Advancing Quantitative Analysis of Targeted Protein Degrader Compounds

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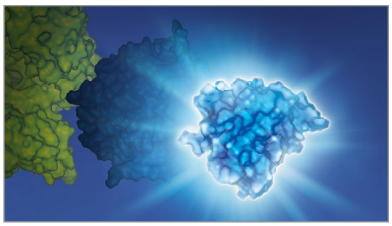

Benefits:

- Sensitive luminescent detection with wide dynamic range
- Endpoint or live-cell kinetic measurements
- No immobilization to plates, beads or other surfaces required
- CRISPR-tagged cell lines available for popular targets

Abstract

Selectively targeting proteins for removal from the cell, instead of inhibiting protein activity, is a newer modality for potential therapy. A range of small-molecule degrader compounds are generating considerable interest in drug development efforts for previously undruggable targets.

Promega products for studying protein degradation include assays to detect ternary complex formation, ubiquitination, compound permeability, E3 ligase target engagement and target protein degradation. These assays are used in many research and drug discovery applications, including profiling of proteolysis-targeting chimera (PROTAC®) degraders, molecular glues and other small molecules or biologics that induce degradation of cellular protein targets. In this white paper, we discuss options and best practices for developing cell-based assays to measure endogenous target protein abundance. We focus on how quantitative, luminescent protein tags can be used, together with CRISPR/Cas9 gene editing, to determine efficacy, rank order and profiles of degrader compound collections. We also address common concerns related to the use of protein tags for measuring target protein levels.

Introduction

Small molecule drug discovery is currently expanding beyond traditional strategies focused on the identification of compounds that inhibit or block the action of disease-causing proteins. Instead, research is focusing on compounds that can target these proteins for degradation and removal from the cell. Immunomodulatory (IMiD) molecular glue compounds and PROTAC® degraders are the best-known examples of these targeted protein degradation (TPD) agents. Both types of degraders function by bringing the target protein into close proximity to the E3 ligase machinery, resulting in target protein ubiquitination and subsequent degradation using the cell's ubiquitin proteasome system (Figure 1). There is currently great interest in these degrader compounds because they are now enabling proteins previously considered "undruggable" to be targeted for therapeutic intervention, expanding our definition of the druggable proteome.

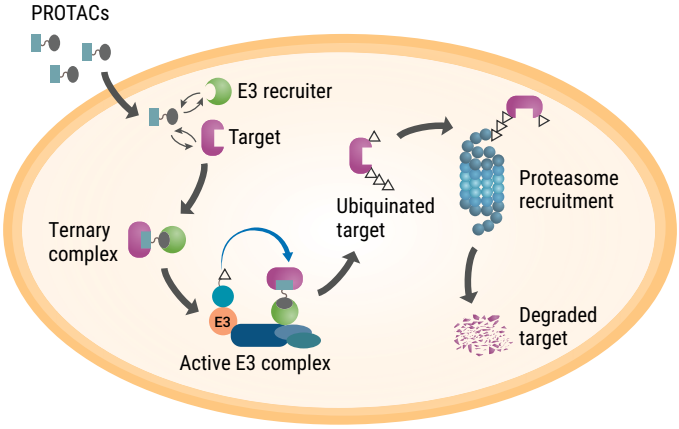

Figure 1. Overview of the cellular targeted protein degradation pathway. PROTAC® compounds facilitate the interaction between the target protein and the cell's E3 ligase complex. This interaction results in target ubiquitination and, ultimately, degradation by the ubiquitin-proteasome system.

Since degrader compounds are designed to achieve loss of target proteins, assays that enable precise protein quantitation are required to identify, characterize and triage small molecule degraders. As with typical small molecule screening approaches, researchers require methods that can be scaled to high-throughput workflows with minimal hands-on time and yield reproducible results. However, there are also unique assay considerations. Because degrader compounds utilize the cell's own machinery as the degradation mechanism, in vitro biochemical approaches are not feasible. In addition, understanding both the protein degradation and recovery rates are required for complete compound characterization.

Strategies for Protein-Level Detection in TPD

The most common approach for quantitating cellular protein abundance is the traditional Western blot that relies on a primary antibody against the target protein of interest. This approach allows for measurement of the endogenous protein within its native environment and requires no cellular engineering. It can be commonly employed in the laboratory setting with generally available research tools. However, the requirement for a high-quality antibody against the target protein can limit this approach. Further, Western blot methods provide only semi-quantitative results, with low-throughput processing and significant hands-on time.

Mass spectrometry methods are also an option, but they are limited in achievable throughput. These methods provide only a snapshot of protein levels at a specific time after compound treatment. They rely on knowledge about when the protein should be measured, or performing repeated sample collection to evaluate multiple timepoints.

To overcome the limitations of primary antibody-based methods, researchers often employ protein tags that can be readily measured. Most commonly, these fall into two categories: 1) fluorescent (e.g., green fluorescent protein [GFP], HaloTag® ligands, monomeric red fluorescent proteins [mRFPs] such as mCherry, or yellow fluorescent protein [YFP]); and 2) luminescent (firefly and *Renilla* luciferase, NanoLuc® luciferase, or HiBiT technology).

Fluorescent tags allow high-content imaging applications, but they are not as sensitive as luminescent options. The luminescent tags, HiBiT and NanoLuc® luciferase, offer the greatest dynamic range of detection, over 7 log orders of magnitude; therefore, they are particularly well suited to measure target protein abundance. In addition, they can easily be monitored using luminometers in plate-based, high-throughput format, yielding highly quantitative results. The assays offer lytic and live-cell detection options, and allow for calculation of key degradation parameters to rank order and triage compounds. NanoLuc® luciferase is a 19kDa protein that generates a bright luminescent signal upon substrate addition. The HiBiT tagging system is a derivative of NanoLuc® technology based on structural complementation. It is composed of the 11 amino acid HiBiT peptide and its high-affinity complementation partner, LgBiT, which can be delivered as part of the detection reagent or expressed in the cell (Figure 2). Upon treatment with a degradation compound, NanoLuc® or HiBiT fusion proteins will be degraded by the proteasome, resulting in a loss of luminescence (Figure 2). These tagging options are compared in Table 1.

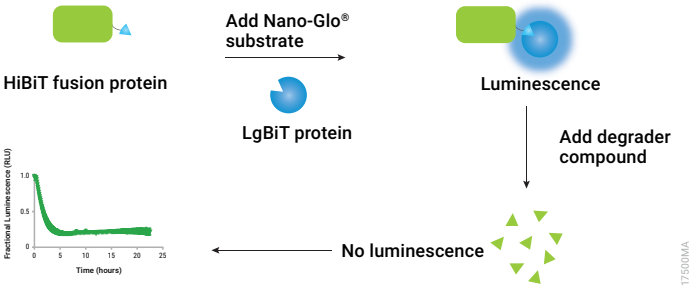

Figure 2. Detecting targeted protein degradation using NanoBiT® complementation. The target HiBiT fusion protein interacts with LgBiT (either provided in solution or expressed in cells) to generate a luminescent signal. In the presence of a degrader compound, the luminescent signal decreases as the target is degraded over time. A similar degradation profile can be obtained using NanoLuc® fusions.

Table 1. Comparison of luminescent tags for protein degradation studies.

Feature	HiBiT Tag	NanoLuc® Tag
Tag Size	11 aa	171 aa (19.1 kDa)
Luminescent Detection	Yes	Yes
Fluorescence Detection	No	No
Kinetic Analysis of Intracellular Proteins	Yes, requires intracellular LgBiT	Yes
CRISPR Donor Template Used for Endogenous Tagging	ssODN	dsDNA
Recommended Homology Arm Length for CRISPR Insertion	50–80bp	300–500bp
Initial CRISPR Insertion Efficiency	5–70%	0.5–10%
CRISPR Cell Lines Available	Yes	Yes

Considerations for Expression of a Tagged Target Protein

While HiBiT and NanoLuc® tags facilitate detection of target protein abundance, the choice of ectopic or endogenous expression is an important consideration that depends on the goal of the project. If transient or stable ectopic expression is done, we recommend keeping expression levels as low as possible. Overexpression can mask or compress the ability to detect weakly active degraders (1).

Although fluorescent or luminescent tagging allows for detection of target protein abundance, endogenous expression of the tagged protein provides the most physiologically relevant results, especially when evaluating degrader compound potency and rank ordering.

We recommend an approach that combines the advantages of luminescent tagging with the ability to measure endogenous protein levels: incorporating the tag at an endogenous locus, using CRISPR/Cas9 gene editing. The HiBiT tag is particularly advantageous for CRISPR/Cas9 insertion, because the small, 11 amino acid tag can be inserted with high efficiency using synthesized single-stranded oligonucleotide donors (ssODNs) (2).

NanoLuc® luciferase can also be used as an endogenous protein tag. However, the larger tag size requires the generation of dsDNA for the donor template. This method results in a lower initial insertion knock-in rate (Table 1), which makes isolating a CRISPR clone more challenging. The success of CRISPR insertions, either with HiBiT or NanoLuc® tags, ultimately depends on both target accessibility and the cell line used. For target accessibility, both N- and C-terminal tagging can be tested to increase the chance of success. When considering a choice of cell lines, we recommend those that can be transfected efficiently by electroporation or Nucleofector™ technology. In addition, it is important to work in a cell line where both your target protein (or any relevant mutations) and the E3 ligase are expressed.

Understanding the Kinetics of Protein Abundance and Calculating Degradation Parameters

For many TPD studies, measuring the level of target protein at a single timepoint can provide sufficient information for compound screening and triaging. However, because target protein levels are dependent on both degradation and resynthesis rates, measurements at only a single timepoint are insufficient to fully understand the dynamic degradation profile. To develop a complete picture, live-

cell kinetic analysis allows for examination of multiple parameters. These parameters include the initial rate of protein degradation, degradation maximum (Dmax), time at Dmax and protein recovery rates for various concentrations of degraders. Plotting Dmax for each concentration yields a degradation potency curve and calculation of a $Dmax_{50}$ value that accounts for any difference in time that each degrader concentration takes to reach its Dmax (Figure 3). The parameters calculated from kinetic analysis can be used to develop better profiles and rank order different PROTAC® compounds, or compare responses from different protein family members to the same PROTAC® compound.

The HiBiT and NanoLuc® tags are particularly well suited to kinetic measurement of target protein levels, because live-cell detection reagents can be added and the luminescence read sequentially over time from the same well. This method provides high-resolution data with only a single reagent addition and minimal hands-on time. When using either of these tags, we have found that endogenous expression of the tagged protein is critical for calculation of accurate degradation parameters, and so tag knock-in at the endogenous locus is preferred. In addition, live-cell detection of HiBiT-tagged proteins requires intracellular expression of the LgBiT complementation partner, and this should be considered as part of the assay design. Intracellular LgBiT expression can be achieved using a cell line that expresses LgBiT constitutively, or LgBiT could be introduced transiently using a vector or viral expression approaches, such as baculovirus (BacMam) reagents (3).

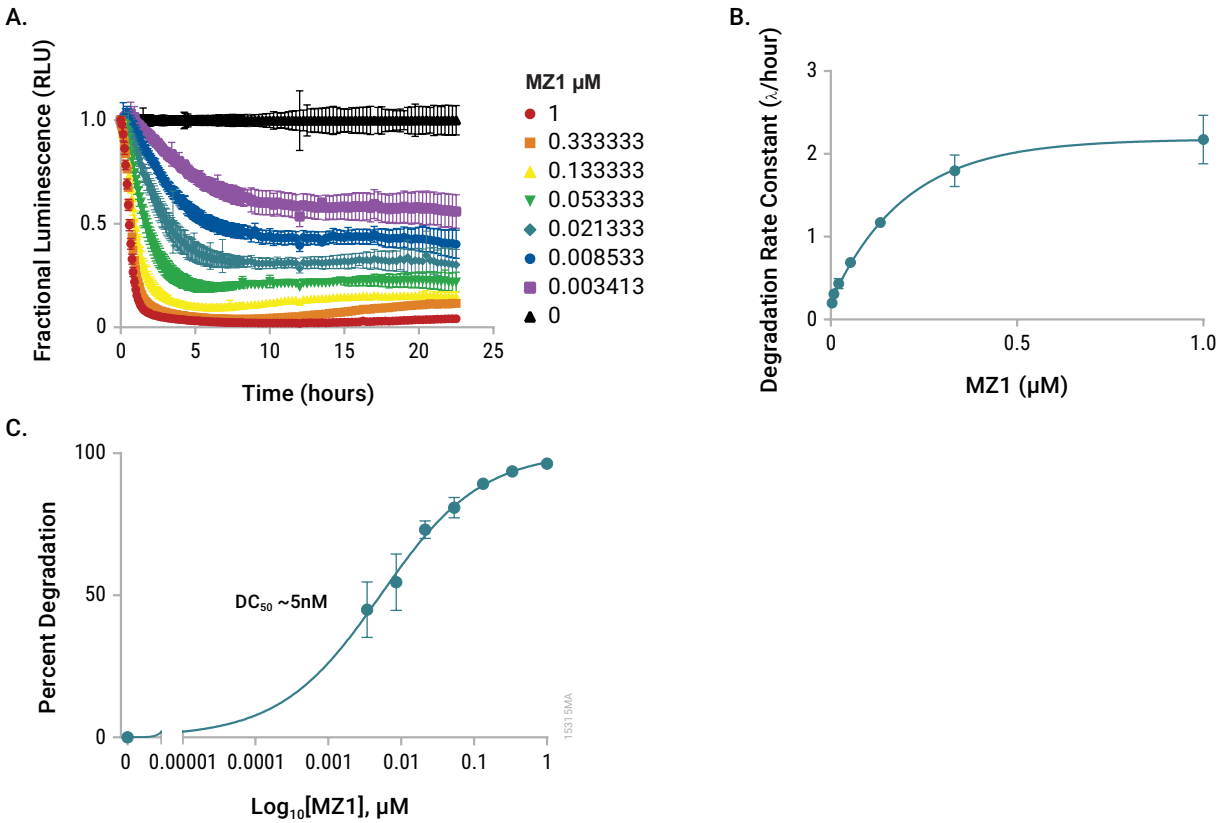

Figure 3. Endogenous HiBiT-BRD4 kinetic degradation assay. HEK293 cells stably expressing LgBiT were engineered using CRISPR-Cas9 to express endogenous HiBiT-BRD4 and plated as described in *HEK293 LgBiT Cell Line and LgBiT Expression Vector*, Technical Manual #TM620. Medium was replaced with CO_2-independent medium containing Nano-Glo® Endurazine™ substrate and incubated for 2.5 hours before adding a titration of MZ1 (3). Kinetic luminescence measurements of degradation at each PROTAC® concentration (Panel A) were collected on a GloMax® Discover, and degradation rate (Panel B) and Dmax (Panel C) were calculated.

Understanding the Influence of the Tag on the Biology Being Measured

Using endogenously tagged target proteins overcomes artefacts associated with overexpression, allows for the study of proteins under native regulatory conditions, and enables the detection benefits that come with protein tags. for studying biology. However, this approach still involves modification of the endogenous protein to include additional amino acids. The addition of tags, small or large, at specific termini can sometimes interfere with complex formation or localization; therefore, it is important to consider the appropriate terminus for endogenous tagging.

For protein degradation, another concern is that the tag may increase or decrease the degradation rate or extent, as compared to untagged endogenous protein. To demonstrate that HiBiT CRISPR insertions show both loss and recovery to similar extents and within the same time frames as the untagged protein, we compared HiBiT-BRD2 and HiBiT-BRD4 CRISPR targets to endogenous BRD2 and BRD4 after treatment with dBET1 degrader (Figure 4). Figure 4A shows Western blots of native, untagged BRD2 and BRD4 protein levels in HEK293 cells following treatment with increasing concentrations of the dBET1 degrader. From these blots, we plotted protein levels and calculated DC_{50} values (Figure 4B). We performed identical dBET1 treatment with HiBiT-BRD2 and HiBiT-BRD4 HEK293 CRISPR cells, plotted luminescence, and determined resulting DC_{50} values (Figure 4C). We observed similar dose-dependent decreases in BRD2 and BRD4 protein levels upon dBet1 treatment, with both methods of measurement yielding similar compound DC_{50} values. For more extensive analysis to determine if the degradation kinetics were similar, we monitored BRD2 and BRD4 protein levels following treatment with 1µM dBET1 across 24 hours using both methods (Figures 4D and 4E). While we could not do continual measurement with Western blot analysis, since this is a lytic method, we observed that the overall dynamic profile of degradation—from initial loss to Dmax and then recovery—was similar (Figures 4D and 4E).

Further, we wanted to understand if the size of the tag influenced degradation rates. Therefore, we compared degradation of CRISPR HiBiT-BRD4 in lytic format, cellular HiBiT-BRD4 complemented with intracellularly expressed LgBiT, and CRISPR NanoLuc®-BRD4 (Figure 5). As HiBiT-BRD4 complemented with LgBiT is effectively similar to NanoLuc®-BRD4, it was important also to show these separate CRISPR cell lines behaved similarly. Figure 5 shows the degradation profiles over 24 hours after treatment of these three possible variations with 1µM MZ1 degrader. These results show that, for this target, degradation rate and extent are not changed by the size of the tag or the choice of HiBiT or NanoLuc® tags.

As PROTAC® or other degraders promote the ubiquitination of lysine residues within the target protein, it is important to determine if the inclusion of tags, which carry their own lysines, will artificially promote or drive degradation themselves. The HiBiT peptide contains two lysines, while HiBiT:LgBiT and NanoLuc® tags contain several more. To study if HiBiT or HiBiT:LgBiT could drive artificial degradation, a CRISPR HiBiT-KRasG12C MIA-Paca2 cell lines was treated with a KRas degrader previously shown to degrade only a GFP-KRasG12C fusion, but not native untagged KRasG12C. The authors of this study postulate degradation of the GFP-KRasG12C fusion was only due to ubiquitination of GFP (4). Our results showed that the HiBiT-KRasG12C treated with this KRas degrader—monitored in either lytic or live-cell formats, complemented with LgBiT—showed no signs of degradation (Figure 6). These results were consistent with native, untagged KRasG12C Western blot and mass spectrometry analysis in this same cell line as used by Zeng et al. (4).

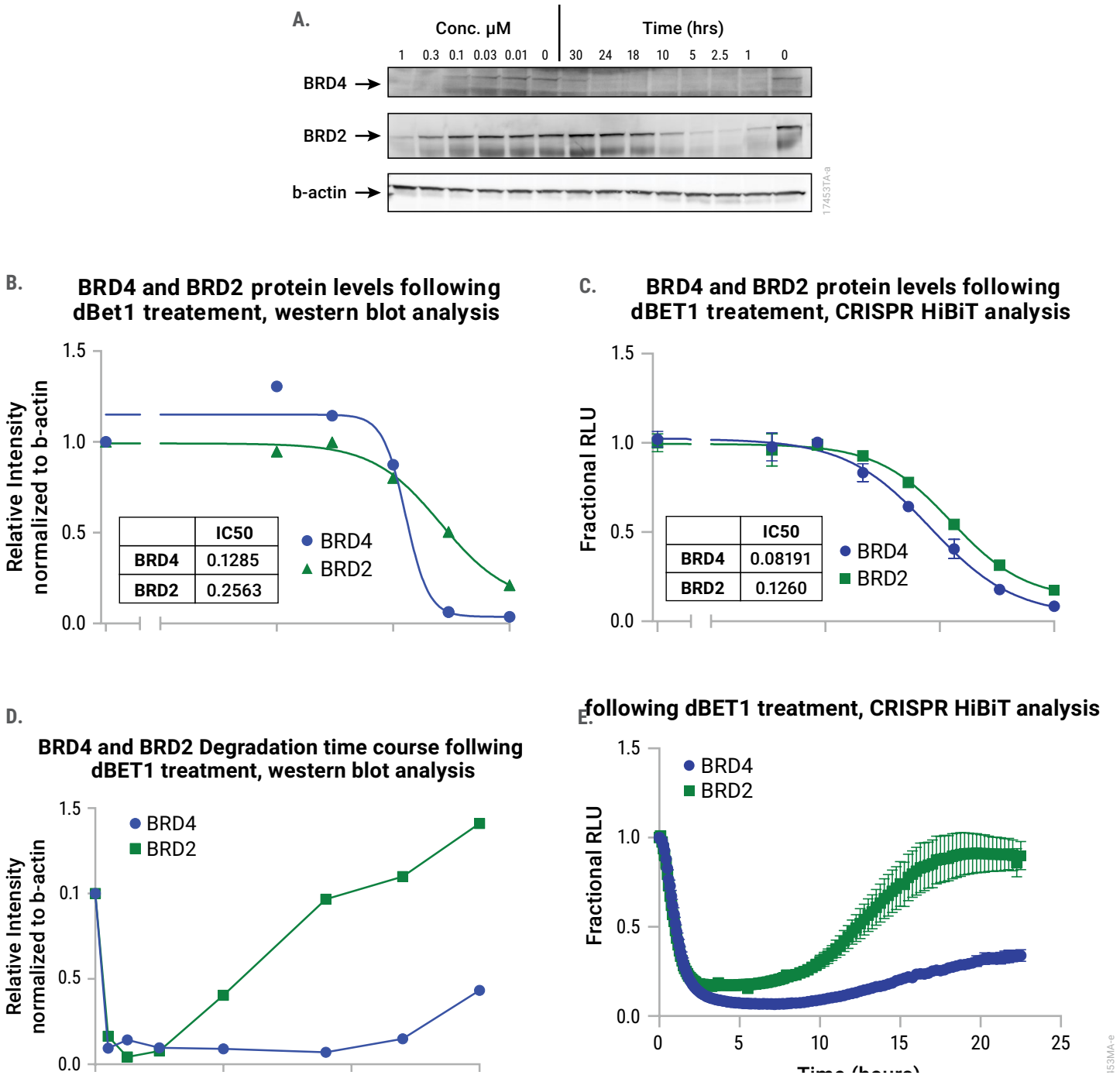

Figure 4. Comparison of HiBiT-BRD2 and HiBiT-BRD4 CRISPR targets to endogenous BRD2 and BRD4 after treatment with dBET1. Panel A: Western blots of native BRD2 and BRD4 protein levels in HEK293 cells after dBET1 treatment at the indicated concentrations and timepoints. β-actin was included for subsequent normalization of protein levels. Panel B: BRD4 and BRD2 protein levels from Western blots, following 4-hour dBET1 treatment. Panel C: HiBiT-BRD2 and HiBiT-BRD4 protein levels after 4-hour dBET1 treatment, measured by luminescence. Panel D: Native BRD2 and BRD4 protein level time course following treatment with 1µM dBET1 across 24 hours, measured by Western blots. Panel E: HiBiT-BRD2 and HiBiT-BRD4 protein level time course following treatment with 1µM dBET1 across 24 hours, measured by luminescence.

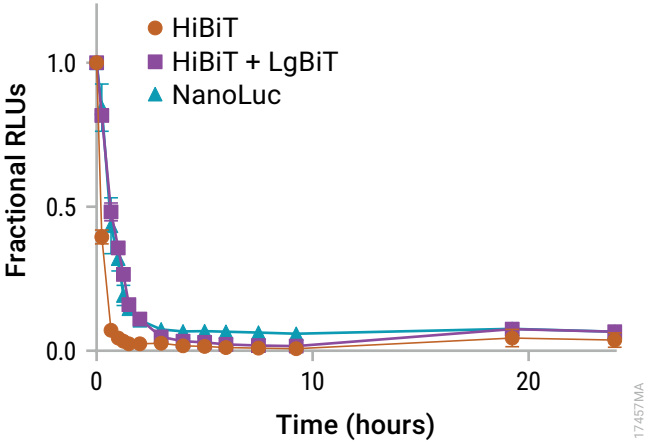

Figure 5. Fusion tag size does not affect BRD4 degradation activity. Degradation profiles were developed for HiBiT-BRD4 in lytic format, cellular HiBiT-BRD4 complemented with intracellularly expressed LgBiT, and NanoLuc®-BRD4.

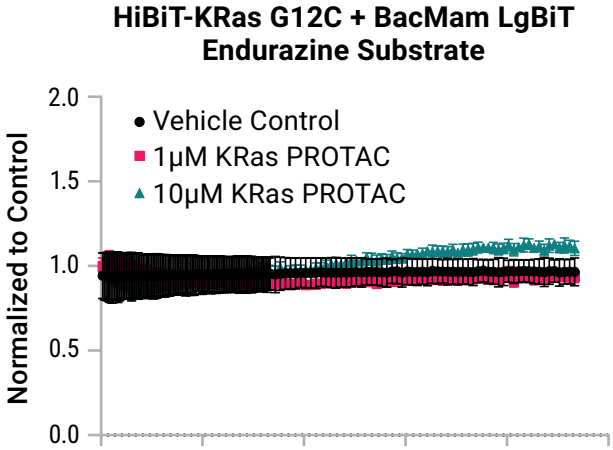

Figure 6. The HiBiT tag itself does not drive degradation activity. A CRISPR HiBiT-KRasG12C cell line was treated with the indicated concentrations of a KRas degrader previously shown to degrade only a GFP-KRasG12C fusion, but not native untagged KRasG12C. No degradation of the KRas fusion protein was observed.

While both HiBiT and HiBiT:LgBiT contain lysines, it is possible that they did not promote artificial degradation because they are less numerous than those in GFP and may be less available for ubiquitination. To further explore any role lysines in the HiBiT tag might have, a mutated HiBiT sequence wherein the two lysines were changed to arginines (KK_RR) was used to generate a CRISPR HiBiT(KK_RR)-BRD4 clonal cell line in HEK293 cells. These results were compared directly to the HiBiT(WT)-BRD4 CRISPR HEK293 clone after treatment with 1μM MZ1 degrader. From this quantitative analysis, Dmax values for HiBiT(WT)-BRD4 could be compared to HiBiT(KK_RR)-BRD4, and no differences were observed (Figure 7). Although we have not studied all possible HiBiT protein fusions, this example demonstrates that the inclusion of lysine residues in the HiBiT sequence does not promote target degradation upon treatment with target-specific PROTAC® degraders.

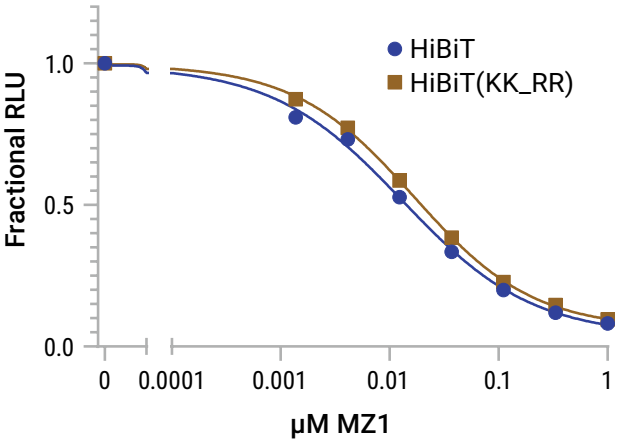

Figure 7. Lysine residues in the HiBiT tag do not promote target degradation by target-specific PROTAC® compounds. A HiBiT(WT)-BRD4 HEK293 clonal cell line was compared to one in which two lysines were changed to arginines (KK_RR). No difference in BRD4 Dmax was observed across the concentration series up to 1μM MZ1.

Interpreting TPD Assays in the Context of Cell Health

When interpreting the results of any cell-based assay to measure loss of signal, care needs to be taken to understand if the signal loss is specific to the event being measured or, more generally, caused by a change in cell viability. This is an important consideration when developing assays to measure target protein loss, because any reduction in cell viability or increased cytotoxicity caused by compound treatment can also cause a global reduction in protein abundance. Although the bioluminescent signal from the tagged protein will be reduced, it will not be specific to target protein loss.

Multiple options exist for multiplexing HiBiT- or NanoLuc®-tagged protein abundance assays with a cell health endpoint that can be incorporated into both endpoint and real-time TPD assays (Figure 8). If performing an endpoint degradation assay, a replicate plate of cells can be used for a bioluminescent cell viability assay, such as the CellTiter-Glo® Assay. Alternatively, a fluorescent cell viability assay, such as the CellTiter-Fluor™ Assay, can be used for same-well multiplexing. If a kinetic degradation assay is used, multiple options are available for same-well multiplexing with a cell health endpoint (5). For example, we multiplexed the CellTox™ Green Cytoxicity Assay with a kinetic degradation assay of endogenously CRISPR HiBiT-tagged Ikaros in Jurkat cells (Figure 9). This method provided same-well monitoring of both protein levels and cellular toxicity, following treatment with a concentration series of the molecular glue compound, iberdomide. The results confirmed that target protein loss observed with compound treatment was not due to increased cytoxicity. Alternatively, either the CellTiter-Fluor™ or CellTiter-Glo® assays could be multiplexed with the degradation assay, following kinetic analysis. Detailed protocols have been described for multiplexing endpoint and kinetic HiBiT TPD assays with various cell health endpoints (6).

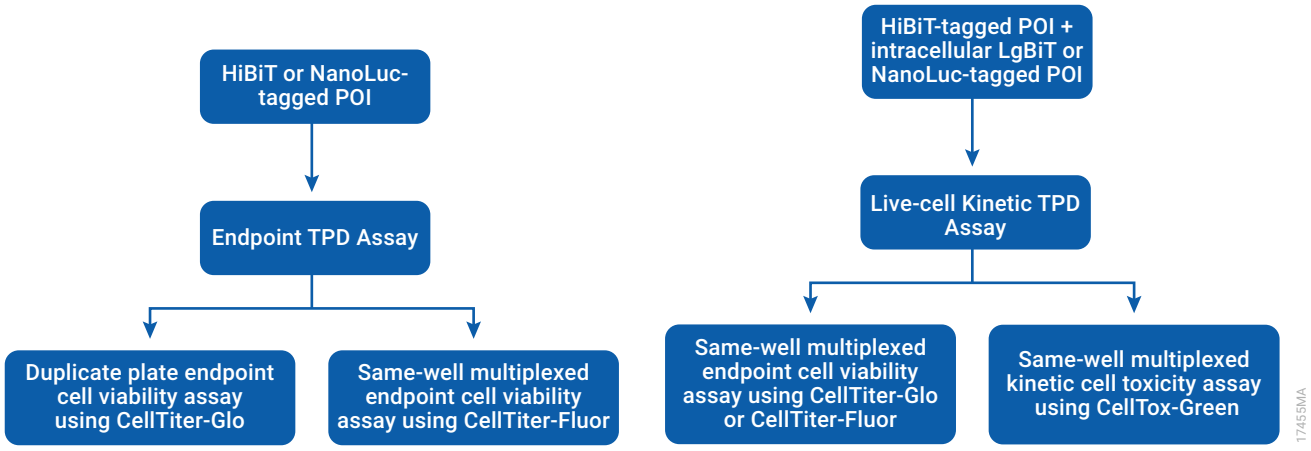

Figure 8. Options for multiplexing degradation assays with indicators of cell health.

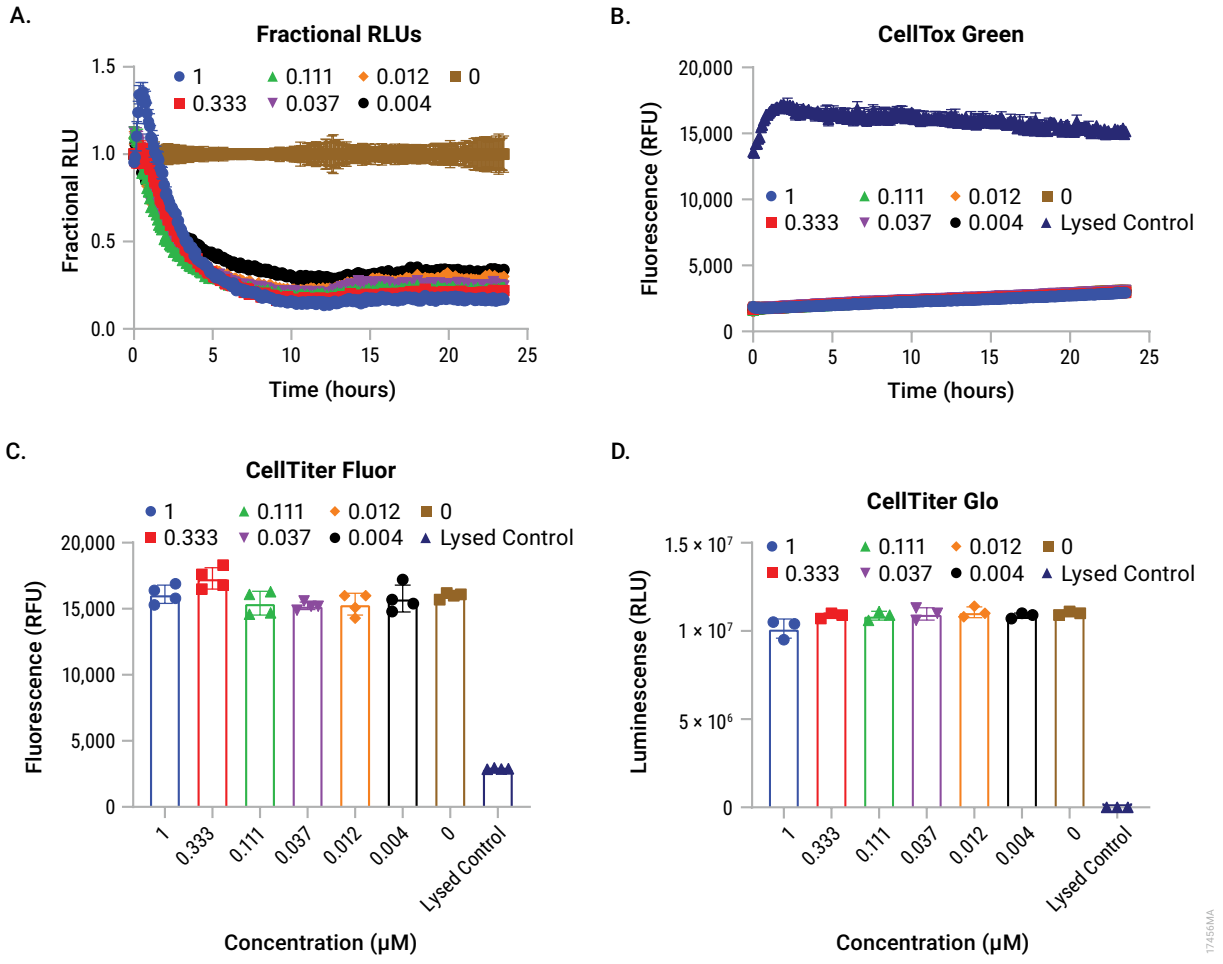

Figure 9. Multiplexing an iberdomide-mediated Ikaros degradation assay with cell health assays. Panel A: Kinetic degradation assay of HiBiT-tagged Ikaros after treatment with the molecular glue compound, iberdomide. Panel B: Degradation assy multiplexed with the CellTox™-Green cytotoxicity assay. Panel C: Degradation assay multiplexed with the CellTiter-Fluor™ assay after a 24-hour kinetic read. Panel D: Degradation assay multiplexed with the CellTiter-Glo® assay after a 24-hour kinetic read.

Conclusions

HiBiT and NanoLuc® luciferase fusion tags offer a sensitive and convenient method to quantitatively assess target protein degradation in the presence of small-molecule degradation compounds. These assays can be performed either in endpoint or live-cell kinetic formats. We have shown that the choice of tag does not substantially affect degradation parameters. The HiBiT tag is particularly advantageous for CRISPR/Cas9 insertion due to its small size, which allows analysis of endogenous proteins without the requirement for target-specific antibodies. TPD assays using these tags are easily integrated into a high-throughput workflow and can be multiplexed with various cell health assays to obtain a comprehensive picture of protein degradation dynamics in an endogenous context.

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More Information

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