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# One-step expression and purification of single-chain variable antibody fragment using an improved hexahistidine tag phagemid vector

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ABSTRACT

Millions of candidate clones are commonly obtained following rounds of phage-displayed antibody library panning, and expression of those selected single-chain variable fragment (scFv) is required for secondary functional screening to identify positive clones. Large scale functional screening is often hampered by the time-consuming and labor-intensive subcloning of those candidate scFv clones into a bacterial expression vector carrying an affinity tag for scFv purification and detection. To overcome the limitations and to develop a multiplex approach, an improved hexahistidine tag phagemid vector was constructed for one-step scFv expression and purification. By using hexahistidine as an affinity tag, soluble scFvs can be rapidly and cost-effectively captured from *Escherichia coli* periplasmic extracts. For proof-of-concept, feasibility of the improved phagemid vector was examined against two scFvs, L17E4d targeting a cell surface antigen and L18Hh5 recognizing a monoclonal antibody (mAb). Using 1 ml of Ni–NTA agarose, 0.2–0.5 mg of soluble scFv was obtained from 1 L of bacteria culture, and the purified scFvs bound specifically to their target antigens with high affinity. Moreover, using two randomly selected hapten-specific scFv phage clones, it was demonstrated that the display of scFvs on phage surface was not affected by the hexahistidine affinity tag. These results suggest the improved phagemid vector allows the shuttle of phage-displayed antibody library panning and functional scFv production. Importantly, the improved phagemid vector can be easily adapted for multiplex screening.



## Introduction

Phage-displayed antibody library provides a flexible selection platform that can be adapted under many specific and rigorous conditions, including selections on whole cells [1–3], tissues [4] and even animals *in vivo* [5]. As for its simplicity, robustness and high versatility, phage-displayed antibody library has also been successfully applied for antibody engineering, such as isolation of high-affinity antibodies [6,7], humanization of antibodies [8,9], affinity maturation of antibodies [10,11], and discovery of tumor markers targeted by antibodies [12,13]. Indeed, at least 21 phage library-derived antibodies have been developed for treating human diseases [14].

A large number of scFv candidates are selected from phage-displayed antibody library after several rounds of panning. Generally, a secondary functional assay will be used to identify scFv clones with optimal affinity and specificity [15,16]. To save time and cost, rescued complete phage particles displaying the scFvs are commonly used for secondary functional assay directly. However, the use of scFv-phage particles in secondary functional assay is troubled by technical problems, including imprecision in quantifying phages [17,18], non-specific background binding, and loss of viability of fusion phages [19]. Hence, to achieve high-throughput screening, it is preferable to express and to purify those candidate scFv proteins for secondary functional assays following rounds of library panning [20].

Conventionally, production of scFv antibodies is achieved by subcloning the candidate scFvs into a bacterial expression vector containing an affinity tag, and bacterially expressed scFvs are purified from *Escherichia coli* extract by affinity chromatography [13,21]. However, production of large number of scFvs for secondary functional assay is limited by the time-consuming and labor-intensive subcloning.

Although many different phage-display systems have been developed for selection of target-specific antibodies and antibody engineering, filamentous phage (fd[1] and M13 bacteriophages) is the most popular vehicle for displaying scFv library [22]. To facilitate

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[1] *Abbreviations used:* scFv, single-chain variable fragment; mAb, monoclonal antibody; fd, filamentous phage; IMAC, immobilized metal-affinity chromatography; IPTG, isopropyl-thio-β-D-galactopyranoside; PBS, phosphate-buffered saline.

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expression and purification of soluble scFv antibodies following phage-displayed antibody library screening, we have developed a hexahistidine tag phagemid vector, pCANTAB His, based on a commercially available phagemid vector. Phage clones targeting a soluble protein, a membrane protein, or a hapten were used to examine the feasibility of the improved phagemid vector. Results showed that no detrimental effect was found in displaying scFvs on phage surface, and soluble scFv antibodies were rapidly expressed and purified from bacteria periplasm using immobilized metal-affinity chromatography (IMAC). Purified scFvs were soluble and functional, recognizing corresponding target antigens with high affinity.

## Materials and methods

### *Plasmids, bacterial strains and enzymes*

Phagemid pCANTAB 5E, *E. coli* strain TG1 and HB2151 were obtained from GE Healthcare (Piscataway, NJ). T4 DNA ligase and *pfu* DNA polymerase were purchased from Promega (Madison, WI). All restriction endonucleases were purchased from New England Biolabs (Ipswich, MA). Reagents of molecular biology grade or highest grade were from Sigma (St. Louis, MO).

### *Construction of pCANTAB His phagemid vector*

For construction of pCANTAB His vector, the SfiI–NotI fragment of pCANTAB 5E vector was amplified by PCR using a forward primer containing an SfiI site (underlined) with sequence 5′-ATGCGGCCCAGCCGGCC-3′ and a reverse primer containing a NotI site (underlined), a hexahistidine tag sequence (italic) and a PspoMI site (double underlined) with sequence 5′- GATC-GGGCCC*TGTGGTGGTGGTGGTGT*GCGGCCGCCCGTTTTC-3′. PCR was performed with *pfu* polymerase. Samples were subjected to 25 cycles of 3-step amplification protocol consisting of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 90 s. The PCR products were separated by electrophoresis in a 1% agarose gel. After purification by gel-extraction (Qiagen, Hilden, Germany), the PCR amplicons were digested with SfiI and PspoMI. As PopMI and NotI have compatible cohesive ends, the digested fragment was subsequently cloned into SfiI- and NotI-linearized pCANTAB 5E vector to generate pCANTAB His.

### *Periplasmic expression of scFvs*

Two pCANTAB His/scFv constructs, His-Hh5 (an anti-idiotypic mouse scFv antibody) and His-E4d (a mouse scFv specifically against a membrane protein), were transformed into *E. coli* strain HB2151 competent cells. A single colony of bacteria was inoculated into 50 ml of 2xYT medium containing 2% glucose and 100 μg/ml ampicillin and grown overnight at 37 °C with shaking at 250 rpm. Overnight culture was diluted to 500 ml with 2xYT medium containing 2% glucose and 100 μg/ml ampicillin. The bacteria culture was further incubated for 1 h. The culture was centrifuged at 1500*g* for 15 min at 4 °C. After removal of the supernatant, the bacteria pellet was resuspended in 500 ml of freshly prepared 2xYT containing 1 mM isopropyl-thio-β-D-galactopyranoside (IPTG) and 100 μg/ml ampicillin and incubated for 3 h at 37 °C with shaking at 250 rpm. The IPTG-treated bacteria cells were collected by centrifugation at 1500*g* for 15 min at 4 °C, and periplasmic scFvs were extracted by osmotic shock. Briefly, the bacterial cell pellet was resuspended in 10 ml of ice-cold 1× TES buffer (0.2 M Tris–HCl; pH 8.0, 0.5 mM EDTA, 0.5 M sucrose) and added to 15 ml of ice-cold 1/5× TES buffer. The mixture was agitated for 1 min and incubated for 30 min on ice. After centrifugation at 10,000*g* for 20 min at 4 °C, the supernatant was dialyzed against 1/2× TS buffer (0.1 M Tris–HCl; pH 8.0, 0.25 M sucrose) at 4 °C.

### *Purification of scFvs using Ni–NTA agarose beads*

The soluble scFv proteins of periplasmic extract were purified using Ni–NTA agarose beads (Invitrogen, Carlsbad, CA). Briefly, 1 ml of Ni–NTA agarose was packed in a poly-Prep column (Bio-Rad, Hercules, CA) and equilibrated with 10 ml of an equilibrating buffer (50 mM $NaH_2PO_4$, 300 mM NaCl, and 10 mM imidazole; pH 8.0). Dialyzed and filtered periplasmic extract (25 ml) was loaded onto the equilibrated columns. After loading, the column was washed with 10 ml of washing buffer (50 mM $NaH_2PO_4$, 300 mM NaCl, and 20 mM imidazole). The bound scFvs were eluted with 5 ml of elution buffer (50 mM $NaH_2PO_4$, 300 mM NaCl, and 250 mM imidazole), and 1 ml-fractions were collected. The fractions were then dialyzed against phosphate-buffered saline (PBS) for 3 h. The protein concentration was determined using BCA protein assay kit (Pierce, Rockford, IL).

### *Protein electrophoresis and Western blot*

Purity of the Ni–NTA-purified His-Hh5 and His-E4d scFvs were examined by SDS–PAGE and Western protein analysis. Purified proteins were separated in a 12% polyacrylamide gel (10 × 10 cm) at a constant voltage of 100 V for 2 h and visualized by Coomassie blue staining. For Western blot, proteins were transferred onto nitrocellulose membranes using wet transfer method at a constant voltage of 70 V for 1 h. The protein blot was pre-incubated with an immunoblotting buffer (50 mM Tris–Cl; pH 7.4, 2 mM $CaCl_2$, 80 mM NaCl, 5% non-fat milk) at room temperature for 1 h to block non-specific protein binding sites, then the blot was probed overnight with a mouse anti-His tag antibody at 1:5000 dilution (GE Healthcare, Piscataway, NJ). Following incubation with a secondary AP-conjugated goat anti-mouse IgG(H+L) antibody at 1:2000 dilution (Zymed, South San Francisco, CA) for 1 h at room temperature, immunoreactive bands were visualized by adding NBT/BCIP (Roche, Basel, Switzerland) as chromogenic substrate (0.1 M NaCl, 0.1 M Tris–HCl; pH 9.5, 50 mM $MgCl_2$, 0.5 mg/ml NBT, 0.19 mg/ml BCIP).

### *Capture ELISA*

ELISA plates were coated with the purified anti-idiotypic His-Hh5 scFv at the concentration of 10 μg/ml in 100 μl of a carbonate–bicarbonate buffer (15 mM $Na_2CO_3$, 35 mM $NaHCO_3$, pH 9.6) per well overnight at 4 °C. After a wash with a borate buffer (26 mM $Na_2B_4O_7$, 100 mM $H_3BO_3$, 0.1% BSA, 100 mM NaCl, 3 mM KCl and 0.5% Tween 20, pH 8.0), non-specific binding sites were blocked with the borate buffer at 37 °C for 1 h. After incubation with 100 μl of serially diluted idiotype or control antibodies at 37 °C for 1 h, bound antibodies were detected by incubation with a HRP-conjugated anti-human Fc antibody at 1:5000 dilution in a final volume of 100 μl (Jackson ImmunoResearch, West grove, PA) at 37 °C for 1 h. Activity of bound HRP was determined by a colorimetric assay with o-phenylenediamine/$H_2O_2$ as substrates, and absorbance ($\lambda$ = 450 nm) was measured with a μQuant® micro-plate reader (Bio-Tek Instruments Inc., Winooski, VT).

### *Flow cytometry*

Activity of His-E4d scFvs against specifically a membrane protein was assayed by a flow cytometry-based cell binding assay. Purified His-E4d scFvs were diluted to a concentration of 10 μg/ml with complete IMDM medium supplemented with 10% FBS, 1% P/S, and 1% BSA. Then the diluted His-E4d scFvs were incubated