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# Combining Yeast Display and Competitive FACS to Select Rare Hapten-Specific Clones from Recombinant Antibody Libraries

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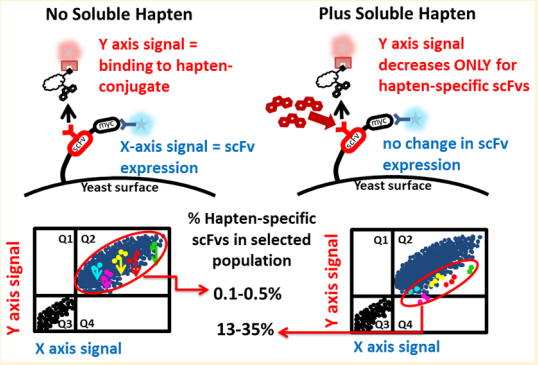
Supporting Information

**ABSTRACT:** The development of antibodies to low molecular weight haptens remains challenging due to both the low immunogenicity of many haptens and the cross-reactivity of the protein carriers used to generate the immune response. Recombinant antibodies and novel display technologies have greatly advanced antibody development; however, new techniques are still required to select rare hapten-specific antibodies from large recombinant libraries. In the present study, we used a combination of phage and yeast display to screen an immune antibody library (size, $4.4 \times 10^6$) against hapten markers for petroleum contamination (phenanthrene and methylphenanthrenes). Selection via phage display was used first to enrich the library between 20- and 100-fold for clones that bound to phenanthrene−protein conjugates. The enriched libraries were subsequently transferred to a yeast display system and a newly developed competitive FACS procedure was employed to select rare hapten-specific clones. Competitive FACS increased the frequency of hapten-specific scFvs in our yeast-displayed scFvs from 0.025 to 0.005% in the original library to between 13 and 35% in selected pools. The presence of hapten-specific scFvs was confirmed by competitive ELISA using periplasmic protein. Three distinct antibody clones that recognize phenanthrene and methylphenanthrenes were selected, and their distinctive binding properties were characterized. To our knowledge, these are first antibodies that can distinguish between methylated (petrogenic) versus unmethylated (pyrogenic) phenanthrenes; such antibodies will be useful in detecting the sources of environmental contamination. This selection method could be generally adopted in the selection of other hapten-specific recombinant antibodies.




Antibodies to low molecular weight haptens are invaluable tools for many analytical applications. In drug analysis, competitive immunoassays are still the mainstay in the screening and semiquantitative analysis of hundreds of different xenobiotics and drugs of abuse.[1] In addition, fully automated, high-throughput antibody-based systems are available in laboratories to help physicians to make timely decisions about drug dosage and safe therapeutic levels.[1,2] The demand for diagnostic immunoassays to monitor the safe and effective use of prescribed drugs will continue to increase as health care evolves to more personalized interventions and to products tailored to the individual patient.[3] In addition to their utility in clinical diagnostics, hapten-specific antibodies also play an important role in environmental monitoring, where immunoassays are most often used on-site to provide near real-time information on the extent of environmental contamination or on the progress of site remediation. Thus, antibodies directed toward low molecular weight contaminants, including pesticides,[4] PCBs,[5] biotoxins,[6] PAHs,[7−9] and metals[10−12] have proven useful to assess the safety of food, water, and the ecosystem.

The generation of high-quality antibodies for low molecular weight haptens has never been straightforward. Antigens smaller than 1000 Da are usually not immunogenic, but can induce a T cell-dependent immune response when conjugated to protein. Because these carrier proteins are often more immunogenic than haptens alone, the antibodies thus generated often have an extended binding sites that includes, in addition to the hapten, portions of the protein used in conjugation. Thus, most antihapten antibodies bind much more tightly to the hapten-protein conjugates than to the soluble hapten, because of the greater number of interactions at the binding site (for specific examples, see refs 13 and 14). Antibodies with primary specificity for soluble haptens are often very rare in the antibody repertoire of immunized animals or from monoclonal antibodies prepared from immune tissue.

Recombinant antibodies such as single-chain fragment variable antibodies (scFvs) have greatly advanced antibody development.[15] Recombinant antibodies can be manipulated at

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molecular level to modify their binding properties[16,17] and they can be shuffled between different expression systems during the selection and production processes.[18] In addition, given the concerns about the reproducibility of many published studies that utilize antibody-based reagents,[19] new requirements for rigor in biomedical research may ultimately demand that all antibodies be sequenced and expressed as recombinant proteins.[20] Antibody libraries of high diversity can be created using recombinant technology,[21] and the large numbers ($10^6$–$10^{11}$) of distinct antibody clones from which to select theoretically improves the chances of discovering rare clones, including hapten-specific antibodies. When suitable selection procedures can be employed, even antibodies present at very low frequency in the original library can be highly enriched and become visible in the subpopulations.

In this study we describe a novel selection procedure for the identification and subsequent isolation of rare, hapten-specific recombinant antibodies from a relatively large immune library ($\sim 4.4 \times 10^6$). We have developed a new, competitive fluorescence activated cell sorting (FACS) protocol that, when combined with preselection via phage and yeast display, yields high percentages (20–40%) of hapten-specific scFvs in the final pool of selected cells, even though no binding to soluble hapten could be detected using standard selection strategies. In the present study, we used competitive FACS to isolate antibody populations that could distinguish between methylated and unmethylated phenanthrene, because antibodies for alkylated PAHs can serve as markers for environmental petroleum contamination.[22,23] However, this general method should be widely applicable to the isolation of a wide variety of scFvs directed toward soluble antigens.

## EXPERIMENTAL SECTION

**Materials.** Chemicals (purities at 98% or higher) were purchased from the following sources: phenanthrene (Phen, Sigma-Aldrich), 2-methylphenanthrene (2-MePhen, Sigma-Aldrich), 3-methylphenanthrene (3-MePhen, BOC Sciences), 4-methylphenanthrene (4-MePhen, Chem Service), 9-methylphenanthrene (9-MePhen, Crescent Chemical). Each compound was dissolved as 10 mM stock in DMSO. 9-Carboxyphenanthrene was purchased from Sigma-Aldrich. 9-Carboxy-2-methylphenanthrene and 9-carboxy-2,7-dimethylphenanthrene were synthesized in-house by the Synthetic Organic Chemistry Core Laboratory (NIEHS supported) at the University of Texas Medical Branch in Galveston, TX. Phage display plasmid pComb3XSS was obtained from The Scripps Research Institute. Both the yeast display plasmid pDNL6-GFP-myc (originally generated from pPNL6 plasmid[18,24]) and scFv expression plasmid POE-myc (generated from a pET based plasmid, pEP-D1.3[25]) were modified in our laboratory to replace the V5 tag with a myc tag. Monoclonal antimyc antibody 9E10 was purified in-house from the culture supernatant of 9E10 hybridoma cells (Developmental Studies Hybridoma Bank, University of Iowa).

**Protein Conjugates, Mouse Immunization, and Immune Library Preparation.** 9-Carboxyphenanthrene, 9-carboxy-2-methyphenanthrene, and 9-carboxyl-2,7-dimethyphenanthrene) were conjugated with protein carriers (either BSA or KLH) using a mixed anhydride method.[26] Details of conjugate synthesis, purification and characterization are provided in Supporting Information. Immunization of mice with these conjugates and the subsequent construction of an scFc immune library are also available in Supporting Information.

**Phage Selection.** Antibody phage library or output pools from previous rounds of selection were grown and infected with helper phage M13KO7 for phage production as described in Supporting Information.

Two selection protocols were performed toward two different targets (Phen or 2MePhen). A 96-well high binding plate (Corning, NY) was coated with 50 $\mu$L of antigen (5 $\mu$g/mL, either Phen-BSA or 2MePhen-BSA) at 4 °C overnight. The next day, the plate was washed three times with PBS containing 0.05% Tween 20 (PBST) and blocked with PBS containing 3% BSA at 25 °C for 1 h. An aliquot of amplified phage (about $10^{12}$ cfu, see Supporting Information) was also blocked with 3% BSA at 25 °C for 1 h in PBS or PBS plus 1% DMSO. The blocked phage were then added into the antigen-coated plate, and incubated for 1 h. Unbound phage were removed by extensive washing (15× with PBST followed by 2× with PBS) and the bound phage were eluted by one of two methods: (1) Acid elution with 200 $\mu$L glycine·HCl at pH 2.2 for 8 min and immediate neutralization with 2 M Tris to yield a final pH of ~7.5. Early in the selection process, this method ensures that selection is comprehensive and does not exclude any rare binders; (2) Competitive elution with 100 $\mu$M soluble analyte (either Phen or 2-MePhen) in 1% DMSO for 30 min. This method was used in the final selection step to enrich a specific population which preferentially recognized soluble analytes rather than protein conjugates.

Half of the eluted phage were used to infect 10 mL log-phase OmniMAX 2 T1$^R$ *E. coli* (Invitrogen, CA, U.S.A.) at 37 °C for 30 min. An aliquot of infected culture was serially diluted and spread on 2xYT-AG plates to calculate the number of phage eluted (as "output" of this round of panning), and the rest of culture was incubated in fresh 2xYT-AG medium overnight at 37 °C. The next day, phage particles were amplified from this culture and used as "input" for next round of panning.

**Phage ELISA.** Phage ELISA was performed in 96-well high binding plates (Corning, Corning, NY). Each well was coated with Phen-BSA or 2MePhen-BSA (2 $\mu$g/mL) at 4 °C overnight and blocked with 3% BSA in PBS. Phage pools recovered from each selection were incubated in the antigen-coated well with solvent control (1% DMSO), 100 $\mu$M soluble hapten (Phen or 2-MePhen) or 50 $\mu$g/mL soluble protein conjugate (Phen-BSA or 2MePhen-BSA). The binding of phage particle to the immobilized antigens on the plate was measured using anti-M13-HRP antibody (GE Healthcare, Little Chalfont, U.K.). The HRP signal was detected by adding Sureblue TMB microwell peroxidase substrate (KPL, Gaithersburg, MD) and the reaction was stopped after 5 min using 1 N HCl. All incubation steps were performed at room temperature for an hour. The plate was washed three times with PBST between steps.

**Yeast Display and Competitive FACS.** After the final phage selection, the phagemids containing the scFv genes were isolated with a miniprep kit (Qiagen, Valencia, CA), and scFv genes were amplified with a pair of transfer primers (Table S2, Supporting Information). Yeast display plasmid pDNL6-GFP-myc was digested with BssHII and NheI restriction enzymes and the linear plasmid was gel-purified without the GFP insert. Digested pDNL6 plasmid (500 ng) and purified scFv gene PCR product (1 $\mu$g) were transformed into EBY$_{100}$ yeast competent cells with Yeast Transformation System 2 kit (Clontech, Mountain View, CA). The homologous region on the plasmid


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and PCR product flanking region led to the formation of circular plasmids carrying scFv insert by the yeast homologous repair mechanism.[27,28]

For flow cytometry using a Beckham FACS Aria, the yeast library was incubated in growth medium SD-CAA at 30 °C overnight for activation. The next day, activated yeast cells were diluted in induction medium SG/R-CAA at $OD_{600}$ = 0.5, and cultured again at 30 °C for 16 h. After induction, $10^7$ induced yeast cells ($OD_{600}$ = 0.5) were washed twice with 0.5 mL wash buffer I (PBS supplemented with 0.5% BSA and 2 mM EDTA), and once with 0.5 mL wash buffer II (PBS supplemented with 0.5% BSA). Yeast cells were first incubated with 50 μL competitor (200 μM Phen or 2-MePhen in PBS containing 1% DMSO) for 30 min with rotation at 25 °C. An additional aliquot (50 μL) of biotinylated protein-conjugate (BSA-biotin, Phen-BSA-biotin, or 2MePhen-BSA-biotin at concentrations between 60 and 200 nM) containing 2 μg/mL antimyc antibody 9E10 was then added and the cells were incubated for another 30 min. Yeast cells were washed 3 times with wash buffer II and stained with 4 μg/mL goat-antimouse-PE (Life Technologies, Grand Island, NY) and 10 μg/mL streptavidin-Alexa633 (Life Technologies, Grand Island, NY) in the dark at 4 °C for an hour. The stained yeast cells were washed three times with wash buffer II and resuspended in 1 mL PBS for flow-cytometry analysis. An identical procedure was used to stain pools after sorting by flow cytometry. In each flow cytometry experiment, five controls were included for compensation tests: (1) yeast cells without any primary and secondary antibodies; (2) yeast cells with goat-antimouse-PE only; (3) yeast cells with streptavidin-Alexa633 only; (4) yeast cells with 9E10 and goat-antimouse-PE; (5) yeast cells with biotinylated protein-conjugate and streptavidin-Alexa633.

**Monoclonal Analysis.** The yeast cells collected from the final FACS sort were serially diluted and spread onto SD-CAA agar plate in 30 °C incubator to form single colonies. After 2−3 days, individual colonies from the plate were selected, inoculated into 0.5 mL of SD-CAA medium in a 96 deep-well plates, and incubated at 30 °C overnight. On the second day, a 50 μL aliquot of the culture was transferred into 500 μL SG/R-CAA medium and induced overnight at 30 °C. An aliquot (50 μL) of the induced monoclonal yeast cells was subsequently transferred into a 96-well vacuum filter plate, and washed twice with 150 μL wash buffer I and once with 150 μL wash buffer II. The cells were stained as described above and suspended in 200 μL PBS for FACS analysis using a Guava easyCyte Flow cytometer (EMD Millipore, Billerica MA).

**Soluble scFv Expression and Purification.** Flow-cytometry-positive scFvs were cloned into expression vector POE-myc. Details of cloning, bacterial transformation, and induction are available in Supporting Information. Soluble scFv from periplasmic extract was purified using HisPur Cobalt Resin (Life Technology, Grand Island, NY). The periplasmic extract (40 mL) was first incubated with 1 mL resin for an hour with rotation, and then the resin was gravity-packed in a column (1 × 1 cm). The resin column was washed with equilibration buffer (50 mM sodium phosphate, 300 mM sodium chloride, 10 mM imidazole, PH 7.4) until the $A_{280}$ of flow through reached a baseline. The scFv was subsequently eluted with 50 mM sodium phosphate, 300 mM sodium chloride, 150 mM imidazole, pH 7.4. A few fractions (1 mL each) of eluate were collected to ensure all protein had been eluted, then those fractions with protein were pooled and concentrated using an Amicon ultra-15 device (EMD Millipore, Billerica, MA). The purified scFv was analyzed by SDS-PAGE and the protein concentration was determined using the BCA protein assay (Pierce, Rockford, IL).

**Binding Characterization of Soluble scFvs.** Competitive ELISA was performed in 96-well high binding plates (Corning, Corning, NY). Each well was coated with 2 μg/mL antigen at 4 °C overnight and blocked with 3% BSA. Antibody scFv (A10, 5.32 μg/mL; D7, 8.76 μg/mL; G8, 3.81 μg/mL) was incubated in the antigen-coated well with or without soluble competitor, and the binding of scFv was accessed using antimyc antibody (9E10) and goat-antimouse-HPR antibody (Sigma-Aldrich, MO). The HRP signal was detected as described for Phage ELISA, above. All incubation steps were performed at room temperature for an hour. The plate was washed three times with PBST between steps.

## RESULTS AND DISCUSSION

In preliminary studies, we attempted to select antibodies against methylphenathrenes from a naïve library with a diversity of approximately $3 \times 10^{11}$.[21] However, the scFvs selected from this library recognized Phen-protein conjugates, but not soluble Phen (data not shown). We therefore prepared an immune phage display library for selection of antibodies that could recognize soluble phenanthrenes. Antibodies selected from immune libraries generally have higher specificity and affinity than those selected from naïve libraries.[29,30] After immunization, serum antibodies to Phen-BSA conjugates were confirmed by indirect ELISA (Figure S1, Supporting Information). Mice were sacrificed 7 days after the final injection and splenic tissue was used as the starting material for cDNA synthesis. DNA sequences coding for antibody variable domains from heavy chains (VH) and light chains (VL) were amplified using a pool of degenerate primers.[31] These primers were adapted to our display system as shown in Tables S1 and S2 (Supporting Information). The flanking regions of VH-reverse primers and VL-forward primers contain the same flexible linker sequence, and were used as complementary regions in a second overlap extension PCR step to form a full-length scFv fragment. The final DNA fragments encoded scFv products that had a VH-linker-VL structure, with a total length of ∼800bp. After gel purification and restriction endonuclease digestion, the 800bp PCR fragments were inserted into the pComb3 plasmid. The differences between two 5′-overhang sequences in the *SfiI* digested fragments ensured that the DNA fragments were inserted in the correct orientation. Phagemids bearing DNA fragments that encoded these scFvs were transformed into SS320 *E. coli* cells by electroporation to provide maximal transformation efficiency. The size of this immune library was $6.2 \times 10^6$. Clones with DNA fragments encoding full-length scFvs (∼800bp) comprised 87.5% of the sequences, and 81.25% of clones had distinct sequences, as confirmed by *BstNI* fingerprint analysis (Figure S2, Supporting Information). The final library size was calculated to be $4.41 \times 10^6$.

Because the library contained ∼4.4 million distinct scFv sequences, sophisticated selection strategies were required to identify the clones that expressed the rare antibodies that recognize soluble hapten (Phen and/or MePhen) in this large pool. We therefore employed a combination of phage and yeast display selections to identify the rare clones that expressed scFvs with the ability to bind these soluble haptens, as shown in Figure 1. Phage display is useful for exploring a relatively large library and permits rapid removal of undesirable scFvs from the library. However, the output of selection always contains a


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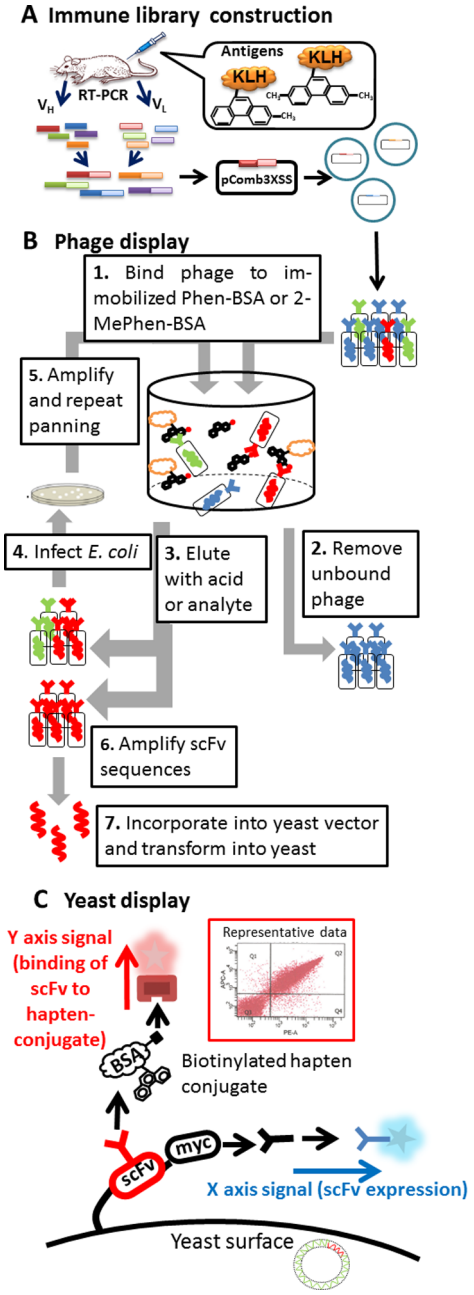


**Figure 1.** General strategy for developing recombinant antibodies against unsubstituted and methylphenanthrenes. (A) Immune library construction. (B) Phage display selection. Phage panning (3 rounds) was performed and final output pools were transferred into the yeast display plasmid (steps 6 and 7). (C) Yeast display analysis. The expression of scFv was measured by an antimyc antibody-phycoerythrine-labeled antimouse antibody (*X* axis signal). The binding of the scFv to the biotinylated phenanthrene-protein conjugate was measured by streptavidin-Alexa633 fluorescence (*Y* axis signal).

relatively high level of nonspecific binders due to the intrinsic "stickiness" of phage particles. Preliminary experiments had indicated that sequences encoding antihapten antibodies were present at very low frequency in the library; thus any nonspecific background noise could overwhelm the true binding signals and make it very difficult to detect antihapten antibodies using competitive phage ELISA.[32,33] The yeast display system is limited to smaller scFv libraries, but this display system can be coupled with Fluorescence-Activated Cell Sorting (FACS) to provide very strict controls over selection parameters.[34,35] Initial selection of our library using phage display excluded undesirable scFvs and reduced the overall size of the library. Subsequent yeast display allowed us to further analyze the output pool and sort for expressed scFvs that not only bound to the hapten-BSA-conjugates but also recognized soluble Phen and/or MePhen haptens.

**Phage Selection.** Preliminary studies showed that the number of panning steps had to be limited in order to efficiently select specific binders while maintaining the diversity of output pools. Multiple rounds of selection not only selected for the antibodies that bound with highest affinity, but also allowed clones with the highest growth rates to predominant during subsequent bacterial amplification.[36] In addition, when selecting for hapten-specific antibodies, high affinity to hapten-protein conjugates does not necessarily translate to high affinity to soluble hapten.[13] In preliminary studies, a seven-step selection process led to the selection of a single scFv that bound to the Phen-BSA conjugate with high affinity ($K_D$ = 10 nM) but did not recognize soluble Phen or MePhen (data not shown). We therefore adjusted our selection protocol as shown in Table 1. Total of three rounds of panning were performed against two different capture antigens, Phen-BSA (Protocol 1) or 2MePhen-BSA (Protocol 2). Each panning step was designed to provide a specific selective pressure. The first round enriched for phage that bound to the hapten-protein conjugate; the second panning step introduced a solvent condition (1% DMSO) to exclude antibodies that were unstable in this solvent and, hence, would not be useful when soluble haptens were applied later in the selection process. In the final round of panning, soluble hapten was added to elute the pool of binders that preferentially bound to soluble hapten. The input-to-output ratio (yield) was used to determine enrichment at each selection step. The last round of panning enriched our populations by 20- and 108-fold, respectively, for Protocols 1 and 2. We confirmed that phage from round 3 bound soluble Phen-BSA and/or 2MePhen-BSA using by competitive phage ELISA, as shown in Figure 2; however, we were not able to detect any inhibition by soluble Phen or 2MePhen in these pooled phage populations.

**Yeast Display, Preliminary FACS Selection, and Competitive FACS.** The sequences that encoded the scFv fragments from round 3 phage (Table 1) were amplified, transferred into the yeast display plasmid, and transformed into two independent yeast pools. The transformed yeast cells were induced to activate the expression of cell surface Aga2p-scFv-myc fusion proteins and the scFv-bearing yeast cells were stained with two fluorescent dyes and analyzed by flow-cytometry (see schematic in Figure 3A). The expression level of scFv was monitored by the signal from the myc tag and is shown on the *X* axis of the flow plot, while the binding capacity of expressed scFv to hapten-conjugates was detected by the signal from the biotinylated antigen and is shown on the *Y* axis of flow plot. The strategy for competitive FACS is shown in Figure 3. The yeast cell population that both expressed scFv (as assessed by the signal on the *X* axis) and bound to the biotinylated Phen-BSA conjugate (as assessed by the *Y* axis signal) is incubated in the presence of soluble hapten (either Phen or 2MePhen). Those clones that bind to soluble antigen will show lower signals in the *Y* axis and would be enriched in the area of the Q2 quadrant circled in Figure 3D.


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**Table 1. Phage Selection**

| Protocol I | | | | | |
|---|---|---|---|---|---|
| round | coating | selective pressure | input | output | yield |
| 1 | Phen-BSA 5 $\mu$g/mL | enrichment | $3.0 \times 10^{12}$ | $10^6$ | $3.3 \times 10^{-7}$ |
| 2 | | 1% DMSO solvent | $1.5 \times 10^{12}$ | $4.4 \times 10^4$ | $2.9 \times 10^{-8}$ |
| 3 | | elute with 100 $\mu$M Phen | $2.4 \times 10^{12}$ | $1.4 \times 10^6$ | $5.8 \times 10^{-7}$ |
| Protocol 2 | | | | | |
| round | coating | selective pressure | input | output | yield |
| 1 | 2MePhen-BSA, 5 $\mu$g/mL | enrichment | $3.0 \times 10^{12}$ | $10^6$ | $3.3 \times 10^{-7}$ |
| 2 | | 1%DMSO solvent | $1.3 \times 10^{12}$ | $3.6 \times 10^4$ | $2.8 \times 10^{-8}$ |
| 3 | | elute with 100 $\mu$M 2MePhen | $1.4 \times 10^{12}$ | $4.2 \times 10^6$ | $3.0 \times 10^{-6}$ |

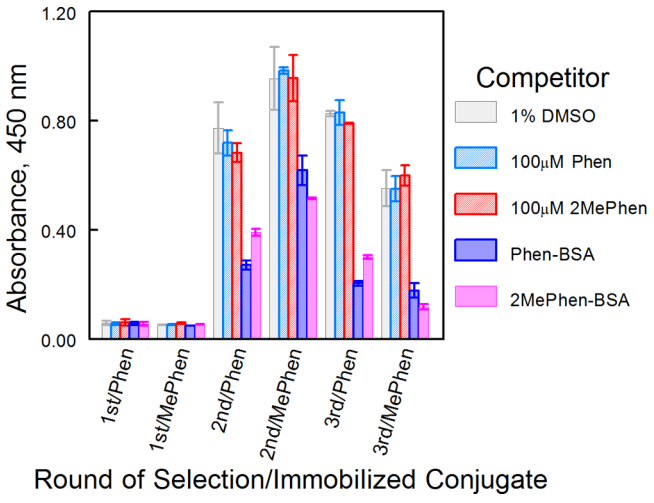


**Figure 2.** Competitive phage ELISA. Phage were tested for binding to immobilized Phen-BSA (Phen) or 2MePhen-BSA (MePhen) in the presence of DMSO, soluble phenanthrenes in DMSO or soluble phenanthrene-protein conjugates (0.75 $\mu$M).

We first explored the yeast population derived from phage selected via Protocol 1. Selection of yeast cells that expressed hapten-specific antibodies involved two rounds of sorting. The first round removed cell populations that could cause interference during subsequent selection steps. As shown in Figure 4A, when yeast cells derived from the Protocol 1 selection were stained with antimyc antibody and BSA conjugated with biotin but not Phen (a negative control in the FACS assay), a significant number of cells migrated in the Q2 quadrant. These biotin-binding cells in Q2 quadrant would interfere with the competitive sorting planned for subsequent selections. We removed these biotin-binding clones by collecting only the cells in Q4, in order to exclude nonspecific binders (see boxed area in Figure 4A). These negatively sorted cells, when re-examined for their binding to BSA-biotin, now showed a much smaller population of cells in the Q2 quadrant (data not shown). A competitive cell sort was then performed, according to the strategy shown in Figure 3. Yeast pools from the negatively sorted cells were divided into two groups and incubated with either 2% DMSO (Figure 4B) or soluble competitor (200 $\mu$M Phen in 2% DMSO) in the presence of 30 nM biotin-labeled Phen-BSA (Figure 4C). Because the cell population was split after induction of cell surface scFvs, there was no change in the expression level of scFv fragments and the *X* axis signal should remain the same for the two groups. However, those cell-surface antibodies that recognized soluble hapten would be competitively inhibited from binding to the biotinylated hapten–protein conjugate, and cells carrying antibodies with these binding characteristics should show a decreased signal on the *Y* axis of the flow plot. Thus, by

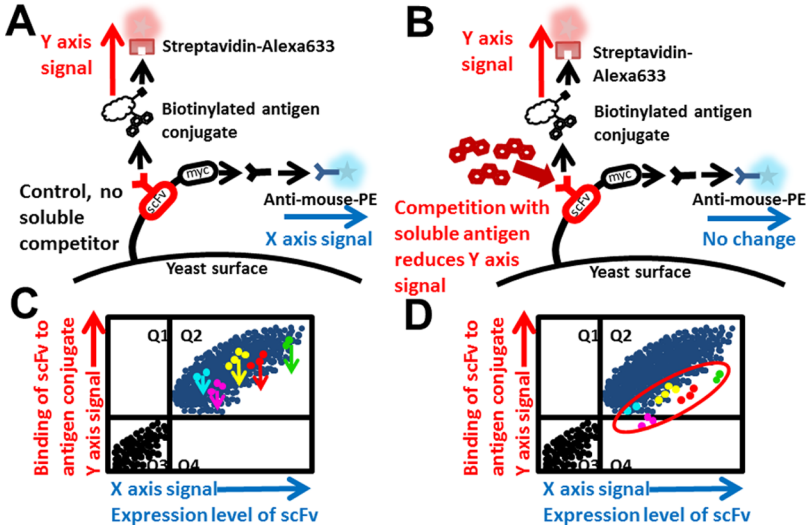


**Figure 3.** Strategy for selection by competitive FACS. The yeast cell population that both expresses scFv (the *X* axis the signal) and binds to the biotinylated Phen-BSA conjugate (the *Y* axis signal) is incubated in the presence of soluble hapten (either Phen or 2MePhen). Those clones that bind to soluble antigen will show lower signals in the *Y* axis and will be enriched in the area of the Q2 quadrant circled in Figure 3D.



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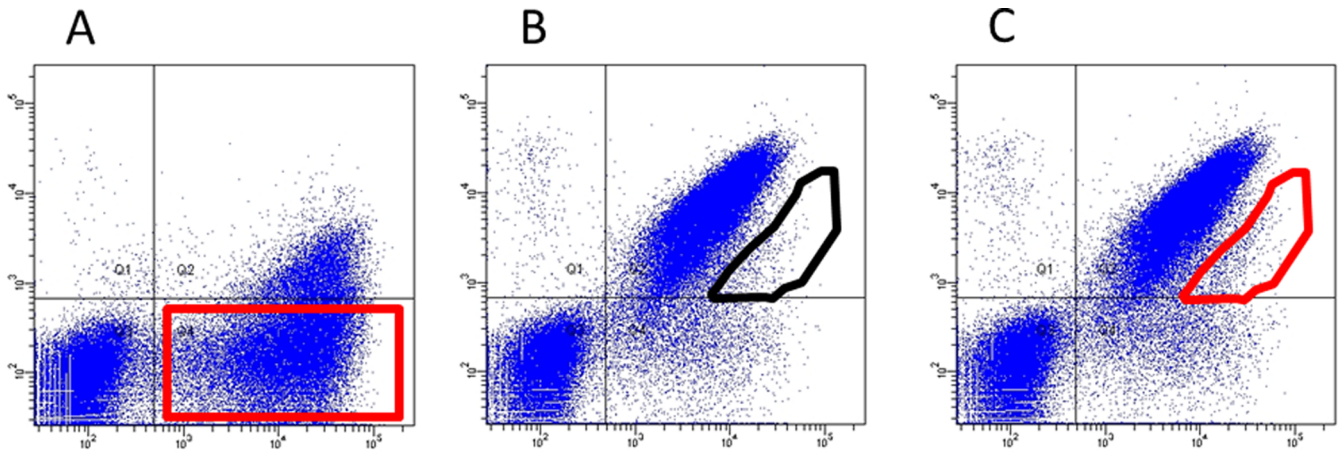


**Figure 4.** FACS of the yeast cells derived from Protocol 1. (A) Cells showed significant background signal when incubated with biotin-BSA (see Q2). Clones outlined in red were carried forward for subsequent selections. (B) Yeast cells from A were incubated with biotin-BSA-Phen. (C) Yeast cells from A were incubated with biotin-BSA-Phen +200 $\mu$M soluble Phen. Cells in the red circle were collected for further analysis.

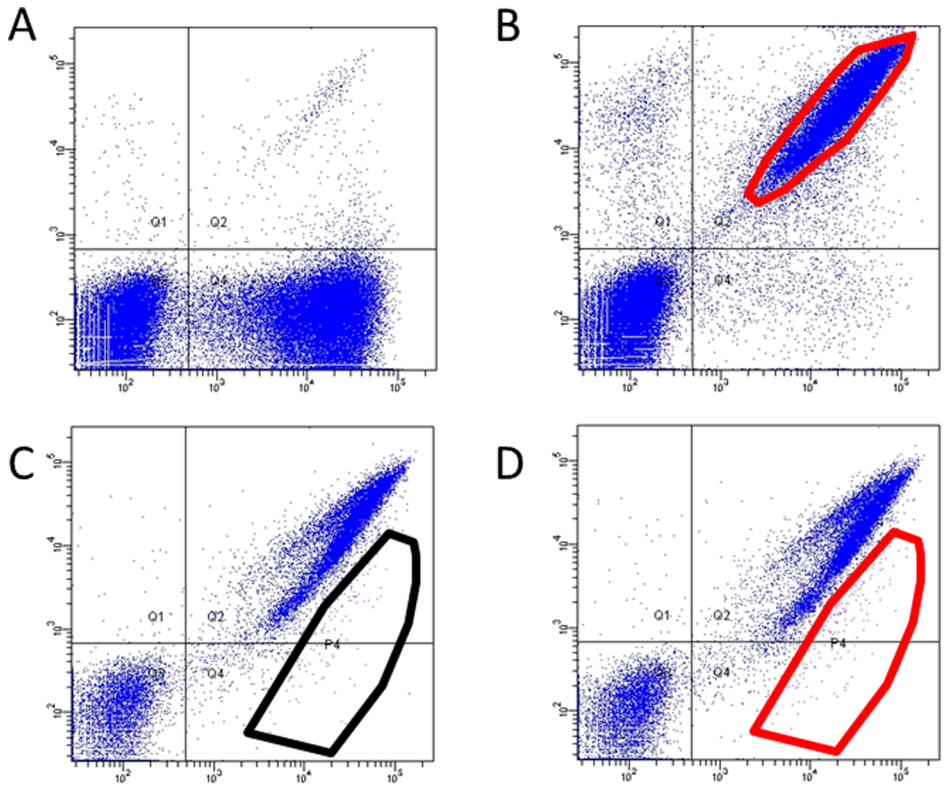


**Figure 5.** FACS of yeast cells derived from Protocol 2. (A) Cells incubated with BSA-biotin (negative control). (B) Cells incubated with biotin-BSA-2MePhen. High affinity binders from this pool were selected as outlined in red. (C, D) Yeast cells from the positive sort shown in B were incubated with biotin-BSA-2MePhen in the absence (C) or presence (D) of soluble 2MePhen.

selecting the cell population that shifted down in the presence of soluble competitor (Figure 4C, red circle), we were able to enrich for those rare surface-displayed antibodies that were specifically inhibited by the soluble haptens. The total cells selected in this gate was ~0.6% of the total cells in the absence of soluble hapten (yellow circle, Figure 4B) and ~1% of cells in the presence of soluble hapten (red circle, Figure 4C). Thus, we hypothesized that ~40% of the yeast clones in this very small population should contain antibodies that would recognize soluble hapten.

When we examined the yeast cells derived from Protocol 2, the first FACS analysis (as shown in Figure 5A) looked very different from what was observed with Protocol 1 cells. The Q2 quadrant of Pool 2 was relatively clean in the presence of BSA-Biotin (Figure 5A), so a negative sort was not required. However, relatively few cells in this population bound to the antigen (biotin-labeled 2MePhen-BSA) and the scattered populations in Q2 and Q4 would overwhelm the small population that might be inhibited by soluble hapten in the subsequent competitive FACS selection. Therefore, the cells derived from Protocol 2 were first selected for those clones that bound tightly to the biotin labeled 2-MePhen-BSA, as shown in P5 gate of Figure 5B. After this selection, a competitive FACS strategy was employed. In this competitive selection, 200 $\mu$M


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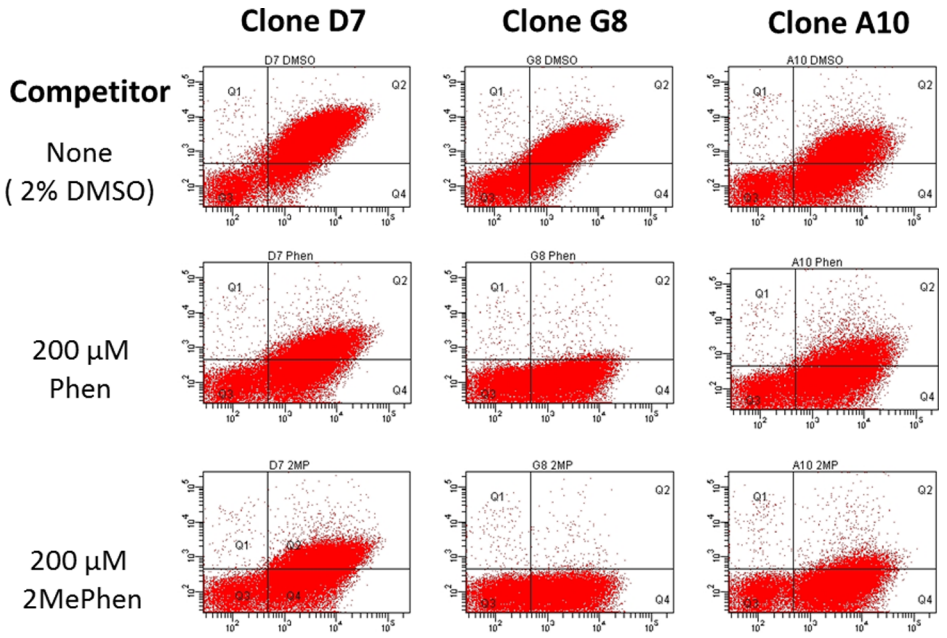


**Figure 6.** Monoclonal FACS of three representative clones is shown in three columns. (Top) Cells were incubated with biotin-labeled Phen-BSA in buffer+2% DMSO (solvent control); (Middle) Cells were incubated with biotin labeled Phen-BSA plus 200 $\mu$M phenanthrene in buffer + 2% DMSO; (Bottom) Cells incubated with biotin labeled Phen-BSA plus 200 $\mu$M 2-methyl-phenanthrene in buffer + 2%DMSO.

soluble 2MePhen was used as the soluble competitor and 10 nM biotinylated 2MePhen-BSA was used as the protein conjugate. In the absence of soluble hapten, the cells selected in P4 gate comprised ~0.7% of total cells (Figure 5C); cells in this gate population increased to ~1% when soluble hapten was added (Figure 5D). Similarly to Pool 1, these results suggest that roughly 30% yeast clones from this population may recognize the soluble hapten.

**Competitive FACS of Monoclonal Yeast Cells, Sequence Analysis, and Competitive ELISA of Periplasmic Extracts.** Single clones were induced individually from the cells collected in the gates shown in Figure 4C and 5D. We picked 184 individual clones from cells gated as shown in Figure 4C and analyzed their binding to biotinylated Phen-BSA in the presence of soluble Phen or 2MePhen. When we performed competitive FACS on yeast cell populations derived from these individual clones, the presence of soluble inhibitor (either Phen or 2MePhen) caused a downward shift in the *Y* axis signal in 70 of the 184 clones (~38%). FACS of three representative clones (D7, G8, and A10) is shown in Figure 6. The presence of 200 $\mu$M Phen or 2MePhen caused a similar downward shift of the signal on the *Y* axis when Clones D7 and G8 were analyzed by competitive FACS; thus, these two clones did not seem to be able to distinguish between the Phen and 2MePhen. However, when clone A10 was analyzed, it appeared to show some preference for 2MePhen, since this competitor caused a larger downward shift of the *Y* axis signal. All 70 of the clones identified via monoclonal FACS were subsequently cloned into an expression vector to produce soluble scFv protein, and the crude periplasmic extracts were used to confirm binding of scFv to soluble hapten by competitive ELISA without the avidity or other interferences that might arise from the yeast display system. Of the 70 clones identified by competitive flow cytometry, 65 also showed activity in the competitive ELISA format. When these 65 clones were sequenced, 7 distinct scFv sequences were found. Such duplication is to be expected in immune libraries, where significant in vivo selection has already occurred. The fact that we have independently isolated duplicate clones with similar binding properties demonstrates the selectivity of the method.

Similar selection protocols were applied to cells derived from Protocol 2, where analysis of 48 individual clones revealed 8 clones that were positive by competitive flow cytometry, 6 that were positive by competitive ELISA, and 3 distinct scFvs with different binding properties. These data are summarized in Table 2. Sequence alignment (see Figure S3 in Supporting Information) indicated that all positive clones appeared to fall into three family lines, related to the A10, D7, and G8 clones that showed distinct patterns during competitive flow cytometry.

**Table 2. Hapten-Specific scFvs after Competitive FACS**

| | Protocol 1 | Protocol 2 |
|---|---|---|
| tested clones | 184 | 48 |
| positives by flow | 70 (38%) | 8 (17%) |
| positives by cELISA | 65 (35%) | 6 (13%) |
| diversity | 7 | 3 |

**Competitive ELISA with Purified scFvs.** Finally, we explored the binding characteristics of the scFvs expressed by these three clones, as models of the antibodies present in the immune library. Purified scFvs were analyzed by competitive ELISA for their abilities to recognize unsubstituted Phen or MePhens, as shown in Figure 7. Clone D7 bound to all Phen analogues with no significant difference, while G8 and A10 were able to differentiate among various methylated phenanthrenes. G8 scFv bound to 4MePhen most tightly, and A10 preferred 2MePhen over other isomers. The position of methyl substituents is shown in the structure above the graphs.

## ■ CONCLUSIONS

In this study, we demonstrate that the FACS selections described herein provide a powerful new tool for the isolation



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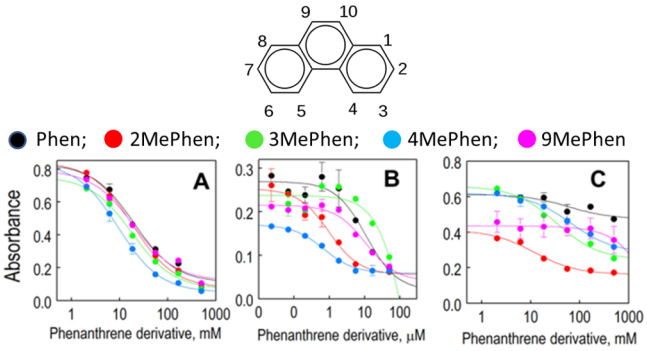


**Figure 7.** Competitive ELISA. Purified scFvs from clones D7 (A), G8 (B), and A10 (C) were incubated in microwell plates coated with Phen-BSA in the presence of varying concentrations of soluble competitors. (Top) Position of methylation of the alkylated phenanthrenes used in these experiments.

of rare hapten-specific scFvs Two FACS procedures were required after transfer of the phage-selected library to the yeast display system. The first selection either removed clones that bound nonspecifically to our antigen (Protocol 1) or enriched the library for those clones that bound efficiently to our protein antigen (Protocol 2). In both cases, ∼50% of the total phage-selected clones were eliminated. The second, competitive FACS allowed us to isolate a very small pool of clones (∼1% of total sorted cells) that were highly enriched (13−35%) in clones that bound to soluble hapten. Thus, after the two FACS selections, sequence analysis and competitive ELISA of periplasmic extracts could be limited to ∼0.5% of the original phage-selected population. When combined with the 20−100-fold enrichment achieved during the panning steps, these methods increased the chances of finding hapten-specific scFvs in from 0.025 to 0.005% in the original immune library to 13−35% in the final selected pool. Such enrichment will greatly reduce the workload of investigators who wish to utilize recombinant technology to generate hapten-specific antibodies, and hopefully will increase the total hapten-specific scFvs available to the general public in the future.

Experiments are currently in progress to further study the binding properties of the 10 distinct PAH-specific scFvs isolated in these experiments and to utilize these unique reagents in new sensors to distinguish the sources of environmental PAH contamination. These antibodies could also be used to evaluate the relative toxicity of PAH mixtures from oil spills since increases in the relative proportions of methylated PAHs in such mixtures are likely to increase the activation of the human aryl hydrocarbon receptor[37] and thus lead to increases in reactive oxygen species and protein- and DNA-PAH adducts.

## ■ ASSOCIATED CONTENT

### Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.6b02334.

Supplemental experimental procedures, Tables S1−S3, and Figures S1−S3 (PDF).

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### Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

### Notes

The authors declare the following competing financial interest(s): The Administrators of the Tulane Educational Fund have filed a patent application (PAH antibodies and uses thereof) on portions of the work described in this publication.

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