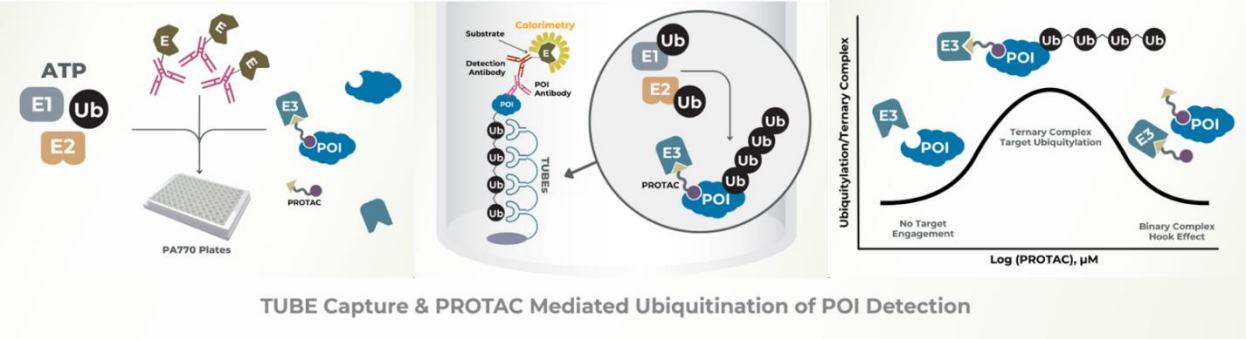
LifeSensors from genomics to proteomics

PROTAC® In Vitro Ubiquitination Assay Kit

Instruction Manual

Cat. No. PA770C



TUBE Capture & PROTAC Mediated Ubiquitination of POI Detection

BACKGROUND

Ubiquitin and Ubiquitin Conjugation Machinery

Ubiquitin is a small polypeptide (8.6 kDa) that can be conjugated via its C-terminus to amine groups of lysine residues on target proteins. This conjugation is called monoubiquitination. 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 polyubiquitination. The most characterized polyubiquitylation is lysine 48 linked (K48-linked) polyubiquitylation. Monoubiquitination 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.

Ubiquitination requires the coordinated function of three distinct ligases, **E1** (ubiquitin activating enzyme), **E2** (ubiquitin conjugating enzyme), and **E3** (ubiquitin ligase). Ubiquitin E3 ligases act as scaffold proteins, providing docking sites for a ubiquitin-conjugating enzyme (E2), and a target substrate (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 can lead to polyubiquitylation and proteasomal degradation of the target protein. However, this scenario does not always play out, and the PROTAC discovery strategy faces several challenges and pitfalls. The current assay addresses a lot of these challenges in evaluating PROTACs and serve as an effective tool to report true PROTAC efficiency by monitoring PROTAC mediated ubiquitination *in vitro*.

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 a PROTAC dependent reaction. For the assay, PROTACs are added first to the assay plate followed by E3 ligase and target protein to enable ternary complex formation. Subsequently, an E1-E2 enzyme cocktail with WT-ubiquitin and ATP is added to the wells to initiate PROTAC mediated ubiquitination. Polyubiquitin chains generated by the E1-E2-E3 machinery are captured in the wells. After washing unbound proteins, the captured polyubiquitylated target protein detected with antibody against protein of interest (not included) and secondary antibody conjugated with-HRP (included) allowing for detection by colorimetric method. The signal generated 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 freezing and thawing cycles. 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. **Assay Buffer (10X), Size: 1x 1.2 mL**
   Note: Add β-mercaptoethanol fresh to final concentration of 1mM in 1X assay buffer.
3. **E3 Mix (20X), Size: 1 x 250 µl**
   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 (20X), Size:1 x 250 µl**
5. **Secondary Antibody (100X), Anti-Mouse or Anti-Rabbit HRP conjugate, Size:1x 60µL**
6. **Colorimetric Reagent (CR), Size: 1x 5.0mL**
7. **Stopping Reagent (SR), Size: 1x 5.0 mL**
8. **Blocking Concentrate (5X), Size: 1x 5.0 mL**
9. **Positive Control Reagents**
   a. PROTAC in DMSO (50X): 10 µL (MZP54, dBET6, A1874)
   b. Target Protein positive control BRD3 (20X): 20 µL
   c. Anti-BRD3 antibody (100X): 5 µL
   d. Anti-Mouse HRP Conjugate (100X): 5 µL

ADDITIONAL ITEMS REQUIRED BUT NOT PROVIDED

1. **Wash Buffer(s),** 1X Phosphate Buffered Saline, 0.1% Tween (PBST)
2. **Absorbance 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

The 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 in this mix – prepare 200 µL

1. Add 20 µl **of E3 ubiquitin ligase** for a (2X) concentration.
2. Add desired volume, µL of target substrate to make final concentration of 40-80 nM.
3. Make the final volume to 200 µL with 1X **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 in µL of ATP to make final concentration to 800 µM.
3. Make the final volume to 200 µL with 1X **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 396 µL 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.

Colorimetric Reagent and Stopping Reagent

Add 50 µL per well of colorimetric reagent for color development followed by equal volume (50 µL per well) of stopping reagent to stop the reaction prior to reading absorbance at 450 nm.

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 50 µL, 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 1.0 µL into appropriate Eppendorf tube.
4. Add 20 µL 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 200 µL.
7. Add 25 µL 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 20 µL 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 200 µL.
12. Add 25 µL of E1-E2-Ubiquitin mix to the Eppendorf tubes with the PROTAC of interest and E3 Mix is located in.
13. Transfer 50 µL 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 120 µL 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
17. *Note:* If eight (8) wells are being used you would need 80µL of 5X BC, and 320µL PBS-T.
18. Dilute the primary antibody according to antibody manufacturer instructions in (1X) BC.
19. Pipette 50 µL of (1X) BC with diluted primary into each well.
20. Incubate for 1 hour and 30 minutes at room temperature with gentle orbital shaking.
21. Wash the assay plate four (4) times with PBS-T using 120 µL per well, tap out excess PBS-T from wells between washes.
22. Dilute 4 µL of anti-mouse-HRP or anti-rabbit-HRP conjugate as needed in 396 µL of (1X) blocking concentrate. For the positive control use 2 µL of anti-mouse-HRP conjugate in 98 µL of (1X) BC.
23. Pipette 50 µL of (1X) BC with diluted primary into each well.
24. Incubate the assay pate for 60 minutes at room temperature with gentle orbital shaking.
25. Wash the assay plate four (4) times with PBS-T using 120 µL per well, tap out excess PBS-T from wells between washes.
26. For colorimetric detection, add 50 µl of Colorimetric Reagent (CR) to each well, and incubate for 1-30 minutes. A blue color will develop and can be read at Absorbance 650 nm using the plate reader. When the solution has turned to a bright blue color at ~30 min (absorbance 650 = ~1.0), proceed to step 26.
    Note: prolonged incubation which makes the color darker blue might saturate the signal after adding stop solution. Do not let the signal saturate.
27. To stop the reaction, add 50 µl of Stopping Reagent (SR) in each well (Note: The stop solution is added on top of the colorimetric reagent). The solution will change color from blue to yellow. Measure the Absorbance at 450nm within 30 minutes of adding the stopping reagent.

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. For colorimetric detection, add 50 µl of Colorimetric Reagent (CR) to each well, and incubate for 1-30 minutes. A blue color will develop and can be read at Absorbance 650 nm using the plate reader. When the solution has turned to a bright blue color at ~30 min (absorbance 650 = ~1.0), proceed to step 26.
22. *Note: prolonged incubation which makes the color darker blue might saturate the signal after adding stop solution. Do not let the signal saturate.*
23. To stop the reaction, add 50 µl of Stopping Reagent (SR) in each well (Note: The stop solution is added on top of the colorimetric reagent). The solution will change color from blue to yellow. Measure the Absorbance at 450nm within 30 minutes of adding the stop solution.

REFERENCES

1. Hershko, A. and A. Ciechanover, The ubiquitin-proteasome pathway. *Annu Rev Biochem*, 1998. **67**: p. 425-479.
2. Welchman, R.L., C. Gordon, and R.J. Mayer, Ubiquitin and ubiquitin-like proteins as multifunctional signals. **Nat Rev Mol Cell Biol**, 2005. **6**(8): p. 599-609.
3. Ciechanover, A., The ubiquitin-proteasomal pathway: on protein death and cell life. Embo J, 1998. **17**(24): p. 7151-7160.
4. Raasi, S., *et al.*, Diverse polyubiquitin interaction properties of ubiquitin-associated domains. Nat Struct Mol Biol, 2005. **12**(8): p. 708-14.

LIMITED USE LABEL LICENSE

The PROTAC® *In Vitro* Ubiquitination Assay Kit and associated technologies are licensed from LifeSensors, Inc. For information on obtaining a license for commercial purposes, contact Director of Business Development, info@lifesensors.com, LifeSensors, Inc., Malvern PA 19355, Phone: 610.644.8845 or www.lifesensors.com.

The product and/or its derivatives are being furnished to the purchaser under the following conditions: By purchasing the product, the purchaser agrees to comply with the terms of this Limited Use Label License. The product is provided to purchaser for research use only. The purchaser of this product may not transfer or otherwise sell this product or its derivatives to a third party and no rights are given to the purchaser to use the product or its derivatives for commercial purposes as defined below.

Commercial purposes mean any activity for which a party receives consideration and may include, but is not limited to,

1. use of the product or its derivatives in manufacturing,
2. use of the product or its derivatives to provide a service, information, or data,
3. use of the product or its derivatives for diagnostic purposes,
4. transfer or sale of protein or chemical component made with the product or derivatives of the product,
5. re-sale of the product or its derivatives, whether or not such product or its derivatives are resold for use in research.

Product use must follow compliance with all laws and regulations, including but not limited to current EPA, FDA, USDA, and NIH guidelines. THE MATERIALS WILL NOT BE USED IN HUMANS.

Purchaser acknowledges that the product is experimental in nature and provided WITHOUT WARRANTY OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE OR ANY OTHER WARRANTY, EXPRESS OR IMPLIED. LIFESENSORS, INC. MAKES NO REPRESENTATION OR WARRANTY THAT THE USE OF THE PRODUCT WILL NOT INFRINGE ANY PATENT OR OTHER PROPRIETARY RIGHTS.

In no event shall LifeSensors, Inc. be liable for any use of the product by the purchaser. Purchaser agrees to defend, indemnify, and hold harmless LifeSensors, Inc. its officers, employees, and agents from any loss, claim, injury, damage, expense, or liability (including attorney's fees), of whatsoever kind or nature, which may arise from or in connection with this Agreement, including but not limited to purchaser use, handling or storage of the product.