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PHOPHABS: ANTIBODY-PHAGE-ALKALINE PHOSPHATASE CONJUGATES FOR ONE STEP ELISA'S WITHOUT IMMUNIZATION

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Abstract.

PhoPhabs have been created by incorporating alkaline phosphatase and antibody Fab's from combinatorial libraries on a filamentous phage framework. These PhoPhabs are antigen specific and can replace antibodies and eliminate the need for immunizations in ELISA (enzyme linked immunoabsorbent) methods.

Introduction.

Combinatorial antibody libraries are rapidly replacing conventional hybridoma techniques.[1] In order to further this change, and expedite immunoassay procedures, a PhoPhab (phosphatase-filamentous phage-antibody Fab fragment) system was developed. Unlike more time consuming traditional methods, the PhoPhab method does not require expensive cell culture and it is possible to perform immunochemical techniques such as ELISA's without isolating soluble antibodies. Most importantly, when PhoPhabs are produced from a semi-synthetic phab library,[2] *no immunizations are required to produce an antigen specific reagent.*

Phage display systems have been used to expedite the screening of large libraries (10^7-10^8 members) of randomly combined heavy and light chain fragments for the ability to bind antigen.[1,3] Such libraries are constructed by PCR cloning of separate variable heavy and light chains from tissue of immunized subjects or from previously constructed libraries with randomized sequences. These chains are randomly paired in the phagemid vector pComb3, resulting in a fusion of the heavy chain to a fragment of the filamentous phage tip coat protein gIIIp. Phage extruded from *E. coli* containing the vector with heavy and light chains have the Fab fragment of the antibody molecule fused to one end of the phage particle, and also contain a single strand of the vector DNA encoding the nucleotide sequence of the displayed

Fab. In this way the processes of recognition and replication are linked in a single phage particle.

In a process called panning,[4] the phage expressing antigen binding pairs of heavy and light chains are enriched and isolated. Human Fab's to HIV,[5] other pathogens, as well as mouse Fab's[6,7] to a variety of antigens have been isolated by these methods. In addition to antibodies, bacterial alkaline phosphatase (E.C. 3.1.3.1, the *Pho*A gene product) has been attached to the gene III protein and shown to be active.[8]

In addition to the gene III protein site at the tip of filamentous phage particles, the gene VIII protein (gVIIIp), which comprises the shaft of the particle, is an attractive site for expression of proteins. Depending on the phage length, there are approximately 2700 copies of the gVIII protein, vs. 5 copies of the gIII protein.[9] Indeed, we have already incorporated functional antibodies along the phage particle via the gVIII coat protein.[6,10] Given these previous results it seemed possible to use the phage as a framework to link a Fab-gIIIp fusion to multiple copies of alkaline phosphatase-gVIIIp fusion, illustrated in Fig. 1. Here we demonstrate that such Fab-phage-alkaline phosphatase conjugates, dubbed PhoPhabs, are specific one step reagents for ELISA's.

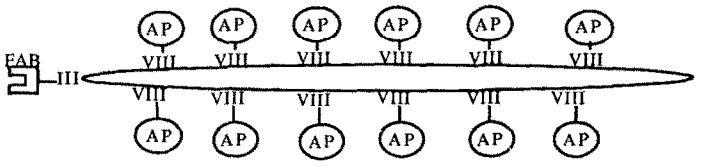


Figure 1. A cartoon of a phage displaying an Fab fused to the gene III coat protein and multiple copies of alkaline phosphatase (AP) conjugated to coat protein VIII.

Results.

To produce the doubly conjugated phage, a second vector pPho8cat was constructed so that antigen-binding clones from the pComb3 system could be directly converted into PhoPhabs. The pPho8cat vector is illustrated in Figure 2.

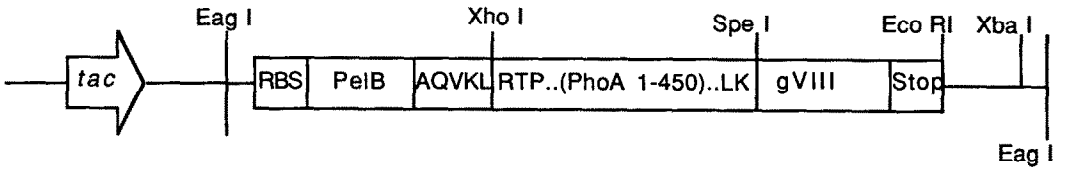
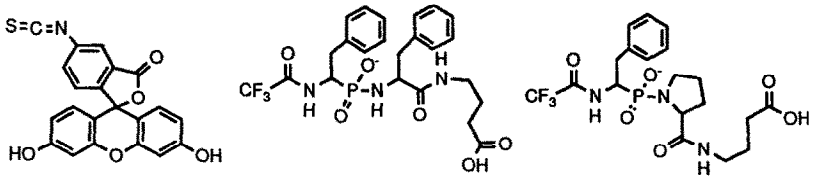


Figure 2. The pPho8cat vector. Vector construction: the alkaline phosphatase gene *phoA* was cloned from *E. coli* strain XL1-blue[17] by PCR with primers PHO5 5'-CAGCTGCTCGAGCGGACACCAGAAATGCCTGTT-3' and PHO3 5'-AGGCTTACTAGTTTTCAGCCCCAGAGCGGCTTT-3' (synthesized by Operon Technologies, Alameda, CA, XhoI and SpeI sites underlined), which were based upon the published sequence of the alkaline phosphatase gene.[11] After digestion with XhoI and SpeI, the insert was ligated into pComb8,[1] introducing the pelB signal peptide and gVIII fused to the C-terminus. The transformants in XL1-blues were selected by plating on LB agar containing 100 µg/mL carbenicillin, 0.1 mM phosphate, and 5-bromo-4-chloroindoyl phosphate (X-P) 40 µg/mL. A blue colony was selected and its plasmid digested with Eag I and the 2458 bp fragment ligated into the Eag I site in pFL261.[12] A clone with the correct orientation was identified by restriction digests with Xba I and the ability to hydrolyze X-P, resulting in the final plasmid pPho8cat. This plasmid contains the p15A origin of replication and the chloramphenicol acyl transferase gene, as well as the *phoA*-gVIIIp fusion under control of the *tac* promoter. The p15A origin is compatible with the colE1 origin on pComb3, allowing stable double transformants to be created.

To test the viability of PhoPhabs as analytical reagents, PhoPhabs specific for several different antigens were created from single clones or libraries enriched for the antigen by panning as previously described. The antigens used were fluorescein isothiocyanate conjugated to BSA[2] **1**, phosphonamidate haptens PPC[13] **2** and Pro1[13] **3**, and tetanus toxoid. The Pro1 and PPC libraries were constructed from immunized mice by published procedures[14,15] and the tetanus toxoid clone P313 was isolated from a library[16] constructed from an immunized human. However, the fluorescein library was constructed without an immunization with hapten by a semi-synthetic approach in which the CDR3 region of tetanus binder 7E was randomized. Panning the randomized library against Fluorescein-BSA gave fluorescein specific binders.[2] These semi-synthetic libraries make it possible to obtain antigen specific Fab's without immunizations. Therefore, PhoPhabs constructed from this anti-fluorescein library are synthetic reagents.



1 **2** **3**

PhoPhabs were produced by cotransforming pPho8cat with the respective pComb3 libraries and selecting double transformants on chloramphenicol and carbenicillin. Cultures of one double transformant (monoclonal) or approximately 20 (polyclonal) were infected with helper phage VCSM13[17] and induced with IPTG. Phage from the supernatants were precipitated, resuspended, and used directly in an ELISA shown in Fig. 3. As expected, the PhoPhabs were specific for the same antigen as the antigen against which the library was panned.

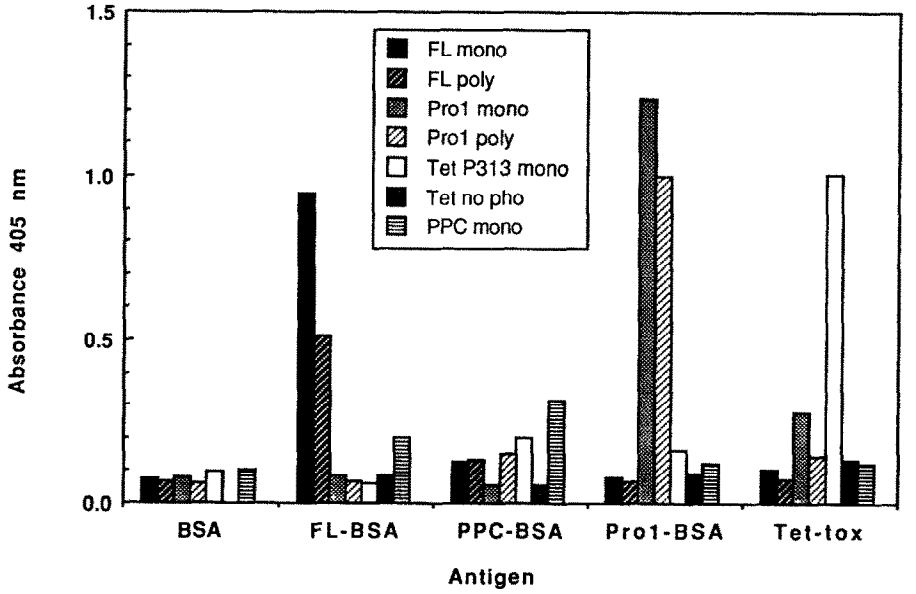


Figure 3. An ELISA performed with PhoPhabs against antigens FL-BSA=fluorescein-BSA conjugate, Pro1-BSA= hapten **2**, PPC-BSA= hapten **3**, Tet-tox=tetanus toxoid protein, Tet no pho=phage displaying P313 Fab-gIII but no AP-gVIII conjugate (BSA=bovine serum albumen). Antigens were coated onto Costar brand EIA plates #3690 at a concentration of 0.2 μg in 25 μL of 0.1 M $NaHCO_3$ pH 8.6 or PBS pH 7.4 at 4 °C overnight. The wells were blocked with 1% BSA in PBS for 1 h at 37 °C, washed with water, and then 10 μL of 1%BSA and 25 μL of concentrated phage was added for 30 min. at 37°C. After washing 10 cycles on a plate washer, 50 μL developer (1 mg/mL *p*-nitrophenyl phosphate, 10% v/v diethanolamine, 1 mM $MgCl_2$, 3 mM NaN_3 pH 9.8) was added and the plate incubated at 37°C overnight. The phage were produced by inoculating 10 mL of super broth (30 g tryptone, 20 g yeast extract, 10 g MOPS/L, pH 7.0, containing 50 μg/mL carbenicillin, 15 μg/mL chloramphenicol, 10 μg/mL tetracycline, 20 mM $MgCl_2$, 1 μM $ZnCl_2$) with 1 mL of a fresh overnight culture of the double transformants in strain XL1-blue[17] (grown in antibiotic medium #3 (Difco Laboratories) same additives). After 1 h at 37 °C, 10^9 cfu of VCSM13 helper phage were added and incubated at 37° C. Twenty minutes later, kanamycin and IPTG were added to concentrations of 70 μg/mL and 1 mM, respectively. After 3 h, the phage were precipitated from the culture supernatants with final concentrations of 4% PEG-8000 and 500 mM NaCl for 1 h 4°C. The phage were pelleted at 15,000 rpm (Beckman JA-20) and resuspended in 400 μL of 10 mM tris, 20 mM $MgCl_2$, 1 mM $ZnCl_2$ pH 8.0.

All of the PhoPhabs used in Fig. 3 gave clear signals, except for the one directed against PPC-BSA, labeled PPC mono. Whether this one case is due to poor expression or is instead a true negative result is presently being explored.

Discussion.

The PhoPhab system which links an antigen-specific Fab to multiple copies of alkaline phosphatase has advantages of simplicity and time over conventional techniques. Traditional methods for immunochemical techniques such as ELISA's and Western blots require an antibody against the antigen of interest and a secondary antibody-enzyme conjugate for detection, which requires two immunizations, as well as the production of hybridomas if monoclonal specificity is desired. Instead, this new technique uses phage display to isolate the desired binding specificity and eliminates the need for a secondary reagent, while still achieving amplification of the antigen signal.

The specificity of the reagent is determined by the panning selection process, and either monoclonal or polyclonal mixtures can be used. The desired specificity can be selected by panning a small amount of Phabs, and then simply growing the desired reagent with bacterial culture techniques. The possibility exists to remove unwanted cross reactivity by subtractive panning. When coupled with a pre-made synthetic library, antigen specific reagents can be produced without immunization in only a few days, compared to months for the production of monoclonal antibodies. The phage reagents are also anticipated to be less expensive to produce. Presently work is under way to improve the expression of the PhoPhab system and apply it to other immunochemical techniques.

Filamentous phage frameworks can be extended beyond the PhoPhab example illustrated here. Other enzymes and proteins could be linked on filamentous phage, using the different coat proteins to vary the number of attachments. For example, a binder-gIIIp-phage-(gVIIIp-enzyme)$_n$ system could be used to deliver multiple copies of an enzyme to a specific site *in vitro,* and perhaps *in vivo,* allowing for antigenic responses. The use of filamentous phage need not be limited to single phage, but networks of phage frameworks could be linked. One way would be to direct Fab#1-phage-enzyme against Fab#2-phage-antigen#1, thereby creating a large amplification in the number of enzyme molecules bound to antigen#2.

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