Description

The PROTAC® Optimization Kit for BRD9-Cereblon Binding is designed for the testing and profiling of PROTACs® directed against BRD9 (Bromodomain-containing protein 9) and Cereblon (CRBN). This kit comes in a convenient AlphaLISA™ format, with the BRD9 Degrader dBRD9 (PROTAC®) added as positive control, BRD9 buffer, and purified BRD9 and CRBN proteins for 400 reactions. The BRD9 inhibitor BI-7273 is included as a control inhibitor of PROTAC® binding to BRD9.

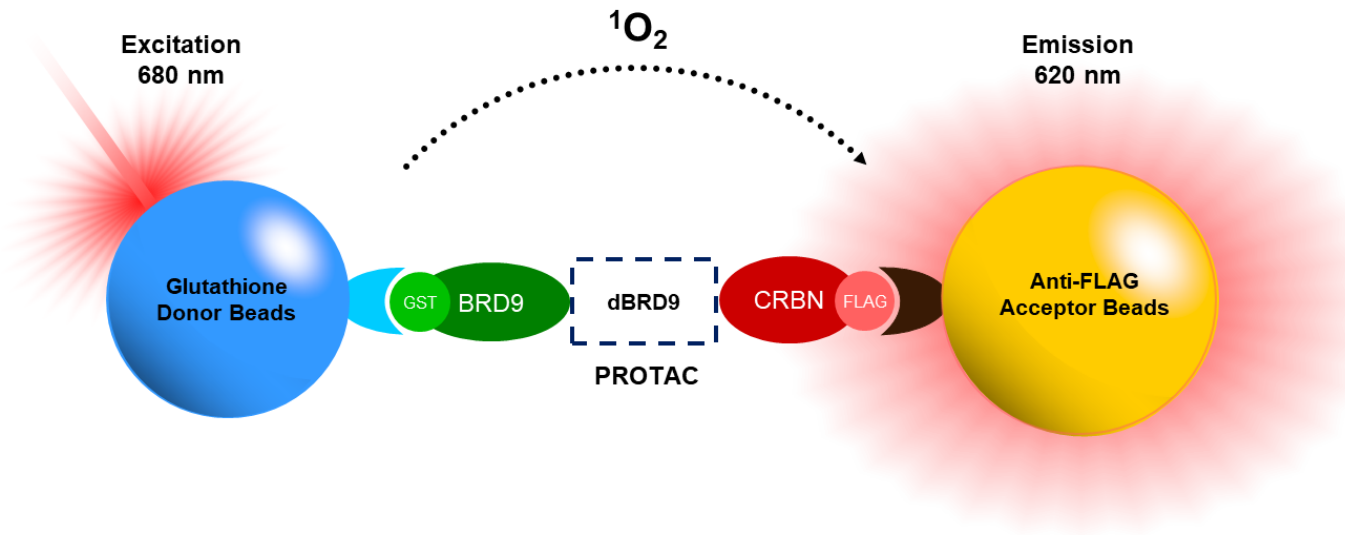

Figure 1:Illustration of the assay principle.

A PROTAC® of interest or positive control dBRD9 (PROTAC®) interacts with both BRD9 and CRBN, bringing them in close proximity. BRD9 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. Cereblon binds to DDB1 (Damaged DNA binding protein 1), to the scaffolding protein CUL4A (Cullin 4A), and its regulator RBX1 (RING-Box protein 1). 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®. BRD9 (Bromodomain-containing protein 9) functions as a transcriptional regulator and is a component of a chromatin remodeling complex. It also regulates the formation of the RAD51-RAD54 complex, involved in homologous recombination. BRD9 plays a role in cancer and is a potential therapeutic target for cancer drugs.

Applications

- Discover and optimize PROTACs® targeting BRD9.
- Design novel molecules targeting CRBN.
- Compare the activities of different PROTACs®.

Supplied Materials

Catalog #	Name	Amount	Storage
100255-KC5	Cereblon, FLAG-Tag*	5 µg	-80°C
31091-KC40	GST-BRD9*	40 µg	-80°C
84081-KC250	dBRD9 PROTAC® (MW=884 Da)	250 µg	-80°C
84082-KC4	3x BRD9 PROTAC® Buffer**	4 ml	-20°C
84083-KC15	10 mM BI-7273 (MW=353 Da)	15 µl	-20°C
82735-KC200	0.5 M DTT	200 µl	-20°C

**The concentration of the proteins is lot-specific and will be indicated on the tube.*
***Add 30 µl of 0.5 mM DTT to the assay buffer before experiment.*

Materials Required but Not Supplied

Name	Catalog #
AlphaLISA™ anti-FLAG Acceptor Beads, 5 mg/ml	PerkinElmer #AL112C
Alpha™ GSH Donor Beads, 5 mg/ml	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

Kit components should be considered hazardous and is 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 (NaN_3) 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 protocol 1 - Optimization of Bromodomain-Cereblon Binding

- This protocol is designed to test the binding affinity of various PROTACs® of interest to BRD9 or Cereblon.
- All samples and controls should be performed in duplicate.
- The assay should include "Blank", "Positive Control" and "Test PROTAC" conditions.
- We recommend maintaining the diluted protein on ice during use.
- For detailed information on protein handling please refer to Protein FAQs (bpsbioscience.com).
- All incubations should be performed with slow agitation on a rotator platform.

STEP 1

1. Prepare 1x BRD9 PROTAC® Buffer by diluting 3x BRD9 PROTAC® Buffer 3-fold with distilled water. Add 30 µl of 0.5M DTT.

   Note: Prepare only the amount needed for the experiment. Aliquot the remaining undiluted BRD9 PROTAC® Buffer and store at -20°C.

2. Add 283 µl of distilled water to the vial of dBRD9 PROTAC®. This makes a 1 mM stock solution.

3. Dilute 1 mM dBRD9 PROTAC® solution 25-fold with 1x BRD9 PROTAC® Buffer to obtain a 40 µM solution. Dilute the 25-fold intermediate solution an additional 100-fold to obtain a 0.4 µM solution.

   Note: Prepare only the amount needed for the experiment. Aliquot the remaining undiluted stock dBRD9 and store at -80°C. The final concentration of dBRD9 in the assay is 100 nM.

4. Thaw Cereblon and BRD9 on ice. Briefly spin the tubes containing the proteins to recover their full content.

5. Prepare the following dilutions (2.5 µl/well):

   a. Dilute Cereblon with 1x BRD9 PROTAC® Buffer to 5 ng/µl.

   b. Dilute BRD9 in 1x BRD9 PROTAC® Buffer to 40 ng/µl.

6. Prepare a **Master Mix** (7.5 µl/well): N wells × (2.5 µl of diluted Cereblon + 2.5 µl of diluted BRD9 + 2.5 µl 1x BRD9 PROTAC® Buffer).

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

8. 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.

8.1 If the Test PROTAC is water-soluble, prepare serial dilutions 4-fold more concentrated than the desired final concentrations in 1x BRD9 PROTAC® Buffer.

For the positive and negative controls, use 1x BRD9 PROTAC® Buffer (Diluent Solution).

OR

8.2 If the Test PROTAC is soluble in DMSO, prepare the test PROTAC at 100-fold the highest desired concentration in 100% DMSO, then dilute the PROTAC 25-fold in 1x BRD9 PROTAC® Buffer to prepare the highest concentration of the 4-fold intermediate dilutions. The concentration of DMSO is now 4%.

Using 1x BRD9 PROTAC® Buffer with 4% DMSO, prepare serial dilutions of the Test PROTAC at 4-fold the desired final concentrations to keep the concentration of DMSO constant.

For positive and negative controls, prepare 4% DMSO in 1x BRD9 PROTAC® 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%.

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

10. Add 2.5 µl of diluted dBRD9 to each well designated "Positive Control".

11. Add 2.5 µl of Diluent Solution to the "Blank" wells.

Component	Blank	Positive Control	Test PROTAC
Master Mix	7.5 µl	7.5 µl	7.5 µl
Diluent Solution	2.5 µl	-	-
Test PROTAC®	-	-	2.5 µl
Diluted dBRD9 (0.4 µM)	-	2.5 µl	-
Total	**10 µl**	**10 µl**	**10 µl**

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

STEP 2

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

1. Dilute the anti-FLAG Acceptor Beads 250-fold with 1x BRD9 PROTAC® Buffer (10 µl/ well).

2. Add 10 µl per well.

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

4. Dilute the GSH Donor Beads 125-fold with 1x BRD9 PROTAC® Buffer (10 µl/ well).

5. Add 10 µl per well.

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

7. Read the Alpha-counts.

8. 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 BRD9. The protocol can be easily modified to study inhibitors of PROTAC® directed to Cereblon.
- All samples and controls should be performed in duplicate.
- The assay should include "Blank", "Positive Control", 'Inhibitor Control" and "Test Compound" conditions.
- We recommend maintaining the diluted protein on ice during use.
- For detailed information on protein handling please refer to Protein FAQs (bpsbioscience.com).
- All incubations should be performed with slow agitation on a rotator platform.

STEP 1

1. Prepare 1x BRD9 PROTAC® Buffer by diluting 3x BRD9 PROTAC® Buffer 3-fold with distilled water. Add 30 µl of 0.5M DTT.

   Note: Prepare only the amount needed for the experiment. Aliquot the remaining undiluted BRD9 PROTAC® Buffer and store at -20°C.

2. Add 283 µl of distilled water to the vial of dBRD9 PROTAC®. This makes a 1 mM stock solution.

3. Dilute 1 mM dBRD9 PROTAC® solution 25-fold with 1x BRD9 PROTAC® Buffer to obtain a 40 µM solution. Dilute the 25-fold intermediate solution an additional 100-fold to obtain a 0.4 µM solution.

   Note: Prepare only the amount needed for the experiment. Aliquot the remaining undiluted stock dBRD9 and store at -80°C. The final concentration of dBRD9 in the assay is 100 nM.

4. Thaw Cereblon and BRD9 on ice. Briefly spin the tubes containing the proteins to recover their full content.

5. Prepare the following dilutions (2.5 µl/well):

   c. Dilute Cereblon with 1x BRD9 PROTAC® Buffer to 5 ng/µl.

   d. Dilute BRD9 in 1x BRD9 PROTAC® Buffer to 40 ng/µl.

6. Prepare a **Master Mix** (5 µl/well): N wells × (2.5 µl of diluted Cereblon + 2.5 µl of diluted BRD9).

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

8. 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.

   8.3 If the Test Inhibitor is water-soluble, prepare serial dilutions 4-fold more concentrated than the desired final concentrations in 1x BRD9 PROTAC® Buffer.

   For the positive and negative controls, use 1x BRD9 PROTAC® Buffer (Diluent Solution).

   OR

   8.4 If the Test Inhibitor is soluble in DMSO, prepare the test PROTAC at 100-fold the highest desired concentration in 100% DMSO, then dilute the PROTAC 25-fold in 1x BRD9 PROTAC® Buffer to prepare the highest concentration of the 4-fold intermediate dilutions. The concentration of DMSO is now 4%.

   Using 1x BRD9 PROTAC® Buffer with 4% DMSO, prepare serial dilutions of the Test P 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 BRD9 PROTAC® 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%.

9. Add 2.5 µl of diluted Test Inhibitor to each well designated “Test Inhibitor”.

10. Add 2.5 µl of Diluent Solution to the “Blank” and “Positive Control” wells.

11. Add 10 µl of 10 mM BI-7273 to 240 µl of 1x BRD9 PROTAC® Buffer to obtain a 400 µM solution.

12. For the wells labeled as “Inhibitor Control", add 2.5 µl of diluted BI-7273.

13. Preincubate for up to 30 minutes at RT with slow agitation.

14. Initiate the reaction by adding 2.5 µl of diluted dBRD9 (0.4 µM) to the wells labeled “Positive Control", “Inhibitor Control” and “Test Inhibitor”.

15. Add 2.5 µl of 1x BRD9 PROTAC® Buffer to the “Blank” wells.

16. Incubate at RT for one hour with slow agitation.

Component	Blank	Positive Control	BI-7273	Test Inhibitor
Master Mix	5 µl	5 µl	5 µl	5 µl
1x BRD9 PROTAC® Buffer	2.5 µl	-	-	-
Test Compound	-	-	-	2.5 µl
Diluent Solution	2.5 µl	2.5 µl	-	-
Diluted BI-7273	-	-	2.5 µl	-
Diluted dBRD9 (0.4 µM)	-	2.5 µl	2.5 µl	2.5 µl
Total	**10 µl**	**10 µl**	**10 µl**	**10 µl**

STEP 2

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

1. Dilute the anti-FLAG Acceptor Beads 250-fold with 1x BRD9 PROTAC® Buffer (10 µl/ well).
2. Add 10 µl per well.
3. Shake on a rotator platform for 30-60 minutes at RT.
4. Dilute the GSH Donor Beads 125-fold with 1x BRD9 PROTAC® Buffer (10 µl/ well).
5. Add 10 µl per well.
6. Shake on a rotator platform for 30 minutes at RT.
7. Read the Alpha-counts.
8. The “Blank” value should be subtracted from all readings.

Example Results

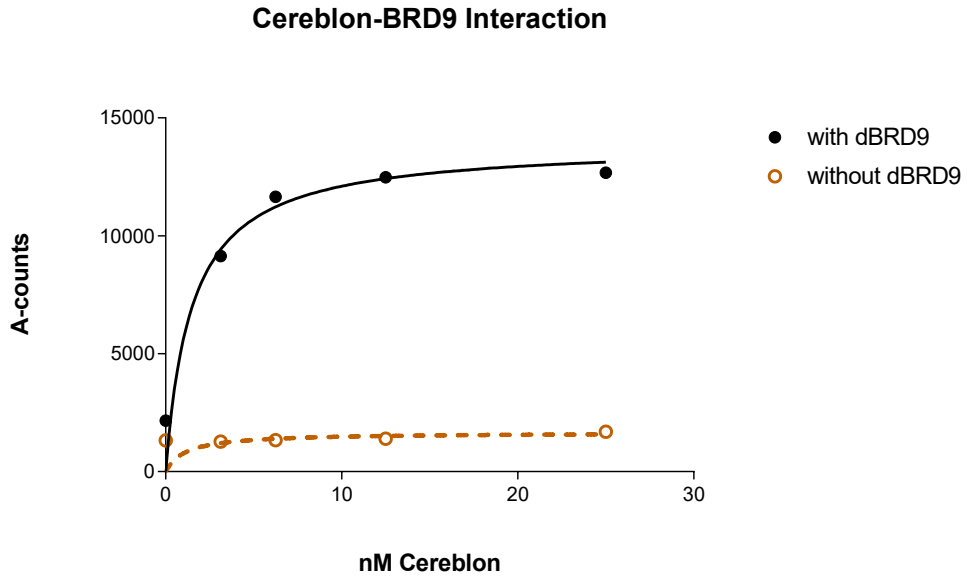

Figure 2: Titration of CRBN at fixed concentration of BRD9.
A fixed amount of BRB9 was added to increasing concentrations of Cereblon in the presence or in the absence of dRBD9 (PROTAC®).

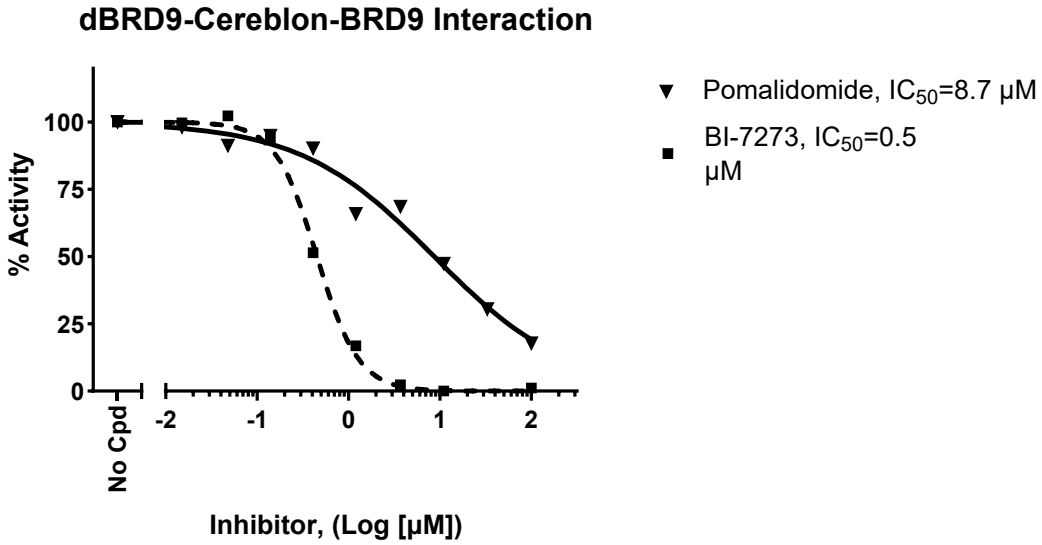

Figure 3: Inhibition by Pomalidomide or BI-7273 of PROTAC®-mediated interaction of Cereblon to BRD9.
Inhibition of PROTAC® mediated interaction of Cereblon with BRD9 was measured in the presence of increasing concentrations of Pomalidomide (BPS Bioscience #82026) and BI-7273.

Data shown is representative.

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 luminescence in the assay. We recommend doing these in duplicate.

"Positive Control": The "Positive Control" is the maximum signal determined by the addition of a PROTAC® molecule known to bind BRD9 and CRBN, i.e. dBRD9 (PROTAC).

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
Remillard D., *et al.,* 2017 *Angew Chem Int Ed Engl*. 56(21): 5738–5743.
Clark, P.G., *et al*., 2016 *Drug Discov Today Technol*. 2016; 19: 73-80.

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
BRD9 (BD1) Inhibitor Screening Assay Kit	32519	384 reactions
BRD9 TR-FRET Assay Kit	32621	384 reactions
BRD9, His-tag Recombinant	31090	100 µg
BRD9, GST-tag Recombinant	31091	100 µg

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