



# Purification and New Cell Biology Assays Using a Stabilized Luciferase Exhibiting Red Luminescence.

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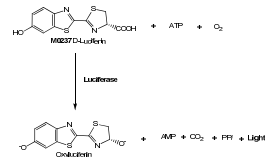
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## Introduction

Bioluminescence is the light produced in certain organisms as a result of enzyme mediated oxidation of a luciferin substrate. This oxidation is dependent on the presence of adenosine triphosphate (ATP). We have developed a codon optimized luciferase based on the gene sequence of the natural luciferase gene isolated from *Luciola cruciata* (Japanese firefly). This new gene codes for a modified amino acid sequence which exhibits improved expression levels in mammalian cells, shifted emission wavelengths (green to red) as well as improved thermal stability. Stability, emission and expression level analyses were performed in NIH3T3 cells transfected with this new luciferase construct. Furthermore, we cloned this new luciferase into a pET vector containing a His-tag at the N-terminus and the resulting construct was transformed into *E. coli* strain BL21 (DE3) for overexpression. The expressed protein was purified to homogeneity with a Ni-sepharose resin. Further study indicated that the new luciferase expressed in *E. coli* retained all the characteristics as observed in mammalian cells. Luciferase expression was used as a genetic marker in cell extracts when mixed with D-Luciferin, Mg<sup>2+</sup>, various concentrations of ATP and resulting luminescence measured using luminescent detection. We also utilized the purified protein to measure ATP levels in biological samples. Cell number could be quantitated using the purified luciferase. We found the cell number of sarcoma (MES-SA) and breast carcinoma (MDA-MB-231T) cells in culture to be proportional to light emission over a large range (10<sup>2</sup> to 5 x 10<sup>4</sup>). We also used the purified luciferase to measure cell viability as well as cytotoxicity effects with chemotherapeutic drugs (Paclitaxel and Doxorubicin). In addition, this new luciferase has a red-shift in light emission (619 nm) which has advantages in applications such as tissue imaging and multiplexed analysis.



**Figure 1: Luciferase reaction to produce light:** Luciferase mediates oxidation of D-luciferin (M2037) with ATP to produce light.

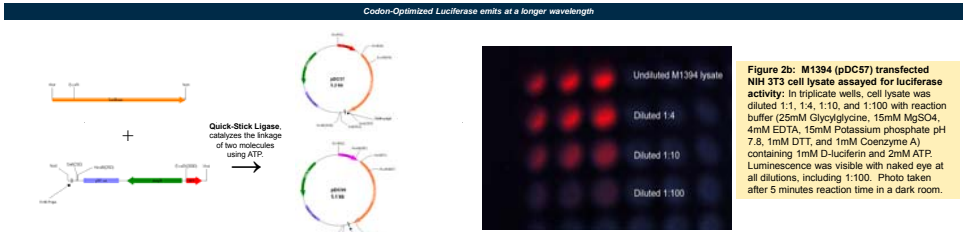
## Methods

### Vector Construction and Protein Purification.

- A codon optimized and stabilized (COS) luciferase gene from *Luciola cruciata* (the Japanese firefly) was cloned into vectors pCD27 (M1394) and pCD99 (M1398) containing the Cytomegalovirus Immediate Early Gene (CMV IE) and the SmaI virus 40 Early Core (SV40) promoters respectively.
- The codon optimized and stabilized (COS) luciferase gene was sub-cloned into a pET-His vector (ATCC) for expression in *E. coli*.
- The His-tagged luciferase protein was purified Ni-sepharose (GE healthcare) chromatography.

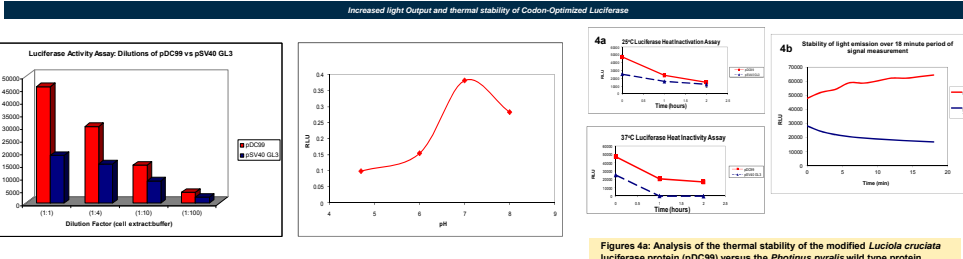
### Luciferase Assays

- NIH 3T3 cells were transfected with M1234 (Promega), M1394, M1395 using IpefectAMINE (Sigma). DNA was pre-complexed with PLUS reagent (Sigma), incubated and added to IpefectAMINE. DNA, PLUS reagent, IpefectAMINE mixture was then added to NIH 3T3 cells. Cells were grown overnight and lysed using lysis buffer (20mM Tris-phosphate (pH 7.8) containing 10% glycerol, 1% Triton X-100, 1 mg/ml BSA, 2 mM EGTA and 2mM DTT). Cell lysate was diluted 1:1 with reaction buffer (25mM Glycylglycine, 15mM MgSO<sub>4</sub>, 4mM EDTA, 15mM Potassium phosphate pH 7.8, 1mM DTT, and 2mM Coenzyme A) containing 1mM D-luciferin and 2mM ATP. Luminescence was recorded using a Perkin-Elmer HTS7000 Plus Bio Assay Reader (200 ms integration time).
- Thermal stability of the modified *Luciola cruciata* luciferase protein versus wild type protein was analyzed using cell lysate of transfected cells incubated at 25°C, 37°C, and 42°C. At 1 hour and 2 hour intervals, aliquots of each temperature-incubation were removed and assayed for activity using the method described above.
- ATP Assays were performed as follows: 0.2mM ATP in reaction buffer containing 1mM D-luciferin was mixed with purified luciferase (4μg). Luminescence was recorded using a BioTek Mx Synergy Reader at an integration time of 5s.
- Cell number assays were performed as follows: 40,000 cells per well were seeded in triplicate wells, incubated at 37°C overnight, then lysed with 60μl lysis buffer (described above) for 30 min on ice. 50μl of the lysate was mixed with equal volume of Luciferase/Luciferin mixture (10μg luciferase and 1mM Luciferin dissolved in reaction buffer) and read immediately using a BioTek Synergy Mx reader with an integration time of 5 s.
- Cell Viability/Cytotoxicity assays were performed using cells seeded in 96-well plates with incubation at 37°C overnight before treating with Doxorubicin. For viability assay, cells were allowed to reach 40% confluency before treatment with Doxorubicin for 48 h before assay. For cytotoxicity assays, cells were allowed to reach 70% confluency and then are treated with Doxorubicin for 24 h. Cells were lysed and assayed as described in "cell number assay".
- A coupled luciferase-β-galactosidase assays were carried out as following: 16-100mM of D-Luciferin-6-O-β-D-galactopyranoside (M1087) was incubated with saturating β-galactosidase (~0.50U) for 30 min, at room temperature. The generated D-luciferin in the reaction was measured by mixing with cos-luciferase (4μg per μl) and 2mM ATP mixture in reaction buffer. The light output was recorded using a BioTek Synergy Mx plate reader with an integration time of 5s.



**Figure 2a: Construction of codon optimized *L. cruciata* luciferase gene mammalian expression vectors.**

**Figure 2b: M1394 (pDC57) transfected NIH 3T3 cell lysate assayed for luciferase activity:** In triplicate wells, cell lysate was diluted 1:1, 1:4, 1:10, and 1:100 with reaction buffer (25mM Glycylglycine, 15mM MgSO<sub>4</sub>, 4mM EDTA, 15mM Potassium phosphate pH 7.8, 1mM DTT, and 1mM Coenzyme A) containing 1mM D-luciferin and 2mM ATP. Luminescence was visible with naked eye at all dilutions, including 1:100. Photo taken after 5 minutes reaction time in a dark room.

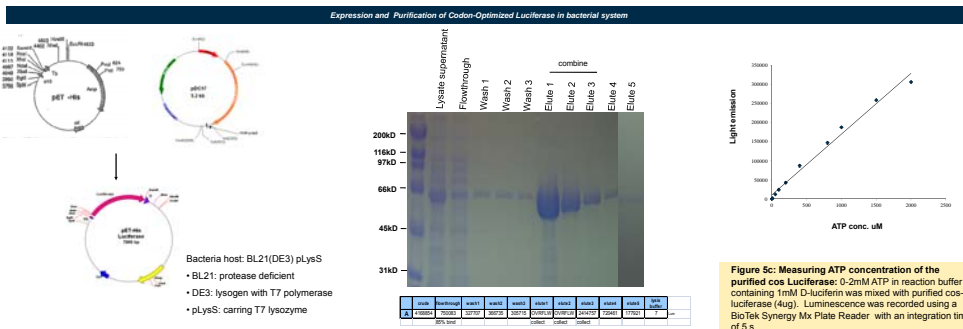


**Figure 3a: Comparison of luciferase expression in mammalian cell lines:** the pGL3- vector containing *Photinus pyralis* luciferase gene from Promega, the pDC99 vector containing a codon-optimized (cos) *L. cruciata* vector from Marker Gene Technologies, Inc. Both vectors utilize SV40 promoter for expression.

**Figure 3b: pH optimum for luciferase:** Cell lysate of NIH 3T3 transfected with M1394 was diluted 1:1 with reaction buffer. Emission wavelength of M1394 was measured at different pHs (4.7, 6.0, 7.0, 8.0) using the Hitachi Fluorescence Spectrophotometer F-2500.

**Figure 4a: Analysis of the thermal stability of the modified *Luciola cruciata* luciferase protein (pDC99) versus the *Photinus pyralis* wild type protein (pSV40GL3):** (upper) at 25°C and (lower) at 37°C. Assay conditions: in triplicate wells, cell lysate was diluted 1:1 with reaction buffer containing 1mM D-luciferin and 2mM Blank (reaction buffer) was subtracted from wells. Luminescence was recorded using a Perkin-Elmer HTS7000 Plus Bio Assay Reader (200 ms integration time).

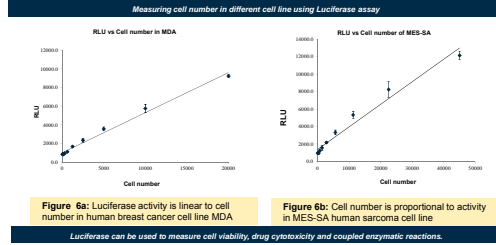
**Figure 4b: Stability of light emission over time of modified *Luciola cruciata* luciferase protein (pDC99) versus the *Photinus pyralis* wild type protein (pSV40GL3):** Cell lysate was diluted 1:1 with reaction buffer containing 1mM D-luciferin and 2mM ATP at indicated time. Luminescence was recorded using a Perkin-Elmer HTS7000 Plus Bio Assay Reader (200 ms integration time).



**Figure 5a: Cloning of Luciferase gene into a bacterial expression vector**

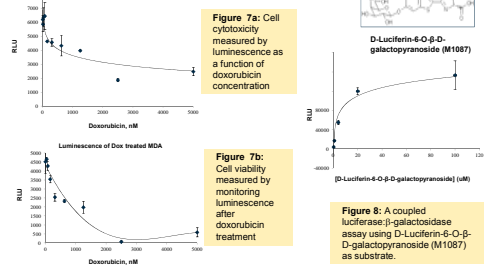
**Figure 5b: Purification of His-tagged luciferase by Ni-sepharose (GE healthcare)**

**Figure 5c: Measuring ATP concentration of the purified cos Luciferase:** 0.2mM ATP in reaction buffer containing 1mM D-luciferin was mixed with purified cos-luciferase (4μg). Luminescence was recorded using a BioTek Synergy Mx Plate Reader with an integration time of 5 s.



**Figure 6a: Luciferase activity is linear to cell number in human breast cancer cell line MDA**

**Figure 6b: Cell number is proportional to activity in MES-SA human sarcoma cell line**



**Figure 7a: Cell cytotoxicity measured by luminescence as a function of doxorubicin concentration**

**Figure 7b: Cell viability measured by monitoring luminescence after doxorubicin treatment**

**Figure 8: A coupled luciferase-β-galactosidase assay using D-Luciferin-6-O-β-D-galactopyranoside (M1087) as substrate.**

## Discussion and Conclusions

In order to develop improved luciferase enzyme systems with long wavelength bioluminescence, we developed a set of codon optimized luciferase vectors that code for a (COS) luciferase enzyme with long-wavelength (red) light (Em: 605nm) emission that has improved expression, thermostability and light production, allowing detection of expression at extremely low levels. The vectors can be used for transfection of cell and tissues from various mammalian and plant species.

After overexpression in bacteria and column purification, the COS-luciferase enzyme was utilized in a variety of ultrasensitive assay systems. It was found that this new enzyme could be useful for monitoring ATP levels, cell number, cell viability, cellular cytotoxicity as well as for monitoring a second enzyme activity in a coupled assay format. The enzyme was found to have the ability to measure quantitatively ATP levels over several orders of magnitude. The new COS-luciferase could also be used to accurately measure cell number for both MES-SA and MDA-MB-231T cell lines over several orders of magnitude. The enzyme also found utility in ultrasensitive cytotoxicity assays for monitoring the effect of compound or drug application (Paclitaxel and Doxorubicin). Finally, the new COS-luciferase enzyme could be utilized in a coupled assay format to accurately measure the levels of a second enzyme, *E. coli* β-galactosidase, in test samples. The combined utility of the new COS-luciferase for enzyme assay development, in multiplexed assays with other bioluminescence assays, and for cellular or tissue analysis *in vivo* are significant.

## References

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**COS-Luciferase based kits available**  
 M1575: Lumi-Lite™ Cell Viability Assay Kit  
 M1576: Lumi-Lite™ Cytotoxicity Assay Kit