

# Chapter 25

## Antibody Fragment Expression and Purification

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### Abstract

Interest in the potential of monoclonal antibodies (mAbs) to serve as therapeutic agents has surged in the past decade with a major emphasis on human viral diseases. There has been much attention in this area directed towards the human immunodeficiency virus type-1 (HIV-1) and promising research developments have emerged on the inhibition of HIV-1 infection by mAbs and the identification of several highly conserved neutralizing epitopes. More recently, potent fully-human neutralizing mAbs have been developed against a variety of important human viral disease agents including the paramyxoviruses Hendra virus and Nipah virus, and human or humanized mAbs have been developed against severe acute respiratory syndrome coronavirus (SARS CoV), and West Nile virus, among others. Most of these more recently developed antiviral mAbs have come from the use of antibody phage-display technologies and the implementation of simplified, inexpensive yet efficient methods, for expressing and purifying the initially selected fragment antibodies is of prime importance in further facilitating this area of research.

**Key words:** Monoclonal, antibody, recombinant, phage, purification.

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### 1. Introduction

Interest in the potential of monoclonal antibodies (mAbs) as therapeutic agents has surged in the past decade. Currently, however, the main therapeutic targets of antibodies remain cancer and autoimmune diseases (1, 2) and only one antibody targeting viral infection, Synagis (palivizumab), has been approved by FDA for prophylaxis against respiratory syncytial virus (3). Recently, potent neutralizing human monoclonal antibodies have been selected against the emerging paramyxoviruses Hendra virus and Nipah virus (4) which are now

being evaluated *in vivo* for efficacy in a ferret model. Newly discovered human mAbs against the human immunodeficiency virus type-1 (HIV-1) have been developed which target highly conserved epitopes on the HIV-1 envelope glycoprotein and have not demonstrated any affinity to human proteins or lipids (5). In addition, humanized or fully human mAbs have been selected against severe acute respiratory syndrome coronavirus (SARS CoV) (6) and West Nile virus (7), among others (8). Perhaps among the most significant advances in facilitating the development of specific antiviral mAbs has been the implementation of bacterial phage-display platforms using combinatorial antibody libraries (9, 10). Such phage libraries can be prepared to encode human antibodies as fragment antibodies (Fabs) containing the light chain and the first two domains of the heavy chain. To meet increasing research needs, the implementation of new methods for expressing and purifying these recombinant antibodies is of primary importance to facilitate their efficient production while decreasing production costs.

The recombinant antibody production methods usually consist of three major steps: transformation, expression, and purification. In the transformation step, plasmid DNA coding for a His-tagged Fab is transformed into HB2151 bacterial cells, and successfully transformed cells are selected using ampicillin. During this step, Fab expression is suppressed by a high glucose concentration in the medium. In the next step, isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) in the absence of glucose is used to induce Fab expression. In the purification step, Polymyxin B is used to release the Fab from the bacterial periplasm. Finally, Ni-NTA affinity chromatography is used to specifically purify the His-tagged Fab. In addition to the use of such mAbs as antivirals, these Fabs can serve as important tools as specific detection reagents in various other techniques, such as enzyme-linked immunosorbent assays (ELISA), western blots, immunoprecipitation, and flow cytometry analysis.

For research purposes it is often convenient to express and purify these Fabs in smaller amounts. However, quite often the quantities of these Fabs are insufficient for other purposes and there is necessity of rapid methods for their expression and purification. Here, we have devised such a rapid method, in which at first a stock of monoclonal recombinant phage harboring the plasmid (phagemid) encoding the desired Fab is prepared and then this phage is used for a multiple rapid Fab expression and purification procedure.

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## 2. Materials

### 2.1. Preparation of Recombinant Monoclonal Phage

1. TGI competent cells (Stratagene, San Diego, CA).
2. *Plasmid DNA*: Phagemid encoding the Fab clone, which has been selected against specific antigen from a phage library (*see Note 1*).
3. *SOC medium*: 20 g/l tryptone (Bacto, Voigt Global Distribution, Lawrence, KS), 5 g/l yeast extract (Bacto, Voigt Global Distribution), 0.5 g/l NaCl, 10 mM MgCl<sub>2</sub>, 10 mM MgSO<sub>4</sub>, 0.4% (w/v) glucose in water.
4. *2YT medium*: 10 g/l yeast extract (Bacto, Voigt Global Distribution), 16 g/l tryptone (Bacto, Voigt Global Distribution), and 5 g/l NaCl in deionized water. Autoclave and store at room temperature for no longer than 1 month.
5. *Supplemented 2YT agar plates*: 15 g/l Select agar (Invitrogen, Carlsbad, CA) and 10 g/l glucose in 2YT medium. Autoclave. When the medium cools down to about 45°C but not solidified, add 100 µg/ml ampicillin (Sigma, St. Louis, MO). Pour 20 ml per 9-cm plate (BD Biosciences, San Jose, CA), and store plates at 4°C for no longer than 1 month.
6. M13KO7 helper phage (NEB, Ipswich, MA).
7. Kanamycin B sulfate (Sigma).
8. *PEG/NaCl solution*: 20% (v/v) PEG6000 and 2.5 M NaCl in water.

### 2.2. Expression of the Fab

1. *Escherichia coli*, strain HB2151, stored at -80°C (11, 12).
2. *Supplemented 2YT expression medium*: 10 g/l yeast extract, 16 g/l tryptone, 5 g/l NaCl, and 2 g/l (0.2%) glucose in deionized water. Autoclave. Add 100 µg/ml ampicillin. Store at 4°C for no longer than 1 month.
3. IPTG (Invitrogen), stored at -20°C.
4. Inoculation loops (Fischer Scientific, Pittsburgh, PA).

### 2.3. Purification

1. 100 mM Polymyxin B sulfate (Invitrogen), diluted 1:50 in PBS.
2. Ni-NTA resin column, prepared by adding 500 µl Ni-NTA agarose (Qiagen, Valencia, CA) to a 10 ml PolyPrep Chromatography column (Bio-Rad, Hercules, CA).
3. 5 M NaCl stock.
4. 1 M Imidazole (Sigma) stock.
5. *Washing buffer*: 0.3 M NaCl, 5 mM imidazole, in PBS. Store at room temperature.
6. *Elution buffer*: 150 mM Imidazole in PBS. Store at room temperature.

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### 3. Methods

#### 3.1. Preparation of Recombinant Monoclonal Phage

##### 3.1.1. Transformation of TG1 Cells with Phagemid Encoding the Fab

1. Thaw a 30  $\mu$ l aliquot of frozen TG1 competent cells on ice (*see Note 2*).
2. Add 2  $\mu$ l plasmid DNA. Mix by gentle stirring. Keep on ice for 30 min.
3. Heat shock at 42°C waterbath for 90 s.
4. Cool on ice for 1–2 min and add 200  $\mu$ l SOC pre-warmed at 37°C.
5. Place in a 37°C shaker at 250 rpm for 1 h.
6. Spread on a supplemented 2YT agar plate.
7. Incubate the plate at 37°C overnight.

##### 3.1.2. Packing the Fab Phagemid into Recombinant Phage

1. Pick up a single colony of the transformed TG1 cells and grow in 40 ml 2YT medium supplemented with 100  $\mu$ g/ml ampicillin and 2% glucose at 37°C until OD<sub>600</sub> reaches 0.8. It usually takes several hours.
2. Add M13KO7 helper phage at multiplicity of infection (moi) 10 to the bacteria and incubate at 37°C for another 30 min.
3. Spin down the bacteria infected with helper phage, resuspend the bacterial pellet into 40 ml 2YT medium supplemented with 100  $\mu$ g/ml ampicillin and 25  $\mu$ g/ml of kanamycin, and grow the bacteria at 30°C overnight with shaking at 250 rpm.
4. Spin down the bacteria at 8000 *g* for 15 min at 4°C.
5. Collect the supernatant and add 10 ml PEG/NaCl solution. Incubate the mixture on ice for 1 h.
6. Centrifuge at 10,000 *g* for 15 min. Discard the supernatant.
7. Resuspend the phage pellet in 2 ml PBS.
8. Centrifuge at 10,000 *g* for 10 min to eliminate the bacterial contamination in the phage pellet. Collect the supernatant and discard the pellet.
9. To further purify the phage, add 0.5 ml phage precipitation PEG/NaCl solution. Incubate on ice for 1 h and repeat Steps 6 and 7 above.
10. Measure the phage concentration by OD at 280 nm (*see Note 3*) and keep in the refrigerator, or for long-term storage add glycerol to a final concentration of 50% aliquot and freeze at –80°C.

#### 3.2. Fab Expression

1. Inoculate HB2151 from the frozen stock in 5 ml 2YT medium and grow the bacteria at 37°C shaker 250 rpm until OD<sub>600</sub> reaches 0.8.

2. Add recombinant phage previously prepared in **Section 3.1.2** at a moi of 10 to the bacteria and incubate at 37°C for 30 min.
3. Centrifuge the bacteria at 3000 *g* for 15 min, discard the supernatant, and resuspend the bacterial pellet into a clean 500 ml bottle or flask filled with 200 ml 2YT expression medium (*see Note 4*).
4. Incubate the culture in a 37°C shaker at 250 rpm until OD<sub>600</sub> reaches between 0.5 and 0.8. This usually takes around 2 h.
5. Add IPTG to a final concentration of 0.5 mM, to induce Fab expression in the culture. At this stage, it is supposed that the glucose in the medium has been already consumed.
6. Incubate the induced culture in a 30°C shaker at 250 rpm between 12 h and 14 h. This incubation time may vary for different Fabs between 9 h and 18 h.

### 3.3. Purification

1. Transfer the culture to a 250 ml centrifuge bottle and centrifuge for 20 min at 3000 *g* and 4°C.
2. Resuspend the pellet in 15 ml of Polymyxin B-supplemented PBS. Transfer the suspension into 50 ml centrifuge tubes.
3. Shake for 30 min at room temperature to lyse the cells. This can be done using an orbital rotator or a shaker (*see Note 5*).
4. Centrifuge the lysate at 22,000 *g* for 30 min at 4°C, and transfer the supernatant to a clean 50 ml Falcon tube. Discard the pellet.
5. During the centrifugation, prepare the purification column. The column must be stabilized on the lab bench in a convenient position, with a container underneath to collect the waste products prior to elution. Add 500 µl Ni-NTA resin to the empty purification column at this point.
6. Wash the prepared column with 10–15 ml PBS.
7. Add 0.3 M NaCl and 5 mM imidazole to the Fab supernatant obtained by centrifugation above in Step 4. Mix well and pass through the column.
8. Wash with 20–30 ml washing buffer.
9. Elute the Fab with two portions of 0.7 ml elution buffer into a clean 1.5- or 2.0 ml tube (*see Note 6*). Determine the Fab concentration, and store at 4°C.
10. Wash the column with water and 20% EtOH. Column may be stored at 4°C in 20% EtOH for reuse. Reusing the column decreases the quantity of the purified Fab by approximately one-half.

### 3.4. Quantification

1. Set the spectrophotometer to measure absorbance at 280 nm. Use elution buffer as the reference to zero the spectrophotometer reading.
2. The approximate molecular extinction constant of human Fabs is 1. Thus, the absorbance readings are equal to the antibody concentration in mg/ml. Because of impurities, results for Fab productions are considered trustworthy if the concentration is at least 0.5 mg/ml. The maximum Fab concentrations in our experiments reached up to 4 mg/ml.

## 4. Notes



1. For methods of selecting and purification of the Fab clone plasmid DNA please refer to the corresponding chapters in this book. In the present protocol, the Fab clones were encoded into the phagemid vector pComp 3X (13) that has 6-His and FLAG tags attached to the Fab C-terminus.
2. Transformation of the bacteria can be alternatively performed by electroporation according to the protocol by Stratagene. However, if there is no available electroporator, prepare competent cells as follows: inoculate TG1 cells in 10 ml 2YT (*see Section 2.1*) and grow overnight. Dilute the overnight culture of wild-type bacteria with 100 ml fresh 2YT medium. Grow until bacteria reach an optical density at 600 nm of 0.35–0.45, then keep bacteria on ice for a minimum of 15 min before centrifugation. Centrifuge at 3000 *g* for 15 min at 4°C. Discard the supernatant. Resuspend the bacterial pellet into 50 ml ice-cold CaCl<sub>2</sub> and keep on ice for 20 min. Cells must remain cold at all times and be treated gently in the presence of CaCl<sub>2</sub>. Centrifuge at 3000 *g* for 10 min at 4°C. Discard the supernatant and gently resuspend the pellet in 2 ml ice-cold CaCl<sub>2</sub>. Incubate on ice for at least 1 h before using for transformation, or store in 30 µl aliquots at –80°C for later use.
3. Measure the OD<sub>280</sub> of prepared phage stock, usual dilution for this measurement is 20 times. OD<sub>280</sub> ~ 1.0 corresponds to 2.33 × 10<sup>12</sup> phages.
4. The volume of medium inoculated in the expression step can be increased up to 300 ml per column used in the purification step. If inoculating a larger volume of medium, increase the volume of Ni-NTA agarose placed in the column by 100 µl per 50 ml increase in culture used.
5. Alternatively, instead of manually resuspending and then shaking/rotating for 30 min at room temperature, one can shake/rotate for 1 h at room temperature. If using an orbital rotator,

be sure to stabilize the tubes properly so that a rotation speed of at least 150 rpm can be used. At the end of the shaking or rotating, the pellet should be completely resuspended.

6. The first portion usually yields a slightly higher concentration, and it may be useful to elute the two portions into separate tubes in order to see the difference. However, the concentrations are seldom very different, and the two portions may be eluted together instead.

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