TOYOBO

MultiReporter Assay System -Tripluc™-
Tripluc™ Luciferase Assay Reagent

For 100 reactions (Code No. MRA-301)

Handling Instructions

TOYOBO CO., LTD.
Biotechnology Overseas Sales and Marketing Department
OSAKA JAPAN

- Contents -

Caution:

This reagent is for research purpose only. Do not use for diagnosis or other clinical purposes. When using this reagent, follow general laboratory precautions strictly and take adequate care to ensure safety.

The luciferase genes for this system were used with the permission of the National Institute of Advanced Industrial Science and Technology (AIST). The AIST has filed an application for patents covering the luciferase genes and the technology related to these genes for measuring transcriptional regulation of the multiple genes simultaneously. Industrial utilization of this system requires a separate permission. Please contact us if you intend to utilize this system for industrial purposes.

[1] Introduction

Analysis of gene expression regulation using reporter genes (hereinafter referred to as “reporter assay”) is designed to evaluate the regulation of gene expression using the activity of a reporter enzyme (which is expressed under certain conditions) after introducing a vector into an appropriate host cell line. The vector used contains elements of the cis-acting sequence (i.e., probable gene expression regulatory sequences, such as promoter, enhancer and silencer) coupled to the reporter gene. The samples used in the reporter assay involve factors that can cause a difference in the absolute amount of the reporter and that are independent of the cis-acting sequence elements. These factors include transfection efficiency, number of cells, state of cell growth and apoptosis. To avoid any influence from these factors, it is necessary to add another reporter (reporter with varying substrate specificity or reactivity, coupled to the control promoter) as internal standards, together with the reporter gene coupled to the test sequence, with the goal of achieving standardization of multiple samples.

Dr. Ohmiya (National Institute of Advanced Industrial Science and Technology) and colleagues succeeded in expressing green-emitting luciferase (**G**reen, maximum wavelength 550 nm), orange-emitting luciferase (**O**range, 580 nm) and red-emitting luciferase (**R**ed, 630 nm) in mammalian cells on a practical level [1,2]. The light-emitting spectra of these luciferases have a small half-band width; they are relatively sharp and are stable against pH (**S**table **L**uciferases). These luciferases are therefore suitable for measurement by splitting emissions[3]. Jointly with Dr. Ohmiya and his colleagues, our company has developed a MultiReporter Assay System -Tripluc^{TM}- Tripluc^{TM} Luciferase Assay Reagent and Vector Series (capable of measuring expression of three genes simultaneously).

Tripluc^{TM} Luciferase Assay Reagent is optimized for the reporter assay with MultiReporter Assay System -Tripluc^{TM}- Vector Series.

[2] Product content

Product	Package	Code No.	Storage
MultiReporter Assay System -Tripluc^{TM}- Tripluc^{TM} Luciferase Assay Reagent	For 100 reactions	MRA-301	-80°C

As a rule the assay reagent may be subjected to two or less cycles of freezing and thawing. It is advisable to divide the reagent into small adequate volume of fractions.

[3] Apparatus and instruments

Measurement of three color luciferases requires devices such as a luminometer equipped with a function of measurement by splitting emissions. The devices need to satisfy the following requirements:

(1) Allow measurement for the wavelength range between 450 and 750 nm
(2) Able to be combined with two or three appropriate optical filters
(3) Preferably allow measurement while switching between filters

[4] Assay Procedure

1 Preparation of the reagent

- The assay reagent is thawed at room temperature, or in a water bath.
- The reagent is equilibrated to room temperature.

2. Assay Procedure

(1) Remove plates containing mammalian cells from CO_2 incubator. The cultured cells are equilibrated to room temperature.

(2) To each plate well, add a volume of Tripluc™ Luciferase Assay Reagent equal to the volume of culture medium in the well, and mix.
For 96-well plates, typically 100μL of reagent is added to cells grown in 100μL of medium.

(3) Wait at least 10 minutes to allow cell lysis.

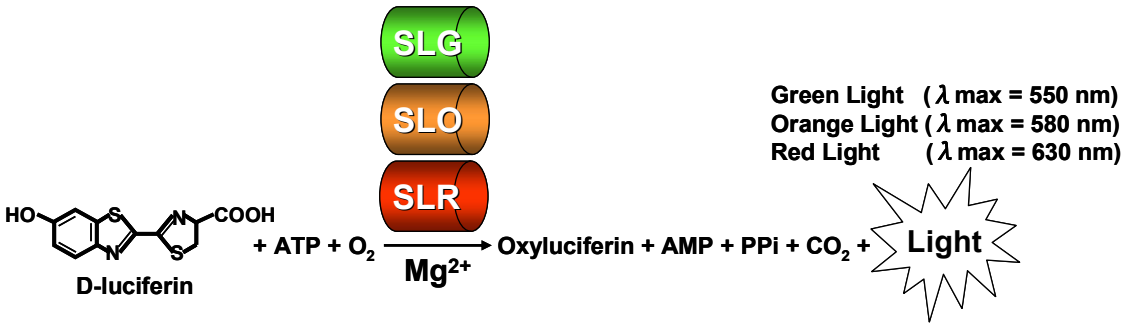
(4) Measure luminescence in a luminometer.

[5] Appendix

1. Luciferase gene

Light-emitting beetles belong to the order Lepidoptera of the class Insecta under the phylum Arthropodae. They are light-emitting land organisms and can be divided into four groups (Lampyridae, Pyrophorus, Phengodidae and Rhagophthalmus). The light emitted from these species is a result of an enzymatic reaction, i.e., oxidation of the light-emitting substrate (luciferin) under the catalytic activity of luciferase. A characteristic of these insect luciferases is that although the firefly luciferase undergoes a shift in light-emitting spectrum under the influence of pH, luciferases of Pyrophorus, Phengodidae and Rhagophthalmus are not affected by pH.

The vector series for the MultiReporter Assay System -Tripluc^{TM}- include green-emitting luciferase (SLG, maximum wavelength 550 nm), orange-emitting luciferase (SLO, 580 nm) and red-emitting luciferase (SLR, 630 nm). Fig. 2 shows the light-emitting spectrum of each luciferase.



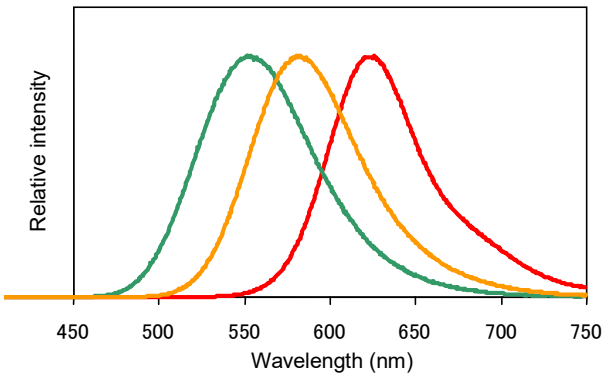


Fig. 2. Reaction mechanisms for SLG, SLO and SLR (upper) and the light-emitting spectrum of each luciferase (left)

Red-emitting luciferase is derived from *Phrixothrix hirtus* [4]. *Phrixothrix hirtus* is a light-emitting beetle originating from South America. It possesses red light emitters in the head and green light emitters in the ventral side.

Green-emitting luciferase is derived from *Rhagophthalmus ohbai* [5]. *Rhagophthalmus ohbai* originates from Iriomote Island and Ishigaki Island in Japan. It is analogous to *Phrixothrix hirtus* from South America. Orange-emitting luciferase is a single amino acid (base) mutant of the green light-emitting luciferase [6].

Since these luciferases represent insect type codon usage, their expression in mammalian cells is low. This has been a problem with these luciferases. For our products SLG, SLO and

SLR, the genes have been modified to those with a codon sequence frequently found in mammals, without changing the amino acid sequence, to increase their expression in mammals while preserving their light-emitting properties. SLG, SLO and SLR are thus optimized for assaying mammalian cells.

2. Emission color separation

Samples composed of proteins emitting multiple color lights can be subjected to measurement by splitting emissions using a luminometer equipped with color separating filters.

For measurement by splitting emissions color spectrometry of MultiReporter Assay System -Tripluc™-, it is recommended to use the Pelios AB-2350(ATTO Corporation), or AB2270 luminescencer Octa(ATTO Corporation). These luminometers are equipped with a 560 nm long path filter (560 nm LP, Filter 1) and a 600 nm long path filter (600 nm LP, Filter 2).

* Atto Corporation has filed an application for a patent covering the principle of measurement shown below (Patent Application No. JP20030162987).

(1) Emission color separation for three colors

Cells expressing one of SLG, SLO and SLR are used for the following measurements: (1) measurement in the absence of filter (total light), (2) measurement of transmission in the presence of the 560 nm LP (Filter 1), and (3) measurement of transmission in the presence of the 600 nm LP (Filter 2). The following coefficients are then calculated, with the values obtained under each filtering condition denoted as F0, F1 and F2.

	Coefficient	Abbreviation	Method of calculation
SLG	Filter 1 transmission rate	T1g	Percentage of Filter 1 transmission relative to total SLG light (= F1/F0)
	Filter 2 transmission rate	T2g	Percentage of Filter 2 transmission relative to total SLG light (= F2/F0)
SLO	Filter 1 transmission rate	T1o	Percentage of Filter 1 transmission relative to total SLO light (= F1/F0)
	Filter 2 transmission rate	T2o	Percentage of Filter 2 transmission relative to total SLO light (= F2/F0)
SLR	Filter 1 transmission rate	T1r	Percentage of Filter 1 transmission relative to total SLR light (= F1/F0)
	Filter 2 transmission rate	T2r	Percentage of Filter 2 transmission relative to total SLR light (= F2/F0)

Then, measurements of the cell lysates expressing three luciferases are conducted. If the level of light emitted from SLG, SLO and SLR in a given sample is denoted as G, O and R, respectively, F0 (total light measurement without a filter), F1 (light after transmission through the 560 nm LP (Filter 1) and F2 (light after transmission through the 600 nm LP (Filter 2)) can be defined as follows:

$$F0 = G + O + R$$
$$F1 = T1g * G + T1o * O + T1r * R$$
$$F2 = T2g * G + T2o * O + T2r * R$$

If each coefficient is determined and F0, F1 and F2 are measured, G, O and R can be calculated as follows.

$$G = \frac{(T1o * T2r - T1r * T2o) * F0 + (T2o - T2r) * F1 + (T1r - T1o) * F2}{T1g * T2o + T1r * T2g + T1o * T2r - T1g * T2r - T1r * T2o - T1o * T2g}$$

$$O = \frac{(T1r * T2g - T1g * T2r) * F0 + (T2r - T2g) * F1 + (T1g - T1r) * F2}{T1g * T2o + T1r * T2g + T1o * T2r - T1g * T2r - T1r * T2o - T1o * T2g}$$

$$R = \frac{(T1g * T2o - T1o * T2g) * F0 + (T2g - T2o) * F1 + (T1o - T1g) * F2}{T1g * T2o + T1r * T2g + T1o * T2r - T1g * T2r - T1r * T2o - T1o * T2g}$$

(2) **Emission color separation** for two colors

Emission Separation for two colors can be done by using either the 560 nm LP (Filter 1) or the 600 nm LP (Filter 2) or by means of total light measurement. The 600 nm LP (Filter 2) is recommended for the pairs of SLG + SLR and SLO + SLR. The 560 nm LP (Filter 1) is recommended for SLG + SLO.

[6] Troubleshooting

Trouble	Cause and Countermeasure
No or too weak signals detectable	• The promoter used may be one that expresses little luciferase. • The reagent or conditions for transfection may be inappropriate. Change the reagent or conditions. • The purity of plasmid may be low. Purify it again to reduce endotoxin contamination. • Cell lysis may be insufficient. Check that the reagent has spread adequately over the cells. In addition, extend the incubation time 5 to 10 minutes. • Luciferase may have become inactive. Where possible, avoid leaving the sample at room temperature. • The reagent may have deteriorated. Change the fresh reagent.
Poor quantitative performance	• The signals may be too low and affected by noise. Optimize the experimental conditions to elevate the signals. • The difference in signal level between different colors emitted may be too large. Adjust the plasmid mixture ratio for transfection (within 1:50). • Lack of correction or inappropriate correction among multiple samples. Correction with an internal standard is necessary. Under some particular conditions, the control promoter transcription activity may change. Repeat the experiment using another control promoter plasmid. • The temperature of the reagent may not have equilibrated to room temperature. Elevate the assay reagent temperature to room temperature. • The reagent may have deteriorated. Change the fresh reagent.
Lack of reproducibility	• There may be variation among experiments in terms of the conditions of cell incubation, conditions of treatment and so on. Check the conditions of individual experiments. • The luminometer may be out of order. Check whether or not the luminometer is functioning normally.

[7] References

1. Nakajima, Y., Kimura, T., Suzuki, C., and Ohmiya, Y. (2004) *Biosci. Biotechnol. Biochem.* **68**, 948-951
2. Nakajima, Y., Ikeda, M., Kimura, T., Honma, S., Ohmiya, Y. and Honma, K. (2004) *FEBS Letters* **565**, 122-126
3. Nakajima, Y., Kimura, T., Sugata, K., Enomoto, T., Asakawa, A., Kubota, H., Ikeda, M. and Ohmiya, Y. (2005) *Biotechniques*, **38**, 891-894
4. Vadim, R.V., Etelvino, J.H., and Ohmiya, Y. (1999) *Biochemistry,* **38**, 8271-8279
5. Ohmiya, Y., Sumiya, M., Viviani, V.R., and Ohba, N. (2000) *Sci. Rept. Yokosuka City Mus.* **47***,* 31-38
6. Vadim, V., Uchida, A., Suenaga, N., Ryufuku, M., and Ohmiya, Y.(2001) *Biochem. Biophys. Res. Commun.* **280**, 1286-1291

[8] Related products

Product	Contents	
SLG vector for promoter insertion **pSLG-test**	20 μg	MRV-101
SLO vector for promoter insertion **pSLO-test**	20 μg	MRV-102
SLR vector for promoter insertion **pSLR-test**	20 μg	MRV-103
SLG SV40 control vector **pSLG-SV40 control**	20 μg	MRV-201
SLO SV40 control vector **pSLO-SV40 control**	20 μg	MRV-202
SLR SV40 control vector **pSLR- SV40 control**	20 μg	MRV-203
SLG HSVtk control vector **pSLG-HSVtk control**	20 μg	MRV-301

[Manufactured and distributed by]

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