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A method for sorting and pairwise selection of nanobodies for the development of highly sensitive sandwich immunoassays

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Abstract

Single domain heavy-chain binders (nanobodies) obtained from camelid antibody libraries hold a great promise for immunoassay development. However, there is no simple method to select the most valuable nanobodies from the crowd of positive clones obtained after the initial screening. In this paper, we describe a novel nanobody-based platform that allows comparison of the reactivity of hundreds of clones with the labeled antigen, and identifies the best nanobody pairs for two-site immunoassay development. The output clones are biotinylated *in vivo* in 96-well culture blocks and then used to saturate the biotin binding capacity of avidin coated wells. This standardizes the amount of captured antibody allowing their sorting by ranking their reactivity with the labeled antigen. Using human soluble epoxide hydrolase (sEH) as a model antigen, we were able to classify 96 clones in four families and confirm this classification by sequencing. This provided a criterion to select a restricted panel of five capturing antibodies and to test each of them against the rest of the 96 clones. The method constitutes a powerful tool for epitope binning, and in our case allowed development of a sandwich ELISA for sEH with a detection limit of 63 pg/mL and four log dynamic range, which performed with excellent recovery in different tissue extracts. This strategy provides a systematic way to test nanobody pairwise combinations and would have a broad utility for the development of highly sensitive sandwich immunoassays.

INTRODUCTION

Since their serendipitous discovery in camelids and later in shark, the heavy-chain-only antibodies have aroused a growing interest due to the unique properties of their antigen binding variable domain (VHH). The recombinant VHH domains, also known as nanobodies (Nb), can be easily obtained from VHH phage display libraries, which, unlike to conventional antibody libraries, are not affected by the heavy/light chain shuffling that occurs during the construction of conventional libraries, and thus have a comprehensive

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representation of the original antibody specificities[1]. Despite, the reduced complexity of their binding site, nanobodies can bind their cognate antigen with similar affinity as conventional antibodies, and equilibrium dissociations constants (K_D) in the nanomolar or even picomolar range are frequently attained[2]. In addition, the recombinant VHHs are produced with high yields in *E. coli*, possessing in most cases an outstanding stability that allows them to withstand extreme conditions of temperature[3,4], or organic solvents[5].

VHHs hold a great potential as versatile reagents for immunoanalytical applications. Their modular structure allows increasing their avidity by multimerization[6], they can be fused to tracer enzymes to produce recombinant conjugates[7,8], be biotinylated *in vivo*[9,10], and some approaches have been developed for their *en masse* screening[11]. The metabolic biotinylation of nanobodies is particularly attractive for immunoassay or biosensor development because of the oriented immobilization that can be attained on avidin/streptavidin coated surfaces[12], and this method of immobilization has been adapted for the selection of immunoassay-ready nanobody pairs against biothreats[13]. Recently, a number of sandwich ELISAs have been developed based on this principle to detect procalcitonin[14], the *Bacillus thuringiensis* Cry1Ac and Cry1Fa toxins[15,16], and the influenza H5N1virus[17]. The sensitivity of these tests was moderate, in the ng/mL range. These assays have been developed on the basis of a trial-and-error approach to find the most promising pairs out of a limited number of Nbs. Considering the comprehensive representation of the antibody response that typically harbors a VHH immune library, a more systematic high-throughput method for the selection of the capturing and detecting antibodies would surely push the boundaries of the sensitivity that can be achieved with two-Nb tests. Using surface plasmon resonance instruments or the Luminex xMAP technology the initial affinity screening of antibody fragments has been automatized[18,19], in this work, we present a simple method that standardizes the conditions to compare a large number of Nb clones biotinylated *in vivo* and classifies them according to their reactivity with a tracer antigen conjugate. This reduces the initial number of capturing antibodies candidates, and thus performs a massive one-against-all comparison of capacity of the nanobody pairs to detect a limiting amount of antigen.

As a model antigen we used human soluble epoxide hydrolase (sEH). The enzyme is a homodimeric protein composed of two 62.5 kDa monomers[20] that is highly expressed in liver, kidney, heart, lungs and brain[21]. sEH is a major regulator of the formation of epoxyeicosatrienoic acids by the epoxygenase CYP enzymes through the hydrolysis of their epoxide group to the corresponding dihydrodiols[22]. Epoxyeicosatrienoic acids have anti-inflammatory and vasoactive actions and thus their hydrolysis by sEH contributes to inflammation, pain and the rise of blood pressure[23]. The inhibition of sEH is therefore a potential therapeutic strategy to treat inflammation and hypertension, and there are some promising results[24]. Less is known about possible variations in the enzyme levels in different disease conditions and its diagnostic value, mostly due to the lack of quantitative test other than Western blots. We recently described a polyclonal/nanobody sandwich assay for human soluble epoxide hydrolase[25] using the IgG fraction from a hyperimmune anti-human sEH rabbit serum as the capture antibody, and a VHH selected from a human sEH immunized llama nanobody library. Using the same VHH library, the method developed in

this work allowed the systematic and rational selection of Nb pairs that performed with a 100 fold increase in sensitivity.

MATERIALS AND METHODS

Materials and reagents

Bovine serum albumin (BSA), Tween 20, IPTG (isopropyl β-D-1-thiogalactopyranoside), polyethylene glycol 8000 (PEG), Histopaque-1077, 3,3',5,5'-tetramethylbenzidine (TMB), D-biotin, and other common chemicals were purchased from Sigma (St. Louis, MO). Helper phage M13KO7 was purchased from New England Biolabs (Ipswich, MA). Anti-HA tag antibody-HRP was purchased from Roche (Madison, WI, USA) and anti-6xHis antibody coupled to peroxidase was purchased from Abcam (Cambrige, MA, USA). Primers used for the library construction were obtained from Integrated DNA Technologies (IDT, Coralville, IA, USA). ER2738 *E. coli* electrocompetent cells were purchased from Lucigen Corporation (Middleton, WI, USA). HisPur Ni-NTA Spin Plates, EZ-Link™ Plus Activated Peroxidase Kit, NHS (N-Hydroxysuccinimide)-biotin, streptavidin magnetic beads, avidin and streptavidin peroxidase were purchased from Pierce (Rockford, IL, USA).

Library construction

The VHH phage display library was built as previously described[4,25]. A three-year-old llama (*Lama glama*) from the Montevideo municipal zoo was immunized every two weeks with 6 doses of 500 μg of recombinant, affinity purified human sEH in incomplete Freund adjuvant by sub-cutaneous injection. One week after the final booster, 150 mL of blood was drawn and about 10^8 lymphocytes were obtained by Ficoll-Histopaque-1077 (Sigma, St. Louis, MO, USA) gradients. The cells were washed three times with PBS and total RNA was extracted with TRIZOL reagent (Invitrogen, Carlsbad), 10 μg were reverse transcribed using the primer JH, and the VH and VHH genes were PCR amplified using the forward primers VH1, VH3, VH4 (CAT GCC ATG ACT CGC GGC CCA GGC GGC C ATG GCC CAG GTGCAG CTG GTG CAG TCT GG, CAT GCC ATG ACT CGC GGCCCAGGCGGCC ATG GCC GAG GTG CAG CTG GTG GAG TCT GG, CAT GCC ATG ACT CGC GGCCCAGGCGGCC ATG GCC CAG GTG CAG CTG CAG GAG TCG GG, respectively) and the reverse primer JH (CCA CGA TTC TGG CCG GCC TGG CCT GAG GAG ACR GTG ACC TGG GTC C) as previously described[4,25]. The 400 bp fragments were digested with SfiI and cloned in the phagemid vector pComb3X (a kind gift from Dr. Barbas, The Scripps Research Institute, La Jolla, USA). Ten μg of the ligated vector were electroporated into highly competent ER2738 *E. coli* cells. The cells were resuspended in SOC medium and incubated with agitation at 37°C for one hour. Then the transformed cells were cultured in LB containing 100 μg/mL of ampicillin, after 3 hours the bacterial cells were super infected with helper phage M13KO7 and cultured overnight in the presence of 35 μg/mL of kanamycin. Phage was precipitated two times with 0.2 volumes of 20% polyethylene glycol 8000, 2.5 M NaCl, and resuspended in PBS.

Panning with human sEH

Recombinant human soluble epoxide hydrolase produced in the baculovirus expression system[26] was labeled with NHS (N-Hydroxysuccinimide)-biotin and then coupled to

streptavidin magnetic beads for the selection process. The beads were blocked with phosphate buffer saline (PBS) containing BSA (3%), washed with PBS, and then incubated with 100 μL of the VH/VHH library for 1 hour with gentle rotation in a 1.5-mL microfuge tube. Then, the tube was placed in the magnetic particle separator and allowed to stand for 15–30 s to remove the supernatant containing the unbound phages. The particles were washed 10 times by cycles of centrifugation and resuspention in PBS. Finally, the phages were collected by acidic elution and used for titration and amplification in ER2738 for one additional round of selection.

"*En masse*" cloning into pINQ-BtH6 and nanobody production

The pool of selected VH/VHHs genes from the second round of panning against sEH were gel purified from the SfiI digested phagemid pComb3X DNA and cloned *en masse* into the pINQ-BtH6 vector[27]. Downstream of the cloning site this vector encodes a biotin acceptor peptide and a 6xHis tag for metal affinity purification. The ligated vector was electroporated in BL21(DE3) *E. coli* cells containing the pCY216 vector for overexpression of the *E. coli* biotin ligase BirA[28]. The next day, single colonies were picked and inoculated in 500 μL of biotinylation medium (Luria Bertani broth (LB) containing 50 μg/ml kanamycin, 35 μg/ml chloramphenicol, 0.04% of L-arabinose and 100 μM D-biotin) in 96 deep well culture blocks (Greiner Bio-One, Monroe, NC, USA). The expression of the nanobodies was induced at OD_{600} = 0.6 AU with 3 μM IPTG for 4 h at 37°C with shaking. Then the culture block was centrifuged 20 minutes at 1200 × g at 4°C, the supernatants were collected and used for the screening.

Screening of positive clones against human sEH

Microtiter ELISA plates (Maxisorp, Nunc) were coated overnight at 4°C with human sEH (10 μg/ml) in PBS. After blocking for 1 hour at 37°C with PBS-BSA 1%, 100 μL of a 1/200 dilution of the bacterial supernatants in PBS Tween 0.05% (PBS-T) were added, and incubated for an additional hour at room temperature with shaking. VHH detection was performed using streptavidin peroxidase. After extensive washing, the peroxidase activity was developed by adding 100 μL of TMB peroxidase substrate (0.4 mL of 6 mg of 3,3´,5,5´-tetramethylbenzidine in 1 mL of DMSO + 0.1 mL of 1% H_2O_2 in water, in a total of 25 mL of 0.1 M acetate buffer, pH 5.5) and incubated for 15 min at room temperature. The enzyme reaction was stopped by addition of 50 μL of 2 N H_2SO_4, and the absorbance was read at 450 nm using a Fluostar Optima Reader (BMG, Ortenberg, GE).

Preparation of biotin free biotinylated nanobodies in 96 well format

Individual colonies of pINQ-BtH6/ pCY216 co-transfected cells were grown in 96 well culture blocks as described above. After centrifugation the cell pellets were resuspended in 600 μL of 100 μM D-biotin in PBS and lysed by three freeze-and-thaw cycles, then the culture plate was briefly incubated for 30 minutes at 37 °C to maximize the enzymatic biotinylation of the monodomain antibodies[27]. The soluble fraction obtained by a 15 minute centrifugation at 4°C and 1200 × g was applied to 96 well HisPur Ni-NTA Spin Plates for individual purification of the his-tagged biotinylated nanobodies (BtNb), following the manufacturer instructions. The bound antibodies were eluted with 3×330 μL of 250 mM

imidazole in PBS and collected by centrifugation at 1000 × g for 1 min in a deep-well "Master Plate".

Reactivity sorting test

Nunc plate wells were coated overnight at 4°C with 1 μg/mL avidin in PBS (100 μL). After blocking for 1 hour at 37°C with PBS-BSA 1%, 100 μL of the purified BtNb collected in the "Master Plate" were added, and incubated for an additional hour at room temperature with shaking. After washing the bound biotinylated nanobodies were detected with an anti-6xHis antibody coupled to peroxidase, and the activity was measured as described above. Alternatively, the immobilized BtNb were incubated with 100 μL of sEH conjugated to peroxidase (600 ng/mL) for 1 hour, at room temperature, and after washing the peroxidase activity was measured. The sEH conjugate was prepared using the EZ-Link™ Plus Activated Peroxidase Kit following the manufacturer instructions.

Binding analysis of nanobody interactions by bio-layer interferometry

Binding interactions were analyzed using the Octet system (Fortebio, Inc., Menlo Park, CA) by measuring the wavelength shift in nanometers. Kinetic rate constants were determined with streptavidin sensor (SA) in the advanced kinetic mode. The BtNb were immobilized on the sensor at 2 μg/mL in kinetic buffer 10X (Fortebio, Inc., Menlo Park, CA), and were exposed for 300 seconds to various concentrations of sEH covering at the expected value of the dissociation constant (K_D) followed by a 600 seconds dissociation step in kinetic buffer 10× with 2200 rpm shaking. Interferometry data were globally fitted to a 1:1 binding ratio for calculating kinetic parameters using the Octet ForteBio Software.

Sandwich pair selection

The selected capturing nanobodies were grown in 100 mL shaking flasks using identical conditions to those used for 96 well culture plates. The cell pellet was resuspended in 5 mL of 100 μM D-biotin in PBS, lysed by sonication and incubated for 30 minutes at 37 °C. After centrifugation (14000 × g) the BtNb were purified using 1 mL Ni-NTA columns (GE Health Care, Pittsburgh, USA). Three avidin coated plates (1 μg/mL) were blocked with PBS-BSA 1% for 1 hour at 37°C and then dispensed with 10 μg/mL of each of the purified capturing BtNb (100 μL/well), and after washing, each plate was incubated with 0, 4.0 or 20 ng/mL of sEH in PBS (100 μL/well) for 1 hour at room temperature. After washing, each plate was incubated with a 1/200 dilution of the "Master Plate" BtNb in PBS-T. The binding of the secondary antibody was detected using streptavidin peroxidase.

Nanobody sandwich ELISA for the detection of human sEH

Avidin coated plates (1μg/mL) were blocked as described above and then dispensed with 10 μg/mL of each of the purified capturing BtNb (100 μL/well), and after washing, 100 μL of the sEH standards in PBS or the samples were loaded and incubated for 1 hour at room temperature. After washing the HA (Influenza hemagglutinin peptide YPYDVPDYA) tagged Nb S43 was added (0.5 μg/mL) for 1hour at room temperature and then the HA-tag was detected using an anti-HA peroxidase conjugate (Roche, Madison, WI, USA). After extensive washing, the peroxidase activity was developed as described above.

RESULTS AND DISCUSSION

Nanobody selection and initial screening

A library of single domain antibodies was generated from the blood of a llama immunized with human sEH, with an estimated size of 2×10^9 independent clones. In order to select nanobodies reacting with native epitopes of the enzyme, the library was panned against chemically biotinylated sEH immobilized on streptavidin coated magnetic beads. After two rounds of panning, the DNA sequence of the VHHs were transferred "*en masse*" to the pINQ-BtH6 vector, which allows high yield expression of the nanobodies fused to a 15-mer biotin-acceptor-peptide (BtAP) tag[27]. The reactivity of 96 clones with sEH was assayed by ELISA, figure 1. The figure shows the typical outcome of the screening of an immune library after panning, where due to differences in the level of expression (concentration) of the VHHs, the test does not provide any information other than positive or negative binding results.

In order to increase the information obtained in the initial screening, we devise a simple and rapid "sorting test" that allowed grouping the VHHs according to their relative reactivity with an antigen-tracer. The strategy is outlined in figure 2, due to big differences in the level of expression of individual clones, the first step seeks to normalize the amount of *in vivo* biotinylated nanobody (BtNb) to be tested in each well. This is accomplished through the quantitative saturation of the capture capacity of the coating avidin. In that regard, the high-affinity of the biotin-avidin interaction favors that saturation, even for low-expression clones. In addition, we optimized the amount of avidin used for coating in order to minimalize the amount of BtNb required to attain saturation. This is shown in figure 3, since the biotin-binding capacity of the coated avidin drastically drops below 1 μg/mL, this concentration was adopted for coating. Under these conditions, the biotin binding capacity of the wells was saturated with BtNb concentrations ≥ 0.5 μg/mL, figure 3. We recently reported the optimization of the *in vivo* biotinylation of nanobodies in 96 well culture plates[27], and typically, the yield of expression of different VHHs in 96 culture plates is well above this value, however to compensate for loses during the purification step and assure full saturation, six replicas of a culture block with 96 sEH-ELISA positive clones were grown in parallel and the cell extracts were combined in the Ni-NTA spin plates. This step serves a double purpose; it concentrates the BtNb and eliminates the free biotin before their incubation in the avidin wells. The bound nanobodies were eluted with 250 mM imidazole in a final volume of 1000 μL in deep wells of a "Master Plate" that was used as the sole source of purified BtNb for the reactivity sorting test and the pairwise analysis of the nanobodies.

We then analyzed the degree of saturation attained when 100 μl of the purified BtNb were transferred to the wells of avidin coated plates, by detecting the 6xHis tag of the bound BtNb, Figure 4. Except for clones 53 and 86, the readout produced by detection of the 6xHis tag was roughly the same, indicating that equal amounts of BtNb were retained in each well. Once this was verified, a similar plate was saturated with the BtNb and incubated with an optimized amount (600 ng/mL) of sEH conjugated to peroxidase (sEH-HRP) to compare the relative reactivity of the nanobodies. Under these conditions, the signal of each nanobody is

expected to be proportional to its affinity for the tracer, in our case the sEH-HRP conjugate, figure 5. According to their readouts, the nanobodies were grouped into four families (F-I to F-IV). To assess the sorting ability of the test, 34 representative clones were sequenced and the results are summarized in **figure S-1**. Thirteen unique sequences were identified, and as expected, nanobodies with identical sequences yielded readouts within a limited range that was characteristic of each family. Supporting that, none of the sequences appear in more than one family at the same time, showing the robust ranking capacity of the method. All clones have the hallmark residues of the VHH domains in framework 2 (FR2) at positions 42, 50 and 52 (International ImMunoGeneTics (IMGT) amino acid numbering), except for clone S33 that respectively has the amino acids V, L and W at those positions, which are typically found in VH domains[29]. In addition this clone has an unusually long CDR2 of 19 amino acids, and four of the five clones belonging to the F-I family corresponded to this sequence. The selection of VH-like single domain antibodies that have good solubility and are good binders has been reported in the past[30], some structural modification such as the Trp103Arg substitution or a long CDR3 that collapses on the erstwhile VL-interface promote their stability[31]. A larger sequence diversity was found in the other families, and clones sharing the same sequence were hit several times within each family.

The assay provides a simple yet useful criterion to select an initial panel of Nb for further characterization, on the basis of their relative affinity to a particular tracer. In our case, this allowed to spot low frequency clones, such as S33 (4 out of 96 clones) and S73 (1 out of 96 clones) which could have otherwise been easily missed by random picking of a few clones. On this basis we chose the two high-readout (S33, S73) and three intermediate-signal (S7, S43 and S76) clones to perform a comprehensive selection of VHH pairs for sEH detection.

Selection of best antibody pairs for sEH detection

The biotin handle also provides a convenient way of finding appropriate nanobody pairs for antigen detection. To this end, the most promising BtNb are used to saturate the binding capacity of avidin coated wells, which in turn are used to capture limiting amounts of the unlabeled antigen. These wells can then be assayed in parallel with a large number of secondary BtNb to find the best pairs for antigen detection. Using our selected clones, each capture antibody was tested in combination with the 96 clones from the “Master Plate” using 20 and 4 ng/mL of sEH, figure 6. There was a fairly good correlation between the clones that produced the highest readouts at both concentrations. Clone S43 seems to define an epitope that does not overlap with the great majority of the clones and thus pairs with a large number of clones. Conversely, clones S7 and S76 (which have very similar sequences) define epitopes that highly overlapping with the rest of the clones and can only be combined with just a few other clones. Curiously, Nb S73 did not form valuable pairs with any of the other antibodies. Based on these experiments, five secondary nanobodies were selected for each of the catching antibodies and were further analyzed by titration curves, figure 6. In order to avoid the selection of the same or similar clones, we chose these secondary Nb within different readouts.

The sEH concentration values causing 50% signal saturation (SC_{50}) are summarized in table 1 and were used as an indicator of assay sensitivity. All capturing antibodies are in the high

affinity range (table 2), however, the best SC_{50} values obtained depended on their combination. It seems that within a certain range of affinity, the most critical aspects are: a) the absolute absence of steric hindrance for the simultaneous binding of the two antibodies, and b) the eventual conformational changes in the antigen induced upon binding to the capture antibody, which may explain the poor pair-forming capacity of Nb S73. This highlights the importance of an extensive empirical pairwise analysis to maximize the sensitivity that can be attained with two-antibody sandwich tests.

Development of a sandwich ELISA for measurement of sEH

Base on overall results of the pairwise screening we chose to optimize the S7/S43 assay. While the streptavidin peroxidase used to characterize the binding of the BtNb to the sEH provides a convenient way of performing the large scale affinity and pair-forming screening from a single “Master Plate”, we found that the expression of the detecting nanobodies with the hemagglutinin epitope (HA) tag and subsequent detection with an anti-HA peroxidase conjugate provided an increased sensitivity. Figure 7 shows the sandwich ELISA developed with the BtNb S7 and HA-tagged Nb S43 as capturing and detecting antibodies. The assay has an extended dynamic range of about four logs with a SC_{50} of 3.97 ± 0.15 ng/mL, and the limit of detection and quantitation (LOD and LOQ), defined as the value of the blank plus 3 or 10 standard deviations[32], were 0.063 and 0.094 ng/mL, respectively.

The usefulness of the test to determine sEH in biological matrices was initially studied using the cytoplasmic fraction of human red blood cells (RBC), where sEH is expressed and regulates the epoxidation of arachidonic acid catalyzed by hemoglobin[33,34]. The extract was prepared as described before and was found to have a sEH concentration of 33.6 ng/mL. A 1/80 dilution of the extract was then spiked with various concentrations of the enzyme, and the samples were analyzed in a blind fashion. Excellent recoveries were found in the 0.25–4.0 ng/mL range, Table 3.

The NbS7/NbS43 sandwich assay was also used to analyze the occurrence of sEH in the S9 fractions of pooled (4–50 individuals) human tissue samples (Xenotech LLC, Lenexa, KS). The data was compared with the determination of sEH in these tissues measuring the enzymatic activity and by Western blot[35], table 4.

CONCLUSIONS

By standardization of the amount of biotinylated nanobody to be assayed, we developed a simple method that can reliably sort out their reactivity against a labeled form of the antigen. Except for the use of peptide tags in recombinant antigens, the labelling process modifies some epitopes of the antigen and thus the reactivity sorting assay does not necessarily correlate with the relative affinity of the nanobodies. Still, as demonstrated in our case, the test is a useful tool to sort out a large number of clones and choose a representative number of capturing Nb that includes low frequency clones. The use of the biotin handle also facilitates the assay of these Nb with a large number of potential detecting Nb in a simple manner. In the particular case of the human sEH, this helped to develop a highly sensitive sandwich immunoassay with excellent performance in highly complex matrices. This highlights the benefits of the large scale screening of nanobody pairs together with the

additional advantage of the oriented immobilization of the capturing antibody that provides the method. Nanobodies possess advantageous properties for immunodetection and the selection method described here could be of broad utility to develop highly sensitive sandwich immunoassays.

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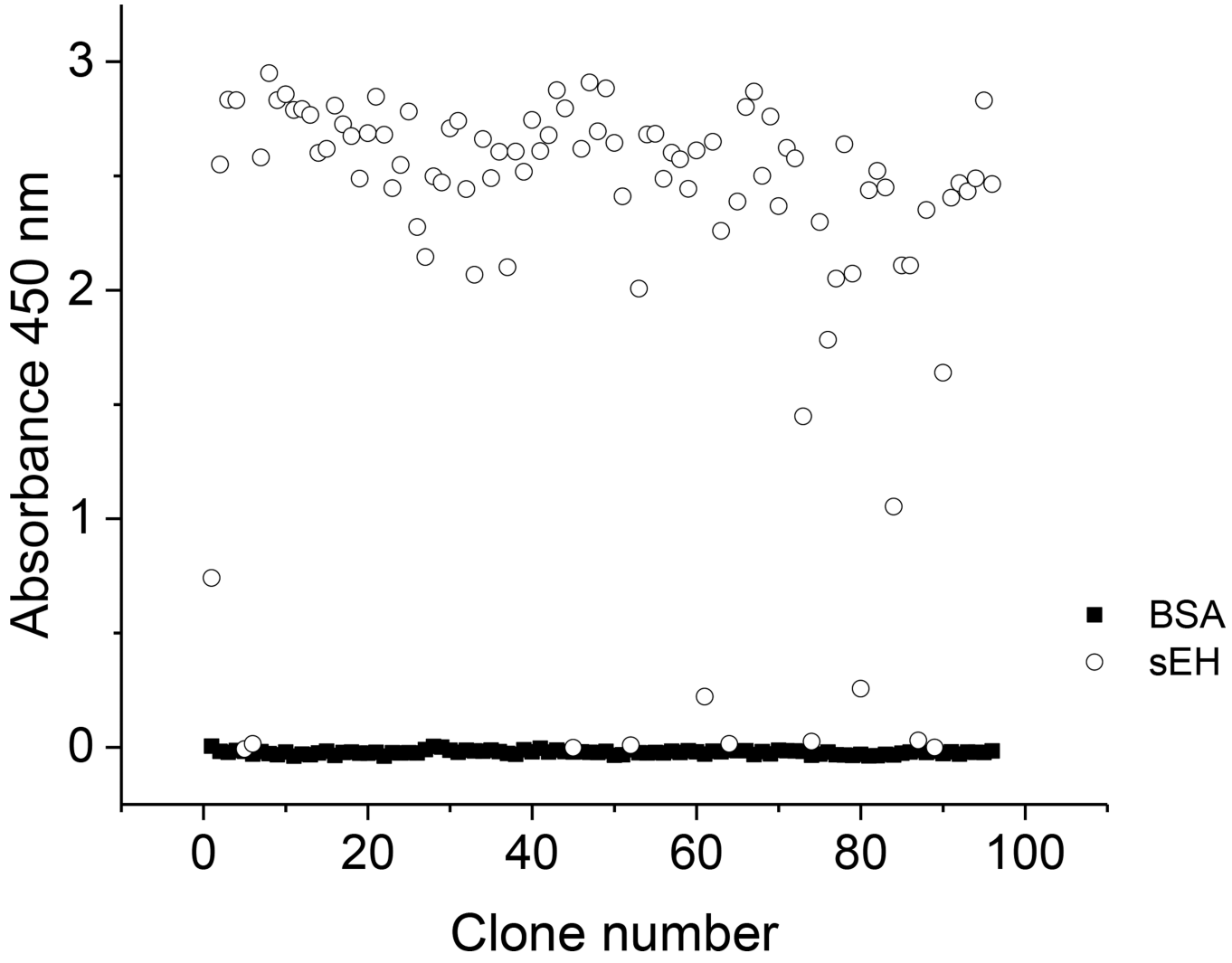

Figure 1. ELISA reactivity of selected clones against sEH

The *in vivo* biotinylated nanobodies (1/200 dilution) were detected using streptavidin peroxidase conjugate. Human sEH, circles; BSA, squares; clone 5 is an unrelated biotinylated VHH against triclocarban[4] used as negative control.

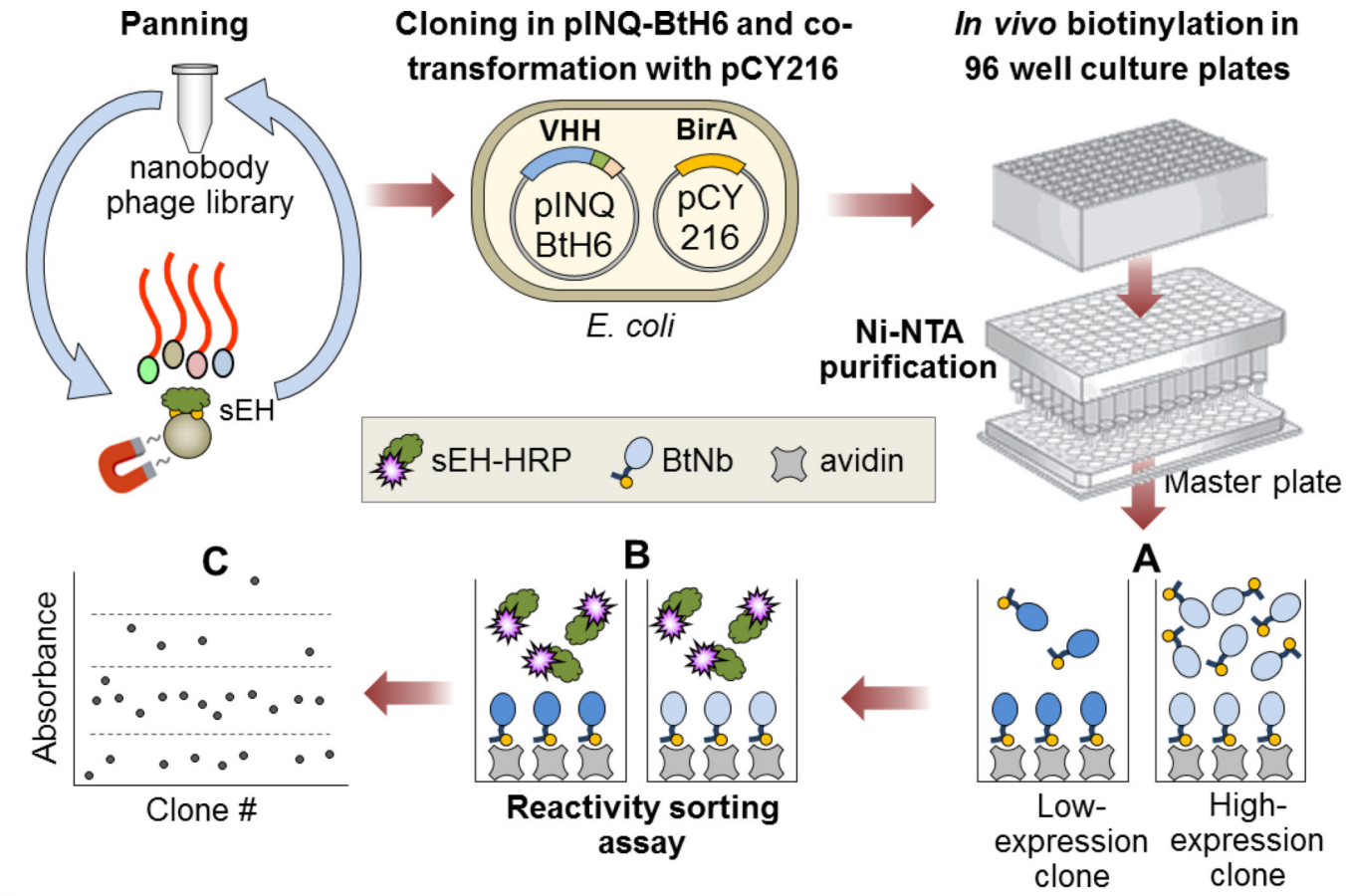

Figure 2. Schematic representation of the strategy to produce the BtNb "Master Plate" and perform the reactivity sorting test

After panning, the VH/VHH pool is cloned *en masse* in the pINQ-BtH6 vector. The resulting library is co-transformed with pCY216 and individual clones are grown in 96 culture wells to produce the *in vivo* biotinylated nanobodies which are further purified/ concentrated in 96 Ni-NTA column plates to eliminate the unconjugated biotin. The purified BtNb are then used to saturate the biotin binding capacity of avidin coated wells (**A**) and thus standardize the amount of captured antibody (**B**). The nanobodies are then probed with the same optimized amount of labeled antigen, under which conditions, the readout correlates with their relative affinity for the tracer antigen (**C**).

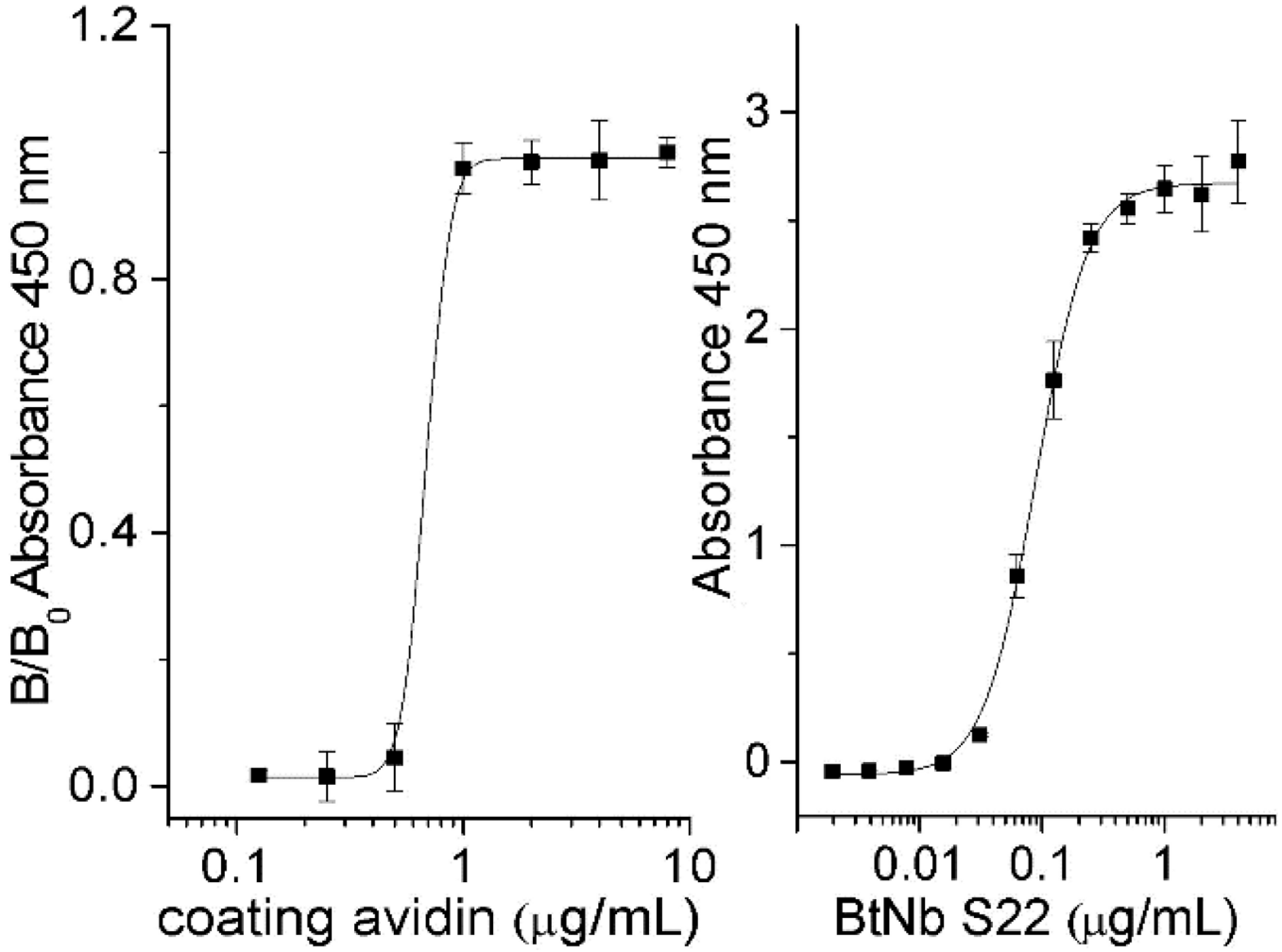

Figure 3. Saturation of the binding capacity of avidin coated wells

Left, wells coated with different concentrations of avidin were saturated with an excess (10 μg/mL) of the biotinylated nanobody S22. Right, saturation of wells coated with 1 μg/mL of avidin, with different amounts of biotinylated S22. The binding of the nanobody was detected with an anti-6xHis HRP conjugate.

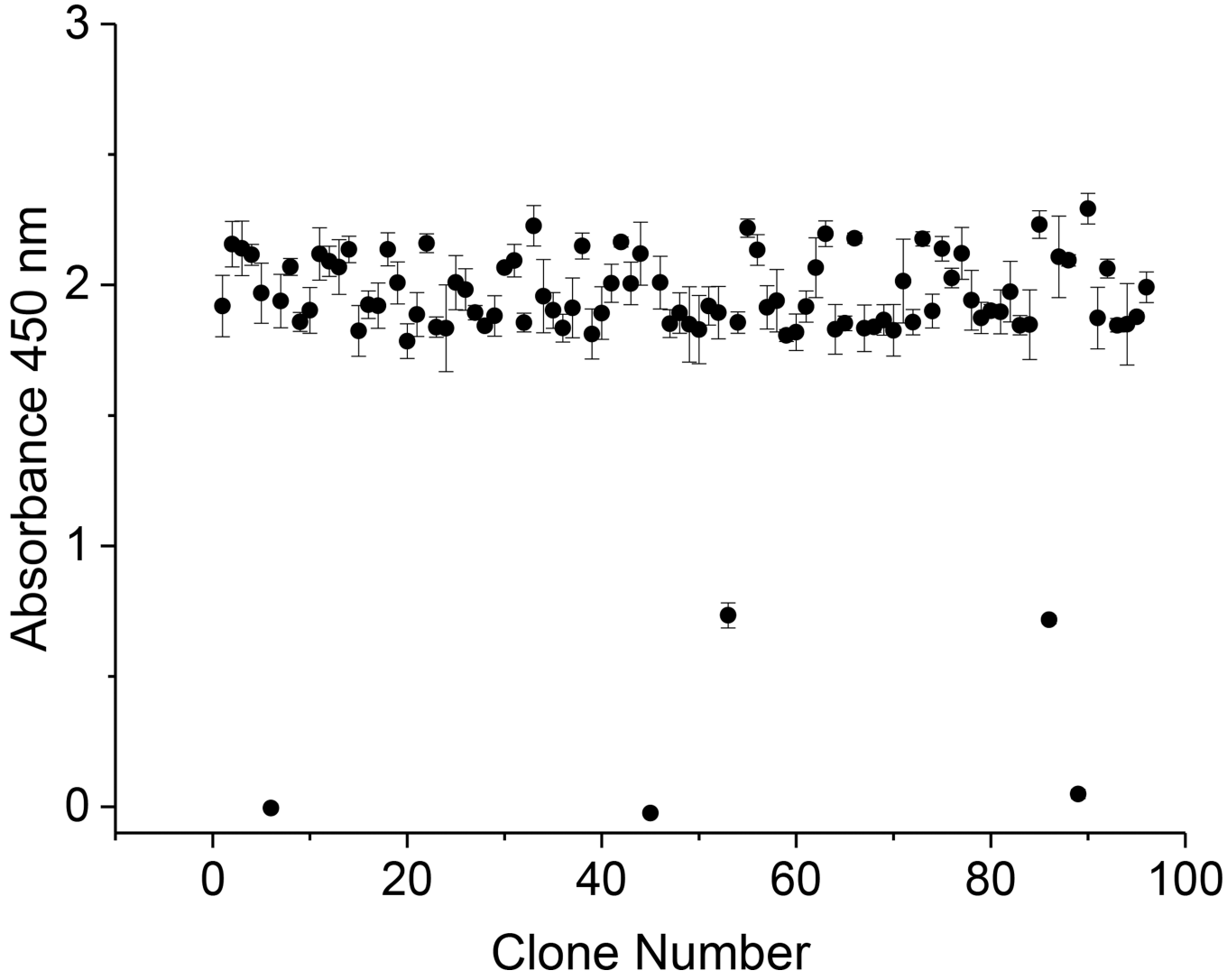

Figure 4. Saturation of avidin coated wells with different BtNb clones

The 6xHis tag of the BtNb bound to the coated avidin was detected using a mAb anti-6xHis conjugated to horseradish peroxidase (HRP); incubation with the substrate was shortened to obtained readouts well below the saturation signal. Each value represents the average of three independent experiments, error bars represent the SD. Clone 5 (VHH TC9), an unrelated BtNb against triclocarban[4] was added at 5 μg/mL as positive control of saturation. Clone 6, 45 and 89 were substituted by PBS as negative controls.

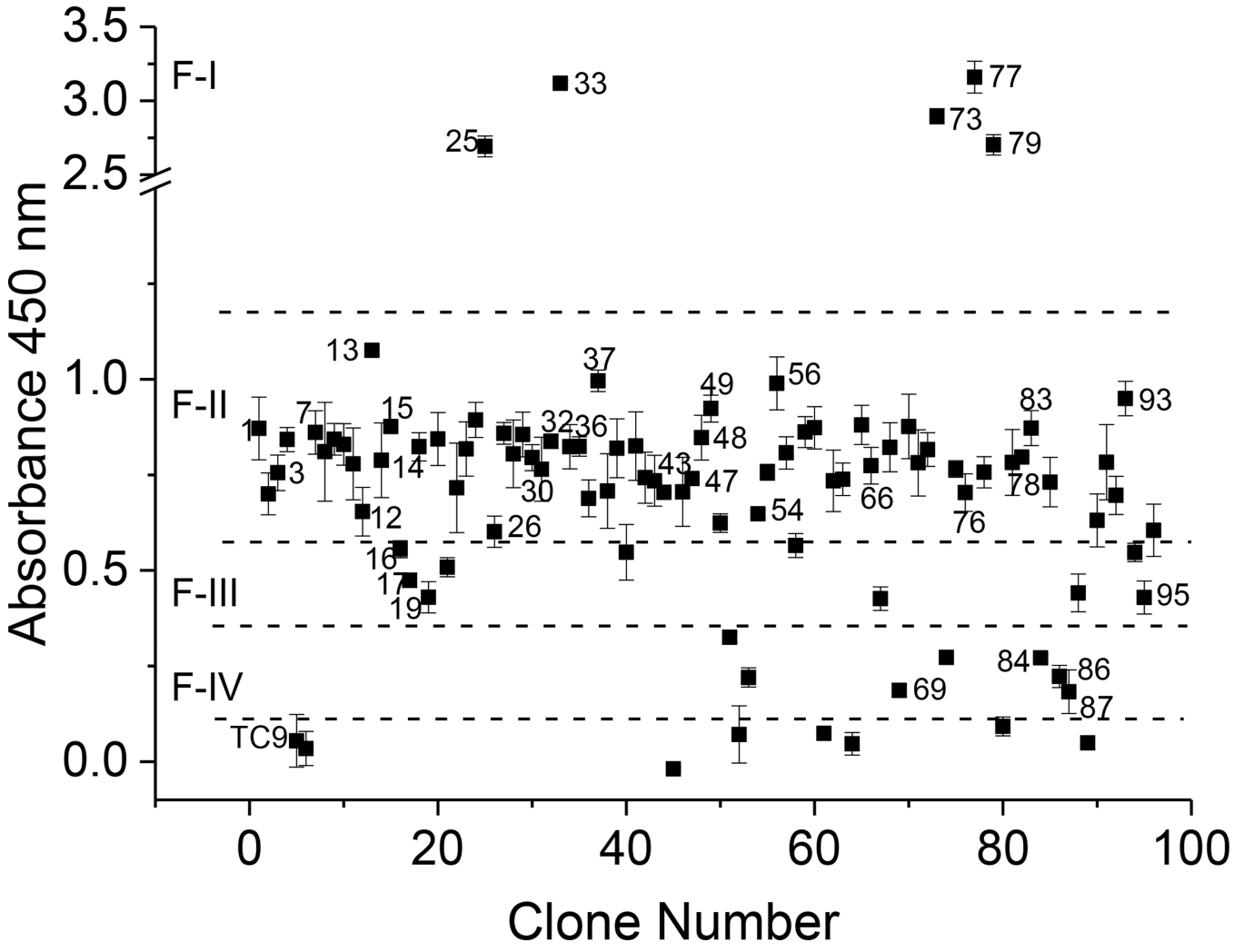

Figure 5. Reactivity sorting assay

Avidin coated wells were saturated with BtNb as described above and then probed with 600 ng/mL of sEH-HRP. According to different absorption ranges, clones were grouped in families (F I–IV) as denoted by dashed lines. Each value represents the average reading of three independent experiments, error bars represent the SD. Clone 5 (TC9, an unrelated BtNb against triclocarban[4]), and clones 6, 45 and 89 (PBS) are negative controls. The numbers close to each point indicate clones that were sequenced.

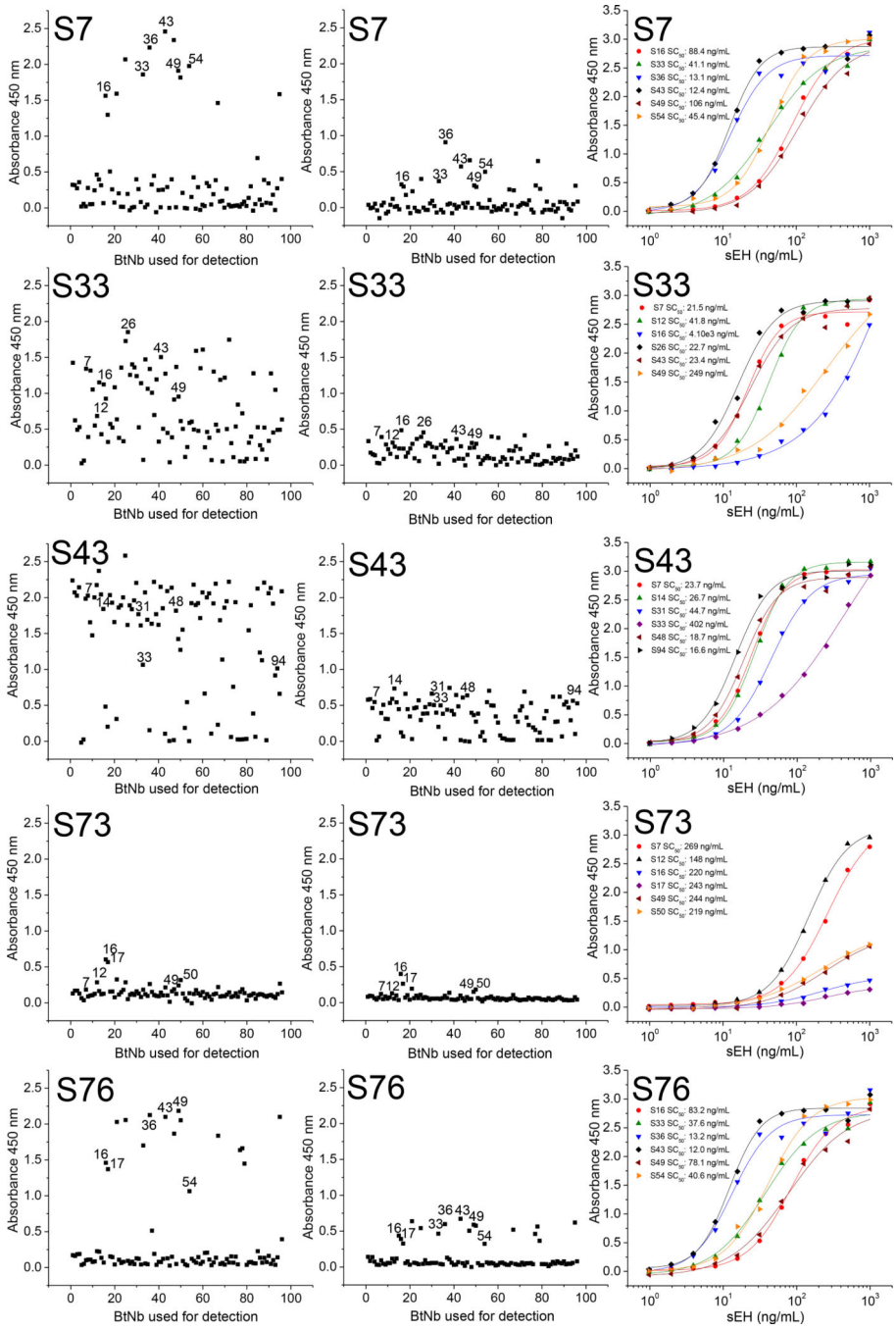

Figure 6. Parallel screening of nanobody pairs for sEH detection

Five capturing antibodies ranked as high or medium affinity clones (indicated in the upper left corner of the graphs) were paired with the 96 monodomain antibodies collected in the "Master Plate" for the detection of 20 (left) or 4 (middle) ng/mL of sEH. The titration curves performed with some of the best performing pairs (numbered) are shown on the right.

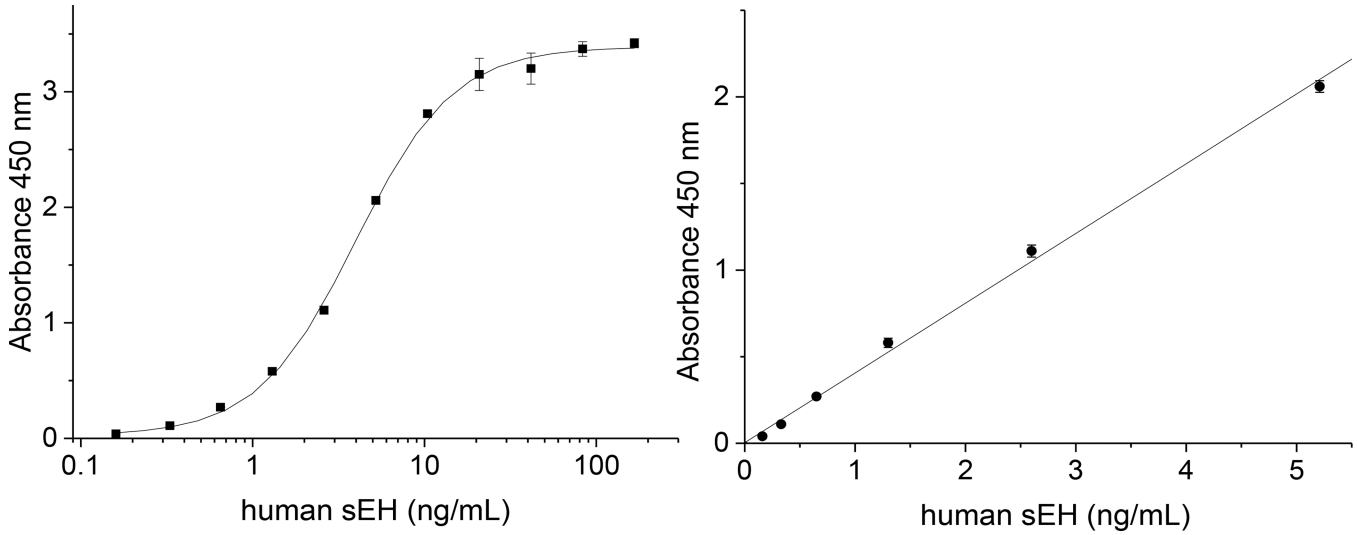

Figure 7. Nanobody sandwich ELISA for the detection of human sEH using nanobodies S7 and S43

Titration curves obtained in the 0.16–160 ng/mL (left) or 0–5.2 ng/mL (right) range. The results are the average values of triplicate measurements, error bars represent the SD.

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Table 1

SC_{50} values (ng/mL) of sEH titration with selected nanobody pairs

Capture antibody		S7	S76	S33	S43	S73
		SC_{50} values (ng/mL)				
	S7			**21.5**	23.6	269
	S12			41.7		**147**
	S14				26.6	
	S16	88.4	83.2	4096		220
	S17					243
	S26			22.7		
	S31				44.2	
Detecting antibody	S33	41.1	37.6			
	S36	13.0	13.2			
	S43	**12.0**	**12.2**	25.4	422	
	S48					
	S49	106	78.1	248.8	**18.6**	246
	S50					
	S54	44.9	40.7			221
	S94				31.6	

The lowest SC_{50} values for each capturing antibody are in bold.

Table 2

Biolayer interferometry kinetic measurements

Nb	K_D (nM)	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})
S7	0.42	2.55×10^5	1.07×10^{-4}
S33	0.35	1.97×10^5	6.85×10^{-5}
S43	0.21	7.35×10^4	1.56×10^{-5}
S73	0.71	1.40×10^5	1.00×10^{-4}

Table 3

Recovery of sEH from RBC spiked samples (n=3)

spiked (ng/mL)	Measured (ng/mL)	Mean recovery (%)
4.0	4.14 ± 0.02	93
2.0	2.44 ± 0.01	101
1.0	1.44 ± 0.01	103
0.5	0.94 ± 0.01	103
0.25	0.67 ± 0.02	99
0	0.42 ± 0.03	-

The cytoplasmic fraction of the RBC was diluted 1/80 in PBS and was spiked with different concentrations of sEH. Results are average ± SD (n=3). Notice that the measure amounts are the combination of natural and spiked sEH.

Table 4

Comparison of the sEH ELISA with the enzymatic activity and Western blot determination

	sEH activity*	Western-blot*	ELISA NbS7/NbS43
Tissue	**average ± SD**	**average ± SD**	**average ± SD**
Liver	420 ± 89	500 ± 150	468 ± 22
Kidney	44 ± 3	80 ± 15	37 ± 3
Lungs (NS)	3.0 ± 0.3	22 ± 6	2.9 ± 0.1
Lungs (S)	2.8 ± 0.3	21 ± 4	2.1 ± 0.2
Intestine	80 ± 9	110 ± 10	121 ± 6

NS, non-smokers; S, smokers.

Results (nM) are average ± SD (n=3)

*Data from reference[35].