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A mix-and-read nanobody-based sandwich homogeneous split-luciferase assay for the rapid detection of human soluble epoxide hydrolase

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Abstract

The soluble epoxide hydrolase (sEH) is possibly both a marker and target of numerous diseases. Herein, we describe a homogenous mix-and-read assay for the detection of the human sEH, based on using split-luciferase detection coupled with anti-sEH nanobodies. Selective anti-sEH nanobodies were individually fused with NanoLuc Binary Technology (NanoBiT) which consists of a large and small portion of NanoLuc (LgBiT and SmBiT respectively). Different orientations of the LgBiT and SmBiT nanobody fusions were expressed and investigated for their ability to reform the active NanoLuc in the presence of the sEH. After optimization, the linear range of the assay could reach 3 orders of magnitude with a limit of detection (LOD) of 1.4 ng/mL. The assay has a high sensitivity to human sEH and reached a similar detection limit to our previously reported conventional nanobody-based ELISA. The procedure of the assay was faster (30 minutes total), and easy to operate, providing a more flexible and simple way to monitor human sEH levels in biological samples. In general, the immunoassay proposed here offers a more efficient detection and quantification approach that can be easily adapted to numerous macromolecules.

Graphical Abstract

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Conflict of interest

The authors declare that there is no conflict of interest to publish this paper.
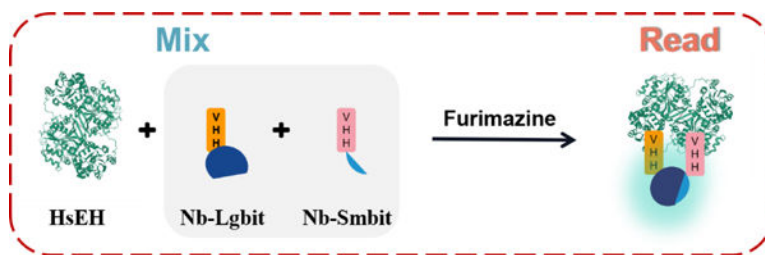
Introduction

In mammals, human soluble epoxide hydrolase (sEH) protein is a homodimer consisting of two 62.5 kDa, antiparallel monomers, encoded by the EPHX2 gene[1]. The protein has a phosphatase activity at the N-terminus coupled by a proline-rich bridge to the C-terminus epoxide hydrolase activity[2]. The sEH is widely expressed in multiple human tissues and involved in the regulation of many physiological processes due to its rapid hydrolysis of epoxy-fatty acids (EpFA)[3]. sEH and its EpFA substrates are associated with diseases such as chronic central nervous system disorders, pain, cardiovascular disease, cancer, and diabetes[2]. The sEH is present in high concentrations in various human tissues, including the kidneys, liver, intestine, adrenal gland, heart, and pancreas, with concentrations ranging from 12 to 440 nM (720–26,400 ng/mL) in tissue extracts[4]. In healthy woman, sEH expression in white blood cells was measured at 76 ± 44 ng/mL (n = 96) during fasting.[5] The sEH acts both as a biomarker and as a pharmaceutical target for potential drug development and disease mechanistic studies[4, 6]. Therefore, the rapid and quantitative measurement of sEH is important for the investigation of disease processes, to give guidance for drug development, and may become a valuable biomarker of health. In a previous study, nanobodies specific to human sEH were developed and applied in ELISAs with different types of capture-detect antibodies and signaling strategies[7–10].

Recently, nanoluciferase (NanoLuc) has been attracting the rising attention of researchers as a reporter enzyme due to its smaller size but stronger and superior luminescence compared with traditional firefly (Fluc) or Renilla (Rluc)[11–12]. Lately, NanoLuc was split into two fragments for the investigation of protein interaction. A typical binary split luciferase technology brings two subunits, the 17.6 kDa (159 amino acids) large portion of the nanoluciferase (LgBiT) and the complimentary 11 amino acids small portion of the nanoluciferase (SmBiT), into close proximity to form a functional NanoLuc and generate a bright and luminescent signal[13]. The favorable characteristics of the split luciferase offer a direct and intuitive way to investigate protein-protein interactions and it is widely applied in biosensing in vivo and in vitro[14–16]. One of the applications of the split luciferase assay is a homogeneous bioluminescence detection based on the interaction between antibody and antigen. Generally, for small molecules, antibodies and hapten are conjugated with either LgBiT or SmBiT, respectively, to develop competitive homogeneous immunoassays[17–18].

There were also several studies applying split luciferase in macromolecules targeting in COVID-19 antibodies and antigen analysis by using chemical coupling between protein and NanoBiT[19–21]. Nevertheless, nanobody fused NanoBiT-based sandwich split luciferase assays are rare in the literature for the detection of macromolecules and to date not heavily used in drug development or for clinical practice. Nanobodies are a type of recombinant protein derived from antibodies that are small and intrinsically monomeric and have been shown to have unique properties compared to conventional antibodies. These include increased stability in extreme temperature and solvent conditions, as well as efficient tissue penetration and relatively low immunogenicity[22–23].

Taking the characteristic of split luciferase and the easy-to-edit function of the recombinant nanobodies, toward obtaining a simplified and rapid method for the detection and quantification of human sEH, a novel sandwich homogenous assay based on nanobody fused NanoBiT was investigated and showed in Figure 1. Toward this goal, herein, we are describing our strategy and results to (I) find a pair of nanobodies that recognized two separate epitopes of sEH; (II) design vectors and fuse nanobodies with the separate peptides of NanoBiT; (III) select the combination that responds best to the target protein; (IV) optimize the assays and establish the quantification range, and (V) validate the assay performances.

2. Experimental Section

2.1 Materials

Vector encoding anti-human sEH nanobodies A1 and A9 were prepared as described in the previous work[9]. The oligo primers for vectors construction were synthesized by Invitrogen, Thermo Fisher Scientific (IL, USA). Restriction enzymes *EcoRI* and *HindIII*, T4 DNA ligase were obtained from New England Biolabs (Beverly, MA). Vector pBiT1.1-C [TK-LgBiT], Nano-Glo™ Luciferase Assay System (N1110) was purchased from Promega (Madison, WI, USA). 96 well half-areas white microplate was obtained from Corning Inc. (Corning, NY, USA).

The luminescence signal was read using both the Tecan Infinite M200 Pro plate reader (Tecan, Switzerland) and the Molecular Devices spectraMax M2 microplate readers (San Jose,CA, USA).

2.2 Vectors construction and protein expression

Previously, nanobodies highly selective for the human sEH named A1 and A9 were employed to establish a double sandwich ELISA for sEH[9]. Considering that these nanobodies were verified to recognize different epitopes of the human sEH, the split nanoluciferase including LgBiT and SmBiT were fused with A1 and A9, respectively. As depicted in Figure 2, both LgBiT and SmBiT were designed to be integrated in the C-terminal and N-terminal of each nanobody to study orientation influences on the assay. Eight vectors contained nanobody and split nanoluciferase named SN1, SN9, NS1, NS9, LN1, LN9, NL1, NL9 which correspond to different orientations of the nanobodies and split nanoluciferase. First, the target genes with kinds of sequences were constructed by

PCR with the primers (shown in Table S1). Second, both pET22b and the target gene were digested by *EcoRI* and *HindIII*, followed by ligation with T4 ligase at 4°C overnight. The ligation products were then transferred to *E. coli* TOP10F'. The positive clones were selected for further DNA sequencing.

To express the protein, the recombinant vectors were transferred to *E. coli* BL21(DE3). Briefly, a single clone was picked and cultured in the 5 mL Super Broth (SB) medium with 50 μg/mL carbenicillin under 250 rpm shaking overnight at 37°C. Next, 1mL overnight was added to 100mL SB medium with carbenicillin and then shaken at 37°C, 250 rpm. 1M IPTG (50 μL) was subsequently added for induction until the OD_{600} reached 0.8–1.0. After overnight incubation, the culture was centrifuged at 4°C, 5000 rpm for 20 min. The bacterial sediment was lysed by B-PER solution at RT for 30 min. After centrifugation, the supernatant was incubated with Ni-NTA resin according to the manufacturer's protocol. All the purified proteins were tested by SDS-PAGE.

2.3   Homogeneous split-luciferase assay

The nanobody-NanoBiT sandwich homogeneous assay was based on the two interactions including nanobody-analyte-nanobody and LgBiT-SmBiT association. After expressing nanobody-NanoBiT protein, the homogeneous assay was developed to determine the human sEH. Briefly, 5 μL/well of each component (Nanobody-LgBiT and Nanobody-SmBiT) was added to the 96-well half-area white microplate. 10μL/well of the samples were then added and incubated at RT for 20 min under a 600 rpm shaker. Then, 10 μL/well of the Nano-Glo luciferase substrate solution was introduced to spark the luminescent signal. Luminescence signals were recorded by a microplate reader.

Sixteen kinds of combinations for the Nanobody-NanoBiT biosensor were conducted as stated. Solutions of different SmBiT and LgBiT fused proteins (0.5 mg/mL)were incubated with 1 μg/mL of human recombinant and affinity purified sEH [24] or PBS buffer. The changes in the signal were recorded and the optimal combination was chosen for further study. The optimizations including each component concentration, incubation time, and incubation type were tested.

2.4   Selectivity

The selectivity of the nanobody-NanoBiT sandwich homogeneous assay was evaluated by testing the cross-reactivity with other epoxide hydrolases including denatured human sEH, human mEH, rat sEH, mouse sEH, and pig sEH. 5 μL of SN9 and NL9 were equally mixed and added to the plate. Then, 10 μL of 1 μg/mL human sEH, denatured human sEH, human mEH, rat sEH, mouse sEH, pig sEH in PBS were added and incubated at RT for 20 min. Subsequently, 10 μL of the Nano-Glo luciferase substrate solution was added to develop the luminescence signals.

2.5   Matrix effects

Brain tissue extraction from sEH global knock out mice[25] was applied to the analysis matrix effect for the proposed method. Different dilutions of the brain tissue extraction were spiked

with a series of human sEH (500, 250, 125, 62.5, 31.25, 15.60 ng/mL) and tested with the biosensor.

2.6 Analysis of human tissue extraction for human sEH

In order to verify the practicality of the assay, five samples from S9 fractions of pooled (4–50 individuals) human tissues from Xenotech LLC (Kansas City, KS, USA) were tested. Different dilutions of each sample were set up to reach the linear range of the generated standard curve. Their sEH levels were determined using the assay described above. As a comparison, enzyme activity[4] and ELISAs[8–9] were tested to validate the method and detailed in supporting information.

3. Results and discussion

3.1 Vector design and protein expression

The nanoluciferase reporter gene has been widely used in developing immunoassays in a conventional sandwich ELISA for proteins determination [26–28]. The nanoluciferase can split into non-functional large binary subunit (LgBiT) and small binary subunit (SmBiT) and reassemble and recover the enzymatic activity when approaching. Two nanobodies that had been previously used to successfully establish a double nanobody sandwich ELISA were chosen as the model to generate the homogeneous split-luciferase assay. In this case, the split nanoluciferase were fused with two different recombinant nanobodies targeting different epitopes on the human sEH. LgBiT and SmBiT were inserted into the C and N terminus of each nanobody, respectively. Considering the steric hindrance, linker $(G_4S)_3$ was inserted between the nanobody and NanoBiT. These vectors were transferred and expressed in *E. coli* BL21(DE3). The SDS-PAGE analysis of eight fusion protein preparations are shown in Figure S1a. For nanobody-LgBiT fusion, the band at approximately 35 kDa was observed in the Ni-NTA purified product while a band at approximately 17kDa in nanobody-SmBiT fusion protein, corresponded to the predicted molecular weights. The existence of the fusion protein after purification was also confirmed by Western Blot assay (Figure S1b) using an HRP-anti-His tag antibody. The yields of the fusion protein expressions are summarized in Table S2. It is notable that all fusion proteins derived from VHHA1 had higher yields than those from VHH A9 which was related to the expression level of their parental nanobodies.

3.2 Nanobody-based sandwich homogeneous split-luciferase assay

The orientation of NanoBiT fragments may interfere with nanobody-analyte recognizing function or result in unpredictable protein folding. Hence, multiple combinations were investigated including the same nanobody with different NanoBiT in human sEH determination. As depicted in Figure 3, the performance of the different formats varies greatly. Most forms of nanobody-SmBiT protein fusion did not show any signals in the presence and absence of human sEH, which might be caused by the interference with the His tag at the C terminus or improper protein folding. With the SmBiT-nanobody fusion proteins, all combinations showed luminescence signals regardless if the human sEH was present or not, indicating background association between the LgBiT and SmBiT fusion nanobodies. Among them, significant changes were observed in the pair of SmBiT-

VHHA9 (SN9) and VHHA9-LgBiT (NL9). Since sEH is a homodimer and each monomer contained globular N- and C-terminal domains linked by a proline-rich bridge[1], it could explain why a single strain nanobody worked in the sandwich homogeneous split-luciferase assay. The luminescence spectra for the pair of SN9 and NL9 were tested and is shown in Figure 4. Single SN9 and NL9 in the presence and absence of human sEH did not catalyze furimazine activation while the mixture including SN9 and NL9 with human sEH oxidized the substrate furimazine and resulted in a large increase in luminescence at 450 nm. Negligible background can be observed in the present of SN9 and NL9 without sEH.

To test the binding properties of the recombinant proteins, a sandwich ELISA was performed. Anti-human sEH rabbit serum was used to capture the sEH protein on the well surface. Both the fusion proteins and their parental nanobodies were used as detection antibodies. As depicted in Figure S2, all formats of the fusion proteins retained their binding function, except for NS1 and NS9. This result was consistent with the combination test, which showed that NS1 and NS9 did not exhibit any luminescence signal when combined in different formats. The loss of function observed in NS1 and NS9 could be attributed to the poor folding of the proteins in the Nanobody-SmBiT form. The combination of NS1 and NS9 did not generate any luminescence, as consistent with the results of combination tests.

To evaluate the feasibility of the proposed biosensor, optimizations including incubation format, incubation time, and optimized volume and concentration of each component were tested. Three types of incubation were tested to find an optimal binding approach. NL9 and SN9 were mixed sequentially with the human sEH: the first one was incubated for 10 min in the presence of sEH, before the second component was added and incubated for an additional 10 min. The result (Figure S3) revealed that the sequential addition did not increase the luminescence compared to an addition of SN9 and NL9 with a total incubation of 20 min before measurement. Incubation time was also tested when the mixture (SN9+NL9+samples) was incubated for 10, 20, 30, 40, 50 min. The result (Figure S4) showed that as time goes on, not only the luminescence value of the positive sample increased but the background also. Therefore, 20 min was chosen as the optimal incubation time as the Signal/Noise (S/N) ratio reached 8.83 (the highest ratio). The S/N was calculated using the following formula: S/N = luminous intensity (with HsEH) / luminous intensity (without HsEH). Figure S5 illustrates the result of the immunoreaction volume. When the same amount of nanobody-NanoBiT fusion protein was used, a weaker signal was observed with increasing volume. This outcome may be due to the increasing volume of the buffer, which can cause signal decline in the system[27]. To obtain the strongest signal, 10 μL of immune-reagents, 10 μL of substrate solution, and 10 μL of samples were selected for further analysis. The effect of the reaction time on the assay performance was also tested (Figure S6.) Within 60 minutes, the immunoreaction maintained a good linear range and limit of detection, indicating the temporal stability of the proposed method. To have a good operational efficiency, a detection time of 10 min was chosen.

In addition, the concentration of NL9 and SN9 were investigated. The result is shown in Figure 5. NL9 in different concentrations (1, 0.5, 0.1, 0.05 mg/mL) was mixed with 0.5 mg/mL SN9, then a series of human sEH concentrations were added to establish the assay. Similarly, SN9 in different concentration (0.5, 0.2, 0.1, 0.05 mg/mL) were mixed

with 0.1 mg/mL NL9 and tested for the calibration curve. The signal increased with the higher concentrations of NL9 or SN9 until a balance was reached resulting in a near maximum luminescence signal. As expected, the background also increased along with the increase of NL9 and SN9. LOD was calculated based on 3×standard deviation of the three blank measurements/slope. The assay was optimized based on minimizing the usage of the reagents, the background, and detection limit. Therefore, 0.1 mg/mL for NL9 and SN9 was chosen as a near optimal concentration for the assay.

Based on the optimal conditions for the homogeneous split-luciferase assay, a calibration curve for homogeneous nanobody sandwich assay was established. A series of human sEH samples (2000, 1000, 500, 250, 125, 62.50, 31.20, 15.60, 7.81, 3.91 ng/mL) were added to establish the standard curve. The linear equation was $Y = 890713X - 13903$ ($R^2=0.9973$) from 0 to 2 μg/mL is depicted in Figure 6. The LOD was calculated as 1.4 ng/mL ($\approx$ 11pM). The relatively wide linear range provided a more flexible way to detect the human sEH. Even with low concentrations of human sEH, the proposed assay maintained a good linear calibration.

The comparison of immunoassays for the detection of human sEH are summarized in Table S3. It only takes 30 minutes to complete the split-luciferase assay in this format. As a homogeneous assay, the proposed split-luciferase method had a great advantage in test duration compared to the conventional ELISA, which can substantially improve detection efficiency and shorten the detection duration. Removing steps improves the reproducibility of any analytical method. In this study, antibody labeling was constructed through genetic fusion and expressed in *E. coli,* which provided relatively inexpensive assays and good batch uniformity. Table S4 provided a rough estimate of the cost comparison between ELISA and split-luciferase assays. While the proposed method has a higher reagent cost compared to the conventional ELISA, it is less labor-intensive and more convenient, thus, it is more cost effective overall.

3.3 Selectivity

To verify the selectivity of the split-luciferase assay, other epoxide hydrolases including denatured human sEH, human mEH, rat sEH, mouse sEH, and pig sEH were tested. As shown in Figure 7, negligible signals were found in other epoxide hydrolases, which revealed the nanobody-NanoBiT probes were highly specific to the human sEH, corresponding to the previously results that A1 and A9 did not recognize other epoxide hydrolases based on the double nanobody sandwich ELISA. The high selectivity of course results from two independent nanobodies having to bind to the same sEH protein to yield a positive signal.

3.4 Sample analysis and validation

The feasibility of the split-luciferase assay was further tested by spike and recovery tests with real samples. A series of concentrations of human sEH (500, 250, 125, 62.50, 31.30, 15.60 ng/mL) were spiked into different dilutions (1:10, 1:50, 1:100 dilutions) of brain tissue sample from sEH knockout mice. As shown in Table 1, the rates of recovery ranged from

82% to 135% were tested in the proposed method. These recovery rates were generally high, indicating that the matrix had limited and acceptable impact on the homogeneous assay.

The proposed method was applied to the detection of human sEH in S9 fractions of pooled (4–50 persons) human tissue samples. Several dilutions were set to measure the levels of human sEH in different tissues samples in order to fit the linear range of the assays. The results (Table 2) were thoroughly compared with the previous assays including enzyme activity, PolyHRP ELISA, and double nanobody sandwich ELISA (dsNb ELISA). The correlations between other methods and split-luciferase assay reflected highly consistency with $r^2 > 0.99$ (Figure S7). The results support the proposed split-luciferase assay were reliable and can be used for the detection of human sEH in actual samples.

Conclusion

In this study, a rapid, simple, and sensitive homogenous assay for the determination of human sEH concentration was developed and optimized. Using the SmBiT-VHHA9 and VHHA9-LgBiT pair, an assay with a three order of magnitude linear range (0.002 to 2 μg/mL) was obtained, with a limit of detection of 1.4 ng/mL (defined as 3×standard deviation of blank measurements). The assays demonstrated good selectivity and spike-and-recovery results. The feasibility of the method was validated by testing human tissue samples compared with enzyme activity and ELISAs. The proposed one-step assay gives a template to establish a sandwich homogenous split luciferase assay for macromolecules. Hopefully this test will be of value in the study of the sEH in regulatory biology and clinical chemistry. More broadly it illustrates the many advantages of nanobodies in providing sensitive, specific and highly adaptable assays with an unlimited supply of nanobodies for clinically relevant assays. From a technological standpoint, exploring modifications or improvements to luminescence substrates could lead to a reduction in the cost of the kit and broaden the range of applications for the assay. As a proof of concept, the proposed strategy provided a cost-effective and easy-to-operate approach to accomplish the rapid quantitative immunoassay of macromolecules and could be easily adapted to many protein targets.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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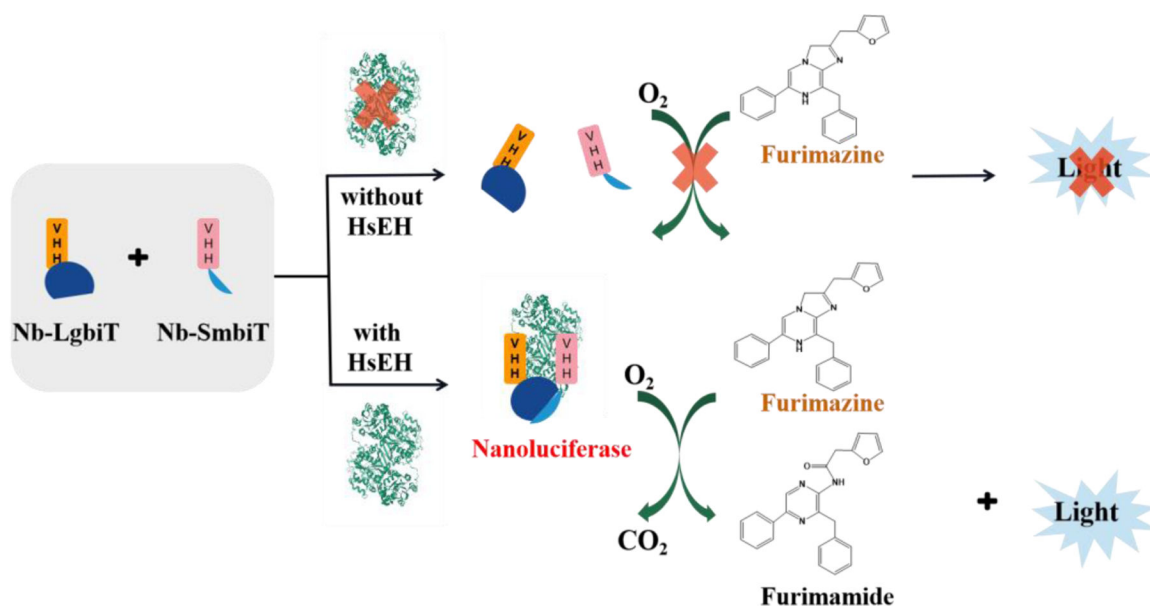
Figure 1.
Scheme of the nanobody-based sandwich homogeneous split-luciferase assay for the rapid detection of human soluble epoxide hydrolase
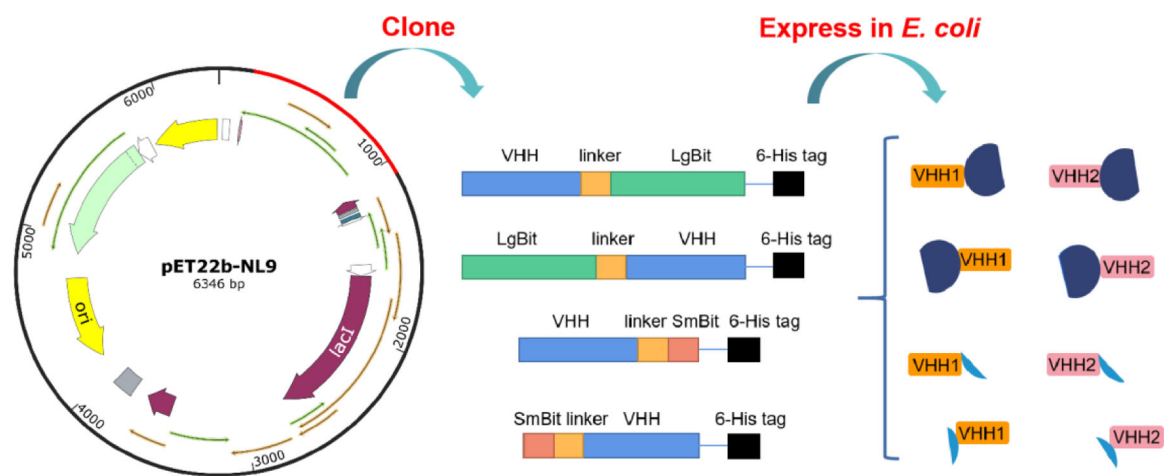
Figure 2.
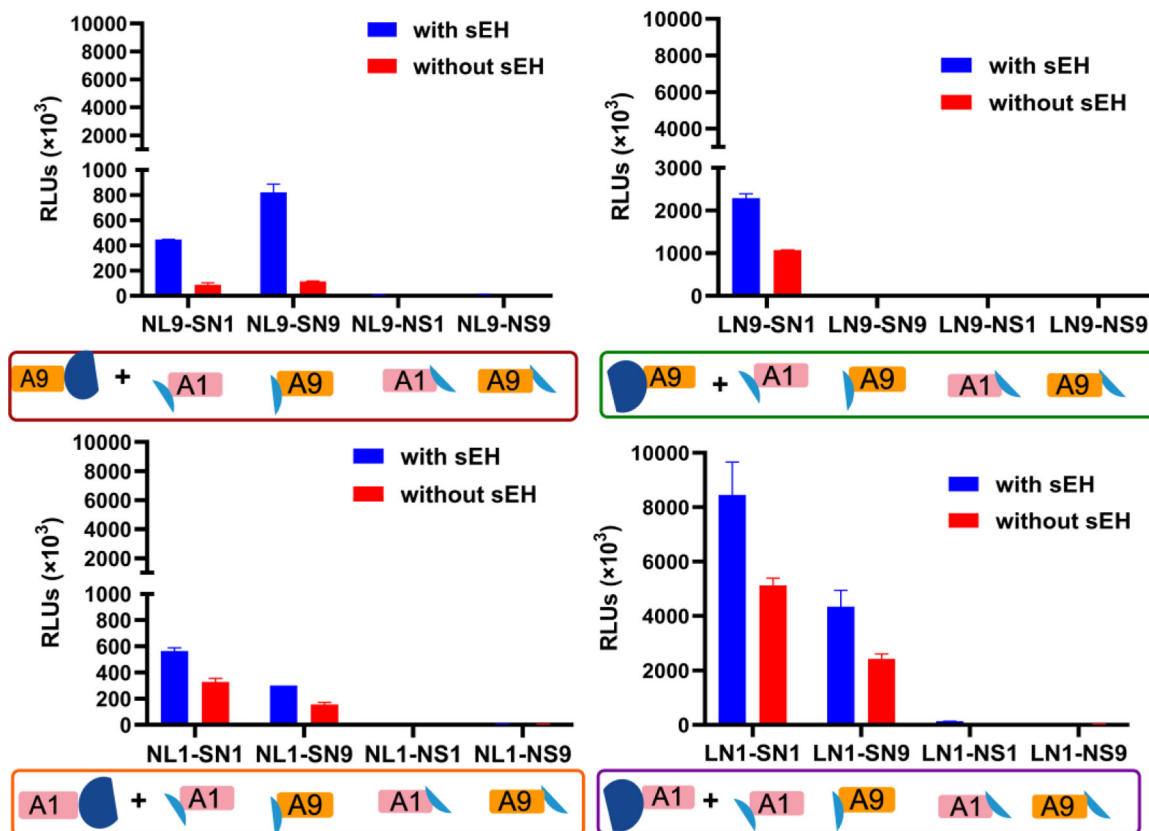Schematic plot of vector construction for the nanobody and NanoBiT fusion protein based on pET22b.

Figure 3.
The performance of different orientations of nanobody-NanoBiT combinations in homogeneous sandwich assay with 1 μg/mL human sEH or PBS. Error bars indicate the standard deviations (SDs) of triplicate tests. The concentrations of all fusion proteins were 0.5 mg/mL. NL1, NL9, LN1, LN9, NS1, NS9, SN1, SN9 represented A1-LgBiT, A9-LgBiT, LgBiT-A1, LgBiT-A9, A1-SmBiT, A9-SmBiT, SmBiT-A1, SmBiT-A9, respectively. Inserts are the schematic diagrams of different combinations.
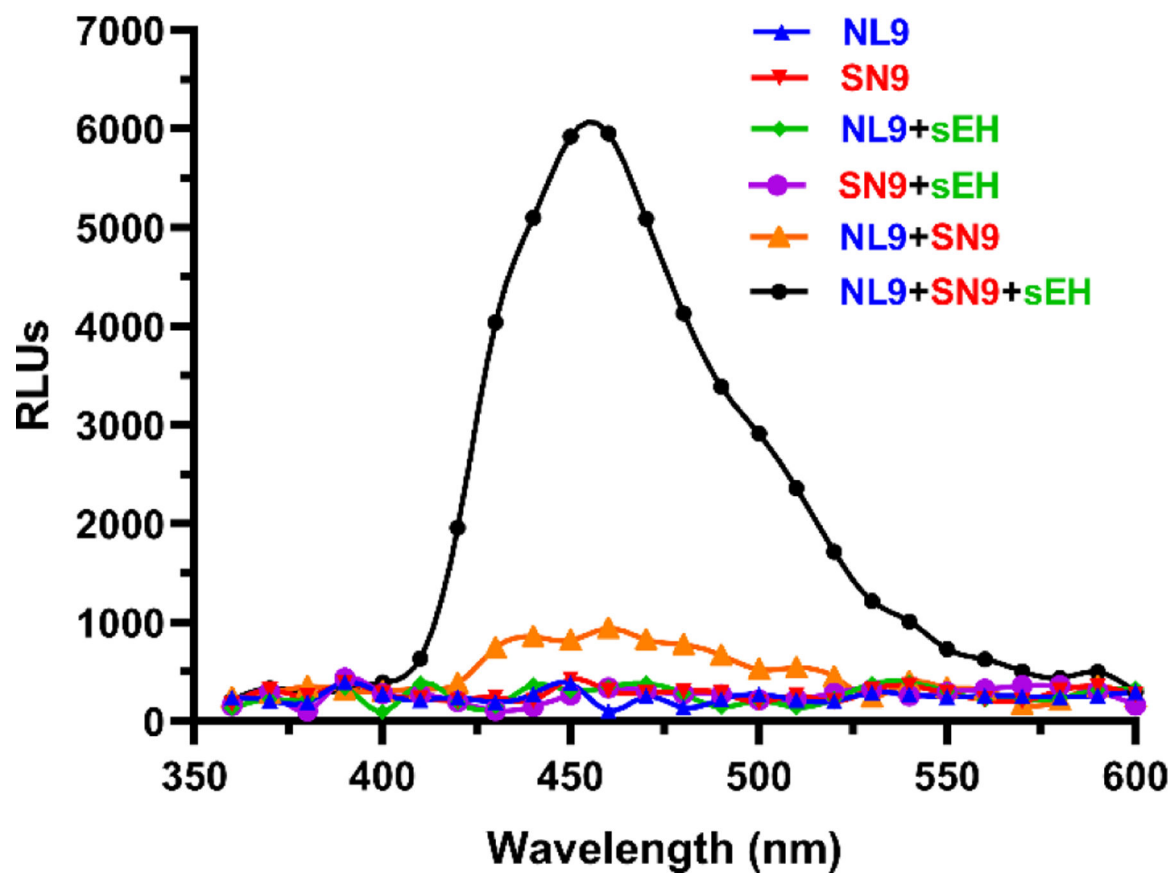
Figure 4.
The luminescence spectra of NL9-SN9 pair in the presence and absence of 1 μg/mL human sEH by adding the substrate furimazine.
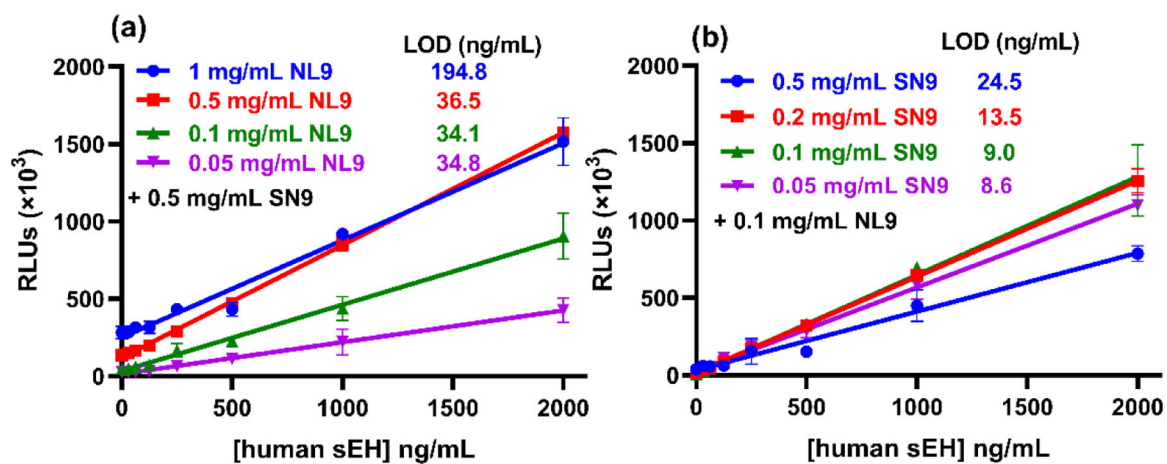
Figure 5.
Optimization of the concentration for NL9 and SN9 for homogeneous nanobody sandwich assay. A series concentration of NL9 (a) and SN9 (b) were mixed with a series of human sEH and incubated for 20 min, then added Nano-Glo luciferase substrate solution to develop the luminescence signals. Error bars indicate the standard deviations of triplicate tests.
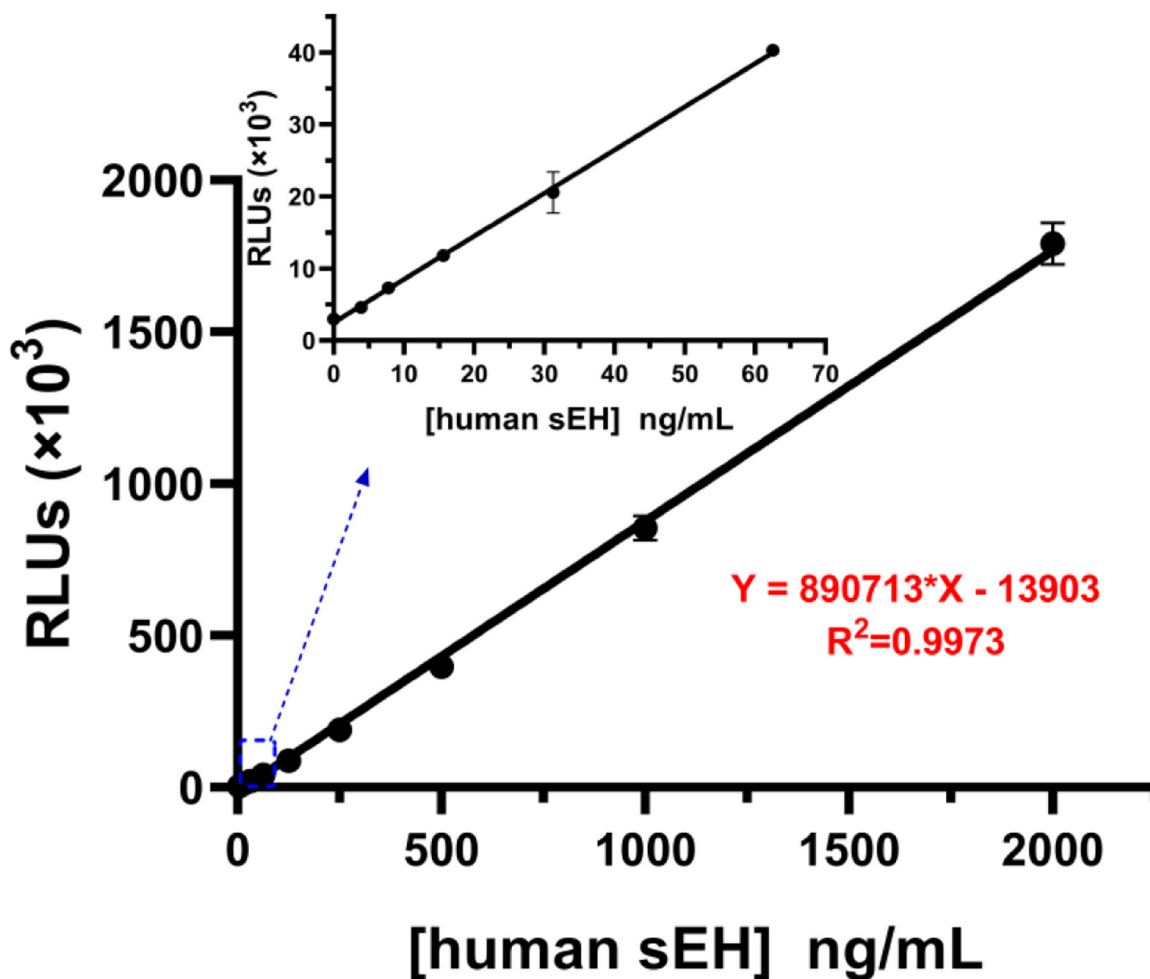
Figure 6.
Calibration curve for homogeneous nanobody sandwich assay. Insert was the expanded low concentration range of human sEH. 0.1 mg/mL NL9 and SN9 were mixed with 2000, 1000, 500.0, 250.0, 125.0, 62.50, 31.25, 15.63, 7.812, 3.906 ng/mL of human sEH and incubated for 20 min, then added Nano-Glo luciferase substrate solution to develop the luminescence signals. Error bars indicate the standard deviations of triplicate tests.

Figure 7.
Selectivity test for the homogeneous nanobody sandwich assay with human sEH and others epoxide hydrolysis. 0.1 mg/mL NL9 and SN9 were mixed with 1 μg/mL of human sEH, denatured human sEH, mouse sEH, rat sEH, pig sEH and human mEH. After incubating for 20 min, the mixtures were added Nano-Glo luciferase substrate solution to develop the luminescence signals. Error bars indicate the standard deviations of triplicate tests.
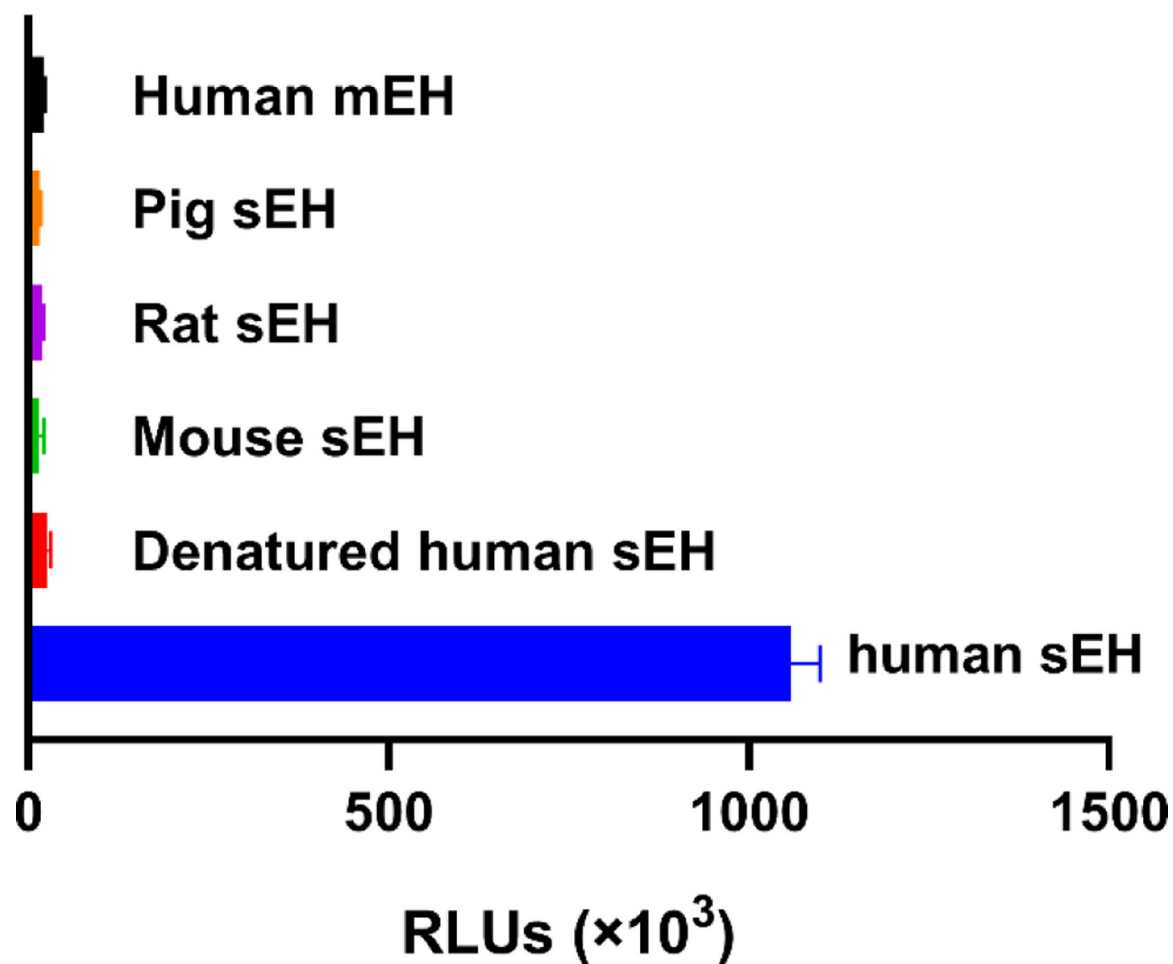
Table 1.

Split-luciferase assay recovery for pure recombinant human sEH spiked into a brain tissue homogenate sample from sEH knockout mice ($n = 3$).

Spiked (ng/mL)	1:10 dilution		1:50 dilution		1:100 dilution	
	detected (ng/mL)	recovery	detected (ng/mL)	recovery	detected (ng/mL)	recovery
15.6	17.6± 0.8	114%	18.8 ± 2.1	120%	15.2 ± 1.2	97%
31.3	39.0± 1.7	127%	39.5 ± 4.3	126%	23.7 ± 3.7	82%
62.5	73.8 ± 6.4	118%	84.0 ± 8.7	134%	80.9 ± 6.5	130%
125	159 ± 3.9	129%	162 ± 5.4	129%	169 ± 2.7	135%
250	316 ± 12	126%	317 ± 15	127%	253 ± 22	101%
500	599 ± 28	122%	620 ± 7.9	124%	660 ± 16	132%

Table 2.

Comparison of split-luciferase assay and three other methods for the detection of human sEH in the S9 fraction of pooled (4–50 persons) human tissues samples ($n = 3$).

Human tissues	sEH concentration (nM) estimated[a]			
	Enzyme activity	PolyHRP ELISA	dsNb ELISA	This work[b]
Liver	86	152 ± 11 (7%)	114 ± 7 (6%)	122 ± 2 (1%)
Kidney	7.5	9.0 ± 0.5 (5%)	7.0 ± 0.7 (10%)	11 ± 0.3 (3%)
Lung (nonsmoker)	0.5	0.4 ± 0.02 (6%)	0.4 ± 0.01 (2%)	0.6± 0.01 (1%)
Lung (smoker)	0.4	0.4 ± 0.01 (2%)	0.3 ± 0.02 (5%)	0.7± 0.01 (2%)
Small intestine	14	17 ± 0.1 (5%)	14± 0.8 (5%)	17 ± 0.8 (5%)

[a] Results are average ± SD (CV, n=3); 1 nM sEH corresponds to 62.5 ng/mL sEH.

[b] Dilution of 100-, 10-, 2-, 2-, and 10- fold was found to be optimal in the detection of tissues of liver, kidney, lung (nonsmoker), lung (smoker), intestine, respectively.