Technical Manual

EnduRen™ Live Cell Substrate

INSTRUCTIONS FOR USE OF PRODUCTS E6481, E6482 AND E6485.

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EnduRen™ Live Cell Substrate

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1. Description

EnduRen™ Live Cell Substrate[a,b,c] is a proprietary compound that generates *Renilla* luciferase-dependent luminescence from live cells under normal growth conditions. The substrate produces luminescence with high signal-to-background ratios (high signal readings with low autoluminescence) that is very stable, allowing luminescence measurement for at least 24 hours after substrate addition. EnduRen™ Substrate may be used in a variety of cell analysis techniques including reporter gene analysis, RNAi analysis and Bioluminescence Resonance Energy Transfer (BRET) analysis, because the substrate permits real-time measurement in multiwell plates (1–4).

Note: EnduRen™ Substrate is for use in **live cells** only.

Selected Citation Using the EnduRen™ Live Cell Substrate

- Dinh, D.T. *et al.* (2005) Helix I of b-arrestin is involved in postendocytic trafficking but is not required for membrane translocation, receptor binding, and internalization. *Mol. Pharmacol.* **67**, 375–82.

  Type 1 angiotensin II receptor-*Renilla* luciferase (AT1R-Rluc), and b-arrestin1 and 2 GFP fusion constructs (barr1-GFP and barr2-GFP) were created for BRET protein interaction assays. Combinations of AT1R-Rluc and b-arrestin-GFP constructs were transfected into COS-7 cells. The COS-7 cell cultures were then activated with 100nM angiotensin II in the presence of 60µM EnduRen™ Live Cell Substrate, and BRET readings were taken at 475 and 515nm over a 1-hour period. Data were displayed as a ratio with both constructs compared to luminescence from the AT1R-Rluc construct alone.

2. Product Components and Storage Conditions

Product	Size	Cat.#
EnduRen™ Live Cell Substrate	0.34mg	E6481

Contains sufficient substrate to dilute into 10ml of cell growth medium. Includes:

- 1 vial EnduRen™ Substrate,

Product	Size	Cat.#
EnduRen™ Live Cell Substrate	3.4mg	E6482

Contains sufficient substrate to dilute into 100ml of cell growth medium. Includes:

- 1 vial EnduRen™ Substrate, dried

Product	Size	Cat.#
EnduRen™ Live Cell Substrate	34mg	E6485

Contains sufficient substrate to dilute into 1L of cell growth medium. Includes:

- 1 vial EnduRen™ Substrate, dried

Storage Conditions: Store the dried EnduRen™ Live Cell Substrate at or below –20°C. No change in potency has been observed when substrate reconstituted in DMSO is stored at 22°C, 4°C or –20°C for up to 4 weeks.

3. Performing the Assay

3.A. General Considerations

EnduRen™ Live Cell Substrate is 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, EnduRen™ Live Cell Substrate can be multiplexed in assays with lysed cells, with minimal *Renilla* luminescence carryover into the lysed cell signals.

Because it is used with live cells, EnduRen™ Live Cell Substrate is diluted directly into the experimental cell cultures. Depending on how the dilutions are performed, the volume per well may increase by as little as 0.1% with the addition of EnduRen™ Substrate.

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

EnduRen™ Substrate generates stable luminescence approximately 1.5 hours after substrate addition, and this luminescence will continue for >24 hours (Figures 6 and 7, Section 6.B). For incubations longer than 24 hours, increases in cell number must be anticipated to maintain consistent luminescence. Generally, confluent cells make less protein, and luminescence will decrease if the cells become confluent during the exposure time.

3.B. EnduRen™ Live Cell Substrate Resuspension

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

- Cat.# E6481 (0.34mg): Resuspend in 10µl of DMSO.
- Cat.# E6482 (3.4mg): Resuspend in 100µl of DMSO.
- Cat.# E6485 (34mg): Resuspend in 1ml of DMSO.

Vortex to resuspend. Resuspension may require repeated vortexing and take up to 10 minutes to complete. Warm the solution to 37°C, if necessary, to assist resuspension. The EnduRen™ Live Cell Substrate is very stable in DMSO and shows no discernible decay after 7 hours at 37°C. The final concentration will be 60mM of EnduRen™ Substrate in DMSO.

Note: DMSO is the only suitable diluent for EnduRen™ Live Cell Substrate. Other diluents may result in suboptimal resuspension of the substrate and are not recommended.

4. EnduRen™ Live Cell Substrate Assay Procedure

EnduRen™ Live Cell Substrate can be delivered to cells in a variety of ways depending on the experimental protocol and the ability to pipet small volumes. Several possible methods exist for the delivery of EnduRen™ Substrate to cells. Methods 1.c. and 1.d. are recommended as the easiest and most convenient.

Note: We recommend diluting EnduRen™ Substrate immediately before each experiment. The substrate should be used within 6 hours of dilution if stored at 37°C or within 12 hours if stored at 22°C.

1. Dilute the EnduRen™ Live Cell Substrate (resuspended at 60mM, Section 3.B) 1:1,000 to a final concentration of 60µM. Diluents can include medium, medium + serum or PBS. The diluent should be prewarmed to 37°C. If the EnduRen™ Substrate is diluted into room temperature or chilled solutions, it may precipitate but will return to solution upon heating to 37°C. The following options are recommended means of preparing the 1:1,000 dilution:

4. EnduRen™ Live Cell Substrate Assay Procedure (continued)

a. Dilute the 60mM EnduRen™ Live Cell Substrate stock 1:10 into pre-warmed medium, medium + serum or PBS. Prepare subsequent 1:100 dilutions in the medium in each well containing cells to be tested. This dilution scheme may also be performed with the initial 1:10 dilution in DMSO if the experimental cells will tolerate culture medium with 1% DMSO. **Or,**

b. Dilute the 60mM EnduRen™ Substrate stock 1:100 into prewarmed medium, medium + serum or PBS. Prepare subsequent 1:10 dilutions into the medium in each well containing cells to be tested. **Or,**

c. Dilute the 60mM EnduRen™ Substrate stock 1:1,000 into prewarmed medium, medium + serum or PBS. Replace the cell culture medium in each plate with medium containing the EnduRen™ Substrate. **Or,**

d. Dilute the 60mM EnduRen™ Substrate stock 1:1,000 into prewarmed medium, medium + serum or PBS containing cells just before dispensing cells into individual wells.

2. Place plates in the incubator. Measure luminescence after at least 1.5 hours of exposure to the EnduRen™ Live Cell Substrate. The luminescent signal is extremely stable and can be measured for at least 24 hours after adding EnduRen™ Substrate (see Section 6.B). Substrate may be added to cells before or after experimental treatment has been initiated depending on convenience and cell tolerance to EnduRen™ Substrate (see Section VI.B).

5. Related Products

Luciferase Assay Systems for *Renilla* Quantitation

Product	Size	Cat.#
ViviRen™ Live Cell Substrate	0.37mg	E6491
	3.7mg	E6492
	37mg	E6495
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 Glo-Kinetics Reporter Assay Systems

Product	Size	Cat.#
Steady-Glo® Luciferase Assay System	10ml**	E2510
Bright-Glo™ Luciferase Assay System*	10ml**	E2610
Beta-Glo® Assay System	10ml**	E4720

*For Laboratory Use.
**Available in Additional Sizes.

pGL4 Luciferase Reporter Vectors

Product	Size	Cat.#
pGL4.70[*hRluc*] Vector	20µg	E6881
pGL4.71[*hRlucP*] Vector	20µg	E6891
pGL4.72[*hRlucCP*] Vector	20µg	E6901
pGL4.73[*hRluc*/SV40] Vector	20µg	E6911
pGL4.74[*hRluc*/TK] Vector	20µg	E6921
pGL4.75[*hRluc*/CMV] Vector	20µg	E6931
pGL4.76[*hRluc*/Hygro] Vector	20µg	E6941
pGL4.77[*hRlucP*/Hygro] Vector	20µg	E6951
pGL4.78[*hRlucCP*/Hygro] Vector	20µg	E6961
pGL4.79[*hRluc*/Neo] Vector	20µg	E6971
pGL4.80[*hRlucP*/Neo] Vector	20µg	E6981
pGL4.81[*hRlucCP*/Neo] Vector	20µg	E6991
pGL4.82[*hRluc*/Puro] Vector	20µg	E7501
pGL4.83[*hRlucP*/Puro] Vector	20µg	E7511
pGL4.84[*hRlucCP*/Puro] Vector	20µg	E7521

Additional pGL4 Vectors are available. Please see our catalog or visit: **www.promega.com** for more information.

Luminometers

Product	Cat.#
GloMax®-Multi Detection System	E7031
GloMax®-Multi+ Detection System Base Instrument	E8031
GloMax®-Multi+ Detection System with Heating and Shaking	E9031
GloMax™ 20/20 Luminometer	E5311
GloMax™ 20/20 Luminometer with Single Auto-Injector	E5321
GloMax™ 20/20 Luminometer with Dual Auto-Injector	E5331
GloMax™ 96 Microplate Luminometer	E6501
GloMax™ 96 Microplate Luminometer with Single Injector	E6511
GloMax™ 96 Microplate Luminometer with Dual Injectors	E6521

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 co-substrates are required. This simplicity is one of the reasons that *Renilla* luciferase is used in BRET (Bioluminescence Resonance Energy Transfer) experiments, whole animal imaging and live cell analysis (1–4).

Measurement of *Renilla* luciferase in live cells using coelenterazine has been hampered by three factors: First is the instability of coelenterazine in aqueous solutions, especially at 37°C; second is the enzyme-independent luminescence generated by coelenterazine in medium containing serum and the subsequent decrease in signal-to-background ratio; and third is the kinetics of the *Renilla* luciferase luminescent signal.

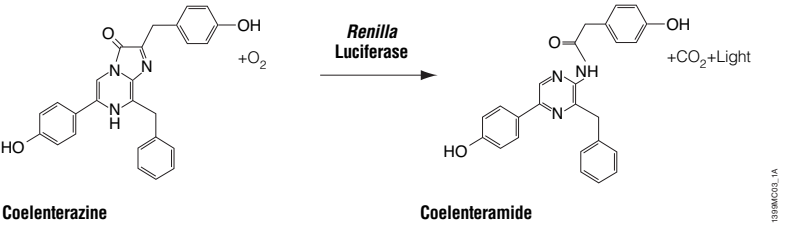

Figure 1. Bioluminescent reaction catalyzed by *Renilla* luciferase. *Renilla* luciferase catalyzes the mono-oxygenation of coelenterazine to coelenteramide, creating a photon of light. Coelenterazine and molecular oxygen are the only substrates required for this reaction.

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). In its native environment, *Renilla reniformis* coelenterazine is protected from degradation as a sulfonated prosubstrate until required for the luminescence reaction (6). This enzyme-independent breakdown of coelenterazine generates autoluminescence and limits the ability to measure luciferase.

We have protected the site of oxygenation within coelenterazine, which reduces the rate of degradation. This protection, however, blocks the availability of the EnduRen™ Live Cell Substrate as a luciferase substrate. To ensure that the Substrate is readily available for *Renilla* luciferase, the protecting group can be cleaved by esterases inside the cells. The EnduRen™ Substrate moves from the growth medium into the cells where its protecting group is cleaved, generating coelenterazine, the substrate for *Renilla* luciferase (Figure 2).

Autoluminescence occurs when coelenterazine is placed into an aqueous environment. Neutral or basic pH or lipids such as those contained in serum or detergents greatly increase the amount of autoluminescence (7). Autoluminescence can limit signal-to-background ratios even at low concentrations of coelenterazine. In the absence of autoluminescence, higher coelenterazine concentrations can be used, resulting in more sensitive luminescence detection (Figure 3). EnduRen™ Live Cell Substrate is a protected coelenterazine derivative and generates such low autoluminescence in medium containing 10% serum that the autoluminescence often cannot be detected. The maximum signal-to-background ratios for these samples is therefore often 10 times greater than that generated by coelenterazine.

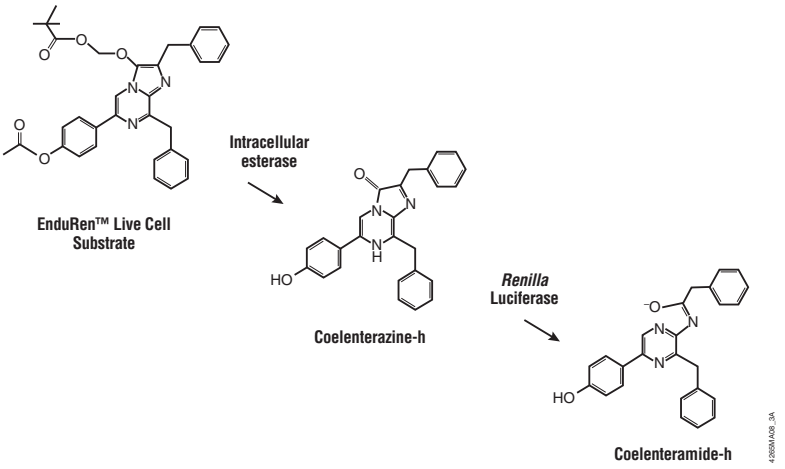

Figure 2. EnduRen™ Live Cell Substrate is converted inside cells to coelenterazine, the substrate for *Renilla* luciferase.

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

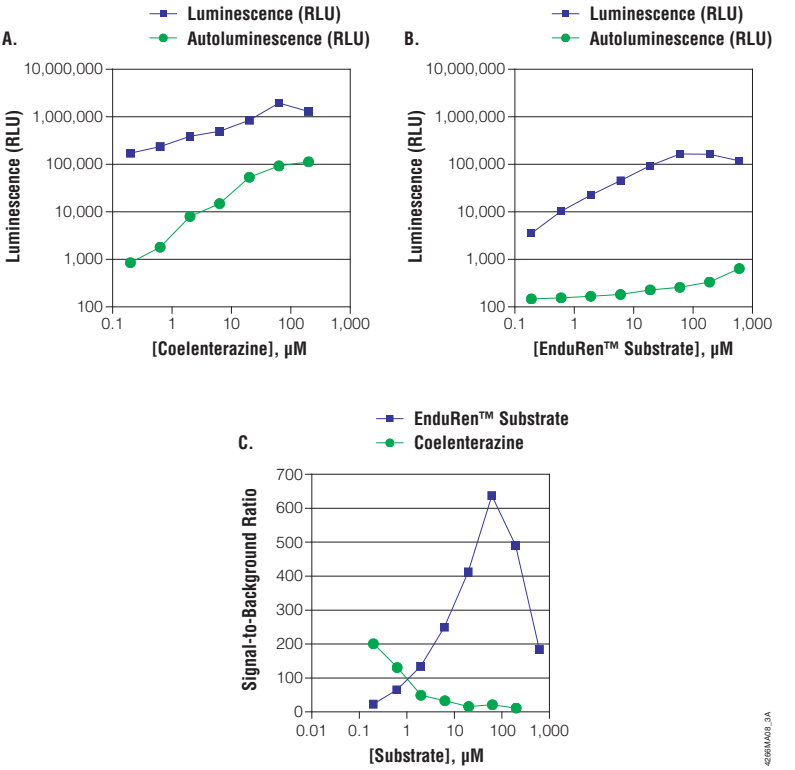

Figure 3. Sensitivity of in situ luminescence measurement increases dramatically in the absence of autoluminescence. Luminescence was measured from CHO cells that were stably transfected with *Renilla* luciferase; autoluminescence was measured similarly from nontransfected CHO cells. Coelenterazine (**Panel A**) or EnduRen™ Substrate (**Panel B**) was titrated into the cell culture medium (F12 + 10% FBS). Luminescence and autoluminescence were measured with a Berthold Mitras luminometer approximately 1 minute and 90 minutes later, respectively. Signal-to-background ratios (background-subtracted luminescence divided by background) were calculated from the data in Panels A and B and presented in **Panel C**.

The kinetics of the *Renilla* luciferase luminescent signal generated by live cells in the presence of coelenterazine make it difficult to measure luminescence in a multiwell plate (Figure 4). The plateau of maximal luminescence in living cells is approximately 1 minute long and starts about 20 seconds after substrate addition, depending on the cell line. Due to this phenomenon, luminometers with injectors incorporated or luminometers that have

extremely reproducible timing of plate movement and measurement are required to monitor luminescence from living cells. Protection of coelenterazine changes the kinetics of coelenterazine availability within cells and extends the plateau of maximal luminescence to >24 hours for the EnduRen™ Substrate (see Section 6.B., Characteristics of *Renilla* Luciferase Luminescence using EnduRen™ Live Cell Substrate).

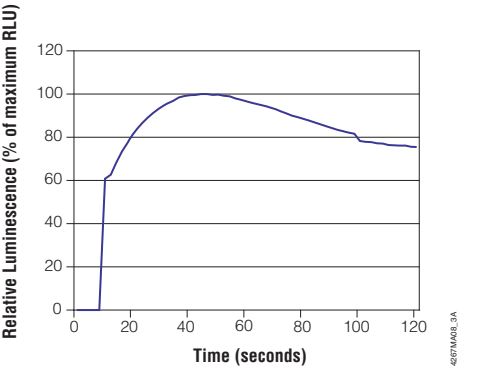

Figure 4. *Renilla* luciferase luminescence in live cells changes quickly when coelenterazine is the substrate. *Renilla* luciferase luminescence was measured from stably transfected CHO cells. Ten seconds after luminescence measurement was initiated, coelenterazine (20µM final concentration) in growth medium was added to the cells using injectors incorporated into a plate luminometer (Berthold Technologies Orion). Luminescence reached 90% of its maximum approximately 18 seconds after coelenterazine addition and remained at or above this level for approximately 50 seconds. Background luminescence was subtracted from the data, which was then presented as a percent of the maximal background-subtracted luminescence measured. Data points represent a single well measured continuously for 2 minutes. The experiment was repeated 3 times with peak luminescence values for each experiment occurring within 10 seconds of each other.

6.B. Characteristics of *Renilla* Luciferase Luminescence Using EnduRen™ Live Cell Substrate

EnduRen™ Live Cell Substrate is a protected *Renilla* luciferase substrate that is extremely stable in cell culture growth conditions, such as medium plus 10% fetal bovine serum. EnduRen™ Substrate generates almost no enzyme-independent luminescence, with maximal enzyme-dependent luminescence observed in many cell lines at 60µM (Figure 5). Unlike coelenterazine, where the luminescence is at maximum within a few minutes of substrate addition but begins to decrease very rapidly, luminescence generated by the EnduRen™ Substrate reaches its peak about 1.5 hours after substrate addition, then remains constant for greater than 24 hours (Figure 6). Depending on the cell type and number, the luminescence may remain constant for almost three

6.B. Characteristics of *Renilla* Luciferase Luminescence Using EnduRen™ Live Cell Substrate (continued)

days. This long-term signal stability permits real-time measurement of intracellular changes within a single sample as the luminescence changes due to experimental treatment (Figure 7).

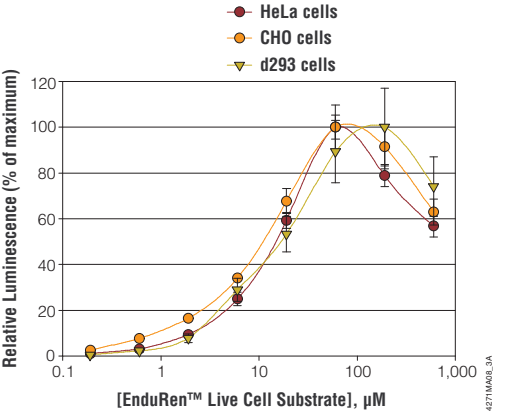

Figure 5. EnduRen™ Live Cell Substrate generates maximal luminescence at approximately 60µM. HeLa, CHO and d293 cells were transiently transfected with *Renilla* luciferase using the phRL-SV40 Vector (Cat.# E6261) and plated at 3,000 cells per well in a 96-well plate. EnduRen™ Live Cell Substrate was titrated into aliquots of the growth medium for each cell line at 600µM–0.19µM. These solutions then replaced the medium covering the cells, and the cells were returned to the incubator. HeLa and d293 cells were grown in DMEM + 10% fetal bovine serum, and CHO cells were grown in F12 + 10% fetal bovine serum. One and one-half hours after exposure to the EnduRen™ Substrate, luminescence was measured using a Berthold Technologies Mitras luminometer. Averages of background-subtracted luminescence, normalized to the maximum for each cell line, are shown. For each data point, n = 6, and standard deviations are plotted. **Note:** d293 cells are a subset of HEK 293 cells, observed in our laboratories to induce more efficiently than other 293 cells.

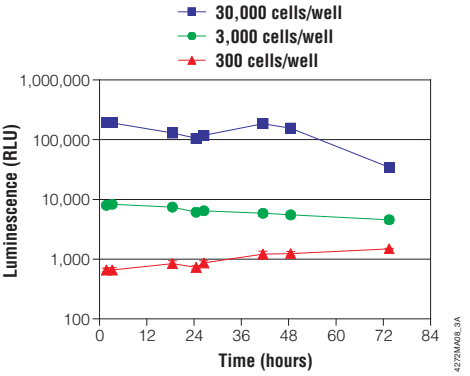

Figure 6. EnduRen™ Substrate generates sustained luminescence of more than 24 hours, but cell number must be considered before starting long exposures. A total of 30,000, 3,000 or 300 CHO cells, stably transfected with *Renilla* luciferase, were plated into a 96-well plate and exposed to EnduRen™ Substrate for three days. The luminescence for the wells containing 3,000 and 300 cells remained relatively stable or increased due to cell growth; however, the 30,000-cell wells decreased in luminescence, likely because of overcrowding. The luminescence was measured periodically over the three days; the plate of cells was returned to the incubator between measurements. Luminescence was measured for 1 second per sample on a Berthold Technologies Mithras luminometer. For each data point, n = 6, and averages with standard deviations are shown.

Luminescence generated by EnduRen™ Substrate in cells expressing *Renilla* luciferase is approximately 10- to 25-fold lower than that generated by coelenterazine, depending on the cell line. The sustained luminescence is a reflection of the steady-state amount of *Renilla* luciferase generated by the cell, degraded by cellular mechanisms and inactivated by *Renilla* luciferase turnover.

Cell type affects the stability of the luminescent signal. Different cell types have different sensitivities to the breakdown products of the protected coelenterazine. Experiments with CHO, HEK 293, NIH3T3 and HeLa cells have shown minimal impact on cell number (<15%), as determined by ATP content after a 24-hour exposure. After a two-day exposure, however, wells containing HeLa cells and the EnduRen™ Substrate tend to decrease in ATP by approximately 80%, implying that the health of the cells has been affected. Conversely, NIH3T3 cells exposed to EnduRen™ Substrate for 72 hours showed no change in ATP content compared to control cells that were grown in the same plate. Since cell lines differ in their response to EnduRen™ Substrate exposure for >24 hours, the health or number of cells should be checked the first time that a new cell line is used with this substrate.

6.B. Characteristics of *Renilla* Luciferase Luminescence Using EnduRen™ Live Cell Substrate (continued)

As seen in Figure 6, cell number affects the general trend in data generation. EnduRen™ Substrate generates luminescence in living cells that are transfected with *Renilla* luciferase. Because the exposure of these cells to EnduRen™ Substrate may extend for up to three days, the growth of the cells may impact the luminescence that can be generated (Figure 6). This effect also exists when lysing cells and measuring luminescence, but it may not have been noticed, since time courses are performed much less frequently with lytic assays because of the high sample numbers required. The long-term stability of the *Renilla* luminescent signal generated by the EnduRen™ Substrate permits a single set of samples to be measured at multiple times (Figure 7) instead of multiple sets of samples harvested at different times.

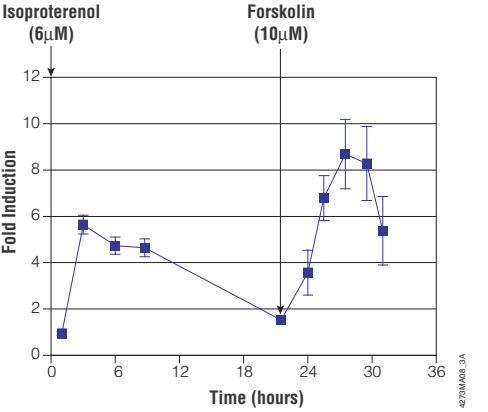

Figure 7. Real-time measurement of *Renilla* luminescence from live cells. Luminescence was monitored from HEK 293 cells for over 24 hours, permitting the measurement of the effects of sequential treatment of the cells with isoproterenol and forskolin. The cells were transiently transfected with a vector containing the *Renilla* luciferase gene with a PEST degradation sequence, then EnduRen™ Live Cell Substrate was added. Approximately 16 hours later (time = 0), cells were treated with 6µM isoproterenol (Calbiochem, LaJolla, CA), which upregulates *Renilla* luciferase expression in this vector. Luminescence was measured periodically from the same 6 samples. Approximately 20 hours later, the same cells were treated with 10µM forskolin (Sigma Chemical, St. Louis, MO), and luminescence was measured periodically for an additional 10 hours. Cells were returned to the incubator between measurements. Data is presented as fold induction. Fold induction is calculated as the average luminescence of treated cells divided by the average luminescence of untreated cells. For each data point, n = 6, and standard deviation is shown.

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. *siLentGene™-2 U6 Cloning Systems (Human) Technical Manual* #TM247, Promega Corporation.
3. Angers, S. *et al*. (2000) Detection of ъ2-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.
5. Hawkins, E.M. *et al*. (2002) *In: Bioluminescence & Chemiluminescence Progress & Current Applications*, Stanley, P.E., and Kricka, L.J., eds. World Scientific, Singapore, 149.
6. Inoue, S. *et al*. (1977) Complete structure of *Renilla* luciferin and luciferyl sulfate. *Tetrahedron Letters* **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.