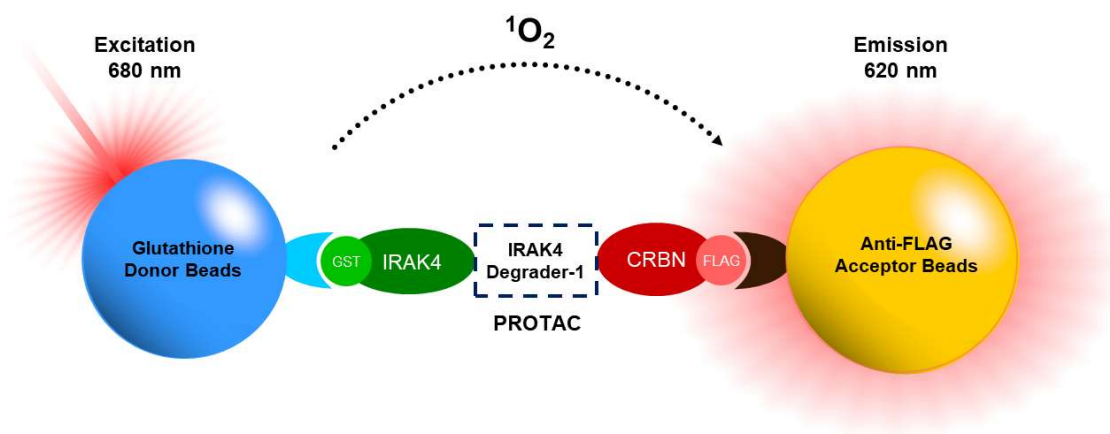


## Description

The PROTAC® Optimization Kit for IRAK4-Cereblon Binding is designed for the testing and profiling of PROTACs® directed against IRAK4 and Cereblon (CRBN). This Kit comes in a convenient AlphaLISA™ format, with the IRAK4 Degradator-1 (PROTAC) added as a positive control, an optimized PROTAC® Assay buffer, purified IRAK4 and CRBN proteins for 384 reactions. The IRAK4 ligand-1 is included as a control that blocks PROTAC® binding to IRAK4. With this kit, only three simple steps are required for the measurement of PROTAC® activity. First, the PROTAC® of interest is incubated with CRBN and IRAK4. Next, acceptor beads are added, then donor beads, followed by reading of the Alpha-counts.



*Figure 1. Illustration of the assay principle:* A PROTAC® of interest or positive control IRAK4 Degradator-1 (PROTAC®) interacts with both IRAK4 and CRBN, bringing them in close proximity. IRAK4 contains a GST-tag, recognized by the GSH donor bead, while CRBN contains a FLAG-tag that binds to the AlphaLISA™ acceptor bead conjugated with an anti-FLAG antibody. Upon excitation of the donor bead, a singlet oxygen is generated by the donor bead, which excites the acceptor bead and emits light proportionally to the level of interaction. AlphaLISA™ immunoassays are a no-wash alternative to ELISA immunoassays. These assays are robust and ideal for a minimal hands-on approach.

## Background

CRBN (Cereblon) is the substrate-binding component of the E3 protein ligase complex DDB1-CUL4A-RBX1 involved in the ubiquitination and proteasomal degradation of target proteins. Binding of CRBN to a substrate protein engages the E3 ligase activity of the complex and results in the ubiquitination and ultimate degradation of the protein substrate. Many proteins are known targets of CRBN, including several transcription factors, growth factors, kinases and more. CRBN has become a target of choice for the development of many therapeutic PROTACs®.

IRAK4 (interleukin-1 receptor-associated kinase 4), a member of the IRAK family, is a protein kinase involved in signaling innate immune responses from Toll-like receptors. It also mediates signaling from T-cell receptors. Human and rodent genetics support the role of IRAK4 in immune function and the involvement of IRAK4-dependent signaling in certain cancers. IRAK4 protein degraders have recently entered clinical trials.

## Applications

- Discover and optimize PROTACs® targeting IRAK4
- Design novel molecules targeting CRBN
- Compare the activities of different PROTACs®

### Supplied Materials

Catalog #	Name	Amount	Storage
100329	FLAG-Cereblon*	5 µg	-80°C
40064	GST-IRAK4*	20 µg	-80°C
	PROTAC® IRAK4 Degradator-1 (solid, MW=905 Da)	20 µg	-80°C
	5X PP-02 Buffer	4 ml	-20°C
	IRAK4 Ligand-1 (solid, MW=613 Da)	20 µg	-20°C

\* The concentration of the proteins is lot-specific and will be indicated on the tube.

### Materials Required but Not Supplied

Name	Catalog #
AlphaLISA™ anti-FLAG acceptor beads, 250 µg	PerkinElmer #AL112C
Alpha™ GSH donor beads, 1 mg	PerkinElmer #6765300
Optiplate 384	PerkinElmer #6007290
AlphaScreen™ microplate reader	
Adjustable micropipettor and sterile tips	

### Storage Conditions



This assay kit will perform optimally for up to 6 months from date of receipt when the materials are stored as directed.

### Safety



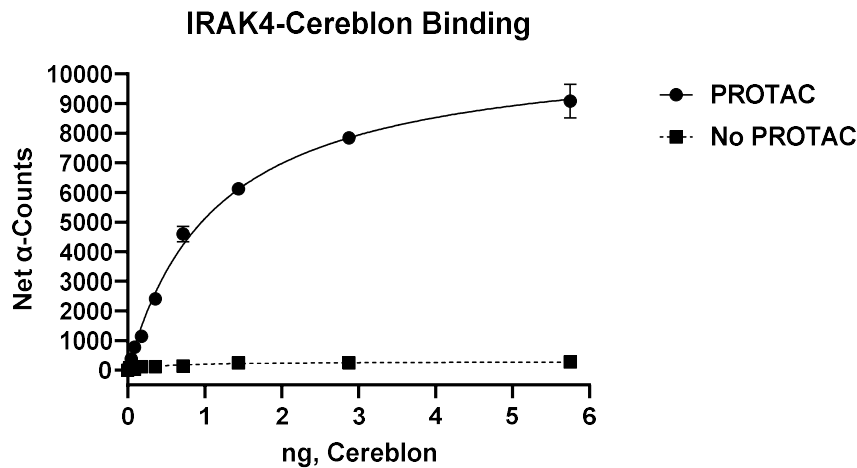
**IRAK4 Degradator-1 is a pomalidomide-derivative, which is known to cause severe birth defects in humans. Use all appropriate precautions when handling this compound!**

Kit components should be considered hazardous and harmful by inhalation, in contact with skin, eyes, clothing, and if swallowed. If contact occurs, wash thoroughly. This product is for research purposes only and not for human or therapeutic use.

### Contraindications

Green and blue dyes, such as Trypan Blue, absorb light in the AlphaScreen™ signal emission range (520-620 nm). Avoid the use of the potent singlet oxygen quenchers such as sodium azide (NaN<sub>3</sub>) or metal ions (Fe<sup>2+</sup>, Fe<sup>3+</sup>, Cu<sup>2+</sup>, Zn<sup>2+</sup> and Ni<sup>2+</sup>). The presence of >1% RPMI 1640 culture medium leads to a signal reduction due to the presence of excess biotin and iron in this medium. MEM, which lacks these components, does not affect AlphaScreen™ assays.

## Example of Assay Results



*Figure 2. Titration of CRBN at fixed concentration of IRAK4.*

A fixed amount of IRAK4 was added to increasing concentrations of Cereblon in the presence or in the absence of a fixed concentration of IRAK4 Degrader-1 (PROTAC®). The IRAK4 Degrader-1-mediated interaction of Cereblon with IRAK4 was quantified using the PROTAC® Optimization Kit for IRAK4-Cereblon Binding.

### Assay Protocols

All samples and controls should be tested in duplicate. All incubations are performed with slow shaking on a rotator platform.

#### ASSAY PROTOCOL 1 - Optimization of IRAK4-Cereblon Binding

This protocol is designed to test the binding affinity of various PROTACs® of interest to IRAK4 or Cereblon.

*Prepare the reagents:*

1. Prepare **1x Assay Buffer** by adding 1 volume of stock Assay Buffer PP-02 and 4 volumes of distilled water.
  - a. Prepare only the amount needed for the experiment.
  - b. Aliquot the remaining undiluted Assay Buffer PP-02 and store at -20°C.
2. Prepare IRAK4 Degrader-1.
  - a. Dissolve 20 µg of **IRAK4 Degrader-1** with 22 µl of DMSO to obtain a 1 mM stock solution.
  - b. Prepare an intermediate solution by diluting the 1 mM stock IRAK4 Degrader-1 solution 125-fold with 1x Assay Buffer to obtain an 8 µM solution.
  - c. Prepare only the amount needed for the experiment.
  - d. Aliquot the remaining undiluted stock IRAK4 Degrader-1 and store at -80°C.

*Note: The final concentration of IRAK4 Degradar-1 in the assay is 2 mM.*

3. Thaw **Cereblon** and **IRAK4** proteins on ice.
  - a. Briefly spin the tubes containing the proteins to recover their full content.
  - b. Use only the amount of protein required for your assay (as described in steps 4 and 5).
  - c. Aliquot the remaining undiluted proteins for single use and store at -80°C immediately.

*Example: If you will use the plate on 4 occasions, aliquot the remaining proteins in 3 aliquots each.*



*Both IRAK4 and Cereblon are sensitive to freeze/thaw cycles. Do not re-use thawed aliquots more than once and do not reuse the diluted proteins.*

4. Dilute Cereblon in 1x Assay Buffer at 2.3 ng/μl. Keep the diluted protein on ice until use. Discard the diluted protein after use.
5. Dilute IRAK4 in 1x Assay Buffer at 20 ng/μl. Keep the diluted protein on ice until use. Discard the diluted protein after use.

*Prepare the reaction:*

1. Prepare the Master Mix (**7.5 μl/well**): N wells × (2.5 μl of diluted Cereblon + 2.5 μl of diluted IRAK4 + 2.5 μl of 1x Assay Buffer). Add 7.5 μl of master mix to every well.
2. For the wells labeled as "Blank", add 2.5 μl of 1x Assay Buffer.
3. Prepare the **Test PROTAC® (2.5 μl/well)**: For a titration, prepare serial dilutions at concentrations 4-fold higher than the desired final concentrations. The final volume of the reaction is 10 μl.

*Without DMSO*

- 3.1. If the Test Inhibitor is water-soluble, prepare serial dilutions in the 1x Assay Buffer, 4-fold more concentrated than the desired final concentrations.

*With DMSO*

- 3.2. If the Test inhibitor is soluble in DMSO, prepare the test inhibitor at 100-fold the highest desired concentration in DMSO, then dilute the inhibitor 25-fold in 1x Assay Buffer to prepare the highest concentration of the 4-fold intermediate dilutions. The concentration of DMSO is now 4%.
  - a. Using 1x Assay Buffer in 4% DMSO, prepare serial dilutions of the Test Inhibitor at 4-fold the desired final concentrations to keep the concentration of DMSO constant.
  - b. For positive and negative controls, prepare 4% DMSO in 1x Assay Buffer (vol/vol) so that all wells contain the same amount of DMSO (Diluent Solution).

*Note: The final concentration of DMSO in the assay should not exceed 1%.*

4. Add 2.5 µl of diluted Test PROTAC® to each well designated “Test PROTAC®”.
5. Add 2.5 µl of diluted IRAK4 Degradar-1 to each well designated “Positive Control”.

Component	Blank	Positive Control	Test PROTAC®
Master Mix	7.5 µl	7.5 µl	7.5 µl
1x Assay Buffer	2.5 µl	-	-
Test PROTAC®	-	-	2.5 µl
IRAK4 Degradar-1 (8 µM)	-	2.5 µl	-
<b>Total</b>	<b>10 µl</b>	<b>10 µl</b>	<b>10 µl</b>

6. Incubate at room temperature for 30 minutes.



**Protect your samples from direct exposure to light for the remaining of the protocol. Photobleaching will occur.**

7. Dilute the anti-FLAG Acceptor beads (PerkinElmer #AL112C) 250-fold with 1x Assay Buffer.
8. Add 10 µl per well. Shake on a rotator platform for 30-60 minutes at room temperature.
9. Dilute the GSH donor beads (PerkinElmer #6765300) 125-fold with 1x Assay Buffer.
10. Add 10 µl per well. Shake on a rotator platform for 30 minutes at room temperature.
11. Read the Alpha-counts. The “Blank” value should be subtracted from all readings.

#### **ASSAY PROTOCOL 2 - Competitive Inhibition of the test PROTAC®**

This protocol is designed to measure the inhibition of the test PROTAC® binding to IRAK4. The protocol can be easily modified to study inhibitors of PROTAC® directed to Cereblon.

*Note: All samples and controls should be tested in duplicate. All incubations are performed with slow shaking on a rotator platform.*

*Prepare the reagents:*

1. Prepare **1x Assay Buffer** by adding 1 volume of stock PROTAC® Assay Buffer PP-02 and 4 volumes of distilled water.
  - a. Prepare only the amount needed for the experiment.
  - b. Aliquot the remaining undiluted PROTAC® Assay Buffer PP-02 and store at -20°C.
2. Prepare **IRAK4 Degradar-1** (PROTAC®).
  - a. Dissolve 20 µg of IRAK4 Degradar-1 with 22 µl of DMSO to obtain a 1 mM stock solution.

- b. Prepare an intermediate solution by diluting the 1 mM stock IRAK4 Degradator-1 solution 125-fold with 1x buffer to obtain an 8 µM solution.
  - c. Prepare only the amount needed for the experiment.
  - d. Aliquot the remaining undiluted stock IRAK4 Degradator-1 and store at -80°C.
3. Thaw **Cereblon** and **IRAK4** proteins on ice. Briefly spin the tubes containing the proteins to recover their full content.
    - a. Use only the amount of protein required for your assay (as described in steps 4 and 5).
    - b. Aliquot the remaining undiluted proteins for single use and store at -80°C immediately

*Example: If you will use the plate on 4 occasions, aliquot the remaining proteins in 3 aliquots each.*



*Both IRAK4 and Cereblon are sensitive to freeze/thaw cycles. Do not re-use thawed aliquots more than once and do not reuse the diluted proteins.*

4. Dilute Cereblon in 1x Assay Buffer at 2.3 ng/µl. Keep the diluted protein on ice until use. Discard any unused diluted enzyme after use.
5. Dilute IRAK4 in 1x Assay Buffer at 20 ng/µl. Keep the diluted protein on ice until use. Discard any unused diluted enzyme after use.

*Prepare the reaction:*

1. Prepare the Master Mix (**5 µl/well**): N wells × (2.5 µl of diluted Cereblon + 2.5 µl of diluted IRAK4). Add 5 µl of master mix to every well.
2. For the wells labeled as "Blank", add 2.5 µl of 1x Assay Buffer.
3. Prepare the **Test Inhibitor (2.5 µl/well)**: for a titration, prepare serial dilutions at concentrations 4-fold higher than the desired final concentrations. The final volume of the reaction is 10 µl.

*Without DMSO*

- 3.1. If the Test Inhibitor is water-soluble, prepare serial dilutions in the 1x Assay Buffer, 4-fold more concentrated than the desired final concentrations.

*With DMSO*

- 3.2. If the Test inhibitor is soluble in DMSO, prepare the test inhibitor at 100-fold the highest desired concentration in DMSO, then dilute the inhibitor 25-fold in 1x Assay Buffer to prepare the highest concentration of the 4-fold intermediate dilutions. Final concentration of DMSO is 4%.

- a. Using 1x Assay Buffer in 4% DMSO, prepare serial dilutions of the Test Inhibitor at 4-fold the desired final concentrations to keep the concentration of DMSO constant.

- b. For positive and negative controls, prepare 4% DMSO in 1x Assay Buffer (vol/vol) so that all wells contain the same amount of DMSO (Diluent Solution).

*Note: The final concentration of DMSO in the assay should not exceed 1%.*

- c. Add 2.5 µl of the Diluent Solution (1x Assay Buffer with 4% DMSO) to the "Positive Control" and "Blank".
4. Add 2.5 µl of Test Compound to each well designated "Test Inhibitor".
  5. Prepare the **IRAK4 Ligand-1** dilution: Dissolve 20 µg of IRAK4 Ligand-1 with 13 µl of DMSO to obtain a 2.5 mM stock solution. Dilute this stock solution 25-fold in 1x Assay Buffer to obtain a 100 µM solution. For the wells labeled as "**IRAK4 Ligand-1**", add 2.5 µl of the diluted IRAK4 Ligand-1.

*Note: The recommended final concentration of IRAK4 Ligand-1 in the reaction is 25 µM.*

6. Preincubate for up to 30 minutes at room temperature with slow shaking.
7. Initiate the reaction by adding 2.5 µl of diluted **IRAK4 Degrader-1** (PROTAC®, 8 µM) prepared as described above to the wells labeled "Positive Control", "IRAK4 Ligand-1" and "Test Inhibitor". **Do NOT add IRAK4 Ligand-1 or IRAK4 Degrader-1 to the "Blank"**.

Component	Blank	Positive Control	IRAK4 Ligand-1	Test Inhibitor
Master Mix	5 µl	5 µl	5 µl	5 µl
1x Assay Buffer	2.5 µl	-	-	-
Test Inhibitor	-	-	-	2.5 µl
Diluent Solution	2.5 µl	2.5 µl	-	-
IRAK4 Ligand-1	-	-	2.5 µl	-
IRAK4 Degrader-1 (8 µM)	-	2.5 µl	2.5 µl	2.5 µl
<b>Total</b>	<b>10 µl</b>	<b>10 µl</b>	<b>10 µl</b>	<b>10 µl</b>

8. Incubate at room temperature for 30 minutes with slow shaking.



**Protect your samples from direct exposure to light for the remaining of the protocol. Photobleaching will occur.**

9. Dilute the anti-FLAG Acceptor beads (PerkinElmer #AL112C) 250-fold with 1x Assay Buffer.
10. Add 10 µl per well.
11. Shake on a rotator platform for 30-60 minutes at room temperature.
12. Dilute the GSH donor beads (PerkinElmer #6765300) 125-fold with 1x Assay Buffer. Add 10 µl per well.
13. Shake on a rotator platform for 30 minutes at room temperature.

14. Read the Alpha-counts. The “Blank” value should be subtracted from all readings.

### Example of Assay Results

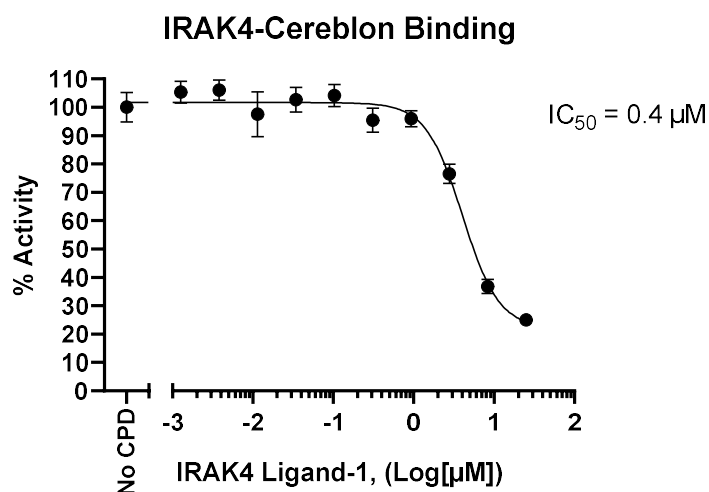


Figure 3. Effect of IRAK4 inhibitor on PROTAC®-mediated IRAK4-CRBN binding.

Inhibition of IRAK4 Degrader-1 (PROTAC®)-mediated interaction of Cereblon with IRAK4 was measured in the presence of increasing concentrations of IRAK4 Ligand-1 (IRAK4 inhibitor) using the PROTAC® Optimization Kit for IRAK4-Cereblon Binding.

For lot-specific information, please contact BPS Bioscience, Inc. at [support@bpsbioscience.com](mailto:support@bpsbioscience.com)

### General Considerations

**Plates and Instruments:** A plate reader capable of Alpha technology detection is required. We recommend using PerkinElmer 384-Optiplate #6007290.

**“Blank” Control:** The “Blank” control is important to determine the background signal in the assay. We recommend doing these at least in duplicate.

**“Positive Control”:** The “Positive Control” is the maximum signal determined by the addition of a PROTAC® molecule known to bind IRAK4 and CRBN, i.e. IRAK4 Degrader-1 (PROTAC®).

### Troubleshooting Guide

Visit [bpsbioscience.com/assay-kits-faq](https://bpsbioscience.com/assay-kits-faq) for detailed troubleshooting instructions. For all further questions, please email [support@bpsbioscience.com](mailto:support@bpsbioscience.com)

### References

Nunes, J., *et al.* Targeting IRAK4 for Degradation with PROTACs. *ACS Med Chem Lett* 2019; **10(7)**: 1081-1085.

Version 010324



**Related Products**

<i>Products</i>	<i>Catalog #</i>	<i>Size</i>
PROTAC® Optimization Kit for BET Bromodomain-Cereblon Binding	79770	384 rxns.
PROTAC® Optimization Kit for CDK Kinase-Cereblon Binding	79924	384 rxns.
PROTAC® Optimization Kit for BRD9-Cereblon Binding	78420	384 rxns.
PROTAC® Optimization Kit for BET Bromodomain-Von Hippel Lindau (VHL) Binding	79790	384 rxns.
PROTAC® Optimization Kit for PARP1-Cereblon Binding	78441	384 rxns.
Cereblon Intrachain TR-FRET Assay Kit	78301	384 rxns.
Cereblon Ubiquitination Homogeneous Assay Kit	79881	384 rxns.
Cereblon Binding Assay Kit	79899	96 rxns.