Supporting information for

Development of a Highly Sensitive Enzyme-Linked Immunosorbent Assay for Mouse Soluble Epoxide Hydrolase Detection by Combining a Polyclonal Capture Antibody with a Nanobody Tracer

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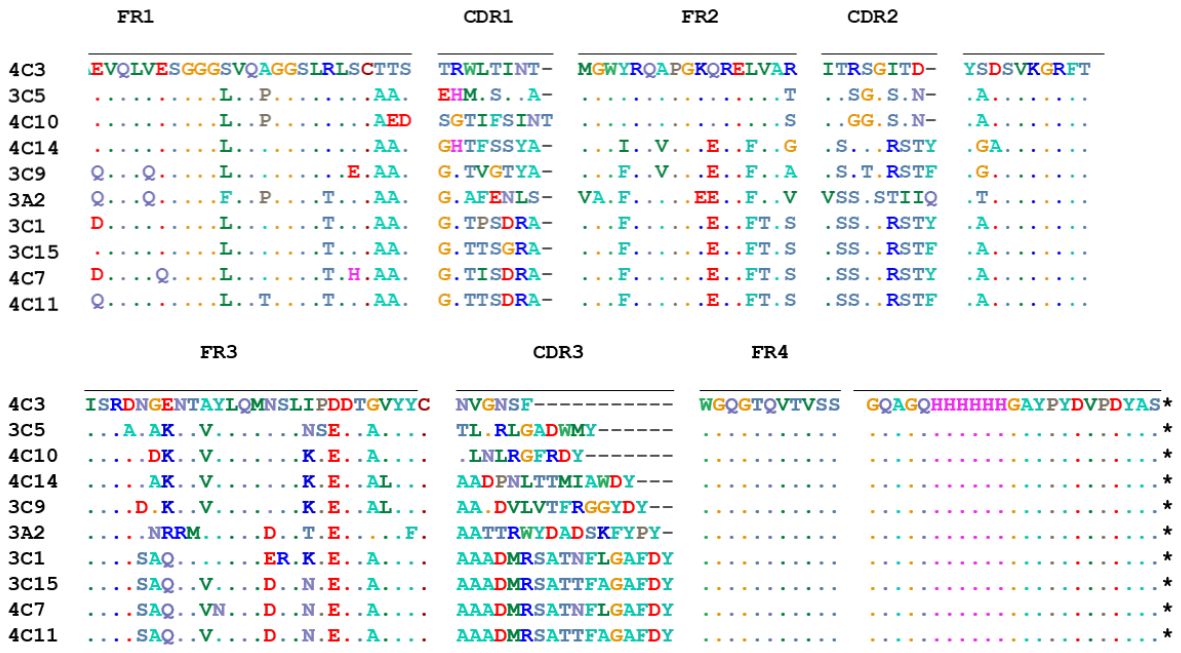


Figure S1. Alignment of amino acid sequences of the re-biopanned nine anti-mouse sEH VHH phage clones.

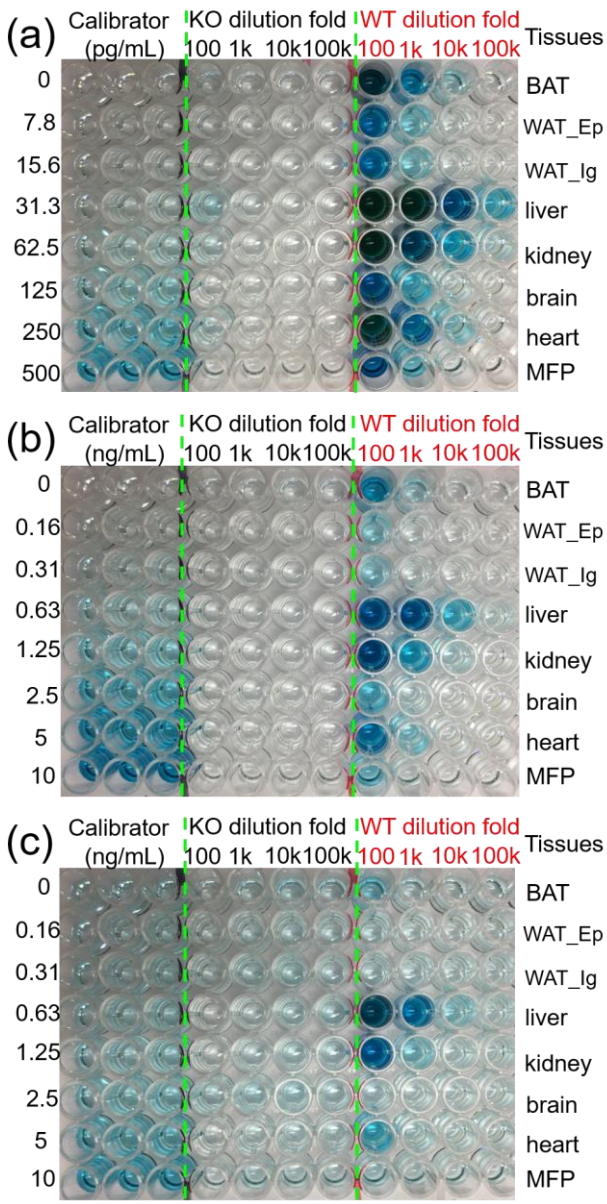


Figure S2. **The selectivity of the other three sandwich ELISAs (format B, C, and D) against eight kinds of tissues from sEH KO and WT mice.** (a) format B (pAb/biotin-4C3/SA-PolyHRP), (b) format C (pAb/HRP-4C3), and (c) format D (SA/biotin-3C1/HRP-4C3).

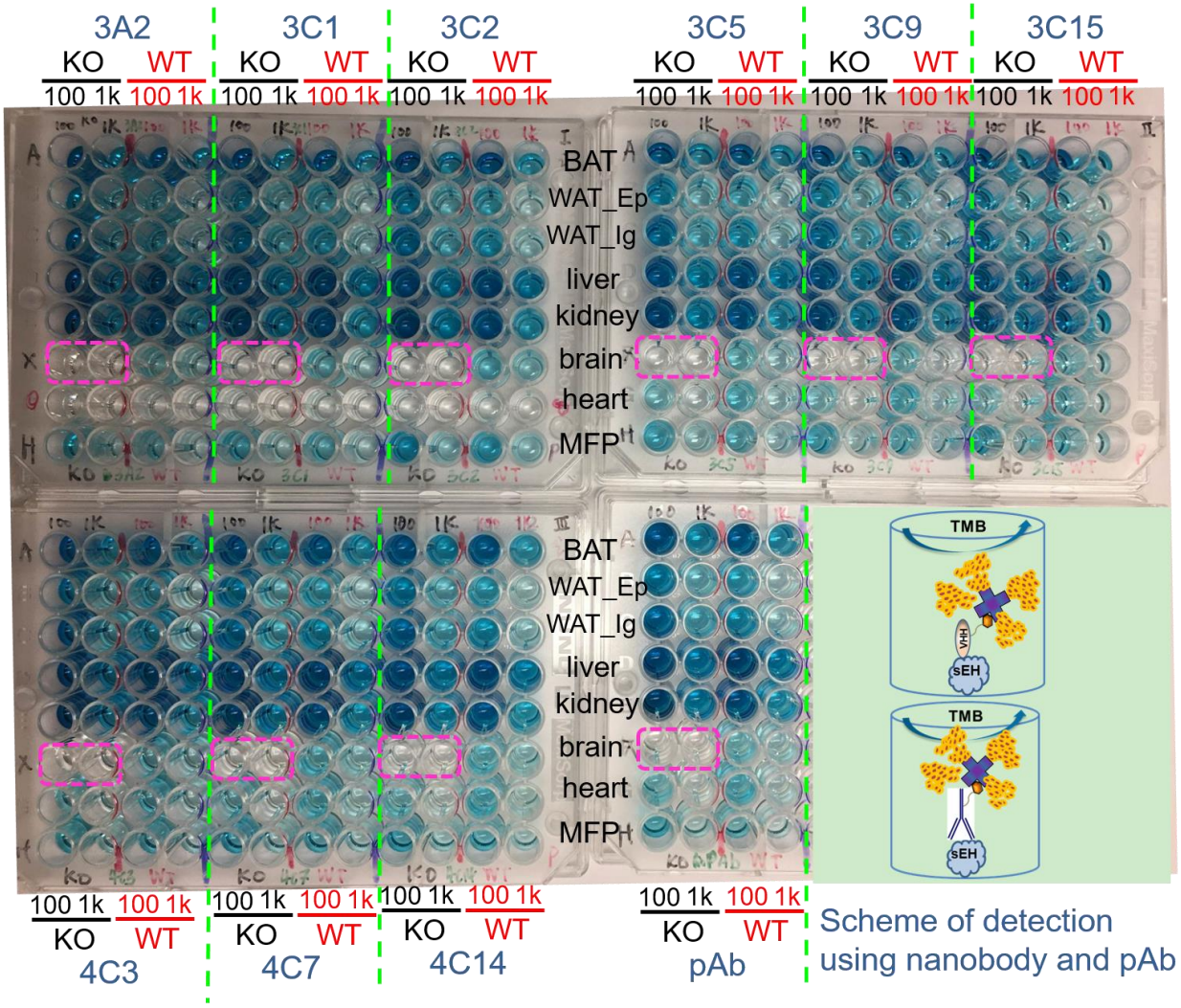
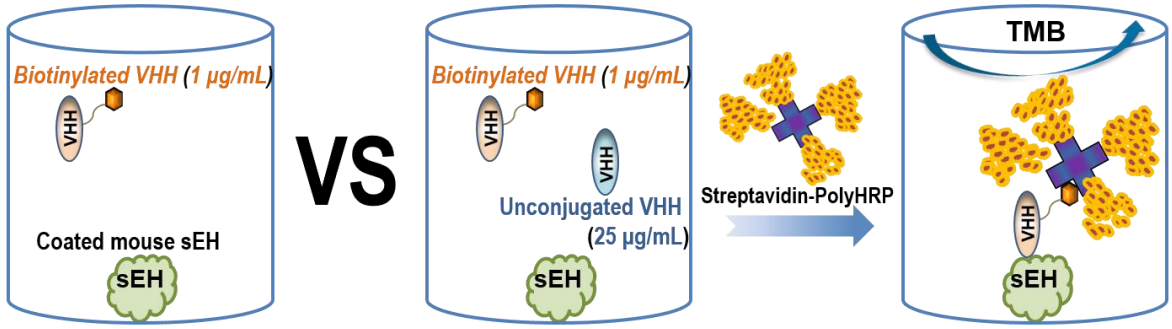


Figure S3. **The selectivity of nine anti-mouse sEH nanobodies and the anti-mouse sEH pAb against eight kinds of tissues from sEH KO and WT mice based on indirect ELISA.** KO and WT tissues diluted 100- and 1000-fold with PBS were coated overnight at 4 °C on high-binding Nunc Maxisorp microplates (100 μL/well). After blocking the plate with 3% skim milk (250 μL/well) for 1 h and washing, 100 μL biotinylated anti-mouse sEH antibody (nanobody, 1 μg/mL; pAb, 0.25 μg/mL) was added to each well. After 1 h immunoreaction and washing, streptavidin-PolyHRP40 conjugate (25 ng/mL, 100 μL/well) was added and incubated for 30 min. After washing, color was developed for 15 min with TMB and stopped with sulfuric acid. OD was recorded at 450 nm. KO brain sample was not added to the wells circled in purple because of its exhaustion. The no color of row G of the first plate in the photo was due to no addition of TMB by accident. The later addition of TMB showed similar color pattern to the row G of other plates.

(a) Schematic representation of the competition assays based on indirect ELISA



(b) Percentage of inhibition (PI) values obtained from competition assays

Unconjugated VHH (25 μg/mL)	*Biotinylated VHH (1 μg/mL)*								
	bio3A2	*bio3C1*	*bio3C2*	*bio3C5*	*bio3C9*	*bio3C15*	*bio4C3*	*bio4C7*	*bio4C14*
3A2	77%	70%	73%	74%	60%	58%	76%	62%	69%
3C1	64%	76%	59%	68%	72%	69%	77%	74%	74%
3C2	61%	62%	59%	64%	52%	45%	73%	50%	59%
3C5	71%	61%	60%	61%	47%	46%	70%	48%	53%
3C9	60%	79%	62%	65%	71%	65%	76%	72%	71%
3C15	61%	75%	62%	62%	68%	62%	74%	74%	71%
4C3	63%	61%	60%	58%	56%	51%	73%	54%	60%
4C7	65%	76%	63%	64%	70%	67%	78%	72%	73%

Figure S4. **(a) Schematic epitope mapping of the competition assays based on indirect ELISA.** Mouse sEH (179 ng/mL) in PBS generating sufficient OD signals for the nine biotinylated nanobodies alone was coated overnight at 4 °C on Nunc microplate. After washing, 3% skim milk/PBS was used to block the plate for 1 h and subsequently washed. Then, unconjugated nanobody (25 μg/mL) or PBS was first added to each well (50 μL/well) and followed immediately by adding biotinylated nanobody (1 μg/mL, 50 μL/well). The immunoreaction was allowed to proceed for 1 h. After washing, streptavidin-PolyHRP40 conjugate was added to the plate (25 ng/mL, 100 μL/well) and incubated for 30 min. After washing, TMB was added and incubated for 15 min. OD was then recorded at 450 nm after stopping the color development; **(b) Percentage of inhibition (PI) values derived from competition assays**. PI = [1-(Experimental – background) / (Control – background)]×100%. Background is the OD of control wells but without sEH coated. Inhibition was considered significant when the PI was over 50%;

Epitope mapping indicates that the nine nanobodies recognize the same epitope of mouse sEH. These nanobodies may have different physicochemical properties due to their difference in sequences of amino acids. Mouse sEH is a dimer. Thus, there are 81 possible combinations of

double nanobody based ELISA. It is impractical to test each combination. From Figure 2b, we inferred that the affinities of these nanobodies are in the same order of magnitude. The clones 3A2, 3C1, and 4C3 have highest yield in expression and were considered for labeling HRP. Two of them (3A2 and 4C3) were used for conjugating HRP due to the limitation of simultaneous handling capacity. As we mentioned in the manuscript, 3A2 lost its functionality after conjugation to HRP. Thus, 4C3-HRP was used throughout the work. In the pilot experiment, we found biotinylated 3C1, 3C2, 3C9, 3C15, 4C3, 4C7, and 4C14 worked well as capture antibodies in format D with comparable sensitivities achieved, whereas 3A2 worked poorly and 3C5 worked moderately (data not shown). Finally, 3C1 was chosen for further validation considering its high yield and excellent performance.

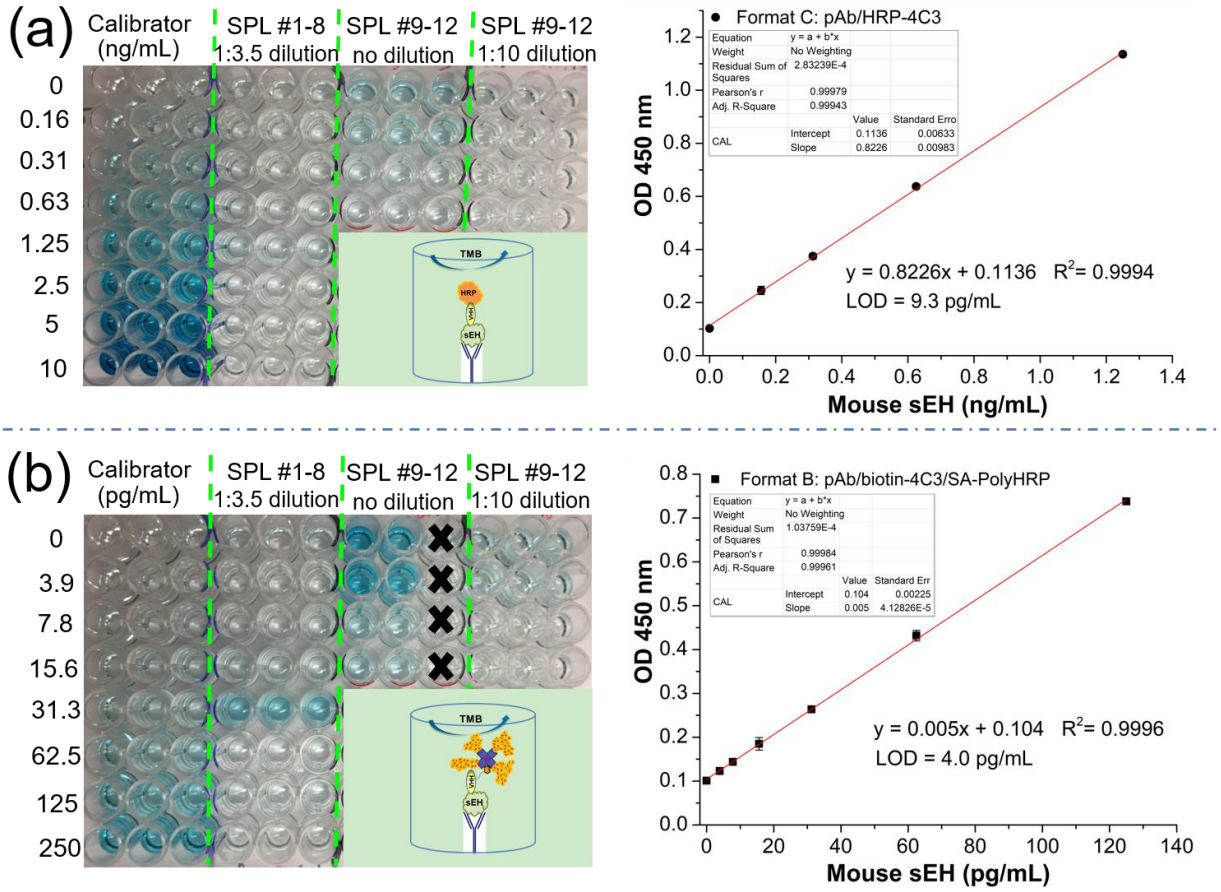


Figure S5. Photos before color stopping and the corresponding calibration curves after color stopping for the analysis of 12 samples of cancer cells using ELISA. (a) format C, and (b) format B. For better sensitivity, the incubation time for antigen and detection antibody in format C and B was extended from 1 h to 2 h and 90 min, respectively; the working concentration of HRP-4C3 was increased from 1:4000 to 1:2000. Samples were added to the wells from top to bottom in sequence of the sample number. The samples (#9-12) at no dilution were added in duplicate in format C due to their exhaustion.

Table S1. Sensitivity comparison of format B in linear range using different affinity-purified pAb (2.5 μg/mL) as capture antibody: rabbit anti-human sEH pAb and anti-mouse sEH pAb.

detection nanobody	anti-human sEH pAb		anti-mouse sEH pAb		sensitivity ratio
	sensitivity (OD•mL/ng)	R^2	sensitivity (OD•mL/ng)	R^2	latter : former
biotin-3A2	0.0569	0.9932	4.7	0.9998	82.6
biotin-3C1	0.0374	0.9967	4.99	0.9936	133.3
biotin-3C2	0.0245	0.9980	2.94	0.9974	120.0
biotin-3C5	0.0202	0.9880	2.4	0.9969	118.7
biotin-3C9	0.0467	0.9881	6.93	0.9970	148.5
biotin-3C15	0.0205	0.9964	2.59	0.9980	126.5
biotin-4C3	0.0330	0.9973	3.67	0.9940	111.1
biotin-4C7	0.0233	0.9961	3.08	0.9978	132.4
biotin-4C14	0.0410	0.9989	5.27	0.9989	128.6
biotin-A1	0.0162	0.9964	1.35	0.9949	83.3

Table S2. Summary of sEH expression in eight strains of mouse cancer cells obtained with ELISA (format C) and activity level with radioactive assay (n = 3).

sample No.	cell name	supersensitive ELISA (format B)					radioactive assay	
		sEH in diluted sample (ng/mL)	CV	+/-[a]	working dilution	sEH in neat sample (pg/mL)	activity	CV
1	Lewis lung cancer	-0.006	5%	-	3.5	-0.021	3.8	8%
2	T241 fibrosarcoma	-0.010	7%	-	3.5	-0.036	2.3	15 %
3	B16F10 melanoma	0.002	5%	-	3.5	0.006	0.8	7%
4	4T1 breast adenocarcinoma	0.014	4%	+	3.5	0.048	2.9	9%
5	E0771 breast adenocarcinoma	0.117	4%	+	3.5	0.410	12.0	5%
6	MC38 colon adenocarcinima	0.013	16 %	+	3.5	0.044	1.0	15 %
7	CT26 colon adenocarcinoma	0.006	4%	-	3.5	0.021	26.8	4%
8	ID8 ovarian adenocarcinoma	-0.003	4%	-	3.5	-0.011	3.1	8%
9	E0771 breast adenocarcinoma	0.340	3%	+	1	0.340	10.3	5%
10	E0771 breast adenocarcinoma	0.335	6%	+	1	0.335	8.1	4%
11	CT26 colon adenocarcinoma	0.100	10 %	+	1	0.110	26.5	3%
12	CT26 colon adenocarcinoma	0.075	5%	+	1	0.075	35.0	3%

[a]diluted samples with calculated value below LOD (9.3 pg/mL) of the ELISA were recognized as negative.

[b]activity (pmol•min^{-1}•mL^{-1}) was measured using *t*-DPPO as substrate.