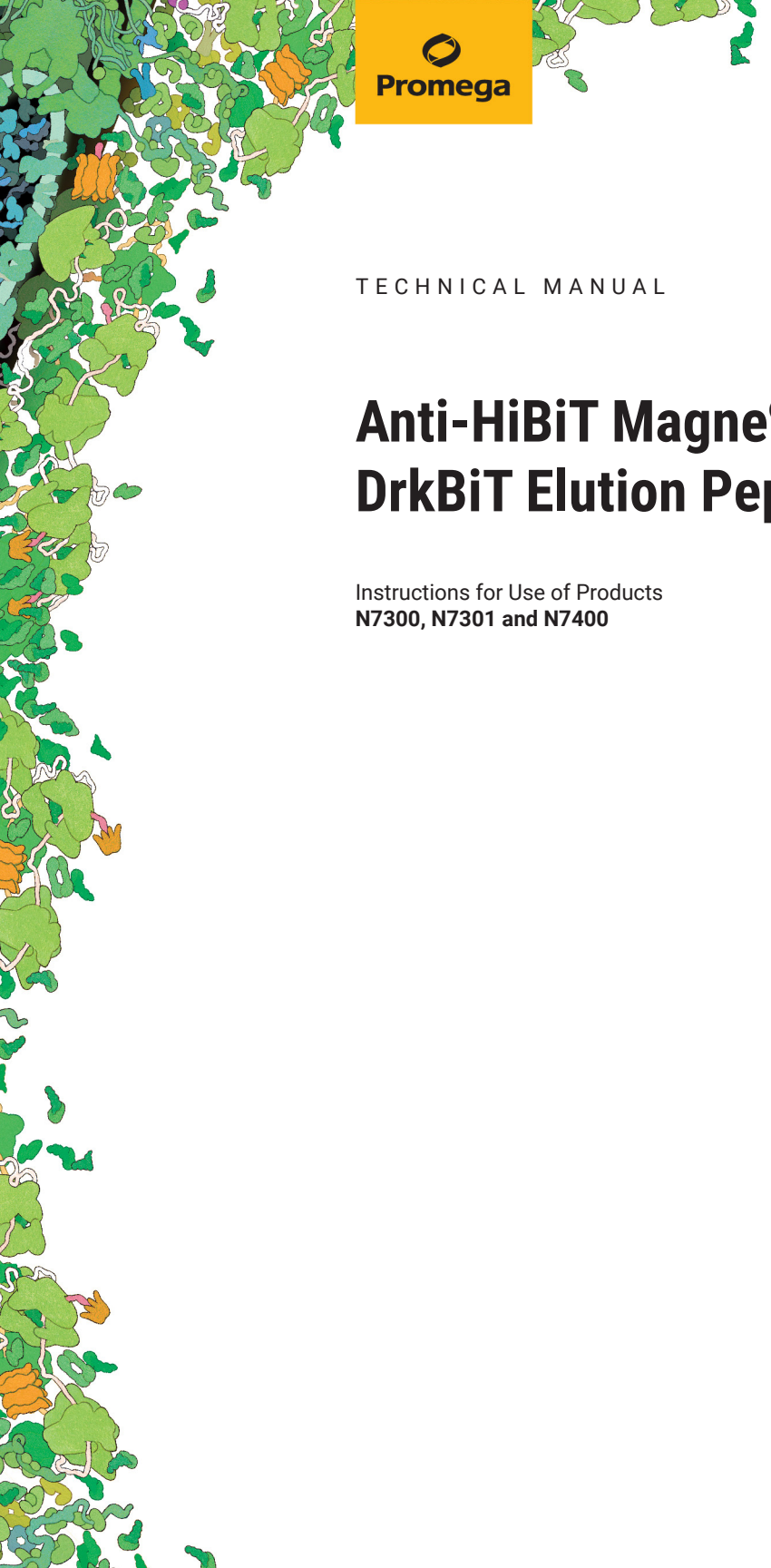

TECHNICAL MANUAL

Anti-HiBiT Magne® Beads and DrkBiT Elution Peptide

Instructions for Use of Products
N7300, N7301 and N7400

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Anti-HiBiT Magne® Beads and DrkBiT Elution Peptide

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

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1. Description

The High BiT (HiBiT) protein tag is an eleven-amino-acid peptide that binds with high affinity to Large BiT (LgBiT) in NanoLuc® Binary Technology (NanoBiT®) to reconstitute NanoBiT® Luciferase, a bright, luminescent enzyme (1,2). Proteins tagged with HiBiT can be easily quantified by homogeneous luminescent assays in multiple formats. Because of its small size and sensitive detection, HiBiT makes an ideal tag for insertion by genome-editing techniques like CRISPR-Cas9, resulting in endogenous expression of HiBiT-tagged proteins (3). The HiBiT tag can also be added to proteins using available HiBiT cloning vectors or directly to existing protein expression constructs by PCR-based or gene synthesis methods. The sequence and rights to synthesize the HiBiT tag can be obtained by reviewing and accepting the Terms and Conditions of Use at: **www.promega.com/HiBiT-Synthesis**

Anti-HiBiT Monoclonal Antibody (Cat.# N7200, N7210) is a potent and specific mouse monoclonal antibody (mAb) that binds to the HiBiT tag, enabling applications such as immunoblotting, immunofluorescence, immunoprecipitation and fluorescence-activated cell sorting (FACS). The antibody binds to HiBiT with high affinity, with a K_D in solution of approximately 6pM. Because of this high affinity and low background binding, many HiBiT-tagged proteins can be detected at endogenous expression levels.

For Anti-HiBiT Magne® Beads[a] (Cat.# N7300, N7301) the Anti-HiBiT Monoclonal Antibody is covalently attached to magnetic particles, enabling capture and elution of HiBiT-tagged proteins in standard immunoprecipitation workflows. This Technical Manual includes example protocols and representative data for use of the beads in immunoprecipitation and co-immunoprecipitation applications.

Table 1. Anti-HiBiT Magne® Bead Specifications.

Bead composition	Magnetic particles encapsulated with microporous cellulose
Bead chemistry	Covalent attachment of Anti-HiBiT Monoclonal Antibody
Bead particle size	30–50µm
Bead binding capacity	≥1mg HaloTag®-HiBiT (36kDa) per ml of settled beads or ≥0.2mg HaloTag®-HiBiT (36kDa) per ml of bead slurry
Bead formulation	20% slurry in 1X phosphate-buffered saline (PBS) containing 0.02% sodium azide
Antibody Host Species	Mouse
Antibody Isotype	IgG2c with kappa light chain

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
Anti-HiBiT Magne® Beads	**1ml**	**N7300**

Includes sufficient reagents for 100 reactions.

PRODUCT	SIZE	CAT.#
Anti-HiBiT Magne® Beads	**5ml**	**N7301**

Includes sufficient reagents for 500 reactions.

PRODUCT	SIZE	CAT.#
DrkBiT Elution Peptide, 100X	**100µl**	**N7400**

Storage Conditions: Anti-HiBiT Magne® Beads are provided as a 20% slurry in PBS containing 0.02% sodium azide. Store the beads at +2°C to +10°C. **Do not freeze**, as this will compromise bead integrity and performance. Additionally, do not allow beads to dry during storage or use. DrkBiT Elution Peptide[(a)] is provided as a 100X solution in water. Store the DrkBiT Elution Peptide at –30°C to –10°C. Upon thawing, aliquot the peptide to minimize freeze-thaw cycles.

3. Immunoprecipitation and Co-Immunoprecipitation

3.A. General Considerations

Anti-HiBiT Magne® Beads are used for immunoprecipitation to capture, enrich and purify HiBiT-tagged proteins from cell lysates or other samples. The covalently immobilized antibody on magnetic particles allows easy sample binding, washing and elution. HiBiT-tagged proteins can be eluted using low pH, DrkBiT Elution Peptide or by heating with SDS loading buffer. **Note:** SDS loading buffer may cause some antibody to dissociate from the beads, particularly if reducing agent (e.g., DTT) is present. Eluted antibody may interfere with certain downstream applications, such as immunoblotting with anti-mouse secondary antibodies. For antibody-free detection, the Nano-Glo® HiBiT Blotting System (Cat.# N2410) provides an alternative for visualizing HiBiT-tagged proteins in eluates.

When more gentle elution conditions are required (e.g., to preserve function of the HiBiT-tagged protein), DrkBiT Elution Peptide (Cat.# N7400; 14 amino acids) can be used. The DrkBiT Peptide is a variant of HiBiT that binds to the LgBiT subunit to form a nonluminescent complex. Although DrkBiT has lower affinity for the antibody than HiBiT, the concentrations used for elution are well above the K_D, effectively blocking re-association of HiBiT-tagged proteins that dissociate from the immobilized antibody. Use of DrkBiT Peptide for elution will interfere with downstream HiBiT luminescent assays unless the peptides are first separated (e.g., by SDS-PAGE) or removed (e.g., by size exclusion columns). Due to the slow dissociation rate of HiBiT/mAb complexes, elution with peptide may require extended incubation periods (e.g., 2 hours at room temperature or overnight at 4°C).

3.A. General Considerations (continued)

Notes:

a. For high-throughput applications, automated systems such as the KingFisher® (Thermo Fisher Scientific) are recommended. Application note "Automation of Immunoprecipitation with Anti-HiBiT Magne® Beads on the KingFisher® Apex Instrument" includes protocols and guidance for use of Anti-HiBiT Magne® Beads in automated systems (4). For further assistance, contact Promega Technical Services: **techserv@promega.com**

b. Ensure that the Anti-HiBiT Magne® Beads remain in suspension during binding, wash, and elution steps. Use a tube shaker (e.g., Eppendorf Thermomixer® set at 1,100rpm) or end-over-end mixing to prevent bead settling. A tube shaker may provide more even mixing under low volume conditions (e.g., <300µl). If some bead solution remains in the cap after mixing, spin tubes briefly at low speeds (≤1,000 × *g*) before placing samples on magnetic stand.

c. For best results, optimize immunoprecipitation conditions (e.g., incubation times and temperatures) for each HiBiT-tagged protein. For co-immunoprecipitation, the lysis, binding, and wash buffers may require additional optimization to maintain the protein-protein interactions of interest.

d. The interaction between the Anti-HiBiT antibody and HiBiT is stable in many lysis buffers, including ones with relatively harsh detergent conditions (e.g., RIPA Buffer).

e. In preparing samples for immunoprecipitation-mass spectrometry (IP-MS), avoid Tween® detergents throughout the immunoprecipitation workflow, since these can interfere with mass spectrometry-based analysis. Use low-pH elution to maximize release of HiBiT-tagged protein while minimizing release of nonspecifically bound proteins.

Materials to Be Supplied by User

- Tris-buffered saline solution (25mM Tris-HCl, 150mM NaCl [pH 7.5])
- Tween® 20 (Cat.# H5152)
- Mammalian Lysis Buffer (Cat.# G9381)
- RQ1 RNase-Free DNase (Cat.# M6101)
- Protease Inhibitor Cocktail (Cat.# G6521)
- magnetic stand (e.g., MagneSphere® Technology Magnetic Separation Stand, Cat.# Z5342)
- **optional:** Nano-Glo® HiBiT Lytic Detection System (Cat.# N3030)
- **optional:** SDS loading buffer without DTT (e.g., 60mM Tris-HCl, 1% SDS, 10% glycerol, 0.75mM bromophenol blue, pH 6.8)
- **optional:** low-pH elution buffer (100mM glycine-HCl, pH 2.5)
- **optional:** neutralization buffer (2M Tris-HCl, pH 7.5)
- **optional:** DrkBiT Elution Peptide (Cat.# N7400)
- **optional:** Nano-Glo® HiBiT Blotting System (Cat.# N2410)
- **optional:** Magnetic Proteomics Sample Prep Kit (available through Early Access at: **www.promega.com/products/mass-spectrometry/protein-enrichment-and-sample-cleanup/magnetic-proteomics-sample-prep-mpsp-kit/**)

3.B. Example Protocol

1. Generate HiBiT-containing lysates (e.g., prepare cell lysates in Mammalian Lysis Buffer supplemented with RQ1 RNase-Free DNase and Protease Inhibitor Cocktail). Clear lysate by centrifuging for 10 minutes at 16,000 × *g* at 4°C and transferring supernatant to a new tube.

   Notes:

   a. The recommended lysate concentration for immunoprecipitation is ~1mg protein/ml (~5 million cells/ml of lysis buffer, depending on cell type). Depending on expression levels of the HiBiT-tagged protein, it may be desirable to adjust the volume of lysate used for immunoprecipitation to ensure efficient target capture, elution, and analysis (refer to bead binding capacity listed in Table 1). HiBiT-tagged protein expression levels can be quantified in cell lysates using the Nano-Glo® HiBiT Lytic Detection System.

   b. For best results, optimal lytic conditions should be determined for a given HiBiT-tagged protein. For co-immunoprecipitation, lytic conditions (e.g., buffer, temperature, incubation times) may require additional optimization to maintain the protein-protein interactions of interest.

2. Dispense 10µl of Anti-HiBiT Magne® Beads (20% slurry) into a new tube.

   Notes:

   a. Ensure the beads are fully resuspended to a homogenous suspension before pipetting.

   b. For HiBiT-tagged proteins that are highly expressed or have a high molecular weight (e.g., >100kDa), more beads may be required for complete target capture (see bead binding capacity listed in Table 1).

3. Wash Anti-HiBiT Magne® Beads twice to equilibrate beads as follows:

   a. Add 1ml of buffer (e.g., lysis buffer or TBST [Tris-buffered saline plus 0.1% Tween® 20]) to beads.

   b. Mix by pipetting or end-over-end rotation.

   c. Place tube in magnetic stand to collect beads. Remove buffer.

   d. Repeat for a total of two washes.

4. Add a lysate sample to the equilibrated Anti-HiBiT Magne® Beads (e.g., 200–1,000µl of cleared cell lysate).

5. Incubate for 1 hour at room temperature with agitation or end-over-end rotation to bind target to beads.

   Note: For some HiBiT-tagged proteins, capture may be complete in as little as 15 minutes, while others may require longer incubation times. Binding can also be performed at 4°C overnight. Conditions should be optimized for a given target and the desired downstream analysis. For instance, co-immunoprecipitations may require additional optimization to preserve stability of protein complexes.

6. Collect beads by placing tube in the magnetic stand.

   Note: Save supernatant to a new tube for later analysis, if desired.

7. Wash beads three times with 1ml of buffer (e.g., lysis buffer or TBST). Remove supernatant after each wash. Refer to Step 3 for detailed wash instructions.

3.B. Example Protocol (continued)

8. Remove wash buffer and elute (e.g., in 50µl) using **one** of the following conditions:

   a. Add SDS loading buffer without DTT (see Section 3.A. for formulation) to beads and heat at 70°C for 10 minutes. Place the sample on a magnetic stand and remove the eluate from the beads.

      Note: Including DTT in the loading buffer may cause antibodies to dissociate from the beads.

   b. Add low-pH buffer (100mM glycine-HCl [pH 2.5]) to beads and incubate at room temperature for 5–10 minutes with agitation or end-over-end rotation. Remove the eluate from the beads on a magnetic stand. Neutralize the eluate with a 20% volume of 2M Tris-HCl (pH 7.5).

      Note: Do not incubate beads at low pH for extended periods of time, as this may cause bead degradation and antibody dissociation.

   c. Prepare competitive peptide elution buffer by diluting DrkBiT Elution Peptide 100-fold in TBST (e.g., add 5µl of DrkBiT Elution Peptide stock to 495µl of TBST). Add competitive peptide elution buffer to beads and incubate overnight at 4°C in a tube shaker (e.g., Eppendorf Thermomixer® set at 1,100rpm) or with end-over-end rotation.

      Note: When using DrkBiT Elution Peptide for competitive peptide elution, incubation at room temperature and/or for shorter amounts of time is possible. The extent of elution will depend on the dissociation rate of HiBiT from the immobilized antibody, which may vary for different fusion proteins.

9. Analyze samples by Nano-Glo® HiBiT Blotting System, Western blotting, mass spectrometry (e.g., IP-MS), or desired assay.

   Notes:

   a. When reducing conditions are required for SDS-PAGE and blotting, reducing agent (e.g., DTT) can be added to SDS loading buffer eluates and samples can be reheated at 70°C before gel loading.

   b. For Western blotting, avoid use of anti-mouse secondary antibodies, as low levels of Anti-HiBiT antibody in the eluates may interfere with analysis. Alternatively, use Nano-Glo® HiBiT Blotting System for antibody-free detection of HiBiT-tagged proteins.

   c. To prepare samples for mass spectrometry, use the Magnetic Proteomics Sample Prep Kit (MPSP; **www.promega.com/products/mass-spectrometry/protein-enrichment-and-sample-cleanup/magnetic-proteomics-sample-prep-mpsp-kit/**) or any generic SP3®/PAC-based sample prep method.

3.C. Example Data

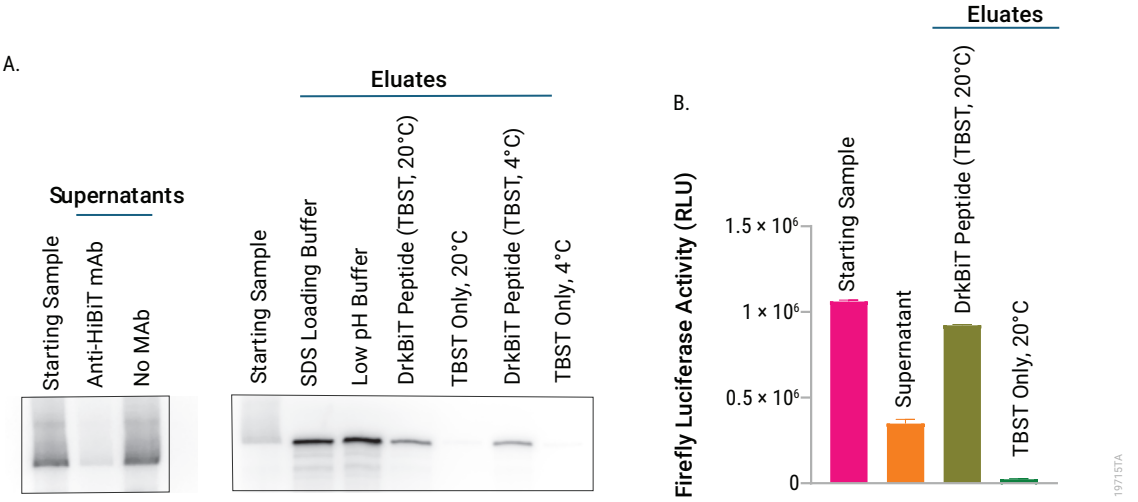

Figure 1. Comparison of elution strategies by immunoprecipitation of HiBiT-tagged firefly luciferase (Fluc-HiBiT) from transiently transfected HEK293 cells. Starting samples consisted of lysate containing 5 × 10^6 cells/ml in Mammalian Lysis Buffer (Cat.# G9381) supplemented with Protease Inhibitor Cocktail (Cat.# G6521) and RQ1 RNase-Free DNase (Cat.# M6101). Cleared lysate was incubated with beads for 2 hours at 4°C. Eluates were generated using four different elution conditions: 1) SDS loading buffer; 2) low-pH buffer; 3) 2-hour DrkBiT Elution Peptide (Cat.# N7400, diluted 100-fold in TBST) incubation at room temperature; or 4) overnight DrkBiT Elution Peptide incubation at 4°C. TBST-only buffer controls were performed for comparison to DrkBiT Peptide eluates. Eluates were generated using a fourfold lower volume (100µl) than the starting lysate samples (400µl). Equivalent volumes of the starting lysate sample, supernatants and eluates were used for analysis. **Panel A** shows analysis of samples using the Nano-Glo® HiBiT Blotting System (Cat.# N2410). The left blot is scaled to lower intensities to illustrate effective clearance of Fluc-HiBiT from supernatants by Anti-HiBiT Magne® Beads but not control beads without antibody. The right blot is scaled to higher intensities to demonstrate increased concentrations of Fluc-HiBiT in the eluates compared to the starting sample. In **Panel B**, the enzymatic activity of firefly luciferase was measured using the ONE-Glo™ Assay (Cat.# E6110), highlighting the ability to gently elute HiBiT-tagged proteins and maintain enzyme activity using the DrkBiT Elution Peptide.

3.C. Example Data (continued)

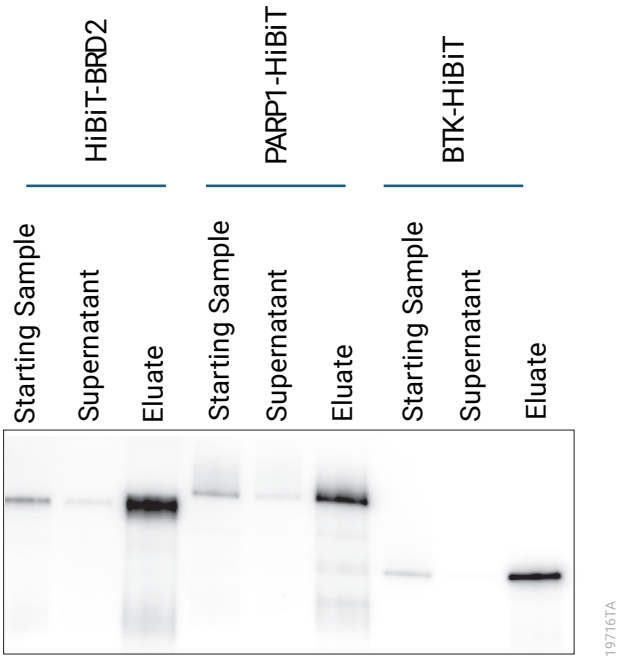

Figure 2. CRISPR-mediated HiBiT-tagging enables immunoprecipitation of proteins expressed at endogenous levels. Using CRISPR/Cas9 genome-editing technology, the HiBiT tag was added to termini of BRD2, PARP1 and BTK in HEK293, DLD-1 and K562 cells, respectively. The standard protocol in Section 3.B was used to immunoprecipitate the proteins. Samples were analyzed to monitor protein clearance from the supernatant and protein recovery in the eluate. Starting samples of cleared cell lysate (1ml; 5×10^6 cells/ml) were immunoprecipitated by binding overnight at 4°C and eluting in 80µl of 0.1M glycine (pH 2.5). Eluates were removed from beads and neutralized with 20µl of 2M Tris (pH 7.5). Equivalent volumes of each starting lysate sample, supernatant and eluate were analyzed using the Nano-Glo® HiBiT Blotting System (Cat.# N2410).

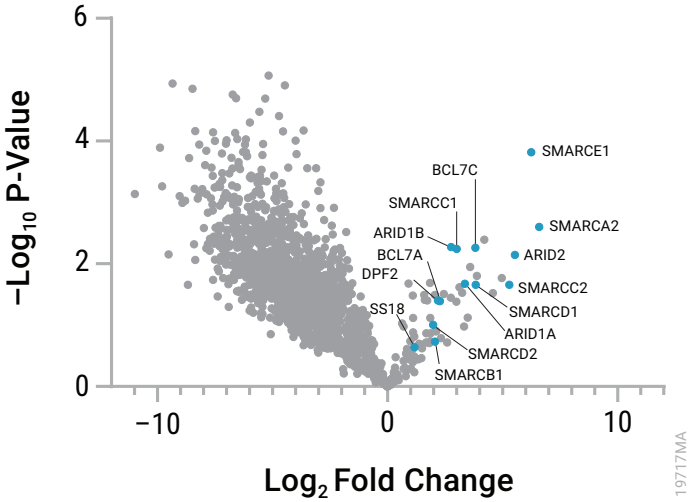

Figure 3. HiBiT-based IP-MS identifies SMARCA2-associated SWI/SNF proteins. The HiBiT tag was inserted at the C-terminus of endogenous SMARCA2 in HeLa cells using CRISPR/Cas9 genome editing. Triplicate immunoprecipitations were performed from lysates of both edited and parental cells using the standard protocol described in Section 3.B. Eluates were processed with the Magnetic Proteomics Sample Prep Kit and digested with Trypsin/Lys-C (Cat.# V5071). Peptides were analyzed by LC-MS/MS on a Thermo Fisher Scientific Orbitrap Exploris™ 240. Data were searched using Proteome Discoverer 3.1 against the Uniprot Human database using the Sequest search algorithm. Blue circles in the volcano plot highlight known SMARCA2 interactors, including members of the SWI/SNF chromatin remodeling complex.

4. Antibody Binding Affinity and Competition with LgBiT

Anti-HiBiT Monoclonal Antibody binds with high affinity to form a stable complex with HiBiT peptide or HiBiT-tagged proteins. Antibody binding largely blocks productive interaction of HiBiT with the LgBiT subunit, so the presence of antibody in a sample can inhibit the signal from HiBiT in bioluminescent assays in which LgBiT is a component of the reagent (e.g., the Nano-Glo® HiBiT Lytic Detection System). Similarly, the presence of LgBiT could inhibit binding of the Anti-HiBiT Monoclonal Antibody (e.g., lysates from cells expressing both HiBiT-tagged protein and LgBiT), but because the antibody binds about two orders of magnitude more tightly to HiBiT than LgBiT does, it can often outcompete low concentrations of LgBiT.

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5. Troubleshooting

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

Symptoms	Causes and Comments
There is low clearance of HiBiT-tagged protein from the supernatant	The amount of HiBiT-tagged protein may exceed the binding capacity of the immobilized antibody. Increase the volume of beads used.
	The cell lysis or buffer conditions may not be optimal. Ensure that cell lysis and protein solubilization is efficient; the high affinity of the HiBiT/mAb interaction helps maintain the interaction even in relatively harsh detergent conditions, like RIPA buffer. If the lysate is too viscous, treat with DNase, but make sure there is no EDTA present during treatment.
	The binding time may not be sufficient. Increase the binding time (e.g., overnight at 4°C).
	The HiBiT tag on the target protein may not be fully accessible (e.g., because it is buried in a protein complex). A lower-than-expected HiBiT bioluminescent signal upon addition of LgBiT may help confirm this. Increasing the stringency of the binding buffer could help make the tag more accessible. Consider increasing the linker length between the target protein and the HiBiT tag or append it to the other protein terminus.
Co-elution of antibody complicates downstream analysis	Elution of immunoprecipitated proteins by heating in SDS loading buffer may cause antibody dissociation into the eluate, particularly if reducing agent (e.g., DTT) is present. Incubation with competing DrkBiT Peptide will retain the antibody on the beads and elute only HiBiT-tagged proteins. Elute more specifically using low-pH buffer or DrkBiT Peptide, rather than SDS loading buffer.

Symptoms	Causes and Comments
Low amount of HiBiT-tagged protein in eluate	The HiBiT fusion protein dissociated during the wash steps. Because of the stable complex between HiBiT and the mAb, this is unlikely unless a particularly harsh wash buffer was used.
	The protein may have degraded. Add Protease Inhibitor Cocktail (Cat.# G6521) to the lysis buffer and, if necessary, add to wash or elution buffers.
	Perform all immunoprecipitation steps at 4°C.
	The protein may be unstable or precipitating irreversibly onto the beads. Optimize buffer conditions to maintain protein solubility.
	Perform a second elution step heating with SDS loading buffer to see what protein may still be stuck to the resin.
An expected coprecipitating protein is not observed in the eluate	If a significant amount of HiBiT-tagged protein is immunoprecipitated, but an expected binding partner is not observed in the eluate, the protein complex may not be stable. Dissociation may occur during the binding or washing steps. Optimizing buffer conditions, using shorter incubation times, and/or keeping samples cold may help preserve protein complexes.
	Optimize buffer conditions to maintain the protein-protein interaction of interest (e.g., adding glycerol to stabilize the complex).
	Perform all immunoprecipitation steps at 4°C.
	The stoichiometry of the protein complex in cells may mean that only a small percentage of the HiBiT-tagged protein is bound to the second protein at the point of lysis. Consider overexpressing the coprecipitating protein.
The eluate appears to contain nonspecifically bound proteins	Increase wash stringency by raising the salt concentration, adding detergents to the wash buffer or increasing the wash volume.
	Elute more specifically using low-pH buffer or DrkBiT Elution Peptide, rather than SDS loading buffer.

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6. References

1. Dixon, A.S. *et al.* (2016) NanoLuc complementation reporter optimized for accurate measurement of protein interactions in cells. *ACS Chem. Biol.* **11**, 400–8.
2. Hall, M.P. *et al.* (2012) Engineered luciferase reporter from a deep sea shrimp utilizing a novel imidazopyrazinone substrate. *ACS Chem. Biol.* **7**, 1848–57.
3. Schwinn, M.K. *et al.* (2018). CRISPR-mediated tagging of endogenous proteins with a luminescent peptide. *ACS Chem. Biol.* **13**, 467–74.
4. *Automation of Immunoprecipitation with Anti-HiBiT Magne® Beads on the KingFisher® Apex Instrument* Applications Note (2025). #PA1177; **www.promega.com/results#q="PA1177"**.