



TECHNICAL MANUAL

Nano-Glo[®] Dual-Luciferase[®] Reporter Assay System

Instructions for Use of Products
N1610, N1620, N1630 and N1650

Nano-Glo[®] Dual-Luciferase[®] Reporter Assay System

All technical literature is available at: www.promega.com/protocols/
 Visit the web site to verify that you are using the most current version of this Technical Manual.
 E-mail Promega Technical Services if you have questions on use of this system: techserv@promega.com

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1. Description

Luciferases are commonly used to monitor gene expression because of their broad dynamic range and extreme sensitivity (1–3). Often, a dual-luciferase assay format is utilized in which firefly (*Photinus pyralis*) luciferase is used as the experimental reporter to measure the biological response and *Renilla* luciferase is used as a constitutively expressed control reporter. Dual assays provide a convenient way to normalize experimental reporter luminescence to that of a co-transfected control, improving data quality by minimizing or eliminating experimental variability arising from such factors as differences in transfection efficiency, cell number, cell viability, temperature and measurement time.

NanoLuc[®] luciferase is a 19.1kDa engineered enzyme developed to be brighter and more versatile than other reporter proteins, and to improve and extend the applications of luciferase enzymes (4). When used as an experimental reporter, NanoLuc[®] luciferase fused to a PEST degradation sequence (Nluc-PEST) provides both a brighter and more responsive reporter gene than firefly luciferase (*luc2*) or *luc2*-PEST.

The Nano-Glo[®] Dual-Luciferase[®] Reporter (NanoDLR[™]) Assay System^(a-e) allows sensitive detection of both firefly luciferase and NanoLuc[®] luciferase in the same sample. The add-read-add-read, or “Stop & Glo”, format is amenable to assays or screens in 96-, 384- or 1,536-well plates. In the Nano-Glo[®] Dual-Luciferase[®] Reporter Assay, firefly luciferase activity is measured first using the supplied ONE-Glo[™] EX Luciferase Assay Reagent. NanoDLR[™] Stop & Glo[®] Reagent is then added to quench the firefly signal (>1,000,000-fold) and to provide the furimazine substrate needed to measure NanoLuc[®] luciferase activity. Both reagents provide stable glow-type luminescence signals with half-lives of approximately 2 hours. Potent inhibition of firefly luciferase coupled with the high-intensity luminescence of NanoLuc[®] luciferase maximizes sensitivity for detection of both reporters. This allows either or both reporters to be used to measure a biological response. For example, you can exploit the brightness and responsiveness of NanoLuc[®]-PEST as the experimental reporter and use firefly luciferase as a constitutive control, use firefly luciferase as the experimental reporter and NanoLuc[®] luciferase as the control, or use both luciferases as experimental reporters (see Section 4.C).

For high-throughput screening (HTS), the NanoDLR[™] Assay can be used in the coincidence reporter system developed by Inglese and co-workers (5,6). This approach utilizes transcription of a firefly-P2A-NanoLuc-PEST coincidence circuit from the same promoter, allowing expression of two unfused forms of luciferase with distinct compound interaction profiles. False hits directly affecting one of the luciferase reporters are distinguished from true hits, which show a similar response for both luciferases (Section 4.C).

See Section 4 for an overview of the NanoDLR[™] Assay, and for information about experimental design and factors affecting assay performance.

For a complete listing of NanoLuc[®] and firefly luciferase vectors suitable for use with the NanoDLR[™] Assay, please visit: www.promega.com/luciferase-vectors

2. Product Components and Storage Conditions

Product	Size	Cat.#
Nano-Glo® Dual-Luciferase® Reporter Assay System	10ml	N1610

Each system contains sufficient material to prepare 10ml of each reagent. Includes:

- 10ml ONE-Glo™ EX Luciferase Assay Buffer
- 1 vial ONE-Glo™ EX Luciferase Assay Substrate (lyophilized)
- 10ml NanoDLR™ Stop & Glo® Buffer
- 100µl NanoDLR™ Stop & Glo® Substrate

Product	Size	Cat.#
Nano-Glo® Dual-Luciferase® Reporter Assay System	100ml	N1620

Each system contains sufficient material to prepare 100ml of each reagent. Includes:

- 100ml ONE-Glo™ EX Luciferase Assay Buffer
- 1 vial ONE-Glo™ EX Luciferase Assay Substrate (lyophilized)
- 100ml NanoDLR™ Stop & Glo® Buffer
- 1,000µl NanoDLR™ Stop & Glo® Substrate

Product	Size	Cat.#
Nano-Glo® Dual-Luciferase® Reporter Assay System	10 × 10ml	N1630

Each system contains sufficient material to prepare 100ml of each reagent. Includes:

- 10 × 10ml ONE-Glo™ EX Luciferase Assay Buffer
- 10 vials ONE-Glo™ EX Luciferase Assay Substrate (lyophilized)
- 10 × 10ml NanoDLR™ Stop & Glo® Buffer
- 10 × 100µl NanoDLR™ Stop & Glo® Substrate

Product	Size	Cat.#
Nano-Glo® Dual-Luciferase® Reporter Assay System	10 × 100ml	N1650

Each system contains sufficient material to prepare 1,000ml of each reagent. Includes:

- 10 × 100ml ONE-Glo™ EX Luciferase Assay Buffer
- 10 vials ONE-Glo™ EX Luciferase Assay Substrate (lyophilized)
- 10 × 100ml NanoDLR™ Stop & Glo® Buffer
- 10 × 1,000µl NanoDLR™ Stop & Glo® Substrate

Storage Conditions: Store the Nano-Glo® Dual-Luciferase® Reporter Assay System components at –30°C to –10°C. Do not thaw above 25°C. The ONE-Glo™ EX Luciferase Assay Buffer may be stored at +2°C to +10°C for 1 year or at room temperature for 6 months. The NanoDLR™ Stop & Glo® Buffer may be stored at +2°C to +10°C for 6 months or at room temperature for 3 months.

Prepare the NanoDLR™ Stop & Glo® Reagent (NanoDLR™ Stop & Glo® Buffer + Substrate) fresh for each use. Store unused reconstituted ONE-Glo™ EX Reagent and NanoDLR™ Stop & Glo® Reagent at 4°C or –20°C, protected from light. Warm or thaw at temperatures below 25°C (e.g., in a room-temperature water bath) and mix by inversion before use. At 22°C, ONE-Glo™ EX Reagent will lose 10% activity in ~18 hours and 50% activity in ~6 days. At 4°C, ONE-Glo™ EX Reagent will lose 10% activity in ~3.5 days and 50% activity in ~1 month. At 22°C, NanoDLR™ Stop & Glo® Reagent will lose 10% activity in ~32 hours and 50% activity in ~6 days. At 4°C, NanoDLR™ Stop & Glo® Reagent will lose 10% activity in ~3.5 days and 50% activity in ~18 days.

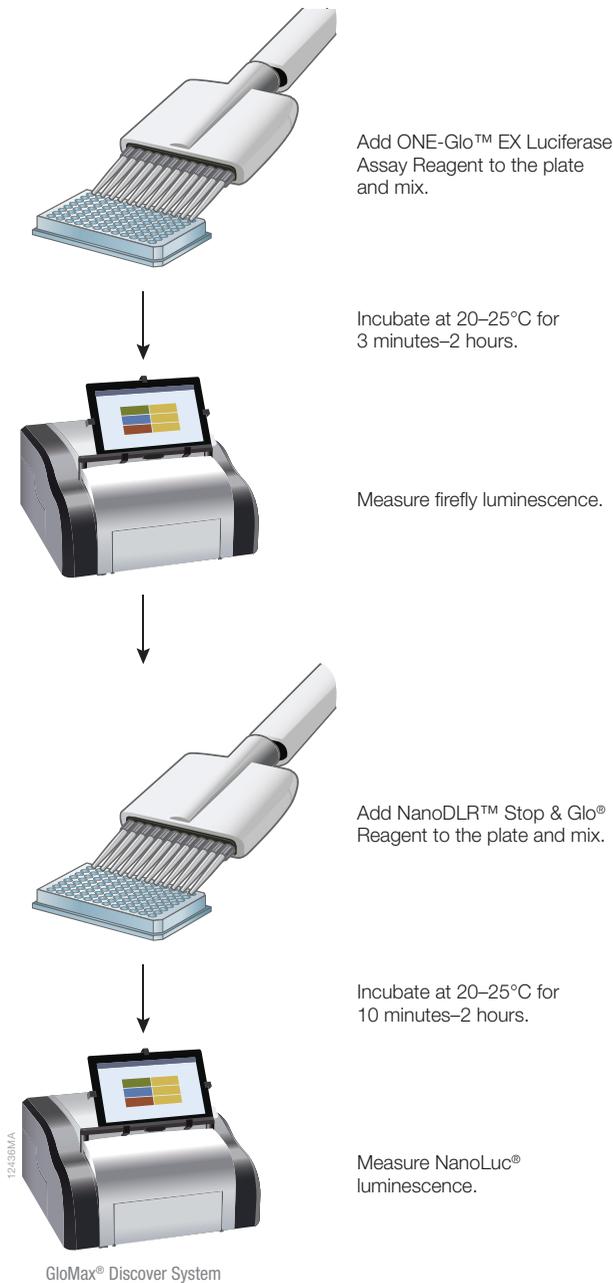


Figure 1. Nano-Glo® Dual-Luciferase® Reporter Assay protocol. Addition of ONE-Glo™ EX Reagent lyses cells and provides the substrates to measure firefly luminescence. Addition of the NanoDLR™ Stop & Glo® Reagent potentially quenches the firefly signal and provides the substrate for NanoLuc® luminescence.

3. Performing the Nano-Glo® Dual-Luciferase® Reporter Assay

3.A. General Considerations

The Nano-Glo® Dual-Luciferase® Reporter Assay System is designed to be used with many media types and has been validated for use with the following culture media containing 0–10% serum: DMEM, RPMI 1640, McCoy's 5A and F-12. The NanoDLR™ assay reagents should give a signal half-life of approximately 2 hours at 22°C for each reporter in many media types. However, different combinations of media and serum may affect luminescence or signal decay rate (see Section 4.F). Luminescence can also be affected by the presence of phenol red or organic solvents, and by temperature changes (see Section 4.F).

Because luminescent signals are affected by assay conditions, raw results should be compared only between samples measured at the same time using the same medium and serum combination. For analysis of multiple plates, greatest accuracy can be obtained by incorporating a common control sample in each plate. Depending on the experiment setup, normalized ratios of experimental reporter luminescence to control reporter luminescence may provide better comparisons across plates by correcting for small variations in luminescence due to differences in incubation times for luminescence measurement, differences in temperature between plates, or other factors. Incorporating positive and negative control wells within a plate or experiment provides the ability to calculate a Relative Response Ratio (RRR), which can be used to compare results from experiments under different conditions (Section 4.E). See Section 4.C for a discussion of various assay formats, experimental setup, and data normalization. See Section 4.D for important considerations and example protocols for performing transient transfections.

3.B. Reagent Preparation

1. To prepare the ONE-Glo™ EX Reagent, transfer the contents of one bottle of ONE-Glo™ EX Luciferase Assay Buffer to one bottle of ONE-Glo™ EX Luciferase Assay Substrate. Replace the stopper, and mix by inversion until the substrate is thoroughly dissolved. This should take less than 10 seconds.
2. Prepare the NanoDLR™ Stop & Glo® Reagent fresh for each use. Calculate the amount of NanoDLR™ Stop & Glo® Reagent needed to perform the desired experiments. In a new container, dilute the NanoDLR™ Stop & Glo® Substrate 1:100 into an appropriate volume of room-temperature NanoDLR™ Stop & Glo® Buffer. Mix by inversion. (If the NanoDLR™ Stop & Glo® Substrate has collected in the cap or on the sides of the tube, briefly spin the tubes in a microcentrifuge before dispensing).

Example: If you need 4ml of NanoDLR™ Stop & Glo® Reagent, transfer 4ml of NanoDLR™ Stop & Glo® Buffer to a disposable container, such as a 15ml centrifuge tube, and add 40µl of NanoDLR™ Stop & Glo® Substrate.



Notes:

- a. Luciferase activity is temperature dependent. The temperature of the samples and reagents should be kept constant while measuring luminescence. This is most easily accomplished by using reagents that are equilibrated to room temperature. Transferring the buffer solutions to room temperature at least a day before experiments can facilitate this process. Equilibrate cultured cells to room temperature before adding the reagents.



Notes (continued):

- b. Quenching of the firefly signal is accomplished in part by using a potent firefly luciferase inhibitor in the NanoDLR™ Stop & Glo® Buffer. This inhibitor may bind reversibly to surfaces like plastics. To prevent carryover of the inhibitor into subsequent assays, we recommend using disposable or dedicated containers for the NanoDLR™ Stop & Glo® Buffer. If you are using larger volumes and need to store the reagent in a Pyrex® bottle, reserve that bottle for the NanoDLR™ Stop & Glo® Buffer. Although the inhibitor displays dramatically less binding to Pyrex® than to plastic, the bottle should be rinsed thoroughly with water 7 times before performing standard glass washing to remove all inhibitor solution. When transferring the NanoDLR™ Stop & Glo® Buffer, use disposable plastic tips—preferably aerosol-resistant or positive-displacement tips. Perform the assay in disposable assay plates.
- c. Once reconstituted, the ONE-Glo™ EX Reagent will lose 10% activity in approximately 18 hours and 50% activity in approximately 6 days at 22°C. Unused reconstituted reagent can be placed at 4°C or -20°C for later use, protected from light. At 4°C, the ONE-Glo™ EX Reagent will lose 10% activity in approximately 3.5 days and 50% activity in approximately 1 month.
- d. We recommend preparing the NanoDLR™ Stop & Glo® Reagent fresh for each use. Once reconstituted, the reagent will lose 10% activity in approximately 32 hours and 50% activity in about 6 days at room temperature. If leftover reconstituted reagent remains after an experiment, it can be stored at 4°C or -20°C for later use, protected from light. Multiple freeze-thaw cycles should be avoided, and some loss of performance may be observed. At 4°C, the NanoDLR™ Stop & Glo® Reagent will lose 10% activity in approximately 3.5 days and 50% activity in approximately 18 days.
- e. If reconstituted ONE-Glo™ EX Reagent or NanoDLR™ Stop & Glo® Reagent is stored at 4°C or frozen, it must be warmed or thawed at temperatures below 25°C to ensure optimal performance. This is best accomplished by placing the reagent in a room-temperature water bath. Mix by inversion after thawing.

3.C. Assay Procedure for 96-Well Plates using Multichannel Pipettes

1. Remove plates containing mammalian cells expressing firefly and NanoLuc[®] luciferases from the incubator and equilibrate to room temperature (see Sections 4.C and 4.D for information about experimental setup). Use a small enough volume of cells in medium so that two equivalent volumes can be easily added to each well without risk of overflow. For 96-well plates, we recommend a starting volume of 80µl per well. Use an opaque, white tissue-culture plate to minimize cross-talk between wells and absorption of the emitted light.



Ensure that the plates used are compatible with the instrument used to measure luminescence.

2. **Measure firefly luciferase activity:**

- Add a volume of ONE-Glo[™] EX Reagent equal to the culture medium.
- Incubate the samples for at least 3 minutes to allow the cells to lyse and the samples to equilibrate. For optimal results, mix the samples by placing the plate on an orbital shaker (300–600 rpm) for 3 minutes.
- Measure firefly luminescence using settings specific to your instrument (consult the user manual). For 96-well plates on GloMax[®] instruments, integration times of 0.5–1 seconds are recommended. The luminescence intensity will decay with a signal half-life of approximately 2 hours.

3. **Measure NanoLuc[®] luciferase activity:**

- Add a volume of NanoDLR[™] Stop & Glo[®] Reagent equal to the original culture medium volume to each well and mix thoroughly. For complete quenching of the firefly luciferase signal and for an optimal NanoLuc[®] signal, it is important that the reagents are mixed well. For best results, incubate the plate on an orbital shaker at 600–900 rpm for at least 3 minutes after addition of NanoDLR[™] Stop & Glo[®] Reagent. If you do not have access to an orbital shaker, good results can be achieved by pipetting up and down twice to ensure sample mixing.
- After at least 10 minutes (including mixing time), measure NanoLuc[®] luminescence using settings specific to your instrument (consult the user manual). For 96-well plates on GloMax[®] instruments, integration times between 0.5–1 seconds are recommended. The luminescence intensity will decay with a signal half-life of approximately 2.5–3 hours. However, very high expression levels can lead to more rapid rates of signal loss due to substrate depletion or may cause the signal to exceed the linear range of detection for the instrument (see Section 4.D).

Note: If multiple plates are being compared and will be measured at different times, measure the luminescence for a given plate the same amount of time after addition of each reagent so that the signal decay of both reporters will be similar. Inclusion of a control on each plate will also help normalize data for plate-to-plate variation.

3.D. Assay Procedure for 96-Well Plates using Dual-Sample Injection

 Because of the possibility of carryover of the firefly luciferase inhibitor, we do not recommend using a single-injector system with the NanoDLR™ Assay. If desired, the NanoDLR™ Assay can be performed using a luminometer equipped with a dual auto-injection system. Injectors #1 and #2 should be dedicated to the delivery of ONE-Glo™ EX Reagent and NanoDLR™ Stop & Glo® Reagent, respectively, for every NanoDLR™ experiment run on a given instrument. Furthermore, injector #2 should not be used to deliver firefly luciferase reagents or reagents with assay chemistries that use Ultra-Glo™ recombinant luciferase (e.g., CellTiter-Glo® Assays). See Section 3.H for information on cleaning injectors after use.

Because of the detergent present in the ONE-Glo™ EX Reagent, injecting at too high a speed may cause excessive foaming, which could increase signal variability or reduce signal intensity. Verify that injectors and injection settings provide good sample mixing and data reproducibility without excessive foaming. The default settings on GloMax® instruments generally work well.

Protocol for Dual-Injection Assays in 96-Well Format

1. Prime injector #1 with ONE-Glo™ EX Reagent and injector #2 with NanoDLR™ Stop & Glo® Reagent.
2. Remove plates from the incubator and equilibrate to room temperature. The samples should be in a starting volume of 80µl per well.

 Ensure that the plates used are compatible with the instrument used to measure luminescence.

3. Inject all sample wells with 80µl of ONE-Glo™ EX Reagent from injector #1.
4. Incubate for 3 minutes.
5. Read the firefly luminescence in all sample wells using a 1-second integration time.
6. Inject all sample wells with 80µl of NanoDLR™ Stop & Glo® Reagent from injector #2.
7. Incubate for 5 minutes. (This incubation time is reduced compared to the 10 minutes recommended in the manual protocol in Section 3.C due to the additional time necessary to inject the reagent and because of good mixing achieved by the injectors.)
8. Read the NanoLuc® luminescence of all sample wells using a 1-second integration time.
9. Clean the dispensing lines as described in Section 3.H.

Notes:

- a. Program files to perform the above protocol are available for the GloMax® 96 Luminometer, the GloMax®-Multi+ Detection System and the GloMax® Discover System. Please contact Promega technical services (techserv@promega.com) for information. Alternatively, for the GloMax® Discover System and luminometers running Instinct™ Software, the protocol can be entered manually using the protocol composer feature and saved for later use.
- b. Using a dual-injector system allows the user to load a plate and walk away, while achieving excellent reproducibility of injection volumes and mixing. However, maximal throughput of a large number of samples is achieved by batch processing of plates and reagent delivery using a multichannel pipette.

3.E. Using the NanoDLR™ Assay with Cell Lysates Prepared with Passive Lysis Buffer

NanoDLR™ reagents are designed to be added directly to cells in media. However, the assay is also compatible with cell lysates made using Passive Lysis Buffer (PLB; Cat.# E1941). If performing experiments in dishes or lower density multiwell plates (6-well, 12-well, etc.), prepare cell lysates in PLB and transfer a portion of the lysate to a 96-well plate to perform the NanoDLR™ Assay. Instructions for preparing cell lysates using Passive Lysis Buffer can be found in the *Dual-Luciferase® Reporter (DLR™) Assay System Technical Manual #TM040* (available at www.promega.com/protocols), with the following change:

The DLR™ Assay protocol calls for 100µl of reagents to be added to 20µl of cell lysate; the NanoDLR™ protocol can be used with 20–80µl of cell lysate in PLB, with subsequent addition of 80µl ONE-Glo™ EX Reagent and 80µl NanoDLR™ Stop & Glo® Reagent. For the same number of moles of enzyme, the volume of PLB has little effect on the initial luminescence or signal half-life for either luciferase. The firefly luciferase signal tends to be somewhat brighter in PLB than in culture media, with a shorter signal half-life (see Section 4.F).

3.F. Considerations for Use of the NanoDLR™ Assay in 384- and 1,536-Well Formats

Use a modified version of the protocol given in Section 3.C for experiments in 384-well plates using reagent delivery with multichannel pipettes. A small enough volume of cells in medium should be used so that two equivalent volumes can be easily added to each well. We recommend a starting volume of 20µl per well for standard-volume, 384-well plates (total well volume about 100µl). Mixing may be more challenging in 384-well plates, so it is particularly important that plates be well-shaken, e.g., on an orbital shaker at 600–900 rpm for at least 3 minutes.

For automated high-throughput protocols, we recommend using a dedicated dispensing line (dispensing cassette and tubing) for delivery of the NanoDLR™ Stop & Glo® Reagent to minimize the possibility of carryover of the potent firefly luciferase inhibitor. If the dispensing line will be reused for other assays, materials that have come into contact with the firefly luciferase inhibitor should be thoroughly washed and the possibility for residual carryover understood (see Section 3.G) before subsequent use to deliver reagents for firefly luciferase assays or other assay chemistries that use Ultra-Glo™ recombinant luciferase. The assay protocol should generally follow that given in Section 3.D, but minimal incubation times should be identified during assay validation depending on the extent of sample mixing that can be achieved with a given system. For example, optimize injection speeds to give good mixing without excessive foaming.

3.G. Important Considerations for Preventing Carryover of the Firefly Inhibitor

The firefly luciferase inhibitor in the NanoDLR™ Stop & Glo® Reagent exhibits a reversible association with surfaces like plastic tubing, injectors, and bottles. If a surface that has come in contact with NanoDLR™ Stop & Glo® Reagent is not thoroughly cleaned after use, small amounts of the inhibitor may leach into other solutions that come into contact with that surface. Because of the potency of the inhibitor, even a small release of adsorbed compound can cause noticeable inhibition of firefly luciferase. This is particularly true of the ONE-Glo™ EX Reagent. Other firefly luciferase detection reagents such as ONE-Glo™ (Cat.# E6110, E6120, E6130) and Steady-Glo® Reagents (Cat.# E2510, E2520, E2550) are somewhat less sensitive to the inhibitor, and assays based on Ultra-Glo™ recombinant luciferase, like the CellTiter-Glo® Assay (Cat.# G7570, G7571, G7472, G7573), are significantly less sensitive.

For low-throughput protocols, the best way to eliminate the possibility of firefly luciferase inhibitor carryover is to deliver the NanoDLR™ Stop & Glo® Reagent using a multichannel pipette with disposable tips and reagent containers. When using automated dispensing, the best practice is to dedicate a particular injector, dispenser, or dispenser line to the NanoDLR™ Stop & Glo® Reagent. If they will be reused, any materials that have come into contact with the firefly luciferase inhibitor should be thoroughly washed prior to delivery of reagents for measuring firefly luciferase activity or other assay chemistries that use Ultra-Glo™ recombinant luciferase. Even after thorough washing, trace amounts of firefly luciferase inhibitor may still be present, potentially leading to a slight decrease in the initial luminescence and/or a decrease in the signal half-life for the first wells injected. Prime the firefly reagent immediately before use, and avoid prolonged incubation of the primed line to minimize the potential for leaching.

The design and materials used in injector systems vary greatly, so some systems may be more prone to inhibitor binding than others. In-line filters may represent a potentially difficult surface to clean off the adsorbed compound, so a disposable or dedicated filter is recommended. Different types of plastic tubing can have widely varying propensities for binding the firefly luciferase inhibitor. The following tubing types are ranked from best to worst for compatibility with the NanoDLR™ Assay:

Teflon® > PEEK (Polyetheretherketone)>>> silicone > PVC > Tygon®

A 1-hour ethanol soak should remove all detectable inhibitor from Teflon® and PEEK tubing. Other types of tubing, such as Tygon® or PVC, may require more stringent washing methods and should be avoided unless they can be dedicated to assays not involving firefly luciferase. GloMax® luminometers have Teflon® tubing in their injectors.

Binding to other surfaces, like those of containers, also varies widely. The following materials are ranked from best to worst for their ability to resist inhibitor binding:

Pyrex® or borosilicate >>> polycarbonate > polystyrene > polypropylene > HDPE

Ideally, containers used for NanoDLR™ Stop & Glo® Reagent should be dedicated for that use or disposable. However, Pyrex® bottles showed virtually no adsorption of the firefly inhibitor to their surface. Bottles used to store NanoDLR™ Stop & Glo® Reagent should be thoroughly rinsed 7 times with water or ethanol to remove all traces of the inhibitor before standard glass washing.

3.H. Wash Protocols for Automated Sample Injection Systems



Wash automated dispensing equipment immediately after use.

Protocol for Washing the Injector used to Dispense ONE-Glo™ EX Reagent on GloMax® Instruments

1. Immediately after completing the assay, flush the injector with three consecutive cycles (pump volumes) of water to remove the ONE-Glo™ EX Reagent.
2. Flush the injector with three consecutive cycles of 70–100% ethanol.
3. Flush the injector with three consecutive cycles of water.
4. Flush the injector with three consecutive cycles of air to purge the line of liquid.

This less-stringent ONE-Glo™ EX wash protocol can also be used for a dedicated injector dispensing NanoDLR™ Stop & Glo® Reagent, provided that the injector will never be used to deliver reagents for firefly luciferase or reagents that contain Ultra-Glo™ recombinant luciferase (e.g., CellTiter-Glo® Reagent).

If the injector is not dedicated to the delivery of the NanoDLR™ Stop & Glo® Reagent, the following more stringent wash protocol should be used after every experiment to prevent carryover of the firefly luciferase inhibitor to other assays. You can use the more stringent wash protocol for the ONE-Glo™ EX injector also.

Protocol for Washing the Injector used to Dispense NanoDLR™ Stop & Glo® Reagent on GloMax® Instruments

1. Immediately after completing the assay, flush the injector with nine consecutive cycles (pump volumes) of water to remove all the NanoDLR™ Stop & Glo® Reagent.
2. Flush the injector with nine consecutive cycles of ethanol, preferably warmed to at least 37°C in a water bath. Although 95–100% ethanol works best, 70% ethanol solutions can also be used.
3. Allow the injector system to soak in the ethanol for at least 60 minutes, preferably overnight.
4. Flush the injector with another three cycles of ethanol.
5. Flush the injector with at least three cycles of water to remove the ethanol.
6. Flush the injector with three cycles of air to remove liquid from the line.
7. If possible, use a dedicated injector tip and tubing for NanoDLR™ Stop & Glo® Reagent if the injector is likely to be used frequently to inject firefly luciferase reagents.

Cleaning Bulk Reagent Dispensers

1. Immediately after completing the assay, flush the line thoroughly with water (at least 10ml).
2. Flush the line thoroughly with 70–100% ethanol (at least 10 ml).
3. After using NanoDLR™ Stop & Glo® Reagent, soak the dispensing line overnight in ethanol. If compatible with the system and with the tubing material, remove the plastic tubing line from the dispenser and soak in ethanol at elevated temperature, e.g., in a 37°C water bath. Flush the line again with ethanol after any prolonged soak.
4. Flush the line with water to remove the ethanol.
5. Flush the line with air to remove liquid.

4. Assay Features and Experimental Design Considerations

4.A. Overview of the Nano-Glo® Dual-Luciferase® Reporter Assay System

The Nano-Glo® Dual-Luciferase® Reporter (NanoDLR™) Assay System is optimized for maximum detection sensitivity and ease-of-use. It offers a number of advantages compared to dual-luciferase assays that use firefly luciferase coupled to *Renilla* luciferase.

Comparison to Dual-Luciferase® (DLR™) Reporter Assay (Cat.# E1910): In contrast to the Dual-Luciferase® Reporter (DLR™) Assay System, the NanoDLR™ Assay provides a homogeneous assay format with only two reagent addition steps and glow-type signal kinetics. The DLR™ protocol requires a wash and two aspiration steps prior to adding Passive Lysis Buffer. After an extended incubation, sequential sample injection is required to measure the flash-type response from each luciferase (signal half-lives of 12–15 and 3 minutes, respectively), greatly limiting assay throughput. In contrast, the NanoDLR™ Assay reagents can be added to all samples at once followed by much faster cycling of plates for luminescence measurement. When comparing dynamic reporters in transfected cells, the glow-type luminescence of NanoLuc®-PEST can be as bright as the flash-type luminescence from firefly luciferase *luc2*-PEST in the DLR™ Assay.

Comparison to Dual-Glo® Luciferase Assay System (Cat.# E2920): Compared to the Dual-Glo® Luciferase Assay System, the NanoDLR™ Assay provides orders of magnitude greater sensitivity for NanoLuc® luciferase compared to *Renilla* luciferase. This is achieved by using a firefly luciferase inhibitor that is 100-fold more potent and by exploiting the inherent differences in brightness and stability of NanoLuc® luciferase. The sensitive measurement of low concentrations of NanoLuc® luciferase, even in the presence of very high concentrations of firefly luciferase, allows either or both of the reporters to be used to measure a biological response. Additionally, the ONE-Glo™ EX Reagent has a number of advantages over the Dual-Glo® Reagent with respect to stability, odor, brightness, and quenching by phenol red (see Section 4.B).

4.B. NanoDLR™ Assay Features and Advantages

An ideal dual-luciferase assay maximizes detection sensitivity for both reporters using a protocol that is easy to perform. The NanoDLR™ Assay exploits the inherent differences between the structure and enzymology of firefly and NanoLuc® luciferases (Figure 2) to allow measurements of both luciferases from the same sample using an add-read-add-read, or "Stop & Glo", format. The addition of ONE-Glo™ EX Luciferase Assay Reagent promotes cell lysis and provides the necessary substrates to measure firefly luciferase activity. After measuring the firefly signal, the NanoDLR™ Stop & Glo® Reagent is added to both quench firefly luciferase activity (>1,000,000-fold) and provide the substrates needed to measure the high-intensity luminescence from NanoLuc® luciferase.

Alternative formats exist for dual-luciferase assays, including those that exploit differences in the emission wavelengths of both luciferases, but these assays can suffer from poor sensitivity due to substantial signal overlap and the loss of signal associated with the use of filters. With the NanoDLR™ Assay, the most popular luciferase (firefly) is coupled to our brightest and most dynamic luciferase (NanoLuc®) to create the next-generation dual-luciferase assay. The NanoDLR™ Assay maximizes the potential of both reporters in assay development, expanding the possible options for experimental formats (see Section 4.C).

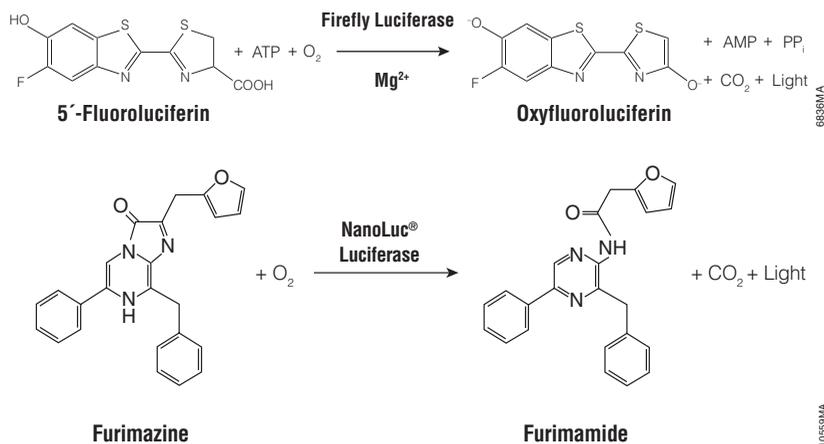


Figure 2. Bioluminescent reactions catalyzed by firefly and NanoLuc® luciferases.

ONE-Glo™ EX Reagent: Improved Chemistry for Detection of Firefly Luciferase Activity

In the NanoDLR™ Assay protocol, firefly luciferase activity is read first (after addition of the ONE-Glo™ EX Luciferase Assay Reagent). Firefly luciferase is a 61kDa monomer that catalyzes the mono-oxygenation of beetle luciferin (7,8). The enzyme uses ATP and molecular oxygen as co-substrates and is fully functional upon translation, making it ideally suited as a genetic reporter (9,10).

ONE-Glo™ EX Luciferase Assay Reagent retains many of the beneficial traits of ONE-Glo™ Reagent (Cat.# E6110), but uses new assay chemistry that increases both the stability of the reconstituted reagent prior to use and the stability of the luminescence signal in cell lysates. The firefly luciferase signal decays with a half-life of approximately 2 hours (compared to ~70 minutes using ONE-Glo™ Reagent). When stored at room temperature or 4°C, reconstituted ONE-Glo™ EX Luciferase Assay Reagent shows a 50% decrease in reagent potency after approximately 6 days or 1 month, respectively, a substantial increase in stability compared to ONE-Glo™ Reagent (Figure 6).

Increases in both signal and reagent stability make ONE-Glo™ EX Reagent even better suited to HTS protocols that require glow-type signal decay kinetics and stable reconstituted reagents for batch processing of plates. Like ONE-Glo™ Reagent, ONE-Glo™ EX Reagent uses 5'-fluoroluciferin as a substrate (Figure 2), allowing lower pH reaction conditions that help to maximize reagent stability and minimize quenching by phenol red. ONE-Glo™ EX Reagent contains no thiol compounds, which can give other firefly luciferase reagents a strong odor.

NanoDLR™ Stop & Glo® Reagent Advantages

After firefly luminescence is measured, the NanoDLR™ Stop & Glo® Reagent is added to both inhibit the firefly luciferase signal and to provide the NanoLuc® substrate. NanoLuc® luciferase is a 19.1kDa enzyme engineered for optimal performance as a luminescent reporter (4). The enzyme uses a novel coelenterazine analog, furimazine, as substrate in an ATP-independent reaction (Figure 2). NanoLuc® luciferase was developed for both enzyme stability and high intensity luminescence, being brighter than both firefly and *Renilla* luciferases (4). NanoLuc® luciferase is fully functional upon translation and performs exceptionally well as a reporter gene. When fused to a PEST sequence (NanoLuc®-PEST), the intracellular protein half-life decreases from several hours to tens of minutes. The shorter protein half-life compared to firefly or firefly-PEST allows NanoLuc®-PEST to respond more quickly to changes in transcription, typically with greater signal-to-background. In addition, because of the inherent brightness of NanoLuc® luciferase, NanoLuc®-PEST luciferase remains brighter than firefly luciferase-PEST (*luc2*-PEST).

Reduced Background Signal

The NanoDLR™ Stop & Glo® Reagent is optimized for reduced background, allowing maximal sensitivity for measuring NanoLuc® luciferase activity. Besides the normal background signal associated with the luminometer, the two sources of background for the NanoLuc® signal are: 1) furimazine autoluminescence and 2) residual firefly luminescence. The NanoDLR™ Stop & Glo® Reagent is designed to minimize autoluminescence caused by the auto-oxidation of furimazine. This autoluminescence can be reduced further by decreasing the amount of serum in samples. By using a 100-fold more potent inhibitor, the NanoDLR™ Assay provides far greater quenching of firefly luciferase signal compared to the Dual-Glo® Assay. The background-subtracted firefly luminescence is quenched at least 1,000,000-fold after a 10-minute incubation in a well-mixed sample, providing a quenched signal very close to the autoluminescence background or machine background (depending on the luminometer used), even when starting with an exceptionally high firefly luciferase signal (Figure 3).

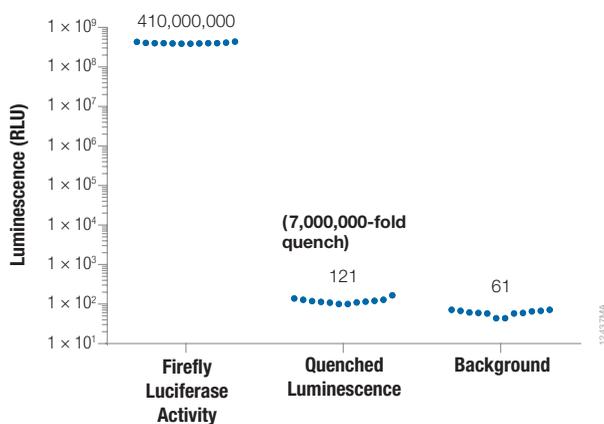


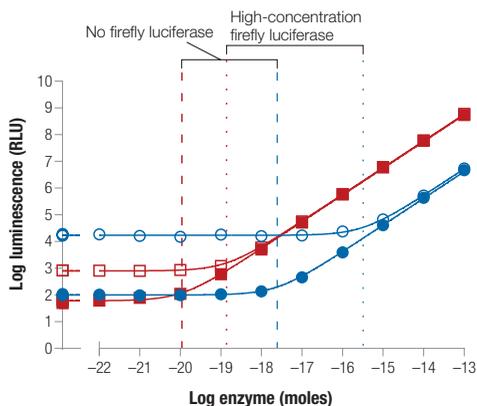
Figure 3. Measurement of luciferase activities before and after addition of NanoDLR™ Stop & Glo® Reagent. DMEM containing 0.1% Prionex® was supplemented with either 1.38 µg/ml purified firefly luciferase or nothing, and 80µl of each was added to 12 wells of a 96-well plate. ONE-Glo™ EX Reagent (80µl) was added to all wells and mixed, and firefly luminescence was measured after 3 minutes. NanoDLR™ Stop & Glo® Reagent (80µl) was then added to all wells and mixed. After 10 minutes, the quenched firefly luminescence and the background luminescence (no luciferase) were measured. “Fold-quench” was calculated by dividing the initial luminescence by the background-subtracted quenched luminescence. All 12 data points are shown for each condition.

Sensitivity

Improved quenching of the firefly luciferase signal and use of a much brighter and more stable enzyme yields orders of magnitude greater sensitivity for measurements of NanoLuc[®] luciferase in the NanoDLR[™] Assay compared to *Renilla* luciferase in the Dual-Glo[®] Assay. Using purified protein *in vitro*, NanoLuc[®] luciferase can be detected above background at concentrations greater than 100-fold lower than *Renilla* luciferase (Figure 4, closed symbols). When starting with a high firefly luciferase signal, NanoLuc[®] luciferase can be detected above background at concentrations greater than 1,000-fold lower than *Renilla* luciferase (Figure 4, open symbols). Because of its enhanced stability, the intracellular accumulation of NanoLuc[®] luciferase also can be expected to contribute to increased sensitivity over *Renilla* luciferase. In contrast, intracellular protein levels are kept lower with NanoLuc[®]-PEST luciferase to improve dynamics, but the luminescence is still substantially higher than that of *Renilla* luciferase.

A. Comparison to Dual-Glo[®] Assay

- *Renilla* luciferase detected by Dual-Glo[®] Assay, no firefly luciferase
- *Renilla* luciferase detected by Dual-Glo[®] Assay, firefly luciferase present
- NanoLuc[®] luciferase detected by NanoDLR[™] Assay, no firefly luciferase
- NanoLuc[®] luciferase detected by NanoDLR[™] Assay, firefly luciferase present



B. Comparison to DLR[™] Assay

- *Renilla* luciferase detected by DLR[™] Assay, no firefly luciferase
- *Renilla* luciferase detected by DLR[™] Assay, firefly luciferase present
- NanoLuc[®] luciferase detected by NanoDLR[™] Assay, no firefly luciferase
- NanoLuc[®] luciferase detected by NanoDLR[™] Assay, firefly luciferase present

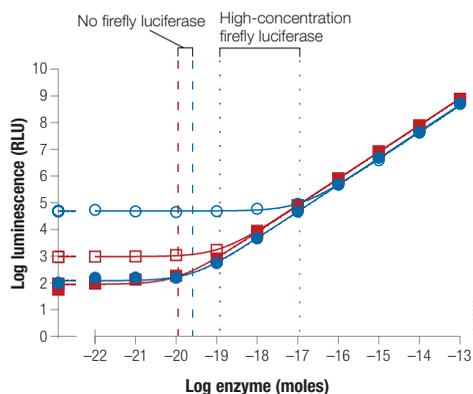


Figure 4. Brightness of NanoLuc[®] luciferase and improved quenching of firefly luciferase in the NanoDLR[™] Assay give greater sensitivity than *Renilla* luciferase in the Dual-Glo[®] and DLR[™] Assays. Luminescence from a titration of purified *Renilla* or NanoLuc[®] luciferase was compared in the presence or absence of a high concentration (100nM) of purified firefly luciferase. The amount of enzyme necessary to achieve a signal double the background luminescence was used as a measure of sensitivity. **Panel A.** Enzymes were diluted into DMEM supplemented with 0.1% Prionex[®], and luminescence of 80µl of each dilution measured 10 minutes after addition of Dual-Glo[®] Reagents to *Renilla* samples and NanoDLR[™] Reagents to NanoLuc[®] samples; n = 4. The NanoDLR[™] Assay was >200-fold more sensitive than the Dual-Glo[®] Assay in the absence of firefly luciferase (dashed lines), and >2,000-fold more sensitive in the presence of 100nM firefly luciferase (dotted lines). **Panel B.** The enzymes were diluted into Passive Lysis Buffer supplemented with 0.1% Prionex[®], and 20µl transferred to a plate. DLR[™] reagents (100µl) were added to *Renilla* samples by injector, and luminescence measured according to the standard DLR[™] protocol. NanoLuc[®] luminescence was measured following injection of 80µl NanoDLR[™] reagent according to the NanoDLR[™] injector protocol (Section 3.D); n = 4. In the absence of firefly luciferase, the glow-type NanoLuc[®] signal of the NanoDLR[™] Assay was about two times more sensitive than the flash-type *Renilla* signal of the DLR[™] Assay (dashed lines). In the presence of a high firefly signal, the NanoDLR[™] Assay was nearly 100-fold more sensitive (dotted lines).

Signal Intensity and Stability

The signal intensity and stability of the NanoDLR™ Assay compare favorably to existing single or dual-luciferase assay reagents with glow-type signals. Figure 5, Panel A illustrates the differences in initial luminescence and signal stability for glow-type reagents used to measure firefly luciferase. Compared to ONE-Glo™ Reagent, ONE-Glo™ EX Reagent provides a slightly dimmer signal (approximately 2/3 that of the ONE-Glo™ Reagent), but with compensating increases in both signal half-life (~115 versus ~75 minutes on average) and reagent stability (see next page). Compared to Dual-Glo® Reagent, the signal from ONE-Glo™ EX Reagent is approximately 1.5- to 2-fold brighter with a correspondingly shorter signal half-life. For NanoLuc® luciferase, the NanoDLR™ signal is usually slightly brighter than the signal in the Nano-Glo® Assay, with a longer signal half-life (~2.5-3.0 versus ~2 hours; Figure 5, Panel B).

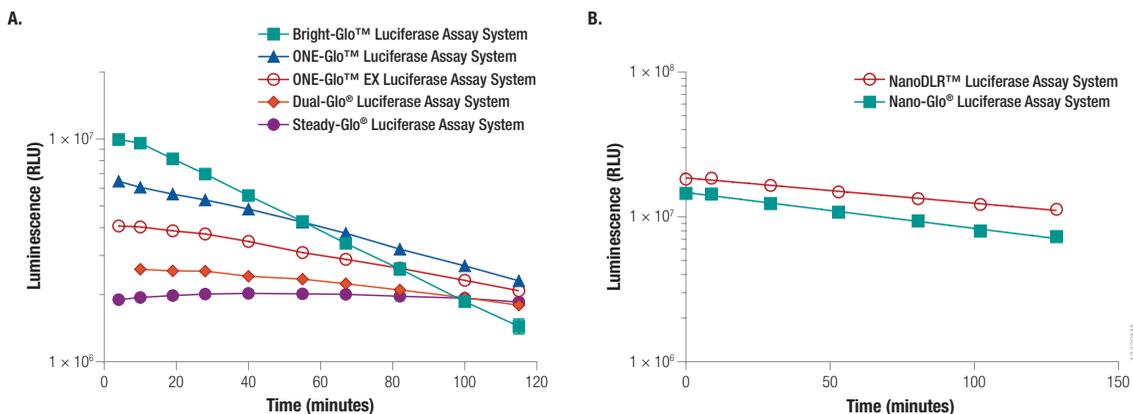


Figure 5. ONE-Glo™ EX and NanoDLR™ Stop & Glo® Reagents provide bright and stable luminescence that can be measured for hours. Panel A. One hundred microliters of purified firefly luciferase (13.8ng/ml in DMEM with 0.1% Prionex® as carrier) was combined with 100µl of the respective reagent in a 96-well plate. Luminescence was measured periodically over 2 hours, n = 8. **Panel B.** Eighty microliters of purified NanoLuc® luciferase (1ng/ml in DMEM with 0.1% Prionex® as carrier) was combined with either 80µl of Nano-Glo® Reagent or 80µl of ONE-Glo™ EX Reagent followed by 80µl of NanoDLR™ Stop & Glo® Reagent in a 96-well plate. Luminescence was measured periodically over 2 hours; n = 6.

Reagent Stability

The NanoDLR™ Reagents are designed to provide extended stability once reconstituted compared to similar reagents. This means that luminescence will remain more constant over long experiments. It also makes the reagents more convenient to use, store, and reuse, reducing waste from leftover reagent. Once reconstituted, ONE-Glo™ EX Reagent will lose 10% of its activity after ~18 hours and 50% of its activity after ~6 days at 22°C (Figure 6). Even after prolonged incubation, ONE-Glo™ EX Reagent may still retain about 20–25% of its original activity for some time. Unused ONE-Glo™ EX Reagent can be stored at –20°C, but multiple freeze-thaw cycles should be avoided. The reagent can also be stored at 4°C, at which temperature it will lose 10% activity after ~3.5 days and 50% activity after about a month. Once the NanoDLR™ Stop & Glo® Reagent is reconstituted, it will lose 10% activity in ~32 hours and 50% activity in ~6 days at room temperature. Only the amount of reagent needed for an experiment should be reconstituted. If leftover NanoDLR™ Stop & Glo® Reagent remains after an experiment, it can be stored frozen at –20°C, but multiple free-thaw cycles should be avoided, and some loss in performance may be observed. The reagent can also be stored at 4°C, at which temperature it will lose 10% activity after ~3.5 days and 50% activity after ~18 days.

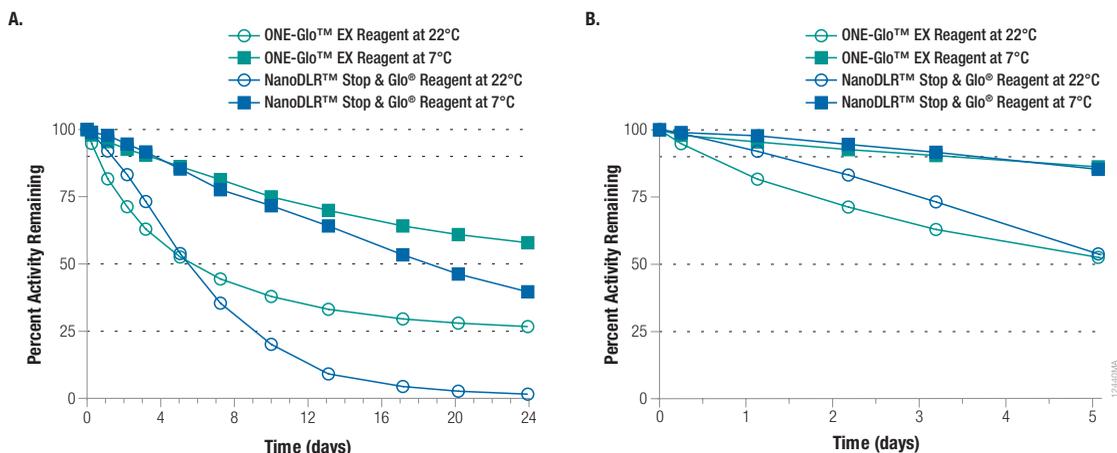


Figure 6. The NanoDLR™ Reagents display extended stability once reconstituted. Reconstituted reagents were stored at 22°C or 7°C and frozen at –70°C at defined times. Upon thawing and equilibrating to room temperature, the ONE-Glo™ EX samples were combined with an equal volume of 13.8 ng/ml purified firefly luciferase in DMEM supplemented with 10% FBS. The NanoDLR™ Stop & Glo® Reagent samples were combined with double the volume of a 1:1 mixture of 1ng/ml purified NanoLuc® luciferase in DMEM plus 10% FBS and ONE-Glo™ EX Reagent. The relative functionality was calculated as the luminescence signal intensity for each sample (measured 3 minutes after enzyme addition) relative to the signal intensity of the sample placed at –70°C with no 22°C incubation; n = 4. **Panel A.** Time course over 24 days. **Panel B.** Time course over the first 5 days only.

4.C. Assay Formats and Design of Experiments

The sensitivity of the NanoDLR™ Assay permits its use in multiple assay formats. Like the DLR™ and Dual-Glo® Assays, firefly luciferase can be used as the experimental reporter to measure a biological response, with NanoLuc® luciferase replacing *Renilla* luciferase as the constitutive control. Alternatively, users can exploit the beneficial traits of NanoLuc®-PEST luciferase as the experimental reporter and use firefly as the constitutive control. The extreme sensitivity of the NanoDLR™ Assay also enables use of two experimental reporters, with each monitoring a different biological response.

Experimental Reporter Plus Control Reporter

A common dual-luciferase assay configuration is to use one luciferase as the experimental reporter and the second luciferase as a constitutively expressed control. Normalizing the activity of the experimental reporter to the activity of the control reporter minimizes well-to-well and experiment-to-experiment variability caused by such factors as differences in cell number, lysis efficiency, cell viability, transfection efficiency, temperature, and measurement time. Thus, dual-luciferase assays can often allow more reliable interpretation of experimental data by providing an internal standard for experimental parameters that can be difficult to control.

Firefly luciferase or firefly luciferase-PEST (*luc2P*) can be used as the experimental reporter and NanoLuc® luciferase used as the constitutive control. The high-intensity luminescence and stable nature of the NanoLuc® protein distinguish NanoLuc® from *Renilla* luciferase. When expressed in mammalian cells under equivalent conditions, NanoLuc® luminescence using the NanoDLR™ Assay is often about 1,000-fold brighter than *Renilla* luminescence using the Dual-Glo® Assay. As a result, lower amounts of control plasmid can be transfected for NanoLuc® luciferase, potentially minimizing transcriptional squelching of the experimental reporter. If sensitivity is an issue for the control, NanoLuc® and the NanoDLR™ Assay will provide the brightest possible signal. See Section 4.D for considerations on the choice of constitutive expression construct and the amounts of DNA to transfect.

For the brightest possible signal and maximal response, use NanoLuc®-PEST luciferase as the experimental reporter and firefly luciferase as the constitutive control. When expressed in mammalian cells under equivalent conditions, the signal from NanoLuc®-PEST using the NanoDLR™ Assay is often more than tenfold brighter than the signal from *luc2*-PEST using the Dual-Glo® Assay. Because of its short intracellular protein half-life, the NanoLuc®-PEST fusion will also respond more quickly and typically with a greater signal-to-background ratio compared to firefly luciferase or *luc2*-PEST. Unfused NanoLuc® luciferase also can be used as the experimental reporter to maximize luminescence, but the longer protein half-life can provide a slower response to changes in gene transcription and lower signal-to-background ratios compared to firefly luciferase, *luc2*-PEST or NanoLuc®-PEST.

Normalizing data by calculating the ratio of the experimental reporter over the control reporter can help minimize some types of experimental variability and permit better comparisons between measurements on different luminometers or at different times. If multiple plates are measured at varying times after the addition of reagents, order the plates so that the luminescence is measured the same amount of time following addition of each respective reagent. Because the firefly and NanoLuc® luciferase signals decay at similar rates, the ratio of their activity should change little over time, as long as both are measured the same amount of time after reagent addition, e.g., measuring firefly luminescence 40 minutes after ONE-Glo™ EX Reagent addition and measuring NanoLuc® luminescence 40 minutes after NanoDLR™ Stop & Glo® Reagent addition.

Two Experimental Reporters

The excellent sensitivity of the NanoDLR™ Assay enables experimental formats where both luciferases are used as experimental reporters. The potent inhibition of firefly luciferase in the NanoDLR™ Assay assures that basal levels of NanoLuc®-PEST expression can be detected without the risk of signal bleedthrough, even when *luc2*-PEST expression levels are very high. This assay format can increase the amount of information that is obtained from a given sample. However, without the internal control reporter, it is important that the experiment is designed to minimize variability from cell number, transfection efficiency, pipetting volumes, etc. The most responsive reporters are the PEST-destabilized forms of firefly and NanoLuc® luciferase.

Coincidence Reporter System

High-throughput screens using reporter gene assays can have a significant number of non-relevant hits generated due to direct interaction of compounds with the reporter enzyme. Compounds truly modulating the biological pathway of interest can be differentiated from those affecting the stability or activity of the reporter enzyme by using a coincidence reporter system in which transcriptional activation leads to stoichiometric expression of two orthologous reporters with dissimilar profiles of compound interference (5). Firefly and NanoLuc® luciferases make a particularly attractive pair of orthologous reporters because of the differences in their respective substrates and their low overlap for interfering compounds (11).

In the second-generation coincidence reporter system, firefly luciferase and NanoLuc®-PEST luciferase are expressed from the same promoter, and stoichiometric amounts of the non-fused reporter enzymes are generated using ribosome skipping mediated by the viral P2A peptide sequence (6). Following insertion of the promoter of interest into the *luc2*-2A-NlucP construct (Cat.# N1461 or N1471), transcriptional activation is measured using the NanoDLR™ Assay. Compounds increasing transcription will show a coincident increase in the activity of both reporters, while compounds affecting the stability or activity of a reporter will generally show an effect on only one reporter. Obtaining an independent readout from two distinct reporters can also improve the signal-to-noise ratio and increase confidence in hits that cause only small effects on transcription.

Firefly Transcriptional Reporter Plus Protein Fusion to NanoLuc® Luciferase

Another assay format enabled by the NanoDLR™ Assay is coupling transcriptional readout of a firefly reporter gene to measurement of the stability of a protein fusion to NanoLuc® luciferase. For instance, many stress response pathways are regulated by dynamic protein turnover of transcription factors. Monitoring the luminescence of NanoLuc® fusions can be a simple way to follow this regulated event that occurs upstream of transcription and translation. Because of its brightness and small size, NanoLuc® luciferase is an ideal fusion partner to act as a protein stability sensor. Brightness is critical because it allows the NanoLuc® fusion protein to be expressed at much lower concentrations than fusions to other luciferases, i.e., at more physiologically relevant levels. Therefore, the most relevant biological response will generally be obtained by expressing the NanoLuc® fusion protein off a relatively weak constitutive promoter, transfecting low amounts of DNA, or diluting the DNA into promoterless carrier DNA (e.g., Transfection Carrier DNA; Cat.# E4881).

4.D. Considerations for Transfection

Precautions for High Expression Levels of NanoLuc® Luciferase

NanoLuc® luciferase is able to maintain a glow-type luminescent signal over a broad concentration range, providing a signal half-life of approximately 2.5–3.0 hours. However, the signal half-life can decrease significantly at high concentrations of enzyme, likely due to rapid depletion of substrate. If you see aberrant results and suspect this may be the cause, we suggest taking several additional measurements over time to determine whether the signal decay is similar in all wells. If the high luminescence signals are decaying much more rapidly than lower signals, this provides an estimate of the luminescence value for a given instrument and experimental setup above which rapid substrate depletion may occur. This value is typically near or often can exceed the linear dynamic range of the luminometer used for detection.

If you have confirmed samples that have an increased signal decay rate or exceed the linear dynamic range of the instrument, modify your experimental protocol to reduce the amount of NanoLuc® luciferase present in the cell lysate:

1. Use a less active promoter for constitutive expression. For example, rather than using full-length CMV promoter, switch to another promoter such as PGK (pNL1.1.PGK[*Nluc*/PGK] Vector; Cat.# N1441) or TK (pNL1.1.TK[*Nluc*/TK] Vector; Cat.# N1501) or a CMV deletion mutant.
2. For transient transfection assays, transfect less NanoLuc® DNA per well. For example, if co-transfecting 50ng total DNA into a well, instead of transfecting 25ng firefly DNA and 25ng NanoLuc® DNA, transfect 49.5ng firefly DNA and 0.5ng NanoLuc® DNA. Alternatively, the NanoLuc® DNA can be diluted with carrier DNA and transfected using the same lipid:DNA ratio (e.g., transfect 5ng luciferase-expressing DNA + 45ng carrier DNA per well). Ensure that the carrier DNA plasmid does not contain eukaryotic regulatory sequences or express proteins that would affect the experimental results (e.g., Transfection Carrier DNA, Cat.# E4881).
3. Plate fewer cells per well.
4. Use a NanoLuc®-PEST expression vector instead of unfused NanoLuc® luciferase.

Dilution of Control Plasmids

In general, the control reporter should be expressed at a level high enough to be easily and accurately measured, but low enough that substrate is not rapidly depleted and the luminescence is not outside the linear range of the detection instrument. Furthermore, transfection of high amounts of control plasmid may lead to transcriptional squelching or other interference with the experimental promoter, i.e., *trans* effects between promoters (12). Therefore, control reporter constructs are often co-transfected at levels 10-1,000 times lower than the experimental reporter. Especially with NanoLuc[®] luciferase, we recommend expressing the control reporter from a constitutive promoter that is less active than a CMV promoter, such as PGK or TK. The appropriate promoter or dilution of DNA for transfection will depend largely on the experimental conditions, i.e., the size of the wells, the number of cells, and the ability of the cells to be transfected.

For example, to study the NF- κ B response in HEK293 cells using firefly luciferase as the experimental reporter and NanoLuc[®] luciferase as the constitutively expressed control reporter, you might transfect a well of cells with a total of 50ng DNA as follows:

- 49.5ng NF- κ B-RE firefly-PEST (pGL4.32[*luc2P*/NF- κ B-RE/Hygro] Vector, Cat.# E8491)
- 0.5ng TK-NanoLuc[®] (pNL1.1.TK[*Nluc*/TK] Vector, Cat.# N1501)

If, instead, you wish to use NanoLuc[®]-PEST as the experimental reporter and firefly luciferase as the constitutive control, we recommend increasing the strength of the constitutive promoter for firefly luciferase and its relative amount of DNA compared to the NanoLuc[®] luciferase experimental reporter. With a high number of easily transfected cells like HEK293, however, we suggest diluting the DNA into carrier DNA to avoid exceeding the linear range of the detector for the bright NanoLuc[®] signal. For example, for a total of 50ng DNA per well, you might transfect the following:

- 5ng NF- κ B-RE NanoLuc[®]-PEST (pNL3.2. NF- κ B-RE[*NlucP*/ NF- κ B-RE/Hygro] Vector, Cat.# N1111)
- 5ng CMV-firefly (pGL4.50[*luc2*/CMV/Hygro] Vector, Cat.# E1310)
- 40ng Transfection Carrier DNA (Cat.# E4881)

Figure 7 shows the expected relative luminescence of firefly and NanoLuc[®] luciferases expressed from various constitutive promoters in the NanoDLR[™] Assay.

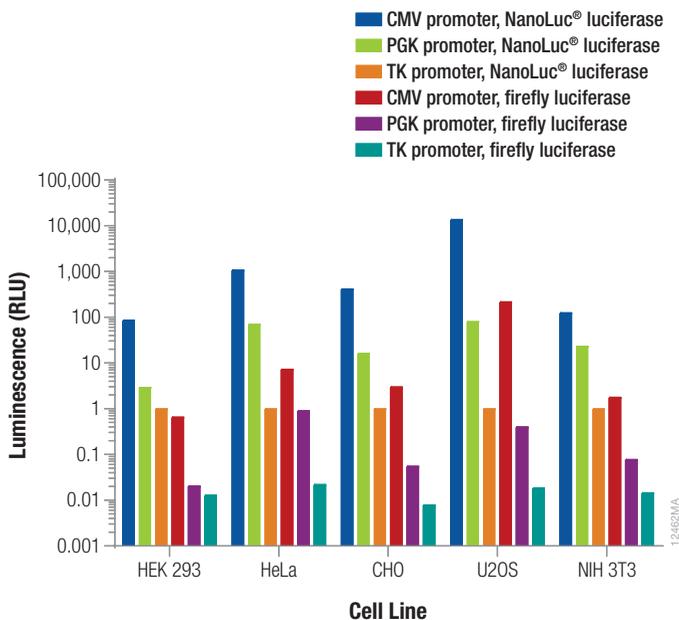


Figure 7. Relative luminescence for firefly and NanoLuc® luciferases expressed from constitutive promoters in multiple cell types. Cells were transiently transfected with varying amounts of DNA containing firefly or NanoLuc® luciferase expressed from CMV, PGK, or TK constitutive promoters. After 24 hours expression, luminescence was measured using the NanoDLR™ Assay, and the approximate relative luminescence compared to TK-NanoLuc® was calculated for those DNA amounts giving usable signals. These values can inform the choice of control reporter construct and the appropriate amount or dilution of DNA to transfect to achieve a desired signal.

4.E. Relative Response Ratios

A Relative Response Ratio (RRR) can be determined to quantitate the effect of an experimental treatment on reporter gene expression. The RRR permits the comparison of multiple treatments from different experiments because it provides a framework to compare treatment effects.

Calculation of RRR requires the inclusion of two sets of controls on each plate: a positive control that provides maximal luminescence and a negative control that provides minimal luminescence. If these two samples are included on every plate, the effect of each new compound can be quantitatively evaluated by its influence on the experimental reporter within the context of the positive and negative control.

If the ratio of experimental reporter luminescence to control reporter luminescence is 53 for the positive control, 1.3 for the negative control, and 22 for the experimental treatment, all of these values can be scaled so that the positive control is assigned the value of 1 and the negative control is assigned the value of 0. The RRR for each experimental treatment can then be calculated using this formula:

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

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

$$\text{RRR} = \frac{22 - 1.3}{53 - 1.3} = 0.40 \text{ or } 40\%$$

The experimental compound is 40% as effective as the positive control at increasing expression of the experimental reporter at this concentration.

Note: If the absolute luminescence values are close to background, the luminescence values will need to have background subtracted before the ratios are calculated.

4.F. Conditions Affecting Assay Performance

Culture Medium

When performing the NanoDLR™ Assay, the culture medium and any compounds added to the medium make up half of the volume of the firefly luciferase reaction, and one third of the volume of the NanoLuc® luciferase reaction. Although the reagents are designed to work with many common culture media, compositional differences between different media (e.g., the concentration of phenol red) may affect the intensity and duration of the luminescent signal (Figure 8). These differences are generally small and do not diminish the utility of the NanoDLR™ Assay; however, controls should be incorporated into every batch of plates to correct for this variability.

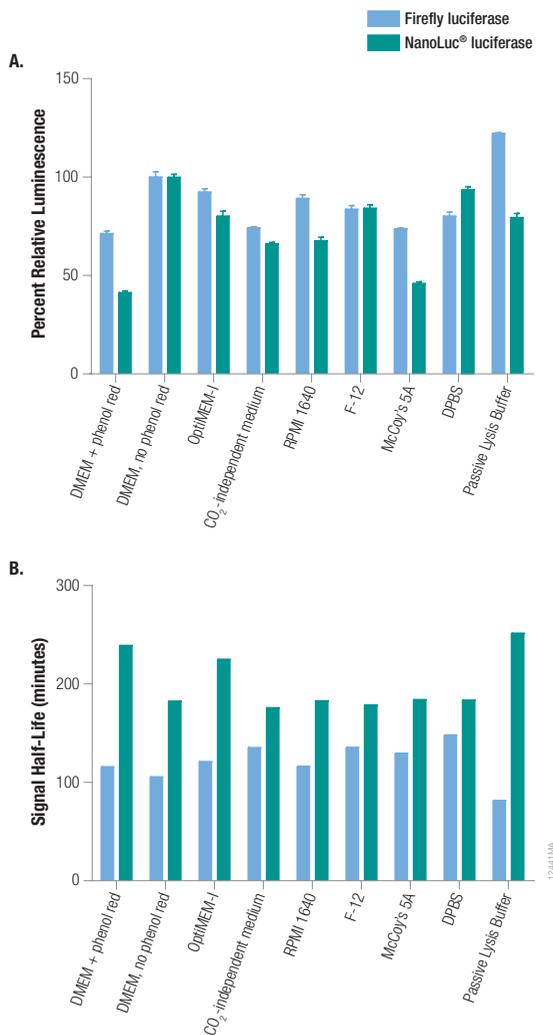


Figure 8. Relative luminescence intensity and signal stability of firefly and NanoLuc[®] luciferases in common media types. HEK293 cells were transiently transfected with either firefly luciferase or NanoLuc[®] luciferase expression constructs. Cells were harvested, split equally among tubes, centrifuged, and resuspended to 3.4×10^5 cells/ml in various media types supplemented with 10% FBS, with the exception of Passive Lysis Buffer, which contained no serum and was incubated with the cells for 15 minutes. Eighty microliters of cells were added to the wells of a 96-well plate, and 80 μ l of ONE-Glo[™] EX Reagent was added to all wells. Then, 80 μ l of NanoDLR[™] Stop & Glo[®] Reagent was added to the wells containing NanoLuc[®] luciferase. Luminescence was measured periodically over 3 hours.

Panel A. Firefly (n = 4) and NanoLuc[®] luminescence (n = 8) at 3 minutes are shown relative to the measurements from DMEM without phenol red. **Panel B.** The signal stability in different media is expressed as the half-life of the signal decay over 3 hours.

Serum

The NanoDLR™ Assay Reagents are designed for use with serum concentrations from 0 to 10%, and the luminescent signals generated are minimally affected by the presence of serum (Figure 9). Serum can, however, increase the background autoluminescence of furimazine in the NanoDLR™ Stop & Glo® Reagent. Therefore, if maximal sensitivity of the NanoLuc® signal is required near background levels, we recommend performing a media exchange with serum-free medium before adding NanoDLR™ Reagents. (See Section 4.G for suggestions on increasing sensitivity.)

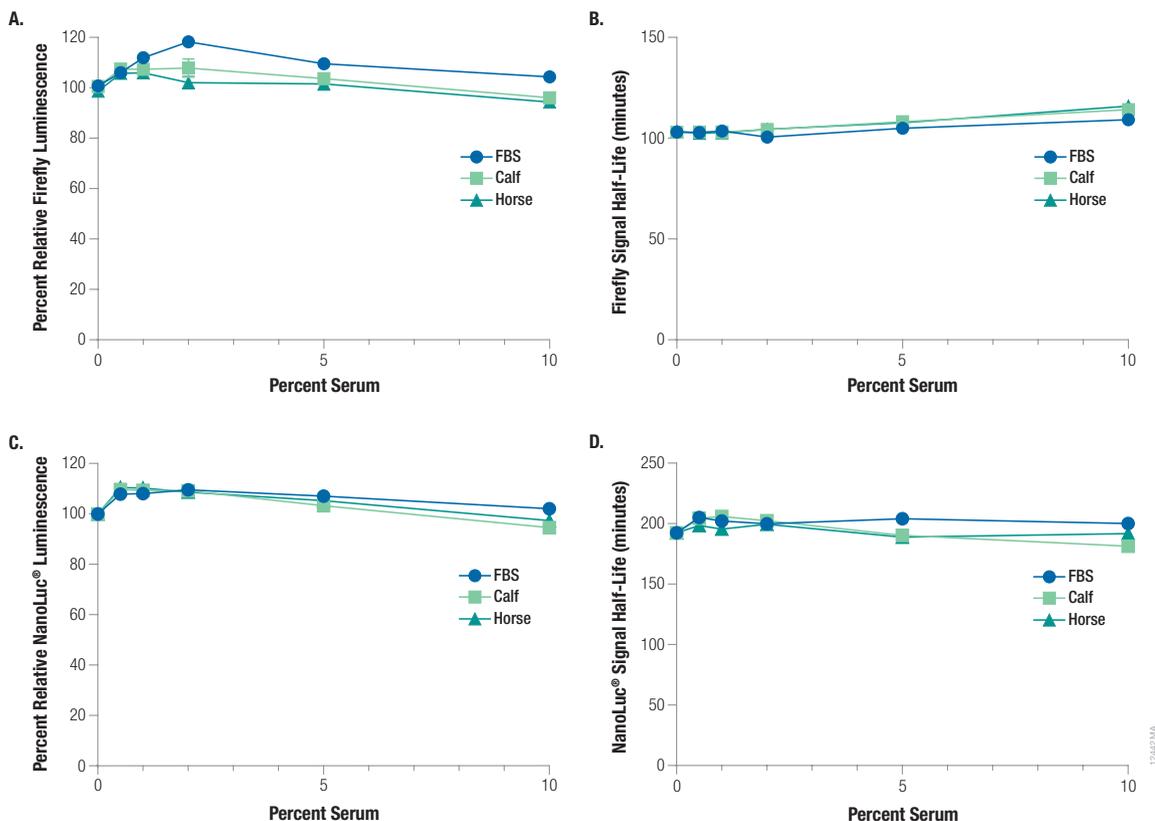


Figure 9. Effect of serum on luminescence intensity and signal stability. Purified firefly (6.88 ng/ml) or NanoLuc® luciferase (0.50 ng/ml) was diluted into DMEM plus 0.1% Prionex® with varying concentrations of fetal bovine serum (FBS), calf serum, or horse serum. Enzyme solutions (80µl per well) were added to 96-well plates, and 80µl of ONE-Glo™ EX Reagent added to all wells. Then, 80µl of NanoDLR™ Stop & Glo® Reagent was added to the wells containing NanoLuc® luciferase. Luminescence was measured periodically over 2 hours to calculate the signal stability (half-life); n = 4. **Panel A.** Firefly luminescence relative to no serum. **Panel B.** Firefly signal half-life. **Panel C.** NanoLuc® luminescence relative to no serum. **Panel D.** NanoLuc® signal half-life.

Organic Solvents

Organic solvents may be present in reporter gene assays because they are used to solubilize screening compounds. DMSO, ethanol and methanol in concentrations up to 3% have little effect on luciferase luminescence or signal kinetics (Figure 10).

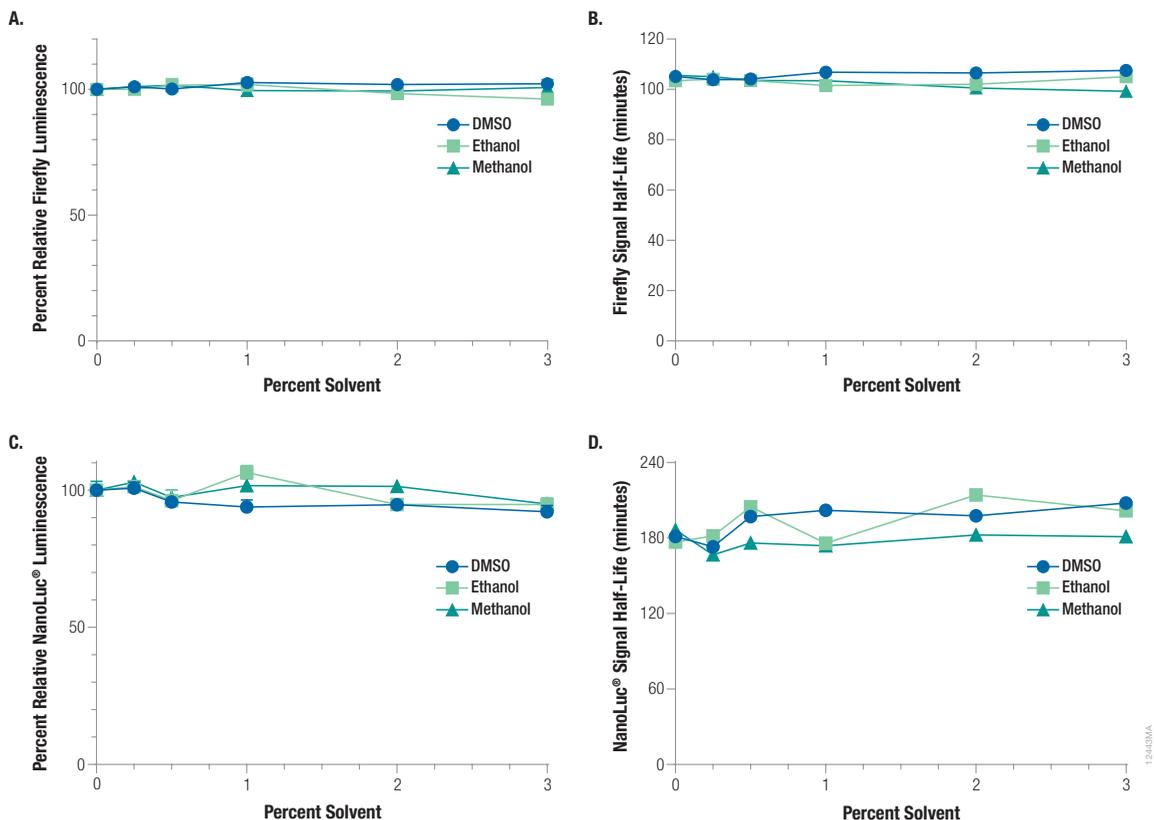


Figure 10. The effect of organic solvents on luminescence intensity and signal stability. Purified firefly (6.88 ng/ml) or NanoLuc[®] luciferase (0.50 ng/ml) were diluted into DMEM plus 0.1% Prionex[®] with varying concentrations of dimethyl sulfoxide (DMSO), ethanol, or methanol. Enzyme solutions (80 μ l per well) were added to 96-well plates, and 80 μ l of ONE-Glo[™] EX Reagent was added to all wells. Then, 80 μ l of NanoDLR[™] Stop & Glo[®] Reagent was then added to the wells containing NanoLuc[®] luciferase. Luminescence was measured periodically over two hours to calculate the signal stability (half-life); n = 4. **Panel A.** Firefly luminescence relative to no solvent. **Panel B.** Firefly signal half-life. **Panel C.** NanoLuc[®] luminescence relative to no solvent. **Panel D.** NanoLuc[®] signal half-life.

Phenol Red

Phenol red is a pH indicator commonly used in cell culture media. Many commercial media formulations contain 5–15mg/l phenol red, causing the characteristic red color. Because it can absorb light, phenol red can reduce assay sensitivity. However, the lower pH of the NanoDLR™ Assay Reagents makes them less sensitive to phenol red than other luciferase reagents (Figure 11). In most applications the presence of phenol red will not significantly affect the utility of the NanoDLR™ Assay, but to maximize the luminescent signal use as little phenol red as possible in culture media.

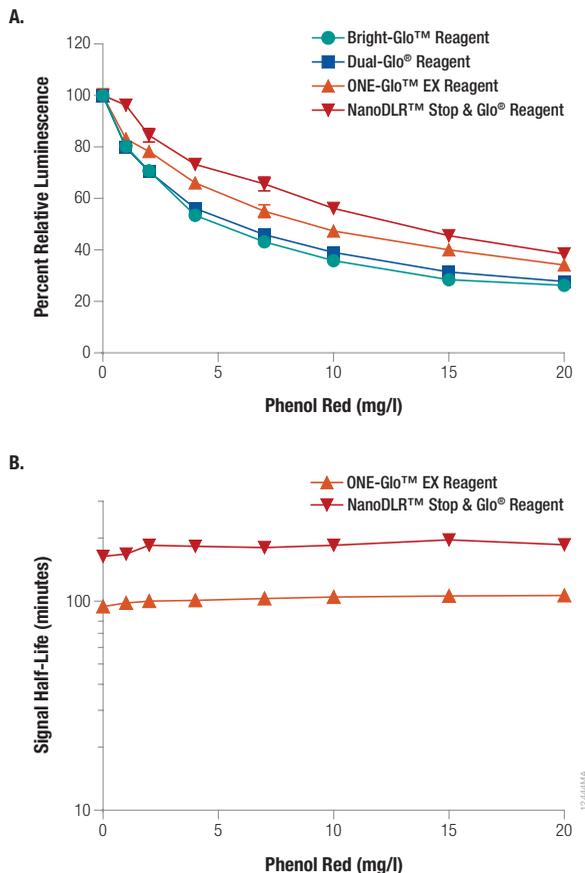


Figure 11. The effect of phenol red on luminescence intensity and stability. Purified firefly (6.88ng/ml) or NanoLuc® luciferase (0.50ng/ml) was diluted into DMEM plus 0.1% Prionex® with varying concentrations of phenol red. Enzyme solutions (80µl per well) were added to 96-well plates. Bright-Glo™, Dual-Glo® or ONE-Glo™ EX Reagent (80µl) was added to the firefly luciferase-containing wells. Eighty microliters of ONE-Glo™ EX Reagent, followed by 80µl of NanoDLR™ Stop & Glo® Reagent, were added to the wells containing NanoLuc® luciferase. Luminescence was measured periodically over 2 hours to calculate the signal stability (half-life); n = 3. **Panel A.** Luminescence relative to no added phenol red. **Panel B.** Signal half-life in NanoDLR™ Reagents.

Temperature

Both firefly and NanoLuc[®] luciferases are temperature-sensitive, so maintaining a consistent temperature is an important factor in experimental precision (Figure 12). Precision can be achieved most easily by performing all experiments at room temperature. The assay reagents should be at room temperature before measuring luminescence, and the culture plate should be equilibrated to room temperature before adding reagents.

The NanoDLR[™] Buffers can be stored at room temperature prior to the experiment to avoid the need for temperature equilibration before use. The heat capacity of the substrates is low, so reconstitution of the substrates with room temperature buffers will produce reagents ready for use. If temperature equilibration is needed to bring reagents to room temperature, incubate reagents in a water bath at room temperature (the water bath should not be set higher than 25°C).

Lower temperatures result in increased signal stabilities, but lower luminescence intensities. If cold reagent is used, the luminescence will slowly increase during the experiment as the reagent warms. Some luminometers run at a higher temperature than the ambient environment. To prevent signal gradients across a plate due to uneven warming of a plate during measurement, equilibrate plates and reagents to the internal temperature of the luminometer (e.g., in a water bath set to that higher-than-ambient temperature).

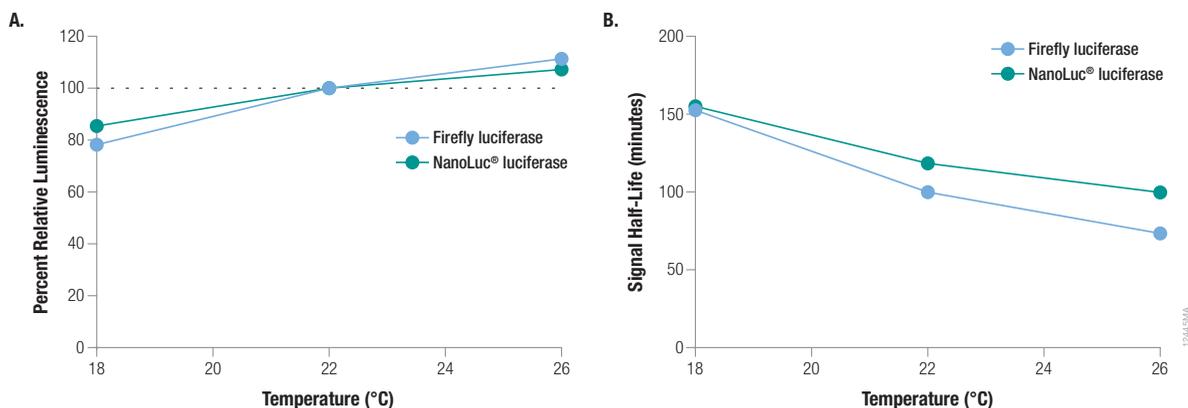


Figure 12. The effect of temperature on luciferase luminescence. Purified firefly (13.8ng/ml) or NanoLuc[®] luciferase (1.0ng/ml) was diluted into DMEM plus 0.1% Prionex[®]. Enzyme solution (200µl) was added to 200µl of ONE-Glo[™] EX Reagent in a luminometer tube, and 200µl of NanoDLR[™] Stop & Glo[®] Reagent was added to the sample containing NanoLuc[®] luciferase. Luminescence was measured periodically over two hours on a Turner Biosystems 20/20n luminometer. The enzyme solutions and reagents were pre-equilibrated at 18, 22, and 26°C prior to mixing and were incubated at those same temperatures between measurements. **Panel A.** Luminescence after 3 minutes relative to the value at 22°C. **Panel B.** Signal half-life.

4.G. Maximizing Sensitivity

At very low concentrations of enzyme, luminescence may approach background levels. For maximal accuracy, the luminescence measurements of both firefly and NanoLuc[®] luciferase should be background-subtracted. Neither enzyme is endogenously expressed in mammalian cells; therefore, the source of background luminescence is either a characteristic of the luminometer or of the luminescent substrate. Background measurements should be taken from samples consisting of nontransfected cells treated with NanoDLR[™] Reagents in the same manner as the test samples. Sample volumes for background measurements must be the same as experimental sample volumes and contain the same media/sera combinations as the experimental samples. To improve sensitivity, the following steps may decrease the background luminescence:

- Plastic assay plates can exhibit a certain amount of phosphorescence when exposed to light. Dark-adapting the plate for several minutes in the luminometer before measuring luminescence can decrease background signal.
- Coelenterazine-like compounds, such as the furimazine substrate used in NanoDLR[™] Stop & Glo[®] Reagent, have a natural rate of autoluminescence. While the reagent is designed to minimize this effect, serum present in media increases the furimazine autoluminescence. Therefore, to make the most sensitive measurements of NanoLuc[®] luciferase, you may wish to reduce or eliminate serum from the sample, i.e., perform a medium exchange.
- Part of the background for the NanoLuc[®] luciferase signal consists of any uninhibited firefly luminescence in the sample. Because of the brightness of NanoLuc[®] luciferase and the >1 million-fold quench of the firefly signal, there should typically be no effect from the firefly luciferase signal on the NanoLuc[®] signal. However, at very high firefly luciferase expression levels and very low NanoLuc[®] luciferase expression levels, you can ensure the best signal separation by making certain that the samples are completely mixed so that all the firefly luciferase solution comes in contact with the NanoDLR[™] Stop & Glo[®] Reagent. Orbital shaking at 600–900 rpm is generally effective at ensuring that all firefly luciferase solution comes in contact with the NanoDLR[™] Stop & Glo[®] Reagent. Incubating the sample with NanoDLR[™] Stop & Glo[®] Reagent for longer than 10 minutes (e.g., 30 minutes) may also decrease any residual firefly signal several fold. Reducing the expression level of firefly luciferase (e.g., switching from CMV to a less active promoter) can also permit more sensitive measurement of NanoLuc[®] luciferase.

5. Related Products

5.A. Luciferase Reporter Vectors

The following is a sampling of the NanoLuc[®] and firefly luciferase vectors available from Promega. Please visit www.promega.com/luciferase-vectors to see a complete listing of luciferase reporter vector options.

NanoLuc[®] Luciferase Reporter Vectors

Product	Size	Cat.#
pNL1.1.PGK[<i>Nluc</i> /PGK] Vector	20µg	N1441
pNL1.1.TK[<i>Nluc</i> /TK] Vector	20µg	N1501
pNL1.1.CMV[<i>Nluc</i> /CMV] Vector	20µg	N1091
pNL1.1[<i>Nluc</i>] Vector	20µg	N1001
pNL1.2[<i>NlucP</i>] Vector	20µg	N1011
pNL1.3[<i>secNluc</i>] Vector	20µg	N1021
pNL3.1[<i>Nluc</i> /minP] Vector	20µg	N1031
pNL3.2[<i>NlucP</i> /minP] Vector	20µg	N1041
pNL3.3[<i>secNluc</i> /minP] Vector	20µg	N1051

Firefly Luciferase Reporter Vectors

Product	Size	Cat.#
pGL4.53[<i>luc2</i> /PGK] Vector	20µg	E5011
pGL4.54[<i>luc2</i> /TK] Vector	20µg	E5061
pGL4.10[<i>luc2</i>] Vector	20µg	E6651
pGL4.11[<i>luc2P</i>] Vector	20µg	E6661
pGL4.13[<i>luc2</i> /SV40] Vector	20µg	E6681
pGL4.23[<i>luc2</i> /minP] Vector	20µg	E8411
pGL4.50[<i>luc2</i> /CMV/Hygro] Vector	20µg	E1310

Coincidence Reporter Vectors Containing Firefly and NanoLuc[®] Luciferases

Product	Size	Cat.#
pNLCol1[<i>luc2</i> -2A- <i>NlucP</i> /Hygro] Vector	20µg	N1461
pNLCol2[<i>luc2</i> -2A- <i>NlucP</i> /minP/Hygro] Vector	20µg	N1471
pNLCol3[<i>luc2</i> -2A- <i>NlucP</i> /CMV/Hygro] Vector	20µg	N1481
pNLCol4[<i>luc2</i> -2A- <i>NlucP</i> /PGK/Hygro] Vector	20µg	N1491

NanoLuc[®] Luciferase Fusion Vectors

Vectors that enable simple generation of N- or C-terminal fusions of NanoLuc[®] Luciferase.

Product	Size	Cat. #
pFN31A <i>Nluc</i> CMV-Hygro Flexi [®] Vector	20µg	N1311
pFN31K <i>Nluc</i> CMV-neo Flexi [®] Vector	20µg	N1321
pFC32A <i>Nluc</i> CMV-Hygro Flexi [®] Vector	20µg	N1331
pFC32K <i>Nluc</i> CMV-neo Flexi [®] Vector	20µg	N1341
pNLF1-N [CMV/Hygro] Vector	20µg	N1351
pNLF1-C [CMV/Hygro] Vector	20µg	N1361
pNLF1-secN [CMV/Hygro] Vector	20µg	N1371

5.B. Accessory Products

Reporter Assays

Product	Size	Cat. #
ONE-Glo [™] EX Luciferase Assay System (detects firefly luciferase)	100ml	E8120
Nano-Glo [®] Luciferase Assay (detects NanoLuc [®] Luciferase)	100ml	N1120

Additional Sizes Available.

Product	Size	Cat. #
Passive Lysis Buffer	30ml	E1941

Luminometers

Product	Cat. #
GloMax [®] Discover System	GM3000
GloMax [®] Explorer System	GM3500

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7. Summary of Changes

The following changes were made to the 2/24 revision of this document:

1. Updated font and cover image.
2. Made minor text edits.
3. Updated patent statements.

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^(c)U.S. Pat. Nos. 7,078,181 and other patents.

^(d)Patent Pending.

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