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Novel functional *Renilla* luciferase mutant provides long-term serum stability and high luminescence activity

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ABSTRACT

Fluorescent and luminescent chemical probes are essential in recent medical diagnostics. However, the use of these probes *in vivo* has raised concerns due to their low sensitivity, background signal interference, and non-biocompatibility. Therefore, biological chromophores have received much attention as new alternatives. In particular, luciferase, a class of oxidative enzyme with bioluminescence, has emerged as a promising fluorophore due to its improved biocompatibility. However, the enzyme usually possesses weaker luminescence and stability relative to its chemically-based competitors. Here, we report a novel functional mutant luciferase with both enhanced luminescence and long-term serum stability. For the preparation of the modified *Renilla* luciferase, a new bacterial subcloning design was established. The luciferase coding DNA sequence was redesigned so that mutant luciferase could be easily expressed in an *Escherichia coli* system. The mutant *Renilla* luciferase, which we called "m-Rluc," demonstrated characteristic enzymatic functions and showed a 5.6-fold increase in luminescence activity. In addition, the enzyme's physiological stability remained >80% for more than 5 days, in contrast to conventional luciferase, termed "*hrluc*," which disappeared within a few hours. We suggest that this novel biological luciferase probe may be a great tool for both *in vitro* and *in vivo* medical diagnostics.

Introduction

For the past two decades, the development of diagnostic tool kits for advanced bioimaging has been an emerging issue in the field of medicine [1–3]. In addition to the development of detection instruments with higher performance, several imaging reagents (e.g., the quantum dot) based on chemical chromophores have been proposed [4]. However, these chemical probes have had some difficulty when applied *in vivo* to biological systems because of their reduced sensitivity and non-biocompatibility. To overcome these obstacles, the development of bio-inspired engineered materials has been recommended. For instance, green fluorescent protein (GFP) is a representative natural fluorescent probe [5]. Ever since GFP was first used as an *in vivo* gene expression marker in the 1990s [5], many bio-fluorescence mutants have been extensively prepared via genetic recombination technology, expanding the color spectrum of original GFPs to include green, yellow, red, and blue [6]. Using the same methods, other biological fluorophores such as luciferase can also be produced. Auxiliary methods, such as a simple conjugation method, have been proposed to design mutant fluorophores with characteristic physiochemical features [7,8].

Several attempts have been made to enhance the functionality of the *Renilla* luciferase protein [9,10]. For example, the *Renilla* luciferase mutant Rluc8 was created by substituting eight amino acids at specific sites of synthetic *Renilla* luciferase (*hrluc*) [10]. The enzyme *hrluc* is a human codon-optimized version of native *Renilla* luciferase (*rluc*), with the same encoding sequences as rluc except for the replacement of threonine with alanine in position 2. The eight mutations in Rluc8 were generated by substituting alanine (A) to threonine (T) at position 55 (A55T), cysteine to alanine at position 124 (C124A), serine to alanine at position 130 (S130A), lysine to arginine at position 136 (K136R), alanine to methionine at position 143 (A143M), methionine to valine at position 185 (M185V), methionine to leucine at position 253 (M253L), and serine to leucine at position 287 (S287L) in the amino acid sequence. According to previous literature [10], the light emission of mutated luciferase increased, and its biological activity was more stable in murine serum as compared to the characteristics of non-mutated

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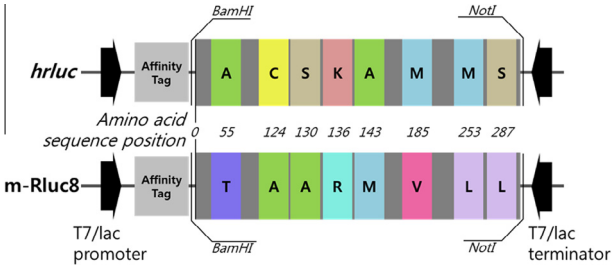

Fig. 1. Schematic comparison of *hrluc* and m-Rluc8. m-Rluc8 differs from *hrluc* in eight amino acid mutations: A55T, C124A, S130A, K136R, A143M, M185V, M253L, and S287L.

luciferase. These results suggest the potential of using mutated luciferase proteins in biomedical diagnostics.

In this study, we redesigned *hrluc* to function favorably inside *Escherichia coli* and then developed a new method to accelerate its activity with enhanced light output and stability under physiological conditions (Fig. 1). The redesigned luciferase is simply called m-Rluc8. A bacteria-based system for protein expression has many advantages, including its ease of handling, fast growth profile, and higher production yields of recombinant proteins. To induce the expression of m-Rluc8 in a bacterial system, *E. coli* codon-optimized nucleotides were adapted (Fig. S1) [11]. m-Rluc8 can be prepared via conventional sub-cloning methods and differs from Rluc8 by a combination of specific amino acid sequence changes, although it retains a similar amino acid phenotype. Compared with *hrluc*, the light output of m-Rluc8 exhibits a 5.6-fold enhancement in luminescence activity with coelenterazine-h as a substrate. This is substantially greater than the activity of Rluc8, which shows only a 3-fold enhancement in luminescence activity compared to *hrluc* [10]. Furthermore, the long-term stability of m-Rluc8 in fetal bovine serum media remained >80% for over five days.

Materials and methods

Preparation of recombinant plasmids (hrluc_pPAL7 and m-Rluc8_pPAL7)

To amplify the *hrluc* gene from the psiCHECK2 plasmid (Promega, Madison, WI, USA), polymerase chain reaction (PCR) was used. Two primers were designed for the bacterial expression vector construct: one for adding the *BamHI* restriction enzyme site at the 5 end of *hrluc* and the other for excluding the start and stop codons. PCR was carried out in the following component mixtures: psiCHECK2 plasmid (2 μg), each primer (400 pmol), $MgCl_2$ (1.5 mM), each dNTP (0.4 mM), 20 μl reaction buffer, and 2 μl Go Taq polymerase (Promega, Madison, USA). The PCR mixtures were then filled with nuclease-free water to a final volume of 100 μl. The PCR cycle consisted of three steps: an initial denaturation at 95 °C for 3 min, 35 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min.

The PCR product of *hrluc* was consecutively ligated into the TA cloning plasmid pGEM®-T Easy vector (Promega, Madison, WI, USA) via the enzymatic activity of T4 ligase. The ligated *hrluc*_T was transformed to the *E. coli* DH5a strain by heat shock at 42 °C for 1 min. The transformed *E. coli* was then grown on a LB agarose plate containing 100 μg/ml ampicillin (Sigma–Aldrich, St. Louis, USA), 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-gal, Ducefa, Haarlem, Netherlands) and isopropyl β-D-1-thiogalactopyranoside (IPTG, Ducefa, Haarlem, Netherlands) for 16 h. The white colonies were selected and further grown on LB media containing 100 μg/ml ampicillin for 12–14 h. The plasmids were isolated by mini-prep and successive nucleotide sequencing was completed.

The *hrluc*_T and pPAL7 plasmids were digested by both *BamHI* and *NotI* restriction enzymes. The expected fragments of the plasmids were isolated via gel-purification (QIAquick Gel extraction Kit, QIAgen, Hilden, Germany). The plasmid fragments (100 ng of pPAL7 and 47.3 ng of *hrluc*) were ligated by T4 ligase to construct the *hrluc*_pPAL7 plasmid.

The amino acid sequence of Rluc8 was obtained from the literature [7]. The m-Rluc8 sequences were optimized for the high-yield expression of modified luciferase proteins in an *E. coli* system. The customized restriction enzyme sites, which were recognized by *BamHI* in the 5′ direction and by *NotI* in the 3′ direction, were added to the m-Rluc8 genes (Fig. S1). The m-Rluc8 encoding plasmid (simply termed m-Rluc8_pUC57) and its codon optimization were commercially available from GenScript Corporation (Piscataway, NJ, USA). The m-Rluc8_pUC57 was confirmed through DNA sequencing and was digested and ligated into pPAL7 with the former *hrluc*_pPAL7 through *BamHI* and *NotI* restriction enzyme sites.

In vivo m-Rluc8 protein production

The expression and purification of m-Rluc8 proteins were performed with the Profinity eXact™ Protein Purification System (BIO-RAD, Hercules, CA, USA). This system works via four steps; (1) Expression of recombinant protein inside *E. coli*, (2) Binding of affinity-tagged target protein to column resin, (3) Washing away non-tagged materials, and (4) Elution and simultaneous cleavage of target protein from the affinity tag. The 8 kDa tag moiety is co-expressed with the target protein attached to the N-terminus of the target protein. The tag allows the protein-tag complex to bind to the affinity column which consisted of agarose-based matrix with conjugated proteins. After the washing step, the protease subsequently performs a specific cleavage of the tagging moieties from the complex triggered by sodium fluoride in the elution buffer. The free target proteins are then released resulting in a highly purified solution. To express m-Rluc8 in the *E. coli* system, m-Rluc8_pPAL7 was transformed into *E. coli* BL21 strain cells by heat shock at 42 °C for 1 min. The bacterial cells containing m-Rluc8_pPAL7 plasmids were grown at 20 °C on 300 ml of LB media containing 100 μg/ml of ampicillin until the optical density at a wavelength of 600 nm (OD_{600}) reached 0.4. According to the previous study by Guennadi et al., the number of *E. coli* when OD at 600 nm reaches 0.4 is approximately 5×10^8 cells/ml [12]. The cell products were induced by the addition of IPTG in a final concentration of 0.1 mM. After 16 h of cultivation, OD_{600} in 10-fold dilution reached 0.95 (9×10^9 cells/ml) and wet weight grams of cells were 9 g per liter [12]. The cells were resuspended with 30 ml of lysis buffer, which consists of 100 mM sodium phosphate (pH 7.2) and 1% Triton X-100, in a 1:10 volume ratio compared to the LB media. The cells were lysed by additional sonication and then centrifuged for debris removal.

Before the cell lysate was purified, 1 ml of tag affinity beads slurry (Profinity eXact Purification Resin, BIO-RAD, Hercules, CA, USA) was injected into the 5 ml Piers Centrifuge Column (Thermo Scientific, Rockford, IL, USA), and the column was washed with 4 ml of wash buffer (100 mM sodium phosphate, pH 7.2). A total of 4 ml of cell lysate was added to the column and incubated at 4 °C for 40 min. Afterward, the column was washed twice with the washing buffer and then incubated with 1 ml elution buffer (100 mM sodium fluoride and 100 mM sodium phosphate) at 4 °C for 16 h. The eluted solution was centrifuged and desalted by an Amicon® Ultra-4 Centrifugal Filter Unit (Millipore, Eschborn, Germany). The purified protein concentrations were measured by Bradford assay with bovine serum albumin (BSA, Sigma–Aldrich,

St. Louis, USA) as the standard. The purified protein products were aliquoted and stored at −20 °C.

Evaluation for the functionality of m-Rluc8

For evaluating the functional activity of m-Rluc-8, we focused on (1) luminescent output intensities and (2) fetal serum stability. (1) Luminescent output intensity: m-Rluc8 proteins were diluted to a final concentration of 20 μM. m-Rluc8 (1 μl) was mixed with 100 μl of the washing buffers containing 10 mg/ml of human serum albumin (HSA, Sigma–Aldrich, St. Louis, USA). Each sample was prepared in triplicate and loaded onto a 96-well plate. Shortly before the assay, 0.5 μg of coelenterazine-*h* (1 μl of 0.5 mg/ml stock in methanol, Promega, Madison, WI, USA) was added. Measurements were performed from 350 to 650 nm using SpectraMax M5 Multi-mode Microplate Readers (Molecular Devices, USA) for the luminescence intensity of each luciferase. (2) Fetal serum stability: m-Rluc8 protein (0.35 μg) was suspended with 500 μl serum-containing media (RPMI 1640 with 10% FBS). Samples were placed in a 37 °C incubator and 10 μl aliquots were removed after 0, 2, 4, 6, 9, 12, 24, 48, 72, 96, 120, 144, and 168 h. The aliquots were mixed with 90 μl of the washing buffer containing 10 mg/ml of HSA. All samples was prepared in triplicate and loaded onto a 96-well plate. Shortly before the assay, coelenterazine-*h* (0.5 μg) was added as previously described. Measurements were performed at 482 nm for *hrluc* and 487 nm for m-Rluc8.

Results and discussion

Set-up of PCR (polymerase chain Reaction) parameters

The luciferase *hrluc* was used as a control in this study, with the same protein prototype as m-Rluc8 but lacking any restriction sites. In contrast, m-Rluc8 was customized by adding restriction enzyme sites with *hrluc* as a template plasmid. PCR was utilized to amplify and add a *BamHI* restriction enzyme site at the 5′ end of *hrluc*. The first steps of PCR were designing a pair of primers (Table 1) and setting the conditions for the reaction (e.g., the annealing temperature). The forward and backward primers consisted of 18 bases of oligonucleotides that were complementary to the 5′ and 3′ ends of *hrluc*, respectively. Using these two primers, a total of four annealing temperatures (63.8, 59.1, 55.7 °C, and 52.9 °C) were tested for optimal PCR conditions. Each temperature was chosen based on the melting temperatures of the two primers: 61.4 °C for the forward primer and 48.4 °C for the backward primer. All of the PCR reactants were mixed, and three-step PCR was run as described in the experimental section. In the electrophoretic assay results of the PCR products shown in Fig. 2, the annealing temperatures of 52.9 and 59.1 °C were found to be broadly acceptable for PCR performance with large volumes. Although we selected the specific annealing temperatures of 59.1 and 52.9 °C, we finally decided to use the intermediate temperature of 58 °C for the thermodynamic stabilization of the forward primer. The forward primer was composed of DNA strands with

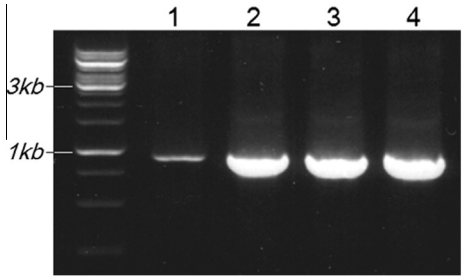

Fig. 2. PCR condition optimization for *hrluc*. Gel electrophoretic image of PCR products of *hrluc*. Each lane represents one of four annealing temperatures; Lane 2: 63.8 °C, Lane 3: 59.1 °C, Lane 4: 55.1 °C, Lane 5: 52.9 °C.

24 bases and DNA strands with only 18 of the 24 bases complementary to the template plasmid. The remaining sequences were utilized for the recognition of the *BamHI* restriction enzyme. The backward primer was completely complementary to the template plasmid.

hrluc-positive colony selection via TA cloning

Some thermophilic DNA polymerases have terminal transferase activity [13]. One example is *Thermus aquaticus* (Taq) polymerase, used in our PCR cycling, which preferentially adds a single adenosine to the 3′ end of a DNA strand and has thermo-resistance at temperatures over 80 °C. Generally, a T-vector is used for the subcloning of preparative PCR products, a process just called TA cloning [14]. Since TA cloning uses two complementary overhangs, the 3′-adenosine of the PCR products and the 5′-thymidine of the T-vector, it is one of the most simple and efficient methods for cloning PCR products. In addition, the T-vector which encodes the '*LacZ*' gene sequence allows for the visual discrimination of colonies with the desirable recombinant plasmids based on blue/white color screening. The *lacz*-product activates β-galactosidase, resulting in blue colonies. However, if the inserted gene (the PCR product) is ligated to the T-vector, the lacz sequence is divided and loses its functionality, resulting in white colonies. A total of eight 'white' colonies were selected, and their plasmids were isolated by mini-prep and digested with *BamHI* and *NotI* restriction enzymes. Through an agarose gel electrophoresis migration assay, it was confirmed that the plasmids from colonies number 2, 3, and 7 were digested into two fragments by *BamHI* and *NotI* restriction enzymes, spanning 942 base pairs for *hrluc* and 3015 base pairs for the T vector (Fig. 3). As a result, it could be deduced that the blue/white color screening was not sufficient, and only colonies 2, 3, and 7 were successfully transformed. Colonies 1, 4, 5, 6, and 8 appeared white but were not digested by the expected restriction enzymes. This result may be due to reverse-ligated PCR products. As previously mentioned, the PCR product has a single adenosine on its 3′ end. Therefore, it can be ligated in two directions with the T-vector. Thus, a white colony does not by itself indicate a successful clone, though blue/white color screening offers greater probability

Table 1
A summary of total protein, target protein, yield and purity for *hrluc* and m-Rluc8 luciferase.

Purification step	*hrluc*			m-Rluc8		
	Cell lysate of soluble fraction	After purification	Purification yield	Cell lysate of soluble fraction	After purification	Purification yield
Total protein (mg)	845	~15.5	–	1160	~19	–
Target protein (mg)	100	15.5	15.5 %	175	19	11%
Purity (%)	12	~99	–	15	~99	–

Grams of wet weight cells were 9 g per 1 l cultivation.
1 ml of tag affinity beads was used.

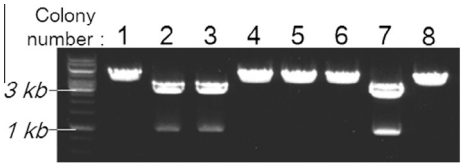

Fig. 3. Gel electrophoretic evaluation of eight white colonies from TA cloning. Their plasmids were purified by mini-prep and digested by *BamHI* and *NotI* restriction enzymes.

of selecting the transformed colonies. By comparing the *hrluc* band intensities, measured by Image J software provided by the NIH, we determined that colony 7 contained the most *hrluc*-positive clones. Consequently, colony 7 was selected as the template colony for further experiments.

Evaluation of optimized Escherichia coli culture conditions for in vivo protein expression

The metabolic activities of most bacterial cells are affected by various parameters such as temperature [15], feed concentration [16], humidity, and culture time. Therefore, several parameters were tested in our study, including the temperature and IPTG (isopropyl β-D-1-thiogalactopyranoside) concentration. IPTG binds to the '*lac* repressor' and could be used in the T7/*lac* promoter system as an inducer to promote the overexpression of target proteins [17]. As shown in Fig. 4, six culture conditions were tested to identify the appropriate conditions for efficient m-Rluc8 luciferase expression. It was noted that IPTG concentrations were 0.1 and 1 mM when the reaction temperatures were 20, 30, or 37 °C. Each cell lysate of soluble fraction was prepared and then assessed through sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). The most intense band of m-Rluc8 appeared in the lane at 44 kDa when the sample conditions consisted of 0.1 mM IPTG at 20 °C, suggesting that the bacterial cells were most efficiently expressing m-Rluc8 *in vivo*. The expression of recombinant proteins in *E. coli* often results in a fraction of insoluble protein complex, forming the inclusion bodies. Through the m-Rluc8 expression and purification, the substantial amount of m-Rluc8 was also included in the insoluble fractions (Fig. S2). Therefore, by adapting a lower cultivation temperature, it was possible to reduce the rate of the inclusion body formation while increasing the amount of target proteins that are soluble in cytoplasm [18,19]. The soluble proteins preserved their inherent functionality. Accordingly, these optimized conditions were used for the expression of large amounts of m-RLuc8 protein.

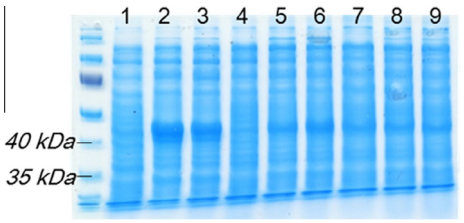

Fig. 4. Optimization of *E. coli* culture conditions for *in vivo* m-Rluc8 expression. Several culture conditions were tested and each cell lysate of soluble fraction was assessed. Each condition was different with regard to the concentration of IPTG (0.1 and 1 mM) and culture temperature (20, 30, and 37 °C). lane 1: IPTG 0 mM and 20 °C, lane 2: IPTG 0.1 mM and 20 °C, lane 3: IPTG 1 mM and 20 °C, lane 4: IPTG 0 mM and 30 °C, lane 5: IPTG 0.1 mM and 30 °C, lane 6: IPTG 1 mM and 30 °C, lane 7: IPTG 0 mM and 37 °C, lane 8: IPTG 0.1 mM and 37 °C, lane 9: IPTG 1 mM and 37 °C.

Evaluation of the isolation of m-Rluc8 products

The m-Rluc8 luciferase in the *E. coli* systems was produced in culture volumes of 300 ml of LB media. After the optical density at a wavelength of 600 nm (OD600) reached 0.4, 0.1 mM IPTG was added to the cell. During the purification process, described in the experimental section above, four intermediate samples were collected and examined by SDS–PAGE to compare the degree of luciferase expression. The four samples were: (1) cell lysate, (2) flow-through (the flow-through of cell lysate from the tag affinity bead column), (3) wash (the wash buffer from the tag affinity bead column) and (4) elution (the elution from the tag affinity bead column) (Fig. S3). We also determined if the target proteins were correctly expressed and were attached to the tag affinity bead during the entire process (Fig. 5). The expected size for both the *hrluc* and the m-Rluc8 was 44 kDa including the affinity tag size of 8 kDa. Specifically, the size of the luciferase band (44 kDa) in the flow-through samples indicated that the target luciferase was successfully bound to the tag affinity beads. The decrease in the protein size to 36 kDa after the elution step was due to the cleavage of the tagging moieties (8 kDa) from the protein products (44 kDa for both *hrluc* and m-Rluc8). The final products *hrluc* and m-Rluc8 exhibited different recovery quantity: 15.5 mg/l for *hrluc* and 19 mg/l for m-Rluc8. The target protein mass of *hrluc* and m-Rluc8 were 100 and 175 mg/l, respectively. Informatively, the total amounts of each unpurified protein were 845 and 1160 mg/l, respectively. To convert into percent yield, each target protein was divided by unpurified target proteins in each case and then multiplied by hundred, giving the percent yield of 15.5% for *hrluc* and 11% for m-Rluc8 (Table 1). As a remark, by seeing the SDS–PAGE result showing only some faint side bands other than the distinct band of the target protein, we concluded that the purified target protein was highly homogeneous. Even if the purification yield of m-Rluc8 appeared to be lower than that of *hrluc* because of the overexpressed m-Rluc8s, the ultimate purified mass was higher in m-Rluc8 than *hrluc*. This may be due to the fact that

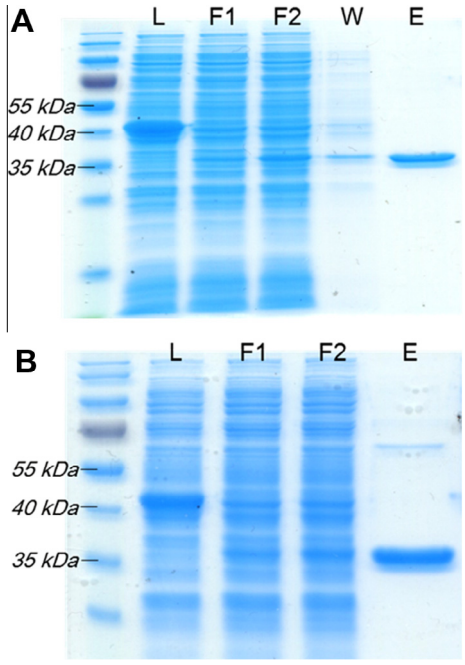

Fig. 5. Coomassie-stained SDS–PAGE gel for the verification of the protein purification process. Expected size for both (A) *hrluc* and (B) m-Rluc8 is 44 kDa with the affinity tag size of 8 kDa. L: cell lysate of soluble fraction, F: flow-through from tag affinity column, W: wash from column, E: elution from column.

hrluc had coding genes derived from psiCHECK2 and was optimized for mammalian systems, whereas m-Rluc8 originated from m-Rluc8_pUC57 and used codons optimized for bacterial environments. Therefore, m-Rluc8 was expected to be more efficient than *hrluc* for translation in *E. coli* systems. If a mammalian system was chosen to express the target proteins, the recovery efficiencies may have been reversed [20].

In this study, 1 ml of tag affinity binding beads slurry was used and affinity binding capacities of target proteins with beads slurry were defined. Several repetitive experiments have been performed and constant tendencies in the experimental results were observed. Beads slurry used here (1 ml) had a capacity to capture 4.7 mg of *hrluc* and 5.7 mg of m-Rluc8. The difference on binding capacity of target proteins would be ascribed to structural stability of two proteins. Due to the more stable quaternary structure of m-Rluc8 resulting from eight amino acids mutations as discussed, m-Rluc8 may be able to bind tightly the tag affinity beads when compared with *hrluc* retaining a less stable quaternary structure. Therefore, m-Rluc8s in even lower concentrations were attached to the beads slurry. Consequently, if additional proteins are required, the amounts of beads slurry would be re-adjusted depending on the tag affinity binding capacity defined in this study.

Evaluation of the functional activities of m-Rluc8

To evaluate the functional activities of m-Rluc8, we measured the protein's luminescence light output and serum stability. With respect to bioluminescence, the luminescent activity of m-Rluc8 was compared with that of *hrluc*. Human serum albumin (HSA) was used as a carrier protein to prevent the loss of luciferase mass from its adsorption to the surrounding surfaces [7]. A coelenterazine is a substrate for bioluminescent enzymes derived from marine organisms, with more than ten coelenterazine analogs commercially available. Benzyl-coelenterazine (coelenterazine-h) is a representative substrate of *Renilla* luciferase for strong bioluminescence activity [21]. With the addition of coelenterazine-h, luminescence wavelength peaks were observed at 482 nm for *hrluc* and 487 nm for m-Rluc8. m-Rluc8 exhibited strong luminescence activity that was up to 5.6-fold greater than that of *hrluc* (Fig. 6A).

Rluc8 has been reported to show enhanced stability in mouse and rat serum [10]. Our study compared the stability of *hrluc* and m-Rluc8 in fetal bovine serum (FBS). Both *hrluc* and m-Rluc8 demonstrated a decline of luminescent activity in FBS. However, compared to *hrluc*, m-Rluc8 showed relatively longer-term stability. Fig. 6B shows the results of both proteins as the percentages of initial activity.

There is a characteristic α/β-hydrolase fold sequence in the amino acid sequence of *hrluc* which originated from *Renilla reniformis* [22]. Interestingly, *hrluc* shows a substantial similarity with haloalkane dehalogenase, which also contains an α/β-hydrolase fold sequence [23]. Based on this similarity, eight mutations were proposed [10] that enhanced the light output and serum stability of m-Rluc8. These results may be due to enhanced quantum yield, kinetics, and structural stability. For example, the position of valine (M185V) in the quaternary structure is near the substrate recognition site. A mutation in this position could affect the turn-over rate of m-Rluc8 enzyme and the quantum yield. Another substitution mutation was alanine for cysteine in position 124 (C124A), which is located in the hydrophobic core of m-Rluc8. Cysteine has a thiol group on its side chain, and its repulsion in the hydrophobic core could cause an unstable state. In contrast with cysteine, alanine does not have an electrophilic residue on its side chain. In addition, the side chain length of alanine is shorter than that of cysteine. Therefore, the replacement from cysteine to alanine at position 124 allows for the better packing of the hydrophobic core both electrically and sterically, resulting in the increased stability of m-Rluc8. The combination of these eight mutations enables a 5.6-fold enhancement in the luminosity and the stability of the luciferase in serum.

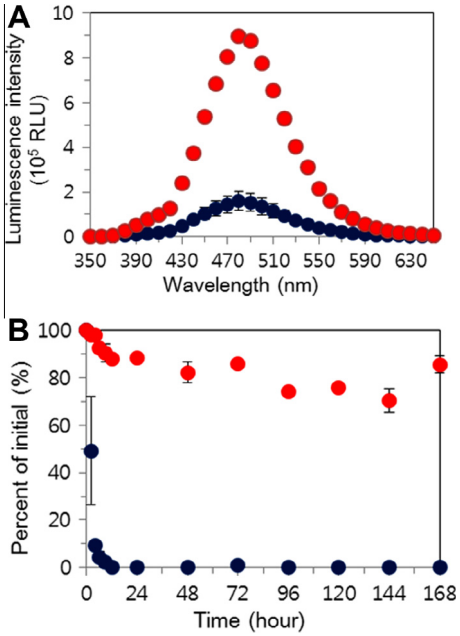

Fig. 6. Functional assay of m-Rluc8 (red circles) and *hrluc* (blue circles). (A) Relative luminescence output; The luminescence intensity of m-Rluc8 was 5.6-fold higher than that of *hrluc*. Emission wavelength peak was at 482 nm for *hrluc* and 487 nm for m-Rluc8. (B) Relative serum stability; m-Rluc8 showed substantial longer stability in bovine serum compared with *hrluc*. 'Percent of initial' is defined by the ratio of protein activity at the determined time to the initial protein activity.

Conclusions

In this study, we developed a functionally new *Renilla* luciferase (m-Rluc8) with a higher luminescence output and longer serum stability. This luciferase differed from *hrluc* in eight amino acid mutations: A55T, C124A, S130A, K136R, A143M, M185V, M253L, and S287L. These selected mutations enabled the protein to emit stronger bioluminescence activity and to be more stable in serum media. In addition, we established the optimized conditions for protein expression. Overall, the redesigned m-Rluc8 exhibited an enhancement in protein expression and showed a 5.6-fold improvement in light output, with increased stability in serum media confirmed to last for over 5 days.

The enhanced activity of m-Rluc8 could mitigate some of the drawbacks of conventional luciferase and offer a useful tool for biomedical applications such as *in vitro* and *in vivo* diagnostics.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.pep.2013.08.004.

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