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Genetically engineered secretory horseradish peroxidase is a sensitive, stable, and affordable non-lytic reporter gene system for real-time promoter activity management

Mu-Shen Chang[1†], Chia-Yi Lee[1†], Yu-Yen Chang[2], Pin-Jie Li[3], Hsin-Yu Wu[2], En-Shuo Liu[2], Hsin-Kai Huang[4] and Wen-Wei Lin[1,2,5,6,7,8*]

Abstract

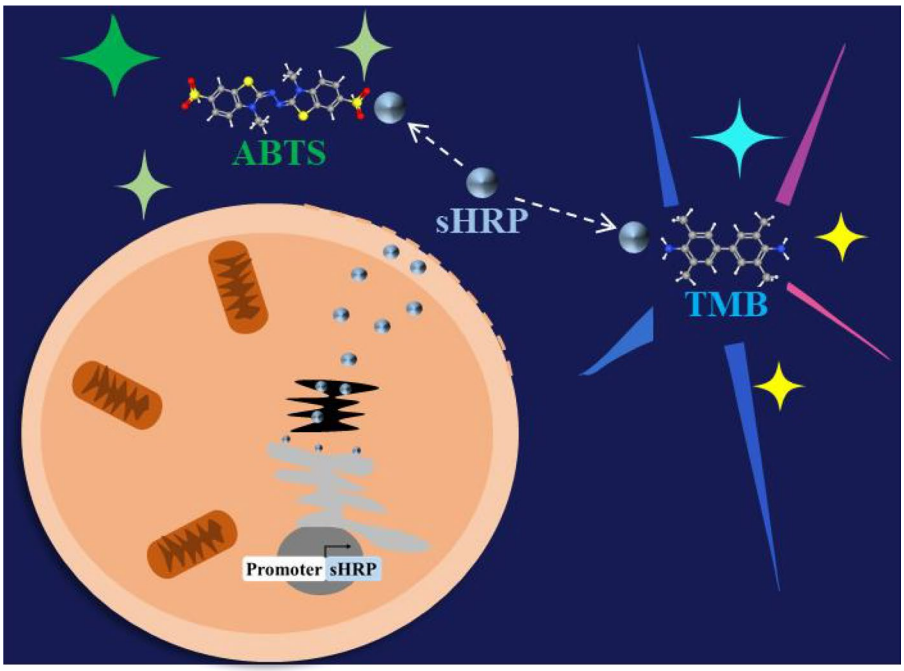
A light-producing secretory protein that is collectible through the supernatant of a culture medium is essential in a cell-based reporter gene system and allows for real-time monitoring of upstream events of a promoter. Compared to other secretory luciferases, Cypridina luciferase (CLuc) coupled with vargulin emits the brightest signal; however, the signal half-life suffers constantly from the fast oxidation process of the substrate, resulting in a rapid signal depletion, which makes the detection signal short and unstable. In this study, we aimed to develop a new reporter gene system with a more stable signal and lower cost, whilst retaining sensitivity comparable to the CLuc reporter gene system. To this end, we genetically engineered horseradish peroxidase (HRP) to be secreted with mammalian cells. The secreted form HRP (sHRP) was then used as a proof-of-concept of real-time cell signaling management. First, we made sure that HRP retained its enzymatic function with our genetic engineering process and confirmed that it was collectable and suitable for side-by-side comparison with CLuc. sHRP showed comparable sensitivity, 7 to 80 times more signal half-life compared to CLuc, and precisely reported NF-κB-regulated promoter in response to stimulation with TNF-α. sHRP was not affected by multiple cell culturing media and was calculated to be at least 9 times cheaper than the CLuc reporter gene system. Thus, sHRP offers new insight into the reporter gene system for drug screening and intracellular signaling management and provides a precise, sustainable and affordable operating environment.

Keywords Secreted form horseradish peroxidase, Reporter gene system, Real-time monitoring, Stable signal, Low cost

[†]Mu-Shen Chang and Chia-Yi Lee shared co-first authorship.

*Correspondence:
Wen-Wei Lin
weber06272000@gmail.com
Full list of author information is available at the end of the article

Graphical Abstract



Introduction

The reporter gene assay is an essential tool for identifying and managing the dynamics of gene expression through the inspection of the final functional product, to facilitate the analysis of the complex gene networks. A reporter gene is a gene sequence that is typically placed downstream of a promoter in a genetic construct, often in-frame with the promoter. It is designed to produce a measurable protein product, that can serve as an indicator of promoter activity. The reporter gene allows researchers to monitor the regulation of gene expression by tracking the changes in its product in response to different signaling molecules or exogenous stimulation. This enables the study of gene expression dynamics, including the timing and extent of gene activation or inhibition, under various treatment conditions [1–4]. The well-known reporter gene firefly luciferase (FLuc), discovered from the *Photinus pyralis,* demonstrates a high dynamic range (D.R.) in detection, with a robust linear relationship between luminescence intensity and enzyme concentration across several orders of magnitude. This broad D.R. facilitates accurate quantification even at low signal levels, which is frequently engineered to be expressed in the cytosolic compartment of mammalian cells. The intensity of the exogenous stimulation to the cells can then be examined by the light signal emitted by assaying FLuc with $_{D}$-luciferin ($_{D}$-Luc). However, the intracellular expression of FLuc requires lysis of cells, to provide a relatively homogenous environment that speeds up the enzyme catalysis process and generates a linear signal. This additional step increases operational time and hinders real-time monitoring of live cells [5–8].

To overcome these limitations, researchers have attempted to create secreted forms of intracellular enzymes. For instance, a secreted version of FLuc was engineered; however, the extracellular expression of FLuc remains suboptimal, with less FLuc in the extracellular compartment compared to the intracellular compartment. Nevertheless, the major concern of such a design is that FLuc-$_{D}$-Luc catalysis requires ATP and Mg^{2+}, which is significantly reduced without the environment built up by intracellular metabolism [9, 10]. Later, another luciferase, Renilla luciferase (RLuc), which is not ATP and Mg^{2+} dependent, was discovered, this property allows RLuc to exert its enzymatic function outside the cell. However, RLuc has significant drawbacks, as its stability is no more than an hour [11, 12] and it loses 50% of activity within 100 s and degrades rapidly after catalysis [13]. While previous luciferases relied on genetic engineering to achieve extracellular expression, Cypridina and Gaussia luciferases (CLuc and GLuc) are relatively new luminescent enzymes found in *Cypridina noctiluca* and

Gaussia princeps. Both are natural secretory enzymes that react with vargulin (cypridina luciferin) and coelenterazine, consecutively, producing approximately tenfold more bioluminescence [14, 15]. These enzymes provide a broad D.R., allowing for the detection of a wide range of signal intensities with high sensitivity, and mitigating some of the variation seen with genetic engineered luciferases. Among them, CLuc is even brighter than GLuc, suggesting that the luminescent signal generated by CLuc may be the most sensitive among secretory luciferases, and their intensity and sensitivity have been studied [15–17]. However, there has been relatively less research regarding their stability. Furthermore, the cost of vargulin (substrate of CLuc) per unit weight can be more than 300 times that of $_{D}$-Luc (substrate of FLuc), and coelenterazine (substrate of GLuc/RLuc) may be 1000 times more expensive [18–20]. Given these high costs, we suggested that there is room for an improved approach to produce equally sensitive, reliable experimental results at an affordable price.

Our strategy was to engineer a new form of enzyme that is able to provide precise and real-time gene expression management without compromising its stability and sensitivity, while at the same time being cost-friendly. We used Horseradish peroxidase (HRP), engineered it into a secreted form of HRP (sHRP), and chose the most sensitive CLuc as our standard for comparison. We then investigated the difference in enzyme stability, sensitivity, and price of substrates, to provide a convenient tool for long-term gene expression analysis.

Materials and methods

Plasmid construction

Briefly, signal peptide (SP)-HRP (secreted HRP (sHRP)) was cloned from pLNCX-Optimized mHRP-myc-mB7 vector, as described in our previous work [21], by PCR with 5′HindIII and 3′ClaI flanking SP-HRP-myc and the transmembrane mB7 sequence was removed to obtain pLNCX-Optimized sHRP-myc. pTEAD1-sHRP-myc was generated by PCR with 5′NcoI and 3′FseI flanking sHRP-myc, removing mB7 and subcloning into 8xGTIIC-luciferase (Addgene plasmid #34,615). pAP- 1-sHRP-myc was generated by PCR with 5′NcoI and 3′FseI flanking sHRP-myc and subcloned into 3× AP- 1 in pGL3-basic (pAP- 1-Luciferase, Addgene plasmid #40,342). pNF-κB-sHRP-myc was generated by PCR with 5′HindIII and 3′EcoRI flanking sHRP-myc from pLNCX-Optimized mHRP-myc-mB7 and subcloned into pGL4.32[luc2P/NF-κB-RE/Hygro] (E8491, Promega, Madison, US). cHRP was generated by PCR with 5′HindIII and 3′SalI to remove SP and subcloned into pLNCX-Optimized-mHRP-myc-mB7. pcDNA3.1-Renilla luciferase was a kind gift from Dr. Tian-Lu Cheng (Kaohsiung Medical University). pCMV-Cypridina Luc Vector was obtained from ThermoFisher (16,150, Waltham, US), by adding 5′HindIII and 3′SalI with PCR, Cypridina luciferase (CLuc, without stop codon) was subcloned into pLNCX-Optimized sHRP-myc vector (with myc-tag and stop codon) to generate pLNCX-CMV-CLuc-myc. pCMV-Cypridina Luc Vector underwent two subsequent PCR procedures to add SalI, myc tag and stop codon, and 5′NcoI and 3′FseI to subclone CLuc-myc to generate pTEAD1-CLuc-myc or pAP- 1-CLuc-myc.

sHRP reporter gene chromogenic assay

Forty-eight hours after transient transfection, 10 µL of supernatants were aspirated from cultured cells and mixed with 150 µL of ABTS (0.4 mg/mL, Sigma-Aldrich, Burlington, US) with 0.01% hydrogen peroxide (Sigma-Aldrich, Burlington, US) or TMB (0.1 mg/mL, 100 µL, Promega, Madison, US), and rotated at 50 rpm, at RT on a rotator. After 60 min of TMB incubation, the reaction was stopped with the addition of 100 µL 1 M HCl (Sigma-Aldrich, Burlington, US). The ABTS group was reacted for 1 h without the addition of a stop solution. Results were read by SpectraMax ABS Plus Absorbance ELISA Microplate Readers (Molecular Devices, San Jose, US) to detect absorbance at 405 nm for the ABTS group, and at 450 nm and 650 nm (background) for the TMB group. The supernatant (100 µL) was then transferred to a new 96-well plate to detect the signals.

Interference of medium components to chromogenic assay analysis

pLNCX-sHRP-myc transfected 293 T cells were replaced with either DMEM, RPMI1640, OptiMEM, or DMEM-F12 with 1% P/S with or without 10% BCS 4–6 h post transfection. Forty-eight hours post-transfection, 10 µL of supernatants were collected and assayed into groups as follows. ABTS groups: (A) 10 µL CMV-sHRP supernatant + 150 µL ABTS, (B) 10 µL 293 T supernatant + 150 µL ABTS, (C) 10 µL medium + 150 µL ABTS, (D) 10 µL CMV-sHRP supernatant + 150 µL medium, (E) 10 µL 293 T supernatant + 150 µL medium, and (F) 160 µL medium. TMB groups: (A) 10 µL CMV-sHRP supernatant + 100 µL TMB + 100 µL 1 M HCl, (B) 10 µL 293 T supernatant + 100 µL TMB + 100 µL 1 M HCl, (C) 10 µL medium + 100 µL TMB + 100 µL 1 M HCl, (D) 10 µL CMV-sHRP supernatant + 200 µL medium, (E) 10 µL 293 T supernatant + 200 µL medium, and (F) 210 µL medium. Signals were reacted for 30 min and detected from 350 to 750 nm at 5 nm intervals. For analysis, C was the background of A or B, while F was the background of D or E, which were subtracted.

Results

Engineering a secreted form of HRP to explore the enzyme functionality

We previously engineered a membrane-tethered HRP (mHRP) that retained catalytic ability after being brought up to the outer cell membrane [21]. We reasoned that it is also possible to generate a secreted form of HRP without having it anchored to the cell membrane. We added a c-myc tag to both HRP and CLuc, then engineered both enzymes with a signal peptide downstream into a CMV expression vector. Our goal was to grant HRP the ability to secrete into the cell supernatant, to compare it side-by-side with CLuc in terms of functionality and collectability (Fig. 1). HEK-293 T (293 T) cells were transiently transfected with pLNCX-CMV-sHRP-myc or pLNCX-CMV-CLuc-myc, by collecting the cells and supernatants. Anti-c-myc staining showed that the molecular weight of sHRP and CLuc was approximately 74 kDa and 85 kDa, respectively. This was larger than the 38 kDa and 63 kDa predicted from the peptide sequences (Fig. 1A and B). sHRP showed an increase in molecular weight from 38 kDa to larger than 72 kDa, from the results of the computed approach (Figure S1) and previous research [22–24], suggesting that post-translational glycosylation may still exert an effect, altering the molecular weight (estimated at 2–4 kDa ($Man_8GlcNAc_2$ to $Man_{20}GlcNAc_2$) for each N-glycosylation site, a total of 7–9 sites) to become close to 74 kDa and might aid the catalyzation ability of sHRP (Fig. 1A).

We performed functional analyses using serial dilution of supernatants in the presence of serum or serum-free media (SFM). sHRP was reacted with either ABTS

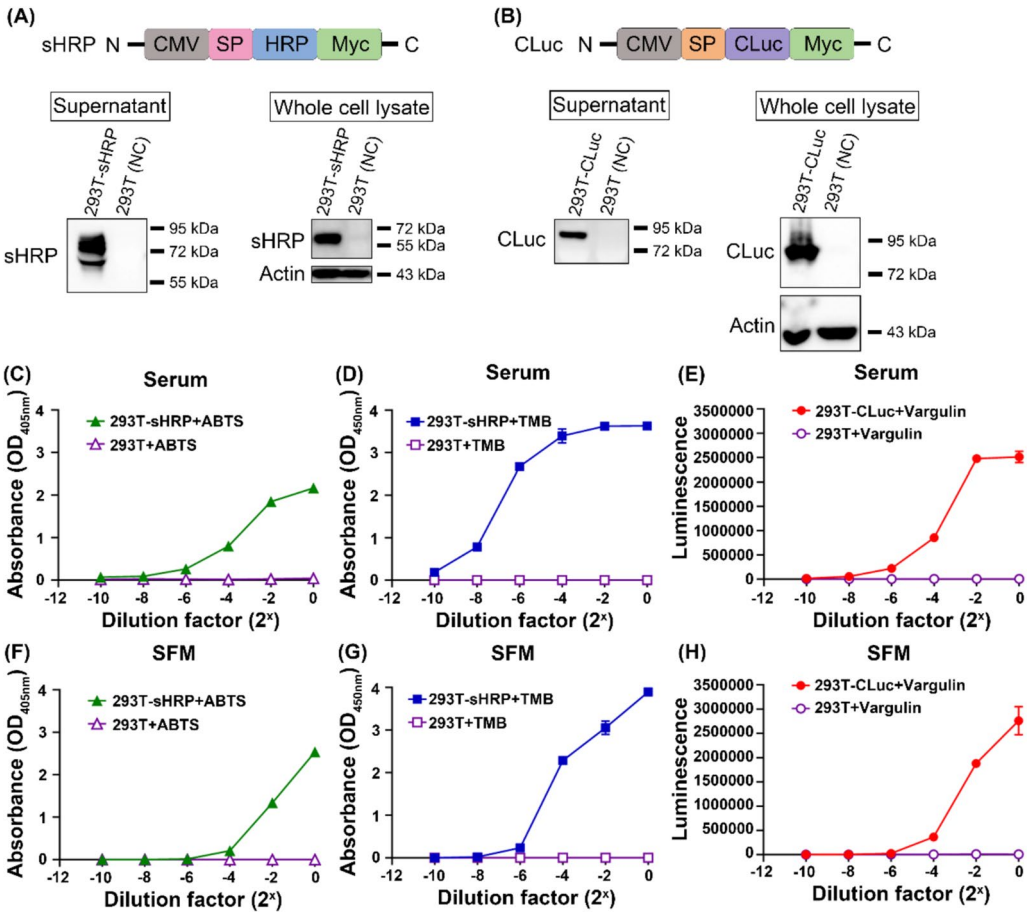


Fig. 1 Characterization of sHRP and CLuc. Protein sequence schematics and molecular weight analysis. **A** HRP was driven by CMV promoter with a myc-tag and a SP for secretion. Supernatants and whole cell lysate from transfected cells were collected and analyzed with Western blotting. **B** CLuc was engineered with a similar sequence design to HRP. 293 T-sHRP; HEK- 293 T cells transiently transfected with sHRP, 293 T (NC); untransfected 293 T cells as a negative control (NC). Actin was used as a loading control. **C**-**E** serum-containing media groups, **F**–**H** SFM groups; untransfected 293 T cells served as a negative control. (▲ and △) sHRP-ABTS, (■ and □) sHRP-TMB, (● and ○) CLuc-vargulin. All error bars show SD; all x-axes were converted into exponential of 2, $n = 3$, with 3 independent repeats

or TMB (Fig. 1C, D, F and G) and CLuc with vargulin (Fig. 1E and H). The control group (supernatants collected from 293 T cells) had negligible OD value throughout the dilution, while the chromogenic signal of sHRP and CLuc decreased with dilution, reaching roughly 1000-fold (2^{-10}) dilution, sHRP-TMB (Fig. 1D and G) was the most sensitive (the most distinguishable curve) enzyme/substrate group compared to sHRP-ABTS (Fig. 1C and F) and CLuc-vargulin (Fig. 1E and H) with and without the presence of serum. We concluded that the engineered sHRP or CLuc were expressible and fully functional, with sHRP being generally more sensitive than CLuc.

The secretion denotes the importance of the sHRP reporter gene system

To prove that the sHRP reporter gene system relies on secretion for the downstream application, we engineered a cytosolic HRP (cHRP) from mHRP with the removal of SP. 293 T cells were transiently transfected with pLNCX-CMV-cHRP-myc-mB7 and pLNCX-CMV-sHRP-myc, and cells and supernatants were collected and analyzed with western blot and ELISA. Both cHRP and sHRP showed positive staining of c-myc from the cell lysates; however, only the supernatant of sHRP showed positive staining in Western blotting (Fig. 2A-B). In the SFM condition, supernatants of cHRP and sHRP were analyzed with ABTS and TMB, and significantly enhanced signals of sHRP were observed from the supernatant collected (Fig. 2C-D). We concluded that sHRP was able to provide a better resolution in terms of the color intensity compared to cHRP. While the supernatant indirectly represented the promoter activity from transfected 293 T cells, it may also serve as an additional tool for monitoring promoter activity without cell lysis, making it suitable for our subsequent experimental design.

Comparison of the sensitivity and signal dynamics of sHRP and the CLuc reporter system in cancer signaling pathways

Because the HRP reporter gene system had the choice of multiple substrates (e.g., ABTS and TMB), and each substrate had its advantages, we sought to compare the signal sensitivity of sHRP to CLuc with different substrates (ABTS/TMB to vargulin) and also the D.R. TEAD1 and AP- 1 were selected due to their relevance in cancer signaling. TEAD1, a key transcription factor in the Hippo pathway, regulates organ size, tissue growth, and proliferation, with dysregulation contributing to

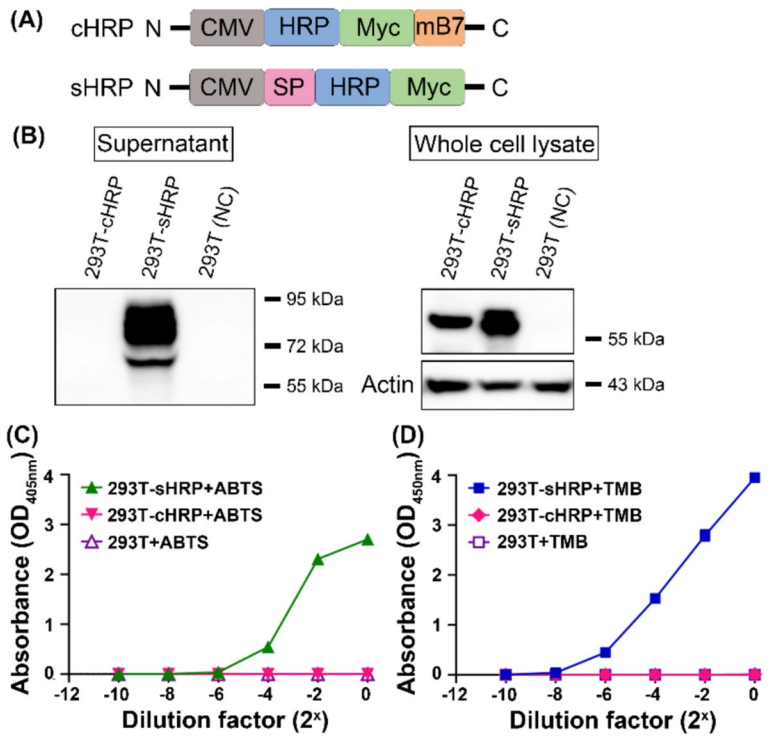


Fig. 2 Analysis of sHRP and cHRP secretion and catalyzation ability. **A** Protein sequence schematics, SP of cHRP was removed to prevent secretion. **B** Molecular weight comparison by western blotting, Lane 1; 293 T cells transfected with cHRP (293 T-cHRP), Lane 2; 293 T cells transfected with sHRP (293 T-sHRP), Lane 3; untransfected 293 T cells as a negative control. Actin was used as the loading control. **C**-**D** (▲, ▼ or △) for ABTS group and (■, ◆ or □) for the TMB group. All x-axes were converted into an exponential of 2, $n = 3$, with 3 independent repeats

cancer development and metastasis [25]. Similarly, AP-1, activated by MAPK and NF-κB pathways, plays a crucial role in cell proliferation, survival, and metastasis, making it significant in cancer biology [26]. These factors provide a relevant context for evaluating reporter systems in cancer research. Therefore, we generated multiple plasmid designs with vectors containing two different transcription factors as TEA domain transcription factor 1 (TEAD1) (pTEAD1-sHRP-myc and pTEAD1-CLuc-myc) and Activator protein 1 (AP- 1) (pAP- 1-sHRP-myc and pAP- 1-CLuc-myc) with sHRP and CLuc using PCR or subcloned from previously engineered pLNCX-CMV-sHRP-myc and pCMV-CLuc from ThermoFisher (Fig. 3A). We employed a serial dilution of (1) plasmids (pTEAD1-sHRP-myc, pTEAD1-CLuc-myc, pAP- 1-sHRP-myc and pAP- 1-CLuc-myc), and (2) supernatants. As the results show, overall, the chromogenic signal produced in AP- 1 was stronger than that in TEAD1 (Fig. 3B-M). sHRP that reacted with TMB provided a similar, if not more sensitive chromogenic signal (measured by the lowest detectable signal) compared to CLuc-vargulin in AP- 1 plasmid design followed by either plasmids or supernatant dilution (Fig. 3F, G, L and M), while ABTS had an acceptable signal range under unsaturated conditions (Fig. 3B, E, H and K). A calculation of the D.R. of both groups showed that sHRP-TMB had the best D.R. in plasmid dilution of TEAD1 and supernatant dilution of AP- 1 (Fig. 3C and L), while CLuc-vargulin had its best D.R. in plasmid dilution of AP- 1 and supernatant dilution of TEAD1 (Fig. 3G and J). In summary, we proved that with the right choice of substrate, sHRP is able to produce a comparable signal sensitivity and D.R. to CLuc-vargulin.

Comparison of signal and enzyme stability in the sHRP and CLuc reporter gene system

The reporter gene assay is a powerful tool that shows the slightest difference in signals generated from the cells with multiple inducing agents, different environments, cell metabolic conditions, etc [27, 28]. A reporter gene assay is usually carried out with a large number of samples, to compare diverse elements in parallel and find the top affecting factors. The assay speed and the stability of the light signal produced by substrates that are catalyzed by an enzyme determine the fidelity of the results; the lower the half-life of the catalyzed substrate, the longer the time span between the first sample and the last sample, resulting in a bigger variation, implying that when the number of samples is increased, the half-life of the light signal should be very important across samples from a single batch of an experiment. For this reason, we conducted a stability analysis to compare the half-life of the ABTS/TMB of the sHRP system with vargulin from the CLuc system (Fig. 4). The 293 T cells were transiently transfected with either pLNCX-CMV-sHRP-myc or pLNCX-CMV-CLuc-myc followed by supernatant collection. Supernatants were applied into two groups: (1) a signal stability group, signals generated from catalyzed substrates were subsequently measured at room temperature (25℃) every 20 min up to 120 min, then every 60 min up to 480 min, and then every 24 h up to 96 h; and (2) an enzyme stability group, enzymes-containing supernatants were stored at either 4 or 25℃ for 0, 24, 48, 72, and 96 h, and mixtures were aspirated and measured at the same time point as the signal stability group. As the results show, either with or without the presence of serum, the sHRP group had better signal stability than the CLuc group, and when the CLuc signal was eliminated, sHRP still preserved more than 80% of signals (Fig. 4A-F). When catalysis was exerted in the serum environment, sHRP-TMB had $t_{1/2}$ of 3500 min, followed by 299.7 min of sHRP-ABTS and 39.38 min of CLuc-vargulin (Fig. 4A-C). In the SFM condition, sHRP-ABTS took first place, with $t_{1/2}$ of 1410 min compared to 441.2 min for sHRP-TMB and 39.47 min for CLuc-vargulin (Fig. 4D-F). Of note, the CLuc-vargulin signal was eliminated within 40 min; at the same time, TMB or ABTS retained more than 80% of the total signal. In terms of enzyme stability, the ABTS group showed better enzyme activity in the SFM condition (Fig. 4G-J), while TMB showed an eightfold increase in the serum condition (Fig. 4K-N), CLuc was not affected by serum (4O- 4R), and the designated storage time points had no impact on either sHRP or CLuc. In summary, we determined the properties of enzymes and substrates from both the sHRP and CLuc systems. From the observations, we ascertained that the signal stability of sHRP and substrates was considerably more stable than CLuc-vargulin.

Determining the potential of sHRP to a reporter gene application

We demonstrated that sHRP was able to produce a sensitive chromogenic signal under the control of different response elements, correlating to the promoter and transcription factor. Therefore, we were next interested in evaluating the applicability of sHRP to reporter gene assay with a well-known transcription factor (NF-κB), to investigate whether sHRP provides enough fidelity in the presence of the corresponding inducer (tumor necrosis factor-α (TNF-α)) at different dosages. We transfected pNF-κB-RE containing sHRP (pNF-κB-sHRP) or pNF-κB-sHRP but NF-κB-RE removed vector control (p△NF-κB-sHRP) plasmids DNA into 293 T cells and treated them with serially diluted TNF-α and cell culture. Supernatants were collected at the designated time points and assayed with ABTS or TMB (Fig. 5). As shown

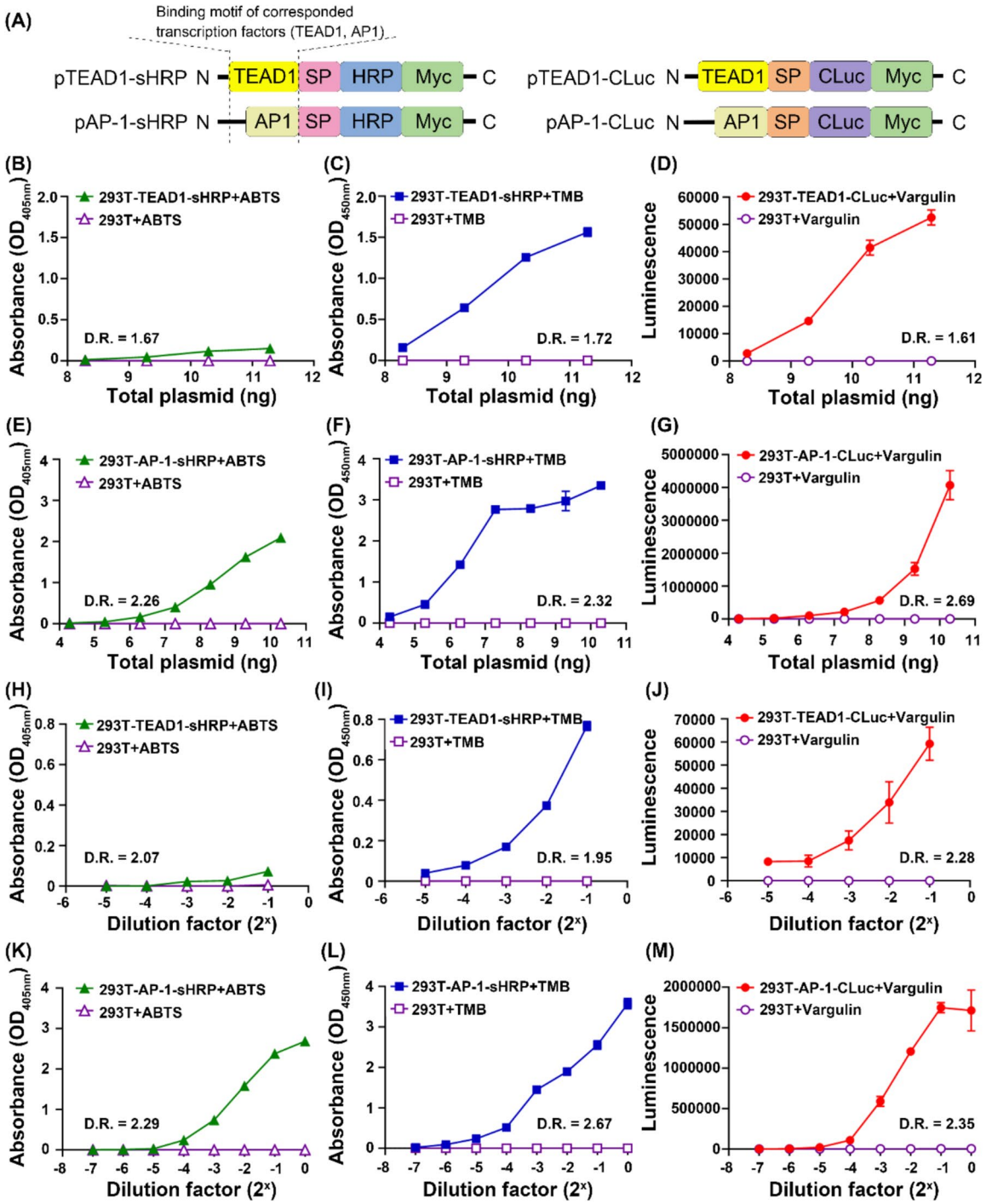


Fig. 3 Comparison of the sensitivity of sHRP and the CLuc system. **A** Protein sequence schematics of pTEAD1-sHRP, pTEAD1-CLuc, pAP- 1-sHRP, and pAP- 1-CLuc. **B**-**M** Signal sensitivity of sHRP and CLuc with different substrates, (▲ and △) sHRP-ABTS, (■ and □) sHRP-TMB, (● and ○) CLuc-vargulin, **B**-**G** plasmid dilution assays, **B**-**D** using pTEAD1 plasmid, **E**–**G** using pAP- 1 plasmid. **H**-**M** supernatant dilution assays, **H**-**J** with pTEAD1, **K**-**M** with pAP- 1. All the error bars show SD, $n = 3$, with 3 independent repeats

in the Fig. 5, supernatants from 293 T-NF-κB-sHRP produced a chromogenic signal positively correlated to the concentration of TNF-α, and the TMB group showed a better response signal than the ABTS group (Fig. 5B and C). To determine whether the sHRP reporter gene system may also be suitable in real-time management and to

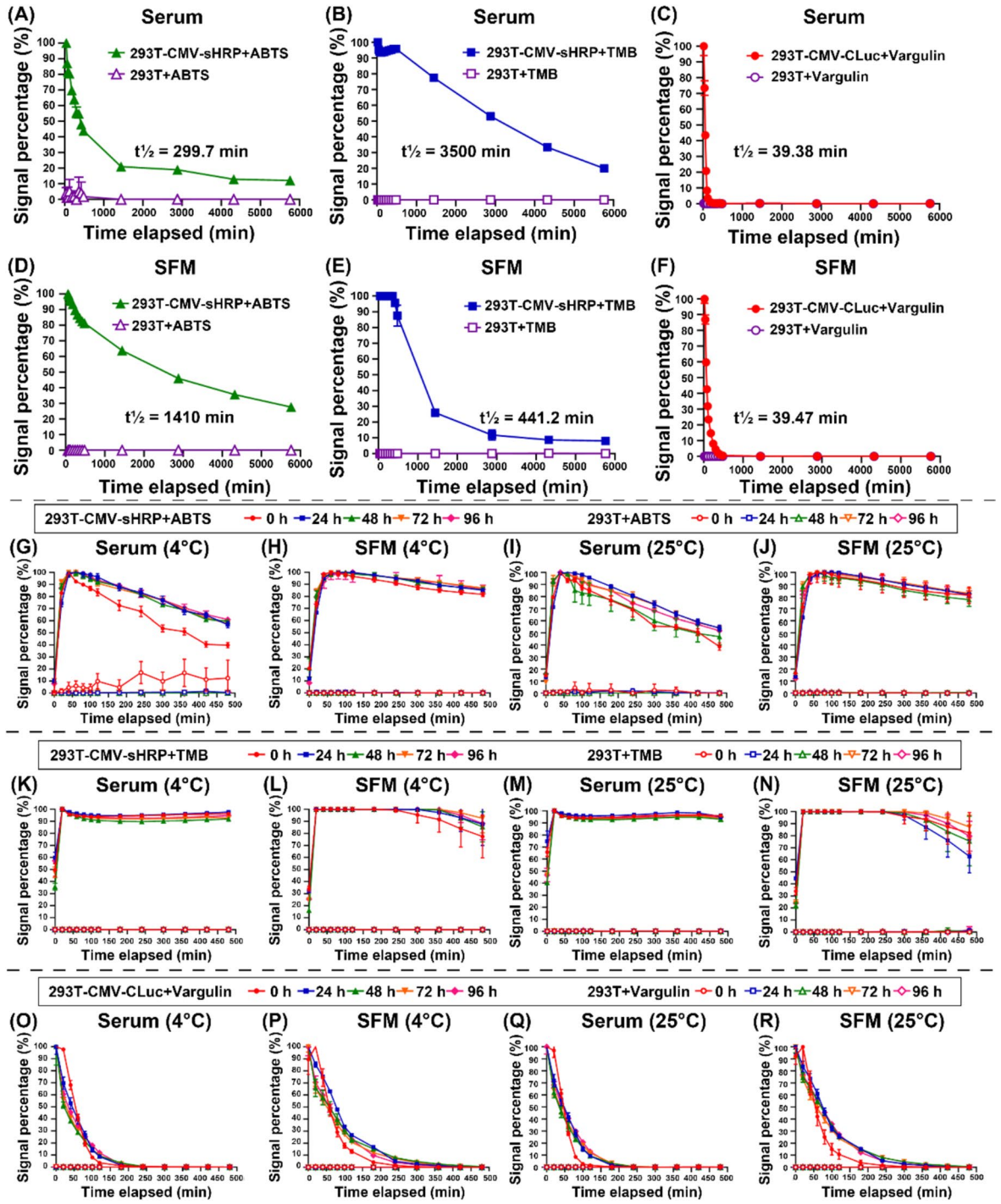


Fig. 4 Stability analysis of sHRP-ABTS, TMB, and CLuc-vargulin. Signal stability analysis, **A**-**C** serum group, **D**-**F** SFM group. (▲ and △) sHRP-ABTS, (■ and □) sHRP-TMB, (● and ○) CLuc-vargulin. Enzyme stability analysis, **G**-**J** sHRP-ABTS group, **K**-**N** sHRP-TMB group, **O**-**R** CLuc-vargulin group. (● 0 h, ■ 24 h, ▲ 48 h, ▼ 72 h, ◆ 96 h) enzymes with corresponding substrates, (○ 0 h, □ 24 h, △ 48 h, ▽ 72 h, ◇ 96 h) supernatants from untransfected 293 T with substrates. All error bars show SD, $n = 5$ for the ABTS group, $n = 6$ for the TMB group, $n = 3$ for the CLuc group, with 3 independent repeats

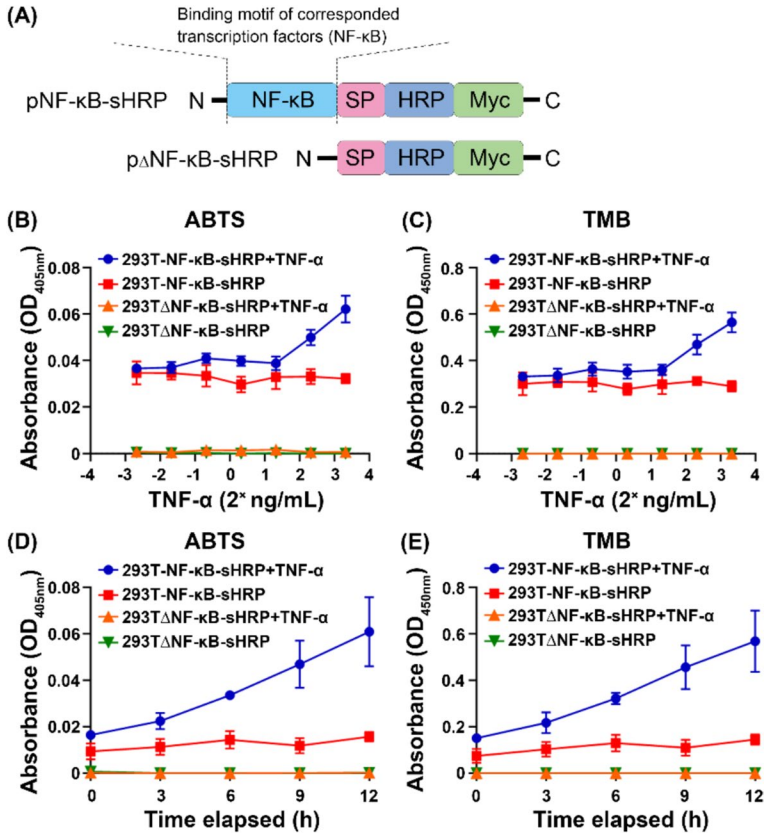


Fig. 5 Simulation of a real-world reporter gene application. **A** Protein sequence schematics for the NF-κB response element (NF-κB-RE) plasmid containing sHRP. **B**-**C** Supernatants from 293 T cells transfected with NF-κB-sHRP, treated with serially diluted TNF-α (10 ng/mL to 0.156 ng/mL) and substrates (ABTS or TMB). The X-axis was converted in the exponential of 2. **D**-**E** Supernatants from 293 T-NF-κB-sHRP cells stimulated with 5 ng/mL TNF-α and assayed at 0, 3, 6, 9, and 12 h. (●) Supernatants from 293 T-NF-κB-sHRP cells with TNF-α or (■) without TNF-α. (▲) Supernatants from 293 TΔNF-κB-sHRP cells with TNF-α or (▼) without TNF-α. All error bars showed SD, $n = 3$, with 3 independent repeats

identify the maximum cumulative signal, we collected 10 uL supernatants 3, 6, 9, and 12 h after the stimulation of 5 ng/mL of TNF-α. The results showed that the activation of the promoter of NF-κB-RE lasted for approximately 12 h after stimulation (Fig. 5D and E). Based on our observation, sHRP showed high fidelity in representing the signal of promoter activity followed by the corresponding inducer of transfected plasmid independent of cell lysis, providing continuous real-time detection, which we found it be suitable as a novel reporter gene system in simulating a real application case.

Evaluation of environmental factors in the media that may affect quantitative measurements

Because we had repeatedly proved that sHRP is superior to CLuc as a reporter gene system, we next focused on the accuracy/variation of chromogenic signals generated by sHRP-ABTS/TMB. Secretion of endogenous enzymes such as peroxidase in cell culture that may affect the background signal of HRP by reacting with ABTS and TMB has been reported previously [29–32]. Therefore, we sought to analyze the effect of the supernatant components on the effect of catalyzed ABTS/TMB from OD350 to 750 nm. A comparison of supernatants secreted from both 293 T-CMV-sHRP and untransfected 293 T cells assayed with substrates, is shown in Fig. 6. The sHRP group (blue line) showed a significantly higher chromogenic signal than the 293 T group (red line), while the O.D. value of the 293 T group was no higher than 0.10 (Fig. 6A and M), which was at least 20-fold lower compared to the lowest sHRP-substrate signal (Fig. 6F and J). Moreover, the culture media contained multiple components such as salts, vitamins, pH indicators (phenol red) [33–35], that are able to interfere with light absorbance. Therefore, we also explored the effect of culture media on the detection wavelength. We tested four media, DMEM, RPMI1640, Opti-MEM, and DMEM/F12, with or without substrates and or serum. To determine the full effect of phenol red and serum, we assayed supernatants with similar volumes of substrates or culture medium. The results indicated that both phenol red

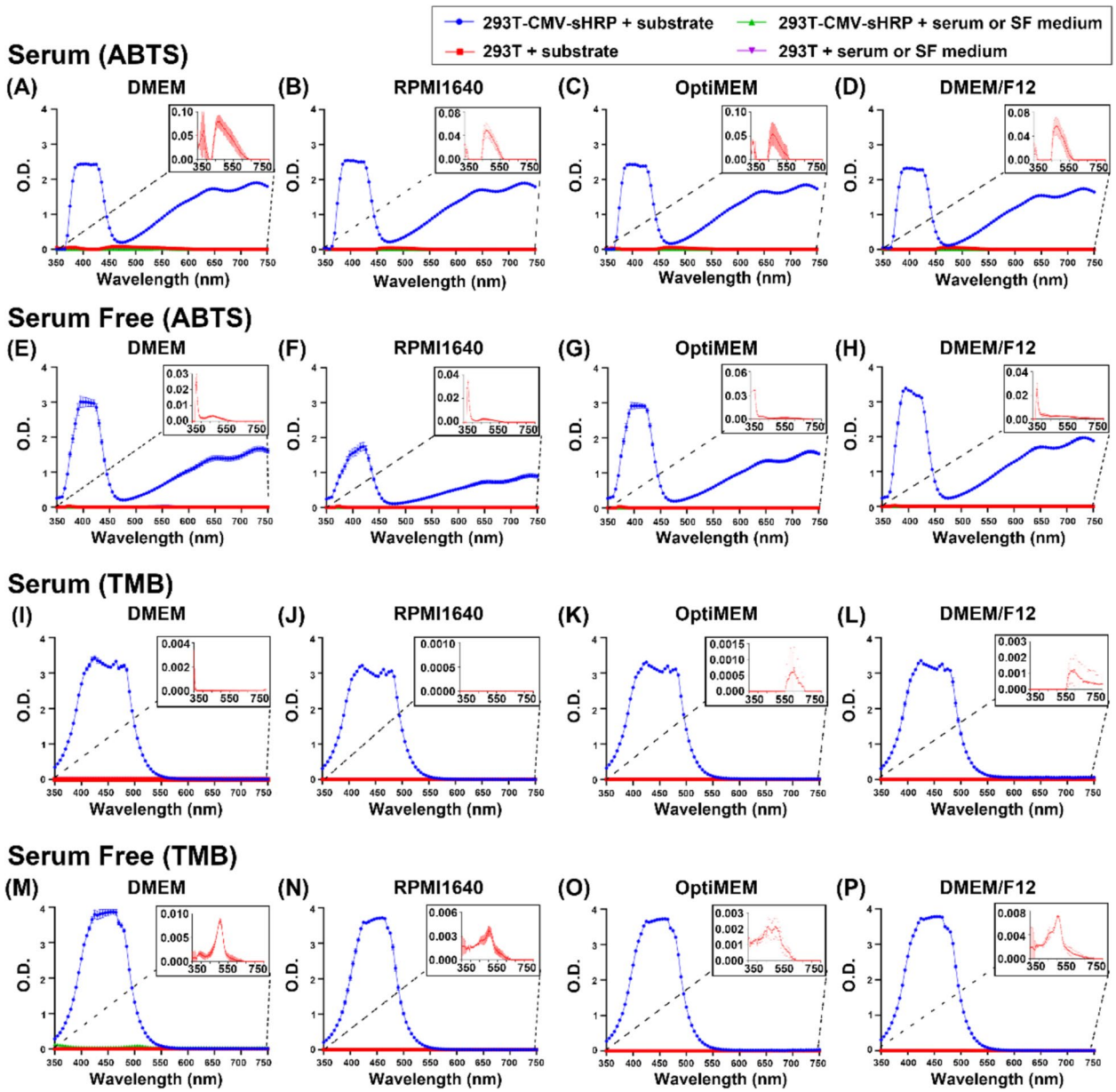


Fig. 6 Analysis of the interference of the chromogenic signal with the presence of serum and phenol red. The ABTS group, **A**-**D** with serum, **E**–**H** without serum, and the TMB group, **I**-**L** with serum, and (**M**-**P**) without serum. (●) Supernatants from CMV-sHRP transfected cells. (■) Supernatants from untransfected 293 T cells. (▲) Supernatants from CMV-sHRP with the indicated media, with or without serum. (▼) Supernatants of untransfected 293 T cells with the indicated media, with or without serum. Blue lines at 405/450 nm indicate the recommended detection signals (●); other symbols (■, ▲, and ▼) show negligible background, the highest background highlighted by dotted lines for better visualization. All error bars show SD, $n = 3$

and serum had nearly no impact on the HRP-substrate groups (Fig. 6, green and purple lines). Of note, there was no significant variation in light absorbance with the catalyzed or uncatalyzed substrates (Fig. 6, blue and green lines) in different media, which proves the low interference, high specificity, and sensitivity of sHRP. Together these features suggest the potential of the diverse applicability of sHRP.

Discussion

In this study, first, we generated a secreted form of HRP that granted HRP the ability to secrete into the culture medium, and then we conducted protein expression analysis. Molecular weight and chromogenic assays indicated proper protein production and retention of the functional catalytic site in sHRP (Fig. 1). Utilizing a cHRP design with SP removed, denoted the obligation of sHRP in terms of secretion dependency, using the recommended transient transfection protocol from the distributor of Lipofectamine 2000; 10 μL of supernatants was able to produce a signal that exceeded the detection limit of ELISA reader (Fig. 2). In our parallel comparison of sHRP and CLuc, sHRP showed a comparable linear signal and retained its D.R. with different folds of dilution factors, while sHRP-TMB provided better sensitivity than sHRP-ABTS and CLuc-vargulin (Fig. 3). ABTS and TMB were able to deliver a half-life of 1,410 min and 3,500 min; these half-lives were significantly longer than that of vargulin, which was 39.38 min. These lead to the conclusion that ABTS and TMB were able to withstand prolonged storage (Fig. 4). To test whether the sHRP reporter gene system was able to reflect the factual situation regarding inducer-promoter interaction, we used TNF-α to induce NF-κB activity and promote sHRP production. We found a positive correlation between the chromogenic signal and TNF-α concentration (Fig. 5). To understand the specificity and possible environmental factors inducing chromogenic events, we selected a multiple cell culture environment made of different compositions of commercially available media. The catalyzed substrate of sHRP eliminated the interference of chromogenic components such as endogenous peroxidase and phenol red in the media and showed negligible background between OD values obtained from different culture media (Fig. 6). Together these results suggest the low interference and wide applicability of the sHRP reporter gene system.

A stable and secretory reporter gene system that is able to monitor continuously of the target cells and withstand a prolonged operation processing time offers numerous advantages, including reducing cellular disturbance that may alter physiology or cause cell lysis, as well as minimizing signal variation between samples or batches. Secreted embryonic alkaline phosphatase (SEAP) is a widely used secretory reporter known for its versatility in detection through colorimetric and chemiluminescent assays, providing high signal sensitivity and stability. Its cost-effectiveness and robustness make it an excellent choice for high-throughput screening systems, suggesting it could serve as a strong comparator to sHRP [36, 37]. However, this study focuses primarily on signal sensitivity. GLuc, which shares similarities with CLuc as a naturally secreted luminescent enzyme, has been reported to be 20,000-fold more sensitive than SEAP in vitro [38]. Furthermore, CLuc has been demonstrated to be brighter than GLuc [15], making it a more suitable comparator for evaluating the sensitivity of our engineered sHRP. Additionally, CLuc's superior signal intensity also provide a valuable reference point for assessing the performance of sHRP.

In a signal stability test, the sHRP-ABTS group had a longer half-life without the presence of serum, but sHRP-TMB had a longer half-life in the presence of serum. We first considered that this might be the result of different enzyme kinetics affecting the catalytic process between HRP-ABTS and TMB. The V_{max} of HRP to ABTS and TMB were analyzed previously as 33 and 100 nM/s, 0.7 and 0.1 mM of Km and the turnover rate of HRP to TMB is 6 times faster than that of ABTS [39–41], which implies that the catalysis and oxidation process of HRP-TMB was faster than HRP-ABTS. Because oxidized ABTS and TMB have been shown to negatively impact the half-life of chemicals, we then inspected their corresponding chemical properties [42–44]. If substrate oxidation played a major role, TMB should be oxidized and depleted faster compared to ABTS; however, our result did not support this hypothesis. The sHRP-TMB group had the longest half-life. We reasoned that the components of serum such as bovine serum albumin might have interfered with the catalytic process of TMB, but not ABTS. It was also noted that, to have the catalysis process be operated until it reached the determined time point, there was no stop solution assayed with TMB. Stop solution can also affect half-life through an early termination of a saturation signal. However, in terms of half-life between the sHRP and CLuc substrates, the natural advantages of ABTS and TMB were that the chromogenic signal relies on the absorption of light and resonance stabilization of benzene rings, while the oxidation of vargulin resulting in the subsequent emission of photons, occurs upon direct contact of CLuc and the 2-methylbutanamide group from imidazopyrazinone ring that produced cypridina oxyluciferin, that may have contributed to the faster degradation of the bioluminescent signal [45, 46]. To sum up, even though the substrates of sHRP employed in this study have different half-lives regarding the presence of serum, both substrates were still able to produce a significantly longer half-life compared to the CLuc reporter gene system. We thus propose each substrate is suitable for different experimental designs, endorsing the stable and diverse practicability of sHRP.

For an application of the reporter gene system such as drug screening, the cost of the substrates determines the scale of screening and limits the selection of candidates. Vargulin was first synthesized using a three-step route comprising condensation of reduced ethioluciferin with

(+)-α-keto acid in three steps, with less than 2% yield. However, the 3-methyl- 2-oxopentanal method was able to achieve close to 85% of the total yield but still requires ethioluciferin (1-(3-(6-(1H-indol- 3-yl)− 3-methylpyrazin- 2-yl)propyl)guanidine) as the primary source, which may directly affect the final cost of the product [47–50]. Because of the nature of luciferin, cypridina luciferin is easily degraded and hard to store, so we reasoned that a reliable and productive synthesis method is not enough to provide a lower price to allow the continual use of the reporter system [51, 52]. The cost of the luciferase assay has long been understood to be a limiting factor in its use, and CLuc, which we utilized in this study, has a relatively higher price compared to the common FLuc and RLuc assays. As prices are mainly determined by the substrates, we compared the cost of substrates (Figure S2) and found that vargulin was 27-fold and ninefold more expensive than ABTS and TMB, respectively. We, thus reasoned that the sHRP reporter gene system is more suitable for large-scale drug screening procedures (e.g., high-throughput drug screening) or cell signaling analyses.

With regard to the limitations of this study, when performing serial dilution, there was an inconsistent CLuc signal produced by the undiluted point that was frequently lower than the first dilution point (Fig. 3M), which failed to produce a linear bioluminescent signal. In addition, a total of four kits were dead on arrival (from two different lots number). Such a phenomenon was not described in the troubleshooting section of the manufacturer's datasheet, but we suggest that this might be the rapid oxidization and depletion of the substrate by CLuc, that is represented by a much smaller Km (11.5 μM) compared to HRP-ABTS or TMB, which means the catalysis process is at least a 9 times faster [53–55]. In the sHRP model, we did not observe a decreased signal at the undiluted point but a saturated chromogenic signal, indicating a large amount of enzyme sped up the conversion of substrates; however, the chemical nature of ABTS and TMB were able to withstand such a process. The normal shelf life, which was 3 months, and two years, respectively, may help to explain this phenomenon [56, 57]. We also need to clarify that, in this study, we focused on the application of sHRP as a reporter gene in cell-based assays, specifically for drug screening and promoter activity analysis. While the use of sHRP has shown considerable promise in cell-based applications, particularly in the context of real-time monitoring of promoter activity and inducible gene expression, it has not yet been explored in in vivo studies. The primary reason for this limitation is that our research primarily concentrates on developing and optimizing cell-based reporter assays for high-throughput drug screening, thereby, whether this design is suitable for in vivo application remain explored. Nevertheless, ABTS and TMB were able to sustain a longer signal degradation compared to vargulin (Fig. 4), before and after catalysis of corresponding enzymes, which confers one more advantage to sHRP for bulk assay and experimental analyses.

Conclusion

In summary, our data illustrate that sHRP is a very stable enzyme when used with its substrates, ABTS and TMB, and the system was able to deliver a sensitive, rapid chromogenic reaction and a stable signal that could withstand a prolonged assay time and is applicable across different culture media. We also showed that the substrates are an affordable option. We believe that sHRP is suitable for use as a new reporter gene system providing an alternative to luciferase.

Abbreviations

ABTS	2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
cHRP	Cytosolic horseradish peroxidase
CLuc	Cypridina luciferase
$_D$-Luc	$_D$-luciferin
FLuc	Firefly luciferase
GLuc	Gaussia luciferase
HRP	Horseradish peroxidase
mHRP	Membrane-tethered horseradish peroxidase
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
RLuc	Renilla luciferase
sHRP	Secreted form horseradish peroxidase
TEAD1	TEA domain family member 1
TNF-α	Tumor necrosis factor-α

Supplementary Information

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Supplementary Material 1

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Authors' contributions

W.W. Lin designed the research; C.Y. Lee performed the research; W.W. Lin, M.S. Chang and C.Y. Lee analyzed and interpreted the data; M.S. Chang wrote the paper; Y.Y. Chang, P.J. Li, H.S. Wu, E.S. Liu and H.K. Huang assisted in troubleshooting during the experimental process and provided suggestion and correction for the manuscript; and all authors approved of the final manuscript.

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Data availability
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
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Competing interests
The authors declare no competing interests.

Author details
[1]College of Life Science, Kaohsiung Medical University, Kaohsiung, Taiwan. [2]Graduate Institute of Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan. [3]Department of Biomedical Science and Environmental Biology, College of Life Science, Kaohsiung Medical University, Kaohsiung, Taiwan. [4]Department of Medical Laboratory, Kaohsiung Armed Forces General Hospital, Kaohsiung, Taiwan. [5]Drug Development and Value Creation Research Center, Kaohsiung Medical University, Kaohsiung, Taiwan. [6]Department of Laboratory Medicine, Post Baccalaureate Medicine, College of Medicine, Kaohsiung Medical University, 100 Shih-Chuan 1 st Road, Kaohsiung 80708, Taiwan. [7]Department of Laboratory Medicine, School of Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan. [8]Department of Medical Research, Kaohsiung Medical University Hospital, Kaohsiung, Taiwan.

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