TECHNICAL MANUAL

Nano-Glo® HiBiT Extracellular Detection System

Instructions for Use of Products
N2420, N2421 and N2422

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Nano-Glo® HiBiT Extracellular Detection 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

The Nano-Glo® HiBiT Extracellular Detection System[a,b,c] sensitively quantifies HiBiT-tagged proteins expressed in the plasma membrane or secreted into the extracellular medium using a simple add-mix-read assay protocol. HiBiT is an 11-amino-acid peptide tag that is fused to the N or C terminus of the protein of interest or inserted into an accessible location within the protein structure. The amount of a HiBiT-tagged protein present on the cell surface or secreted is determined by adding a nonlytic detection reagent containing the substrate furimazine and Large BiT (LgBiT; Figure 1), the large subunit used in NanoLuc Binary Technology (NanoBiT®; 1). Unlike Small BiT (SmBiT, 11 a.a.), which binds to LgBiT with low affinity (K_D = 190μM), HiBiT binds tightly to LgBiT (K_D = 0.7nM), promoting complex formation to generate a bright, luminescent enzyme. The amount of luminescence generated after adding the Nano-Glo® HiBiT Extracellular Reagent is proportional to the amount of HiBiT-tagged protein accessible to the extracellular medium over seven orders of magnitude (Figure 2).

Proteins of interest can be tagged with HiBiT at the N or C terminus using HiBiT expression vectors. Alternatively, the HiBiT tag can be added by standard methods to existing expression constructs. Finally, the HiBiT tag can be added to an endogenous locus by use of a genome-editing tool like CRISPR/Cas9, where the small size enables efficient integration using single-stranded donor DNA, and the bright signal permits quantification of even low abundance proteins at endogenous levels of expression.

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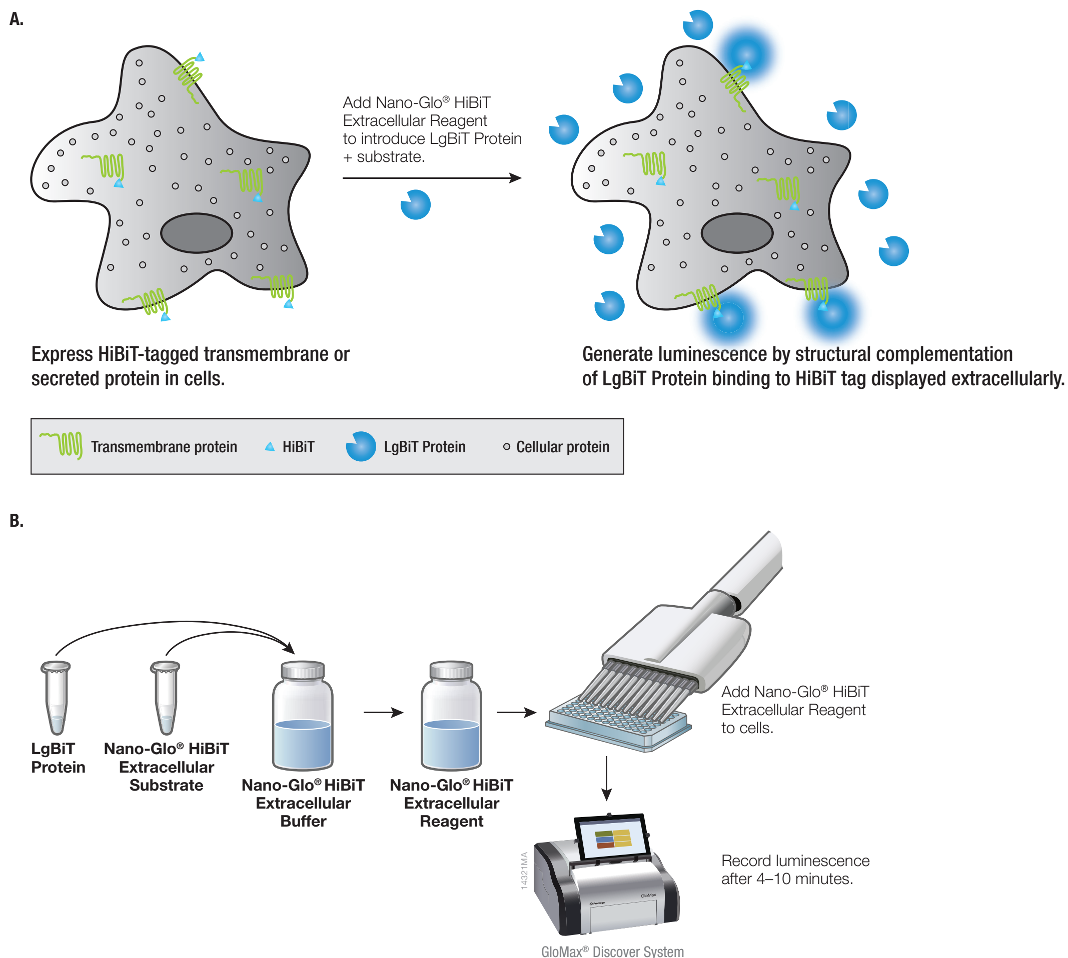

Figure 1. Nano-Glo® HiBiT Extracellular Detection System. Panel A. Adding the Nano-Glo® HiBiT Extracellular Reagent to mammalian cells introduces the cell-impermeable LgBiT protein present in the nonlytic reagent. LgBiT binds to HiBiT tag that is accessible to the extracellular medium and generates light proportional to the amount of cell-surface or secreted HiBiT-tagged protein. **Panel B.** The Nano-Glo® HiBiT Extracellular Reagent is reconstituted by adding LgBiT Protein and substrate to a nonlytic buffer. After adding the reagent to cells, the luminescence is measured after 2–10 minutes on a luminometer.

1. Description (continued)

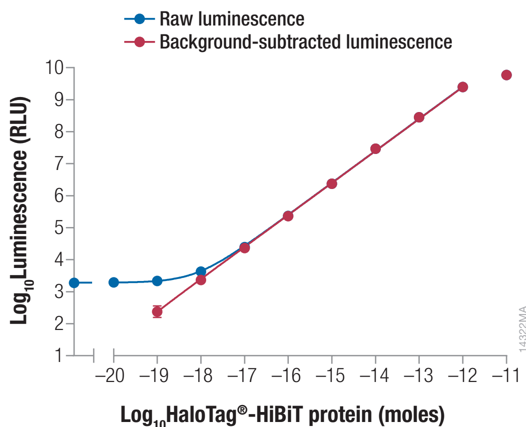

Figure 2. Titrating purified HiBiT-tagged protein. Nano-Glo® HiBiT Extracellular Reagent was added to a titration of purified HaloTag®-HiBiT protein (4) and luminescence measured after 2 minutes. The red curve is a best-fit line for the background-subtracted data, demonstrating at least 7 orders of magnitude of linear dynamic range (r^2 = 0.9982). Error bars represent standard deviation for n = 4.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
Nano-Glo® HiBiT Extracellular Detection System	**10ml**	**N2420**

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

- 10ml Nano-Glo® HiBiT Extracellular Buffer
- 0.2ml Nano-Glo® HiBiT Extracellular Substrate
- 0.1ml LgBiT Protein

PRODUCT	SIZE	CAT.#
Nano-Glo® HiBiT Extracellular Detection System	**100ml**	**N2421**

Each system contains sufficient components to prepare 100ml reagent. Includes:

- 100ml Nano-Glo® HiBiT Extracellular Buffer
- 2 × 1ml Nano-Glo® HiBiT Extracellular Substrate
- 1ml LgBiT Protein

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PRODUCT	SIZE	CAT.#
Nano-Glo® HiBiT Extracellular Detection System	**10 × 100ml**	**N2422**

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

- 10 × 100ml Nano-Glo® HiBiT Extracellular Buffer
- 5 × 4ml Nano-Glo® HiBiT Extracellular Substrate
- 10 × 1ml LgBiT Protein

Storage Conditions: Store the Nano-Glo® HiBiT Extracellular Detection System components at –20°C. Do not thaw above 25°C. The Nano-Glo® HiBiT Extracellular Buffer may be stored at room temperature for 1 year. The Nano-Glo® HiBiT Extracellular Substrate and LgBiT Protein will not freeze at –20°C.

Available Separately

PRODUCT	SIZE	CAT.#
Nano-Glo® HiBiT Blotting System	**100ml**	**N2410**
Nano-Glo® HiBiT Lytic Detection System	**10ml**	**N3030**
	100ml	**N3040**
	10 × 100ml	**N3050**
HiBiT Control Protein	**100µl**	**N3010**

HiBiT Cloning Vectors

Vector Name	Cloning Format	Tag Orientation	Cat.#
pBiT3.1-N [CMV/HiBiT/Blast]	MCS	HiBiT-POI	N2361
pBiT3.1-C [CMV/HiBiT/Blast]	MCS	POI-HiBiT	N2371
pBiT3.1-secN [CMV/HiBiT/Blast]	MCS	IL6-HiBiT-POI	N2381
pFC37K HiBiT CMV-neo Flexi® Vector	Flexi	POI-HiBiT	N2391
pFN38K HiBiT CMV-neo Flexi® Vector	Flexi	HiBiT-POI	N2401
pFN39K secHiBiT CMV-neo Flexi® Vector	Flexi	IL6-HiBiT-POI	N2411

3. Nano-Glo® HiBiT Extracellular Assay Protocols

3.A. Overview of the Nano-Glo® HiBiT Extracellular Detection System

The Nano-Glo® HiBiT Extracellular Detection System quantifies the amount of HiBiT tag that is accessible to the extracellular medium. The assay is compatible with most commonly used cell culture medium containing 0–10% serum and has been tested with DMEM, RPMI 1640, McCoy's 5A, MEMα, Opti-MEM® I, F-12 and CO_2-Independent media. While the reagents have been designed to give a signal half-life of greater than 60 minutes at 22°C, different combinations of medium and serum may affect the background, signal, or signal decay rate (see Section 6.H). Additional information about the Nano-Glo® HiBiT Extracellular Detection System can be found in Section 6.

To achieve a linear assay performance at low light levels, the background luminescence must be subtracted from all readings. Background luminescence generally originates from two main sources: 1) reagent background from autoluminescence of the furimazine substrate and low levels of activity associated with the LgBiT protein, and 2) machine background from the luminometer. Reagent background can vary with media type, and is increased by both serum and cells (see Section 6.H). Therefore, for the most accurate measurements of low-abundance proteins, include a sample of untransfected or mock-transfected cells in the same medium to measure the assay background. For the greatest sensitivity, reduce the amount of serum by exchanging medium prior to the assay (see Section 6.I). 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 measuring luminescence.

3.B. Preparing the Nano-Glo® HiBiT Extracellular Reagent

Calculate the amount of Nano-Glo® HiBiT Extracellular Reagent needed to perform the desired experiments. This usually constitutes a volume equal to the total amount of medium in wells, plus any extra required for dispensing. Dilute the LgBiT Protein 1:100 and the Nano-Glo® HiBiT Extracellular Substrate 1:50 into an appropriate volume of room temperature Nano-Glo® HiBiT Extracellular Buffer in a new tube. Mix by inversion.

For example, if 4ml of Nano-Glo® HiBiT Extracellular Reagent is needed, transfer 4ml of Nano-Glo® HiBiT Extracellular Buffer to a 15ml centrifuge tube and add 40µl of LgBiT Protein and 80µl of Nano-Glo® HiBiT Extracellular Substrate.

Notes:

1. The LgBiT Protein stock contains glycerol, which prevents it from freezing at –20°C. The viscosity of this solution may make accurate pipetting difficult. Pipet slowly and avoid excess solution clinging to the outside of the pipette tip. Use a positive displacement pipette, if possible.
2. If the Nano-Glo® HiBiT Extracellular Substrate or LgBiT Protein has collected in the cap or on the sides of the tube, briefly spin the tubes in a microcentrifuge.
3. We recommend preparing the Nano-Glo® HiBiT Extracellular Reagent fresh for each use. Once reconstituted, the reagent will lose about 15% activity over 8 hours and about 60% activity over 24 hours at room temperature. Unused reconstituted reagent may be stored at –80°C, –20°C or 4°C for later use, although there will be some loss of performance relative to freshly prepared reagent. At 4°C, the reconstituted reagent should lose less than 20% activity over 24 hours.

3.C. General Protocol for Adding Nano-Glo® HiBiT Extracellular Reagent to Cells

1. Reconstitute the Nano-Glo® HiBiT Extracellular Reagent as described in Section 3.B.
2. Remove plates containing mammalian cells expressing a HiBiT-tagged protein from the 37°C incubator.

   Optional: To minimize well-to-well variability caused by differences in temperature, equilibrate the plate to room temperature (e.g., 5 minutes on a metal block).
3. Add a volume of Nano-Glo® HiBiT Extracellular Reagent equal to the culture medium present in each well, and mix. For example, add 100µl of Nano-Glo® HiBiT Extracellular Reagent to 100µl of cell culture medium.

   Note: Mix the samples by gently pipetting samples or placing the plate on an orbital shaker (300–500 rpm) for 3–10 minutes.
4. Measure luminescence 10 minutes after adding reagent. For a HiBiT tag placed within the protein sequence, longer incubation times may be necessary compared to terminal protein tagging. Measure luminescence using settings specific to your instrument. For 96-well plates on GloMax® instruments, integration times of 0.5–2 seconds are recommended. Longer integration times may improve data quality at lower levels of expression. The luminescence intensity will generally decay in a well-mixed sample with a signal half-life of 1–2.5 hours, depending on conditions (see Figure 8, Panel B; Figure 9 Panel B; and Figure 10, Panel B).

Notes:

1. To ensure luminescence is proportional to the amount of HiBiT-tagged protein present, subtract the assay background, especially when measuring low amounts of protein. Include untransfected or mock-transfected cells as background controls in your experiment (see Section 6.I).
2. Placing HiBiT in internal protein positions, like extracellular surface loops, may slow LgBiT and HiBiT equilibration. Increase reagent incubation times to compensate, if necessary.

3.D. Alternative Protocol for Rapid Measurements

This protocol rapidly quantifies the amount of HiBiT tag on the surface of adherent cells. This is useful if the biological state of the cell is changing quickly (e.g., agonist-induced internalization of a cell surface receptor).

1. Remove plates containing mammalian cells expressing HiBiT-tagged protein from the 37°C incubator.
2. Immediately add a volume of room-temperature Nano-Glo® HiBiT Extracellular Reagent equal to the volume of culture medium present in each well using low-speed reagent dispensing. Do **not** mix. The reagent is slightly denser than cell culture media for rapid equilibration of HiBiT-tagged proteins with LgBiT Protein and furimazine substrate. Incubate in a room temperature or 37°C luminometer as described below.
   a. **Room-temperature luminometer:** After adding the room-temperature reagent, incubate plates at room temperature until you measure luminescence. Keep the plate inside the room-temperature luminometer or outside of it (e.g., on a metal block) to equilibrate all samples to the same temperature and reduce well-to-well variability from temperature gradients.
   b. **37°C luminometer:** Alternatively, you may wish to keep cells at 37°C by placing plates in a 37°C luminometer. After adding reagent, immediately place plates inside the luminometer and incubate until you measure luminescence. Plates can also be incubated in a 37°C incubator after adding reagent and then transferred to the luminometer. To prevent temperature fluctuations during plate handling, we recommend using only the inner 60 wells of 96-well plates. Pipet 150µl of sterile buffer into the empty outer wells and inner-well spaces during cell plating to help maintain sample temperatures when removed from the 37°C incubator. Warming the reagent to 37°C immediately before dispensing is generally not necessary and will adversely affect reagent stability.
3. Measure luminescence 2–10 minutes after adding reagent. Generally, incubating for 4 minutes is sufficient for maximal luminescence with low well-to-well variability between replicates. Measure luminescence using settings specific to your instrument. For 96-well plates on GloMax® instruments, we recommend integration times of 0.5–2 seconds. Short integration times may measure a plate more quickly to reduce variability across the plate for experimental systems where conditions are changing rapidly, while longer integration times may improve data quality at low expression levels.

Notes:

1. While luminescence intensity in a well-mixed sample has a signal half-life of 1–2.5 hours, gently adding reagent without mixing, as described above, leads to more rapid changes in signal intensity. With this protocol, the signal generally peaks about 4 minutes after reagent addition, but then decays more quickly as the sample mixes, yielding a signal half-life of about 30 minutes. For this reason, we recommend minimizing the amount of time to measure the entire plate by decreasing the integration time to reduce variability caused by signal decay between the first and last measurement on the plate.
2. When assaying surface expression changes, the internalization of the LgBiT/HiBiT complex with the fusion partner generally causes few problems. The short incubation time used in this protocol minimizes any LgBiT internalization. In addition, internalized LgBiT/HiBiT complexes often have significantly lower activity than surface complexes, possibly due to reduced substrate availability in endosomes compared to surface protein.

4. Representative Data

Monitoring β2-adrenergic receptor internalization following stimulation by agonists

The β2-adrenergic receptor (β2AR) is a seven-transmembrane, G-protein coupled receptor (GPCR) that binds adrenaline and synthetic agonists to trigger a range of physiological responses in organisms. Agonist binding results in the activation of Gs, the stimulatory G protein, promoting an increase in intracellular cAMP and activating protein kinase A. The signal is propagated through the phosphorylation of downstream targets, and receptor phosphorylation leads to recruitment of β-arrestin-2. The binding of β-arrestin-2 decouples the receptor from G proteins and promotes clathrin-mediated endocytosis, after which most receptors rapidly recycle back to the plasma membrane.

The Nano-Glo® HiBiT Extracellular Detection System can quantify the activation and internalization of GPCRs. Using β2AR as a model system, we monitored the internalization of endogenous and overexpressed HiBiT-β2AR following treatment with a panel of full and partial agonists. HiBiT was inserted following the initiating methionine either with no linker or with a 10 a.a. Gly/Ser linker sequence (Figure 3). Varying amounts of a plasmid DNA construct were transfected to achieve varying levels of expression, and these conditions were compared to stable expression and expression from the endogenous locus. For endogenous expression, the CRISPR/Cas9 gene-editing approach was used to rapidly make a cell pool (5), followed by the isolation of individual clones.

As expected, known agonists of β2AR promoted receptor internalization with the expected differences in rank order potency. Additionally, the partial agonists salbutamol and salmeterol displayed the expected reduction in the extent of internalization (7). These experiments were performed in <5 minutes after removing agonist-treated cells from the 37°C, 5% CO_2 incubator. Endogenous expression, stable expression and transient transfection of small amounts of DNA produced greater fold responses than transfection of higher amounts of DNA (Section 6.C). In general, lower levels of expression are preferred for receptor internalization experiments.

4. Representative Data (continued)

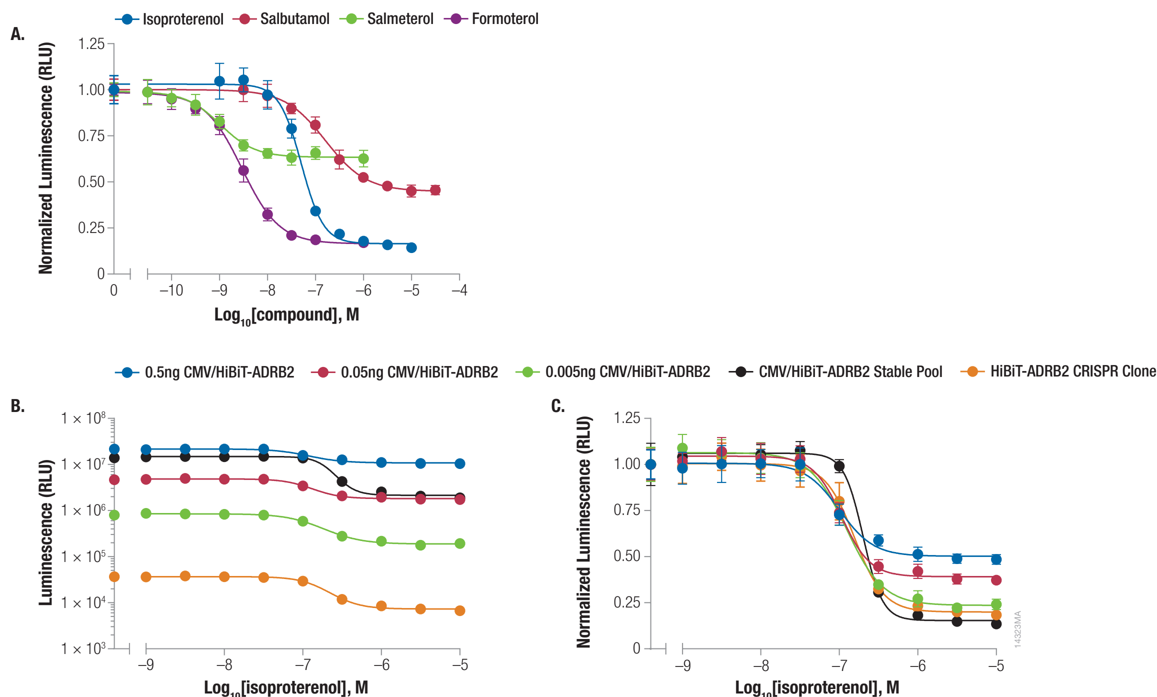


Figure 3. HiBiT-β2AR internalization following stimulation by agonists. Panel A. A stably transfected pool of HEK 293 cells was generated using a construct expressing HiBiT-GGSGGGGSGG-β2AR from a CMV promoter. The cells were treated for 30 minutes with a titration of various ligands, Nano-Glo® HiBiT Extracellular Reagent was added to each well, and luminescence was measured after 4 minutes using the protocol in Section 3.D. The background-subtracted luminescence was normalized to untreated cells. **Panel B.** HEK 293 cells were transiently transfected with different amounts of a CMV HiBiT-β2AR expression construct diluted in carrier DNA. These cells were compared to the stably transfected pool described in Panel A and a HEK 293 clone expressing HiBiT-β2AR from its endogenous locus. Cells were plated in 96-well plates and treated the following day for 30 minutes with a titration of isoproterenol. The Nano-Glo® HiBiT Extracellular Reagent was added to all wells and luminescence measured after 4 minutes using the protocol in Section 3.D. Background-subtracted luminescence was plotted to highlight the varying expression levels. **Panel C.** The normalized luminescence from the experiment in Panel B is plotted relative to the control with no isoproterenol added. Error bars represent standard deviation for n = 6.

Monitoring Epidermal Growth Factor Receptor internalization following stimulation by agonists

The epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase (RTK) that binds EGF family growth factors. Agonist binding leads to receptor autophosphorylation and recruitment of adapter proteins containing SH2 domains, resulting in the activation of downstream signaling pathways. Activated receptor undergoes clathrin-mediated endocytosis, followed by recycling back to the plasma membrane or degradation in the late endosome/lysosome.

The Nano-Glo® HiBiT Extracellular Detection System can quantify the activation and internalization of RTKs. For example, using EGFR as a model system, we monitored the internalization of endogenous and overexpressed HiBiT-EGFR following treatment with EGF. The HiBiT tag was inserted between the native signal peptide and the mature EGFR, but in some cases, the native signal peptide was replaced with the signal peptide from IL-6. For some clones, a small Gly/Ser linker fused HiBiT and mature EGFR. CMV- or HSV-TK-driven expression constructs were diluted into carrier DNA to reduce expression levels when transfected into cells. The results were compared to expression of HiBiT-EGFR from the endogenous locus using the CRISPR/Cas9 gene editing approach to rapidly make a cell pool (5).

As expected, EGF promoted receptor internalization of HiBiT-EGFR (Figure 4). These measurements were performed in <5 minutes after removing agonist-treated cells from the 37°C, 5% CO_2 incubator. The greatest fold decrease in surface expression was seen using endogenous expression, showing a greater than tenfold decrease in luminescence.

4. Representative Data (continued)

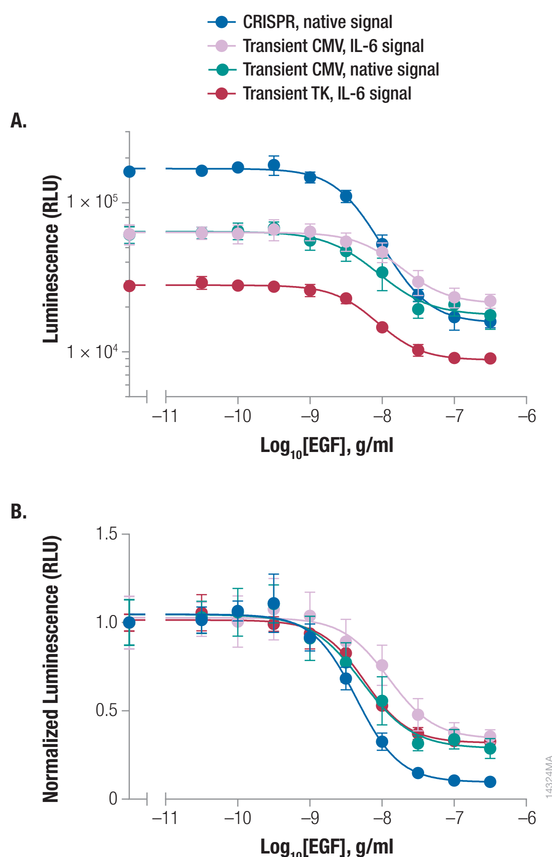


Figure 4. Internalization of HiBiT-EGFR following EGF stimulation. Panel A. HeLa cells were transiently transfected with 0.005ng/well of CMV-based or 0.05ng/well of HSV-TK-based expression constructs diluted in carrier DNA. Transient transfection was compared to expression from the endogenous locus using a CRISPR-derived pool of cells. Expression constructs had the HiBiT tag inserted immediately following the native signal sequence or replaced the native signal sequence with the IL-6 signal sequence and HiBiT. Cells were plated in 96-well plates and treated the following day for 30 minutes with titrated EGF. The Nano-Glo® HiBiT Extracellular Reagent was added to all wells, and luminescence measured after 4 minutes using the protocol in Section 3.D. Background-subtracted luminescence was plotted, showing similar average expression levels for the four conditions. **Panel B.** The normalized luminescence from the experiment of Panel A is plotted relative to the control with no EGF added. The plasmid constructs used for transfection were transient CMV, IL-6 signal = CMV/IL6-HiBiT-GSSG-EGFR(25–1210); transient CMV, native signal = CMV/EGFR(1–24)-HiBiT-GSSG-EGFR(25–1210); and transient TK, IL-6 signal = HSV-TK/IL6-HiBiT-GSSG-EGFR(25–1210). Error bars represent standard deviation for n = 6.

Secretion of PCSK9-HiBiT

Proprotein convertase subtilisin/kexin type 9 (PCSK9) undergoes auto-proteolytic processing in the endoplasmic reticulum, followed by trafficking to the plasma membrane and secretion. Secreted PCSK9 promotes degradation of the LDL receptor, and loss-of-function mutations of PCSK9 are associated with protection from coronary disease. The C679X mutation, for instance, causes misfolding that blocks secretion without impairing proteolytic activity (2). Mutating the catalytic histidine of the protease domain with H226A, on the other hand, prevents autoproteolysis and inhibits protein secretion (6).

The Nano-Glo® HiBiT Extracellular Detection System can be used to quantify the secretion of HiBiT-tagged proteins of interest within minutes without multiple steps like separation of medium from cells, making it ideal for high-throughput screening of compounds that alter expression, processing or trafficking of secreted proteins. Furthermore, parallel measurement of total protein with the Nano-Glo® HiBiT Lytic Detection System allows users to distinguish changes in protein expression from changes in protein trafficking.

We monitored the amount of PCSK9-HiBiT secreted into the medium over time using the Nano-Glo® HiBiT Extracellular Detection System and assessed total protein levels over time using the Nano-Glo® HiBiT Lytic Detection System. The wild-type protein was compared both to mutant forms with defective secretion and to treatment with brefeldin A (BFA) to inhibit secretion. HEK 293 cells were transiently transfected in bulk with CMV-driven expression constructs. The following day, the cells were dissociated, resuspended and plated for immediate treatment with either BFA or vehicle control. At time zero, the mutant forms of PCSK9 generated extracellular signals near assay background levels, while the wild-type protein had a signal well above background even though the lytic assay showed similar total protein levels for all PCSK9 forms. This highlights the common observation that medium exchange or cell washing does not remove all of the extracellular HiBiT signal. This signal may be caused by protein present in damaged cells or cell debris, protein bound to the outside of cells, or protein found in residual cell medium. For experiments measuring differences in the rate or extent of HiBiT-tagged protein secretion, an inducible expression system may help lower this protein background by reducing extracellular accumulation prior to cell treatment.

As seen in Figure 5, the extracellular signal from wild-type PCSK9 steadily increases over time as the protein is expressed and secreted. BFA treatment, however, effectively blocks secretion of the protein while having almost no effect on protein expression as measured by the lytic assay. Similarly, the mutant forms of the protein show very little accumulation of extracellular signal over the course of the experiment. The total levels of the mutant proteins change little over time, presumably because protein levels within the cell had already reached near steady-state levels at the beginning of the experiment.

4. Representative Data (continued)

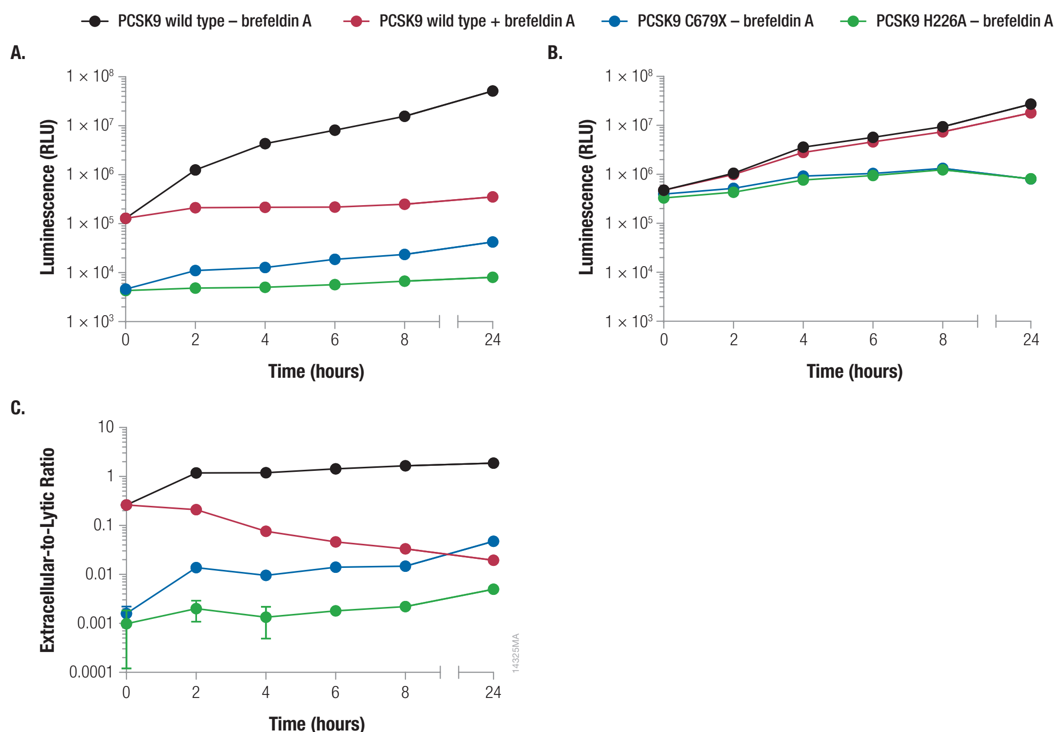


Figure 5. Effect of brefeldin A or mutations on PCSK9-HiBiT secretion. HEK 293 cells were transiently transfected with CMV/PCSK9-HiBiT expression constructs containing either wild-type sequence or a mutation, H226A or C679X, which inhibits secretion and causes the protein to accumulate in the endoplasmic reticulum. Transfected cells were dissociated, washed, resuspended in medium, plated, and then immediately treated at time zero with either vehicle or 1µM brefeldin A (BFA) to inhibit secretion. At various time points after plating and treatment, replicate plates were removed from the 37°C incubator, and either the Nano-Glo® HiBiT Extracellular or Lytic Reagent was added to respective wells. Luminescence was measured after 10 minutes following the protcol in Section 3.C. **Panel A.** Raw luminescence from the Nano-Glo® HiBiT Extracellular Assay increased over time for wild-type PCSK9 as the protein was expressed and secreted, but BFA effectively inhibited secretion. Both mutant proteins showed very low levels of extracellular protein, near reagent background. **Panel B.** Raw luminescence from the Nano-Glo® HiBiT Lytic Assay shows that BFA does not inhibit protein expression of wild-type PCSK9, just its secretion. At time zero, the mutant forms of PCSK9 are present at nearly the same level as wild-type PCSK9, but the total amount of protein changes little over the course of the experiment. **Panel C.** The ratio of the background-subtracted extracellular and lytic signals increases initially for wild-type PCSK9, but after two hours, nearly all the protein is extracellular, leading to a relatively constant, high value. Likewise, the mutant forms of the proteins remain intracellular throughout the time course, so the ratio stays relatively constant at a low level. BFA treatment of wild-type PCSK9 causes increasing accumulation of intracellular protein, so the ratio decreases continually over time until it is similar to that of the mutant forms of the protein. Error bars represent standard deviation for n = 6.

5. Related Products

Product	Size	Cat.#
Mammalian Lysis Buffer	40ml	G9381
Digitonin (20mg/ml)	40µl	G9441
NanoBiT® PPI MCS Starter System	1 each	N2014
NanoBiT® PPI Flexi® Starter System	1 each	N2015
FuGENE® HD Transfection Reagent	1ml	E2311
	5 × 1ml	E2312
ViaFect™ Transfection Reagent	0.75ml	E4981
	2 × 0.75ml	E4982
Transfection Carrier DNA	5 × 20µg	E4881
GloMax® Discover System	1 each	GM3000
GloMax® Explorer Fully Loaded	1 each	GM3500
GloMax® Explorer with Luminescence and Fluorescence	1 each	GM3510

6. Appendix

6.A. Overview of the Nano-Glo® HiBiT Extracellular Assay

Changes in the surface expression of membrane proteins can play a large role in biological regulatory mechanisms and cellular responses to changing environments. However, precisely quantifying the amount of protein at the plasma membrane separately from intracellular stores can be difficult. A commonly used approach to monitor changes in surface expression is to perform a cell-based ELISA, a labor-intensive procedure requiring fixation of cells, followed by multiple steps of antibody binding and washing, which can increase well-to-well variability and decrease throughput. Furthermore, the process requires high-quality antibodies to detect proteins at endogenous levels of expression.

At 11 amino acids in length, the High BiT (HiBiT) peptide tag is comparable to commonly used epitope tags. When used in combination with cell-impermeable Large BiT (LgBiT, 17.6 kDa), surface-expressed or secreted HiBiT-tagged proteins are sensitively quantified over a linear dynamic range with seven orders of magnitude (Figure 2). In contrast to cell-based ELISAs, the Nano-Glo® HiBiT Extracellular Assay can quantify surface expression of HiBiT-tagged proteins at endogenous levels in less than 5 minutes, using a simple assay protocol that is amenable to high-throughput screening.

Luciferases are commonly used to monitor gene expression and protein levels because of their broad dynamic range and sensitivity. NanoLuc® luciferase is a 19.1 kDa engineered enzyme that was developed to be brighter and more versatile than other reporter proteins (3). NanoLuc® luciferase was subsequently used as the basis for an optimized two-component structural complementation system called Nano-Glo® Binary Technology (NanoBiT®). In this system, the LgBiT subunit has little activity on its own, but binding to an 11 amino acid peptide leads to enzyme complementation that restores nearly the entire NanoLuc® Luciferase activity (1). Because it does not readily interact with LgBiT on its own, the low-affinity peptide, termed Small BiT (SmBiT), can be used to study protein:protein interactions of fusion partners with the NanoBiT® PPI System. By contrast, the HiBiT peptide binds spontaneously to LgBiT with high affinity (~1nM).

6.A. Overview of the Nano-Glo® HiBiT Extracellular Assay (continued)

HiBiT, therefore, makes an excellent tag for proteins because its small size reduces any effect on protein function. The high-affinity binding of HiBiT to the excess LgBiT protein present in the Nano-Glo® HiBiT Extracellular Reagent converts the HiBiT tag into a bright, luminescent enzyme for quantifying protein expression levels.

The Nano-Glo® HiBiT Extracellular Detection System was designed to rapidly quantify HiBiT tag that is accessible to cell-impermeable LgBiT Protein in the extracellular medium using a live-cell, nonlytic assay format. The brightness of the signal quantifies many proteins at endogenous expression levels, while a dynamic range with seven orders of magnitude ensures that highly overexpressed proteins can also be quantified (Figures 2, 3 and 5). The HiBiT signal is stable, with a typical signal half-life of 1–2.5 hours, depending on conditions (Figure 6). While the Nano-Glo® HiBiT Extracellular Detection System measures surface or secreted HiBiT-tagged proteins, the Nano-Glo® HiBiT Lytic Detection System quantifies the total amount of HiBiT-tagged protein present in a sample (Cat.# N3030, N3040, N3050). In addition, HiBiT-tagged proteins can be detected on membranes following SDS-PAGE using the Nano-Glo® HiBiT Blotting System (Cat.# N2410).

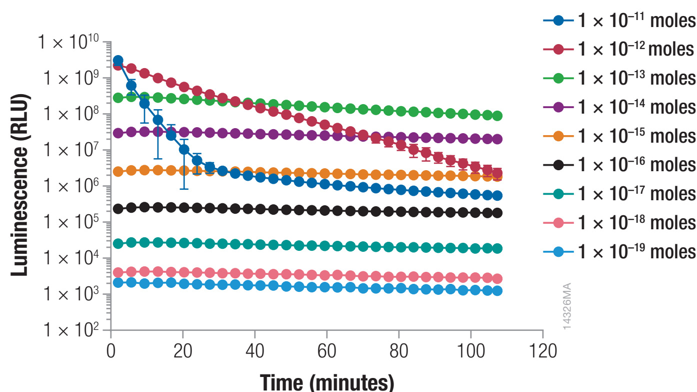


Figure 6. Signal decay kinetics for the Nano-Glo® HiBiT Extracellular Assay. The signal decay kinetics are shown for the titration of purified HaloTag®-HiBiT from Figure 2. Error bars represent standard deviation for n = 4.

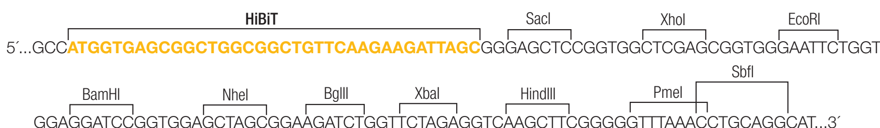
6.B. Appending HiBiT to Proteins of Interest

Multiple Cloning Site (MCS) Vectors

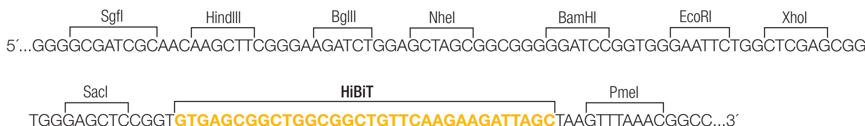
Follow standard cloning protocols to introduce genes of interest (GOI) into the HiBiT-containing vectors pBiT3.1-N [CMV/HiBiT/Blast], pBiT3.1-C [CMV/HiBiT/Blast] and pBiT3.1-secN [CMV/HiBiT/Blast] Vectors. Figure 7 displays the unique restriction enzyme sites present in the MCS of each vector. XhoI and SacI sites are present within the Gly/Ser linker that fuses the POI to the HiBiT tag. The XhoI site can be used to introduce the standard 8 Gly/Ser linker, while the SacI site can be used to introduce the smallest linker, Gly-Ser-Ser-Gly. Use of other restriction sites will generate longer linker sequences. See the section "Adding HiBiT to Other Expression Vectors" for more information about linkers or adding the HiBiT tag after native signal sequences. The HiBiT tag or ORF can be transferred from these vectors into any other expression system as long as the HiBiT coding sequence is not altered and HiBiT is used in conjunction with the Nano-Glo® detection reagents.

Table 1 lists the fusion protein created when choosing each of the four restriction sites closest to the HiBiT tag, generating different linker lengths. When designing PCR primers, incorporate the nucleotide sequences given in Table 2 to produce the correct in-frame protein and linker sequence. For pBiT3.1-N [CMV/HiBiT/Blast] and pBiT3.1-secN [CMV/HiBiT/Blast] Vectors, ensure that the 3´ primer contains a stop codon. For pBiT3.1-C [CMV/HiBiT/Blast] Vector, ensure that the 5´ primer contains an initiating ATG codon. These vectors carry kanamycin resistance in bacteria and blasticidin resistance in mammalian cells. The pBiT3.1-secN [CMV/HiBiT/Blast] Vector includes the IL-6 signal sequence N-terminal to the HiBiT tag and the POI to drive efficient secretion or plasma membrane targeting of proteins. Design the 5´ PCR primer to begin with the residue following any native cleavable signal sequence for the pBiT3.1-secN [CMV/HiBiT/Blast] Vector. This will replace the native signal sequence with the strong IL-6 sequence that, after signal peptide cleavage, will leave a mature protein with HiBiT on the N terminus.

pBiT3.1-N [CMV/HiBiT/Blast] Vector



pBiT3.1-C [CMV/HiBiT/Blast] Vector



pBiT3.1-secN [CMV/HiBiT/Blast] Vector

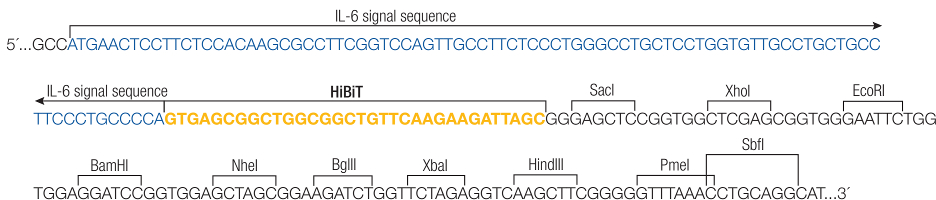


Figure 7. Available Restriction Sites in MCS Entry Vectors.

6.B. Appending HiBiT to Proteins of Interest (continued)

Table 1. Linker Sequences Associated with SacI, XhoI, EcoRI or BamHI Sites in the pBiT3.1 Vectors.

HiBiT Entry Vector	Fusion Protein	MCS Restriction Site
pBiT3.1-N [CMV/HiBiT/Blast] Vector	HiBiT-GSSG-POI	SacI
	HiBiT-GSSGGSSG-POI	XhoI
	HiBiT-GSSGGSSGGNS-POI	EcoRI
	HiBiT-GSSGGSSGGNSGGGS-POI	BamHI
pBiT3.1-C [CMV/HiBiT/Blast] Vector	POI-GSSG-HiBiT	SacI
	POI-GSSGGSSG-HiBiT	XhoI
	POI-GNSGSSGGSSG-HiBiT	EcoRI
	POI-GSGGNSGSSGGSSG-HiBiT	BamHI
pBiT3.1-secN [CMV/HiBiT/Blast] Vector	IL6-HiBiT-GSSG-POI	SacI
	IL6-HiBiT-GSSGGSSG-POI	XhoI
	IL6-HiBiT-GSSGGSSGGNS-POI	EcoRI
	IL6-HiBiT-GSSGGSSGGNSGGGS-POI	BamHI

Table 2. Primer Sequences for Restriction Enzyme Sites in the MCS of the pBiT3.1 Vectors.

HiBiT Entry Vector	Restriction Site	Primer Sequence
pBiT3.1-N [CMV/HiBiT/Blast] Vector	SacI	5´-NNNNNNGAGCTCCGGT(GOI)-3´
	XhoI	5´-NNNNNNCTCGAGCGGT(GOI)-3´
	EcoRI	5´-NNNNNNGAATTCT(GOI)-3´
	BamHI	5´-NNNNNNGGATCC(GOI)-3´
pBiT3.1-C [CMV/HiBiT/Blast] Vector	SacI	5´-NNNNNNGAGCTCCC(RC GOI)-3´
	XhoI	5´-NNNNNNCTCGAGCC(RC GOI)-3´
	EcoRI	5´-NNNNNNGAATTCCC(RC GOI)-3´
	BamHI	5´-NNNNNNGGATCC(RC GOI)-3´
pBiT3.1-secN [CMV/HiBiT/Blast] Vector	SacI	5´-NNNNNNGAGCTCCGGT(GOI)-3´
	XhoI	5´-NNNNNNCTCGAGCGGT(GOI)-3´
	EcoRI	5´-NNNNNNGAATTCT(GOI)-3´
	BamHI	5´-NNNNNNGGATCC(GOI)-3´

5´ N nucleotides represent the 5–10 bases to be added to ensure efficient restriction enzyme digestion.
GOI = gene of interest; RC GOI = reverse complement of the gene of interest

Flexi® Entry Vectors

The Flexi® Vector System rapidly transfers a single DNA insert containing the protein coding sequence between multiple Flexi® Vectors for adding both N- and C-terminal fusions. Follow the instructions in the *Flexi® Vector Systems Technical Manual* #TM254 to clone protein-coding sequences into the pFC37K HiBiT, pFN38K HiBiT or pFN39K secHiBiT CMV-neo Flexi® Vectors. The Flexi® Vectors contain a universal lethal gene, barnase, which must be replaced with an insert for survival of the desired clone. This lethality ensures high-efficiency transfer of a protein-coding region between vector backbones. It is impossible to propagate Flexi® Vectors in strains commonly used for plasmid propagation and protein expression without replacing the barnase gene. The HiBiT Flexi® Vectors carry kanamycin resistance in bacteria and neomycin resistance in mammalian cells. The pFN39K secHiBiT CMV-neo Flexi® Vector includes the IL-6 signal sequence N-terminal to HiBiT and the POI for efficient secretion or plasma membrane targeting of proteins. When designing PCR primers for this vector, remove any native signal sequence so that the HiBiT tag is appended to the N terminus of the mature protein. The HiBiT tag or ORF can be transferred from these vectors into any other expression system as long as the HiBiT coding sequence is not altered and HiBiT is used in conjunction with the Nano-Glo® detection reagents.

There are different options for cloning PCR products into the Flexi® Vectors:

- For adding HiBiT to the N terminus (pFN38K HiBiT and pFN39K secHiBiT CMV-neo Flexi® Vectors), follow the protocol in Section 4 of TM254.
- When adding HiBiT to the C terminus (pFC37K HiBiT CMV-neo Flexi® Vector), substitute the Flexi® Vector digestion protocol in Section 5.B, Step 3, of TM254 when digesting acceptor Flexi® Vectors in Section 4.B, Step 2. The Carboxy Flexi® Enzyme Blend is used only for acceptor C-terminal Flexi® Vectors.
- First clone the protein-coding sequence into pF4A CMV Flexi® Vector (Cat.# C8481); once the sequence is verified, transfer the ORF into the HiBiT Flexi® Vectors.

When designing PCR primers, make sure to add 1 base between the SgfI recognition site and the start codon. Do not include a stop codon at the end of the protein coding region if the insert will be used for C-terminal fusions (see TM254, Section 9.A).

Our Find My Gene resource (**www.promega.com/findmygene/search.aspx**) contains nearly 10,000 ready-to-use constructs from the Kazusa DNA Research Institute that can be used as Flexi® donor DNA. Each construct from Kazusa can be transferred directly into HiBiT Flexi® Vectors without the need for PCR.

Table 3. Linker Sequences Associated with the HiBiT Flexi® Vectors.

Vector Name	Fusion Protein
pFC37K HiBiT CMV-neo Flexi® Vector	POI-VSQGSSGGSSG-HiBiT
pFN38K HiBiT CMV-neo Flexi® Vector	HiBiT-GSSGGSSGAIA-POI
pFN39K secHiBiT CMV-neo Flexi® Vector	IL6-HiBiT-GSSGGSSGAIA-POI

6.B. Appending HiBiT to Proteins of Interest (continued)

Adding HiBiT to Other Expression Vectors

The HiBiT tag can be added directly to existing protein expression constructs by PCR-based or gene-synthesis methods. The rights to synthesize the HiBiT tag can be obtained by reviewing and accepting the Terms and Conditions of Use at: **www.promega.com/HiBiT-Synthesis**. The HiBiT tag may be transferred into any expression system as long as the peptide sequence is not altered and HiBiT is used in conjunction with the Nano-Glo® detection reagents.

In designing HiBiT expression constructs, you may choose to reduce or eliminate the size of the linker between the protein of interest and HiBiT. We use an eight-residue Gly/Ser linker to ensure accessibility of the HiBiT tag when fused to a wide variety of proteins. However, in most cases we found reducing the linker to just a few residues or eliminating the linker completely has not significantly impaired HiBiT tag function. When a protein of interest has an N-terminal signal sequence that is cleaved from the mature protein, the HiBiT tag can be placed immediately following the signal cleavage site to fuse HiBiT to the N terminus of the mature protein.

In some cases, a HiBiT tag can be successfully placed at an internal site of a protein rather than on one of the termini. Whether the internal HiBiT tag can effectively complement LgBiT depends primarily on its accessibility and is specific to the particular protein structure and tag placement. When placed internally, the HiBiT tag may require linker sequences on either side of HiBiT because efficient complementation requires that the tag adopt an extended conformation. Placing HiBiT in a tight loop may generate conformational strain, reducing signal intensity. Generally, internal tags give somewhat reduced signals and take longer to equilibrate with LgBiT. When first using an internally tagged protein, we recommend monitoring luminescence for up to 3 hours after adding reagent to determine the optimal incubation time for measurement.

CRISPR/Cas9 Knock-In of HiBiT at the Endogenous Locus

Because of its small size and brightness, HiBiT makes an ideal tag for CRISPR/Cas9-mediated genomic editing, which involves introducing (knocking-in) the tag at the endogenous gene locus. The bright luminescence generated by HiBiT facilitates measuring proteins expressed at endogenous levels. Tagging endogenous proteins with HiBiT can ensure that no overexpression artifacts are introduced and that more physiologically relevant biological responses are observed (see Figures 3 and 4).

Because the HiBiT sequence is only 33 nucleotides long, homology-directed repair to insert the HiBiT tag can be accomplished with a single-stranded DNA oligonucleotide, eliminating the need to generate any DNA constructs. A ribonucleoprotein particle (RNP) containing the guide RNA (gRNA) and Cas9 protein can be electroporated into cells along with the donor ssDNA to generate high-efficiency insertion of HiBiT. This insertion event can be measured by the Nano-Glo® HiBiT Extracellular Detection System in 1–2 days (5). Contact Promega Technical Services for more information about using CRISPR/Cas9 to add HiBiT to endogenous proteins. The rights to synthesize the HiBiT tag on the donor ssDNA can be obtained by reviewing and accepting the Terms and Conditions of Use at: **www.promega.com/HiBiT-Synthesis**.

6.C. Effects of Expression Level on Assay Performance

Differences in expression level can affect assay performance in two main ways: Enzyme signal kinetics and the observed biological response. The Nano-Glo® HiBiT Extracellular Detection System should maintain a glow-type luminescent signal over a broad concentration range, providing a signal half-life of 1–2 hours for a well-mixed sample. However, the signal half-life can decrease significantly at extremely high concentrations of HiBiT, likely due to rapid depletion of substrate (see Figure 6). Samples in this range will not maintain the same relative levels of light output over time compared to samples at lower concentrations. If you suspect that you may have an extremely high concentration of HiBiT in your samples, monitor the rate of signal decay over time after adding the Nano-Glo® HiBiT Extracellular Reagent. The concentration of HiBiT that leads to rapid substrate depletion typically is near or exceeds the linear dynamic range of the luminometer used for detection in 96-well format.

In contrast, very low amounts of HiBiT can be difficult to measure because the signal will be near the reagent background. This may increase well-to-well variability. Under these circumstances, you need to subtract the background luminescence so that the value is proportional to the concentration of HiBiT. Because cells and medium can both affect the background, the proper background control would be cells not expressing a HiBiT-tagged protein, grown in the same medium. See Sections 6.H and 6.I for more information on maximizing sensitivity near assay background.

To make sure HiBiT-tagged proteins behave similarly to their endogenous counterparts, express the proteins at low levels, while still maintaining a sufficiently high signal for accurate measurement. If proteins are expressed at very high levels, they may become inappropriately regulated due to altered stoichiometry with endogenous binding partners or regulatory machinery. Figures 3 and 4 demonstrate how increasing overexpression of HiBiT-tagged proteins can lead to reduced fold response to treatments affecting surface expression. By contrast, expressing proteins at endogenous levels using CRISPR/Cas9 knock-in of the HiBiT tag promotes excellent fold response, presumably because the proper stoichiometry with endogenous proteins is maintained.

Because of the brightness of the HiBiT/LgBiT complex, proteins can generally be monitored at their endogenous levels. When transiently transfecting DNA constructs expressing HiBiT-tagged proteins from a strong promoter like CMV, we recommend diluting the DNA construct with carrier DNA (e.g., Transfection Carrier DNA [Cat.# E4881]), even as much as 1,000-fold or more. The optimal amount of DNA to transfect will depend upon the cell type used and the transfection efficiency. Transfecting low amounts of expression constructs helps ensure that high enzyme levels will not rapidly deplete substrate or exceed the linear detection range of the luminometer. More importantly, expression construct dilution means that the protein is expressed at more physiological levels for proper biological regulation. However, diluting CMV expression constructs does not lead to uniformly low expression levels across all cells, but rather tends to reduce and broaden the distribution of expression levels. Therefore, much of the luminescence may derive from a few cells expressing high levels of protein. To achieve more uniform low-level expression of HiBiT-tagged proteins, you can 1) transiently transfect an expression construct with a weaker promoter like TK, PGK or a CMV deletion; 2) stably transfect a CMV expression construct and select a clone with lower expression levels; or 3) use CRISPR/Cas9 to add the HiBiT tag at the endogenous locus of the protein.

6.D. Transfecting HiBiT Constructs

The following protocol is recommended for transient transfection in 96-well plates of constructs for CMV-promoter-driven expression of HiBiT-tagged proteins. Alternative protocols, such as mixing lipid and DNA with cells prior to plating or bulk transfection and replating, can be used but are not described here. As detailed in Section 6.C, optimizing a HiBiT assay may involve determining the expression level that achieves both an easily measured signal and the expected biological response. In commonly used cultured cells, we suggest transfecting CMV expression constructs at 0.5 or 0.05ng/well. The protocol below describes diluting an expression construct in carrier DNA to transfect 5–0.005ng/well of the expression construct. For constructs with weaker promoters, higher amounts of DNA may be optimal.

1. Plate cells in white 96-well tissue culture plates (e.g., Corning Cat.# 3917) in a total volume of 100ul per well.
2. Incubate in a 37°C, 5% CO_2 incubator for 16–24 hours for cell attachment.
3. Dilute Transfection Carrier DNA (Cat.# E4881) and the HiBiT expression construct to 6.25ng/µl using Opti-MEM® I Reduced Serum Medium (Life Technologies Cat.# 11058).
4. Serially dilute the expression construct with 6.25ng/µl solution of Transfection Carrier DNA as shown in the table below.

Tube number	Dilution	Expression Construct Transfected/Well
1	1/10 dilution of 6.25ng/µl construct	5ng
2	1/10 dilution of Tube #1	0.5ng
3	1/10 dilution of Tube #2	0.05ng
4	1/10 dilution of Tube #3	0.005ng

5. Add FuGENE® HD or ViaFect™ Transfection Reagent at a lipid-to-DNA ratio appropriate for the cell type of interest. Incubate at ambient temperature for 10 minutes.

   Note: Consult the FuGENE® HD database (**www.promega.com/resources/tools/fugene-hd-protocol-database/**) for recommended lipid-to-DNA ratios.
6. Add 8µl of lipid:DNA mixture to respective wells. Manually mix the plate in a circle for 2–3 seconds.
7. Incubate the plates at 37°C in a 5% CO_2 incubator for 20–24 hours.

6.E. Alternative Protocol for Removing Medium and Adding 1X Nano-Glo® HiBiT Extracellular Reagent

Removing the medium from adherent cells and replacing with 1X Nano-Glo® HiBiT Extracellular Reagent may be advantageous when:

- The signal is near the assay background, and a higher signal-to-background ratio is desired (see Sections 6.H and 6.I).
- Removing one or more components in the medium (e.g., a receptor agonist) prior to measuring surface expression is necessary.

1. Reconstitute the Nano-Glo® HiBiT Extracellular Reagent as described in Section 3.B.
2. Add an equal volume of a minimally buffered saline solution, such as PBS, to the reagent and mix by inversion. This creates the 1X reagent.

3. Remove plates containing mammalian cells that express a HiBiT-tagged protein from the 37°C incubator.
4. Remove medium from each well (e.g., by aspiration) without disturbing the adherent cells or letting them dry out.
5. Add an appropriate amount of the 1X reagent to the plate wells. In general, we suggest adding 1X reagent at 1–2 times the original sample volume.
6. Incubate for 10 minutes at room temperature to equilibrate LgBiT and HiBiT. If the HiBiT tag is placed within the protein sequence, longer incubation times may be necessary compared to terminal protein tags. Measure luminescence at room temperature using settings specific to your instrument. For 96-well plates on GloMax® instruments, we recommend integration times of 0.5–2 seconds. Longer integration times may improve data quality at lower levels of expression. The luminescence intensity will generally decay with a signal half-life of 1–2.5 hours.

Notes:

1. After adding the 1X reagent, plates also can be incubated at 37°C for 10 minutes, prior to measuring luminescence in a 37°C luminometer.
2. Even when HiBiT is not fused to a surface protein, it may be difficult to remove all extracellular HiBiT signal by exchanging medium or washing cells. This may be due to a number of potential causes, including the presence of dead or damaged cells with permeable membranes, protein that has remained bound to the outside of cells, or protein that has remained associated with the plate in some other way. Inducible expression systems may help lower this background by reducing extracellular accumulation prior to cell treatment.

6.F. Alternative Protocol for Real-Time Measurements

In some cases, you may wish to measure changes in extracellular HiBiT levels over time, rather than measuring a single endpoint. For example, differences in the rate of protein trafficking or secretion can be measured over the span of 1–2 hours in a defined medium. The following protocol describes adding a 1X reagent made with cell culture medium, rather than the Nano-Glo® HiBiT Extracellular Buffer.

1. Select an appropriate volume of a HEPES-buffered medium to replace the medium currently in the plates. This is usually a volume equal to the total amount of medium in all wells, plus any extra volume required for dispensing. For optimal signal-to-background ratios and signal stability, minimize the amount of serum added to the medium (see Sections 6.H and 6.I).
2. Dilute the LgBiT Protein 1:200 and the Nano-Glo® HiBiT Extracellular Substrate 1:100 into an appropriate volume of medium in a new container, and mix by inversion to generate the 1X reagent. **Example:** If 4ml of 1X reagent is needed, transfer 4ml of buffered medium to a disposable container and add 20µl of LgBiT Protein and 40µl of Nano-Glo® HiBiT Extracellular Substrate.

   Note: Warming the reagent to 37°C before dispensing is generally not necessary and will adversely affect reagent stability.
3. Remove plates containing mammalian cells that express HiBiT-tagged protein from the incubator.
4. Remove medium from each well (e.g., by aspiration) without disturbing the adherent cells or letting them dry out.

6.F. Alternative Protocol for Real-Time Measurements (continued)

5. Add an appropriate volume of 1X reagent to the plate wells. In general, we suggest adding 1X reagent at 1–2 times the original sample volume.
6. Insert the plate into a luminometer set to the desired temperature (usually 37°C or room temperature), and measure luminescence over the desired time period (up to 2 hours). After 10–15 minutes, the plate temperature generally has stabilized and the LgBiT and HiBiT have equilibrated. Signal changes after this point should be due to the combination of changes in the level of extracellular HiBiT-tagged protein and the natural signal decay rate for the enzyme/substrate system. To quantify changes in HiBiT levels over time, take this signal decay into account by normalizing to control wells with an unchanging amount of HiBiT-tagged protein.

Notes:

1. When measuring the rate of trafficking of HiBiT-tagged protein from intracellular to extracellular locations, we suggest setting t = 0 as the first time point after signal equilibration, usually 10–15 minutes after adding reagent. Subtracting this value from subsequent measurements would give the signal from HiBiT-tagged protein that was trafficked to the surface during the experimental measurements. To account for signal decay, include a sample with a constant amount of extracellular HiBiT-tagged protein for normalization of experimental values.
2. In some instances, HiBiT-tagged protein internalization can be measured in real time by prebinding LgBiT. We suggest following the protocol for real-time measurement, allowing the signal to equilibrate, adding receptor agonists or other compounds that drive internalization, and then monitoring changes in signal compared with a vehicle control. Although LgBiT would be expected to internalize with the HiBiT-tagged protein and the furimazine substrate is cell permeable, the complex often displays a substantial drop in activity upon internalization. This offers a real-time measurement of internalization. Normalizing on a per-well basis by dividing luminescence values by the value prior to adding compound (or an average of the last few measurements) can be effective at reducing variability caused by cell number, expression level or other factors. Calculating the fold response on a per-well basis can enable precise measurement of small changes in surface expression.
3. Under certain circumstances, you may want to incubate LgBiT Protein with cells over longer periods and then add substrate just prior to an endpoint measurement. For instance, when measuring the trafficking of a protein containing an internal HiBiT tag that requires additional time to equilibrate, replace the cell medium at the beginning of the experiment with medium containing LgBiT but no substrate (e.g., an equivalent volume of HEPES-buffered medium with a 100-fold dilution of LgBiT Protein). After the desired amount of time, add an equivalent volume of a 2X reagent lacking LgBiT (50-fold dilution of substrate into Nano-Glo® HiBiT Extracellular Buffer), mix and measure luminescence after 10 minutes per Section 3.C, Step 4.
4. Even when HiBiT is not fused to a surface protein, removing all extracellular HiBiT signal by exchanging medium or washing cells may be difficult. This could be due to a number of potential causes, including the presence of dead or damaged cells with permeable membranes, protein that has remained bound to the outside of cells, or protein that has remained associated with the plate in some other way. Inducible expression systems may help lower this background by reducing extracellular accumulation prior to cell treatment.

6.G. Comparing Extracellular and Lytic HiBiT Signals

When measuring the amount of HiBiT-tagged protein on the cell surface or secreted into the medium, you may be interested in the ratio of extracellular to total protein present in a sample. This can be particularly important for determining whether changes in extracellular protein levels are due to changes in trafficking efficiency or protein expression or degradation. Two approaches may be considered for measuring both extracellular and total HiBiT signal: Adding a lytic reagent to replicate wells or multiplexing in the same well by adding detergent after the extracellular measurement.

Comparing the Nano-Glo® HiBiT Lytic and Extracellular Reagents in Replicate Wells

The Nano-Glo® HiBiT Lytic Detection System (Cat.# N2030, N2040, N2050) is the ideal way to measure the total amount of HiBiT-tagged protein in a sample. Add the Extracellular and Lytic Reagents to replicate wells and follow the protocol listed in Section 3.C of Technical Manual #TM516, mixing by orbital shaking and measuring luminescence at 10 minutes. A given amount of HiBiT will produce a different amount of light in each reagent because of the different buffer conditions combined with various media. These differences in signal brightness can be corrected by comparing the signal from a HiBiT-tagged protein that is 100% extracellular, such as HiBiT Control Protein (Cat.# N3010), diluted in the assay medium (e.g., at 100pM).

To determine the extracellular fraction of HiBiT-tagged protein in experimental samples, add the Nano-Glo® HiBiT Extracellular and Lytic Reagents to replicate wells containing the following sample types:

1. **Background:** Untransfected cells not expressing HiBiT
2. **Test:** Experimental cells expressing HiBiT-tagged protein
3. **Control:** 100% extracellular HiBiT (HiBiT Control Protein in medium)

The fraction of extracellular protein in the test sample is determined by taking the ratio of the background-subtracted extracellular and lytic values and multiplying by the fold-difference in signal for the Control sample (100% extracellular HiBiT) in the lytic reagent compared to the extracellular reagent:

$$\frac{(\text{Extracellular Test} - \text{Extracellular Background})}{(\text{Lytic Test} - \text{Lytic Background})} \times \frac{(\text{Lytic Control} - \text{Lytic Background})}{(\text{Extracellular Control} - \text{Extracellular Background})}$$

Example: The extent of secretion from transfected cells of a HiBiT-tagged protein of interest (POI) is being studied by comparing its signals in the Nano-Glo® HiBiT Extracellular and Lytic Reagents. Untransfected cells, as well as purified HiBiT Control Protein in medium, are also included on the plate for comparison, generating the following luminescence data:

Sample	Extracellular Signal (RLU)	Lytic Signal (RLU)
Untransfected cells	6×10^3	1×10^3
Cells transfected with POI-HiBiT	2×10^5	3×10^6
HiBiT Control Protein	2×10^7	1×10^7

$$\frac{(2 \times 10^5 - 6 \times 10^3)}{(3 \times 10^6 - 1 \times 10^3)} \times \frac{(1 \times 10^7 - 1 \times 10^3)}{(2 \times 10^7 - 6 \times 10^3)}$$

$0.0647 \times 0.500 = 0.0323$, or 3.2% secretion of the POI

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Adding Detergent to the Nano-Glo® HiBiT Extracellular Reagent for Comparing Replicate Wells

While we recommend using the Nano-Glo® HiBiT Lytic Reagent to measure total HiBiT-tagged protein, you may want to perform the lytic and extracellular measurements in similar buffer conditions to easily compare the luminescence values. Therefore, a small volume of concentrated detergent solution may be added to reconstituted Nano-Glo® HiBiT Extracellular Reagent to generate a 2X lytic reagent.

While high concentrations of detergent inhibit the HiBiT signal, many intracellular compartments can be made accessible to LgBiT protein with relatively mild detergent conditions that yield very little signal inhibition. This permits a more straightforward comparison of extracellular and total protein measurements in replicate wells. For instance, supplementing the reagent with 200µg/ml digitonin causes little inhibition of the HiBiT signal but is generally sufficient for equilibrating LgBiT with proteins in the cytosol and in subcellular compartments like the endoplasmic reticulum.

Low final concentrations of Triton® X-100 are also generally able to permeabilize many subcellular compartments without significant inhibition of the HiBiT signal. For example, dilute a 10% (v/v) stock of Triton® X-100 into reconstituted Nano-Glo® HiBiT Extracellular Reagent 100-fold for a final concentration on cells of 0.05% (v/v).

The reagent background often differs significantly in the presence and absence of detergent, so determine the background for each reagent with untransfected cells. Adding detergent may also affect the signal decay rate, so the signal from control samples may start to diverge with longer incubations.

Example protocol for using digitonin to lyse cells:

1. Reconstitute the desired volume of Nano-Glo® HiBiT Extracellular Reagent, following the protocol in Section 3.B. Transfer half of the volume to a separate container to make the lytic reagent.
2. To make the 2X lytic reagent, add digitonin to a final concentration of 0.2 mg/ml in the reagent (e.g., dilute a 20mg/ml digitonin stock in DMSO [Cat.# G9441] 100-fold. This concentration should cause little effect on the HiBiT signal.
3. Following the protocol in Section 3.C, add the extracellular and 2X lytic reagents to replicate wells of the plate. The samples should include untransfected cells for determining background, as well as a 100% extracellular control sample like HiBiT Control Protein.
4. Determine the fraction of extracellular protein as described in the previous section, “Comparing to the Nano-Glo® HiBiT Lytic Reagent in Replicate Wells”. If this concentration of digitonin causes no change in signal for the 100% extracellular control, normalization may be unnecessary for future plates.

Multiplexed Extracellular and Lytic Measurements in the Same Well

Depending on the protein of interest being investigated and its subcellular localization, sequential extracellular and lytic measurements can be made in the same well. In this approach, the Nano-Glo® HiBiT Extracellular Detection System is performed using the protocol in Section 3.C. After incubating for 10 minutes at room temperature and measuring the extracellular signal, a concentrated stock of detergent is added to each well to lyse cells. After mixing the samples and incubating at least 10 minutes, the luminescence is again measured. Subtract the reagent background from both measurements because adding detergent usually changes the reagent background signal. Because of signal decay between the two measurements, the 100% extracellular control sample will show a decrease in signal under lytic conditions, even in the absence of detergent inhibition of signal. While this per-well normalization can reduce some sources of experimental variability (e.g., cell number or expression levels), there may also be increased well-to-well variability in lysis compared to adding a 2X reagent to replicate wells.

See the above section on considerations for balancing membrane permeabilization with enzyme inhibition. Adding a 10X or 20X aqueous stock of detergent to provide final concentrations of 100µg/ml digitonin or 0.05% Triton® X-100 often effectively releases HiBiT-tagged proteins without substantial inhibition of the HiBiT signal. Mammalian Lysis Buffer (Cat.# G9381) works well as a 20X detergent stock to effectively lyse many cellular compartments with minimal HiBiT signal inhibition. Effective mixing is important for complete lysis and reducing well-to-well variability. To determine the extracellular fraction of HiBiT-tagged protein, perform the analysis described in the preceding sections. The 100% extracellular control serves to correct for both inhibition of the HiBiT signal by detergent and for signal decay between the two measurements.

6.H. Effects of Typical Assay Components

Culture Medium

Although the Nano-Glo® HiBiT Extracellular Detection System is designed to work with many common culture media, compositional differences among different media 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 assay. Most notably, phenol red in medium somewhat decreases the luminescent signal by absorbing light. Some media, like Opti-MEM® I Reduced Serum Medium or McCoy's 5A, may exhibit higher background luminescence with the reagent, which could reduce the signal-to-background ratio. Proper controls should be used to ensure comparisons are being drawn between similar conditions.

6.H. Effects of Typical Assay Components (continued)

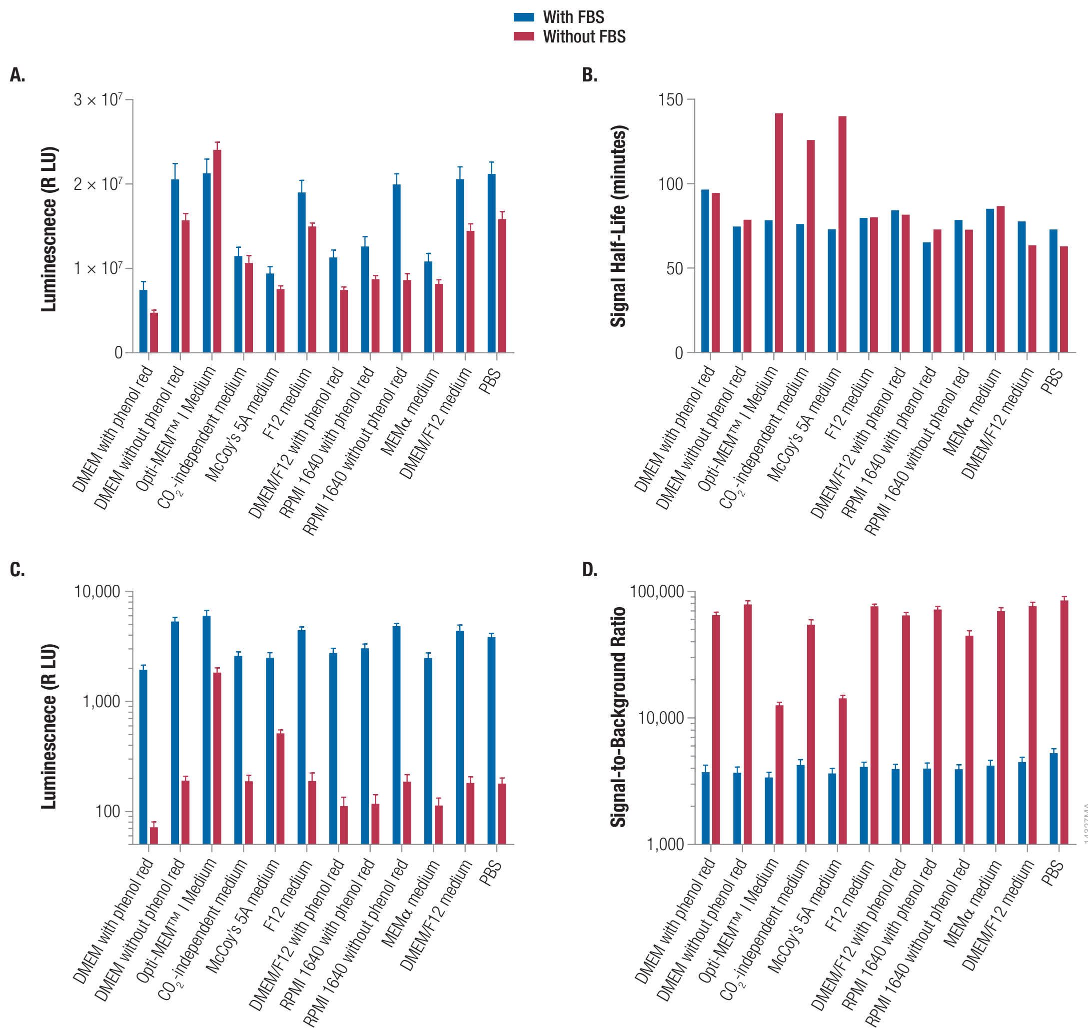

Figure 8. Effect of medium type on signal intensity, signal stability and background. Purified HaloTag®-HiBiT protein (100pM final concentration) was diluted into various cell culture media in either the presence or absence of 10% fetal bovine serum. **Panel A.** Luminescence was measured 10 minutes after adding the Nano-Glo® HiBiT Extracellular Reagent. **Panel B.** Luminescence was measured repeatedly over 2 hours at room temperature. Signal half-life was determined by fitting data to a single exponential curve. **Panel C.** Reagent was added to the various media without any HaloTag®-HiBiT protein to measure the reagent background. **Panel D.** The signal-to-background ratio for 100pM HaloTag®-HiBiT protein was calculated by dividing the signal at 10 minutes by the background luminescence in the absence of HiBiT. Error bars represent standard deviation for n = 4.

Serum

The Nano-Glo® HiBiT Extracellular Reagent is designed for use with 0–10% serum with minimal effects on luminescent signal (Figure 9, Panel A). The signal half-life may have minor reductions with serum, but luminescence is stable for 1–2 hours (Figure 9, Panel B). Serum does increase the reagent luminescent background in the absence of HiBiT (Figure 9, Panel C), due to both increased substrate autoluminescence and a small amount of LgBiT Protein activation. When measuring tiny amounts of HiBiT-tagged proteins, there may be an advantage to reducing serum levels in the medium (see Section 6.I).

6.H. Effects of Typical Assay Components (continued)

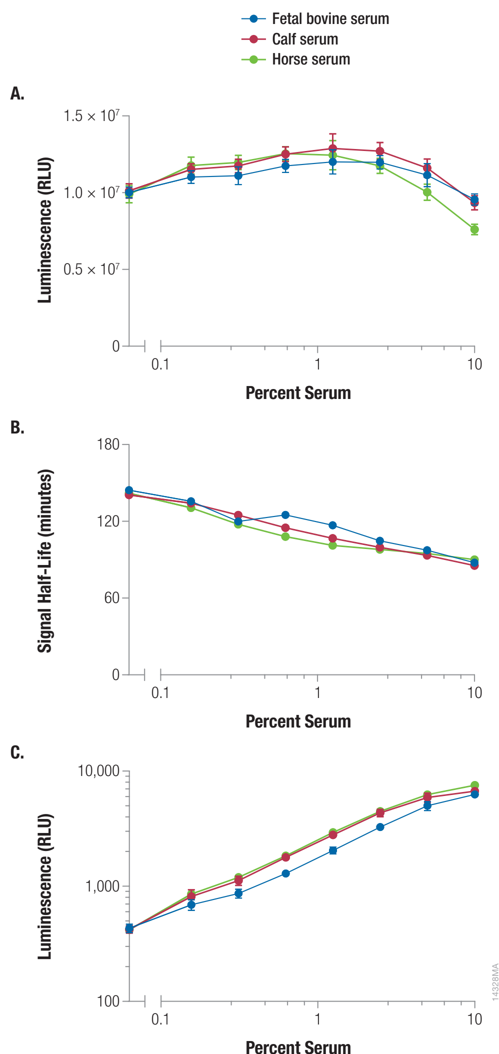

Figure 9. Effect of serum on signal intensity, signal stability and background. Fetal bovine serum (FBS), calf serum or horse serum were serially diluted into DMEM containing 0.1mg/ml BSA carrier. **Panel A.** HaloTag®-HiBiT protein (100pM) was diluted into medium with different serum concentrations, and luminescence was measured 10 minutes after adding the Nano-Glo® HiBiT Extracellular Reagent. **Panel B.** Luminescence was measured repeatedly over 2 hours at room temperature. Signal half-life was determined by fitting data to a single exponential curve. **Panel C.** Reagent was added to serum titrations in the absence of HaloTag®-HiBiT protein to measure reagent background. Error bars represent standard deviation for n = 4.

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Organic Solvents

Organic solvents may be present in assays because they are used to solubilize compounds for adding to cells. DMSO, ethanol and methanol in concentrations up to 3% have little effect on assay intensity, signal kinetics or background (Figure 10).

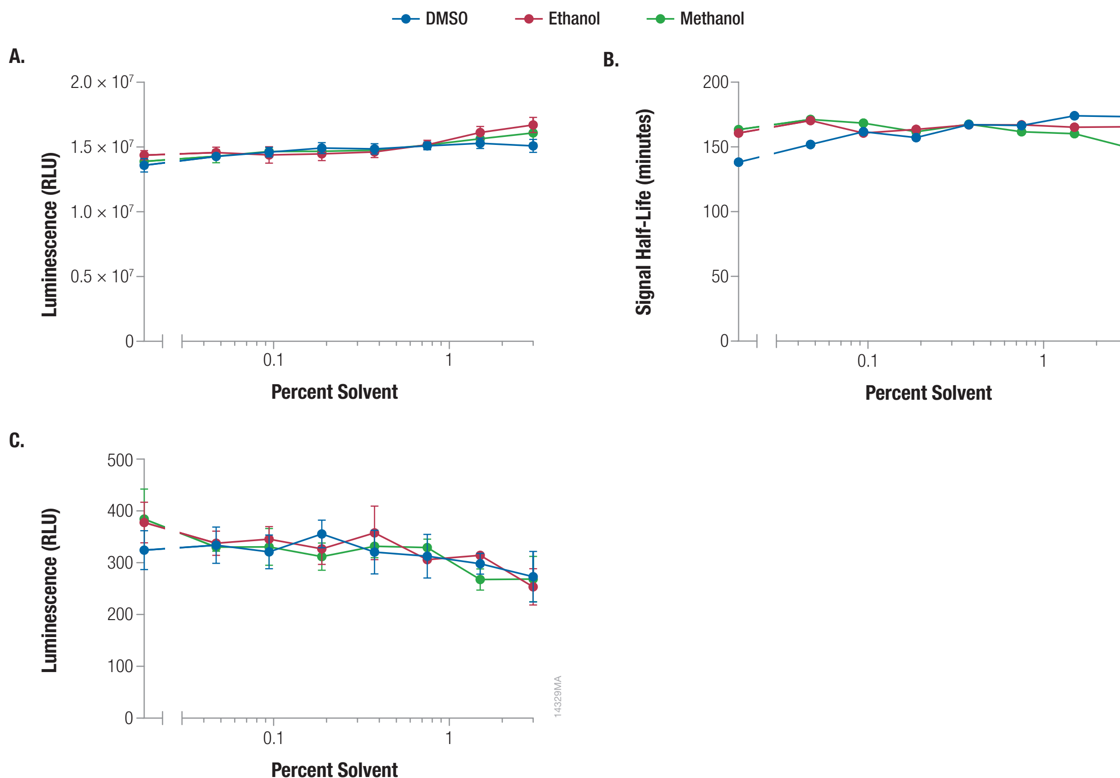

Figure 10. Effect of organic solvents on signal intensity, signal stability and background. DMSO, ethanol and methanol were serially diluted into DMEM containing 0.1mg/ml BSA carrier. **Panel A.** HaloTag®-HiBiT protein (100pM) was diluted into medium with different concentrations of solvents and luminescence measured 10 minutes after adding Nano-Glo® HiBiT Extracellular Reagent. **Panel B.** Luminescence was measured repeatedly over 2 hours at room temperature. Signal half-life was determined by fitting data to a single exponential curve. **Panel C.** Reagent was added to the solvent titrations in the absence of HaloTag®-HiBiT protein to measure the reagent background. Error bars represent standard deviation for n = 4.

Factors That Increase Assay Background

The Nano-Glo® HiBiT Extracellular Reagent contains LgBiT Protein at a concentration sufficient to quickly saturate the HiBiT tag present in a sample. While the HiBiT peptide is able to activate LgBiT luminescence greater than 10^8-fold, other peptide or protein sequences may bind and activate LgBiT to some extent. Generally, this occurs with much lower affinity and fold-activation than with HiBiT, but high concentrations of LgBiT-activating peptides or proteins may increase the assay background. This possible activation may be more pronounced with the Nano-Glo® HiBiT Extracellular Reagent compared to the Nano-Glo® HiBiT Lytic Reagent because of the assay conditions.

Cells not expressing HiBiT-tagged proteins can raise the assay background compared to medium alone (Figure 11). This background is generally lower than the amount of signal generated from 100fM HiBiT. The presence of serum in the medium can also increase background levels (Figure 8, Panel C).

Users adding high concentrations of peptides to samples (e.g., when screening peptide libraries) might find that peptides similar to HiBiT may activate LgBiT Protein. This LgBiT activation can be easily determined by performing a secondary assay in which the peptide is added to Nano-Glo® HiBiT Extracellular Reagent in the absence of cells or HiBiT. While small molecules may activate LgBiT Protein, initial library screening results suggest such compounds are very rare.

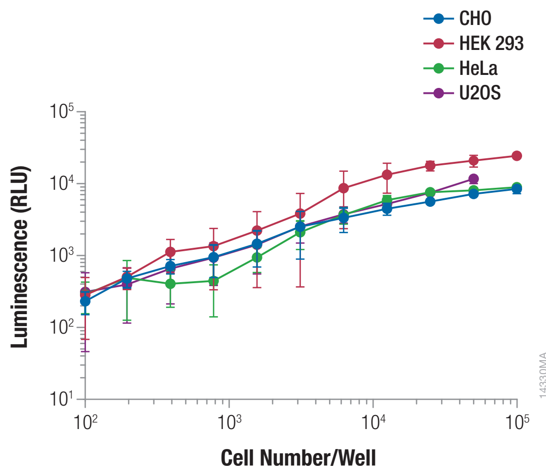

Figure 11. Effect of cell number on assay background. CHO, HEK 293, HeLa or U2OS cells were dissociated, counted and serially diluted into DMEM medium. After transferring cell titrations to a 96-well plate, the Nano-Glo® HiBiT Extracellular Reagent was added, and luminescence measured after 10 minutes. The background-subtracted luminescence was plotted. Error bars represent standard deviation for n = 4.

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6.I. Maximizing Assay Sensitivity

When HiBiT-tagged proteins are present at very low levels, the most accurate measurement is achieved by increasing the signal and reducing the background. To ensure that the HiBiT signal is proportional to the amount of HiBiT-tagged protein in the sample, subtract the assay background. This background consists of luminometer machine background, autoluminescence of the furimazine substrate, intrinsic LgBiT activity and nonspecific activation of LgBiT by proteins, peptides or other compounds in the sample. An appropriate background control would consist of the same number and type of cells, grown in the same medium but lacking expression of any HiBiT-tagged proteins. The luminescence obtained when reagent is added to these background control cells is subtracted to generate a value that is proportional to the amount of HiBiT-tagged protein in wells.

Choose a cell medium without phenol red to achieve higher signals (see Figure 8, Panel A). Reduce assay background by lowering or eliminating the amount of serum in the medium and choosing a medium with lower background (see Figure 8, Panel C). If compatible with your workflow, consider removing the medium during or at the end of the experiment and replacing it with medium lacking phenol red and serum. Alternatively, because the Nano-Glo® HiBiT Extracellular Reagent is a 2X reagent, you can dilute it 1:1 with PBS, and add this 1X reagent to wells after removing the medium at the desired time point (see protocol in Section 6.E). This is only possible if measuring surface expression of a HiBiT-tagged protein with adherent cells, not for secreted proteins or suspension cells.

6.J. Troubleshooting

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

Symptoms	Causes and Comments
Signal gradient across the plate or rapid drop in signal	The signal may drop more quickly if samples are not mixed thoroughly beforehand. Include a 30-second orbital shake after adding the reagent to better mix samples or decrease the amount of time needed to read a plate (e.g., by decreasing the integration time) or both.
Rapid signal decay observed	Substrate is depleted rapidly by a high HiBiT signal. Lower the protein expression level by decreasing the amount of DNA transfected, using a weaker promoter on the transfected vector, or reducing the number of cells per well.
Potential LgBiT activators in libraries of small molecules or peptides causing false hits	Perform a secondary screen in the absence of cells or HiBiT to test whether compounds may activate LgBiT. Add putative activators in buffer to the Nano-Glo® HiBiT Extracellular Reagent. This experiment will test if the activation is unrelated to the HiBiT-tagged POI.
Potential luciferase inhibitors in libraries of small molecules or peptides causing false hits	Perform a secondary screen in the absence of cells with 100pM of HiBiT Control Protein (Cat.# N3010) in buffer. Add the protein to the Nano-Glo® HiBiT Extracellular Reagent to determine if inhibition is unrelated to the HiBiT-tagged POI. Avoid contaminating dispensers or reagents with the HiBiT sample.

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6.J. Troubleshooting (continued)

Symptoms	Causes and Comments
High background signal is seen in samples lacking HiBiT	Because of the HiBiT detection sensitivity, avoid contaminating reagents or dispensing lines with samples containing HiBiT-tagged proteins. For instance, if a cell lysate containing HiBiT tag was dispensed with an automated injection system, a small portion of the protein may adsorb to surfaces of the dispenser even after cleaning. This protein may later release in small amounts into other solutions dispensed, such as the Nano-Glo® HiBiT Extracellular Reagent. This might cause an increase to the background of the assay due to the transferred HiBiT tag. Use caution when handling HiBiT Control Protein to avoid contamination of other surfaces and materials. We recommend single-use equipment when transferring solutions containing the HiBiT Control Protein to minimize contamination and elevation of background signal.
	Background luminescence can be increased by cells, serum or certain media. See Sections 6.H and 6.I for advice on decreasing reagent background.
Signal is too low to measure accurately	Increase signal-to-background by switching to a phenol red-free medium with low serum. Consider removing medium and replacing with 1X reagent diluted 1:1 with PBS. If cells are transiently transfected, use higher amounts of expression construct.
	Specific properties of a given fusion partner or tag placement could result in particularly low accessibility of the HiBiT tag with reduced complementation and luminescence. Try adding HiBiT to the other protein terminus or using a different linker length between the protein and HiBiT tag.
Low biological response to treatment	The HiBiT-tagged POI may be expressed too highly. Dilute the expression construct in carrier DNA, switch to a weaker promoter, or consider using CRISPR/Cas9 to express the HiBiT-tagged protein at endogenous levels.
High plate-to-plate variability	Measure each plate the same amount of time after adding reagent. Make sure conditions are the same for each plate (e.g., medium, cell number and temperature). Incorporate a common control sample on each plate that can be used to normalize the batch of plates.

Symptoms	Causes and Comments
Neither protein terminus is available for tagging	Add HiBiT to an internal protein position in an accessible surface loop. The signal and equilibration rate with LgBiT may be reduced compared to terminal tags. Adding linkers to both sides of HiBiT (e.g., GSSGGSSG) can help with protein accessibility.
HiBiT-tagged proteins that should be intracellular generate high signals in the assay	Under normal cell culture conditions, a certain amount of cell lysis is common. HiBiT-tagged proteins may release from lysed cells into medium or may bind LgBiT within cell debris. With transiently transfected cells, a subpopulation that greatly overexpresses the HiBiT-tagged protein may be more likely to lyse, causing a high fraction of the total protein in the well to be extracellular under basal conditions. Washing cells may not be very effective or even counterproductive for removing this extracellular background because the cell remnants or debris containing HiBiT signal remain bound in the well.
Starting signal for trafficking or secretion experiment is too high	The basal signal from HiBiT-tagged protein already extracellular at the beginning of cell treatment may represent unwanted background. Washing cells or replacing medium may not completely alleviate this problem. Using an inducible expression system may be advantageous for measuring the extent of protein trafficking under particular conditions because it reduces protein accumulation prior to treatment.

6.K. References

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