Real-Time Monitoring of Protein Degradation Using Bioluminescent Tagging and the GloMax® Discover

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Materials Required

- cell line endogenously tagged with HiBiT via CRISPR/Cas9 and co-expressing LgBiT
  Note: If target protein is ectopically expressed, we recommend low expression levels.
- white, 96-well (Costar® Cat.# 3917) or 384-well plates (Corning® Cat.# 3570)
- tissue culture equipment and reagents
- DPBS (Invitrogen Cat.# 25300)
- DMEM (Gibco Cat.# 11995)
- Fetal bovine serum (Seradigm Cat.# 1500-050)
- Nano-Glo® Endurazine™ Live Cell Substrate (Cat. N2570) for extended kinetic monitoring
- CO_2-independent medium (Gibco Cat.# 18045-088)
- test PROTAC(s)

Media Composition

Cell Culture Medium

- 90% DMEM
- 10% Fetal bovine serum

Assay Medium

- 90% CO_2-independent medium
- 10% Fetal bovine serum

Protocols: *GloMax® Discover System Technical Manual* #TM397 and Application Note #AN331 *Kinetically Detecting and Quantitating PROTAC-Induced Degradation of Endogenous HiBiT-Tagged Proteins* are available at **www.promega.com/protocols/**

Introduction

Protein degradation and turnover via the ubiquitin-proteasomal pathway is a key regulatory step for most mammalian cellular proteins. Recently, this process has been co-opted by small molecules called proteolysis targeting chimeras (PROTACs), a newly developed class of therapeutic compounds which exploit the cell's degradation machinery to rapidly eliminate a target protein from the cell. PROTACs are heterobifunctional molecules that contain two binding ligands and result in the simultaneous engagement of a target protein with an E3 ubiquitin ligase. Upon formation of this ternary complex, the E3 ligase ubiquitinates the protein, marking it for degradation by the proteasome. Whether proteins are degraded through their native intracellular pathways or targeted for degradation using PROTACs, monitoring their levels in terms of loss and subsequent recovery is a significant challenge, especially when attempting to capture these processes in real time.

To address these challenges and increase the throughput capacity of screening, we used our NanoLuc® Luciferase-based tagging technologies in combination with kinetic analysis on the GloMax® Discover instrument. With this approach, we can detect the levels of any protein tagged with full-length NanoLuc or binary complemented NanoBiT (consisting of HiBiT peptide complemented with LgBiT) in living cells. The high sensitivity and dynamic range allow detection of endogenously tagged proteins, even those with low expression levels. The development of the stabilized furimazine substrates Endurazine and Vivazine enables continual luminescent detection over a 24–72 hour range without need to replenish substrate.

Here we show kinetic dose-response degradation curves for the bromodomain protein, BRD4, endogenously tagged using CRISRP/Cas9 with the 11-amino-acid peptide HiBiT in HEK293 cells stably expressing the LgBiT fragment. Because of the high affinity of HiBiT for LgBiT, HiBiT-BRD4 is constitutively complemented with this design, and therefore constitutively produces luminescence in the presence of substrate. Treatment of these cells with different concentrations of BRD4 PROTAC compound and Endurazine™ substrate allows for continuous, real-time monitoring of BRD4 levels over a 24-hour time period. From the kinetic degradation profiles, we can determine several quantitative parameters that describe the degradation efficacy for rank-ordering different PROTAC compounds, including degradation rate, D_{max} and DC_{50}. This same approach can be used to monitor the dynamics of any target protein in response to any type of cellular stimuli, such as transcriptional feedback or perturbation of upstream signaling.

With the GloMax® Discover, it is easy to set up long kinetic experiments with 37°C temperature incubation and user-defined read intervals—without worry of condensation forming on the plate lid. Visualization and analysis of the data is easily achieved with different export formats that compare kinetic traces from each well across the plate or with all RLU values compiled in a table for computing statistics and plotting. Below is an example protocol to monitor protein degradation by PROTACs in real time using the GloMax® Discover System.

Degradation Assay Protocol

1. Trypsinize cells to dissociate from flask.
2. Neutralize trypsin with cell culture medium and adjust density to 2×10^5 cells/ml in cell culture medium.
   Note: To cover an entire 96-well plate, prepare at least 10ml (96-well) or 16 ml (384-well) of cells.
3. Plate 100µl (96-well) or 40µl (384-well) of cells per well.
4. Incubate plates overnight (18—24 hours) at 37°C, 5% CO_2.
5. Prepare a 1X solution of Endurazine™ by diluting 1:100 in assay medium.
6. Aspirate cell culture medium from plate and add 90µl (96-well) or 45µl (384-well) of Endurazine™ solution to each well.
7. Incubate the plate for 2.5 hours at 37°C, 5% CO_2 to equilibrate the luminescence signal.
8. Prepare a 10X concentration of test PROTAC titration (starting at 10µM) in assay medium and add 10µl (96-well) or 5µl (384-well) to each well for a final concentration of 1µM at the highest point.
9. Collect kinetic measurements of luminescence on a GloMax® Discover pre-equilibrated to 37°C.
   Note: Include a heating step in the kinetic loop after the luminescence read to prevent condensation from forming on the plate lid.
10. From the kinetic luminescence measurements, normalize the raw luminescence (measured in relative light units, RLUs) for each PROTAC concentration to the replicate averaged "No PROTAC" condition at every time point to account for the steady decline in RLU due to substrate depletion. This displays the data as fractional RLU and can be used for further quantitation by fitting to a single-component exponential decay model to obtain the parameters rate, degradation maximum (D_{max}) and concentration at which 50% of target protein is degraded (DC_{50}).

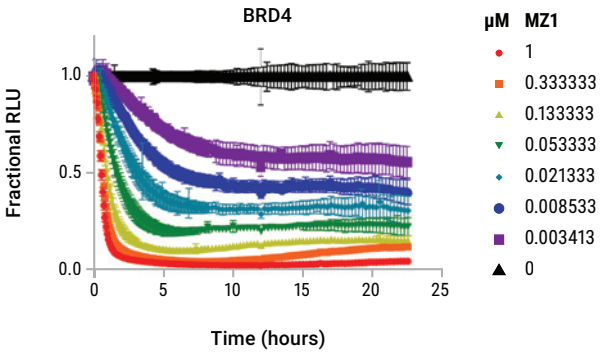

Figure 1. Degradation kinetics of endogenous HiBiT-BRD4 following PROTAC treatment. HEK293 cells stably expressing LgBiT were engineered using CRISPR-Cas9 to express endogenous HiBiT-BRD4 and plated as described. Cell culture medium was replaced with CO_2-independent medium containing Nano-Glo® Endurazine™ substrate for 2.5 hours, before adding a titration of 1µM MZ1 PROTAC. Kinetic measurements of BRD4 degradation at each PROTAC concentration were collected on the GloMax® Discover instrument.

Conclusion

The GloMax® Discover can collect continuous luminescence measurements, enabling real-time monitoring of protein degradation as shown in Figure 1. Rapid degradation of endogenously tagged HiBiT-BRD4 was observed in a dose-dependent fashion with MZ1 PROTAC. At the highest concentration, almost complete degradation of BRD4 was obtained and sustained over the 24-hour period.

The GloMax® Discover System

The GloMax® Discover System offers superior sensitivity and dynamic range as well as limited well-to-well cross-talk. The instrument was developed and optimized with Promega cell and gene reporter assays, and may be integrated into low- and medium-throughput automation workflows. The GloMax® Discover System allows flexible use of filters to measure fluorescence intensity, filtered luminescence, BRET, FRET and UV-visible absorbance for a wide variety of laboratory applications. The instrument is operated by an integrated tablet PC, which provides quick and easy navigation through the control options. Exporting your results is made seamless with a variety of options, including the option to export data to your local network.

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