Description

The PROTAC-Driven Ubiquitination Assay Kit for BET Bromodomains is designed for the testing and profiling of PROTACs targeting the bromodomains on the BET (bromodomain and extraterminal) protein family. After tertiary complex formation between the E3 protein, the PROTAC and the protein of interest, ubiquitination of the target protein is a critical step preceding its degradation. The PROTAC-Driven Ubiquitination Assay Kit for BET Bromodomains comes in an AlphaLISA® format with enough optimized assay buffer, purified recombinant E1 (UBE1), E2 (UbcH5b), and E3 enzymes (Cereblon complex), BRD3 (BD2) (bromodomain-containing protein 3) (amino acids 305-417) as target protein, ATP, and biotinylated ubiquitin for 384 reactions. This kit also contains the PROTAC dBET1 as the control, and JQ1 as control inhibitor.

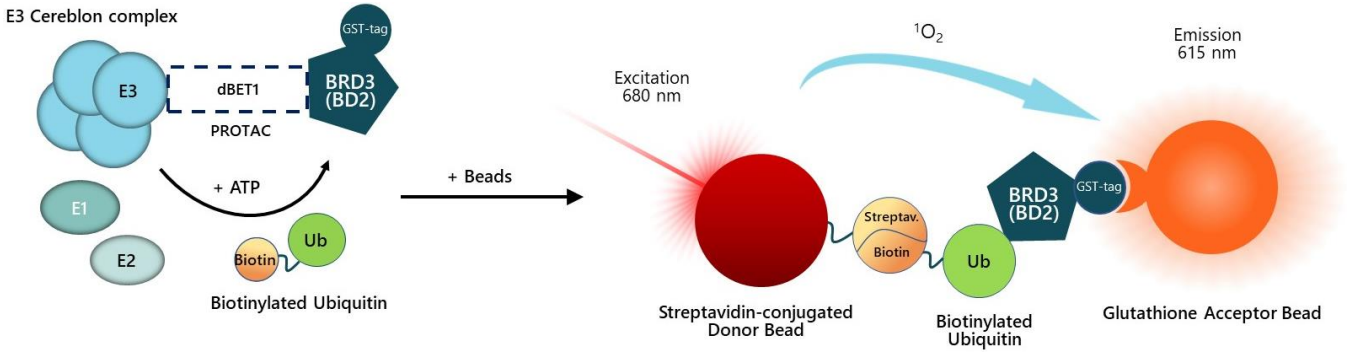

Figure 1. Schematic representation of the principle of the PROTAC-Driven Ubiquitination Assay Kit for BET Bromodomains.

dBET1 or a PROTAC® of interest is incubated with Cereblon complex and BRD3 (BD2), bringing them in close proximity. Ubiquitination reaction occurs following the addition of E2 enzyme, ATP, biotinylated ubiquitin, and, finally, E1 enzyme. Once ubiquitination has taken place, AlphaLISA® donor and acceptor beads are added. BRD3 (BD2) contains a GST-tag, which is recognized by GSH-AlphaLISA™ acceptor beads. Ubiquitinated BRD3 (BD2) contains biotin-ubiquitin, that binds to Streptavidin donor beads. Upon excitation of the donor bead, a singlet oxygen is generated. The singlet oxygen excites the acceptor bead, which emits light. The resulting signal is directly proportional to the level of ubiquitination of BRD3 (BD2).

Background

Cereblon is the substrate-binding component of the E3 protein ligase complex Cereblon-CUL4A (cullin-4A)-RBX1 (RING-box protein 1) that targets proteins for degradation through the proteasome system. The binding of Cereblon to a substrate protein engages the E3 ligase activity of the complex and results in the ubiquitination and ultimate degradation of the substrate protein. PROTACs are bifunctional molecules that bring together a therapeutic target and an E3 ligase to promote the ubiquitination and ultimately the degradation of the target. Efficiency of ubiquitination is an important factor for optimization of heterobifunctional degraders. BRD3, also known as RING3L (RING3-like protein) belongs to the BET family of proteins and is involved in transcription by associating with acetylated lysines present in histones and transcription factors. Chromosomal translocations of BRD3 with NUT (nuclear protein in testis) can lead to cancer, and it has been shown that the use of BET inhibitors or depletion of BRD3 can slow down cancer progression in models of prostate cancer. The use of PROTAC technology for degradation of BRD proteins is an active field of research. Development of novel PROTAC molecules involved in the recruitment of BRD3 (BD2) provides a framework for future efforts to utilize Cereblon to degrade other proteins of interest.

Application(s)

- Identify and optimize functional activity of PROTACs targeting bromodomains of the BET family.
- Design novel molecules linking bromodomains of the BET family and the Cereblon complex.
- Directly compare ubiquitination of target proteins by different PROTACs.
- Identify potential inhibitors of ubiquitination.

Supplied Materials

Catalog #	Name	Amount	Storage
80301	UBE1 (UBA1), FLAG-Tag*	25 µg	-80°C
80314	UbcH5b, His-Tag (Human)*	60 µg	-80°C
100329	Cereblon/DDB1/Cul4A/Rbx1 Complex*	60 µg	-80°C
31033	BRD3 (BD2), GST-Tag*	5 µg	-80°C
	dBET1 (MW=785 Da)	2 x 0.31 µg	-20°C
27403	10 mM (+)-JQ1	10 µl	-20°C
	U2 Assay Buffer	10 ml	-20°C
	10 mM ATP	400 µl	-80°C
	Biotin-Ubiquitin	400 µl	-80°C
	Plate sealer	1	Room Temp

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

Materials Required but Not Supplied

Component	Ordering Information
AlphaLISA GSH Acceptor Beads, 250 µg	PerkinElmer #AL109C
AlphaScreen Streptavidin-Conjugated Donor Beads, 5 mg/ml	PerkinElmer #6760002S
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. dBET1 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 – Comparison of target ubiquitination driven by various PROTACS designed to link BET Bromodomain and Cereblon

- This protocol is designed to test the ubiquitination of BRD3 (BD2) driven by various PROTACs. BRD3 (BD2) can be replaced by other GST-tagged bromodomains of the BET family as target.
- All samples and controls should be performed in duplicate.
- The assay should include "Blank" (no PROTAC), "Positive Control" and "Test PROTAC" conditions.
- We recommend maintaining the diluted proteins 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. Thaw a vial of **U2 Assay Buffer**.

   Note: Use only the amount needed for the experiment. Aliquot the remaining U2 Assay Buffer and store at -20°C.

2. Add 700 µl of U2 Assay Buffer to the vial of dBET1. This makes a 0.55 mM dBET1 stock solution.

   Note: The final concentration of dBET1 in the assay will be 110 nM. Store the remaining solution for up to 2 days at 4°C. Use the second vial provided if running experiments more than 2 days later.

3. Thaw **Cereblon Complex** and **BRD3 (BD2)** on ice. Briefly spin the tube containing the protein to recover the full content of the tube.

4. Prepare the following dilutions:

   a. Dilute **Cereblon Complex** to 150 ng/µl with U2 Assay Buffer (1 µl/well);

   b. Dilute **BRD3 (BD2)** to 12 ng/µl with U2 Assay Buffer (1 µl/well).

5. Prepare a **Master Mix 1** (3 µl/well): N wells × (1 µl of diluted Cereblon Complex + 1 µl of the diluted BRD3 (BD2) + 1 µl of U2 Assay Buffer).

6. Add 3 µl of **Master Mix 1** to every well.

7. Prepare **Test PROTAC** (2 µl/well): for a titration prepare serial dilutions at concentrations 5-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 5-fold more concentrated than the desired final concentrations in U2 Assay Buffer.

For the positive and negative controls, use U2 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 20-fold with U2 Assay Buffer to prepare the highest concentration of the 5-fold intermediate dilutions. The concentration of DMSO is now 5%.

Using U2 Assay Buffer with 5% DMSO, prepare serial dilutions of the Test PROTAC at 5-fold the desired final concentrations to keep the concentration of DMSO constant.

For positive and negative controls, prepare 5% DMSO in U2 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 µl of Test PROTAC to the "Test PROTAC" wells.
9. Add 2 µl of Diluent Solution to the "Blank" wells.
10. Add 2 µl of 0.55 µM dBET1 to the "Positive Control" wells.
11. Incubate for 30 minutes at room temperature (RT) with slow agitation.
12. During the 30 minutes incubation, thaw **ATP**, **Biotin-Ubiquitin**, **UBE1** and **UbcH5b** on ice. Briefly spin the tubes containing the proteins to recover the full content of the tube.
13. Prepare the following dilutions:
    a. Dilute **UBE1** to 24 ng/µl with U2 Assay Buffer (2 µl/well)
    b. Dilute **UbcH5b** to 90 ng/µl with U2 Assay Buffer (1 µl/well)
14. Prepare a **Master Mix 2** (3 µl/well): N wells × (1 µl of diluted UbcH5b + 1 µl of ATP + 1 µl of Biotin-Ubiquitin).

    Note: Use only the amount of ATP and Biotin-Ubiquitin needed for the experiment. Aliquot the remaining reagents and store at -80°C in single use aliquots (minimum volume of 5 µl/aliquot).
15. Add 3 µl of Master Mix 2 to each well.
16. Mix well using slow agitation for 2-5 minutes at RT.
17. Initiate ubiquitination by adding 2 µl of diluted UBE1 to each well.

Component	Blank	Positive Control (dBET1)	Test PROTAC
Master Mix 1	3 µl	3 µl	3 µl
Diluent Solution	2 µl	-	-
Test PROTAC	-	-	2 µl
Diluted dBET1 (0.55 µM)	-	2 µl	-
	Incubate 30 minutes at RT		
Master Mix 2	3 µl	3 µl	3 µl
Diluted UBE1 (24 ng/µl)	2 µl	2 µl	2 µl
Total	**10 µl**	**10 µl**	**10 µl**

18. Seal the plate (10 µl in each well) and incubate at 30°C for 90 minutes.

Step 2

Note: Protect your samples from direct exposure to light!

1. Dilute **GSH Acceptor Beads** 250-fold with U2 Assay Buffer (10 µl/well).
2. Add 10 µl of diluted GSH Acceptor Beads per well.
3. Shake on a rotator platform for 30 minutes at RT.
4. Dilute **Streptavidin-Conjugated Donor Beads** 125-fold with U2 Assay Buffer (10 µl/well).
5. Add 10 µl of diluted Streptavidin-Conjugated Donor Beads per well.
6. Shake on a rotator platform for 15-45 minutes at RT.
7. Read Alpha-counts.
8. The “Blank” value should be subtracted from all readings.

Protocol 1 Example Results

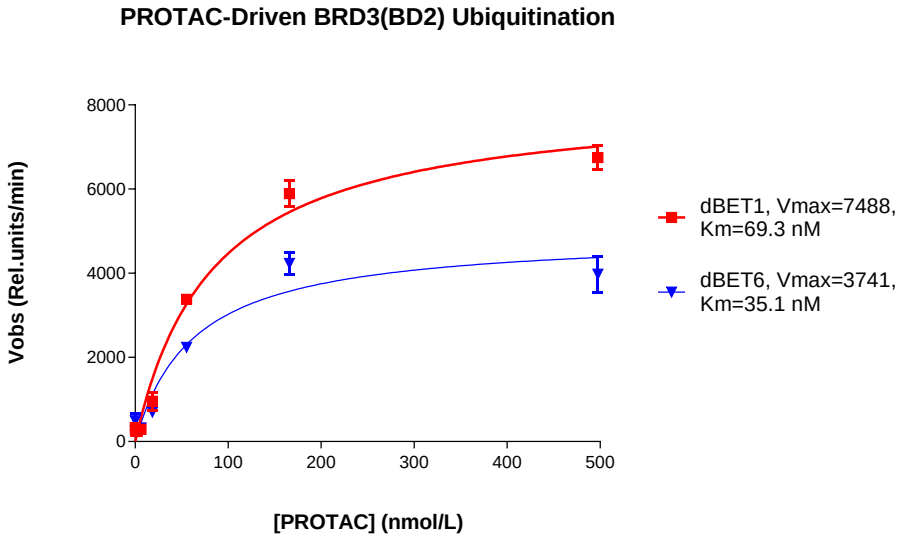

Figure 2: dBET1 and dBET6 PROTAC-Driven BRD3 (BD2) ubiquitination, two similar PROTACS with different linker sizes.
PROTAC-driven BRD3 (BD2) ubiquitination was measured in the presence of increasing concentrations of dBET1 and dBET6 (similar PROTACS with different linker sizes). dBET6 showed higher affinity compared to dBET1, whereas dBET1 showed higher efficiency since a higher maximal level of BRD3 ubiquitination was observed when using dBET1 compared to dBET6.

Assay Protocol 2 – Competitive inhibition of PROTAC linked BET ubiquitination

- This protocol is designed to measure inhibition of the PROTAC-driven ubiquitination of BRD3 (BD2). BRD3 (BD2) can be replaced by other GST-tagged bromodomains of the BET family.
- dBET1 can be replaced by another PROTAC of interest.
- The protocol can be used to study inhibitors of UBE1, UcbH5b, and Cereblon complex as well as ubiquitination inhibitors.
- All samples and controls should be performed in duplicate.
- The assay should include “Blank” (no UBE1), “Positive Control”, “Inhibitor Control” and “Test Compound” conditions.
- We recommend maintaining the diluted proteins 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.
- We recommend using (+)-JQ1 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.

Step 1

1. Thaw a vial of **U2 Assay Buffer**.

   Note: Use only the amount needed for the experiment. Aliquot the remaining U2 Assay Buffer and store at -20°C.

2. Add 350 µl of U2 Assay Buffer to the vial of dBET1. This makes a 1.1 mM dBET1 stock solution.

   Note: The final concentration of dBET1 in the assay will be 110 nM. Store the remaining solution for up to 2 days at 4°C. Use the second vial provided if running experiments more than 2 days later.

3. Thaw **Cerebron Complex** and **BRD3 (BD2)** on ice. Briefly spin the tubes containing the proteins to recover the full content of the tube.

4. Prepare the following dilutions:

   a. Dilute **Cereblon Complex** to 150 ng/µl with U2 Assay Buffer (1 µl/well);

   b. Dilute **BRD3 (BD2)** to 12 ng/µl with U2 Assay Buffer (1 µl/well).

5. Prepare a **Master Mix 1** (3 µl/well, except "Blank" wells): N wells × (1 µl of diluted Cereblon Complex + 1 µl of the diluted BRD3 (BD2) + 1 µl of the 1.1 µM dBET1).

6. Add 3 µl of **Master Mix 1** to every well, except "Blank" wells.

7. Prepare **Test Compound** (2 µl/well): for a titration prepare serial dilutions at concentrations 5-fold higher than the desired final concentrations. The final volume of the reaction is 10 µl.

   7.3 If the Test Compound is water-soluble, prepare serial dilutions 5-fold more concentrated than the desired final concentrations in U2 Assay Buffer.

   For the positive and negative controls, use U2 Assay Buffer (Diluent Solution).

   OR

   7.4 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 20-fold with U2 Assay Buffer to prepare the highest concentration of the 5-fold intermediate dilutions. The concentration of DMSO is now 5%.

   Using U2 Assay Buffer with 5% DMSO, prepare serial dilutions of the Test Compound at 5-fold the desired final concentrations to keep the concentration of DMSO constant.

   For positive and negative controls, prepare 5% DMSO in U2 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. Dilute 10 mM (+)-JQ1 to 100, 10, and 1 mM with DMSO.

9. Dilute those solutions a further 20-fold with U2 Buffer to make 5, 0.5, and 0.05 mM solutions.

10. Add 2 µl of diluted (+)-JQ1 the wells labeled as "Inhibitor Control". The final concentrations in the assay will be 1, 0.1, and 0.01 mM (corresponding to 10X IC_{50}, the IC_{50} and 0.1X IC_{50} values).

11. Add 2 µl of Test Compound to the "Test Compound" wells.

12. Add 2 µl of Diluent Solution to the "Blank" and "Positive Control" wells.

13. Incubate for 30 minutes at room temperature (RT) with slow agitation.

14. During the 30 minutes incubation, thaw **ATP**, **Biotin-Ubiquitin**, **UBE1**, and **UbcH5b** tubes on ice. Briefly spin the tube containing the proteins to recover the full content of the tube.

15. Prepare the following dilutions:

    a. Dilute **UBE1** to 24 ng/µl with U2 Assay Buffer (2 µl/well);

    b. Dilute **UbcH5b** to 90 ng/µl with U2 Assay Buffer (1 µl/well).

16. Prepare a **Master Mix 2** (3 µl/well): N wells × (1 µl of diluted UbcH5b + 1 µl of ATP + 1 µl of Biotin-Ubiquitin).

    Note: Use only the amount of ATP and Biotin-Ubiquitin needed for the experiment. Aliquot the remaining reagents and store at -80°C in single use aliquots (minimum volume of 5 µl/aliquot).

17. Add 3 µl of Master Mix 2 to each well.

18. Mix well using slow agitation for 2-5 minutes at RT.

19. Add 2 µl of the diluted UBE1 to the "Positive Control", "Inhibitor Control", and "Test Compound" wells.

20. Add 2 µl of U2 Assay Buffer to the "Blank" wells.

Component	Blank	Positive Control	Inhibitor Control (JQ1)	Test Compound
Master Mix 1	3 µl	3 µl	3 µl	3 µl
Diluent Solution	2 µl	2 µl	-	-
Test Compound	-	-	-	2 µl
Diluted (+)-JQ1	-	-	2 µl	-
	Incubate for 30 minutes at RT			
Master Mix 2	3 µl	3 µl	3 µl	3 µl
U2 Assay Buffer	2 µl	-	-	-
Diluted UBE1 (24 ng/µl)	-	2 µl	2 µl	2 µl
Total	**10 µl**	**10 µl**	**10 µl**	**10 µl**

21. Seal the plate (10 µl in each well) and incubate at 30°C for 90 minutes.

Step 2

Note: Protect your samples from direct exposure to light!

1. Dilute **GSH Acceptor Beads** 250-fold with U2 Assay Buffer (10 µl/well).
2. Add 10 µl of diluted GSH Acceptor Beads per well.
3. Shake on a rotator platform for 30 minutes at RT.
4. Dilute **Streptavidin-Conjugated Donor Beads** 125-fold with U2 Assay Buffer (10 µl/well).
5. Add 10 µl of diluted Streptavidin-Conjugated Donor Beads per well.
6. Shake on a rotator platform for 15-45 minutes at RT.
7. Read Alpha-counts.
8. The "Blank" value should be subtracted from all readings.

Protocol 2 Example Results

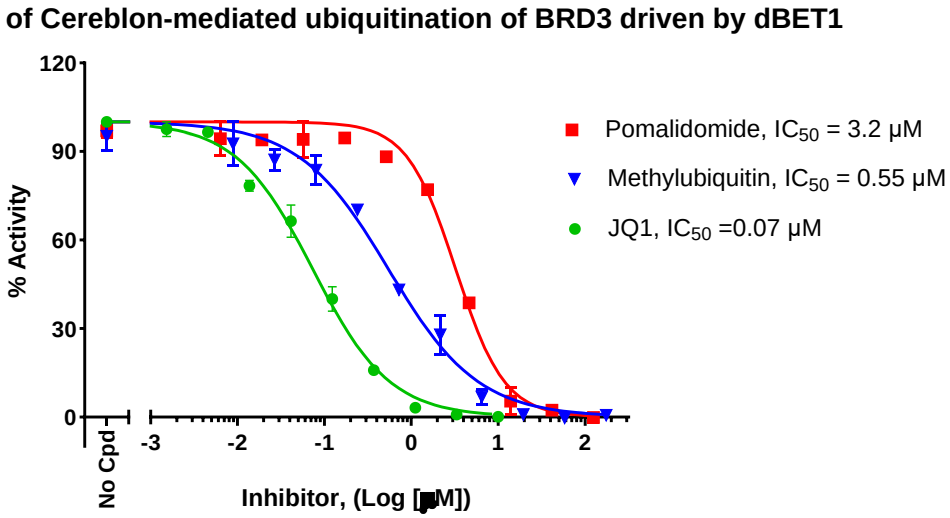

Figure 3: Inhibition of dBET1 mediated ubiquitination of BRD3 (BD2) by Cereblon in the presence of various inhibitors.
dBET1 mediated ubiquitination of BRD3 (BD2) by Cereblon Complex was measured in the presence of increasing concentrations of Pomalidomide (Cereblon binder), (+)-JQ1 (BET bromodomain inhibitor), and Methylubiquitin (inhibitor of ubiquitination).

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

Reference

Kaur, S.D., *et al.* 2023 *Cancer Lett*; 556: 216065.
Wang C., *et al.*, 2022 *J Enzyme Inhib Med Chem* 37(1): 1694-1703.

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

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