Description

The PROTAC® Optimization Kit for CDK-Cereblon Binding is designed for the testing and profiling of PROTACs directed against the CDK (Cyclin-dependent kinase family) and Cereblon. The PROTAC® Optimization Kit for CDK-Cereblon Binding comes in a convenient AlphaLISA® format, with enough BSJ-03-204 PROTAC®, CDK PROTAC® buffer, purified CDK4, CDK6, and CRBN for 384 reactions. The CDK inhibitor Palbociclib is included as a control inhibitor of PROTAC binding to CDK (internal control).

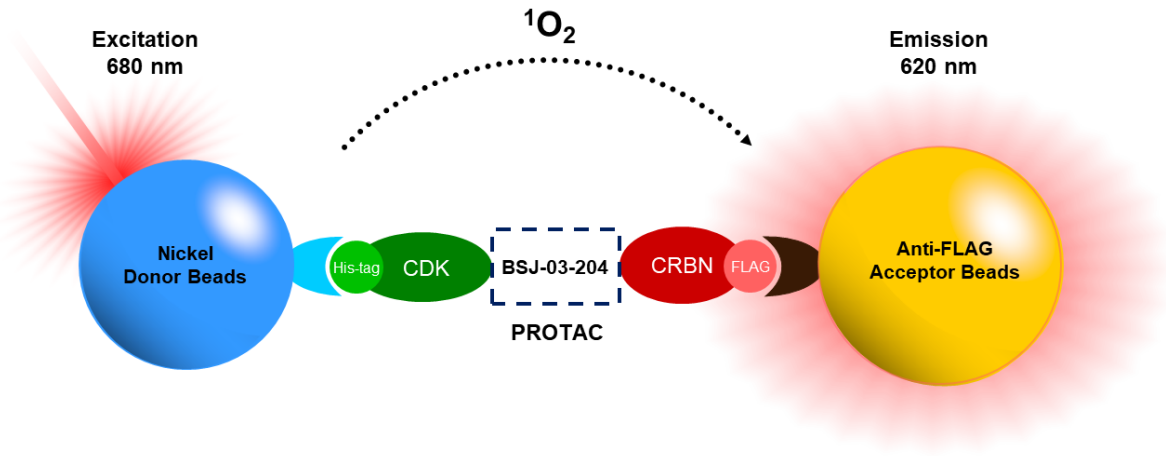

Figure 1. Schematic representation of CDK-Cereblon complex formation via BSJ-03-204 PROTAC®.

The PROTAC® of interest is incubated with CRBN and CDK4 or CDK6, bringing them in close proximity. CDK contains an His-tag, which is recognized by the donor bead. CRBN contains a FLAG tag that binds to the AlphaLISA™ acceptor bead, which is conjugated to an anti-FLAG antibody. Upon excitation of the donor bead, a singlet oxygen is generated by the donor bead. The singlet oxygen excites the acceptor bead, which emits light proportionally to the level of interaction.

Background

CDKs (cyclin-dependent Kinases) are a family of 20 kinases known for their role in regulating cell cycle, transcription and splicing. CDKs are organized in a pathway that safeguards the replication of DNA in each cell during cell division. The activity of CDKs is regulated by cyclins. Together CDK and cyclins, drive the cell cycle by phosphorylating other proteins. Several members of the CDK family have unique tissue specific functions and dysregulation of CDKs and their cyclin partners are associated with a range of tumor types. CDKs have emerged as potential therapeutic targets.

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

Application(s)

- Identify and optimize PROTACs targeting the CDK family of kinases.
- Design novel molecules targeting CRBN.
- Directly compare the activity of different PROTACs.

Supplied Materials

Catalog #	Name	Amount	Storage
100255	Cereblon, FLAG-Tag*	2 x 10 µg	-80°C
40104	CDK4/Cyclin D3, GST-His-Tag*	50 µg	-80°C
40206	CDK6/Cyclin D3, His-Tags*	30 µg	-80°C
	200 µM BSJ-03-204 PROTAC®	2 x 20 µl	-80°C
	3x CDK PROTAC® Buffer	4 ml	-20°C
	Palbociclib (MW = 574 Da)	120 µg	-80°C

**The initial concentration of enzyme is lot-specific and will be indicated on the tube containing the protein.*

Materials Required but Not Supplied

Component	Ordering Information
AlphaLISA anti-FLAG acceptor beads, 5 mg/ml	PerkinElmer #AL112C
Alpha Nickel donor beads, 5 mg/ml	PerkinElmer #AS101D
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

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

BSJ-03-204 is a thalidomide-derivative, which is known to cause severe birth defects in humans. It is very important to use all appropriate precautions when handling this compound.

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 to the CDK-Cereblon complex.
- 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 Assay Buffer by diluting 3x CDK PROTAC® Buffer 3-fold with distilled water.

   Note: Prepare only the amount needed for the experiment. Aliquot the remaining undiluted 3x CDK PROTAC® Buffer and store at -20°C.

2. Dilute BSJ-03-204 PROTAC® by adding 980 µl of Assay Buffer to 20 µl of 200 µM BSJ-03-204 PROTAC® to make a 4 µM solution.

   Note: The final concentration of BSJ-03-204 PROTAC® in the assay will be 1 µM. Alternatively, PROTAC XY028-140 can be used at a final concentration of 250 nM in the assay.

3. Thaw **Cereblon** and the desired **CDK** on ice. Briefly spin the tubes containing the proteins to recover the full content of the tube.

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

   a. Dilute **Cereblon** to 20 ng/µl with Assay Buffer (400 nM, the final concentration in the reaction will be 100 nM);

   b. Dilute **CDK4/Cyclin D3** to 49.2 ng/µl with Assay Buffer (400 nM, the final concentration in the reaction will be 100 nM);

   c. Dilute **CDK6/Cyclin D3** to 28.4 ng/µl with Assay Buffer (400 nM, the final concentration in reaction will be 100 nM).

5. Prepare a **Master Mix** (7.5 µl/well): N wells × (2.5 µl of diluted Cereblon + 2.5 µl of the diluted desired CDK + 2.5 µl of Assay Buffer).

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

7. 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.

   7.1 If the Test PROTAC is water-soluble, prepare serial dilutions 4-fold more concentrated than the desired final concentrations in Assay Buffer.

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

OR

7.2 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 Assay Buffer to prepare the highest concentration of the 4-fold intermediate dilutions. The concentration of DMSO is now 4%.

Using Assay 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 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%.

8. Add 2.5 µl of Test PROTAC to the "Test PROTAC" wells.

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

10. For the wells labeled as "Positive Control" add 2.5 µl of diluted BSJ-03-204 PROTAC® (4 µM).

Component	Blank	Positive Control	Test
Master Mix	7.5 µl	7.5 µl	7.5 µl
Diluent Solution	2.5 µl	-	-
Test PROTAC	-		2.5 µl
Diluted BSJ-03-204 PROTAC® (4 µM)	-	2.5 µl	-
Total	**10 µl**	**10 µl**	**10 µl**

11. Incubate at Room Temperature (RT) for one hour.

STEP 2

Note: Protect your samples from direct exposure to light!

1. Dilute **anti-FLAG Acceptor beads** 250-fold with Assay Buffer (10 µl/well).

2. Add 10 µl per well.

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

4. Dilute **Nickel donor beads** 250-fold with Assay Buffer (10 µl/well).

5. Add 10 µl per well. Shake on a rotator platform for 60-90 minutes at RT.

6. Read Alpha-counts.

7. The "Blank" value should be subtracted from all readings.

Assay Protocol 2 - PROTAC Competitive Inhibition

- This protocol is designed to measure inhibition of the PROTAC binding to CDK kinases. The protocol can be easily modified to study inhibitors of the binding of PROTAC to the cereblon complex.
- 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 shaking on a rotator platform.

STEP 1

1. Prepare Assay Buffer by diluting 3x CDK PROTAC® Buffer 3-fold with distilled water.

   Note: Prepare only the amount needed for the experiment. Aliquot the remaining undiluted 3x CDK PROTAC® Buffer and store at -20°C.

2. Thaw **Cereblon** and the desired **CDK** on ice. Briefly spin the tubes containing the proteins to recover the full content of the tube.

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

   a. Dilute **Cereblon** to 20 ng/µl with Assay Buffer (400 nM; the final concentration in the reaction will be 100 nM);

   b. Dilute **CDK4/Cyclin D3** to 49.2 ng/µl with Assay Buffer (400 nM; the final concentration in the reaction 100 nM);

   c. Dilute **CDK6/Cyclin D3** to 28.4 ng/µl with Assay Buffer (400 nM; the final concentration in the reaction 100 nM).

4. Prepare a **Master Mix** (5 µl/well): N wells × (2.5 µl of diluted Cereblon + 2.5 µl of the desired diluted CDK).

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

6. Prepare the **Test Compound** (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.

   6.1 If the Test Compound is water-soluble, prepare serial dilutions in Assay Buffer, 4-fold more concentrated than the desired final concentrations.

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

   OR

   6.2 If the Test Compound is soluble in DMSO, prepare the test compound at 100-fold the highest desired concentration in 100% DMSO, then dilute the test compound 25-fold in Assay Buffer to prepare the highest concentration of the 4-fold intermediate dilutions. The concentration of DMSO is now 4%.

Using Assay Buffer in 4% DMSO, prepare serial dilutions of the test compound at 4-fold the desired final concentrations to keep the concentration of DMSO constant.

For positive and negative controls, prepare 4% DMSO in 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%.

7. Add 2.5 µl of diluted Test Compound to each well designated “Test Compound”.
8. Add 2.5 µl of Diluent Solution to the "Positive Control" and "Blank" wells.
9. Resuspend the tube containing Palbociclib with 1000 µl of Assay Buffer to make a 200 µM solution (2.5 µl/well).
10. Add 2.5 µl of diluted Palbociclib to the "Inhibitor Control" wells.
11. Preincubate the test compound with the Cereblon and CDK for up to 30 minutes at RT with slow agitation.
12. Dilute BSJ-03-204 PROTAC® by adding 980 µl of Assay Buffer to 20 µl of BSJ-03-204 PROTAC® (200 µM) to make a 4 µM solution.

    Note: The final concentration of BSJ-03-204 PROTAC® in the assay will be 1 µM. Alternatively, PROTAC XY028-140 can be used with a final concentration of 250 nM in the assay.

13. Initiate the reaction by adding 2.5 µl of diluted BSJ-03-204 PROTAC® to wells labeled “Positive Control", “Inhibitor Control” and "Test Inhibitor".
14. Add 2.5 µl of Assay Buffer to the “Blank” wells.

Component	Blank	Positive Control	Inhibitor Control	Test Compound
Master Mix	5 µl	5 µl	5 µl	5 µl
Diluent Solution	2.5 µl	2.5 µl	-	-
Diluted Test Compound	-	-	-	2.5 µl
Diluted BSJ-03-204 PROTAC® (4 µm)	-	2.5 µl	2.5 µl	2.5 µl
Assay Buffer	2.5 µl	-	-	-
Diluted Palbociclib	-	-	2.5 µl	-
Total	**10 µl**	**10 µl**	**10 µl**	**10 µl**

15. Incubate at RT for one hour.

STEP 2

Note: Protect your samples from direct exposure to light!

1. Dilute **anti-FLAG Acceptor beads** 250-fold with Assay Buffer (10 µl/well).
2. Add 10 µl per well.

3. Shake on a rotator platform for 30 minutes at RT.
4. Dilute **Nickel donor beads** 250-fold with Assay Buffer (10 µl/well).
5. Add 10 µl per well.
6. Shake on a rotator platform for 60-90 minutes at RT.
7. Read Alpha-counts.
8. The "Blank" value should be subtracted from all readings.

Example Results

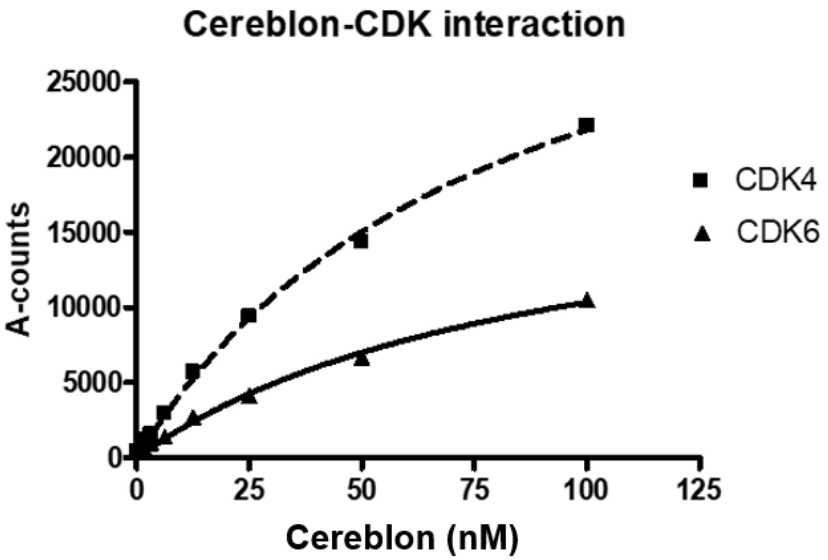

Figure 2: BSJ-03-204 PROTAC®-mediated interaction of Cereblon with CDK.
BSJ-03-204 PROTAC® activity was measured in the presence of increasing concentrations of Cereblon.

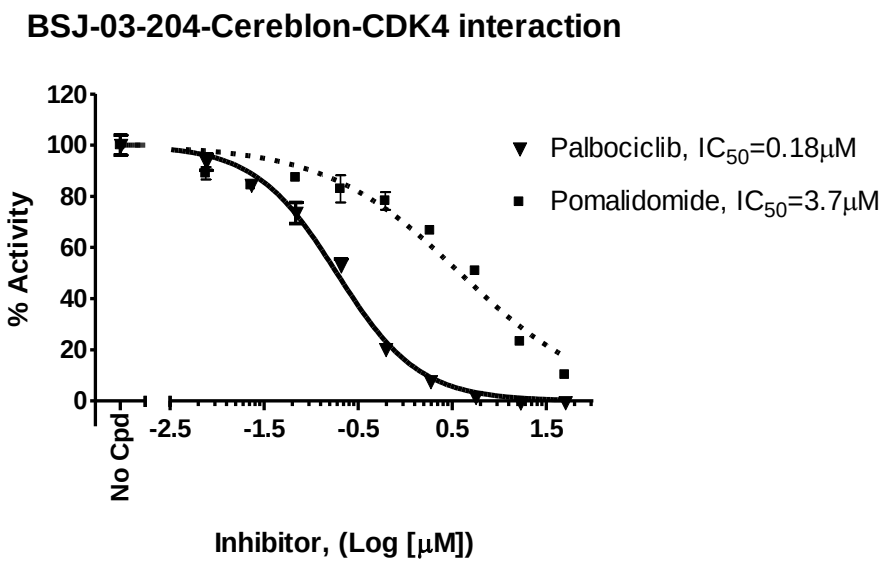

Figure 3: Inhibition by Palbociclib or Pomalidomide of BSJ-03-204 PROTAC®-mediated interaction of Cereblon with CDK4.
Inhibition of BSJ-03-204- PROTAC® mediated interaction of Cereblon with CDK4 was measured in the presence of increasing concentrations of Palbociclib and Pomalidomide (BPS Bioscience #82026).

Data shown is representative. For lot-specific information, please contact BPS Bioscience, Inc. at info@bpsbioscience.com

Reference

Brand M., *et al.*, 2019 Cell Chem. Biol. 26(2):300-306.
Nunes J., *et al.*, 2019 *ACS Med Chem Lett* 10(7): 1081-1085.
Łukasik P., *et al.*, 2021 *Int J Mol Sci. Mar* 13;22(6):2935.

Troubleshooting Guide

Visit bpsbioscience.com/assay-kits-faq for detailed troubleshooting instructions. For all further questions, please email support@bpsbioscience.com

Related Products

Products	*Catalog #*	*Size*
PROTAC® Optimization Kit for BET Bromodomain-Cereblon Binding	79770	384 reactions
PROTAC® Optimization Kit for BET Bromodomain-Von Hippel Lindau (VHL) Binding	79790	384 reactions
PROTAC® Optimization Kit for BRD9-Cereblon Binding	78420	384 reactions
PROTAC® Optimization Kit for CDK Kinase-Cereblon Binding	79924	384 reactions
PROTAC® Optimization Kit for IRAK4-Cereblon Binding	78512	384 reactions
PROTAC® Optimization Kit for PARP1-Cereblon Binding	78441	384 reactions

Version 122023