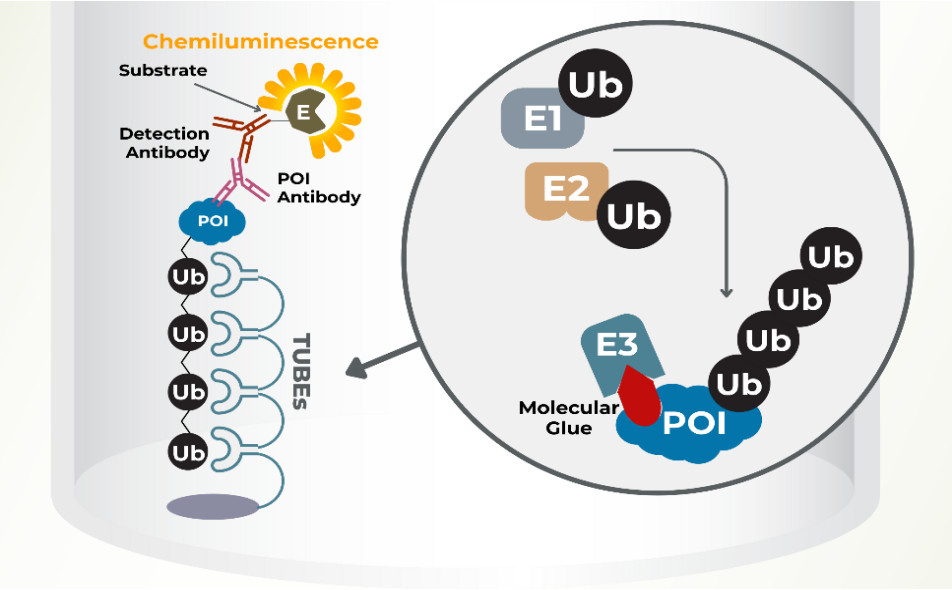
Molecular Glue® *In Vitro* Ubiquitination Assay Kit: Customizable Ubiquitin Ligase Kit

MANUAL

Molecular Glue In Vitro Ubiquitination Assay Kit
Catalog Number: **MG780**



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Cat. #MG780

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 of Interest (POI) requires the coordinated function of 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 POI. Ubiquitin E3 ligases act as scaffold proteins, providing docking sites for 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. In addition to the ubiquitylation of substrates, E3 ligases can also “auto ubiquitylate” themselves.

Molecular Glue®

Molecular Glues (Proteolysis-targeting chimeric molecules) artificially hijack the components of the UPS to degrade the target protein. Molecular Glue drugs are monovalent small molecules that binds to the E3 ligase making it to ubiquitinate a neo-substrate or a target protein of interest. 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 Molecular Glue discovery strategy faces several challenges and pitfalls. The current assay addresses a lot of these challenges in evaluating Molecular Glue and serves as a tool to effectively report true Molecular Glue efficiency by monitoring Molecular Glue mediated ubiquitination.

INTENDED USE

The In vitro ubiquitination kit has been developed to establish a high throughput approach that can accurately predict Molecular Glue efficiency by monitoring the target protein’s intrinsic ability to get ubiquitinated. We offer this kit for three E3 ubiquitin ligases, Cereblon, VHL and HDM2 to monitor Molecular Glue mediated ubiquitination for target of choice.

PRINCIPLE OF THE ASSAY

Microtiter plate strips, pre-coated with a proprietary TUBE reagent (assay plate) are used to capture polyubiquitin chains formed in a Molecular Glue dependent reaction. In the assay, Molecular Glues are incubated with E3 ligase and target Protein Of Interest (POI, not included) to enable ternary complex formation. Next, an E1-E2 enzyme cocktail with ubiquitin and ATP is added to initiate Molecular Glue mediated ubiquitination. Polyubiquitin chains generated by the E1-E2-E3-Molecular Glue-POI machinery are captured in the wells, washed, and the ubiquitinated POI is detected with antibodies against the POI (not included) and secondary antibody conjugated with-HRP (included) allowing for detection by chemiluminescence. Thus, the signal generated in this "sandwich" ELISA-like assay is a quantitative measure of Molecular Glue activity.

BENEFITS

1. Monitor Molecular Glue activity by monitoring ubiquitination, with the POI 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 rationally designing Molecular Glues that rely on functional assays that report protein's "ubiquitination potential".
5. This detection strategy does not require non-native tagging or labeling of ubiquitin, which could lead to experimental artifacts.

SUGGESTED USES

1. Testing and comparing multiple Molecular Glues for activity and establishing correlation with DC_{50}.
2. For comparing Molecular Glues activity with CRBN or other E3 ligase of choice.
3. Test Molecular Glue POI specificity and Isoform selectivity.

COMPONENTS INCLUDED IN THE KIT

1. ***In Vitro* Ubiquitination Assay Plate**
   Note: Do not thaw the assay plate rapidly to room temperature. We recommend placing the assay plate at 4°C for 30 minutes prior to transferring to room temperature.
2. **Assay Buffer (10X)** – 1 x 1.2 mL
   Important Note: *Add β-mercaptoethanol to a final concentration of 1 mM in 1X assay buffer*
3. **E3 Mix (20X)** – 1 x 250 µL
4. **E1-E2-Ubiquitin Mix (20X)** – 1 x 250 µL
5. **Secondary Ab (Anti-Mouse or Anti-Rabbit HRP conjugate, 100X)** –1 x 60µL
6. **Detection Reagents DR1 & DR2 (2X)** – 1 x 1 mL of Hydrogen Peroxide and 1x 1mL of Luminol
7. **Blocking Concentrate (5X)** –1 x 5 mL

Positive Control Reagents:

8. **Biotin-Poly-Ubiquitin Chain (20X): -** 1 x 25 µL
9. **Streptavidin HRP (100X):** 1 x 10 µL

STORAGE AND STABILITY

Store all materials at -80ºC. Avoid multiple freeze/thaw cycle. All components are stable for at least 6 months if stored in the proper temperature.

ADDITIONAL ITEMS REQUIRED BUT NOT INCLUDED IN THE KIT

1. **Purified Target Protein Of Interest**
2. **Molecular Glues (including known positive controls if needed)**
3. **Anti-POI Specific Primary Antibodies (ELISA compatible and highly specific)**
4. **WASH BUFFERS:** 1X Phosphate Buffered Saline, 0.1% Tween (PBS-T)
5. **LUMINESCENCE CAPABLE PLATE READER**
6. **β-mercaptoethanol (Typical stock is 14.4M, dilute in ultrapure water before adding to 1X assay buffer at 1mM final concentration). See the table below.**
7. **100 mM ATP (freshly prepared)**
8. **15 mL centrifuge tubes**
9. **Plate sealing film.**
10. **Polypropylene plate (Optional)**
11. **Multi-channel Pipettors and Automatic Plate Washer (Optional)**

PREPARATION OF DILUTION FOR IN VITRO UBIQUITINATION REACTION

Note: It is essential that the entire manual is thoroughly reviewed before starting the procedure.

Assay Components	Final Concentration	2X Concentration	Volume Required for 8-wells or 1 strip	Volume Required for 96-wells
Assay Buffer (10X)				
10X Assay buffer	1X		40 µL of 10X	500 µL of 10X
β-mercaptoethanol	1mM		3 µL of 0.14M stock	4 µL of 1.4 M stock
Ultra-Pure Water			357 µL	4,496 µL
E3 Mix (20X)				
E3 Mix			20 µL	240 µL
Target Protein Of Interest (-- µM)	10-40nM	20-80nM	-- µL	-- µL
Assay buffer	1x		Bring up to 200 µL	Bring up to 2,400 µL
Molecular Glues (50X) and Vehicle Control (typically 100% DMSO)	0-30µM		1.1 µL/reaction	1.1µL/reaction
E1 + E2 Ubiquitin Mix (20X)				
E1 + E2 Ubiquitin Mix			20 µL	240 µL
ATP (100 mM)	0.5 mM	1 mM	2 µL	24 µL
1x Assay buffer			178 µL	2,136 µL
Blocking Concentrate (5X)				
5X Blocking Concentrate	1X		160 µL	1 mL
PBS-T, 1X			640 µL	4 mL
Anti-POI Primary Antibody				
Anti-POI Primary Ab (per manufacturer's recommendation)	1:1000 to 1:5000		0.4 µL	5 µL
1X Blocking Concentrate			400 µL	5 mL
Secondary Antibody-HRP conjugate				
100X Secondary Ab-HRP			4 µL	50 µL
1X Blocking Concentrate			396 µL	4,500 µL
Biotin-poly-ubiquitin Chain (Positive Control)				
20X Biotin-poly-ubiquitin Chain			7.5 µL	20 µL
1X Blocking Concentrate			142.5 µL	380 µL
Streptavidin HRP for detecting positive control				
100X Streptavidin HRP			4 µL	4 µL
1X Blocking Concentrate			396 µL	396 µL
Detection Reagents				
DR1	1:400 to 1:200 dilutions		1.25 µL	12.5 µL
DR2			1.25 µL	12.5 µL
Ultrapure water			997.5 µL	9.975 mL

DETAILED PROTOCOLS:

Note: This Protocol is suitable for performing reactions in 8-wells. If you use more than 8-wells, scale up accordingly (see the Table above). Additional wells are required if using the positive control (supplied) to verify the plate performance.

1. Allow coated plate wells to equilibrate at 4ºC for at least 30 minutes before transferring to room temperature. Thaw assay components on ice. Gently mix the solutions and quickly spin down to bring all the liquid to the bottom of the tube.
2. Prepare **Molecular Glue** doses at (50X) concentration in a suitable vehicle (typically 100% DMSO). Transfer **1.1 µL of Molecular Glues** and **1.1µL of vehicle** (typically 100% DMSO) into microcentrifuge tube or polypropylene plate.
3. Add 20µL of **E3 Mix** for a (2X) concentration in a labeled microcentrifuge tube.
4. Add appropriate amount (in µL) of **target POI** to make final concentration of 40-80 nM at 2X to the E3 Mix.
5. Add sufficient volume of 1X assay buffer to the "**E3 Mix+POI**" to make the final volume 200µL.
6. Add **25µL** of "**E3 Mix+POI**" to each microcentrifuge tube or well containing the Molecular Glue of interest.
7. Incubate **"E3 Mix+POI+Molecular Glue**" for **30 minute**s at **room temperature** with gentle shaking. This pre-incubation step allows formation of ternary complex.
8. Add 20µL of "E1-E2-ubiquitin mix" for a (2X) concentration into a labeled microcentrifuge.
9. Add 2 µL of ATP to make the final concentration 1mM at 2X into the "**E1-E2-ubiquitin mix**"
10. Add 178µL 1x assay buffer to the "E1-E2-ubiquitin mix+ATP" to make the final volume 200µL.
11. Add **25 µL** of "**E1-E2-ubiquitin mix+ATP**" to the tubes containing "**E3 Mix+POI+Molecular Glue**". This step will start the ubiquitination reaction.
12. **Incubate for 2 hours** in a **37ºC** incubator.
13. Quickly transfer 50µL of the reaction mixes into each of the wells in the in vitro ubiquitination **assay plate strip**. Avoid bubble formation during transfer.
14. Seal the assay plate and **incubate for 60 minutes** at **room temperature** with shaking at 300 RPM on an orbital shaker.
15. Remove the seal, tap out the solution if using manual washing. Wash the assay plate 4 times with PBS-T using 200µL per well, tap out excess PBS-T from wells between washes.
16. Prepare 800µL of 1X Blocking Concentrate (BC) by adding 160µL of 5X BC to 640µL PBS-T
17. *Optional step:* If using the supplied positive control (Biotin-polyubiquitin chain) to verify plate performance, add 7.5 µL the 20X Biotin-polyubiquitin chain into 142.5 µL 1X BC (Separate strip wells and additional 1X BC needs to be prepared for this).
18. Dilute the anti-POI primary antibody according to antibody manufacturer instructions in (1X) BC.
19. Pipette **50 µL** of diluted **anti-POI primary antibody** into each well.
20. *Optional step:* Pipette 50 µL of diluted Biotin-polyubiquitin chain (positive control) into three wells in assay plate. Pipette 50 µL 1X BC into three wells as blank control.
21. Seal the plate and incubate the assay plate for **90 minutes** at **room temperature** with shaking at 300RPM on an orbital shaker.

22. Wash the assay plate 4 times with PBS-T using 200µL per well, tap out (upside down) excess PBS-T from wells between washes on paper towels or other blotting paper. **NOTE:** DO NOT ALLOW WELLS TO DRY COMPLETELY.
23. Dilute the secondary anti-mouse or anti-rabbit antibody as needed in (1X) BC to a final dilution of 1X. Dilute the Streptavidin-HRP in 1X BC for positive control detection.
24. Pipette **50µL of secondary antibody-HRP** diluted in 1X BC into each well. *Optional step:* Pipette 50µL of Streptavidin-HRP in 1X BC into wells containing positive control and blank.
25. Seal the plate and incubate the assay plate for **45-60 minutes** at room temperature with shaking at 300RPM on an orbital shaker.
26. Wash the assay plate 4 times with PBS-T using 200µL per well, tap out excess PBS-T from wells between washes.
27. Just before use, mix 1.25 µL DR1 and 1.25µL DR2 into 1.0 mL (1:400 dilution) of ultrapure water (deionized or distilled). Add **100 µL of Detection Reagent Mix** to each well and read immediately with a plate reader optimized for chemiluminescence for 5-10 reads with 1 min intervals.
Important Note: *DR1 and DR2 amounts needed will vary based on overall signal and plate reader being used. Optimize the amount of DR1 and DR2 needed by reading with a 1:400 dilution of each reagent and then titrating up (1:200 to 1:100) based on initial raw values.*

EXAMPLE DATA

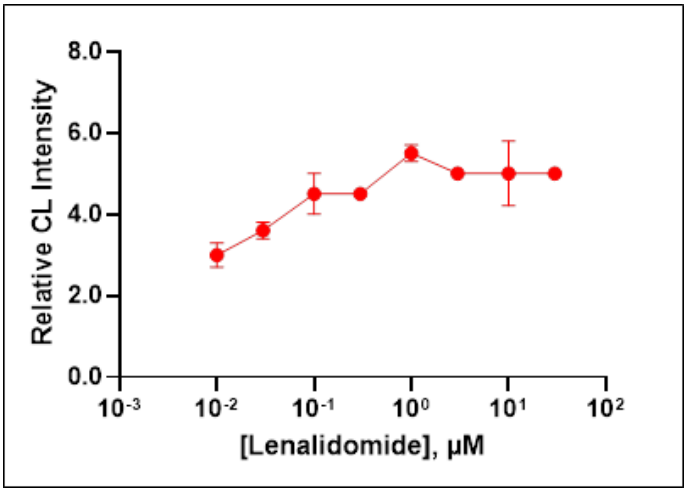

Figure 1. ***Lenalidomide Molecular Glue promotes CK1α ubiquitination by CRBN E3 ligase in vitro***: CRBN E3 ligase was preincubated for 30min with Lenalidomide, a CRBN Molecular Glue at indicated doses and ubiquitination was carried out for 90 min at RT. Ubiquitinated CK1α captured on the assay plate was detected using anti-CK1α 1º Ab followed by washing with PBST and incubation with 1:2000 secondary Ab-HRP conjugate (R&D Systems, HAF007). After washing with PBST, detection reagent diluted to 1:200 was added to the wells and chemiluminescence was detected using ClarioStar (BMG LabTech) plate reader. Raw chemiluminescence intensity was plotted using GraphPad prism. The standard deviation is represented as error bars with triplicate reads (n=3).

OPTIMIZIATION OF Molecular Glue MEDIATED IN VITRO UBIQUITINATION ASSAY

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To ensure accurate and reproducible results when using the In Vitro Ubiquitination Kit, proper optimization of experimental conditions and various assay parameters is essential. Below are key factors to consider for both Molecular Glues.

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1. Understanding Molecular Glues

- Molecular Glues: Monovalent molecules that simultaneously bind a target protein and an E3 ubiquitin ligase, bringing them into proximity for ubiquitination.

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2. Key Optimization Strategies

Protein of Interest (POI) Titration

- We typically recommend titrating the POI between 5 nM and 40 nM to determine the optimal concentration for ubiquitination.
    - Too high POI: Risk of substrate saturation, binary complex formation, hook effect, reducing the ubiquitination efficiency of Molecular Glues
    - Too low POI: Weak signal may not adequately reflect intrinsic ubiquitination potential.

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3. Primary Antibody Titration

- Start at the recommended concentration provided by the antibody manufacturer and titrate at both higher and lower dilutions to optimize signal-to-background ratio.
- Ensure the chosen antibody is validated for ELISA applications and has strong supporting data. Poor antibody quality can compromise the results and lead to reduced signal to background.
    - Excess 1º antibody: Increases background noise and non-specific binding.
    - Insufficient 1º antibody: Weak signal may obscure subtle ubiquitination effects.

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4. Molecular Glue Concentration Optimization

Dose-response curves should be generated to confirm that the Molecular Glue is working effectively and producing the expected ubiquitination profile.

- Molecular Glues: Titrate concentrations (typical range is 0-30µM, half-log dilutions) to balance ubiquitination efficiency. A hook effect can be seen with Molecular Glue mediated POI ubiquitination although this is not always the case.

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5. Reaction Time Course

- Molecular Glues: Ubiquitination kinetics may be rapid if the ternary complex is highly productive. A time course experiment might be needed to find the peak ubiquitination time point.

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6. Controls for Data Interpretation

- Negative Controls: Reactions lacking Molecular Glue, or E3 ligase help assess background signal.
- Positive Controls: Using well-characterized Molecular Glues ensures assay validity.

TROUBLESHOOTING GUIDE: ENSURING SUCCESS WITH THE Molecular Glue IN VITRO UBIQUITINATION KIT

Even with proper optimization, occasional issues may arise during ubiquitination assays. Below are common problems, possible causes, and recommended solutions to help customers achieve reliable and reproducible results.

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1. Weak or No Ubiquitination Signal

Possible Causes & Solutions:

- POI concentration too low → Increase POI concentration within the 5 nM – 40 nM range.
- Ineffective Molecular Glue → Perform a dose-response curve to verify activity. Check compound integrity by LC-MS if possible. Molecular Glues can go bad over multiple freeze-thaws.
- Suboptimal reaction conditions → Ensure ATP is present in the reaction, buffers are fresh and contains β-mercaptoethanol, and all components are at their recommended concentrations.
- Incompatible E3 ligase → Confirm that the chosen E3 ligase can effectively ubiquitinate the POI. Consider testing multiple ligases if unsure.
- Poor primary antibody performance → This can be a major hurdle in getting optimal results. Verify that the 1º antibody is validated for ELISA and titrate as needed. A poor antibody can lead to non-specific signals. Try different lot of antibodies.
- Incomplete protein folding or aggregation → If working with recombinant POI, confirm that it is properly folded and not forming aggregates that prevent efficient ubiquitination.

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2. High Background Signal or Non-Specific Ubiquitination

Possible Causes & Solutions:

- Insufficient washing between steps → Inadequate washing can leave non-specific proteins on the plate, leading to high background. Ensure proper plate washing using the recommended buffer and multiple wash steps.
- Excess POI or E3 ligase → Reduce protein concentrations to prevent non-specific ubiquitination.
- Primary antibody concentration too high → Titrate the antibody to reduce non-specific binding.
- Non-specific ubiquitination of other proteins → Run a negative control (no Molecular Glue) to confirm specificity.
- Contaminated reagents or buffers → Use fresh reagents and ensure proper storage.
- Aggregated or precipitated POI → Centrifuge protein stocks before use and avoid freeze-thaw cycles that may cause aggregation.
- If using a plate washer to wash the plates, use freshly made wash buffer.

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3. Unexpected Ubiquitination Patterns

Possible Causes & Solutions:

- Molecular Glue instability → Ensure the compound is fresh and properly stored (e.g., protected from light, kept at -80°C if needed).
- Non-optimal reaction time → Run a time-course experiment to determine the ideal incubation period.
- E3 ligase degradation or inactivation → Check for protein degradation by running an SDS-PAGE gel to confirm protein integrity.

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4. Inconsistent Results Between Experiments

Possible Causes & Solutions:

- Variation in protein or reagent concentrations → Always prepare master mixes and use consistent protein stocks.
- Temperature fluctuations → Maintain a stable incubation temperature throughout the assay.
- Improper washing of plates → Inconsistent washing can cause variability in signal intensity. Ensure uniform, thorough washing between steps.
- Poor pipetting accuracy → Use calibrated pipettes and low-retention tips to ensure precise reagent additions.
- Cross-contamination between wells → Be cautious when adding reagents to prevent splashing or cross-contamination between wells.

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5. No Change in Ubiquitination with Molecular Glue Treatment

Possible Causes & Solutions:

- Molecular Glue not functional → Run a positive control with a known, well-characterized Molecular Glue.
- Incorrect stoichiometry of Molecular Glue/POI/E3 ligase → Perform dose-response experiments to find the optimal concentration of each component.
- Protein stability issues → Check if the POI is being degraded prematurely by running a Western blot for total protein levels.

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6. Poor Reproducibility Across Experiments or Between Users

Possible Causes & Solutions:

- Inconsistent incubation times → Use a timer to ensure precise reaction timing between experiments.
- Variation in reagent lot numbers → If switching reagent lots, validate the new batch by running side-by-side comparisons.
- Improper mixing of reagents → Always mix reagents gently but thoroughly to avoid gradients in concentration within the wells.
- User variability in pipetting technique → Train all users on consistent pipetting methods and consider using multi-channel pipettes to minimize variability.

Final Tips for Experimental Success

- Run Controls – Always include positive and negative controls to validate results.
- **Use High-Quality Antibodies** – Poor antibodies can produce misleading data. Choose ELISA-validated antibodies with strong supporting data.
- Optimize Titrations – Proper titration of POI, primary antibody, and Molecular Glue is essential for high-quality results.
- Check Reagent Stability – Ensure Molecular Glues and protein stocks are stored correctly to prevent degradation.
- Repeat Key Experiments – If results are inconsistent, repeat experiments with fresh reagents and carefully controlled conditions.
- Wash the Plate Properly – Inadequate washing between steps can lead to high background signals and retention of non-specific proteins. Be sure to follow recommended washing steps, including multiple wash cycles, to ensure cleaner results.
- Confirm Proper POI Folding & Stability – If using recombinant proteins, ensure that they are correctly folded and not forming insoluble aggregates.

By systematically optimizing these parameters and following the troubleshooting steps, the user can maximize the predictive power of the In Vitro Ubiquitination Kit, enabling a more accurate assessment of Molecular Glue-mediated ubiquitination.

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4. transfer or sale of protein or chemical component made with the product or derivatives of the product,
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