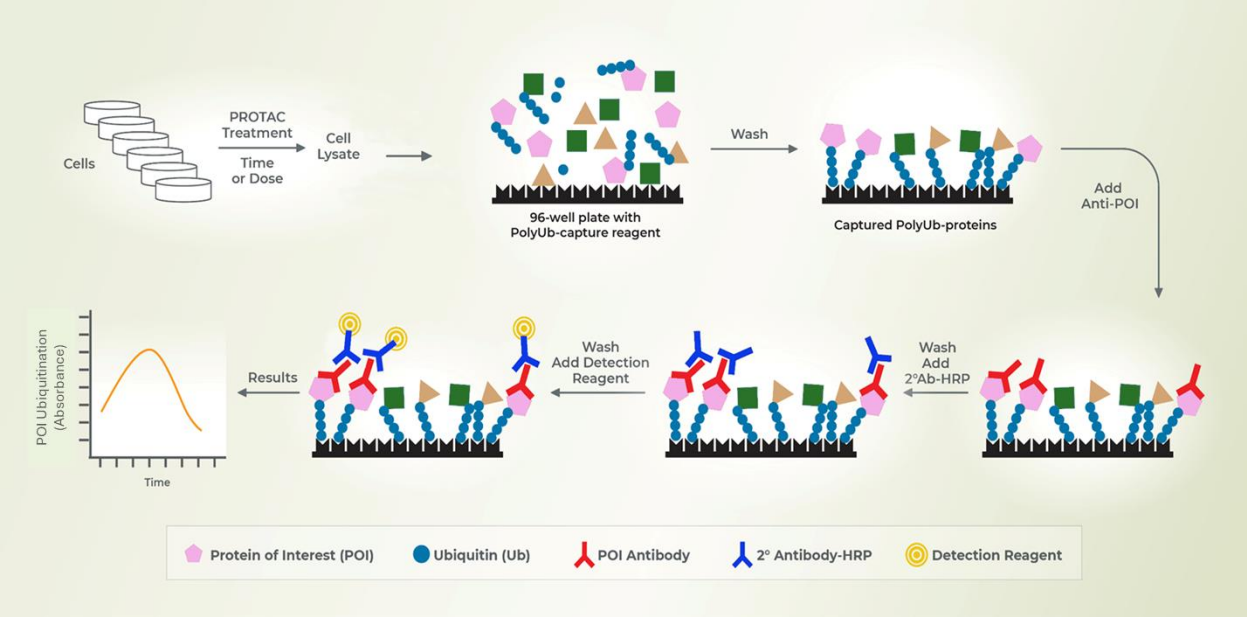
K48 Ubiquitin Linkage ELISA Kit
(Chain Selective)

Instruction Manual

Cat. No. PA480C



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Intended Use

To enrich and quantitate -K48 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-linked polyubiquitination being a crucial post-translational modification that drives protein degradation and maintains cellular homeostasis. Lifesensors' K48 Ubiquitin Linkage ELISA Kit enables relative and absolute quantitation of K48 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 K48 polyubiquitination and protein degradation, offering quantitative, reproducible results and facilitating high-throughput screening for compound library processing and structure-activity relationship (SAR) studies.

*The plate is designed for research use only.

Principle of the assay

The K48 Ubiquitin Linkage Assay Kit is a sandwich ELISA-based assay that captures K48 polyubiquitinated proteins from cell lysates in wells pre-coated with a proprietary K48 polyubiquitin capture reagent. Non-polyubiquitinated proteins or those ubiquitinated on lysine residues other than K48 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 K48-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. For Targeted protein degradation applications - to monitor K48 polyubiquitination of target protein followed by its degradation, please select times before and after Dmax of PROTAC. It is critical to perform an appropriate time-course experiment with optimum dose of PROTAC to enable capturing of ubiquitinated targets before its degraded.
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. For suspension cells, spin down the cells at ~1500 rpm for 3-5 min, gently aspirate media, wash the pellet with ice cold 1X 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)