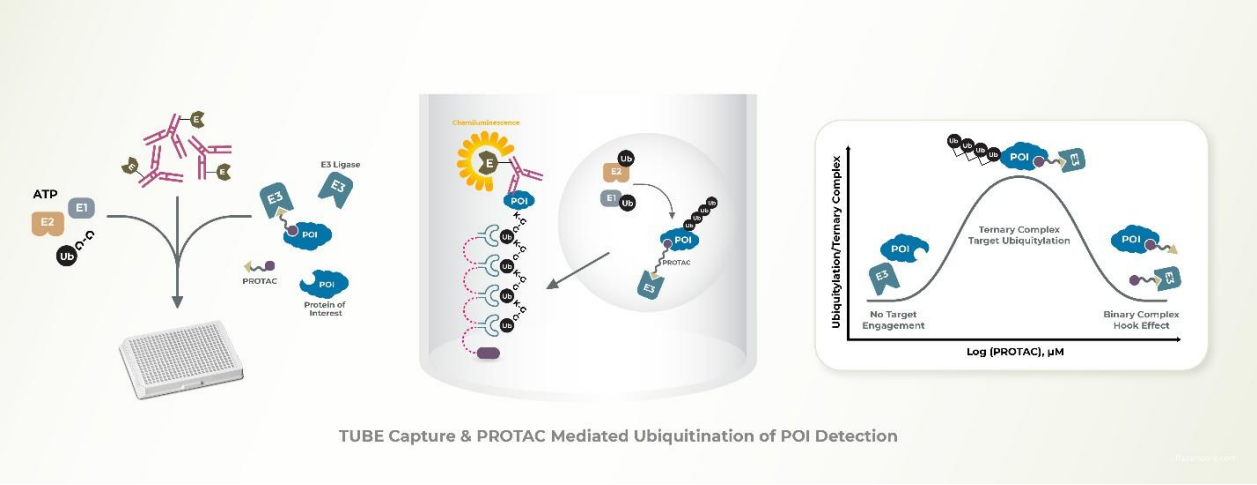
PROTAC® *In Vitro* Ubiquitination Assay Kit: Customizable Ubiquitin Ligase Kit

MANUAL

PROTAC In Vitro Ubiquitination Assay Kit

Catalog Number PA770



TUBE Capture & PROTAC Mediated Ubiquitination of POI Detection

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BACKGROUND

Ubiquitin and Ubiquitin Conjugation Machinery

Ubiquitin is a small polypeptide that can be conjugated via its C-terminus to amine groups of lysine residues on target proteins. This conjugation is referred to as monoubiquitylation. Additional ubiquitin moieties can be conjugated to this initial ubiquitin utilizing any one of the seven lysine residues present in ubiquitin. The formation of these ubiquitin chains is referred to as polyubiquitylation. The most well characterized of this polyubiquitylation is chain formation via lysine at position 48 of ubiquitin (K48-linked chains). Monoubiquitylation has been shown to alter the localization, activity, and/or function of the target protein. The most prevalent consequence of polyubiquitylation is the proteasome-mediated degradation of the target protein.

The conjugation of ubiquitin to a target protein requires the coordinated function of three distinct ligases, **E1** (ubiquitin activating enzyme), **E2** (ubiquitin conjugating enzyme), and **E3** (ubiquitin ligase) resulting in isopeptide bond formation between the C-terminus of ubiquitin and the ε-amino group of the lysine residue on target proteins. Ubiquitin E3 ligases act as scaffold proteins, providing docking sites for an ubiquitin-conjugating enzyme (E2), and a target substrate. Typically, E3 ligases mediate the transfer of ubiquitin from an E2 thioester intermediate to an amide linkage with a substrate protein (Hershko and Ciechanover, 1998). In addition to the ubiquitylation of substrates, E3 ligases can also "auto ubiquitylate" themselves.

PROTAC®

PROTACs (Proteolysis-targeting chimeric molecules) artificially hijack the components of the UPS to degrade a target protein. PROTAC drugs are hetero-bifunctional small molecules that contain two functional ligands connected via a linker; one ligand binds to a target protein and the other ligand binds to an E3 ligase. Bringing these two entities into proximity theoretically leads to polyubiquitylation and proteasomal degradation of the target protein. However, given the complexity this scenario does not always play out, and the PROTAC discovery strategy faces several challenges and pitfalls. The current assay addresses a lot these challenges in evaluating PROTAC and serve as a tool to effectively report true PROTAC efficiency by monitoring PROTAC mediated ubiquitination.

ABOUT THE ASSAY

The *In vitro* ubiquitination kit has been developed to establish a high throughput approach that can accurately predict PROTAC efficiency by monitoring the protein's intrinsic ability to get ubiquitinated. We offer this kit for three E3 ubiquitin ligases Cereblon, VHL and HDM2 to monitor PROTAC mediated ubiquitination for target of choice. At the core of the assay, microtiter plate strips, pre-coated with a proprietary TUBE reagent (assay plate) are used for the capture of polyubiquitin chains formed in an PROTAC dependent reaction. For the assay, PROTACs are added first to the assay plate followed by E3 ligase and target protein under investigation to enable ternary complex formation. As a sequential step, an E1-E2 enzyme cocktail with wt-ubiquitin and ATP is then added to the wells to initiate PROTAC mediated ubiquitination. During the reaction, polyubiquitin chains generated by the E1-E2-E3 machinery are recognized and captured in the wells. Following the reaction and subsequent wash steps, the isolated polyubiquitylated product is incubated with antibodies against protein of interest (not included) and secondary antibody conjugated with-HRP (included) allowing for detection by chemiluminescence. Thus, the signal generated by captured polyubiquitylated product in this "sandwich" ELISA-like assay is a quantitative measure of PROTAC activity. **Furthermore, this detection strategy does not require additional non-native tagging or labeling of ubiquitin, which could lead to experimental artifacts.**

BENEFITS

1. Monitor PROTAC activity by monitoring ubiquitination, with the target of your choice in HTS format.
2. Screen multiple PROTAC variants simultaneously with variable ligands, exit vectors and chemical linkers.
3. Accurately establish rank order potencies to guide medicinal chemists for reliable SAR.
4. Accelerate PROTAC drug discovery and clinical development by rationally designing PROTACs that rely on functional assays that report protein's "ubiquitination potential" since we are hijacking UPS, rather than just relying on simple proximity ligand assays.
5. Amenable for molecular glue drug discovery.

SUGGESTED USES

1. Testing PROTAC and molecular glue (MGs) activity.
2. Compare between multiple PROTAC variants and establish predicitve **DC_{50}** from **Ub_{Max}**.
3. Choose all three ligases for comparing PROTACs / MGs activity to demonstrate selectivity.
4. Test PROTAC substrate specificity and Isoform selectivity.

COMPONENTS

Store all materials at -80°C, avoid cycles of freezing and thawing. All components are stable for at least 2 months.

1. ***In Vitro* ubiquitination Assay Plate**

   Note: Do not thaw the assay plate rapidly to room temperature. We recommend to initially place the assay plate at 4°C for 30 minutes prior to transferring to room temperature.

2. **10X Assay Buffer**

   Size: 1.2 mL

   Note: Add β-mercaptoethanol fresh to final concentration of 1mM in 1X assay buffer.

3. **E3 Mix (2X)**

   Size: 1 x 250 µl (20X)

   Note: Add target protein of choice at concentrations between 40-80 nM to the E3 mix provided to facilitate ternary complex formation with PROTAC and E3 ligase.

4. **E1-E2-Ubiquitin Mix (2X)**

   Size: 1 x 250 µl (20X)

5. **Secondary Antibody (Anti-Mouse or Anti-Rabbit HRP conjugate)**

   Size: 60µL (100X)

6. **Detection Reagent 1 & 2**

   Size: 1 mL of Detection Reagent 1 and 1mL of Detection Reagent 2

7. **Blocking Concentrate (5X)**

   Size: 5 mL

8. **Positive Control Reagents**

PROTAC in DMSO (50X):	10 µL (MZP54, dBET6, A1874)
Target Protein positive control BRD3 (20X):	20 µL
Anti-BRD3 antibody (100X):	5 µL
Anti-Mouse HRP Conjugate (100X):	5 µL

ADDITIONAL ITEMS REQUIRED BUT NOT PROVIDED

1. **Wash Buffer(s)**
   a. 1X Phosphate Buffered Saline, 0.1% Tween (PBST)
2. **Luminescence capable plate reader**
3. **β-mercaptoethanol**
4. **100mM ATP**
5. **PROTACs Under Study, Target Protein & Target Specific Antibodies (ELISA compatible)**
6. **Polypropylene plate (Optional)**
7. **Multi-channel Pipettors and Automatic Plate Washer (Optional)**
8. **15 mL centrifuge tubes**

SOLUTIONS FOR *IN VITRO* UBIQUITINATION REACTION

Volumes listed below are sufficient for 8 reaction wells, or 1 modular strip (scale accordingly). The volumes provided in this Kit should be sufficient for 96 wells. The reaction volume per well is 50µL, hence for 8 wells the calculations provided below are for 400µL.

Assay Buffer

Prepare 400µL of assay buffer by diluting 40µL of 10X assay buffer with 360µL of ultra-pure water. Add β-mercaptoethanol to final concentration of 1mM.

PROTAC Dilutions

Prepare PROTAC doses at 50X concentration and transfer 1 µL per well into the In vitro PROTAC assay plate prior to added 2X E3-Substrate mix and E1-E2-Ubiquitn mix. For positive control, we have provided 10 µL of positive control PROTAC add DMSO or Positive controls as needed to desired number of wells.

E3 Mix (2X) – Add your substrate here – prepare 200 µL

1. Add 20 µl **of E3 ubiquitin ligase** for a (2x) concentration.
2. Add desired µl of target substrate to make final concentration of 40-80 nM.
3. Make the final volume to 200µL with **assay buffer**.

E1-E2-Ubiquitin Mix (2x), prepare 200 µL

1. Add 20 µl **of E1-E2-ubiquitin mix** for a (2x) concentration.
2. Add desired amount of ATP to make final concentration to 800 µM.
3. Make the final volume to 200µL with **assay buffer**.

Blocking concentrate 1X

Add 1 part of 5X blocking concentrate (80 µL) to 4 parts of PBST (320 µL).

Primary Target Antibody, 400µl

Dilute antibody according to antibody manufacturer instructions. For example, the positive control antibody provided at 100X - dilute 4 µL of anti-BRD3 antibody in 396µL of 1X blocking concentrate.

Secondary Antibody – HRP conjugate, 400µl

Secondary control antibody HRP conjugate provided at 100X - dilute 4 µL of anti-mouse / anti-rabbit as needed in 396µL of 1X blocking concentrate. For positive control (anti BRD3) please use anti-mouse HRP.

Detection Reagent 1 and 2

Perform either 1:50 or 1:10 dilution as needed in ultrapure water and add 50 µL per well prior to detection.

PROTOCOLS

PROTAC® *In Vitro* Ubiquitination Assay Kit (Suggested Protocol)

Note: Volumes listed below are sufficient for 8 reaction wells, or 1 modular strip (scale accordingly). The volumes provided in this Kit should be sufficient for 96 wells. The reaction volume per well is 50µL, hence for 8 wells the calculations provided below are for 400µL.

1. As directed in previous sections, prepare all reagents, standards, and samples.
2. Cut and remove aluminum seal from wells and transfer 1 strip of 8 wells to user plate frame. **Transfer of strips may be easier with frozen wells.** Allow coated plate wells to equilibrate at 4ºC for at least 30 minutes before transferring to room temperature.
3. Prepare PROTAC doses at (50X) concentration and transfer 1µL into appropriate Eppendorf tube.
4. Add 20µL of prepared E3 Mix for a (2X) concentration in an Eppendorf tube.
5. Add desired µL of target substrate to make final concentration of 40-80 nM to the same Eppendorf that has your E3 Mix.
6. Add enough assay buffer to the E3 Mix to make the final volume 200µL.
7. Add 25µL of E3 Mix to each Eppendorf tube with the PROTAC of interest.
8. Incubate E3 Mix for 15-30 minutes at room temperature on a shaker
9. Add 20µL of E1-E2-ubiquitin mix for a (2X) concentration into a labeled Eppendorf.
10. Add desired amount of ATP to make the final concentration 800µM into the Eppendorf with the E1-E2-ubiquitin mix
11. Add enough assay buffer to the E1-E2-ubiquitin mix to make the final volume 200µL.
12. Add 25µL of E1-E2-ubiquitin mix to the Eppendorf tubes with the PROTAC of interest and E3 Mix is located in.
13. Transfer 50µL of the mixes into each of the eight (8) wells in the in vitro ubiquitination assay plate.
14. Incubate the assay plate for 1 hour and 30 minutes at room temperature while shaking. Follow by placing the plate at 37°C for 30 minutes.
15. Wash the assay plate four (4) times with PBS-T using 120µL per well, tap out excess PBS-T from wells between washes.
16. Prepare Blocking Concentrate (BC) by adding four (4) parts PBS-T to one (1) part of 5X BC

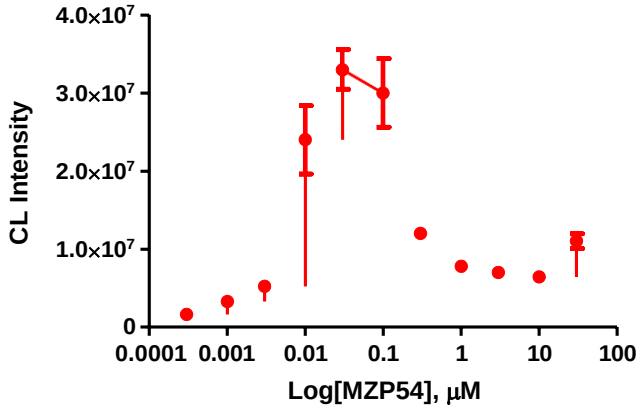
    Note: If eight (8) wells are being used you would need 80µL of 5X BC, and 320µL PBS-T.
17. Dilute the primary antibody according to antibody manufacturer instructions in (1X) BC.
18. Pipette 50µL of (1X) BC with diluted primary into each well.
19. Incubate for 1 hour and 30 minutes at room temperature while shaking.
20. Wash the assay plate four (4) times with PBS-T using 120µL per well, tap out excess PBS-T from wells between washes.
21. Dilute 4 µL of anti-mouse / anti-rabbit as needed in 396µL of (1X) blocking concentrate. For the positive control use 2µL of anti-mouse in 98µL of (1X) BC.
22. Pipette 50µL of (1X) BC with diluted primary into each well.
23. Incubate the assay pate for 45 minutes at room temperature while shaking.
24. Wash the assay plate four (4) times with PBS-T using 120µL per well, tap out excess PBS-T from wells between washes.
25. Just before use, mix 200 µL of DR1 and 200 µL of DR2 into 10 mL of ultrapure water (deionized or distilled). Add 50 µL of this solution to each well
26. Read with a plate reader optimized for detection of chemiluminescence for 5-10 reads with 1 min intervals.

Control for Detection Using BRD3 (Suggested Protocol)

The BRD-3 Target is given as a positive control. One should expect to see a 3- to 20-fold signal to background with the control reagents. We provide MZP-54 PROTAC as positive control for VHL E3 ligase, dBET6 for Cereblon E3 ligase and A1874 for MDM2 E3 ligase. Enhancement in ubiquitination represents kit performing at optimal conditions. In case of issues with positive controls performance please contact LifeSensors technical services (info@lifesensors.com or 610-644-8845) for assistance.

1. Add 2µL of positive control PROTAC to each well of the provided into an Eppendorf tube. The user can modify the number of wells needed for positive control as required. (This example is for 2 wells)
2. Add 5µl of E3 ubiquitin ligase for a (2x) concentration into an Eppendorf.
3. Add 5µL of the BRD3 Target.
4. Make the final volume to 50µL with assay buffer.
5. Add the E3 mixture to the Eppendorf with the PROTAC.
6. Incubate for 15-30 minutes.
7. Add 5µl of E1-E2-ubiquitin mix for a (2x) concentration into an Eppendorf.
8. Add desired amount of ATP to make the final concentration 800µM into the Eppendorf with the E1-E2-ubiquitin mix
9. Add enough assay buffer to the E1-E2-ubiquitin mix to make the final volume 50µL.
10. Add the E1-E2 mixture to the Eppendorf with the incubated E3 -PROTAC Mix.
11. Add 50µL of E3-PROTAC-E1-E2 mix to each well in the assay plate provided.
12. Incubate the assay plate for 1 hour and 30 minutes at room temperature while shaking. Follow by placing the plate at 37°C for 30 minutes.
13. Wash plate four (4) times with PBST using 120µL per well, tap out excess PBST from wells between washes.
14. Dilute 1µL of anti-BRD3 antibody and 99µL of (1X) BC for the primary.
15. Pipette 50µL of (1X) BC with primary to each well.
16. Incubate for1 hour and 30 minutes at room temperature.
17. Wash plate four (4) times with PBST using 120µL per well, tap out excess PBST from wells between washes.
18. Dilute 1µL of anti-mouse antibody and 99µL of (1X) BC for the secondary.
19. Incubate for 45 minutes at room temperature.
20. Wash plate four (4) times with PBST using 120µL per well, tap out excess PBS-T from wells between washes.
21. Dilute DR1 and DR2 to 1:50 or 1:10 as needed in ultra-pure water, 50µL should be added to each well.
22. Read the plate.

EXAMPLE DOSE RESPONSE OF VHL PROTAC MZP54 WITH THE PROTAC® *IN VITRO* UBIQUITINATION ASSAY KIT



MZP54 Dose	Replicate 1	Replicate 2	Replicate 3	Avearage	Standard deviation	RSD	Relative fold change
30	9.6E+06	1.1E+07	1.1E+07	1.1E+07	9.6E+05	9.0	7.4
10	6.0E+06	6.5E+06	6.7E+06	6.4E+06	3.4E+05	5.3	4.4
3	7.0E+06	7.2E+06	6.7E+06	7.0E+06	2.4E+05	3.5	4.8
1	8.2E+06	7.6E+06	7.5E+06	7.8E+06	3.6E+05	4.6	5.3
0.3	1.2E+07	1.2E+07	1.1E+07	1.2E+07	7.4E+05	6.3	8.0
0.1	2.9E+07	3.5E+07	2.6E+07	3.0E+07	4.4E+06	14.9	20.5
0.03	3.6E+07	3.2E+07	3.1E+07	3.3E+07	2.5E+06	7.7	22.6
0.01	2.9E+07	2.1E+07	2.3E+07	2.4E+07	4.4E+06	18.2	16.8
0.003	5.1E+06	5.2E+06	5.3E+06	5.2E+06	9.0E+04	1.7	3.6
0.001	3.2E+06	3.6E+06	3.0E+06	3.3E+06	2.8E+05	8.6	2.2
0.0003	1.5E+06	1.4E+06	1.8E+06	1.6E+06	2.0E+05	12.9	1.1
DMSO	1.7E+06	1.4E+06	1.3E+06	1.5E+06	2.1E+05	14.7	1.0

Premixed E1-E2-Ub mix was added to E3-BRD3 mix incubated with MZP54 a VHL PROTAC in a dose response study to study PROTAC mediated ternary complex and ubiquitination. Ubiquitination on BRD3 was captured using TUBEs on In vitro ubiquitination assay plate provided in kit using anti-BRD3 antibody. Chemiluminescence intensities were plotted against PROTAC doses to evaluate extent of ubiquitination and hook effect. The data set presented above represents overall signal for PROTAC mediated ubiquitination along with relative change in ubiquitination levels with dose of PROTAC. The standard deviation is represented as error bars with triplicate reads (n=3).

REFERENCES

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