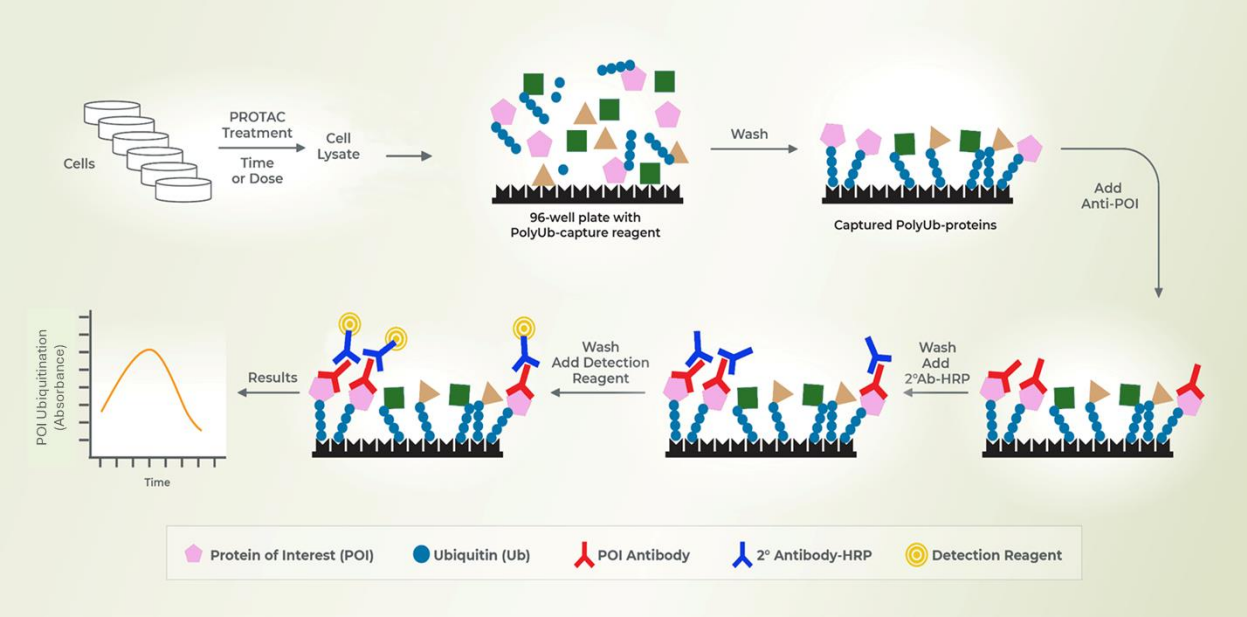
LifeSensors from genomics to proteomics

K63 Ubiquitin Linkage ELISA Kit
(Chain Selective)

Instruction Manual

Cat. No. PA630C



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Intended Use

To enrich and quantitate -K63 ubiquitination in cellular and tissue lysates.

The Ubiquitin Proteasome System (UPS) is a dynamic process involved in various cellular functions, including cell signaling, DNA repair, and proteasomal degradation. Ubiquitin can form diverse chains on its seven lysine residues, with K48 and K63-linked polyubiquitination being a crucial post-translational modification. While K48 polyubiquitination predominantly drives protein degradation and maintains cellular homeostasis. K63 linked polyubiquitination plays pivotal role in various cellular processes, primarily associated with trafficking, signaling pathways, DNA repair mechanisms and the regulation of protein-protein interactions. Lifesensors' K63 Ubiquitin Linkage ELISA Kit enables relative and absolute quantitation of K63 ubiquitination on target proteins in cellular and tissue lysates under various treatments and conditions.

This assay replaces labor-intensive, semi-quantitative Western blot methods for examining K63 polyubiquitination, offering quantitative, reproducible results and facilitating high-throughput screening for compound library processing and structure-activity relationship (SAR) studies in drug discovery applications.

*The plate is designed for research use only.

Principle of the assay

The K63 Ubiquitin Linkage Assay Kit is a sandwich ELISA-based assay that captures K63 polyubiquitinated proteins from cell lysates in wells pre-coated with a proprietary K63 polyubiquitin capture reagent. Non-polyubiquitinated proteins or those ubiquitinated on lysine residues other than K63 are removed by washing. An antibody directed against the target protein is then used for quantitation. A secondary antibody conjugated to horseradish peroxidase (HRP) generates a measurable colorimetric signal on a microplate reader, which directly correlates with the levels of K63-linked polyubiquitinated target proteins.

Components

Coated plates:	One pre-coated and blocked 96-well strip plate (***Store at -80°C***)
Blocking concentrate (BC):	12 mL of 5X blocking agent for antibody dilution (***Store at 4°C***)
Plastic plate seals:	Two provided.
Colorimetric reagent (CR):	5.0 mL vial of CR reagent (***Store at 4°C***)
Stopping reagent (SR):	5.0 mL vial of SR reagent (***Store at 4°C***)
UPS Inhibitor cocktail (10X)	1000 µL vial (in RIPA Lysis Buffer; ***Store at -20°C***)
	(Contains PR-619, MG-132 and 1,10-phenanthroline)
Proteasome Inhibitor (10x)	1000 µL vial (in RIPA Lysis Buffer; **Store at -20°C**)

Components required but not supplied

PBS and PBS containing 0.1% Tween 20 (PBS-T) or TBS / TBS-T for phosphorylated targets.

Phosphatase Inhibitors as needed for studying phosphorylated targets.

Disposable gloves, pipette tips, reagent reservoirs

Multi-channel pipettor, Automatic plate washer (optional), An absorbance microplate reader

Optional

Decomplexing Buffer - SKU: UE-1003-2000. This buffer is available for purchase on the LifeSensors website **here**.

For some targets, the decomplexing agent enhances the signal to background. We recommend the use. of the decomplexing agent prior to analysis with PA950, PA480 & PA630. The use of the decomplexing agent disrupts any native protein complexes that might be part of ubiquitin complexes. This urea based decomplexing agent results in reduced background signal, resulting in a better signal-to-background ratio.

Detailed protocol

1. Remove plate from freezer and reagents from refrigerator and allow them to reach room temperature (RT). Incubations are performed at either 4°C or RT (22°-27°C). To avoid cross contamination, do not re-use plate sealers.
2. Prepare and take sufficient amount of cell lysate and decomplex the lysate with PA950 Decomplexing Buffer for 15 min with vortexing at 5 min interval. Add 10-20 µg/well lysate diluted with PBST (V_t = 50-100 µl/well). Seal the plate.
3. Incubate plate for overnight (~16.0 hours) at 4°C with gentle orbital shaking.
4. Wash plate with PBST (4 x 200 µL/well). After the last wash, remove the residual buffer by lightly tapping the plate (upside down) on paper towels or other blotting paper. DO NOT ALLOW WELLS TO DRY COMPLETELY.
5. Dilute primary antibody in 1x Blocking concentrate (dilution depends on primary antibody efficiency; a good starting dilution is 0.5-1 µg/mL) and add 100 µL/well and incubate plate for 1 hours at RT with shaking.
6. Repeat washing as described above (see step 4).
7. Dilute secondary HRP-conjugated antibody in 1x blocking concentrate (dilution as recommended by manufacturer) and add 50-100 µL/well and incubate plate for 30-45 minutes at room temperature with shaking.
8. Repeat washing as described above (see step 4).
9. 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 10.
    a. *Note: prolonged incubation which makes the color darker blue might saturate the signal after adding stop solution. Do not let the signal saturate.*
10. 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.

Notes

1. It is critical to perform an appropriate time-course experiment with optimum dose of compounds or other treatments to enable capturing of ubiquitinated targets.
2. Optimize antibody dilution and specificity of target protein by western blotting. Selection of a good, clean target-specific antibody suitable for sandwich ELISA is essential for the success of this assay.
3. Optimize lysate concentration depending on target abundance.
4. See suggested lysis buffer below to have the best polyubiquitination profile of target protein.
5. Include appropriate controls for best results and interpretation.

Cell lysis protocol

1. Aspirate medium completely and rinse cells with ice cold 1X PBS. Scrape cells appropriately with 1X PBS, centrifuge to pellet cells and remove PBS.
2. Freeze cell pellet at -80°C for long term storage or proceed with lysis on ice.
3. Add RIPA lysis buffer (5-10 times the volume of the pellet, (i.e. 100 µL of pellet add 500-1000 µL lysis buffer). Vortex intermittently for 10-15 minutes while keeping the sample on ice to allow efficient lysis. RIPA lysis buffer needs to be supplemented with UPS inhibitor cocktail to preserve ubiquitination signature and generate robust signal-to-background.
4. Centrifuge at 13,000 xg for 15-20 minutes at 4°C.
5. Collect the supernatant (lysate) and determine protein concentration using standard methods.

RIPA Lysis Buffer

50 mM Tris-HCl, pH 7.5

150 mM NaCl

1% NP40

1% Sodium deoxycholate

2 mM EDTA

1 mM PMSF

10% Glycerol

20 µM MG132 (proteasome inhibitor)

50 µM PR-619 (pan DUB inhibitor)

5 mM 1,10-phenanthroline (JAMM-type isopeptidase inhibitor)

Protease inhibitor cocktail (Sigma cat #P8849, 1:500)

Representative Data for PA630C – K63 Ubiquitin Linkage ELISA Kit:

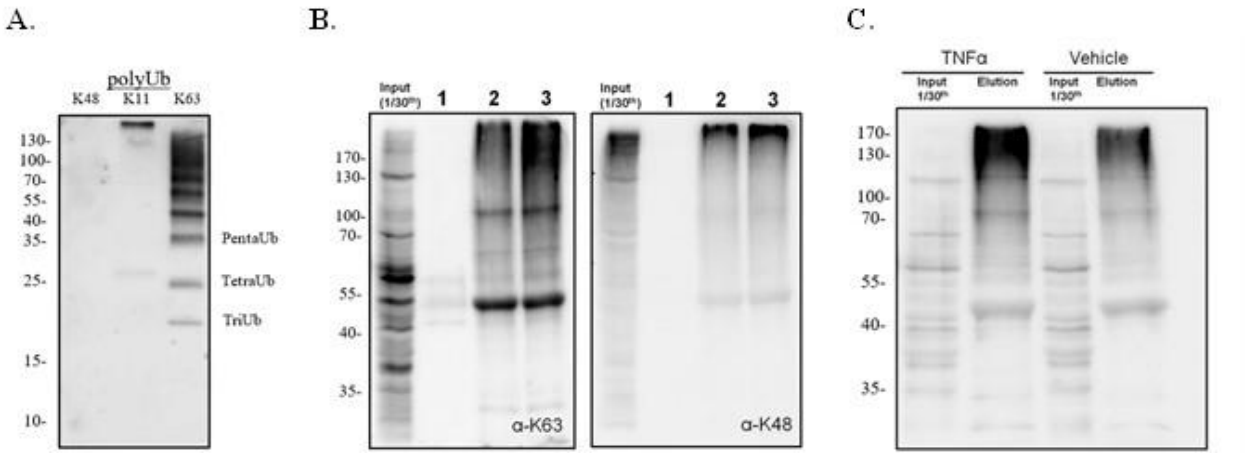

Figure 1: Validation of K63 ubiquitin linkage selective TUBEs (A) K48, K63 and K11 polyubiquitin chains were generated enzymatically followed by far western detection using K63 TUBE to investigate sensitivity and specificity of the TUBEs. Data represents good selectivity with K63 polyubiquitinated chains. (B) HEK293T cellular lysates used for IP applications using 0.5µM (2) or 1µM (3) FLAG® K63-TUBE. ~2mg total protein lysate was used to enrich K63 ubiquitinated proteome using anti-Flag M2 resin (10µl) for 3h (4°C). Enriched and eluted fractions are probed with commercial monoclonal antibodies specific for either K63- or K48-polyUb (Cell Signaling) to demonstrate K63 IP selectivity. Results indicate that K63 polyUb species are clearly enriched (α- K63), while the enrichment of K48 polyUb is relatively poor (α-K48). (C) Macrophages were isolated from murine bone marrow cultured for 8 days in DMEM supplemented with a combination of 10% FCS and conditioned media from the cultured murine L929 fibroblasts. Macrophages were then harvested and treated with either vehicle control or TNFα (20µg/ml) for 15 min to induce up regulation of K63-specific ubiquitylation. Enrichment of K63 ubiquitinated proteome using 500nM FLAG® K63-TUBE was performed analyzed by immunoblot using anti-K63 (α- K63).

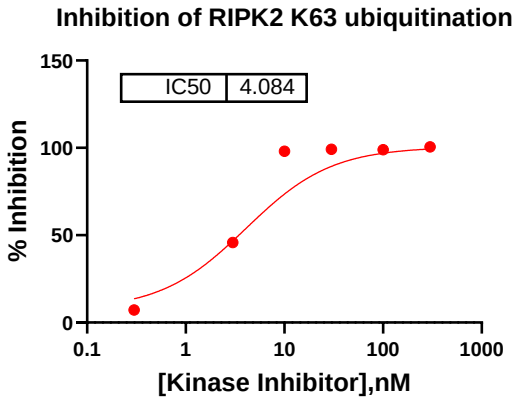

Figure 2: Validation of kinase inhibitor that inhibits K63 polyubiquitination on RIPK2 kinase using LifeSensors K63 Ubiquitin linkage ELISA Kit. RIPK2 K63 polyubiquitination triggered by L18-MDP (Lysine 18-muryldipeptide) treatment for 30 min in THP-1 cells. RIPK2 K63-polyubiquitination mediated by L18-MDP can be blocked by kinase inhibitors. Linkage specific K63-TUBE ELISA kit clearly demonstrated effect of kinase inhibitor on RIPK2 inhibition reaching 100% inhibition at 100-300 nM of kinase inhibitors with IC50 values ~4nM consistent with literature. The data clearly suggests that PA630C can be used to selectively monitor levels of K63 polyubiquitination in the presence of a stimulation like L18-MDP followed by monitoring inhibitory effects to predict IC50s crucial for reliable drug discovery.