Bright Light, No Lysis

Measuring *Renilla* Luciferase Luminescence in Living Cells

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Abstract

The ViviRen™ and EnduRen™ Live Cell Substrates have been developed to make measuring Renilla *luciferase from live cells simpler and more sensitive than was possible with the substrates coelenterazine and coelenterazine-h. ViviRen™ Substrate generates very bright luminescence, at least 3-fold brighter than coelenterazine, while EnduRen™ Substrate generates very stable luminescence, permitting multiple measurements over incubations of 24 hours or more. Both live cell substrates can be multiplexed with CellTiter-Glo® Reagent to normalize for viable cell numbers. Alternatively, the Chroma-Luc™ red Luciferase can be used to normalize for viable cell number or transfection efficiency.*

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Introduction

Researchers strive to monitor cellular activities with as little impact on the cell as possible. However, complete disruption of the cells is required for most cellular reporters to achieve accurate and reliable measurements. *Renilla* luciferase is a notable exception because its substrates, oxygen and coelenterazine, are nontoxic and readily permeable to cellular membranes. Unfortunately, coelenterazine is unstable in aqueous solutions and has been difficult and inconvenient to use under normal cell culture conditions. EnduRen™ and ViviRen™ Live Cell Substrates[(a,b,c)] have been designed to overcome this difficulty and, moreover, can be multiplexed with other luminescence assays to determine viable cell number.

Stabilizing the Live Cell Substrates

The EnduRen™ and ViviRen™ Live Cell Substrates are based on the core structure of coelenterazine-h, an efficient structural analog of coelenterazine commonly used in luminescent cellular assays. Historically, coelenterazine-based compounds have been difficult to work with because they are unstable in the aqueous environment used to maintain live cells. For example, coelenterazine-h in cell culture medium containing 10% fetal bovine serum (FBS) at 37°C, decreases in concentration by 50% in approximately 25 minutes. Coelenterazine concentration decreases even more quickly in this environment, dropping 50% in approximately 17 minutes (1). Auto-oxidation is one means by which this loss in coelenterazine and coelenterazine-h concentration occurs. Auto-oxidation presents a double problem in that it also generates background luminescence, thus decreasing assay sensitivity.

The EnduRen™ and ViviRen™ Live Cell Substrates were designed so that the site of oxidation is protected by esters or oxymethyl ethers. The protecting groups serve to increase the half-life of the live cell substrates in culture medium to 5–11 hours compared to the 17- to 25-minute half-life for the unprotected coelenterazine substrates (1–3). Upon entry into viable cells, esterases and lipases cleave the protecting group from the live cell substrates, generating coelenterazine-h for use by intracellular *Renilla* luciferase (Figure 1). The low concentration of free coelenterazine-h in the culture medium results in a 10- to 100-fold decrease in autoluminescent background and thus an increased luminescent signal-to-background ratio (Figure 2, Panels A and B).

Differences in the Live Cell Substrates

ViviRen™ and EnduRen™ Live Cell Substrates both support *Renilla* bioluminescence, but the characteristics of the resulting luminescent signals are very different. The ViviRen™ Substrate generates a very bright but short-lived luminescent signal. In the example shown (Figure 2, Panel C), the maximum intensity of the ViviRen™ Substrate was approximately 3-fold brighter than coelenterazine. The much reduced autoluminescence from the ViviRen™ Substrate resulted in a signal-to-background ratio that was 288-fold higher than that of coelenterazine. Low autoluminescence is a key feature of both the ViviRen™ and EnduRen™ Substrates.

Whereas ViviRen™ Substrate generates bright luminescence, EnduRen™ Substrate generates long-lasting luminescence. Esterases cleave the oxymethyl ether moiety from the EnduRen™ Substrate more slowly than they cleave the ester group of the ViviRen™ Substrate (structures in Figure 1, esterase data not shown). The lower concentration of coelenterazine-h results in a lower intensity but more stable luminescent signal. The luminescent signal generated by the EnduRen™ Substrate is thus stable for up to 24 hours.

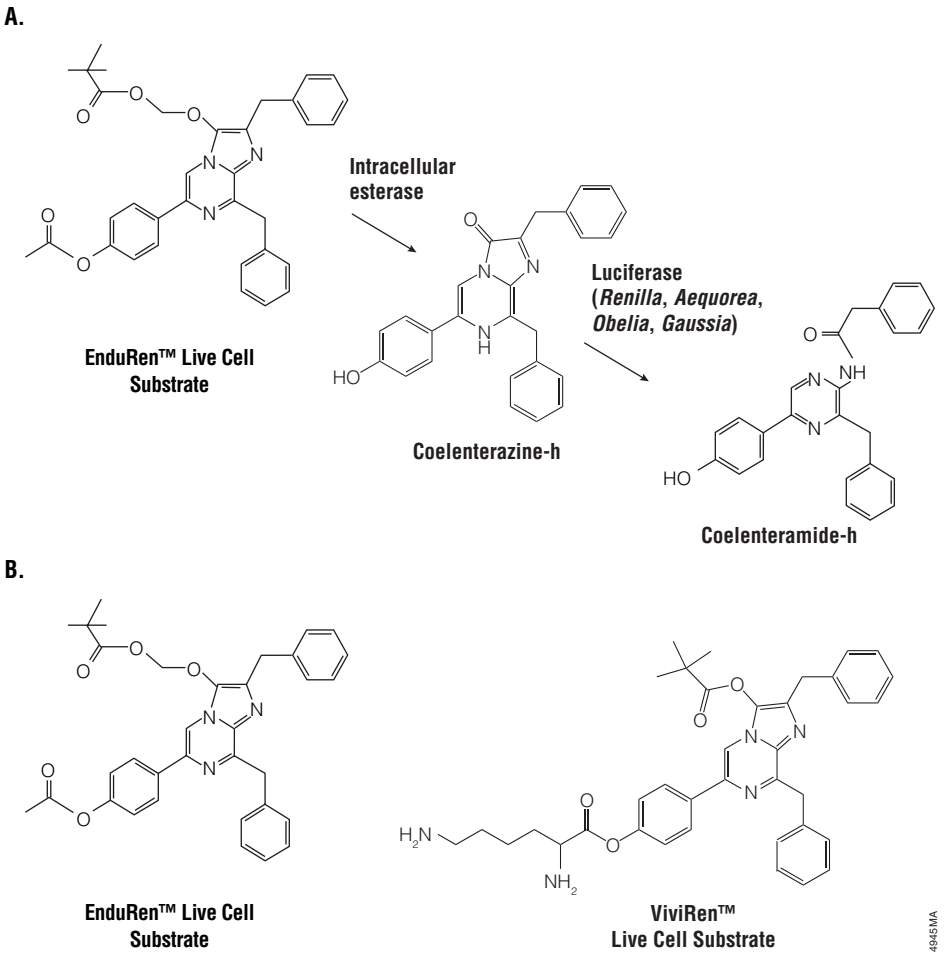

Figure 1. Structural features of the Live Cell Substrates. Panel A. EnduRen™ Live Cell Substrate is converted inside cells to coelenterazine-h, a substrate of *Renilla* luciferase. A similar conversion occurs with ViviRen™ Live cell Substrate changing to coelenterazine-h (not shown). **Panel B.** Structures for EnduRen™ and ViviRen™ Live Cell Substrates.

In typical measurements, the initial luminescent signal generated by the ViviRen™ Substrate was at least 20 times brighter than the EnduRen™ Substrate, but after approximately 45 minutes the EnduRen™ Substrate signal was as bright as the ViviRen™ Substrate signal (Figure 3). The exceptionally stable luminescent signal generated by the EnduRen™ Substrate permits many measurements to be made from a single sample. For example, a single addition of EnduRen™ Substrate permitted sequential measurements of the inhibition of *Renilla* luciferase stably expressed in CHO cells for more than 33 hours after transfection of siRNA for *Renilla* luciferase (Figure 4).

We have demonstrated that the recommended concentrations and exposure times for the ViviRen™ and EnduRen™ Live Cell Substrates were not toxic to CHO, HeLa, HEK 293 or NIH/3T3 cell lines when compared to samples exposed to coelenterazine, to carrier or to no treatment (2,3).

Multiplex Live Cell Substrates with Cell Viability Assays

The assay for *Renilla* bioluminescence in living cells can be multiplexed with cell viability assays, allowing reporter activity to be normalized to the number of viable cells. This can be done most easily by using the CellTiter-Glo® Reagent, which determines viable cell number by assaying for total ATP. Another normalization method uses co-transfection of a control plasmid that constitutively expresses a red-emitting luciferase, such as the Chroma-Luc™ Luciferase contained in the pCBR-Basic Vector (Cat.# E1411) or pCBR-Control Vector (Cat.# E1421).

Multiplexing with CellTiter-Glo® Reagent involves two reagent additions into the same sample. First *Renilla* luciferase is quantitated from the living cells following addition of the EnduRen™ or ViviRen™ Substrate. Second, CellTiter-Glo® Reagent is added to the samples, lysing the cells to generate an ATP-dependent firefly luciferase signal. The CellTiter-Glo® Reagent effectively

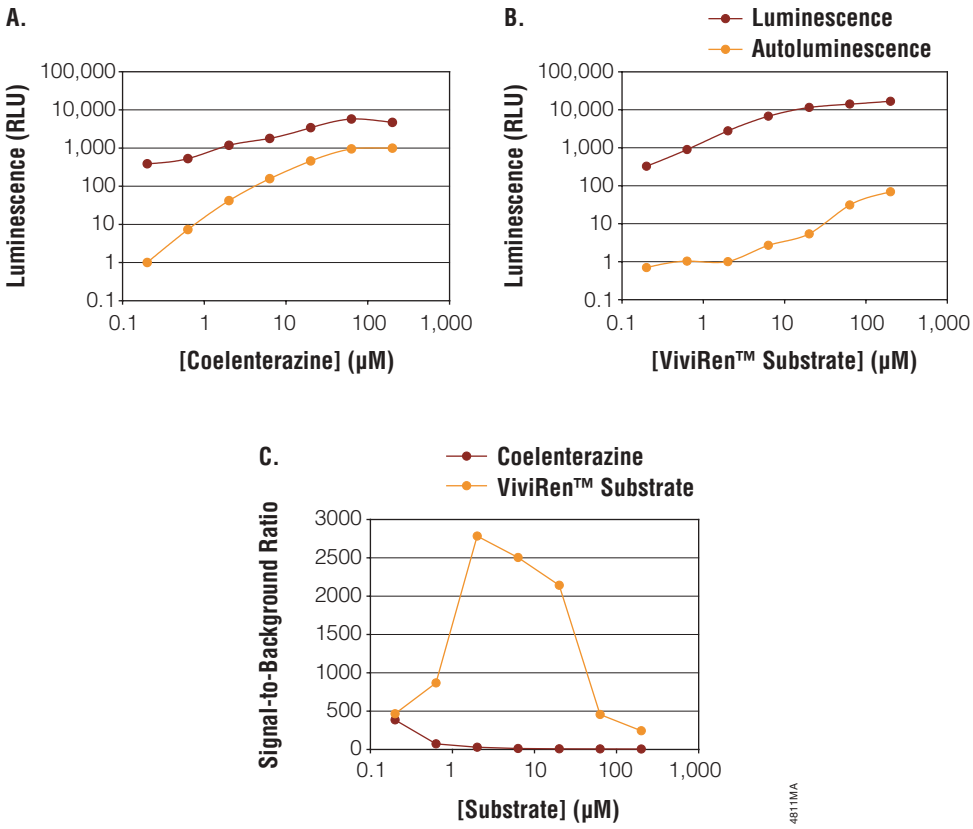

Figure 2. Sensitivity of in situ measurement of luminescence increases dramatically in the absence of autoluminescence. Luminescence was measured from CHO cells stably expressing *Renilla* luciferase luminescence. Similarly, autoluminescence was measured from nontransfected CHO cells. Coelenterazine (**Panel A**) or ViviRen™ Substrate (**Panel B**) was titrated into wells containing CHO cells in F12 media with 10% FBS. Luminescence or autoluminescence was measured with a Turner BioSystems Veritas™ Luminometer approximately 2 minutes after addition of coelenterazine or ViviRen™ Substrate (n = 6). Signal-to-background ratios (luminescence minus autoluminescence divided by autoluminescence) were calculated from the data in Panels A and B and are presented in **Panel C**.

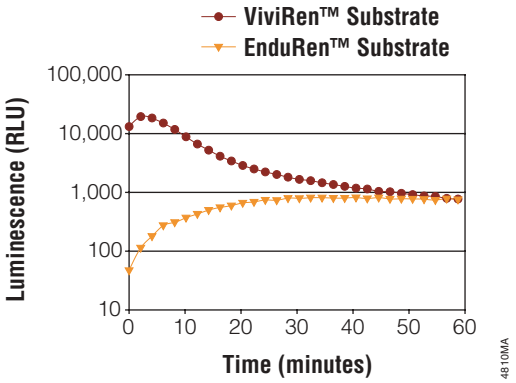

Figure 3. Maximal luminescence intensity generated by ViviRen™ Substrate is ~20-fold higher than that of EnduRen™ Substrate, but they converge approximately 45 minutes after substrate addition. CHO cells stably expressing *Renilla* luciferase were exposed to ViviRen™ or EnduRen™ Substrate at 60µM, and luminescence was measured at intervals of approximately 2 minutes for 1 hour with the Turner BioSystems Veritas™ Luminometer (n = 6). After approximately 45 minutes, the luminescence intensities from the two sample sets were similar.

eliminates the *Renilla* luciferase signal and any enzyme-independent luminescence that may be generated by the residual coelenterazine-h in the sample reaction. Using the most extreme case as an example, the luminescent and autoluminescent signals generated by 2×10^{-12} moles of purified *Renilla* luciferase (over 1,000 times more enzyme than most cellular preparations) exposed to 60µM coelenterazine-h was quenched by 107,000-fold ± 10,900-fold after adding CellTiter-Glo® Reagent. The residual luminescence was only 56% ± 4.7% of the luminescence generated by CellTiter-Glo® Reagent and DMEM medium + 10% serum (in the absence of cells), and so below the level of luminescence generated by cells in this environment.

Multiplexing Live Cell Substrates with an in situ beetle luciferase measurement requires the use of a luminometer with wavelength-discriminating filters. The red Chroma-Luc™ Luciferase generates luminescence with maximal intensity at 611nm, while the *Renilla* luciferase generates maximal intensity at 488nm (Figure 5A). This large spectral difference permits the two signals to be measured from the same sample using the appropriate red and blue filters.

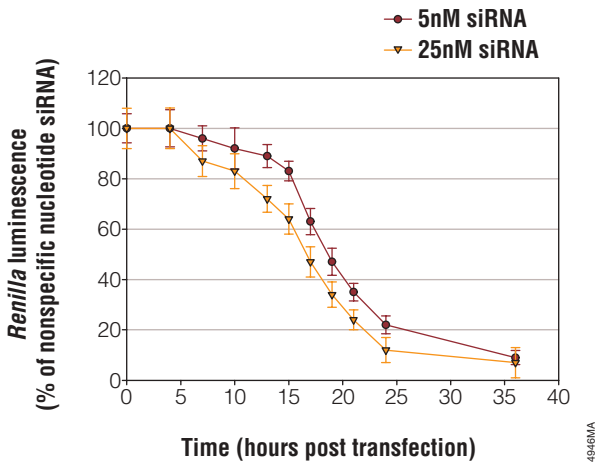

Figure 4. EnduRen™ Substrate permits repetitive measurement of *Renilla* luminescence from live cells, monitoring RNAi over time in the same cell population. Decreasing luminescence can be measured from live cells to monitor the effect of siRNA on *Renilla* luciferase expression in CHO cells stably expressing *Renilla* luciferase. Cells were transfected with 5nM or 25nM siRNA against a nonspecific negative sequence or against a *Renilla* luciferase target sequence. *Renilla* luciferase activity was measured at indicated time points. The data were normalized as percent of *Renilla* luciferase activity in cells transfected with specific targeting sequence compared to a nonspecific control. The results are the average of 12 wells.

The ability to multiplex was demonstrated in an experiment where *Renilla* luciferase expression was controlled with the responsive CRE element as promoter and red Chroma-Luc™ Luciferase expression was controlled with a constitutive SV40 promoter. The addition of EnduRen™ Substrate and beetle luciferin to the cells allowed measurement of the cells' CRE response, while normalizing for viable cell number (Figure 5). As expected, normalizing to an internal control reduced the relative error within the experimental data. As Figure 5B shows, the relative error of the calculated induction decreased from 43% to 24% when the *Renilla* luminescence was normalized to the red Chroma-Luc™ luminescence in the same well.

Conclusion

The *Renilla* luciferase reporter system requires only oxygen and coelenterazine substrates to generate luminescence. For an in situ *Renilla* reporter gene assay, providing coelenterazine is all that is necessary to generate light in living cells. However, the instability of coelenterazine in aqueous culture medium previously limited the sensitivity and utility of this approach. The chemically protected EnduRen™ and ViviRen™ Live Cell Substrates have overcome stability problems, enabling consistent and sensitive measurement of *Renilla* luminescence in real time, in a convenient format.

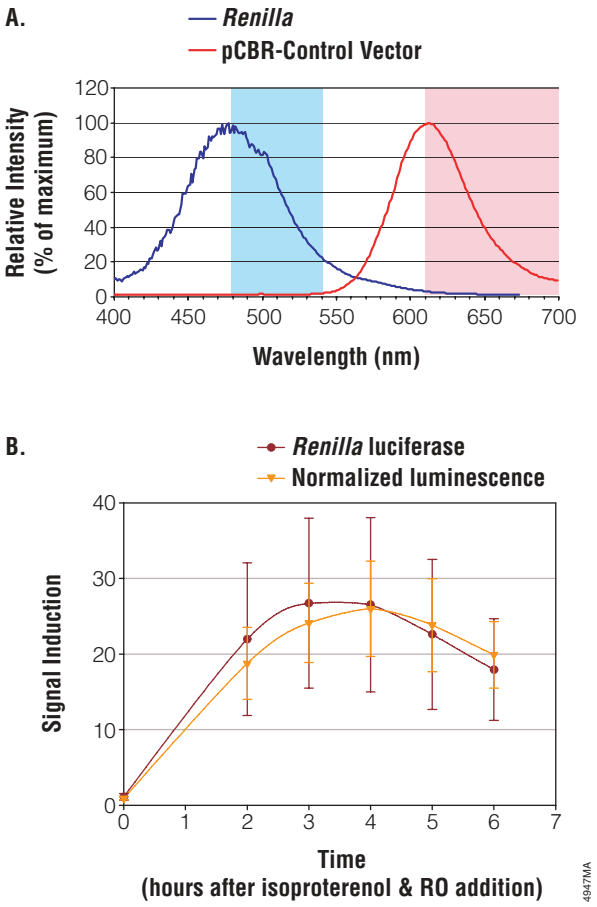

Figure 5. Spectra and luminescence measurements generated by *Renilla* luciferase and red Chroma-Luc™ Luciferase. Panel A. Spectra were measured from purified *Renilla* luciferase and red Chroma-Luc™ Luciferase lysate from CHO cells stably transfected with the pCBR-Control Vector (Cat.# E1421). Spectra were captured using a scanning spectrophotometer configured to collect emission data. The shaded areas represent the maximal transmittance range of the two filters used: a 510/60nm filter and a 610 longpass filter. **Panel B.** *Renilla* luciferase and red Chroma-Luc™ Luciferase luminescence were monitored from HEK 293 cells over 6 hours. Cells were transiently transfected in a 96-well plate with 2 vectors containing: i) the *Renilla* luciferase gene with PEST and CL1 destabilization sequences under control of the CRE promoter, and ii) the pCBR-Control Vector (Cat.# E1421) containing red Chroma-Luc™ Luciferase under the control of the SV40 promoter. Approximately 24 hours post-transfection, cells were exposed to 60µM EnduRen™ Substrate and 2mM beetle luciferin. After 2 hours, cells were treated with 1µM isoproterenol and 100µM RO-20-1724 (both Calbiochem, La Jolla, CA). Luminescence was measured periodically for 6 hours using the Turner BioSystems Veritas™ Microplate Luminometer (Cat.# E6501). For each measurement, *Renilla* luminescence was quantitated using the blue filter (510/60) and the red Chroma-Luc™ luminescence was measured using the red filter (610 longpass). Total signal for each color luminescence was calculated (using the spreadsheet tool at: **www.promega.com/techserv/tools/** select the "Chromma-Luc Calculator") then induction was calculated as the average induced luminescence divided by the average noninduced luminescence (normalized or not normalized to the red luminescence). For each data point, n = 12 and standard deviation is shown.

Measuring *Renilla* Luciferase...continued

References

1. Hawkins, E.M. *et al.* (2002) In: *Bioluminescence & Chemiluminescence Progress & Current Applications*. Stanley, P.E., and Kricka, L.J., eds. World Scientific, Singapore, 149.
2. *EnduRen™ Live Cell Substrate Technical Manual,* #TM244, Promega Corporation.
3. *ViviRen™ Live Cell Substrate Technical Manual,* #TM064, Promega Corporation.

Protocols

- *EnduRen™ Live Cell Substrate Technical Manual*, #TM244, Promega Corporation. (www.promega.com/tbs/tm244/tm244.html)
- *ViviRen™ Live Cell Substrate Technical Manual*, #TM064, Promega Corporation. (www.promega.com/tbs/tm064/tm064.html)
- *CellTiter-Glo® Luminescent Cell Viability Assay Technical Bulletin*, #TB288, Promega Corporation. (www.promega.com/tbs/tb288/tb288.html)
- *Chroma-Luc™ Series Reporter Vectors Technical Manual*, #TM059, Promega Corporation. (www.promega.com/tbs/tm059/tm059.html)
- *Chroma-Glo™ Luciferase Assay System Technical Manual*, #TM062, Promega Corporation. (www.promega.com/tbs/tm062/tm062.html)

Ordering Information

Product	Size	Cat.#
EnduRen™ Live Cell Substrate	0.34mg	E6481
	3.4mg	E6482
	34mg	E6485
ViviRen™ Live Cell Substrate	0.37mg	E6491
	3.7mg	E6492
	37mg	E6495
CellTiter-Glo® Luminescent Cell Viability Assay	10 × 100ml	G7573
	100ml	G7572
	10 × 10ml	G7571

Red Chroma-Luc™ Vectors

Product	Size	Cat.#
pCBR-Basic Vector	20µg	E1411
pCBR-Control Vector	20µg	E1421

[a] Patent Pending.

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

[c] This product does not convey a license to use recombinant *Renilla* luciferase under U.S. Pat. Nos. 5,292,658, 5,418,155 and related patents. Promega sells licensed *Renilla* luciferase vectors, which may be used in conjunction with this product.

Products may be covered by pending or issued patents or may have certain limitations. Please visit our Web site for more information.

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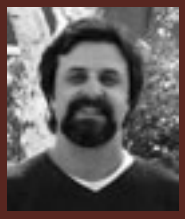

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