TECHNICAL MANUAL

HaloPROTAC3

Instructions for Use of Products
GA3110 and GA4110

1/21
TM642

Promega

HaloPROTAC3

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

Promega

1. Description

Targeted protein degradation has emerged as an exciting new modality for therapeutic treatment by specifically removing key target proteins from the cell using small-molecule degrader compounds (1–3). In addition, these degraders can be used to understand temporal protein-loss phenotype and function without the need to knock out proteins or mRNA using genomic modification approaches or siRNA, respectively (4,5).

One class of degradation compounds are proteolysis-targeting chimeras (PROTACs). These heterobifunctional molecules recruit target proteins to E3 ligase components to ubiquitinate and degrade the target protein via the ubiquitin proteasomal pathway (1–4). HaloPROTAC3[(a–c)] is a small-molecule degrader that specifically binds to and degrades the HaloTag® protein (6) and its fusion partners in live cells (4; Figure 1). HaloPROTAC3 binds irreversibly to the HaloTag® protein and HaloTag®-target fusions, recruiting them via co-engagement with von Hippel Lindau (VHL), an E3 ligase component, to active E2/E3 ubiquitin ligase complexes (4; Figure 1). As a result, the HaloTag® protein and HaloTag®-target fusions are ubiquitinated and subsequently degraded by the proteasome (4; Figure 1).

This approach of using a fusion tag PROTAC to degrade specific targets means broad study of degradation of numerous targets is possible, including phenotypic study of loss of specific isoforms or mutations, without the need for target-specific PROTACs. Furthermore, targeted protein degradation studies can be performed even if no target-specific PROTACs are available (4,5,7,8). HaloPROTAC3 has also been shown to work effectively in mouse studies (7), providing the opportunity to study protein loss in vivo related to disease treatments for cancer or otherwise lethal approaches like embryonic gene knock-outs.

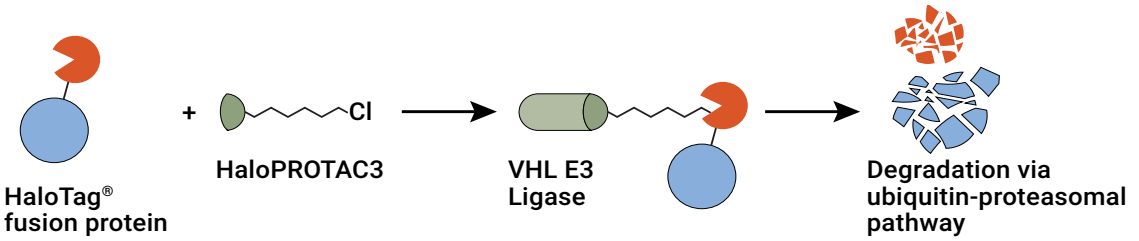

Figure 1. HaloPROTAC3, a cell-permeable degrader of HaloTag® fusions, interacts irreversibly with the 34kDa HaloTag® protein via its chloroalkane moiety. HaloPROTAC3 forms a ternary complex between a HaloTag® fusion protein and the VHL E3 ligase component (4). This induced complex promotes the ubiquitination, predominantly of HaloTag® itself, and the subsequent degradation of the HaloTag® target fusion via the ubiquitin-proteasomal pathway.

In addition to HaloPROTAC3, a negative control compound called *ent*-HaloPROTAC3 (4) is also available. The *ent*-HaloPROTAC3 is the enantiomeric compound of HaloPROTAC3 consisting of the same molecular weight and general molecular structure. However it contains D-hydroxyproline and D-valine residue modifications that significantly disrupt the binding to VHL, but not to HaloTag® protein (4). This enantiomeric compound can be used in parallel with HaloPROTAC3 to confirm that degradation of the HaloTag® fusion protein is mediated through VHL engagement and a PROTAC mechanism.

A positive control NanoLuc®-HaloTag® fusion vector, the NanoBRET™ Positive Control (Cat.# N1581), is available separately for transient transfection expression and degradation with HaloPROTAC3. This vector can be used in any study as a positive plate control and is part of an optional protocol to use the luminescent NanoLuc® enzyme expressed from the vector to quantitate degradation without the use of antibodies.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
HaloPROTAC3, 2.5mM	**20µl**	**GA3110**
***ent*-HaloPROTAC3, 2.5mM**	**20µl**	**GA4110**

GA3110 and GA4110 are suspended in DMSO.

Storage Conditions: Store at –30°C to –10°C. Avoid multiple freeze-thaw cycles and exposure to frequent temperature changes because these fluctuations can greatly alter product stability.

Note: For custom format or bulk amounts of HaloPROTAC3 necessary for in vivo studies, please contact EliteAccess@promega.com

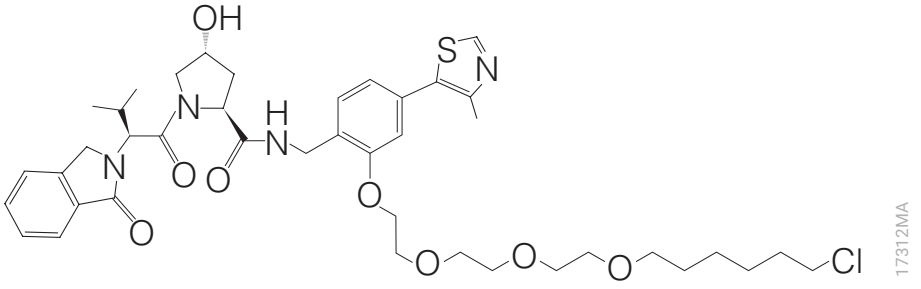

Figure 2. HaloPROTAC3 chemical structure

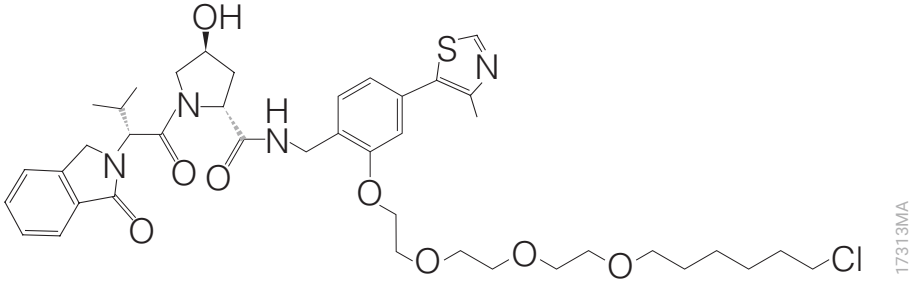

Figure 3. *ent*-HaloPROTAC3 chemical structure

2. Product Components and Storage Conditions (continued)

Available Separately

Positive Control Vector Regents

PRODUCT	SIZE	CAT.#
NanoBRET™ Positive Control	20µg	N1581

HaloTag® Antibody for Protein Detection

PRODUCT	SIZE	CAT.#
Anti-HaloTag® Monoclonal Antibody	200µg	G9211

3. Before You Begin

3.A. Designing and Expressing the HaloTag®-Target Fusion Protein

If using HaloTag® fusion proteins with HaloPROTAC3 to study phenotype after protein knock-out or loss, you must consider the optimal and physiologically relevant expression setting of the HaloTag®-target fusion (Figure 4). To study phenotypic loss using HaloPROTAC3 to remove the endogenous target protein, endogenously tagging the target gene with HaloTag® is required to fully understand the resulting biology.

There have been significant advances using CRISPR-Cas9 technology to introduce larger protein fusion tags into genomic loci though editing efficiency with larger tags remains challenging. This applies to the 34kDa HaloTag® protein (6,9). To overcome initial low efficiency of HaloTag® insertion with a CRISPR pool, use the fluorescent HaloTag® ligands to specifically enrich for HaloTag® edited cells using FACS sorting (9,10). The bright and sensitive Janelia Fluor® 646 HaloTag® Ligand (10) has high signal:background ratio and can readily detect endogenous HaloTag®-target fusions and clones for phenotypic HaloPROTAC3 degradation experiments.

As a general starting point for inserting the HaloTag® protein using CRISPR, we recommend using a dsDNA vector with 500bp 5´ and 3´ homology arms of the target protein. For more detailed protocols on CRISPR editing and tag insertions, please visit: **www.promega.com/products/small-molecule-drug-discovery/crispr-cas9-cell-lines-knock-in-tagging/crispr-diy-overview/**

In addition, a list of pre-existing HaloTag® and HiBiT-HaloTag® target CRISPR cell lines can be found at: **www.promega.com/products/small-molecule-drug-discovery/crispr-cas9-cell-lines-knock-in-tagging/**. See Section 3.B for an explanation on using HiBiT-HaloTag®-fusions.

For experiments where ectopic expression can be used to study phenotype (e.g., dominant mutations; 4), HaloTag®-target fusion vectors are generated and expressed transiently or stably prior to degradation. In addition, ectopic HaloTag® fusions can:

- Determine if PROTAC-mediated degradation is possible for a specific HaloTag®-target fusion due to target protein localization
- Assess VHL in different cell backgrounds
- Examine if the ubiquitin proteasome system (UPS) is used

The results from transfected cells can be helpful for deciding whether to use HaloTag® endogenous tagging. With any type of ectopic expression, degradation may be incomplete due to unregulated expression from nonendogenous promoters (5). If a transient system will be used, we recommend low expression levels and optimizing protein expression by testing multiple dilutions of your HaloTag® vector.

Find prebuilt HaloTag® vectors for your assay by visiting the Kazusa collection:
www.promega.com/products/pm/halotag-technology/kazusa-collection/

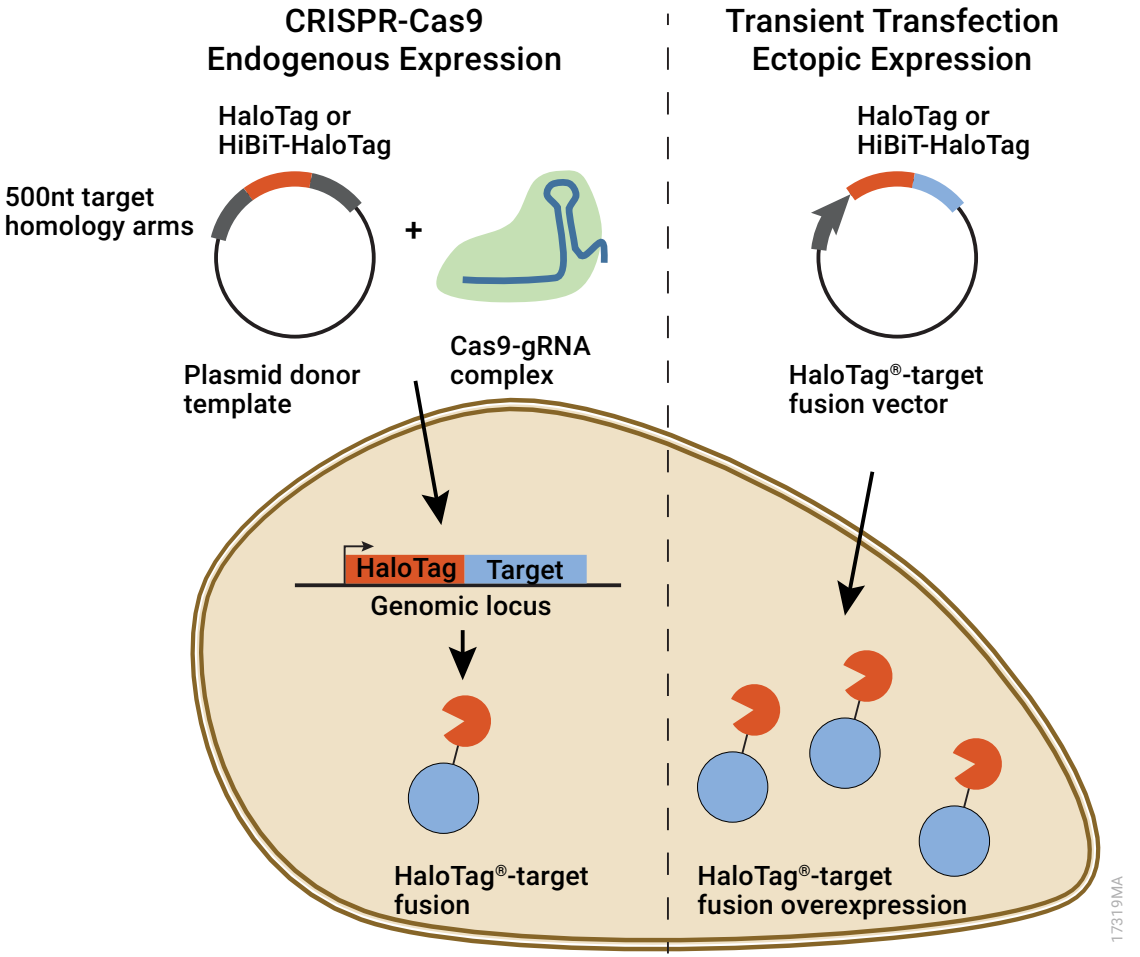

Figure 4. Strategies for designing and expressing HaloTag®-target fusions for HaloPROTAC3 degradation. HaloTag® alone or in combination with the 11-amino-acid HiBiT luminescent reporter (11) can be inserted into the target genomic N or C terminus using CRISPR-Cas9 gene editing. Alternatively, HaloTag® or HiBiT-HaloTag®-target fusions can be expressed ectopically via transient transfection as a N- or C-terminal fusions.

3.B. Detection Strategies for HaloTag®-Target Fusion Protein Degradation

Use Western blot analysis to confirm degradation of the HaloTag®-target fusion protein either using antibodies to the target protein or HaloTag® itself. To detect HaloTag® protein, we recommend using anti-HaloTag® monoclonal antibodies (Table 1). HaloPROTAC3 binding to HaloTag® is irreversible but does not prevent HaloTag® detection in Western blots with the HaloTag® antibodies. In addition, we recommend using HaloTag® antibodies for detection when the HaloTag®-target fusion of interest is highly expressed. This is typically the case with ectopic, transient expression

compared with endogenously tagged HaloTag® proteins, which can be challenging to detect given the low level of expression. In cases where greater sensitivity is required and Western blot detection is preferred, we recommend a target-specific antibody (Table 1).

A strategy to improve detection sensitivity and quantitate degradation involves pairing the HaloTag® protein with the 11-amino-acid HiBiT peptide (9,11,12; Table 1). HiBiT complemented with the LgBiT protein, expressed in cells or added post-lysis, produces luminescence, which can be quantitatively measured and directly correlates to target protein levels (11). For endogenous tagging, HiBiT can be appended to the end of a N-terminal or C-terminal HaloTag® insertion by including its sequence in the dsDNA CRISPR donor vector (Table 1). In a similar fashion, N-terminal HiBiT-HaloTag® and C-terminal HaloTag®-HiBiT vectors can be used for transient or stable ectopic expression (Table 1). This technology coupling yields a multifunctional fusion protein that degrades the target and an orthogonal readout of protein loss without the need of antibodies or performing Western blot analysis.

Table 1. Possible Expression Formats, Recommended HaloPROTAC3 Starting Concentrations, and Detection Methods for Potential HaloTag®-Target Fusion Proteins.

Ectopic Expression	Tagged Terminus	Recommended HaloPROTAC3 Concentration	Detection Method	Assay Type
HaloTag®-Target	N	1µM	Monoclonal HaloTag® Antibodies	Endpoint
Target-HaloTag®	C		Target Specific Antibodies	Endpoint
HiBiT-HaloTag®-Target	N	1µM	Monoclonal HaloTag® Antibodies	Endpoint
Target-HaloTag®-HiBiT	C		Target Specific Antibodies	Endpoint
			Nano-Glo® HiBiT Lytic or Live Cell Detection System	Endpoint
			Nano-Glo® Endurazine™ Live Cell Substrate	Kinetic

CRISPR-Cas9 Endogenous Expression	Tagged Terminus	Recommended HaloPROTAC3 Concentration	Detection Method	Assay Type
HaloTag®-Target	N	300nM	Target Specific Antibodies	Endpoint
Target-HaloTag®	C			
HiBiT-HaloTag®-Target	N	300nM	Target Specific Antibodies	Endpoint
Target-HaloTag®-HiBiT	C		Nano-Glo® HiBiT Lytic or Live Cell Detection System	Endpoint
			Nano-Glo® Endurazine™ Live Cell Substrate	Kinetic

Promega

4. HaloPROTAC3 Degradation of Ectopic HaloTag®-Target Fusion Proteins

Materials to be Supplied by User

- HEK293 or similar mammalian cells able to be transfected
- HaloTag® or HiBiT-HaloTag®-target fusion vector
- NanoBRET™ Positive Control Vector (Cat.# N1581)
- HaloPROTAC3, *ent*-HaloPROTAC3 or both
- tissue culture equipment and reagents
- DMEM (GIBCO Cat.# 11995-065)
- Opti-MEM® I Reduced Serum Medium, no phenol red (GIBCO Cat.# 11058-021)
- DPBS (GIBCO Cat.# 14190-144)
- fetal bovine serum (FBS; Seradigm Cat.# 89510-194)
- 0.05% trypsin/EDTA (GIBCO Cat.# 25300-054)
- DMSO (Sigma Aldrich Cat.# D2650-100ml)
- FuGENE® HD Transfection Reagent (Cat.# E2311)
- Transfection Carrier DNA (Cat.# E4881, E4882)

Optional: Western blotting analysis

- Anti-HaloTag® Monoclonal Antibody (Cat.# G9211) for Western blot analysis
- Mammalian Lysis Buffer (Cat.# G9381)
- SDS-PAGE gel and gel running apparatus for Western blot analysis
- Nitrocellulose or PVDF membrane and Western blotting apparatus
- TBST (1X TBS with 0.1% Tween®)
- 5% bovine serum albumin (BSA) in TBST
- anti-mouse IgG secondary antibody appropriately conjugated for Western blot analysis and corresponding detection substrate (e.g., Anti-Mouse IgG (H+L), AP Conjugate [Cat. # S3721] and Western Blue® Stabilized Substrate for Alkaline Phosphatase [Cat.# S3841])
- rotating platform for gentle agitation

Optional: Luminescence Detection

- white, 96-well plate (Corning® Cat.# 3917)
- plate reader capable of measuring luminescence (e.g., GloMax® Discover System [Cat.# GM3000])
- Nano-Glo® Live Cell Assay System (Cat.# N2011) for NanoBRET™ Positive Control Vector
- Nano-Glo® HiBiT Lytic Detection System (Cat.# N3030) for HiBiT-HaloTag® vectors

In this example protocol, the NanoBRET™ Positive Control Vector, a NanoLuc®-HaloTag® fusion protein, is used as the expression vector.

Notes:

1. If using a cell line that expresses an endogenous HaloTag®-target fusion, proceed to Section 4.B for recommended HaloPROTAC3 concentrations. The overall degradation levels will differ based on cellular expression levels.
2. For ectopic expression, we recommend using 500nM–20µM HaloPROTAC3. At concentrations above 1µM, monitor cell toxicity and ensure a total amount of DMSO does not exceed 0.5% by volume for the cell treatment. Degradation levels with ectopic fusions depends on many factors, including overexpression levels. Start with an initial dilution of 1:100 or 1:1,000 for the HaloTag® or HiBiT-HaloTag®-target fusion vector in a transient transfection with a cell line that has high transfection efficiency. We have observed 20–70% degradation for ectopic expression in transfected cells. The NanoBRET™ Positive Control Vector is diluted 1:1,000.

4.A. Transfecting the NanoBRET™ Positive Control Vector

This protocol describes transfecting HEK293 cells with the NanoBRET™ Positive Control Vector to use for HaloPROTAC3 degradation assays. Use these instructions as a guide for transfecting mammalian cells with any HaloTag® fusion vector.

1. Culture HEK293 cells appropriately prior to assay.
2. Remove medium from cell flask by aspiration, trypsinize and allow cells to dissociate from the flask bottom.
3. Neutralize trypsin using cell culture growth medium (DMEM + 10% FBS), count cells to estimate density and resuspend to a final density of 4×10^5 cells/ml in cell culture growth medium.
4. Plate 2ml of cells (800,000 cells) into one well of a six-well plate.
5. Allow the cells to attach and recover for 4–6 hours at 37°C, 5% CO_2.
6. Prepare a transfection mixture consisting of 0.001µg of the NanoBRET™ Positive Control Vector, 2µg of Transfection Carrier DNA, and 100µl of Opti-MEM® I Reduced Serum Medium, no phenol red.

   Note: This is the recommended starting amount for HaloTag® vectors. However, further optimization of the vector amount may be needed, depending on cell transfection efficiency and protein expression. Total DNA in the transfection mixture should equal 2µg to maintain a 3:1 reagent:DNA ratio.
7. Add 6µl of FuGENE® HD Transfection Reagent and incubate at room temperature for 10 minutes.
8. Add transfection mixture to wells with attached cells, and express proteins overnight for approximately 18–24 hours at 37°C, 5% CO_2.
9. Proceed to Section 4.B, HaloTag® Degradation with HaloPROTAC3.

4.B. HaloTag® Degradation with HaloPROTAC3

1. Prepare 1µM HaloPROTAC3, *ent*-HaloPROTAC3 or both compounds in cell culture growth medium.

   Note: This is the recommended starting concentration for any ectopically expressed HaloTag® or HiBiT-HaloTag®-target fusion. Increasing or decreasing this concentration will be dependent upon target.

2. Prepare a 1X DMSO solution to use as a negative control by adding an equivalent amount of DMSO to cell culture growth medium.

3. Aspirate medium from mammalian cells expressing HaloTag®-target fusion and replace with HaloPROTAC3, *ent*-HaloPROTA3 or DMSO control solutions.

4. Incubate cells at 37°C, 5% CO_2 for 18–24 hours.

   Note: This is the recommended initial incubation time for any ectopically expressed HaloTag® or HiBiT-HaloTag® target fusion. Increasing or decreasing the incubation time will be dependent upon target.

5. Proceed with degradation detection.

   To detect HaloTag®-target fusion degradation using Western blot analysis, proceed to Section 4.C, HaloTag® Antibody Western Blot Detection.

   To detect degradation of the NanoBRET Positive Control Vector or any NanoLuc®-HaloTag® fusion using luminescence, proceed to Section 4.D, Detecting Degradation Using NanoLuc® Luminescence.

   To detect degradation of HiBiT-HaloTag® ectopic fusions using luminescence, proceed to Section 4.E, Detect Degradation Using HiBiT Luminescence.

4.C. HaloTag® Antibody Western Blot Detection

1. Lyse mammalian cells expressing HaloTag®-target fusion in Mammalian Lysis Buffer.

2. Run lysates on SDS-PAGE, and transfer to nitrocellulose or PDVF membrane as per manufacturer protocol.

3. Place resulting nitrocellulose or PVDF membranes in TBST containing 5% BSA to block for 1 hour at room temperature or overnight at 4°C, with gentle agitation.

4. Replace blocking buffer with a solution of Anti-HaloTag® mAb at 1:1,000 dilution in TBST and leave at room temperature for 1 hour or overnight at 4°C with gentle agitation.

5. Replace primary antibody-containing solution with TBST. Wash three times for 15 minutes at room temperature with gentle agitation.

6. Replace wash with anti-mouse IgG conjugated secondary antibody at manufacturer recommended concentration (e.g., for anti-mouse IgG-AP conjugated use at 1:7,500 dilution) in TBST and leave for 30 minutes at room temperature with gentle agitation.

7. Replace secondary solution with TBST. Wash three times for 15 minutes at room temperature with gentle agitation.

8. Detect HaloTag® protein bands using appropriate substrate (e.g., AP substrate).

4.D. Detecting Degradation Using NanoLuc® Luminescence

Use this detection protocol for NanoBRET™ Positive Control Vector or other NanoLuc®-HaloTag® fusion vectors.

1. Split and count cells to estimate density, centrifuge at 200 × *g* for 5 minutes, aspirate medium, and resuspend to a final density of 2×10^5 cells/ml in Opti-MEM® I Reduced Serum Medium, no phenol red.
2. Plate 100µl of cells (20,000 cells) into each well of a white 96-well assay plate.

   Note: Prepare at least 3–4 wells for each treatment.
3. Prepare the desired amount of Nano-Glo® Live Cell Reagent by combining 1 volume of Nano-Glo® Live Cell Substrate with 19 volumes of Nano-Glo® LCS Dilution Buffer (a 20-fold dilution). For example, if the experiment requires 20ml of reagent, add 1ml of substrate to 19ml of dilution buffer.

   Note: Consult the *Nano-Glo® Live Cell Assay System Quick Protocol* #FB195 for detailed protocols at: **www.promega.com/protocols**
4. Add 25µl of Nano-Glo® Live Cell Reagent to each well.
5. Gently mix the plate by hand or with an orbital shaker (e.g., 15 seconds at 300–500 rpm).
6. Measure luminescence immediately after adding the Nano-Glo® Live Cell Reagent. Use an integration time of 0.25–2 seconds.

4.E. Detecting Degradation Using HiBiT Luminescence

Use this detection protocol for HiBiT-HaloTag® Vectors.

1. Split and count cells to estimate density and resuspend to a final density of 4×10^5 cells/ml in cell culture growth medium.
2. Plate 50µl of cells (20,000 cells) into each well of a white 96-well assay plate.

   Note: Prepare at least 3–4 wells for each treatment.
3. Dilute the LgBiT Protein 1:100 and the Nano-Glo® HiBiT Lytic Substrate 1:50 into an appropriate volume of room temperature Nano-Glo® HiBiT Lytic Buffer in a new tube. Mix by inversion.

   Note: Consult the *Nano-Glo® HiBiT Lytic Detection System Technical Manual* #TM516 for detailed protocols at: **www.promega.com/protocols**
4. Add 50µl of Nano-Glo® HiBiT Lytic Reagent to each well.
5. Mix the samples by placing the plate on an orbital shaker (300–600rpm) for 3–10 minutes or by pipetting samples.
6. Incubate plate for at least 10 minutes at ambient temperature to equilibrate LgBiT and HiBiT in the lysate. Measure luminescence using integration times of 0.5–2 seconds.

5. CRISPR-Edited Endogenous HaloTag® Fusion Protein Degradation and Luminescent Detection

Materials to be Supplied by User

- HaloTag® or HiBiT-HaloTag®-target CRISPR cell line
- white, 96-well plate (Corning® Cat.# 3917)
- tissue culture equipment and reagents
- complete cell culture growth medium appropriate for cell type used
- DPBS (GIBCO Cat.# 14190-144)
- trypsin/EDTA
- DMSO (Sigma Aldrich Cat.# D2650-100ml)
- HaloPROTAC3, *ent*-HaloPROTAC3 or both

Optional: Luminescent Detection with HiBiT-HaloTag® CRISPR Cell Lines

- plate reader capable of measuring luminescence (e.g., GloMax® Discover System [Cat.# GM3000])
- Nano-Glo® HiBiT Lytic Detection System (Cat.# N3030)

Notes:

1. Endogenously tagged HaloTag® or HiBiT-HaloTag®-target fusion proteins show greater degradation than ectopic expression. Therefore we recommend using 100nM–1µM HaloPROTAC3 in degradation assays. The total amount of DMSO should not exceed 0.5% by volume in the test sample. Degradation of an endogenously tagged HaloTag® target proteins varies between targets, but on average, the observed degradation maximums were 50–95%, depending on protein, localization, expression level, HaloPROTAC3 concentration and time of treatment.
2. Endogenous HaloTag®-target fusions can have variable levels of expression and will likely require highly sensitive methods for degradation detection. If using antibodies with Western blot detection, we recommend target-specific antibodies and optimizing the number of cells. For increased sensitivity and to remove the need for antibodies, we recommend CRISPR insertion of a HiBiT-HaloTag® dual tag and monitoring the protein level after HaloPROTAC3 treatment using HiBiT luminescence.

5.A. Treating the CRISPR-Edited Cell Line with HaloPROTAC3

1. Culture HaloTag® or HiBiT-HaloTag® CRISPR cells appropriately prior to assay.
2. Remove medium from cells by aspiration, wash cells with DPBS, dispense trypsin-EDTA, and allow cells to dissociate from the flask bottom.

   Note: If using suspension cells, estimate density, resuspend to a final density of 2.2×10^5 cells/ml in cell culture growth medium, and proceed to Step 4.
3. Neutralize trypsin using complete cell culture growth medium. Count cells to estimate density and resuspend to a final density of 2.2×10^5 cells/ml in cell culture growth medium.

4. Plate 90µl of cells (~20,000 cells) into each well of a white 96-well assay plate.

   Note: Prepare at least 3–4 wells for each treatment.

5. Prepare a 3µM concentration of HaloPROTAC3, *ent*-HaloPROTAC3 or both compounds in complete cell culture growth medium. Add 10µl to each well for a final concentration of 300nM.

   Note: We recommend this starting concentration for any endogenous expressed HaloTag® or HiBiT-HaloTag®-target fusion. Increasing or decreasing this concentration depends on the target.

6. Prepare a 10X concentration of DMSO to use as a negative control by adding an equivalent amount of DMSO to cell culture growth medium as performed in Step 5. Add 10µl to each negative control well.

7. Incubate cells at 37°C, 5% CO_2 for 18–24 hours.

   Note: We recommended this incubation time for any endogenous expressed HaloTag® or HiBiT-HaloTag® target fusion. Increasing or decreasing the time will depend on the target.

8. For detecting degradation of any HaloTag® endogenous fusions using Western blot analysis, follow recommended protocols by the respective manufacturer. For detecting degradation of the HiBiT-HaloTag® endogenous fusions using HiBiT lytic luminescence, proceed to Section 5.B, Detecting Degradation Using HiBiT Luminescence.

5.B. Detecting Degradation Using HiBiT Luminescence

Use this detection protocol for HiBiT-HaloTag® endogenous fusions.

1. Calculate the amount of Nano-Glo® HiBiT Lytic Reagent needed to perform the desired experiments. This volume is usually equal to the total amount of medium in wells plus any extra required for dispensing.

2. Dilute the LgBiT Protein 1:100 and Nano-Glo® HiBiT Lytic Substrate 1:50 into an appropriate volume of room temperature Nano-Glo® HiBiT Lytic Buffer in a new tube. Mix by inversion.

   Note: Consult the *Nano-Glo® HiBiT Lytic Detection System Technical Manual* #TM516 for detailed protocols at: **www.promega.com/protocols**

3. Add a volume of Nano-Glo® HiBiT Lytic Reagent equal to the volume of medium already in each well. For example, if 100µl of medium is present in each well, add 100µl of Nano-Glo® HiBiT Lytic Reagent.

4. Mix the samples by placing the plate on an orbital shaker (300–600rpm) for 3–10 minutes or by pipetting samples.

5. Incubate plate for at least 10 minutes at ambient temperature to equilibrate LgBiT and HiBiT in the lysate. Measure luminescence on the appropriate plate reader using integration times of 0.5–2 seconds.

5. Representative Data

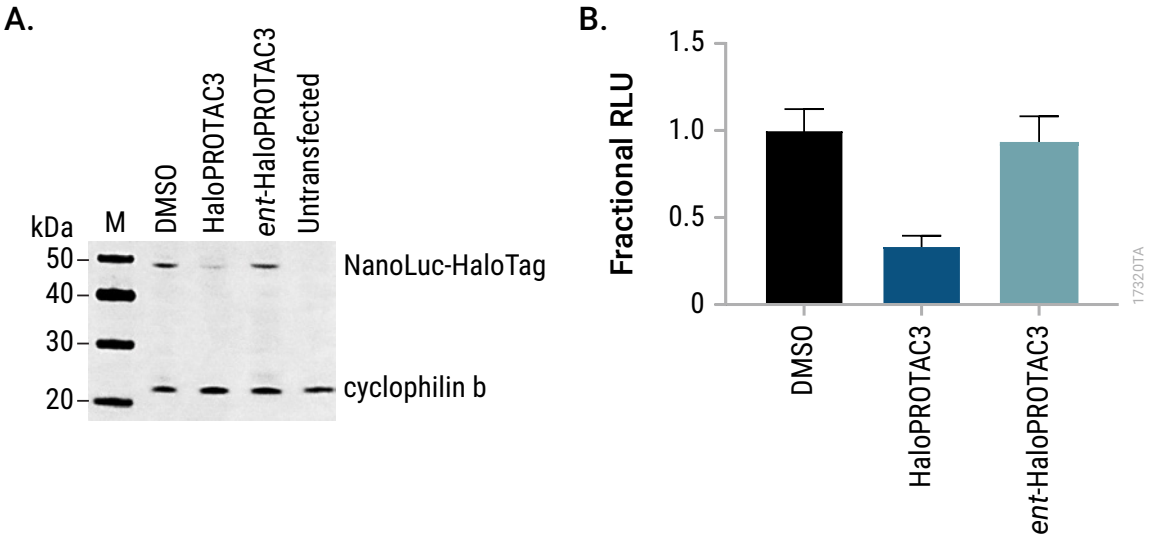

Figure 5. Degradation of NanoLuc®-HaloTag® Fusion with HaloPROTAC3. HEK293 cells were transfected with 0.001µg of the NanoBRET™ Positive Control Vector. After 24 hours of expression, cells were treated with 1µM of HaloPROTAC3 or *ent*-HaloPROTAC3 for 24 hours. Degradation was analyzed by Western blot with Anti-HaloTag® Monoclonal Antibody as described in Section 4.C, HaloTag® Antibody Western Blot Detection (**Panel A**), or with a live-cell luminescence assay described in Section 4.D, Detecting Degradation Using NanoLuc® Luminescence, using a GloMax® Discover System (**Panel B**).

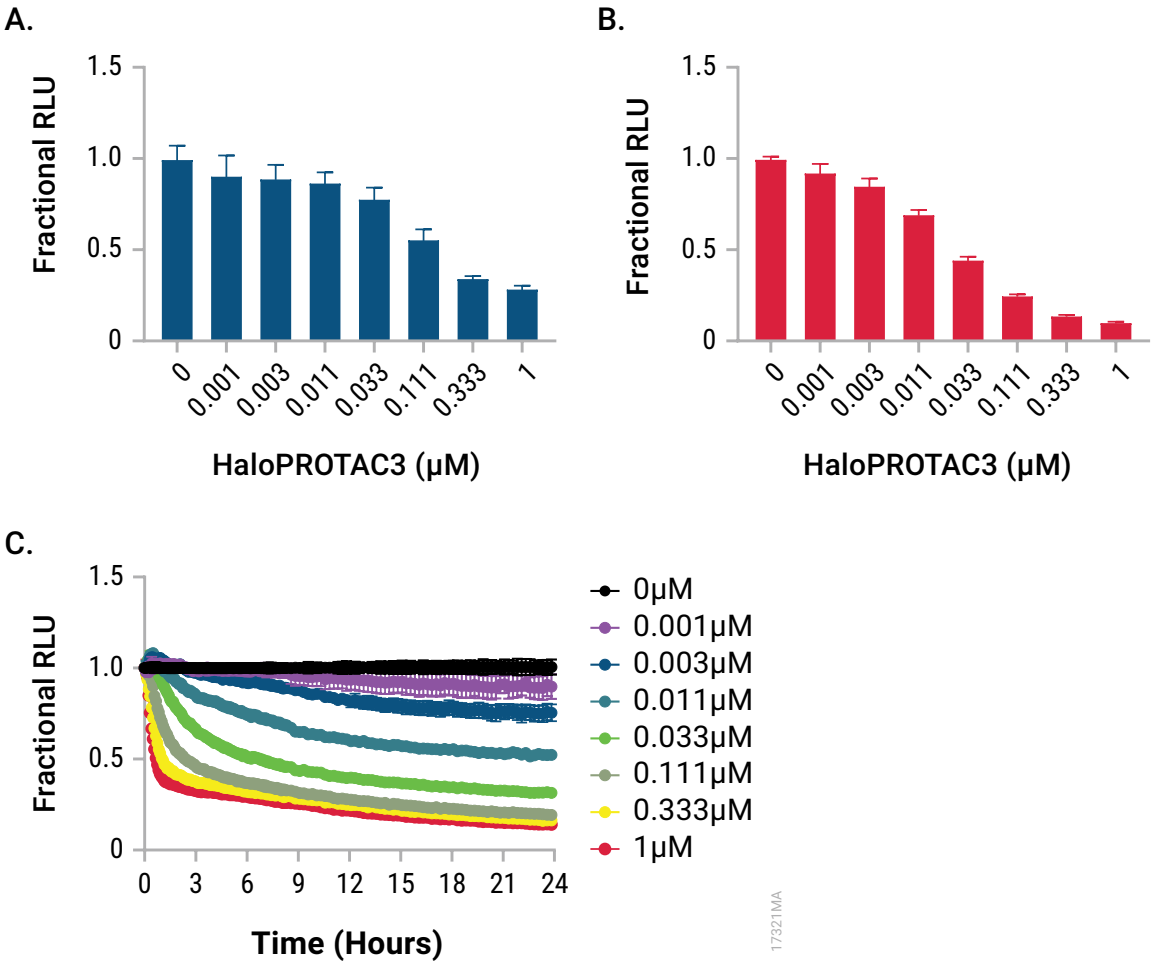

Figure 6. Endpoint lytic and live-cell kinetic degradation analysis of endogenous HiBiT-HaloTag®-BRD4 fusion protein with HaloPROTAC3. Using CRISPR-Cas9 gene editing, the HiBiT-HaloTag® dual tag was inserted into the N terminus of BRD4 in HEK293 cells stably expressing LgBiT protein. HiBiT-HaloTag®-BRD4 cells were treated with increasing amounts of HaloPROTAC3 for 3 hours (**Panel A**) and 24 hours (**Panel B**) to generate a dose-response curve. Loss of BRD4 was assessed by measuring luminescence using the Nano-Glo® HiBiT Lytic Detection Assay with the GloMax® Discover System as described in Section 5.B, Detecting Degradation Using HiBiT Luminescence. **Panel C.** HiBiT-HaloTag®-BRD4 cells were treated with various concentrations of HaloPROTAC3 in a dose-response curve. Luminescence was measured kinetically in live cells over 24 hours. Consult the *HEK293 LgBiT Cell Line and LgBiT Expression Vector Technical Manual* #TM620 for protocols analyzing live-cell kinetic degradation at: **www.promega.com/protocols**

6. Troubleshooting

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

Symptom	Causes and Comments
HaloTag®-target fusion shows minimal or no degradation	If ectopic expression is being used, expression level is too high. Overexpression will outcompete the rate of HaloPROTAC3-mediated degradation. Dilute expression vector or use an ectopic expression vector with a weaker promoter.
	Consider using HaloTag® CRISPR endogenous tagging.
	HaloTag® is not optimally oriented or structurally available in the fusion for ubiquitination. Tag other termini with the HaloTag® fusion.
	The concentration, time of degradation or both parameters were not sufficient. Increase either HaloPROTAC3 concentration, time of treatment or both parameters.
	The HaloTag® fusion is positioned or localized within the cell that is not accessible to VHL recruitment. VHL is present in the cytoplasm and nucleus and not found in other compartments.
	The HaloTag® fusion is a membrane protein. Single-pass transmembrane proteins have been successfully degraded via PROTAC mechanisms. Higher order membrane proteins have not.
	The HaloPROTAC3 compound is not functional. Test the NanoBRET™ Positive Control Vector for activity. If positive control shows activity, test for binding to the HaloTag® target fusion by competitive labeling first with HaloPROTAC3 followed by the red fluorescent Janelia Fluor® 549 or 646 HaloTag® Ligands. HaloPROTAC3 and HaloTag® ligands bind irreversibly to HaloTag® protein. Therefore if HaloPROTAC3 is bound to the HaloTag® target fusion, the target fusion cannot be subsequently labeled with the HaloTag® fluorescent ligand.
	If using luminescence to detect degradation, check that starting RLUs were above 1×10^4. If RLUs are low, increase amount of cells in the assay plate.
Decrease in HaloTag® fusion protein levels using *ent*-HaloPROTAC3	High concentrations of *ent*-HaloPROTAC3 can facilitate some interaction with VHL. Therefore decrease the concentration of *ent*-HaloPROTAC3 below 1µM.
	Test for cell toxicity of *ent*-HaloPROTAC3.

Symptom	Causes and Comments
Cell death is observed after treatment with HaloPROTAC3	Ensure your target protein is not essential for long-term cell survival or growth. Use orthogonal cell viability assays.
	DMSO concentrations added to cells are too high. Remain below 0.5% by volume after adding to the cells.
	Concentrations of HaloPROTAC3 used are too high. HaloPROTAC3 will start to show some toxicity at concentrations above 1µM.
	If you increase the HaloPROTAC3 concentration above 1µM, monitor for increased cell death.
Unable to detect HaloTag® fusion via Western blot	HaloTag®-target fusion protein levels are too low. Increase number of cells used for Western blotting analysis.
	If using the HaloTag® monoclonal antibody, increase amount of antibody used or test using the HaloTag® pAb (Cat.# G9281).
	If using a target-specific antibody, try other antibodies from several different manufacturers if available.

7. References

1. Ciulli, A. and Farnaby, W. (2019) Protein degradation for drug discovery. *Drug Discov. Today Technol.* **31**, 1–3.
2. Cromm, P.M. and Crews, C.M. (2017) Targeted protein degradation: From chemical biology to drug discovery. *Cell. Chem. Biol.* **24**, 1181–90.
3. Deshaies, R.J. (2015) Protein degradation: Prime time for PROTACs. *Nat. Chem. Biol.* **11**, 634–5.
4. Buckley, D.L. *et al.* (2015) HaloPROTACS: Use of small molecule PROTACs to induce degradation of HaloTag fusion proteins. *ACS Chem. Biol.* **10**, 1831–7.
5. Daniels, D.L., Riching, K.M. and Urh, M. (2019) Monitoring and deciphering protein degradation pathways inside cells. *Drug Discov. Today Technol.* **31**, 61–8.
6. Los, G.V. *et al.* (2008) HaloTag®: A novel protein labeling technology for cell imaging and protein analysis. *ACS Chem. Biol.* **3**, 373–82.
7. BasuRay, S. *et al.* (2019) Accumulation of PNPLA3 on lipid droplets is the basis of associated hepatic steatosis. *Proc. Natl. Acad. Sci. USA* **116**, 9521–6.
8. Komatsubara, A.T. *et al.* (2019) Single-cell quantification of the concentrations and dissociation constants of endogenous proteins. *J. Biol. Chem.* **294**, 6062–72.
9. Caine, E.A. *et al.* (2020) Targeted protein degradation phenotypic studies using HaloTag CRISPR/Cas9 endogenous tagging coupled with HaloPROTAC3. *Curr. Protoc. Pharmaco.* **91**, e81.
10. Grimm, J.B. *et al.* (2015) A general method to improve fluorophores for live-cell and single-molecule microscopy. *Nat. Methods* **12**, 244–50.

7. References (continued)

11. Schwinn, M.K. *et al.* (2018) CRISPR-mediated tagging of endogenous proteins with a luminescent peptide. *ACS Chem. Biol.* **13**, 467–74.

12. Riching, K.M. *et al.* (2018) Quantitative live-cell kinetic degradation and mechanistic profiling of PROTAC mode of action. *ACS Chem. Biol.* **13**, 2758–70.

8. Related Products

Product	Size	Cat.#
Janelia Fluor® 646 HaloTag® Ligand	5ug	GA1120
Janelia Fluor® 549 HaloTag® Ligand	5µg	GA1110
Anti-HaloTag® pAb	200µg	G9281
Anti-HaloTag® Monoclonal Antibody	200µg	G9211
Nano-Glo® HiBiT Lytic Detection System	10ml	N3030
	100ml	N3040
	10 × 100ml	N3050
Nano-Glo® Live Cell Assay System	100 assays	N2011
	1,000 assays	N2012
	10,000 assays	N2013
Nano-Glo® Endurazine Substrate	0.1ml	N2570
	1ml	N2571
	10ml	N2572

[a]BY USE OF THIS PRODUCT, RESEARCHER AGREES TO BE BOUND BY THE TERMS OF THIS LIMITED USE LABEL LICENSE. If researcher is not willing to accept the terms of this label license, and the product is unused, Promega will accept return of the unused product and provide researcher with a full refund.

Researcher may use this product for research use only; no transfer or commercial use of this product is allowed. Commercial use means any and all uses of this product by a party in exchange for consideration, including, but not limited to (1) use in further product manufacture; (2) use in provision of services, information or data; and (3) resale of the product or its derivatives, whether or not such product or derivatives are resold for use in research. With respect to any uses outside this label license, including any commercial, diagnostic, therapeutic or prophylactic uses, please contact Promega for supply and licensing information. PROMEGA MAKES NO REPRESENTATIONS OR WARRANTIES OF ANY KIND, EITHER EXPRESSED OR IMPLIED, INCLUDING FOR MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE WITH REGARDS TO THE PRODUCT. The terms of this label license shall be governed under the laws of the State of Wisconsin, USA.

[b]Licensed from Yale University under U.S. Pat. No. 10,730,862.

[c]U.S. Pat. Nos. 8,168,405; 8,257,939; 9,540,402; and other patents and patents pending.

GloMax, HaloTag, Nano-Glo and NanoLuc are registered trademarks of Promega Corporation. NanoBRET is a trademark of Promega Corporation.

Corning is a registered trademark of Corning, Inc. FuGENE is a registered trademark of Fugent, L.L.C., USA. GIBCO and Opti-MEM are registered trademarks of Thermo Fisher Scientific. Janelia Fluor is a registered trademark of Howard Hughes Medical Institute.

Products may be covered by pending or issued patents or may have certain limitations. Please visit our Web site for more information.

All prices and specifications are subject to change without prior notice.

Product claims are subject to change. Please contact Promega Technical Services or access the Promega online catalog for the most up-to-date information on Promega products.