Published in final edited form as:

Anal Biochem. 2011 May 15; 412(2): 210–216. doi:10.1016/j.ab.2011.02.006.

Drop-out phagemid vector for switching from phage displayed affinity reagents to expression formats

Kritika Pershad¹, Mark A. Sullivan², and Brian K. Kay¹

¹Department of Biological Sciences, University of Illinois at Chicago, 900 S. Ashland Ave., Molecular Biology Research Building Rm 4318, Laboratory for Molecular Biology (M/C 567), Chicago, IL 60607 (USA)

²Department of Biochemistry & Biophysics, University of Rochester, 601 Elmwood Avenue, Rochester, NY 14642 USA

Abstract

Affinity reagents that are generated by phage display are typically sub-cloned into an expression vector for further biochemical characterization. This insert transfer process is time consuming and laborious especially if many inserts are to be sub-cloned. To simplify the transfer process, we have constructed a “Drop-out” phagemid vector that can be rapidly converted to an expression vector by a simple restriction enzyme digestion with *Mfe* I (to “drop-out” the gene III coding sequence), which generates alkaline phosphatase (AP) fusions of the affinity reagents upon re-ligation. Subsequently, restriction digestion with *Asc* I drops out the AP coding region and re-ligation generates affinity reagents with a C-terminal six-histidine tag. To validate the usefulness of this vector, four different human single chain Fragments of variable regions (scFv) were tested, three of which show specific binding to three zebrafish (*Danio rerio*) proteins, namely suppression of tumorigenicity 13, recoverin, and Ppib and the fourth binds to human Lactoferrin protein. For each of the constructs tested, the gene III and AP drop-out efficiency was between 90–100%. This vector is especially useful in speeding up the downstream screening of affinity reagents and bypassing the time consuming sub-cloning experiments.

Keywords

Phagemid vector; Restriction enzymes; Re-ligation; Affinity-capture; Alkaline phosphatase fusions

Introduction

Various scaffolds, either immunoglobulin-based or engineered protein scaffolds have been displayed on the surface of filamentous bacteriophage M13 as fusions to the minor or major coat proteins of the phage particle. Examples of successfully displayed scaffolds include scFvs [1;2], Fragments of antigen binding (Fab) [3], Fibronectin type III domain (FN3) [4],

© 2011 Elsevier Inc. All rights reserved

Corresponding Author: Brian K. Kay, Department of Biological Sciences, University of Illinois at Chicago, 900 S. Ashland Ave., Molecular Biology Research Building Rm 4318, Laboratory for Molecular Biology (M/C 567), Chicago, IL 60607 (USA). Phone: 312-996-4249 Fax: 312-264-0547 bkay@uic.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

combinatorial peptides [5], PDZ domains [6], designed ankyrin repeat proteins (DARPs) [7], cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) [8], Affibodies [9] and anticalins [10]. Two types of vectors are commonly used to display proteins on the bacteriophage surface: phage vectors, that allow multivalent display, thus facilitating isolation of weaker binding affinity reagents; and, phagemid vectors, that allow monovalent display, thus promoting the isolation of high affinity binders [11]. The phage genome encodes for the protein displayed on its surface and this advantageous feature makes it feasible to obtain the DNA sequence of the binding phage clones with relative ease.

Non-immune, immune or synthetic antibody libraries [12;13;14] have been displayed on the phage and successively screened against various target proteins. The first step involved in the isolation of an affinity reagent that binds to the target protein of interest is to screen a library of inserts against the target protein by a process called affinity selection or “biopanning” [15;16]. Two or three rounds of affinity selection are usually performed for each target. By this approach, scFvs have been recently generated to a large panel of bacterial as well as mammalian proteins [17], and to 20 different human Src homology 2 (SH2) domains [18]. Once affinity reagents are confirmed to bind to the target, the second step is to transfer inserts from binding clones into expression vectors and evaluate their utility for various applications, such as western blotting and enzyme-linked binding assays, using AP fusions of the affinity reagents. Alkaline phosphatase fusions of scFvs (scFv-AP) were first described 18 years ago [19] and have proven useful as probes for western blotting, immunohistochemistry and affinity ranking of peptide ligands [20;21;22;23]. However, the sub-cloning process for transferring inserts from the phage-display vector to expression vectors is time consuming, laborious and requires expertise.

We have designed a “double drop-out” phage-display vector (pKP300) that can be conveniently converted into one or more expression vectors by simple restriction enzyme digestions to excise pieces of DNA such as the gene III or AP coding sequence in a sequential manner, with re-ligation generating either scFv-AP fusions or scFvs with a C-terminal 6xHis tag. Moreover, a single restriction enzyme digestion can also be performed to directly drop out the gene III and AP coding sequences simultaneously, thereby generating 6xHis tagged scFvs in a single step. This drop-out strategy has an efficiency of 90–100% and eliminates the need for preparing vector and insert DNAs separately, designing primers, performing PCR, and gel or column purifications which are laborious and overall require twice as much time compared to this drop-out approach. The convenience of switching from a phage-displayed format to one or more expression formats, without additional sub-cloning steps, offers an attractive alternative for efficient and faster downstream evaluation of affinity reagents.

Materials and Methods

Construction of the double drop-out vector (pKP300)

The AP coding sequence was amplified by PCR from the plasmid DNA pEZ707 [24], using AccuPrime™ Pfx DNA Polymerase (Invitrogen, Carlsbad, CA). The two oligonucleotide primers: AP-Fw 5'- TGC AGC CTC GAG ATG CCT GTT CTG GAA AAC CGG -3' and AP-Rv 5'- CTA TGA CTC GAG CGG TAC TTT CAG CCC CAG AGC -3' used in PCR created flanking *Xho* I restriction enzyme sites. The PCR product was purified using QIAquick® PCR Purification Kit (QIAGEN Sciences), digested with *Xho* I restriction enzyme and purified again using the same kit. The pAP-III₆ vector [25] was digested with the same restriction enzyme, *Xho* I, dephosphorylated for 1 h using Antarctic Phosphatase (New England Biolabs, MA, USA), and gel-purified using QIAquick® Gel Extraction Kit (QIAGEN Sciences, Valencia, CA). The *Xho* I digested PCR product and the pAP-III₆ vector were ligated overnight at 16°C, using T4 DNA Ligase (New England Biolabs, MA).

The ligated DNA was transformed into the TG1 strain of *E. coli*. DNA was purified using the Wizard® Plus SV Minipreps DNA Purification System (Promega Corporation, Madison, WI) and was sequenced using the following primers: pAP-III₆ Fw 5'- CTC AAT CGG TTG AAT GTC GCC -3' and pAP-III₆ Rv 5'- GCG TTT CAC TTC TGA GTT CGG CAT -3'. Using Kunkel mutagenesis [26], an internal *Nco* I restriction endonuclease site in the AP coding sequence was mutated using the oligonucleotide: KM-X-*Nco*I-AP 5'- GAC TGC GGG CTT ATC GAT ATT GCC GTG GTA CGT TGC TTT CGG TCC TAG C-3'. The *Xho* I restriction site, present towards the end of the gene III coding sequence after the opal and ochre stop codons, was mutated to *Mfe* I restriction enzyme site by Kunkel mutagenesis, using the oligonucleotide: KM *Xho* I-*Mfe* I 5'- CGG TTT TCC AGA ACA GGC ATC AAT TGT CAT TAA GAC TCC TTA TTA CGC AGT A -3'. An *Asc* I restriction endonuclease site was introduced before the 6xHis tag, towards the end of the AP coding sequence by mutating the *Xho* I restriction site to an *Asc* I restriction site. This was performed by Kunkel mutagenesis, using the following oligonucleotide: KM-*Xho*I-*Asc*I-AP 5'- GTG ATG GTG ATG GTG ATG GGA AGC GGC GCG CCC CGG TAC TTT CAG CCC CAG AGC -3'. To introduce a new multiple cloning site, two oligonucleotides (pKP300-PL-Fw 5'- AGC TTG CTA GCG CCA TGG GCT CTG GTG GGT CCG GCG CGG CCG CAG TCG ACG GGC GCG CCC AAT TGA -3', and pKP300-PL-Rv 5'- TCG ATC AAT TGG GCG CGC CCG TCG ACT GCG GCC GCG CCG GAC CCA CCA GAG CCC ATG GCG CTA GCA -3') were annealed to create a double-stranded DNA insert with *Hind* III and *Sal* I sticky ends. The vector DNA, cut with the corresponding *Hind* III and *Sal* I restriction enzymes was ligated with the insert overnight at 16°C using T4 DNA Ligase (New England Biolabs, MA). The final construct-pKP300 was transformed into the TG1 strain of *E. coli*. DNA was purified using the Wizard® Plus SV Minipreps DNA Purification System (Promega Corporation, Madison, WI) and was sequenced using the following primer: *OmpA* Up: 5'- CTG TCA TAA AGT TGT CAC GGC CGA -3'

Protein expression and purification

Luria Bertani (LB) medium (1 mL), supplemented with carbenicillin (50 µg/mL) (Mediatech, Inc., Manassas, VA), was inoculated with TG1 bacterial cells harboring either the pKP300ΔIII-scFv (without gene III coding sequence) or pKP300ΔIIIΔAP-scFv (lacking both, gene III and AP coding sequences) expression vector. The cells were grown overnight at 37°C, at 250 rpm. The next day, low phosphate medium (50 mL in 500 mL baffled flasks) supplemented with carbenicillin (50 µg/mL) was inoculated with the overnight culture (50 µL, i.e., 1:1000 dilution). Proteins were expressed at 30°C for 18–20 h at 250–280 rpm. Composition per liter of low phosphate media: ammonium sulfate (3.57 g); sodium citrate dihydrate (0.71 g); potassium chloride (1.07 g); yeast extract (5.36 g); and, Hy-Case® SF casein acid hydrolysate (5.36 g) from bovine milk (Sigma-Aldrich). The solution's pH was adjusted to 7.3 with KOH. The media was autoclaved, cooled, and MgSO₄ (1 M, 7 mL) and glucose (1 M, 14 mL) were added. After 18–20 hours of protein expression, the culture was centrifuged at 8,000 rpm for 10 min at 4°C. The cell pellet was lysed on ice with lysis reagent (3 mL). Composition of lysis reagent: BugBuster® 10× reagent (Novagen, Madison, WI) diluted to 1× final concentration using 1× Tris Buffered Saline (Amresco, Solon, OH), supplemented with Benzonase® Nuclease (3 µL; Novagen, Madison, WI); and, complete, EDTA-free protease inhibitor cocktail (1× concentration; Roche Applied Science, Indianapolis, IN). The lysed cells were incubated at room temperature on a shaking platform at 40 rpm for 20 min, followed by centrifugation at 8,000 rpm for 10 min, at 4°C, to pellet the cell debris. The clarified cell lysate was used for protein purification. Proteins were purified via the C-terminal 6xHis tag by IMAC, using a KingFisher® (Thermo Electron Corporation, Vantaa, Finland). The solutions were loaded into a plastic strip (KingFisher mL Combi) containing five wells: the first well contained clarified cell lysate (1 mL) and His-Mag™ agarose beads (25 µL) (Novagen®, San Diego, CA); the second and third wells

contained wash buffer #1 (1 mL); the fourth well contained wash buffer #2 (1 mL); and, the fifth well contained elution buffer (150 μ L). (Wash buffer #1: 0.5 M NaCl; 20 mM Tris HCl; 5 mM imidazole; and, 0.5% Tween 20 (v/v), pH 7.9. Wash buffer #2: 0.5 M NaCl; 20 mM Tris HCl; 20 mM imidazole; and, 0.5% Tween 20 (v/v), pH 7.9. Elution buffer: 0.5 M NaCl; 20 mM Tris HCl; and, 500 mM imidazole, pH 7.9). The purified proteins were desalted using Zeba™ Spin Desalting Columns (2 mL; Thermo Scientific, Rockford, IL). Glycerol was added to the scFvs (15% final) and scFv-AP fusions (30% final). The proteins were then aliquoted and stored at -20°C .

Western blots using alkaline phosphatase fusions of scFv as detection reagents

Preparing TG1 cell lysate containing total bacterial proteins—Total bacterial proteins were obtained from an overnight culture of TG1 (40 mL) in LB medium. Clarified cell lysate was prepared as described above and the total protein concentration was determined by measuring the absorbance at 280 nm.

Preparing cell lysates containing specific scFv-AP fusions—The clarified cell lysates containing the scFv-AP fusions that bind specifically to two recombinant zebrafish proteins, ZF130 (suppression of tumorigenicity 13/Hsp70 interacting protein) [27], and ZF656 (recoverin) [28], were prepared in a similar manner as the TG1 bacterial cell lysate, except that protein expression was carried out in the low phosphate medium with carbenicillin (50 $\mu\text{g/mL}$).

Western blotting using scFv-AP fusions as probes—Decreasing amounts of purified recombinant ZF130 and ZF656 proteins (200 to 0 ng) were resolved separately on two 10% SDS-PAGE gels (Bio-Rad Laboratories, Hercules, CA), along with a fixed amount of clarified TG1 bacterial cell lysate (100 μg) per lane. Proteins were transferred to Immobilon-P PVDF transfer membrane (Millipore Corporation, Bedford, MA) and the blocked membrane was probed for 1 h with the clarified cell lysate (20 $\mu\text{g/mL}$) containing the scFv-AP fusion specific for the target protein. The target protein complexed with its scFv-AP fusion was detected by ECF substrate (GE Healthcare, Piscataway, NJ).

Coomassie blue staining of TG1 bacterial cell lysate—TG1 cell lysate (100 μg) was resolved by SDS-PAGE and the gel was stained with Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Inc., Hercules, CA) to reveal the bacterial proteins present in each lane.

Affinity-capture using alkaline phosphatase fusions of scFv

Protein expression and preparation of clarified cell lysates were as described above. Clarified cell lysate (1 mg) containing the scFv-AP fusion specific for Lactoferrin protein (Sigma-Aldrich, Saint Louis, Missouri) was incubated overnight at 4°C with biotinylated anti-AP antibody (20 μg) (Abcam, Cambridge, MA) in a final reaction volume (50 μL) made up using PBST. The next day, this mixture was added to Dynabeads® MyOne™ Streptavidin T1 magnetic beads (50 μL ; Invitrogen Dynal AS, Oslo, Norway) and incubated at room temperature for 1 h on a rotator to capture the anti-AP antibody bound to scFv-AP fusion onto streptavidin beads via the biotin. TG1 bacterial cell lysate (100 μg) mixed with pure Lactoferrin protein (500 ng) in a final reaction volume (50 μL ; made up using PBST) was added to the washed beads and incubated for 1 h at room temperature. Finally, the washed beads were boiled at 95°C for 10 min in the presence of 2 $\times$ Novex® Tris-Glycine SDS sample buffer (50 μL ; Invitrogen, Carlsbad, CA) containing 2-mercaptoethanol (1.5 μL ; Acrosorganics, New Jersey, USA) to elute the bound proteins. The eluted proteins were separated from the beads by spinning at 6,000 rpm for 2 min and half the volume (25 μL) was resolved on a 10% SDS-PAGE gel (Bio-Rad Laboratories, Hercules, CA). The proteins

were transferred to a PVDF membrane (Millipore Corporation, Bedford, MA) and the blocked membrane was probed for 2 h at room temperature with anti-Lactoferrin antibody conjugated to AP (Abcam, Cambridge, MA). Finally, the immune complexes were detected with ECF substrate (GE Healthcare, Piscataway, NJ).

Results & Discussion

High-throughput conversion of a phage-display vector into expression vectors

Generally, when a library of phage-displayed scFvs is screened with a target protein, the binding clones are transferred into expression vectors to overexpress individual scFvs for downstream biochemical characterization. To accomplish this step, various molecular biology techniques (plasmid DNA isolation, primer design, PCR, restriction enzyme digestion, and DNA purification) are employed prior to sub-cloning into a second expression vector. Typically in our hands, DNA purification results in 30–60% physical loss of the DNA fragment. Moreover, the sub-cloning process generally requires each insert to be prepared individually, which makes it time consuming and laborious when dozens or more individual inserts have to be transferred into expression vectors. Sometimes, it is desirable to move them *en masse*, and then to analyze the binding properties of large numbers of scFvs [17;24]. However, both approaches are time consuming (up to 5 days) and the efficiency, in practice, ranges between 60 and 100%, and also requires expertise with the molecular cloning process. To facilitate the conversion of phage-displayed scFvs to enzyme fusions or 6xHis tagged forms, we have developed a “Double drop-out” approach, in which DNA fragments are dropped out of plasmid DNAs through restriction enzyme digestion and plasmid re-circularization by ligation generates expression formats in an iterative process.

The double drop-out vector (pKP300) has been designed with several useful properties (Figure 1) for efficient downstream screening of affinity reagents. The pKP300 vector is a modified version of the phagemid vector pAP-III₆ [25]. A mutant version of the *E. coli* AP, which has 16 fold greater enzymatic activity compared to the wild type AP [24], was cloned in-frame with the gene III coding sequence. The gene III coding sequence can be dropped out by restriction digestion with *Mfe* I enzyme, and re-ligation fuses the coding region of the scFv in-frame with the *E. coli* AP coding sequence. Similarly, by digestion with *Asc* I enzyme, the AP coding region can be dropped out, with re-ligation generating 6xHis tagged scFvs. If preferred, the gene III and AP coding sequences can be dropped out in a single step by restriction digestion with *Asc* I and re-ligation generates 6xHis tagged scFvs. After restriction enzyme digestions, the vector DNA is not further purified before re-ligation. Instead, the digested DNA is diluted 1:20 with water before re-ligation which facilitates intramolecular interaction (i.e., vector re-ligation) over intermolecular interactions (i.e., re-ligation between the vector and dropped out DNA). As this drop-out approach eliminates multiple steps (primer design, PCR of scFv inserts, gel or column purifications of digested vector and insert fragments, and sub-cloning), this increases the efficiency of converting a phage-display vector into one or more expression vectors, and speeds up the overall process of downstream screening of affinity reagents for various applications.

Both gene III and AP drop-out efficiencies were tested for four different scFv constructs; in each case, the efficiency was consistently between 90–100%. Of the 60 clones tested, only one clone did not have the gene III or AP coding sequence dropped out- indicating an efficiency of 98% (Supplementary Figures 1 and 2). As discussed above, the drop-out approach uses only one phagemid DNA into which useful restriction enzyme sites have been incorporated for excision of specific pieces of DNA and vector DNA re-ligation generates expression formats. This makes the drop-out approach faster (2 days) and takes half as much time compared to conventional sub-cloning strategies. In addition, this approach can be applied to other types of affinity reagents, such as the FN3 scaffold [29; 30], as long as they

do not contain *Asc* I or *Mfe* I restriction enzyme sites. We have selected these two restriction enzymes because they do not appear to occur in the scFv clones that we isolated from three human scFv libraries and an FN3 library that we tested. Therefore, these restriction sites occur infrequently in human scFvs and the FN3 domain such that they can be used for constructing such libraries, without cleaving within their coding regions. Before library construction, the AP coding sequence can be replaced with any other fusion partner such as AviTag [31], cutinase [32], Green fluorescent protein [33], or halotag [34] depending upon the downstream application of the affinity reagents.

Functional evaluation of the double drop-out vector

To demonstrate the utility of this double drop-out vector, four scFvs, each recognizing a different antigen, were cloned into the pKP300 phagemid vector, in-frame with the gene III coding sequence (Figure 1). Phage particles displaying the scFvs were incubated with the target proteins immobilized on Nunc MaxiSorp plates and binding was detected with an anti-M13 antibody conjugated to horseradish peroxidase (HRP). Phage ELISA (Figure 2A) demonstrated that each scFv bound specifically to its cognate target protein, and no binding to unrelated/control proteins was evident. To convert the phage-display vector (pKP300-scFv construct) into an expression vector, the phagemid DNA was digested with *Mfe* I restriction enzyme (to excise the gene III coding sequence) and re-ligated using T4 DNA ligase generating in-frame scFv-AP fusions (pKP300ΔIII-scFv construct). All the scFv-AP fusions retained cognate antigen recognition (Figure 2B). This drop-out strategy is extremely efficient, as 90 to 100% of the analyzed clones had their gene III coding sequence dropped out and the scFv fused in-frame to AP (as demonstrated by the presence of AP activity in cell lysates; Supplementary Figure 1). In order to generate scFvs with a C-terminal 6xHis tag (pKP300ΔIIIΔAP-scFv construct), a second restriction digestion with the *Asc* I restriction enzyme was performed prior to religation. Out of the 60 resulting transformants tested, 59 lacked the AP coding sequence as determined by performing a colony PCR (Supplementary Figure 2). Functional protein ELISA (Figure 2C) demonstrated that all the scFvs retained specific binding to their cognate targets. Therefore, the strategy of dropping out the gene III and AP coding sequences in a sequential manner or simultaneously by restriction enzyme digestion and re-ligation provides a simple and quick way of converting the phage-displayed scFvs into different expression formats. Although the pKP300 vector is relatively large in size (5.5 kb), an FN3 library with a diversity of 1.3×10^{10} members was constructed in this vector (data not shown).

An alternative approach for switching a construct from phage-display to expression of an antibody fragment is to use an amber stop codon in the coding region between the antibody fragment and protein III. In a supE bacterial host, there is suppression of the amber stop codon, leading to phage-display of the antibody fragment and, in a non-suppressor host, the antibody fragment accumulates in soluble form in the periplasm. This has been used for both Fragments of antigen binding (Fabs) [35] and scFvs [36]. However, depending on the bacterial strain, the suppression of the amber stop codon can be low (i.e., 16–20%), thus reducing the efficiency of phage-display. Conversely, as the amber stop codon is not a very strong termination codon, there will be read-through in the non-suppressor strain, leading to reduced yields of soluble antibody fragments in the periplasm. This is in contrast to our system in which there is no amber stop codon, phage-display is efficient, and yields of soluble scFvs are high (i.e., 4–14 mg/L). One other difference to note between our work and that of Zhao et al (2009) is that our phage-displayed antibodies lack the 6xHis tag, and thus can be used in affinity selection experiments in which the target is immobilized through its own 6xHis tag.

SDS-PAGE analysis of antibody fragments purified by IMAC

Western blotting revealed ~8–11 fold greater protein expression in the low phosphate medium compared to the high phosphate medium (Supplementary Figure 3). Thus, this drop-out vector is highly regulated, and has very little uninduced scFv expression. In order to confirm that scFvs can be overexpressed at high levels from the drop-out vector, scFvs and their AP fusions were expressed in low phosphate media, under the control of the PhoA promoter, an endogenous *E. coli* promoter. Bacterial cell pellets were lysed with detergent and proteins were purified from clarified cell lysates via the C-terminal 6xHis tag by IMAC [37]. The scFv-AP fusions (Figure 3A) and scFvs (Figure 3B) were resolved on 10% and 12% SDS-PAGE gels, respectively, followed by staining with Coomassie Brilliant Blue. After destaining the gel, the visualized protein bands appeared pure and were of the expected size, i.e., ~76 kDa and ~30 kDa for the scFv-AP fusions and scFvs, respectively. The yields of seven different scFvs and their AP fusions ranged from 4–14 mg/L and 3–8 mg/L, respectively, with purity ranging between 80–100% for scFvs and 60–90% for the scFv-AP fusions (Supplementary Table. 1). We have observed that the scFv-AP fusions exhibit lower yields and purity compared to their scFvs as a consequence of proteolytic cleavage between the scFv and AP proteins. However, this has not posed an obstacle in performing either the microtiter plate assays or western blotting experiments.

Alkaline phosphatase fusions as probes for western blotting

To demonstrate the utility of scFv-AP fusions in western blotting, various amounts of pure recombinant target protein was mixed with a fixed amount of bacterial cell lysate (100 µg) and resolved on a 10% SDS-PAGE gel. The protein bands were then transferred to a polyvinylidene fluorine (PVDF) transfer membrane and probed with a clarified cell lysate containing the scFv-AP fusion specific for the target protein. As shown in Figure 4, immune complexes were readily detected by enhanced chemifluorescence (ECF), even though each lane contained 100 µg of bacterial proteins.

Affinity-capture using scFv-AP fusions

Finally, we decided to take advantage of the scFv-AP fusions in immunocapture experiments. First, the scFv-AP fusion (specific for Lactoferrin) present in the clarified cell lysate was captured on streptavidin magnetic beads via the biotinylated anti-AP antibody. After the beads were washed, they were incubated with a complex mixture of bacterial proteins (100 µg) that had been doped with a small amount (500 ng) of the cognate antigen (Lactoferrin). Figure 5 shows that beads with the scFv-AP fusion (but not the scFv alone) could specifically affinity-capture Lactoferrin even in the presence of 100 µg of bacterial proteins (shown after Coomassie Brilliant Blue staining in lane 5). Immunocapture of Lactoferrin was not observed if either the anti-AP antibody (Lane 2) or the scFv-AP fusion specific for Lactoferrin (Lanes 3 and 4) were not present in the reaction mixture. This affinity-capture approach offers an advantage in that the scFv-AP fusions can be immobilized on beads without having to chemically biotinylate the AP fusions, which can sometimes disrupt the antigen binding site. Also, it is easier and faster to use the anti-AP antibody to immobilize the scFv-AP fusions as opposed to having to biotinylate each of the AP fusions separately.

Summary

Using the “Double drop-out” phagemid vector for constructing scFv or FN3 libraries will simplify and expedite the overall process of downstream screening of affinity reagents. Simple restriction enzyme digestions and re-ligations as opposed to time consuming and laborious sub-cloning procedures make this approach a highly efficient way of converting a phage-display vector into one or more expression vectors. For laboratories performing large

number of affinity selections against various proteins on a proteome scale, this construct will make the technology faster and the conversion of phage-displayed affinity reagents to their expression forms will be just a matter of a few days, with a high success rate of close to 100%.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The recombinant zebrafish proteins and *Rhodobacter sphaeroides* Reaction Center were gifts from Dr. Frank Collart, and Dr. Philip Laible and Dr. Deborah Hanson (Argonne National Laboratory, Argonne, IL), respectively. The scFvs that recognize zebrafish proteins and Reaction Center were from Ms. Sang Thai (UIC), Ms. Zengping Hao (UIC) and Alex Smetana (UIC). This work was supported by grants from the National Institutes of Health (R01 EY016094, R01 GM079096, and P01 GM075913).

Abbreviations

(AP)	alkaline phosphatase
(bp)	base pairs
(kb)	kilobases
(kDa)	kilodaltons
(ECF)	enhanced chemifluorescence
(HRP)	enzyme-linked immunosorbent assay, horseradish peroxidase
(PBS)	Phosphate Buffered Saline
(PBST)	Phosphate Buffered Saline with 0.1% Tween 20 v/v
(PCR)	polymerase chain reaction
(PVDF) membrane	polyvinylidene fluoride
(scFv)	single chain Fragments of variable regions
(scFv-AP)	alkaline phosphatase fusions of single chain Fragments of variable region
(LB)	Luria Bertani
(FN3) domain	Fibronectin type III
(IMAC)	immobilized metal affinity chromatography
(<i>E. coli</i>)	Escherichia coli
(pNPP)	para-Nitrophenyl Phosphate
(6xHis) tag	six-histidine

References

- [1]. Sheets MD, Amersdorfer P, Finnern R, Sargent P, Lindquist E, Schier R, Hemingsen G, Wong C, Gerhart JC, Marks JD. Efficient construction of a large nonimmune phage antibody library: the production of high-affinity human single-chain antibodies to protein antigens. *Proc Natl Acad Sci U S A*. 1998; 95:6157–62. [PubMed: 9600934]

- [2]. Bliss JM, Sullivan MA, Malone J, Haidaris CG. Differentiation of *Candida albicans* and *Candida dubliniensis* by using recombinant human antibody single-chain variable fragments specific for hyphae. *J Clin Microbiol.* 2003; 41:1152–60. [PubMed: 12624045]
- [3]. Rothe C, Urlinger S, Lohning C, Prassler J, Stark Y, Jager U, Hubner B, Bardroff M, Pradel I, Boss M, Bittlingmaier R, Bataa T, Frisch C, Brocks B, Honegger A, Urban M. The human combinatorial antibody library HuCAL GOLD combines diversification of all six CDRs according to the natural immune system with a novel display method for efficient selection of high-affinity antibodies. *J Mol Biol.* 2008; 376:1182–200. [PubMed: 18191144]
- [4]. Koide A, Bailey CW, Huang X, Koide S. The fibronectin type III domain as a scaffold for novel binding proteins. *J Mol Biol.* 1998; 284:1141–51. [PubMed: 9837732]
- [5]. Scholle MD, Kehoe JW, Kay BK. Efficient construction of a large collection of phage-displayed combinatorial peptide libraries. *Comb Chem High Throughput Screen.* 2005; 8:545–51. [PubMed: 16178814]
- [6]. Ernst A, Sazinsky SL, Hui S, Currell B, Dharsee M, Seshagiri S, Bader GD, Sidhu SS. Rapid evolution of functional complexity in a domain family. *Sci Signal.* 2009; 2:ra50. [PubMed: 19738200]
- [7]. Huber T, Steiner D, Rothlisberger D, Pluckthun A. In vitro selection and characterization of DARPin and Fab fragments for the co-crystallization of membrane proteins: The Na(+)-citrate symporter CitS as an example. *J Struct Biol.* 2007; 159:206–21. [PubMed: 17369048]
- [8]. Hufton SE, van Neer N, van den Beuken T, Desmet J, Sablon E, Hoogenboom HR. Development and application of cytotoxic T lymphocyte-associated antigen 4 as a protein scaffold for the generation of novel binding ligands. *FEBS Lett.* 2000; 475:225–31. [PubMed: 10869561]
- [9]. Nilsson FY, Tolmachev V. Affibody molecules: new protein domains for molecular imaging and targeted tumor therapy. *Curr Opin Drug Discov Devel.* 2007; 10:167–75.
- [10]. Skerra A. Alternative binding proteins: anticalins - harnessing the structural plasticity of the lipocalin ligand pocket to engineer novel binding activities. *Febs J.* 2008; 275:2677–83. [PubMed: 18435758]
- [11]. Smith GP, Petrenko VA. Phage Display. *Chem Rev.* 1997; 97:391–410. [PubMed: 11848876]
- [12]. Andris-Widhopf J, Rader C, Steinberger P, Fuller R, Barbas CF 3rd. Methods for the generation of chicken monoclonal antibody fragments by phage display. *J Immunol Methods.* 2000; 242:159–81. [PubMed: 10986398]
- [13]. Yang HY, Kang KJ, Chung JE, Shim H. Construction of a large synthetic human scFv library with six diversified CDRs and high functional diversity. *Mol Cells.* 2009; 27:225–35. [PubMed: 19277506]
- [14]. Vaughan TJ, Williams AJ, Pritchard K, Osbourn JK, Pope AR, Earnshaw JC, McCafferty J, Hodits RA, Wilton J, Johnson KS. Human antibodies with sub-nanomolar affinities isolated from a large non-immunized phage display library. *Nat Biotechnol.* 1996; 14:309–14. [PubMed: 9630891]
- [15]. Kay BK, Kasanov J, Yamabhai M. Screening phage-displayed combinatorial peptide libraries. *Methods.* 2001; 24:240–6. [PubMed: 11403573]
- [16]. Turunen L, Takkinen K, Soderlund H, Pulli T. Automated panning and screening procedure on microplates for antibody generation from phage display libraries. *J Biomol Screen.* 2009; 14:282–93. [PubMed: 19224869]
- [17]. Schofield DJ, Pope AR, Clementel V, Buckell J, Chapple S, Clarke KF, Conquer JS, Crofts AM, Crowther SR, Dyson MR, Flack G, Griffin GJ, Hooks Y, Howat WJ, Kolb-Kokocinski A, Kunze S, Martin CD, Maslen GL, Mitchell JN, O'Sullivan M, Perera RL, Roake W, Shadbolt SP, Vincent KJ, Warford A, Wilson WE, Xie J, Young JL, McCafferty J. Application of phage display to high throughput antibody generation and characterization. *Genome Biol.* 2007; 8:R254. [PubMed: 18047641]
- [18]. Pershad K, Pavlovic JD, Graslund S, Nilsson P, Colwill K, Karatt-Vellatt A, Schofield DJ, Dyson MR, Pawson T, Kay BK, McCafferty J. Generating a panel of highly specific antibodies to 20 human SH2 domains by phage display. *Protein Eng Des Sel.* 23:279–88. [PubMed: 20164216]
- [19]. Wels W, Harwerth IM, Zwickl M, Hardman N, Groner B, Hynes NE. Construction, bacterial expression and characterization of a bifunctional single-chain antibody-phosphatase fusion

- protein targeted to the human erbB-2 receptor. *Biotechnology (N Y)*. 1992; 10:1128–32. [PubMed: 1369487]
- [20]. Martin CD, Rojas G, Mitchell JN, Vincent KJ, Wu J, McCafferty J, Schofield DJ. A simple vector system to improve performance and utilisation of recombinant antibodies. *BMC Biotechnol*. 2006; 6:46. [PubMed: 17156422]
- [21]. Muller BH, Chevrier D, Boulain JC, Guesdon JL. Recombinant single-chain Fv antibody fragment-alkaline phosphatase conjugate for one-step immunodetection in molecular hybridization. *J Immunol Methods*. 1999; 227:177–85. [PubMed: 10485265]
- [22]. Wright RM, Dudas D, Gavin B, Dottavio D, Hexham JM, Lake P. A high-capacity alkaline phosphatase reporter system for the rapid analysis of specificity and relative affinity of peptides from phage-display libraries. *J Immunol Methods*. 2001; 253:223–32. [PubMed: 11384683]
- [23]. Gonzalez-Techera A, Umpierrez-Failache M, Cardozo S, Obal G, Pritsch O, Last JA, Gee SJ, Hammock BD, Gonzalez-Sapienza G. High-throughput method for ranking the affinity of peptide ligands selected from phage display libraries. *Bioconjug Chem*. 2008; 19:993–1000. [PubMed: 18393454]
- [24]. Han Z, Karatan E, Scholle MD, McCafferty J, Kay BK. Accelerated screening of phage-display output with alkaline phosphatase fusions. *Comb Chem High Throughput Screen*. 2004; 7:55–62. [PubMed: 14965261]
- [25]. Haidaris CG, Malone J, Sherrill LA, Bliss JM, Gaspari AA, Insel RA, Sullivan MA. Recombinant human antibody single chain variable fragments reactive with *Candida albicans* surface antigens. *J Immunol Methods*. 2001; 257:185–202. [PubMed: 11687252]
- [26]. Sidhu SS, Lowman HB, Cunningham BC, Wells JA. Phage display for selection of novel binding peptides. *Methods Enzymol*. 2000; 328:333–63. [PubMed: 11075354]
- [27]. Shi ZZ, Zhang JW, Zheng S. What we know about ST13, a co-factor of heat shock protein, or a tumor suppressor? *J Zhejiang Univ Sci B*. 2007; 8:170–6. [PubMed: 17323428]
- [28]. Bazhin AV, Schadendorf D, Philippov PP, Eichmuller SB. Recoverin as a cancer-retina antigen. *Cancer Immunol Immunother*. 2007; 56:110–6. [PubMed: 16444517]
- [29]. Karatan E, Merguerian M, Han Z, Scholle MD, Koide S, Kay BK. Molecular recognition properties of FN3 monobodies that bind the Src SH3 domain. *Chem Biol*. 2004; 11:835–44. [PubMed: 15217616]
- [30]. Koide A, Gilbreth RN, Esaki K, Tereshko V, Koide S. High-affinity single-domain binding proteins with a binary-code interface. *Proc Natl Acad Sci U S A*. 2007; 104:6632–7. [PubMed: 17420456]
- [31]. Scholle MD, Collart FR, Kay BK. In vivo biotinylated proteins as targets for phage-display selection experiments. *Protein Expr Purif*. 2004; 37:243–52. [PubMed: 15294305]
- [32]. Kwon Y, Han Z, Karatan E, Mrksich M, Kay BK. Antibody arrays prepared by cutinase-mediated immobilization on self-assembled monolayers. *Anal Chem*. 2004; 76:5713–20. [PubMed: 15456290]
- [33]. Ohshima M, Inoue K, Hayashi H, Tsuji D, Mizugaki M, Itoh K. Generation of AcGFP fusion with single-chain Fv selected from a phage display library constructed from mice hyperimmunized against 5-methyl 2'-deoxycytidine. *Protein Eng Des Sel*. 23:881–8. [PubMed: 20876190]
- [34]. Los GV, Encell LP, McDougall MG, Hartzell DD, Karassina N, Zimprich C, Wood MG, Learish R, Ohana RF, Urh M, Simpson D, Mendez J, Zimmerman K, Otto P, Vidugiris G, Zhu J, Darzins A, Klaubert DH, Bulleit RF, Wood KV. HaloTag: a novel protein labeling technology for cell imaging and protein analysis. *ACS Chem Biol*. 2008; 3:373–82. [PubMed: 18533659]
- [35]. Hoogenboom HR, Griffiths AD, Johnson KS, Chiswell DJ, Hudson P, Winter G. Multi-subunit proteins on the surface of filamentous phage: methodologies for displaying antibody (Fab) heavy and light chains. *Nucleic Acids Res*. 1991; 19:4133–4137. [PubMed: 1908075]
- [36]. Zhao Q, Chan YW, Lee SS, Cheung WT. One-step expression and purification of single-chain variable antibody fragment using an improved hexahistidine tag phagemid vector. *Protein Expr Purif*. 2009; 68:190–195. [PubMed: 19683057]
- [37]. Hamilton S, Odili J, Pacifico MD, Wilson GD, Kupsch JM. Effect of imidazole on the solubility of a his-tagged antibody fragment. *Hybrid Hybridomics*. 2003; 22:347–55. [PubMed: 14683594]

Figure 1. Strategy for efficiently converting a phage-display vector into different expression vectors

A) The scFvs cloned in-frame with the gene III coding sequence are displayed on the surface of bacteriophage M13. The OmpA signal sequence (ss) directs the recombinant protein into the periplasm of *E. coli*. All the proteins display a Flag peptide (DYKDDDDKL) at their N-termini. Gene III is truncated (~630 bp) and has opal and ochre stop codons at the C-terminus, which are represented by two asterisks. B) After the affinity selection process, the gene III coding sequence is dropped out by restriction digestion with *Mfe* I, followed by re-ligation of the vector, resulting in an in-frame fusion of the AP coding region to the scFv. C) Subsequent digestion of the recombinant plasmid with *Asc* I leads to loss of the AP coding region, with re-ligation generating 6xHis tagged scFvs. The sizes of the three vector DNAs including the scFv coding sequences are represented in kilobases (kb).

Figure 2. Functional tests validating the drop-out strategy

The coding sequences of four scFvs that recognize different protein targets were transferred into the pKP300 vector followed by dropping out the coding regions for gene III and AP. A) ELISA demonstrated specific binding of the phage-displayed scFvs to their cognate targets, with no binding detected for the unrelated targets. Binding of phage particles to immobilized targets was detected using anti-M13 antibody conjugated to HRP. B) To generate scFv-AP fusions, the pKP300-scFv recombinant DNAs were digested with *Mfe* I and re-ligated. The scFv-AP fusions showed specific binding to their cognate targets. Binding was detected by addition of para-Nitrophenyl Phosphate (pNPP), a substrate for AP. C) To generate scFvs with a C-terminal 6xHis tag, the second set of recombinants were generated by digesting the pKP300 Δ III-scFv DNAs with *Asc* I to drop out the AP coding sequence followed by re-ligation. Binding of the 6xHis tagged scFvs to their cognate targets was detected using an anti-His antibody conjugated to HRP. The optical absorbance of microtiter plate wells was recorded at 405 nm. All experiments were conducted in duplicates, with standard error shown.

Figure 3. Purified antibody fragments visualized on SDS-PAGE gels

Five different antibody fragments were purified by IMAC via the C-terminal 6xHis tag. The purified proteins were resolved by SDS-PAGE electrophoresis and stained with Coomassie Brilliant Blue. The first lane shows the molecular weight (MW) ladder with the standard protein sizes marked in kilodaltons (kDa). A) Purified scFv-AP fusions (2 μ g total protein per lane) visualized on a 10% SDS-PAGE gel, with an expected molecular weight of ~76 kDa. B) Purified scFvs (3 μ g total protein per lane) visualized on a 12% SDS-PAGE gel, with an expected size of ~30 kDa. The antibody fragments LF 2.1, RC E12/RC H2 and ZF578 C8/ZF656 #17 were isolated against human Lactoferrin protein, *Rhodobacter sphaeroides* Reaction Center and two proteins from zebrafish.

Figure 4. Demonstrating the utility of scFv-AP fusions in western blotting

Decreasing amounts (200, 150, 100, 50, 25, 5, and 0 ng) of two zebrafish proteins, ZF130 and ZF656, were loaded from left to right on two different SDS-PAGE gels (10%), along with a fixed amount of clarified bacterial cell lysate (100 μ g) per lane, which is shown in the right most lane by Coomassie Blue staining. After electrophoretic transfer to a PVDF membrane, the clarified cell lysate containing the scFv-AP fusion specific for either the ZF130 or ZF656 protein was used as the primary antibody for probing the membrane. Antigen-antibody complexes were detected by ECF. A) The anti-ZF130 scFv-AP fusion could detect as little as 25 ng of ZF130 protein mixed with 100 μ g bacterial cell lysate. B) The ZF656 scFv-AP fusion could detect as low as 5 ng of ZF656 protein mixed with 100 μ g bacterial cell lysate.

Figure 5. Affinity-capture of the target protein from a complex mixture of proteins using the scFv-AP fusion protein

The scFv-AP fusion protein that binds specifically to human Lactoferrin was immobilized on streptavidin magnetic beads via the biotinylated anti-AP antibody. Immunocapture of the target protein (Lactoferrin) from a complex mixture of bacterial proteins was monitored by western blotting. The immunocapture reactions were resolved on a 10% SDS-PAGE gel, transferred to a PVDF membrane and probed with anti-Lactoferrin antibody conjugated to AP. Detection was performed with ECF. Lane 1. Anti-Lactoferrin scFv-AP fusion specifically affinity-captured Lactoferrin (~80 kDa) from a complex mixture of proteins, i.e., ~100 μ g of bacterial proteins which are depicted in Lane 5 after Coomassie Brilliant Blue staining. Lanes 2–4 demonstrate that successful immunocapture of Lactoferrin requires both the anti-AP antibody and the anti-Lactoferrin scFv-AP fusion.