PA480: K48 Ubiquitin Linkage ELISA Kit

Targeted Protein Degradation – PROTAC® MOA

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BACKGROUND

The ubiquitin proteasome system (UPS) is a tightly regulated pathway that eukaryotic cells employ for a myriad of cellular functions. At the center of this system is ubiquitin (Ub), a small (8.5 kDa) protein that is conjugated to target proteins *via* its C-terminal glycine to a lysine residue of the protein of interest (POI). Additional Ub molecules can be attached to the conjugated Ub on the POI at its M1 methionine or any of the seven lysine residues (K6, K11, K27, K29, K33, K48, K63). This site-specificity gives rise to a coded language in the UPS that earmarks the protein for different fates, triggering a different cellular pathway. For example, the most well-studied UPS pathway concerns K48 chains, which flag proteins for proteasomal degradation to maintain protein homeostasis.

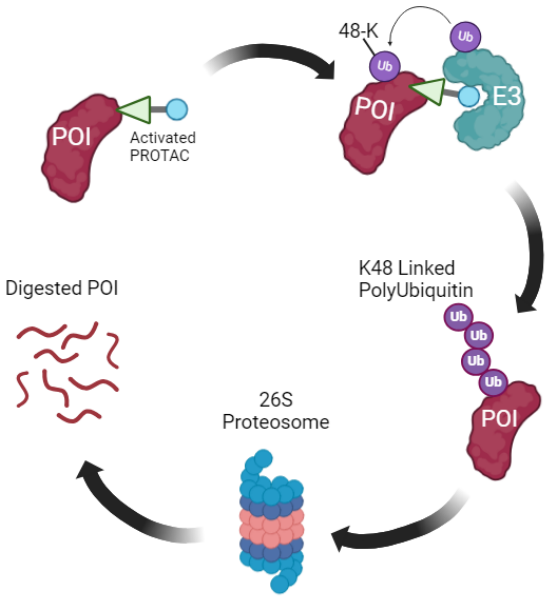


***Figure 1**: Schematic representation of polyubiquitin enrichment on disease causing proteins of interest (POI) mediated by PROTACs, eventually leading to proteasomal degradation.*

In recent years, hijacking the UPS for targeted protein degradation has been a desirable target for small molecule drug discovery. These candidates will often bring the target protein in proximity to an E3 ligase for enhanced degradation. PROTACs (Proteolysis-targeting chimeric molecules) are hetero-bifunctional small molecules that artificially hijack the ubiquitin-proteasome system to degrade target proteins. They consist of two ligands connected by a linker: one ligand binds to the target protein and the other binds to an E3 ligase, facilitating polyubiquitination and proteasomal degradation of the target protein (*Figure 1*).

Protein degradation mediated by PROTACs are typically governed by rates and extent of K48 polyubiquitination on target disease causing proteins. Studying UPS highjack using PROTACs by evaluating kinetics and extent of K48 ubiquitination is crucial for developing potent therapeutic options. Understanding ubiquitin dynamics is crucial for medicinal chemists to rationally PROTACs with improved potencies. LifeSensors PA480 ELISA kit designed specifically to capture, enrich, and quantitatively profile K48 ubiquitinated targets. The kit utilizes a highly sensitive K48 specific capture reagent coated on a plate that can be used to measure the level of K48 ubiquitination or K48 mediated proteasomal degradation of target proteins. This kit enables the researcher to conduct a variety of experiments regarding targeted protein degradation, including (but not limited to) the identification of K48 ubiquitinated proteins, understanding protein degradation pathways, and drug discovery and validation.

Studying changes in target protein ubiquitination is a challenging task that requires highly sensitive reagents. The K48 TUBEs, specially designed for enriching K48 polyubiquitinated proteomes in ELISA-based assays, have demonstrated remarkable sensitivity, with binding affinities reaching sub-nanomolar levels. The multivalency of TUBEs, a key feature that facilitates tight binding to polyubiquitin chains, allows for stringent washing to ensure minimal non-specific binding, resulting in reliable assays. Surface Plasmon Resonance (SPR) studies using new K48 TUBEs designed for ELISA assays have shown impressive sensitivity, with dissociation constants (KD) ranging between 1-2 nM (*Figure 2*).

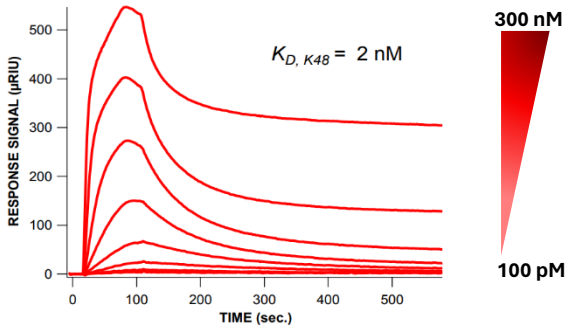


Figure 2: *SPR sensogram of K48 TUBE capture reagent against K48 tri-ubiquitin chains. Biotinylated tri-ubiquitin chains were immobilized on a neutravidin coated surface. The binding affinity of K48 TUBE capture reagent was determined in a dose response study (max dose 300 nM). The capture reagent showed no measurable binding to K6, K11 or K63 chains run in parallel.*

Translating such high-affinity TUBE reagents into user-friendly ELISA-based approaches is crucial for expanding their utility and enhancing drug discovery solutions for targeted protein degradation. To validate the selectivity of K48 TUBE reagents for K48-linked polyubiquitin chains, we performed a dose-response study with recombinant biotinylated di-ubiquitin chains of four different linkages: K6, K11, K48, and K63 with concentrations ranging from 300 pM to 300 nM. Simultaneous analysis of all chains on the same plate revealed remarkable selectivity for K48, with over three orders of magnitude difference in detection sensitivity compared to other linkages (*Figure 3*). Captured biotinylated di-ubiquitin chains using PA480 plates were detected using streptavidin-HRP via chemiluminescence in a compatible plate reader, with signals integrated over 60 seconds prior to analysis.

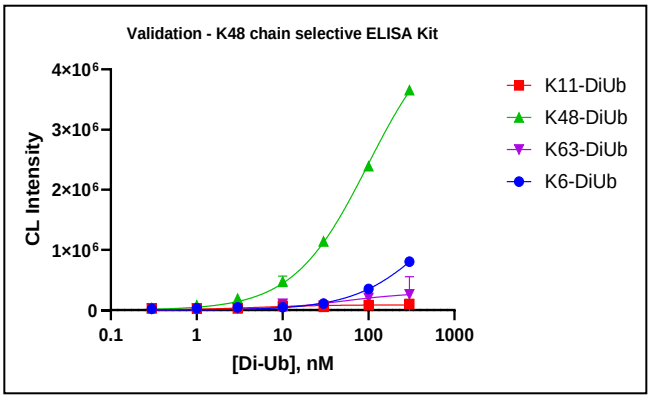
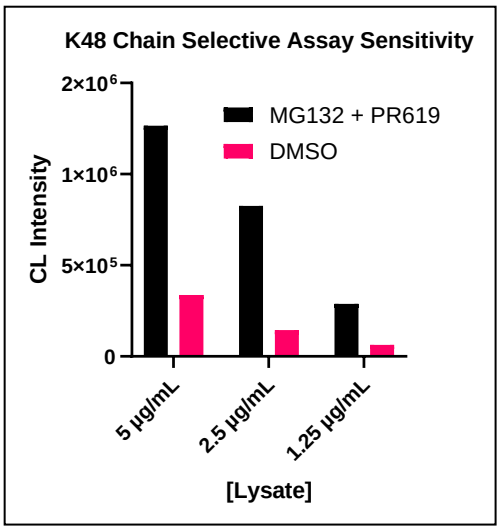


Figure 3: Validation of PA480 ELISA kit. Dose response of K6, K11, K48, and K63 biotinylated Di-Ubiquitin chains captured on ELISA plates coated with K48 selective capture reagent detected using streptavidin HRP. Error bars represent standard deviation, n=3.

Studying PROTAC-mediated ubiquitination of a target protein is akin to finding a needle in a haystack. Assays with remarkable sensitivity in complex matrices such as cellular and tissue lysates, as well as clinical samples, are crucial for accelerating PROTAC discovery. As shown in Figure 4, the remarkable detection sensitivities, even at ~1 µg/mL of protein lysates, establish the utility of this approach in studying complex samples containing ubiquitinated proteins. A robust signal-to-background ratio between the vehicle control (DMSO) and the proteasome + DUB inhibitor sample, which predominantly results in K48 ubiquitin linkage, has been demonstrated. Cellular lysates derived from HeLa cells treated with 3 µM MG-132 and PR-619, along with DMSO as a vehicle control, were evaluated. Detection of cellular K48 ubiquitination was performed using K48 TUBE HF Biotin (UM307) and streptavidin-HRP. The robust signal-to-background ratio (>3-fold) between DMSO control and proteasome inhibitor-treated samples, even at 1.25 µg/mL of lysate concentration, highlights the sensitivity of the ELISA kit. Error bars represent standard deviation, n=3 (*Figure 4*).

Figure 4: Sensitivity of LifeSensors K48 ubiquitin linkage ELISA Kit in



cellular lysates. Cellular lysates derived from HeLa cells treated with 3µM MG-132 and PR-619 along with DMSO as vehicle control were evaluated. Detection of cellular K48 ubiquitination was performed using K48 TUBE HF Biotin (UM307) and streptavidin HRP. Robust signal-to-background between DMSO control and proteasome inhibitor treated samples even at 1.25 µg/mL of lysate concentration represent sensitivity of the ELISA Kit. Error bars represent standard deviation, n=3.

PROTAC® Mechanism of Action - Oncogene KRAS

KRAS (Kirsten rat sarcoma) has garnered significant interest in the field of targeted protein degradation. Mutated in approximately 20% of human cancers, KRAS is a highly sought-after target for small molecule drugs. The KRAS gene encodes a membrane bound GTPase that interacts with receptor tyrosine kinases and plays a critical role in cell proliferation, differentiation, and survival. Mutations in KRAS can lead to hyperactivation of downstream signaling pathways, causing uncontrolled cell proliferation. Notably, the KRAS p.G12C mutation is prevalent in cancers, accounting for over 50% of KRAS mutations in lung adenocarcinoma tumors. PROTACs targeting KRASG12C, such as the small molecule LC-2 from Bond et al., link the KRAS G12C covalent inhibitor MRTX849 with the VHL E3 ligand to achieve robust degradation.

Bond, M. J., Chu, L., Nalawansha, D. A., Li, K., & Crews, C. M. (2020). Targeted degradation of oncogenic KRASG12C by VHL-recruiting PROTACs. ACS central science, 6(8), 1367-1375.

Evaluating PROTAC-mediated ubiquitination and degradation in a high-throughput screening (HTS) format will ensure rational design opportunities. Since PROTACs function by hijacking the UPS, validating the extent of ubiquitination and the types of ubiquitin linkages on target proteins to establish a link with degradation is warranted.

Traditional Western blot approaches to monitor ubiquitination and degradation are not reliable for rapid PROTAC design. Additionally, as seen in studies performed by Bond et al., (*Figure 5*). Time course in NCI-H2030 cells by Bond et al., demonstrated LC-2 and LC-2 Epimer PROTACs engage with KRAS within 1 hour evident by covalent adduct formation on KRAS G12C reaching maximal engagement and significant degradation occurred within 4 hours. of treatment with maximum degradation achieved by 8 hours. that persisted up to 24 hours.

Understanding degradation kinetics is crucial for chemists to design PROTACs with different linkerology or exit vectors to improve the rate of degradation. This assay replaces labor-intensive, semi-quantitative Western blot methods for examining K48 polyubiquitination and protein degradation, offering quantitative, reproducible results and facilitating high-throughput screening for compound library processing and structure-activity relationship (SAR) studies.

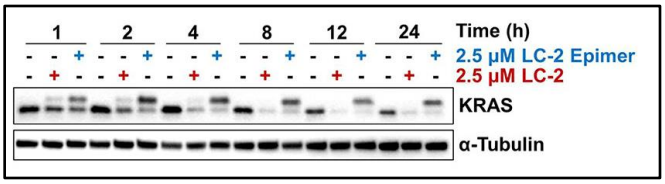


Figure 5: Time course in NCI-H2030 cells. LC-2 and LC-2 Epimer PROTACs engage with KRAS within 1 h. Maximal engagement and significant degradation occurred within 4 h. of treatment with maximum degradation was reached by 8 h. that persisted up to 24 h.

Reference: Bond, M. J., Chu, L., Nalawansha, D. A., Li, K., & Crews, C. M. (2020). Targeted degradation of oncogenic KRASG12C by VHL-recruiting PROTACs. ACS central science, 6(8), 1367-1375.

PA480 K48 chain-selective ELISA assays were used to investigate changes in K48 ubiquitination and degradation kinetics following treatment with LC-2 PROTAC for KRAS in NCI-H358 cells (KRAS G12C +/-). H358 cells were treated with LC-2 PROTAC (1 μM) in a time-course study ranging from 0 to 1020 minutes. Cell lysates were prepared according to the PA480 product manual, and 15 μg/well of cellular lysates were processed to evaluate changes in K48 ubiquitination mediated by LC-2 PROTAC.

Briefly, cellular lysates were incubated in PA480 assay plates for 1.5 hours, followed by washing to remove unbound material as per the manual's instructions. Anti-KRAS mouse antibody at 0.3 μg/mL in 1X blocking concentrate was used to detect K48 ubiquitinated levels in the captured proteome. The anti-KRAS antibody was incubated for 1.5 hours, followed by washing as recommended. Signal detection was achieved using anti-mouse HRP conjugate at 1:1000 dilution in 1X blocking concentrate for 45 minutes, followed by stringent washing as detailed in the manual. Chemiluminescence intensity was integrated using detection reagents 1 and 2, and the intensities were plotted against treatment time in the time-course study.

As shown in *Figure 6*, the PA480 ELISA kit demonstrated that maximum ubiquitination occurred between 2 to 4 hours of LC-2 treatment, consistent with maximal engagement, while significant degradation occurred within 4 hours, and maximum degradation reached by 8 hours, demonstrated in studies by Bond et al. using NCI-H2030 cells.

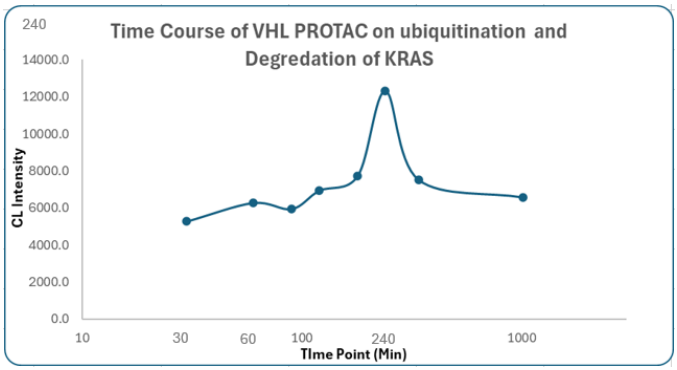


Figure 6: Time course in NCI-H358 cells. Cells were treated with PROTAC (1 μM) in a time course study with times ranging from 0 to 1020 minutes. Enhancement in chemiluminescent intensity by 2 hours represent K48 ubiquitination on KRAS reached maximum followed by protein turnover. Error bars represent standard deviation, n=3.

Overall, the PA480 kit provides a rapid, quantitative, and reproducible alternative to the labor-intensive, semi-quantitative Western blot methods traditionally used to examine K48 polyubiquitination and protein degradation in cells. Additionally, the assay enables high-throughput screening of compound libraries, facilitating rank order potency and aiding chemists in establishing structure-activity relationships (SAR). **PA480 is a pioneering assay platform offering a versatile method for researchers to understand how PROTACs interact with proteins of interest, aiding rational design.**