Supporting Information for:

Isolation of Alpaca Anti-Hapten Heavy Chain Single Domain Antibodies for Development of Sensitive Immunoassay

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1 **Materials**. 3-PBA standard, bovine serum albumin (BSA), thyroglobulin, polyethyleneglycol
2 8000 (PEG 8000), Tween 20, isopropyl-β-D-thiogalactopyranoside (IPTG), Freund's incomplete
3 adjuvant, and 3, 3', 5, 5'-tetramethylbenzidine (TMB) were obtained from Sigma-Aldrich (St.
4 Louis, MO). Helper phage M13KO7, T4 DNA ligase, and SfiI were purchased from New
5 England Biolabs (Ipswich, MA). Mouse anti-M13 phage MAb-horseradish peroxidase (HRP)
6 was purchased from GE Health Care (Piscataway, NJ). Goat anti-HA tag PAb-HRP was
7 purchased from Abcam (Cambridge, MA). 3-PBA immunizing hapten was previously
8 synthesized in our laboratory (Figure S-1)[1]. The phagemid vector pComb3XSS was kindly
9 provided by Dr. Carlos F. Barbas (The Scripps Research Institute, La Jolla, CA). Chemically
10 competent TOP10F' cells, Platinum® Taq DNA Polymerase and The SuperScript III First-Strand
11 Synthesis System were obtained from Invitrogen (Carlsbad, CA). LeukoLOCK™ Total RNA
12 Isolation Kit was obtained from Applied Biosystems (Foster City, CA). Plasmid purification kit,
13 gel purification kit, PCR purification kit, and 6xHis tag purification resins were obtained from
14 Qiagen (Valencia, CA). Electrocompetent ER2738 *E. coli* cells were purchased from Lucigen
15 Corporation (Middleton, WI). B-PER lysis solution was purchased from Thermo Pierce
16 Scientific (Rockford, IL).
17 **Immunization.** A three-year old castrated male alpaca was subcutaneously immunized with
18 200 µg of 3-PBA hapten-thyroglobulin conjugate diluted in 1 mL of PBS mixed with 1 mL of
19 Freund's incomplete adjuvant. Identical booster injections followed every 2 weeks for a total of
20 6 injections. The same hapten was conjugated to BSA and used as a coating antigen for the
21 screening of positive VHH clones and for assay development. A 5 mL serum sample was taken a
22 few days prior to each immunization for titer estimation. A week after the final injection, 10 mL

of blood was withdrawn from a catheter in the neck vein into EDTA-coated Vacutainer® blood collection tubes (Becton Dickinson, Franklin Lakes, NJ).

Library construction. Peripheral blood lymphocytes were isolated from 10 mL of EDTA-treated blood (about 10^7 cells) using the LeukoLOCK™ Total RNA Isolation Kit following the manufacturer's instructions. The concentration of RNA was calculated by measuring the absorbance at 260 nm on a NanoDrop (Thermo Scientific, Fremont, CA) and the RNA integrity was confirmed by denaturing agarose gel electrophoresis. The SuperScript III First-Strand Synthesis System for RT-PCR with oligo(dT)s as the primers was used to synthesize cDNA from 2.5 µg of RNA. DNA fragments encoding the VHH IgG variable domains were amplified by PCR using the forward primer Alp-Vh-F1 SfiI: CAT GCC ATG ACT GTG GCC CAG GCG GCC CAG KTG CAG CTC GTG GAG TCN GGN GG targeting the framework 1 region; and reverse primers AlpVhh-R1 SfiI: CAT GCC ATG ACT CGC GGC CGG CCT GGC CTC GTG GGG GTC TTC GCT GTG GTG CG and AlpVhh-R2 SfiI: CAT GCC ATG ACT CGC GGC CGG CCT GGC CTC GCC TTG TGG TTT TGG TGT CTT GGG corresponding to the short (IgG3) and long (IgG2) hinge region, respectively. The reverse and forward primers were based on previously published alpaca sequences (Maass et al[2]) and also introduced two different SfiI sites (underlined) that were used for subsequent cloning of the fragments into the phagemid pComb3XSS vector. We also shortened the length of the Alp-Vh-F1 annealing region because we found in our first attempt that this introduced frame shifts in some of our VHH sequences. The ligated vector was electroporated into electrocompetent ER2738 cells from *E. coli* using a MicroPulser Electroporation Apparatus (Biorad Laboratories, Hercules, CA). The resulting library had an estimated size of 3.4 x 10^{7} independent clones. For the amplification and isolation of the phage-VHH library, the transformed cells were added into a 1 L culture flask containing

400 mL Super Broth (SB, 10g of MOPS, 30g of tryptone, 20g of yeast extract, pH 7.0) containing tetracycline (20 µg/mL) and ampicillin (100 µg/mL). The flask was shaken vigorously at 37 ^{o}C until O.D.600 reached 0.5. The cells were super infected by adding 2 mL of helper phage M13KO7 (1x10^{12} c.f.u/mL) by 30 min incubation at 37 ^{o}C without shaking. The flask was shaken for 1 h before kanamycin (70 µg/mL) was added into the flask. On the following day, the overnight culture was centrifuged at 11,000 x g for 10 min at 4 ^{o}C. The clear supernatant was transferred to clean bottles and 40 mL of 20% PEG 8000/2.5 M NaCl (PEG/NaCl) was added. The bottles were incubated on ice for 2 h and centrifuged for 15 min at 11,000 x g. The supernatants were discarded and the phage pellets were resuspended with 100 mL of sterilized PBS. Twenty ml of PEG/NaCl was added into each bottle and incubated for 2 h on ice. The bottles were centrifuged again at 11,000 x g for 15 min at 4 ^{o}C. The supernatants were discarded and the phage pellets were resuspended with 10 mL of phage suspension buffer (sterilized PBS containing 0.02% sodium azide, 0.5% BSA, and 1x protease inhibitor cocktail (Roche Bioscience). The phage library solution was filtered through 0.2 µm membrane and stored at -70 ^{o}C until use.

Selection of phage anti- 3-PBA VHH clones. Six wells of an ELISA plate (Nunc MaxiSorp, Thermo Fisher) were coated with 0.5 µg/mL of 3-PBA-BSA by overnight incubation at 4 °C. The plates were blocked with 3% BSA in PBS (350 µL per well) for 1 h at 37 °C. One hundred µL of the phage VHH library (10^{12} c.f.u/mL) was added into each well and the plate was incubated for 2 h at 4 ^{o}C with gentle shaking. After washings five times with PBST, the bound phage-VHHs were competitively eluted with 100 µL of 3-PBA diluted in PBS (1 µg/mL per well) by 30 min incubation at 4 ^{o}C. The phage VHH eluates were transferred to BSA-coated wells followed by 1 h incubation to remove non specific binders. Unbound phage VHHs were

collected and pooled. For amplification of the phage VHH eluates for the next round of panning, 500 µL of overnight culture of the ER2738 cells was added into a 125 mL flask containing 10 mL of SB and the cells were grown by shaking at 37 ^{o}C until O.D.600 reached 0.5-0.6. Three mL of the cells were then infected with 200 µL of the phage VHH eluates at 37 ^{o}C for 30 min without shaking. Five mL of pre-warmed SB containing 200 µg of ampicillin and 60 µg of tetracycline was added to the infected cells and the culture was shaken for 1 h at 37 ^{o}C. Five hundred µg of ampicillin was added and the culture was incubated at 37 ^{o}C for 1 h with shaking. After infecting the cell culture with 1 mL of helper phage by 30 min incubation at 37 ^{o}C without shaking, the culture was transferred to a 500 mL culture flask containing 91 mL SB with 9.4 mg of ampicillin and 920 µg of tetracycline. After shaking 1 h at 37 ^{o}C, kanamycin was added at a final concentration of 70 µg/mL. The flask was shaken overnight by vigorous shaking at 250 rpm. The amplified phages were prepared using the procedures described above in the section of library construction. Four more rounds of panning were carried out with the concentrations of the 3-PBA-BSA and free 3-PBA gradually decreased. For 2nd, 3rd, 4th, and 5th panning, the plate was coated with 3-PBA-BSA at 0.25, 0.1, 0.02, and 0.01 µg/well, respectively and the concentrations of free 3-PBA for the elution of bound phage-VHHs were 1000, 100, 10, 1, and 0.1 ng/mL, respectively. For the 2nd panning, the plates were washed ten times with PBST. For the rest of panning, the plates were washed ten times and the plates were shaken for 30 min with wells filled with the washing buffer, followed by ten more washings prior to elution. Post-absorption of the eluted phage-VHHs was performed throughout the panning under the same condition. After the final round of panning, individual clones were screened to identify positive clones by performing an indirect competitive phage ELISA.

1 **Expression and purification of soluble VHHs.** The pCombi3XSS phagemid vectors encoding
2 VHHs with inhibited binding to the coating antigen and unique DNA sequences were extracted
3 from ER2738 clones and were transformed into the TOP 10F' cells by heat shock following the
4 manufacturer instructions. A single colony was selected and grown in 5 mL of SB overnight.
5 One ml of the overnight culture was added to a 500 mL cultural flask containing 100 mL of SB
6 with tetracycline (100 µg/mL) and ampicillin (100 µg/mL). The flask was shaken until $O.D._{600}$
7 reached 0.5. IPTG was added at final concentration of 1 mM, and the flask was shaken overnight
8 at 250 rpm at 37 ^{o}C. The cell cultures were centrifuged at 3,000 x g for 10 min and the cell
9 pellets were frozen overnight at -70 ^{o}C. The melted cell pellets were lysed by adding 6 mL of B-
10 PER lysis solution containing phenylmethylsulfonyl fluoride (PMSF). The cell lysates were
11 centrifuged at 13,000 x g for 15 min at 4 ^{o}C. The clear supernatant was dialyzed against 6x His
12 tag purification washing buffer (50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl, and 25 mM
13 imidazole). On the following day, the dialyzed solution was centrifuged again at 13,000 x g for
14 15 min at 4 ^{o}C. The clear supernatant was added into a purification column packed with 1 mL of
15 Ni-NTA resin which had been washed with 10 mL of washing buffer. The columns were gently
16 rocked for 1 h at 4 ^{o}C. The columns were washed with 10 mL of the washing buffer. The
17 captured soluble VHHs were eluted with 6 mL of elution buffer (50 mM NaH_2PO_4 (pH 8.0), 300
18 mM NaCl, and 250 mM imidazole). The VHH eluate was loaded onto a PD-10 desalting column
19 for buffer exchange to PBS. The purified soluble VHHs were aliquoted and stored at -70 ^{o}C until
20 use.
21 **VHH ELISA (VELISA) and phage ELISA (PELISA).** One hundred µL of 3-PBA hapten-
22 BSA conjugate (0.05 µg/mL for the VELISAs and 0.4 µg/mL for PELISAs) diluted in 0.1 M
23 carbonate-bicarbonate buffer (pH 9.6) was added into wells of a 96-well ELISA plate followed

1 by an overnight incubation at 4 ^{o}C. The plate was washed and then blocked with 350 μL of 1%
2 BSA in PBS by incubation for 1 h at 37 ^{o}C. Fifty μL of various concentrations of 3-PBA diluted
3 in PBS was mixed with an equal volume of soluble VHH (1/1000 dilution in PBS) or phage-
4 displayed VHH (1/2500 dilution in PBS). One hundred μL of the mixture was added to wells and
5 the plate was incubated for 2 h at room temperature with gentle shaking. The plate was washed
6 (5 times for the VELISA and 10 for the PELISA) with PBST. One hundred μL of goat anti-HA
7 tag PAb-HRP (1/10000 dilution in PBS) or goat anti-M13 phage MAb-HRP (1/5000 dilution in
8 PBS) was added to wells, followed by 1 h incubation at room temperature. After 5 washings, 100
9 μL of HRP substrate solution was added and the reaction was stopped after 15 min by adding 50
10 μL of 2 M sulfuric acid. The plates were read with a plate reader (Molecular device, Sunnyvale,
11 CA) at absorbance of 450 with background reading at 650 nm. The obtained absorbance was
12 plotted against the concentrations of 3-PBA using the Sigma plot software version 11.0.

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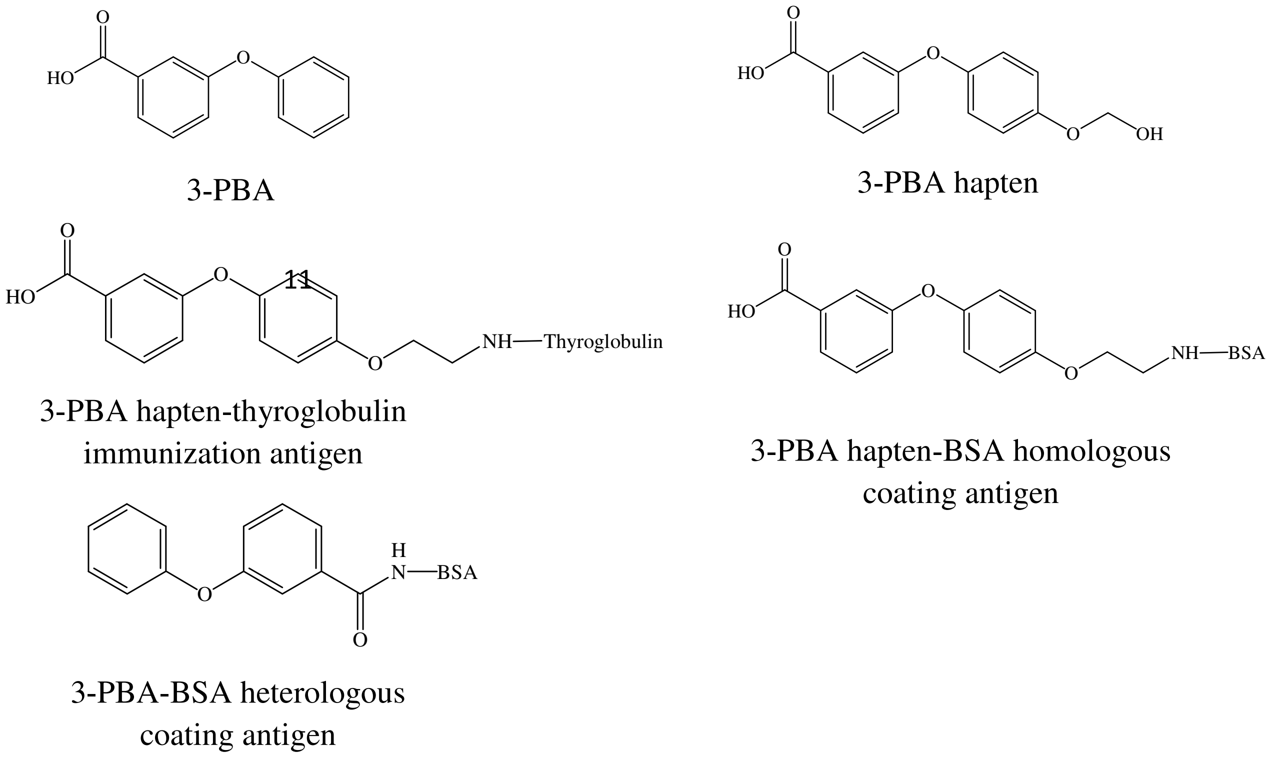

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Figure S-1. Structure of 3-PBA, 3-PBA hapten, homologous, and heterologous coating antigens

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                  FR1                        CDR1      FR2               CDR2      FR3
3P5ThC19  QLQLVESGGGLAQPGGSLRLSCLAS GFTLDSYG IGWFRQAPGKGREPFGC ISSFGGST NYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYC
3P5ThC15  ...........V..........T.. .A..EH.. ..........E....A. F.TLD... ..R...................................
3P5ThC1   .V.........V..........A.. .TS..Y.. ..........E..GVA. ...S..G. .I..---..............F................
3P5ThC12  .V.........V..........A.. ..S.EY.. ..........E..GVA. ...S..R. .IRE---..........E......L.........I...
3P4ThC9   .V.........V..........A.. ..S.EY.. .........MA..GVA. ...R.... .LRE---..........E......L.........I...
3P4ThC6   ...........V..........A.. ..S.EY.. ..........E..GVA. ...G..R. .LRE---..........E......L.........I...
3P4ThC11  .V.........V..........A.. ..S.E... ..........E..GIV. ...Y.... ..R...T..........E........N...........

                 CDR3                 FR4
3P5ThC19  AAAPFYKCGTYNYSAFRS WGQGTQVTVSSEPKTPKPQD
3P5ThC15  .............N.... ....................
3P5ThC1   ....Q.R....R...... ....................
3P5ThC12  ....Q.I....R.T...T ....................
3P4ThC9   ....Q.I....Q.T...T ....................
3P4ThC6   ....K.T....Q.T...T ....................
3P4ThC11  ....Q.I....R...... ....................
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Figure S-2. Amino acid sequence alignment of the isolated clones**.** The deduced amino acid sequences of **t**he seven clones isolated from the phage VHH library are given in the single-letter code. Dots are used to denote residues identical to those of clone 3P5ThC19 that is arbitrarily taken as a reference sequence. Gaps were introduced to improve the alignment and are marked with dashes. Solid-line boxes outline the characteristic amino acid substitutions of VHH FR2.

Table S-1. Recovery of 3-PBA by GC-MS and VELISA

Fortification level (ng/mL)	GC-MS		VELISA	
	Measured (ng/mL)	Average recovery (%)	Measured (ng/mL)	Average recovery (%)
1	[1]ND		1.1±0.04	110
5	3.4±0.3	68	4.9±0.2	98
20	18±0.7	87.5	18±0.4	91
50	51±1.7	101.4	41±1.0	82

[1]Not detected

References

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