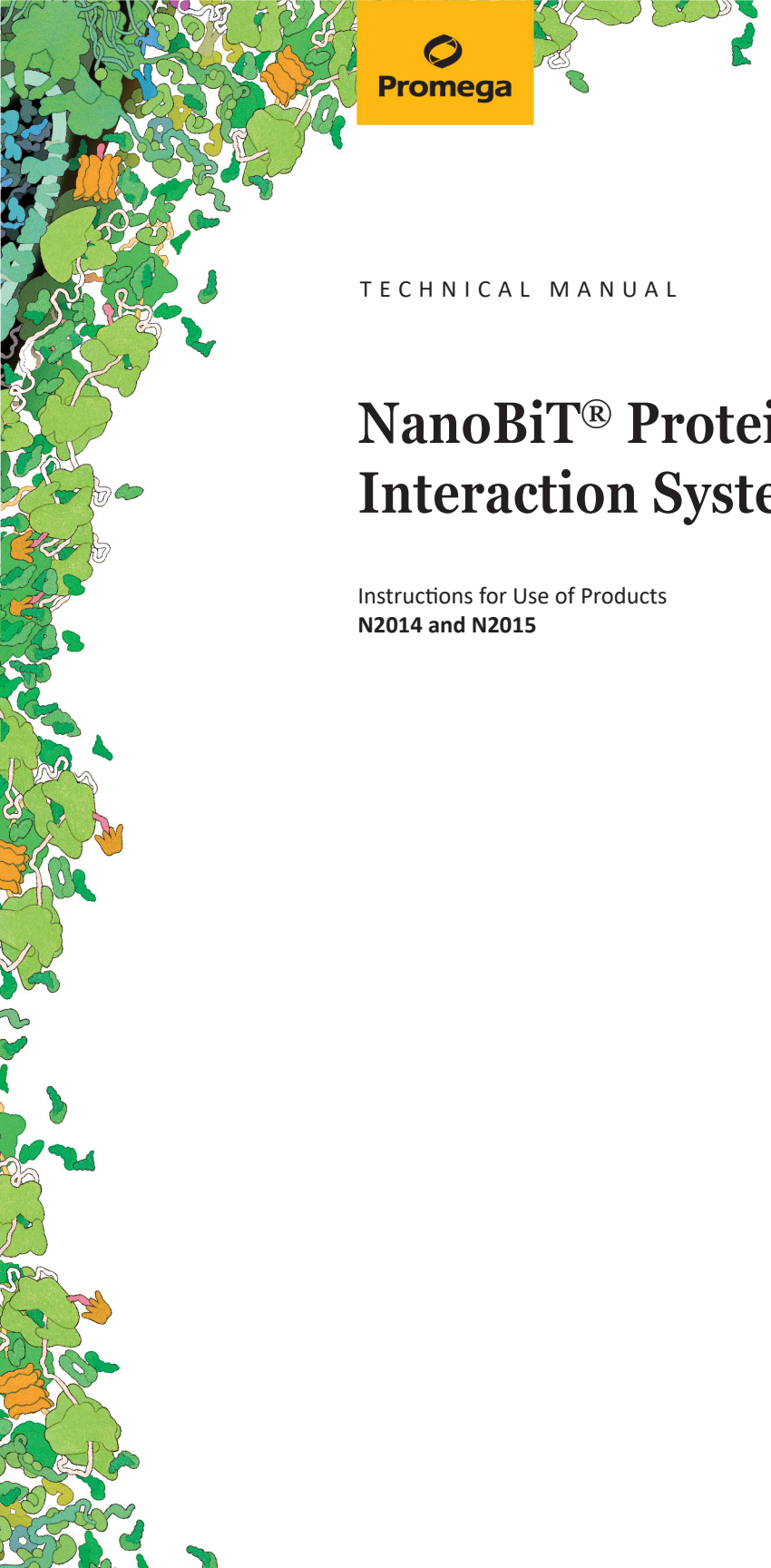

TECHNICAL MANUAL

NanoBiT® Protein:Protein Interaction System

Instructions for Use of Products
N2014 and N2015

Revised 10/21
TM461

NanoBiT® Protein:Protein Interaction System

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

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1. Description

NanoLuc® Binary Technology (NanoBiT®) is a two-subunit system based on NanoLuc® luciferase that can be used for intracellular detection of protein:protein interactions (PPIs; 1). Large BiT (LgBiT; 17.6kDa) and Small BiT (SmBiT; 11 amino acids) subunits are fused to proteins of interest (Figure 1), and when expressed, the PPI brings the subunits into close proximity to form a functional enzyme that generates a bright, luminescent signal. Unlike related approaches, where an enzyme or protein is divided into two fragments, LgBiT was independently optimized for structural stability and SmBiT was selected from a peptide library specifically for the PPI application. The result is a subunit pair that weakly associates (K_D = 190μM) yet retains the bright signal of NanoLuc® luciferase at saturation. The interaction of LgBiT and SmBiT (designated as LgBiT:SmBiT) is reversible, and the system can be used to detect rapidly dissociating proteins. PPI dynamics can be followed in real time inside living cells using the Nano-Glo® Live Cell Assay System, a nonlytic detection reagent that contains the cell-permeable substrate furimazine. Advantages over protein fragment systems include greater sensitivity, reversibility, fusion to a peptide or a small, structurally stable protein domain, real-time measurements using a nonlytic assay format, and subunits with reduced affinity for spontaneous association.

1. Description (continued)

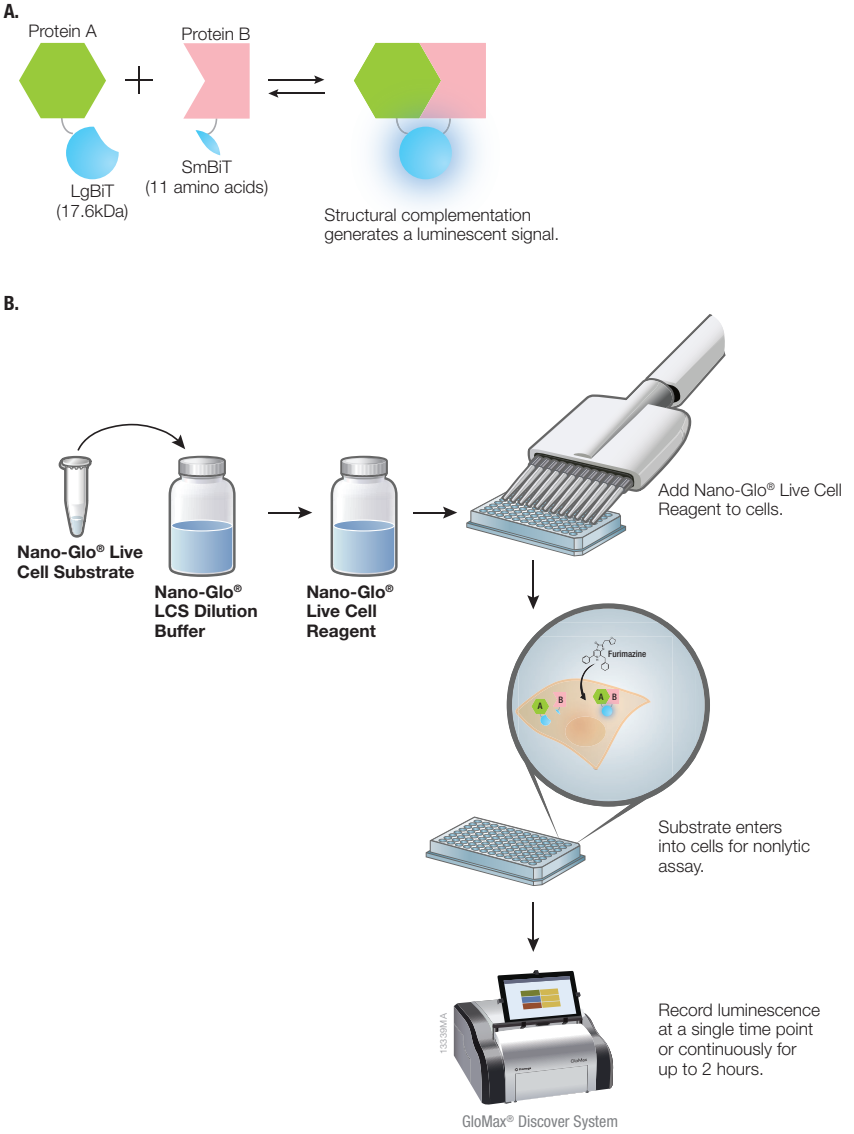


Figure 1. Overview of the NanoBiT® Protein:Protein Interaction System. Panel A. For a PPI of interest, proteins A and B are fused to LgBiT and SmBiT and expressed in cells. Interaction of fusion partners leads to structural complementation of LgBiT with SmBiT, generating a functional enzyme with a bright, luminescent signal. **Panel B.** PPIs can be monitored in real-time inside living cells using the Nano-Glo® Live Cell Reagent, which provides the cell-permeable furimazine substrate.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
NanoBiT® PPI MCS Starter System[a–e]	**1 each**	**N2014**

Each system contains vectors for standard cloning and sufficient reagent for 200 assays in 96-well plates. Includes:

- 2 × 125µl Nano-Glo® Live Cell Substrate
- 2 × 2.5ml Nano-Glo® LCS Dilution Buffer
- 20µg pBiT1.1-C [TK/LgBiT] Vector
- 20µg pBiT2.1-C [TK/SmBiT] Vector
- 20µg pBiT1.1-N [TK/LgBiT] Vector
- 20µg pBiT2.1-N [TK/SmBiT] Vector
- 20µg SmBiT-PRKACA Control Vector
- 20µg LgBiT-PRKAR2A Control Vector
- 20µg NanoBiT® Negative Control Vector

PRODUCT	SIZE	CAT.#
NanoBiT® PPI Flexi® Starter System [a–e]	**1 each**	**N2015**

Each system contains vectors for Flexi® Vector cloning and sufficient reagent for 200 assays in 96-well plates. Includes:

- 2 × 125µl Nano-Glo® Live Cell Substrate
- 2 × 2.5ml Nano-Glo® LCS Dilution Buffer
- 20µg pFC34K LgBiT TK-neo Flexi® Vector
- 20µg pFC36K SmBiT TK-neo Flexi® Vector
- 20µg pFN33K LgBiT TK-neo Flexi® Vector
- 20µg pFN35K SmBiT TK-neo Flexi® Vector
- 20µg SmBiT-PRKACA Control Vector
- 20µg LgBiT-PRKAR2A Control Vector
- 20µg NanoBiT® Negative Control Vector
- 20µg pF4A CMV Flexi® Vector

Storage Conditions: Store the Nano-Glo® Live Cell Substrate at –30°C to –10°C. Thaw the Nano-Glo® LCS Dilution Buffer upon first use and store at room temperature. Store all plasmid DNA constructs at –30°C to –10°C.

Available Separately

PRODUCT	SIZE	CAT.#
Nano-Glo® Live Cell Assay System	**100 assays**	**N2011**
	1,000 assays	**N2012**
	10,000 assays	**N2013**
NanoBiT® PPI Control Pair (FKBP, FRB)	**1 each**	**N2016**

- 20µg FRB-LgBiT Control Vector
- 20µg FKBP-SmBiT Control Vector

3. Strategy and Workflow for Developing a NanoBiT® PPI Assay

3.A. General Considerations

When developing a NanoBiT® assay for a protein pair of interest, we recommend testing all possible combinations of expression constructs to determine the optimal orientation for fusing to LgBiT and SmBiT. For example, if you want to test the interaction of two cytosolic proteins, a total of eight different expression constructs need to be made (Table 1) to test the eight possible combinations of fusion protein pairs (Table 2). The fusion protein pair that provides the best response or the brightest relative signal should be selected for assay development (Sections 3.B and 3.C). Protein-coding sequences can be cloned into NanoBiT® entry vectors using a multiple cloning site (Section 4.A) or cloned into vectors compatible with the Flexi® Vector System, which facilitates the cloning of NanoBiT® expression constructs (Section 4.B).

Depending on your interacting proteins, you may be able to reduce the number of constructs to make and orientations to test. For instance, if the C terminus of a fusion partner undergoes proteolysis, C-terminal fusions to SmBiT or LgBiT are not needed. Structural information, if available, can help guide selection of termini for LgBiT and SmBiT fusions, although we still recommend testing all possible orientations to empirically determine which fusion proteins give the best response.

Table 1. Expression Constructs to Make for a Cytosolic PPI. Each protein is fused at either the N or C terminus with LgBiT and SmBiT. The various protein fusions are depicted below.

Protein A	A-LgBiT	Protein B	B-LgBiT
	LgBiT-A		LgBiT-B
	A-SmBiT		B-SmBiT
	SmBiT-A		SmBiT-B

Table 2. Eight Combinations to Screen for a Cytosolic PPI.

LgBiT-A:SmBiT-B	A-LgBiT:SmBiT-B	LgBiT-B:SmBiT-A	B-LgBiT:SmBiT-A
LgBiT-A:B-SmBiT	A-LgBiT:B-SmBiT	LgBiT-B:A-SmBiT	B-LgBiT:A-SmBiT

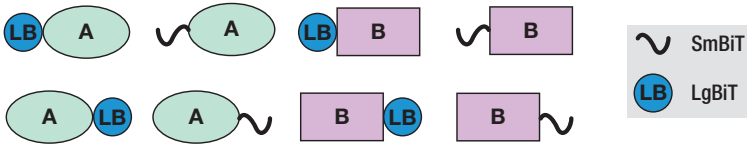
Although the NanoBiT® PPI system was optimized to minimize spontaneous association, high levels of intracellular expression can lead to low levels of LgBiT:SmBiT complex formation. Spontaneous association can lead to a decrease in the available assay window for compound treatment (1) or be a potential source of false positives when determining the interaction status of two proteins. A nonspecific pairing of a LgBiT fusion with a SmBiT fusion will typically show greater luminescence than the LgBiT fusion expressed alone (see data in Section 7.A), a difference resulting from the spontaneous association of LgBiT and SmBiT. LgBiT fusions expressed alone can show low levels of luminescence due to the low level of catalytic activity associated with LgBiT. These levels vary with the fusion partner, likely owing to differences in expression level. Cells transfected with the SmBiT fusion alone or mock-transfected will show a signal near instrument background due to furimazine autoluminescence. This low signal increases in the presence of serum.

Be aware that the NanoBiT® PPI approach monitors changes in the local concentration of SmBiT and LgBiT, not necessarily a PPI between fusion partners. For instance, LgBiT and SmBiT fusion proteins can colocalize to the same subcellular compartment, causing increased luminescence without a direct interaction. Therefore, inferring a direct physical interaction between fusion proteins from an increased luminescent signal may be erroneous.

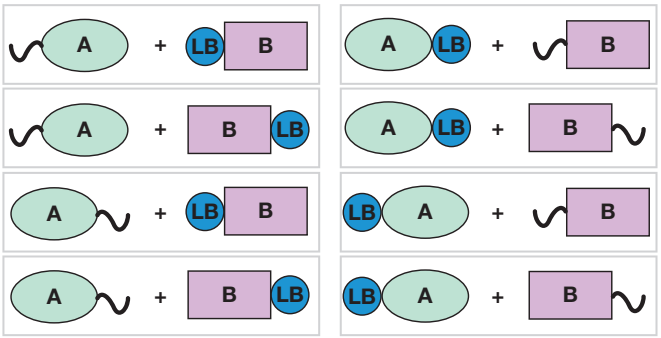
3.B. Developing a NanoBiT® PPI Assay Using a Tool Compound

A tool compound, a molecule that specifically modulates a PPI in a dose-dependent manner, can be used in NanoBiT® assay development. Example tool compounds include ligands that activate cellular signal transduction pathways, leading directly or indirectly to a change in the PPI status, or direct inhibitors of the PPI that bind to one or both fusion partners. When using a tool compound, always compare luminescence in the presence of the compound to vehicle treatment. The interaction should be considered specific if the expected change in luminescence is seen with tool compound treatment, whether it be an increase or a decrease. Figure 2 summarizes the workflow for an orientation screen using a tool compound. For a known interaction pair, multiple orientations of the protein pair typically will have similar levels of luminescence, each showing the expected response to tool compound treatment (Figure 2). Representative data are shown in Section 7.B. Select the plasmid combination showing maximal fold response to tool compound treatment. These constructs can then be used to study the interaction dynamics of the protein pair in follow-up experiments.

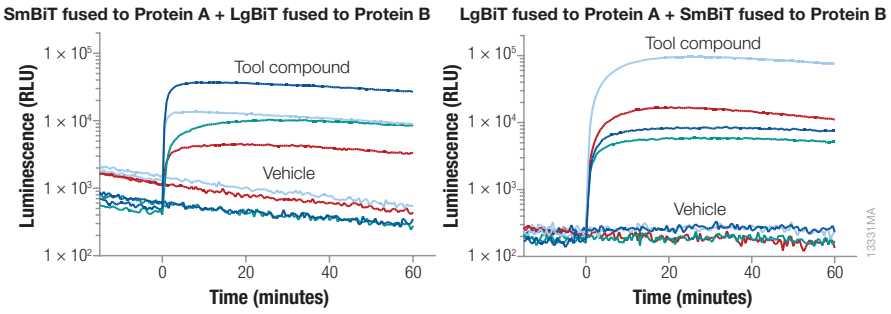
1. Generate constructs encoding LgBiT and SmBiT fusions to protein A and protein B (up to 8 possible constructs).



2. Transiently transfect the different plasmid combinations into cells (up to 8 possible combinations).



3. Screen for an optimal orientation by comparing tool compound treatment to vehicle treatment.



4. Select an orientation showing maximal fold response.

Figure 2. Developing a NanoBiT® PPI assay using a tool compound. Up to eight expression constructs are made encoding LgBiT and SmBiT fused to the N and C termini of proteins A and B (Step 1). Up to eight different plasmid combinations are transiently transfected into cells (Step 2). An orientation screen is performed by comparing the signal in the presence or absence of tool compound for each plasmid combination (tool compound injected at time zero; Step 3). The optimal orientation will be the plasmid combination showing maximal fold response (Step 4).

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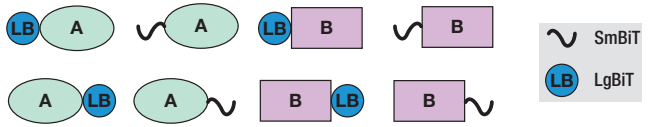
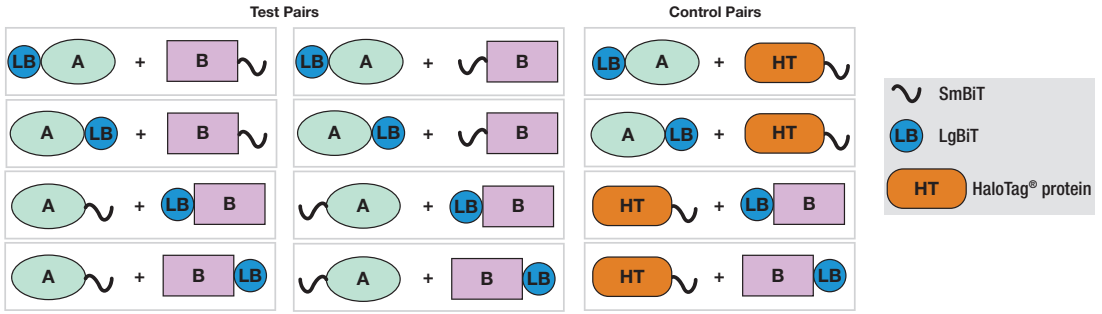
3.C. Developing a NanoBiT® PPI Assay Without a Tool Compound

In the absence of a tool compound for your protein pair, luminescence should be compared to wells transfected with one or more negative control constructs. For instance, include controls with mutations that are known to inhibit the PPI. Alternatively, replace a fusion partner with a noninteracting protein that shows similar levels of expression and subcellular localization, although this type of control may be difficult to identify. In the absence of such controls, the NanoBiT® Negative Control Vector, which encodes HaloTag®-SmBiT, can be used. The HaloTag® protein is a structurally stable fusion partner that is expressed throughout the cell (2). In general, known protein interactions show levels of luminescence 10- to 1,000-fold higher than the LgBiT fusion co-expressed with HaloTag®-SmBiT (Section 7.A). This range is only a guideline and will not be true in every case. Figure 3 summarizes the workflow for an orientation screen done using the NanoBiT® Negative Control Vector. The optimal orientation will be the plasmid combination showing the maximal fold signal increase over the respective LgBiT fusion co-expressed with HaloTag®-SmBiT. These constructs can then be used to study the interaction dynamics of the protein pair in follow-up experiments.

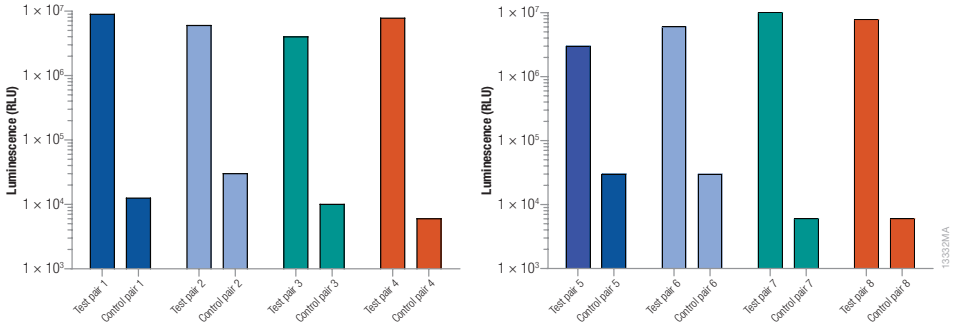
1. Generate constructs encoding LgBiT and SmBiT fusions to protein A and protein B (up to 8 possible constructs).



2. Transiently transfect the different plasmid combinations into cells (up to 8 possible combinations and 4 possible controls).



3. Screen for an optimal orientation by comparing the luminescence of a test pair to respective LgBiT fusion control.



4. Select an orientation showing maximal fold increase over respective control.

Figure 3. Developing a NanoBiT® PPI assay without a tool compound. Up to eight expression constructs are made encoding LgBiT and SmBiT fusions to the N and C termini of proteins A and B (Step 1). Up to eight different plasmid combinations and four respective controls are transiently transfected into a cell type of interest (Step 2). An orientation screen is performed by comparing the signal for a given test pair to the signal of the respective LgBiT fusion co-expressed with HaloTag®-SmBiT (Step 3). An orientation is selected that shows the maximal fold signal increase over the respective control (Step 4).

3.C. Developing a NanoBiT® PPI Assay Without a Tool Compound (continued)

For a protein pair with little or no pre-existing evidence for an interaction, using negative control constructs is required. Without these controls, a signal resulting from spontaneous association of LgBiT and SmBiT could be misinterpreted as a specific protein interaction. As described above, compare the luminescence of the unknown PPI pair to the LgBiT fusion protein co-expressed with HaloTag®-SmBiT. A specific interaction is more likely as the luminescent signal increases above the level of the control, although we recommend follow-up experiments to confirm protein interaction specificity. If the signal from the unknown PPI pair is less than tenfold higher than the corresponding control, this result may indicate a nonspecific interaction between fusion partners.

Confirm the specificity of an interaction using NanoBiT® luminescence or by an independent assay, such as co-immunoprecipitation. If using the NanoBiT® approach, confirm specificity by titrating the expression of untagged fusion partners, i.e., both proteins of the PPI pair not fused to LgBiT or SmBiT. If the interaction is specific, this approach shows decreased luminescence with increasing levels of the expressed untagged protein. See Figure 10 in Section 7.C for example data. If the interaction is the result of nonspecific association between LgBiT and SmBiT, no decrease in luminescence is expected. Complicating factors for the NanoBiT® luminescence analysis include transcriptional squelching, where high levels of expression of one protein reduce the expression levels of another, or cytotoxicity at high levels of expression of an untagged protein. We recommend performing this analysis with both protein fusion partners because a single partner may not express well enough to promote efficient competition with its tagged counterpart.

3.D. Effects of Expression Levels on Assay Performance

To minimize potential nonspecific background, NanoBiT® entry vectors use the HSV-TK promoter, providing constitutive, low-level expression in mammalian cells. When compared to CMV, HSV-TK typically provides >100-fold lower levels of expression across a broad range of mammalian cell types. Although we predict that HSV-TK will be a viable promoter in most cases, a stronger promoter may be needed to express select PPIs or if working in difficult-to-transfect cell types. If necessary, transfer fusion protein ORFs to a vector with a stronger promoter, such as PGK, SV40, eIF1a or CMV. Be aware that CMV-based expression constructs can lead to high levels of nonspecific association, requiring transfection of small amounts of plasmid DNA for optimal assay performance (Section 5.B). For a list of additional NanoBiT® expression vectors, including other promoter options, visit: **www.promega.com/nanobit**

In general, NanoBiT® assays do not require optimization of relative expression levels of the NanoBiT® fusion proteins. We have successfully tested numerous PPIs using transient transfection of an equal mass ratio of plasmid DNA for both fusion proteins.

3.E. NanoBiT® Positive and Negative Control Constructs

Two positive control PPIs are available. The FKBP:FRB pair can be used as an inducible positive control, where adding rapamycin leads to association of the fusion partners (Section 7.B, Figure 8). The PRKACA:PRKAR2A pair can be used as a constitutive positive control, where fusion partners interact without adding a compound (Section 7.A, Figure 7). The PRKACA:PRKAR2A pair can also be modulated; the luminescent signal decreases when adding compounds that increase the intracellular cAMP concentration. Fusion partners for both protein pairs are expressed using the HSV-TK promoter, and both pairs of proteins have been validated in cell types such as HEK293, CHO, HeLa and U2OS using the protocols in Sections 5.A and 6.C. Positive control vectors for FKBP-SmBiT, FRB-LgBiT, SmBiT-PRKACA and LgBiT-PRKAR2A contain an expression cassette for kanamycin resistance in bacteria. Rapamycin can be purchased as a 2.74mM stock in DMSO (Sigma Cat.# R8781).

The NanoBiT® Negative Control Vector encodes HaloTag®-SmBiT, which replaces a SmBiT fusion protein as a negative control (Section 3.C). The HaloTag® protein is a structurally stable fusion partner that is expressed throughout the cell (2). The NanoBiT® Negative Control Vector has an expression cassette for kanamycin resistance in bacteria.

4. Cloning NanoBiT® Expression Constructs

4.A. Multiple Cloning Site Vectors

Follow standard cloning protocols to introduce genes of interest (GOI) into pBiT1.1-C [TK/LgBiT], pBiT2.1-C [TK/SmBiT], pBiT1.1-N [TK/LgBiT] and pBiT2.1-N [TK/SmBiT] Vectors. Figure 4 lists the unique restriction enzyme sites present in the MCS of each vector. SacI, EcoRI or XhoI sites are available in the coding sequence of the Gly/Ser rich linker that fuses the protein of interest (POI) to LgBiT or SmBiT. At least one of these three sites must be selected as one of the two unique restriction enzymes needed for directional cloning due to the presence of an in-frame stop codon that divides the MCS. This requirement limits the number of possible linkers for a given fusion protein to three (Table 3). We have confirmed similar performance using different linker combinations for multiple protein pairs. Incorporate nucleotide sequence into your primers (Table 4) to encode the linker residues shown in bold in Table 3. For pBiT1.1-C [TK/LgBiT] and pBiT2.1-C [TK/SmBiT] Vectors, make sure that your 5´ primer contains an ATG codon. For pBiT1.1-N [TK/LgBiT] and pBiT2.1-N [TK/SmBiT] Vectors, ensure that your 3´ primer contains a stop codon. The pBiT1.1-C [TK/LgBiT], pBiT2.1-C [TK/SmBiT], pBiT1.1-N [TK/LgBiT] and pBiT2.1-N [TK/SmBiT] vectors all use the HSV-TK promoter to minimize nonspecific association and reduce experimental artifacts (Section 3.D), and each vector contains an expression cassette for ampicillin resistance in bacteria.

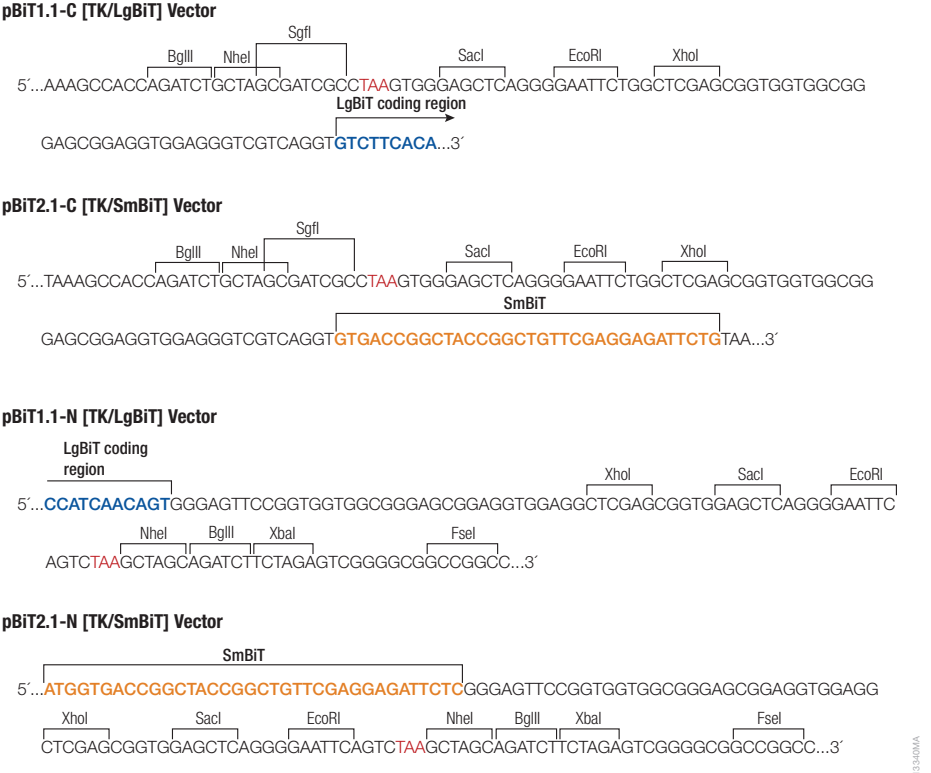


Figure 4. MCS sequences for the pBiT1.1 and pBiT2.1 Vectors. The sequence in red represents the in-frame stop codon that divides the MCS.

Table 3. Linker Sequences Associated with SacI, EcoRI or XhoI Sites in the pBiT1.1 and pBiT2.1 Vectors.

NanoBiT® Vector	Fusion Protein	MCS Restriction Site
pBiT1.1-C [TK/LgBiT] Vector	POI-**G**AQGNS-GSSGGGGSGGGGSSG-LgBiT	SacI
	POI-**G**NS-GSSGGGGSGGGGSSG-LgBiT	EcoRI
	POI-**G**SSGGGGSGGGGSSG-LgBiT	XhoI
pBiT2.1-C [TK/SmBiT] Vector	POI-**G**AQGNS-GSSGGGGSGGGGSSG-SmBiT	SacI
	POI-**G**NS-GSSGGGGSGGGGSSG-SmBiT	EcoRI
	POI-**G**SSGGGGSGGGGSSG-SmBiT	XhoI
pBiT1.1-N [TK/LgBiT] Vector	LgBiT-GSSGGGGSGGGGS**SG**-POI	XhoI
	LgBiT-GSSGGGGSGGGGSSG-GA**Q**-POI	SacI
	LgBiT-GSSGGGGSGGGGSSG-GAQGNS-POI	EcoRI
pBiT2.1-N [TK/SmBiT] Vector	SmBiT-GSSGGGGSGGGGS**SG**-POI	XhoI
	SmBiT-GSSGGGGSGGGGSSG-GA**Q**-POI	SacI
	SmBiT-GSSGGGGSGGGGSSG-GAQGNS-POI	EcoRI

Bolded residues must be encoded by PCR primer (see Table 4). POI = protein of interest.

Table 4. Primer Sequences for the Different Restriction Enzyme Sites in the Coding Sequence of the Linker for the pBiT1.1 and pBiT2.1 Vectors.

NanoBiT® Vector	Restriction Enzyme	Primer Sequence
pBiT1.1-C [TK/LgBiT] Vector	SacI	5´-NNNNNNGAGCTCC(RC GOI)-3´
	EcoRI	5´-NNNNNNGAATTCCC(RC GOI)-3´
	XhoI	5´-NNNNNNCTCGAGCC(RC GOI)-3´
pBiT2.1-C [TK/SmBiT] Vector	SacI	5´-NNNNNNGAGCTCC(RC GOI)-3´
	EcoRI	5´-NNNNNNGAATTCCC(RC GOI)-3´
	XhoI	5´-NNNNNNCTCGAGCC(RC GOI)-3´
pBiT1.1-N [TK/LgBiT] Vector	XhoI	5´-NNNNNNCTCGAGCGGT(GOI)-3´
	SacI	5´-NNNNNNGAGCTCAG(GOI)-3´
	EcoRI	5´-NNNNNNGAATTCA(GOI)-3´
pBiT2.1-N [TK/SmBiT] Vector	XhoI	5´-NNNNNNCTCGAGCGGT(GOI)-3´
	SacI	5´-NNNNNNGAGCTCAG(GOI)-3´
	EcoRI	5´-NNNNNNGAATTCA(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.

4.B. Flexi® Vector Cloning

The Flexi® Vector System allows the rapid transfer of protein coding sequences between Flexi® Vectors, making it easier to create the constructs for empirically determining the proper orientation of a given PPI pair.

Follow the protocol in Section 4 of the *Flexi® Vector Systems Technical Manual* #TM254 to clone protein-coding sequences into the pF4A CMV Flexi® Vector. Make sure to add 1 base between the SgfI recognition site and the start codon, and do not include a stop codon at the end of the protein coding region (TM254, Section 9.A). Once the sequence is verified, transfer both ORFs from pF4A CMV Flexi® Vector to pFC34K LgBiT TK-neo, pFC36K SmBiT TK-neo, pFN33K LgBiT TK-neo and pFN35K SmBiT TK-neo Flexi® Vectors using the protocols in Sections 5.A, TM254 (for pFN33K LgBiT TK-neo and pFN35K SmBiT TK-neo Flexi® Vectors) and 5.B, TM254 (for pFC34K LgBiT TK-neo and pFC36K SmBiT TK-neo Flexi® Vectors).

Alternatively, use our Find My Gene resource (**www.promega.com/findmygene/search.aspx**) to search a list of nearly 10,000 ready-to-use constructs from the Kazusa DNA Research Institute to determine if a Flexi® Vector clone exists for your gene of interest. Each construct from Kazusa can be transferred directly into pFC34K LgBiT TK-neo, pFC36K SmBiT TK-neo, pFN33K LgBiT TK-neo and pFN35K SmBiT TK-neo Flexi® Vectors.

The linkers that result from Flexi® Vector cloning have been tested at Promega using numerous PPI pairs (Table 5). pFC34K LgBiT TK-neo, pFC36K SmBiT TK-neo, pFN33K LgBiT TK-neo and pFN35K SmBiT TK-neo Flexi® Vectors all use the HSV-TK promoter to minimize nonspecific association and reduce experimental artifacts (Section 3.D).

Table 5. Linker Sequences for the NanoBiT® Flexi® Vectors.

Vector Name	Fusion Protein
pFC34K LgBiT TK-neo Flexi® Vector	POI-VSQ-GSSGGGGSGGGGSSG-LgBiT
pFC36K SmBiT TK-neo Flexi® Vector	POI-VSQ-GSSGGGGSGGGGSSG-SmBiT
pFN33K LgBiT TK-neo Flexi® Vector	LgBiT-GSSGGGGSGGGGSSG-AIA-POI
pFN35K SmBiT TK-neo Flexi® Vector	SmBiT-GSSGGGGSGGGGSSG-AIA-POI

POI = protein of interest.

5. Transient Transfection of NanoBiT® PPI Constructs

5.A. Transient Transfection of HSV-TK Expression Constructs

The following protocol is recommended for transient transfection of NanoBiT® expression constructs using the HSV-TK promoter in 96-well plates. Alternative protocols, such as mixing lipid and DNA with cells prior to plating, can be used but are not described here. If you are not using a tool compound, also transfect negative control constructs as described in Section 3.C. Alternatively, transfect a negative control better suited for your system if available, replacing the NanoBiT® Negative Control Vector. Transfect the PRKACA:PRKAR2A pair as a positive control for transfection efficiency and reagent handling.

1. Plate cells in white 96-well tissue culture plates (e.g., Corning Cat.# 3917) in a total volume of 100µl per well.

   Notes:

   1. We recommend using a buffered cell culture medium (e.g., containing HEPES) for measuring NanoBiT® PPI luminescence outside of the CO_2 incubator (see Section 6.C). If appropriate, plate cells in a buffered cell culture medium to avoid a subsequent medium exchange step. Serum can be added to buffered medium at 0–10% (v/v) fetal bovine serum (FBS).
   2. Use only the inner 60 wells to minimize the potential for thermal gradients across the plate and edge effects from evaporation after an overnight incubation. For experiments at 37°C, add a sterile solution to the outside wells and spaces between wells as instructed in Section 6.C.

2. Incubate in a 37°C, 5% CO_2 incubator for 16–24 hours for cell attachment.
3. Dilute plasmid DNA encoding LgBiT and SmBiT fusion proteins in Opti-MEM® I Reduced Serum Medium (Life Technologies Cat.# 11058) to 6.25ng/µl for each construct.
4. 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.
5. Add 8µl of lipid:DNA mixture to designated wells. Manually mix the plate in a circle for 2–3 seconds.

   Note: This results in transfection of both LgBiT and SmBiT expression constructs at 50ng/well.
6. Incubate the plates at 37°C in a 5% CO_2 incubator for 20–24 hours.

   Note: Longer incubation times may lead to higher levels of fusion protein expression, promoting nonspecific association between SmBiT and LgBiT.

5.B. Transient Transfection of Constructs with High Expression Promoters

Expression constructs using the HSV-TK promoter will be appropriate for many PPIs, reducing nonspecific association of LgBiT and SmBiT and providing physiological levels of expression in most cases. However, for some PPIs, a stronger promoter, like CMV, may be required. For a list of additional NanoBiT® expression vectors, including other promoter options, visit: **www.promega.com/nanobit**

The following is a generic protocol for diluting the amount of transfected DNA to reduce intracellular expression levels of NanoBiT® fusion proteins. This protocol for 96-well plates should be used for expression constructs with strong promoters, like CMV. Alternative protocols, such as mixing lipid and DNA with cells prior to plating, can be used but are not described here. When using a strong promoter like CMV, we recommend varying the amount of transfected DNA to optimize the assay response. At high levels of transfected DNA, like 50ng/well for each construct, typically you will see high levels of nonspecific association between LgBiT and SmBiT when using a strong promoter like CMV.

5.B. Transient Transfection of Constructs with High Expression Promoters (continued)

1. Plate cells in white 96-well tissue culture plates (e.g., Corning Cat.# 3917) in a total volume of 100µl per well.

   Notes:

   1. We recommend using a buffered cell culture medium (e.g., containing HEPES) for measuring NanoBiT® PPI luminescence outside of the CO_2 incubator (see Section 6.C). If appropriate, plate cells in a buffered cell culture medium to avoid a subsequent medium exchange step. Serum can be added to buffered medium at 0–10% (v/v) fetal bovine serum (FBS).
   2. Use only the inner 60 wells to minimize the potential for thermal gradients across the plate and edge effects from evaporation after an overnight incubation. For experiments at 37°C, add a sterile solution to the outside wells and spaces between wells as instructed in Section 6.C.

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 each NanoBiT expression construct to 12.5ng/µl using Opti-MEM® I Reduced Serum Medium (Life Technologies Cat.# 11058).
4. Serially dilute the plasmid DNA for each NanoBiT® expression construct with the 12.5 ng/µl solution of Transfection Carrier DNA as shown in Table 6.

Table 6. Serial Dilution of NanoBiT® High Expression Promoter Plasmids.

Tube Number	Dilution
1	1/10 dilution of 12.5ng/µl, LgBiT fusion
2	1/10 dilution of Tube #1
3	1/10 dilution of Tube #2
4	1/10 dilution of 12.5ng/µl, SmBiT fusion
5	1/10 dilution of Tube #4
6	1/10 dilution of Tube #5

5. Combine equal volumes of plasmid DNA dilutions as shown in Table 7.

Table 7. Combining Dilutions of NanoBiT® Expression Plasmids.

Transfection Conditions for Each Construct		
5ng/well	0.5ng/well	50pg/well
Tubes 1, 4	Tubes 2, 5	Tubes 3, 6

Notes:

1. See Sections 3.B and 3.C for recommendations on screening for the optimal orientation for a given PPI. See Section 7.B for example orientation screen data.
2. See Sections 3.C and 3.E for a discussion of recommended controls to include in your NanoBiT® PPI experiment.

5.B. Transient Transfection of Constructs with High Expression Promoters (continued)

6. 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.
7. Add 8µl of lipid:DNA mixture to respective wells. Manually mix the plate in a circle for 2–3 seconds.
8. Incubate the plates at 37°C in a 5% CO_2 incubator for 20–24 hours.

   Note: Longer incubation times may lead to higher levels of fusion protein expression, promoting nonspecific association between SmBiT and LgBiT.

6. Protocols for Measuring NanoBiT® PPI Luminescence

6.A. Overview of the Nano-Glo® Live Cell Assay System

The Nano-Glo® Live Cell Assay System is used for NanoBiT® PPI assays to detect luminescence from living cells using a nonlytic protocol. This system has two components: The Nano-Glo® Live Cell Substrate and the Nano-Glo® LCS Dilution Buffer. The Nano-Glo® Live Cell Substrate is an organic stock solution that contains the cell-permeable furimazine substrate and a proprietary agent to reduce furimazine autoluminescence in the presence or absence of serum. The Nano-Glo® Live Cell Substrate is diluted using the Nano-Glo® LCS Dilution Buffer to make the Nano-Glo® Live Cell Reagent, a 5X stock for delivery to cell culture medium. The Nano-Glo® LCS Dilution Buffer is an aqueous solution that provides enhanced furimazine stability. The Nano-Glo® LCS Dilution Buffer is designed to share similar tonicity to buffered solutions commonly used in cell culture, such as PBS, Hank's Balanced Salt Solution, etc. In addition to NanoBiT® PPI assays, the Nano-Glo® Live Cell Assay System can be used for measurement of intracellular NanoLuc® luciferase and with NanoBRET™ assays as well.

6.B. Preparation of the Nano-Glo® Live Cell Reagent

1. Equilibrate Nano-Glo® LCS Dilution Buffer to ambient temperature if using for the first time.
2. Remove the Nano-Glo® Live Cell Substrate from storage and mix.
3. Prepare the desired amount of reconstituted Nano-Glo® Live Cell Reagent by combining 1 volume of Nano-Glo® Live Cell Substrate with 19 volumes of Nano-Glo® LCS Dilution Buffer (a 20-fold dilution), creating a 5X stock to mix with cell culture medium. For example, if the experiment requires 20ml of reagent, add 1ml of substrate to 19ml of dilution buffer.

   Notes:

   1. If the Nano-Glo® Live Cell Substrate has collected in the cap or on the sides of the tube, briefly spin tubes containing 125µl or 1.25ml of substrate in a microcentrifuge.
   2. The Nano-Glo® Live Cell Reagent should be made fresh for each experiment. We do not recommend long-term storage of this solution at any temperature.
   3. Once reconstituted, the furimazine component of the Nano-Glo® Live Cell Reagent will decay at a rate that depends on the storage temperature. We have quantified reagent stability using purified NanoLuc® luciferase in cell-free experiments. At 20°C, the reagent will lose 10% activity in approximately 3 hours. At 4°C, the reagent will lose 10% activity in approximately 7 hours.

6.C. Measuring NanoBiT® PPI Luminescence

The following example protocols can be used for NanoBiT® PPI assays conducted in 96-well plates (e.g., Corning Cat.# 3917). These protocols can be used to measure luminescence from the FKBP:FRB and PRKACA:PRKAR2A positive control pairs. Figure 8 in Section 7.B shows representative data for the FKBP:FRB positive control pair at 37°C. Figure 7 in Section 7.A shows representative data for the PRKACA:PRKAR2A positive control pair at 37°C.

1. Aspirate medium and replace with 100µl of buffered cell culture medium, such as Opti-MEM® I Reduced Serum Medium (Life Technologies Cat.# 11058).
   a. For experiments at 37°C, warm medium to 37°C prior to medium exchange.

      Note: To maintain a temperature of 37°C throughout the experiment, we recommend using only the inner 60 wells of a 96-well plate to avoid edge-cooling effects, and adding 200µl of a sterile solution to the outside 36 wells. In addition, add 150µl of sterile solution to the spaces between wells. These measures will help maintain a temperature of 37°C if the plate is removed temporarily from a 37°C environment.

   b. For experiments at ambient temperature, replace with ambient temperature medium, and allow the plate to sit at ambient temperature for 10 minutes to equilibrate.

   Notes:

   1. Cells can be plated in buffered medium to avoid the medium exchange, if deemed appropriate.
   2. Fetal bovine serum (FBS) can be added to buffered medium at 0–10% (v/v) FBS.
2. Choose Option #1 or Option #2 to proceed with the protocol.

Option #1: Adding Nano-Glo® Live Cell Reagent Prior to Compound Addition

3. Add 25µl of Nano-Glo® Live Cell Reagent to each well, and gently mix the plate by hand or with an orbital shaker (e.g., 15 seconds at 300–500rpm).

   Note: Be careful not to disturb the cell monolayer.

4. Measure baseline luminescence.
   a. For experiments at 37°C: Measure luminescence for 15–20 minutes to ensure thermal equilibration of the entire plate to 37°C.
   b. For experiments at ambient temperature: Measure luminescence for 5 minutes.

   Note: Using this baseline signal to normalize the response of each well can reduce variability caused by varying numbers of cells plated per well, differences in transfection efficiency per well and other factors. For instance, after adding a compound that modulates the PPI, normalize the response of a given well to the average of the final three luminescence measurements taken for the same well prior to compound addition. Once calculated, average the normalized response from replicate wells for a given treatment. This data transformation will be less effective with baseline signals that approach autoluminescence background.

5. Dilute compounds or vehicle in the same medium used for the medium exchange in Step 1 to make 13.5X stock solutions. Equilibrate compound stocks to ambient temperature.

Option #1: Adding Nano-Glo® Live Cell Reagent Prior to Compound Addition (continued)

6. Dispense 10µl of 13.5X stock solution of compounds or vehicle, and gently mix.
   a. For experiments at 37°C: Use injectors to dispense compounds and mix by using the instrument orbital shaker. Alternatively, if you are not using injectors, remove the plate from the luminometer, add compounds and mix the plate by hand or using an orbital shaker (e.g., 15 seconds at 300–500rpm).

      Note: We recommend the use of injectors and a shaker within the detection instrument to minimize temperature fluctuations associated with removing the plate from the luminometer.

   b. For experiments at ambient temperature: Dispense compounds using injectors or a multichannel pipette and mix the plate by hand or using an orbital shaker (e.g., 15 seconds at 300–500rpm).

   Note: Be careful not to disturb the cell monolayer.

7. Monitor luminescence at a user-defined time point or continuously for up to 2 hours.

   Notes:

   1. Standard benchtop luminometers can be used for NanoBiT® assays, such as the GloMax® Discover and GloMax® Explorer Systems. Use an integration time of 0.25–2 seconds.
   2. Furimazine will degrade in aqueous solution at or near physiological pH, leading to a gradual decrease in luminescence intensity independent of any change in the PPI status. Always include normalization control(s) when continuously monitoring luminescence for extended time periods. The presence of FBS can increase the rate of furimazine degradation.
   3. The signal intensity and stability will vary, depending on experimental conditions. At high levels of luminescence, the rate of signal decay can increase owing to increased enzymatic turnover (Figure 5). At lower levels of luminescence, the rate of signal decay will be primarily determined by furimazine instability (Figure 5).
   4. The Nano-Glo® Live Cell Reagent promotes no significant increase in cytotoxicity in multiple cell types (as determined using the CellTiter-Glo® Luminescent Cell Viability Assay) for incubation times ≤2 hours in the absence of NanoLuc® luciferase or NanoBiT® fusion protein expression.

Option #2: Compound Addition Prior to Adding Nano-Glo® Live Cell Reagent

3. Dilute compounds or vehicle in the same medium used for the medium exchange in Step 1 to make 11X stock solutions. Equilibrate compound stocks to ambient temperature.
4. Dispense 10µl of 11X stock solution of compounds or vehicle, and gently mix plate by hand or using an orbital shaker (e.g., 15 seconds at 300–500rpm).
   a. For experiments at 37°C: Incubate the plate in a 37°C, 5–10% CO_2 incubator.
   b. For experiments at ambient temperature: Incubate the plate on your lab bench.

   Note: Be careful not to disturb the cell monolayer.

6.C. Measuring NanoBiT® PPI Luminescence (continued)

Option #2: Compound Addition Prior to Adding Nano-Glo® Live Cell Reagent (continued)

5. Add 25µl of Nano-Glo® Live Cell Reagent per well, and gently mix plate by hand or using an orbital shaker (e.g., 15 seconds at 300–500rpm).
   a. For experiments at 37°C: Equilibrate the plate inside a 37°C luminometer for 10–15 minutes.
   b. For experiments at ambient temperature: Measure luminescence immediately after adding the Nano-Glo® Live Cell Reagent and mixing.

   Note: Be careful not to disturb the cell monolayer.

6. Monitor luminescence at a user-defined time point or continuously for up to 2 hours.

   Notes:

   1. Standard benchtop luminometers can be used for NanoBiT® assays, such as the GloMax® Discover and GloMax® Explorer Systems. Use an integration time of 0.25–2 seconds.
   2. Furimazine will degrade in aqueous solution at or near physiological pH, leading to a gradual decrease in luminescence intensity independent of any change in the PPI status. Always include normalization control(s) when continuously monitoring luminescence for extended time periods. The presence of FBS can increase the rate of furimazine degradation.
   3. The signal intensity and stability will vary, depending on experimental conditions. At high levels of luminescence, the rate of signal decay can increase owing to increased enzymatic turnover (Figure 5). At lower levels of luminescence, the rate of signal decay will be primarily determined by furimazine instability (Figure 5).
   4. The Nano-Glo® Live Cell Reagent promotes no significant increase in cytotoxicity in multiple cell types (as determined using the CellTiter-Glo® Luminescent Cell Viability Assay) for incubation times ≤2 hours in the absence of NanoLuc® luciferase or NanoBiT® fusion protein expression.

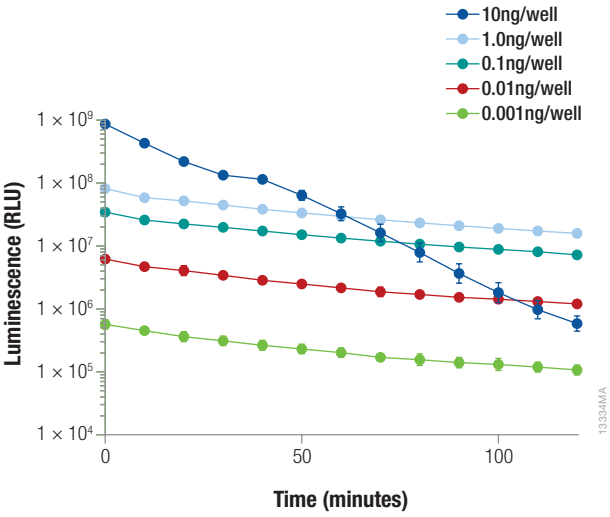


Figure 5. Monitoring signal stability of the Nano-Glo® Live Cell Reagent in HEK293 cells. HEK293 cells were transfected with varying amounts of the pNL1.1.CMV [*Nluc*/CMV] Vector encoding NanoLuc® luciferase. After 24 hours, cell culture medium was exchanged for Opti-MEM® I Reduced Serum Medium, and Nano-Glo® Live Cell Reagent was added according to the protocol in Section 6.C. Luminescence was measured during the indicated time points at 37°C. The total amount of DNA transfected per well (50ng) was kept constant using Transfection Carrier DNA (Cat.# E4881). n = 3; error bars represent standard deviation.

7. Representative Data

7.A. Relative Luminescence of Specific versus Nonspecific Interactions

In our experiments, the luminescence from a known PPI is 10- to 1,000-fold higher than the LgBiT fusion co-expressed with HaloTag®-SmBiT, the NanoBiT® Negative Control. We recommend using this range as a general guideline to determine if a signal is the result of a specific PPI.

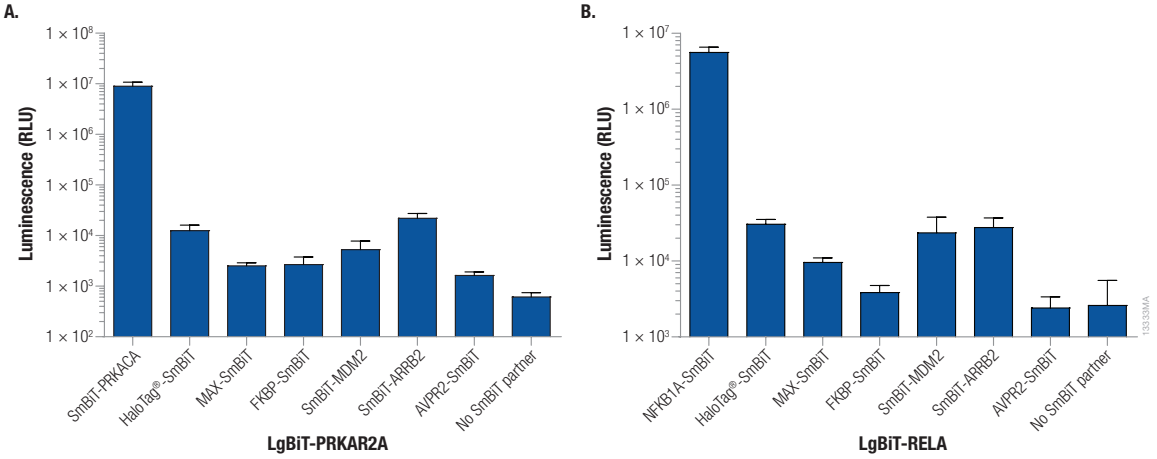


Figure 6. A comparison of specific versus nonspecific NanoBiT® signals. Expression constructs were made encoding the respective fusions to LgBiT and SmBiT (see Section 4.B). Following transient transfection of HEK293 cells as described in Section 5.A, the Nano-Glo® Live Cell Reagent was added and luminescence was measured at 37°C (see Section 6.C). **Panel A.** Luminescence from LgBiT-PRKAR2A fusion protein paired with a specific binding partner, SmBiT-PRKACA fusion protein, or with various nonspecific controls. **Panel B.** Luminescence from LgBiT-RELA fusion protein paired with a specific binding partner, NFKBIA-SmBiT fusion protein, or with various nonspecific controls. The LgBiT fusion protein expressed without its interaction partner is also included in Panels A and B for comparison. n = 6; error bars represent standard deviation.

7.A. Relative Luminescence of Specific versus Nonspecific Interactions (continued)

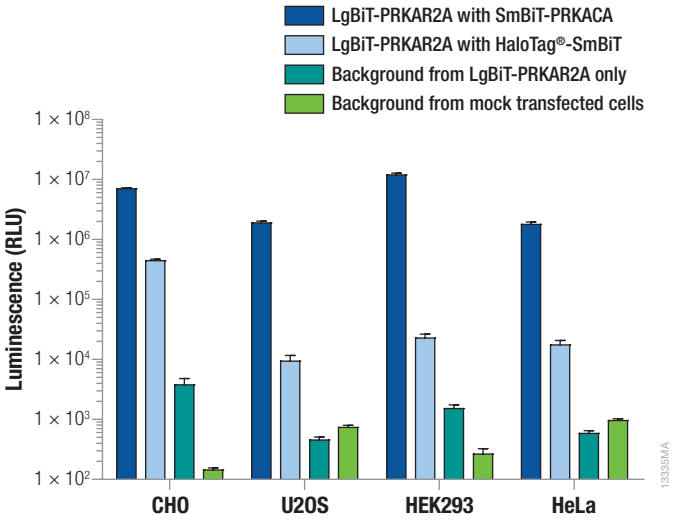


Figure 7. A comparison of the signal from a known PPI to different sources of background signal. The optimal orientation of PRKACA and PRKAR2A (SmBiT-PRKACA and LgBiT-PRKAR2A positive control fusion proteins) was compared to LgBiT-PRKAR2A and HaloTag®-SmBiT fusion proteins, LgBiT-PRKAR2A fusion protein expressed alone or the signal from cells transfected with only carrier DNA in multiple cell types. Luminescence was measured at 37°C. n = 6; error bars represent standard deviation.

7.B. Orientation Screens Using a Tool Compound

The eight different orientations of FKBP and FRB proteins fused to LgBiT and SmBiT showed the expected signal increase after adding the tool compound rapamycin, although the magnitude of the increase did vary between constructs.

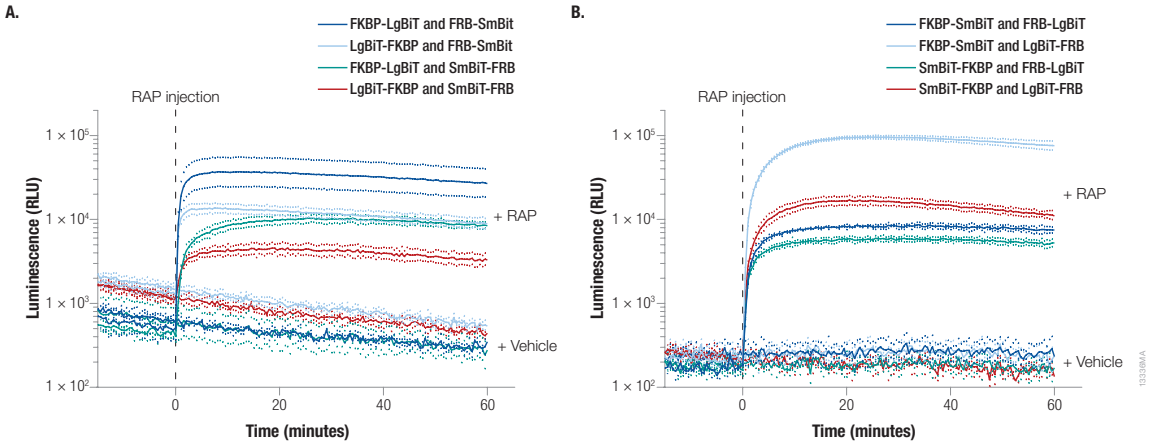


Figure 8. Screening for optimal orientations of the FKBP and FRB pair. Expression constructs were made encoding all possible fusions of LgBiT and SmBiT to the N and C termini of FKBP and FRB (see Section 4.B). Following transient transfection of HEK293 cells as described in Section 5.A, the Nano-Glo® Live Cell Reagent was added and luminescence was monitored continuously at 37°C. At time zero, 30nM rapamycin (RAP) was injected and luminescence measured continuously for 1 hour. **Panel A.** LgBiT fused to FKBP and SmBiT fused to FRB in various orientations. **Panel B.** SmBiT fused to FKBP and LgBiT fused to FRB in various orientations. n = 3; dotted lines represent standard deviation.

All four orientations of LgBiT and SmBiT fused to androgen receptor (AR) showed the expected signal increase with addition of R1881, although the magnitude of the increase varied among the orientations.

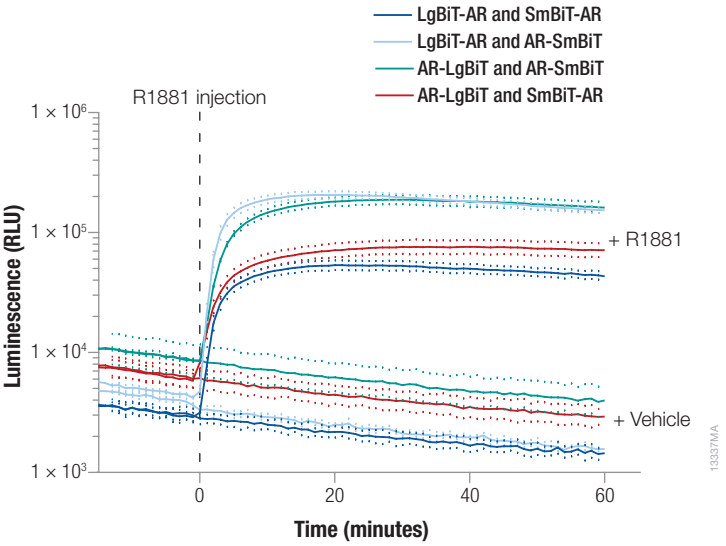


Figure 9. Screening for optimal orientations for androgen receptor dimerization. Expression constructs were made encoding all possible fusions of LgBiT and SmBiT to the N and C termini of the androgen receptor (see Section 4.B). Following transient transfection of HEK293 cells as described in Section 5.A, the Nano-Glo® Live Cell Reagent was added and luminescence was monitored continuously at 37°C. At time zero, 100nM R1881 was injected and luminescence measured continuously for 1 hour. n = 3; dotted lines represent standard deviation.

7.C. Determining Specificity Using an Untagged Fusion Partner

To demonstrate the specificity of the interaction between NanoBiT® fusion proteins, titrate the level of untagged fusion partner present by increasing the amount of vector that expresses the untagged partner. A specific interaction will show decreased luminescence with increasing levels of one or both expressed untagged proteins. Nonspecific interactions will show no change in luminescence.

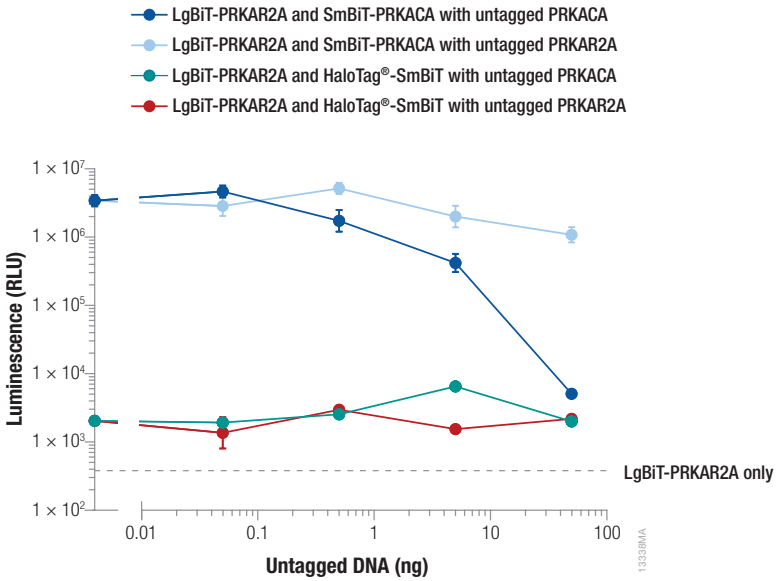


Figure 10. Confirming specificity by expressing untagged fusion partners. The PRKACA:PRKAR2A positive control pair was transfected at 25ng/construct/well using a modified version of the protocol listed in Section 5.A. Varying amounts of CMV-based expression constructs for untagged PRKACA and PRKAR2A were also transfected, keeping the total amount of DNA transfected per well fixed at 100ng using Transfection Carrier DNA (Cat.# E4881). n = 3; error bars represent standard deviation.

8. NanoBiT® Technology on the Web

We will continue to expand our NanoBiT® Technology portfolio. The NanoBiT® web site will be updated with new products, representative data for validated PPI pairs and alternative protocols for transfection or data analysis. Our web site will also list NanoBiT® expression vectors that can be custom ordered, including vectors with varying promoter strength. Find this information and more at: **www.promega.com/nanobit**

9. Troubleshooting

Symptoms	Causes and Comments
Luminescence values for a known PPI are at or near the the levels of mock-transfected cells and much lower than the luminescence from the PRKACA:PRKAR2A constitutive control.	Expression from HSV-TK promoter is too low. Subclone the ORFs encoding LgBiT and SmBiT fusion proteins into expression vectors using the CMV promoter. When switching to a stronger promoter, optimize the amount of transfected DNA to generate the best assay response, as described in Section 5.B.
Bright luminescent signal was measured, but little or no signal change is seen when adding a tool compound known to modulate the interaction.	Fusion protein expression levels may be too high, potentially driving significant levels of nonspecific association of LgBiT and SmBiT. • If you are using CMV or a similarly strong promoter, dilute the amount of transfected DNA to optimize the assay response, as outlined in Section 5.B. • Subclone the ORFs encoding LgBiT and SmBiT fusions to expression vectors with a weaker promoter.
Highly variable data between replicate wells for the same treatment.	Poor mixing of reagents. Make sure to properly mix the wells after adding compounds or vehicle to the Nano-Glo® Live Cell Reagent (e.g., 15 seconds at 300–500rpm).
	Cell numbers vary from well to well or poor transfection efficiency. To reduce well-to-well variability in cell plating or transfection efficiency: • Transfect the cells in a six-well plate and replate after 16–24 hours. • Combine lipid and DNA with cell suspension prior to plating and express for 16–24 hours. • If possible, normalize the data on a per well basis to the baseline signal prior to compound treatment, as described in Section 6.C. This approach can reduce well-to-well variability from differences in transfection efficiency or cell number.

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9. Troubleshooting (continued)

Symptoms	Causes and Comments
Vehicle treatment for a kinetic trace at 37°C did not decay at a steady rate but instead showed a signal increase followed by a signal decrease.	Did not equilibrate multiwell plate prior to experiment. • Allow the plate to equilibrate to 37°C for 15–20 minutes in the luminometer prior to adding test or control compounds. • Use injectors to deliver compound(s) or vehicle to respective wells. • If using a multichannel pipettor to quickly dispense compounds, minimize the amount of time the plate is removed from the luminometer to prevent cooling. • For experiments in 96-well plates, add 200µl of sterile solution to the outside 36 wells and 150µl of sterile solution between wells to provide a buffer to temperature fluctuations.

10. References

1. Dixon, A.S. *et al.* (2015) NanoLuc complementation reporter optimized for accurate measurement of protein interactions in cells. *ACS Chem. Biol.* In press.
2. Los, G.V. *et al.* (2008) HaloTag: A novel protein labeling technology for cell imaging and protein analysis. *ACS Chem. Biol.* **3**, 373–82.

11. Related Products

Product	Size	Cat.#
GloMax® Discover System	1 each	GM3000
GloMax® Explorer Fully Loaded Model	1 each	GM3500
GloMax® Explorer with Luminescence and Fluorescence	1 each	GM3510
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

12. Summary of Changes

The following changes were made to the 10/21 revision of this document:

1. Removed links to the FuGENE® HD database.
2. Text and disclaimer updates were made.
3. The cover image was updated.

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[c]Patents Pending

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

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