TECHNICAL MANUAL

Nano-Glo® Endurazine™ and Vivazine™ Live Cell Substrates

Instructions for Use of Products
N2570, N2571, N2572, N2580, N2581, N2582 and N2590

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Nano-Glo® Endurazine™ and Vivazine™ Live Cell Substrates

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1. Description

Live-cell, nonlytic assays using the Nano-Glo® Live Cell Assay System are limited in duration by degradation of the furimazine substrate, which leads to signal decay that limits assay sensitivity and data quality for experiments lasting several hours or days. Furimazine undergoes both enzymatic and nonenzymatic turnover in these live-cell experiments, and the presence of serum enhances the signal decay rate. The Nano-Glo® Endurazine™ and Vivazine™ Live Cell Substrates[a,b] are versions of furimazine that enable live-cell, nonlytic assays for periods lasting several hours or days. For both substrates, a slow rate of ester hydrolysis leads to the steady release of furimazine throughout the experiment, a process catalyzed by cellular esterases (Figure 1). Once formed, furimazine can serve as a substrate for NanoLuc® or NanoBiT® luciferases. NanoBiT® luciferase consists of Large BiT (LgBiT) bound to Small BiT (SmBiT) or HiBiT peptides. Both substrates contain a proprietary compound that reduces furimazine autoluminescence.

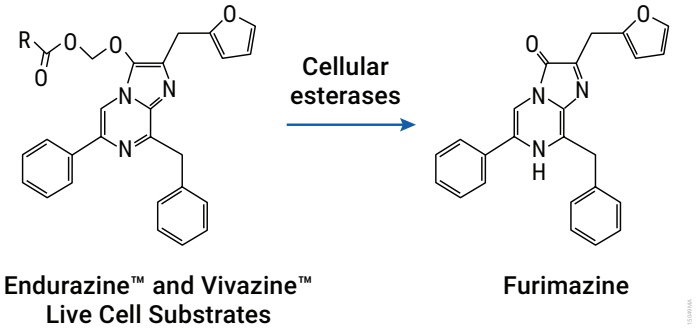

Figure 1. Conversion of Endurazine™ and Vivazine™ to furimazine. Esterase-mediated cleavage of the protecting group (R) liberates furimazine, the substrate for NanoLuc® and NanoBiT® luciferases.

2. Product Components and Storage Conditions

PRODUCTS	SIZE	CAT. #
Nano-Glo® Endurazine™ Live Cell Substrate	0.1ml	N2570
	1ml	N2571
	10ml	N2572
Nano-Glo® Vivazine™ Live Cell Substrate	0.1ml	N2580
	1ml	N2581
	10ml	N2582
Nano-Glo® Extended Live Cell Substrate Trial Pack	0.2ml	N2590

Storage Conditions: Nano-Glo® Endurazine™ and Vivazine™ Live Cell Substrates can be stored at –30°C to –10°C. The Nano-Glo® Endurazine™ and Vivazine™ Live Cell Substrates will not freeze at –20°C. Minimize the amount of time each stock is kept at room temperature and exposed to light.

3. Assay Design Considerations

3.A. Comparing Performance of the Nano-Glo® Endurazine™ and Vivazine™ Live Cell Substrates

When Endurazine™ and Vivazine™ substrates are compared under equivalent conditions (e.g., NanoLuc® or NanoBiT® luciferase, expression level, cell type, etc.), these live-cell substrates display differences in luminescent intensity and signal stability (Figure 2). After approximately 2 hours, Endurazine™ substrate provides a stable signal throughout a 24-hour time course, and, in some cases, over 72 hours or more (see Section 7.A). Endurazine™ substrate has a substantially dimmer signal compared to the Nano-Glo® Live Cell Assay System over the first 1–2 hours of a time-course experiment. Beyond approximately 2 hours, the Endurazine™ signal is substantially brighter (Section 7.B). If Endurazine™ substrate does not provide sufficient sensitivity, Vivazine™ substrate is brighter over the initial hours of a time-course experiment (Figure 2 and Sections 7.A and 7.B). However, the increased brightness of Vivazine™ substrate is associated with an increased rate of signal decay. For time-course experiments lasting several hours or days, Vivazine™substrate will provide a brighter signal early in the time course, but Endurazine™ substrate will provide a brighter signal at later time points (Figure 2).

Endurazine™ and Vivazine™ formulations have been optimized to minimize cytotoxicity for extended incubations in the presence of 10% fetal bovine serum (FBS; Section 7.C).

3.A. Comparing Performance of Endurazine™ and Vivazine™ Substrates (continued)

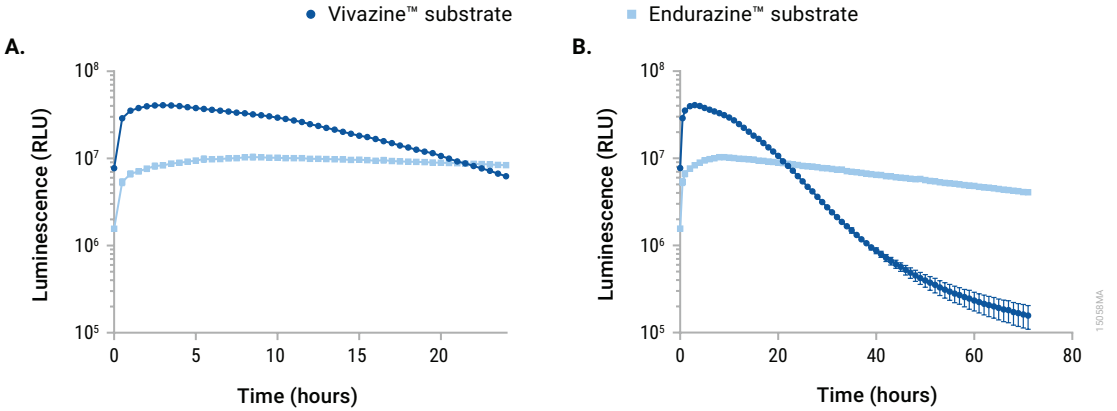

Figure 2. A comparison of Nano-Glo® Endurazine™ and Vivazine™ Live Cell Substrates. A plasmid encoding NanoLuc® luciferase expressed from the HSV-TK promoter was transiently transfected into HEK293 cells using a bulk transfection protocol followed by replating. **Panel A.** A comparison of Endurazine™ and Vivazine™ signal intensity measuring over the first 24 hours. **Panel B.** A comparison of Endurazine™ and Vivazine™ signal intensity over the full 72-hour time course. Luminescence was measured continuously with the lid on using a GloMax® Discover System at 37°C running version 3.1.0 software to prevent lid condensation. The average luminescence is plotted with error bars representing the standard deviation (n = 3).

3.B. Factors that Influence Signal Intensity, Signal Decay and Linearity

Signal intensity will be determined by the amount of active NanoLuc® or NanoBiT® luciferase present and the amount of furimazine available at a given time point. Furimazine is steadily released throughout a time-course experiment, a process catalyzed by cellular esterases. Immediately after adding Endurazine™ or Vivazine™ substrate to cells, furimazine will begin to accumulate, increasing both enzyme-catalyzed luminescence and autoluminescence (Section 3.F). After the initial accumulation, furimazine levels will reach a steady state, where production is balanced by degradation. Both enzymatic and nonenzymatic pathways mediate turnover of furimazine. NanoLuc® and NanoBiT® luciferase act on furimazine, together with serum components like albumin to a far lesser extent. With nonenzymatic turnover, furimazine degrades in aqueous solutions. After achieving a steady state, furimazine levels will decrease when the rate of turnover exceeds the rate of production, leading to signal decay for a constant amount of active luciferase.

The amount of active NanoLuc® or NanoBiT® luciferase will depend on several factors. For NanoLuc® luciferase, when used as a genetic reporter, the expression level determines the amount of active enzyme and is affected by the presence of a PEST sequence that decreases the protein half-life. For NanoBiT® enzyme, which is composed of two subunits that interact to make a functional protein, expression levels and the extent of complementation are both factors. For instance, for a protein:protein interaction assay (e.g., NanoBiT® PPI Assay), fusion partners that interact minimally will generate low levels of NanoBiT® enzyme. Additional factors may influence the amount of active enzyme, including inhibitor accumulation or enzyme inactivation mechanisms.

All of these factors will determine the luminescence over time, the kinetic trace, for a given set of conditions. Kinetic traces will vary with factors that include type of luciferase (e.g., NanoLuc® or NanoBiT® luciferase), expression level and cell type. Over a similar range of expression, rates of signal decay are expected to be similar for a given substrate (Sections 5.A and 5.C).

Live-cell, nonlytic assays with Endurazine™ and Vivazine™ substrates will be limited in linear dynamic range compared to the lytic Nano-Glo® Luciferase Assay System (Sections 5.A, 5.C and 7.E). At higher levels of expression, Endurazine™ and Vivazine™ signals will plateau while the signal will continue to increase for the lytic assay, leading to nonlinearity (Section 7.E). Under these conditions, enzymatic turnover of furimazine is rapid, and furimazine production likely becomes rate limiting.

3.C. Recommendations for Choosing Substrate and Controls

For time-course experiments lasting ≤2 hours, we recommend using the Nano-Glo® Live Cell Assay System, which provides a high concentration of furimazine at the start of the experiment. The extent of signal loss over approximately 2 hours in these experiments is usually tolerated. For longer time-course experiments, the magnitude of signal loss with the Nano-Glo® Live Cell Assay System leads to decreasing assay sensitivity and data quality.

For longer time course experiments, we recommend use of Endurazine™ or Vivazine™ Live Cell Substrate. If possible, we recommend evaluating both substrates in preliminary experiments to determine which performs best for a given application. The Nano-Glo® Extended Live Cell Substrate Trial Pack (Cat.# N2590) contains both substrates to test in your experiment.

If choosing a single substrate, first determine if Endurazine™ substrate provides sufficient luminescent intensity for your application. If so, Endurazine™ substrate will provide a stable baseline signal and support multi-day experiments. To reduce assay noise and improve data quality, luminescent intensity needs to be significantly above machine background (i.e., the luminescence reported by a luminometer for an empty plate). If replicate variability is high, kinetic traces do not appear continuous or both, evaluate whether Vivazine™ Live Cell Substrate provides better data quality with its brighter signal, assuming that the increased signal decay rate can be tolerated for your experiment. In addition, furimazine autoluminescence will produce a background signal in the absence of NanoLuc® or NanoBiT® luciferase (Section 3.F). For experimental systems that express very low levels of NanoLuc® or NanoBiT® luciferase, consider how autoluminescence may contribute to the signal.

For treatments with test or control compounds, include vehicle controls in your experiment. By having these controls, data can be normalized or compared to a vehicle treatment to determine the biological response to a treatment in the context of a common rate of signal decay for all samples.

3.D. Continuous Measurements in a Luminometer at 37°C

Continuous measurements can be performed in a luminometer at 37°C without removing the plate from the instrument. If your luminometer lacks CO_2 control, we recommend using a buffered medium to help maintain physiological pH throughout the experiment. Medium buffered with sodium bicarbonate will undergo a pH increase when removed from a CO_2 incubator, which can be seen if the medium contains phenol red and the color changes from red to pink/purple. We recommend medium buffered with HEPES (e.g., Opti-MEM® I, ThermoFisher Scientific Cat.# 11058) or a medium that is designed for use outside of a CO_2 incubator (e.g., CO_2 Independent Medium, ThermoFisher Scientific Cat.# 18045).

3.D. Continuous Measurements in a Luminometer at 37°C (continued)

Program the luminometer for continuous measurements at 37°C. Measure luminescence at user-defined intervals, e.g., every 15 minutes. For a GloMax® Discover System, set the instrument temperature to 37°C and include a heating step at 37°C in your kinetic loop (Section 7.F). If your luminometer lacks humidity control, measure luminescence with the lid on, if possible, but avoid conditions that promote condensation on the lid. For a GloMax® Discover instrument, software update 3.1.0 adjusts the temperature of heating elements within the instrument to prevent condensation on the lid. If your luminometer requires the lid to be removed, we recommend using 200µl per well (96-well format) to minimize the percent loss of medium in each well. There may be variability in the amount of evaporation per well across the plate, and a mock run with PBS can be used to determine which wells to use in an experiment.

3.E. Cycling Plates from a 37°C, 5% CO_2 Incubator to a 37°C Luminometer

Plates may be cycled between a 37°C, 5% CO_2 incubator and a 37°C luminometer at user-defined time points. If the amount of time outside of the 37°C, 5% CO_2 incubator is kept to a minimum, standard growth medium can be used, even medium buffered solely with sodium bicarbonate. Cycle plates quickly between the 37°C, 5% CO_2 incubator and the 37°C luminometer to limit changes in pH and plate cooling. Temperature gradients across the plate will be a source of well-to-well variability. If using a buffered medium suitable for use outside of a CO_2 incubator, e.g., Opti-MEM®I or CO_2 Independent Medium (Section 3.D), include a five-minute incubation in the luminometer at 37°C to reduce the possibility of thermal gradients. Measure luminescence with the lid on to prevent evaporation, if possible.

3.F. Factors Influencing Autoluminescence Background

Endurazine™ and Vivazine™ substrates steadily release furimazine throughout a multi-hour time course experiment. Furimazine will oxidize in the absence of NanoLuc® or NanoBiT® luciferase to produce autoluminescence. At low levels of luciferase expression, autoluminescence may contribute significantly to the signal, limiting sensitivity. Serum will substantially increase furimazine autoluminescence.

When expressing low levels of NanoLuc® or NanoBiT® luciferase, include control wells in your experiment to measure autoluminescence as a function of time (Section 7.D). Autoluminescence controls should use the same cell type as those expressing NanoLuc® or NanoBiT luciferase, and the wells around the control wells should be empty to prevent signal bleed over from adjacent wells. Autoluminescence magnitude will vary, depending on the furimazine concentration at a given time point. If a sample contains low levels of NanoLuc® or NanoBiT® luciferase, enzymatic turnover will decrease the concentration of furimazine. As a result, the autoluminescence at a given time point in the absence of NanoLuc® or NanoBiT® luciferase will differ from the autoluminescence contribution in the presence of NanoLuc® or NanoBiT® luciferase. As a result, autoluminescence controls will only provide an estimate of the autoluminescence contribution throughout a time-course experiment.

The Endurazine™ and Vivazine™ stock solutions contain a proprietary compound to reduce furimazine autoluminescence in both the presence and absence of serum. In the presence of 10% FBS, the autoluminescence reduction is greater than tenfold. Suppressing autoluminescence background provides increased dynamic range to detect low levels of NanoLuc® or NanoBiT® luciferase.

3.G. Data Analysis

As mentioned in Section 3.C, we recommend including vehicle controls to determine the specific effect of a given treatment. You can also normalize data on a per-well basis to the time point just prior to adding the test compounds or control treatments. Per-well normalization reduces variability between replicates arising from well-to-well differences in the number of cells plated, the transfection efficiency, cells lost during an aspiration step, etc. To normalize on a per-well basis, divide the luminescence for each well at a given time point by the luminescence measurement from that well just prior to adding test compounds or control treatments. Average the normalized response from replicate wells at each time point, and plot error as standard deviation or standard error.

4. Endurazine™ and Vivazine™ Live Cell Substrate Protocols

The following protocols are recommended for experiments in 96-well format. For higher-density formats, scale volumes and modify the protocol accordingly.

4.A. Cell Preparation

Endurazine™ and Vivazine™ substrates are used to detect NanoLuc® or NanoBiT® luciferases expressed in a broad range of cell types. We have validated both substrates using transient or stable expression in HEK293, HeLa, U2OS and CHO cells (Section 7.A). Perform tissue culture in a certified tissue culture hood using personal protective equipment appropriate for the biosafety level of the material be handled (e.g., gloves and a lab coat). Follow standard laboratory practices to avoid sample cross-contamination.

Adherent cells

1. Plate cells expressing NanoLuc® or NanoBiT® luciferase in white 96-well tissue culture plates (e.g., Corning Cat.# 3917). To minimize effects associated with evaporation or thermal gradients, use only the inner 60 wells of a 96-well plate, and add 200µl of sterile medium or PBS to the outer 36 wells and 100µl to the spaces between wells. If you intend to add Endurazine™ or Vivazine™ substrate from a 2X stock solution (Section 4.C), plate cells in the medium that will be used during luminescence measurements. Endurazine™ or Vivazine™ substrate can also be added by replacing medium (Section 4.B), plating the cells in the same medium used for cell propagation.

   Notes:

   1. For multi-day luminescence measurements, plate adherent cells at a low cell density if cells are to grow throughout the experiment.
   2. For transient expression and multi-day luminescence measurements, we recommend using a bulk transfection protocol followed by replating.
   3. Thermal gradients across the plate will be a source of well-to-well variability.
   4. Medium recommendations if delivering Endurazine™ or Vivazine™ from a 2X stock:
      - For continuous measurements in a luminometer, we recommend a buffered medium that will help maintain physiological pH throughout the experiment, unless your luminometer provides CO_2 control (Section 3.D).
      - FBS can be added up to 10% v/v final, but it will increase autoluminescence (Section 3.F).

4.A. Cell Preparation (continued)

2. Place plate containing cells in 37°C, 5% CO_2 incubator and incubate overnight.

   Note: We recommend an overnight incubation for cells to attach to the plate surface, although shorter times may be sufficient for certain cell types.

Suspension cells

1. Plate cells expressing NanoLuc® or NanoBiT® luciferase in white 96-well tissue culture plates (e.g., Corning Cat. # 3917). To minimize effects associated with evaporation or thermal gradients, use only the inner 60X wells of a 96-well plate, and add 200µl of sterile medium or PBS to the outer 36 wells and 100µl to the spaces between wells. If you intend to add Endurazine™ or Vivazine™ substrate from a 2X stock solution (Section 4.C), plate cells in the medium that will be used during luminescence measurements at 2X the desired final concentration of cells per millilter. If you intend to replace with medium containing Endurazine™ or Vivazine™ substrate, see Section 4.B.

   Notes:

   1. Thermal gradients across the plate will be a source of well-to-well variability.
   2. Medium recommendations if delivering Endurazine™ or Vivazine™ substrate from a 2X stock:
      - For continuous measurements in a luminometer, we recommend a buffered medium that will help maintain physiological pH throughout the experiment, unless your luminometer provides CO_2 control (Section 3.D).
      - FBS can be added up to 10% v/v final, but it will increase autoluminescence (Section 3.F).

4.B. Adding Substrate By Replacing the Medium

Endurazine™ and Vivazine™ substrates are provided as 100X stocks in organic solvent. Dilute Endurazine™ or Vivazine™ substrate 100-fold in cell culture medium for delivery to the cells. Serum-free medium may decrease Endurazine™ or Vivazine™ solubility, depending on medium type, and may increase cytotoxicity. If possible, include serum in your experiment to promote solubility and cell health. For serum-free conditions, protein supplements, (e.g. albumin) may increase solubility.

Wear gloves when handling Endurazine™ and Vivazine™ stock solutions, and avoid direct contact with skin. Take appropriate precautions when handling serum-containing medium. Consult the SDS for additional safety information.

1. Dilute Endurazine™ or Vivazine™ substrate 100-fold in cell culture medium and mix.

   Notes:

   1. Replace the cap immediately to avoid evaporation of the volatile organic solvent. Place tube(s) in an ice bucket if uncapped for extended periods.
   2. For continuous measurements in a luminometer, we recommend a buffered medium that will help maintain physiological pH throughout the experiment, unless your luminometer provides CO_2 control (Section 3.D). If measurements will be performed without a lid for several hours, we recommend using 200µl of medium per well (Section 3.D).
   3. FBS can be added up to 10% v/v final, but it will increase autoluminescence (Section 3.F).
   4. Warm medium to 37°C (optional).

2. For adherent cells, aspirate medium from tissue culture plate(s) and replace with the solution from Step 1. For suspension cells, pellet cells at 130 × *g* for 10 minutes and resuspend pellet with the solution from Step 1.

   Notes:

   1. For adherent cells, add medium containing Endurazine™ or Vivazine™ substrate slowly to the side of each well to avoid dislodging cells.
   2. For suspension cells, plate at the desired cell density or incubate cells in suspension (see Step 3).

3. If desired, incubate at 37°C to accumulate furimazine in the medium before adding test compounds and control treatments. In general, approximately 1 hour is required for Vivazine™ substrate to reach a peak signal and approximately 2 hours are required for Endurazine™ substrate to reach a stable signal.

   Notes:

   1. Measure luminescence continuously at 37°C to determine when a signal of sufficient intensity/stability is reached (Section 3.D).
   2. For suspension cells, this incubation can occur prior to cell plating.
   3. Signal intensity for Vivazine™ substrate will be brighter than Endurazine™ substrate at time zero and over the first several hours of a time-course experiment (Section 3.A).

4. Add test compounds and control treatments. For ≥10X stock solutions, use the same medium as Step 1 or similar solution as diluent minus Endurazine™ or Vivazine™ substrate. For less concentrated stock solutions, use a solution containing a 100-fold dilution of Endurazine™ or Vivazine™ substrate as diluent.

   Notes:

   1. Always include a vehicle control in your experiments for normalization or comparison purposes (Section 3.C).
   2. Measure baseline luminescence prior to adding test or control compounds for per-well normalization (Section 3.G).

4.B. Adding Substrate By Replacing the Medium (continued)

5. Measure luminescence at user-defined time points. We recommend using a luminometer that provides enhanced detection sensitivity such as the GloMax® Discover System (Cat.# GM3000). Use integration times of 0.5–2 seconds. When the experiment is complete, dispose of the plates in an approved biohazard container. If needed, dispose of Endurazine™ or Vivazine™ 100X stock solutions in an approved organic waste container (non-halogenated).

   Notes:

   1. When performing continuous measurements in the luminometer (Section 3.D), remove the lid if the luminometer provides humidity control. Otherwise, leave the lid on (if compatible with your instrument) if your luminometer lacks humidity control, but avoid excessive condensation on the lid. Kinetic measurements with the lid on can be performed using the GloMax® Discover System (Cat.# GM3000). GloMax® software update 3.1.0 modifies the temperature of heating elements within the instrument to prevent condensation on the plate lid.
   2. If you are cycling plates between a 37°C, 5% CO_2 incubator and 37°C luminometer (Section 3.E), remove plates from 37°C, 5% CO_2 incubator and place in 37°C luminometer. Remove the plate lid, if necessary. We recommend quickly cycling the plate between the 37°C, 5% CO_2 incubator and the 37°C luminometer to prevent changes in pH (if using a medium buffered solely with bicarbonate) and minimize cooling effects that will cause thermal gradients, which can introduce well-to-well variability in your results. If using a buffered medium (Section 3.D), include a 5-minute incubation in the 37°C luminometer to reduce thermal gradients.
   3. Furimazine will degrade in aqueous solution without enzyme and generate autoluminescence (Section 3.F), which can limit the sensitivity and dynamic range of a live-cell, nonlytic assay. The autoluminescence background can be estimated using cells that do not express NanoLuc® or NanoBiT® luciferase. Examples of autoluminescence for varying cell types are shown in Section 7.D.

4.C. Adding Substrate to Cells Using a 2X Stock Solution

Endurazine™ and Vivazine™ substrates are provided as 100X stocks in organic solvent. Dilute Endurazine™ and Vivazine™ substrates 50-fold in cell culture medium to create a 2X stock solution for delivery to plated cells. Higher concentration stock solutions may precipitate Endurazine™ or Vivazine™ substrate, depending on medium type. Serum-free medium may lead to a decrease in Endurazine™ or Vivazine™ solubility and increased cytotoxicity. If possible, include serum in your experiment to promote substrate solubility and cell health. For serum-free conditions, protein supplements (e.g., albumin) may increase solubility.

Wear gloves when handling Endurazine™ and Vivazine™ stock solutions, and avoid direct contact with skin. Consult the SDS for additional safety information.

1. Dilute Endurazine™ or Vivazine™ substrate 50-fold in cell culture medium and mix to create a 2X stock solution. The substrate should be diluted in the same cell culture medium used to plate the cells (Section 4.A).

   Notes:

   1. Replace the cap immediately to avoid evaporation of the volatile organic solvent. Place tube(s) in an ice bucket if uncapped for extended periods.
   2. Warm medium to 37°C (optional).

2. Add an equal volume of 2X stock solution to the wells of cells plated in Section 4.A, and mix.

   Notes:

   1. For adherent cells, slowly dispense medium containing Endurazine™ or Vivazine™ substrate along the side of each well to avoid dislodging cells.
   2. If performing continuous measurements without a lid for several hours, we recommend a volume of 200µl per well at the start of the experiment (Section 3.D).

3. If desired, incubate at 37°C to accumulate furimazine in the medium before adding test compounds and control treatments. In general, approximately 1 hour is required for Vivazine™ substrate to reach a peak signal and approximately 2 hours are required for Endurazine™ substrate to reach a stable signal.

   Notes:

   1. Measure luminescence continuously at 37°C to determine when a signal of sufficient intensity and stability is reached (Section 3.D).
   2. Signal intensity for Vivazine™ substrate will be brighter than Endurazine™ substrate at time zero and over the first several hours of a time course experiment (Section 3.A).

4. Add test compounds and control treatments. For ≥10X stock solutions, use the same medium as Step 1 or similar solution as diluent without substrate. For less concentrated stock solutions, use a solution containing a 100-fold dilution of Endurazine™ or Vivazine™ substrate as diluent.

   Notes:

   1. Always include a vehicle control in your experiments for comparison/normalization purposes (Section 3.C).
   2. Measure baseline luminescence prior to adding test or control compounds to employ per-well normalization (Section 3.G).

4.C. Adding Substrate to Cells Using a 2X Stock Solution (continued)

5. Measure luminescence at user defined time points. A luminometer that provides enhanced detection sensitivity is recommended, e.g., GloMax® Discover or GloMax® Explorer. Integration times between 0.5-2 seconds will suffice in most cases. When the experiment is complete, dispose of the plates in an approved biohazard container. If needed, dispose of Endurazine™ or Vivazine™ 100X stock solutions in an approved organic waste container (non-halogenated).

   Notes:

   1. When performing continuous measurements in the luminometer (Section 3.D), remove the lid if the luminometer provides humidity control. Otherwise, leave the lid on (if compatible with your instrument) if your luminometer lacks humidity control, but avoid excessive condensation on the lid. Kinetic measurements with the lid on can be performed using the GloMax® Discover System (Cat.# GM3000). GloMax® software update 3.1.0 modifies the temperature of heating elements within the instrument to prevent condensation on the plate lid.
   2. If you are cycling plates between a 37°C, 5% CO_2 incubator and 37°C luminometer (Section 3.E), remove plates from 37°C, 5% CO_2 incubator and place in 37°C luminometer. Remove the plate lid, if necessary. We recommend quickly cycling the plate between the 37°C, 5% CO_2 incubator and the 37°C luminometer to prevent changes in pH (if using a medium buffered solely with bicarbonate) and minimize cooling effects that will cause thermal gradients, which can introduce well-to-well variability in your results. If using a buffered medium (Section 3.D), include a 5-minute incubation in the 37°C luminometer to reduce thermal gradients.
   3. Furimazine will degrade in aqueous solution without enzyme and generate autoluminescence (Section 3.F), which can limit the sensitivity and dynamic range of a live-cell, nonlytic assay. The autoluminescence background can be estimated using cells that do not express NanoLuc® or NanoBiT® luciferase. Examples of autoluminescence for varying cell types are shown in Section 7.D.

5. Representative Data

The following sections contain representative data for the use of Endurazine™ and Vivazine™ substrates in live-cell, nonlytic applications.

5.A. Live-Cell, NonLytic NanoLuc®-PEST Reporter Gene Assay Using Endurazine™ and Vivazine™ Substrates

Endurazine™ and Vivazine™ substrates can be used for live-cell, nonlytic reporter gene assays. After furimazine has accumulated, test compounds and control treatments can be added and the luminescence measured continuously at user-defined time points (Figure 3). We recommend including a vehicle control for normalization or to compare relative rates of signal decay (Section 3.C). At high levels of expression, signals from Endurazine™ and Vivazine™ substrates can plateau while signals from the lytic Nano-Glo® Luciferase Assay System continue to rise (Section 3.B and Figures 3.E and 3.F). This break from linearity can lead to misinterpretating the true biological response. To avoid this, transiently transfect low amounts of plasmid DNA (diluted with Transfection Carrier DNA) or pick clones with lower expression levels. At lower levels of expression, the luminescence with Vivazine™ substrate can approximate the luminescence with the Nano-Glo® Luciferase Assay System (Figure 3.E).

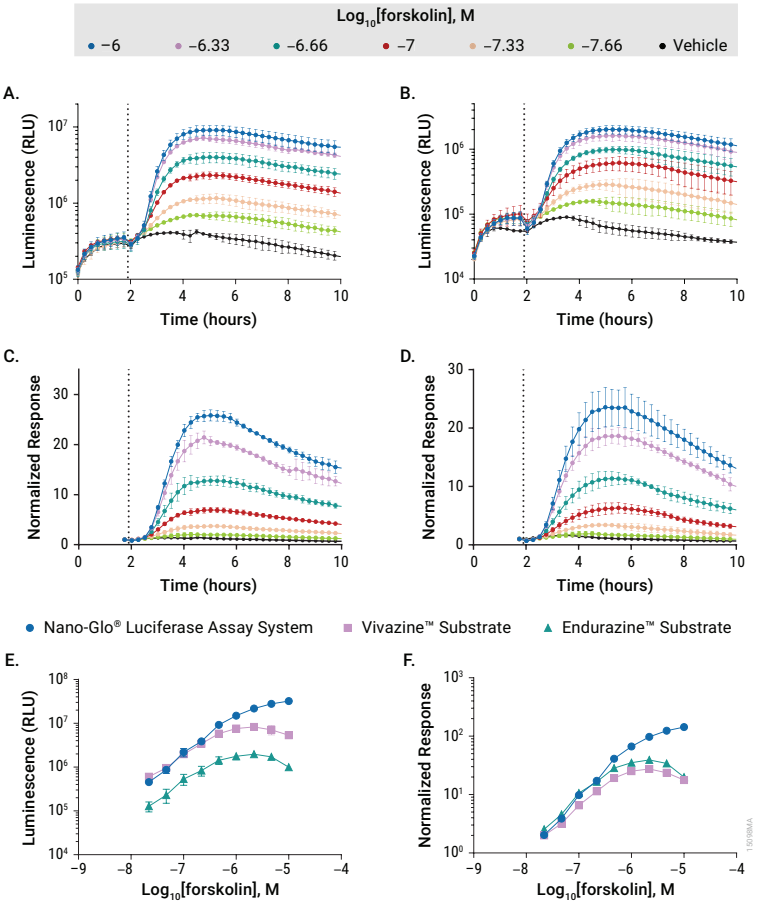

Figure 3. Live-cell, nonlytic reporter gene assay using Endurazine™ and Vivazine™ substrates. HEK293 cells were transiently transfected with a construct expressing NanoLuc®-PEST from a promoter with CRE response elements using a bulk transfection protocol followed by replating. Endurazine™ and Vivazine™ substrates were added to cells at time zero, and luminescence was measured continuously using a GloMax® Discover instrument at 37°C. After approximately 2 hours, varying concentrations of forskolin were added to induce expression of NanoLuc®-PEST by increasing the intracellular cAMP concentration. **Panel A.** Ten hour time course using Vivazine™ substrate. **Panel B.** Ten hour time course using Endurazine™ substrate. For Panels A and B, the average luminescence is plotted (n = 3) with error bars representing the standard deviation. **Panel C.** Per-well normalization (Section 3.G) for the data in Panel A. **Panel D.** Per-well normalization (Section 3.G) for the data in Panel B. For Panels A–D, traces with a saturating concentration of forskolin were removed for increased clarity. **Panel E.** A comparison of Endurazine™ and Vivazine™ substrates to the lytic Nano-Glo® Luciferase Assay System at 5 hours. **Panel F.** Normalized response for the data of Panel E. The average luminescence for a given dose of forskolin was divided by the average luminescence for vehicle treatment. Endurazine™ and Vivazine™ data were collected using a GloMax® Discover System with the lid on at 37°C running software version 3.1.0 to prevent condensation on the lid.

5.B. Live-Cell, NonLytic HiBiT Assay to Measure Protein Degradation Using Endurazine™ and Vivazine™ Substrates

Endurazine™ and Vivazine™ substrates can be used to monitor expression levels of HiBiT-tagged proteins in real time in cells expressing LgBiT, where HiBiT binds to LgBiT to form NanoBiT® Luciferase. In many cases, the HiBiT tag can be introduced via CRISPR-Cas9, and protein levels can be assayed in real time at endogenous levels of expression. For example, HEK293 cells stably expressing LgBiT were modified via CRISPR-Cas9 to create cells expressing a HiBiT-BRD4 fusion. Clonal cell lines were isolated and the integrity of the HiBiT fusion was confirmed by Sanger sequencing. Expression levels of the HiBiT-BRD4 fusion were monitored in real time following treatment with ARV-771, a proteolysis targeting chimeric molecule (PROTAC) that mediates degradation of the HiBiT-BRD4 fusion via interaction with the VHL E3 ligase complex (Figure 4). The Endurazine™ and Vivazine™ substrates can be used for real-time measurements of both the initial increase in protein degradation and the subsequent recovery of protein levels from de novo synthesis.

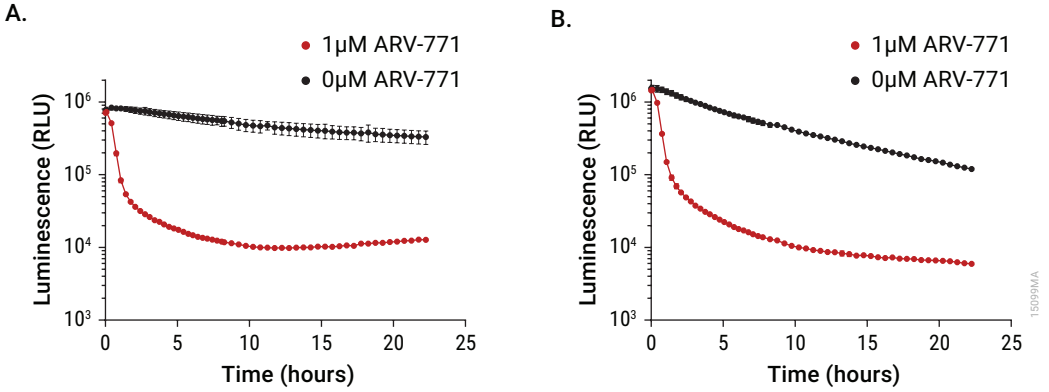

Figure 4. Continuous measurements of PROTAC-mediated protein degradation using Endurazine™ and Vivazine™ substrates. Expression levels of a HiBiT-BRD4 fusion were monitored in real time using Endurazine™ and Vivazine™ substrates following treatment with ARV-771, a BRD4-targeted PROTAC. **Panel A.** Live-cell, nonlytic measurements using Endurazine™ substrate. **Panel B.** Live-cell, nonlytic measurements using Vivazine™ substrate. The average luminescence is plotted with error bars representing the standard deviation (n = 3).

5.C. Comparing Endurazine™ and Vivazine™ Substrate at Varying NanoLuc® Expression Levels in HEK293 Cells

Expression levels will affect luminescence intensity and, to a lesser extent, the rate of signal decay. At higher expression levels, luminescent signals can stabilize and assay linearity can be lost (Section 3.B). This deviation is evident when transiently expressing NanoLuc® luciferase using constitutive promoters of varying strength and transfecting varying amounts of plasmid DNA (Figure 5). At greater expression levels, larger fold changes in signal are seen with the lytic Nano-Glo® Luciferase System compared to use of either live-cell substrate. However, at lower expression levels, fold changes in signal are more similar.

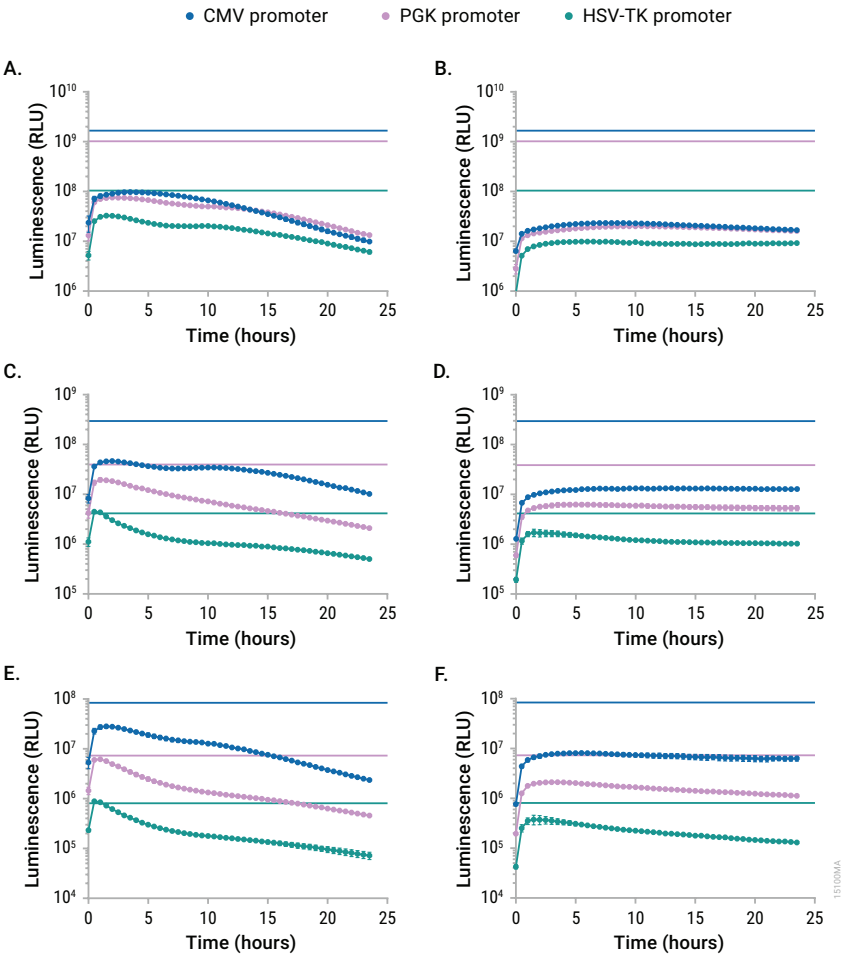

Figure 5. Comparing Endurazine™ and Vivazine™ substrates with NanoLuc® luciferase expressed at various levels in HEK293 cells. NanoLuc® luciferase was transiently expressed in HEK293 cells using different promoters and varying amounts of transfected DNA. Vivazine™ and Endurazine™ substrates were added at time zero and luminescence was monitored continuously with the lid on using the GloMax® Discover instrument at 37°C running version 3.1.0 software to prevent condensation. Solid lines of constant value represent data from a replicate plate treated with the lytic Nano-Glo® Luciferase Assay System at time zero. **Panel A.** Vivazine™ substrate with 10ng of DNA. **Panel B.** Endurazine™ substrate with 10ng of DNA. **Panel C.** Vivazine™ substrate with 1ng of DNA. **Panel D.** Endurazine™ substrate with 1ng of DNA. **Panel E.** Vivazine™ substrate with 0.1ng of DNA. **Panel F.** Endurazine™ substrate with 0.1ng of DNA. The average luminescence is plotted (n = 3) with error bars representing the standard deviation. When using 10ng of DNA, rapid depletion of substrate or luminometer saturation or both are likely causes for the similarity between CMV and PGK promoters using the lytic Nano-Glo® Luciferase Assay System.

6. Related Products

Nano-Glo® Detection Reagents

Products	Size	Cat. #
Nano-Glo® Live Cell Assay System	100 assays	N2011
	1,000 assays	N2012
	10,000 assays	N2013

NanoLuc® Reporter Vectors

For full list of NanoLuc® Luciferase vector options, please visit:
www.promega.com/products/reporter-assays-and-transfection/reporter-vectors-and-cell-lines/

NanoBiT® Protein Interaction Systems

Products	Size	Cat. #
NanoBiT® PPI MCS Starter System	1 each	N2014
NanoBiT® PPI Flexi® Starter System	1 each	N2015

HiBiT Protein Tagging Vectors

Products	Size	Cat. #
HiBiT MCS Cloning Vectors		N2361
		N2371
		N2381
HiBiT Flexi® Cloning Vectors		N2391
		N2401
		N2411

Transfection Reagents

Products	Size	Cat. #
FuGENE® HD Transfection Reagent	1ml	E2311
	5 × 1ml	E2312
ViaFect™ Transfection Reagent	0.75ml	E4981
	2 × 0.75ml	E4982

Luminometers

Products	Size	Cat. #
GloMax® Discover System	1 each	GM3000
GloMax® Explorer System, Fully Loaded	1 each	GM3500
GloMax® Explorer System with Luminescence and Fluorescence	1 each	GM3510

7. Appendix

7.A. Comparing Endurazine™ and Vivazine™ Substrates in HEK293, CHO, U2OS and HeLa Cells

For both Endurazine™ and Vivazine™ substrates, the signal intensity and stability will vary with the type of luciferase being measured (e.g., NanoLuc® or NanoBiT® enzyme), expression level and cell type. Figure 6 compares the profiles obtained in HEK293, CHO, U2OS and HeLa cells for NanoLuc® Luciferase transiently expressed from the HSV-TK promoter. In general, Endurazine™ substrate will provide relatively stable luminescence throughout a 24-hour time course experiment. In some cases, the luminescence intensity with Endurazine™ substrate will be too dim, limiting assay sensitivity. Vivazine™ substrate will provide a brighter signal over the first several hours of a time course, but the rate of signal decay will be greater and sometimes unpredictable. At time points greater than approximately 20 hours, the luminescence intensity for Endurazine™ substrate will be substantially brighter than Vivazine™ substrate.

7.A. Comparing Endurazine™ and Vivazine™ Substrates in HEK293, CHO, U2OS and HeLa Cells (continued)

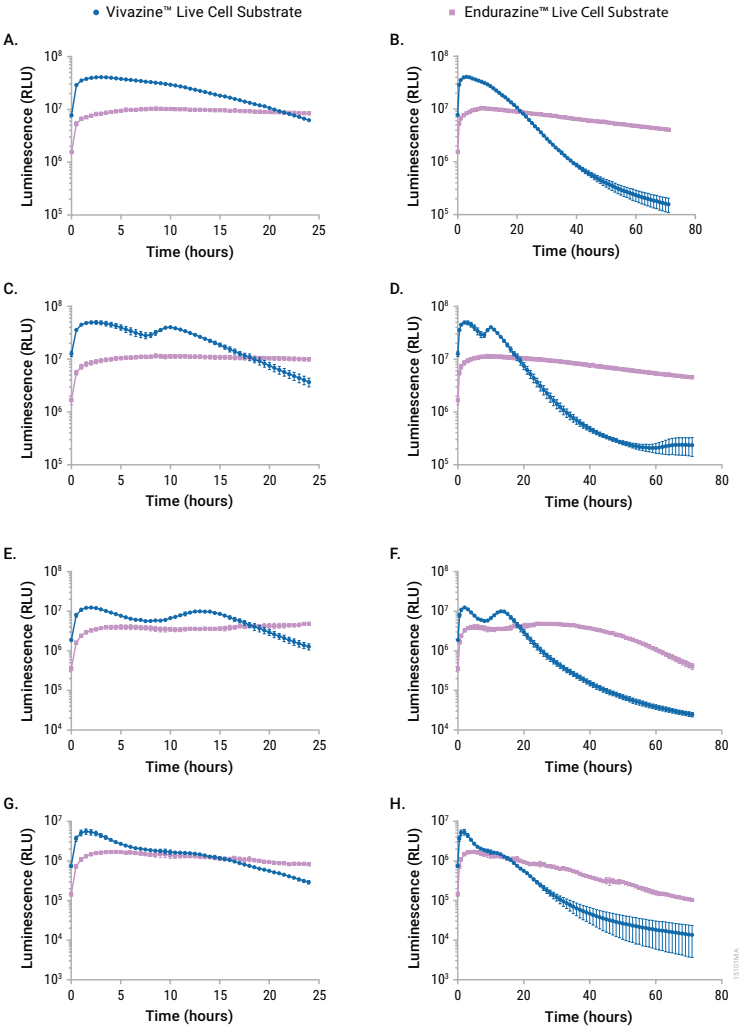

Figure 6. Comparing Endurazine™ and Vivazine™ substrates in HEK293, CHO, U2OS and HeLa cells. NanoLuc® luciferase was transiently expressed from the HSV-TK promoter in HEK293, CHO, U2OS and HeLa cells using a bulk transfection protocol followed by replating. Luminescence was measured from cells with Endurazine™ or Vivazine™ substrate over 72 hours. **Panel A.** HEK293 cells in the first 24 hours. **Panel B.** HEK293 cells for 72 hours. **Panel C.** CHO cells in the first 24 hours. **Panel D.** CHO cells for 72 hours. **Panel E.** U2OS cells in the first 24 hours. **Panel F.** U2OS cells for 72 hours. **Panel G.** HeLa cells in the first 24 hours. **Panel H.** HeLa cells for 72 hours. Luminescence was measured with the lid on using a GloMax® Discover multimode reader at 37°C running version 3.1.0 software to prevent condensation on the lid. The average luminescence is plotted with error bars representing the standard deviation (n = 3).

7.B. Comparing Endurazine™ and Vivazine™ Substrates with the Nano-Glo® Live Cell Assay System in HEK293 Cells

Endurazine™ and Vivazine™ substrates provide substantially brighter luminescence than the Nano-Glo® Live Cell Assay System for time course experiments lasting several hours or days (Figure 7). Both substrates are stable in medium with or without FBS, and both provide a steady release of furimazine throughout a time-course experiment. In contrast, the Nano-Glo® Live Cell Assay System provides a high initial concentration of furimazine at the start of the experiment for a bright signal, but signal stability is poor due to both enzymatic and nonenzymatic turnover during the time course.

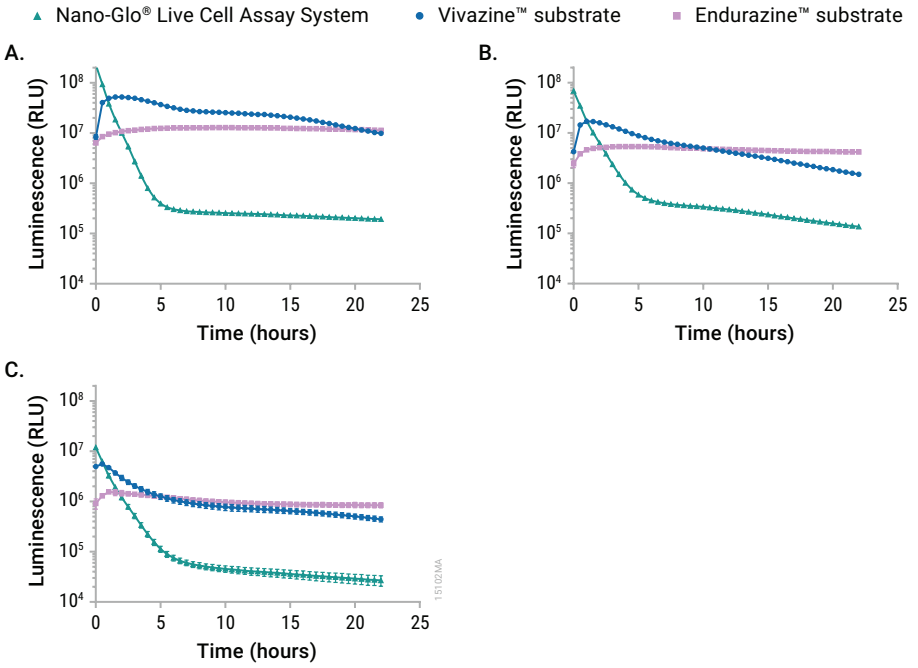

Figure 7. Comparing Endurazine™ and Vivazine™ substrates with the Nano-Glo® Live Cell Assay System in HEK293 cells. NanoLuc® luciferase expressed from CMV, PGK or HSV-TK promoters were transiently transfected into HEK293 cells using 1ng vector DNA/well in a 96-well plate. **Panel A.** NanoLuc® luciferase expression using the CMV promoter. **Panel B.** NanoLuc® luciferase expression using the PGK promoter. **Panel C.** NanoLuc® luciferase expression using the HSV-TK promoter. The Nano-Glo® Live Cell Assay System is a source of unmodified furimazine (Figure 1). The average luminescence is plotted (n = 3) with error bars representing the standard deviation.

7.C. Cytotoxicity Induced by Endurazine™ and Vivazine™ Substrates in HEK293, CHO, U2OS and HeLa Cells

Prolonged exposure to Endurazine™ or Vivazine™ substrate can promote low levels of cytotoxicity with results varying with cell type (Figure 8). HEK293, CHO, U2OS and HeLa cells were exposed to Endurazine™ or Vivazine™ substrates in medium containing 10% FBS. After 24 hours, cell viability was measured using the CellTiter-Glo® Luminescent Cell Viability Assay. Low levels of cytotoxicity were apparent in all cases. Similar results were seen at 48 and 72 hours (not shown).

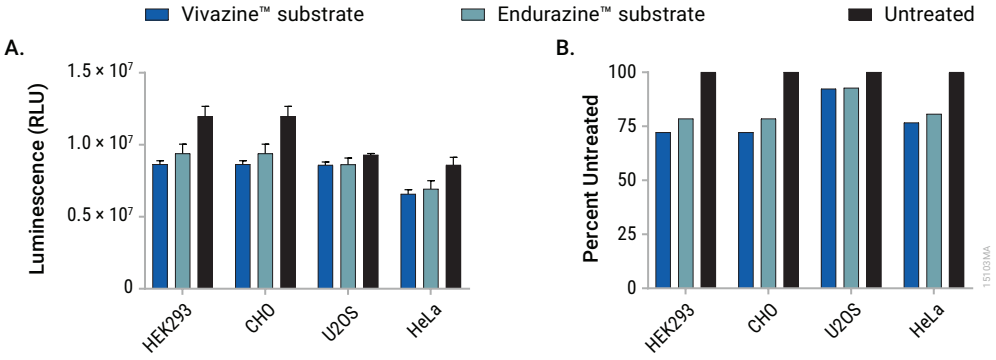

Figure 8. Cytotoxicity induced by Endurazine™ and Vivazine™ substrates in HEK293, CHO, U2OS and HeLa cells. Cells were plated and allowed to attach overnight. Medium was exchanged for Opti-MEM® I + 10% FBS containing either Vivazine™ or Endurazine™ substrates or medium alone. **Panel A.** Cytotoxicity was measured using the CellTiter-Glo® Luminescent Cell Viability Assay (Cat.# G7570) after a 24-hour incubation in a 37°C, 5% CO_2 incubator. The average luminescence is plotted (n = 3) with error bars representing the standard deviation. **Panel B.** Data from Panel A normalized to untreated wells.

7.D. Autoluminescence Background in HEK293, CHO, U2OS and HeLa Cells

Furimazine autoluminescence can limit assay sensitivity when NanoLuc® or NanoBiT® luciferase are expressed at low levels (Section 3.F). Autoluminescence profiles can be similar in different cell types (Figure 9). In general, autoluminescence for Vivazine™ substrate has been higher than Endurazine™ substrate over the first 24 hours of a time course, indicating higher concentrations of furimazine. Beyond 24 hours, the autoluminescence for Endurazine™ substrate will be higher.

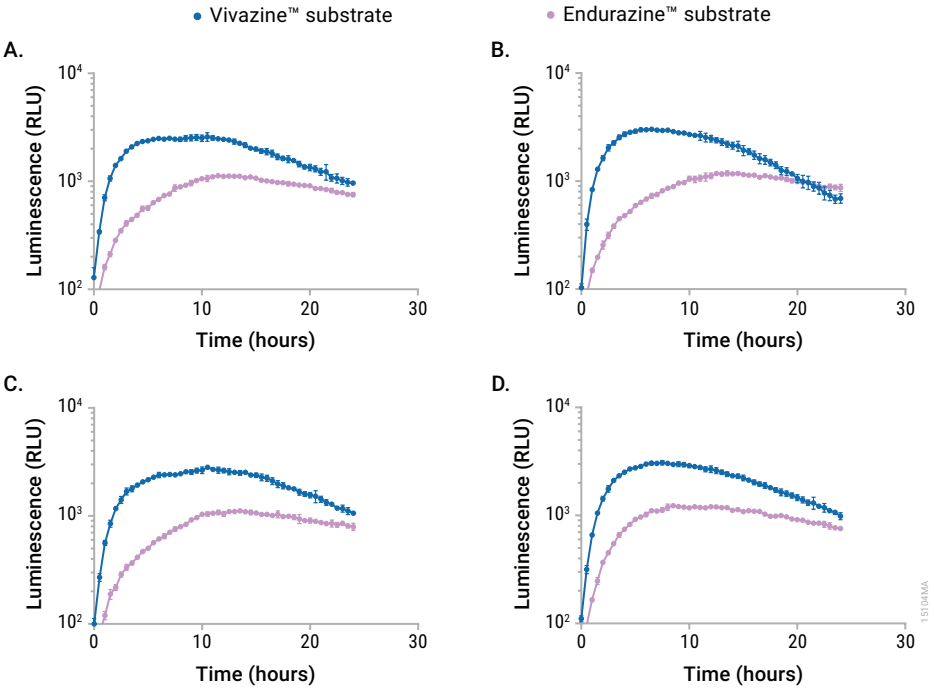

Figure 9. Autoluminescence in HEK293, CHO, U2OS and HeLa cells lacking NanoLuc® or NanoBiT® luciferase. Autoluminescence was measured continuously in a GloMax® Discover multimode reader at 37°C for 24 hours. **Panel A.** HEK293 cells. **Panel B.** CHO cells. **Panel C.** U2OS cells. **Panel D.** HeLa cells. The average luminescence is plotted (n = 3) with error bars representing the standard deviation.

7.E. Linearity Demonstrated Using Purified NanoLuc® Luciferase

Endurazine™ and Vivazine™ substrates will have a reduced linear dynamic range compared to the lytic Nano-Glo® Luciferase Assay System (Section 3.B). At high levels of expression, luminescence becomes nonlinear when signals for both substrates plateau while luminescence from the lytic Nano-Glo® Luciferase Assay System will continue to rise (Section 3.B; Figure 3, Panels E and F). To demonstrate this, we incubated cells with either substrate for 2 hours followed by adding varying concentrations of purified NanoLuc® luciferase (Figure 10). Adding purified NanoLuc® luciferase precisely controlled enzyme levels in each well, which is impossible to achieve using cellular expression. Although NanoLuc® luciferase was present in the extracellular medium, intracellular furimazine is free to diffuse to the extracellular environment. A linear response was seen over a 4–5 log concentration range. However, at high concentrations, this linearity breaks. Under these conditions, enzymatic turnover of furimazine is rapid, and furimazine production likely becomes rate limiting.

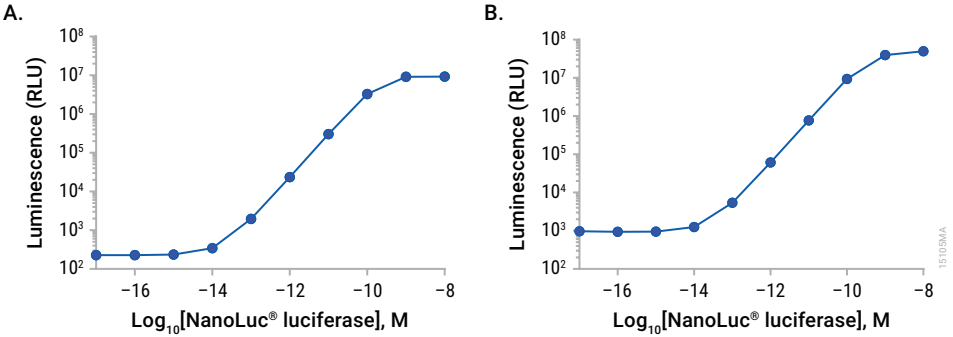

Figure 10. Linearity demonstration using purified NanoLuc® luciferase. Endurazine® and Vivazine® substrates were added to HEK293 cells and furimazine accumulated for 2 hours. Varying concentrations of NanoLuc® luciferase were added, and luminescence was measured using a GloMax® Multi+ luminometer. **Panel A.** NanoLuc® dose response using Endurazine™ substrate. **Panel B.** NanoLuc® dose response using Vivazine™ substrate. The average luminescence is plotted (n = 3) with error bars representing the standard deviation (not visible).

7.F. Programming a GloMax® Discover System for Time Course Experiments at 37°C

To download Endurazine™ and Vivazine™ protocols for 24, 48 and 72-hour incubations at 37°C on the GloMax® Discover System, visit: **www.promega.com/resources/software-firmware/detection-instruments-software/promega-branded-instruments/protocols-for-glomax-discover-and-explorer/**. If you have any questions about the protocols, contact Technical Services.

7.G. Troubleshooting

Causes	Comments
Endurazine™ or Vivazine™ RLUs are significantly reduced compared to the Nano-Glo® Live Cell Assay System.	Endurazine™ and Vivazine™ substrates enable time-course experiments over several hours or days by providing a steady release of furimazine throughout the experiment. As a result, the concentration of furimazine at a given time point will be substantially lower than the concentration of furimazine provided by the Nano-Glo® Live Cell Assay System immediately when added. If signals are too dim, switch to a stronger promoter, if possible. For NanoBiT® PPI applications, use of a stronger promoter may require diluting plasmid DNA for transient transfection experiments. If using the Endurazine™ Live Cell Substrate, try switching to the Vivazine™ Live Cell Substrate, which has a brighter signal but an increased rate of signal decay.
Signals increase then decrease during the time course when a steady signal or a signal decrease is preferred.	Incubate cells with Endurazine™ or Vivazine™ Live Cell Substrates for 2 hours prior to adding test compounds and control treatments. This will allow the furimazine to accumulate and signals to stabilize.
Reduced dynamic range using Endurazine™ or Vivazine™ Live Cell Substrates compared to the lytic Nano-Glo® Luciferase Assay System.	At high levels of expression, the rate of furimazine formation likely becomes rate limiting, showing a signal plateau over a range of NanoLuc® or NanoBiT® luciferase expression levels that show a signal increase using the lytic Nano-Glo® Luciferase Assay System (Section 3.B and Figure 3, Panels E and F). Use weaker promoters or dilute plasmid DNA or both for transient transfections.

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