Technical Manual

Chroma-Glo™ Luciferase Assay System

INSTRUCTIONS FOR USE OF PRODUCTS E4910, E4920 AND E4950.

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1. Description

The Chroma-Glo™ Luciferase Assay System[a–d] is designed to generate luminescence from luciferases emitting two different colors (red and green) within a single plate well (or from a single sample) after the addition of a single reagent. Quantitating multiple reporter gene outputs within a single sample well in a high-throughput setting has traditionally been difficult. Different reporter proteins require different, often incompatible environments. Additionally, sample well size limits the number of reagent additions possible. Filtered measurement of the luminescence produced by Chroma-Luc™ luciferases[a,c,d,e] permits red- and green-emitting luciferases to be measured

independently. This homogeneous assay generates luminescence with signal half-lives generally >30 minutes, which enables the processing of multiple plates without multistep sample handling. Two pieces of information can thus be efficiently and reproducibly gleaned from each well in a typical high-throughput screen.

2. Product Components and Storage Conditions

Product	Size	Cat.#
Chroma-Glo™ Luciferase Assay System	10ml	E4910

Each system contains sufficient reagents to perform 100 assays of 100µl each. Includes:

- 10ml Chroma-Glo™ Assay Buffer
- 1 vial Chroma-Glo™ Assay Substrate

Product	Size	Cat.#
Chroma-Glo™ Luciferase Assay System	100ml	E4920

Each system contains sufficient reagents to perform 1,000 assays of 100µl each. Includes:

- 100ml Chroma-Glo™ Assay Buffer
- 1 vial Chroma-Glo™ Assay Substrate

Product	Size	Cat.#
Chroma-Glo™ Luciferase Assay System	10 × 100ml	E4950

Each system contains sufficient reagents to perform 10,000 assays of 100µl each. Includes:

- 10 × 100ml Chroma-Glo™ Assay Buffer
- 10 vials Chroma-Glo™ Assay Substrate

Note: Cat.# E4950 contains two sleeves: A ten-pack of substrate to be stored at –20°C, and a ten-pack of buffer to be stored unfrozen but **below 25°C.**

Storage Conditions: Store the lyophilized Chroma-Glo™ Assay Substrate at –20°C. Store the Chroma-Glo™ Assay Buffer below 25°C. Room temperature storage is recommended to prevent the need for temperature equilibration when the reagent is reconstituted. Use reconstituted reagent on the day it is prepared. If necessary, freeze reconstituted reagent at –70°C. **Do not** store reconstituted reagent at –20°C, as potency decreases by >20% per week for the red enzyme when stored at –20°C.

Approximate Stability of Chroma-Glo™ Reagent after Reconstitution:

- 10% loss of potency for red-emitting luciferase (CBR) over 4.5 hours at 22°C and over 23 hours at 4°C.
- 3% loss of potency for green-emitting luciferases (CBG68 and CBG99) over 4.5 hours at 22°C

Caution: The lyophilized Chroma-Glo™ Substrate contains dithiothreitol (DTT) and is therefore classified as hazardous. The reconstituted reagent is not known to present any hazards, 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 or any chemical reagents. Promega assumes no liability for damage resulting from handling or contact with these products.

3. Performing the Chroma-Glo™ Luciferase Assay

3.A. General Considerations

The Chroma-Glo™ Luciferase Assay System has been designed for use with the following culture media containing 0–10% serum: RPMI 1640, MEMα, DMEM and Ham's F12. The signal half-life of each Chroma-Luc™ luciferase under these conditions will generally exceed 30 minutes at 22°C. This signal half-life is independent of enzyme concentration. Other medium/serum combinations can also be used, but experimental verification of assay performance is recommended in these cases. The luminescent signal can also be affected by the presence of phenol red and organic solvents or changes in temperature (Section 4.D).

Because the luminescent signals are affected by assay conditions, raw results should be compared only between samples measured using the same medium/serum combination. Incorporation of control wells on each plate allows the user to calculate a normalized red luminescence/green luminescence ratio for each well on a plate. These normalized ratios will remain consistent 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 medium/serum combination or that have been affected by changes in temperature or other variables (Section 4.C).

The red- and green-emitting Chroma-Luc™ luciferases generate signals in Chroma-Glo™ Reagent with half-lives that exceed 30 minutes; however, the luciferases have different signal kinetics (Figure 1). We recommend measuring the luminescence from samples with the red filter **before** measuring luminescence with the green filter because the red Chroma-Luc™ luciferase signal is less stable than the green Chroma-Luc™ signals in Chroma-Glo™ Reagent. The green luciferases generate luminescence with a half-life that often exceeds 5 hours.

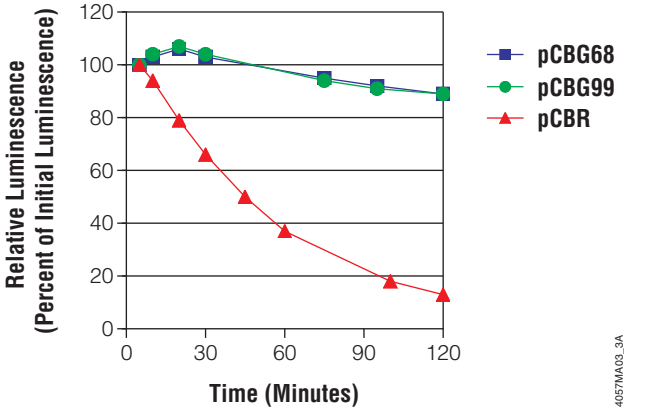

Figure 1. Luminescent signal kinetics for the red- and green-emitting Chroma-Luc™ luciferases. CHO cells were transfected with red (pCBR-Control Vector; Cat.# E1421), green 68 (pCBG68-Control Vector; Cat.# E1441) or green 99 (pCBG99-Control Vector; Cat.# E1461) Chroma-Luc™ luciferases under control of the SV40 promoter. Samples were lysed and the luminescence initiated with Chroma-Glo™ Reagent (100µl/well per sample). Plate contents were mixed with a plate shaker. Five minutes after reagent addition, luminescence was integrated over 0.5 seconds per well periodically for 120 minutes. As the data show, the kinetic profile of the two green-emitting Chroma-Luc™ luciferases is similar, producing a very stable luminescent signal. The red-emitting Chroma-Luc™ luciferase signal decays more quickly but is stable enough to measure an entire plate with little change in luminescent intensity.

The Chroma-Glo™ Reagent should be added to plates at least 5 minutes before quantifying luminescence. We have observed complete cell lysis within 5 minutes of Chroma-Glo™ Reagent addition to HeLa, NIH/3T3 and 293 cells.

For maximal light intensity, samples should be measured within 2 hours of reagent addition. **The Chroma-Glo™ Reagent is not designed for use with the automated reagent injectors that are integrated into some luminometers.**

To achieve linear assay performance at low light levels, subtract the background luminescence from all readings. No background is produced by the Chroma-Glo™ Reagent or by mammalian cells lacking the click beetle luciferase genes, but background luminescence is a characteristic of luminometer performance. Some instruments also require verification of linear response at high light levels. (Consult the instrument manual.)

3.B. Reagent Preparation

To prepare the Chroma-Glo™ Reagent, transfer the contents of one bottle of Chroma-Glo™ Buffer to one bottle of Chroma-Glo™ Substrate. Mix by inversion until the substrate is thoroughly dissolved.

Notes:

1. The temperature of the Chroma-Glo™ Reagent should be held constant while measuring luminescence, since luciferase activity is temperature dependent. This is achieved most easily by using reagent equilibrated to room temperature, which is near the temperature optimum of luciferase (Section 4.D). Equilibration of the reagent prior to use is unnecessary when the Buffer is stored at room temperature.

2. If the Chroma-Glo™ Reagent is stored frozen after reconstitution, it must be thawed at temperatures below 25°C for optimal performance. Mix well after thawing. The most convenient and effective method for thawing or temperature equilibrating cold reagent is by placing it in a room temperature water bath. Best results are obtained by using reagent at room temperature.

3. For maximum reproducibility, equilibrate cultured cells to room temperature before adding the Chroma-Glo™ Reagent.

3.C. Assay Procedure

1. Remove multiwell plates containing mammalian cells from the incubator. The plates must be compatible with the luminometer used.

2. Add a volume of Chroma-Glo™ Reagent equal to that of the volume of culture medium in each well and mix. For 96-well plates, typically 100µl of reagent is added to cells grown in 100µl of medium. For 384-well plates, typically 30µl of reagent is added to cells grown in 30µl of medium.

3. Wait at least 5 minutes to allow complete cell lysis, then measure in a luminometer. (Consult the luminometer operating manual for instructions.) To obtain maximal sensitivity, measure the luminescence from samples with the red filter before measuring the luminescence with the green filter.

   Note: The length of reading time for samples will vary depending on the signal intensity.

   Note: Filters are required for separating and measuring light from the different luciferases. Consult the luminometer operating manual for instructions. See also Section 4.B, Filter Choice.

4. Calculate the ratio of luminescence from the experimental reporter/ luminescence to that from the control reporter. Normalize this ratio to the ratio of the control well/series of control wells (for examples on normalizing data see Section 4.C). This normalization is essential for optimal and consistent results from the Chroma-Glo™ Reagent. Relative Response Ratios can then be calculated from the Normalized Ratios (Section 4.C).

4. Appendix

4.A. Overview of the Chroma-Glo™ Luciferase Assay System

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). This testing is typically achieved through transcriptional assays using genetic reporters coupled to physiologically regulated genetic elements.

Monitoring the upregulation of these genetic elements using luciferase assays is relatively simple because they are extremely sensitive, rapid and easy to perform. Monitoring the downregulation of these genetic elements is more difficult, however, because the generalized effects of cell death can easily be interpreted as a specific decrease in luciferase production. Normalizing the expression of an experimental reporter to the expression of a control reporter can help to differentiate specific from nonspecific effects of cell treatment protocols. This same normalization can also reduce the variability from transiently transfecting cells with an experimental reporter. The Chroma-Glo™ Luciferase Assay System is an ideal dual-reporter system because the red and green reporter enzymes are almost identical (3), and luminescence for each enzyme is generated by adding a single reagent.

Click Beetle Luciferases

Luciferases are widely used as co-reporters for these normalized studies because luciferase assays are quick, easy and sensitive. Promega has introduced three click beetle reporter genes for use in luminescent reporter gene assays. Luminous click beetles produce luciferases that generate light in colors from green to orange. We have evolved and created three synthetic genes that code for luciferases that emit red and green light (maximum emission at 613nm and 537nm, respectively; Figure 2). The *Chroma-Luc™ Series Reporter Vectors Technical Manual*, #TM059, contains details on the Chroma-Luc™ genes and vectors.

These synthetic Chroma-Luc™ luciferases are highly homologous, having over 98% amino acid identity. They are approximately 60kDa each and do not require post-translational modification for activity (3).

Development of the Assay

Like all coleopteran luciferases, the Chroma-Luc™ luciferases catalyze the oxidation of luciferin, using molecular oxygen, ATP and magnesium as co-substrates (Figure 3). The green- and red-emitting Chroma-Luc™ luciferases have different requirements for each of these substrates, and the Chroma-Glo™ Reagent is designed to allow for optimal performance of the Chroma-Luc™ luciferases with their various co-substrate requirements.

The Chroma-Glo™ Reagent has been optimized to facilitate high-throughput analysis of mammalian cells grown in 96-or 384-well plates. The Chroma-Glo™ Reagent can be added directly to cells in growth medium without washing or preconditioning. This reagent lyses the cells and elicits a stable luminescent signal from each Chroma-Luc™ luciferase.

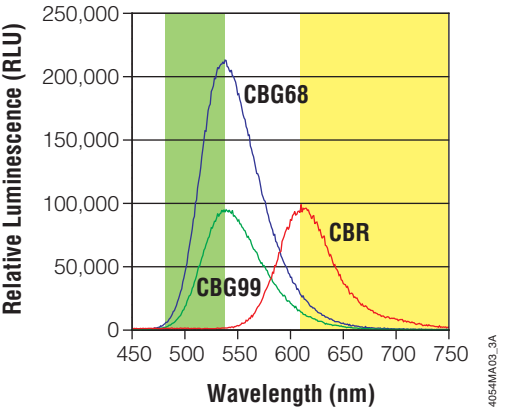

Figure 2. Areas of light capture using recommended 510/60 and 610 long-pass filters. Separate populations of CHO cells were transiently transfected with 1µg of one of the Chroma-Luc™ luciferase vectors. The vectors used include the pCBR-Control Vector (Cat.# E1421), the pCBG68-Control Vector (Cat.# E1441) and the pCBG99-Control Vector (Cat.# E1461), encoding green-emitting luciferases. At 24 hours post-transfection, an equal volume of Chroma-Glo™ Reagent was added to cells in medium. Spectra were captured using a scanning spectrophotometer configured to collect emission data. The shaded area on the right indicates the maximal transmittance range of a 610 long-pass filter. The shaded area on the left indicates the maximal transmittance range of a 510/60 filter. Note that each spectrum is broad, with a range of approximately 75nm at half-maximal intensity. In addition, some luminescence from each luciferase may be captured by both filters. RLU denotes relative light units.

The signal from red-emitting luciferase has the shortest half-life (approximately 30 minutes), so it is advisable to monitor the red signal first. The green luciferase signal is more stable (half-life often exceeds 5 hours), so it may be measured after the red luciferase without substantial loss in luminescence. We recommend that the luminescence be measured within two hours of reagent addition for maximal light intensity. The assay system is designed to work in the common growth media (DMEM, MEMα, F12, RPMI 1640) for mammalian cells with or without added serum.

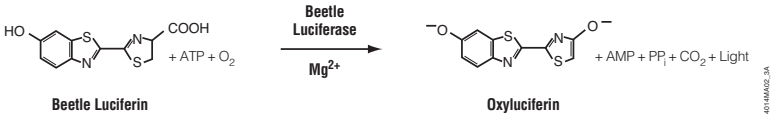

Figure 3. Bioluminescent reaction catalyzed by beetle luciferases. Monooxygenation of beetle luciferin is catalyzed by the Chroma-Luc™ luciferases in the presence of Mg^{2+}, ATP and molecular oxygen.

4.B. Filter Choice

We recommend using 510/60nm and 610 long-pass filters from Chroma Corporation (Chroma Part# D510/60 and E610LP, respectively) to successfully separate the red and green luminescent signals generated by the Chroma-Luc™ luciferases. These filters permit transmittance in the region shown in Figure 2.

The 510/60nm and 610 long-pass filter pair provide a balance between sensitivity and separation. This filter pair has been chosen so that the two colored signals have the least amount of overlap, while permitting transmittance of the largest possible portion of each luminescent spectrum. Other filter pairs can be used if extreme separation or extreme sensitivity is critical. If extreme separation of signal is required, filter pairs may be chosen so that no green spectra tailing is captured in the red signal. A 640 long-pass filter, for example, might be chosen for this application and combined with a 510/60nm filter.

If extreme sensitivity is critical, filter pairs may be chosen so that more of each signal spectrum is transmitted. This means that more of the red luminescent signal will be transmitted through the green filter and more of the green luminescent signal will be transmitted through the red filter. Regardless of the filter set chosen, the luminescent signal transmittance through each filter must be calculated. Section 4.C (below) describes the mathematical formulae that are required to separate the red and green luminescent signals. These calculations must be performed regardless of filter choice and must be performed for each combination of filter pair and luminometer.

4.C. Data Analysis

Note: When analyzing data we recommend (i) performing color separation, (ii) normalizing data and (iii) calculating relative response ratios.

Color Separation

Filter sets provide the ability to measure a consistent portion of the red or green luminescence generated by a sample (Section 4.B). If the same filters are used for all measurements in an experiment, then it is not necessary to calculate the total luminescence generated by each luciferase. The relative proportion that is measured can therefore be used to calculate induction ratios and other experimentally relevant information. (The equations in Section 4.C can be used to calculate total signal.)

Figure 2 shows the overlap of the spectra generated by the Chroma-Luc™ luciferase enzymes in the transmittance window of two filters. Filter corrections can be made to factor out the tail of the green luminescent signal from a red luminescent measurement. A single calculation of the filter corrections is adequate for all measurements using a single luminometer or CCD camera with a particular filter set. However, measurements using a different instrument or filter set require calculation of different correction factors.

Calculating the filter corrections (calibration constants) requires a one-time measurement of two samples of cells, one expressing only a red-emitting luciferase and the other expressing only a green-emitting luciferase. A total of six one-time measurements (Table 1) must be made to determine calibration constants used to normalize filter and detector efficiencies. The calibration constants should not change over time; however, they must be recalculated upon substituting filter sets, luminometers or detectors.

Table 1. Initial Measurements Required to Calculate Calibration Constants.

Name	Symbol	Measurement
Total red luminescence, no filter	R	No filter used. Total luminescence measured from the red-emitting luciferase.
Total green luminescence, no filter	G	No filter used. Total luminescence measured from the green-emitting luciferase.
Red luminescence using the red filter	Rrf	Luminescence measured from the red-emitting luciferase. Red filter used.
Green luminescence using the red filter	Grf	Luminescence measured from the green-emitting luciferase. Red filter used.
Red luminescence using the green filter	Rgf	Luminescence measured from the red-emitting luciferase. Green filter used.
Green luminescence using the green filter	Ggf	Luminescence measured from the green-emitting luciferase. Green filter used.

Once these calibration constants (Table 1) are known, determine the corrected red and green luminescence values (R´ and G´) using the two experimental luminescence values, Lrf and Lgf (Table 2) and the equations provided below.

Note: The amount of red- or green-emitting luminescence generated for filter calibration is not critical, and the red and green amounts need not be equal. The goal is to generate a quantifiable amount of luminescence with each filter.

Table 2. Experimental Luminescence Values Required to Determine Corrected Luminescence Values, R´ and G´.

Name	Symbol	Measurement
Experimental luminescence, red filter	Lrf	Experimental luminescence measured with the red filter.
Experimental luminescence, green filter	Lgf	Experimental luminescence measured with the green filter.

The corrected amount of red luminescence in a mixed sample, R´, is calculated by subtracting the portion of the green luminescence measured using the red filter [Lgf × (Grf/Ggf)] from the amount of total luminescence measured with the red filter (Lrf) that is normalized for filter efficiencies [(Rrf/R) – (Rgf/R) × (Grf/Ggf)].

$$R' = \frac{Lrf - [Lgf \times (Grf/Ggf)]}{(Rrf/R) - (Rgf/R) \times (Grf/Ggf)}$$

The corrected amount of green luminescence from the same sample, G´, is calculated as the amount of total luminescence measured with the green filter (Lgf) minus the amount of corrected red luminescence (R´) measured with the green filter [R´ × (Rgf/R)] that has been normalized for filter efficiencies (Ggf/G).

$$G' = \frac{Lgf - [R' \times (Rgf/R)]}{(Ggf/G)}$$

Measurements for the calibration constants Rrf/R, Rgf/R, Grf/Ggf and Ggf/G must be made every time filter sets and/or detection equipment changes.

To assist you in the determination of corrected luminescence values, we have prepared the Chroma-Luc™ Calculator, a PC-based spreadsheet. This tool is available for download at: **www.promega.com/techserv/tools/**

Normalized Data

The green and red Chroma-Luc™ luciferase signals change intensity at different rates. Depending on the method of measurement utilized (measuring red then green luminescence from each sample, each plate or each stack of plates), the ratio of experimental to control luminescence can change between the first and the last sample measured. In order for these ratios to be compared, experimental samples on each plate must be compared to controls on that same plate. Normalizing the results from each experimental sample to control samples repeated on each plate also minimizes the impact of variables like temperature, plate order and timing on the ratio of experimental/control reporter activity.

Example: Sample 14 on plate 1 in a stack of 20 plates had red luminescence (experimental signal) = 36,000 relative light units (RLU) and green luminescence (control signal) = 540RLU.

The negative control on plate 1 (no experimental compound added) had red luminescence = 18,000RLU and green luminescence = 500RLU.

On plate 20, sample 14 (the same treatment as sample 14 on plate 1) had red luminescence = 27,000RLU and green luminescence = 490RLU.

The negative control on plate 20 had red luminescence = 13,200RLU and green luminescence = 450RLU.

If the red/green luminescence ratios are calculated with raw data, the red/green luminescence ratio for sample 14 is 66.7 on plate 1 (36,000RLU/540RLU) and 55.1 on plate 20 (27,000RLU/490RLU). These values differ by 17.4%, while they should be almost identical.

However, if the red/green luminescence ratios are normalized to the ratio for the negative controls on each plate, the red/green luminescence ratios for the same treatment become much more consistent:

Plate 1: the normalized red/green luminescence ratio is (36,000RLU/540RLU)/(18,000RLU/500RLU) or 1.85.

Plate 20: the normalized red/green luminescence ratio is (27,000RLU/490RLU)/(13,200RLU/450RLU) or 1.87.

As expected, if the experimental ratios are normalized to the negative control ratios on the same plate, the same treatment generates the same ratio regardless of when it is measured. Normalization of the experimental/control reporter activity is essential for easy comparison of samples with minimal variability across an experiment.

If the luminescence values are close to the background levels measured by the luminometer, the background luminescence must be subtracted before the ratios are calculated.

Note: The luminescence from an empty well (background) is dependent on the luminometer used and will vary for different luminometers. This measurement should be subtracted from all samples, especially those that produce low levels of luminescence.

Relative Response Ratios

To assist quantitation of the impact of an experimental treatment on reporter gene expression, a Relative Response Ratio (RRR) can be determined. This RRR permits the comparison of multiple treatments from different experiments by providing a framework within which the impact of the treatment can be calculated.

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 two controls are included on every plate, the impact of each new compound can be evaluated by its impact on the experimental reporter within the context of the positive and negative control. Is the new compound more effective, half as effective, or ineffective at decreasing the expression of the experimental reporter when compared to the compound used as the negative control?

For 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, then 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 the formula:

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

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

$$RRR = \frac{22 - 1.3}{53 - 1.3}$$

$$= 0.40 \text{ or } 40\%$$

This result indicated that the experimental compound is 40% as effective as the negative control at decreasing expression of the experimental reporter at this concentration, and so the hierarchy of efficacy for the experimental system would be that no treatment generated an RRR of 100%; the experimental compound, an RRR of 40%; and the negative control, an RRR of 0%. In this type of experiment, those compounds that are most significant will have RRRs that are negative, since they are more effective inhibitors than the negative control.

4.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 Chroma-Glo™ Luciferase Assay System is uniquely formulated for use with the Chroma-Luc™ Series Reporter Vectors—it is chemically different from the other luciferase assay systems from Promega. As a result, data presented here will not be applicable to other luciferase assay systems.

Lysates containing red- or green-emitting Chroma-Luc™ luciferase were used to generate the data presented in this section. Because the two green Chroma-Luc™ luciferases perform similarly in the Chroma-Glo™ Reagent, only the CBG99*luc* luciferase was used for the examples below. The lysates were generated by transfecting CHO cells with red or green Chroma-Luc™ luciferase under control of the SV40 promoter (pCBR-Control Vector, Cat.# E1421, and pCBG99-Control Vector, Cat.# E1461, respectively) using the

Tfx™-50 Transfection Reagent (Cat. E1811) and lysing at 48 hours with Glo Lysis Buffer (Cat.# E2661). Single-use aliquots were stored at –70°C. Lysates were further diluted in each experiment below using the medium indicated. The diluent contains BSA (1mg/ml) to simulate protein that would normally be contributed by the cells. The addition of BSA is not required when using the Chroma-Glo™ Reagent on intact cells.

Culture Medium

In the Chroma-Glo™ Assay, the culture medium and any other compounds contained therein comprise half of the chemical environment that defines the luminescent reaction. Although the Chroma-Glo™ Reagent is designed to work in conjunction with many common culture media, compositional differences between these media can affect the assay characteristics of each of the Chroma-Luc™ enzymes (e.g., light intensity and stability).

The Chroma-Glo™ Assay was designed to provide relatively high luminescence with extended signal half-lives for the red- and green-emitting Chroma-Luc™ luciferases generally exceeding 30 minutes when using common growth media. However, performance differences are evident between these media (Figure 4) as well as between medium from different manufacturers. Sometimes differences are also evident between lots of media from the same manufacturer. Although these differences are generally small and do not diminish the utility of the Chroma-Glo™ Assay, controls should be incorporated into every batch of plates to correct for this variability.

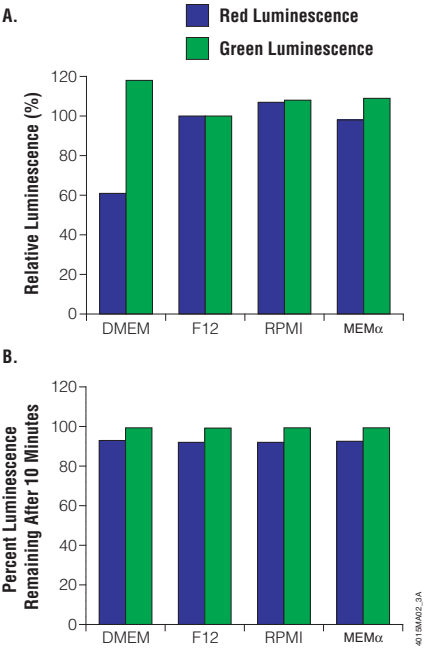

Figure 4. Relative luminescence intensity and signal stability of red- and green-emitting Chroma-Luc™ luciferases in four common media. Cell lysates containing red or green Chroma-Luc™ luciferases were added to a 96-well plate at 100µl per well. Lysate dilutions (1:100) were made in RPMI 1640, DMEM, MEMα, or F12 medium. Chroma-Glo™ Reagent was added and luminescence measurements were integrated over 0.5 seconds per well. **Panel A.** Luminescence measurements were taken 5 minutes after reagent addition. Luminescence is shown relative to the light output generated in F12 medium for both red- and green-emitting luciferases. **Panel B.** Signal stability in various media expressed as percent luminescence remaining after 10 minutes. Number of samples = 3; relative standard error <2.6%.

Serum

The Chroma-Glo™ Luciferase Assay System is compatible with medium containing serum. The Chroma-Glo™ Reagent has been designed for use with serum concentrations from 0–10%, and the luminescent signals generated are generally unaffected by the presence of fetal bovine or calf sera (Figure 5).

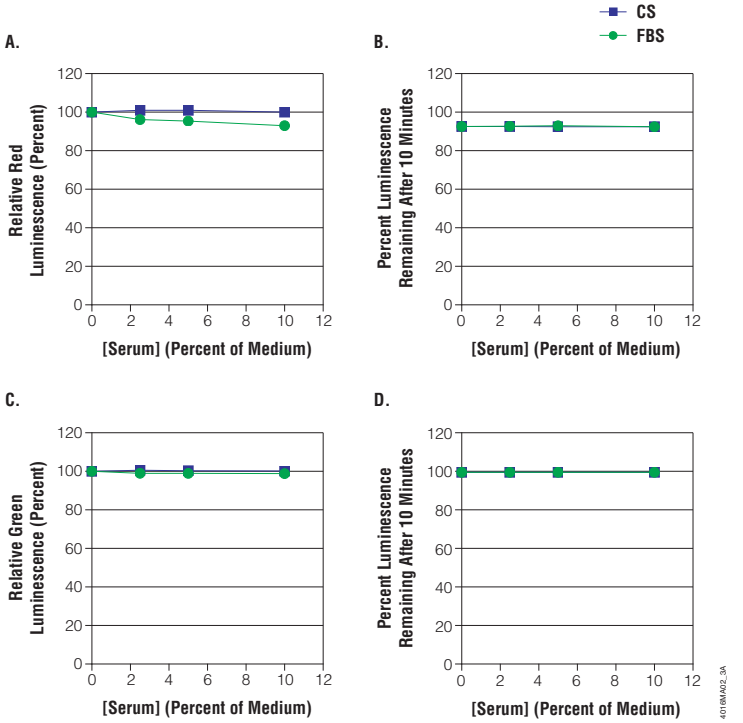

Figure 5. Effect of serum on luminescence intensity and signal stability. Cell lysates containing red- or green-emitting Chroma-Luc™ luciferases were added to a 96-well plate, 100µl per well. F12 medium containing various concentrations of either fetal bovine serum (FBS) or calf serum (CS) was used for the assay. Chroma-Glo™ Reagent was added, and luminescence measurements integrated over 1.0 second per well. Red-emitting Chroma-Luc™ luciferase relative luminescence (**Panel A**) and signal stability (**Panel B**) were measured in medium containing fetal bovine serum (FBS) and calf serum (CS). Green-emitting Chroma-Luc™ luciferase relative luminescence (**Panel C**) and signal stability (**Panel D**) were measured in medium containing FBS and CS. Luminescence was compared for samples with and without serum. Signal stability in various concentrations of serum is expressed as percent luminescence remaining after 10 minutes. Number of samples = 3, relative standard error <3.0%.

Phenol Red

Phenol red is a pH indicator that is routinely added to cell culture media. Many commercially prepared media contain 5–15mg/L phenol red, as evidenced by their red color. This compound will reduce the assay sensitivity of the green luciferase signal but will not significantly reduce the red luciferase signal (Figure 6). In most applications containing appropriate controls, relative changes in luminescence are measured, and these relative changes will not be altered significantly. To minimize the effects of phenol red, minimize its presence in culture media.

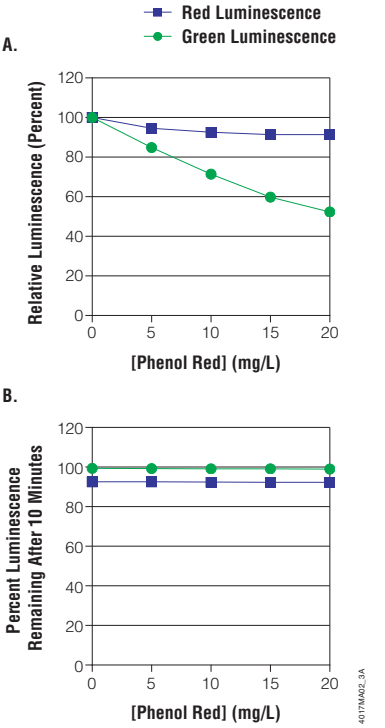

Figure 6. The effect of phenol red on luminescence intensity and signal stability. Cell lysates containing red or green Chroma-Luc™ luciferases were added to a 96-well plate at 100µl per well. MEMα containing various concentrations of phenol red was used for the assay. Chroma-Glo™ Reagent was added, 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 luminescence remaining after 10 minutes. Number of samples = 3; relative standard error <2.6%.

Organic Solvents

Organic solvents are typically present in reporter gene assays since they are used to stabilize and solubilize experimental compounds. DMSO, methanol and ethanol have little effect on the assay (Figure 7). The compatibility of other solvents should be verified prior to use.

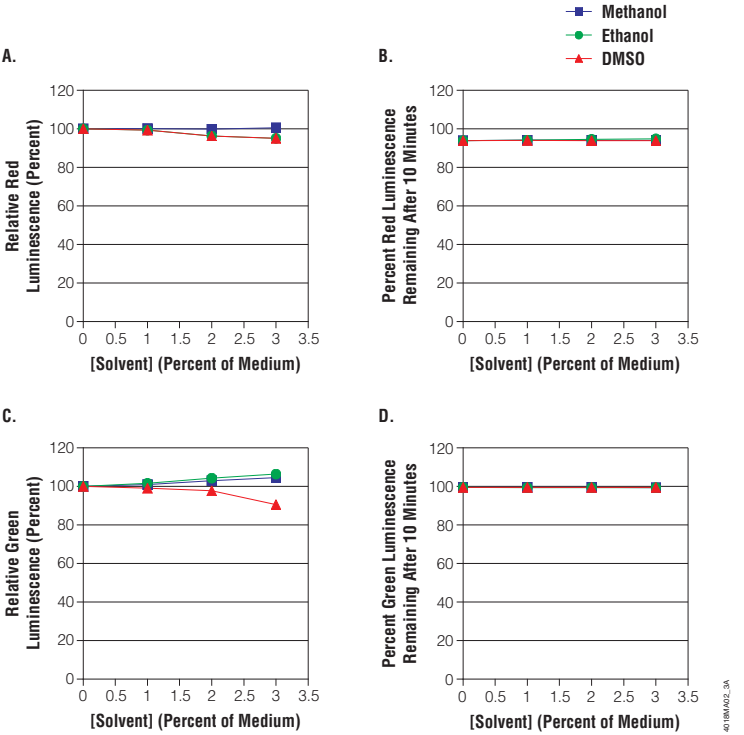

Figure 7. The effect of organic solvents on luminescent intensity and signal stability. Cell lysates containing red- or green-emitting Chroma-Luc™ luciferases were added to a 96-well plate at 100µl per well. F12 medium containing various concentrations of DMSO, methanol or ethanol was used for the assay. Chroma-Glo™ Reagent was added, and luminescence measurements were integrated over 0.5 seconds per well. Red-emitting Chroma-Luc™ luciferase relative luminescence (**Panel A**) and signal stability (**Panel B**), as well as green-emitting Chroma-Luc™ luciferase relative luminescence (**Panel C**) and signal stability (**Panel D**), were determined in various concentrations of solvent. Luminescence is expressed relative to samples containing no organic solvents. Signal stability is expressed as percent luminescence remaining after 10 minutes. Number of samples = 3, relative standard error <2.2%.

Temperature

Chroma-Luc™ luciferase activity is temperature dependent (Figure 8). For this reason, the most precise results are achieved by performing all experiments at room temperature, which is near the luciferase temperature optimum. The assay reagent should be at room temperature before beginning measurements.

As mentioned previously, the Chroma-Glo™ Buffer can be stored at room temperature to avoid the need to bring the reagent to room temperature before use. The heat capacity of the dried substrates is low, thus reconstitution of the Chroma-Glo™ Substrate with room-temperature Chroma-Glo™ Buffer yields a Chroma-Glo™ Reagent that is ready to use. If temperature equilibration is needed, use a room-temperature water bath. Do **not** use a water bath above 25°C.

Lower temperatures result in increased signal stability but also lower luminescent intensity. If cold reagent is used, luminescence slowly increases during the experiment as the reagent warms. Higher temperatures cause an increase in the red luminescence signal, while green luminescent intensity remains relatively consistent. However, red and green luminescent signals become less stable with increased temperatures. This loss of stability can occur if the culture plates are too warm or if the luminometer produces excess heat within the reading chamber.

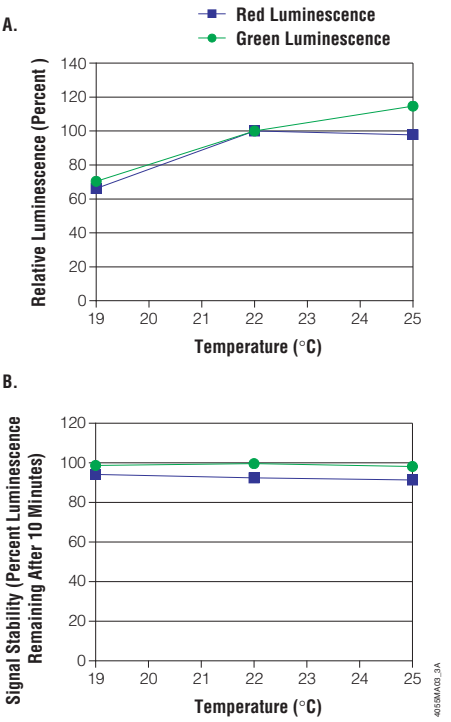

Figure 8. The effect of temperature on luminescent intensity and signal stability. Cell lysates (in F12 medium) containing red- or green-emitting Chroma-Luc™ luciferases were added to luminometer tubes at 100µl per sample. An equal volume of Chroma-Glo™ Reagent was added to each sample, and triplicate samples were incubated at various temperatures. Light emission was measured on a Turner Model 20e luminometer and integrated over 5 seconds after a 2-second preread delay. **Panel A.** Luminescence at 5 minutes is shown relative to that measured at 22°C. **Panel B.** Signal stability at various temperatures is expressed as the percent of luminescence remaining after 10 minutes. Relative standard error is ≤4.8%.

4.E. 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. *Chroma-Luc™ Series Reporter Vectors Technical Manual* #TM059, Promega Corporation.

4.F. Related Products

Chroma-Luc™ Series Reporter Vectors

Product	Size	Cat.#
pCBR-Basic Vector[a,c,d,e]	20µg	E1411
pCBR-Control Vector[a,c,d,e]	20µg	E1421
pCBG68-Basic Vector[a,c,d,e]	20µg	E1431
pCBG68-Control Vector[a,c,d,e]	20µg	E1441
pCBG99-Basic Vector[a,c,d,e]	20µg	E1451
pCBG99-Control Vector[a,c,d,e]	20µg	E1461

Luciferase Assay System

Product	Size	Cat.#
Dual-Glo™ Luciferase Assay System	10ml	E2920
	100ml	E2940
	10 × 100ml	E2980

Luciferase Assay Reagents

Product	Size	Cat.#
Glo Lysis Buffer, 1X	100ml	E2661

Transfection Reagents

Product	Size	Cat.#
TransFast™ Transfection Reagent	1.2mg	E2431
Transfectam® Reagent for the Transfection of Eukaryotic Cells	1mg	E1231
	0.5mg	E1232
Tfx™-20 Reagent	4.8mg	E2391
Tfx™-50 Reagent	2.1mg	E1811
ProFection® Mammalian Transfection System —Calcium Phosphate	40 reactions	E1200
ProFection® Mammalian Transfection System —DEAE-Dextran	40 reactions	E1210

[a]U.S. Pat. Nos. 6,387,675 and 6,552,179 and Australian Pat. No. 698424 have been issued to Promega Corporation for mutants of beetle luciferases. Other patents are pending.

[b]U.S. Pat. Nos. 5,283,179, 5,641,641, 5,650,289 and 5,814,471, Australian Pat. No. 649289, European Pat. No. 0 553 234 and Japanese Pat. No. 3171595 have been issued to Promega Corporation for a beetle luciferase assay method, which affords greater light output with improved kinetics as compared to the conventional assay.

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

[d]The method of recombinant expression of *Coleoptera* luciferase is covered by U.S. Pat. Nos. 5,583,024, 5,674,713 and 5,700,673. A license (from Promega for research reagent products and from The Regents of the University of California for all other fields) is needed for any commercial sale of nucleic acid contained within or derived from this product.

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Product claims are subject to change. Please contact Promega Technical Services or access the Promega online catalog for the most up-to-date information on Promega products.

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