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Abstract

Firefly luciferase is a prominent reporter on molecular imaging with the advantage of longer wavelength on light emission and the ATP linear correlation, which makes it useful in most of current bioluminescence imaging model. However, the utility of this biomaterial was limited by the signal intensity and stability which are respectively affected by enzyme activity and substrate consumption. This study demonstrated a series of novel synthetic bifunctional enzyme complex of Firefly luciferase (Fluc) and Luciferin-regenerating enzyme (LRE). A peptide linker library was constructed for the fusion strategy on biosynthesis. The findings of both experimental data and structural simulation demonstrated that the intervention of fused LRE remarkably improve the stability of in vitro bioluminescence signal through luciferin recycling; and revealed the competitive relationship of Fluc and LRE on luciferin binding: Fluc performed higher activity with one copy number of rigid linker (EAAAK) at the C terminal while LRE acted more efficiently with two copy numbers of flexible linker (GGGGS) at the N terminal. With the advantage of signal intensity and stability, this fused bifunctional enzyme complex may expand the application of firefly luciferase to in vitro bioluminescence imaging.

Keywords	Bioluminescence imaging; Enzyme complex; Structural simulation
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Highlights

1. Fusion strategy on biosynthetic bifunctional enzyme complex using peptide linker library.

2. Optimal enzyme structure with high-efficiency for luciferin-recycling to enhance bioluminescence imaging.

3. 3D protein structural simulation to analyze the functional domains and verify the enzyme properties.

4. Organic reagents to strengthen the bioluminescent property of the optimal enzyme complex.

ABSTRACT

Firefly luciferase is a prominent reporter on molecular imaging with the advantage of longer wavelength on light emission and the ATP linear correlation, which makes it useful in most of current bioluminescence imaging model. However, the utility of this biomaterial was limited by the signal intensity and stability which are respectively affected by enzyme activity and substrate consumption.

This study demonstrated a series of novel synthetic bifunctional enzyme complex of Firefly luciferase (Fluc) and Luciferin-regenerating enzyme (LRE). A peptide linker library was constructed for the fusion strategy on biosynthesis. The findings of both experimental data and structural simulation demonstrated that the intervention of fused LRE remarkably improve the stability of *in vitro* bioluminescence signal through luciferin recycling; and revealed the competitive relationship of Fluc and LRE on luciferin binding: Fluc performed higher activity with one copy number of rigid linker (EAAAK) at the C terminal while LRE acted more efficiently with two copy numbers of flexible linker (GGGGS) at the N terminal. With the advantage of signal intensity and stability, this fused bifunctional enzyme complex may expand the application of firefly luciferase to *in vitro* bioluminescence imaging.

TITLE: Biosynthetic bifunctional enzyme complex with high-efficiency luciferin-recycling to enhance the bioluminescence imaging

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Running title:

Optimal bifunctional enzyme complex to enhance the bioluminescence imaging

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ABSTRACT

Firefly luciferase is a prominent reporter on molecular imaging with the advantage of longer wavelength on light emission and the ATP linear correlation, which makes it useful in most of current bioluminescence imaging model. However, the utility of this biomaterial was limited by the signal intensity and stability which are respectively affected by enzyme activity and substrate consumption.

This study demonstrated a series of novel synthetic bifunctional enzyme complex of Firefly luciferase (Fluc) and Luciferin-regenerating enzyme (LRE). A peptide linker library was constructed for the fusion strategy on biosynthesis. The findings of both experimental data and structural simulation demonstrated that the intervention of fused LRE remarkably improve the stability of *in vitro* bioluminescence signal through luciferin recycling; and revealed the competitive relationship of Fluc and LRE on luciferin binding: Fluc performed higher activity with one copy number of rigid linker (EAAAK) at the C terminal while LRE acted more efficiently with two copy numbers of flexible linker (GGGGS) at the N terminal. With the advantage of signal intensity and stability, this fused bifunctional enzyme complex may expand the application of firefly luciferase to *in vitro* bioluminescence imaging.

Key words: Bioluminescence imaging, Bifunctional enzyme complex, Firefly luciferase, Luciferin-recycling, Structural simulation, Peptide linker library.

1. INTRODUCTION

On molecular imaging, the firefly luciferase (Fluc) was widely studied due to its ATP linear correlative feature on *in vitro* detection of ATP in live cell [1,2], therefore it was used as an indicator for the hygienic index. Meanwhile, the luciferin-regenerating enzyme (LRE) [3] that catalyzed converting process from oxyluciferin to luciferin in the presence of D-cysteine [4] was also reported to improve the luminescence signal generated by exogenous Fluc [5]. However, the application of Fluc catalyzed bioluminescence imaging was still limited by the bioluminescence intensity and signal decaying, especially for the *in vitro* detection of ATP at low concentration.

Fusion strategy has been widely used in a variety of fields to construct artificial multifunction proteins [6,7]. Fusion protein can be designed and synthesized to achieve improved properties or new functionality of multiple proteins by tandem fusion, domain insertion, or post-translational protein conjugation [8], among which, , using connection medium such as peptide linker to produce the combination of two or more protein domainsin order to enhance bioactivities or generate novel functional complex was studied in recent years, with a wide range of biotechnological and (bio)pharmaceutical applications [9–11]. The length of linker and the residues on the structure play an important role in the stability and functionality of a fusion protein by affecting the active domain and the structure of protein [6,12].

In our previous work, we reported that the fusion expressed Firefly luciferase (Fluc) and luciferin-regenerating enzyme (LRE) could enhance the *in vivo*

luminescence imaging [13], mediated by two typical types of peptide linker, a rigid linker (hereinafter referred to as R) with alpha helical structure (sequence of EAAAK) to maintain distance between domains and a flexible linker (hereinafter referred to as S, sequence of GGGGS) that increases spatial separation and allows interaction between domains [14,15].

To study the mediation of peptide linkers on *in vitro* luminescence imaging and luciferin-recycling catalyzed by Fluc-LRE fusion complex, and evaluate the efficiency of designed linkers to separate domains, a peptide linker library containing rigid linker, flexible linker and mixed linker with different length were assessed in this study on the model of Fluc and LRE bifunctional enzyme complex, to determine the optimal structure of bifunctional fusion protein of Fluc and LRE, thus to optimize the catalytic efficiency on *in vitro* luminescence imaging.

2. MATERIAL AND METHODS

2.1 Bacteria, plasmids and reagents

The competent cell of *E. coli* DH5α and *E. coli* BL21 strain, Isopropyl-β-D-thiogalactopyranoside (IPTG) and Kanamycin were purchased from Transgen (Beijing, China). The sequence of *Fluc* was cloned from the template of pGL4.17 [*luc2*/Neo] Vector purchased from Promega (Cat. E672A). The sequence of *Lre* (GenBank: AB062786) was synthesised by Sangon Biotech (Shanghai) Co., Ltd. The primers used for PCR (listed in ~~Table 1~~the appendix) were synthesised by Sangon Biotech (Shanghai) Co., Ltd. The Takara PrimeSTAR Max DNA Polymerase (Cat. R045A), Takara QuickCut restriction enzyme *Nde*I (Cat. 1621) and *Xho*I (Cat. 1635), and

Takara ligation kit (Cat. 6022) were used for the construction of expression clones. The plasmid pET28a expression vector was from Novagen (Cat. 69864-3). The plasmid miniprep kit (Cat. GMK5999) and gel extraction kit (Cat. D2500-02) were purchased from Promega.

2.2 Construction of fusion enzyme complex

The coding sequences ~~A~~ of a series of fusion proteins with different linker (listed in Table 1) were respectively constructed ~~using the templates and primers listed in Table 1~~framed with the restriction enzyme cutting site of *Nde*I and *Xho*I by PCR. The PCR products were respectively digested by the restriction enzyme *Nde*I and *Xho*I, and subsequently ligated between the multiple cloning sites on the pET28a vector by Takara ligation kit. The expression clones were respectively driven by T7 promoter, and the His-tag coding sequence on the plasmid encoded a histidine to the N-terminal of target protein. The engineered plasmids were transferred in *E. coli* BL21 for protein expression.

2.3 Bacterial culture

The bacteria was cultured using LB media (containing 10g/L Tryptone, 5g/L Yeast extract and 10g/L NaCl to pH7.0 at 25 ℃) with shaking at 180 rpm at 37 ℃, 0.2 mM IPTG was injected to induce the protein expression after 2-hour incubation of bacterial culture.

2.4 Protein expression, purification and qualification

The bacteria carrying the recombinated enzyme expression clones were incubated in triplicate with 0.2 mM IPTG induction at 20 ℃ with shaking. The overnight cultured bacteria were washed and concentrated 5:1 with PBS (pH 7.0), performed ultrasonic breaking (3 s×6 s at 300W for 60 times) on ice and centrifuged

at 4℃ at 10000 rpm for 15 min to harvest the crude extract from supernatant. The His-tagged enzyme were purified by affinity chromatography (GE AKTA prime plus) using 5 ml HisTrap™ HP column (GE Healthcare, Sweden). The purified enzyme were qualified by SDS-PAGE and the concentration of protein were analyzed using the Bradford assay.

2.5 *in vitro* assessment on luminescence imaging

The purified enzyme was added in triplicate to the reaction mixture containing 0.25 mM D-luciferin, 4 mM ATP, 10 mM $MgSO_4$ and 2.5 mM D-cysteine in PBS (pH 7.0). The luminescence signal was measured in triplicate at 37 ℃ by Tecan Infinite M200 Pro. to analyze the specific activity of each luciferase and fusion protein. The enzyme activity of Fluc was determined by the relative light unit (RLU) per microgram of protein while the enzyme activity of LRE was determined by luminescence changing ratio in the presence/absence of D-cysteine.

The equation which can be used for the determination of changing rate of luminescence signal to analyze the effect of LRE to the *in vitro* bioluminescence is given as:

$$\text{Luminescence changing ratio} = \frac{(RLU' - RLU)}{RLU} \times 100\%$$

Where RLU' is the bioluminescence intensity in the presence of ~~C~~D-cysteine; RLU is the bioluminescence intensity in the absence of D-cysteine.

2.6 3D Structure simulation

The 3D structure models of enzyme complexes were simulated and predicted by the I-TASSER server [16], the simulated models with a high confidence score were analyzed by the software VMD 1.8.3, focusing on the luciferin binding domain I (LBD

I) and luciferin binding domain II (LBD II) [5, 17].

2.7 Statistic analysis

One-way ANOVA was applied to compare the effect of different enzyme complex to the *in vivo* and *in vitro* bioluminescence, the data of which were analyzed by the software GraphPad Prism 6 and P value was used to determine the difference between each two structures.

3. RESULTS

3.1 Construction of enzyme complex and protein expression

A panel of Fluc expression clones was constructed on the plasmid of pET28a and induced for expression in *E. coli* BL21, respectively encoding the enzyme of Fluc, dual enzyme of Fluc and LRE, and the fusion proteins through different peptide linker. The serial constructions of Fluc expression clones were listed in Table ~~2~~1, and were confirmed by sequencing by Sangon Biotech (Shanghai) Co., Ltd.

The expressed enzyme complexes were purified through affinity chromatography and the concentration of harvested samples were listed in Table ~~2~~1. The samples of extracted enzyme were analyzed by SDS-PAGE. As was shown in Fig 1, the Fluc at 62 kDa were obtained in lane 2&3, LRE at 38 kDa were obtained in lane 3 and the series of bifunctional enzyme complexes at approximately 100 kDa were respectively obtained in lane 4-13.

3.2 Effect of peptide linker on Fluc activity

The luminescence intensity of the various of enzyme complex were measured *in*

vitro with the existence of the substrates D-luciferin. The luminescence intensity, which indicated the activity of fusion expressed Fluc on catalyzing light-generation, were present in Fig 2, compared with the signals produced by the free Fluc and the dual expression of Fluc-LRE. The initial luminescence intensity demonstrated the activity of Fluc in different structure with the effects of linkers and residues. Among the group, the control of Fluc was observed with the strongest initial signal (1.21×10^{10} RLU) but sharp decaying tendency, whilst Fluc-R-LRE demonstrated a signal at 9.66×10^{9} RLU (79.83% of control) with no significant difference (P value was 0.1739 by T-test), which was 22.59% stronger than the free Fluc at the presence of free LRE (7.88×10^{9} RLU, 65.12% of control, P value was 0.9633 to control and 0.1352 to Fluc-R-LRE). With the effect of peptide linker and the presence of fused LRE, the other enzyme complex were observed to have a similar tendency but remarkably lower Fluc activity than that of control, as was shown in the magnified view in Fig 2, from high to low, respectively at the initial luminescence intensity of 1.15×10^{9} RLU to Fluc-RSR-LRE (9.50% of control, P value was 0.0274), 6.96×10^{8} RLU to Fluc-SSS-LRE (5.75% of control, P value was 0.0212), 6.36×10^{8} RLU to Fluc-S-LRE (5.26% of control, P value was 0.0202), 6.01×10^{8} RLU to Fluc-RR-LRE (4.97% of control, P value was 0.0173), 4.83×10^{8} RLU to Fluc-RS-LRE (3.99% of control, P value was 0.0183), 3.27×10^{8} RLU to Fluc-SR-LRE (2.70% of control, P value was 0.0152), 3.01×10^{8} RLU to Fluc-RRR-LRE (2.49% of control, P value was 0.0149), 2.40×10^{8} RLU to Fluc-SS-LRE (1.98% of control, P value was 0.0139) and 1.32×10^{8} RLU to Fluc-SRS-LRE (1.09% of control, P value was 0.0123). The results indicated that all the enzyme complex

showed accordant decaying tendency to luminescence signal under the consumption of the substrate D-luciferin, and the peptide linker and residues on Fluc affected the Fluc activity in most fusion construction, in which the type of one copy rigid linker provided the optimal conformation for the activity domain.

3.3 Effect of D-cysteine on *in vitro* bioluminescence

The substrate of D-cysteine for luciferin-regenerating was added to the purified Fluc solution to assess its effect on the luminescence. As was shown in Fig 3, the presence of D-cysteine increased the initial luminescence intensity generated by the Fluc with 12.40% enhancement, in which there was no LRE existence, though the signals both decayed and showed no difference (P value was 0.9911) in signal emission.

3.4 Effect of linker on LRE activity and substrate recycling

The luminescence intensity of the enzyme complexes were measured *in vitro* in the presence of both D-luciferin and D-cysteine. The term of Luminescence changing ratio was used to indicate the activities of LRE and assess the efficiency of luciferin-recycling in different fusion complex.

As was shown in Fig 4, the changing ratio of Fluc (Panel B) verified the effect of D-cysteine at the absence of LRE, with this as control, the higher changing ratio revealed the higher activity of LRE on luciferin-regeneration that caused extra luminescence signal, while the lower ratio demonstrated low activity of LRE and poor efficiency in luciferin-recycling. In Panel A, the linear ratio on the complex of Fluc-SRS-LRE and Fluc-SS-LRE demonstrated significantly higher effect of LRE than other

complexes, which indicated that 2 copy number of flexible linker enabled the optimal structure of LRE on luciferin-regenerating. The other complexes in Panel B were observed with similar tendency to that of control. Among which, the rigid linker enhanced the activities of fused LRE along with the increasing copy number of rigid linker, while the flexible linker SSS and S also promoted the LRE activity, which all performed better than the free LRE and contributed extract signal during the 6-hour test period. The mixed linker of RS demonstrated less but still positive effect on LRE activity, while the linker of SR and RSR were observed to inhibit the LRE activity and thus decreased the luminescence emission from the bifunctional enzyme.

3.5 Structure feature and bioluminescence kinetics of bifunctional enzyme

The structure feature of enzyme complexes were shown in Fig 5, the luminescence signals indicated the activities of bifunctional enzyme complex whilst the difference of signals in the presence/absence of D-cysteine indicated the effect of peptide linker to luciferin-recycling on *in vitro* bioluminescence.

As for the series of rigid linker in Panel A, the intervention of luciferin-regeneration did not significantly affect the initial luminescence from each enzyme complex, but remarkably slow the decaying in signal recession. The luminescence intensity was varied with the copy number of peptide linker in that the signal became weaker along with the increasing of rigid linker copy number. In addition, the enzyme fused with one copy of rigid linker (Fluc-R-LRE) demonstrated the optimal light emission, which was observed to have lower initial but higher continuous signal than that of control with no linker mediation.

As for the series of flexible linker in Panel B, the luciferin-recycling caused extract signal to increase the luminescence emission, and slow the decaying of signal recession. The enzyme complex mediated with 3 copies of flexible linker generated higher initial luminescence while that with one copy of flexible linker provided more continuous signal after 0.5 h. Remarkably, the enzyme fused through 2 copies of flexible linker significantly increased the signal emission (P value was 0.0189), though its signal was the lowest in the group, which indicated the high efficiency of luciferin-recycling in this type of peptide linker.

With regard to the series of mixed linker in Panel C, the fused enzyme complex through the peptide linker RSR was observed to decrease of signal generation when D-cysteine involving LRE catalyzed luciferin-regeneration (P value was 0.5129). The enzyme complex of Fluc-RS-LRE and Fluc-SR-LRE both presented decreasing of luminescence at the beginning but the luciferin-regenerating led to an enhancement of signal emission, which made no difference in the luminescence generation (P value were respectively 0.9527 and 0.5301). However, the complex of Fluc-SRS-LRE generated significantly stronger signals when the bifunctional enzyme both worked (P value <0.0001).

The bioluminescence kinetics of enzyme complex were analyzed as shown in Table ~~3~~2. The decaying of bioluminescent signals were nonlinearly growing with the reaction period, the decay kinetics were satisfactorily fitted with a two-exponential decay function and consequently described by two rate constants, K1 and K2 as shown in Table ~~3~~2. The initial light intensities and half-life of signals also indicated

the Fluc-R-LRE as optimal structure on light emission while the Fluc-SRS-LRE was the optimal one on luciferin-regeneration.

3.6 3D structural simulation of enzyme complex

The typical enzyme complexes were structural simulated as shown in Fig 6, and the predicted results were consistent with the experimental data. The model of Fluc-R-LRE with an average distance of all residue pairs in two structures (RMSD) at 10.3 $\pm$4.6Å was observed with open LBD I and LBD II located in both Fluc and LRE. In the model of Fluc-RSR-LRE (RMSD at 11.3$\pm$ 4.5Å), the LBD II in LRE was distant to the Fluc domain, which might affect the substrate channeling and thus caused lower LRE activity. The model of Fluc-SRS-LRE (RMSD at 11.8$\pm$ 4.5Å) was observed with a covered LBD I in Fluc that might caused lower Fluc activity, but with wilder open LBD I and LBD II in LRE for better luciferin-regenerating.

3.7 Bioluminescent property of optimal enzyme complex in organic solvents

The bioluminescent property of Fluc-R-LRE was assessed respectively in the solvent of DDT, EDTA, fucose, BSA, Triton X-100 and glycerinum (Fig 7). The results demonstrated that the initial signal was enhanced in the presence of DDT (peaked at 0.4 mmol/L), EDTA (peaked at 0.4 mmol/L), fucose (peaked at 0.8 mol/L) and BSA (peaked at 1.0 mg/L) respectively, whilst decreased along with the increasing concentration of Triton X-100 and glyerinum, which indicated the component of stabilizing agent for the bifunctional enzyme in further application.

4. DISCUSSION

LRE was reported to enhance the bioluminescence imaging by regenerating luciferin for Fluc catalyzing light emission. Many researches such as codon humanization and mutations [18–20], and the replacement of homogenous diverse from other firefly species [17, 21–22], had been processed to increase emission intensity. However, the fast fading of signal catalyzed by free enzyme was still unsolved (shown as half-life in Table ~~3~~2).

In this study, a serial fused protein of Fluc and LRE mediated by different type of peptide linker, were conducted to assess the effect of peptide linker on the enzyme activities and the bioluminescence imaging. The results of *in vitro* assessment demonstrated a relationship of wane and wax between the activity of fused Fluc and LRE, which indicated the competitive binding of oxyluciferin between Fluc and LRE. Generally, the increasing unit of rigid linker caused decreasing of Fluc activity (R>RR>RRR on Fluc activity in Fig 2 and Fig 5) but enhanced the LRE induced luciferin-recycling (R<RR<RRR on LRE activity in Fig 4). As for the series of flexible linker, Fluc activity peaked at one unit of flexible linker and was lowest at two units of flexible linkers (S>SSS>SS in Fig 2 and Fig 5), on the contrary, LRE catalyzed luciferin-regeneration peaked in Fluc-SS-LRE and bottomed in Fluc-S-LRE (S<SSS<SS in Fig 4). As for the series of mixed linker, the rigid linker on C terminal of Fluc showed more effectiveness on Fluc activity than the flexible linker (RSR>RS>SR>SRS in Fig 2), whilst the LRE activity showed more effectiveness with flexible linker on the N terminal (SRS>SR>RS>RSR in Fig 4). Among the group, Fluc showed the highest activity in Fluc-R-LRE, whilst LRE present the most efficiency in luciferin-recycling in Fluc-SRS-LRE.

The results ~~also~~ revealed that the Fluc activity was related to suitable space between functional domains by rigid linker, whilst the LRE activity was related to appropriate folding by the flexible linker. This deduction was consistent with the reported inhibition of firefly luciferase caused by oxyluciferin and dehydroluciferyl adenylate [23], that the spacer increase benefits the luciferyl adenylate [24] transferring and the light emission during coenzyme A (CoA) intermediated dehydrogenation into oxyluciferin [25].

These findings were also verified on the predicted structural models (Fig 6). The biological functions of the enzyme complex were reflected by the exposure of substrate-binding sites and the interactions of luciferin-recycling between domains. The residues in these domains were also concerned in recent years to study the biological feature on bioluminescence imaging [5, 20].

Unlike the products, such as oxyluciferin and dehydroluciferyl adenylate [26], and the substrate pyrophosphate and tripolyphosphate [27], as the inhibitor to the bioluminescence [28], ~~As~~ the regenerated luciferin supplemented the consumed substrate for Fluc, therefore the activity of fused LRE and the efficiency of luciferin-recycling were ~~also~~ important ~~for~~ to the stability of bioluminescence imaging. The role of LRE in luciferin-regeneration is still unclear [~~23~~29]. As the substrate involved the LRE catalyzed luciferin-regeneration, D-cysteine was reported to be characterized as a positive factor to Fluc light-generation in the absence of LRE [13,~~24~~30]. The results in this article also demonstrated that D-cysteine caused an extra increase of luminescence intensity to the Fluc structure (Fig 3). Besides, the

optimal enzyme complex, Fluc-R-LRE, ~~performs~~ performed better bioluminescence in the presence of 0.4 mmol/L DDT, 0.4 mmol/L EDTA, 0.8 mol/L fucose and 1.0 mg/L BSA, and present~~s~~ negative effect by Triton X-100 and glyerinum (Fig 7).

The ATP *in vitro* detection was limited by the bioluminescence intensity and rapid decaying of signal. The intensity of signal refers to precision while the stability of signal refers to the accuracy of detection. In this study, the bifunctional enzyme complex with appropriate structure still generated detectable signal after 6 hours. Meanwhile, the intervention of fused LRE significantly improved the stability of luminescence and prolonged the half-life of signal, which might advance and expand the application of Fluc catalyzed bioluminescence imaging.

5. CONCLUSION

In this study, we demonstrated a novel strategy on fusion expression of Fluc and LRE to improve the bioluminescence imaging using a series of peptide linker. The effect of peptide linker on the bioluminescence imaging was analyzed according to the initial luminescence intensity, decaying kinetic of bifunctional enzyme complex, and the computer simulation of structural feature. The findings revealed the relationship of wane and wax between the activity of fused Fluc and LRE, which indicated the competitive binding of oxyluciferin between Fluc and LRE. The Fluc catalyzed light emission that determined the sensitivity of detection, and the activity was the highest in the space made by one copy number of rigid linker; whilst the LRE catalyzed luciferin regeneration that determined the durability of signal, and the

activity was the most effective with the folding caused by two copy numbers of flexible linker. The bioluminescent property of the optimal structure of Fluc-R-LRE was observed with positive effects in DDT, EDTA, fucose and BSA, besides D-cysteine, and negative effects in Triton X-100 and glyerinum.

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CONFLICT OF INTEREST

No conflict of interest exits in the submission of this manuscript.

AUTHOR CONTRIBUTION

XS and CX contributed to the conception of the study; XS performed the experiments, analyzed the data and wrote the manuscript; BF helped design the enzyme complexes shown in Table 1; PH helped design the experiments shown in Fig 2 & Fig 4; XT and PH contributed significantly to analysis the results; RH and MR helped perform the experiments and analysis with constructive discussions. All authors reviewed the results and approved the final version of the manuscript.

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TABLE LEGENDS

Table 1. Sequences of PCR primers in this study.

Protein	Template	Primers	Sequences
Fluc	*Fluc*	Fluc-FusionP1	GGAATTC**CATATG** GAAGATGCCAAAAACATTAAGAAGG
		Fluc-FusionP2	CCG**CTCGAG**TTA CACGGCGATCTTGCCGCCCTT
LRE	*Lre*	LRE-P1	GGAATTC**CATATG**GGCCCCGTAGTTGAAAAGATCG
		LRE-P2	CCG**CTCGAG**TTACAATTTAACTTTAACACCAGCAAAACCTTTCAC
Fluc-LRE	*Fluc*	Fluc-FusionP1	GGAATTC**CATATG** GAAGATGCCAAAAACATTAAGAAGG
		Fluc-LRE-Rev-P2	CGATCTTTTCAACTACGGGGCCCAT***CTCCTTCTTAAAGTTAAACAAAATTATTTCTAGAGGGGAATTGTTATCCG*** ***CTCACAATTCCCCTTATATAGTGAGTCGTATTA***TTACACGGCGATCTTGCCGC
	Lre	Fluc-LRE-For-P1	GCGGCAAGATCGCCGTGTAA***TAATACGACTCACTATATAAGGGGAATTGTGAGCGGATAACAATTCCCCTCTA*** ***GAAATAATTTTGTTTAACTTTAAGAAGGAG***ATGGGCCCCGTAGTTGAAAAGATCG

		LRE-P2	CCG**CTCGAG**TTACAATTTAACTTTAACACCAGCAAAACCTTTCAC
Fluc-R-LRE	*Fluc*	Fluc-FusionP1	GGAATTC**CATATG** GAAGATGCCAAAAACATTAAGAAGG
		Fluc-R-FusionP2	CGATCTTTTCAACTACGGGGCCCAT**TTTAGCAGCAGCTTC**CACGGCGATCTTGCCGC
	Lre	LRE-R-FusionP1	GCGGCAAGATCGCCGTG**GAAGCTGCTGCTAAA**ATGGGCCCCGTAGTTGAAAAGATCG
		LRE-P2	CCG**CTCGAG**TTACAATTTAACTTTAACACCAGCAAAACCTTTCAC
Fluc-RR-LRE	*Fluc*	Fluc-FusionP1	GGAATTC**CATATG** GAAGATGCCAAAAACATTAAGAAGG
		Fluc-RR-FusionP2	CGATCTTTTCAACTACGGGGCCCAT**TTTAGCAGCAGCTTCTTTAGCAGCAGCTTC**CACGGCGATCTTGCCGC
	Lre	LRE-RR-FusionP1	GCGGCAAGATCGCCGTG**GAAGCTGCTGCTAAAGAAGCTGCTGCTAAA**ATGGGCCCCGTAGTTGAAAAGATCG
		LRE-P2	CCG**CTCGAG**TTACAATTTAACTTTAACACCAGCAAAACCTTTCAC
Fluc-RRR-LRE	*Fluc*	Fluc-FusionP1	GGAATTC**CATATG** GAAGATGCCAAAAACATTAAGAAGG
		Fluc-RRR-FusionP2	CGATCTTTTCAACTACGGGGCCCAT**TTTAGCAGCAGCTTCTTTAGCAGCAGCTTCTTTAGCAGCAGCTTC**CACGG CGATCTTGCCGC
	Lre	LRE-RRR-FusionP1	GCGGCAAGATCGCCGTG**GAAGCTGCTGCTAAAGAAGCTGCTGCTAAAGAAGCTGCTGCTAAA**ATGGGCCCCG

			TAGTTGAAAAGATCG
		LRE-P2	CCG**CTCGAG**TTACAATTTAACTTTAACACCAGCAAAACCTTTCAC
Fluc-S-LRE	*Fluc*	Fluc-FusionP1	GGAATTC**CATATG** GAAGATGCCAAAAACATTAAGAAGG
		Fluc-S-FusionP2	CGATCTTTTCAACTACGGGGCCC**ATAGAACCACCACCACC**CACGGCGATCTTGCCGC
	Lre	LRE-S-FusionP1	GCGGCAAGATCGCCGTG**GGTGGTGGTGGTTCT**ATGGGCCCCGTAGTTGAAAAGATCG
		LRE-P2	CCG**CTCGAG**TTACAATTTAACTTTAACACCAGCAAAACCTTTCAC
Fluc-SS-LRE	*Fluc*	Fluc-FusionP1	GGAATTC**CATATG** GAAGATGCCAAAAACATTAAGAAGG
		Fluc-SS-FusionP2	CGATCTTTTCAACTACGGGGCCC**ATAGAACCACCACCACCATAGAACCACCACCACC**CACGGCGATCTTGCCGC
	Lre	LRE-SS-FusionP1	GCGGCAAGATCGCCGTG**GGTGGTGGTGGTTCTGGTGGTGGTGGTTCT**ATGGGCCCCGTAGTTGAAAAGATCG
		LRE-P2	CCG**CTCGAG**TTACAATTTAACTTTAACACCAGCAAAACCTTTCAC
Fluc-SSS-LRE	*Fluc*	Fluc-FusionP1	GGAATTC**CATATG** GAAGATGCCAAAAACATTAAGAAGG
		Fluc-SSS-FusionP2	CGATCTTTTCAACTACGGGGCCC**ATAGAACCACCACCACCATAGAACCACCACCACCATAGAACCACCACCACC** CACGGCGATCTTGCCGC

	Lre	LRE-SSS-FusionP1	GCGGCAAGATCGCCGTG**GGTGGTGGTGGTTCTGGTGGTGGTGGTTCTGGTGGTGGTGGTTCT**ATGGGCCCCG TAGTTGAAAAGATCG
		LRE-P2	CCG**CTCGAG**TTACAATTTAACTTTAACACCAGCAAAACCTTTCAC
Fluc-RS-LRE	*Fluc*	Fluc-FusionP1	GGAATTC**CATATG** GAAGATGCCAAAAACATTAAGAAGG
		Fluc-RS-FusionP2	CGATCTTTTCAACTACGGGGCCCAT**TTTAGCAGCAGCTTCAGAACCACCACCACC**CACGGCGATCTTGCCGC
	Lre	LRE-RS-FusionP1	GCGGCAAGATCGCCGTG**GAAGCTGCTGCTAAAGGTGGTGGTGGTTCT**ATGGGCCCCGTAGTTGAAAAGATCG
		LRE-P2	CCG**CTCGAG**TTACAATTTAACTTTAACACCAGCAAAACCTTTCAC
Fluc-SR-LRE	*Fluc*	Fluc-FusionP1	GGAATTC**CATATG** GAAGATGCCAAAAACATTAAGAAGG
		Fluc-SR-FusionP2	CGATCTTTTCAACTACGGGGCCCAT**AGAACCACCACCACCTTTAGCAGCAGCTTC**CACGGCGATCTTGCCGC
	Lre	LRE-SR-FusionP1	GCGGCAAGATCGCCGTG**GGTGGTGGTGGTTCTGAAGCTGCTGCTAAA**ATGGGCCCCGTAGTTGAAAAGATCG
		LRE-P2	CCG**CTCGAG**TTACAATTTAACTTTAACACCAGCAAAACCTTTCAC
Fluc-RSR-	*Fluc*	Fluc-FusionP1	GGAATTC**CATATG** GAAGATGCCAAAAACATTAAGAAGG
		Fluc-RSR-FusionP2	CGATCTTTTCAACTACGGGGCCCAT**TTTAGCAGCAGCTTCAGAACCACCACCACCTTTAGCAGCAGCTTC**CACG

~~LRE~~			~~GCGATCTTGCCGC~~
	~~*Lre*~~	~~LRE-RSR-FusionP1~~	~~GCGGCAAGATCGCCGTG**GAAGCTGCTGCTAAAGGTGGTGGTGGTTCTGAAGCTGCTGCTAAA**ATGGGCCCCG TAGTTGAAAAGATCG~~
		~~LRE-P2~~	~~CCG**CTCGAG**TTACAATTTAACTTTAACACCAGCAAAACCTTTCAC~~
~~Fluc-SRS-LRE~~	~~*Fluc*~~	~~Fluc-FusionP1~~	~~GGAATTC**CATATG** GAAGATGCCAAAAACATTAAGAAGG~~
		~~Fluc-SRS-FusionP2~~	~~CGATCTTTTCAACTACGGGGCCCAT**AGAACCACCACCACCTTTAGCAGCAGCTTCAGAACCACCACCACC**CACG GCGATCTTGCCGC~~
	~~*Lre*~~	~~LRE-SRS-FusionP1~~	~~GCGGCAAGATCGCCGTG**GAAGCTGCTGCTAAAGAAGCTGCTGCTAAAGGTGGTGGTGGTTCT**ATGGGCCCCG TAGTTGAAAAGATCG~~
		~~LRE-P2~~	~~CCG**CTCGAG**TTACAATTTAACTTTAACACCAGCAAAACCTTTCAC~~

Table ~~2~~1. Construction and qualification of enzyme complex.

Fusion enzyme	sketch map	abbreviation	Amino acid sequence	Oligonucleotide sequence	Concentration (μg/ml)
Fluc	Fluc	/	/	/	597.26

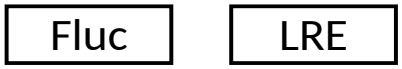
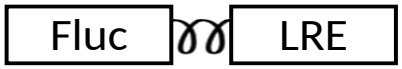
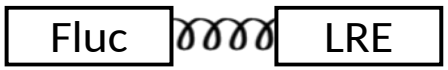
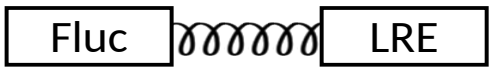
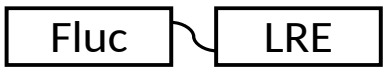
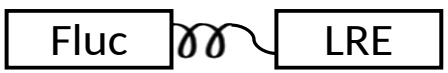
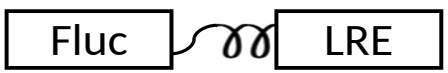
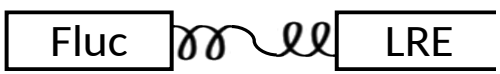
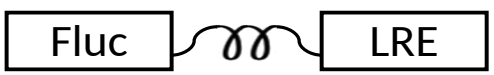
Fluc-LRE		/	/	/	594.04
Fluc-R-LRE		R	(EAAAK)	GAAGCTGCTGCTAAA	885.75
Fluc-RR-LRE		RR	(EAAAKEAAAK)	GAAGCTGCTGCTAAAGAAGCTGCTGCTAAA	580.60
Fluc-RRR-LRE		RRR	(EAAAKEAAAKEAAAK)	GAAGCTGCTGCTAAAGAAGCTGCTGCTAAAGAAGCTGCTGCTAAA	599.31
Fluc-S-LRE		S	(GGGGS)	GGTGGTGGTGGTTCT	610.30
Fluc-SS-LRE		SS	(GGGGSGGGGS)	GGTGGTGGTGGTTCTGGTGGTGGTGGTTCT	551.22
Fluc-SSS-LRE		SSS	(GGGGSGGGGSGGGGS)	GGTGGTGGTGGTTCTGGTGGTGGTGGTTCTGGTGGTGGTGGTTCT	581.81
Fluc-RS-LRE		RS	(EAAAKGGGGS)	GAAGCTGCTGCTAAAGGTGGTGGTGGTTCT	648.61
Fluc-SR-LRE		SR	(GGGGSEAAAK)	GGTGGTGGTGGTTCTGAAGCTGCTGCTAAA	577.09
Fluc-RSR-LRE		RSR	(EAAAKGGGGSEAAAK)	GAAGCTGCTGCTAAAGGTGGTGGTGGTTCTGAAGCTGCTGCTAAA	590.98
Fluc-SRS-LRE		SRS	(GGGGSEAAAKGGGGS)	GGTGGTGGTGGTTCTGAAGCTGCTGCTAAAGGTGGTGGTGGTTCT	959.96

Table ~~3~~2. Kinetic properties of enzyme complexs depending on enzyme structure.

Enzyme structure	Initial light intensity(RLU)	K1	K2	half-life (h^{-1})
Fluc	1.36E+10	1.526±0.056	9.617±0.632	0.14
Fluc-LRE	1.49E+10	3.888±1.106	1.061±0.138	0.37
R	1.06E+10	0.742±0.037	4.357±0.997	0.54
RR	5.99E+08	2.976±0.153	0.661±0.009	0.61
RRR	2.72E+08	0.403±0.016	1.705±0.061	0.64
S	6.66E+08	0.643±0.010	3.057±0.141	0.55
SS	3.21E+08	1.776±0.043	0.300±0.017	0.54
SSS	7.56E+08	2.683±0.114	0.643±0.020	0.44
RS	3.78E+08	0.865±0.015	3.710±0.484	0.61
SR	2.14E+08	4.359±0.532	0.787±0.012	0.64
RSR	9.92E+08	1.096±0.026	6.719±1.173	0.54
SRS	2.04E+08	0.292±0.005	4.803±2.948	2.20

FIGURE LEGENDS

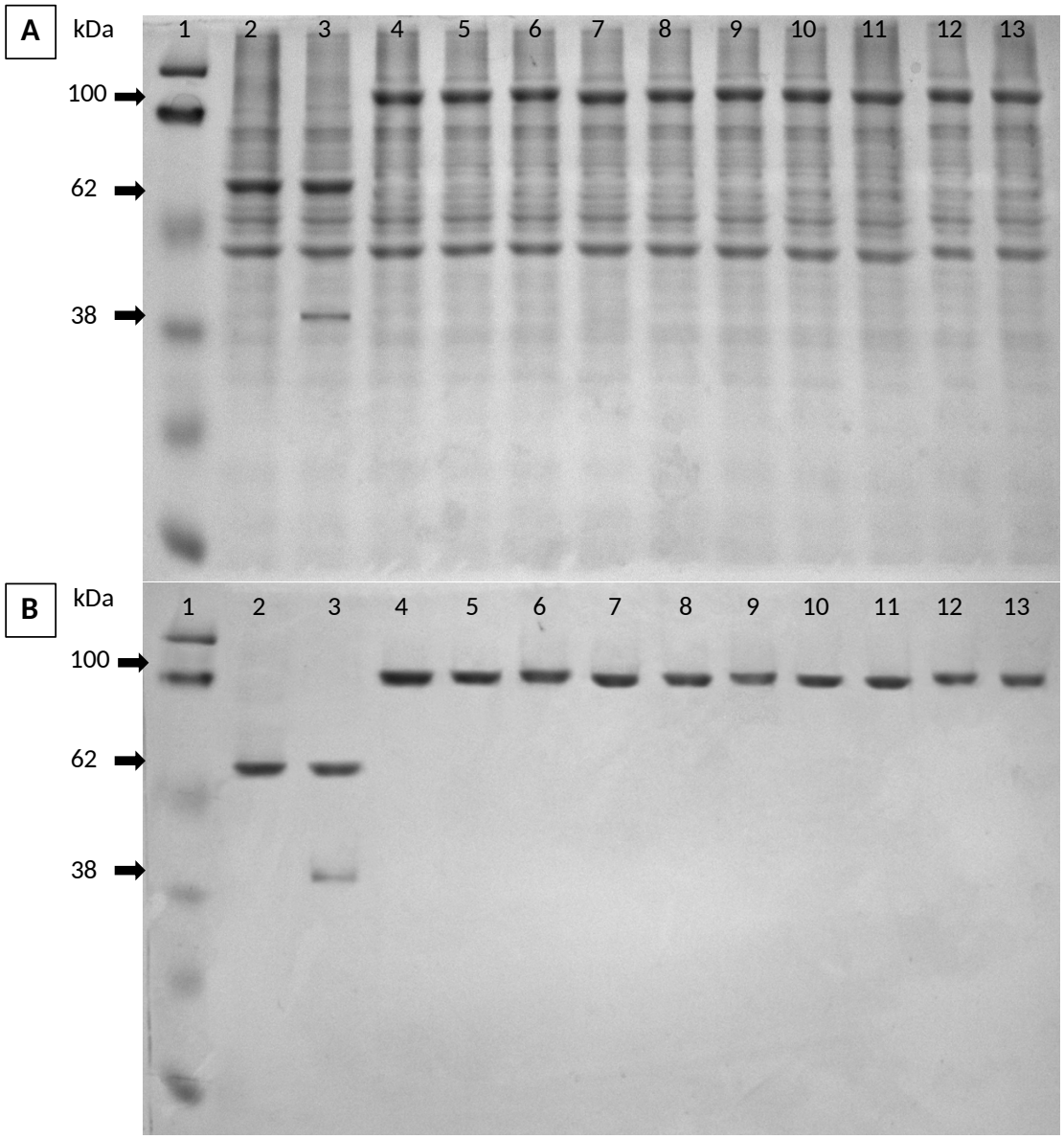


Fig 1. SDS-PAGE analysis of the enzyme complex expressed with IPTG induction.

Panel A: crude samples; Panel B: purified samples. Lane 1, Protein Marker; lane 2, Fluc; lane 3, Fluc & LRE; lane 4, Fluc-R-LRE; lane 5, Fluc-RR-LRE; lane 6, Fluc-RRR-LRE; lane 7, Fluc-S-LRE; lane 8, Fluc-SS-LRE; lane 9, Fluc-SSS-LRE; lane 10, Fluc-RS-LRE; lane 11, Fluc-SR-LRE; lane 12, Fluc-RSR-LRE; lane 13, Fluc-SRS-LRE.

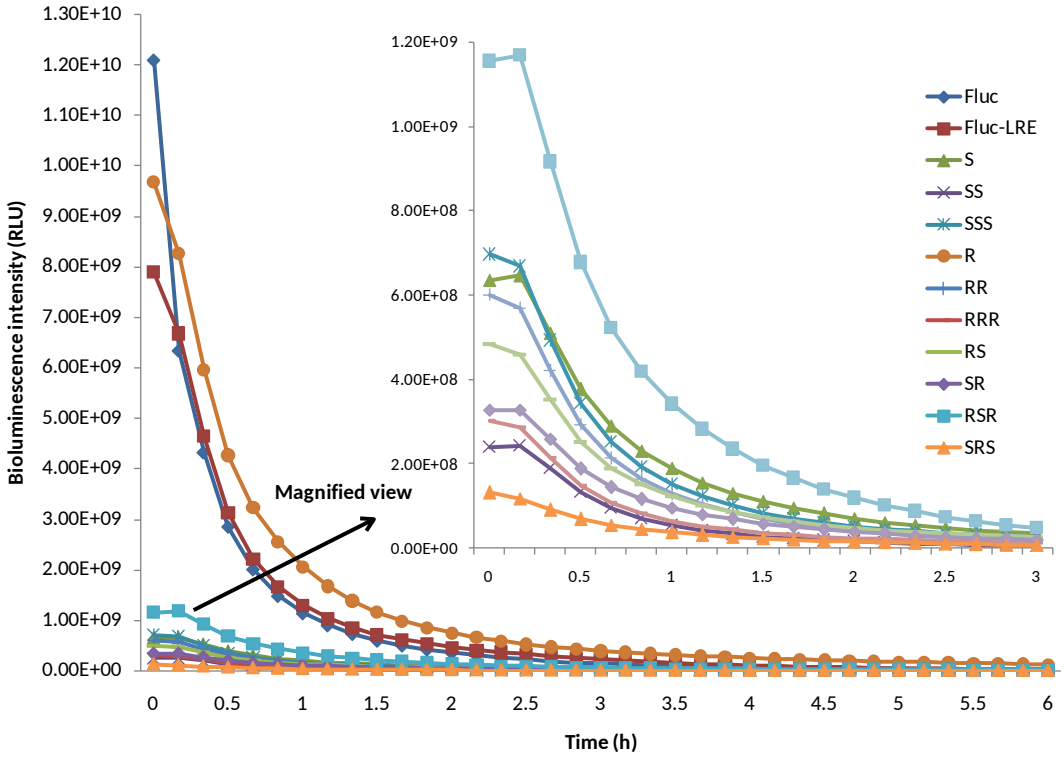


Fig 2. *In vitro* luminescence assessment of signal generated by purified enzyme complex in the absence of D-cysteine.

40 µL of purified enzyme was added to 160 µL reaction mixture containing with 0.25 mM D-luciferin, 2.5mM D-cysteine, 4 mM ATP and 10 mM $MgSO_4$ in microplate. The luminescence generated by each enzyme complex was measured by Tecan Infinite M200 Pro. at 37 ℃.

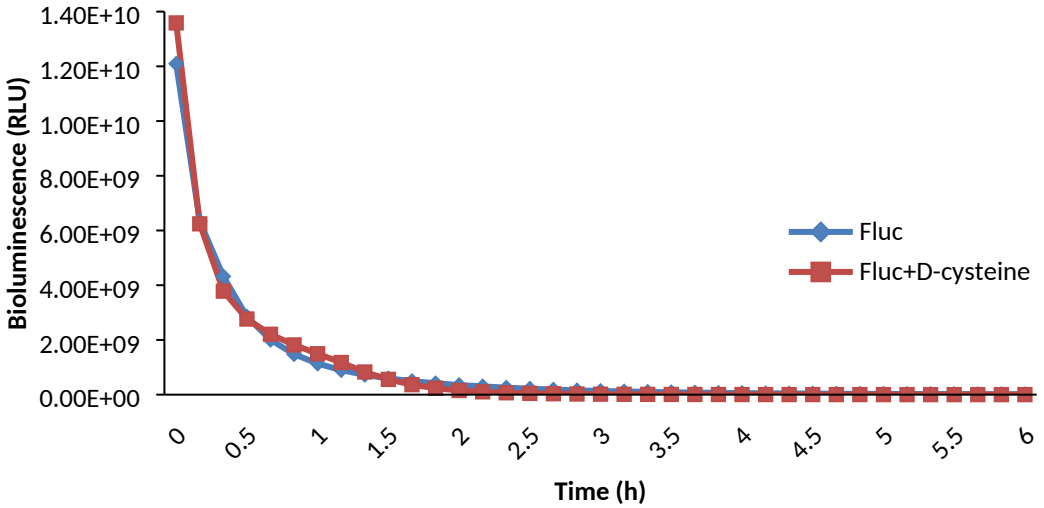
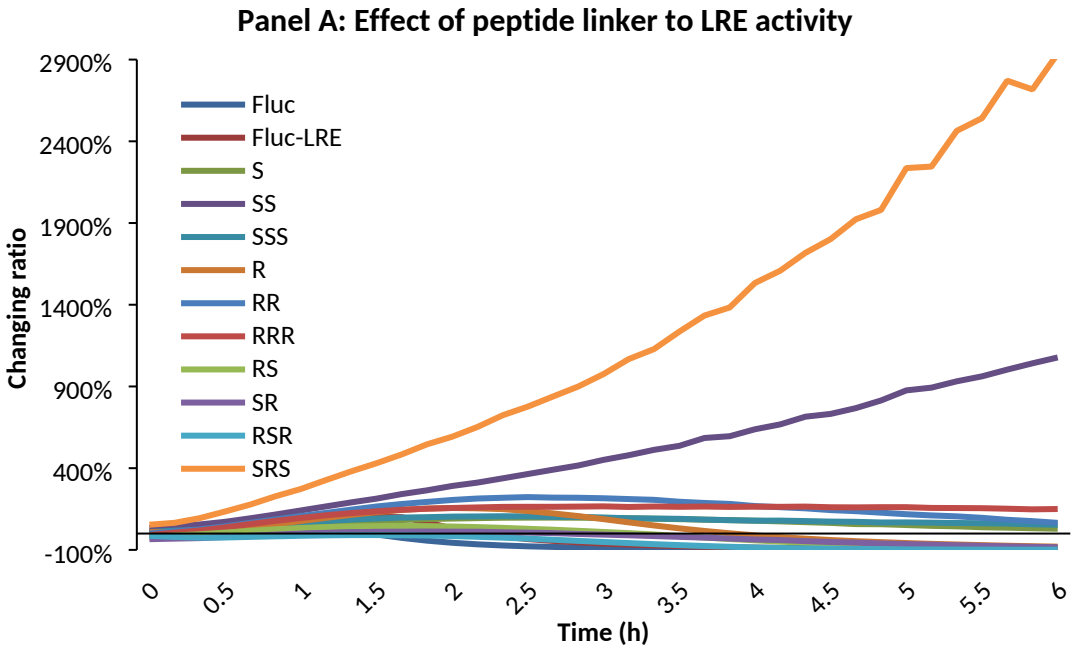


Fig 3. Effect of D-cysteine to the *in vitro* luminescence in the absence of LRE

2.5mM D-cysteine was added to the mixture of purified Fluc containing with 0.25 mM D-luciferin, 4 mM ATP and 10 mM $MgSO_4$. Luminescence generated by Fluc in the absence of D-cysteine and in the presence of D-cysteine was measured by Tecan Infinite M200 Pro. at 37 °C.



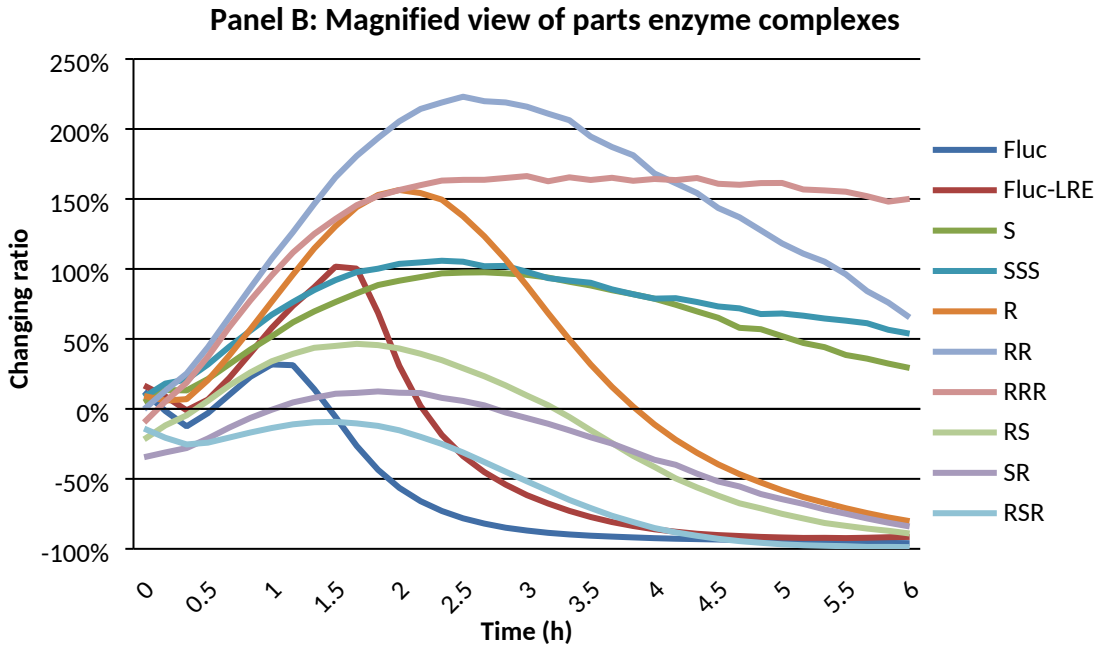
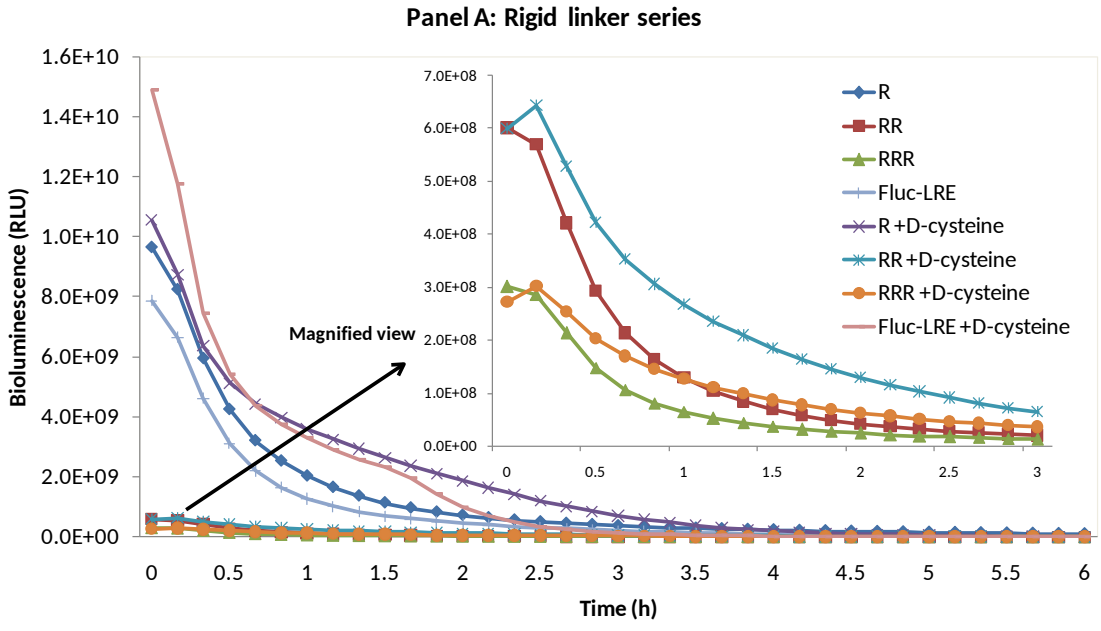
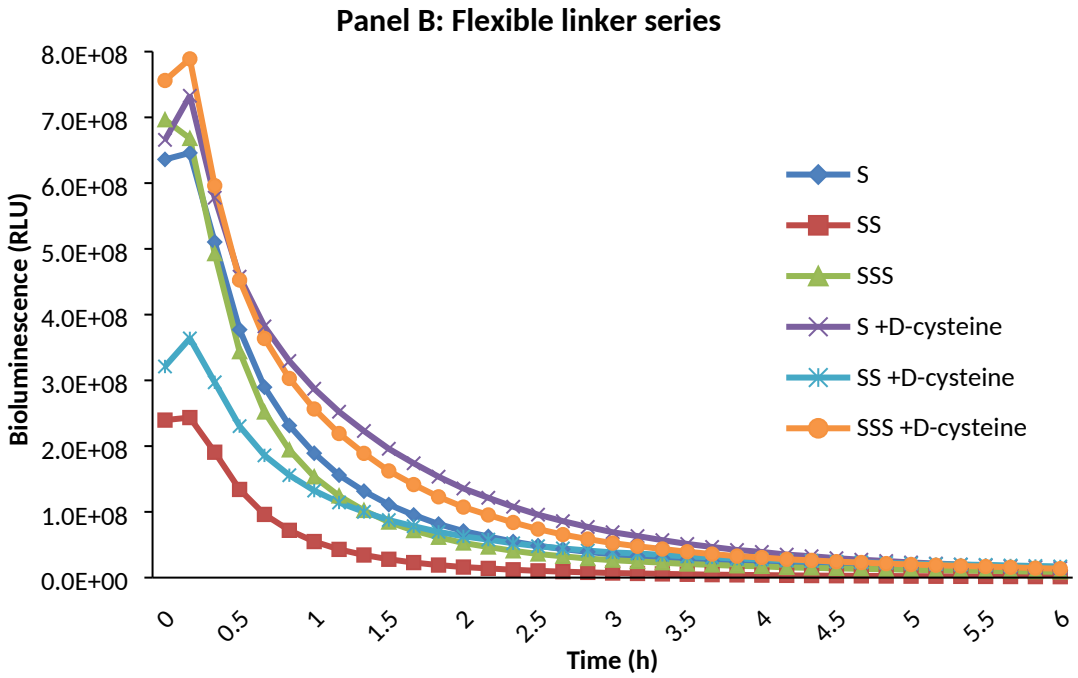


Fig 4. Effect of peptide linker on the *in vitro* activities of fused LRE and the luciferin-regeneration

0.25 mM D-luciferin and 2.5 mM D-cysteine were injected simultaneously to the enzyme mixture and trigger the catalytic reactions by both Fluc and LRE. The luminescence intensity was measured by Tecan Infinite M200 Pro. at 37 ℃.





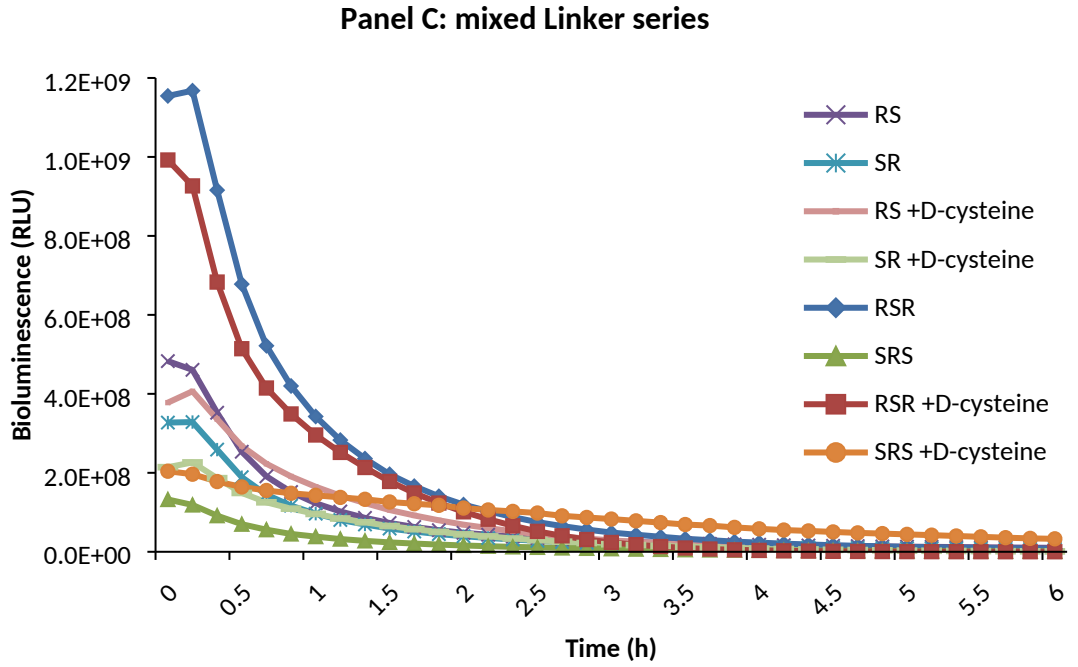


Fig 5. *In vitro* luminescence assessment of signal generated by purified Fluc-LRE enzyme complex in the presence of D-luciferin and D-cysteine.

40 µL of purified enzyme was added to 160 µL reaction mixture containing 0.25 mM D-luciferin, 2.5mM D-cysteine, 4 mM ATP and 10 mM $MgSO_4$ in microplate. The luminescence intensity was measured by Tecan Infinite M200 Pro. at 37 ℃.

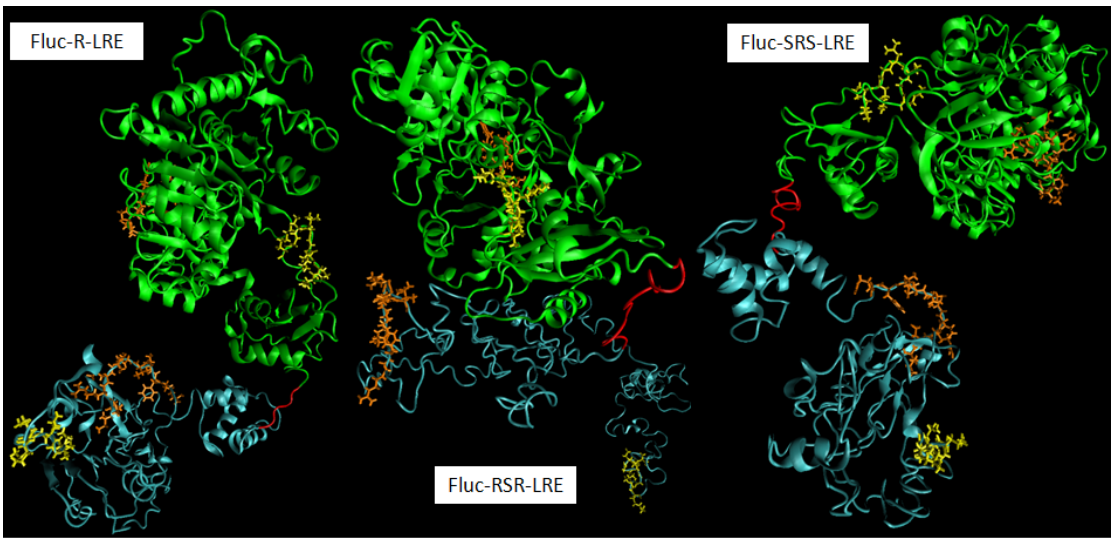
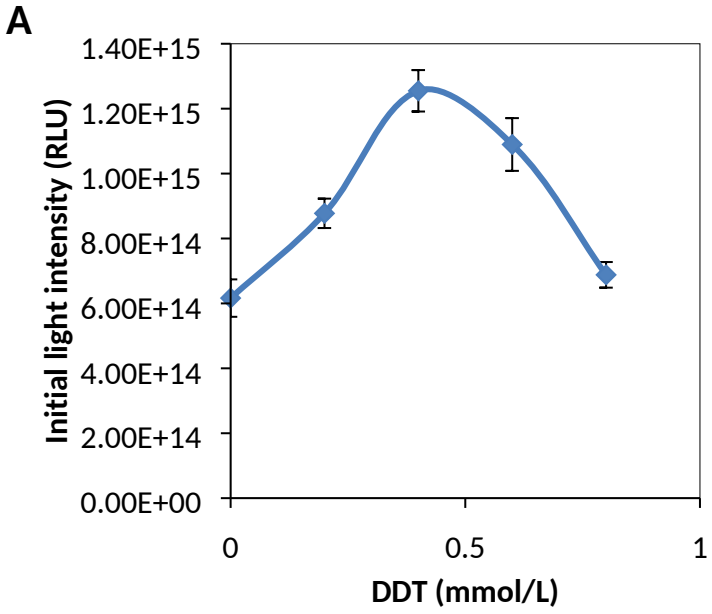
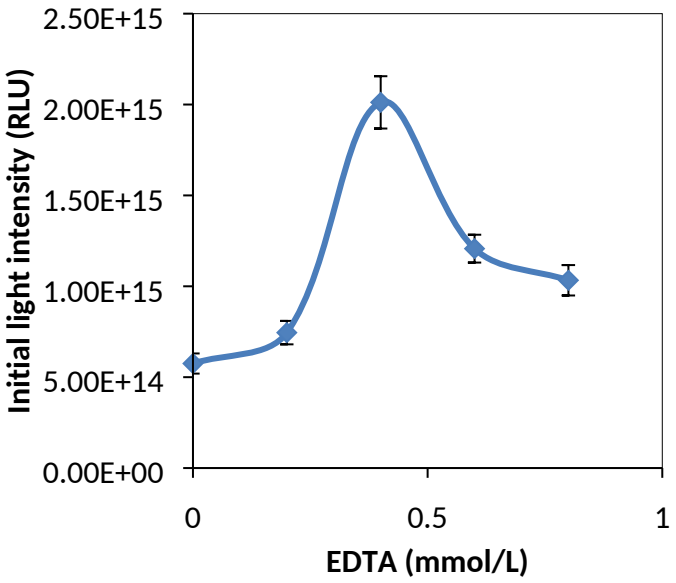


Fig 6. Enzyme complex structural models

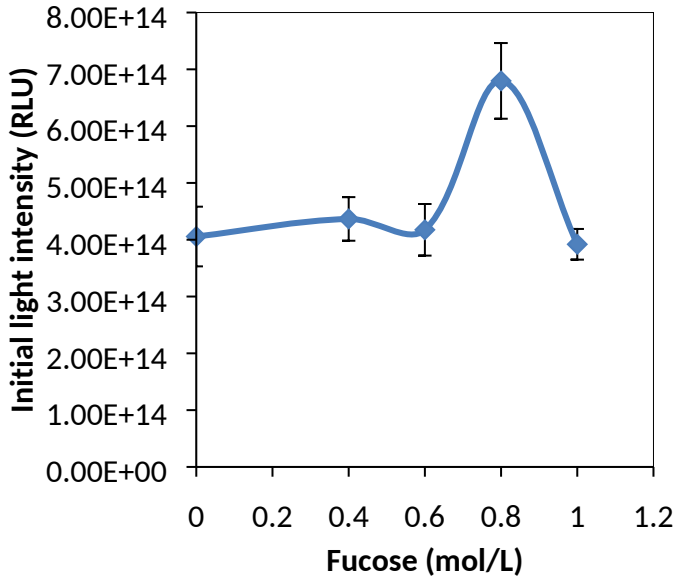
The 3D structure models of enzyme complexes were simulated and predicted by the I-TASSER server and analyzed by the software VMD 1.8.3. Three typical structures with highest confidence were present. The domain of Fluc (in Green), LRE (in Cyan), peptide linker (in Red), LBD I (in Orange) and LBD II (in Yellow) were labeled.



B


2.50E+15
2.00E+15
1.50E+15
1.00E+15
5.00E+14
0.00E+00
Initial light intensity (RLU)
0
0.5
1
EDTA (mmol/L)

C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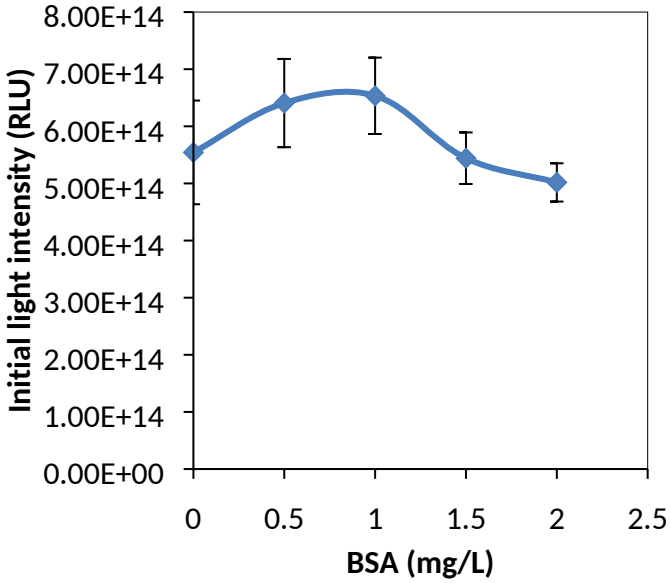
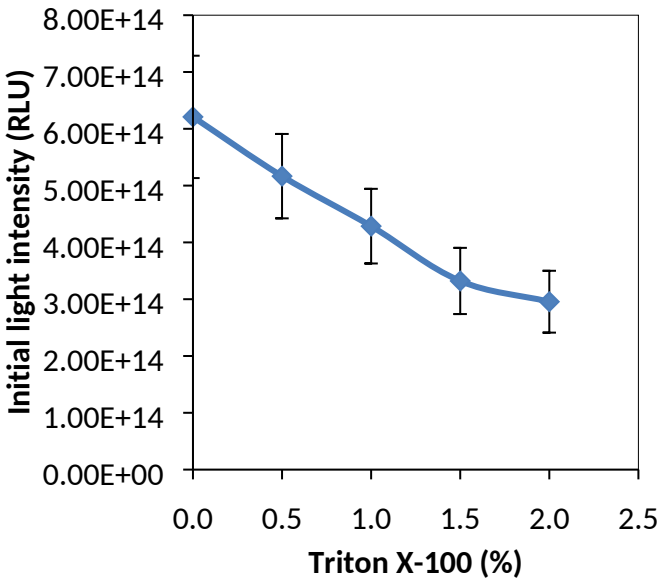
7.00E+14
6.00E+14
5.00E+14
4.00E+14
3.00E+14
2.00E+14
1.00E+14
0.00E+00
Initial light intensity (RLU)
0
0.2
0.4
0.6
0.8
1
1.2
Fucose (mol/L)

D


8.00E+14
7.00E+14
6.00E+14
5.00E+14
4.00E+14
3.00E+14
2.00E+14
1.00E+14
0.00E+00
Initial light intensity (RLU)
0
0.5
1
1.5
2
2.5
BSA (mg/L)

E


8.00E+14
7.00E+14
6.00E+14
5.00E+14
4.00E+14
3.00E+14
2.00E+14
1.00E+14
0.00E+00
Initial light intensity (RLU)
0.0
0.5
1.0
1.5
2.0
2.5
Triton X-100 (%)

F

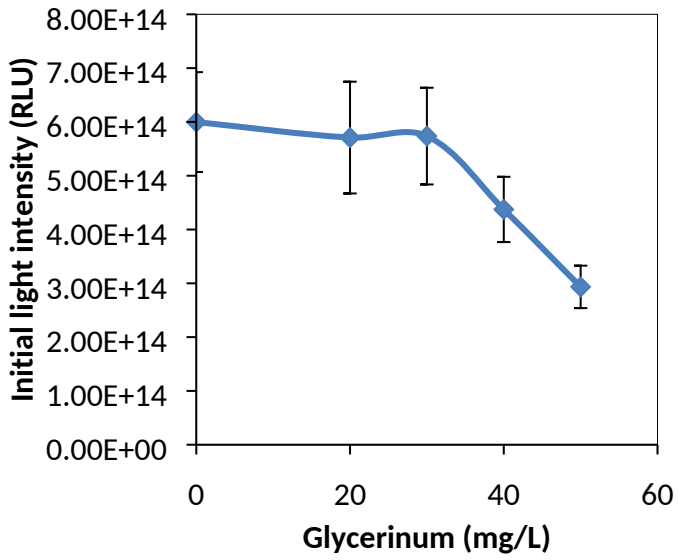


Fig 7. Effect of organic reagents to the bioluminescent property of optimal enzyme complex

Bioluminescent property of the optimal enzyme complex Fluc-R-LRE in organic reagents, different concentration of DDT (A), EDTA (B), fucose (C), BSA (D), Triton X-100 (E) and Glycerinum (F). 180 µL of purified Fluc-R-LRE was added to 20 µL reaction mixture of organic reagents containing 0.25 mM D-luciferin, 2.5mM D-cysteine, 4 mM ATP and 10 mM $MgSO_4$ in microplate. The luminescence intensity was measured by Tecan Infinite M200 Pro. at 37 ℃.

Appendix

Table 1. Sequences of PCR primers in this study.

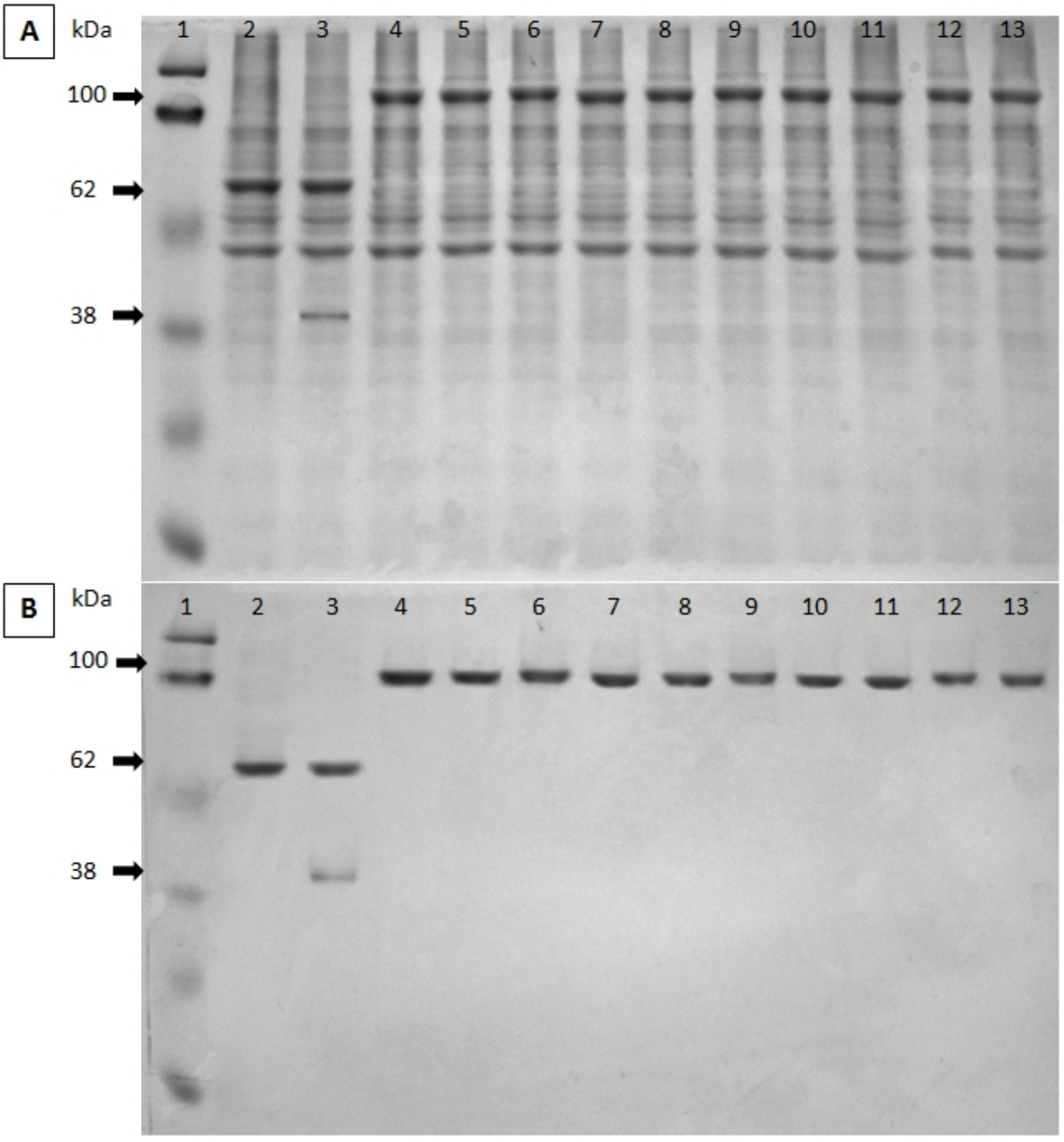
Protein	Template	Primers	Sequences
Fluc	*Fluc*	Fluc-FusionP1	GGAATTC**CATATG** GAAGATGCCAAAAACATTAAGAAGG
		Fluc-FusionP2	CCG**CTCGAG**TTA CACGGCGATCTTGCCGCCCTT
LRE	*Lre*	LRE-P1	GGAATTC**CATATG**GGCCCCGTAGTTGAAAAGATCG
		LRE-P2	CCG**CTCGAG**TTACAATTTAACTTTAACACCAGCAAAACCTTTCAC
Fluc-LRE	*Fluc*	Fluc-FusionP1	GGAATTC**CATATG** GAAGATGCCAAAAACATTAAGAAGG
		Fluc-LRE-Rev-P2	CGATCTTTTCAACTACGGGGCCCAT***CTCCTTCTTAAAGTTAAACAAAATTATTTCTAGAGGGGAATTGTTATCCG*** ***CTCACAATTCCCCTTATATAGTGAGTCGTATTA***TTACACGGCGATCTTGCCGC
	Lre	Fluc-LRE-For-P1	GCGGCAAGATCGCCGTGTAA***TAATACGACTCACTATATAAGGGGAATTGTGAGCGGATAACAATTCCCCTCTA*** ***GAAATAATTTTGTTTAACTTTAAGAAGGAG***ATGGGCCCCGTAGTTGAAAAGATCG

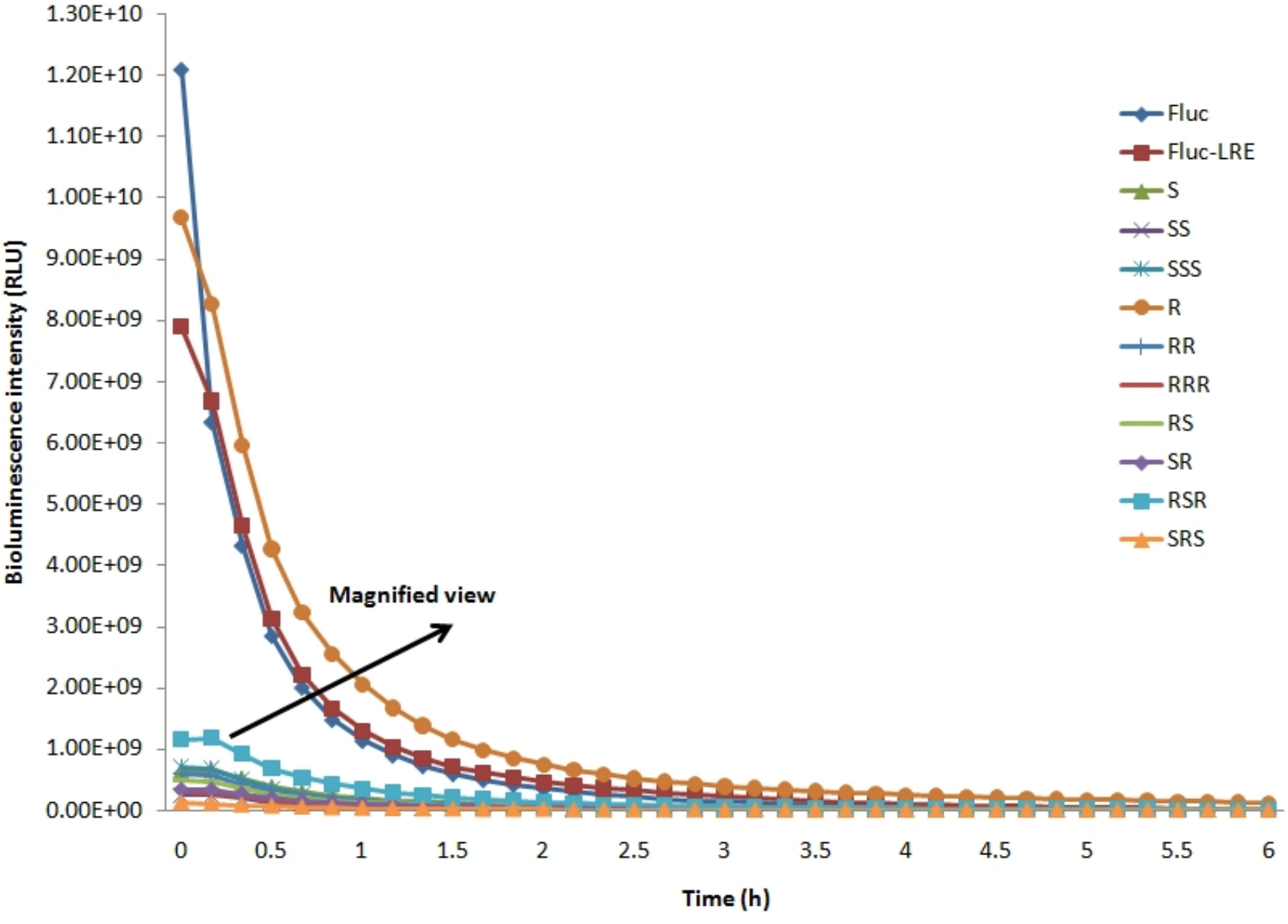
		LRE-P2	CCG**CTCGAG**TTACAATTTAACTTTAACACCAGCAAAACCTTTCAC
Fluc-R-LRE	*Fluc*	Fluc-FusionP1	GGAATTC**CATATG** GAAGATGCCAAAAACATTAAGAAGG
		Fluc-R-FusionP2	CGATCTTTTCAACTACGGGGCCCAT**TTTAGCAGCAGCTTC**CACGGCGATCTTGCCGC
	Lre	LRE-R-FusionP1	GCGGCAAGATCGCCGTG**GAAGCTGCTGCTAAA**ATGGGCCCCGTAGTTGAAAAGATCG
		LRE-P2	CCG**CTCGAG**TTACAATTTAACTTTAACACCAGCAAAACCTTTCAC
Fluc-RR-LRE	*Fluc*	Fluc-FusionP1	GGAATTC**CATATG** GAAGATGCCAAAAACATTAAGAAGG
		Fluc-RR-FusionP2	CGATCTTTTCAACTACGGGGCCCAT**TTTAGCAGCAGCTTCTTTAGCAGCAGCTTC**CACGGCGATCTTGCCGC
	Lre	LRE-RR-FusionP1	GCGGCAAGATCGCCGTG**GAAGCTGCTGCTAAAGAAGCTGCTGCTAAA**ATGGGCCCCGTAGTTGAAAAGATCG
		LRE-P2	CCG**CTCGAG**TTACAATTTAACTTTAACACCAGCAAAACCTTTCAC
Fluc-RRR-LRE	*Fluc*	Fluc-FusionP1	GGAATTC**CATATG** GAAGATGCCAAAAACATTAAGAAGG
		Fluc-RRR-FusionP2	CGATCTTTTCAACTACGGGGCCCAT**TTTAGCAGCAGCTTCTTTAGCAGCAGCTTCTTTAGCAGCAGCTTC**CACGG CGATCTTGCCGC
	Lre	LRE-RRR-FusionP1	GCGGCAAGATCGCCGTG**GAAGCTGCTGCTAAAGAAGCTGCTGCTAAAGAAGCTGCTGCTAAA**ATGGGCCCCG

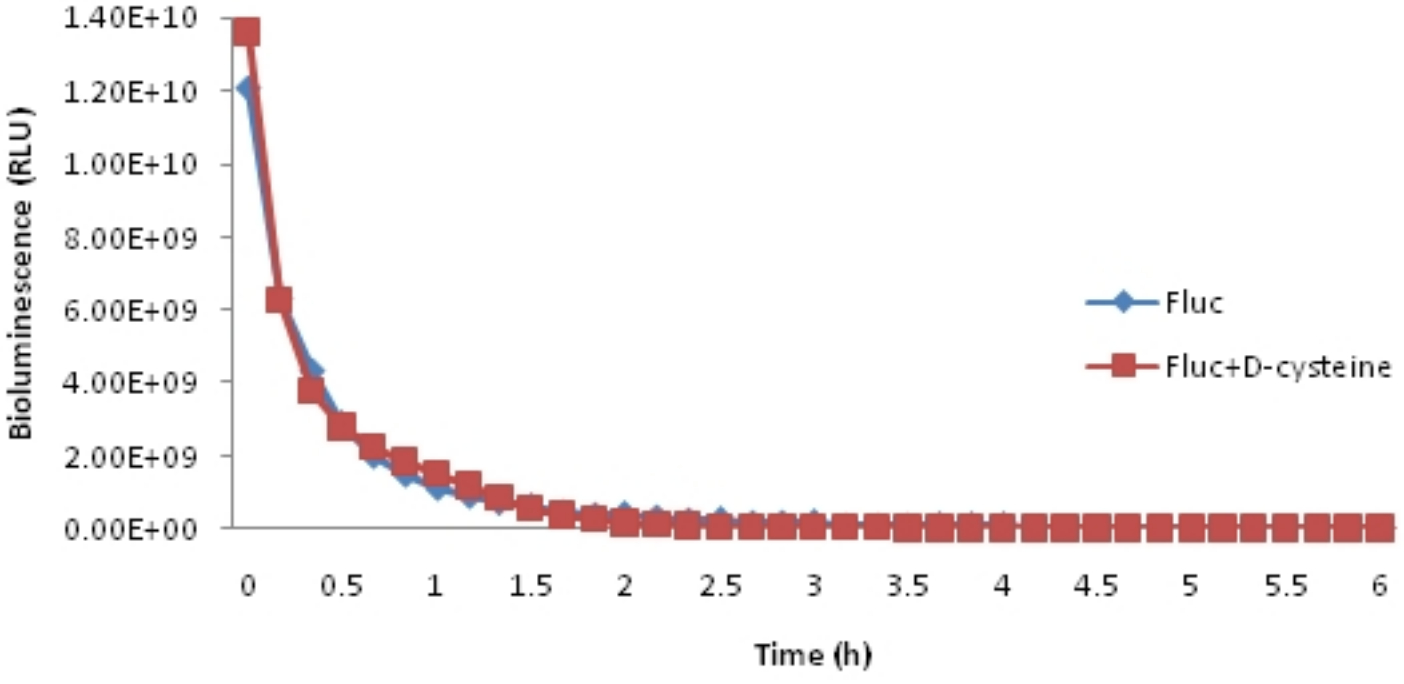
			TAGTTGAAAAGATCG
		LRE-P2	CCG**CTCGAG**TTACAATTTAACTTTAACACCAGCAAAACCTTTCAC
Fluc-S-LRE	*Fluc*	Fluc-FusionP1	GGAATTC**CATATG** GAAGATGCCAAAAACATTAAGAAGG
		Fluc-S-FusionP2	CGATCTTTTCAACTACGGGGCCC**ATAGAACCACCACCACC**CACGGCGATCTTGCCGC
	Lre	LRE-S-FusionP1	GCGGCAAGATCGCCGTG**GGTGGTGGTGGTTCT**ATGGGCCCCGTAGTTGAAAAGATCG
		LRE-P2	CCG**CTCGAG**TTACAATTTAACTTTAACACCAGCAAAACCTTTCAC
Fluc-SS-LRE	*Fluc*	Fluc-FusionP1	GGAATTC**CATATG** GAAGATGCCAAAAACATTAAGAAGG
		Fluc-SS-FusionP2	CGATCTTTTCAACTACGGGGCCC**ATAGAACCACCACCACCATAGAACCACCACCACC**CACGGCGATCTTGCCGC
	Lre	LRE-SS-FusionP1	GCGGCAAGATCGCCGTG**GGTGGTGGTGGTTCTGGTGGTGGTGGTTCT**ATGGGCCCCGTAGTTGAAAAGATCG
		LRE-P2	CCG**CTCGAG**TTACAATTTAACTTTAACACCAGCAAAACCTTTCAC
Fluc-SSS-LRE	*Fluc*	Fluc-FusionP1	GGAATTC**CATATG** GAAGATGCCAAAAACATTAAGAAGG
		Fluc-SSS-FusionP2	CGATCTTTTCAACTACGGGGCCC**ATAGAACCACCACCACCATAGAACCACCACCACCATAGAACCACCACCACC**CACGGCGATCTTGCCGC

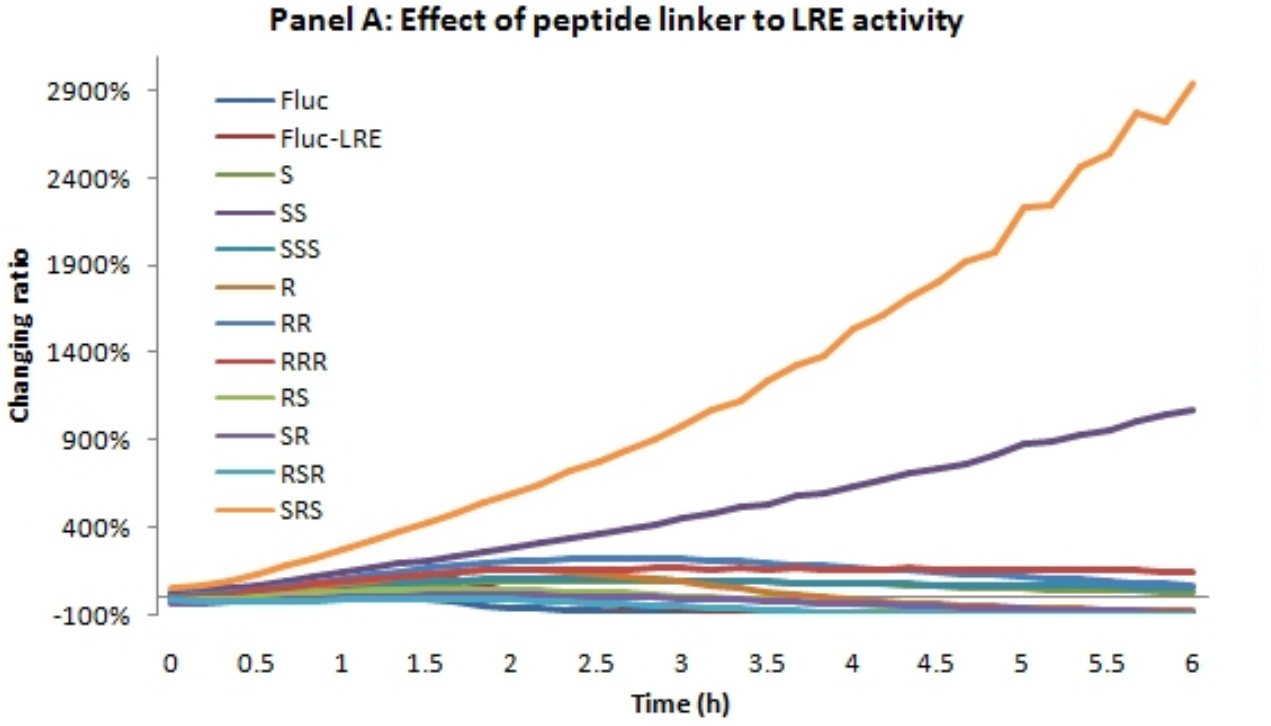
	Lre	LRE-SSS-FusionP1	GCGGCAAGATCGCCGTG**GGTGGTGGTGGTTCTGGTGGTGGTGGTTCTGGTGGTGGTGGTTCT**ATGGGCCCCG TAGTTGAAAAGATCG
		LRE-P2	CCG**CTCGAG**TTACAATTTAACTTTAACACCAGCAAAACCTTTCAC
Fluc-RS-LRE	*Fluc*	Fluc-FusionP1	GGAATTC**CATATG** GAAGATGCCAAAAACATTAAGAAGG
		Fluc-RS-FusionP2	CGATCTTTTCAACTACGGGGCCCAT**TTTAGCAGCAGCTTCAGAACCACCACCACC**CACGGCGATCTTGCCGC
	Lre	LRE-RS-FusionP1	GCGGCAAGATCGCCGTG**GAAGCTGCTGCTAAAGGTGGTGGTGGTTCT**ATGGGCCCCGTAGTTGAAAAGATCG
		LRE-P2	CCG**CTCGAG**TTACAATTTAACTTTAACACCAGCAAAACCTTTCAC
Fluc-SR-LRE	*Fluc*	Fluc-FusionP1	GGAATTC**CATATG** GAAGATGCCAAAAACATTAAGAAGG
		Fluc-SR-FusionP2	CGATCTTTTCAACTACGGGGCCCAT**AGAACCACCACCACCTTTAGCAGCAGCTTC**CACGGCGATCTTGCCGC
	Lre	LRE-SR-FusionP1	GCGGCAAGATCGCCGTG**GGTGGTGGTGGTTCTGAAGCTGCTGCTAAA**ATGGGCCCCGTAGTTGAAAAGATCG
		LRE-P2	CCG**CTCGAG**TTACAATTTAACTTTAACACCAGCAAAACCTTTCAC
Fluc-RSR-	*Fluc*	Fluc-FusionP1	GGAATTC**CATATG** GAAGATGCCAAAAACATTAAGAAGG
		Fluc-RSR-FusionP2	CGATCTTTTCAACTACGGGGCCCAT**TTTAGCAGCAGCTTCAGAACCACCACCACCTTTAGCAGCAGCTTC**CACG

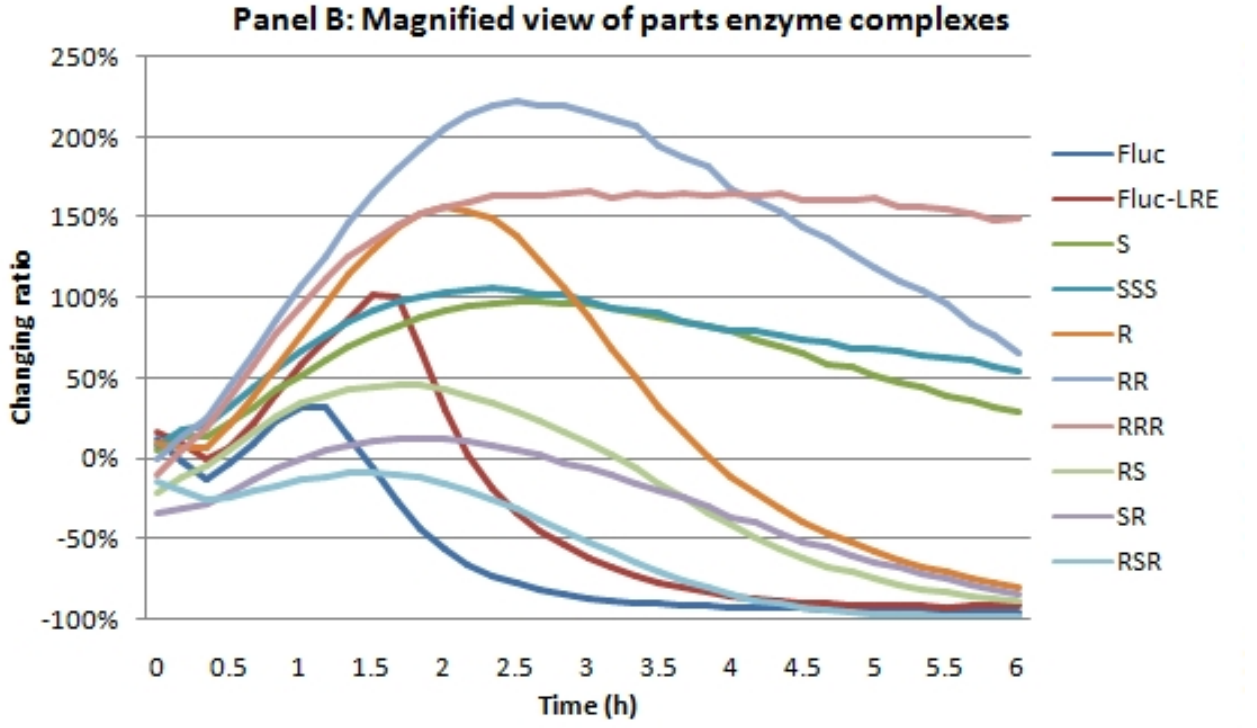
LRE			GCGATCTTGCCGC
	Lre	LRE-RSR-FusionP1	GCGGCAAGATCGCCGTG**GAAGCTGCTGCTAAAGGTGGTGGTGGTTCTGAAGCTGCTGCTAAA**ATGGGCCCCG TAGTTGAAAAGATCG
		LRE-P2	CCG**CTCGAG**TTACAATTTAACTTTAACACCAGCAAAACCTTTCAC
Fluc-SRS-LRE	*Fluc*	Fluc-FusionP1	GGAATTC**CATATG** GAAGATGCCAAAAACATTAAGAAGG
		Fluc-SRS-FusionP2	CGATCTTTTCAACTACGGGGCCCAT**AGAACCACCACCACCTTTAGCAGCAGCTTCAGAACCACCACCACC**CACG GCGATCTTGCCGC
	Lre	LRE-SRS-FusionP1	GCGGCAAGATCGCCGTG**GAAGCTGCTGCTAAAGAAGCTGCTGCTAAAGGTGGTGGTGGTTCT**ATGGGCCCCG TAGTTGAAAAGATCG
		LRE-P2	CCG**CTCGAG**TTACAATTTAACTTTAACACCAGCAAAACCTTTCAC


A
kDa
1
2
3
4
5
6
7
8
9
10
11
12
13
100
62
38
B
kDa
1
2
3
4
5
6
7
8
9
10
11
12
13
100
62
38

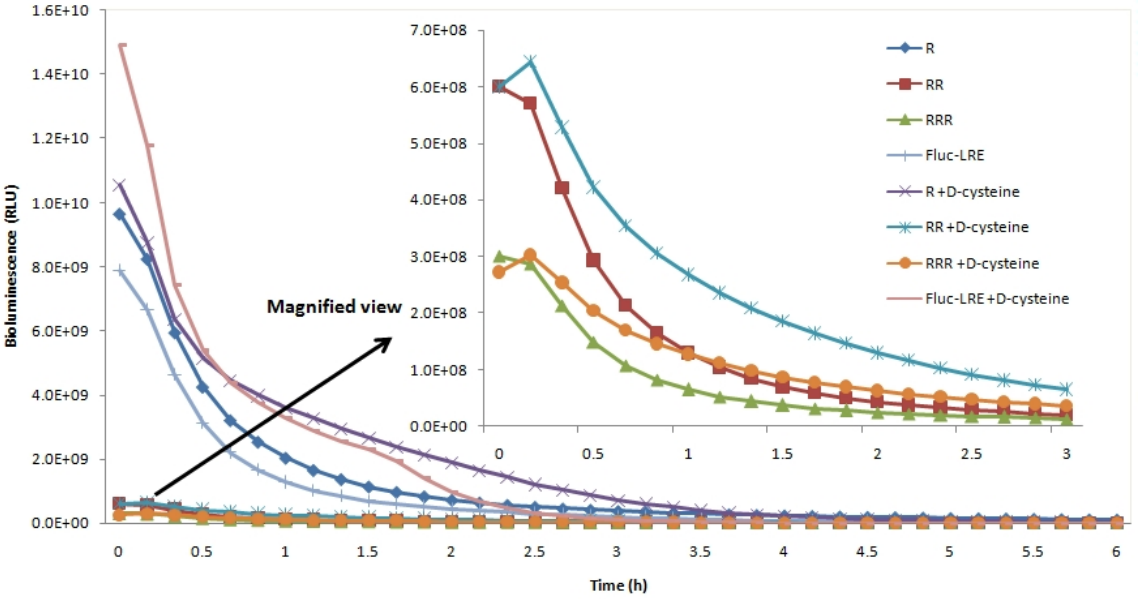

1.30E+10
1.20E+10
1.10E+10
1.00E+10
9.00E+09
8.00E+09
7.00E+09
6.00E+09
5.00E+09
4.00E+09
3.00E+09
2.00E+09
1.00E+09
0.00E+00
Bioluminescence intensity (RLU)
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
5.5
6
Time (h)
Magnified view
Fluc
Fluc-LRE
S
SS
SSS
R
RR
RRR
RS
SR
RSR
SRS


1.40E+10
1.20E+10
1.00E+10
8.00E+09
6.00E+09
4.00E+09
2.00E+09
0.00E+00
Bioluminescence (RLU)
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
5.5
6
Time (h)
Fluc
Fluc+D-cysteine

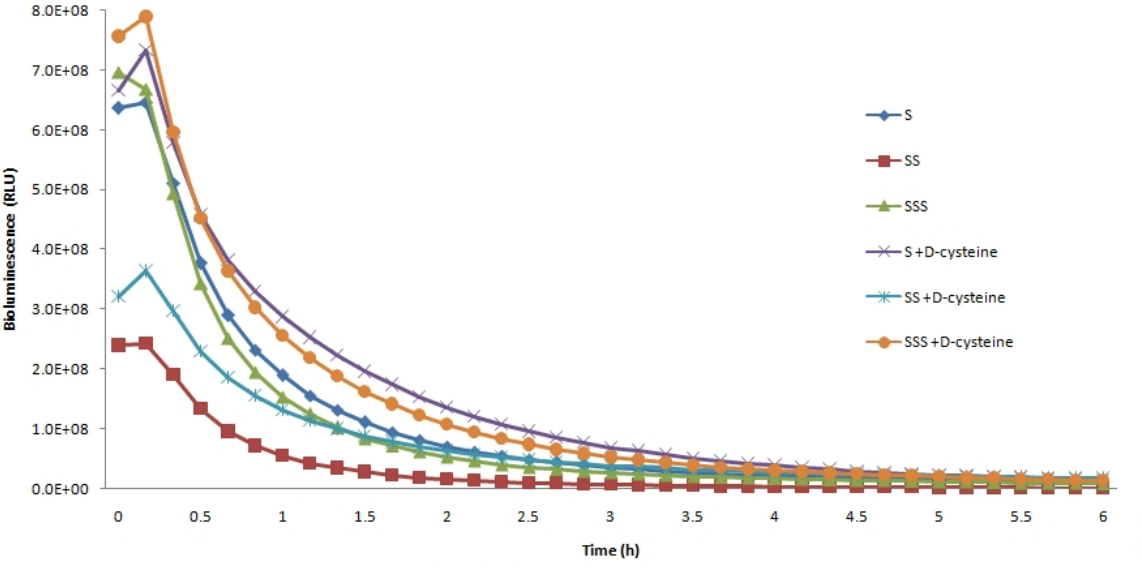

Panel A: Effect of peptide linker to LRE activity
Changing ratio
2900%
2400%
1900%
1400%
900%
400%
-100%
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
5.5
6
Time (h)
Fluc
Fluc-LRE
S
SS
SSS
R
RR
RRR
RS
SR
RSR
SRS


Panel B: Magnified view of parts enzyme complexes
Changing ratio
250%
200%
150%
100%
50%
0%
-50%
-100%
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
5.5
6
Time (h)
Fluc
Fluc-LRE
S
SSS
R
RR
RRR
RS
SR
RSR

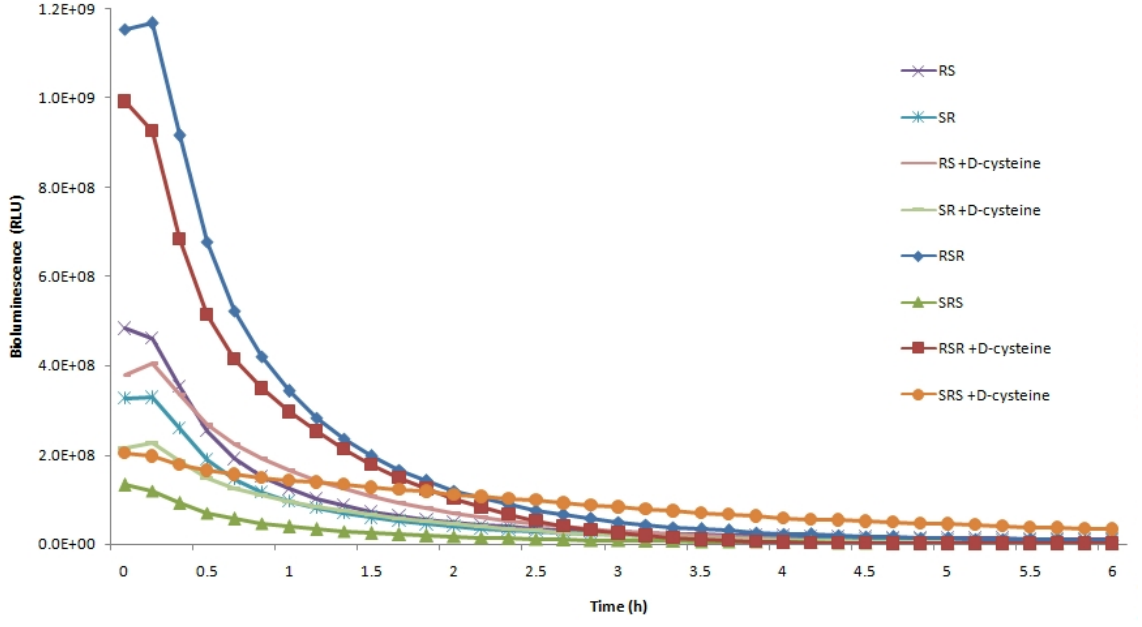
Panel A: Rigid linker series

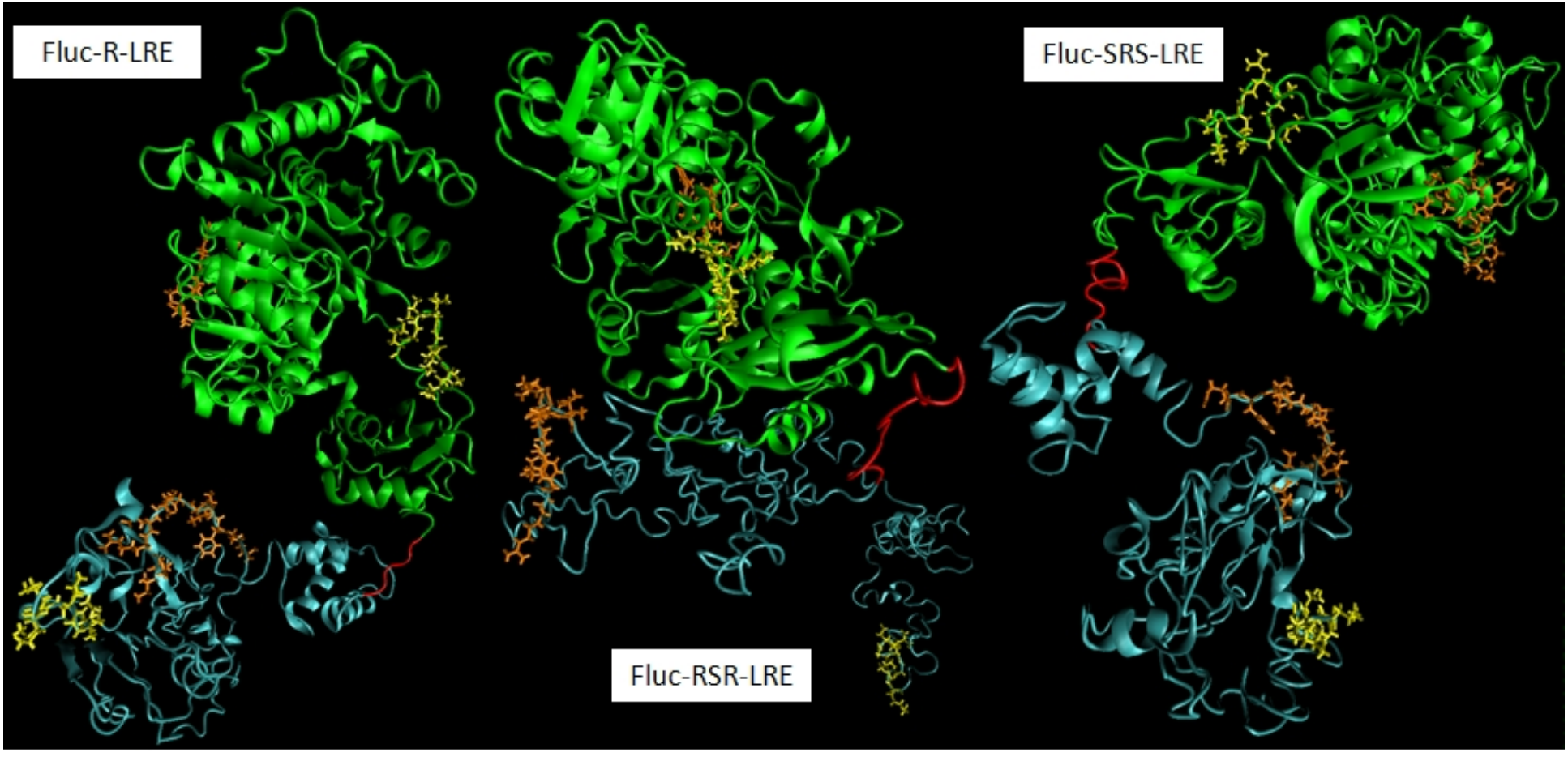


Panel B: Flexible linker series



Panel C: mixed Linker series




Fluc-R-LRE
Fluc-SRS-LRE
Fluc-RSR-LRE

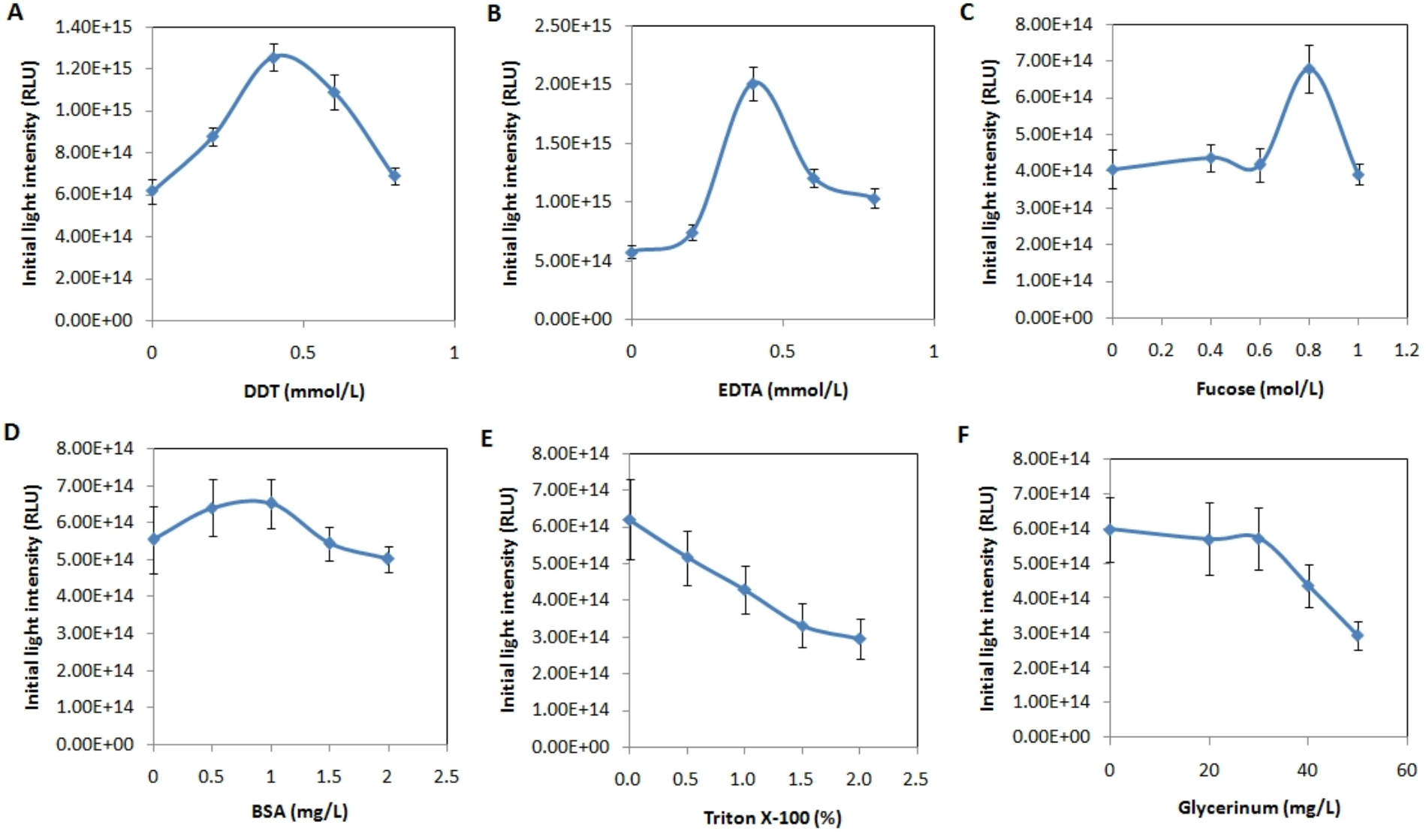
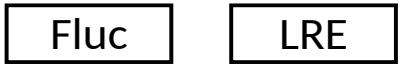
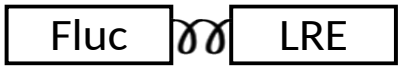
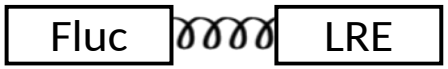
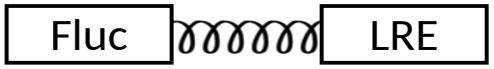
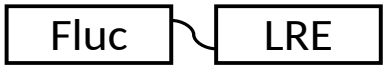
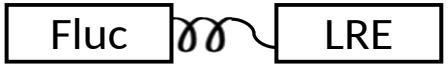
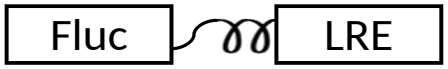
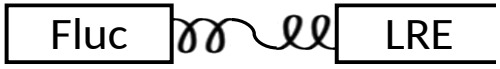

A
Initial light intensity (RLU)
1.40E+15
1.20E+15
1.00E+15
8.00E+14
6.00E+14
4.00E+14
2.00E+14
0.00E+00
0
0.5
1
DDT (mmol/L)
B
Initial light intensity (RLU)
2.50E+15
2.00E+15
1.50E+15
1.00E+15
5.00E+14
0.00E+00
0
0.5
1
EDTA (mmol/L)
C
Initial light intensity (RLU)
8.00E+14
7.00E+14
6.00E+14
5.00E+14
4.00E+14
3.00E+14
2.00E+14
1.00E+14
0.00E+00
0
0.2
0.4
0.6
0.8
1
1.2
Fucose (mol/L)
D
Initial light intensity (RLU)
8.00E+14
7.00E+14
6.00E+14
5.00E+14
4.00E+14
3.00E+14
2.00E+14
1.00E+14
0.00E+00
0
0.5
1
1.5
2
2.5
BSA (mg/L)
E
Initial light intensity (RLU)
8.00E+14
7.00E+14
6.00E+14
5.00E+14
4.00E+14
3.00E+14
2.00E+14
1.00E+14
0.00E+00
0.0
0.5
1.0
1.5
2.0
2.5
Triton X-100 (%)
F
Initial light intensity (RLU)
8.00E+14
7.00E+14
6.00E+14
5.00E+14
4.00E+14
3.00E+14
2.00E+14
1.00E+14
0.00E+00
0
20
40
60
Glycerinum (mg/L)

Table 1. Construction and qualification of enzyme complex.

Fusion enzyme	sketch map	abbreviation	Amino acid sequence	Oligonucleotide sequence	Concentration (μg/ml)
Fluc		/	/	/	597.26
Fluc-LRE		/	/	/	594.04
Fluc-R-LRE		R	(EAAAK)	GAAGCTGCTGCTAAA	885.75
Fluc-RR-LRE		RR	(EAAAKEAAAK)	GAAGCTGCTGCTAAAGAAGCTGCTGCTAAA	580.60
Fluc-RRR-LRE		RRR	(EAAAKEAAAKEAAAK)	GAAGCTGCTGCTAAAGAAGCTGCTGCTAAAGAAGCTGCTGCTAAA	599.31
Fluc-S-LRE		S	(GGGGS)	GGTGGTGGTGGTTCT	610.30
Fluc-SS-LRE		SS	(GGGGSGGGGS)	GGTGGTGGTGGTTCTGGTGGTGGTGGTTCT	551.22
Fluc-SSS-LRE		SSS	(GGGGSGGGGSGGGGS)	GGTGGTGGTGGTTCTGGTGGTGGTGGTTCTGGTGGTGGTGGTTCT	581.81
Fluc-RS-LRE		RS	(EAAAKGGGGS)	GAAGCTGCTGCTAAAGGTGGTGGTGGTTCT	648.61
Fluc-SR-LRE		SR	(GGGGSEAAAK)	GGTGGTGGTGGTTCTGAAGCTGCTGCTAAA	577.09
Fluc-RSR-LRE		RSR	(EAAAKGGGGSEAAAK)	GAAGCTGCTGCTAAAGGTGGTGGTGGTTCTGAAGCTGCTGCTAAA	590.98

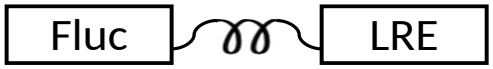
Fluc-SRS-LRE		SRS	(GGGGSEAAAK GGGGS)	GGTGGTGGTGGTTCTGAAGCTGCT GCTAAAGGTGGTGGTGGTTCT	959.96

Table 2. Kinetic properties of enzyme complexs depending on enzyme structure.

Enzyme structure	Initial light intensity(RLU)	K1	K2	half-life (h^{-1})
Fluc	1.36E+10	1.526±0.056	9.617±0.632	0.14
Fluc-LRE	1.49E+10	3.888±1.106	1.061±0.138	0.37
R	1.06E+10	0.742±0.037	4.357±0.997	0.54
RR	5.99E+08	2.976±0.153	0.661±0.009	0.61
RRR	2.72E+08	0.403±0.016	1.705±0.061	0.64
S	6.66E+08	0.643±0.010	3.057±0.141	0.55
SS	3.21E+08	1.776±0.043	0.300±0.017	0.54
SSS	7.56E+08	2.683±0.114	0.643±0.020	0.44
RS	3.78E+08	0.865±0.015	3.710±0.484	0.61
SR	2.14E+08	4.359±0.532	0.787±0.012	0.64
RSR	9.92E+08	1.096±0.026	6.719±1.173	0.54
SRS	2.04E+08	0.292±0.005	4.803±2.948	2.20

Table 1. Sequences of PCR primers in this study.

Protein	Template	Primers	Sequences
Fluc	*Fluc*	Fluc-FusionP1	GGAATTC**CATATG** GAAGATGCCAAAAACATTAAGAAGG
		Fluc-FusionP2	CCG**CTCGAG**TTA CACGGCGATCTTGCCGCCCTT
LRE	*Lre*	LRE-P1	GGAATTC**CATATG**GGCCCCGTAGTTGAAAAGATCG
		LRE-P2	CCG**CTCGAG**TTACAATTTAACTTTAACACCAGCAAAACCTTTCAC
Fluc-LRE	*Fluc*	Fluc-FusionP1	GGAATTC**CATATG** GAAGATGCCAAAAACATTAAGAAGG
		Fluc-LRE-Rev-P2	CGATCTTTTCAACTACGGGGCCCAT***CTCCTTCTTAAAGTTAAACAAAATTATTTCTAGAGGGGAATTGTTATCCG*** ***CTCACAATTCCCCTTATATAGTGAGTCGTATTA***TTACACGGCGATCTTGCCGC
	Lre	Fluc-LRE-For-P1	GCGGCAAGATCGCCGTGTAA***TAATACGACTCACTATATAAGGGGAATTGTGAGCGGATAACAATTCCCCTCTA*** ***GAAATAATTTTGTTTAACTTTAAGAAGGAG***ATGGGCCCCGTAGTTGAAAAGATCG
		LRE-P2	CCG**CTCGAG**TTACAATTTAACTTTAACACCAGCAAAACCTTTCAC

Fluc-R-LRE	*Fluc*	Fluc-FusionP1	GGAATTC**CATATG** GAAGATGCCAAAAACATTAAGAAGG
		Fluc-R-FusionP2	CGATCTTTTCAACTACGGGGCCCAT**TTTAGCAGCAGCTTC**CACGGCGATCTTGCCGC
	Lre	LRE-R-FusionP1	GCGGCAAGATCGCCGTG**GAAGCTGCTGCTAAA**ATGGGCCCCGTAGTTGAAAAGATCG
		LRE-P2	CCG**CTCGAG**TTACAATTTAACTTTAACACCAGCAAAACCTTTCAC
Fluc-RR-LRE	*Fluc*	Fluc-FusionP1	GGAATTC**CATATG** GAAGATGCCAAAAACATTAAGAAGG
		Fluc-RR-FusionP2	CGATCTTTTCAACTACGGGGCCCAT**TTTAGCAGCAGCTTCTTTAGCAGCAGCTTC**CACGGCGATCTTGCCGC
	Lre	LRE-RR-FusionP1	GCGGCAAGATCGCCGTG**GAAGCTGCTGCTAAAGAAGCTGCTGCTAAA**ATGGGCCCCGTAGTTGAAAAGATCG
		LRE-P2	CCG**CTCGAG**TTACAATTTAACTTTAACACCAGCAAAACCTTTCAC
Fluc-RRR-LRE	*Fluc*	Fluc-FusionP1	GGAATTC**CATATG** GAAGATGCCAAAAACATTAAGAAGG
		Fluc-RRR-FusionP2	CGATCTTTTCAACTACGGGGCCCAT**TTTAGCAGCAGCTTCTTTAGCAGCAGCTTCTTTAGCAGCAGCTTC**CACGGCGATCTTGCCGC
	Lre	LRE-RRR-FusionP1	GCGGCAAGATCGCCGTG**GAAGCTGCTGCTAAAGAAGCTGCTGCTAAAGAAGCTGCTGCTAAA**ATGGGCCCCGTAGTTGAAAAGATCG

		LRE-P2	CCG**CTCGAG**TTACAATTTAACTTTAACACCAGCAAAACCTTTCAC
Fluc-S-LRE	*Fluc*	Fluc-FusionP1	GGAATTC**CATATG** GAAGATGCCAAAAACATTAAGAAGG
		Fluc-S-FusionP2	CGATCTTTTCAACTACGGGGCCC**ATAGAACCACCACCACC**CACGGCGATCTTGCCGC
	Lre	LRE-S-FusionP1	GCGGCAAGATCGCCGTG**GGTGGTGGTGGTTCT**ATGGGCCCCGTAGTTGAAAAGATCG
		LRE-P2	CCG**CTCGAG**TTACAATTTAACTTTAACACCAGCAAAACCTTTCAC
Fluc-SS-LRE	*Fluc*	Fluc-FusionP1	GGAATTC**CATATG** GAAGATGCCAAAAACATTAAGAAGG
		Fluc-SS-FusionP2	CGATCTTTTCAACTACGGGGCCC**ATAGAACCACCACCACCATAGAACCACCACCACC**CACGGCGATCTTGCCGC
	Lre	LRE-SS-FusionP1	GCGGCAAGATCGCCGTG**GGTGGTGGTGGTTCTGGTGGTGGTGGTTCT**ATGGGCCCCGTAGTTGAAAAGATCG
		LRE-P2	CCG**CTCGAG**TTACAATTTAACTTTAACACCAGCAAAACCTTTCAC
Fluc-SSS-LRE	*Fluc*	Fluc-FusionP1	GGAATTC**CATATG** GAAGATGCCAAAAACATTAAGAAGG
		Fluc-SSS-FusionP2	CGATCTTTTCAACTACGGGGCCC**ATAGAACCACCACCACCATAGAACCACCACCACCATAGAACCACCACCACC** CACGGCGATCTTGCCGC
	Lre	LRE-SSS-FusionP1	GCGGCAAGATCGCCGTG**GGTGGTGGTGGTTCTGGTGGTGGTGGTTCTGGTGGTGGTGGTTCT**ATGGGCCCCG

			TAGTTGAAAAGATCG
		LRE-P2	CCG**CTCGAG**TTACAATTTAACTTTAACACCAGCAAAACCTTTCAC
Fluc-RS-LRE	*Fluc*	Fluc-FusionP1	GGAATTC**CATATG** GAAGATGCCAAAAACATTAAGAAGG
		Fluc-RS-FusionP2	CGATCTTTTCAACTACGGGGCCCAT**TTTAGCAGCAGCTTCAGAACCACCACCACC**CACGGCGATCTTGCCGC
	Lre	LRE-RS-FusionP1	GCGGCAAGATCGCCGTG**GAAGCTGCTGCTAAAGGTGGTGGTGGTTCT**ATGGGCCCCGTAGTTGAAAAGATCG
		LRE-P2	CCG**CTCGAG**TTACAATTTAACTTTAACACCAGCAAAACCTTTCAC
Fluc-SR-LRE	*Fluc*	Fluc-FusionP1	GGAATTC**CATATG** GAAGATGCCAAAAACATTAAGAAGG
		Fluc-SR-FusionP2	CGATCTTTTCAACTACGGGGCCCAT**AGAACCACCACCACCTTTAGCAGCAGCTTC**CACGGCGATCTTGCCGC
	Lre	LRE-SR-FusionP1	GCGGCAAGATCGCCGTG**GGTGGTGGTGGTTCTGAAGCTGCTGCTAAA**ATGGGCCCCGTAGTTGAAAAGATCG
		LRE-P2	CCG**CTCGAG**TTACAATTTAACTTTAACACCAGCAAAACCTTTCAC
Fluc-RSR-LRE	*Fluc*	Fluc-FusionP1	GGAATTC**CATATG** GAAGATGCCAAAAACATTAAGAAGG
		Fluc-RSR-FusionP2	CGATCTTTTCAACTACGGGGCCCAT**TTTAGCAGCAGCTTCAGAACCACCACCACCTTTAGCAGCAGCTTC**CACG GCGATCTTGCCGC

	Lre	LRE-RSR-FusionP1	GCGGCAAGATCGCCGTG**GAAGCTGCTGCTAAAGGTGGTGGTGGTTCTGAAGCTGCTGCTAAA**ATGGGCCCCG TAGTTGAAAAGATCG
		LRE-P2	CCG**CTCGAG**TTACAATTTAACTTTAACACCAGCAAAACCTTTCAC
Fluc-SRS-LRE	*Fluc*	Fluc-FusionP1	GGAATTC**CATATG** GAAGATGCCAAAAACATTAAGAAGG
		Fluc-SRS-FusionP2	CGATCTTTTCAACTACGGGGCCCAT**AGAACCACCACCACCTTTAGCAGCAGCTTCAGAACCACCACCACC**CACG GCGATCTTGCCGC
	Lre	LRE-SRS-FusionP1	GCGGCAAGATCGCCGTG**GAAGCTGCTGCTAAAGAAGCTGCTGCTAAAGGTGGTGGTGGTTCT**ATGGGCCCCG TAGTTGAAAAGATCG
		LRE-P2	CCG**CTCGAG**TTACAATTTAACTTTAACACCAGCAAAACCTTTCAC