

## Expression of Antibody Fragments by Periplasmic Secretion in *Escherichia coli*

Andrew G. Popplewell, Mukesh Sehdev,  
Mariangela Spitali, and A. Neil C. Weir

### 1. Introduction

Antibody-based drugs are increasingly used in the clinic, and their importance is set to escalate in the coming years as more drugs in this class progress through clinical trials. Although many such drugs utilize whole antibodies, others exploit fragments, e.g., fragment antigen binding (Fab') or single-chain fragment variable (scFv), which retain the antigen-binding specificity without the fragment crystallizable (Fc) element necessary to mediate effector functions. Antibody fragments can be advantageous for many therapeutic uses, owing to the fact that valency and half-life can be tailored through protein engineering approaches to suit the desired mechanism of action (1). Furthermore, antibody fragments are more suited to expression in microbial systems, providing benefits in terms of increased scale and ease of manufacture (2).

*Escherichia coli* is currently the host of choice for producing antibody fragments. For disulfide-bonded proteins (Fab' and scFv), good expression levels have been achieved via soluble production, secreting the Fab' chains into the oxidizing environment of the bacterial periplasm where assembly and disulfide bond formation can occur. Periplasmic secretion is achieved by genetically fusing the signal sequence from an *E. coli* protein onto the N-terminus of the antibody V-region sequence. Numerous systems have been developed for the overexpression of recombinant proteins and can be adapted for antibody fragment production. The method described in this chapter relies on the lactose promoter–repressor–operator system. Other systems may work equally well, and the methods given can be adapted accordingly. While moderate amounts of most, if not all, Fabs can be expressed with these methods, high-level expression may require engineering of the antibody fragment to maximize expressibility in *E. coli* (see **Note 1**). In the following protocols, the expression of a humanized Fab' fragment is used to exemplify the methodology. A description of small-scale production by isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) induction in shake flasks

is followed by a protocol for fermentation using a switch of carbon source from glycerol to lactose as the means of induction. Methods for periplasmic extraction and purification are also given.

## 2. Materials

1. Plasmids: pTTO-1 and pDNAbEng-1 (Celltech R&D).
2. *E. coli* strains: INV $\alpha$ F' (InVitrogen) and W3110 (ATCC).
3. Oligonucleotide primers.
4. Restriction enzymes, T4 DNA ligase and *Taq* DNA polymerase.
5. Agarose gel apparatus and DNA sequencing apparatus.
6. L-broth: 10 g/L bactotryptone, 10 g/L NaCl, 5 g/L yeast extract. Sterilize by autoclaving.
7. Transformation solution: 10 g/L bactotryptone, 10 g/L NaCl, 5 g/L yeast extract, 10 g/L MgCl<sub>2</sub>, 100 g/L polyethylene glycol (PEG)4000, and 50 mL/L dimethylsulfoxide (DMSO), pH 6.5. Filter-sterilize.
8. SOC medium: 2% bactotryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl<sub>2</sub>, 10 mM MgSO<sub>4</sub>, and 20 mM glucose (added from 20% stock solution after autoclaving other components).
9. Tetracycline: 7.5 mg/mL stock solution in 50% ethanol.
10. Glycerol solutions: 80% (w/w), 52.5% (w/w). Sterilize by autoclaving.
11. IPTG: 200 mM stock solution, make fresh. Filter-sterilize.
12. Periplasmic extraction buffer (2X stock): 200 mM Tris-HCl and 20 mM EDTA, pH 7.4.
13. Coating buffer: 1.59 g/L Na<sub>2</sub>CO<sub>3</sub>, 2.93 g/L NaHCO<sub>3</sub>, and 0.2 g/L NaN<sub>3</sub>, pH 9.6.
14. Capture and reveal antibodies.
15. Phosphate-buffered saline (PBS).
16. PBS-Tween (PBST): PBS plus 0.05% (v/v) Tween-20.
17. Sample conjugate buffer: 0.1 M Tris-HCl, 0.1 M NaCl, 0.02% (v/v) Tween-20, and 0.2% (w/v) casein, pH 7.0.
18. Substrate solution: 10 mL sodium acetate/citrate solution (0.1 M, pH 6), 100  $\mu$ L H<sub>2</sub>O<sub>2</sub> solution (0.44% [v/v]), and 100  $\mu$ L tetramethyl benzidine solution (10 mg/mL in DMSO).
19. 2.5 L Braun BiostatB batch fermenter (or equivalent) and associated pH and dissolved oxygen probes.
20. SM6 defined media: 5.2 g/L (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 4.2 g/L NaH<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O, 4.025 g/L KCl, 1.05 g/L MgSO<sub>4</sub>·7H<sub>2</sub>O, 5.2 g/L citric acid, 0.052 g/L CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.0200 g/L ZnSO<sub>4</sub>·7H<sub>2</sub>O, 0.0275 g/L MnSO<sub>4</sub>·4H<sub>2</sub>O, 0.0075 g/L CuSO<sub>4</sub>·5H<sub>2</sub>O, 0.004 g/L CoSO<sub>4</sub>·7H<sub>2</sub>O, 0.1000 g/L FeCl<sub>3</sub>·6H<sub>2</sub>O, 0.0003 g/L H<sub>3</sub>BO<sub>3</sub>, 0.0003 g/L Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O, and 31.1 g/L glycerol. Sterilize by autoclaving.
21. Struktol (antifoam agent) stock solution: 10% (v/v). Sterilize by autoclaving.
22. Lactose solution: 40% (w/w). Sterilize by autoclaving.
23. Sartobrand P capsule (Sartorius) fitted onto a peristaltic pump and tubing.
24. Nalgene steriups: 0.45  $\mu$ m and 0.22  $\mu$ m (Millipore).
25. Affinity chromatography equilibration buffer: PBS.
26. Affinity chromatography elution buffer: 0.1 M glycine-HCl, pH 2.7.
27. Protein G Gammabind Plus Sepharose (Amersham Biosciences).

## 3. Methods

These methods outline the (1) construction of the expression plasmid; (2) small-scale expression of the protein in *E. coli*; (3) large-scale expression by fermentation; and (4) extraction and purification of the protein.

### 3.1. Construction of the Expression Plasmid

The construction of a plasmid for expression of a Fab' fragment by translocation to the *E. coli* periplasm is described. The same principle can be applied to construct plasmids to express other antibody fragments, including scFv. However, the construction is more complicated for those fragments made up of two component polypeptides, such as the Fab'. Such “double gene” expression can be achieved by the use of separate promoters for each gene, but perhaps the simplest solution is to use a dicistronic expression method as described here. This indicates that two genes are produced from one transcript, requiring only a single promoter.

#### 3.1.1. pTTO Expression Vector

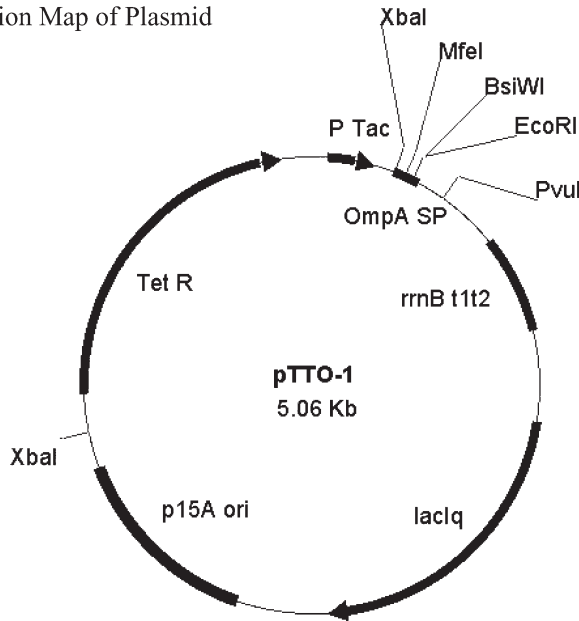
Plasmid pTTO-1 (**Fig. 1**) is a derivative of plasmids pTTQ9 (**3**) and pACYC184 (**4**). The expression unit consists of the strong *Tac* promoter (**5**) and dual *rrnB*<sub>11/12</sub> transcriptional terminator (**6**). The plasmid contains the *lacIq* gene (**7**), giving constitutive expression of the lac repressor protein necessary to keep the *tac* promoter repressed. Derepression (or induction) is mediated by the addition of lactose (which is converted to allolactose, the natural inducer of the *lac* operon in *E. coli*), or IPTG, a non-hydrolyzable synthetic inducer. The plasmid also contains the origin of replication from plasmid p15A (**8**), conferring a low-copy number, and it carries the tetracycline resistance gene. As shown in **Fig. 1**, the plasmid contains DNA encoding a portion of the signal peptide from the *E. coli* OmpA protein (**9**). Insertion of DNA encoding an antibody fragment and the remainder of the OmpA signal sequence creates a gene encoding a protein product that will be translocated to the *E. coli* periplasm.

#### 3.1.2. Insertion of Fab' Light-Chain Gene

The light chain of a Fab' can be inserted into the pTTO-1 polylinker so that an in-frame OmpA signal peptide is created on ligation. A polymerase chain reaction (PCR) strategy is required to “build” DNA encoding the C-terminal portion of the signal peptide onto the 5' end of the light-chain gene. A suggested oligonucleotide to act as a 5' forward PCR primer is shown in **Fig. 1**. Thus, the unique *Mfe*I site within the pTTO-1 polylinker sequence is employed. For the 3' reverse primer, no additional components are necessary, apart from a restriction site for cloning into the vector *Bsi*WI site. The cloning scheme is as follows (*see Note 2*):

1. Use PCR to amplify the light chain, adding a 5' *Mfe*I site and DNA encoding part of the OmpA signal peptide and adding a *Bsi*WI site to the 3' end of the light-chain gene.
2. Purify the amplified DNA, digest with restriction enzymes *Mfe*I and *Bsi*WI, and repurify.
3. Prepare vector pTTO-1 by restriction with *Mfe*I and *Bsi*WI, and purify the cleaved fragment.
4. Ligate the two fragments together, and transform into an appropriate host strain (*see Note 3*).

This creates the light-chain intermediate plasmid. This method can be adapted for creation of a plasmid for single-gene expression (e.g., scFv), in which case the expression construct is now complete. For Fab' expression, the heavy-chain gene must also be inserted.



XbaI RBS MfeI BsiWI EcoRI  
GTTC TAGATAAC GAGG CGTAAAAAATGAAAAAGACAGCTATCGCAATTGCAGTGCCCTTGGCTCTGACGTCAGTAGCAGGAAATTC A

M K K T A I A I A V A L

MfeI  
NNNNNNGCAATTGCAGTGGCCTTGGCTGGTTTCGCTACCGTAGCGCAAGCTXXXXXXXXXXXXXXXXXXXXXXX  
A I A V A L A G F A T V A Q A

Fig. 1. Expression plasmid pTTO-1. **(A)** Map of plasmid. P Tac, *tac* promoter and *lac* operator region; SP, signal peptide; rrnBt12, transcriptional terminator; lacIq, gene constitutively expressing lac repressor protein; p15A ori, origin of replication; Tet R, tetracycline resistance gene. **(B)** Nucleotide sequence of polylinker showing N-terminal portion of OmpA signal peptide amino acid sequence. Restriction enzyme recognition sequences are underlined. The ribosome binding site (RBS) is shown in bold. **(C)** Potential sequence of oligonucleotide to add remainder of OmpA signal peptide sequence to sequence of antibody fragment gene. N, any nucleotide; X, nucleotide identical to that in antibody fragment to be amplified.

To create a dicistronic message expressing both Fab' chains, the gene for the heavy chain also needs to be inserted 3' to the light-chain gene. Another signal sequence is required, in addition to a ribosome-binding site to ensure efficient translational initia-

tion. This can be achieved by cloning the heavy chain through an intermediate vector. One such vector, termed pDNAbEng-1, is shown in **Fig. 2**. Again, this contains part of the OmpA signal peptide, requiring the use of a “forward” oligonucleotide to amplify

the heavy chain and, at the same time, introduce the remaining sequences of the OmpA signal peptide. Once the heavy chain is cloned into this intermediate, it can be excised as an *EcoRI* fragment and inserted into the light-chain vector *EcoRI* site.

1. Use PCR to amplify the heavy chain, adding a 5' *MfeI* site and DNA encoding part of the OmpA signal peptide and adding a *HindIII* site to the 3' end.
2. Purify the amplified DNA, digest with restriction enzymes *MfeI* and *HindIII*, and repurify.
3. Prepare vector pDNAbEng-1 by digestion with restriction enzymes, *MfeI* and *HindIII*, and purify the cleaved fragment.
4. Ligate the two fragments together and transform into an appropriate host strain.
5. Prepare plasmid DNA from a transformed cell.
6. Digest a preparation of DNA with *EcoRI*, run on agarose, and purify the released fragment (containing DNA encoding the heavy chain plus a ribosome-binding site and OmpA signal peptide).
7. Ligate into the light-chain intermediate plasmid cut with *EcoRI*, and transform it into an appropriate host to generate single colonies containing the Fab' double-gene vector. Colonies need to be analyzed for insertion of the heavy-chain fragment in the correct orientation (PCR colony screen or plasmid miniprep; see **Note 4**).

### 3.2. Small-Scale Expression in Shake Flasks

Competent cell preparation is discussed for the *E. coli* expression strain and transformation of the plasmid. Small-scale culture and induction of the plasmid-carrying cells is then described, together with the sampling necessary to give an induction time course—this permits an evaluation of the expression of the antibody fragment before undertaking large-scale expression studies. A simple enzyme-linked immunosorbent assay (ELISA) procedure is also described to enable Fab' quantification.

#### 3.2.1. Competent Cell Preparation

A number of host strains can be used for protein expression; ideally, several should be tried with each recombinant product. One widely used strain is W3110, which has a history of safe use in the production of biopharmaceuticals. The following method (**10**) can be used to make competent W3110 cells.

1. Inoculate 50 mL L-broth in a 500-mL Erlenmeyer flask with a single colony of W3110, and incubate with shaking (250 rpm) at 37°C.
2. Measure the optical density at 600 nm ( $OD_{600}$ ) at intervals until a value of 0.3–0.5 is attained (see **Note 5**).
3. Chill the culture on ice for 5 min, then spin at more than 5000g for 10 min in a centrifuge rotor precooled to 4°C.
4. Discard the supernatant, and resuspend the cells gently in an 5-mL ice-cold transformation solution.
5. The cells are now competent and can be used immediately or alternately can be frozen at –70°C in 50  $\mu$ L aliquots.

#### 3.2.2. Transformation of Cells with Expression Plasmid

To transform the competent cells with expression plasmid, the following protocol is used:

1. Thaw an aliquot of competent cells slowly on ice. Add 50–100 ng plasmid DNA, and mix gently with the pipet tip. Do not pipet up and down.

2. Leave on ice for 30 min.
3. Heat-shock at 42°C for 1 min.
4. Return to ice for a further 3 min.
5. Add 250  $\mu$ L SOC, and incubate at 37°C for at least 1 h.
6. Plate 100  $\mu$ L and 100  $\mu$ L of a 10-fold dilution of the transformation mix (to ensure that single colonies are obtained) onto fresh L-broth agar plates containing 7.5  $\mu$ g/mL tetracycline.
7. Incubate plates overnight at 37°C.

### 3.2.3. Preparation of Glycerol Stock (Working Cell Stock)

This procedure describes the creation of a glycerol stock of the transformed strain.

1. Inoculate 40 mL L-broth (containing 7.5  $\mu$ g/mL tetracycline) in a 0.25-L Erlenmeyer flask with a single colony of transformed cells.
2. Incubate in an orbital shaker at  $30 \pm 1^\circ\text{C}$ , 250 rpm for 8–10 h to an  $\text{OD}_{600}$  of 0.9–1.1.
3. Combine 30 mL culture aseptically with 4.5-mL sterile 80% (w/w) glycerol and divide into 1-mL aliquots. These aliquots are frozen at  $-70^\circ\text{C}$  to form the working cell stock.

### 3.2.4. Shake Flask Expression: Induction Time Course

The following procedure permits the small-scale analysis of expression of a Fab' (or other) fragment prior to production at scale.

1. Thaw 1-mL glycerol stock and transfer to 100-mL L-broth (containing 7.5  $\mu$ g/mL tetracycline) in a 1-L baffled Erlenmeyer flask.
2. Incubate in an orbital shaker at 30°C, 250 rpm overnight (12–16 h). By the following morning, the culture will be at the stationary phase (*see Note 6*).
3. Measure the  $\text{OD}_{600}$  of the overnight culture. Inoculate three 2-L baffled Erlenmeyer flasks containing 200 mL L-broth (containing 7.5  $\mu$ g/mL tetracycline) with an appropriate volume of the culture, such that the  $\text{OD}_{600}$  is approx 0.1. These three flasks should be prewarmed to 30°C; incubation is continued at this temperature while growth is monitored.
4. When the culture reaches an  $\text{OD}_{600}$  of approx 0.5 (about 2 h), all three flasks should be sampled (*see step 5*). Each culture can then be induced by addition of IPTG to 200  $\mu$ M (*see Note 7*). The OD of the cultures should be measured and further samples taken at 0.5, 1, 2, 3, and 4 h postinduction.
5. To sample the cultures so that they are normalized for differing culture ODs, the following protocol is used:
  - a. Measure the  $\text{OD}_{600}$  and use the formula below to calculate the volume of culture to sample:

$$\text{Volume (mL)} = 9/\text{culture } \text{OD}_{600}$$

Remove this volume of culture, chill on ice for 5 min, and harvest the cells by centrifugation ( $>5000g$  for 15 min).

- b. Decant the supernatant, and retain a 1-mL sample (some antibody fragments will “leak” into the culture supernatant; retention of this sample allows analysis of this). Pellets can be stored frozen at this point if desired.
- c. Resuspend the cell pellet in 300  $\mu$ L 1X periplasmic extraction buffer.
- d. Remove an aliquot (50  $\mu$ L) for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of total cells. Incubate the remainder at 30°C overnight (12–16 h) with shaking (*see Note 8*).

- e. Spin the sample in a microfuge ( $>12,000g$  for 10 min). Retain both the supernatant sample (for ELISA, SDS-PAGE, and Western blot), and pellet sample (for SDS-PAGE and Western blot; see **Note 9**).

### 3.2.5. Expression Analysis: Assembly ELISA

The following method describes an ELISA technique to quantify the amount of Fab' material present in soluble extracts (see **Note 10**).

1. Dilute the capture antibody to  $2\text{ }\mu\text{g/mL}$  in coating buffer, add  $100\text{ }\mu\text{L}$  to each well of a 96-well ELISA plate, and leave overnight at  $4^{\circ}\text{C}$ .
2. Wash the wells three times with PBST.
3. Load  $100\text{ }\mu\text{L}$  sample to be quantified in each well of the first column. Include a well of purified Fab' (initially at  $2\text{ }\mu\text{g/mL}$ ) and a well of PBST blank.
4. Serially dilute samples twofold across the plate in a sample conjugate buffer.
5. Incubate at room temperature for 1 h with agitation.
6. Wash and dry the plates. Add  $100\text{ }\mu\text{L}$  reveal antibody (diluted to  $0.2\text{ }\mu\text{g/mL}$  in sample conjugate buffer).
7. Incubate at room temperature for 1 h with agitation.
8. Wash and dry the plates. Add  $100\text{ }\mu\text{L}$  substrate solution. Wait 4–6 min for the color to develop.
9. Read the absorbance at  $A_{630\text{ nm}}$ . Determine concentrations of antibody in the samples by comparison with a standard curve obtained using the control Fab'.

## 3.3. Fermentation

These methods apply to the production of a Fab' fragment and the recovery of this material from the *E. coli* periplasm. The steps outline the (1) preparation of preinoculum and inoculum and (2) fermentation procedure and cell harvest. The induction system employed for this section differs from that used for the shake flask cultures and is based on switching the carbon source from glycerol to lactose. Carbon source switches can be more readily controlled in the defined medium used for fermentation, and the lactose induction appears to support a more gradual rate of Fab' expression that can be sustained over a comparatively long induction phase. Samples should be taken throughout the fermentation. A method for making small-scale periplasmic extractions from these samples is described. In this way, an optimum harvest time for repeat fermentations can be determined.

### 3.3.1. Shake Flask Preinoculum (Culture 1) and Inoculum (Culture 2)

1. Thaw a vial of the working cell stock at room temperature.
2. Transfer  $40\text{ }\mu\text{L}$  to a 0.25-L Erlenmeyer flask containing 40 mL L-broth and  $7.5\text{ }\mu\text{g/mL}$  tetracycline (culture 1).
3. Incubate in an orbital shaker at  $30^{\circ}\text{C}$  for 8–10 h until an  $\text{OD}_{600}$  of 1.0–1.5 is reached.
4. Use 20 mL to inoculate 200 mL sterile SM6 medium (supplemented with  $5\text{ }\mu\text{g/mL}$  tetracycline) in a 2-L Erlenmeyer flask (culture 2).
5. Incubate in an orbital shaker at  $30^{\circ}\text{C}$  (250 rpm). Monitor the  $\text{OD}_{600}$ .
6. When an  $\text{OD}_{600}$  of 3.0–3.5 is attained, use 0.1 L to inoculate the production fermenter.



### 3.3.2. Fermentation Procedure

1. Prepare 1 L SM6 media, transfer to a 2.5-L batch fermenter, and sterilize at 121°C for 30 min; adjust the pH to 7.0 with the addition of 50% (v/v)  $\text{NH}_4\text{OH}$ . Add Struktol antifoam stock solution (2.0 mL/L).
2. Inoculate with 0.1 L culture 2, and incubate at  $30 \pm 1^\circ\text{C}$ . Maintain at  $\text{pH } 7.0 \pm 0.2$  with 50% (v/v)  $\text{NH}_4\text{OH}$  and 20% (v/v)  $\text{H}_2\text{SO}_4$ . Maintain the DO at more than 20% using variable aeration and agitation. Monitor the  $\text{OD}_{600}$ .
3. At  $\text{OD}_{600}$  18–22, add 49.2 mL sterile 52.5% (w/w) glycerol solution.
4. At  $\text{OD}_{600}$  38–42, lower the incubation temperature to  $25^\circ\text{C}$ , and add a further 49.2 mL sterile 52.5% (w/w) glycerol solution, together with 16.7 mL sterile 0.83 M  $\text{MgSO}_4$  and 16.7 mL sterile 0.1 M  $\text{CaCl}_2$ .
5. At  $\text{OD}_{600}$  58–62, add 115 mL sterile 40% (w/w) lactose solution. When the culture reaches  $\text{OD}_{600}$  75–80, glycerol will become depleted, and carbon utilization switches to lactose. This point will be marked by a reduction in oxygen utilization and represents the start of the induction phase.
6. During the induction phase, add lactose as required to maintain the concentration between 20 and 50 g/L. Continue the induction for 24–36 h.
7. To harvest, cool the fermenter to  $20^\circ\text{C}$ , and remove the culture. Dispense into 1-L centrifuge pots and spin at more than 5000g for 1 h.
8. Decant the culture supernatant from the cell pellet, and store the pelleted cells at  $-20^\circ\text{C}$ .
9. Take 1-mL samples throughout the fermentation. Recover cells by centrifugation (12000g for 10 min). Retain both the supernatant (for subsequent analysis of extracellular Fab' leakage) and cell pellet fraction (for small-scale periplasmic extraction and subsequent analysis). Both fractions can be stored at  $-20^\circ\text{C}$ .

### 3.3.3. Small-Scale Periplasmic Extraction

These steps describe the procedure for small-scale recovery of soluble Fab' from the *E. coli* periplasm.

1. Thaw the 1 mL *E. coli* cell pellets at room temperature, and resuspend to the original culture volume in 1X periplasmic extraction buffer.
2. Incubate at  $30^\circ\text{C}$  overnight (12–16 h) with constant agitation in an orbital shaker.
3. Separate the cell debris from the periplasmic extract by centrifugation ( $>15,000\text{g}$  for 10 min).
4. Decant the periplasmic extract from the cell debris, and retain the extract (for assembly ELISA, SDS-PAGE, and Western blot analysis).

**Figure 3** shows the growth profile for the fermentation of W3110 expressing a humanized Fab' fragment, in combination with expression profiles of periplasmic and extracellular Fab' estimated by the ELISA method outlined in **Subheading 3.2.5**.

### 3.4. Primary Recovery and Purification

These methods outline the: (1) extraction of soluble periplasmic Fab' and (2) purification of Fab' using affinity chromatography.

#### 3.4.1. Periplasmic Extraction

This section outlines the procedure for primary recovery of soluble Fab' from the periplasm of the *E. coli* using Tris and EDTA to disrupt the outer cell membrane.

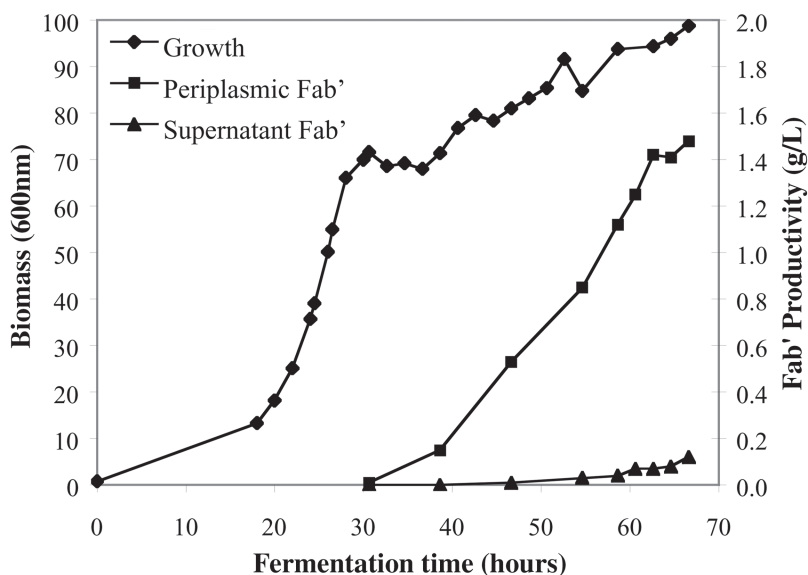


Fig. 3. Fermentation of W3110 expressing a Fab' fragment. Fermentation growth profile and humanized Fab' productivity profile. Samples were taken through the induction phase and analyzed as described in the text. The supernatant Fab' samples were taken from the culture medium. With this particular Fab', the vast majority of Fab' is retained within the periplasm.

1. If frozen, the harvested *E. coli* cell paste should be thawed at ambient temperature before use. Once thawed, the cell paste is resuspended to the original culture volume in 1X periplasmic extraction buffer.
2. The *E. coli* resuspension is incubated at 30°C overnight (12–16 h) with constant agitation by either returning to the fermenter vessel or incubation in an orbital shaker (see **Note 11**).
3. The cell extract is then clarified by centrifugation at more than 15,000g for 45 min, followed by filtration using 0.45  $\mu\text{m}$  followed by 0.22- $\mu\text{m}$  filters. For larger volumes, a Sartobrand P (0.45–0.22  $\mu\text{m}$ ) filter capsule could be used. If filtration difficulties are encountered, a prefilter capsule, e.g., Sartoclean GF (0.8–0.65  $\mu\text{m}$ ), could be implemented.

#### 3.4.2. Affinity Chromatography

Fab' can be purified to greater than 95% using protein G chromatography, as detailed in the following steps. To avoid product loss, aim to load the column at less than 6 mg Fab' per milliliter of resin.

1. Sanitize the protein G column using two column volumes of 6 M guanidine-HCl, followed by two column volumes of 10% (v/v) methanol, then equilibrate in PBS for approximately five column volumes.
2. Adjust the pH of the clarified cell extract to 7.5 using 2 M Tris-HCl stock solution and apply to the prepared column at 75 cm/h.
3. After loading, the column is washed with PBS until the absorbance at 280 nm returns to baseline.

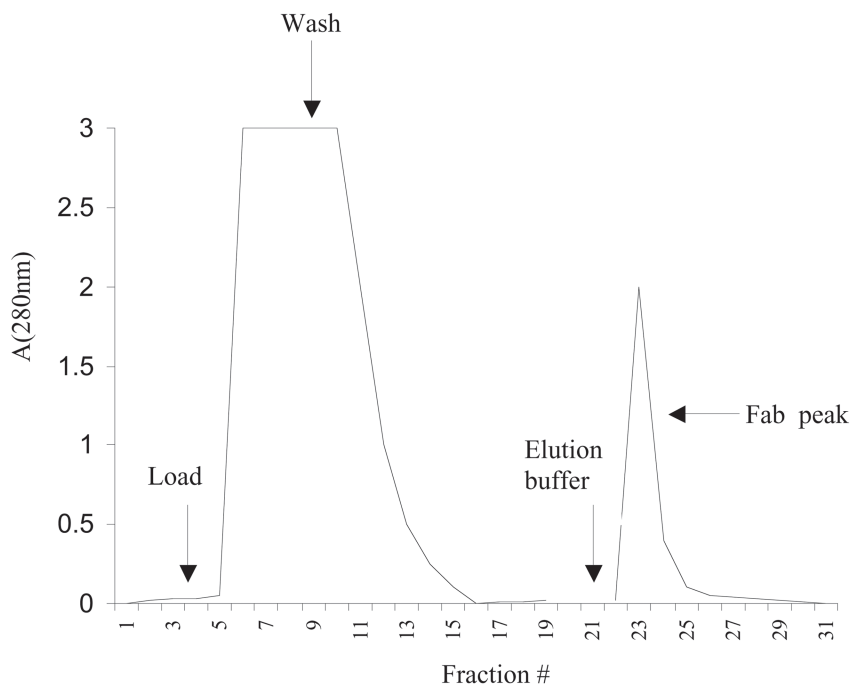
4. Bound Fab' is step eluted with 0.1 M glycine-HCl buffer, pH 2.7. Start product collection when the absorbance at 280 nm goes above baseline, and stop collection when the absorbance returns to baseline.
5. Neutralize the column eluate using 2 M Tris-HCl solution dropwise.
6. After use, the column is washed using 6 M guanidine-HCl and 10% (v/v) methanol as described in **step 1**. When not in use, the column should be stored in 20% ethanol.
7. Filter-sterilize the neutralized column eluate through a 0.22- $\mu$ m filter, and store at 2–8°C (see **Notes 12** and **13**).

**Figure 4A** shows the protein G chromatography profile, whereas **Fig. 4B** shows SDS-PAGE analysis of the affinity purification.

#### 4. Notes

1. This can include removal of rare codons, removal of unfavorable residues or even whole-sale “grafting” of the complementarity determining regions (CDRs) onto optimized frameworks. The balance of expression of the two chains may also need to be optimized. Such engineering is beyond the scope of this chapter.
2. Because of space limitations, the precise details of these molecular biology techniques are not included.
3. Strain W3110, which is used for expression studies, is *Eco*KI restriction-competent, i.e., it will restrict DNA that is not protected by adenine methylation at the appropriate sites. To be transformed into this strain, DNA should be prepared in a host that is competent in *Eco*KI methylation. This phenotype is conferred by the *hsdRMS* alleles, and any host strain used for subcloning work should not carry a *hsdS* allele, which abolishes both restriction and methylation ( $r^-m^-$ ). We recommend strains carrying *hsdR* alleles ( $r^-m^+$ ), such as JM109 or INV $\alpha$ F'.
4. The copy number of the pTTO-1 vector is low and can result in poor yields from plasmid preparations. We routinely use 10-mL L-broth cultures for minipreparations (Qiagen) and up to 500-mL L-broth cultures for maximum preparations (Qiagen).
5. Measurements of OD<sub>600</sub> need to be in the linear range of the curve of cell number vs OD; in practice, this means values in the range of 0.05–0.35. Where the density of the culture is too high, it should be diluted in L-broth so that it falls within this range.
6. In an ideal experiment, the cells would not be allowed to enter the stationary phase, but they would be subcultured in the late log phase of growth. However, this is rarely practical, and allowing the cultures to reach the stationary phase is unlikely to have deleterious effects.
7. An additional flask of culture to which IPTG is not added can be used as a control to highlight changes in the growth profile from induction and fragment production. It is possible to vary the strength of the induction by varying the IPTG concentration added. Although 200  $\mu$ M will give a strong induction, for some antibody fragments this can result in the accumulation of insoluble protein. In such cases, the use of 50  $\mu$ M IPTG can be beneficial in “slowing” the induction, allowing more protein to fold correctly and remain soluble. With thorough sampling and analysis, any insolubility issues can be monitored, and adjustments to inducer concentration can be considered.
8. This extended incubation period works well for most antibody fragments because they are generally very resistant to proteolysis. Western blot analysis of samples pre- and postincubation will provide a check for this.

**A** Protein G chromatography profile



**B** SDS-PAGE analysis of Fab' purification using Protein G chromatography

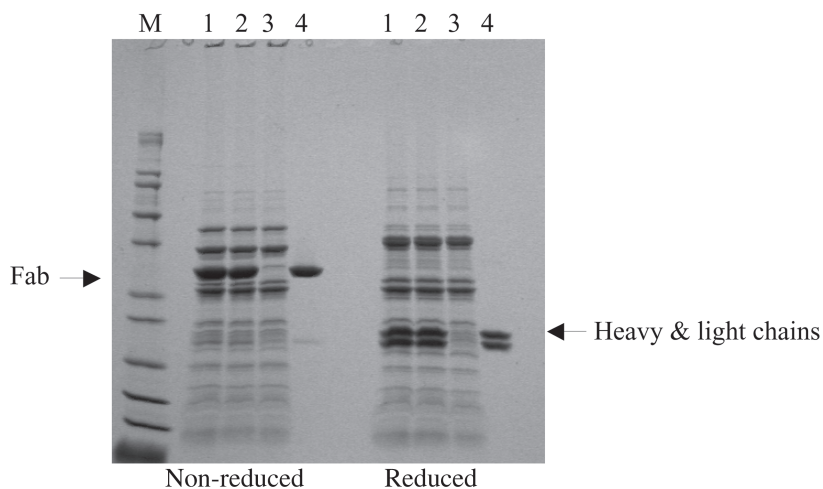


Fig. 4. Fab' purification. (A) Elution profile of protein (A280) against the fraction number, showing the elution of a sharp protein peak after application of elution buffer. (B) SDS-PAGE analysis of Fab' purification. Coomassie blue-stained reducing and nonreducing 4–20% Tris-Glycine SDS-PAGE gel. M, Mark 12 markers (Novex); 1, cell extract, 5  $\mu$ L; 2, column load, 5  $\mu$ L; 3, column flow-through, 10  $\mu$ L; 4, column eluate, 2  $\mu$ g protein.

9. SDS-PAGE and Western blot analysis will give useful information about the integrity of the expressed protein as well as an indication of the level of expression. It is also advisable to analyze medium supernatant, whole cell, periplasmic extract, and cellular fractions to provide information about periplasmic retention and any propensity to form insoluble aggregates.
10. The capture and reveal antibodies used will vary according to the nature of the antibody fragment being expressed. For a humanized Fab' fragment with human constant regions, we use anti-human Fd 6045 (The Binding Site) as the capture antibody and anti-human C- $\kappa$  GD12-peroxidase (The Binding Site) as the reveal antibody. Amounts to use may have to be fine-tuned for other antibody pairings.
11. The purity of the final product may be improved by performing the periplasmic extraction using elevated temperature, e.g., 60°C instead of 30°C (**II**). The heat treatment will ensure the removal of any incorrectly folded nondisulfide Fab' and truncated Fab' species, as well as ensuring the removal of some host cell proteins. Some Fab' constructs may be unstable at 60°C, resulting in loss of product. In this case, the temperature of extraction may need to be reduced to 55°C or 50°C.
12. If high-purity material is required, the affinity chromatography fractionated Fab' can be further processed using anion-exchange chromatography.
13. In most cases, the final purified product will contain some diFab'. If diFab' removal is necessary, it can be reduced to mono-Fab' by incubation in 5 mM  $\beta$ -mercaptoethylamine at 37°C for 30 min. To avoid over-reduction, resulting in the breaking of the disulfide bonds between the heavy and light chains, it is important to remove the reductant immediately after the incubation period.  $\beta$ -mercaptoethylamine can be removed by ultrafiltration or for volumes less than 1 mL by implementation of a desalting column, such as a PD10 (Amersham). Once the Fab' has been reduced, the unpaired hinge thiol can be capped with *N*-ethylmaleimide to avoid oxidation and redimerization. The reduction reaction will also remove any adducts, e.g., glutathione, on the hinge thiol introduced during biosynthesis. Alternatively, any diFab' species could be fractionated from the Fab' using size exclusion chromatography.

## Acknowledgments

We would like to acknowledge the contribution of our many colleagues at Celltech, both past and present, who have helped in the development of these methods.

## References

1. Weir, A. N. C., Nesbitt, A., Chapman, A. P., Popplewell, A. G., Antoniwi, P., and Lawson, A. D. G. (2002) Formatting antibody fragments to mediate specific therapeutic functions. *Biochem. Soc. Trans.* **30**, 512–516.
2. Humphreys, D. P. (2003) Production of antibodies and antibody fragments in *Escherichia coli* and a comparison of their functions, uses and modification. *Curr. Opin. Drug Discov. Devel.* **6**, 188–196.
3. Stark, M. J. (1987) Multicopy expression vectors carrying the lac repressor gene for regulated high-level expression of genes in *Escherichia coli*. *Gene* **51**, 255–267.
4. Chang, A. C. and Cohen, S. N. (1978) Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. *J. Bact.* **134**, 1141–1156.
5. de Boer, H. A., Comstock, L. J., and Vasser, M. (1983) The tac promoter: a functional hybrid derived from the trp and lac promoters. *Proc. Natl. Acad. Sci. USA* **80**, 21–25.

6. Brosius, J. (1984) Toxicity of an overproduced foreign gene product in *Escherichia coli* and its use in plasmid vectors for the selection of transcription terminators. *Gene* **27**, 161–172.
7. Muller-Hill, B., Crapo, L., and Gilbert, W. (1968) Mutants that make more lac repressor. *Proc. Natl. Acad. Sci. USA* **59**, 1259–1264.
8. Selzer, G., Som, T., Itoh, T., and Tomizawa, J. (1983) The origin of replication of plasmid p15A and comparative studies on the nucleotide sequences around the origin of related plasmids. *Cell* **32**, 119–129.
9. Movva, N. R., Nakamura, K., and Inouye, M. (1980) Amino acid sequence of the signal peptide of ompA protein, a major outer membrane protein of *Escherichia coli*. *J. Biol. Chem.* **255**, 27–29.
10. Chung, C. T., Niemela, S. L., and Millar, R. H. (1989) One-step preparation of competent *Escherichia coli*: transformation and storage of bacterial cells in the same solution. *Proc. Natl. Acad. Sci. USA* **86**, 2172–2175.
11. Weir, A. N. C. and Bailey, N. A. (1997) Process for obtaining antibodies utilising heat treatment. US Patent No. 5,665,866.