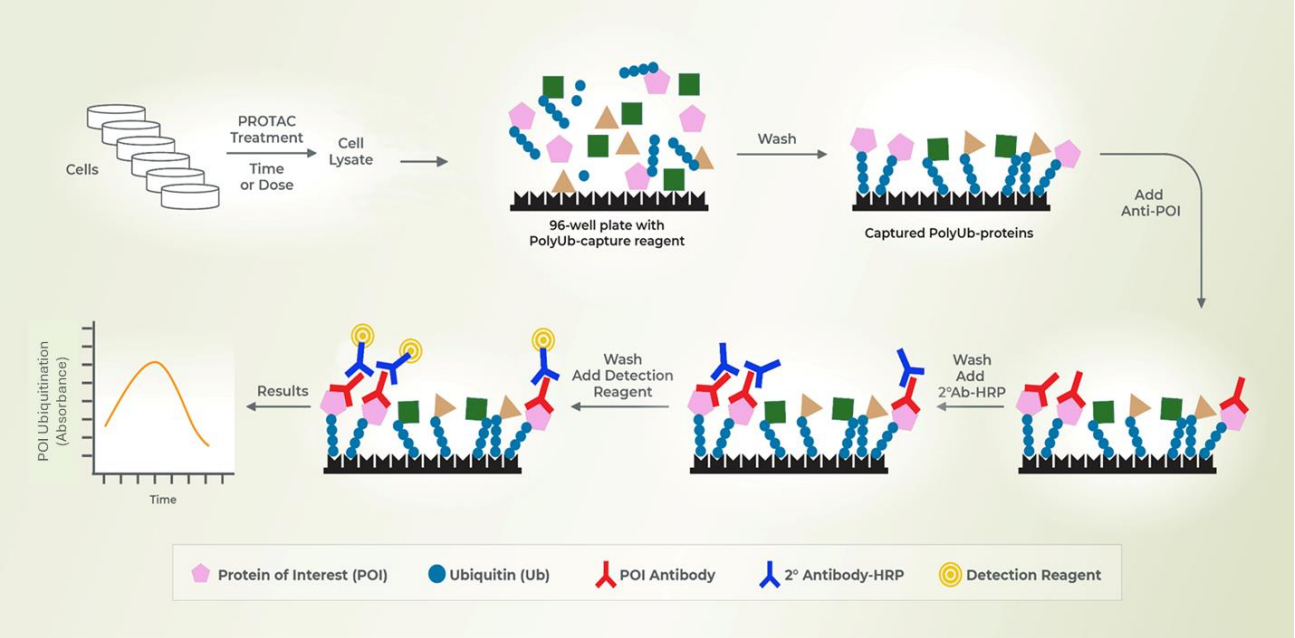
LifeSensors from genomics to proteomics

# PROTAC® Assay Plate

## *Instruction Manual*

## *Cat. No. PA950C*



## Intended Use

LifeSensors' PROTAC® Assay Plate is intended for the relative determination of the ubiquitination of a target protein in cellular lysates after PROTAC treatment. This assay is designed to replace the more laborious, semi-quantitative western blot methods to examine polyubiquitination and degradation of a target protein in cells and provide quantitative and reproducible results. Additionally, this assay allows for high-throughput screening to process compound libraries and establish rank order potency helping chemists establish SAR. The plate is designed for research use only and is not intended for human or animal diagnostic or therapeutic applications.

## Principle of the assay

The PROTAC® Assay Plate is a sandwich-based assay in which polyubiquitinated proteins from cell lysates are captured in the wells of a precoated microtiter plate using a proprietary polyubiquitin binding reagent. Non-ubiquitinated proteins are removed by washing and then an antibody directed against the target protein is added followed by washing. Lastly, a secondary antibody conjugated to horse radish peroxidase (HRP) is used to measure the bound target antibody with colorimetric detection reagents and an absorbance microplate reader.

## 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 mL vial of CR reagent (***Store at 4°C***) |
| Stopping reagent (SR): | 5 mL vial of SR reagent (***Store at 4°C***) |
| MG132 | 25 µL vial (10 mM in DMSO; ***Store at -20°C***) |
| PR-619 | 25 µL vial (22 mM in DMSO; ***Store at -20°C***) |
| 1,10-phenanthroline | 100 µL vial (500 mM in DMSO; ***Store at -20°C***) |

## Components required but not supplied

Phosphate buffered saline (PBS) and PBS containing 0.1% Tween 20 (PBST)

Disposable gloves, pipette tips, reagent reservoirs

Multi-channel pipettors

Automatic plate washer (optional)

A microplate reader capable of reading Absorbance

## Optional

**PA950 Decomplexing Buffer** - SKU: UE-1003-2000

For some targets, the decomplexing agent enhances the signal to background. We recommend the use of the decomplexing agent prior to analysis with PA950C. The use of the decomplexing agent disrupts any

native protein complexes that might be part of ubiquitin complexes. Using this urea based decomplexing agent results in reduced background signal, resulting in a better signal-to-background ratio.
This buffer is available for purchase on the LifeSensors website **here**.

## Detailed protocol

1. Remove the plate from freezer and reagents from the refrigerator and allow them to reach room temperature (RT). Incubations are performed at either 4ºC 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 of decomplexed lysate diluted with PBST (Total volume = 50-100 µL/well). Seal the plate.
3. Incubate plate for overnight (~16 hours) at 4ºC with gentle orbital shaking (~120 rpm).
4. Wash plate with PBST (4 x 180 µL/well). After the last wash, remove the last droplets of 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 agent (dilution depends on primary antibody efficiency; a good starting dilution is 1 µg/mL) and add 100 µL/well. Incubate plate for 90 minutes at RT with gentle orbital shaking (~300 rpm).
6. Repeat washing as described above (see step 4).
7. Dilute secondary HRP-conjugated antibody in 1x Blocking agent (dilution as recommended by manufacturer) and add 100 µL/well and incubate plate for 1.0 hour at room temperature with gentle orbital shaking (~300rpm).
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, protected from light. 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.

   *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 450 nm within 30 minutes of adding the stopping reagent.

## Notes

1. To monitor polyubiquitination of target protein, which is typically followed by its degradation, select time points before and after the 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 it gets 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 at RT, 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.
4. Centrifuge at 13,000 x g for 15-30 min 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)

## Example of PROTAC assays

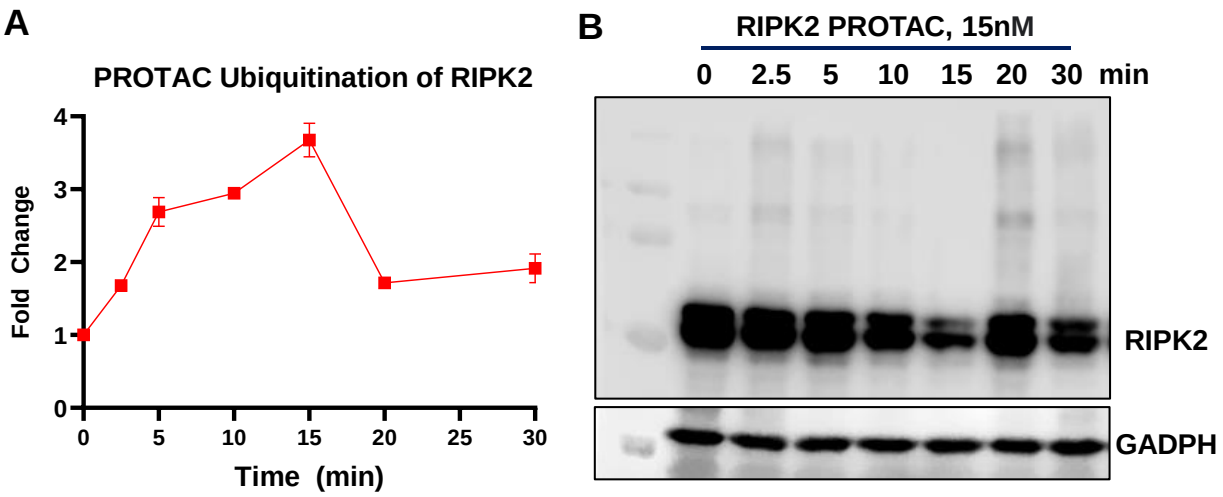



**Figure 1. Quantification of PROTAC mediated RIPK2 ubiquitination in cells**. **A)** K562 cells (1.5x10^6 cells/ml) were treated with RIPK2 PROTAC at 15nM for indicated time points. Cells were harvested, lysed, decomplexed and polyubiquitinated proteins were captured onto PA950 PROTAC Assay plate and detected with anti-RPIK2 antibody (Santa Cruz Cat# Sc-166765, 1:250 dilution). Fold change in RIPK2 ubiquitination relative to time point zero was plotted using GraphPad Prism. **B)** Cell lysates (15µg) were separated on 10% SDS-PAGE and probed with anti-RIPK2 antibody (Santa Cruz Cat# Sc-166765, 1:1000). GAPDH was used as loading control.