aggregates within the periplasm or interference with the export of essential outer membrane components. Proteins expressed without an N-terminal signal sequence remain in the cytoplasm and form aggregates of insoluble folding intermediates or inclusion bodies. This can result in high protein yields, protection from proteolysis, and protection of the host cell against the toxic effects of export. The protein is produced in a non-functional form, and must be solubilized and re-folded using time-consuming and labor-intensive procedures. Final yields of biologically active material are often very low when using this approach.

A significant advance in the development of Ab engineering came about when it was demonstrated that Ab fragments could be exported to the bacterial

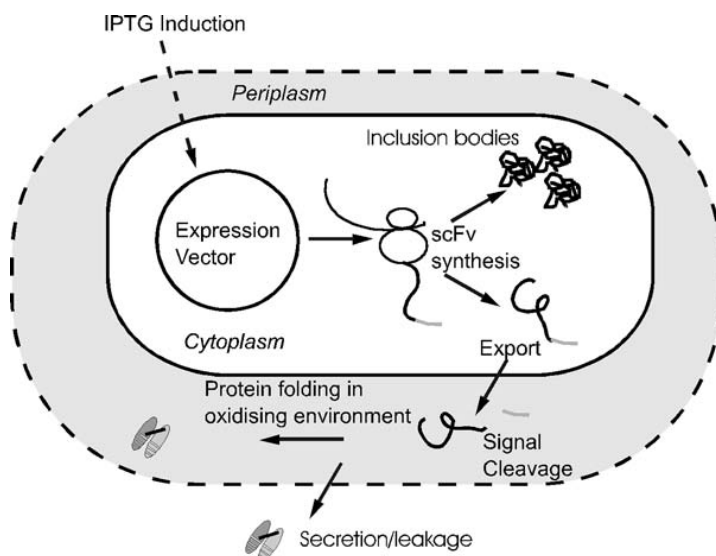


Fig. 1. Schematic representation of the localization of recombinant Ab fragments expressed in *E. coli* using different vectors and under different culture conditions.

periplasm by the inclusion of an N-terminal secretion signal sequence. Within the periplasmic space, Ab fragments are able to fold correctly, aided by the formation of intra-domain disulfide bonds made possible by the oxidative environment, and remain in a soluble form (7,8). The choice of bacterial signal sequence does not appear to be of major importance, as several have been used successfully, such as OmpA, PelB, PhoA, OmpF, β -lactamase, and many others (9–14). Upon transport into the periplasmic space, signal peptides are cleaved leaving the correct N-terminal sequence. High-level expression of recombinant proteins in *E. coli*, and subsequent export to the periplasm often result in the formation of insoluble aggregates (15). This not only reduces protein (functional) yield, but can also lead to growth arrest and cell lysis. The accumulation of insoluble material was once believed to reflect the solubility and stability of the Ab fragments involved. More recent research suggests that the solubility and stability of folding intermediates determine the extent of aggregation (16).

It is generally accepted that such effects can be reduced by induction and expression of Ab fragments (scFv, scAb, and Fab) at 20–25°C (16,17). This does not prevent aggregation completely, but does appear to increase the proportion of correctly folded functional protein produced. Significant improvements in the yields of soluble recombinant Ab fragments have also been

obtained by identifying and overexpressing *E. coli* periplasmic chaperones such as Skp (18,19) and other proteins involved in modulating folding, such as the peptidylprolyl *cis,trans*-isomerase FkpA (20). Periplasmic aggregation can also be reduced by induction of cultures in growth medium supplemented with osmotic stress-inducing concentrations of non-metabolized compounds. These include 1 M sorbitol and 2.5 mM glycine betaine (3,21), or 0.4 M sucrose (22).

In a number of studies of individual recombinant Ab fragments, specific residues have been identified that contribute to poor protein folding. Some of these are exposed hydrophobic residues located at the interface between variable and constant domains (23), and thus are not problematic for Fab fragments, while others are located in both framework and CDR regions (24–26). In each case, mutation of the residue(s) resulted in improved solubility and decreased toxicity. A final consideration is that of codon usage. Many antibody fragments include codons that may not be effectively used in the host organism chosen for expression (27). Generally, with *E. coli*, there is little to be gained by engineering Ab fragment genes to include only those codons used most frequently. However, there may be advantages to precluding those codons used least frequently.

Recombinant Ab fragments expressed in the periplasm of *E. coli* can be readily released by lysozyme/EDTA treatment and mild cold osmotic shock. The crude Ab fragment is most conveniently purified from the periplasmic release fraction or culture supernatant by immobilized metal affinity chelate chromatography (IMAC) via a C-terminal poly-histidine tag incorporated into the expression vector. Detailed methods for purification of expressed recombinant Ab fragments are given in Chapter 22.

2. Materials

All media and buffers are prepared with ultrapure water (>18 MΩ/cm) and autoclaved. Antibiotics and glucose are added after cooling media to 50°C. Quantities given are for 1 L of medium.

2.1. Expression of Recombinant Antibody Fragments (for Subheadings 3.1., 3.2., and 3.4.)

1. *E. coli* strain XL1-Blue {*supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lacF'*[*proAB + lacI^q lacZΔM15 Tn10(ter^r)*]} (Stratagene).
2. *E. coli* strain TG1 {K12 D(*lac-pro*) *supE thi hsdD5/F' traD36 proA⁺B⁺ lacI^q lacZΔM15*} (Stratagene).
3. 2TY medium: 16 g Bacto-peptone, 5 g Bacto-yeast extract, 5 g NaCl, pH 7.5.
4. 2TY-amp/glu: 2TY medium supplemented with 100 μg/mL ampicillin and 2% (w/v) glucose.
5. 2TY-amp/suc: 2TY medium, 0.4 M sucrose (see Note 1), 100 μg/mL ampicillin.

6. TB (Terrific broth): 12 g Bacto-tryptone, 24 g Bacto-yeast extract, 4 mL glycerol dissolved in 800 mL milli-Q water, made up to 900 mL and autoclaved. Separately make up and sterilize a 100-mL solution containing 2.31 g KH_2PO_4 and 12.54 g K_2HPO_4 , and add to the cooled broth.
7. TB-amp/glu: TB medium supplemented with 100 $\mu\text{g/mL}$ ampicillin and 2% (w/v) glucose.
8. TB-amp/suc: TB medium, 0.4 M sucrose, 100 $\mu\text{g/mL}$ ampicillin (*see Note 1*).
9. LB (Luria-Bertani) broth: 10 g Bacto-peptone, 5 g Bacto-yeast extract, 5 g NaCl, pH 7.5.
10. LB-amp/glu: LB medium supplemented with 100 $\mu\text{g/mL}$ ampicillin and 2% (w/v) glucose.
11. 1 M IPTG stock solution (store at -20°C).

2.2. Isolation of Recombinant Antibody Fragments (for Subheadings 3.3. and 3.5.)

1. Amicon high-performance stirred ultrafiltration cell (Millipore).
2. Amicom YM 10 membrane with 10-kDa cut-off (Millipore).
3. ST buffer: 200 mM Tris-HCl pH 7.5, 20% (w/v) sucrose.
4. 1 M EDTA stock solution.
5. Hen egg lysozyme (Sigma-Aldrich; Cat. #L-6876).
6. Dialysis buffer: 50 mM Tris-HCl, 1 M NaCl, 10 mM imidazole, pH 7.0.
7. STE buffer: 20% sucrose, 100 mM Tris-HCl, pH 7.4, 10 mM EDTA.
8. TE buffer: 10 mM Tris HCl, pH 7.4, 1 mM EDTA.

3. Methods

A variety of methods for expressing and isolating recombinant Ab fragments in *E. coli* are available in the literature, which can lead to confusion among those who are not familiar with this area. The experience of our lab (and others) is that different protocols are best applied to different Ab fragment formulations. With this in mind, separate methods are given for scFv, scAb, and Fab fragments. The reader should also note that variations in the sequences of different antibodies and the use of different vectors will affect protein expression. It is therefore recommended that some small-scale experiments be carried out to optimize conditions for each clone.

3.1. Expression of scFv Fragments

1. Inoculate 10 mL of 2TY-amp/glu with a single fresh colony of XL1-Blue transformed with the scFv expression vector and grow at 37°C overnight.
2. Dilute the overnight culture 1:50 in fresh 2TY-amp/glu (*see Note 2*), and incubate at 37°C with vigorous shaking (200–250 rpm) to an optical density 600 nm (OD_{600}) of 0.6–0.8.
3. Pellet the cells by centrifugation at 3,000g for 20 min at 20°C .

4. Resuspend the cells in the same volume of 2TY-amp/suc (*see Note 1*) and incubate at 20–25°C for 1 h (*see Note 3*).
5. Add IPTG to a final concentration of 0.1 mM (*see Note 4*) and continue incubation at 20–25°C (*see Note 5*) overnight (16 h) with shaking (*see Notes 6, 7*).
6. Collect the cells by centrifugation at 6,000g for 30 min. Retain the culture supernatant and keep on ice (*see Note 8*).

3.2. Expression of scAb Fragments

1. Inoculate 10 mL of TB-amp/glu with a single fresh colony of XL1-Blue transformed with the scAb expression vector and grow overnight at 37°C.
2. Dilute the overnight culture 1:50 in fresh TB-amp/glu, and incubate at 37°C with vigorous shaking (200–250 rpm) for 7–8 h (*see Note 2*).
3. Pellet the cells by centrifugation at 3,000g for 20 min at 20°C and resuspend in fresh TB-amp/glu. Incubate overnight at 20–25°C with shaking (*see Note 9*).
4. Pellet the cells as in **step 3** and resuspend in the same volume of fresh TB-amp or TB-amp/suc (*see Note 8*). Incubate for 1 h at 20–25°C for cells to recover.
5. Induce the culture by addition of IPTG to a final concentration of 0.1 mM (*see Note 4*), and continue incubation for 4–16 h (*see Notes 6, 7*).
6. Collect the cells by centrifugation at 6,000g for 30 min. Retain the culture supernatant and keep on ice (*see Note 8*).

3.3. Isolation of Soluble scFv/scAb Fragments

1. Remove any remaining cells or cell debris from the retained culture supernatant by centrifuging at 20,000g for 45 min at 4°C.
2. Concentrate the solution using an Amicon ultrafiltration cell and YM 10 membrane (*see Note 11*).
3. Remove any remaining culture supernatant from cell pellets by pipetting or blotting with tissue paper.
4. Prepare fractionation buffer by adding 1 M EDTA to ice-cold ST buffer to a final concentration of 1 mM, and lysozyme to 500 µg/mL (*see Note 12*).
5. Resuspend the cell pellet in 10% culture volume ice-cold fractionation buffer (*see Note 13*) and shake gently on ice for 15 min (*see Note 14*).
6. Add an equal volume of ice-cold water and continue shaking gently on ice for an additional 15 min.
7. Pellet the spheroplast suspension by centrifugation at 20,000g for 30 min at 4°C, and carefully decant the supernatant containing released periplasmic proteins into a clean tube (*see Note 15*).
8. Pass the periplasmic release fraction through a 0.45-µm disposable filter.
9. The periplasmic release fraction can be combined with the concentrated culture supernatant (**step 2**), and dialyzed against 2–3 changes 50 mM Tris-HCl, 1 M NaCl, 10 mM imidazole, pH 7.0 (*see Note 16*).
10. For a detailed description of immobilized metal affinity chromatography (IMAC) purification, *see* Chapter 22.

3.4. Expression of Fab Fragments

1. Inoculate 10 mL of LB-amp^{glu} with a single fresh colony of XL1-Blue or TG1 transformed with the Fab expression vector and grow overnight at 37°C (*see Note 17*).
2. Inoculate the overnight culture into 1 L of fresh LB-amp (*see Note 2*) and incubate with vigorous shaking at 37°C to OD₆₀₀ 0.6–0.8.
3. Induce the culture by addition of IPTG to a final concentration of 0.1 mM (*see Note 4*), and continue incubation at 20–25°C for 16–24 h.
4. Collect the cells by centrifugation at 6,000g for 30 min at 4°C.

3.5. Isolation of Fab Fragments

1. Remove any remaining culture supernatant from cell pellets by pipetting or blotting with tissue paper.
2. Resuspend the cell pellet in 10% culture volume ice-cold STE buffer and incubate on ice with gentle shaking for 1 h (*see Notes 14, 18*).
3. Pellet the cells by centrifugation at 6,000g for 30 min at 4°C.
4. Resuspend the pellet in 10% culture volume ice-cold TE buffer and incubate on ice with gentle shaking for 15 min.
5. Pellet the cells by centrifugation at 6,000g for 30 min at 4°C and carefully decant the supernatant containing released periplasmic proteins into a clean tube (*see Note 15*).
6. Dialyze the periplasmic release fraction against 2–3 changes 50 mM Tris-HCl, 1 M NaCl, 10 mM imidazole, pH 7.0 (*see Note 16*).
7. For a detailed description of IMAC purification, *see* Chapter 22.

4. Notes

1. 2TY-amp/suc (and TB-amp/suc) are prepared by adding 137 g sucrose to 1 L sterile 2TY-amp (TB-amp) medium immediately before use. The inclusion of 0.4 M sucrose can significantly increase yields of some Ab fragments, particularly scFvs (**22**).
2. Improved growth of *E. coli* (and protein yields) in shake-flask cultures are obtained when cells are grown in baffled flasks that increase oxygenation of the growth medium. Single 500-mL cultures can be grown in 2-L flasks or multiple 50-mL cultures in 250-mL flasks.
3. When the growth medium is replaced, the culture will enter lag phase. Incubation in fresh medium for 1 h allows the culture to begin exponential growth prior to induction.
4. This concentration of IPTG was found to be optimal when low-density cultures are induced (e.g., induction at OD₆₀₀ 0.6–0.8). For very high cell densities (**Subheading 3.2**), higher concentrations (1 mM) can be used. Because IPTG is an expensive reagent, it is recommended that small-scale expressions are carried out to optimize conditions for each vector/antibody used.
5. Induction of antibody fragments at suboptimal growth temperatures reduces the accumulation of insoluble partially folded aggregates in the periplasmic space, thus increasing yields of functional protein.
6. Antibody fragments are a very heterologous group of proteins, and factors such as folding efficiency and host-cell toxicity will vary considerably. Cell lysis causes a

significant proportion of the antibody to leak into the culture supernatant, and the release of proteases and nucleic acids that reduce protein yields and make subsequent isolation and purification more complex. It is advisable to generate growth curves for each antibody under inducing conditions to determine the time at which cell lysis begins, indicated by a reduction in culture OD₆₀₀.

7. Increasingly, antibody fragments are generated using phage display technology. The repeated expression of scFvs in TG1 cells as fusions to the phage minor coat protein, and export to the periplasm, drives selection to favor those antibodies that express at least reasonably well. In comparison, antibody fragments derived from hybridoma cell lines exhibit a much wider range of expression yields, and can be very toxic to *E. coli*.
8. The inclusion of sucrose in growth medium results in an increase in the amount of protein localizing in the culture supernatant, particularly when prolonged induction times are used. In the case of scFvs, it may also promote the formation of dimeric antibody fragments (diabodies), in which the V_H of one molecule associates with the V_L of a second, and *vice versa* (3).
9. We have found that the use of very rich (TB) medium and growth of cultures to high cell density before induction gives high yields of scAb fragments over short induction times (≤ 4 h). Prolonged induction can lead to cell lysis.
10. Although the addition of sucrose to the growth medium can significantly increase yields of scFvs, we find improvements in yield of scAbs to be very variable. It is advisable to determine its effect for each different scAb.
11. Any other protein concentrator with a mW cutoff of 10–12 kDa may be used.
12. A high concentration of Tris-HCl together with EDTA serves to destabilize the bacterial outer membrane and facilitate release of periplasmic proteins. Lysozyme cleaves the peptidoglycan matrix that makes up the cell wall, releasing recombinant protein held behind or within the matrix (29).
13. When expressing scAbs at high cell density (Subheading 3.2.), it may be necessary to use 20% culture volume fractionation buffer because of the large cell pellet.
14. The cell pellet is best resuspended using a 10 mL Gilson with the end of the tip cut-off to increase the hole size or a disposable pipet and hand pump.
15. The cell pellet will be quite loose after fractionation and osmotic shock. It may be necessary to extend the spin time to firm up the pellet.
16. The addition of 10 mM imidazole to the dialysis buffer helps to prevent nonspecific binding of *E. coli* proteins to the Ni²⁺ resin during IMAC purification. It does not inhibit binding of recombinant proteins with a hexa-histidine tag. Other buffers can be substituted, as suggested by the nickel column supplier used.
17. Fab fragments can be successfully expressed in either XL1-Blue or TG1 cells. TG1 cells are reported to give yields as much as 10 $\times$ greater than those observed with the same Fab fragment expressed in XL1-Blue (28). The improved yields result in part from the faster growth rate of TG1 and resulting higher cell densities, but they also express higher levels of soluble protein (Fab) per g wet cell weight.
18. Incubation in excess of 1 h can lead to leakage of the Fab fragments into the supernatant.

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