Description

The PROTAC® Optimization Kit for IRAK4-Cereblon Binding is designed for the testing and profiling of PROTACs® (proteolysis targeting chimera) directed against IRAK4 (interleukin-1 receptor-associated kinase 4) and Cereblon (CRBN). This kit comes in a convenient AlphaLISA™ format, with the IRAK4 Degrader-1 (PROTAC) as a positive control, an optimized PROTAC® Assay buffer, purified IRAK4 and CRBN complex proteins for 384 reactions. The IRAK4 Ligand-1 is included as a control that blocks PROTAC® binding to IRAK4.

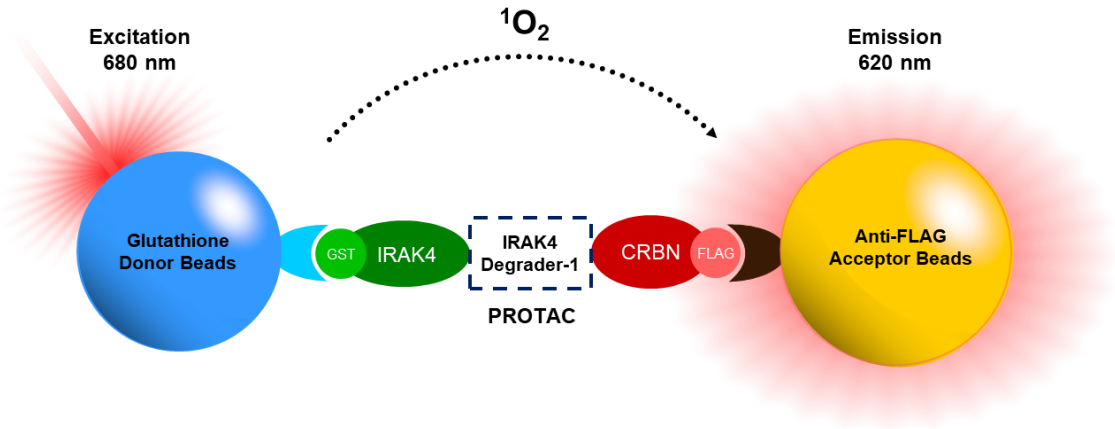

Figure 1. Illustration of the assay principle.

A PROTAC® of interest or positive control IRAK4 Degrader-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(damage-specific DNA-binding protein 1)-CUL4A (cullin 4)-RBX1 (RING-box protein 1) 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 (TLR). It also mediates signaling from T-cell receptors (TCRs). 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 and hold great promise in the cancer therapy.

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	Cereblon/DDB1/Cul4A/Rbx1 Complex*	5 µg	-80°C
40064	IRAK4, GST-Tag*	20 µg	-80°C
83521	PROTAC® IRAK4 Degrader-1 (solid, MW=905 Da)	20 µg	-80°C
82620	5x PP-02 Buffer	4 ml	-20°C
83522	IRAK4 Ligand-1 (solid, MW=613 Da)	20 µg	-80°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 Degrader-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

- The final concentration of DMSO in the assay should not exceed 1%.
- Avoid green and blue dyes that absorb light in the AlphaScreen signal emission range (λ=520-620 nm), such as Trypan Blue.
- Avoid the use of potent singlet oxygen quenchers such as sodium azide (NaN3) or metal ions (Fe^{2+}, Fe^{3+}, Cu^{2+}, Zn^{2+} and Ni^{2+}).
- 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. Media like MEM, which lacks these components, does not affect AlphaScreen assays.

Assay Protocols

- This protocol is designed to test the binding affinity of various PROTACs to the IRAK4 or Cereblon complex.
- All samples should be run in duplicate while controls should be performed in quadruplicate.
- The assay should include “Blank”, “Positive Control” and “Test PROTAC”/“Test Compound” conditions.
- We recommend maintaining the diluted protein on ice during use.
- For detailed information on protein handling please refer to.
- All incubations should be performed with slow shaking on a rotator platform.
- We recommend using IRAK4 Ligand-1 as internal control. If not running a dose response curve for the control inhibitor, we recommend running the control inhibitor at 0.1X, 1X and 10X the IC_{50} value shown in the validation data below.
- For instructions on how to prepare reagent dilutions please refer to Serial Dilution Protocol (bpsbioscience.com).

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 diluting 5x PP-02 Buffer 5-fold with distilled water.

   Note: The remaining undiluted 5x PP-02 Buffer can be aliquoted and stored at -20°C.

2. Add 22 µl of DMSO to the 20 µg of **IRAK4 Degrader-1**. This makes a 1 mM stock solution.

3. 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.

   Note: The remaining undiluted stock IRAK4 Degrader-1 can be aliquoted and stored at -80°C (minimum 5 µl per aliquot).

4. Thaw **Cereblon** and **IRAK4** proteins on ice. Briefly spin the tubes containing the proteins to recover their full content.

5. Dilute Cereblon with 1x Assay Buffer to 2.3 ng/µl (2.5 µl/well).

6. Dilute IRAK4 with 1x Assay Buffer to 20 ng/µl (2.5 µl/well).

Prepare the reaction:

1. Prepare a **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).

2. Add 7.5 µl of Master Mix to every well.

3. For the wells labeled as "Blank", add 2.5 µl of 1x Assay Buffer.

4. 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.

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

   For the positive and negative controls, use 1x Assay Buffer (Diluent Solution).

   OR

   4.2. 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%.

   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.

   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%.

5. Add 2.5 µl of diluted Test PROTAC® to each well designated "Test PROTAC®".

6. Add 2.5 µl of diluted IRAK4 Degrader-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
Diluted IRAK4 Degrader-1 (8 µM)	-	2.5 µl	-
Total	**10 µl**	**10 µl**	**10 µl**

7. Incubate at Room Temperature (RT) for 30 minutes.

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

8. Dilute the **Anti-FLAG Acceptor Beads** 250-fold with 1x Assay Buffer (10 µl/well).

9. Add 10 µl of diluted Anti-FLAG Acceptor Beads per well.

10. Shake on a rotator platform for 30-60 minutes at RT.

11. Dilute the **GSH Donor Beads** 125-fold with 1x Assay Buffer (10 µl/well).

12. Add 10 µl per well of diluted GSH Donor Beads.

13. Shake on a rotator platform for 30 minutes at RT.

14. Read the Alpha-counts.

15. 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.

Prepare the reagents:

1. Prepare **1x Assay Buffer** by diluting 5x PP-02 Buffer 5-fold with distilled water.

   Note: The remaining undiluted 5x PP-02 Buffer can be aliquoted and stored at -20°C.

2. Add 22 µl of DMSO to the 20 µg of **IRAK4 Degrader-1**. This makes a 1 mM stock solution.

3. 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.

   Note: The remaining undiluted stock IRAK4 Degrader-1 can be aliquoted and stored at -80°C (minimum 5 µl volume per aliquot).

4. Thaw **Cereblon** and **IRAK4** proteins on ice. Briefly spin the tubes containing the proteins to recover their full content.

5. Dilute Cereblon with 1x Assay Buffer to 2.3 ng/µl (2.5 µl/well).

6. Dilute IRAK4 with 1x Assay Buffer to 20 ng/µl (2.5 µl/well).

Prepare the reaction:

1. Prepare a **Master Mix** (5 µl/well): N wells × (2.5 µl of diluted Cereblon + 2.5 µl of diluted IRAK4).

2. Add 5 µl of Master Mix to every well.

3. For the wells labeled as "Blank", add 2.5 µl of 1x Assay Buffer.

4. 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.

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

For the positive and negative controls, use 1x Assay Buffer (Diluent Solution).

OR

4.2 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%.

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.

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%.

5. Add 2.5 µl of the Diluent Solution to the “Positive Control" and "Blank” wells.
6. Add 2.5 µl of Test Compound to each well designated “Test Compound”.
7. Add 13 µl of DMSO to the 20 µg of **IRAK4 Ligand-1**. This makes a 2.5 mM stock solution.
8. Dilute this stock solution 25-fold with 1x Assay Buffer to obtain a 100 µM solution (2.5 µl/well).
9. Add 2.5 µl of the diluted IRAK4 Ligand-1 to the wells labeled as "**IRAK4 Ligand-1**".

   Note: The recommended final concentration of IRAK4 Ligand-1 in the reaction is 25 µM.
10. Preincubate for up to 30 minutes at RT with slow agitation.
11. Initiate the reaction by adding 2.5 µl of diluted **IRAK4 Degrader-1** (PROTAC®, 8 µM) the wells labeled “Positive Control", “IRAK4 Ligand-1” and “Test Compound”.

Component	Blank	Positive Control	IRAK4 Ligand-1	Test Compound
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	-	-
Diluted IRAK4 Ligand-1	-	-	2.5 µl	-
Diluted IRAK4 Degrader-1 (8 µM)	-	2.5 µl	2.5 µl	2.5 µl
Total	**10 µl**	**10 µl**	**10 µl**	**10 µl**

12. Incubate at RT for 30 minutes with slow shaking.

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

13. Dilute the **Anti-FLAG Acceptor Beads** 250-fold with 1x Assay Buffer (10 µl/well).
14. Add 10 µl per well of diluted Anti-FLAG Acceptor Beads.
15. Shake on a rotator platform for 30-60 minutes at RT.
16. Dilute the **GSH Donor Beads** 125-fold with 1x Assay Buffer (10 µl/well).
17. Add 10 µl per well of diluted GSH Donor Beads.
18. Shake on a rotator platform for 30 minutes at RT.
19. Read the Alpha-counts.
20. The "Blank" value should be subtracted from all readings.

Example of Assay Results

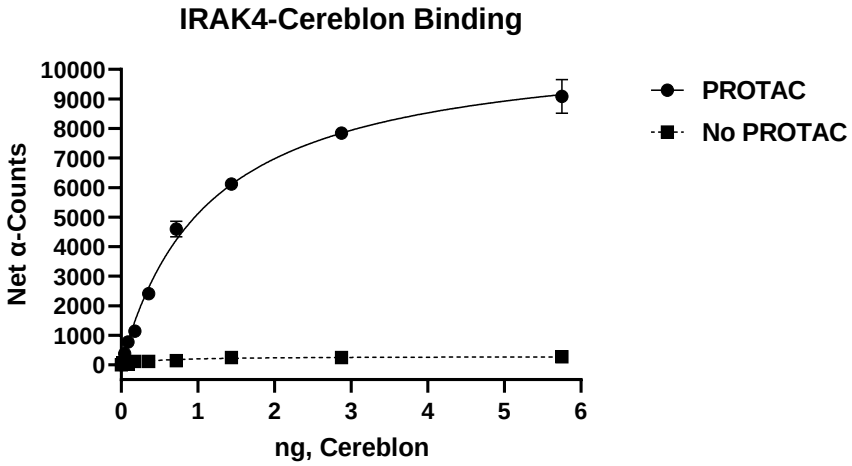

Figure 2. Titration of CRBN in the presence and absence of a PROTAC.
A fixed amount of IRAK4 was added to increasing concentrations of Cereblon in the presence or absence of a fixed concentration of IRAK4 Degrader-1 (PROTAC®).

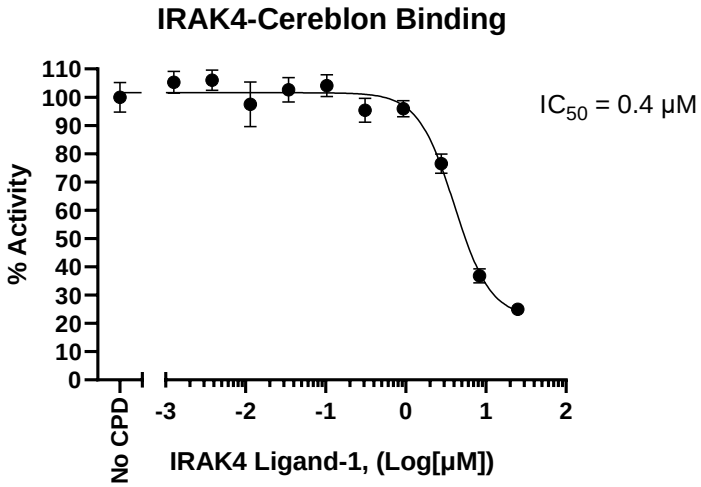

Figure 3. Effect of IRAK4 Ligand-1, an IRK4 inhibitor, on PROTAC®-mediated IRAK4-CRBN binding. Inhibition of IRAK4 Degrader-1 (PROTAC®)-mediated interaction of Cereblon with IRAK4 was measured in the presence of increasing concentrations of IRAK4 Ligand-1 (IRAK4 inhibitor).

Data shown is representative.

Troubleshooting Guide

Visit bpsbioscience.com/assay-kits-faq for detailed troubleshooting instructions. For lot-specific information and all other questions, please visit https://bpsbioscience.com/contact.

References

Nunes J., *et al.,* 2019 *ACS Med Chem Lett* 10(7): 1081-1085.

Related Products

Products	*Catalog #*	*Size*
PROTAC® Optimization Kit for BET Bromodomain-Cereblon Binding	79770	384 reactions
PROTAC® Optimization Kit for CDK Kinase-Cereblon Binding	79924	384 reactions
PROTAC® Optimization Kit for BRD9-Cereblon Binding	78420	384 reactions
PROTAC® Optimization Kit for BET Bromodomain-Von Hippel Lindau (VHL) Binding	79790	384 reactions
PROTAC® Optimization Kit for PARP1-Cereblon Binding	78441	384 reactions
Cereblon Intrachain TR-FRET Assay Kit	78301	384 reactions

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