TECHNICAL MANUAL

Nano-Glo® HiBiT Blotting System

Instructions for Use of Product
N2410

8/17
TM524

Nano-Glo® HiBiT Blotting 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 Blotting System[a,b,c] simply and sensitively detects HiBiT-tagged proteins on nitrocellulose or PVDF membranes following SDS-PAGE and transfer (Figure 1). HiBiT is an 11-amino-acid peptide tag that can be 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 HiBiT-tagged protein transferred to the membrane is determined by adding a reagent containing the substrate furimazine and Large BiT (LgBiT), the large protein 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 spontaneous complex formation to generate a bright, luminescent enzyme. When added to a protein blot, LgBiT only generates luminescence when bound to immobilized HiBiT, which virtually eliminates background caused by nonspecific binding (Figures 2 and 3). HiBiT-tagged proteins generate a proportional signal over five orders of magnitude, down to femtogram amounts (Figure 4). The result is a blotting system that yields sensitive detection of HiBiT-tagged proteins with greater speed and simplicity than protocols that use multiple steps for antibody blocking, binding and washing (see Section 6 for more details).

Proteins of interest can be tagged with HiBiT at the N or C terminus using HiBiT expression vectors (Section 2). Alternatively, the HiBiT tag can be added by standard molecular biological 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 means many proteins can be visualized at endogenous expression levels.

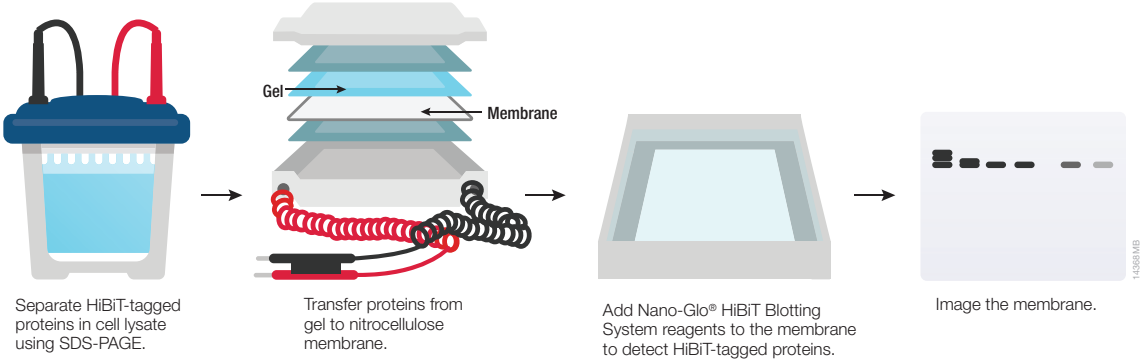

Figure 1. Nano-Glo® HiBiT Blotting System. Samples including a HiBiT-tagged protein are subjected to SDS-PAGE and transferred to nitrocellulose or PVDF membranes. LgBiT Protein in the reagent binds to immobilized HiBiT tag to generate a luminescent enzyme in the presence of substrate.

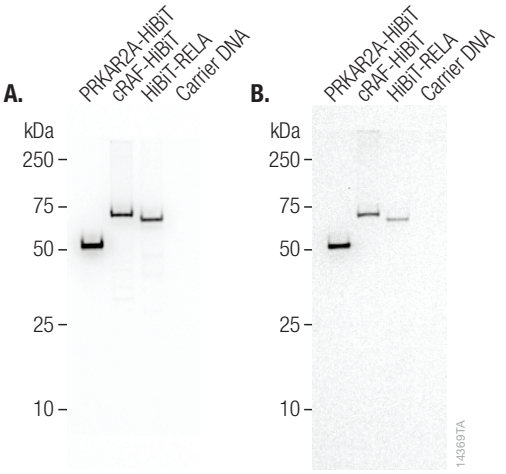

Figure 2. HiBiT blotting of transiently transfected HeLa cells. HeLa cells were plated at 10^5/ml and transfected the following day with carrier DNA (mock transfection control) or a CMV-driven expression construct for PRKAR2A-HiBiT, cRAF-HiBiT or HiBiT-RELA that was diluted 50-, 5- or 3.6-fold into carrier DNA, respectively. The following day, cells were washed and lysed in ¼ volume of Mammalian Lysis Buffer (Cat.# G9381) supplemented with Protease Inhibitor Cocktail (Cat.# G6521) and RQ1 RNase-free DNase (Cat.# M6101). After adding SDS loading buffer, the equivalent of about 12,000 cells was added to each well of duplicate 4–12% gradient Bis-Tris gels and subjected to SDS-PAGE. The proteins were transferred to nitrocellulose membranes using the iBlot™ 2 Gel Transfer Device (ThermoFisher), and the membranes were treated according to the protocols in Sections 3.B (**Panel A**) and 3.C (**Panel B**). The membrane in Panel A was rinsed briefly in TBST and then incubated in LgBiT/buffer solution for 1 hour. Nano-Glo® Luciferase Assay Substrate was added and incubated 5 minutes, and the blot was exposed for 7.4 minutes on an ImageQuant™ LAS 4000 (GE Healthcare). The membrane in Panel B was rinsed briefly in TBST and then added immediately to reconstituted Nano-Glo® HiBiT Blotting Reagent, incubated 5 minutes, and exposed on the imager for 7.4 minutes. Due to its higher signal, the Panel A image was scaled to a fourfold higher intensity than that of Panel B.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
Nano-Glo® HiBiT Blotting System	**100ml**	**N2410**

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

- 10ml Nano-Glo® Blotting Buffer (10X)
- 0.2ml Nano-Glo® Luciferase Assay Substrate
- 0.5ml LgBiT Protein

Storage Conditions: Store the Nano-Glo® HiBiT Blotting System components at –20°C. The Nano-Glo® Luciferase Assay Substrate and LgBiT Protein solutions will not freeze at –20°C. The Nano-Glo® Blotting Buffer (10X) may be stored at 4°C for 6 months. For best results, prepare fresh 1X solutions using Nano-Glo® Blotting Buffer (10X) before each use. 1X Nano-Glo® Blotting Buffer can be stored at 4°C, protected from light, for up to 1 month, but there may be a small decrease in signal stability compared to freshly diluted buffer.

Available Separately

PRODUCT	SIZE	CAT.#
Nano-Glo® HiBiT Lytic System	**10ml**	**N3030**
	100ml	**N3040**
	1,000ml	**N3050**
Nano-Glo® HiBiT Extracellular Detection System	**10ml**	**N2420**
	100ml	**N2421**
	1,000ml	**N2422**
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 Blotting Protocols

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

The Nano-Glo® HiBiT Blotting System is an antibody-free method for characterizing HiBiT-tagged proteins that have been separated by SDS-PAGE and transferred to nitrocellulose or PVDF membranes. The method is based on enzyme complementation between LgBiT Protein in the reagent and the HiBiT tag fused to the immobilized protein, generating a bright, luminescent enzyme. Because LgBiT is not luminescent until it binds to HiBiT, there is no need to block or wash the membrane to generate an exceptionally clean blot. Immobilized HiBiT-tagged proteins generate light proportional to the amount of protein in a three-step process:

1. The HiBiT tag on a transferred protein becomes accessible to solution by incubating in TBST [Tris-buffered saline solution with 0.05–0.1% (v/v) Tween® 20] or the Nano-Glo® Blotting Buffer.
2. LgBiT Protein binds spontaneously to accessible HiBiT tag to form a stable complex.
3. Furimazine substrate reacts with the LgBiT/HiBiT complex, generating luminescence.

These three steps can be accomplished by sequentially adding reagent components for the strongest signal (Sections 3.B and 6.C), or by adding a fully reconstituted reagent for the quickest results (Section 3.C).

Although both nitrocellulose and PVDF membranes can be used for HiBiT Blotting, we recommend transferring proteins to nitrocellulose for two reasons:

1. Proteins appear to bind more tightly to PVDF membranes, making the HiBiT tag less accessible for LgBiT complementation. Consequently, obtaining a maximal signal with PVDF membranes requires a longer preincubation in Nano-Glo® Blotting Buffer or TBST for the detergent to make the tag fully accessible. Due to reduced accessibility, the signal intensity with PVDF may be several fold weaker than with nitrocellulose.
2. The furimazine substrate can bind to PVDF membranes over time in a manner that leads to increased autoluminescence. This can cause a signal background on the membrane that may lower sensitivity.

We recommend using TBST [Tris-buffered saline solution with 0.05–0.1% (v/v) Tween® 20] buffer for rinsing membranes, as well as for preincubating membranes prior to adding LgBiT Protein. If TBST is not available, water and Nano-Glo® Blotting Buffer can be used as described in Section 3.B, but handling may be simpler using TBST.

3.B. General Detection Protocol for Nitrocellulose Membranes

The following protocol uses sequentially added reagent components with a nitrocellulose membrane to maximize detection sensitivity. Ample incubation times are included to solubilize the HiBiT tag and maximize LgBiT interacting with HiBiT. If a brighter signal is needed, incubation times can be increased further for greater binding of LgBiT to the HiBiT tag.

1. Separate proteins by SDS-PAGE. We recommend including prestained molecular weight markers.
2. Transfer proteins from the gel to a nitrocellulose membrane. We recommend using semi-dry transfer systems for strong signals with minimal transfer times.

3.B. General Detection Protocol for Nitrocellulose Membranes (continued)

3. Transfer the membrane to a container with TBST, and rock gently to rinse away transfer buffer and solubilize the HiBiT tag. Use enough solution to completely cover the membrane. Do **not** allow the membrane to dry out. If no TBST is available, rinse the membrane in water instead.

   Note: Keep membrane in TBST or water until Step 6. TBST will promote increased accessibility of the HiBiT tag, but extended incubation in water will have little effect.

4. Determine the volume of Nano-Glo® HiBiT Blotting Reagent necessary to completely cover the membrane. Dilute the 10X Nano-Glo® Blotting Buffer tenfold with water to generate the required amount of 1X Nano-Glo® Blotting Buffer, and mix by inversion. For example, add 1ml of 10X Nano-Glo® Blotting Buffer to 9ml of water in a 15ml conical tube.

5. Dilute the LgBiT Protein 200-fold with the 1X Nano-Glo® Blotting Buffer, and mix by inversion (e.g., add 50µl of LgBiT Protein to 10ml of 1X Nano-Glo® Blotting Buffer in a 15ml conical tube). Remove the TBST or water on the membrane and replace with the LgBiT/buffer solution.

   Note: 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.

6. Incubate the membrane with the LgBiT/buffer solution for 1 hour at room temperature with gentle rocking. Longer incubation times may increase signal, but prolonged incubation at room temperature may also cause a small decrease in signal. The strongest signal is usually achieved by an overnight incubation at 4°C; equlibrate the blot to room temperature before adding substrate in this case.

7. Dilute the Nano-Glo® Luciferase Assay Substrate 500-fold into the LgBiT/buffer solution by tilting the container to one side, dispensing the substrate into the gathered liquid and immediately mixing by rocking the container back and forth several times. For instance, if the membrane is in a container with 10ml of LgBiT/buffer solution, add 20µl of substrate solution, and mix. For best results, do not add the concentrated substrate solution directly onto the membrane or onto the container surface but rather dilute it into the bulk of the solution and quickly mix by gentle rocking. Incubate for 5 minutes at room temperature.

8. With the membrane submerged in the reagent, transfer the container with the blot to an appropriate chemiluminescence imager with a CCD camera. Alternatively, remove the membrane from the solution and place it between transparent plastic sheets or plastic wrap for imaging. Make sure that the membrane was incubated with the substrate for at least 5 minutes before removal. The signal will decrease with a half-life of about 60 minutes (Figure 4, Panel B).

9. Image the blot with appropriate settings for the desired sensitivity, resolution and exposure times. Greater sensitivity will be achieved with longer exposures (e.g., 30 minutes). If compatible with the imager, we recommend using an imaging program that automatically takes increasing exposure times (e.g., 1 second, 3 seconds, 10 seconds, 30 seconds, 100 seconds, 300 seconds, 1,000 seconds) and can integrate the various exposures for maximal signal. In this way, very bright signals are imaged with short exposures that do not exceed the detector limits, while weak signals are visible during longer exposures.

3.C. Rapid Detection Protocol for Nitrocellulose Membranes

When maximal sensitivity is not required, you can image your blot within minutes by placing the membrane directly into fully reconstituted reagent containing buffer, LgBiT Protein and substrate.

1. Separate proteins by SDS-PAGE. We recommend including prestained molecular weight markers.
2. Transfer proteins from the gel to a nitrocellulose membrane. We recommend using semi-dry transfer systems for strong signals with minimal transfer times.
3. Transfer the membrane to a container with TBST and rock gently to rinse away transfer buffer and solubilize the HiBiT tag. Use enough solution to completely cover the membrane. Do **not** allow the membrane to dry out. If no TBST is available, rinse the membrane in water instead. Keep the membrane in TBST or water until Step 6. Incubating the nitrocellulose membrane in TBST up to about 30 minutes will increase accessibility of the HiBiT tag, but extended incubation in water will have little effect.
4. Determine the volume of Nano-Glo® HiBiT Blotting Reagent necessary to completely cover the membrane. Dilute 10X Nano-Glo® Blotting Buffer tenfold in water to generate the required amount of 1X Nano-Glo® Blotting Buffer, and mix by inversion. For example, add 1ml of 10X Nano-Glo® Blotting Buffer to 9ml of water in a 15ml conical tube.
5. Add LgBiT Protein and Nano-Glo® Luciferase Assay Substrate to the 1X Nano-Glo® Blotting Buffer to make the Nano-Glo® HiBiT Blotting Reagent. Dilute the LgBiT Protein 200-fold and the Nano-Glo® Luciferase Assay Substrate 500-fold, and mix by inversion. For example, add 50µl of LgBiT Protein and 20µl of Nano-Glo® Luciferase Assay Substrate to 10ml of 1X Nano-Glo® Blotting Buffer in a 15ml conical tube.

   Note: 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.
6. Remove the TBST or water from the membrane and replace with the reconstituted Nano-Glo® HiBiT Blotting Reagent. Incubate for 5 minutes with gentle rocking.
7. Keeping the membrane submerged in the reagent, transfer the container with the blot to an appropriate chemiluminescence imager with a CCD camera. We recommend keeping the membrane in the solution to image because the immobilized HiBiT will continue to bind LgBiT Protein over time.
8. Image the blot with appropriate settings for the desired sensitivity, resolution and exposure times. The signal will likely increase over the course of about an hour as LgBiT Protein continues to bind to immobilized HiBiT (Figure 4, Panel B). Greater sensitivity will be achieved with longer exposures (e.g., 30 minutes). If compatible with the imager, we recommend using an imaging program that automatically takes increasing exposure times (e.g., 1 second, 3 seconds, 10 seconds, 30 seconds, 100 seconds, 300 seconds, 1,000 seconds) and can integrate the various exposures for maximal signal. In this way, very bright signals are seen with short exposures that do not exceed the detector limits, while weak signals are visible during the long exposures.

3.C. Rapid Detection Protocol for Nitrocellulose Membranes (continued)

9. If the signal is not bright enough, continue incubating the membrane in reagent for 1–2 hours. Immediately prior to re-imaging the blot, tilt the container with the membrane and dilute the Nano-Glo® Luciferase Assay Substrate 500-fold into the bulk of the solution. Mix by rocking the container back and forth. Repeat Steps 7 and 8.

Note: We recommend preparing the Nano-Glo® HiBiT Blotting Reagent fresh for each use. Once reconstituted, the reagent will lose 50% activity over about 8 hours at room temperature or about 24 hours at 4°C.

4. Representative Data

Processing and 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 Blotting Assay can be used to monitor proteolytic processing of PCSK9 and to quantify PCSK9 secretion. Constructs expressing HiBiT-tagged wild-type, H226A or C679X PCSK9 from a CMV promoter were transiently transfected into HEK293 cells. The following day, medium was removed and transferred to a tube. Cells were washed and lysed in a volume of lysis buffer equivalent to the cell medium. Samples of cell lysate and medium were subjected to SDS-PAGE and transferred to nitrocellulose. Treatment with Nano-Glo® HiBiT Blotting Reagent showed accumulation of HiBiT-tagged protein in the medium only for wild-type PCSK9. In the cell lysate, PCSK9 was present in both its unprocessed and processed forms for the wild-type and C679X forms of the protein, but the H226A mutant displayed the expected loss of autoproteolytic processing. These results highlight the ability to quickly monitor both processing and secretion of HiBiT-tagged proteins using HiBiT Blotting.

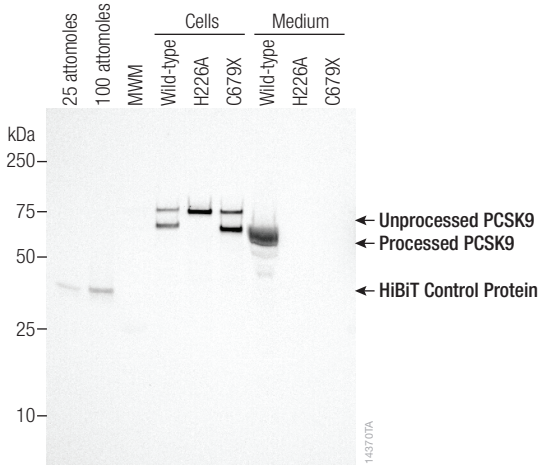

Figure 3. Processing and secretion of HiBiT-tagged PCSK9. HEK 293 cells were transiently transfected with CMV-driven expression constructs expressing PCSK9-HiBiT with either the wild-type, H226A or C679X sequence, diluted 1000-fold into carrier DNA. One day after transfection, medium was removed from cells. The cells were then washed and lysed in Mammalian Lysis Buffer (Cat.# G9381), supplemented with Protease Inhibitor Cocktail (Cat.# G6521) and RQ1 RNase-free DNase (Cat.# M6101), in a volume equivalent to the original medium. Samples from cell lysate and medium, as well as 25 and 100 attomoles of HiBiT Control Protein (Cat.# N3010; about 0.9 and 3.6pg protein, respectively), were run on a 4–20% gradient gel and transferred to nitrocellulose using the iBlot™ 2 Gel Transfer Device (ThermoFisher). The Nano-Glo® HiBiT Blotting protocol was performed as detailed in Section 3.B and imaged using an ImageQuant™ LAS 4000 (GE Healthcare). An integrated exposure over about 74 minutes is shown. Bands for the prestained molecular weight standards were added by overlaying with the illuminated light image. The H226A mutation disrupts autoproteolytic processing of PCSK9, leading to cellular accumulation of PCSK9 in its unprocessed form. The C679X mutant is capable of proteolytic processing, but it is incapable of effective secretion into the medium. Only the wild-type protein accumulates in the medium, where bovine serum albumin causes some bulging of the PCSK9-HiBiT band on the gel.

5. Related Products

Product	Size	Cat.#
Mammalian Lysis Buffer	40ml	G9381
Protease Inhibitor Cocktail	1ml	G6521
RQ1 RNase-Free DNase	1,000u	M6101
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 Blotting System

One of the most common ways to characterize protein samples is to separate the proteins by molecular weight using SDS-PAGE, transfer them to a membrane and perform Western blotting (immunoblotting). Western blotting depends on the availability of high-quality antibodies for sensitive and selective detection of proteins, and blotting performance can be quite variable depending on the conditions used (e.g., type of blocking agent, number and duration of washes, antibody concentrations, length of incubations, etc.). At 11 amino acids in length, the High BiT (HiBiT) peptide tag is comparable in size to commonly used epitope tags and can be detected on membranes through structural complementation with LgBiT Protein present in a reagent. HiBiT and LgBiT were derived from NanoLuc® luciferase, a 19.1kDa engineered enzyme that was developed to be brighter and more versatile than other reporter proteins (3). Because a luminescent enzyme is generated only upon complementation, the Nano-Glo® HiBiT Blotting protocol needs no blocking or washing steps to produce extremely low background luminescence (Figure 1).

The HiBiT tag can be added to proteins of interest in multiple ways. See Section 6.B in the *Nano-Glo® HiBiT Lytic Detection System Technical Manual* #TM516 or *Nano-Glo® HiBiT Extracellular Detection System Technical Manual* #TM523 for details. Genes of interest can be added to HiBiT entry vectors, using either multiple cloning site-based vectors or the Flexi® Vector Cloning System (see Section 2 for a list of available vectors). Additionally, the HiBiT tag can be added to existing protein expression constructs by PCR-based or gene-synthesis methods. The rights to synthesize the HiBiT tag can be obtained at: **www.promega.com/HiBiT-Synthesis**. Finally, the HiBiT sequence can be easily added at the endogenous locus using CRISPR/Cas9. The small size of the HiBiT tag means that a single-stranded DNA oligonucleotide can serve as the donor DNA for homology-directed repair, eliminating the need for cloning (5).

When loading gels with samples containing HiBiT-tagged proteins, we recommend also loading prestained molecular weight markers. In addition, the HiBiT Control Protein (Cat.# N3010) can be included on the gel as a positive control to verify that the transfer and assay are working properly. This 20µM solution of HaloTag®-HiBiT protein (4) will generally require significant dilution before use. For example, Figure 4, Panel A, represents the loading of 10µl of HiBiT Control Protein with a range of 3pM–300nM (1pg–100ng). Do not to contaminate dispensers, surfaces or components of the Nano-Glo® HiBiT Detection Systems with HiBiT Control Protein, because this could cause unwanted background luminescence. The ability of SDS-PAGE to resolve two proteins of different molecular weight means that the HiBiT Blotting System can measure multiple HiBiT-tagged proteins in the same sample. For instance, one could monitor changes in the expression level of one HiBiT-tagged protein relative to a control protein of different molecular weight that is not expected to change in expression. This could help normalize for the amount of protein loaded onto different lanes or different expression levels.

With its low background and bright signal, the Nano-Glo® HiBiT Blotting System can detect small amounts of HiBiT-tagged protein on a membrane (subpicogram amounts of a 30kDa protein). However, blot detection with CCD camera-based imagers will not be as sensitive as luminometer-based plate assays like the Nano-Glo® HiBiT Lytic Detection System. Luminescence on the blot is proportional to the amount of protein transferred over a range of about five orders of magnitude (Figure 4, Panel A). However, on a given exposure of a single blot, it may be possible to quantify only over about two orders of magnitude due to carryover of light from surrounding lanes. Different proteins may display different intensities due to tag placement and the surrounding sequence. For instance, internal placement of a HiBiT tag in a protein may result in significantly reduced blotting intensity compared to a terminal tag. Prestained molecular weight markers will be visible upon transfer to the membrane. Image the blot using epi-illumination before or after the luminescence image, so that the two images can be overlaid to estimate the molecular weight of your HiBiT-tagged protein. Some imagers enable epifluorescence imaging, which can be particularly useful for overlays since many prestained molecular weight standards are intrinsically fluorescent.

The HiBiT signal is stable, with a half-life of around 1 hour. When fully reconstituted reagent containing both LgBiT Protein and substrate is added to a blot, the signal will generally increase over the first 30–60 minutes as LgBiT protein binds to immobilized HiBiT tag (Figure 4, Panel B). Preincubation of the membrane with LgBiT Protein more completely saturates the HiBiT tag, leading to increased initial signal. Preincubating the membrane with TBST or 1X Nano-Glo® Blotting Buffer prior to adding LgBiT can also speed the rate of LgBiT binding by making the immobilized HiBiT more accessible. This preincubation is particularly important when using PVDF membranes, whereas with nitrocellulose membranes, LgBiT and buffer preincubation can be accomplished simultaneously.

6.A. Overview of the Nano-Glo® HiBiT Blotting System (continued)

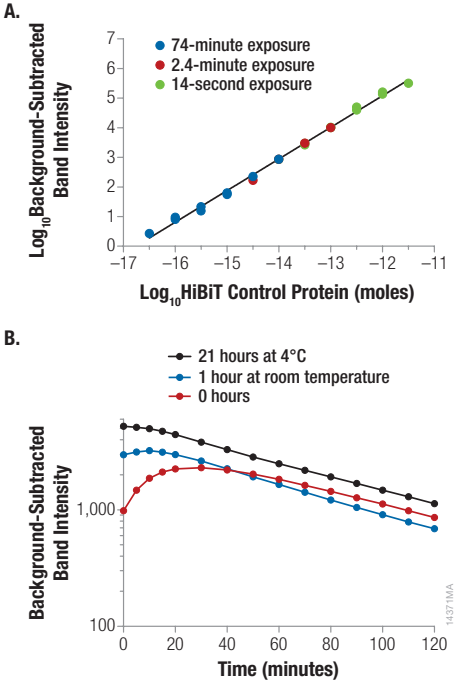

Figure 4. Dynamic range and signal decay kinetics of the Nano-Glo® HiBiT Blotting System. Panel A. HiBiT Control Protein (Cat.# N3010) was serially diluted by half-logs in buffer, subjected to SDS-PAGE, transferred to nitrocellulose using the iBlot™ 2 Gel Transfer Device (ThermoFisher) and cut into six separate strips with overlapping titrations of 3–5 HiBiT Control Protein amounts. The six blots were imaged separately to ensure accurate quantification, each with no more than a 100-fold range of protein levels. Blots were incubated overnight with LgBiT Protein, and Nano-Glo® Luciferase Assay Substrate was added just before imaging on an ImageQuant™ LAS 4000. After imaging with geometrically increasing exposure times, the blot bands were quantified with either 74 minutes, 2.4 minutes or 14 seconds of exposure time. The relative background-subtracted band intensities for the 74 minute and 14 second exposures were normalized to those of the 2.4 minute exposures using an overlapping common point of equal protein loads. A best-fit line through all of the normalized points gave a slope very near 1, indicating proportionality of signal over about five orders of magnitude. **Panel B.** Equivalent wells of 2fmol HiBiT Control Protein were subjected to SDS-PAGE, transferred to nitrocellulose and cut into indentical strips. After pre-incubation for more than 30 minutes in TBST, one strip was incubated with LgBiT for 21 hours at 4°C, one was incubated with LgBiT for 1 hour at room temperature, and one was not pre-incubated with LgBiT at all. Nano-Glo® Luciferase Assay Substrate was added to the first two strips, and Nano-Glo® HiBiT Blotting Reagent was added to the third blot. After 5 minutes, a series of 5-minute exposures were taken over two hours. Overnight pre-incubation with LgBiT at 4°C led to higher signal than 1 hour incubation at room temperature. With no pre-incubation, the signal started out lower and rose over the first 30 minutes as LgBiT bound to the HiBiT tag on the blot. Once equilibrated, the blots displayed a steady signal decay rate with a half-life of about 60 minutes.

6.B. Maximizing Assay Sensitivity

As described in Section 3, the HiBiT Blotting protocol can be adjusted for either greater sensitivity or for greater speed. The two factors most affecting signal intensity for a given band are accessibility of the HiBiT tag and the extent of LgBiT binding to the tag. For maximal sensitivity, we recommend nitrocellulose membranes over PVDF, because nitrocellulose offers improved HiBiT tag accessibility to LgBiT in solution with lower substrate autoluminescent background than PVDF. Incubating a membrane after transfer in TBST or 1X Nano-Glo® Blotting Buffer prior to adding LgBiT can help the HiBiT tag become more accessible to solution and increase the signal. With nitrocellulose membranes, HiBiT appears to reach maximal accessibility in about 30 minutes, whereas with PVDF it can take 4 hours or more to generate maximal signal. With nitrocellulose, this process is rapid enough that Nano-Glo® Blotting Buffer incubation can occur jointly with LgBiT Protein incubation, but with PVDF we recommend performing the buffer and LgBiT incubations separately.

In many cases, LgBiT Protein may be able to equilibrate with the immobilized HiBiT tag within 1 hour, especially if the membrane has been previously incubated with Nano-Glo® Blotting Buffer or TBST. However, the strongest signals are generally seen with overnight incubation with LgBiT Protein at 4°C. LgBiT incubations longer than 1 hour can also be performed at room temperature, but in some cases incubation times longer than 2 hours have actually shown slight decreases in signal, possibly from partial inactivation of the LgBiT/HiBiT complex over time.

Background signal on a Nano-Glo® HiBiT blot can occur from multiple sources:

1. Background signal from the imager.
2. Substrate autoluminescence, which may increase upon binding to the membrane.
3. Intrinsic LgBiT activity.
4. Activation of LgBiT by endogenous cellular proteins lacking a HiBiT tag.

In general, blotting assays with imagers based on CCD cameras will not be as sensitive as plate-based assays on PMT-based luminometers, which also demonstrate less cross-talk between samples. Therefore, HiBiT Blotting will not be as sensitive in most cases as the Nano-Glo® HiBiT Lytic Assay. The reagent background from substrate autoluminescence and intrinsic LgBiT activity is usually below the sensitivity of most imagers, but substrate autoluminescence on PVDF membranes can increase to noticeable levels over time. We therefore recommend adding the substrate within 5–10 minutes of imaging.

Activating bands from endogenous mammalian proteins are generally only visible when loading high amounts of cell lysate (e.g., >20,000 cell equivalents) and imaging with long exposures (e.g., 1 hour). Under these conditions, several weak bands have been observed. Additionally, *E. coli* lysates have been observed to contain several strong activators. These activating bands probably represent proteins with sequences similar to HiBiT, which interact more weakly with LgBiT and with lower activity. Such background bands can often be reduced or eliminated by removing the LgBiT solution or reconstituted reagent after incubation with the membrane and washing/incubating with TBST. Nano-Glo® Luciferase Assay Substrate can then be diluted 500-fold into 1X Nano-Glo® Blotting Buffer in the absence of LgBiT, added to the membrane and imaged. Because the affinity of LgBiT is much higher for HiBiT than for the background bands, imaging the membrane in the absence of free LgBiT can generate a cleaner blot.

6.C. Detection Protocol for PVDF Membranes

While we recommend using nitrocellulose membranes for high signal-to-background values, you may prefer to use PVDF for other reasons. Follow a protocol using sequentially added reagent components to maximize sensitivity when using PVDF.

1. Separate proteins by SDS-PAGE. We recommend inclusion of prestained molecular weight markers.
2. Transfer proteins from the gel to a PVDF membrane. We recommend semi-dry transfer systems for strong signals with minimal transfer times.
3. Transfer the membrane to a container with TBST and rock gently to rinse away transfer buffer and solubilize the HiBiT tag. Use enough solution to completely cover the membrane. Do **not** allow the membrane to dry out. Incubate in the TBST for at least 4 hours for best results. If incubating longer than 8 hours, store the membrane at 4°C if desired.

   Note: If no TBST is available, rinse the membrane briefly in water and incubate at least 4 hours in 1X Nano-Glo® Blotting Buffer. Dilute LgBiT Protein 200-fold directly into the buffer on the membrane, mix well by gentle rocking and proceed to Step 6.
4. Determine the volume of Nano-Glo® HiBiT Blotting Reagent necessary to completely cover the membrane. Dilute the 10X Nano-Glo® Blotting Buffer tenfold into water to generate the required volume of 1X Nano-Glo® Blotting Buffer, and mix by inversion. For example, add 1ml of the 10X Nano-Glo® Blotting Buffer to 9ml of water in a 15ml conical tube.
5. Dilute the LgBiT Protein 200-fold in the 1X Nano-Glo® Blotting Buffer, and mix by inversion (e.g., add 50µl of LgBiT Protein to 10ml of 1X Nano-Glo® Blotting Buffer in a 15ml conical tube). Remove the TBST on the membrane and replace with the LgBiT/buffer solution.

   Note: 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.
6. Incubate the membrane with the LgBiT/buffer solution at room temperature for 2 hours with gentle rocking. Longer incubation times may give an increased signal. The strongest signal is usually achieved by an overnight incubation at 4°C. Equilibrate the blot to room temperature before adding substrate.
7. Dilute the Nano-Glo® Luciferase Assay Substrate 500-fold into the LgBiT/buffer solution at the side of the membrane by tilting the container to one side, dispensing the substrate into the gathered liquid and immediately mixing by rocking the container back and forth several times. For instance, if the membrane is covered with 10ml of LgBiT/buffer solution, add 20µl of substrate solution, and mix. For best results, do not add the concentrated substrate solution directly onto the membrane or onto the container surface but rather dilute it into the bulk of the solution and quickly mix by gentle rocking. Incubate for 5 minutes at room temperature.
8. Keeping the membrane submerged in the reagent, transfer the container with the blot to an appropriate chemiluminescence imager with a CCD camera. Alternatively, remove the membrane from the solution and place it between transparent plastic sheets or plastic wrap to image. Make sure that the membrane has incubated with the substrate for at least 5 minutes before removing it from the solution. The signal will decrease with a half-life of about 60 minutes.

9. Image the blot with appropriate settings for the desired sensitivity, resolution and exposure times. Greater sensitivity will be achieved with longer exposures (e.g., 30 minutes). If compatible with the imager, we recommend using an imaging program that automatically takes increasing exposure times (e.g., 1 second, 3 seconds, 10 seconds, 30 seconds, 100 seconds, 300 seconds, 1,000 seconds) and can integrate the various exposures for maximal signal. In this way, very bright signals are seen with short exposures that do not exceed the detector limits, while weak signals are visible with longer exposures.

6.D. Reprobing Blots

Because of the high-affinity interaction between HiBiT and LgBiT, HiBiT blots that have been incubated with LgBiT or reconstituted reagent can often be reimaged at a later time, even when excess LgBiT has been washed off and the blots incubated in TBST. In this case, just dilute Nano-Glo® Luciferase Assay Substrate 500-fold into 1X Nano-Glo® Blotting Buffer, add the substrate solution to the membrane, incubate 5 minutes, and then proceed with imaging as described in Section 3.B, Step 8. There may be a decrease in band signal intensity over time from LgBiT/HiBiT complex inactivation (e.g., from binding directly to the membrane in a nonactive conformation).

Because HiBiT blotting uses a different assay chemistry than Western blotting, performing both antibody-based and HiBiT-based detection is straightforward to do on the same membrane. While either order is possible, we recommend performing the immunoblotting first, especially if using PVDF membranes. This ensures that the membrane will have already been preincubated with detergent-containing buffers like TBST, making that step unnecessary for the HiBiT Blotting protocol. After the Western blot, you will need to remove any light-producing enzyme/substrate complexes (e.g., by washing away substrate or inactivating the enzyme-linked antibody). The presence of bound antibodies should not affect HiBiT blotting. After the Western blot is completed and any stripping procedures are used, wash the blot several times with TBST and then follow the HiBiT Blotting protocol in Section 3.B or 6.C, starting at Step 4.

When Western blotting is performed after HiBiT Blotting, thoroughly wash away the substrate and ensure enough time has passed that substrate molecules bound to LgBiT/HiBiT complexes have been degraded. Depending on the relative brightness of the HiBiT blot and the Western blot, this may mean multiple hours of incubation to be certain that absolutely no HiBiT signal remains that would interfere with the Western. Check the blot by imaging for residual HiBiT signal after the blocking step of the Western blot before continuing with the immunodetection.

6.E. 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
Low band intensities observed	PVDF membranes were used. Switch to nitrocellulose membranes for greater HiBiT accessibility.
	HiBiT tag was not accessible. To ensure the HiBiT tag is fully accessible to solution and maximize the amount of LgBiT bound, incubate the membrane in TBST for 30 minutes with nitrocellulose or at least 4 hours with PVDF before incubating with LgBiT/buffer solution overnight at 4°C.

6.E. Troubleshooting (continued)

Symptoms	Causes and Comments
Low band intensities observed (continued)	A problem may exist with protein transfer or blot imaging. Use the HiBiT Control Protein (Cat.# N3010) to verify that the SDS-PAGE, protein transfer and HiBiT Blotting steps are behaving as expected and determine the sensitivity achieved with a particular imager and assay format. Loading 0.1–10fmol of HiBiT Control Protein (e.g., 10µl of 10pM–1nM solution) is generally an appropriate control, depending on the brightness of the other samples. Sensitive imaging systems should be able to detect <1 pg of HiBiT Control Protein (~0.03 fmoles). Avoid contamination of solutions, surfaces or dispensing lines with HiBiT Control Protein because this could lead to high background in future HiBiT experiments.
	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.
Signal decays quickly	Blotting solution may be old. Make a fresh dilution of 1X Nano-Glo® Blotting Buffer from the 10X stock, and keep the 1X buffer at 4°C for greater stability.
Background bands are present in samples lacking HiBiT	Proteins present in cell lysates may weakly activate LgBiT. Long exposures of blots with high amounts of cellular proteins may show faint background bands from cells not expressing HiBiT-tagged proteins. Because LgBiT has weaker affinity for these proteins, the HiBiT Blot can be cleaned up by prebinding LgBiT Protein as described in Section 3.B or 6.C, and then washing away unbound LgBiT with multiple washes/incubations with TBST. Substrate/buffer solution without LgBiT can then be added to the blot, and the subsequent blot should show an improved signal-to-background ratio for HiBiT bands.
Difficult to determine the molecular weight of HiBiT bands	Molecular weight markers not used or are unmarked. While there are no molecular weight standards that luminesce in the Nano-Glo® HiBiT Blotting Reagent, prestained molecular weight markers can be used and transferred to the membrane. An illuminated image can be taken before or after the luminescent image so that the positions of the molecular weight standards can be determined. If available on the imager, use epifluorescence imaging of prestained markers for particularly easy overlay of images.

6.F. References

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