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# Phage-borne Peptidomimetics Accelerate the Development of Polyclonal Antibody-based Heterologous Immunoassays for the Detection of Pesticide Metabolites

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Competitive immunoassays for the detection of small analytes, such as pesticides and their metabolites, use haptens that compete with the target compounds for binding to the antibody. This competing hapten can be either the same as the immunizing hapten (homologous assay) or structurally modified mimics of the immunizing hapten (heterologous assay). Polyclonal antibody-based heterologous immunoassays have shown superior sensitivities to homologous ones, but the synthesis of heterologous haptens may be time-consuming, requiring expertise in synthetic chemistry. In this work we demonstrate that phage display peptide libraries can be used as a source of phage-borne peptidomimetics to facilitate the development of sensitive heterologous assays. Different strategies for the isolation of these peptides were explored using two metabolites of pyrethroid insecticides. The sensitivities of the best competitive phage heterologous enzyme-linked immunosorbent assays were 13 fold and 100 fold better than the homologous assay, for the glycine conjugate of *trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid and 3-phenoxybenzoic acid, respectively. The phage particles were highly versatile as tracer reagents, allowing the use of enzymatic, chemiluminescent, or immuno-polymerase chain reaction detection. The data presented here shows a new systematic procedure that enables the fast generation of several competing haptens for the rapid development of sensitive heterologous immunoassays.

## Introduction

Immunoassays are practical analytical tools for detecting a large variety of biological and environmental substances in clinical diagnosis, home testing, and environmental monitoring. Due to the fact that they are fast, low cost assays that allow parallel processing of a large number of samples, immunoassays are particularly useful in numerous environmental applications (*1*–*3*). In addition, the antibody-driven high selectivity and sensitivity for a target molecule makes it possible to eliminate or to simplify the steps of sample cleanup, making the assay rapid and cheaper.

High molecular weight compounds are conveniently detected by the so-called sandwich assay, in which one antibody immobilized on a solid support captures the target molecule and a second antibody is later used to detect the formation of the immune complex. When the analyte is a small molecule (hapten), the simultaneous recognition by two antibodies is seldom possible, and the formation of the immune complex is indirectly detected using a competitive format. In this layout, the analyte competes with a tracer compound, (i.e., enzyme, fluorophore, etc.) coupled to a competing hapten for binding to the antibody. In the simplest setup (homologous assay) the competing hapten is the same as the hapten used to elicit the analyte-specific antibodies (immunizing hapten). However, when polyclonal antibodies (PAbs), and often monoclonal antibodies, are used, the sensitivity of the assay can be improved by orders of magnitude if structural variants of the immunizing hapten are used for competition (heterologous assay) (*4, 5*). The main principle underlying this observation is related to the fact that different competing haptens might cross-react with different antibody subpopulations within the serum. Thus, the best competing hapten will be the one that binds to the subpopulation of antibodies that has the highest affinity for the analyte, which will translate to higher assay sensitivity. These heterologous assays may require a considerable effort in chemical synthesis to generate a panel of candidate haptens from which the best competing hapten needs to be selected. Depending on the analyte, the synthesis may be particularly challenging. Thus, new approaches for the development of PAbs heterologous assays are needed.

We have recently found that analyte peptidomimetics isolated from phage display libraries can be used as surrogate competing haptens in monoclonal antibody-based immunoassays for herbicides molinate and atrazine (*6*). The concept has a high potential to be of practical use in the development of heterologous PAb-based immunoassays. Since phage peptide display was first reported (*7*), it has been a powerful tool for a variety of applications, including the isolation of peptide ligands for antibodies and enzymes (*8*–*10*), antibody engineering (*11, 12*), and the isolation of receptor peptides for small molecules (*13, 14*). In this work, we tested the hypothesis that the enormous diversity of phage display peptide libraries would facilitate the easy isolation of phage-borne peptide haptens specific for antianalyte PAbs, avoiding the laborious synthesis of chemical heterologous competing haptens. The different peptide sequences, which cross-react with different antibody subpopulations, would be used to recruit the higher affinity population of antibodies, accelerating the development of highly sensitive competitive immunoassays.

To test this concept, we used a phage display peptide library displaying cyclic peptides of eight amino acids to select phage-borne peptides competitively binding to two PAbs that are specific for two major metabolites of pyrethroid insecticides: 3-phenoxybenzoic acid (3-PBA) and the glycine conjugate of *t*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid (*t*-DCCA-glycine). A novel strategy for isolation of phage-borne peptide haptens with the well-established biopanning method and comparison of isolation efficiencies with two different PAbs purification methods are

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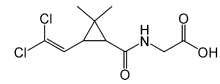
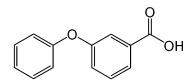
**TABLE 1. Peptide Sequences Isolated Using Immobilized Hapten or Protein A Purified PAbs[a]**

| | | |
|---|---|---|
| PAbs to DCCA-glycine | |  |
| Purification method | Clone | Sequence |
| Hapten purified | D8 | C A Q F M G Y S R C |
| | D5, D6, D9, D10 | C **S** **W** F L E W S L C |
| | D3 | C **S** **W** **L** N H **F** I D C |
| | D1, D2 | C **S** **W** **L** D F **F** S H C |
| Protein A purified | 7D, 11F | C **S** **W** **L** T G **F** V T C |
| | 2A, 5C,8F | C N N S H S L L F C |
| | 2D, 1F | C S Y V W D L W K C |
| PAbs to 3-PBA | |  |
| | P1, P2, P3 | C T Y V W G P L W C |
| | P4 | C G L T D M L N F C |
| Hapten purified | P5, P6, P7 | C I P Y L Q W W N C |
| | P8, P9 | C P G Y Q N H S R C |
| | P10 | C P E C D L D C Y C |

[a] Bold letters are used to denote consensus residues.

presented. We further show the high throughput screening of phage clones and the downstream applications of the isolated phages for the development of phage enzyme-linked immunosorbent assays (ELISAs) using conventional enzymatic and nonenzymatic detection, as well as an alternative detection principle based on the genetic information of the phage (i.e., phage real-time PCR (PrPCR)).

## Materials and Methods

**Materials.** All reagents were of analytical grade unless specified otherwise. The synthesis of *t*-DCCA-glycine, *t*-DCCA-glycine hapten, and 3-PBA hapten and the production of antiserum 3703 for *t*-DCCA-glycine and 294 for 3-PBA were previously presented in this laboratory (*4, 5*). Mouse anti-M13 monoclonal antibody-horse radish peroxidase (HRP) conjugate and PD-10 desalting columns were purchased from GE Health Care (Piscataway, NJ), and helper phage M13KO7 was purchased from New England Biolabs (Ipswich, MA). BCA protein assay kit for the quantification of purified antibody, protein A affinity column for IgG purification, and Aminolink Plus Coupling Gel for the immobilization of hapten-bovine serum albumin (BSA) conjugate were purchased from Pierce (Rockford, IL). BSA, polyethyleneglycol (PEG), Tween 20, 3,3′,5,5′-tetramethylbenzidine (TMB), and 3-PBA standard were obtained from Sigma (St. Louis, MO). Streptavidin coated magnetic beads and magnetic extractor were purchased from Invitrogen (Carlsbad, CA). Real-time PCRs were carried out with the ABI 7500 instrument (Foster City, CA). The primers and TaqMan probe were designed with ABI's software. Acridinium NHS ester was purchased from Assay Designs (Ann Arbor, MI). Chemiluminescent signals were obtained with a Veritas with dual injectors manufactured by Turner Biosystems (Sunnyvale, CA).

**Phage Display Peptide Libraries and Biopanning.** A random phage display peptide library with an estimated diversity of $3 \times 10^9$ independent clones was constructed on the phagemid vector pAFF/MBP as previously described (*15*).The peptide library used in this study expresses cyclic 8 mer random peptides flanked by two cysteines (−AS-GSACX8CGP6−) and fused to the phage coat protein pIII. The 96 well microtiter ELISA plates (Maxisorp Nunc) were coated with the analyte-specific PAbs (0.1 $\mu$g/well) purified with 3-PBA or *t*-DCCA-glycine hapten affinity column or PAb 3703 (0.5 $\mu$g/well) purified with protein A column in carbonate-bicarbonate buffer (pH 9.6) by overnight incubation at 4 °C. Nonspecific binding sites were blocked with 250 $\mu$L of 1% BSA in phosphate buffered saline (PBS) containing 0.05% Tween 20 (PBST) for 1 h at room temperature, and then 100 $\mu$L of phage library ($1 \times 10^{12}$ transducing units) containing 1% BSA in PBS was added into each of 6 wells (600 $\mu$L total volume) followed by incubation for 2 h at 4 °C with gentle shaking. Unbound phages were removed by 10 washes with PBST, and the plate was incubated again for 30 min with 250 $\mu$L of PBS. This step was repeated twice. Bound phages were eluted by incubation with 0.1 N glycine solution (pH 2.2) for 15 min at room temperature and were immediately neutralized with 35 $\mu$L of 2 M Tris base. For an amplification of eluted phage, 1 mL of ARI 292 cells (Affymax Research Institute, Palo Alto, CA) that had been cultured overnight in 5 mL Luria–Bertani (LB) media was added into the 100 mL culture flask containing 20 mL of LB medium, and the cells were cultured again until the optical density at 600 nm (OD600) reached 0.4. The medium was centrifuged at 12 000*g* for 15 min. The cell pellet was resuspended with 2 mL of LB media, and 600 $\mu$L of cell suspension was mixed

with 300 $\mu$L of phage eluate. After incubation at 37 °C for 30 min without shaking, the entire mixture was added into another 100 mL culture flask containing 10 mL of SOP medium (LB containing 0.25% $K_2HPO_4$, 0.1% $MgSO_4$, 0.1% glucose, and 100 $\mu$g/mL ampicillin). When OD600 reached 0.2, the M13KO7 helper phage was added at a multiplicity of infection of 10:1. After a period of 30 min at 37 °C without shaking, arabinose and kanamycin were added to a final concentration of 0.02% and 40 $\mu$g/mL, respectively, and the cultures were incubated overnight at 37 °C with vigorous shaking. Cell cultures incubated overnight were centrifuged at 12 000*g* for 15 min. Phages were obtained by incubation of the clear supernatants mixed with 0.2 volume of 20% PEG in 2.5 M NaCl (PEG-NaCl) on ice and were centrifuged as above. Phage pellets were resuspended with 2 mL of sterile PBS and were titrated in ARI 292. This entire panning procedure was then repeated twice, except that 5 and 2.5 ng/mL concentrations of *t*-DCCA-glycine and 3-PBA standard were used to elute bound phage for the second and third panning, respectively, and for the elution of phages bound to protein A purified 3703, 100 $\mu$L of 16 $\mu$g/mL of *t*-DCCA-glycine standard was added for the second and third panning and 2.5 ng/mL for the final round of panning. After the final round of panning, phage isolates were obtained by randomly culturing phage clones from titration plates, and they were screened for their competitive binding to the purified antibodies by the phage ELISA.

**Screening of Phage Clones.** After the last round of phage panning, ARI 292 was infected with phage and was grown on LB-agar plates with 100 $\mu$g/mL ampicillin. Phages were obtained from 10 clones for each hapten affinity-purified antibody. For protein A purified 3703, 100 colonies were picked up from the titration plate into the 96 well tissue culture plates containing 100 $\mu$L of LB medium with 100 $\mu$g/mL ampicillin, and then the plates were incubated overnight at 37 °C with shaking. The following day, 25 $\mu$L of each culture was transferred to a 96 deep-well plate with a multichannel pipet for which each well contained 600 $\mu$L of SOP medium. The plates were incubated until OD600 = 0.2. Twelve microliters of the helper phage (1:10 dilution of phage stock ($1 \times 10^{12}$ t.u/mL) in SOP) was added to the plates with the multichannel pipet, followed by incubation at 37 °C for 30 min with no shaking. Ten microliters of the mixture of arabinose and kanamycin (1:5 dilution of arabinose (20%) and kanamycin (20 mg/mL) in SOP medium) was added to the plates with the multichannel pipet, and the plates were incubated overnight at 37 °C with vigorous shaking. On the following day, the plate was centrifuged at 3000 rpm for 30 min, and then the supernatants were used for screening.

**Phage ELISA and Cross-reactivity (CR).** Phage ELISA was performed for the screening of positive phage clones and for the development of phage ELISA. Concentrated phage suspensions were prepared from positive clones on the basis of their performance in the screening assay. The selectivity of PAb 3703 and 294 in the phage ELISA were estimated with several structurally related pyrethroids and their metabolites. Methods for these experiments were previously presented (*6, 16*).

**Matrix Effect and Validation of Phage ELISA with Real Urine Samples.** Urine samples were collected from persons who had no known exposure to pyrethroid insecticides and were filtered through a 0.45 $\mu$m membrane. The effect of urine on the phage ELISA was estimated by measuring the recoveries of 3-PBA spiked in different concentrations of urine (0, 2, 5, 10, or 20% in PBST). To each preparation, 3-PBA was added at 0.5 and 1.0 ng/mL. Spiked urine was mixed with the same volume of phage solution and was added to PAb-coated plates for competition. For assay validation, two urine samples were used. Each urine sample was spiked with 3-PBA at 0, 10, 20, and 40 ng/mL and was diluted 5-fold with PBST.

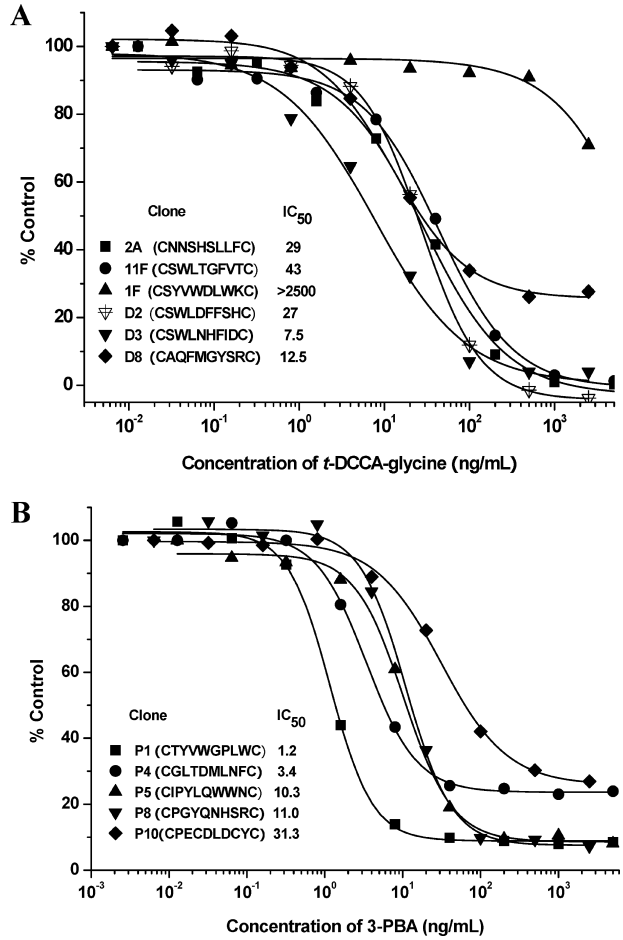


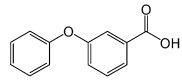
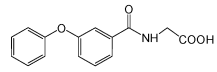
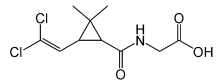
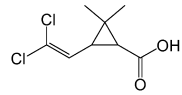
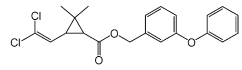
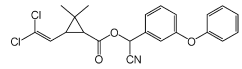
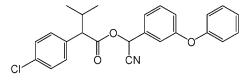
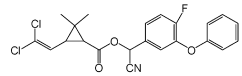
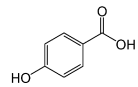

**FIGURE 1. Competitive phage ELISA. (A) Inhibition of different phages by *t*-DCCA-glycine. (B) Inhibition of different phages by 3-PBA. Microtiter plates were coated with protein A purified 3703 (A) or 294 (B) at 0.2 $\mu$g/well. Appropriate dilution of the selected phage-borne peptides in PBST was incubated with various concentrations of analytes diluted in PBST for competition. Bound phages were detected using HRP-conjugated anti-M13 monoclonal antibody. Phage clones 2A, 11F, and 1F were selected using protein A purified 3703, and clones D2, D3, and D8 were from hapten-affinity purified 3703. Phage clones P1, P4, P5, P8, and P10 were isolated with hapten-affinity purified 294.**

The recovered concentrations were obtained from the standard curves generated with PBST containing no urine.

**PrPCR.** Biotinylated PAb 294 was immobilized on streptavidin-coated magnetic beads by 40 min incubation with gentle agitation at room temperature. The beads were washed four times with 500 $\mu$L of PBST, and then beads were blocked with 3% BSA in PBST by shaking for 1 h at 37 °C. Five microliters of beads were placed in each well of an 8-well strip, and 100 $\mu$L of phage (1:5000 dilution in PBST) and 3-PBA standard diluted in PBST were added. The strip was placed on the microplate shaker at room temperature for 1 h. The beads were washed five times with PBST. The beads were transferred to the 96 well real-time PCR plate. PBST buffer was aspirated while holding the beads with the magnetic extractor. Twenty micoliters of PCR premix was added. The PCR program was as follows: 95 °C for 5 min 20 s, followed by 30 cycles of 95 °C for 3 s and 60 °C for 30 s. The PCR products were quantified by determining the numbers of PCR cycles that give the fluorescent signal of 0.13 as the threshold cycles (Ct). Linear regression for the Ct values versus the concentrations of 3-PBA was carried out with the software OriginPro 7.5.

**Phage Labeling with Acridinium.** Three hundred microliters of phage stock ($2 \times 10^{12}$) diluted with 1.5 mL PBS

**TABLE 2. Cross-Reactivity (CR) of Different Pyrethroid Insecticides and Their Metabolites in a Phage Competitive ELISA with Two Phage-Borne Peptides[a]**

| Compound | Structure | D3 CSWLNHFIDC | | P1 CTYVWGPLWC | |
|---|---|---|---|---|---|
| | | $IC_{50}$ (ng/mL) | CR(%) | $IC_{50}$ (ng/mL) | CR(%) |
| 3-PBA |  | ND[b] | | 2.8 | 100 |
| 3-PBA-glycine |  | >5000 | <0.16 | 252 | 1.1 |
| *t*-DCCA-glycine |  | 7.8 | 100 | ND | |
| *t*-DCCA |  | >5000 | <0.16 | ND | |
| Permethrin |  | >5000 | <0.16 | >5000 | <0.06 |
| Cypermethrin |  | >5000 | <0.16 | >5000 | <0.06 |
| Esfenvalerate |  | >5000 | <0.16 | >5000 | <0.06 |
| Cyfluthrin |  | >5000 | <0.16 | >5000 | <0.06 |
| 4-hydroxybenzoic acid |  | ND | | >5000 | <0.06 |

[a] Microtiter plates were coated with 100 $\mu$l of PAbs (2 $\mu$g/mL). The appropriate dilutions of each phage-borne peptide were incubated with various concentrations of the compounds listed in PBST. All data are the mean values of four independent experiments. [b] ND, not determined.

buffer was precipitated by adding 0.2 mL of PEG/NaCl, followed by 1 h on ice and centrifugation for 10 min at 4 °C. The phage pellet was resuspended with 1 mL of sodium carbonate buffer (0.1 M, pH 9.3). Twenty microliters of acridinium stock solution (2 mg/mL dry DMF) was slowly added. The tube was rotated at room temperature for 30 min. The conjugation reaction was quenched by adding 10 $\mu$L of 10% L-glycine solution and rotating for another 10 min. Labeled phage was purified on a PD-10 desalting column following manufacturer's instruction. Purified phage was aliquoted and stored at −70 °C.

## Results

**Phage Panning and Screening.** We previously reported two sensitive PAb-based ELISAs for the detection of *t*-DCCA-glycine and 3-PBA, which exhibited considerably improved assay sensitivities when the homologous competitor haptens were replaced by chemically synthesized heterologous ones (*4*, *5*). For this reason, we used these two PAbs to test our hypothesis. Immunoglobulin fractions specific to target compounds were purified with the hapten affinity column. To avoid antibody denaturation and increase specificity, we used a high analyte concentration to competitively elute the specific antibodies. This was followed by intensive dialysis to remove the analyte. Alternatively, to explore the possibility of using a less laborious panning procedure, total IgG from serum 3703 (specific for *t*-DCCA-glycine) was also purified with a protein A column. For the first round of panning, all bound phages were eluted nonspecifically with glycine buffer (pH 2.2) and then (second and third rounds) phages were competitively eluted with analyte to promote specificity and to exert control on the specific elution from particular target antibody subpopulations. In that regard, the concentration of analyte used in the last round of panning was reduced (2.5 ng/mL) to promote the release of phage-borne peptides specific for the antibodies with the highest affinity for the analyte. As expected, the number of isolated phages gradually decreased as the concentration of analyte was diminished (data not shown). In the case of the panning performed with protein A purified total IgG from serum 3703, only competitive elution was used using a high concentration (16 $\mu$g/ml of *t*-DCCA-glycine) for first, second, and third rounds, and 2.5 ng/mL for the fourth round.

**Phage Screening for Positive Clones.** After the final round of panning, individual phage supernatants were assayed with the immobilized antibody by phage ELISA. Positive clones

**TABLE 3. Matrix Effect of Urine on The Performance of Phage ELISA[a]**

| final concentration of urine (%)[b] | PBA spiked (ng/mL)[c] | detected (ng/mL)[d] | mean recovery (%, $n = 4$) |
|---|---|---|---|
| 0 | 0.5 | 0.40 ± 0.07 | 80 |
| | 1 | 0.89 ± 0.20 | 89 |
| 2 | 0.5 | 0.62 ± 0.25 | 124 |
| | 1 | 0.91 ± 0.09 | 91 |
| 5 | 0.5 | 0.38 ± 0.08 | 76 |
| | 1 | 1.15 ± 0.21 | 115 |
| 10 | 0.5 | 0.38 ± 0.21 | 76 |
| | 1 | 0.94 ± 0.30 | 94 |
| 20 | 0.5 | 0.64 ± 0.20 | 128 |
| | 1 | 0.99 ± 0.18 | 99 |

[a] Appropriate dilution of phage-borne peptide in PBST was incubated with different concentrations of spiked urine. [b] Urine concentrations before mixing with phage-borne peptide. [c] Concentrations of 3-PBA spiked to each urine preparation before mixing with phage-borne peptide. [d] Mean recovery values of four replicates and standard deviation.

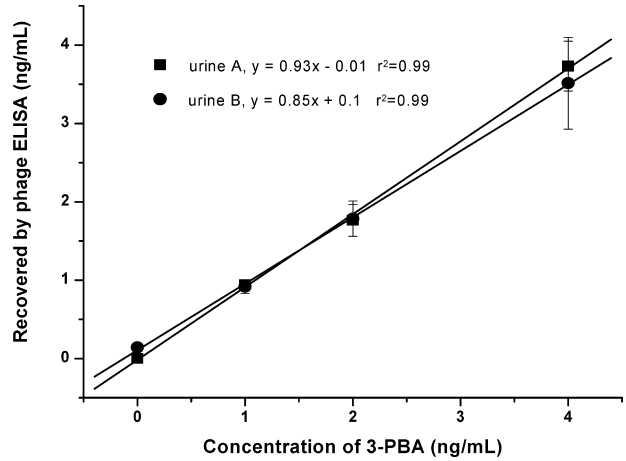



**FIGURE 2. Validation of phage ELISA with two urine samples. Urine samples were spiked with 3-PBA at 0, 10, 20, and 40 ng/mL. Each spiked sample was diluted 5-fold with PBST and was mixed with the solution of phage isolate to give a final 10-fold dilution.**

were further confirmed by competition experiments using 100 ng/mL of their respective analyte. For hapten affinity-purified PAbs, 8 positive phage clones out of 10 were identified for 3703, and 10 clones were positive for 294, but only 7 out of 66 clones were positive from the panning performed with the Protein A purified antibodies. Sequencing results of all positive clones are shown in Table 1. Four and five sequences were identified from hapten purified antibodies from serum 3703 and 294, respectively, and three sequences were identified from protein A purified antibodies from serum 3703. Sequencing results showed no distinctive consensus motif, except for the SWLxxF motif found for some of the clones isolated with the anti *t*-DCCA-glycine antibodies.

**Competitive Phage ELISA.** Concentrated phage supernatants from clones expressing different peptides sequences were prepared for performing competitive phage ELISAs. The optimal dilution of phage and coating antibodies was first determined by two-dimensional checkerboard titration. The competitive inhibition curves are presented in Figure 1. In general, assay sensitivity obtained with clones selected with hapten purified antibodies appeared to be better than that obtained with clones isolated with protein A purified antibodies. The $IC_{50}$ values of the previously reported chemically synthesized homologous hapten-based ELISAs for *t*-DCCA-glycine and 3-PBA were 100 and 121 ng/mL, respectively. Compared to the homologous ELISA for *t*-DCCA-glycine, the best improvement in assay sensitivity using phage-borne peptides was achieved with clone D3, with a 13-fold enhancement in $IC_{50}$. In the case of 3-PBA, this improvement was much better, achieving a 100-fold increase in assay sensitivity with clone P1. The lowest $IC_{50}$ values of previously reported heterologous ELISA using chemically synthesized competing haptens were 1.2 and 1.3 ng/mL for *t*-DCCA-glycine and 3-PBA, respectively. The $IC_{50}$ for the clone D3 phage was 6-fold higher than that of the best chemical heterologous ELISA for *t*-DCCA-glycine. For 3-PBA, the lowest $IC_{50}$ obtained with the phage-borne peptide was the same as that achieved with the best heterologous chemical hapten.

**Cross-reactivity.** In our previous report, cross reactivity tests with serum 3703 and 294 for structurally related pyrethroids and their metabolites showed that these antibodies were very specific to their target compounds. In this study, phage-borne peptides had no effect on the assay specificity (Table 2), showing an overall cross-reactivity of less than 1% when 5000 ng/mL of the test compound were used.

**Matrix Effect and Assay Validation.** Matrix effects on assay accuracy were evaluated to avoid false positive/negative results in the application of phage ELISA to crude urine samples. For this purpose, we analyzed the urine matrix effects in the 3-PBA assay set up with clone P1 by comparing recoveries of spiked 3-PBA in PBST buffer containing various urine concentrations (0, 2, 5, 10, and 20% urine) spiked with 0.5 and 1.0 ng/mL of 3-PBA into each preparation. The recoveries were calculated with a calibration curve generated with 1× PBST with no urine. Average recoveries for all dilutions of the urine sample ranged from 76 to 128% (Table 3). Because no adjustment of the buffer solution was needed for the generation of the standard curve (e.g., the same dilution of control urine), this assay could be suitable for use with up to a 10-fold dilution of crude urine. The feasibility of the application of phage ELISA to analyze 3-PBA in real urine samples was further validated by analysis of the recovery from two urine samples spiked with various concentrations of the analyte. Urine samples were spiked with 0, 10, 20, and 40 ng/mL and were diluted 5-fold with PBST, and each preparation was then mixed with the same volume of phage isolate for competition. The linear regression analysis of the phage ELISA results showed a very good correlation between spiked and detected levels (Figure 2). The concentrations in the figure refer to the final concentrations after 10-fold dilution. All recoveries were over 86% of the spiked values. These results demonstrate that this assay was able to accurately detect pyrethroid metabolites over a wide range of concentrations in urine samples.

**Phage Detection by PrPCR.** Because of the unique nature of the phage particle, the phage ELISA has the potential to be adapted into different formats. In one of the applications, clone P1 was used to develop nonenzymatic phage assays. Bound phages were detected by quantitative real-time PCR by amplifying the arabinose promoter region of the heat-released phagemid DNA. For this, phages were reacted with PAb immobilized on magnetic beads in the presence of different concentrations of 3-PBA. The bead-bound phages were transferred to a real-time PCR plate for amplification, and the trend of fluorescent signals is shown in Figure 3. The number of PCR cycles giving a fluorescent signal of 0.13 fluorescence units were gradually increased up to 200 ng/mL of 3-PBA. Figure 3B shows the linear regression of the Ct values versus the logarithmic concentrations of 3-PBA added for the competition. The PrPCR method could detect 3-PBA concentrations that ranged from 0.06 to 200 ng/mL, meaning

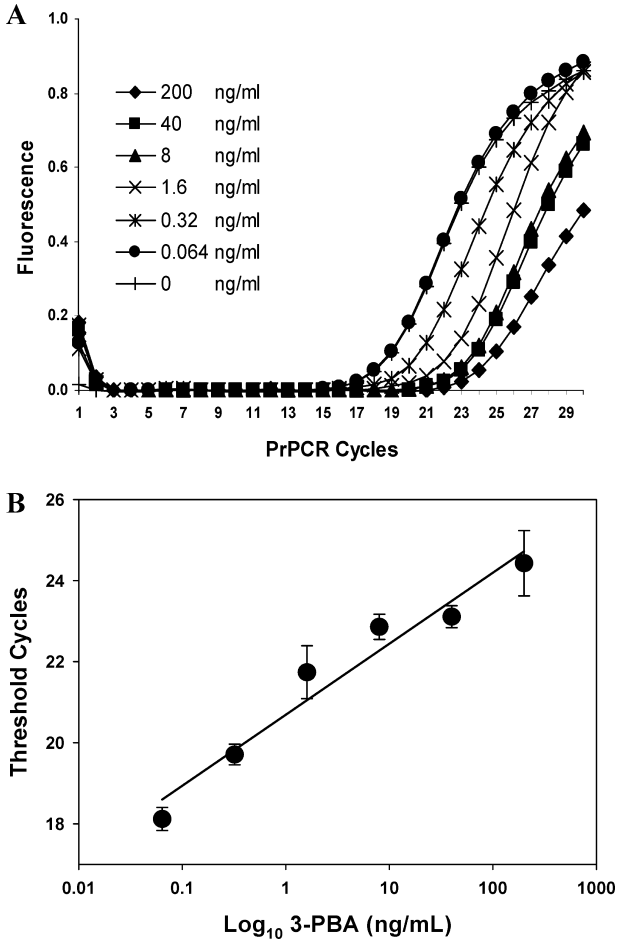



**FIGURE 3. Quantitative PrPCR assay for the detection of 3-PBA. Representative quantitative curves for the 3-PBA PrPCR assay (A). The mixture of 50 $\mu$L of a 1:2500 dilution of clone P1 phage isolate ($1 \times 10^{12}$ t.u/mL) with 50 $\mu$L of various concentrations of 3-PBA was incubated for competition with protein A purified 294 immobilized on magnetic beads. PrPCR was carried out to detect bound phages after transferring beads suspended in PBST washing buffer to the 96 well real-time PCR plate with a multichannel pipet and replacing the washing buffer with 20 $\mu$L of PCR reagent. (B). Linear regression of Ct values against logarithmic concentrations of 3-PBA. The $R^2$ value of the linear calibration curve is 0.95. The number of PCR cycles giving a fluorescent signal of 0.13 fluorescence units for each concentration of 3-PBA was determined as the Ct value. Each value represents the mean (±SD) of four replicates.**

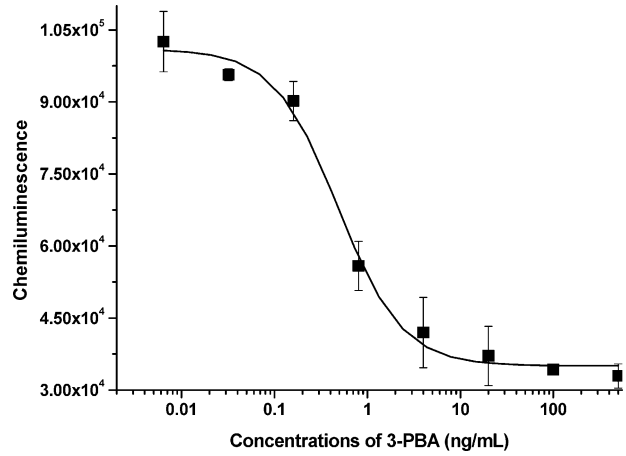



**FIGURE 4. Competitive 3-PBA chemiluminescent assay. Opaque high binding 96 well plates were coated with protein A purified 294 (0.5 $\mu$g/well). A 1:8000 dilution of acridinium-labeled clone P1 phage was incubated with different concentrations of 3-PBA for competition. Bound phages were detected by reading chemiluminescence immediately after sequentially adding acridinium trigger solution I and II. Each value represents the mean of two independent assays with four replicates for each. The $IC_{50}$ value of the assay is 0.5 ng/mL of 3-PBA. The linear range of the assay is approximately 0.2–2 ng/mL of 3-PBA.**

that the PrPCR significantly extended the detectable linear range in comparison with the phage ELISA.

**Chemiluminescence Phage ELISA.** The phage particle was also conjugated to an acridinium ester to develop a faster and simpler assay. For this, protein A affinity purified PAbs were immobilized on a high-binding opaque plate by overnight incubation at 4 °C. A checkerboard titration was performed to optimize the dilution of phage for the chemiluminescence phage ELISA. The acridinium labeled phages were added with various concentrations of 3-PBA, and the captured phages were detected by reading chemiluminescence signals immediately after adding signal generating reagents. Figure 4 shows the representative inhibition curve. An $IC_{50}$ value of 0.5 ng/mL was observed, which corresponds to a 2.4-fold improvement of sensitivity as compared to the phage ELISA.

## Discussion

In this study, we demonstrated that phage-borne peptides selected from phage libraries can be used as convenient reagents and can facilitate the development of sensitive PAb-based heterologous immunoassays for the detection of environmentally relevant compounds, thereby avoiding the synthesis of a panel of competing haptens and the preparation of hapten-protein conjugates that are frequently required in conventional ELISA for assay optimization. The technology utilized for this study has been well established as being straightforward to perform, with an additional advantage in the ready availability of phage peptide libraries. Thus, this method can easily be incorporated into most biochemical laboratories. We compared panning efficiency with PAbs purified with hapten or protein A affinity column, and in both cases there was an improvement in sensitivity with regard to the homologous assay. Although it would be desirable to use protein A purified antibodies because they are easier to prepare, the use of clones selected with hapten affinity purified PAbs yielded more sensitive assays. The reasoning behind the fact that different peptides sequences result in different assay sensitivities is that different peptides react with different antibody subpopulations (heterologous principle). Therefore, peptides that react with subpopulations with increased affinity for the analyte result in more sensitive assays. Following this reasoning, the use of a low concentration of analyte in the last round of panning was intended to promote the selection of peptides that react with fractions of antibodies with high affinity for the analyte. At limiting concentrations, the competitive elution of the peptides with the analyte will occur with the highest affinity antibody fraction. In the two models examined, this resulted in an improvement of 13- and 100-fold in the $IC_{50}$ values for *t*-DCCA-glycine and 3-PBA, respectively, when compared to the homologous assay.

Cross reactivity tests showed that phage-borne peptide D3 and P1 clones had similar specificities for the target compounds, as previously obtained in the heterologous chemical hapten-based ELISAs. Recovery tests indicated that the phage ELISA was suitable to accurately detect analyte in unknown urine samples that had been diluted 10-fold. Phage ELISA was validated by analyzing two urine samples spiked

with three different concentrations of 3-PBA. A good correlation ($r^2 = 0.99$) between spiked and detected level was observed.

We also demonstrated the versatility of the phage particle as a universal platform for developing different assay formats. In that regard, we used the DNA packed in the phage as a template for amplification by immuno real-time PCR. The main advantage of this approach was that it significantly extended the dynamic linear range of detection. In another example, direct conjugation of the phage particle to acridinium ester allowed chemiluminescent detection of the bound phage, which further improved the sensitivity of the assay 2.4-fold as compared to the phage ELISA.

In summary, phage-borne analyte peptidomimetics selected from a phage peptide display library can be a useful alternative to the chemical competing hapten for developing a sensitive heterologous ELISA for the detection of low molecular weight environmental contaminants such as pesticides or their metabolites. Our experimental data demonstrate that the phage-borne competing peptide hapten can easily be selected with well-established phage panning technology, thereby greatly improving assay sensitivity; the phage-based assay is robust with a tolerance up to 20% urine concentration, and phage particles can be used for different detection methods by real-time PCR amplifying phage DNA and by direct conjugation of a signal producing molecule that not only enhances assay performance but also significantly shortened assay time. In addition, despite accurate and reliable detection for low molecular environmental contaminants by instrumental analysis, the phage-based ELISA can be a cost-effective tool for the detection of such contaminants because instrumental analysis requires multistep sample cleanup and trained personnel for operation.

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