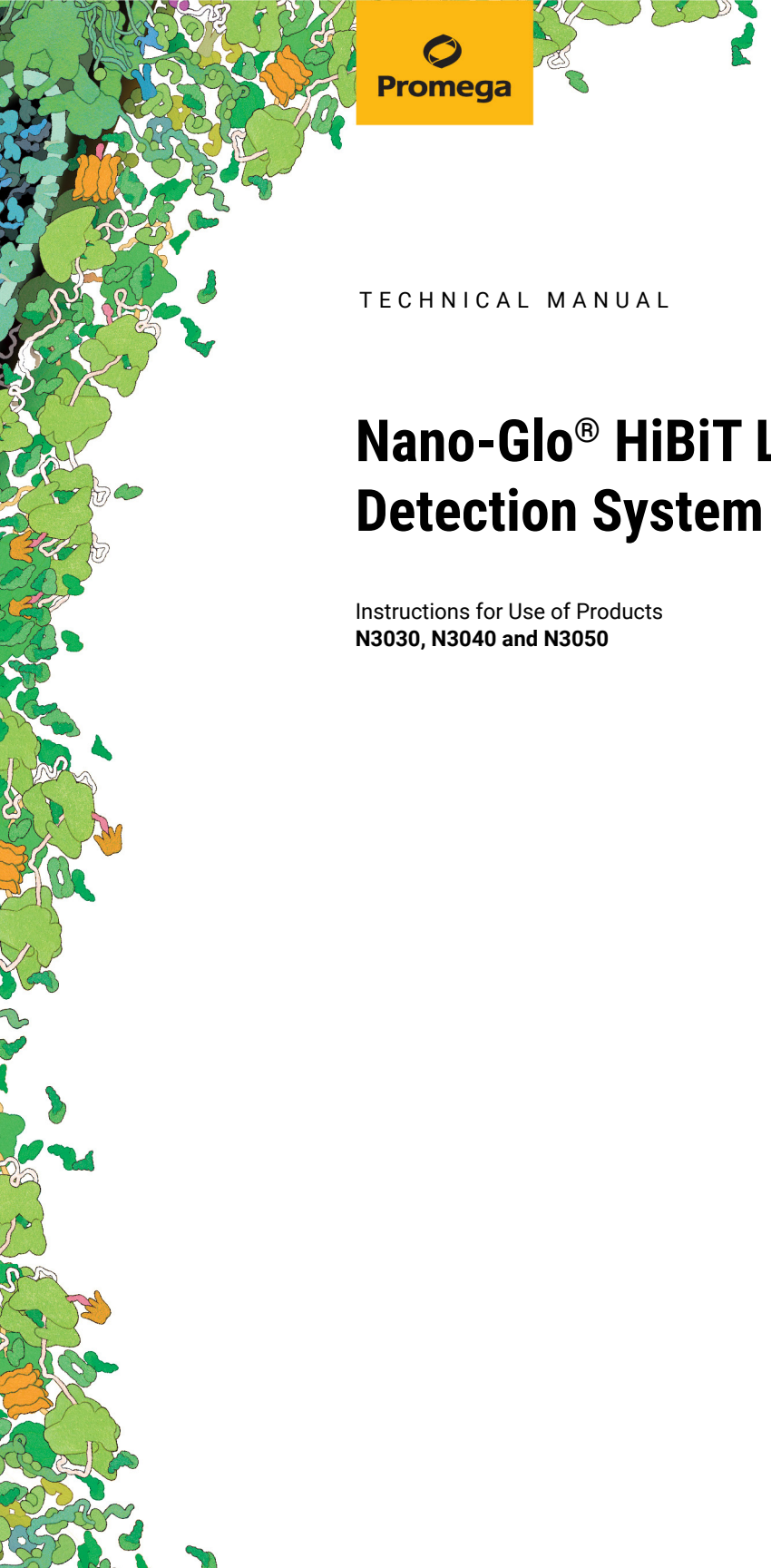

TECHNICAL MANUAL

Nano-Glo® HiBiT Lytic Detection System

Instructions for Use of Products
N3030, N3040 and N3050

Revised 6/23
TM516

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Nano-Glo® HiBiT Lytic 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

1. Description

The Nano-Glo® HiBiT Lytic Detection System[a–d] sensitively quantifies HiBiT-tagged proteins in cell lysates 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 expressed in a cell is determined by adding a lytic detection reagent containing the substrate furimazine and Large BiT (LgBiT), 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 in the cell lysate to generate a bright, luminescent enzyme (Figure 1). The amount of luminescence is proportional to the amount of HiBiT-tagged protein in the cell lysate over seven orders of magnitude (Figure 2), with a glow-type luminescent signal that is stable for hours (Figure 5, Panels B and C).

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 (5), and the bright signal permits quantification of even low abundance proteins at endogenous levels of expression.

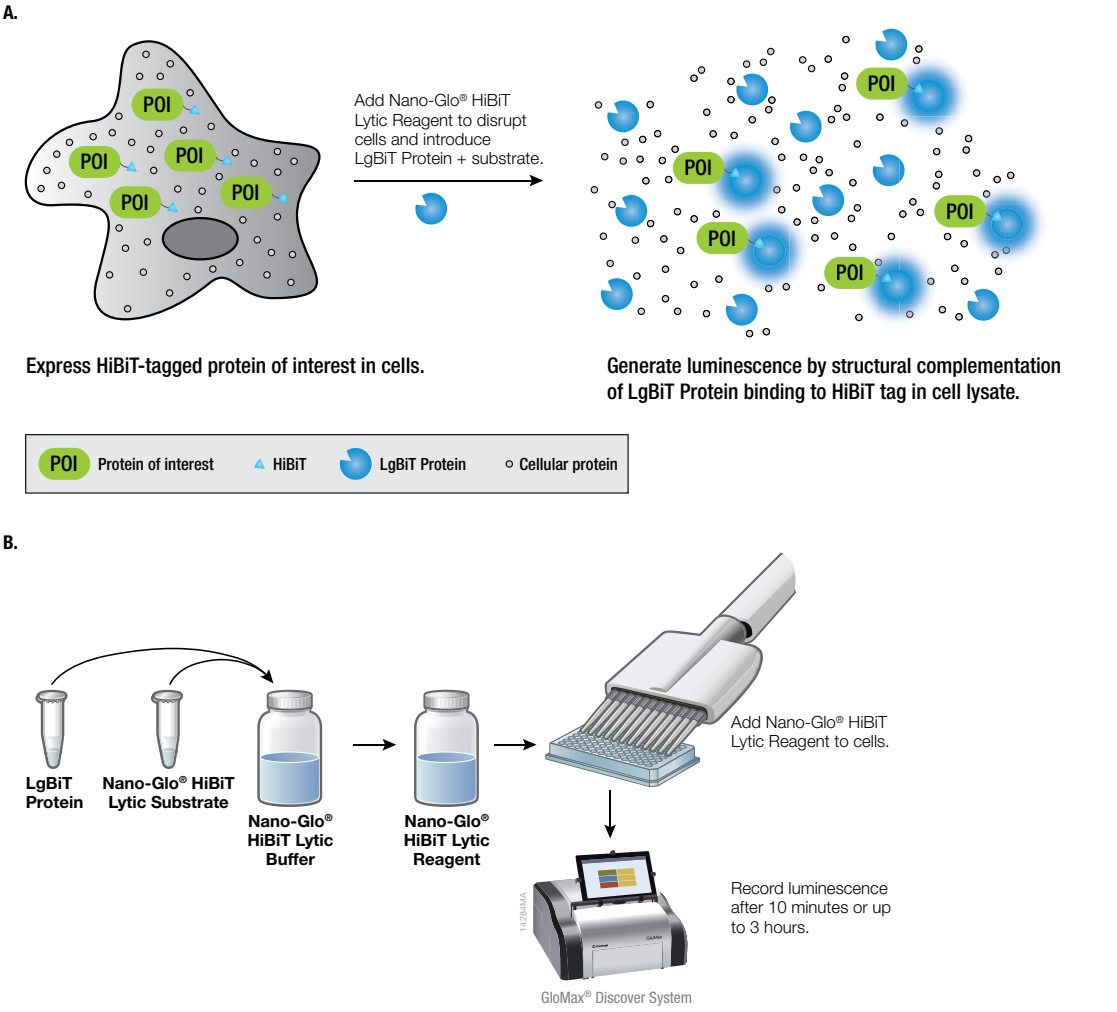

Figure 1. Nano-Glo® HiBiT Lytic Detection System protocol. Panel A. Principle of HiBiT tagging and detection. **Panel B.** The Nano-Glo® HiBiT Lytic Reagent is reconstituted by adding LgBiT Protein and substrate to a detergent-containing buffer. When added to mammalian cells, the luminescence generated is detected on a luminometer. This light signal is proportional to the amount of HiBiT-tagged protein in the sample.

1. Description (continued)

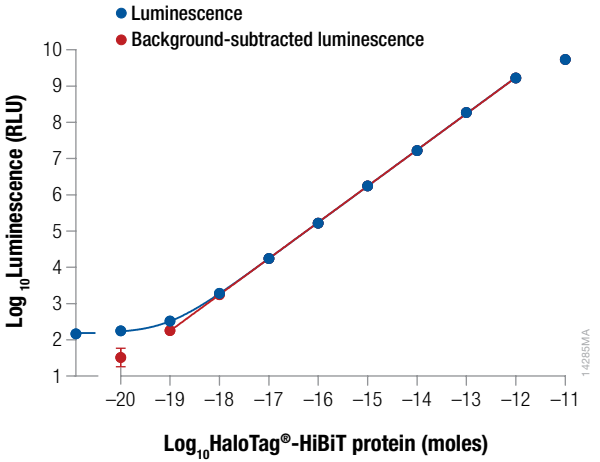

Figure 2. Titrating purified HiBiT-tagged protein. Nano-Glo® HiBiT Lytic Reagent was added to a titration of purified HaloTag®-HiBiT protein (4) and luminescence measured after 10 minutes. The blue and red symbols represent the luminescence and background-subtracted luminescence, respectively. The red 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 = 8.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
Nano-Glo® HiBiT Lytic Detection System	**10ml**	**N3030**

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

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

PRODUCT	SIZE	CAT.#
Nano-Glo® HiBiT Lytic Detection System	**100ml**	**N3040**

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

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

PRODUCT	SIZE	CAT.#
Nano-Glo® HiBiT Lytic Detection System	**10 × 100ml**	**N3050**

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

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

Storage Conditions: Store the Nano-Glo® HiBiT Lytic Detection System components at –10°C to –30°C. Do not thaw above 25°C. The Nano-Glo® HiBiT Lytic Buffer may be stored at +2°C to +8°C for 1 year or at room temperature for 3 months, and the buffer can be frozen and thawed at least ten times without any change in performance.

3. Nano-Glo® HiBiT Lytic Assay Protocol

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

The Nano-Glo® HiBiT Lytic Detection System measures the total amount of HiBiT-tagged protein in a sample using a detergent-containing buffer to lyse cell membranes. 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 3 hours at 22°C, different combinations of medium and serum may affect the background, signal, or signal decay rate (see Section 6.F). Additional information about the Nano-Glo® HiBiT Lytic 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 is generally present due to three sources: 1) autoluminescence of the furimazine substrate, 2) a small amount of activity of the LgBiT protein in the absence of HiBiT, and 3) machine background from the luminometer. Reagent background can vary with medium type and is increased by serum, cells or cell lysates (see Section 6.F). Therefore, for the most accurate measurements of low-abundance proteins, include a sample of untransfected cells in the same medium to calculate the assay background. For the greatest sensitivity, reduce the amount of serum by exchanging medium prior to the assay (see Section 6.G).

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

Calculate the amount of Nano-Glo® HiBiT Lytic Reagent needed to perform the desired experiments. This volume is usually 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 Lytic Substrate 1:50 into an appropriate volume of room temperature Nano-Glo® HiBiT Lytic Buffer in a new tube. Mix by inversion.

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

Notes:

a. If the Nano-Glo® HiBiT Lytic Substrate or LgBiT Protein has collected in the cap or on the sides of the tube, briefly spin the tubes in a microcentrifuge.

b. The LgBiT Protein stock contains glycerol, which prevents it from freezing at –20°C. The solution viscosity 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.

c. Because luciferase activity is temperature dependent, the temperature of the samples and reagents should be kept constant while measuring luminescence. We recommend equilibrating reagents to room temperature. For ease of use, store the Nano-Glo® HiBiT Lytic Buffer at room temperature at least a day before experiments. Equilibrate cultured cells to room temperature before adding reagents.

d. We recommend preparing the Nano-Glo® HiBiT Lytic Reagent fresh for each use. Once reconstituted, the reagent will lose about 10% activity over 8 hours and about 30% 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 10% activity over 24 hours.

3.C. Detecting HiBiT-Tagged Proteins in Mammalian Cells

1. Remove plates containing mammalian cells expressing a HiBiT-tagged protein from the incubator and equilibrate to room temperature. Use an opaque, white tissue-culture plate to minimize cross-talk between wells and absorption of the emitted light.

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

2. Add a volume of Nano-Glo® HiBiT Lytic Reagent equal to the culture medium present in each well, and mix. For optimal results, mix the samples by placing the plate on an orbital shaker (300–600rpm) for 3–10 minutes or by pipetting samples. At a minimum, employ 15–30 seconds of orbital shaking to reduce variability between replicates.

3. Wait at least 10 minutes for equilibration of LgBiT and HiBiT in the lysate. Measure luminescence using settings specific to your instrument. When using 96-well plates on the GloMax® instruments, we recommend integration times of 0.5–2 seconds. The luminescence intensity will usually decay with a signal half-life of greater than 3 hours. While 10 minutes of incubation is typically sufficient for maximal signal and low variability, longer incubation times may be necessary for internal fusions of HiBiT (e.g., surface loops) or when the HiBiT-tagged protein is expressed under conditions where the tag is not readily accessible upon cell lysis.

Note: 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 control samples on the assay plate, consisting of cells not expressing HiBiT-tagged proteins (see Section 6.G).

4. Representative Data

HiBiT tagging is particularly useful when studying the regulated expression or degradation of proteins of interest. For instance, cells expressing the HiBiT-tagged protein may be incubated with compounds that increase or decrease the rate of protein degradation. After incubation, the Nano-Glo® HiBiT Lytic Reagent is added to measure changes in protein stability.

HiBiT-BRD4 Degradation by dBET1

The bromodomain-containing protein, BRD4, represents an attractive target for cancer therapeutics. The compound dBET1 is an example of a protein-targeting chimeric molecule (PROTAC) that links BRD4 to the E3 ligase, cereblon, to induce its degradation (2). HiBiT-tagged BRD4 was expressed in HEK293 cells, either by transiently transfecting different dilutions of expression constructs into carrier DNA or by introducing the HiBiT tag at the endogenous locus using CRISPR/Cas9. As seen in Figure 3, transfection of lower amounts of CMV-promoter-driven expression constructs produced the expected lower amounts of total protein expression and luminescence, but also increased the fold response after 4 hours incubation with dBET1. Importantly, both transiently transfected cells expressing HiBiT-BRD4 from the weaker TK promoter and the CRISPR/Cas9-derived cell pool expressing HiBiT-BRD4 at endogenous levels displayed significantly greater degradation compared to cells transfected with dilutions of CMV expression constructs, even at similar luminescence levels. This highlights the importance of stoichiometry with proteins in the degradation machinery for regulating protein levels and the possible artifacts from expression driven by a strong promoter. See Section 6.C for more on the importance of protein expression levels.

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4. Representative Data (continued)

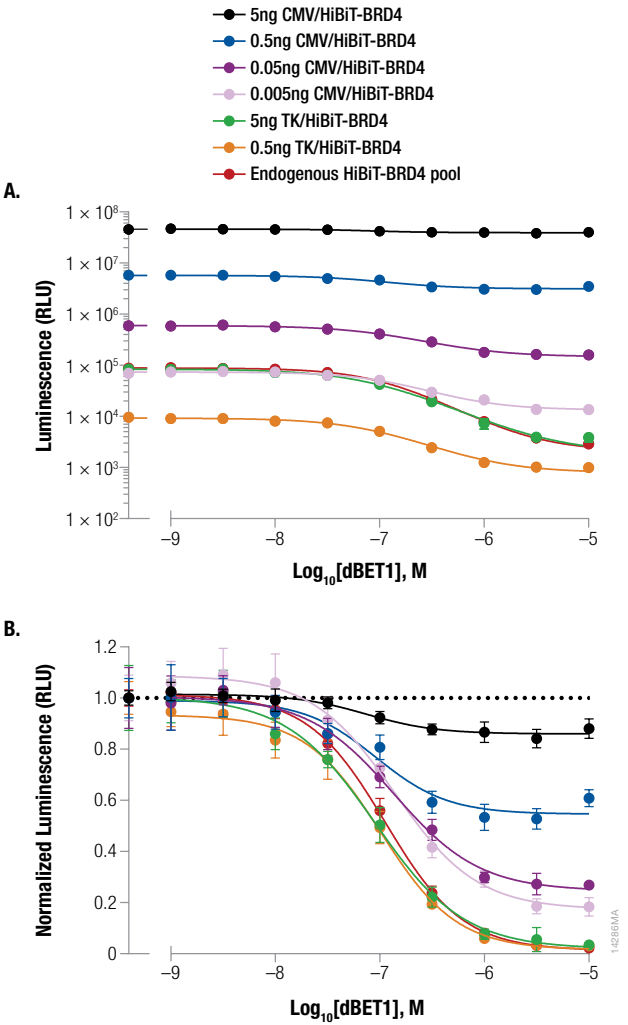

Figure 3. Induced degradation of HiBiT-BRD4 by incubating with the PROTAC compound, dBET1. HEK293 cells were transiently transfected with different amounts of HiBiT-BRD4 expression constructs with either the strong CMV promoter or the weak HSV-TK promoter, diluted in carrier DNA to keep the DNA amount constant. In parallel, a pool of HEK293 cells expressing HiBiT-BRD4 from the endogenous locus were generated using CRISPR/Cas9. Cells were plated in 96-well plates and treated the following day for 4 hours with a titration of dBET1 to induce degradation. The Nano-Glo® Lytic Reagent was added to all wells, and luminescence was measured after 10 minutes. **Panel A.** Raw luminescence data that illustrates the varying expression levels. **Panel B.** Background-subtracted luminescence was normalized to untreated cells to show the importance of low expression levels for the magnitude of dBET1-mediated protein degradation. Error bars represent the standard deviation for n = 6.

HIF1A-HiBiT Stabilization by Phenanthroline

Hypoxia-inducible factor-1A (HIF1A) is a transcription factor involved in the response to hypoxic conditions. Protein levels are kept low under basal conditions by prolyl hydroxylation and VHL-directed ubiquitination. Hypoxic conditions or chemical inhibition of prolyl hydroxylases with compounds like 1,10-phenanthroline lead to accumulation of HIF1A protein (6). HIF1A-HiBiT was expressed in HeLa cells by either transient transfection of varying amounts of CMV or PGK-promoter-driven expression constructs or by CRISPR/Cas9-mediated HiBiT tagging at the endogenous locus. Incubation of cells with a titration of 1,10-phenanthroline for 4 hours led to stabilization and accumulation of HIF1A-HiBiT (Figure 4). As seen with HiBiT-BRD4, lower levels of protein expression led to a greater fold response of accumulation, with endogenous expression displaying the largest fold change. Use of the weaker promoter, PGK, resulted in enhanced fold responses compared to CMV, even at similar luminescence. This result suggests that transfecting higher amounts of an expression construct with a weaker promoter may promote a more uniform distribution of low expression across cells, compared to diluted CMV expression constructs. See Section 6.C for more discussion on optimizing protein expression levels.

4. Representative Data (continued)

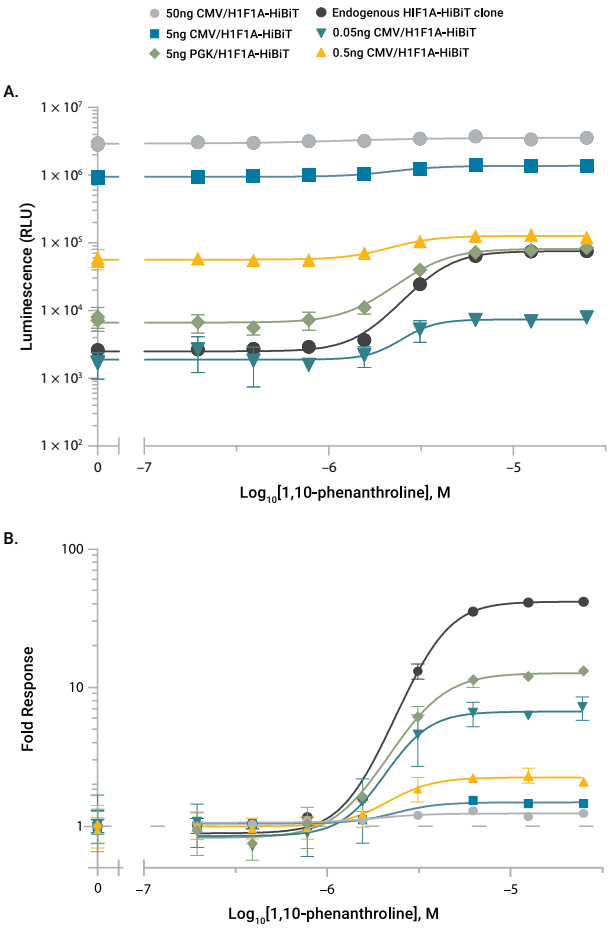

Figure 4. Stabilizing HIF1A-HiBiT by incubation with the hypoxia mimetic 1,10-phenanthroline. HeLa cells were transiently transfected with different amounts of CMV- or PGK-driven expression constructs for HIF1A-HiBiT, diluted in carrier DNA. In parallel, HiBiT was tagged to the endogenous locus in HeLa cells using CRISPR/Cas9, and a clone was isolated. Untransfected cells, transfected cells and the endogenously tagged cell line were plated in 96-well plates and treated the following day for 4 hours with a titration of 1,10-phenanthroline. The Nano-Glo® HiBiT Lytic Reagent was added to all wells, and luminescence was measured after 10 minutes. **Panel A.** Background-subtracted luminescence shows the varying expression levels of HIF1A-HiBiT in transiently-transfected cells compared to expression from the endogenous promoter. **Panel B.** Data normalized to untreated cells shows how overexpression of HIF1A-HiBiT reduces the fold response from treatment with 1,10-phenanthroline. Error bars represent the standard deviation for n = 6.

5. Related Products

Product	Size	Cat.#
Nano-Glo® HiBiT Blotting System	100ml	N2410
Nano-Glo® HiBiT Extracellular Detection System	10ml	N2420
	100ml	N2421
	10 × 100ml	N2422
HiBiT Control Protein	100µl	N3010
Anti-HiBiT Monoclonal Antibody	100µg	N7200
	5 × 100µg	N7210
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

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

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6. Appendix

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

The desire to precisely quantify proteins of interest is ubiquitous in biological research, because cells commonly respond to changes in their environment through changes in protein expression, stability or degradation. Dysregulation of protein expression is a key mechanism for tumorigenesis, and compounds affecting target protein levels could be useful for drug development. The most commonly used approach to monitor changes in expression is to perform SDS-PAGE followed by immunoblotting, a labor-intensive process that 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, but acts like a bright luciferase for simple and sensitive quantification of tagged proteins over a linear dynamic range with seven orders of magnitude (Figure 2).

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 used as the basis for an optimized two-component structural complementation system called NanoLuc® Binary Technology (NanoBiT®). In this system, the Large BiT (LgBiT, 17.6 kDa) 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 using the NanoBiT® PPI System. By contrast, the High BiT (HiBiT) peptide binds spontaneously to LgBiT with high affinity (~1nM). 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 Lytic Reagent converts the HiBiT tag into a bright, luminescent enzyme for quantifying protein expression levels.

The Nano-Glo® HiBiT Lytic Detection System was designed to provide the optimal combination of brightness and low background for accurate quantification of proteins across a wide range of expression (Figures 2 and 5). The luminescent signal is extremely stable, usually decaying with a half-life of more than three hours (Figure 5, Panels B and C). This makes the Nano-Glo® HiBiT Lytic Detection System ideal for batch processing of multiple plates for high-throughput applications. The combination of the detergent-containing buffer and high concentration LgBiT Protein mean that cell lysis and equilibration of the excess LgBiT with HiBiT in the sample typically is complete within 10 minutes of adding reagent. While the Nano-Glo® HiBiT Lytic Detection System provides the best way to measure the total amount of HiBiT-tagged protein in a sample, the Nano-Glo® HiBiT Extracellular Detection System (Cat.# N2420, N2421, N2422) can be used to quantify the amount of HiBiT-tagged protein present on the cell surface or secreted from the cell in a nonlytic live-cell assay. In addition, HiBiT-tagged proteins can be detected on membranes following SDS-PAGE using the Nano-Glo® HiBiT Blotting System (Cat.# N2410).

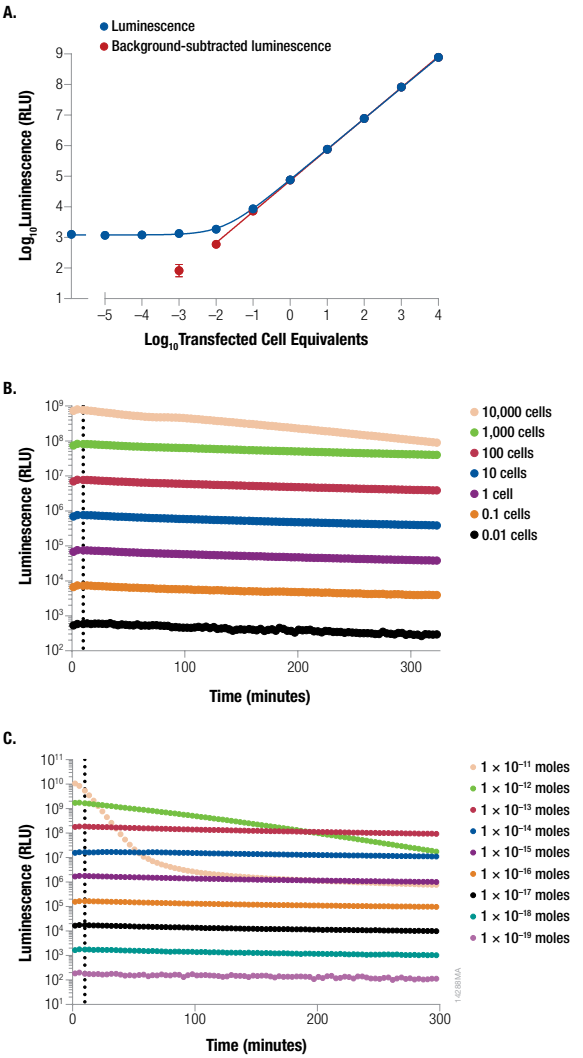


Figure 5. Titration of lysate from cells expressing a HiBiT-tagged protein. Panel A. HEK 293 cells were transiently transfected with a CMV construct expressing HaloTag®-HiBiT protein. After 24 hours, cells were dissociated, counted, lysed and serially diluted into lysate from untransfected cells for a constant lysate equivalent to 10,000 cells in each well. Nano-Glo® HiBiT Lytic Reagent was added and equilibrated for 10 minutes before luminescence was measured. **Panel B.** The signal decay kinetics over five hours are shown for the HaloTag®-HiBiT cell lysate titration from Panel A with the dotted line showing the measurement at 10 minutes. **Panel C.** The signal decay kinetics over 5 hours for the titration of purified HaloTag®-HiBiT protein from Figure 1 are shown with the dotted line showing the measurement at 10 minutes.

6.B. Appending HiBiT to Proteins of Interest

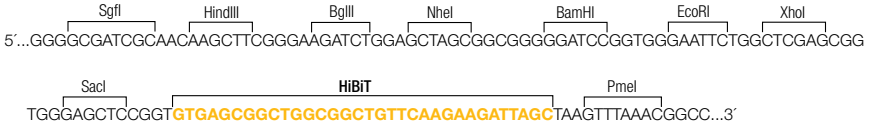
Multiple Cloning Site (MCS) Vectors

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 6 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. For pBiT3.1-secN [CMV/HiBiT/Blast] Vector, design the 5´ PCR primer to begin with the residue following any native cleavable signal sequence, effectively replacing the native signal sequence with the strong IL-6 signal sequence.

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

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



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

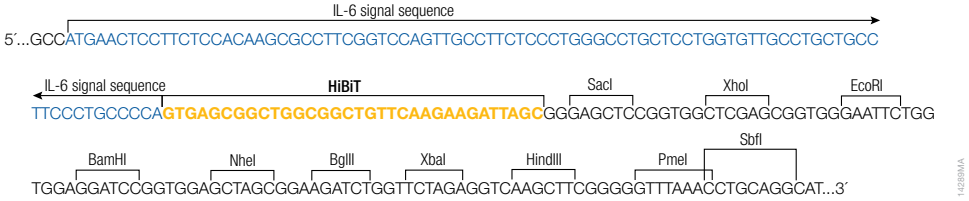


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Figure 6. Available Restriction Sites in MCS Entry Vectors.

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. You cannot 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.

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

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).

The 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

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 have frequently used 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 sensitivity of the Nano-Glo® HiBiT Lytic Detection System 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, a single-stranded DNA (ssDNA) oligonucleotide can be used as the donor template for homology-directed repair to insert the HiBiT tag, eliminating the need to generate any DNA constructs. The Ribonucleoprotein (RNP) Complex 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 Lytic 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 Lytic Detection System should maintain a glow-type luminescent signal over a broad concentration range, providing a signal half-life usually exceeding 3 hours. However, the signal half-life can decrease significantly at extremely high concentrations of HiBiT, likely due to rapid depletion of substrate (see Figure 5.C). 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 starting 10 minutes after adding the Nano-Glo® HiBiT Lytic 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 Section 6.G for more information on maximizing sensitivity near assay background.

6.C. Effects of Expression Level on Assay Performance (continued)

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 changes in protein level upon treatments that alter protein stability. By contrast, expressing proteins at endogenous levels using CRISPR/Cas9 knock-in of the HiBiT tag promotes the greatest 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 HSV- 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. Alternative Protocol Using 1X Nano-Glo® HiBiT Lytic Reagent

The protocol in Section 3.C can be used to quantify the amount of HiBiT-tagged protein in any sample, not just cells in medium. As a 2X reagent, an equal volume of Nano-Glo® HiBiT Lytic Reagent is always added to the volume of a given sample. However, there may be some instances when a 1X reagent is desired because the buffer or medium has been removed from the HiBiT sample. For instance, you may wish to quantify co-precipitated HiBiT-tagged protein after wash solutions have been removed, or the medium may be removed from cells immediately prior to HiBiT-tagged protein quantification (see Sections 6.F and 6.G). The following protocol can be used for adding a 1X reagent:

1. Reconstitute the Nano-Glo® HiBiT Lytic 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.
3. Add an appropriate amount of the 1X lytic reagent to the sample, and mix. We recommend a volume 1–2 times the original sample volume.
4. After incubating 10 minutes, measure luminescence as described in Section 3.C.

6.E. 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 100µl 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 to the cell type of interest. Incubate at ambient temperature for 10 minutes.
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.F. Effects of Typical Assay Components

Culture Medium

Although the Nano-Glo® HiBiT Lytic Assay 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 7). These differences are generally small and do not diminish the utility of the assay. Most notably, phenol red in medium decreases the luminescent signal by absorbing light. Some media, like Opti-MEM® I Reduced Serum Medium, may exhibit higher background luminescence with the reagent, which could reduce sensitivity. Proper controls should be used to ensure comparisons are being drawn between similar conditions.

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

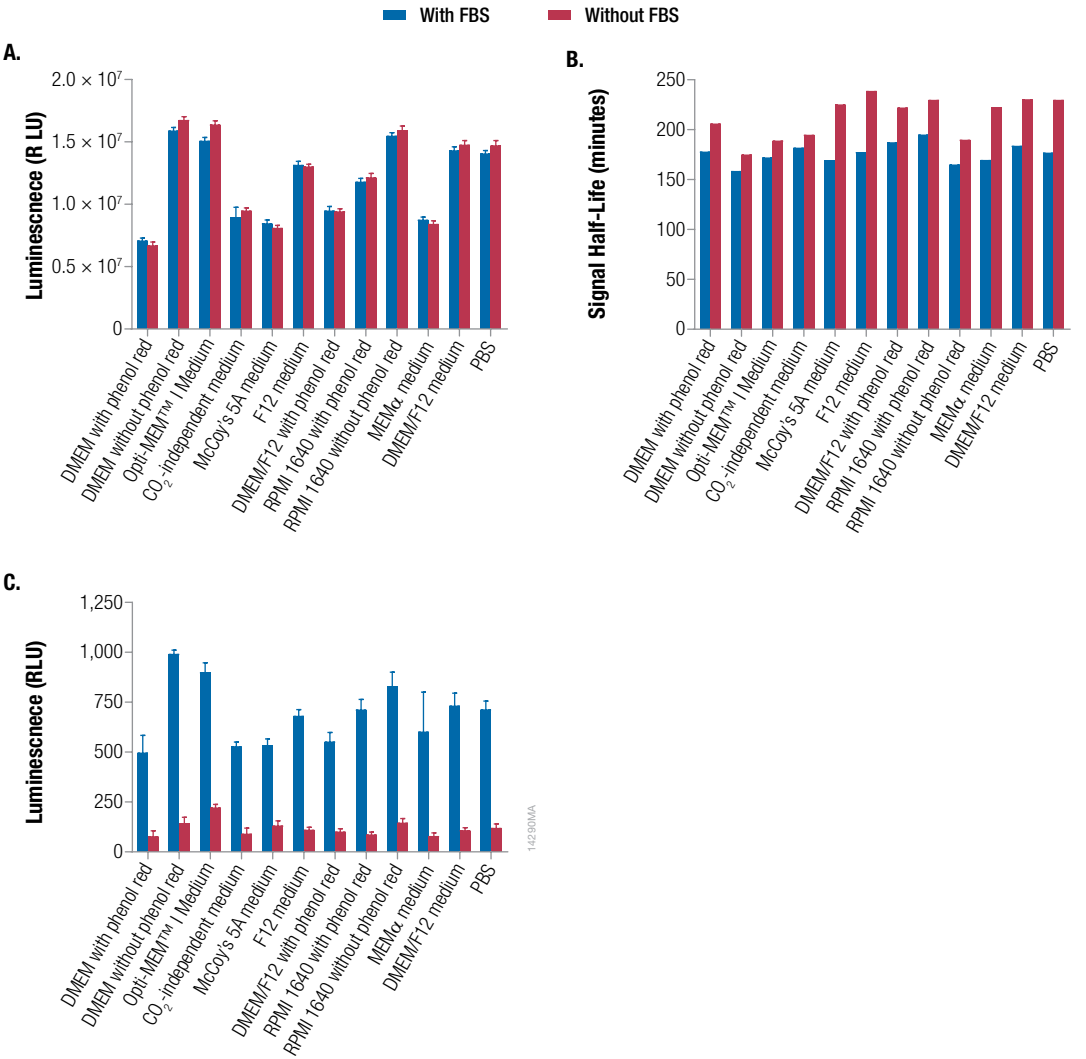

Figure 7. Effect of medium type on signal intensity, signal stability and background. Purified HaloTag®-HiBiT protein was diluted into various cell culture media either in the presence or absence of 10% fetal bovine serum. **Panel A.** Luminescence was measured 10 minutes after adding the Nano-Glo® HiBiT Lytic 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. Error bars represent standard deviation for n = 4.

Serum

The Nano-Glo® HiBiT Lytic Reagent is designed for use with 0–10% serum with minimal effects on luminescent signal or signal half-life (Figure 8, Panels A and B). Serum does increase the reagent luminescent background in the absence of HiBiT (Figure 8, Panel C), although this background is still very low. When measuring small amounts of HiBiT-tagged proteins, however, there may be an advantage to reducing serum levels in the medium (see Section 6.G).

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

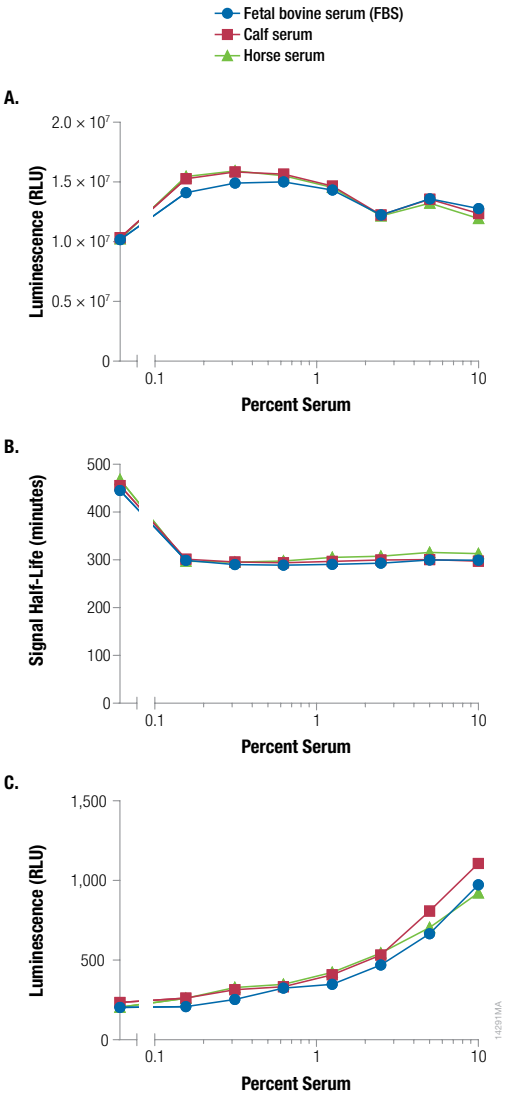

Figure 8. Effect of serum on signal intensity, signal stability and background. Fetal bovine serum (FBS), calf serum or horse serum were serially diluted into DMEM containing 0.1mg/ml BSA carrier. **Panel A.** HaloTag®-HiBiT protein was diluted into medium with different serum concentrations, and luminescence was measured 10 minutes after adding the Nano-Glo® HiBiT Lytic 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.

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 9).

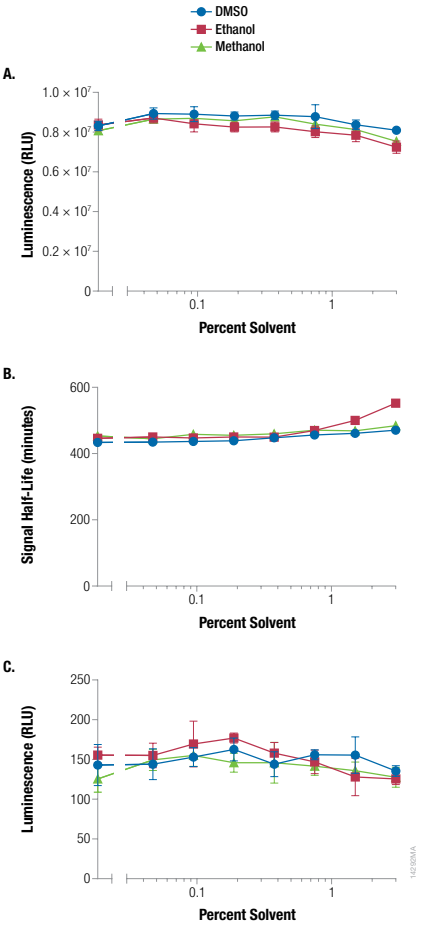

Figure 9. Effect of organic solvents on signal intensity, signal stability, and background. DMSO, ethanol, and methanol were serially diluted into DMEM containing 0.1mg/ml BSA carrier. **Panel A.** HaloTag®-HiBiT protein was diluted into medium with different concentrations of solvents and luminescence measured 10 minutes after adding Nano-Glo® HiBiT Lytic 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.

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

Factors That Increase Assay Background

The Nano-Glo® HiBiT Lytic Reagent contains concentrations of LgBiT Protein sufficient to saturate the HiBiT tag present in a sample. While the HiBiT peptide is able to activate LgBiT Protein 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 activating peptides or proteins may increase the assay background.

Cell lysates often contain some weak LgBiT activators that can raise the assay background compared to medium alone (Figure 10). This background is generally lower than the amount of signal generated from 10fM 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 with similarity to HiBiT may activate LgBiT Protein. This sort of activation can be easily determined by performing a secondary assay in which the peptide is added to Nano-Glo® HiBiT Lytic Reagent in the absence of cells or HiBiT. While small molecules may activate LgBiT Protein, initial library screening results have suggested such compounds are very rare.

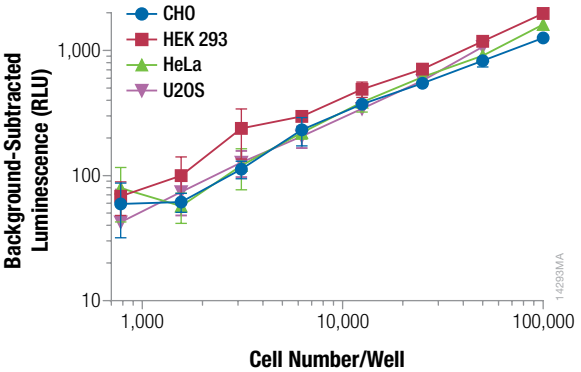

Figure 10. Effect of cell number on assay background. CHO, HEK 293, HeLa or U2OS cells were dissociated, counted and serially diluted into DMEM medium. After transferring cell titrations to a 96-well plate, the Nano-Glo® HiBiT Lytic Reagent was added, and luminescence was measured at 10 minutes. Background-subtracted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

6.G. Maximizing Assay Sensitivity

When HiBiT-tagged proteins are present at very low levels, achieving the most accurate measurement involves increasing the signal, reducing the background, and subtracting the background. Higher signals may be achieved by choosing a cell medium without phenol red (see Figure 7, Panel A). The assay background can be reduced by lowering or eliminating the amount of serum in the medium and choosing a medium with lower background (see Figure 7, Panel C). Consider removing the medium at the end of the experiment and replacing it with medium lacking phenol red and serum immediately prior to adding reagent. Alternatively, because the Nano-Glo® HiBiT Lytic Reagent is a 2X reagent, you could dilute it 1:1 with PBS, and add the resulting 1X reagent to wells after removing the medium.

To ensure that the HiBiT signal is proportional to the amount of HiBiT-tagged protein in the sample, subtract the assay background when working with low signals. This background consists of luminometer machine background, autoluminescence of furimazine substrate, intrinsic LgBiT activity and any 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 control cells can be subtracted to generate a value that is proportional to the amount of HiBiT-tagged protein in wells.

6.H. 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
Increasing signal past 10 minutes incubation or high well-to-well variability	If the HiBiT tag is internal or the HiBiT-tagged protein is in a relatively inaccessible subcellular compartment, it may take longer to equilibrate with LgBiT protein. Increase the incubation time before measuring luminescence to 30 or 60 minutes.
High well-to-well variability	Make sure samples are well-mixed by orbital shaking at least 3 minutes or pipetting repeatedly. However, if adding reagent is too forceful, foaming may occur, which can increase variability. Decrease the injection speed of the reagent or increase incubation time or both to allow foaming to subside before measurement. Rather than transiently transfecting cells in each well, use bulk- or stably transfected cells.
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 Lytic Reagent. This experiment will test if the activation is unrelated to the HiBiT-tagged POI.

6.H. Troubleshooting (continued)

Symptoms	Causes and Comments
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 Lytic Reagent to determine if inhibition is unrelated to the HiBiT-tagged POI. Avoid contaminating dispensers or reagents with the HiBiT sample.
Rapid signal decay is observed	The HiBiT signal may be too high, leading to rapid substrate depletion. Lower the expression level of the protein by decreasing the amount of DNA transfected, using a weaker promoter on the transfected vector or reducing the number of cells used.
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 solution containing HiBiT Control Protein 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 Lytic Reagent. This might cause an increase to the background of the assay due to the transferred HiBiT tag.
	Background luminescence can be increased by cells, serum or certain media. See Sections 6.F and 6.G for advice on decreasing reagent background.
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.
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 (Section 6.D). 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.

Symptoms	Causes and Comments
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.

6.I. References

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7. Summary of Changes

The following changes were made to the 6/23 revision of this document:

1. Edited Sections 2 and 5.
2. Updated patent statements.
3. Changed font and cover image.
4. Made minor text edits.

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[b]Patent Pending.

[c]U.S. Pat. No. 8,809,529, European Pat. No. 2635582, Japanese Pat. No. 5889910 and other patents and patents pending.

[d] U.S. Pat. Nos. 9,797,889 and 9,797,890 and other patents pending.

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