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Improved Red-emitting Firefly Luciferase Mutant for Biotechnical Applications

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Improved Red-emitting Firefly Luciferase Mutant for Biotechnical Applications

**Audrey L. Davis
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**Presents an Honors Thesis
under the supervision of Dr. Bruce R. Branchini**

May, 2009

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Abstract

Luciferase from the North American firefly *Photinus Pyralis* catalyzes a highly efficient bioluminescent reaction that produces yellow-green light (557 nm at pH 7.8). This bioluminescence system has been adapted for a variety of applications including gene reporter assays, detection of bacteria and toxins, whole-cell biosensor measurements, and *in vivo* imaging. A luciferase variant with a red-shifted bioluminescence spectra and high specific activity, two qualities that are challenging to achieve simultaneously, would be especially useful when paired with a green-emitting enzyme in a dual-color reporter assay, or used alone for improved imaging in living animals.

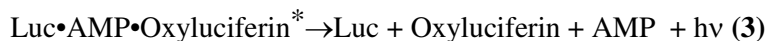
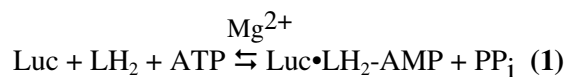
Our lab previously reported a red-emitting mutant named Ppy RE, which contained a single mutation at S284T that shifted the emission maximum from the 557 nm to 615 nm at pH 7.8. In a subsequent publication, Ppy RE was enhanced with five mutations to make Ppy RE-TS, which exhibited improved thermostability at 37°C, but at the expense of reducing the red-shift to 610 nm. In this report, we further improve upon Ppy RE-TS with four rational point mutations that red-shift the emission maximum to 617 nm, dramatically increase the enzyme thermostability, and improve the total light emission during 8-second assays. This new enzyme, named PRET7, is characterized and evaluated for performance in *E. Coli* and HEK293 mammalian cell lines. Compared to a commercially available enzyme called CBR, which has been codon-optimized for expression in mammalian cells, PRET7 produces a stronger signal over 8-second assays in which the least expensive reagents available are used. Aiming to improve the already impressive performance of PRET7, we are currently awaiting results from collaborators who are evaluating a human codon-optimized version of PRET7 in HEK293 cells.

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Introduction

The North American firefly *Photinus pyralis*, known for its flash of yellow-green light, houses one of the most efficient bioluminescent systems ever studied¹ (Fig. 1). The light-producing reaction is catalyzed by the luciferase enzyme (Luc)², a ~62 kDa oxygenase that has been well-characterized (1, 2). To begin the reaction, Luc adenylates the substrate firefly luciferin (LH₂), a heterocyclic carboxylic acid, with Mg-ATP (Eq (1)). The resultant luciferyl adenylate combines with molecular oxygen at the luciferase active site to produce an electronically excited state product (Eq. (2)). Upon relaxation to the ground state, a photon of visible light is emitted (Eq (3)).



This bioluminescence system has a quantum yield of $41.0 \pm 7.4\%$ (3), which is impressive compared to most chemiluminescent reactions (4). Also, unlike fluorescence systems, it does not require an external light source for excitation, and there is virtually

¹ This thesis research was presented at the National Conference for Undergraduate Research at the University of Wisconsin-La Crosse, on April 17, 2009.

² *Abbreviations used:* Luc, *photinus pyralis* luciferase; LH₂, D-firefly luciferin; Ppy WT, recombinant *photinus pyralis* luciferase containing the additional N-terminal peptide GlyProLeuGlySer; Ppy RE, Ppy WT containing S284T; Ppy GR, Ppy WT containing V24I/G246A/F250S; Ppy RE-TS, Ppy RE containing T214A/A215L/I232A/F295L/E354K; Ppy GR-TS, Ppy GR containing T214A/A215L/I232A/F295L/E354K; PRET7, Ppy RE-TS containing R330G/I351V/K354E/ F465R; MAR, minimal assay reagents of 400 μM LH₂, 2 mM Mg-ATP, and 25 mM glycylglycine buffer (pH 7.8); LAR, Promega's luciferase assay reagent; BSA, bovine serum albumin; CB, 50 mM Tris-HCl (pH 7.0) containing 150 mM NaCl, 1 mM EDTA, and 1 mM dithiothreitol; CBA, CB with 0.8 M ammonium sulfate and 2% glycerol; EDTA, ethylenediaminetetraacetic acid; IPTG, 100 mM isopropyl-β-d-thiogalactopyranoside; LB-amp, Luria Bertani broth with 100μg/ml ampicillin; PBS, phosphate-buffered saline; PMT, photomultiplier tube.

no non-specific light production induced in the cellular environment. As such, it is possible to detect even trace amounts of luciferase using photomultiplier tubes (PMTs) or charge-coupled devices (CCDs). It has been estimated that attomole levels of luciferase can be quantified (5), making the enzyme an attractive candidate for bioanalytical uses where high sensitivity is desired. Among a vast variety of applications, firefly luciferase has been used in probes for detecting bacteria and environmental toxins(6), bioluminescence resonance energy transfer (BRET) assays to detect protein-protein interactions(7), whole-cell based biosensors(8), *in vivo* bioluminescence imaging(4,9), and gene expression assays(10, 11).

Many gene expression assays require two reporters, with one functioning as a control to monitor nonspecific interferences. For these assays it is necessary to employ two luminescent signals with spectral bandwidths that are easily separable by optical filters (12). Several commercial options are available for bioluminescent dual-analyte assays. The Promega Dual-Luciferase Reporter Assay System

monitors two distinct bioluminescent reactions from firefly and *Renilla* luciferases, which are carried out sequentially using two different substrates and a stop solution. More elegant reporter systems require only one substrate to initiate multiple reactions simultaneously. A dual-color system based on *Phrixothrix* railroad worms (13, 14), and a tricolor enzyme system derived from Japanese beetle and railroad worm enzymes (15)

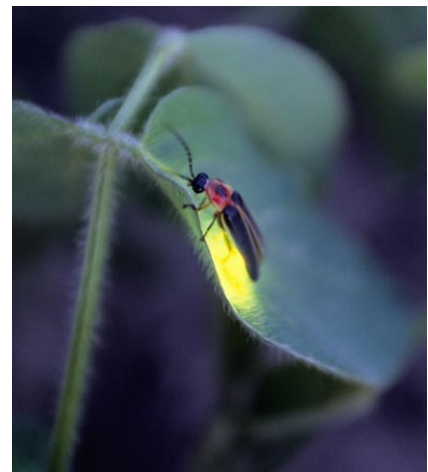


Figure 1. North American firefly *photinus pyralis* with flashing lantern. (<http://static.howstuffworks.com/gif/firefly.jpg>)

both use only D-luciferin as a substrate, but may not be adequately sensitive for demanding assays.

A drawback of both the above assay methods is that they require co-expression of dissimilar proteins. It has been shown that assays that combine luciferases of different species, like click beetle and firefly luciferases, can be ineffective because of differences in expression and in specific activity (12). The Promega Chroma-Luc™ vectors, which use a single reagent called Chroma-Glo, encode red- and green-emitting luciferase mutants from a single species, the Jamaican click beetle *Pyrophorus plagiophthalmus* (16). The reporter vectors included with this system are called CBR, CBG99, and CBG68, all of which are structurally and chemically alike.

Our laboratory has already published two sets of red- and green-emitting firefly luciferase mutants to be used in dual-color assays. The first two mutants developed were named Ppy RE and Ppy GR (17). Ppy RE contained the single point mutation Ser284Thr, which had been discovered by random mutagenesis and which shifted the bioluminescence maximum from 557 nm to 615 nm at pH 7.8. Ppy GR, originally designed for a different project, contained the mutations Val241Ile/Gly246Ala/Phe250Ser, and was effectively blue-shifted to 549 nm. Considering the 66 nm difference in the emission maxima of these enzymes, as well as the sufficiently narrow bandwidths of the bioluminescence curves, we predicted minimal spectral overlap if the two enzymes were to be used simultaneously (Fig. 2).

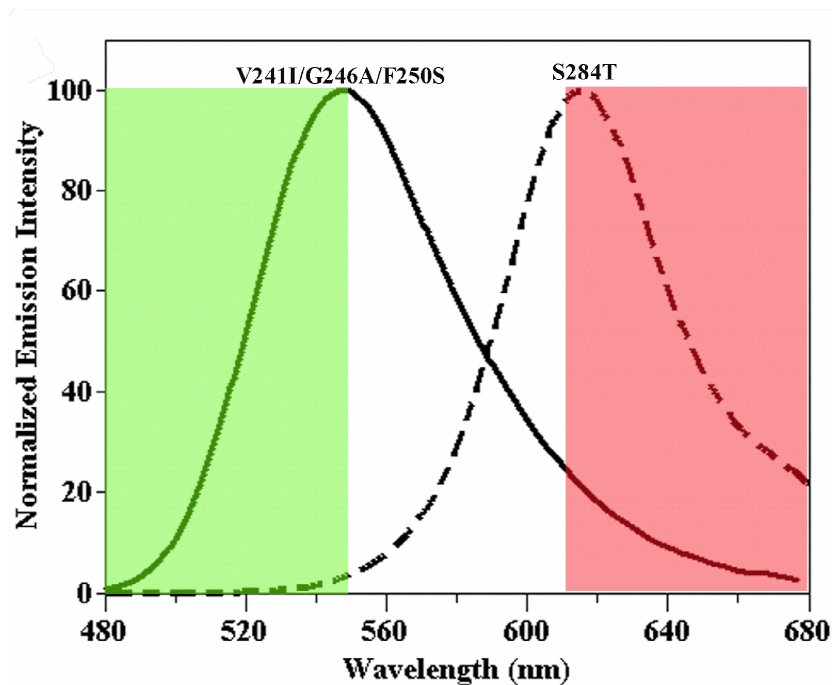


Figure 2. Normalized bioluminescence emission spectra produced by the Ppy GR and Ppy RE luciferases at pH 7.8, obtained as previously reported (17).

Indeed, model experiments showed that the red and green signals could be separated by optical filters, such that the enzymes were distinguishable from one another at femtomolar quantities. But Ppy RE and GR, like wild-type firefly luciferase, required refrigeration to remain active. At 37°C, the activity of Ppy GR dropped to 50% in under 15 minutes and Ppy RE dropped to 50% in under an hour. Concerned that these enzymes might have limited utility in experiments conducted at physiological or even just room temperature, we created thermostable versions named Ppy RE-TS and Ppy GR-TS (18).

The same five mutations were added to both Ppy RE and GR to increase their stability at 37°C: Thr214Ala, Ala215Leu, Ile232Ala, Phe295Leu, and Glu354Lys. These mutations were discovered in the lab of L.C. Tisi, who introduced them into wild-type firefly luciferase and reported a significant increase in thermostability (19, 20). After

mutating our enzymes, Ppy GR-TS exhibited a 46-fold increase in thermostability, and Ppy RE-TS increased 7-fold. But the additional mutations also had the undesired effect of shifting the bioluminescence spectra of both the enzymes. Though the green-emitter became slightly greener, shifted from 548 to 546 nm, the red-emitter dropped from 615 to 610 nm, creating more overlap between the two bioluminescence curves.

The work presented here is our third attempt to engineer firefly luciferases suitable for dual-color applications. Having already improved on enzyme thermostability, we aimed to improve on the separation between the red and green bioluminescence curves. The bioluminescence profile of the green-emitter has a tail that encroaches on the red area of the spectrum, and cannot be eliminated by modifying the enzyme. As such, we chose to modify Ppy RE-TS alone, aiming to further red-shift its bioluminescence spectrum. Mutations that cause red-shifts in luciferases often compromise the specific activity of the enzyme. For example, Ppy RE-TS exhibits a flash-height based specific activity only 23% of *P. pyralis* wild-type luciferase (Ppy WT). We hoped to rationally incorporate point mutations into Ppy RE-TS to achieve a redder-emitting enzyme while retaining or improving its specific activity, favorable kinetic properties, and thermostability.

Our lab has developed a new red-emitting mutant, PRET7, which is 7 nm red-shifted compared to the precursor Ppy RE-TS, has improved specific activity and exhibits prolonged stability at 37°C. The pure protein was characterized and compared to Promega's red-emitting commercial enzyme CBR, a click beetle luciferase that has been codon-optimized for expression in mammalian cells. To predict the performance of the

PRET7 and CBR in reporter assays, luciferase-expressing *E. Coli* and mammalian HEK293 cells were lysed and assayed for luminescence. To complete the study, a codon-optimized variant of PRET7 was designed, and at the time of this writing is being tested in HEK293 cells by collaborators at Promega. Here we present evidence for possible advantages and disadvantages of using PRET7, as compared to CBR, for bioanalytical applications.

Materials and Methods

Materials. The following materials were obtained from the sources indicated: Bacterial Mg-ATP and GenElute DNA Miniprep Kit from Sigma-Aldrich (St. Louis, MO); restriction endonucleases from New England Biolabs (Beverly, MA); mutagenic oligonucleotides from Invitrogen and Integrated DNA Technologies (Carlsbad, CA and Coralville, IA); Glutathione Sepharose 4B media and pGEX-6P-2 expression vector from GE Healthcare (Piscataway, NJ); QuikChange® Site-Directed Mutagenesis kit from Stratagene (La Jolla, CA); QIAquick PCR purification kit and QIAquick gel extraction kit from Qiagen (Valencia, CA); DMEM and Opti-MEM® I reduced-serum medium from Invitrogen (Carlsbad, CA); 10% FBS from Thermo Scientific Hyclone (Logan, UT); *TransIT*®-LTI transfection reagent from Mirus Bio (Madison, WI); D-Firefly luciferin, the pCBR-Basic Vector, Luciferase Assay Reagent (LAR), were generous gifts from Promega (Madison, WI). The Passive Lysis Buffer and Flexi® Vector pF9a were provided by Promega as well.

General Methods. Luciferase genes in the pGEX-6p-2 vector were altered by site-directed mutagenesis (described below) and transformed into *E. Coli* XL10 gold cells. Luciferase-expressing clones were selected by a dark-room screening and the plasmid was retrieved using Sigma's GeneElute DNA Miniprep Kit. Mutations were confirmed by DNA sequencing at the W.M. Keck Biotechnology Laboratory at Yale University.

After transformation into *E. Coli* BL21 cells, the proteins were expressed at 22 °C and purified by a method described below. Mass-shifts of pure proteins were verified by tandem HPLC-electrospray ionization mass spectrometry with a ThermoFinnigan Surveyor HPLC system and a ThermoFinnigan LCQ Advantage mass spectrometer. The found molecular masses (Da) of the proteins were: CBR, 60 596 and Ppy PRET7 (Ppy RE TS/Arg330Gly/ Ile351Val/ Lys354Glu/ Phe465Arg), 61 000. The determined mass values were all within the allowable experimental error (0.01 %) of the calculated values. The concentrations of pure protein solutions were determined with the Biorad Protein Assay system, using BSA as the standard. The recombinant Ppy WT, Ppy RE and Ppy RE-TS proteins were expressed and purified as previously reported (17, 18, 21).

Site-directed mutagenesis. Starting with the designated DNA sequences in the pGEX-6P-2 vector as templates, the QuikChange® Site-Directed Mutagenesis kit was used to create the PRET7 mutant from the Ppy RE-TS template. These primers and their respective reverse complements were used: Arg330Gly5' - G GTT GCA AAA **GGC**

TTC CAT CTT CCA GGG ATA CGC CAA GGA TAT G- 3' [*StyI*];
Ile351Val/Lys354Glu, 5' - GAG ACT ACT AGC GCT ATT CTG **GTA** ACA CCC
GAG GGG GAT GAT AAA C- 3' [*SpeI*]; Phe465Arg, 5' - GAA TTG GAA TCC ATA
TTG TTA CAA CAC CCC AAC ATC **CGG** GAC GCG GGC- 3' [*ClaI*] (bold
represents the mutated codon, underline represents silent changes to create a unique
screening endonuclease site and brackets indicate the screening endonuclease).

Insertion of Promega's CBRluc into the pGEX-6P-2 vector. The following
primer set was used to amplify *CBRluc* from Promega's pCBR-Basic: 5' - GGT AAA
GGA TCC ATG GTA AAG CGT GAG AAA AAT GTC-3' [*BamHI*] and 5'-ACT CAT
CTC GAG ATC TTA TCA TGT CTG CTC GAA G-3' [*XhoI*] (underline represents
endonuclease site introduced for cloning into the pGEX-6P-2 vector and brackets indicate
the endonuclease). PCR amplification conditions were: initial denaturation at 95°C for
2 min, amplification for 30 cycles (each cycle was 95 °C for 30s, 55 °C for 30s and 68 °C
for 1.75 min) and a final extension at 68 °C for 5 min. PCR products were cleaned with
the QIAquick PCR purification kit and then digested with the restriction endonucleases
listed above. Digested products were purified from an agarose gel with the QIAquick gel
extraction kit and ligated into the corresponding sites on the pGEX-6P-2 vector.

Protein expression and purification. Luciferases were expressed as GST-fusion
proteins in *E. Coli* strain BL21 at 22°C for 18-20 hours in a 500ml culture with LB-amp
media. The cells were harvested by centrifugation at 4°C and the pellets were frozen at

-80°C for at least 15 minutes. They were resuspended in 50 ml of phosphate-buffered saline (PBS) containing 0.1 mM phenylmethylsulfonyl fluoride. Cell membranes were weakened with the addition of a lysozyme solution (5 ml of 10 mg/ml lysozyme in PBS) and lysed by sonication. To solubilize membrane components, Triton X-100 (1% final volume) was added. Whole cell extracts were isolated by centrifugation at 20,000×g for 45 minutes at 4°C.

Proteins were further purified using glutathione Sepharose4B affinity chromatography according to the manufacturer's instructions. During this purification, luciferases were released from the GST tags by incubation in Prescission protease in CB (50 mM Tris-HCl (pH 7.0) containing 150 mM NaCl, 1 mM EDTA, and 1 mM dithiothreitol) for 18-20 hours at 4°C with gentle mixing. Proteins were eluted with CB and were stored at 4°C in this buffer with additives 0.8 M ammonium sulfate and 2% glycerol (CBA).

For studies in bacterial lysates, the GST-luciferase fusion proteins were expressed in *E. coli* strain BL21, grown in 10 ml LB-amp at 37 °C to mid log phase ($A_{600} = 0.6-0.9$) and induced with 0.1 mM IPTG. Following expression for 16 hrs at 37 °C, the cells were harvested by centrifugation at 4 °C and the resulting pellets were resuspended in 0.5 ml of PBS containing 0.1 mM PMSF and 0.5 mM DTT. Lysozyme (5 µl of a 10 mg/ml solution) was added to the cell suspensions and they were incubated on ice for 10 minutes. The cell suspensions were then frozen in a dry ice-isopropanol bath and rapidly thawed in a 37°C water bath. After 10 additional freeze/thaw cycles, the lysates were treated with DNase (5 µg/ml) and RNase (10 µg/ml) for 5 min on ice. Triton X-100 (1% final volume) was added and the soluble cell lysates were isolated by

centrifugation at 13,000×g for 30 min at 4°C. These extracts were assayed within several hours of isolation, and could not be stored with additives as the pure proteins were.

Bioluminescence activity-based light assays. A description of the equipment used to perform bioluminescence activity-based light assays has been published previously(22). Reactions were carried out in polypropylene tubes that contained 2-3 µl pure luciferase in CBA and 400 µl LH₂ in glycylglycine buffer (pH 7.8). A final assay concentration 70 µM LH₂ was adequate for all luciferase mutants except for PRET7, which required 300 µM LH₂ due to its elevated K_m constant. Reactions were initiated by injecting 120µl of Mg-ATP (in glycylglycine buffer, pH 8.0, final concentration 2.0 mM) for a total assay volume of 522-523 µl. A Hamamatsu R928 PMT was used to measured specific activity based on flash height, as well as integrated activities based on light emission over 15-minute intervals, and corrections were applied for the spectral response of the PMT (Table 1).

Heat inactivation studies. Pure luciferases in CBA were diluted to 0.08-0.10 mg/ml, transferred to thin-walled PCR tubes and incubated at 37°C in a thermocycler without heating the lid. Bioluminescence activity-based light assays were performed in duplicate to track enzyme inactivation. Control samples were kept at 4°C and did not drop below 90% activity throughout the assays. Enzymes were tested until a 50% activity loss was observed, with the exception of PRET7 and Ppy RE-TS (Table 1). After 24 hours of incubation, PRET7 did not drop below 50% activity, so a value of >24 hours was reported. The 8.8 hour value for Ppy RE-TS was extrapolated from data points taken up to two hours.

Bioluminescence emission spectra. Bioluminescence emission spectra for luciferases were obtained at 25°C and 37°C in a Perkin-Elmer LS55 luminescence spectrometer with the excitation source turned off, using previously described software and methods (22). Reactions contained 20 µl CBA, 120 µl Mg-ATP(2.0 mM), 70 µl LH₂, and a reaction buffer for a 525µl total assay volume upon the addition of 5 µl luciferase to the cuvette. The reaction buffers used to vary pH were 25 mM Mes (pH 6.0), 25 mM glycylglycine (pH 7.0 and 7.8), and 50 mM Tris (pH 8.6). The spectra at 37 °C were obtained 30 min after the addition of the luciferase to the buffered reagent solutions maintained at 37 °C. To ensure that the spectra were not shifting during measurement, reactions were allowed to proceed for at least one minute before the spectra were measured. Results were corrected for the spectral response of the R298 photomultiplier tube.

Microplate luminometer assays. A Luminoskan Ascent Microplate Luminometer (Thermo Fisher Scientific, Waltham, MA) was used to measure the light emission from PRET7 and CBR pure protein samples, and from GST-proteins expressed at 37°C in *E. Coli* and extracted as soluble cell lysates. Measurements were performed in triplicate in 96-well plates (LUMITRAC). In studies using minimal assay reagents (MAR), each well contained 2 µl of sample in 200 µl LH₂ in 25 mM glycylglycine buffer (pH 7.8). For wild-type controls and CBR samples, 91 µM LH₂ was used to saturate the enzyme. For PRET7, 400 µM LH₂ was needed. Assays were initiated by injection of 60 µl Mg-ATP (9mM) in the same buffer. Light output was measured immediately and integrated using the monitor measurement function with an integration time of 20 ms and a measurement

count of 200. For assays in which the initial flash was not needed, a lag time of 2s was used. Data were collected using a PMT voltage of 300 and a 635 nm long-pass filter in place. The data were scaled by 100 and no corrections were applied for the spectral response of the PMT.

Similar activity assays were also performed using Promega's Luciferase Assay Reagent (LAR). Each assay consisted of 2 μ l sample in 130 μ l of 25 mM glycylglycine buffer (pH 7.8) and assays were initiated by injection of 130 μ l of LAR. For the LAR assay using bacterial lysates, an additional luminometric measurement with an integration time of 5000 ms preceded the usual measurement with 20 ms integration. This programming change altered the relative light units reported by the Ascent software, which explains the difference in the y-axis values plotted in Figure 6.

To find detection limit assays of purified proteins, stock solutions were prepared by diluting all proteins to 0.65 mg/ml using CBA containing 1mg/ml BSA. The stock solutions were serially diluted and 2 μ l (2.1 amol– 4.3pmol) aliquots were assayed as above, using a lag time of 2s and a PMT voltage of 900. The values obtained were corrected for background bioluminescence and plotted. Detection limits were defined as a S/N (signal:noise) ratio greater than or equal to 2.0.

Mammalian cell experiments by Promega collaborators. Promega technicians cloned luciferase genes into the Flexi® Vector pF9a CMV mammalian expression vector. Incorporation into the vector was confirmed by sequencing at Agencourt Bioscience Corporation (Beverly, MA).

Transfection of mammalian cells with Luc mutants. HEK293 (ATCC) cells were transfected with luciferase genes in the Flexi® Vector pF9a CMV mammalian expression vector as follows. HEK293 cells were plated into 6 well plates at a concentration of ~500,000 cells per well in DMEM + 10% FBS. Cells were allowed to recover overnight at 37 °C in 5% CO₂. For each well of mammalian cells to be transiently transfected, 95 ul Opti-MEM® I reduced-serum medium was combined with 6 ul *TransIT*®-LTI transfection reagent. The mixture was then vortexed at the highest setting for 1 s and incubated for 20 min at ambient temperature. Next, 2 ug of plasmid DNA was added to the Opti-MEM® I + *TransIT*®-LTI solution. The mixture was incubated at ambient temperature for 20 min, then added to the mammalian cells. Transfections were allowed to proceed for roughly 20 hr at 37 °C in the presence of 5% CO₂.

Preparation of soluble cell lysates from HEK293 cells expressed at 37 °C. Following transfection, HEK293 cells (600,000 cells) were harvested by trypsinization and pelleted by centrifugation at 1000 rpm for 5 minutes. The mammalian cell pellets were then resuspended in 500 µl PBS. Cell lysis was carried out by subjecting the resuspended cells to 5 freeze-thaw cycles at -80 °C and 30 °C. The cell lysates were finally treated with 1% Triton X-100.

Bioluminescence in mammalian cells. Luminescence measurements of soluble cell lysates from HEK293 mammalian cells were performed in triplicate in 96-well tissue culture plates (Costar) using a Varioskan Flash spectral scan multimode plate reader (Thermo Fisher Scientific, Waltham, MA) programmed with SkanIT Software version

2.4. For studies using MAR, each well contained 10 ul of soluble cell lysate in 200 ul of 300 uM LH₂ in 25 mM glycylglycine buffer (pH 7.8). Assays were initiated by injection of 60 ul Mg-ATP (2mM) in 25 mM glycylglycine buffer (pH 7.8). Using the kinetic measurement function, light output was measured immediately and integrated at 500 ms/measurement with each sample being measured continuously for 30 measurements. Light output was measured using a PMT voltage that was automatically adjusted using the AutoRange default setting and filtered through a 600 nm long-pass filter. The Varioskan Flash spectral scan multimode plate reader has a built in delay of 0.8s before the first data point is recorded. No corrections were applied for the spectral response of the PMT.

Bioluminescence activity assays were also performed using LAR. For these experiments, the trypsinized transfected mammalian cells were plated in 96 well plates at 20,000 cells per well and allowed to recover overnight at 37 °C in the presence of 5% CO₂. The media was removed and 20ul 1x Passive Lysis Buffer was added to each well and the cells were allowed to lyse for 10 min while shaking at 500 rpm. Following the cell lysis, plates were placed into the Varioskan Flash plate reader and the reaction was initiated by auto injection of 100ul LAR. Measurements were made as described above.

Results and Discussion

Rationale for mutagenesis. Using Ppy RE-TS as a template that was already characterized (18), we logically introduced point mutations into the gene with the intention of creating a further red-shifted mutant. In the course of a different project in our lab, the mutation Arg330Gly, discovered by a random mutagenesis screening, was found to produce a 9 nm red-shift in other mutants. When introduced into Ppy RE-TS, the mutation shifted its bioluminescence an additional 4 nm, resulting in maximum emission at 614 nm. However, we observed a two-fold decrease in the protein yield from cultures grown at 37°C, and in the longevity of the protein activity when incubated at 37°C, indicating that Arg330Gly had decreased the thermostability of the enzyme. To remedy this problem, we altered residue Phe465, which was identified in L.C. Tisi's lab as yet another residue important to thermostability (19, 20). After mutating this residue to an Arg, the protein yield at 37 °C increased 3.5-fold (a 1.6-fold improvement over Ppy RE-TS), while the bioluminescence color dropped to 613 nm.

Hoping to compensate for the 1 nm loss in the red-shift, we chose to modify one of the five thermostability mutations that had been adapted from Tisi's work to engineer Ppy RE-TS. Of these mutations, Glu354Lys was seen to cause a 6-nm blue-shift in the bioluminescence emission spectrum (data not published), so it was reverted with the aim of regaining red color. Reverting the Lys354 to the native Glu residue caused a promising 4 nm red-shift in the bioluminescence emission maxima, resulting in a mutant with a λ_{\max} of 617 nm.

The final mutation, Ile351Val, had been observed previously by our lab to increase the integrated specific activity of Ppy WT 1.2-fold (data not published) while not affecting the color of Luc wild-type. When introduced into the red-emitting mutant, the 8-second integrated specific activity surpassed that of the Ppy RE-TS template. All mutations and reversions accounted for, this resultant enzyme could be identified as Ppy WT with eight changes: T214A, A215L, I232A, S284T, R330G, I351V, F465R, and F295L (Fig. 3). It can alternately be viewed as Ppy RE with seven changes, and was therefore named PRET7 (**P**py, **R**ed-emitter, **T**hermostable, **7** mutations).

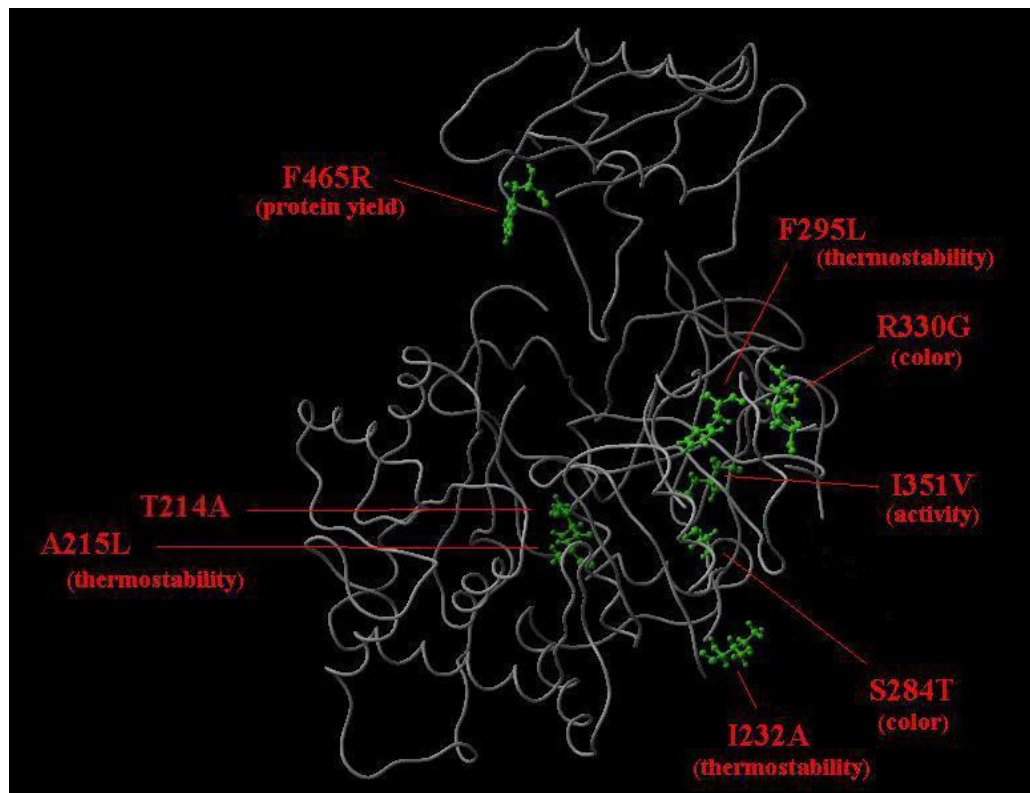


Figure 3. Eight mutations added to wild-type *P. Pyralis* firefly luciferase to create red-emitting mutant PRET7.

Bioluminescence color of luciferase mutants. The new red-emitting mutant PRET7 was characterized and compared with both previous templates, Ppy RE and Ppy RE-TS, as well as Promega's commercial enzyme CBR. Improved bioluminescence color had been the primary goal in engineering PRET7, and this goal was met. When the five thermostability mutations were added to Ppy RE to make Ppy RE-TS, a slight green-shift occurred at both 25°C and 37°C, resulting in emission maxima of 610 nm and 612 nm, respectively (Table 1). The four alterations in PRET7 caused red-shifts at both assayed temperatures, resulting in emission maxima at 617 nm and 618 nm. Therefore, the impressive 615 nm maximum of Ppy RE was not only restored, but also exceeded by PRET7 (Fig. 6). Also, the PRET7 emission maximum matches that of CBR at 25°C and is only one nm lower at 37°C. At both temperatures, PRET7 has a slightly smaller bandwidth at full-width half-maximum than CBR, indicating a more narrow emission profile, which is necessary to minimize spectral overlap with a green counterpart in dual-analyte assays.

The improved red-shift of PRET7 might also make it a good candidate for *in vivo* bioluminescence imaging. The expression of bioluminescent proteins inside live mammals can be used to visualize events such as tumor growth and metastasis, cell trafficking, apoptosis, and to confirm gene delivery (23, 24, 25). One challenge in these applications is the scattering and absorption of light due to hemoglobin and myoglobin (23). Because light of 600 nm or longer wavelength penetrates tissue better than do shorter wavelengths (23), PRET7 should be well-suited for *in vivo* imaging. To investigate its potential as an imaging reporter, PRET7 has been sent to the lab of Aldo G. Roda (University of Bologna, Italy), where it is currently being evaluated in murine models.

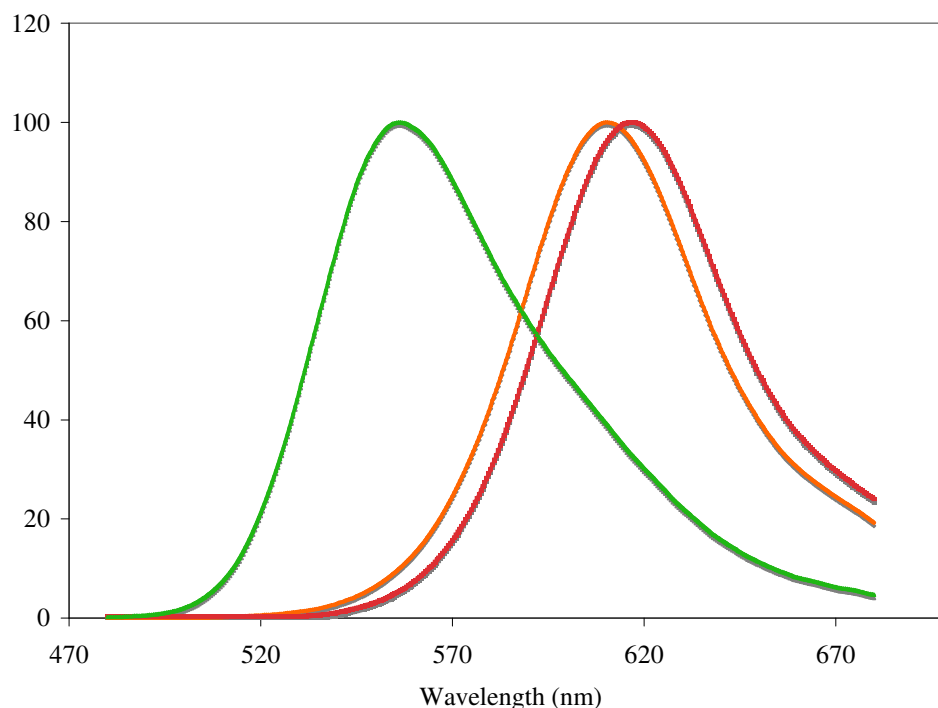


Figure 4. Bioluminescence spectra of Ppy WT (green curve), Ppy RE-TS (orange curve) and PRET7 (red curve) were obtained at pH 7.8 and 25°C using a Perkin-Elmer LS55 luminescence spectrometer with the excitation source turned off, as described in Materials and Methods.

Specific activities of luciferase mutants. Red-shifted bioluminescence is often difficult to achieve without incurring losses in the flash-height based specific activity of the luciferase. For example, Ppy RE, which contains only the single S284T mutation, lost 77% of its flash-height based specific activity relative to Ppy WT (Table 1). As anticipated, the flash-height specific activity of PRET7 dropped relative to Ppy RE-TS. But this activity loss was not too dramatic, considering that PRET7 is still over 7-fold more active than CBR in terms of flash-height.

For dual-analyte assays that are carried out for several seconds or minutes, the sustained light-emission of the luciferase may be more important than the initial flash.

Unlike the case with flash-height, mutations to red-shift a luciferase do not necessarily have predictable effects on the sustained light emission of an enzyme. When light output was integrated over 8-second and 15-minute intervals, PRET7 appeared to have respectively gained and lost specific activity compared to Ppy RE-TS. But more importantly, the advantage over CBR was not lost, since PRET7 emits ~7-fold more light than CBR in the 8s assay, and ~9-fold more light in the 15-minute assay. In summary, our objective of making PRET7 red-shifted without significantly compromising specific activity was achieved. PRET7, like Ppy RE-TS, provides a stronger signal during the initial flash and throughout 8-second and 15-minute assays compared to commercially available CBR.

Table 1. Properties of pure luciferases at pH 7.8

Enzyme	Relative specific activity ^b			Thermal inactivation Time to 50% activity loss, 37 °C (h)	Bioluminescence emission maximum ^c	
	Flash Height	Integration (8s)	Integration (15 min)		25 °C λ max (fwhm)	37 °C λ max (fwhm)
Ppy WT	100	100	100	0.26	557 (66)	563, 610(sh) (92)
Ppy RE ^a	23	18	32	0.80	615 (57)	616 (64)
Ppy RE-TS	31	15	61	8.8	610 (59)	612 (60)
PRET7	22	21	34	>24	617 (60)	618 (62)
CBR	3	3	4	2.1	617 (62)	619 (66)

^a Characterization of Ppy RE and Ppy RE-TS was previously reported (17, 18).

^b Flash height-based and 15 min integrated specific activities were obtained as described in Materials and Methods and were corrected for the spectral response of the Hamamatsu R928 PMT. Enzyme activity values used to calculate 8s integration values were obtained as described in Materials and methods using a Luminoskan microplate luminometer to integrate light output for 8 sec after a 2-sec delay, employing a 610 nm cut-off filter. All activity values are expressed relative to Ppy WT at pH 7.8, defined as 100.

^c Bioluminescence emission spectra of purified proteins were obtained as described in Materials and methods, with bandwidths at full-width half-maximum (fwhm) and shoulders (sh) indicated.

Heat inactivation studies. In addition to enzyme color, specific activity, and kinetics, thermostability at 37°C was another property of PRET7 that was analyzed. *In vivo* expression of luciferases requires enzymes that remain stable at physiological temperature, approximately 37°C. While *in vitro* reporter assays can be carried out at any controlled temperature, the expression that is monitored often takes place at 37°C, and should be assayed at this temperature to serve an accurate reporter function. For both *in vivo* and *in vitro* experiments, it is predicted that luciferases with greater stability at 37°C are more likely to be expressed as properly-folded proteins in higher yield, and will remain active for longer time periods, resulting in more sensitive reporter assays overall.

Ppy RE-TS was much more stable at 37°C than its template Ppy RE, retaining at least 50% activity for up to 8.8 hours at 37°C, compared to 0.8 hours, and it was anticipated that PRET7 would share this characteristic (Table 1). The PRET7 inactivation assay results surpassed expectations, since PRET7 did not drop below 50% activity even after 24 hours of incubation. It is uncertain whether the outstanding thermostability of PRET7 will translate to a significant advantage in reporter assays compared to Ppy RE-TS or CBR, since it is possible that a 24 hour period of stability is far greater than is necessary for a sensitive assay. But in theory, PRET7 should be a generally more versatile enzyme to work with at room temperature and physiological temperature, since refrigeration is less imperative. The possibility of protein degradation due to heat-exposure, which might be a problem for some researchers (26), will be avoided if PRET7 is used.

Microplate luminometer studies. To compare the performances of PRET7 and CBR, 8-second plots of light-emission were generated and integrated for pure proteins,

bacterial cell lysates, and HEK293 mammalian cells using 96-well microplate luminometers (Figs. 5-7, Table 2). These studies were carried out with both minimal assay reagents (MAR) and Promega's Luciferase Assay Reagent (LAR), so as to assess the feasibility of using these reagents for reporter assays with these enzymes.

Minimal assay reagents include the bare minimum of substrates required for the reaction, LH_2 and Mg-ATP, as well as 25mM glycylglycine buffer at pH 7.8. The performance of PRET7 in minimal assay reagents was superior to CBR under all three assayed conditions. The intrinsic properties of the PRET7 protein resulted in 6-fold greater activity than CBR (Fig. 5, Table 2). However, in bacterial cell lysates the advantage dropped to 3.5-fold, and in mammalian cells it dropped to 2.5-fold, suggesting that CBR is expressed in higher yield in these cells lines compared to PRET7 (Figs. 6 and 7). It was expected that CBR, because it is human-codon optimized, would be expressed more efficiently in mammalian cells than bacterial cells, which may explain the difference between the 3.5-fold and 2.5-fold advantages. But it is unclear why CBR expression is better than that of PRET7 in *E. Coli* at 37°C, considering the thermostability exhibited by PRET7 at that temperature. It is possible that the codon-optimization of CBR for expression in human cell lines has favorable effects in bacterial cells as well. Regardless, when minimal assay reagents are used the specific activity of PRET7 is sufficiently high to outweigh disadvantages in the expression.

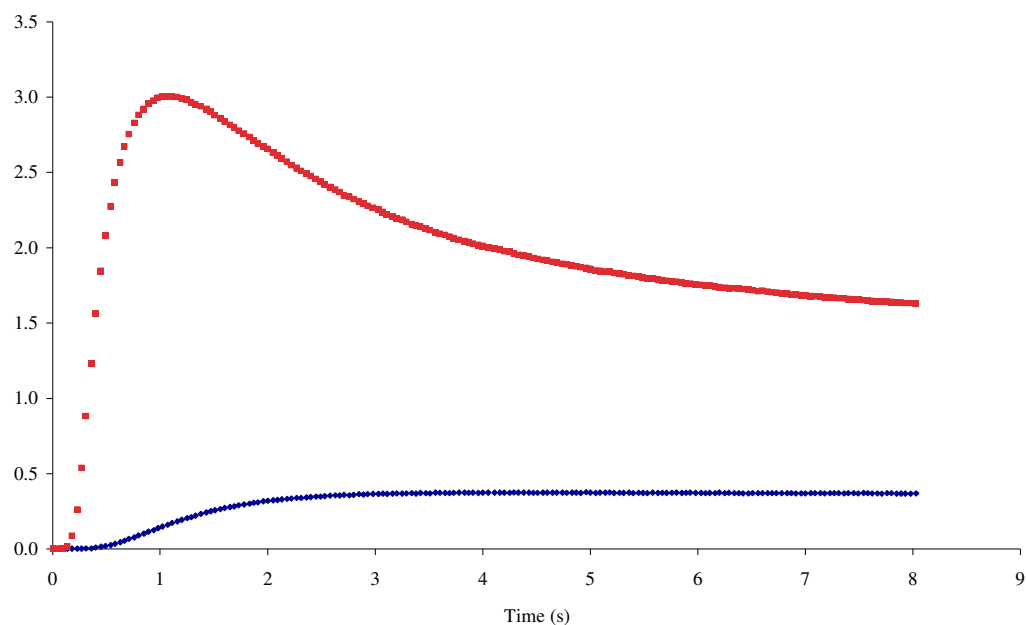
When LAR was used, the superior performance of PRET7 over CBR was diminished in the pure protein assay, dropping to a 2-fold advantage (Fig. 5, Table 2). In bacterial and mammalian cell lysates, LAR assays actually favored CBR, which had a 2.7-fold greater activity when expressed in bacteria and a 1.3-fold greater activity when

expressed in mammalian cells (Figs. 6 and 7, Table 2). According to the pure protein data, LAR appears to decrease PRET7 integrated activity, in part by dampening the initial flash, and also elicits a steady rise in light emission from CBR (Fig. 5). Because the full formula of LAR is proprietary, it is not possible to determine precisely how the enzyme kinetics and activity are affected biochemically. It is likely that additional chemicals, such as coenzyme A (27), have been added to the minimal substrates, and it appears that these components favor Promega's enzyme while impairing PRET7.

Using two different reagents also affected the linear ranges of detection for these luciferases, but not dramatically (Fig. 8). In MAR, PRET7 provides a detectable signal when assayed at 3×10^{-18} mol quantities, whereas CBR is only detectable to 1×10^{-16} mol. In LAR, the detection limits for the two luciferases begin to converge, such that both enzymes reach their detection limit close to 1×10^{-17} mol, though PRET7 still appears to provide a slightly stronger signal at all assayed concentrations.

In summary, the microplate luminometer assays indicate that PRET7, though it has lower expression levels in cell cultures than CBR, has comparatively impressive light-emission over an 8-second time period when minimal assay reagents are used. It is also detectable in lower quantities than CBR when assayed as a pure protein in MAR, which may translate to greater sensitivity in an applied bioluminescence assay system. Also promising is the finding that PRET7 performed best in MAR instead of the commercial reagent LAR. An enzyme like PRET7 that performs well with the simple, relatively inexpensive reagents Mg-ATP and LH_2 may be appreciated by researchers who do not want to purchase entire assay kits.

A. MAR



B. LAR

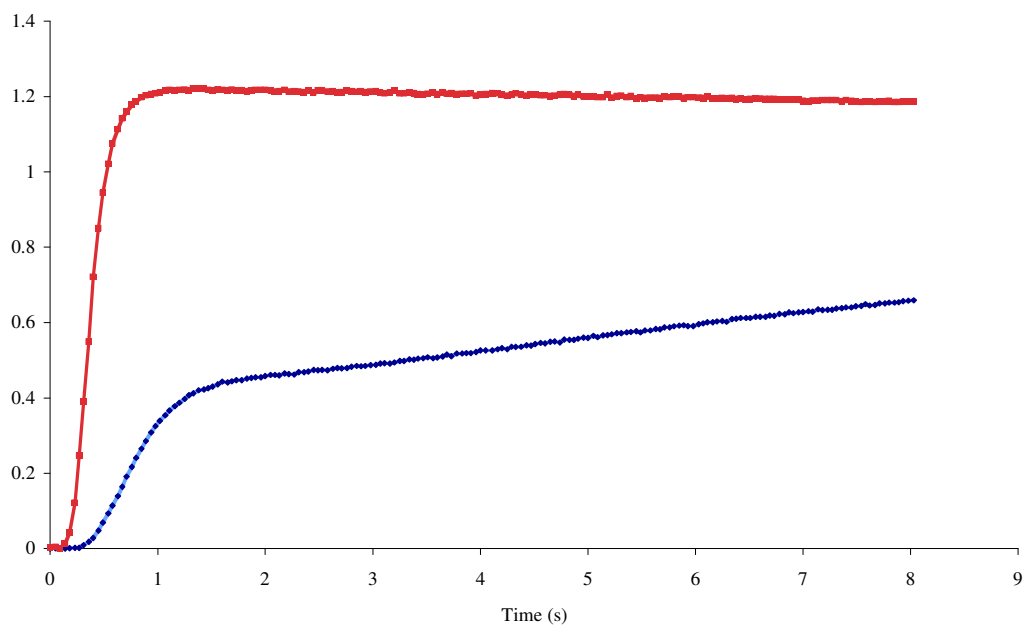
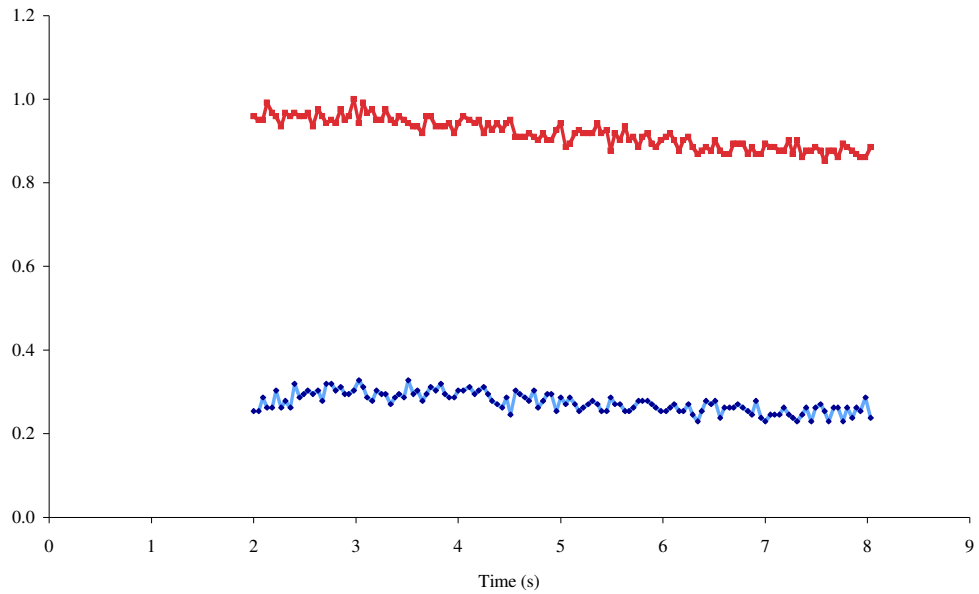


Figure 5. Bioluminescence of pure red-emitting luciferases reacted with A) minimal assay reagent (MAR) and B) Luciferase Assay Reagent (LAR). Relative light emission of PRET7 (red curve) and CBR (blue curve) was measured in a Luminoskan Ascent Microplate Luminometer. Assays (0.262 ml) were performed in triplicate at pH 7.8 and ambient temperature as described in Materials and Methods, and mean values were plotted.

A. MAR



B. LAR

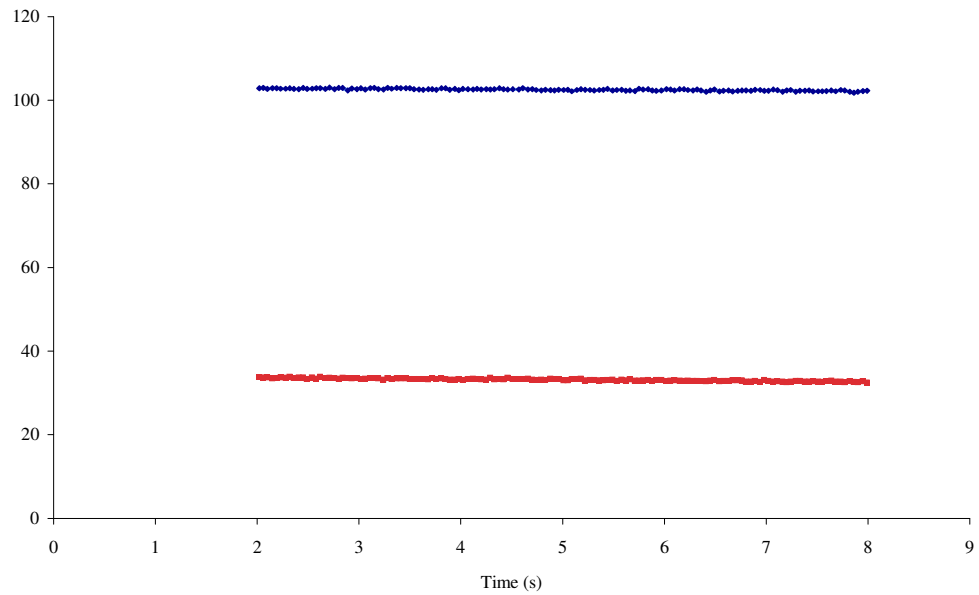
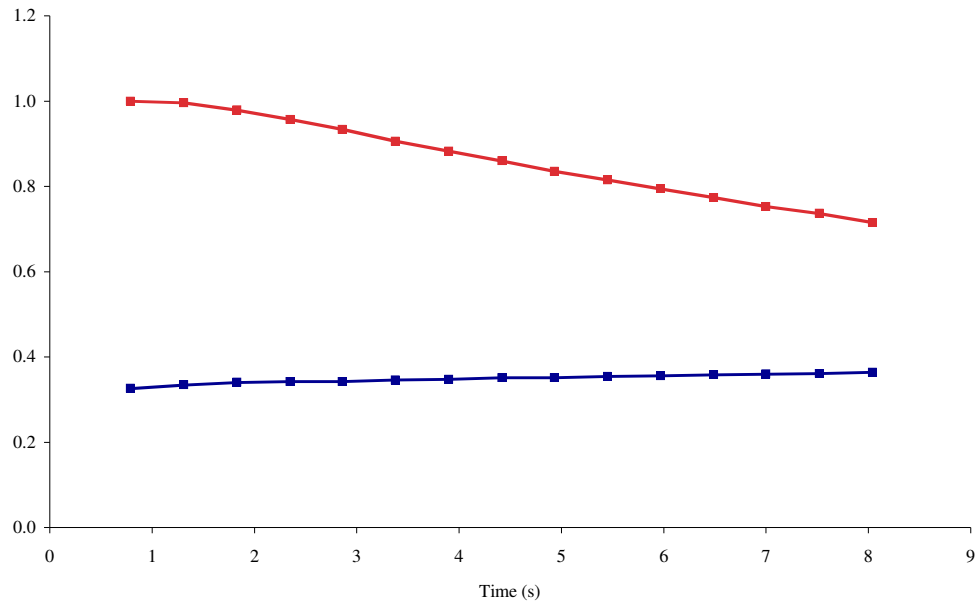


Figure 6. Bioluminescence of soluble cell lysates from *E. Coli* which expressed red-emitting GST-fusion luciferases PRET7 and CBR at 37°C. Cell lysates were reacted with A) minimal assay reagent (MAR) and B) Luciferase Assay Reagent (LAR). Relative light emission of PRET7 (red curve) and CBR (blue curve) was measured in a Luminoskan Ascent Microplate Luminometer using a 2s delay. Assays (0.262 ml) were performed in triplicate at pH 7.8 and ambient temperature, and mean values were plotted. Plots differ in appearance because the LAR assay was performed with an additional luminometric measurement, as explained in Materials and Methods, that resulted in higher relative light unit values and a cleaner line.

A. MAR



B. LAR

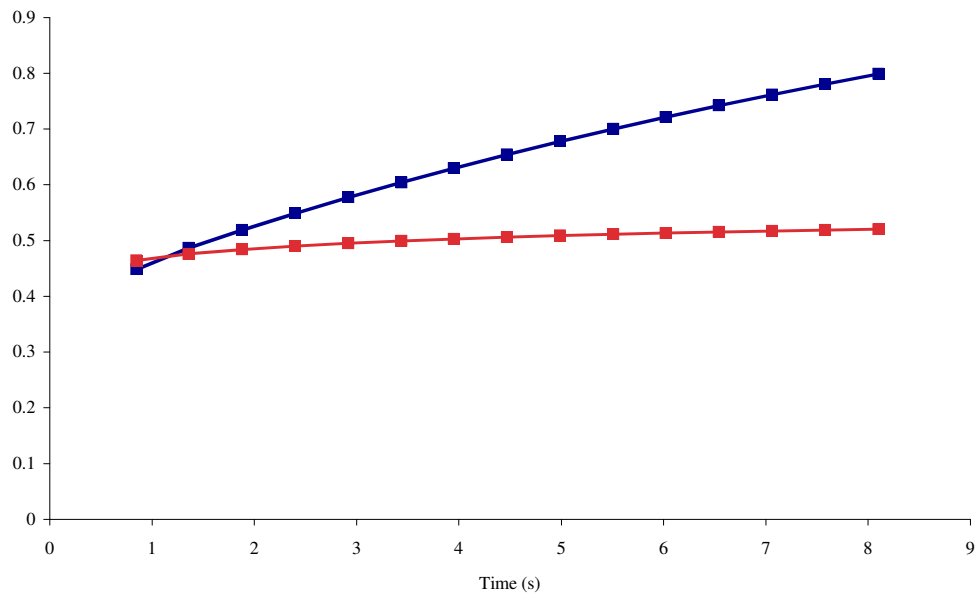


Figure 7. Bioluminescence of soluble cell lysates from HEK293 cells which expressed red-emitting GST-fusion luciferases PRET7 and CBR at 37°C. Cell lysates were reacted with A) minimal assay reagent (MAR) and B) Luciferase Assay Reagent (LAR). Relative light emission of PRET7 (red curve) and CBR (blue curve) in pH 7.8 buffer was measured with a Varioskan Flash plate reader with a built-in delay of 0.8s, as described in Materials and Methods.

Table 2. Integrated relative luminescence of luciferase proteins^a

Enzyme	pure protein (Fig. 5)		bacterial soluble cell extract (Fig. 6)		mammalian soluble cell extract (Fig. 7)	
	MAR	LAR	MAR	LAR	MAR	LAR
PRET7	397	229	176	6933	9 494 047	192 945 440
CBR	63	101	48	18706	3 954 664	254 490 861

^aIntegrated values are reported in relative light units that are dependent on programming and instrumentation.

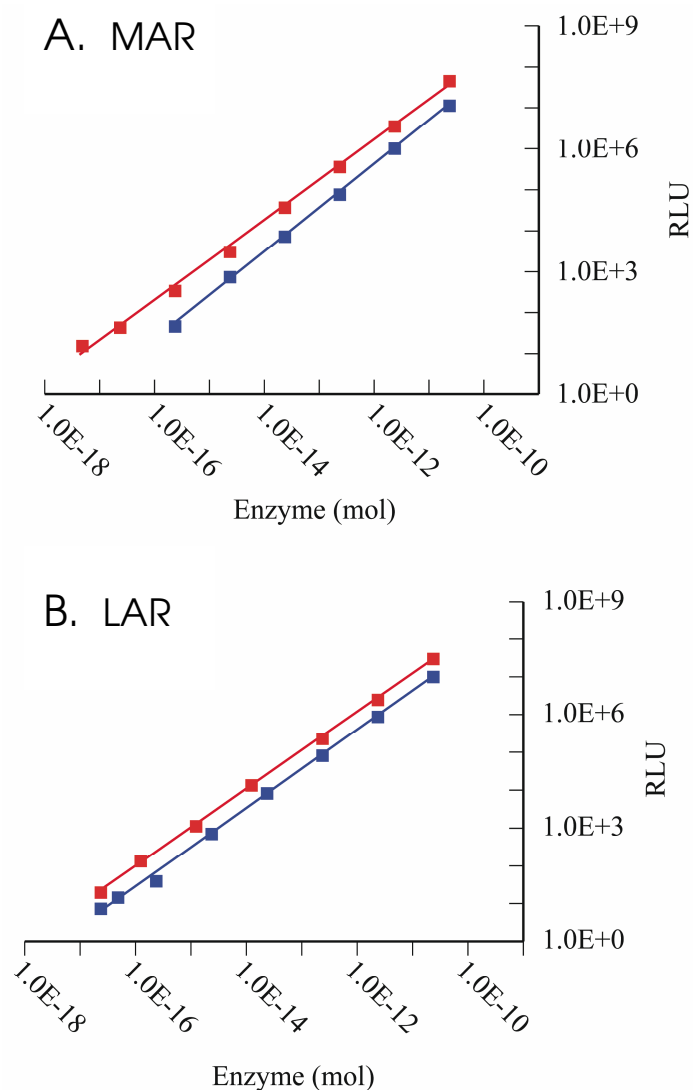


Figure 8. Relationship of relative bioluminescence activity to enzyme concentration for purified red-emitting luciferases in (A) minimal assay reagent (MAR) and (B) Luciferase Assay Reagent (LAR). Luciferase activity (relative emission intensity) of PRET7 (red line, $R^2=0.99$), and CBR (blue line, $R^2=0.99$) were measured in a Luminoskan Microplate Luminometer. Activity assays (0.262 ml) were performed in triplicate at pH 7.8 and ambient temperature as described in Materials and methods. The mean values are plotted.

Codon-optimization for mammalian cells. Though PRET7 performance in minimal reagents was superior to CBR even in mammalian cells, we anticipate that human-codon optimization of PRET7 will lead to further improvements. The PRET7 gene has just recently been codon-optimized by collaborators Amit P. Jathoul and Martin A. Pule (University College London). To tailor the enzyme for mammalian cell expression, silent mutations were made throughout the DNA sequence to favor the codon usage bias of human cells, rather than firefly cells. The sequence was also altered to eliminate stretches of G and C repeats, local hairpins, and cryptic splice sites, and to remove the C-terminal targeting sequence that directs peroxisomal degradation. Additionally, the GC content was increased to 70%, a strategy known to increase the efficiency of expression (28). With optimized sequence, we presume that more properly-folded protein will remain in the cytosol for a greater length of time, increasing the overall light-emission (29). Aside from Promega's CBR, luciferases from *Renilla*, *P. Pyralis*, and *Gaussia* fireflies have been codon-optimized for mammalian cells with success (30, 31, 32).

The PRET7 human codon-optimized plasmid has been sent to Promega collaborators for reevaluation in mammalian cells, and will again be compared to CBR. It is anticipated that codon-optimizing PRET7 will improve the 2.5-fold advantage that was observed relative to CBR. The 6-fold advantage observed in the pure protein assays represents the maximum value that would be achieved if the proteins were expressed in equal quantities. The end result will be a red-emitting enzyme that, when compared with a marketed enzyme, has near-identical color, at least 10 times more longevity at 37°C,

and emits a stronger bioluminescent signal in bacterial and mammalian cells lysates when assayed with the least expensive reagents. We look forward to receiving *in vivo* imaging results from collaborators in Bologna, Italy to conclusively determine whether PRET7 is a viable candidate for imaging studies, as well.

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