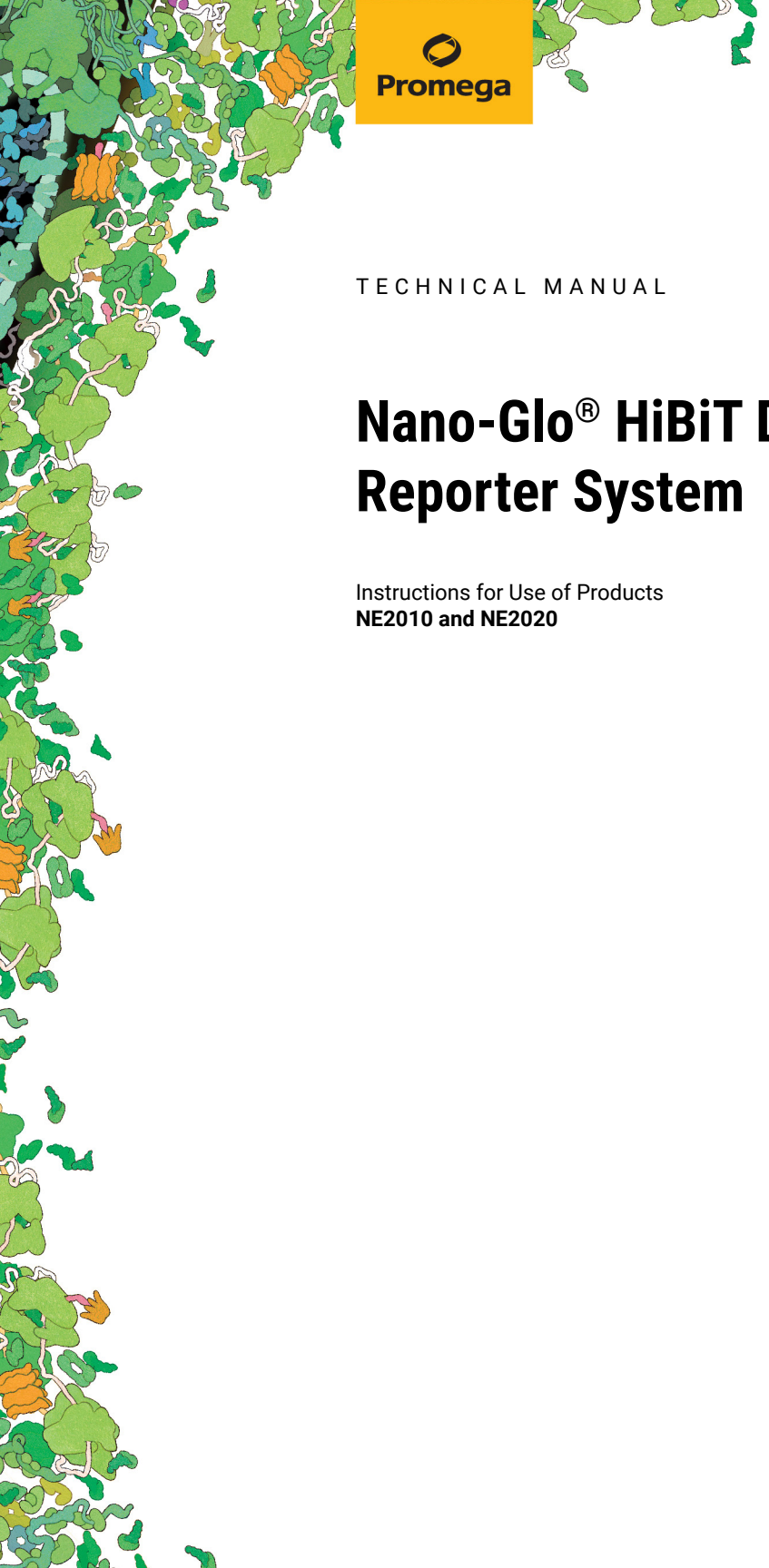

TECHNICAL MANUAL

Nano-Glo® HiBiT Dual-Luciferase® Reporter System

Instructions for Use of Products
NE2010 and NE2020

4/26
TM775

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Nano-Glo® HiBiT Dual-Luciferase® Reporter System

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

1. Description

The Nano-Glo® HiBiT Dual-Luciferase® Reporter System (HiBiT NanoDLR) sequentially quantitates firefly luciferase and HiBiT-tagged proteins in the same well using a simple add–read–add–read workflow.

The HiBiT NanoDLR™ assay is well suited for applications in which changes in the levels of a HiBiT-tagged protein must be interpreted relative to a second reporter. A common format involves co-expression of a HiBiT-tagged target protein with constitutively expressed firefly luciferase to distinguish specific modulation of the tagged protein from nonspecific effects on global protein expression or cell viability.

In the first step, the ONE-Glo™ EX+LgBiT reagent lyses cells, provides the substrate and buffer conditions needed for measuring firefly luciferase activity and allows LgBiT to complement HiBiT, forming the NanoBiT® luciferase. In the second step, NanoDLR™ Stop & Glo® reagent quenches firefly luciferase activity and provides the substrate for NanoBiT® luminescence to quantitate the HiBiT-tagged protein.

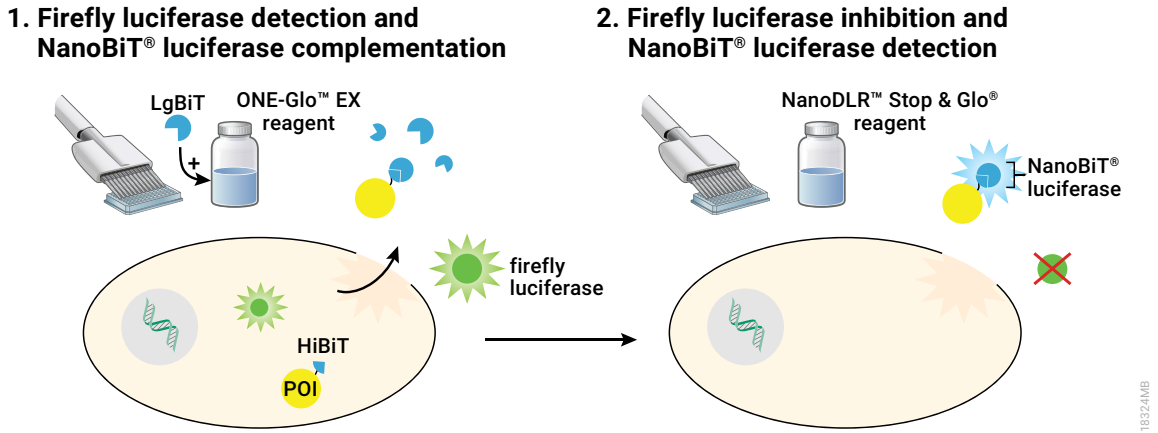

Figure 1. Assay principle and workflow. The ONE-Glo™ EX+LgBiT reagent lyses cells and provides the substrate necessary for measuring firefly luciferase, while also providing the LgBiT Protein necessary to complement HiBiT, forming the NanoBiT® luciferase. NanoDLR™ Stop & Glo® reagent is then added to quench the firefly luciferase signal and provide NanoBiT® luciferase with the substrate required for HiBiT quantification.

2. Product Components and Storage Conditions

2.A. Product Components List

PRODUCT	SIZE	CAT.#
Nano-Glo® HiBiT Dual-Luciferase® Reporter Assay System	**10ml**	**NE2010**

Contains sufficient reagents to perform ~100 assays at 80µl/well in a 96-well plate format. Includes:

- 1 × 10ml ONE-Glo™ EX Luciferase Assay Buffer
- 1 vial ONE-Glo™ EX Luciferase Assay Substrate (lyophilized)
- 1 × 10ml NanoDLR™ Stop & Glo® Buffer
- 1 × 0.1ml NanoDLR™ Stop & Glo® Substrate
- 1 × 0.1ml LgBiT Protein

PRODUCT	SIZE	CAT.#
Nano-Glo® HiBiT Dual-Luciferase® Reporter Assay System	**100ml**	**NE2020**

Contains sufficient reagents to perform ~1,000 assays at 80µl/well in a 96-well plate format. Includes:

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

2.B. Component and System Storage Conditions

Store all components at –30°C to –10°C. Avoid thawing buffers above +25°C. LgBiT Protein and NanoDLR™ Stop & Glo® Substrate remain liquid at –30°C to –10°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.

2.C. Prepared Solutions Storage Conditions

For optimal performance, prepare the needed volumes of both the ONE-Glo™ EX+LgBiT reagent and NanoDLR™ Stop & Glo® reagent fresh immediately before each experiment (see Section 4.A). Once prepared, these reagents are stable enough for high-throughput screening and will maintain at least 90% activity after 8 hours at 22°C.

If unused reconstituted ONE-Glo™ EX reagent (without LgBiT) or NanoDLR™ Stop & Glo® reagent remains after an experiment, it can be stored at 2–10°C for use within a few days. Reconstituted ONE-Glo™ EX reagent (without LgBiT) stored at 4°C will lose approximately 10% activity after 3.5 days and 50% activity after 1 month. Add LgBiT Protein to reconstituted ONE-Glo™ EX reagent immediately before each experiment. Reconstituted NanoDLR™ Stop & Glo® reagent stored at 4°C will lose approximately 10% activity after 3.5 days.

Alternatively, unused reconstituted reagents may be stored at –30°C to –10°C for longer-term storage, protected from light. Avoid multiple freeze/thaw cycles and thaw at temperatures below +25°C (e.g., in a room-temperature water bath); mix by inversion before use.

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3. Before You Begin

3.A. Assay Considerations

Firefly Luciferase Inhibitor Carryover

Quenching of the firefly luciferase signal is accomplished in part by a potent firefly luciferase inhibitor present in the NanoDLR™ Stop & Glo® Buffer. This inhibitor can bind reversibly to plastic, tubing, injectors and other surfaces, creating a risk of carryover into subsequent luciferase-based assays if materials are reused without thorough cleaning. Because of the potency of the inhibitor, even trace amounts carried over from previous use can noticeably inhibit firefly luciferase, particularly when measuring firefly luciferase activity with ONE-Glo™ EX reagent. To minimize this risk, use disposable or dedicated containers and pipette tips when handling the NanoDLR™ Stop & Glo® reagent. Additional guidance on preventing inhibitor carryover is provided in Section 7.C.

Well Volume and Sequential Reagent Addition

The HiBiT NanoDLR™ assay involves sequential addition of two reagent volumes to each well. To prevent overflow and potential cross-contamination, ensure that the initial culture medium volume provides sufficient capacity for adding both reagents. For example, when performing assays in 96-well plates, cells can be plated in a reduced medium volume (e.g., 70–80µl per well) to accommodate subsequent addition of ONE-Glo™ EX+LgBiT reagent and NanoDLR™ Stop & Glo® reagent at equal volumes. Adjust initial plating and transfection volumes accordingly to maintain appropriate final well volumes.

Temperature Control

Luciferase activity is temperature-dependent, and maintaining consistent assay temperature is critical for obtaining reproducible luminescence measurements. All reconstituted reagents, assay plates and cell samples should be equilibrated to room temperature (20–25°C) before beginning the assay. Transferring buffer solutions to room temperature at least 1 day prior to use can help minimize temperature-related variability. If reconstituted reagents have been stored at 2–10°C or frozen, they should be warmed or thawed at temperatures below +25°C, preferably in a room-temperature water bath, and thoroughly mixed by inversion before use.

Media Compatibility and Plate-to-Plate Consistency

The HiBiT NanoDLR™ assay is compatible with many commonly used culture media containing 0–10% serum, including DMEM, RPMI 1640, McCoy's 5A and Ham's F-12. However, media composition, serum concentration, phenol red, organic solvents and temperature fluctuations can affect luminescence intensity and signal decay kinetics. Because luminescence is sensitive to these variables, raw luminescence values should be compared only between samples measured under identical conditions. For experiments spanning multiple plates or time points, we recommend including common positive and negative control wells on each plate (see Section 5.C). When processing multiple plates, each plate should be measured at a consistent time interval after reagent addition. Because both firefly and HiBiT signals exhibit long glow-type half-lives under recommended conditions, small variations in read timing are unlikely to substantially affect results; however, consistent timing improves reproducibility across plates.

Control Wells for Background Subtraction

We recommend including appropriate control wells in each experiment for calculating background-subtracted luminescence values. Parental cells or cells that do not express either firefly luciferase or a HiBiT-tagged protein, treated identically to experimental samples, provide the preferred estimate of non-reporter signal. Including these controls at the experimental design stage ensures accurate normalization and interpretation of results (see Section 5.B).

3.B. Materials to Be Supplied by the User

This assay requires cells expressing both a HiBiT-tagged protein of interest and firefly luciferase. HiBiT-tagged proteins can be generated using HiBiT expression vectors by adding the HiBiT peptide sequence to existing constructs, or by integrating HiBiT at the endogenous gene locus using CRISPR-Cas9 genome editing. HiBiT expression vectors are available at: **www.promega.com/products/protein-detection/protein-quantification/** and firefly luciferase vectors at: **www.promega.com/luciferase-vectors**. Researchers can obtain rights to synthesize the HiBiT tag by reviewing and accepting the Terms and Conditions found at: **www.promega.com/HiBiT-Synthesis**

Additional Materials:

- appropriate cell culture medium
- solid white, opaque 96-well tissue culture plates (e.g., Corning® Cat.# 3917) or other culture plates compatible with your luminometer
- orbital shaker (300–900rpm)
- multichannel pipettes and tips (preferably aerosol-resistant)
- GloMax® Discover System (Cat.# GM3000) or compatible luminometer
- disposable polypropylene tubes or Pyrex® bottles for reagent preparation

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4. Protocols

4.A. Preparing Solutions

To minimize temperature-related variability, transfer ONE-Glo™ EX Luciferase Assay Buffer and NanoDLR™ Stop & Glo® Buffer to room temperature well before use, ideally the day before the experiment. If same-day equilibration is necessary, buffers may be warmed in a room-temperature water bath; do not thaw or warm above 25°C. To ensure accurate volume transfer of LgBiT Protein, transfer to 0–10°C ahead of use (to reduce viscosity) and pipette slowly. Keep NanoDLR™ Stop & Glo® Substrate and LgBiT Protein at 0–10°C during handling (e.g., in an ice bucket).

Preparing ONE-Glo™ EX Reagent

To reconstitute the ONE-Glo™ EX reagent, transfer the entire contents of one bottle of ONE-Glo™ EX Luciferase Assay Buffer to one vial of ONE-Glo™ EX Luciferase Assay Substrate. Replace the stopper and mix by gentle inversion until the substrate is fully dissolved (approximately 10 seconds). Do not vortex.

Preparing ONE-Glo™ EX+LgBiT Reagent

Prepare the ONE-Glo™ EX+LgBiT reagent immediately before use by adding LgBiT Protein to the required volume of reconstituted ONE-Glo™ EX reagent at a 1:100 dilution. For example, to prepare 4ml of reagent, add 40µl of LgBiT Protein to 4ml of ONE-Glo™ EX reagent. Mix gently by inversion.

Preparing NanoDLR™ Stop & Glo® Reagent

Prepare the NanoDLR™ Stop & Glo® reagent fresh for each experiment by diluting the NanoDLR™ Stop & Glo® Substrate 1:100 into the appropriate volume of NanoDLR™ Stop & Glo® Buffer. For example, to prepare 4ml of reagent, add 40µl of substrate to 4ml of buffer. Mix by gentle inversion.

Notes:

a. If the substrate or LgBiT Protein has collected in the cap or on the sides of the tube, briefly centrifuge the tube before dispensing.

b. Luciferase activity is temperature dependent; equilibrate all prepared reagents to room temperature before use.

4.B. Assay Protocol

1. Remove assay plates from the incubator and equilibrate to room temperature (e.g., leave on the benchtop for 15 minutes).
2. Add a volume of ONE-Glo™ EX+LgBiT reagent equal to the initial culture medium volume (typically 80µl for a 96-well plate) to each well.
3. Incubate for 10 minutes on an orbital shaker at 300–600rpm to lyse cells and allow LgBiT to equilibrate with HiBiT.
4. Measure firefly luminescence using an appropriate plate reader. Standard luminescence settings that collect all wavelengths using no filters and integration times of 0.5–1 seconds are typically sufficient.
5. Add a volume of NanoDLR™ Stop & Glo® reagent to each well that is equal to that of the added ONE-Glo™ EX+LgBiT reagent (typically 80µl for a 96-well plate).
6. Incubate for 10 minutes on an orbital shaker at 600–900rpm to ensure complete mixing and quenching of the firefly signal.
7. Measure HiBiT luminescence using the same instrument settings.

5. Data Analysis

Table 1. Summary of Assay Performance Characteristics.

Assay Parameter	Value
Detection limit	$<10^{-18}$ moles for both firefly luciferase and HiBiT using a GloMax® Discover System
Signal half-life	2.0–2.5 hours for firefly luciferase with moderate expression; 4–6 hours for HiBiT with low-to-moderate expression
Quenching efficiency	>1,000,000-fold reduction of firefly signal
Dynamic range	Linear response from ~1fM to 10nM for HiBiT; 10fM to 100nM for firefly luciferase using a GloMax® Discover System

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5.A. Luminescent Signal Characteristics

Firefly luciferase and NanoBiT® luciferase (composed of HiBiT + LgBiT) use chemically distinct substrates supplied in separate assay reagents for sequential measurement of both reporters in the same well without cross-reactivity.

Firefly luminescence generated using ONE-Glo™ EX reagent typically displays an apparent signal half-life of approximately 2–2.5 hours across measurable concentrations. HiBiT luminescence measured after adding NanoDLR™ Stop & Glo® reagent exhibits long apparent half-lives (approximately 4–6 hours) at subnanomolar HiBiT concentrations, supporting batch processing and high-throughput workflows. At substantially higher HiBiT concentrations (approximately ≥10nM), more rapid signal decay consistent with substrate depletion can occur. These high concentrations typically produce luminescence values approaching the upper limits of most plate luminometers and define the practical upper boundary of the assay's quantitative range (see Figure 2).

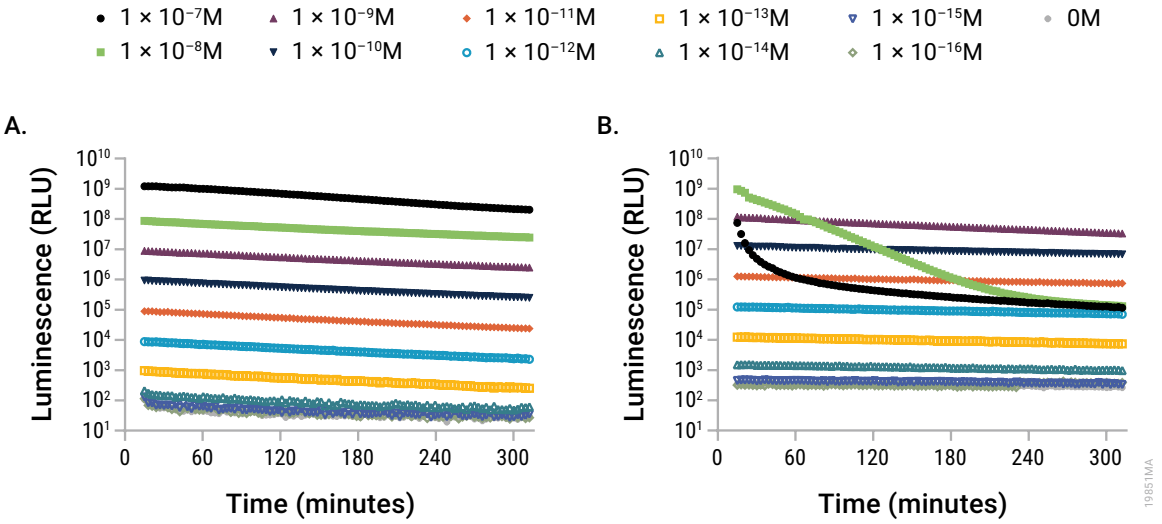

Figure 2. Signal decay across the dynamic range. Panel A shows signal decay measured using ONE-Glo™ EX reagent across a titration of purified firefly luciferase. **Panel B** shows signal decay measured following addition of NanoDLR™ Stop & Glo® reagent across a titration of HaloTag-HiBiT (HiBiT Control Protein; Cat.# N3010). Stable glow-type kinetics are observed within the recommended operating range. At ≥10nM HiBiT, rapid signal decay consistent with substrate depletion is observed, defining the upper boundary of the assay's quantitative range.

5.B. Linearity and Background Subtraction

Raw luminescence values include contributions from instrument background, assay reagents and cellular components and should be background-subtracted prior to quantitative analysis. Background wells (e.g., parental cells that do not express either firefly luciferase or a HiBiT-tagged protein and that are treated identically to experimental samples) provide the preferred estimate of non-reporter signal.

Within the recommended operating range, background-subtracted relative light units (RLUs) scale proportionally with reporter concentration, enabling quantitative comparisons and ratio-based normalization (see Figure 3). At very high HiBiT concentrations (approximately ≥10nM under the conditions tested), signal may deviate from linearity due to rapid substrate depletion and, in some cases, detector saturation. Users should therefore confirm that measured RLUs remain within the linear detection range of the instrument.

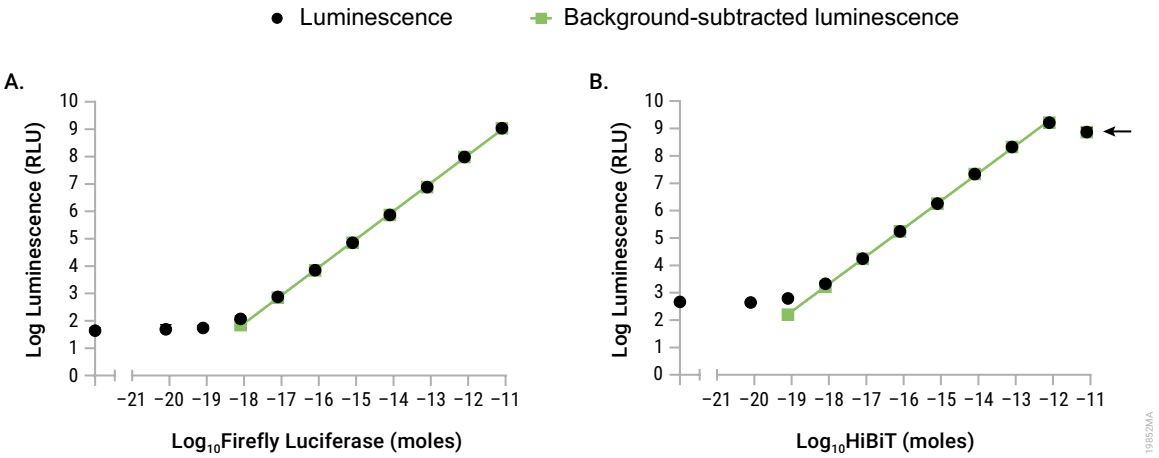

Figure 3. Background-subtracted linearity for firefly luciferase and HaloTag-HiBiT. Panel A shows firefly luciferase measured using ONE-Glo™ EX+LgBiT reagent. **Panel B** shows NanoBiT® luminescence from HaloTag-HiBiT (HiBiT Control Protein, Cat.# N3010) measured following addition of NanoDLR™ Stop & Glo® reagent. Raw luminescence and background-subtracted luminescence measured 10 minutes after reagent addition are plotted versus the moles of reporter present in the well (log-log scale). Both reporters exhibit linear responses across the recommended operating range. At the highest HiBiT concentration tested (highlighted with arrow in Panel B), deviation from linearity is observed 10 minutes after reagent addition due to substrate depletion (see Figure 2).

Efficient quenching of firefly luciferase by the NanoDLR™ Stop & Glo® reagent reduces residual firefly signal to near-background levels before HiBiT measurement, enabling accurate background subtraction and subsequent normalization (Figure 4). Detailed considerations for preventing unintended inhibition of firefly luciferase in subsequent assays are described in Section 7.C.

5.B. Linearity and Background Subtraction (continued)

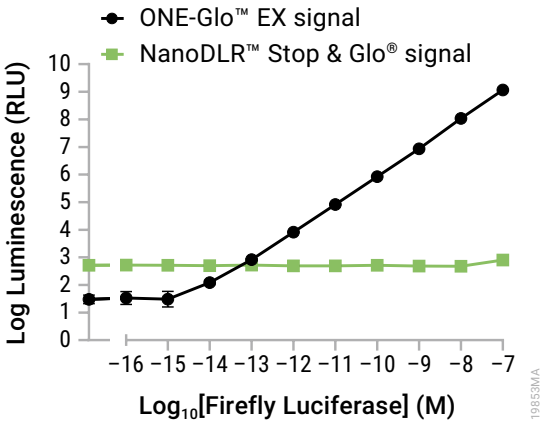

Figure 4. Efficient quenching of firefly luciferase signal by NanoDLR™ Stop & Glo® reagent. Firefly luminescence was measured before and after adding NanoDLR™ Stop & Glo® reagent. The potent firefly luciferase inhibitor in the Stop & Glo® formulation reduces residual firefly signal to near-background levels for accurate background subtraction and subsequent HiBiT measurement.

5.C. Normalization and Comparative Analysis

The HiBiT NanoDLR™ system can sequentially measure signals from a constitutively expressed firefly luciferase and a HiBiT-tagged protein in the same well. Ratios should be calculated for each well using background-subtracted RLUs for both reporters (see Section 5.B) to ensure proportionality to relative protein abundance.

Normalized Ratio = (Experimental Reporter HiBiT RLU – Background RLU) ÷ (Control Reporter Firefly RLU – Background RLU)

Normalizing background-subtracted HiBiT signal to background-subtracted firefly signal can reduce variability arising from differences in cell number, transfection efficiency or global changes in protein expression. In many experimental contexts, parallel decreases in both reporters suggest nonspecific effects such as cytotoxicity or general inhibition of protein synthesis, whereas selective reduction of the HiBiT signal in the presence of a relatively stable firefly signal is consistent with a specific effect on the abundance or stability of the HiBiT-tagged protein. Representative examples illustrating these relationships are shown in Section 7.A.

A simple analysis method is to average the normalized ratios for replicate wells of each treatment and then determine the fold change in activity between two test groups. Typically, one compares the experimental test sample to a negative control (e.g., vehicle-treated cells), using the following equation:

Fold Change = (Experimental Sample Average Normalized Ratio) ÷ (Negative Control Average Normalized Ratio)

Including positive controls with maximal fold change on each plate can help ensure that results are reproducible over time or from experiment to experiment, or they can provide an internal reference for comparing treatment effects.

6. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: **www.promega.com**. Email: **techserv@promega.com**

Symptom	Causes and Comments
Low HiBiT signal	Confirm LgBiT Protein was added to ONE-Glo™ EX reagent at the correct dilution.
	Ensure use of a plate reader designed for sensitive luminescence detection. Luminometers report relative light units (RLUs), so the actual values may vary greatly between instruments.
	Verify that the Stop & Glo® reagent was prepared fresh.
	For CRISPR knock-in experiments, target protein expression may be inherently low or knock-in efficiency in pooled cell populations may be limited; enriching edited cells or isolating clones may improve signal.
	Consider extending incubation time if the HiBiT tag is internal to the protein (not fused to terminus) or might be poorly accessible.
	High HiBiT concentrations may rapidly deplete substrate, yielding a low signal at the time of measurement. Measure the luminescence of the plate several times over 10 minutes to determine if the well displays rapid signal decay.
Rapid decay of HiBiT signal	HiBiT concentration can exceed the recommended operating range; concentrations ≥10nM lead to substrate depletion. Dilute samples or reduce input levels to return signals to the linear range.
Loss of linearity at high signal levels	Substrate depletion at high HiBiT concentrations can cause deviation from linearity.
	Detector saturation may occur at high luminescence values. Verify instrument dynamic range and consider sample dilution.
High variability between replicate wells	Inconsistent reagent dispensing, incomplete mixing, presence of bubbles or uneven cell plating may contribute to variability.
	Confirm consistent timing of reagent addition and measurement.
	Strong luminescent signals in adjacent wells can carry over to neighboring wells with much lower signal (cross-talk). Use solid white plates with the lids off and ensure that the plate reader settings are correctly set for the type of plate and absence of lid. If possible, separate wells with very high signals from those with very low signals (e.g., separate background wells from others using blank wells).

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6. Troubleshooting (continued)

Symptom	Causes and Comments
Low firefly luciferase signal across all wells	Reconstituted ONE-Glo™ EX reagent may have lost activity due to improper storage or repeated freeze–thaw cycles.
	Ensure reagents were equilibrated to room temperature.
	Confirm correct reagent volumes were added.
	Use a plate reader designed for sensitive luminescence detection. Luminometers report relative light units (RLUs), so the actual values may vary greatly between instruments.
	Low transfection efficiency or low basal expression level for transiently transfected firefly construct can reduce signal.
Residual firefly luciferase signal after adding Stop & Glo® reagent	Insufficient mixing after adding Stop & Glo® reagent. Test whether current mixing protocol is sufficient by comparing to replicate wells mixed by pipetting several times after reagent addition.
Low or variable firefly luminescence	Possible carryover of firefly luciferase inhibitor from previous dispensing of NanoDLR™ Stop & Glo® reagent. Follow cleaning and handling recommendations in Section 7.C.
High background signal is seen in sample lacking HiBiT	Contamination of detection reagents or dispensing lines with HiBiT-tagged proteins.
	High concentrations of proteins or peptides from cells, serum or other sources can cause some nonspecific LgBiT Protein activation in the detection reagent. Ensure the plate has background wells containing equivalent cells not expressing HiBiT or firefly luciferase.

7. Appendix

7.A. Representative Application Data: Distinguishing Specific Protein Degradation from Nonspecific Effects

HiBiT tagging of proteins provides a sensitive and quantitative approach for monitoring changes in protein abundance. When combined with CRISPR-Cas9 genome editing, HiBiT can be integrated at the native locus for measuring target protein levels under more physiologically relevant regulatory control without overexpression artifacts. This approach is particularly useful in studies of induced protein degradation, where maintaining native protein levels avoids saturating cellular degradation machinery.

In screening environments, compounds that reduce cell viability or globally suppress transcription or translation can decrease reporter signal, mimicking target degradation. Incorporating a constitutively expressed firefly luciferase reporter provides an internal control that discriminates between selective loss of the HiBiT-tagged protein and nonspecific effects on global protein expression or cell health.

Figure 5 demonstrates this principle using a representative experimental system in which HEK293 cells expressing HiBiT-tagged BRD4 and firefly luciferase were treated with test compounds prior to dual measurement using the HiBiT NanoDLR™ assay.

Panels A and B show 6-hour incubations with the BRD4-degrading compounds dBET1 and MZ1, respectively. In both cases, selective reduction of the HiBiT signal with minimal change in firefly signal is consistent with compound-induced degradation of BRD4. Panel C shows 24-hour incubation with the cytotoxic compound staurosporine. In contrast to the degrader compounds, staurosporine produces parallel decreases in both HiBiT and firefly luciferase signals, consistent with nonspecific toxicity. These data demonstrate how dual measurement can identify selective degrader activity while flagging false positives arising from general cytotoxic or global effects.

7.A. Representative Application Data: Distinguishing Specific Protein Degradation from Nonspecific Effects (continued)

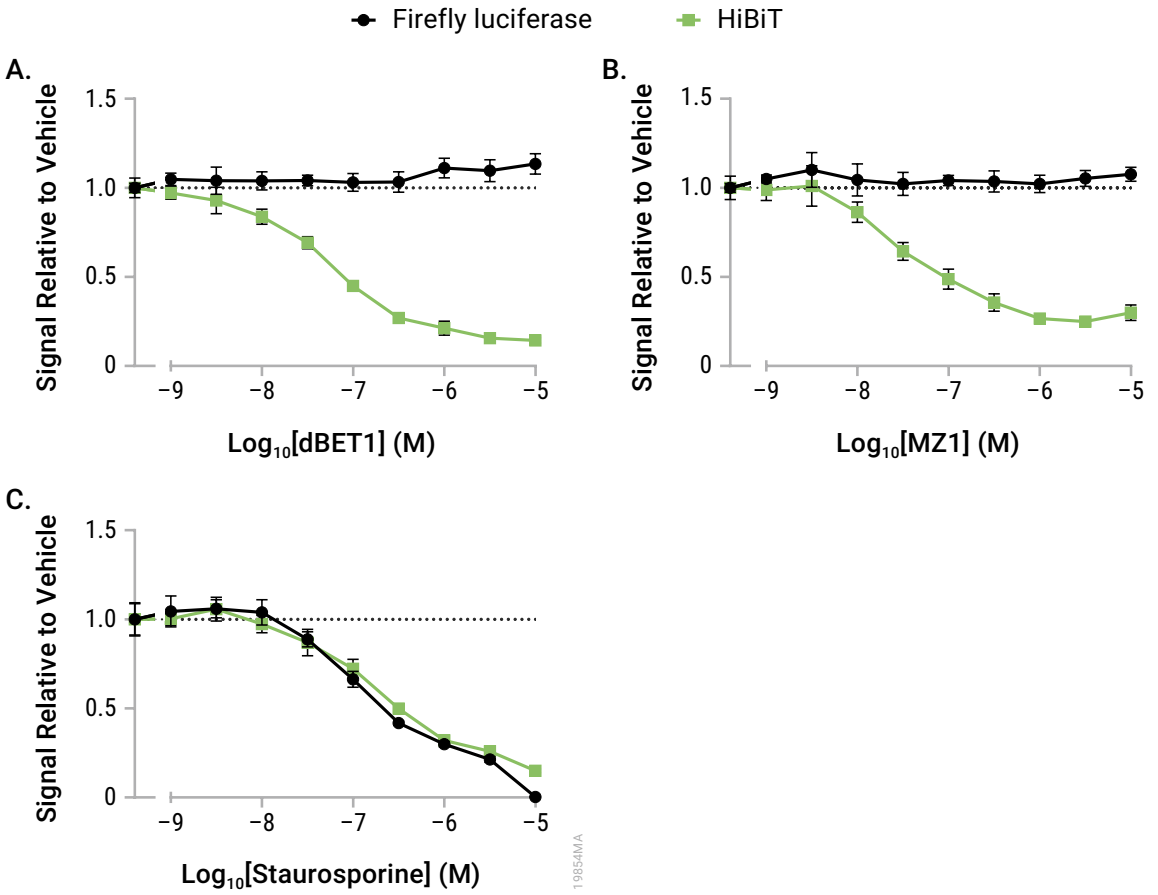

Figure 5. Selective and nonselective compound effects on HiBiT and firefly luciferase signals. HEK293 cells expressing HiBiT-tagged BRD4 and constitutive firefly luciferase were treated with compounds prior to dual measurement using the HiBiT NanoDLR™ assay. Six-hour incubation with the BRD4-degrading compounds dBET1 (**Panel A**) or MZ1 (**Panel B**) demonstrates selective reduction of the HiBiT signal with minimal change in firefly luminescence, consistent with compound-specific degradation of BRD4. In contrast, 24-hour incubation with staurosporine (**Panel C**) produces parallel decreases in both reporters, consistent with nonspecific cytotoxic effects. Signals are plotted relative to vehicle-treated controls. Data represent the mean ± standard deviation (n = 6).

7.B. Additional Assay Formats

While the HiBiT NanoDLR™ assay is commonly used to monitor changes in a HiBiT-tagged protein normalized to constitutive firefly luciferase expression, alternative dual-reporter configurations are possible, depending on experimental objectives.

In transient expression studies, firefly luciferase may be co-expressed with a HiBiT-tagged protein from the same construct or from parallel constructs. In this configuration, firefly luminescence serves as a normalization control for transfection efficiency and overall expression levels for more accurate comparison of compound effects on the HiBiT-tagged protein.

In other applications, firefly luciferase may function as an independent experimental reporter rather than solely as a normalization control. A common example is the use of firefly luciferase driven by a regulated promoter to report transcriptional activation of a signaling pathway. This configuration is particularly useful when screening compounds that target transcription factors or nuclear receptors such as estrogen receptor or androgen receptor. In these systems, HiBiT luminescence reports protein abundance, while firefly luminescence reports transcriptional activation of a response-element-driven reporter. Compounds may alter transcriptional activity without substantially changing protein levels, or they may induce degradation of the target protein, reducing both abundance and reporter activation. Measurement of both reporters discriminates between effects on protein stability and functional activity.

When firefly luciferase is used as a second experimental reporter rather than a normalization control, you may find it beneficial to have an independent measure of cell viability. CellTiter-Fluor™ Cell Viability Assay is a live-cell fluorescent assay that can be performed immediately before the HiBiT NanoDLR™ lytic assay. This multiplexed format was demonstrated using the NanoDLR™ assay for NanoLuc®-labeled HIF1A transcription factor together with a firefly luciferase transcriptional reporter and the CellTiter-Fluor™ assay, but the same approach could be used with a HiBiT-tagged protein using HiBiT NanoDLR™ assay (see Application Note: "Multiplexed Measurement of Protein Levels, Transcriptional Activation and Cell Viability in a Single Well using NanoDLR™ and CellTiter-Fluor™ Assays on the GloMax® Discover System" at: **www.promega.com/resources/pubhub/multiplexing-protein-levels-transcription-and-cell-viability-with-nanodlr-and-celltiter-fluor/**).

HiBiT can also be integrated at the native locus of a gene whose expression is transcriptionally induced by activating a defined signaling pathway. In this configuration, increases in HiBiT luminescence directly reflect pathway-driven changes in endogenous protein levels under native regulatory control. Firefly luciferase can be used either as a constitutive normalization control or as a promoter-driven reporter for the same or a related pathway. This can be implemented either by introducing HiBiT knock-ins into an existing stable firefly reporter line, or by transiently transfecting firefly reporter constructs into a HiBiT knock-in cell line. In both cases, dual measurement enables monitoring of both endogenous protein induction and pathway activity within the same well.

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7.C. Preventing Carryover of the Firefly Luciferase Inhibitor

The NanoDLR™ Stop & Glo® reagent contains a potent firefly luciferase inhibitor that efficiently quenches firefly luminescence prior to HiBiT measurement. This inhibitor exhibits reversible adsorption to plastic, tubing, injectors and container surfaces. If surfaces that have come into contact with NanoDLR™ Stop & Glo® reagent are not thoroughly cleaned or dedicated to that reagent, small amounts of inhibitor may leach into subsequent assays, resulting in unintended inhibition of firefly luciferase.

Because of the high sensitivity of the ONE-Glo™ EX reagent to the firefly luciferase inhibitor, even trace carryover can lead to a reduction in initial firefly luminescence or a decrease in signal half-life. Other firefly luciferase detection reagents may be less sensitive to this inhibitor, and assays based on Ultra-Glo™ recombinant luciferase are significantly less affected. However, you should assume that any assay measuring firefly luciferase activity is potentially susceptible to inhibitor carryover.

For low-throughput experiments, the most effective way to prevent inhibitor carryover is to dispense NanoDLR™ Stop & Glo® reagent using disposable reagent containers and pipette tips. For higher-throughput or automated workflows, best practice is to dedicate specific injectors, dispenser lines, tubing and filters to NanoDLR™ Stop & Glo® reagent whenever possible. If components must be reused, thorough cleaning with water and ethanol is required prior to subsequent use with firefly luciferase detection reagents. Even after extensive cleaning, trace inhibitor may remain, potentially causing reduced signal in the first wells dispensed.

The propensity of the firefly luciferase inhibitor to bind to materials varies widely. Tubing materials are ranked from most to least compatible with NanoDLR™ Stop & Glo® reagent as follows: Teflon > PEEK (polyetheretherketone) >>> silicone > PVC > Tygon. Containers are ranked from most to least resistant to inhibitor binding as follows: Pyrex® or borosilicate glass >>> polycarbonate > polystyrene > polypropylene > HDPE. Pyrex® or borosilicate glass containers exhibit minimal adsorption of the inhibitor and are preferred when reusable containers are required. Containers used with NanoDLR™ Stop & Glo® reagent should be rinsed thoroughly with water or ethanol at least seven times prior to routine washing.

Users employing automated dispensing or injection systems should consult Technical Manual #TM426 for detailed injector wash protocols and additional guidance.

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(i) use NanoBRET®-branded energy acceptors (e.g., BRET-optimized HaloTag® ligands) for all determinations of energy transfer activity by this product; or

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[b]U.S. Pat. No. 10,077,244 and other patents.

[c]U.S. Pat. No. 9,487,814, European Pat. No. 2959010 and other patents and patents pending.

[d]U.S. Pat. No. 9,797,889 and other patents pending.

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