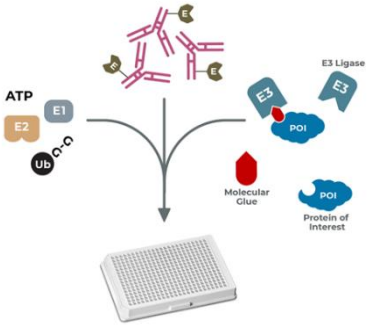
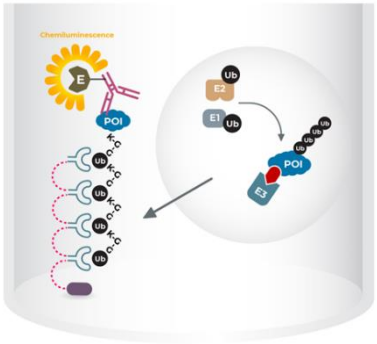
Molecular Glue® *In Vitro* Ubiquitination Assay Kit: Customizable Ubiquitin

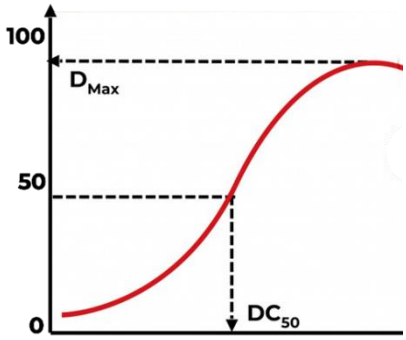
MANUAL

Molecular Glue In Vitro Ubiquitination Assay Kit

Catalog Number MG780







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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.

Molecular Glues®

Molecular glues are biologically active small molecules that induce specific interactions between E3 ligases and substrates, often by stabilizing otherwise non-natural protein-protein interactions. By bridging a target protein - commonly one linked to diseases - with an E3 ubiquitin ligase, molecular glues harness the cell's ubiquitin-proteasome pathway to selectively degrade the target protein. This mechanism works by facilitating the ubiquitination of the target protein, effectively tagging it for degradation by the proteasome. This assay is designed to address challenges in studying molecular glue where traditional proximity assays are incapable of studying weak interactions induced by prospective molecular glues. Our vision is that through this approach, one can effectively capture both proximity and catalytic nature of molecular glues by studying compound mediated ubiquitination, thus demonstrating the true function - enabling design and discovery of novel molecular glues.

ABOUT THE ASSAY

The *In vitro* ubiquitination kit has been developed to establish a high throughput approach that can accurately predict Molecular Glue efficiency by monitoring the protein's intrinsic ability to get ubiquitinated. We offer this kit for multiple E3 ubiquitin ligases. 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 Molecular Glue dependent reaction. For the assay, Molecular Glues 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 Molecular Glue 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 Molecular Glue 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 Molecular Glue activity by monitoring ubiquitination, with the target of your choice in HTS format.
2. Screen multiple Molecular Glue variants simultaneously
3. Accurately establish rank order potencies to guide medicinal chemists for reliable SAR.
4. Accelerate Molecular Glue drug discovery and clinical development by testing different combinations of Molecular Glues and ligases 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.

SUGGESTED USES

1. Testing molecular glue (MGs) activity.
2. Compare between multiple Molecular Glues and establish predictive **DC_{50}** from **Ub_{Max}**.
3. Choose all three ligases for comparing MGs activity to demonstrate selectivity.
4. Test Molecular Glue 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. **Cereblon 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 Molecular Glue 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**

Molecular Glue in DMSO (50X):	10 µL (Lenalidomide)
Target Protein positive control CK1α (20X):	20 µL
Anti-CK1α 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 (PBS-T)
2. **Luminescence capable plate reader**
3. **β-mercaptoethanol**
4. **100mM ATP**
5. **Molecular Glues 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.

Molecular Glue Dilutions

Prepare Molecular Glue doses at 50X concentration and transfer 1 µL per well into the In vitro Molecular Glue 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 Molecular Glue 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 1000 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 PBS-T (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-CK1α 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 (ant-CK1α) 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

Molecular Glue® *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.

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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 Molecular Glue doses at (50X) concentration and transfer 1.1µL into appropriate Eppendorf tube or a polypropylene plate for a larger number of samples.
4. Add 20µL of (20X) E3 Mix for a final dilution of (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 Molecular Glue of interest.
8. Incubate E3 Mix for 15-30 minutes at room temperature on a shaker
9. Add 20µL of (20X) E1+E2-Ubiquitin Mix for a final dilution of (2X) concentration in an Eppendorf tube.
10. Add desired amount of ATP to make the final concentration 1000µM 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 Molecular Glue of interest and E3 Mix is located in.
13. Incubate for 2 hours in a 37°C incubator.
14. Transfer 50µL of the mixes into each of the eight (8) wells in the in vitro ubiquitination assay plate.
15. Incubate the assay plate for 1 hour at room temperature while shaking.
16. Wash the assay plate four (4) times with PBS-T using 200µL per well, tap out excess PBS-T from wells between washes.
17. Prepare Blocking Concentrate (BC) by adding four (4) parts PBS-T to one (1) part of 5X BC

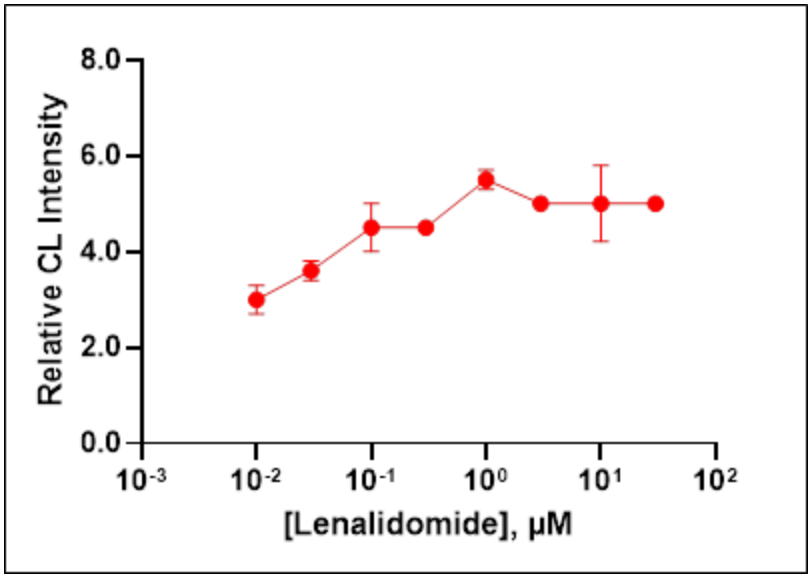
    Note: If eight (8) wells are being used you would need 80µL of 5X BC, and 320µL PBS-T.
18. Dilute the primary antibody according to antibody manufacturer instructions in (1X) BC.
19. Pipette 50µL of (1X) BC with diluted primary into each well.
20. Incubate for 1 hour and 30 minutes at room temperature while shaking.
21. Wash the assay plate four (4) times with PBS-T using 200µL per well, tap out excess PBS-T from wells between washes.
22. 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.
23. Pipette 50µL of (1X) BC with diluted primary into each well.
24. Incubate the assay pate for 45 minutes at room temperature while shaking.
25. Wash the assay plate four (4) times with PBS-T using 200µL per well, tap out excess PBS-T from wells between washes.
26. 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
27. Read with a plate reader optimized for detection of chemiluminescence for 5-10 reads with 1 min intervals.

Control for Detection Using CK1α (Suggested Protocol)

The CK1α 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 Lenalidomide Molecular Glue as positive control for CRBN 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 Molecular Glue 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 CK1α Target.
4. Make the final volume to 50µL with assay buffer.
5. Add the E3 mixture to the Eppendorf with the Molecular Glue.
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 1000µM 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 -Molecular Glue Mix.
11. Incubate for 2 hours in a 37°C incubator.
12. Add 50µL of E3-Molecular Glue-E1-E2 mix to each well in the assay plate provided.
13. Incubate the assay plate for 1 room temperature while shaking.
14. Wash plate four (4) times with PBS-T using 200µL per well, tap out excess PBS-T from wells between washes.
15. Dilute 1µL of anti-CK1α antibody and 99µL of (1X) BC for the primary.
16. Pipette 50µL of (1X) BC with primary to each well.
17. Incubate for 1 hour and 30 minutes at room temperature.
18. Wash plate four (4) times with PBS-T using 200µL per well, tap out excess PBS-T from wells between washes.
19. Dilute 1µL of anti-mouse antibody and 99µL of (1X) BC for the secondary.
20. Incubate for 45 minutes at room temperature.
21. Wash plate four (4) times with PBS-T using 200µL per well, tap out excess PBS-T from wells between washes.
22. Dilute DR1 and DR2 to 1:50 or 1:10 as needed in ultra-pure water, 50µL should be added to each well.
23. Read the plate.

EXAMPLE DOSE RESPONSE OF CRBN CK1α MOLECULAR GLUE LENALIDOMIDE *IN VITRO* UBIQUITINATION ASSAY KIT



Premixed E1-E2-Ub mix was added to E3-CK1α mix incubated with Lenalidomide a CRBN Molecular Glue in a dose response study to study Molecular Glue mediated ternary complex and ubiquitination. Ubiquitination on CK1α was captured using TUBEs on In vitro ubiquitination assay plate provided in kit using anti-CK1α antibody. Relative chemiluminescence intensities were plotted against Molecular Glue doses to evaluate extent of ubiquitination. The data set presented above represents overall signal for Molecular Glue mediated ubiquitination along with relative change in ubiquitination levels with dose of Molecular Glue. The standard deviation is represented as error bars with triplicate reads (n=3).

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. Daniel St-Cyr et al. ,Identification and optimization of molecular glue compounds that inhibit a noncovalent E2 enzyme–ubiquitin complex.Sci. Adv.7,eabi5797(2021).DOI:10.1126/sciadv.abi5797
5. Petzold, G., Fischer, E. & Thomä, N. Structural basis of lenalidomide-induced CK1α degradation by the CRL4CRBN ubiquitin ligase. Nature 532, 127–130 (2016). https://doi.org/10.1038/nature16979
6. Raasi, S., *et al.*, Diverse polyubiquitin interaction properties of ubiquitin-associated domains. Nat Struct Mol Biol, 2005. **12**(8): p. 708-14.

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