Technical Manual

# ViviRen™ Live Cell Substrate

INSTRUCTIONS FOR USE OF PRODUCTS E6491, E6492 AND E6495.

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Part# TM064

# ViviRen™ Live Cell Substrate

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## 1. Description

ViviRen™ Live Cell Substrate[a,b,c] is a proprietary compound that generates *Renilla* luciferase luminescence from live cells under normal growth conditions. The substrate produces *Renilla* luciferase luminescence that is 3- to 5-fold brighter than coelenterazine in live cells. Low autoluminescence from the substrate permits signal-to-background ratios that are 100-fold higher than those for coelenterazine. ViviRen™ Substrate can be used in a variety of cell analysis techniques including reporter gene assays, RNA interference and bioluminescence resonance energy transfer (BRET), because the substrate permits real-time measurement in multiwell plates (1–4).


**Promega Corporation** · 2800 Woods Hollow Road · Madison, WI 53711-5399 USA
Toll Free in USA 800-356-9526 · Phone 608-274-4330 · Fax 608-277-2516 · **www.promega.com**
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## 2. Product Components and Storage Conditions

| Product | Size | Cat.# |
|---|---|---|
| ViviRen™ Live Cell Substrate | 0.37mg | E6491 |

Contains sufficient substrate to perform 100 assays of 100µl each. Includes:

- 1 vial ViviRen™ Live Cell Substrate in 10µl DMSO (60mM)

| Product | Size | Cat.# |
|---|---|---|
| ViviRen™ Live Cell Substrate | 3.7mg | E6492 |

Contains sufficient substrate to perform 1,000 assays of 100µl each. Includes:

- 1 vial ViviRen™ Live Cell Substrate (solid)

| Product | Size | Cat.# |
|---|---|---|
| ViviRen™ Live Cell Substrate | 37mg | E6495 |

Contains sufficient substrate to perform 10,000 assays of 100µl each. Includes:

- 1 vial ViviRen™ Live Cell Substrate (solid)

**Storage Conditions:** Store the dry ViviRen™ Live Cell Substrate (Cat.# E6492 and E6495) at –20°C. After resuspension in DMSO, store Cat.# E6492 and E6495 at –20°C for up to 2.5 months. Similary, store Cat.# E6491, provided in DMSO, at –70°C for up to 12 months. The substrates may decrease 10% in concentration at –20°C over 2.5 months. ViviRen™ Live Cell Substrate in DMSO can be stored at room temperature (22°C) for 3 days or at 4°C for approximately 2 weeks with a 10% decrease in concentration. ViviRen™ Live Cell Substrate in DMSO will decrease <10% in concentration after 10 freeze-thaw cycles, thus you may wish to store in aliquots after resuspension.

**Caution:** The ViviRen™ Live Cell Substrate (Cat.# E6491) contains trifluoroacetate salt (1.2% w:w; not included in the "Size" shown above) and DMSO and is therefore classified as harmful. The diluted substrate is not known to present any hazards, as the concentrations of DMSO and trifluoroacetate are less than 0.1%. Cat.# E6492 and E6495 are classified as irritants. Data showing a lack of toxicity in cell cultures is included in Section 6.B. 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. General Considerations

ViviRen™ Substrate has been designed to generate *Renilla* luciferase luminescence in live cells with high signal-to-background ratios. The substrate will generate luminescence only in live cells, not in dead or lysed cells. By generating luminescence in live cells only, ViviRen™ Live Cell Substrate can be multiplexed with assays that lyse cells with minimal *Renilla* luciferase luminescence carryover into the lysed cell signals.

Because it is used with live cells, the ViviRen™ Substrate is diluted directly into the cell culture growth medium. Depending on how the dilutions are performed, the volume per well may increase by as little as 0.1% with the addition of this substrate.

Intracellular luminescence is affected by cell type, luciferase expression and experimental treatment, thus results should be directly compared only between samples from the same cell line that have undergone similar treatments (e.g., treatments with compounds that permeabilize cells). For analysis of multiple plates or for comparison between treatments, the most accurate results can be obtained by incorporating a common control sample on each plate. Luminescence measurements on each plate can be normalized to the control well on that plate, which allows comparisons across different treatments and corrects for small variations in luminescence that can result from other variables such as temperature or timing.

ViviRen™ Substrate will generate nearly maximal luminescence approximately 2 minutes after addition, and the luminescent signal will then decrease in intensity, with a half-life of 8–15 minutes (see Section 6.B).

## 4. Performing the ViviRen™ Live Cell Substrate Assay

### 4.A. ViviRen™ Live Cell Substrate Resuspension (Cat.# E6492, E6495)

Resuspend the dry substrate in tissue culture-grade dimethylsulfoxide (DMSO) as follows:

- Cat.# E6492 (3.7mg): Resuspend in 100µl of DMSO. Vortex to mix.
- Cat.# E6495 (37mg): Resuspend in 1ml of DMSO. Vortex to mix.

The final concentration of the resuspended substrates will be 60mM of ViviRen™ Substrate in DMSO. (Cat.# E6491 is provided in DMSO at 60mM.)

### 4.B. ViviRen™ Live Cell Substrate Assay Protocol

ViviRen™ Substrate can be delivered to cells in a variety of ways, depending on the experimental protocol and the ability to pipette small volumes. Several methods exist for the delivery of ViviRen™ Substrate to cells.

**Note:** We recommend diluting ViviRen™ Substrate immediately before each experiment. The substrate should be used within 8 hours of dilution if stored at room temperature (22°C).

1. Dilute the ViviRen™ Substrate 1:1,000 to a final concentration of 60µM, or more than 1:1,000, to a final concentration of less than 60µM. A variety of diluents can be used, including medium, medium + serum or PBS. If using medium without serum or PBS, a protein carrier should be included to prevent precipitation. For example, 0.5% (w:v) gelatin or Prionex® (Pentapharm Ltd., Switzerland) can be used. The following are recommended means of preparing the 1:1,000 dilution:

   a. Dilute the ViviRen™ Substrate 1:50 into room temperature medium + serum, medium + carrier or PBS + carrier. Prepare subsequent 1:20 dilutions in the medium in each well containing cells to be tested.

   Or,

   b. Dilute the ViviRen™ Substrate 1:1,000 into room temperature medium + serum, medium + carrier or PBS + carrier. Replace the cell culture medium in each plate with the solution containing the diluted ViviRen™ Substrate.

2. Wait at least 2 minutes to allow the luminescence to peak, then measure luminescence using a luminometer or CCD unit (consult owner's manual).

## 5. Related Products

### Assay Systems for *Renilla* Luciferase Quantitation

| Product | Size | Cat.# |
|---|---|---|
| EnduRen™ Live Cell Substrate | 0.34mg | E6481 |
| | 3.4mg | E6482 |
| | 34mg | E6485 |
| *Renilla* Luciferase Assay System | 100 assays | E2810 |
| | 1,000 assays | E2820 |
| Dual-Luciferase® Reporter Assay System | 100 assays | E1910 |
| Dual-Luciferase® Reporter Assay System, 10-Pack | 1,000 assays | E1960 |
| Dual-Luciferase® Reporter 1000 Assay System | 1,000 assays | E1980 |
| Dual-Glo™ Luciferase Assay System | 10ml | E2920 |
| | 100ml | E2940 |
| | 10 × 100ml | E2980 |

## Other Homogeneous Reporter Assay Systems

| Product | Size | Cat.# |
|---|---|---|
| Steady-Glo® Luciferase Assay System | 10ml | E2510 |
| | 100ml | E2520 |
| | 10 × 100ml | E2550 |
| Bright-Glo™ Luciferase Assay System | 10ml | E2610 |
| | 100ml | E2620 |
| | 10 × 100ml | E2650 |
| Beta-Glo® Assay System | 10ml | E4720 |
| | 100ml | E4740 |
| | 10 × 100ml | E4780 |

## Luminometers

| Product | Cat.# |
|---|---|
| GloMax™ 20/20 Luminometry System | E5311 |
| GloMax™ 96 Microplate Luminometer | E6501 |

## pGL*4 Renilla* Luciferase Reporter Vectors

| Product | Size | Cat.# |
|---|---|---|
| pGL4.70[*hRluc*] Vector | 20µg | E6881 |
| pGL4.71[*hRluc*P] Vector | 20µg | E6891 |
| pGL4.72[*hRluc*CP] Vector | 20µg | E6901 |
| pGL4.73[*hRluc*/SV40] Vector | 20µg | E6911 |
| pGL4.74[*hRluc*/TK] Vector | 20µg | E6921 |
| pGL4.75[*hRluc*/CMV] Vector | 20µg | E6931 |

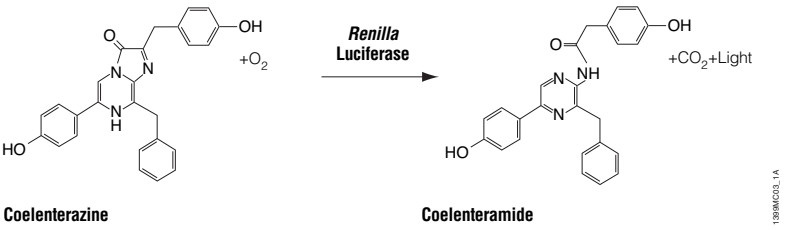


**Figure 1. Bioluminescent reaction catalyzed by *Renilla* luciferase.** *Renilla* luciferase catalyzes the mono-oxygenation of coelenterazine to coelenteramide, creating a photon of light. Coelenterazine and molecular oxygen are the only substrates required for this reaction.

## 6. Appendix

### 6.A. Overview of in situ *Renilla* Luciferase Luminescence Measurements

The *Renilla* luciferase reaction is one of the simplest luminescent reactions, requiring only two substrates: coelenterazine and molecular oxygen (Figure 1). In situ measurement of *Renilla* luciferase luminescence, therefore, requires only the addition of coelenterazine to the cell culture medium to initiate luminescence. No additional cosubstrates are required. This simplicity is one of the reasons that *Renilla* luciferase is used in BRET experiments, whole animal imaging and live cell analysis (1–4).

Measurement of *Renilla* luciferase in live cells using coelenterazine has been hampered by the instability of coelenterazine in aqueous solutions, especially at 37°C. Furthermore, the enzyme-independent luminescence generated by the coelenterazine in medium + serum causes a significant decrease in the signal-to-background ratio.

**Protected Coelenterazines**

Coelenterazine and many of its analogs are extremely unstable in aqueous environments. In medium containing 10% fetal bovine serum (FBS) at 37°C, coelenterazine concentration will decrease by 50% in 17 minutes (5). Even in its native environment, *Renilla reniformis* coelenterazine is protected from degradation as a sulfonated prosubstrate until the substrate is required for the luminescence reaction (6). It is the enzyme-independent breakdown of coelenterazine that generates autoluminescence and limits the ability to measure enzyme-dependent luminescence.

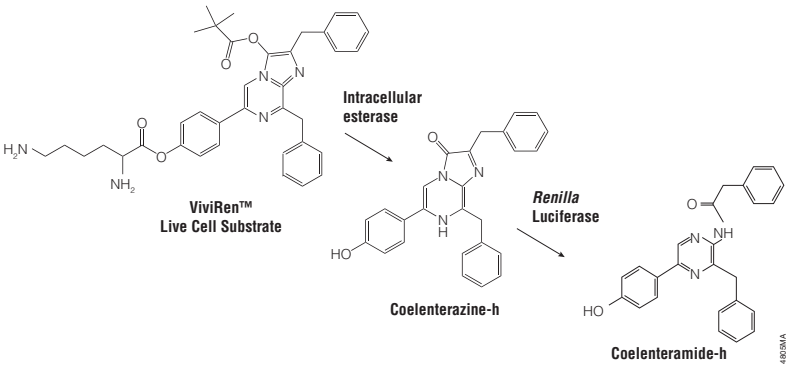


**Figure 2. ViviRen™ Live Cell Substrate is converted inside cells to coelenterazine-h, a substrate for *Renilla* luciferase.**

For the ViviRen™ and EnduRen™ Live Cell Substrates (EnduRen™ Substrate was previously introduced in Technical Manual #TM244), we have protected the site of oxygenation within coelenterazine, which reduces the rate of degradation. This protection, however, blocks the availability of the ViviRen™ and EnduRen™ Live Cell Substrates as luciferase substrates. To ensure that they are available to act as substrates for *Renilla* luciferase, the protecting group on ViviRen™ and EnduRen™ Live Cell Substrates can be cleaved by esterases within cells. As both substrates move from the growth medium into the cells, the protecting groups are cleaved, generating coelenterazine, the substrate for *Renilla* luciferase (Figure 2).

Autoluminescence occurs whenever coelenterazine is placed into an aqueous environment. Solutions at neutral or basic pH, or lipids such as those contained in serum or detergents, greatly increase the amount of autoluminescence that is measured (7). Autoluminescence can limit signal-to-background ratios even at low coelenterazine concentrations. In the absence of autoluminescence, higher coelenterazine concentrations can be used, resulting in more sensitive luminescence detection (Figure 3). ViviRen™ Live Cell Substrate is a protected coelenterazine derivative and generates such low autoluminescence and bright luminescence that the maximum signal-to-background ratios for these samples can therefore be 100 times greater than the signal-to-background ratios generated by coelenterazine.

## 6.A. Overview of in situ *Renilla* Luciferase Luminescence Measurements (continued)

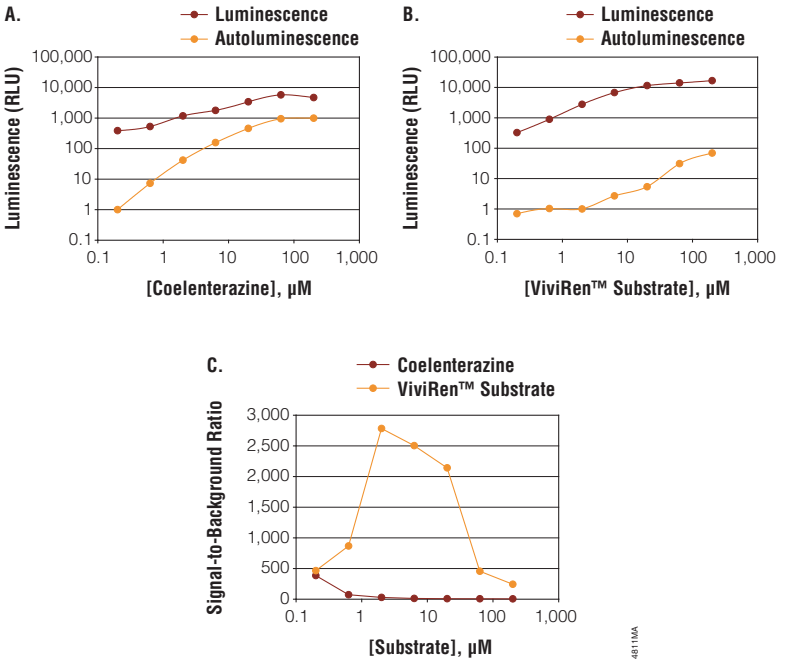


**Figure 3. Sensitivity of in situ measurement of luminescence increases dramatically in the absence of autoluminescence.** Luminescence was measured from CHO cells stably expressing *Renilla* luciferase. Similarly, autoluminescence was measured from nontransfected CHO cells. Coelenterazine (**Panel A**) or ViviRen™ Live Cell Substrate (**Panel B**) was titrated into the medium covering the cells (F12 with 10% FBS). Luminescence or autoluminescence was measured with a GloMax™ 96 Microplate Luminometer approximately 2 minutes after adding 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**.

## 6.B. Characteristics of *Renilla* Luciferase Luminescence using ViviRen™ Live Cell Substrate

ViviRen™ Live Cell Substrate is a protected *Renilla* luciferase substrate that is stable in cell culture growth conditions, such as medium plus 10% fetal bovine serum (50% of the tertiary butyryl ester is removed from the coelenterazine-h base of ViviRen™ Substrate in ~5 hours). ViviRen™ Substrate generates low enzyme-independent luminescence with maximal enzyme-dependent luminescence observed in many cell lines at >60µM (Figure 4). Because the

autoluminescence begins to increase above 60µM, with a subsequent decrease in signal-to-background ratio, we recommend using ViviRen™ Substrate at 20–60µM. As with coelenterazine, luminescence generated by the ViviRen™ Substrate is at maximum within a few minutes of substrate addition (Figure 5).

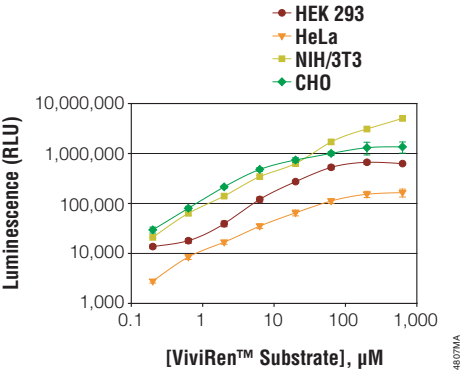


**Figure 4. Luminescence increases with increasing concentrations of ViviRen™ Live Cell Substrate.** Luminescence was measured from HeLa, NIH/3T3, CHO and HEK 293 cells transiently expressing *Renilla* luciferase under the control of the SV40 promoter. Forty-eight hours after transfection, ViviRen™ Live Cell Substrate was titrated onto cells, and luminescence was measured for 0.5 seconds per well using a GloMax™ 96 Microplate Luminometer (approximately 4 minutes after substrate addition). Values shown are averages of 6 replicate wells.

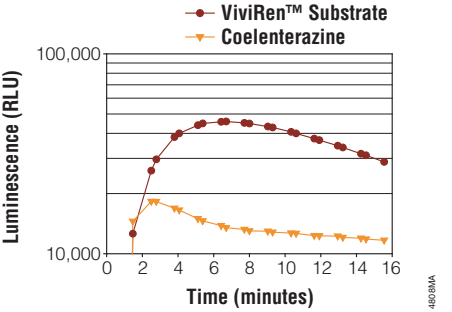


**Figure 5. Luminescence reaches near-maximum levels within minutes of ViviRen™ Live Cell Substrate or coelenterazine addition.** CHO cells stably expressing *Renilla* luciferase were exposed to 20µM of ViviRen™ Live Cell Substrate or coelenterazine approximately 1.5 minutes after continuous luminescent reading was initiated. Measurements were made using the Berthold Detection Systems Orion, integrating each well for 0.5 seconds. Values are averages of 12 replicate wells measured repeatedly for 15 minutes.

### 6.B. Characteristics of *Renilla* Luciferase Luminescence using ViviRen™ Live Cell Substrate (continued)

The luminescence generated by ViviRen™ Substrate in cells expressing *Renilla* luciferase is greater than that generated by coelenterazine by approximately 3- to 5-fold, depending on the cell line used. The brighter luminescence is a reflection of the higher intracellular coelenterazine-h concentration.

Cell types vary in their sensitivity to the breakdown products of the protected coelenterazines, and testing should be done if using different cell lines. Experiments with CHO, NIH/3T3, HEK 293 and HeLa cells have shown minimal impact on cell viability (<15%), as determined by ATP content after ViviRen™ Substrate exposure for 45 minutes (Figure 6). If measurement of samples is expected to take longer than 45–60 minutes, EnduRen™ Substrate, which generates similar intensity but more stable luminescence by that time, is recommended (Figure 7).

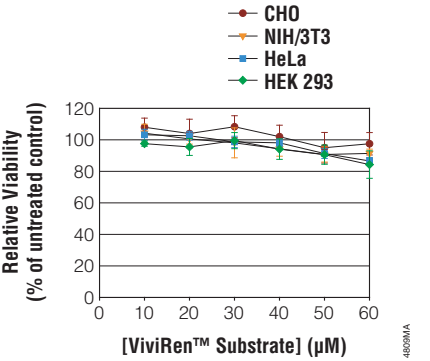


**Figure 6. ViviRen™ Live Cell Substrate does not affect cell viability in CHO, NIH/3T3, HeLa or HEK 293 cells over 45 minutes.** ATP was measured as an indicator of cell viability using CellTiter-Glo® Reagent with 4 cell lines exposed to ViviRen™ Substrate for 45 minutes. Data represents averages (n = 7) normalized to values measured for untreated cells. Samples were integrated over 0.5 seconds/well in a GloMax™ 96 Microplate Luminometer.

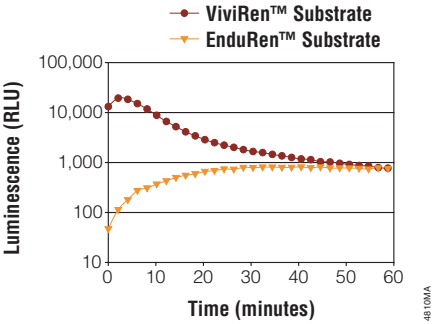


**Figure 7. Luminescent intensity generated by EnduRen™ and ViviRen™ Live Cell Substrates converges approximately 45 minutes after substrate addition.** CHO cells stably expressing *Renilla* luciferase were exposed to ViviRen™ or EnduRen™ Substrate at 60µM, and luminescence was measured at intervals of approximately 2 minutes for 1 hour with the GloMax™ 96 Microplate Luminometer (n = 6). After approximately 45 minutes, the luminescence intensities from the two sample sets were similar. Therefore, measurement of samples requiring more than 45–60 minutes should be made using EnduRen™ Substrate to maintain the brightest, most consistent signal throughout the entire measurement period.

### 6.C. References

1. Bhaumik, S. and Gambhir, S.S. (2002) Optical imaging of *Renilla* luciferase reporter gene expression in living mice. *Proc. Natl. Acad. Sci. USA* **99**, 377–82.

2. Vidugiriene, J. *et al.* (2004) Shedding light on RNAi. The use of bioluminescent reporter genes for RNAi optimization. *Promega Notes* **87**, 2–6.

3. Angers, S. *et al.* (2000) Detection of β2-adrenergic receptor dimerization in living cells using bioluminescence resonance energy transfer (BRET). *Proc. Natl. Acad. Sci. USA* **97**, 3684–9.

4. Lavine, N. *et al.* (2002) G protein-coupled receptors form stable complexes with inwardly rectifying potassium channels and adenylyl cyclase. *J. Biol. Chem.* **277**, 46010–9.

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6. Inoue, S. *et al.* (1977) Complete structure of *Renilla* luciferin and luciferyl sulfate. *Tetrahedron Lett.* **31**, 2685–8.

7. Renilla *Luciferase Assay System Technical Manual*, #TM055, Promega Corporation.

[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.