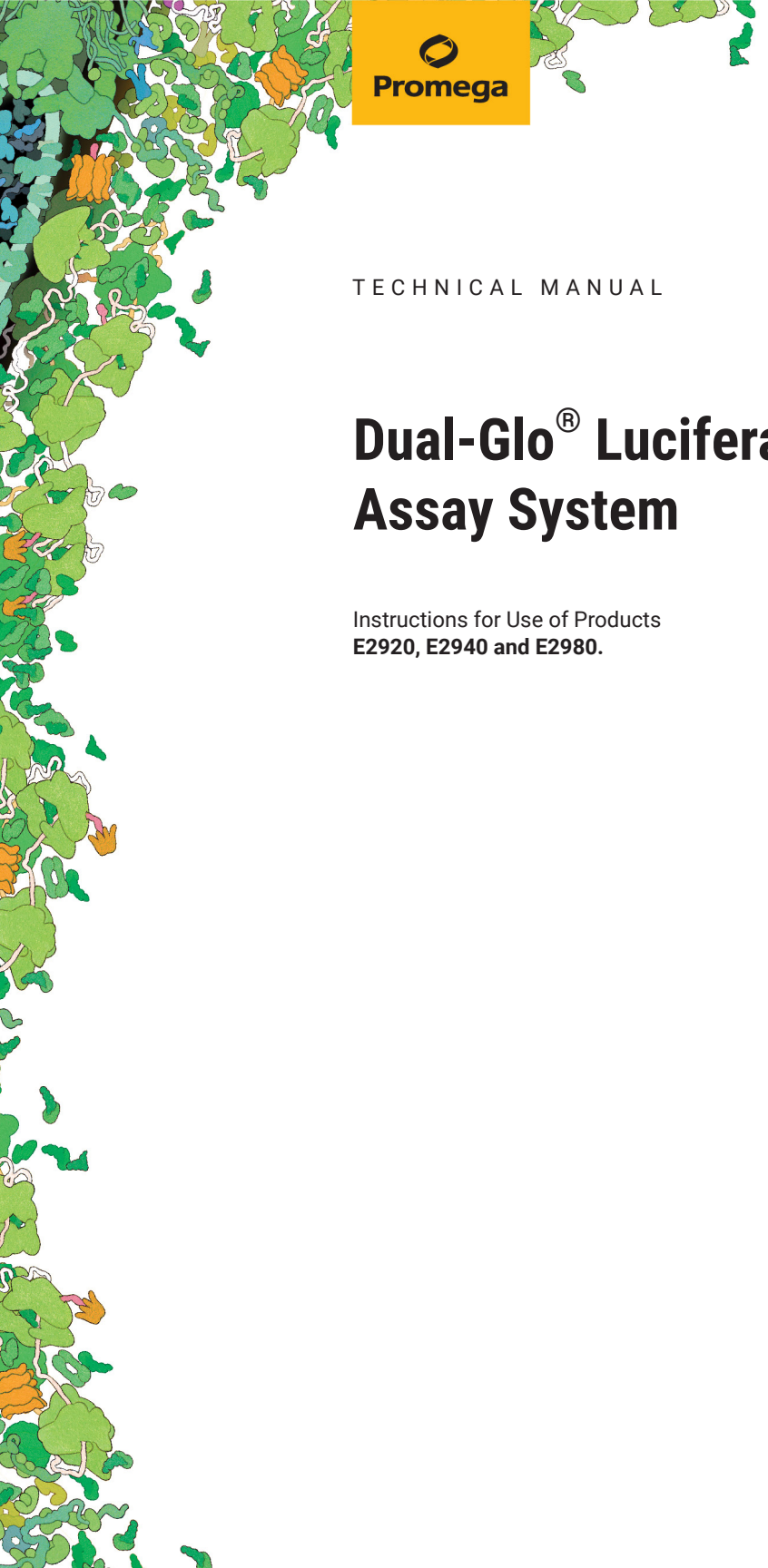

TECHNICAL MANUAL

Dual-Glo® Luciferase Assay System

Instructions for Use of Products
E2920, E2940 and E2980.

Revised 1/23
TM058

Dual-Glo® Luciferase Assay System

All technical literature is available at: **www.promega.com/protocols/**
Visit the web site to verify that you are using the most current version of this Technical Manual.
E-mail Promega Technical Services if you have questions on use of this system: **techserv@promega.com**

Promega

1. Description

In cell biology research and pharmaceutical discovery, it is common to test a wide variety of experimental conditions or a large number of chemical compounds for their effects on cellular physiology (1,2). Traditionally, the ease and sensitivity of firefly luciferase assays have made it relatively simple to monitor the upregulation of genetic elements. However, it has been more difficult to measure downregulation of genes because of the difficulty in discriminating between cell death and cellular downregulation. Normalizing the expression of an experimental reporter to the expression of a control reporter can help differentiate between specific and nonspecific cellular responses. This normalization can also control for transfection efficiencies.

Firefly and *Renilla* luciferases are widely used as co-reporters for these normalized studies because both assays are quick, easy and sensitive. Firefly luciferase is a 61kDa and *Renilla* luciferase a 36kDa protein (3–5). Both are monomeric and neither requires post-translational processing, so they can function as genetic reporters immediately upon translation.

The Dual-Glo® Luciferase Assay System[a,b] is designed to allow high-throughput analysis of mammalian cells containing genes for firefly and *Renilla* luciferases grown in 96- or 384-well plates (Figure 1). The Dual-Glo® Luciferase Reagent can be added directly to cells in growth medium without washing or preconditioning. This reagent induces cell lysis and acts as a substrate for firefly luciferase, which has a half-life of approximately 2 hours. Addition of the Dual-Glo® Stop & Glo® Reagent quenches the luminescence from the firefly reaction by at least 10,000-fold and provides the substrate for *Renilla* luciferase in a reaction that can also be read within 2 hours (with a similar retention in signal). The Dual-Glo® Luciferase Assay System is designed to work in growth media commonly used for mammalian cells with or without added serum. For an overview and information on the development of the Dual-Glo® Luciferase Assay System, see Sections 6.A and B.

To see articles that cite the use of the Dual-Glo® Luciferase Assay System, visit:
www.promega.com/resources/tools/citations/

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
Dual-Glo® Luciferase Assay System	**10ml**	**E2920**

Each system contains sufficient components to prepare 10ml of each reagent. Includes:

- 10ml Dual-Glo® Luciferase Buffer
- 1 vial Dual-Glo® Luciferase Substrate (lyophilized)
- 10ml Dual-Glo® Stop & Glo® Buffer
- 100µl Dual-Glo® Stop & Glo® Substrate

PRODUCT	SIZE	CAT.#
Dual-Glo® Luciferase Assay System	**100ml**	**E2940**

Each system contains sufficient components to prepare 100ml of each reagent. Includes:

- 100ml Dual-Glo® Luciferase Buffer
- 1 vial Dual-Glo® Luciferase Substrate (lyophilized)
- 100ml Dual-Glo® Stop & Glo® Buffer
- 1,000µl Dual-Glo® Stop & Glo® Substrate

PRODUCT	SIZE	CAT.#
Dual-Glo® Luciferase Assay System	**10 × 100ml**	**E2980**

Each system contains sufficient components to prepare 1,000ml of each reagent. Includes:

- 10 × 100ml Dual-Glo® Luciferase Buffer
- 10 vials Dual-Glo® Luciferase Substrate (lyophilized)
- 10 × 100ml Dual-Glo® Stop & Glo® Buffer
- 10 × 1,000µl Dual-Glo® Stop & Glo® Substrate

Storage Conditions: Store the Dual-Glo® Stop & Glo® Substrate and the lyophilized Dual-Glo® Luciferase Substrate at –30°C to –10°C. The substrates also may be stored at 4°C for up to two weeks. Store the Dual-Glo® Stop & Glo® Buffer and the Dual-Glo® Luciferase Buffer below 25°C. Buffer storage at room temperature is recommended to prevent the need for temperature equilibration when the reagents are reconstituted. Use the reconstituted Dual-Glo® Luciferase Reagent on the day it is prepared or store at –70°C after preparation for up to six months. Prepare the Dual-Glo® Stop & Glo® Reagent on the day it is to be used.

Caution: The lyophilized Dual-Glo® Luciferase Substrate contains dithiothreitol (DTT) and is therefore classified as hazardous. The reconstituted reagent is not known to present a hazard, as the concentration of DTT is less than 1%. However, we recommend the use of gloves, lab coats and eye protection when working with these and all chemical reagents. In addition, the Dual-Glo® Stop & Glo® Substrate contains a highly volatile solvent. Please pipet carefully and close the cap tightly after use.

Note: If Dual-Glo® Stop & Glo® Buffer precipitates upon freezing, it can be resolubilized without affecting solution performance. To resolubilize precipitate, store the Dual-Glo® Stop & Glo® Buffer at room temperature for 3 days or at 4°C for 2 weeks before use. Precipitate can also be resolubilized by heating to 37°C for up to 2 hours with vigorous shaking. Dual-Glo® Stop & Glo® Buffer should be equilibrated to room temperature for use.

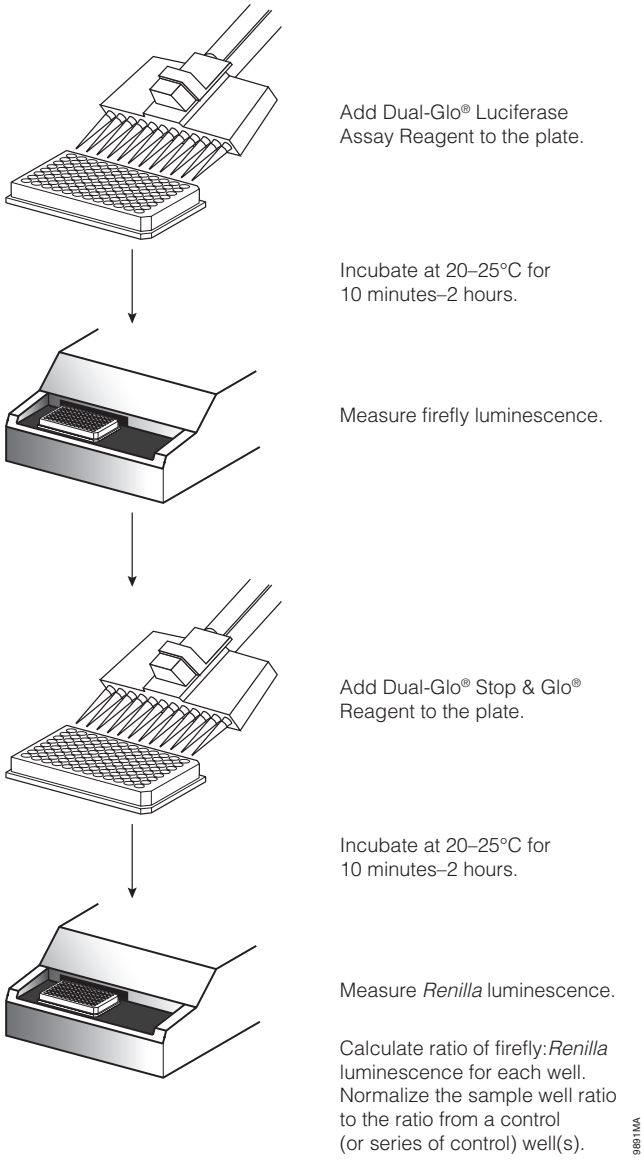

Figure 1. Dual-Glo® Luciferase Assay protocol. Firefly luciferase is produced, measured and quenched, followed by *Renilla* luciferase, in the same well.

3. Performing the Dual-Glo® Luciferase Assay

3.A. General Considerations

The Dual-Glo® Luciferase Assay System is designed for use in mammalian cell culture medium and is optimized for use with the following types of media containing 0–10% serum: RPMI 1640, DMEM, MEMα, and F12. The reagents have been developed so that the signals for firefly and *Renilla* luciferases are relatively stable and have a half-life of approximately 2 hours. Media/sera combinations affect the stability of each luminescent signal, so experimental determination of assay performance is recommended for media/sera combinations not listed above (see Section 6.D). The luminescence signal of each reporter also can be affected by the presence of phenol red, organic solvents or changes in temperature (Section 6.D).

Because the luminescent signals are affected by assay conditions, raw results should be compared only between samples measured at the same time and using the same medium/serum combination. Incorporation of consistent control wells on each plate provides the ability to calculate a normalized firefly luminescence/*Renilla* luminescence ratio for each sample well. These normalized ratios will remain essentially constant (±10%) for samples in a plate measured during the recommended 2-hour measurement window. Incorporating positive and negative control wells within a plate or experiment provides the ability to calculate a Relative Response Ratio (RRR). The RRR can be used to compare results between experiments that do not use the same media/sera combination or have been affected by changes in temperature or other variables (see Section 6.C).

Because a small amount of time is required for complete cell lysis and enzyme equilibration, the reagents of the Dual-Glo® Luciferase Assay System should be added to plates 10 minutes before quantifying luminescence. For maximal light intensity, samples should be measured within 2 hours of reagent addition. The Dual-Glo® Luciferase Reagents are not designed for use with the automated injectors that are integrated into some luminometers, as excessive reagent foaming may occur.

To achieve linear assay performance at low light levels, the background luminescence must be subtracted from all readings. Some luminometers also require verification of linear response at high light levels (consult your luminometer instrument manual for usage information).

Promega

3.B. Reagent Preparation

1. Transfer the contents of one bottle of Dual-Glo® Luciferase Buffer to one bottle of Dual-Glo® Luciferase Substrate to create the Dual-Glo® Luciferase Reagent. Mix by inversion until the substrate is thoroughly dissolved.
2. Calculate the amount of Dual-Glo® Stop & Glo® Reagent needed to perform the desired experiments. Dilute the Dual-Glo® Stop & Glo® Substrate 1:100 into an appropriate volume of Dual-Glo® Stop & Glo® Buffer in a new container.

Example: If 6ml of Dual-Glo® Stop & Glo® Reagent is needed, dilute 60µl of Dual-Glo® Stop & Glo® Substrate into 6ml of Dual-Glo® Stop & Glo® Buffer. For Cat.# E2940 and E2980, if the entire bottle of Dual-Glo® Stop & Glo® Buffer is to be used at one time, transfer the entire contents of the Dual-Glo® Stop & Glo® Substrate into the buffer. For Cat.# E2920, combine 10ml of Dual-Glo® Stop & Glo® Buffer and 100µl of Dual-Glo® Stop & Glo® Substrate.

Notes:

a. Assay reagents are stable at room temperature for several hours (see Notes 2 and 3). Freezing the reagent can reduce the loss of activity of the Dual-Glo® Luciferase Reagent. **Do not** thaw the reconstituted reagent at temperatures above 25°C. Mix well after thawing. The most convenient and effective method for thawing is to place the reagent in a room temperature water bath. Prepare only the amount of Dual-Glo® Stop & Glo® Reagent required. For best results, prepare the Dual-Glo® Stop & Glo® Reagent immediately before use.

b. Dual-Glo® Luciferase Reagent Stability: Liquid reagent has approximately a 10% loss of firefly RLU after 8 hours at room temperature and after 48 hours at 4°C. Frozen reagent has approximately a 10% loss of firefly RLU after 1 week at –20°C and after 6 months at –70°C. Do not store the reagent at –20°C for longer than 1 week. The reagent can be exposed to 5 freeze-thaw cycles with approximately a 15% loss in firefly RLU. Holding or storing the reconstituted reagent may cause *Renilla* RLU to rise.

c. Approximate stability of Dual-Glo® Stop & Glo® Reagent after reconstitution: 8.1% loss after 8 hours at room temperature, 8.5% loss after 24 hours at 4°C. We recommend that the Dual-Glo® Stop & Glo® Reagent always be prepared immediately before use.

d. Light intensity is a measure of the rate of catalysis by the luciferases and is therefore temperature sensitive. The temperature optimum for the activity of both luciferases is approximately room temperature (20–25°C), so it is important that the reagents be equilibrated to room temperature before beginning measurements. To avoid the need to temperature equilibrate reagents before use, store the Dual-Glo® Luciferase Buffer and the Dual-Glo® Stop & Glo® Buffer at room temperature. If reagents are colder than room temperature, place them in a room temperature water bath to equilibrate before use.

e. To achieve maximum reproducibility, equilibrate cells in media to room temperature before performing luciferase measurements.

3.C. Assay Procedure

1. Remove multiwell plates containing mammalian cells from the incubator. Make certain that the plates are compatible with the type of luminometer being used.
2. **Measuring firefly luciferase activity:** Add a volume of Dual-Glo® Luciferase Reagent equal to the culture medium volume to each well and mix. For 96-well plates, typically 75µl of reagent is added to cells grown in 75µl of medium. For 384-well plates, typically 20µl of reagent is added to cells grown in 20µl of medium.
3. Wait at least 10 minutes, then measure the firefly luminescence (consult your luminometer manual for proper use of the instrument). Optimal results will be generated if the luminescence is measured within 2 hours of addition of Dual-Glo® Luciferase Reagent.
4. **Measuring *Renilla* luciferase activity:** Add a volume of Dual-Glo® Stop & Glo® Reagent equal to the original culture medium volume to each well and mix. As noted in Step 2, this volume is typically 75µl for 96-well plates and 20µl for 384-well plates.

   Note: Dual-Glo® Stop & Glo® Reagent should be added to plate wells within 4 hours of addition of Dual-Glo® Luciferase Reagent.
5. Wait at least 10 minutes, then measure luminescence. *Renilla* luminescence should be measured in the same plate order as the firefly luminescence was measured (Step 3). For optimal results, measure luminescence within 2 hours of addition of Dual-Glo® Stop & Glo® Reagent.
6. Calculate the ratio of luminescence from the experimental reporter to luminescence from the control reporter. Normalize this ratio to the ratio of a control well or series of control wells that are treated consistently on all plates (see Section 6.C). This normalization provides optimal and consistent results from the Dual-Glo® Luciferase Assay System. Relative Response Ratios can then be calculated from the Normalized Ratios. See Section 6.C for more information and sample calculations.

Promega

4. Related Products

pGL4 Luciferase Reporter Vectors

Please visit **www.promega.com** to see a complete listing of reporter vectors.

Vector	Multiple Cloning Region	Reporter Gene	Protein Degradation Sequence	Reporter Gene Promoter	Mammalian Selectable Marker	Cat.#
pGL4.10[*luc2*]	Yes	*luc2*[A]	No	No	No	E6651
pGL4.11[*luc2P*]	Yes	"	hPEST	No	No	E6661
pGL4.12[*luc2CP*]	Yes	"	hCL1-hPEST	No	No	E6671
pGL4.13[*luc2*/SV40]	No	"	No	SV40	No	E6681
pGL4.14[*luc2*/Hygro]	Yes	"	No	No	Hygro	E6691
pGL4.15[*luc2P*/Hygro]	Yes	"	hPEST	No	Hygro	E6701
pGL4.16[*luc2CP*/Hygro]	Yes	"	hCL1-hPEST	No	Hygro	E6711
pGL4.17[*luc2*/Neo]	Yes	"	No	No	Neo	E6721
pGL4.18[*luc2P*/Neo]	Yes	"	hPEST	No	Neo	E6731
pGL4.19[*luc2CP*/Neo]	Yes	"	hCL1-hPEST	No	Neo	E6741
pGL4.20[*luc2*/Puro]	Yes	"	No	No	Puro	E6751
pGL4.21[*luc2P*/Puro]	Yes	"	hPEST	No	Puro	E6761
pGL4.22[*luc2CP*/Puro]	Yes	"	hCL1-hPEST	No	Puro	E6771
pGL4.23[*luc2*/minP]	Yes	"	No	minP	No	E8411
pGL4.24[*luc2P*/minP]	Yes	"	hPEST	"	No	E8421
pGL4.25[*luc2CP*/minP]	Yes	"	hCL1-PEST	"	No	E8431
pGL4.26[*luc2*/minP/Hygro]	Yes	"	No	"	Hygro	E8441
pGL4.27[*luc2P*/minP/Hygro]	Yes	"	hPEST	"	Hygro	E8451
pGL4.28[*luc2CP*/minP/Hygro]	Yes	"	hCL1-PEST	"	Hygro	E8461
pGL4.29[*luc2P*/CRE/Hygro]	No	"	hPEST	CRE	Hygro	E8471
pGL4.30[*luc2P*/NFAT-RE/Hygro]	No	"	hPEST	NFAT-RE	Hygro	E8481
pGL4.31[*luc2P*/*GAL4*UAS/Hygro]	No	"	hPEST	*GAL4*UAS	Hygro	C9351
pGL4.32[*luc2P*/NF-κB-RE/Hygro]	No	"	hPEST	NF-κB-RE	Hygro	E8491

[A]*luc2* = synthetic firefly luciferase gene. [B]*hRluc* = synthetic *Renilla* luciferase gene.

pGL4 Luciferase Reporter Vectors (continued)

Vector	Multiple Cloning Region	Reporter Gene	Protein Degradation Sequence	Reporter Gene Promoter	Mammalian Selectable Marker	Cat.#
pGL4.33[*luc2P*/SRE/Hygro]	No	"	hPEST	SRE	Hygro	E1340
pGL4.34[*luc2P*/SRF-RE/Hygro]	No	"	hPEST	SRF-RE	Hygro	E1350
pGL4.36[*luc2P*/MMTV/Hygro]	No	"	hPEST	MMTV	Hygro	E1360
pGL4.50[*luc2*/CMV/Hygro]	No	"	No	CMV	Hygro	E1310
pGL4.51[*luc2*/CMV/Neo]	No	"	No	CMV	Neo	E1320
pGL4.70[*hRluc*]	Yes	*hRluc*[B]	No	No	No	E6881
pGL4.73[*hRluc*/SV40]	No	"	No	SV40	No	E6911
pGL4.74[*hRluc*/TK]	No	"	No	HSV-TK	No	E6921
pGL4.75[*hRluc*/CMV]	No	"	No	CMV	No	E6931
pGL4.82[*hRluc*/Puro]	Yes	"	No	No	Puro	E7501
pGL4.83[*hRlucP*/Puro]	Yes	"	hPEST	No	Puro	E7511
pGL4.84[*hRlucCP*/Puro]	Yes	"	hCL1-hPEST	No	Puro	E7521

[A]*luc2* = synthetic firefly luciferase gene. [B]*hRluc* = synthetic *Renilla* luciferase gene.

Luciferase Assay Systems

Product	Size	Cat.#
Steady-Glo® Luciferase Assay System*	100ml	E2520
Bright-Glo™ Luciferase Assay System*	100ml	E2620
Dual-Luciferase® Reporter Assay System*	100 assays	E1910
Luciferase Assay System*	100 assays	E1500
Luciferase Assay Reagent	1,000 assays	E1483
Renilla Luciferase Assay System*	100 assays	E2810
QuantiLum® Recombinant Luciferase*	1mg	E1701
EnduRen™ Live Cell Substrate*	0.34mg	E6481
ViviRen™ Live Cell Substrate*	0.37mg	E6491

*Additional Sizes Available.

4. Related Products (continued)

Transfection Reagent

Product	Size	Cat.#
FuGENE® 4K Transfection Reagent	1ml	E5911
	5 × 1ml	E5912
FuGENE® HD Transfection Reagent	1ml	E2311
	5 × 1ml	E2312

Luminometers

Product	Size	Cat.#
GloMax® Discover System	1 each	GM3000
GloMax® Navigator System	1 each	GM2000
GloMax® Explorer System	1 each	GM3500

Plasmid DNA Purification System

Product	Size	Cat.#
PureYield™ Plasmid Midiprep System	25 preps	A2492
	100 preps	A2495
	300 preps	A2496
PureYield™ Plasmid Maxiprep System	10 preps	A2392
	25 preps	A2393

5. References

1. Alam, J. and Cook, J.L. (1990) Reporter genes: Application to the study of mammalian gene transcription. *Anal. Biochem.* **188**, 245–54.
2. Wood, K.V. (1991) In: *Bioluminescence and Chemiluminescence: Current Status*, Stanley, P., and Kricka, L., eds., John Wiley and Sons, Chichester, NY, 543.
3. Wood, K.V. *et al.* (1984) Synthesis of active firefly luciferase by in vitro translation of RNA obtained from adult lanterns. *Biochem. Biophys. Res. Comm.* **124**, 592–6.
4. deWet, J.R. *et al.* (1985) Cloning of firefly luciferase cDNA and the expression of active luciferase in *Escherichia coli. Proc. Natl. Acad. Sci. USA* **82**, 7870–3.
5. Matthews. J.C. *et al.* (1977) Purification and properties of *Renilla reniformis* luciferase. *Biochemistry* **16**, 85–91.

6. Appendix

6.A. Overview of the Dual-Glo® Luciferase Assay System

Transcriptional regulation coupled to reporter gene expression is routinely used to study a wide range of physiological responses. A common example is the analysis of receptor function by quantitating the action of specific reporter response elements on gene expression. Firefly luciferase has been used as a simple, convenient and sensitive reporter gene for this type of analysis.

An increase in the transcription or translation of a reporter molecule, like firefly luciferase, is easily tracked in a biological system. Firefly luciferase is immediately functional upon translation. Thus if the amount of luminescence from an experimental sample is greater than the luminescence from a control sample, an increase in transcription or translation has occurred.

Studies of antagonists or other factors that decrease transcription or translation are difficult to interpret when using a single reporter gene. A decrease in an experimental reporter response (firefly luciferase in this case) can be caused by a specific effect on the reporter or by a global effect such as cell death. Normalization of an experimental reporter with a control reporter from the same sample, such as *Renilla* luciferase, allows a distinction to be made between specific and global effects. A decrease in firefly luminescence with *Renilla* luminescence remaining unchanged indicates a specific impact of the experimental condition. A decrease of both luminescences indicates a global impact on the cell or cell population (i.e., cell death, inhibition of cell growth, variable initial cell numbers).

6.A. Overview of the Dual-Glo® Luciferase Assay System (continued)

The Dual-Luciferase® Reporter Assay System (DLR™ Assay System, Cat.# E1910) allows measurement of both firefly and *Renilla* luciferases from a single sample. This assay system, however, generates luminescence that rapidly decreases in intensity. The firefly luciferase signal decreases 50% in approximately 12–15 minutes, and the *Renilla* luciferase signal decreases 50% in less than 3 minutes. These signal kinetics make the measurement of firefly and *Renilla* luciferases difficult if large numbers of samples are to be measured in 96- or 384-well plates. Although robotic systems can easily add reagents to all the samples on a plate at the same time, the signals are too unstable to easily measure the luminescence from the entire plate without having to calculate time corrections.

The Dual-Glo® Luciferase Assay System, with its stabilized luminescent signals and half-life of approximately 2 hours, easily can be used to measure both firefly and *Renilla* luminescence in multiwell plates. Many plates containing experimental samples can be batch processed; all plates can be filled with reagent at one time, and the luminescence from each plate can be measured in series. In this way, the Dual-Glo® Luciferase Assay System can easily and rapidly quantitate both firefly and *Renilla* luciferases, allowing one to distinguish between global and specific effects.

6.B. Development of the Assay

Firefly and *Renilla* luciferases have distinct evolutionary origins and have very different enzyme structures and substrates. Promega has been able to exploit these differences so that the two luciferases can be measured in succession, with firefly luciferase luminescence elicited by one reagent, while a second reagent simultaneously quenches the firefly luciferase and elicits *Renilla* luciferase luminescence.

Firefly and *Renilla* luciferases do not require post-translational modification and thus are fully functional enzymes immediately after translation (3,4). In order to generate luminescence firefly luciferase requires beetle luciferin, ATP, magnesium and molecular oxygen. *Renilla* luciferase requires only coelenterate luciferin (coelenterazine) and molecular oxygen (Figure 2). Firefly and *Renilla* luciferases undergo spontaneous inactivation after generating luminescence. This inactivation causes the "flash"-type kinetics seen in assays that have been optimized for maximal assay sensitivity, like the DLR™ Assay System (Figure 3). In order to generate luminescence with signal stability that is amenable to robotic measurements, the rate of inactivation and subsequently the rate of catalysis must be slowed. For this reason, "glo"-type reagents developed for use in high-throughput screening, like the Steady-Glo® and Dual-Glo® Reagents, have lower luminescences than "flash"-type reagents that have been developed for maximal sensitivity, like the Luciferase and DLR™ Assay Reagents.

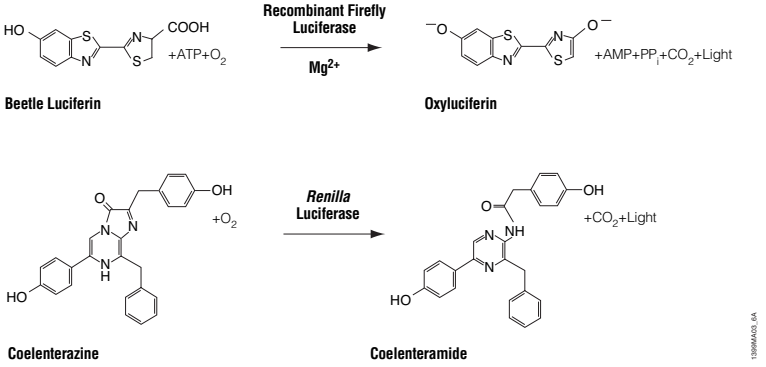

Figure 2. Bioluminescent reactions catalyzed by firefly and *Renilla* luciferases. Mono-oxygenation of beetle luciferin is catalyzed by firefly luciferase in the presence of Mg^{2+}, ATP and molecular oxygen. Unlike beetle luciferin, coelenterazine undergoes mono-oxygenation catalyzed by *Renilla* luciferase but requires only molecular oxygen.

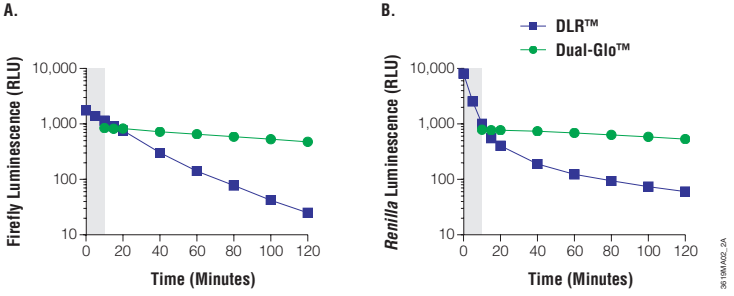

Figure 3. Comparison of the signal decay of luciferases in Dual-Glo® Reagent and DLR™ Assay Reagent. The two luciferase assay reagents were compared using 1.67×10^{-9}M (plus 1mg/ml gelatin) firefly and *Renilla* luciferase (20µl and 100µl of enzyme mix were used with DLR™ and Dual-Glo® Reagents, respectively). The luciferases were diluted in RPMI 1640 before assaying with Dual-Glo® Luciferase Reagent and in Passive Lysis Buffer (Cat.# E1941) before assaying with DLR™ Assay Reagent. The DLR™ Assay measurements were taken immediately after reagent addition, while the Dual-Glo® measurements were taken after a 10-minute incubation at room temperature. Luminescence was integrated over 0.5 seconds per well at regular intervals until 2 hours after reagent addition. **Panel A.** Firefly luciferase activity in Dual-Glo® and DLR™ Assays. **Panel B.** *Renilla* luciferase activity in Dual-Glo® and DLR™ Assays.

6.B. Development of the Assay (continued)

Quenching Effect

Providing the ability to measure firefly luminescence is only one of the requirements for the Dual-Glo® Luciferase Assay System. After measurement of the firefly luminescence, the Dual-Glo® Stop & Glo® Reagent must simultaneously quench firefly luminescence and generate *Renilla* luminescence.

The Dual-Glo® Stop & Glo® Reagent has been designed to decrease the luminescence of the firefly luciferase reaction by at least 10,000-fold (Figure 4). Thus if the firefly luciferase luminescence generated 530,000 Relative Light Units per second, the maximum firefly luminescence measured 10 minutes after the Dual-Glo® Stop & Glo® Reagent addition would be 53 Relative Light Units per second. The promoter for the control reporter (either firefly or *Renilla* luciferase) should be chosen so that the luminescence from the firefly luciferase is no more than 100 times the luminescence from the *Renilla* luciferase. If this is the case, the firefly luciferase luminescence will contribute no more than 1% to the *Renilla* luciferase luminescence, and variability in the firefly luciferase signal will not noticeably affect the *Renilla* luciferase signal.

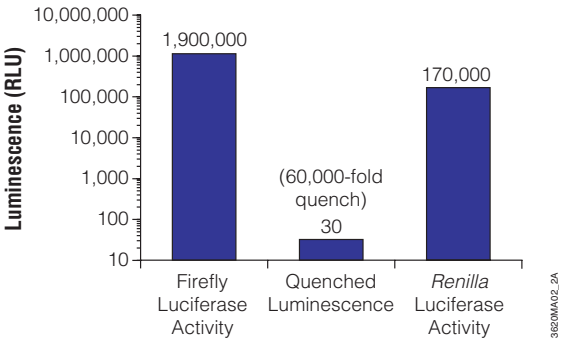

Figure 4. Measurement of luciferase activities before and after addition of Dual-Glo® Stop & Glo® Reagent. Both firefly and *Renilla* luciferase reporter activities were quantitated using a 100µl sample of purified firefly or *Renilla* luciferase (1.67×10^{-9}M and 1.67×10^{-10}M, respectively, with 1mg/ml gelatin). The concentration of *Renilla* luciferase is tenfold less than that of firefly luciferase. To demonstrate the efficient quenching of firefly luciferase by Dual-Glo® Stop & Glo® Reagent, an equal volume of Dual-Glo® Stop & Glo® Buffer (which does not contain the substrate for *Renilla* luciferase) was added to the Dual-Glo® Luciferase Reagent with firefly luciferase. Firefly luciferase luminescence was quenched by greater than 4 orders of magnitude with 0.0016% residual activity. RLU = relative light units.

Dual-Glo® Stop & Glo® Reagent has been designed to provide simultaneous quenching of the firefly luciferase signal and sustained luminescence for the *Renilla* luciferase reaction. *Renilla* luciferase does not use any of the substrates for the firefly reaction except molecular oxygen. This permits the Dual-Glo® Stop & Glo® Reagent to both quench the firefly reaction and generate the *Renilla* luciferase reaction. *Renilla* luciferase is similar to firefly luciferase in that the enzyme inactivates itself. As with the firefly luciferase, the rate of catalysis must be slowed if the luminescent signal is to be stabilized. "Glo"-type *Renilla* assays (Dual-Glo® Luciferase Assay) are therefore not as sensitive as "flash"-type assays (DLR™ and *Renilla* Luciferase Assays).

Unlike the firefly luciferase substrate (beetle luciferin), coelenterazine will auto-oxidize in the absence of *Renilla* luciferase. This enzyme-independent reaction is termed autoluminescence. Reaction conditions such as high detergent levels created in homogeneous assays can increase the level of autoluminescence, which can limit the sensitivity of the *Renilla* luciferase reporter system. The Dual-Glo® Luciferase Assay System uses unique technology that reduces autoluminescence to levels that are often undetectable, thereby increasing the sensitivity of the *Renilla* luciferase reagent of the Dual-Glo® Luciferase Assay System by >100-fold over nonoptimized reagents (Figure 5).

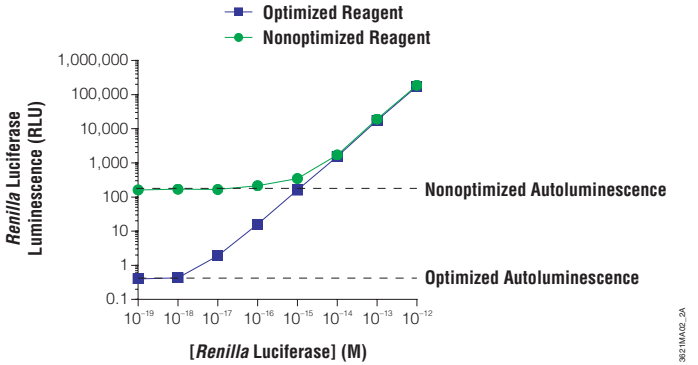

Figure 5. The Dual-Glo® Luciferase Assay System is optimized for minimal autoluminescence from *Renilla* luciferase substrate. Luminescence from a titration of *Renilla* luciferase was compared in Dual-Glo® Reagent before and after optimization for homogeneous assay formats. Luciferase concentrations varied over a titration range of 1×10^{-12} to 1×10^{-19} moles/reaction. One hundred microliters of *Renilla* luciferase in RPMI 1640 (containing 1mg/ml gelatin) was added to 100µl of Dual-Glo® Luciferase Reagent and 100µl of Dual-Glo® Stop & Glo® Reagent, either optimized or nonoptimized for homogeneous assay format. Samples were incubated for 10 minutes at 22°C, then measured on a Turner Designs Model 20e luminometer. Light emission was integrated over 5 seconds after an initial 2-second preread delay. Limit of detection values shown (horizontal lines labeled Optimized and Nonoptimized) represent background plus three standard deviations and were determined for each assay by performing the assay without enzyme.

6.C. Data Analysis

Background Subtraction

For maximal accuracy, the luminescence measurements of both firefly and *Renilla* luciferases should be background-subtracted. Neither enzyme is endogenously expressed in mammalian cells, so the source of background luminescence is either a characteristic of the luminometer or of the luminescent substrate. Beetle luciferin, one of the firefly luciferase substrates, does not generate light in the absence of luciferase in the Dual-Glo® Reagents. A proprietary chemistry minimizes the enzyme-independent luminescence (autoluminescence) exhibited by coelenterate luciferin, the substrate for *Renilla* luciferase in the Dual-Glo® Stop & Glo® Reagent. Background luminescence for both luciferase reagents therefore may not be measurable above the background on many luminometers.

Background measurements for firefly luciferase should be taken from samples consisting of nontransfected cells and Dual-Glo® Luciferase Reagent. For *Renilla* luciferase, background measurements should be taken from samples containing nontransfected cells and both Dual-Glo® Luciferase Reagent and Dual-Glo® Stop & Glo® Reagent. Sample volumes for background measurements must be the same as experimental sample volumes and contain the same media/sera combinations as the experimental samples.

Normalizing Ratios

Normalizing the results from each experimental sample to control samples repeated on each plate minimizes the impact of variables like temperature, plate order and timing on the ratio of experimental/control reporter activity.

Example 1:

An experiment includes 20 plates. If the timing of measurement of the plates is changed between the firefly and *Renilla* luminescence measurements, then the ratio of firefly luminescence to *Renilla* luminescence will be different on each of the plates because the two reporter signals would have decayed by different amounts (changing the order of the plates changes the length of time between reagent addition and plate reading). For instance, the luminometer jammed 20 minutes after the Dual-Glo® Stop & Glo® Reagent was added to a large stack of plates:

Ratio of plate 1 control firefly luminescence/*Renilla* luminescence = 1.7.
Ratio of plate 1 well A1 firefly luminescence/*Renilla* luminescence = 3.4.

Both firefly luciferase and *Renilla* luciferase were measured 10 minutes after the reagents were added.

Relative ratios are: control = 1.7/1.7 = 1 and well A1 = 3.4/1.7 = 2.

However, for plate 12 the firefly luciferase was measured 40 minutes after Dual-Glo® Luciferase Reagent was added, but because of the luminometer jam, the *Renilla* luciferase was not measured until 75 minutes after the Dual-Glo® Stop & Glo® Reagent was added.

Ratio of plate 12 control firefly luminescence/*Renilla* luminescence = 2.1.
Ratio of plate 12 well A1 firefly luminescence/*Renilla* luminescence = 4.2.
Relative ratios are: control = 2.1/2.1 = 1 and well A1 = 4.2/2.1 = 2.

Example 2:

The Dual-Glo® Assay is being performed in a cell culture medium (MEMα) that causes the firefly and *Renilla* luciferases to decay at different rates (firefly luciferase retains ~90% activity over 2 hours, *Renilla* luciferase retains ~75% activity over 2 hours). Normalizing the ratios permits comparison of these ratios between plates in the same experiment.

Ratio of plate 1 control firefly luminescence/*Renilla* luminescence = 6.4.
Ratio of plate 1 well A1 firefly luminescence/*Renilla* luminescence = 1.6.

Both firefly luciferase and *Renilla* luciferase were measured 10 minutes after the reagents were added.

Relative ratios were: control = 6.4/6.4 = 1 and well A1= 1.6/6.4 = 0.25.

For plate 12, both firefly luciferase and *Renilla* luciferase were measured 65 minutes after the reagents were added, but the two signals do not decay at the same rate. Thus the raw ratio changes.

Ratio of plate 12 control firefly luminescence/*Renilla* luminescence = 6.9.
Ratio of plate 12 well A1 firefly luminescence/*Renilla* luminescence = 1.7.
Relative ratios are: control = 6.9/6.9 = 1 and well A1 = 1.7/6.9 = 0.25.

Other factors may affect the ratio of experimental/control reporter. As seen in Section 6.D and mentioned above, chemical components of the medium/serum combination affect the firefly luciferase signal stability differently than they affect the *Renilla* luciferase signal stability. This is also true of changes in temperature and other parameters that affect enzyme reactions. Normalization of the experimental/control reporter activity is essential for easy comparison of samples with minimal variability across an experiment.

Relative Response Ratios

A Relative Response Ratio (RRR) can be determined to assist in quantitation of the impact of an experimental treatment on reporter gene expression. The RRR permits the comparison of multiple treatments from different experiments because it provides a framework within which the effect of the treatment can be placed.

Calculation of RRR requires the inclusion of 2 sets of controls on each plate: a positive control that provides maximal luminescence, and a negative control that provides minimal luminescence. For an experiment that monitors down-regulation of the experimental reporter by chemical treatments, the positive control for the experimental series might be no treatment. The negative control would be treatment with some compound that had previously been shown to drastically inhibit the experimental reporter. If these 2 samples are included on every plate, the impact of each new compound can be quantitatively evaluated by its influence on the experimental reporter within the context of the positive and negative control.

6.C. Data Analysis (continued)

Example:

If the ratio of experimental reporter luminescence/control reporter luminescence is 53 for the positive control, 1.3 for the negative control, and 22 for the experimental treatment, all of these values can be scaled so that the positive control is assigned the value of 1 and the negative control is assigned the value of 0. The RRR for each experimental treatment can then be calculated using this formula:

$$\text{RRR} = \frac{(\text{experimental sample ratio}) - (\text{negative control ratio})}{(\text{positive control ratio}) - (\text{negative control ratio})}$$

The RRR for the experimental treatment example listed above would be:

$$\text{RRR} = \frac{22 - 1.3}{53 - 1.3} = 0.40 \text{ or } 40\%$$

The experimental compound is 40% as effective as the negative control at decreasing expression of the experimental reporter at this concentration. A hierarchy of efficacy for the experimental system would be: i) no treatment generated a RRR of 100%; ii) the experimental compound generated a RRR of 40% and; iii) the negative control generated a RRR of 0%. Those compounds that are most significant will have RRRs that are negative, since they would be more effective inhibitors than the negative control.

Note: If the absolute luminescence values are close to background, the luminescences will need to be background subtracted before the ratios are calculated.

6.D. Conditions Affecting Assay Performance

The data presented in this section are intended to provide a general overview of assay characteristics under a wide range of experimental conditions. The cell culture medium accounts for 50% of the firefly luciferase and 33% of the *Renilla* luciferase reaction volumes. Thus medium can affect assay performance. The chemistry used for the Dual-Glo® Luciferase Assay System is unique when compared to other assays developed for high-throughput analysis and compared to the DLR™ Assay System. Therefore the data presented in this technical manual may not be applicable to other luciferase assay systems.

Purified firefly and *Renilla* luciferases were diluted into culture medium to generate the data presented in this section. This was done to illustrate the performance characteristics without adding the experimental complexities common to cell culture. However, as shown in Figure 6, purified luciferases diluted into culture medium show little or no difference when compared to enzymes expressed in transfected cells. Gelatin (1mg/ml) was added to the wells to simulate the protein that would normally be contributed by cells. Addition of gelatin is not required when using the Dual-Glo® Luciferase Assay System with cells.

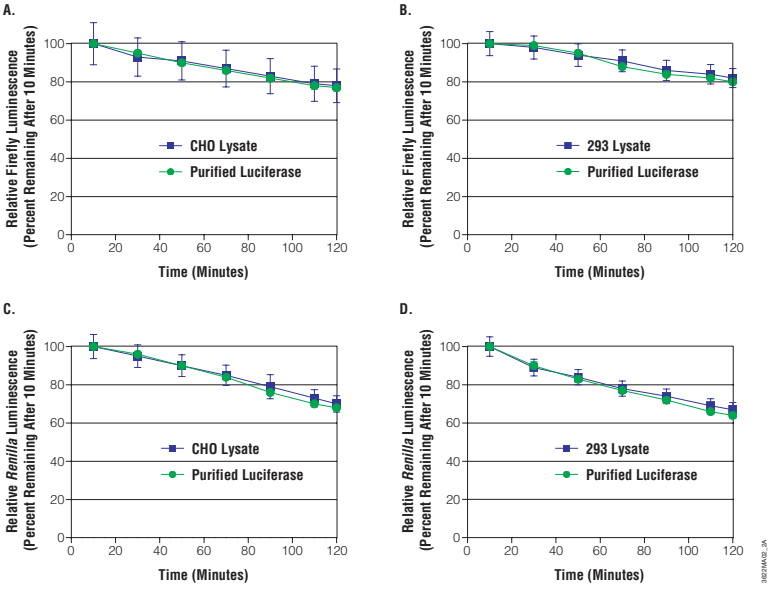

Figure 6. Reaction kinetics for purified firefly and *Renilla* luciferase and for luciferases expressed by transiently transfected mammalian cells. Samples in 96-well plates consisted of 75µl of either purified firefly and *Renilla* luciferases (both at 1.67×10^{-9}M, 1mg/ml gelatin as a protein carrier) or mammalian cells transfected with firefly and *Renilla* luciferase genes (using pCI Mammalian Expression Vector [Cat.# E1731] with *luc*+ inserted at XbaI/XhoI sites for firefly luciferase and the phRL-SV40 Vector for *Renilla* luciferase [Cat.# E6261]) in the same growth medium. Seventy-five microliters of Dual-Glo® Luciferase Reagent was added to plate wells and mixed on a plate shaker. After a 10-minute room temperature incubation, luminescence was integrated over 0.5 seconds per well, periodically, for 2 hours after reagent addition. Stop & Glo® Reagent was then added to wells and mixed on a plate shaker, and luminescence was integrated as noted above. Firefly luciferase was measured in CHO cells in F12 medium (**Panel A**) and in 293 cells in DMEM (**Panel B**). *Renilla* luciferase was measured in CHO cells in F12 medium (**Panel C**) and in 293 cells in DMEM (**Panel D**). As these data show, very little difference in relative luminescence is apparent over time between transfected cells and purified enzyme in the same culture medium. Number of samples = 6 for cell lysate data, 3 for purified enzyme data.

6.D. Conditions Affecting Assay Performance (continued)

Culture Medium

When performing the Dual-Glo® Luciferase Assay, the culture medium and any compounds added to the medium make up half of the chemical environment of the firefly luciferase reaction, and one-third of the environment of the *Renilla* luciferase reaction. Although the Dual-Glo® Reagents are designed to work with many common culture media, compositional differences between the different media may affect the assay characteristics (i.e., light intensity and signal stability).

The Dual-Glo® Luciferase Reagents were designed to provide relatively high luminescence, with a half-life of approximately 2 hours when used with common growth medium. However, performance differences are evident between these media (Figure 7), as well as between the same media from different manufacturers. Although these differences are generally small and do not diminish the utility of the Dual-Glo® Luciferase Assay System, controls should be incorporated into every batch of plates to correct for this variability.

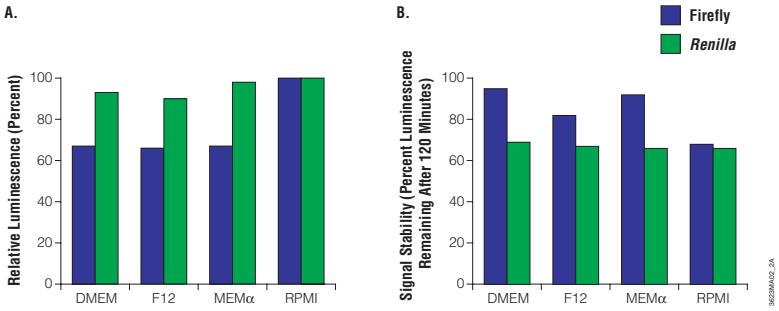

Figure 7. Relative luminescence intensity and signal stability of firefly and *Renilla* luciferase in four common media. Purified firefly or *Renilla* enzyme (1.67×10^{-9}M, 1mg/ml gelatin) was added to a 96-well plate at 100µl per well. Dilutions were made in RPMI 1640, DMEM, MEMα or F12 medium. Either Dual-Glo® Luciferase Reagent (for firefly luciferase) or Dual-Glo® Luciferase Reagent plus Dual-Glo® Stop & Glo® Reagent (for *Renilla* luciferase) was added, and luminescence measurements were integrated over 0.5 seconds per well. **Panel A.** Luminescence measurements were taken 10 minutes after reagent addition. Luminescence is shown relative to the light output generated in RPMI 1640 for both firefly and *Renilla* luciferases. **Panel B.** Signal stability in various media expressed as percent luminescence remaining after 2 hours. Number of samples = 3; relative standard error ≤3.4%.

Serum

The Dual-Glo® Reagents are compatible with medium containing serum. The reagents have been designed for use with serum concentrations from 0–10%, and the luminescent signals generated are minimally affected by the presence of fetal bovine or calf serum (Figure 8).

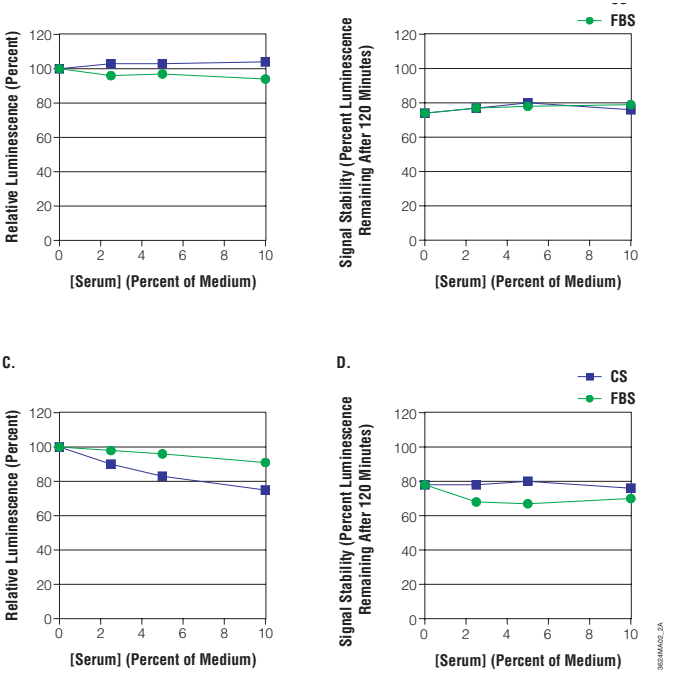

Figure 8. Effects of serum on luminescence intensity and signal stability. Purified firefly or *Renilla* luciferase (1.67×10^{-9}M, 1mg/ml gelatin) was added to a 96-well plate at 100µl per well. F12 medium containing various concentrations of either fetal bovine serum (FBS) or calf serum (CS) was used for the assay. Either Dual-Glo® Luciferase Reagent (for firefly luciferase) or Dual-Glo® Luciferase Reagent plus Dual-Glo® Stop & Glo® Reagent (for *Renilla* luciferase) was added, and luminescence measurements were integrated over 0.5 seconds per well. Firefly luciferase relative luminescence (**Panel A**) and signal stability (**Panel B**) were measured in medium containing fetal bovine serum (FBS) and calf serum (CS). *Renilla* luciferase relative luminescence (**Panel C**) and signal stability (**Panel D**) were measured in medium containing various concentrations of FBS and CS. Luminescence was compared for samples containing serum and for samples containing no serum. Signal stability expressed as percent of luminescence remaining after 2 hours. Number of samples = 3; relative standard error ≤3.9%.

6.D. Conditions Affecting Assay Performance (continued)

Phenol Red

Phenol red is a pH indicator commonly used in cell culture media. Many commercial media formulations contain 5–15mg/L phenol red, causing the characteristic red color. This compound can reduce assay sensitivity (Figure 9). However, in most applications the presence of phenol red will not significantly affect the utility of the Dual-Glo® Luciferase Assay System. To minimize its effect, use as little phenol red as possible in culture medium.

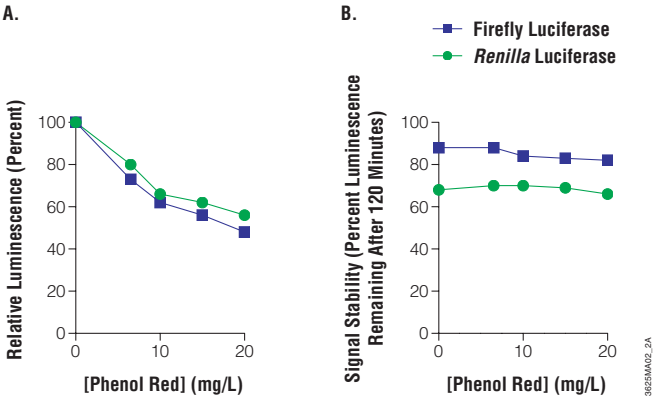

Figure 9. The effect of phenol red on luminescence intensity and signal stability. Purified firefly or *Renilla* luciferase (1.67×10^{-9}M, 1mg/ml gelatin) was added to a 96-well plate at 100µl per well. MEMα containing various concentrations of phenol red was used for the assay. Either Dual-Glo® Luciferase Reagent (for firefly luciferase) or Dual-Glo® Luciferase Reagent plus Dual-Glo® Stop & Glo® Reagent (for *Renilla* luciferase) was added, and luminescence measurements were integrated over 0.5 seconds per well. Luminescence is compared for samples containing phenol red and for samples containing no phenol red. **Panel A.** Luminescence is shown relative to that measured without phenol red. **Panel B.** Signal stability in various concentrations of phenol red expressed as percent of luminescence remaining after 2 hours. Number of samples = 3; relative standard error ≤4.0%.

Organic Solvents

Organic solvents are typically present in reporter gene assays, because they are used to stabilize and solubilize screening compounds. DMSO, ethanol and methanol have little effect on the assay (Figure 10). The compatibility of other solvents should be verified prior to use.

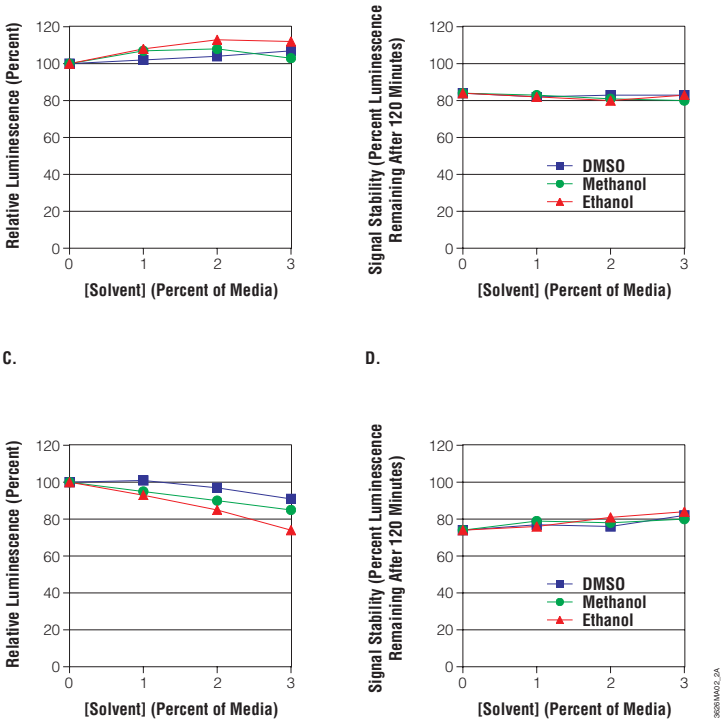

Figure 10. The effect of organic solvents on luminescence intensity and signal stability. Purified firefly or *Renilla* luciferase (1.67×10^{-9}M, 1mg/ml gelatin) was added to a 96-well plate at 100µl per well. F12 medium containing various concentrations of DMSO, methanol or ethanol was used for the assay. Either Dual-Glo® Luciferase Reagent (for firefly luciferase) or Dual-Glo® Luciferase Reagent plus Dual-Glo® Stop & Glo® Reagent (for *Renilla* luciferase) was added, and luminescence measurements were integrated over 0.5 seconds per well. Firefly luciferase relative luminescence (**Panel A**) and signal stability (**Panel B**), as well as *Renilla* luciferase relative luminescence (**Panel C**) and signal stability (**Panel D**) were determined in various concentrations of solvent. Luminescence is expressed relative to samples without organic solvents. Signal stability is expressed as percent of luminescence remaining after 2 hours. Number of samples = 3; relative standard error ≤4.0%.

6.D. Conditions Affecting Assay Performance (continued)

Temperature

Both firefly and *Renilla* luciferases are temperature sensitive, thus temperature is an important factor in experimental precision (Figure 11). Good precision can be achieved most easily by performing all experiments at room temperature, which is near the temperature optima for both firefly and *Renilla* luciferases. The assay reagents should be at room temperature before beginning measurements.

As mentioned previously, the Dual-Glo® Buffers can be stored at room temperature to avoid the need for temperature equilibration before use. The heat capacity of the substrates is low; therefore reconstitution of the Dual-Glo® Substrates with the room temperature Dual-Glo® Buffers produces reagents that are ready for use. If temperature equilibration is needed, incubate reagents in a water bath at room temperature. Do not use a water bath set higher than 25°C.

Lower temperatures result in increased signal stabilities but lower luminescent intensities. If cold reagent is used, the luminescence will slowly increase during the experiment as the reagent warms. High temperatures affect firefly and *Renilla* luciferases differently; however, both signals become less stable. This can occur if the culture plates are too warm or if the luminometer produces excess heat within the reading chamber. As shown in Figure 11, firefly luciferase is more sensitive to changes in temperature than *Renilla* luciferase.

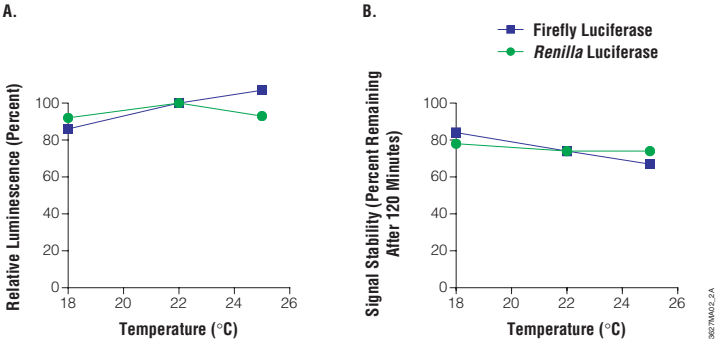

Figure 11. The effect of temperature on luciferase luminescence. Purified firefly or *Renilla* luciferase (1.67×10^{-9}M, 1mg/ml gelatin) was added to a luminometer tube containing 100µl of Dual-Glo® Luciferase Reagent (for firefly luciferase) or Dual-Glo® Luciferase Reagent plus Dual-Glo® Stop & Glo® Reagent (for *Renilla* luciferase). Samples were incubated at various temperatures, and light emission was measured on a Turner Designs Model 20e luminometer, integrated over 10 seconds after a 2-second preread delay. **Panel A.** Luminescence at 10 minutes is shown relative to that measured at 22°C. **Panel B.** Signal stability at various temperatures is expressed as percent of luminescence remaining after 2 hours. Number of samples = 3; relative standard error ≤3.1%.

7. Summary of Changes

The following changes were made to the 1/23 revision of this document:

1. Expired patent statement was removed, and another statement updated.
2. Discontinued products were removed.
3. Miscellaneous nontechnical text edits.
4. Cover image and font were updated.

[a]U.S. Pat. Nos. 7,078,181, 7,108,996, 7,118,878 and other patents.

[b]Certain applications of this product may require licenses from others.