Application Note
Simple Western HiBiT Assay

Highly Specific Protein Quantification Using an Anti-HiBiT Monoclonal Antibody and Simple Western Technology

Automated Analysis of HiBiT-Protein Expression, Isoforms, Targeted Protein Degradation, and More

Introduction

Accurate quantification of tagged proteins plays a pivotal role in a broad array of biological applications, including protein expression profiling, isoform characterization, assay validation, and targeted protein degradation (TPD) studies. The HiBiT protein tagging system, developed by Promega, provides a sensitive and versatile method for protein detection through an 11–amino acid peptide tag. The HiBiT tag binds to its complementary subunit LgBiT to reconstitute NanoBiT® luciferase, a bright, luminescent enzyme, enabling rapid protein quantification in multiple formats, including lytic and live-cell plate-based assays. A high-affinity anti-HiBiT monoclonal antibody expands the utility of the HiBiT-tag, enabling traditional epitope tag analysis of HiBiT-tagged proteins.

While plate-based HiBiT assays are fast and high-throughput, they lack the molecular-weight resolution required to verify tagged-protein identity and to distinguish isoforms or degradation products. **Simple Western™ Technology** addresses this gap with automated capillary-based immunoassays that integrate protein separation, immunoprobing, and detection on a single platform. Compared with traditional western blots, Simple Western offers markedly higher sensitivity along with excellent reproducibility, total-protein normalization, and multiplexing capabilities, making it an effective orthogonal complement to plate-based assays.

In this application note, we evaluate the performance of the Anti-HiBiT Monoclonal Antibody from Promega using Simple Western Technology **(Fig. 1)**. Simple Western assays that utilize Anti-HiBiT Monoclonal Antibody provide an advanced solution for validating hits from high-throughput plate-based HiBiT screens. This study provides protocol guidance for the Simple Western HiBiT mAb Assay and demonstrates the use of the Simple Western for downstream DC_{50} and D_{max} determination to characterize hits identified via HiBiT plate screens.

Key Takeaways:

- High-throughput, high-specificity antibody-based detection of HiBiT-tagged proteins, offering a powerful orthogonal approach to plate-based luminescent HiBiT assays.
- Capable of processing up to 96 samples in ~3 hours, combining throughput with molecular weight resolution, ideal for efficient screening follow-up or standalone protein analysis
- Demonstrated sensitivity exceeding traditional Western blot and broad dynamic and linear ranges
- Quantitative DC_{50} and D_{max} characterization of degradation hits identified in HiBiT screens, providing enhanced resolution and isoform specificity

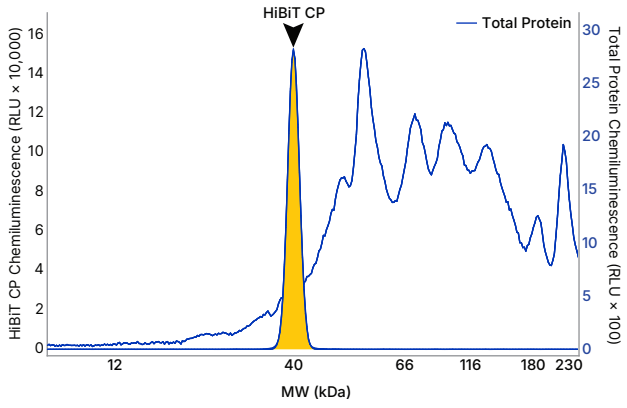

Figure 1. Detection of HiBiT Control Protein (CP) in human lysate using the Anti-HiBiT Monoclonal Antibody (Promega) with Simple Western Technology (Bio-Techne). Total protein (blue trace) was detected in the same capillary using the RePlex Module.

Materials and Methods

Establishing the Simple Western HiBiT Assay

HiBiT detection was performed using the Anti-HiBiT Monoclonal Antibody (clone 30E5). Simple Western analysis was performed using **Leo**™ and **Jess**™ Systems. Additional consumables and reagents for protein analysis were provided by Promega **(Table 1)** and Bio-Techne **(Table 2)**.

TABLE 1.

Promega materials used in this study.

Name	Cat. #
Anti-HiBiT Monoclonal Antibody	N7200
HiBiT Control Protein	N3010
Mass Spec-Compatible Human Protein Extract	V6941
Anti-Mouse IgG (H+L), HRP Conjugate	W4021
ECL Western Blotting Substrate	W1015

TABLE 2.

Bio-Techne materials used in this study.

Name	Cat. #
12-230 kDa Separation Module (Jess)	**SM-W001**
12-230 kDa Separation Module (Leo)	**SWSM-W014**
EZ Standard Pack 1	**PS-ST01EZ-8**
Anti-Mouse Detection Module	**DM-002**
Anti-Rabbit Detection Module	**DM-001**
Total Protein Detection Module	**SWDM-TP21**
RePlex™ Module	**RP-001**
Anti-Mouse HRP Secondary Antibody 100X	**040-655**
Anti-BRD4 rabbit pAb (C-terminus)	**NBP1-86640**
Anti-BRD4 rabbit mAb (N-terminus)	**NBP3-15452**
Anti-GAPDH mouse mAb	**MAB5718**

To optimize antibody performance, HiBiT Control Protein (CP) was diluted to 10 ng/mL in 0.1 mg/mL human protein extract to simulate complex biological samples. Samples were heat-denatured under reducing conditions and analyzed on both Leo and Jess. For Leo, the Anti-HiBiT Monoclonal Antibody was tested in a 12-point, 2-fold titration series ranging from 100 – 0.049 µg/mL with four replicates. Jess experiments used the antibody in an 8-point, 3-fold titration series from 100 – 0.046 µg/mL with three replicates. HiBiT signal was normalized to total protein measured in the same capillary using the RePlex Module.

To define assay sensitivity, range, standard curve, and recovery, HiBiT CP was prepared in a 10-point, 5-fold dilution series from 5 µg/mL down to 2.56 pg/mL in 0.1 mg/mL human protein extract. The same batch of sample was prepared for analysis on Leo and Jess Systems. Anti-HiBiT Monoclonal Antibody concentration was fixed at 10 µg/mL, the saturation point determined in earlier optimization.

Comparison to Traditional Western Blot

For comparison to traditional Western blot, the HiBiT CP was prepared in a 7-point, 2-fold titration ranging from 2 – 0.031 ng/mL in 0.1 mg/mL human protein extract in triplicate. The same batch of sample was split and loaded in equivalent volumes for analysis by Western blot and Simple Western. For detection by Western blot, Anti-HiBiT Monoclonal Antibody was diluted to 1 µg/mL and incubated overnight at 4 ˚C with gentle shaking. Anti-Mouse IgG (H+L), HRP Conjugate was diluted to 0.2 µg/mL and incubated 1 hour at room temperature with gentle shaking. ECL Western Blotting Substrate was added and then the membrane was imaged on a FluorChem R Imager. Detection with Simple Western was performed using the Anti-Mouse Detection Module. Simple Western and traditional Western blot exposures were 128 seconds for direct comparison.

BRD4 Targeted Protein Degradation

A549 cells edited via CRISPR/Cas9 to insert the HiBiT tag at the N-terminus of the BRD4 gene were treated for 6 hours with vehicle only or treated with Compound 8, a BRD4-targeting PROTAC® Degrader, at concentrations (nM) of 0.1, 0.5, 2, 4, 8, 32, and 125. Samples were analyzed at concentrations (mg/mL) of 2.0, 0.5, and 0.1 for detection using antibodies targeting HiBiT, N-terminal BRD4, and C-terminal BRD4, respectively. Primary antibodies were prepared at concentrations (µg/mL) of 10, 40, and 20 targeting HiBiT, N-terminal BRD4, and C-terminal BRD4, respectively. Protein expression measurements were normalized to GAPDH detected in the same capillary using the RePlex Module using mouse anti-GAPDH antibody prepared at 50 µg/mL concentration. Due to high (2 mg/mL) lysate concentration, Anti-Mouse HRP Secondary Antibody 100X diluted to 1X was used for HiBiT detection, while the Anti-Mouse HRP Detection Module was used for BRD4 (N- and C-term) and GAPDH detection. DC_{50} and D_{max} values were calculated using GraphPad Prism with a 4-parameter logistic (4-PL), unweighted curve fit.

Results

Defining the Simple Western HiBiT Assay

Optimization experiments demonstrated that the Anti-HiBiT Monoclonal Antibody reached saturation at 10 µg/mL on both Leo and Jess platforms **(Fig. 2)**. Signal intensity increased with antibody concentration before plateauing, indicating effective saturation for subsequent quantitative experiments.

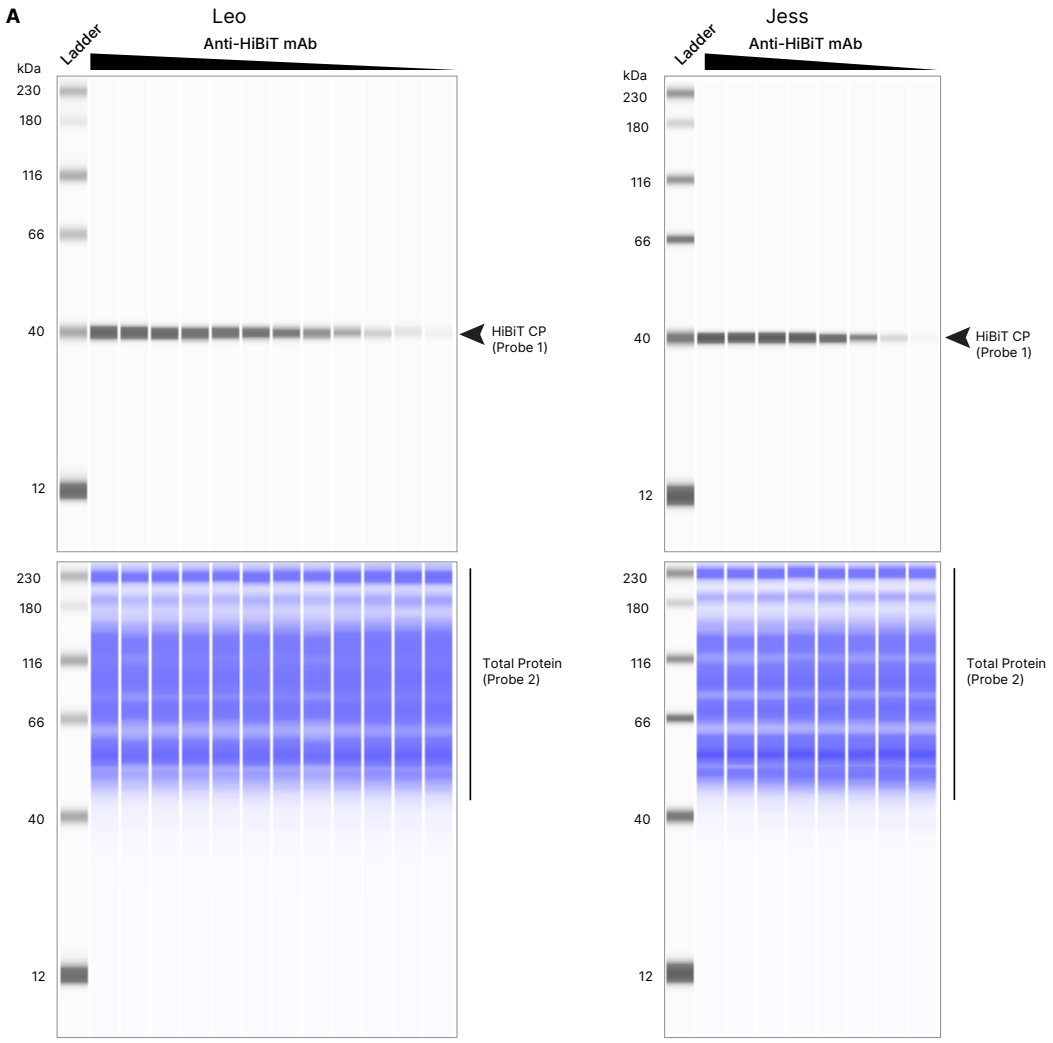

Figure 2: Optimization of HiBit CP Detection using the Anti-HiBit Monoclonal Antibody on Leo (Left) and Jess (Right). Lane view

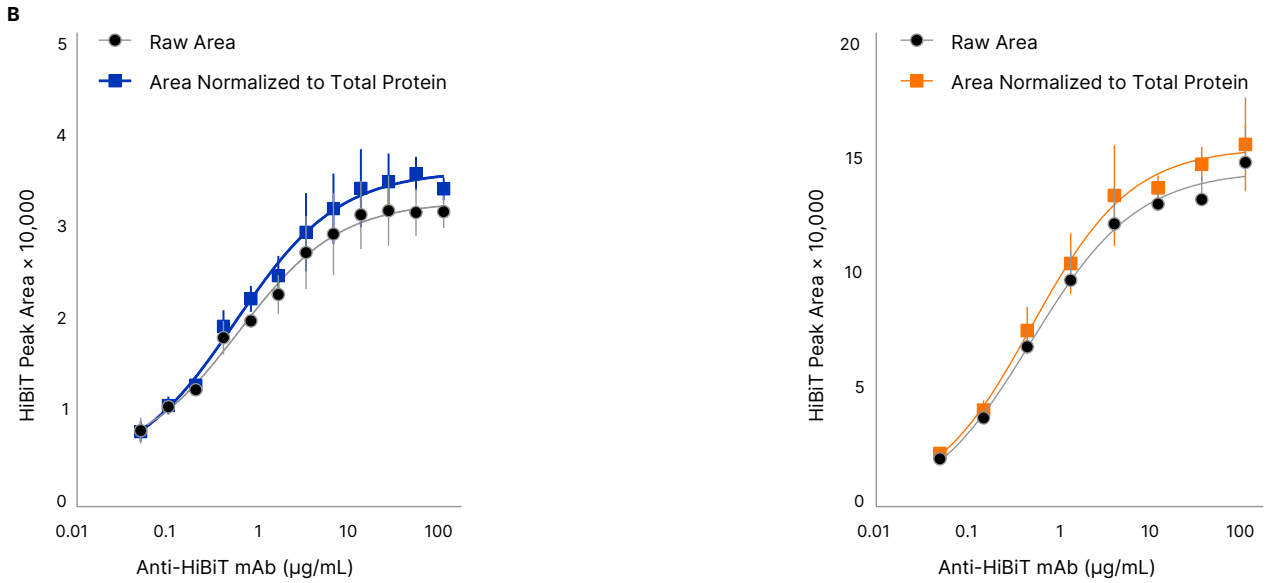

Figure 2. Optimization of HiBiT CP detection using the Anti-HiBiT Monoclonal Antibody on Leo (left) and Jess (right). (A) Lane view of HiBiT detection using the Anti-HiBiT Monoclonal Antibody and Total Protein Detection performed during the same run using the RePlex feature on Leo. (B) Antibody saturation curves of average peak areas resulting from HiBiT detection using the Anti-HiBiT Monoclonal Antibody, including raw values (black trace) and values normalized to total protein, shown in the blue trace and orange traces for Leo and Jess, respectively. Error bars represent standard deviations from the means.

The Simple Western HiBiT assay achieved a dynamic range exceeding four orders of magnitude (0.32 – 5000 ng/mL), with a linear range of approximately 2.5 logs (0.32 – 200 ng/mL) **(Fig. 3)**. This broad quantitative range allows for flexible assay design across varied expression levels.

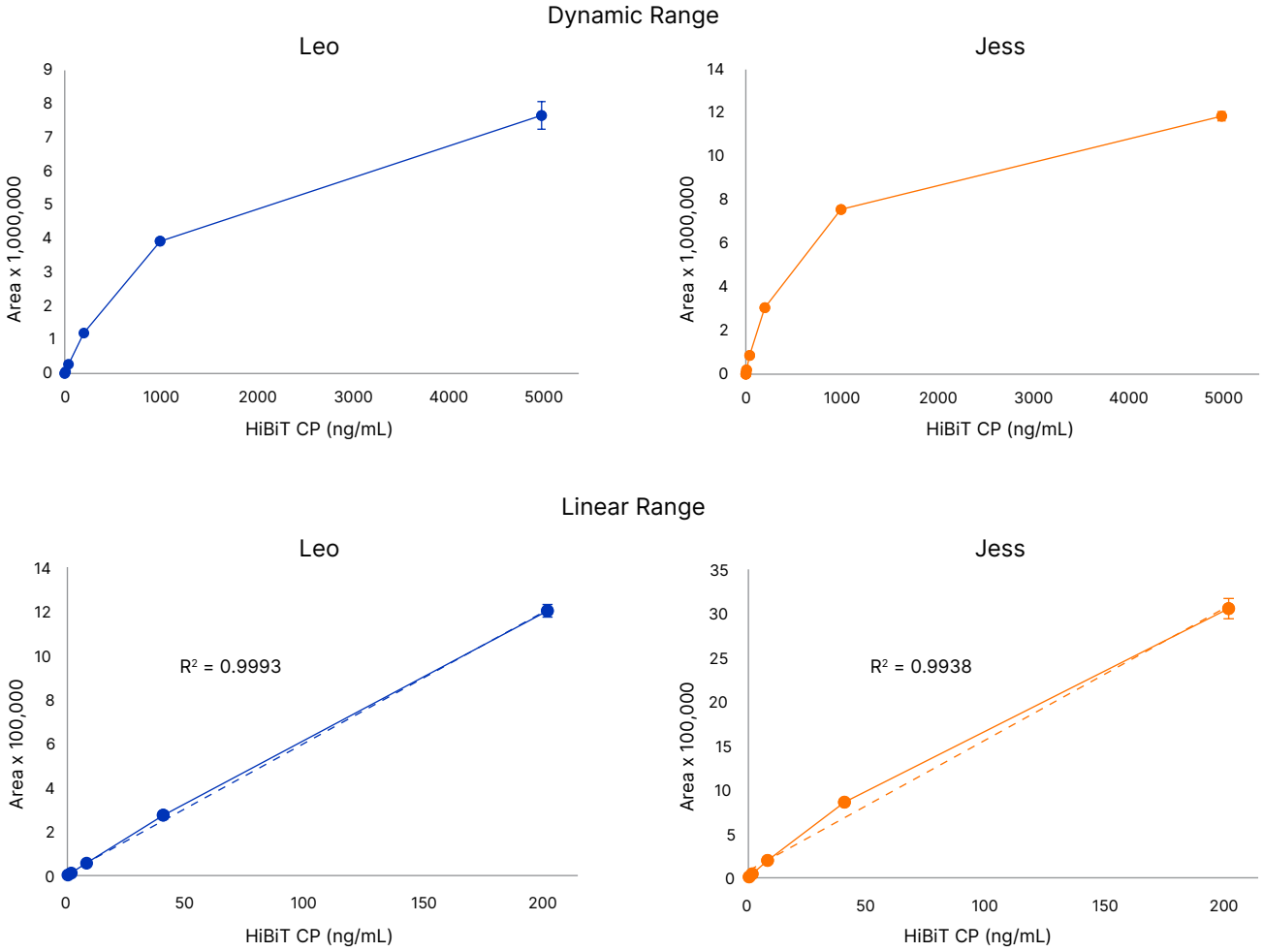

Figure 3. Dynamic and linear ranges of HiBiT detection using the Anti-HiBiT Monoclonal Antibody on Leo and Jess.

Comparisons with traditional Western blot showed that both Leo and Jess were significantly more sensitive, detecting HiBiT CP at concentrations as low as 0.1 ng/mL, compared to 0.5 ng/mL for traditional blotting **(Fig. 4)**. Limit of detection (LOD) values were 0.11 ng/mL for Jess and 0.24 ng/mL for Leo, compared to 0.66 ng/mL for Western blot **(Table 3)**. Similarly, limit of quantification (LOQ) values further underscored the improved sensitivity of Simple Western over conventional methods **(Table 3)**.

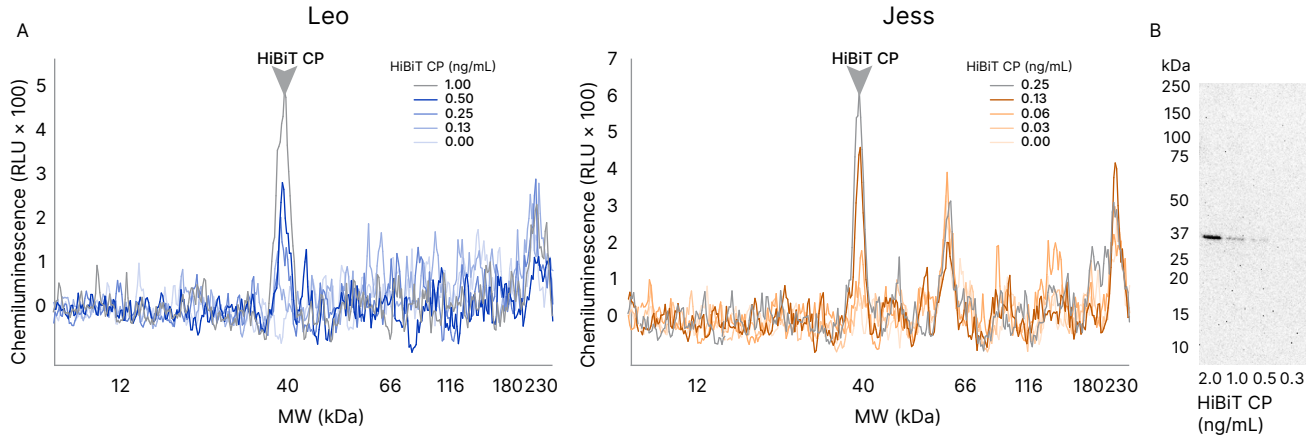

Figure 4. Sensitivity of HiBiT CP detection using the Anti-HiBiT Monoclonal Antibody by Simple Western Leo and Jess and traditional Western blot. (A) Electropherograms of HiBiT CP Detection using Leo and Jess (B) traditional Western blot detection of HiBiT CP. The exposure for both Simple Western and traditional Western blot was 128 seconds.

Parameter	Leo	Jess	Western blot
LOD ng/mL (pg/well)	0.24 (0.72)	0.11 (0.33)	0.66 (1.98)
LOQ ng/mL (pg/well)	0.71 (2.13)	0.34 (1.02)	1.62 (4.86)

Table 3. LOD and LOQ of the HiBiT detection using the Anti-HiBiT Monoclonal Antibody on Leo and Jess and comparison to traditional Western blot.

Standard curve analysis demonstrated strong assay reproducibility, with log-log nonlinear regression ($1/y^2$ weighting) yielding R^2 values greater than 0.99 and recovery rates near 100% **(Fig. 5)**. These data confirm that Simple Western can reliably and quantitatively detect HiBiT-tagged proteins with high precision.

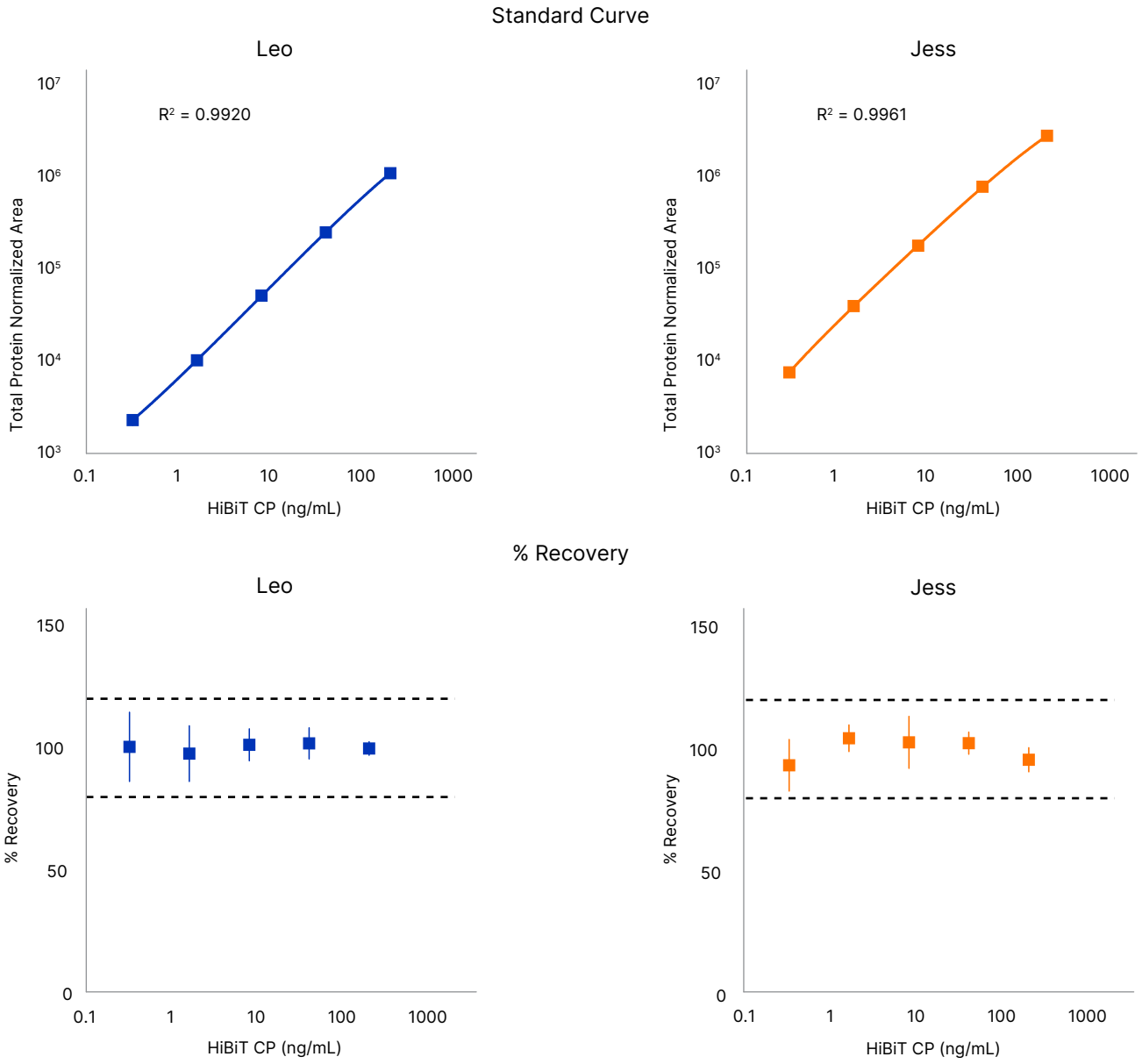

Figure 5. Standard curve and recovery of HiBiT CP detection using the Anti-HiBiT Monoclonal Antibody on Leo and Jess.

Utility of the Simple Western HiBiT Assay in Targeted Protein Degradation

To illustrate the assay's relevance in targeted protein degradation workflows, we applied the method to monitor BRD4 degradation, a transcriptional co-activator implicated in cancer, in response to Compound 8, a next-generation PROTAC® Degrader.[1] A similar dose-dependent decrease in HiBiT-BRD4 signal in a lung carcinoma cell line (A549) was observed with all three antibodies targeting HiBiT, N-terminal BRD4, and C-terminal BRD4 **(Fig. 6)**. Importantly, Simple Western separates full-length from truncated BRD4 isoforms, adding molecular-weight context that complements plate-based luminescent readouts.

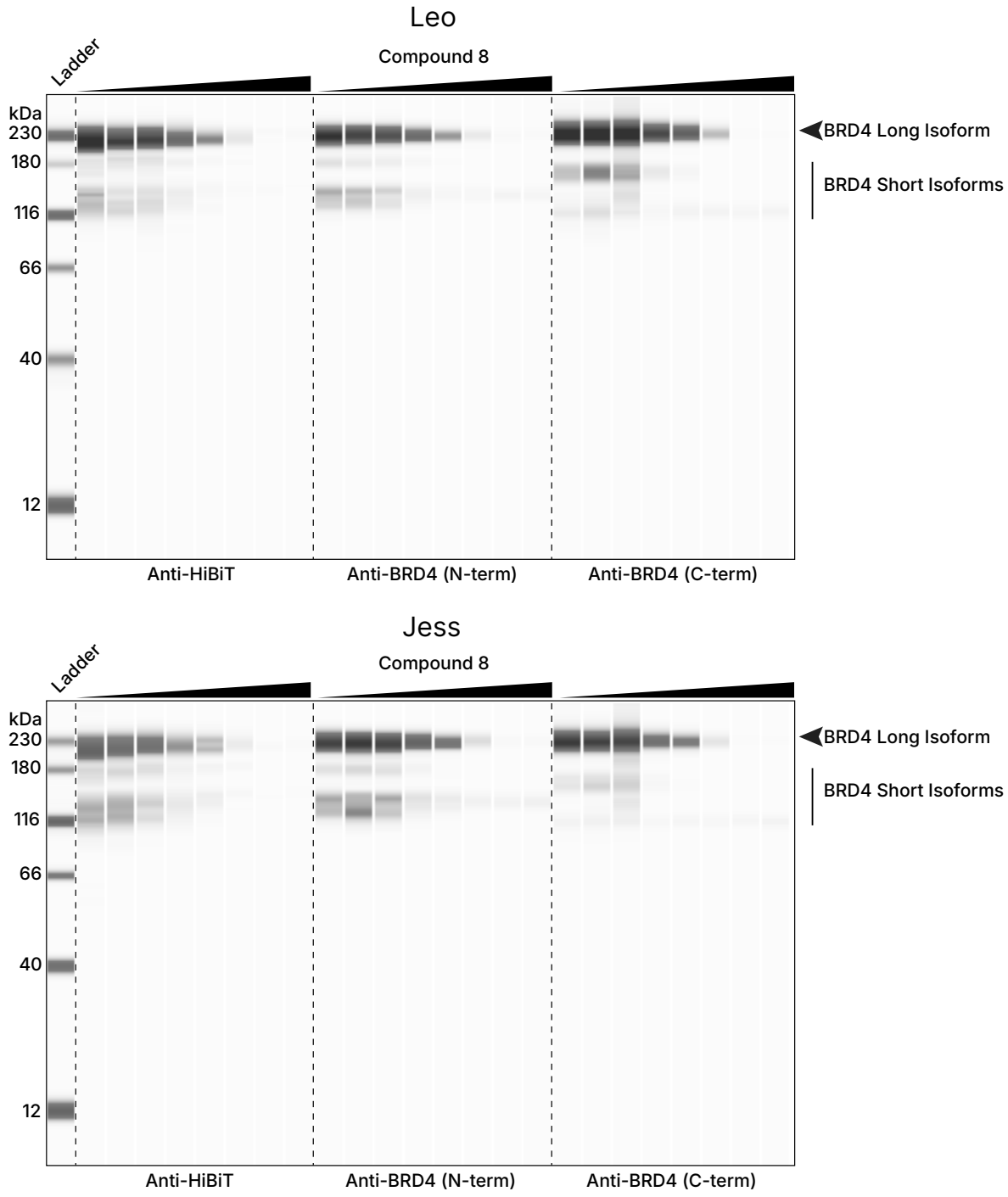

Figure 6. BRD4 degradation by dosing with Compound 8 detected using the Anti-HiBiT Monoclonal Antibody and antibodies targeting N-terminal BRD4 and C-terminal BRD4. Cells were treated with Compound 8, a BRD4-targeting degrader, at concentrations of 0.1, 0.5, 2, 4, 8, 32, and 125 nM (shown left to right).

Dose-response curves derived from long BRD4 isoform signals normalized to the vehicle-only control produced nearly identical trends across detection strategies **(Fig. 7)**. Consistent DC_{50} and D_{max} values were obtained, validating the Anti-HiBiT Monoclonal Antibody detection method for characterizing BRD4 degradation **(Fig. 8)**. Together, these results demonstrate that HiBiT tagging provides results consistent with traditional BRD4 antibody detection, while Simple Western adds the ability to resolve isoform-specific responses, offering deeper insight into degrader-driven protein dynamics.

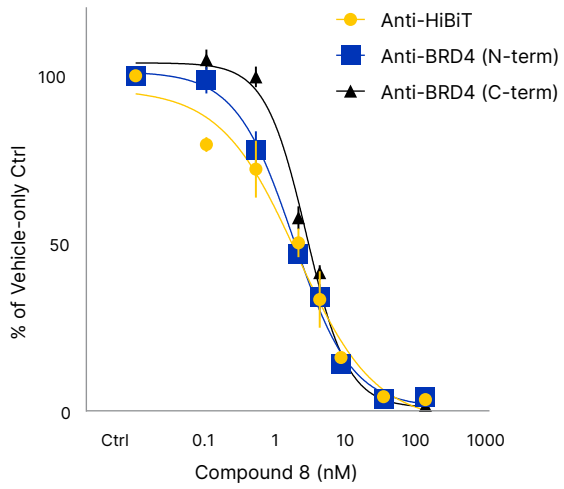

Figure 7. Dose-response analysis of BRD4 degradation by Compound 8 detected using 3 antibodies targeting HiBiT, N-terminal BRD4, and C-terminal BRD4 normalized to the vehicle-only control.

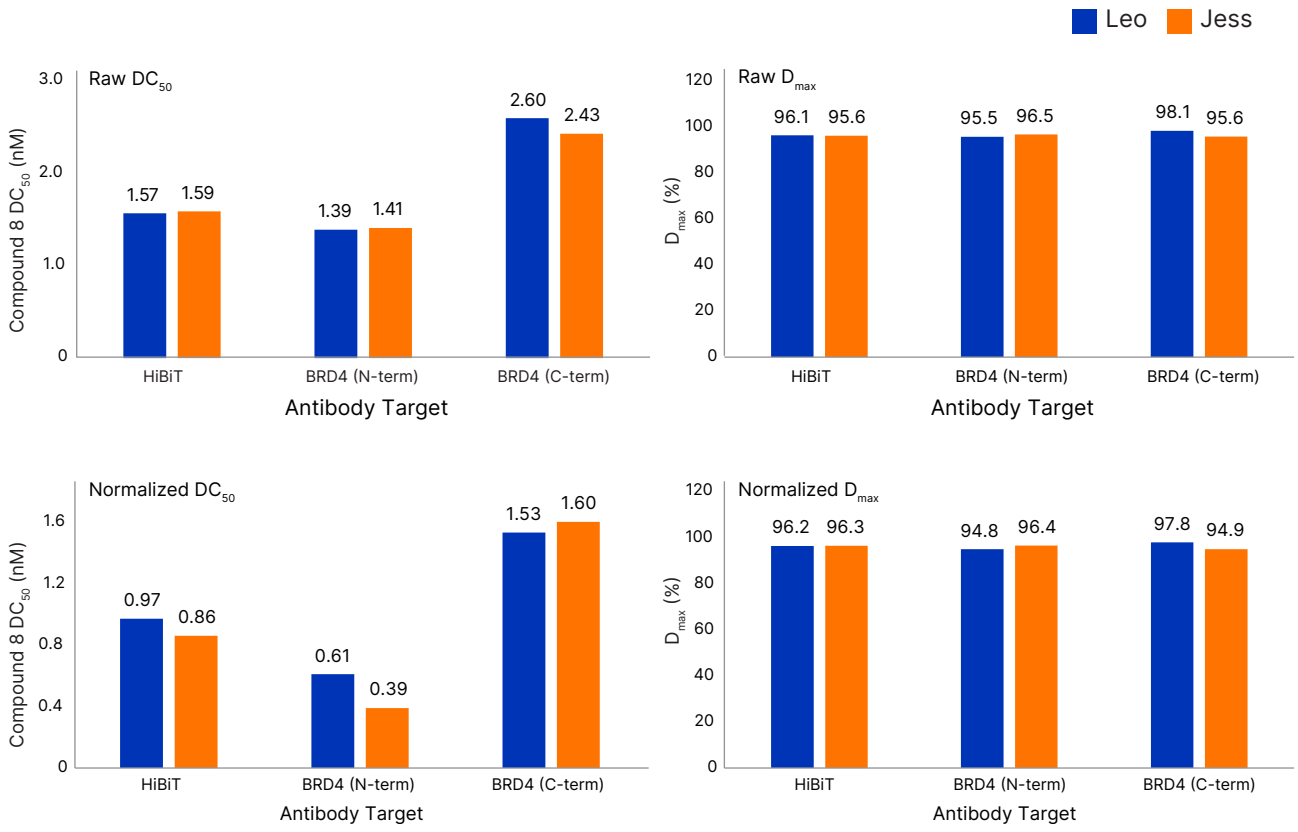

Figure 8. DC_{50} and D_{max} values calculated from BRD4 degradation by Compound 8 detected using 3 antibodies targeting HiBiT, N-terminal BRD4, and C-terminal BRD4 on Leo (blue bars) and Jess (orange bars). Values were normalized to GAPDH.

Conclusion

Simple Western Technology provides a robust, highly specific platform for the quantitative detection of HiBiT-tagged proteins using the Anti-HiBiT Monoclonal Antibody from Promega. Simple Western provides molecular weight resolution to determine molecular weight variants and protein isoforms, complementing plate-based assays. Furthermore, Simple Western provides superior sensitivity, reproducibility, and automation compared to traditional Western blot methods, supporting a wide dynamic range and enables sub-picogram protein quantification. Its utility extends from protein expression and isoform analysis to TPD workflows, where accurate monitoring of degradation kinetics is essential. The Anti-HiBiT Monoclonal Antibody may also be used with Simple Western in co-immunoprecipitation studies for identifying protein-protein interactions.[2]

When paired with the Anti-HiBiT Monoclonal Antibody, Simple Western becomes a valuable orthogonal tool for confident characterization of tagged proteins in both basic and translational research, offering a unified workflow where Simple Western is directly integrated into target screening using Promega's Anti-HiBiT Monoclonal Antibody, yielding higher specificity with up to 96 samples analyzed in just 3 hours.

REFERENCES

1. Actis M, Cresser-Brown J, Caine EA, *et al.* Evaluation of Cereblon-Directing Warheads for the Development of Orally Bioavailable PROTACs. *J Med Chem.* 2025 Feb 13;68(3):3591-3611.

2. Prindle V, Richardson AE, Sher KR, *et al.* Synthetic lethality of mRNA quality control complexes in cancer. *Nature.* 2025 Feb;638(8052):1095-1103.