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TITLE:

High-Throughput Cellular Profiling of Targeted Protein Degradation Compounds Using HiBiT CRISPR Cell Lines

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PROTACs, targeted protein degradation, HiBiT, CRISPR, kinetics, live cell

SUMMARY:

This protocol describes the quantitative luminescent detection of protein degradation kinetics in living cells that have been engineered using CRISPR/Cas9 to express antibody free endogenous protein detection tag fused to a target protein. Detailed instructions for calculating and obtaining quantitative degradation parameters, rate, Dmax, DC_{50}, and $Dmax_{50}$ are included.

ABSTRACT

Targeted protein degradation compounds, including molecular glues or proteolysis targeting chimeras, are an exciting new therapeutic modality in small molecule drug discovery. This class of compounds induces protein degradation by bringing into proximity the target protein and the E3 ligase machinery proteins required to ubiquitinate and ultimately degrade the target protein through the ubiquitin-proteasomal pathway (UPP). Profiling of target protein degradation in a high-throughput fashion, however, remains highly challenging given the complexity of cellular pathways required to achieve degradation. Here we present a protocol and screening strategy based on the use of CRISPR/Cas9 endogenous tagging of target proteins with the 11 amino acid HiBiT tag which complements with high affinity to the LgBiT protein, to produce a luminescent protein. These CRISPR targeted cell lines with endogenous tags can be used to measure compound induced degradation in either real-time, kinetic live cell or endpoint lytic modes by monitoring luminescent signal using a luminescent plate-based reader. Here we outline the recommended screening protocols for the different formats and, also describe the calculation of key degradation parameters of rate, Dmax, DC_{50}, $Dmax_{50}$, as well as multiplexing with cell viability assays. These approaches enable rapid discovery and triaging of early stage compounds while maintaining endogenous expression and regulation of target proteins in relevant cellular backgrounds, allowing for efficient optimization of lead therapeutic compounds.

INTRODUCTION:

Targeted protein degradation has emerged as one of the fastest growing areas in small molecule drug discovery, bolstered greatly by the therapeutic success of immunomodulatory molecular glue compounds (e.g., IMiD) for cancer treatment, and promising early clinical trial data of Proteolysis Targeting Chimera compounds[1-12]. Targeted protein degradation compounds function by bringing into proximity a target protein with E3 ligase machinery proteins[1-12]. This compound-induced recruitment of the target protein to the E3 ligase leads to the target protein ubiquitination and degradation via the ubiquitin proteasomal pathway (UPP)[1-12]. Historically, small molecule drug discovery screening programs have relied upon initial biochemical assays to assess activity and rank order compounds. This, however, has presented a significant challenge for targeted protein degraders whose ultimate activity, degradation via the proteasome, is dependent upon a cascade of cellular events[1,2,4-6,11-18]. The multiple pathways and complexity of protein complexes required for the successful target degradation necessitate cellular assay approaches for early screening and triaging of initial compounds. Currently, the availability of technologies to monitor target protein degradation in a high-throughput fashion in the context of the cellular environment is severely lacking[14]. Here we will present protocols for real-time kinetic live cell or endpoint lytic degradation activity assessment using CRISPR/Cas9 endogenously tagged HiBiT target cell lines[18-20] to monitor the loss of the target protein via luminescent measurement after treatment with degrader compounds[10,11,18,19].

To achieve successful degradation of therapeutic targets and to expand the druggable proteome, numerous approaches and types of degraders have emerged which can target a broad range of proteins for destruction, including those localized at or in the plasma membrane, lysosomes, mitochondrial membranes, cytoplasm, and the nucleus[21-57]. The two primary classes of compounds most extensively studied are molecular glues and protein targeting cimeras[2,4-7,12,26]. Molecular glues are monovalent, thus typically smaller in size, and facilitate a novel protein:protein interaction interface with a target protein upon binding to an E3 ligase component[2,12,26]. They are most commonly degraders that bind to the Cereblon (CRBN) E3 ligase component[2,12,26,55-57]. Recently though exciting new examples utilizing other E3 ligase machinery such as DCAF15[58-60] and CDK/Cyclin recruitment to DDB1[45] show the potential for expansion of this class of compounds. In contrast, PROTACs are larger, bivalent molecules, consisting of a target binding ligand, most often an inhibitor, bridged via a chemical linker to a E3 ligase handle[1,3-5,7,13]. As such, these compounds are capable of direct binding to both the E3 ligase and the target protein[1,3-5,7,13]. Numerous proteins have been shown to be degraded via these bivalent molecules, and the most used E3 ligase handles recruit either CRBN or Von Hippel Lindau (VHL)[1,3-5,7,13]. However, the number of available handles for E3 ligase recruitment in chimeras targeting proteolysis design is rapidly growing, expanding the capabilities of this class of compounds with the potential to degrade diverse target classes as well as enhance cell- or tissue-type specificity[24,48,61,62]. Combined with the minimal requirement to engage a target protein, even with marginal affinity, degradation compounds hold promise for expanding the druggable proteome.

Characterizing the cellular dynamics of protein loss, as well as potential protein recovery post-

treatment, is critical for understanding degradation compound function and efficacy. While it is possible to study endogenous protein level changes in relevant cellular systems with western blot antibody assays or mass spectrometry, these approaches are difficult to adapt to high-throughput screening formats, have limited quantification capability, or ability to measure kinetic changes at many timepoints[14]. To address these challenges, we have developed a plate-based cellular luminescent system for monitoring changes in endogenous protein levels, which utilizes genomic insertion via CRISPR/Cas9 of the 11 amino acid tag, HiBiT, to the loci of any key degradation targets[18-20]. This peptide complements with high affinity to its binding partner, LgBiT, to produce bright luminescence in the presence of its substrate[18-20,63], thereby making these tagged endogenous proteins luminescent in cells or lysates [18-20,63]. The relative light units (RLUs) measured with a luminometer instrument are directly proportional to the tagged target protein levels[18-20,63]. With the development of stabilized luciferase substrates, real-time kinetic protein level measurements over 24-48 h time frames are possible[18,53,64]. This allows for the determination of a complete degradation profile for any given target at any given compound concentration, including quantitative analysis of initial degradation rate, degradation maximum (Dmax), and recovery after compound treatment[18,53]. If screening large libraries of degradation compounds, however, endpoint analysis can also readily be performed in 384-well format at various drug concentrations and designated times.

The protocols presented in this manuscript represent cellular screening strategies for targeted protein degradation compounds, applicable for all types of degraders. The use of HiBiT CRISPR cell lines along with these protocols, however, are not limited to protein degradation, rather they are general tools for monitoring any endogenous target protein level which could be modulated post-treatment to study impact of compounds or even resistance mechanisms[20,65,66]. A pre-requisite for these luminescent-based detection methods is a CRISPR endogenously tagged HiBiT target cell line, which is critical as it enables sensitive luminescent detection, while still maintaining endogenous target expression and native promoter regulation[18-20]. Significant advances have been made in utilizing CRISRP/Cas9 for insertion of genomic tags, particularly in scalability[20] and with the high sensitivity of detection, in various formats including CRISPR pools or clones with either heterozygous or homozygous allelic insertions[18-20]. Use of exogenous expression of HiBiT or other reporter fusions in cells in lieu of endogenous tagging is possible, but significant caution should be taken using systems with protein overexpression[14,18]. These can lead to artefacts in understanding true compound potency and protein recovery dynamics[14,18], including potential transcriptional feedback loops activated post target degradation. In addition, early stage compounds with low potency could be missed, and present themselves as false negatives in screening. As protein loss could result from compound-induced toxicity and cell death, the protocols described here contain highly recommended, but optional cell viability luminescent or fluorescent assays paired with the degradation protocol. There are two major sections to the protocol, lytic endpoint, and live cell kinetic screening. Within each of those sections, options are included for multiplexed cell viability measurements in endpoint or kinetic formats. Monitoring the changes of the tagged endogenous protein requires complementation with LgBiT in cells. Therefore, the kinetic screening section references important protocols for the introduction of this, which can be achieved via transient or stable expression and is essential for performing the live cell luminescent measurements. All approaches presented here allow

rapid rank ordering and activity assessment of compounds, enabling early stage compound screening efforts and more rapid identification of lead degraders.

This protocol is designed for the study of degradation compounds in conjunction with a HiBiT CRISPR cell line. Protocols for generation of HiBiT CRISPR insertions for numerous targets have been outlined in several recent publications[18-20].

PROTOCOL:

1. Endpoint degradation studies with HiBiT CRISPR target proteins in lytic format with optional cell viability fluorescence analysis

1.1. Preparation and plating of mammalian adherent or suspension cell line

1.1.1. Adjust the cell density to 2.22 x 10^5/mL by dilution in appropriate cell media used for passaging and cell growth.

1.1.2. Dispense cells into plates with a minimum of 3 wells per experimental and control condition. Dispense 90 µL (20,000 cells) per well of cell suspension into 96-well white plates. For 384-well format, dispense 36 µL (8,000 cells) per well of cell suspension into 384-well white plates.

1.2. Preparation and addition of compounds

1.2.1. Prepare serially diluted PROTAC or degrader test compound plates at 1,000x final concentration in 100% DMSO. Then dilute it to 10x final concentration in the cell culture medium. Add an equal volume of DMSO to the medium, to be used as a no compound DMSO control.

1.2.2. For 96-well format add 10 µL of 10x compound and control solutions to 90 µL of cells. For 384-well format add 4 µL of 10x compound and control solutions to 36 µL of cells.

1.2.3. Incubate the plates in an incubator at 37 °C and 5% CO_2 for the desired amount of time or in conditions that are optimal for their growth.

NOTE: As this is an endpoint assay, testing of multiple time points will require preparation of separate degradation plates for each time point, as described in above step 1.1.2. Incubation times to detect compound-mediated degradation are highly variable and are also likely dependent on compound concentration. Suggested initial time points would be 6 h and 24 h.

1.2.4. If measuring endpoint luminescent detection without the optional CTF, proceed directly to step 1.3 below. If performing multiplexing with CTF, proceed to the next section 1.4 below.

1.3. Lytic measurement of cells

1.3.1. Immediately prior to HiBiT lytic measurements, prepare 2x lytic detection reagent by adding 20 µL of lytic substrate and 10 µL of LgBiT protein per every 1 mL of the lytic buffer. Prepare enough 2x detection reagent for number of wells to be assayed, including extra volume to account for pipetting error (i.e., number of wells + 10%).

1.3.2. Add prepared lytic detection reagent to cells. For 96-well format, add 100 µL of 2x lytic detection reagent to each well containing 100 µL of cells. For 384-well format, add 40 µL of 2x lytic detection reagent to each well containing 40 µL of cells. Mix the plate on a microplate vortex mixer for 10-20 min at 350 rpm.

1.3.3. Measure luminescence on a luminometer capable of reading luminescence in a 96- or 384-well plate.

1.4. Optional cell viability multiplexing

NOTE: This step is performed using a commercially available CTF kit (see **Table of Materials**).

1.4.1. 30-40 min prior to the desired endpoint measurement, prepare a 6x cell viability detection reagent solution by adding 10 µL of the substrate to 2 mL of the assay Buffer. Prepare enough 6x reagent for each well to be assayed, including extra volume for pipetting error (i.e., number of wells + 10%).

1.4.2. Add the prepared reagent to wells. For 96-well format add 20 µL of 6x reagent to each well already containing a 100 µL volume. For 384-well format add 8 µL of 6x reagent to each well containing 40 µL of cells. Mix briefly on a microplate vortex mixer, then incubate the plate for 30 min in a 37 °C incubator.

1.4.3. At the desired endpoint of measurement (i.e., 6 or 24 h post treatment, step 1.2.3), measure fluorescence on an instrument capable of reading fluorescence (380-400nm_{Ex}/505nm_{Em}) in 96- or 384- well format.

1.4.4. Prepare 2x lytic detection reagent by adding 20 µL of lytic substrate and 10 µL of LgBiT protein per 1 mL of lytic buffer. Prepare enough 2x detection reagent for number of wells to be assayed, including extra volume to account for pipetting error (e.g., number of wells + 10%).

1.4.5. Add the prepared lytic detection reagent to wells. For 96-well format add 120 µL of 2x lytic detection reagent to each well already containing a 120 µL volume. For 384-well format add 48 µL of 2x lytic detection reagent to each well already containing a 48 µL volume. Mix the plate on a microplate vortex mixer for 10-20 min.

1.4.6. Measure luminescence on a luminometer capable of reading luminescence in 96- or 384-well plates.

1.5. Quantification of degradation and cell viability

1.5.1. Average the relative light units (RLU) from the DMSO control at the measured time point. Use this value as the baseline protein level of the target to calculate fractional degradation by normalizing all other treatments tested at this same time point to this value. For example, if the average RLU for the DMSO control wells at 6 h was 10,000, and the RLU for a given compound treatment at 6 h was 5,000, the fractional degradation would be calculated as 5,000 ÷ 10,000 = 0.5 (Equation 1).

Equation 1:

$$\frac{RLU_{PROTAC}}{RLU_{DMSO}} = Fractional\ RLU$$

1.5.2. Determine the percentage degradation from Fractional RLU:

Equation 2:

$$(1 - Fractional\ RLU) * 100\ =\ \%\ Degradation$$

1.5.3. Plot fractional RLU or % degradation at specific time points to rank the activity of compounds.

1.5.4. Optionally, analyze RFU for the cell viability assay measurement by comparing the values from all treatments to the DMSO control. If a significant decline in RFU is observed for any treatment relative to the DMSO control, the degradation data can be additionally normalized to the cell viability assay data to determine changes in protein level relative to losses in cell viability.

2. Real-time kinetic degradation of HiBiT CRISPR target proteins and optional cell viability luminescence assay

NOTE: The ability to perform kinetic screening and degradation requires LgBiT protein co-expression in the cell, which has been described previously[18,19,63]. This can be achieved via transient transfection of a LgBiT vector, use of BacMam LgBiT, or by performing HiBiT CRISPR insertion into a LgBiT stable cell line.

2.1. Plating of adherent cell lines.

2.1.1. Remove medium from cell flask by aspiration, wash cells with DPBS, dissociate cells with 0.05% trypsin-EDTA, and allow cells to dissociate from the flask bottom. For suspension cell lines, proceed to section 2.1.2.

2.1.2. Neutralize trypsin using serum-containing cell culture medium, mix to collect and resuspend cells, and transfer cell suspension to a conical tube.

2.1.3. Spin down cells at 125 *x g* for 5 min. Discard the cell culture medium and resuspend in an equal volume of fresh cell culture medium.

2.1.4. Plate cells into assay plates with a minimum of triplicate wells per experimental and control condition. For 96-well format count to estimate cell density, adjust density to 2 x 10^5 cells/mL in assay medium and dispense 100 µL (20,000 cells) per well in a 96-well plate. For 384-well format count to estimate cell density, adjust density to 4.44 x 10^5 cells/mL in the assay medium and dispense 18 µL (8,000 cells) per well.

2.1.5. Incubate plates at 37 °C, 5% CO_2 overnight or in conditions which are optimal for their growth.

2.2. Plating of suspension cells

2.2.1. Adjust cell density to 2.22 x 10^5 cells/mL in CO_2-independent medium supplemented with 10% FBS and 1x Endurazine (1:100 dilution of the stock reagent).

2.2.2. Plate cells into assay plates with a minimum of 3 wells per experimental and control condition. For 96-well format dispense 90 µL (20,000 cells) per well. For 384-well format dispense 36 µL (8,000 cells) per well.

NOTE**:** For suspension cell lines which have low signal to background (S:B) luminescence, e.g., when working with CRISPR pools rather than clones, it is possible to increase the luminescence by increasing the number of cells plated, up to 100,000 cells/well in 96-well format, or 40,000 cells/well in 384-well format.

2.3. Kinetic degradation assays using HiBiT CRISPR cells expressing LgBiT

2.3.1. For suspension cells already containing Endurazine, which was included at the plating step in 2.2., proceed directly to step 2.3.3. For adherent cell lines prepare Nano-Glo Endurazine solution. For 96-well format, prepare a 1x solution of Endurazine by diluting stock reagent 1:100 into CO_2-independent medium supplemented with 10% FBS. For 384-well format, prepare a 2x solution of Endurazine by diluting stock reagent 1:50 into CO_2-independent medium supplemented with 10% FBS.

2.3.2. Add Endurazine solution to each well of adherent cells. For 96-well format aspirate medium and add 90 µL of 1x Endurazine solution. For 384-well format, add 18 µL of 2x Endurazine solution to 18 µL of cells. Do not aspirate medium as the degradation assay is performed in a 50:50 mixture of culture medium and CO_2 independent medium in 384-well format.

2.3.3. Incubate suspension or adherent cell plates containing Endurazine for 2.5 h in an incubator at 37 °C and 5% CO_2 to allow luminescence to equilibrate.

2.3.4. Prepare a 10x concentration of test PROTAC titration in CO_2-independent medium and add 10 µL to each well of 96-well plate or 4 µL for 384-well plate. For compounds with unknown efficacy, a final concentration of 1-10 µM at the highest point is recommended as a starting point.

2.3.5. Collect kinetic measurements of luminescence in luminometer pre-equilibrated to 37 °C for a period between 0-48 h. The time increments of measurement can be customized for each experiment, but a recommended initial experiment would be luminescence measurements every 5-15 min for 24 h or the desired amount of time.

2.4. Optional cell viability same-well multiplex analysis after final kinetic measurement

NOTE: This assay is performed with a commercially available CTG kit (see **Table of Materials**).

2.4.1. Equilibrate CTG reagent to room temperature.

2.4.2. Following degradation measurement at the last time point of the kinetic analysis, add 100 µL (96-well plate) or 40 µL (384-well plate) of the reagent per well of the plate, and mix on a plate shaker at 500-700 rpm (96-well plate) or an microplate vortex mixer (384-well plate) for 5 min.

2.4.3. Incubate the plate at room temperature for 30 min to allow for cell lysis and quenching of HiBiT signal.

2.4.4. Measure total luminescence on a luminometer by following manufacturer's recommendation.

2.5. Quantification of kinetic degradation profiles

2.5.1. Using the kinetic luminescence measurements collected, normalize the raw RLUs for each PROTAC concentration to the replicate averaged DMSO condition at every time point to account for changes in free furimazine concentration over time. Calculate Fractional RLU using Equation 1.

Equation. 1

$$\frac{RLU_{PROTAC}}{RLU_{DMSO}} = Fractional\ RLU$$

2.5.2. From the degradation curves, fit a single-component exponential decay model using Equation 2 to the initial degradation portion of each curve to the point where the data reaches a plateau.

NOTE: It may be helpful to exclude from the fit the first few data points as there may be a brief lag before degradation is observed.

Equation 2.

$$y = (y_0 - Plateau)e^{-\lambda t} + Plateau$$

2.5.3. From Equation 2, determine the parameter λ, which represents the degradation rate constant and the Plateau, which represents the lowest amount of protein remaining.

2.5.4. Calculate Dmax, which is the maximum fractional amount of degraded protein and is calculated as 1-Plateau.

2.5.5. Plot Dmax for each concentration of PROTAC to determine a time-independent degradation potency curve.

2.5.6. Determine the *$Dmax_{50}$* value for the plot in 2.3.5 to analyze efficacy of compounds.

NOTE: To determine a DC_{50} at a specific time point, plot the calculated percent degradation for each concentration at the chosen time. This can be specified as DC_{50} t=4 h or DC_{50} t=12 h.

REPRESENTATIVE RESULTS:

To demonstrate single concentration endpoint lytic degradation analysis, several CDK target proteins; CDK2, CDK4, CDK7, and CDK10 were endogenously tagged with HiBiT at their C-terminus in HEK293 cells and treated with a 1 µM concentration of the pan-kinase Cereblon-based PROTAC, TL12-186[54] (**Figure 1A**). The level of CDK protein was measured at different time points and the fractional RLU relative to the DMSO control was determined (**Figure 1A**). Each CDK protein showed different extents of degradation in response to the compound treatment and the various time points (**Figure 1A**). To understand how CDK proteins compared to each other directly in terms of protein loss, the fractional RLUs in **Figure 1A** were calculated as total % degradation and plotted for each time point in **Figure 1B**. This shows that even at early time points, 2 or 4 h, some of the CDK family members show high levels of degradation which continue to trend upwards over time (**Figure 1B**).

To demonstrate kinetic degradation analysis, each of the BET family member proteins; BRD2, BRD3, and BRD4 were endogenously tagged with HiBiT at their N-terminus in HEK293 cells stably expressing the LgBiT protein[18]. These were then treated with three different concentrations of the pan-BET PROTACs; the Cereblon-based dBET6[50] (**Figure 2A**) and the VHL-based ARV-771[41] (**Figure 2B**). Kinetic measurements were collected over a 24 h period, and from the profiles at each concentration, the differences in BET family member response are readily apparent. The ability of BRD2 to initiate a more rapid recovery response post-degradation compound treatment (**Figure 2A,B**) has been previously observed with other pan-BET PROTACs and is likely due to a transcriptional feedback response competitive to the degradation process[18].

Both endpoint and kinetic analysis can be done with full compound dose response treatments. Shown in **Figure 3** are kinetic dose response degradation profiles of treatment of Ikaros/IKZF1-HiBiT CRISPR Jurkat cells stably expressing LgBiT protein with four different molecular glue compounds[2,26,55,57]; lenalidomide (**Figure 3A**), iberdomide (CC-220) (**Figure 3B**), thalidomide (**Figure 3C**), and pomalidomide (**Figure 3D**). These degraders show significant differences in degradation response amongst the compounds as well as across the concentration series (**Figure 3**).

To quantitatively assess degradation and rank order the compounds in **Figure 3** the dose

response profiles were used to calculate key degradation parameters including the degradation rate (**Figure 4A**), Dmax (**Figure 4B**), and $Dmax_{50}$ values (**Figure 4B**). These analyses show that iberdomide (CC-220) and pomalidomide have very similar rapid initial degradation rates (**Figure 4A**), yet iberdomide (CC-220) has the highest potency as has been previously seen in orthogonal studies[55,57] (**Figure 4B**). Since Iberdomide exhibits such high potency, and all concentrations tested show greater than 50% degradation, the $Dmax_{50}$ value obtained for Iberdomide represents an estimate based on the limitation in accurately fitting the data. From the graphs in **Figure 3C**,**D** and **Figure 4B**, neither lenalidomide nor thalidomide degrade the Ikaros/IKZF1 target to completion at the highest concentrations tested. Due to very little degradation observed with thalidomide, the degradation traces could not be accurately fit to an exponential decay model, therefore, degradation rate was not quantified for this treatment. For the most potent degrader, iberdomide (CC-220)[55,57] (**Figure 4B**). Cell viability multiplex assays showed no loss in cell viability for the concentrations tested (**Figure 4C**).

FIGURE AND TABLE LEGENDS:

Figure 1: CDK endpoint degradation and toxicity with pan-kinase PROTAC, TL12-186[54]. (**A**) Select panel of endogenous CDK target proteins fused with HiBiT on the C-terminus via CRISPR/Cas9 and assessed for degradation with 1 µM TL12-186 PROTAC[54] at 2 h, 4 h, 8 h, and 24 h treatment. Values are represented as Fractional RLU relative to a DMSO control measured at each time point. Error bars represent SD of the mean of 3 technical replicates. (**B**) Percent degradation of panel of CDK target proteins calculated from (A) representing the amount of degradation of each family member observed at 2, 4, 8, and 24 h time points. Error bars represent SD of the mean of 3 technical replicates.

Figure 2: Profiling kinetic degradation selectivity of BET family members with BET degraders, dBET6[50] and ARV-771[41]. Kinetic degradation profiles of endogenous BET family members, BRD2, BRD3, and BRD4, tagged with HiBiT on the N-terminus via CRISPR/Cas9 with treatment of single concentrations of 1 nM (left), 10 nM (middle), or 100 nM (right) dBET6[50] (**A**) or ARV-771[41] (**B**) PROTACs. Values are represented as Fractional RLU calculated from a DMSO control at each kinetic time point. Error bars represent SD of the mean of 4 technical replicates.

Figure 3: Live cell kinetic degradation dose response profiles of Ikaros/IKZF1-HiBiT with a molecular glue panel[2,26,55,57]. Jurkat cells stably expressing the LgBiT protein were engineered using CRISPR/Cas9 to tag the C-terminus of Ikaros/IKZF1 with the HiBiT peptide. Cells were treated with an 8 point dose response concentration series including DMSO of four different molecular glue compounds[2,26,55,57]: (**A**) lenalidomide, (**B**) iberdomide (CC-220), (**C**) thalidomide, or (**D**) pomalidomide. Luminescence was measured every 5 min for a total of 19.5 h. Relative light unit (RLU) data from (A-D) were converted to fractional RLU as described in Step 2.4.1 and graphed as a function of time. Error bars represent SD of 3 technical replicates.

Figure 4: Calculation of degradation rate and Dmax50 for Ikaros/IKZF1-HiBiT, and multiplexing cell health assays. Kinetic degradation data from Figure 3 were used to calculate quantitative degradation parameters. (**A**) Degradation rates and (**B**) degradation maximum values (Dmax) are graphed at each drug concentration for the indicated molecular glue compounds[2,26,55,57]. (**B**)

$Dmax_{50}$ values for each compound were calculated using a dose-response model with constrained Hill slope of 1, which can be used to rank order degradation compounds for a target. (**C**) Cell viability assays with the iberdomide (CC-220)[55,57] degradation dose response from Figure 3B was performed as an endpoint measurement upon completion of the kinetic degradation measurements. Error bars represent SD of 3 technical replicates.

DISCUSSION:

We present here two methods of screening degradation compound activity in either endpoint lytic format or live-cell kinetic mode. These approaches are based upon the same luminescent measurement principles yet provide different levels of detail and understanding. The choice of either approach will likely be dependent upon screening goals and size of compound library. For large compound screening decks or primary screens to observe any detectable degradation, endpoint lytic screening offers sensitive and efficient high-throughput compatibility where other endpoint approaches, such as western blot or mass spectrometry can be either impractical or difficult to adapt[14]. A starting point for these screens could be performed with a limited number of concentrations and timepoints. Recommended initial concentrations to test are in the range of 100 nM-10 µM, to account for initial degraders having low potency, poor permeability, or in some cases with highly potent compounds, the presence of a hook effect. It is further recommended that a minimum of two different time points should be tested to establish early onset degradation at 4-6 h and latent or sustained degradation at 18-24 h. Compounds that exhibit high degradation potency and on-target mechanism are readily observed within a 4-6 h timeframe, whereas degradation or apparent protein loss observed only at later time points could be due to a variety of mechanisms. It is highly recommended to monitor cell viability at both early and late time points, such that protein loss can be uncoupled from loss due to cell death. Similar to any type of luminescent or fluorescent assay, there is a potential for compounds within libraries to interfere or inhibit signal, therefore, orthogonal follow-up experiments with lead compounds using unrelated fusions or alternative approaches to monitoring protein level will be important for assessing that loss of RLU in these assays is associated directly with target protein degradation.

The ability to screen in live cell kinetic format over extended periods of time relies heavily on the assay signal to background (S:B). Factors that contribute to S:B include the expression level of the target protein itself, which can span several orders of magnitude, the efficiency of LgBiT expression in the cell line chosen for peptide insertion, and the availability of the tagged target for complementation in its various native complexes. We have established a general cutoff requirement consisting of a S:B of 15 to successfully measure degradation in kinetic mode with either Endurazine or Vivazine. The S:B is determined by measuring the baseline signal of the HiBiT-edited cells co-expressing LgBiT relative to unedited parental cells expressing LgBiT alone in the presence of either Endurazine or Vivazine live cell substrates. Vivazine will produce a higher luminescent signal but will decay faster than Endurazine and may limit signal acquisition to 24 hours or less. Furthermore, S:B can also be highly dependent on whether CRISPR pools or clones are used. For targets in cell lines which are more amenable and have high efficiency for CRISPR/Cas9 engineering, a heterogeneous CRISPR pool population of edited cells may have sufficient S:B for kinetic analysis. For targets in more difficult cell lines where less efficient

genomic integration via CRISPR results in pools with low S:B, isolating CRISPR clones might be necessary to enrich edited populations and achieve a sufficiently high S:B for kinetic analysis. For any of these scenarios, if S:B is less than 15 with either Endurazine or Vivazine substrates, endpoint lytic screening is advised.

For better understanding and characterization of compounds, including determination of a degradation profile with quantitative parameters, real-time kinetic analysis in live cells is the recommended screening approach[14,18]. Like endpoint analysis discussed above, initial kinetic screening can be done with a limited number of concentrations in the range of 100nM-10µM in high-throughput fashion. In 384-well format, over 100 compounds can readily be screened in triplicate at one concentration on a single plate. The resulting degradation profiles will provide guidance not only on the extent of degradation observed, but the rate of degradation, the duration of degradation, and potential recovery of the protein[14,18] (**Figure 2** and **Figure 3**). The shapes of the degradation profile also yield valuable information. Specific and potent degraders often show an initial rapid loss of the target protein to a plateau in a matter of hours[18,53], whereas other mechanisms such as transcriptional feedback or compound toxicity typically result in more linear loss of the protein over time. These details and nuances are missed with endpoint lytic analysis, and with real-time analysis over 24-48 hours, one does not have to predict the time to capture the true Dmax within sets of new or unknown compounds.

Real-time kinetics also allows for efficient dose response screening to better understand compound efficacy, how compound concentration impacts initial degradation rate, and offers possibilities to rank compounds based on more than one parameter. Classical measurements of degradation potency involve DC_{50} calculations at a specific point in time based on apparent degradation maxima. In contrast, our kinetic approach to evaluate potency incorporates the true degradation maximum at each concentration regardless of when it occurs in time[18]. We call this measurement of kinetic degradation potency, the $Dmax_{50}$[18]. Analysis in this fashion accounts for compounds that may initiate degradation more slowly at lower concentrations and therefore take a longer time post-treatment to reach their Dmax. It can be especially informative to rank compounds on both degradation rate and Dmax. For the most potent degraders this will further differentiate slow, but potent degraders from those which are both fast and potent. Together, both lytic and live cell kinetic screening utilizing HiBiT CRISPR cell lines are powerful approaches which yield a more comprehensive picture of targeted protein degradation, compound function, and enable the screening process from initial activity assessment to downstream chemical optimization through enhancement of key degradation parameters.

ACKNOWLEDGMENTS:

K.M.R, S.D.M, M.U. and D.L.D are all employees of Promega Corporation

DISCLOSURES:

Promega Corporation is the commercial owner by assignment of patents of the HiBiT and NanoLuc technologies and applications.

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Figure 1.

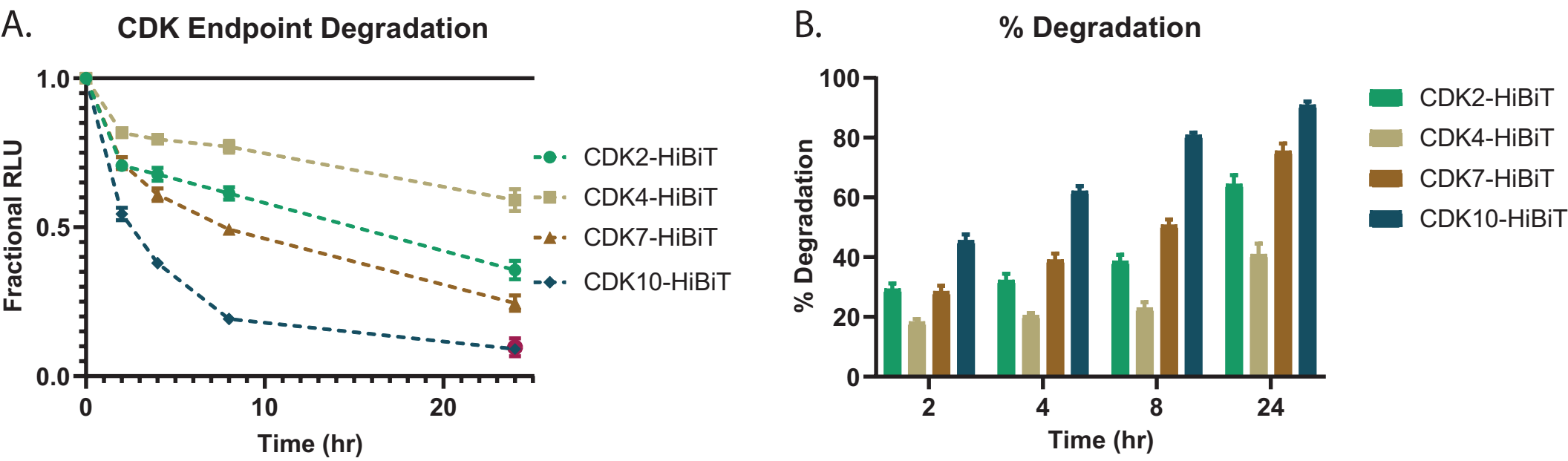


Figure 2.

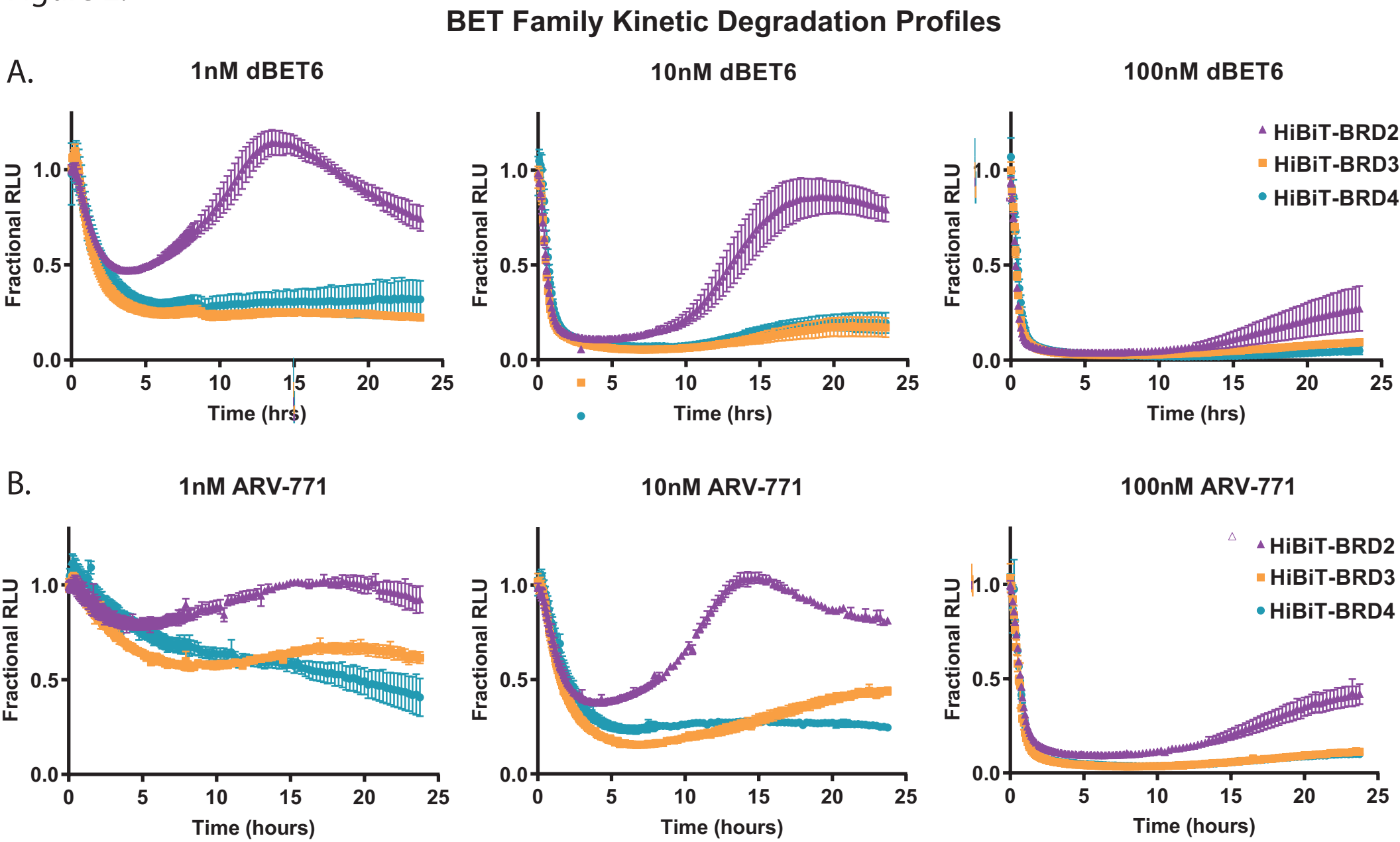


Figure 3.

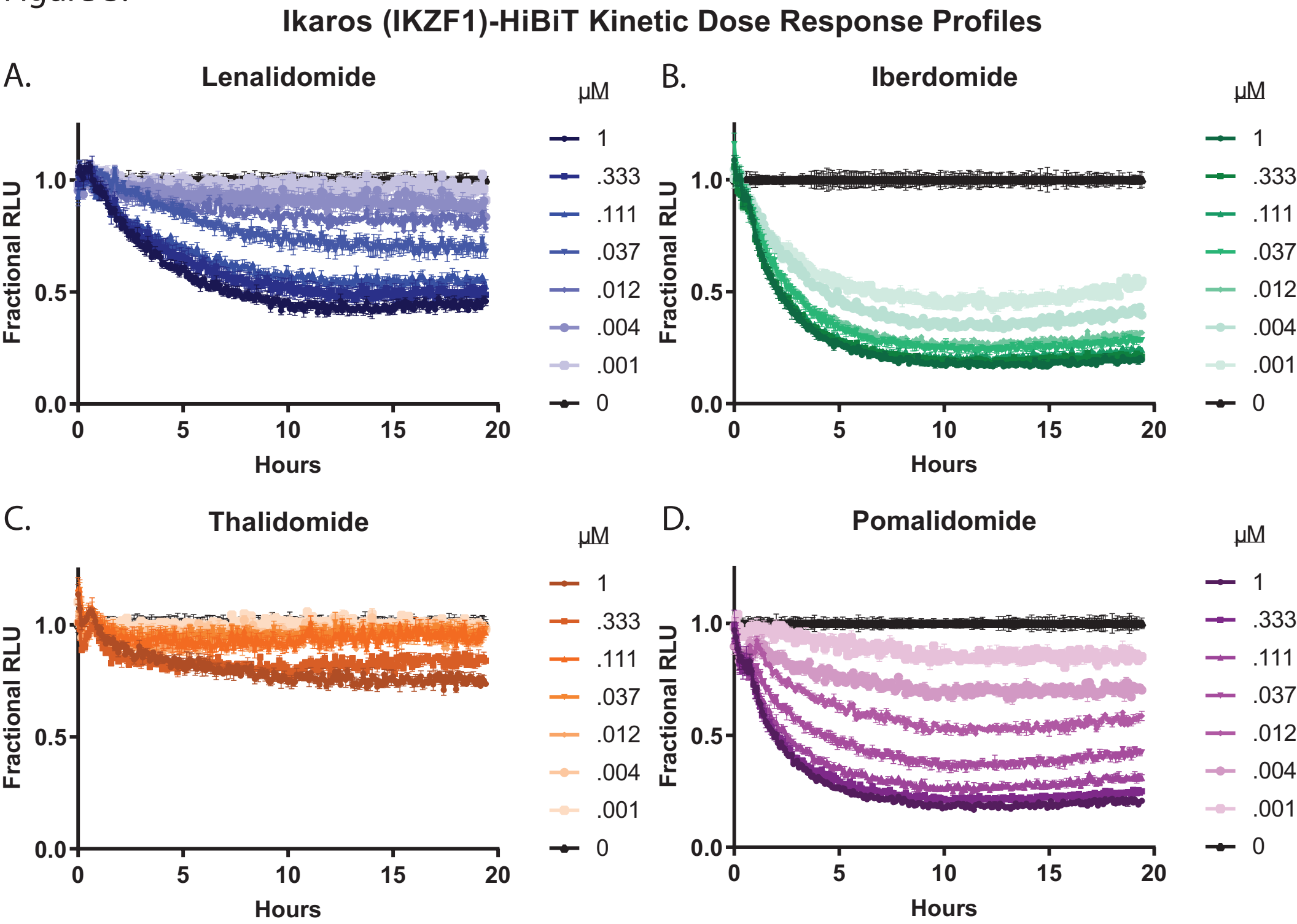
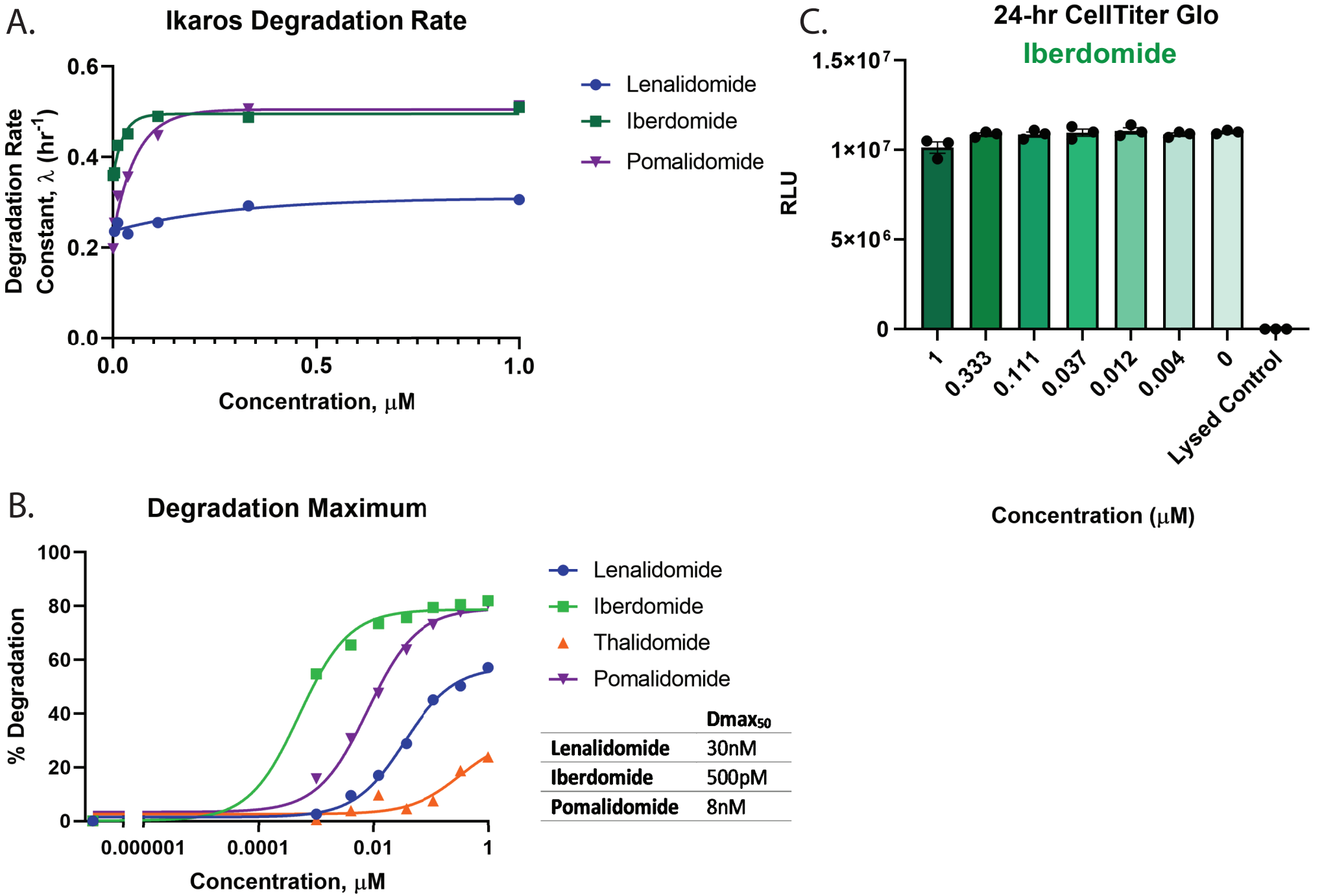


Figure 4.



	Dmax$_{50}$
Lenalidomide	30nM
Iberdomide	500pM
Pomalidomide	8nM

Table of Materials

Click here to access/download;Table of Materials;Table_of_Materials_Revised.xlsx

Name of Material/ Equipment	Company	Catalog Number
CellTiter-Glo 2.0 reagent	Promega	G9241
CellTiter-Fluor Cell Viability Assay	Promega	G6080
CO_2-independent medium	ThermoFisher	18045-088
DMSO	Sigma Aldrich	D2650
DPBS	Gibco	14190
Fetal Bovine Serum	Seradigm	89510-194
HEK293 LgBiT stable cell line	Promega	N2672
HiBiT CRISPR mammalian cell line	Promega	
Hygromycin B solution	Gibco	10-687-010
LgBiT BacMam	Promega	CS1956C01
LgBiT Expression Vector	Promega	N2681
Luminometer Plate Reader		
NanoGlo Endurazine live cell substrate	Promega	N2570
NanoGlo Vivazine live cell substrate	Promega	N2580
NanoGlo HiBiT Lytic Detection system	Promega	N3030
Opti-MEM Reduced Serum Medium, no phenol	ThermoFisher	11058-021
Tissue culture plates, white, 96 well plate	Costar	3917
Tissue culture plates, white, 384 well plate	Corning	3570
Trypsin/EDTA	Gibco	25300

Comments/Description

Cell Viability luminescent assay
Cell Viability fluorescent assay
Cell culture
For compound dilution and control
Cell culture
Cell culture
For complementation with HiBiT to generate luminescence
https://www.promega.com/crispr-tpd
Cell culture
For complementation with HiBiT to generate luminescence
For complementation with HiBiT to generate luminescence
Luminomenter capable of measuring luminescence and fluorescence (e.g. GloMax Discover System, Pror
Kinetic HiBiT reagent
Kinetic HiBiT reagent
Enpoint lytic HiBiT reagent
Cell culture
Cell culture
Cell culture
Cell culture

ɱega GM3000)

Dear Dr. Vineeta Bajaj,

Thank you for sending your editorial comments as well as those from the reviewers for our manuscript JoVE61787 "High-throughput profiling of targeted protein degradation compounds using HiBiT CRISPR cell lines in endpoint or live cell kinetic screening formats." We are pleased to read these and here are our detailed responses.

Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

1. We have done this.

• **Textual Overlap:** Significant portions show significant overlap with previously published work. Please re-write the text indicated on lines 324-336, 342-344, 349-359, 382-391, 483-490, this overlap.

2. These sections of text have been removed and their available protocols referenced. As they cover the addition of LgBiT vector into the cell, which can be done in a variety of ways we did not find them necessary to reiterate here.

• **Protocol Detail:**
The JoVE protocol should be almost entirely composed of numbered short steps (2-3 related actions each) written in the imperative voice/tense (as if you are telling someone how to do the technique, i.e. "Do this", "Measure that" etc.). Any text that cannot be written in the imperative tense may be added as a brief "Note" at the end of the step (please limit notes). Please re-write protocol section 2.4 accordingly. Descriptive sections of the protocol can be moved to Representative Results or Discussion. The JoVE protocol should be a set of instructions rather a report of a study. Any reporting should be moved into the representative results.

3. Section 2.4 is now 2.3 and has been modified in the imperative voice/tense.

Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please ensure that all specific details (e.g. button clicks for software actions, numerical values for settings, etc) have been added to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

4. We believe there is enough detail for this.

Define CTG.

5. We have defined this when we first mention it. CellTiter Glo=CTG

• **Protocol Numbering:**
All steps should be lined up at the left margin with no indentations.

6. This has been completed.

Please add a one-line space after each protocol step.

7. This has been completed

• **Protocol Highlight:** After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3- page limit for filmable content. If your protocol is longer than 3 pages, please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.

1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
4) Notes cannot be filmed and should be excluded from highlighting.

8. The portion in yellow for filming is highlighted – this is the endpoint lytic portion of the protocol

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

9. We have addressed these points.

• **References:** Please spell out journal names.

10. I have installed in EndNote the JoVE format. If this in incorrect and I should use a different format in EndNote, please let me know.

• **Commercial Language:**JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are HiBiT, CellTiter, NanoGlo H, . GloMax Discover Instrument, etc
1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the

generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names.

11. All TM/R symbols have been removed. We cannot rename HiBiT, CellTiter Glo, or NanoGlo as there are not generic names for these. These are the only reagents that can be added at these steps and the only names available.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

12. We are not using any figures or tables previously published.

Reviewer #1:
Manuscript Summary:
The manuscript provides a comprehensive, extremely detailed and accurate description of the hibit-based method aimed at quantifying a protein of interest.

Major Concerns:
None, the method is very accurately described and largely utilized in the field of targeted degradation. The here described protocol is extremely comprehensive

Minor Concerns:
The manuscript is very well written although a but "heavy", if would be a bit easier for the reader if it could be shortened/condensed.

1. We agreed with this assessment. To make the manuscript more concise we have separated out the cell viability assays from the main endpoint and kinetic protocols, as well as chosen only the best cell viability assay to be paired with either approach. We have also removed the section in the kinetic assay detailing LgBiT introduction and cited references for this instead as it is published.

On a separate note, The authors focus the hibit assay in the setting of targeted protein degradation and molecular glues which are certainly the main and most obvious utilization of the method, however, this assay could be in principle used in any application in which a protein level needed to be monitored, I guess highlighting this point in the intro might also be important and could further extend the purpose for which this protocol could be used. For example this method was also used to monitor protein dynamics (Sci Rep 2020 Jun 2;10(1):8953. doi: 10.1038/s41598-020-65832-1.), to just monitor protein expression of a targeted protein (Biochem Biophys Rep. 2017 Dec; 12: 40-45.) or even to interrogate

biology to identify mechanisms of resistance to targeted degradation (ACS Chem Biol. 2019 Oct 18;14(10):2215-2223. doi: 10.1021/acschembio.9b00525).

2. Yes it is a universal tool to monitor changes in protein levels and we would like to acknowledge this. We have added a comment to this in the introduction and referenced these papers.

Reviewer #2:
Manuscript Summary:
The manuscript illustrates the use of luminescent approaches to profile target degradation. The protocol is well written and with sufficient detail to allow effective use.

I have a few minor concerns but would like more detail with regards to the genome-engineering that is required to establish the cell lines used in these approaches as detailed below.

Major Concerns:
Given the title and scope of the protocol and as stated by the authors the relative importance of the CRISPR genome-editing more detail is required on the CRISPR engineering than is provided to fully demonstrate the method.

1. Yes the HiBiT CRISPR engineering is a pre-requisite for these studies and critical for the success of the protocols. We feel that protocol for HiBiT CRISPR insertion to this screening protocol would be a significant addition, worthy of its own separate JoVE paper with all of the considerations for this process. Several publications exist with protocols and guidance of HiBiT CRISPR insertion as well as tag insertion using CRISPR in general. We have added two additional statements, the first into the introduction and the second in the Note before beginning the protocol to guide people to these manuscripts. The screening protocols here already are lengthy with the endpoint and kinetic options and addition of the HiBiT CRISPR protocol would detract from these.

Minor Concerns:
1.1.1.2.2.1. Is an optional step however 1.1.1.2.1 and 1.1.1.2.2 require two plates to be seeded. When two or one plate is seeded should be clarified.

1. This step was for CTG cell viability, and as this protocol has been removed, so has this option which we agree is confusing.
   1.1.1.2.1 requires white plates where no mention of optimal colour etc is mentioned
2. We have clarified the plate color to be white and the brand (Corning) is indicated in the Table of Materials
   1.1.1.3. "Store plates in a 37°C + 5% CO2 incubator" also ongoing eg. 1.1.2.2 Use of CO2 incubator will be dependent on the cell medium used by the operator for passaging and / seeding therefore may CO2 may not be required for pH maintenance.
3. Yes this is correct and we have indicated that cells should be stored at conditions required for their growth at Sections 1.1.2.2 and 2.2.1.5.

1.2.2.3. "If measuring endpoint luminescent detection without the optional CTF,
go to step 1.1.3 above. If performing multiplexing with CTF, proceed to the next section

1.2.3 below."Section 1.2 deals specifically with multiplexing ""If measuring endpoint luminescent detection without the optional CTF, go to step 1.1.3 above." appears redundant and was somewhat confusing when reading.

4. Yes we have clarified this and moved the CTF to an optional step 1.1.4

Transfection in step 2.1.1.7. could be performed by a range of transfection reagents that may be optimal for the cell line of interest.

5. We have removed the transfection of LgBiT vector section.

Section 2.1.3. requires additional detail

6. We have removed this section of LgBiT stable cell line generation.

In Figure 4B it appears sigmoidal concentration response curves have been fitted however for some compounds e.g. Iberdomide this fit appears in appropriate as most to the data sits in <50% of the fitted curve. All curves also appear to include '0' when being fit.

7. We agree that at best the fit for Iberdomide represents an estimate due to a lack of more data points at lower concentrations. We have clarified this in the text and also constrained the curve fits for all compounds to a Hill slope of 1. This change did not significantly impact the Dmax50 values calculated for Lenalidomide or Pomalidomide, but it did increase slightly the estimate of Dmax50 for Iberdomide from 300pM to 500pM. We feel this is a good estimate based on agreement with other published reports demonstrating Iberdomide potency in the high pM to single-digit nM range.

The potential for cell toxicity has been established and accounted for within the protocol however as the intended/potential use of the protocol is in screening compounds other potential confounds may deserve discussion (e.g. compounds that inhibit luminescent output by interfering with the luciferase)

8. We have addressed this in the Discussion at the end of the first paragraph and indicated that addition controls are needed to understand this with any type of hit or lead compounds identified.

Reviewer #3:

Manuscript Summary:

The manuscript details why an how HiBit//LgBit based degradation assays, both in endpoint and live cell format, may be performed and can have significant impact for profiling small molecule degraders. This manuscript will serve to widen access and understanding of a method for robust, high throughput and informative set off assays within the targeted protein degradation field. By providing useful and informed recommendations to contextualise when each assay format is most useful and or most practical the authors have done a nice job in providing what will surely be a useful resource to both existing practitioners within the TPD field and those who are looking for a route in. The authors have also provided, based on their own experiences, some key recommendations with respect to critical

experimental and material elements required to obtain meaningful data from these assays, for example a clear recommendation to use CRISPR/Cas9 introduction of the Hibit tag to proteins of interest.

Major Concerns:
No major concerns.

Minor Concerns:
I highlight the following minor points which, if the authors chose to incorporate, may serve to help communicate the statements already provided by the authors:

- First line of the abstract ''small molecule targeted degradation compounds'' - i would suggest rewording this as the proteins are targeted by the small molecules.

1. This has been changed to be more clear.

- line 74 : I suggest removing 'IMiD' here as you specify most (but therefore not all) are IMiD based a little later in the paragraph.

2. Removed IMiD

- line 78: As far as i'm aware CELMoD is a phrase from Genentech, not sure how much others in the field use that term?

3. Agreed and removed

- line 114: perhaps no need to specify ImiDs? molecular glues more generally are all applicable

4. Agreed and removed

- line 597-599:It may help readers to know what you consider is the lowest dmax recommended to warrant rate quantification?

5. We do not have a lowest Dmax recommendation as this depends on the quality of the data. We have fit data showing only ~10% degradation, but this may not always be possible.

- line 662-666: I think this is good advice personally as well, but i do think the requirement to have a minimum of two time points is an opinion rather than hard fact. Perhaps phrase to make it clearer that this is your recommendation rather than a technical necessity? It is a very useful recommendation to make though so please keep it in.

6. We have kept this statement in, though have clarified it to be a recommendation.

Reviewer #4:

Manuscript Summary:

Riching and colleagues present comprehensive protocols to discuss various uses of their HiBiT technology. These HiBit systems have been highly influential and their usage is growing rapidly in the community. As such, this is a very timely protocol that will be read and employed extensively. This manuscript is well written and should be accepted upon addressing the minor points noted below. I look forward to using these protocols in my lab.

Minor Concerns:

1. It would be useful to clarify the sequence of LgBit plasmid; the catalog number provided did not yield a result on Promega's website. Can the sequence be made as investigators may desire cloning LgBit into alternate plasmids that might be more adaptable for other cell lines of interest. Our lab typically re-sequences any plasmid to ensure its correct, whether commercial or otherwise, so having this information would be needed.

1. We have changed the LgBiT section to include the references and information of the LgBiT protein and options for introduction.

2. Can the authors comment on extensibility across cell lines of HiBit? A limitation seems to be where CRISPR knock-in is feasible but if there's a catalog of successful cell types available, that would be helpful as users decide cell lines to employ. Also, is homozygous knock-in necessary or advised?

2. We have expanded on this in the Introduction and Discussion section, as well as referenced the publications which has most recently shown HiBiT CRISPR insertions done at scale in many different cell types. We have also referenced the paper that have used successfully both heterozygous and homozygous CRISPR insertions for degradation studies.

3. For the CDK result in Figure 1, do the authors have a sense for why CDK10 was most efficiently degraded? This is likely beyond the scope of the manuscript but if there is a reference that supports the results similar to the discussion of results noted in Figure 2, that would be nice to include. A speculative comment would also be fine or a note on further evaluation using nanoBRET or other technologies that could be used to help clarify.

3. We are preparing a separate manuscript with detailed description of CDK family degradation with this compound, but yes there could be many reasons for the differences. To discuss the biology of these results are outside the scope of this manuscript and the data we present are consistent with previous published analysis of this tool compound PROTAC, which is cited in the Results section and the Figure legend.

4. S:B is not defined until the discussion but is mentioned in the protocol, can it be defined earlier?

4. It is defined in Section 2 at the start of the Kinetic assays in Note 2 and we have expanded upon the importance of this in the Discussion.

5. I would interested in the author's perspective on limitations of the approaches mentioned and future directions. A comment in the discussion would be appreciated.

5. We have added to the Discussion the limitation of compound interference for the assay and need for additional controls. We also discuss the limitations of CRISPR, dependence upon a minimal signal needed for these assays, and the difficulties in genome engineering of difficult lines where key mutations for disease might only be present.