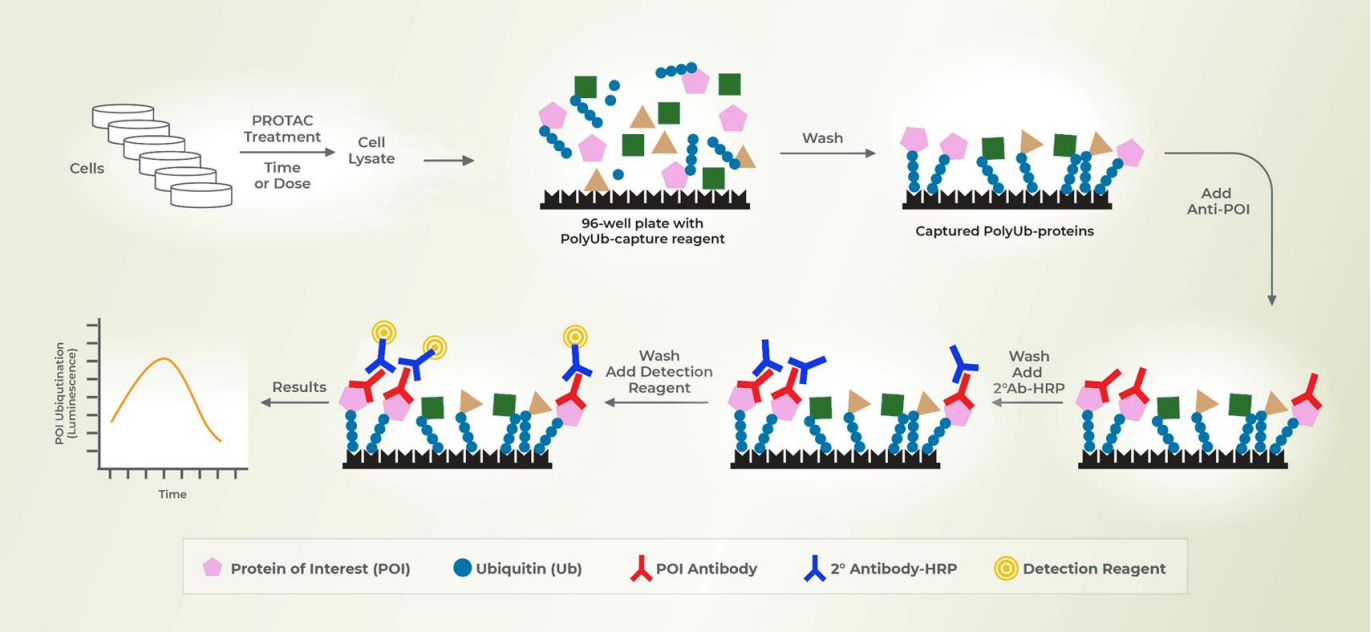
LifeSensors from genomics to proteomics

PROTAC® Assay Plate

Instruction Manual

Cat. No. PA950



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. Proteins that are not polyubiquitinated/unbound 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 detection reagents and a luminescence 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
Detection reagent 1 (DR1):	1.0 mL vial of DR1 reagent (***Store at 4°C***)
Detection reagent 2 (DR2):	1.0 mL vial of DR2 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 luminescence microplate reader

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 PA950. 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 plate from freezer and reagents from refrigerator and allow them to reach room temperature (RT). All incubations are performed at RT (22°-27°C). To avoid cross contamination, do not re-use plate sealers.
2. Prepare cell lysate and use 15-20 µg/well diluted with PBS (V_t = 50-100 µl/well).
3. Incubate plate for 2 h at RT with shaking.
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 50-100 µL/well and incubate plate for 1 h at RT with shaking.
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 50-100 µL/well and incubate plate for 1 h at room temperature with shaking.
8. Repeat washing as described above (see step 4).
9. Just before use, mix 800 µL of DR1 and 800 µL of DR2 into 10 mL of ultrapure water (deionized or distilled). Add 50-100 µL of this solution to each well and read with a plate reader optimized for chemiluminescence for 5-10 reads with 1 min intervals.

Notes

1. To monitor polyubiquitination of target protein followed by its degradation, please select times before and after Dmax of PROTAC.
2. Optimize antibody dilution and specificity of target protein by western blotting. Selection of target antibody should be suitable for sandwich ELISA-based assays.
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.
4. Centrifuge at 13,000 xg for 15-20 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)

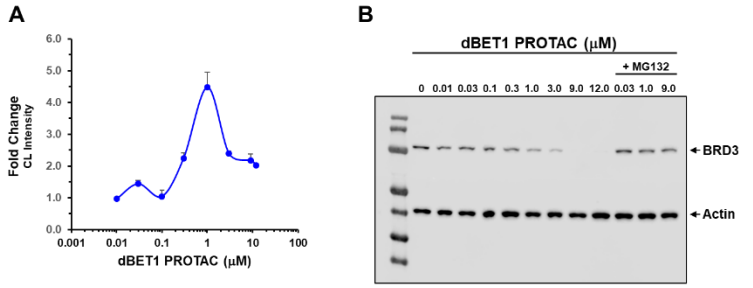
5 mM 1,10-phenanthroline (JAMM-type isopeptidase inhibitor)

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

Aprotinin (10 mg/ml stock) 20 µL/10 mL

Examples of PROTAC assays

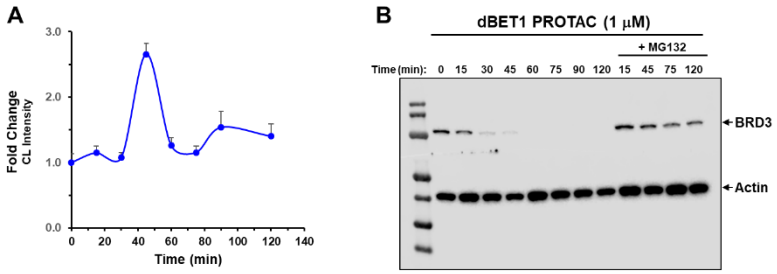
Dose response of dBET1 PROTAC on ubiquitination and degradation of BRD3



(A) Jurkat cells were untreated (DMSO) or treated with dBET1 PROTAC (0.01 - 12.0 µM) for 45 min. Cell lysates (15 µg/well) were processed as described in the manual for PROTAC® Assay Plate (Cat# PA950). Results are expressed as Fold Change of average chemiluminescent intensity ± Std Dev (n=3).

(B) Cell lysates (25 µg) from Jurkat cells treated as in (A) were immunoblotted for BRD3 and β-Actin. The proteasomal inhibitor MG132 was used as a control to prevent BRD3 degradation.

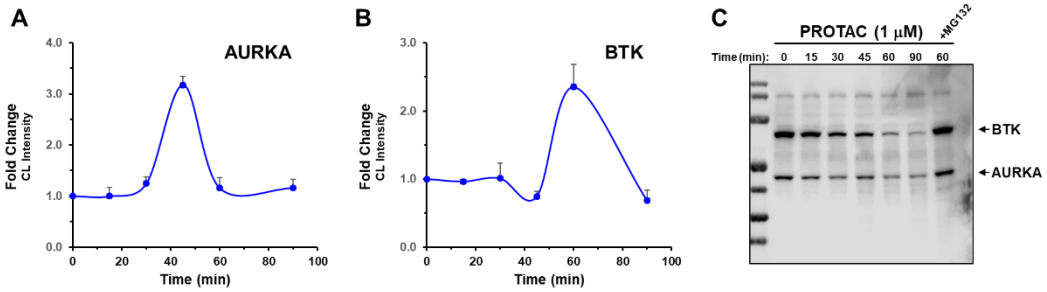
Time course of dBET1 PROTAC on ubiquitination and degradation of BRD3



(A) Jurkat cells were treated with dBET1 PROTAC (1 µM) for various times (0-120 min). Cell lysates (15 µg/well) were processed as described in the manual for PROTAC® Assay Plate (Cat# PA950). Results expressed as Fold Change of average chemiluminescent intensity ± Std Dev (n=3).

(B) Cell lysates (25 µg) from Jurkat cells treated as in (A) were immunoblotted for BRD3 and β-Actin. The proteasomal inhibitor MG132 was used as a control to prevent BRD3 degradation.

Time course of cereblon-based pan kinase PROTAC on ubiquitination and degradation of selected kinases Aurora Kinase A and BTK



(A-B) K562 cells were treated with PROTAC (1 µM) for various times. Cell lysates (15 µg/well) were processed as described in the manual for PROTAC® Assay Plate (Cat# PA950). Results are expressed as Fold Change of average chemiluminescent intensity ± Std Dev (n=3).

(C) Cell lysates (25 µg) from K562 cells treated as in (A) were immunoblotted for BTK and AURKA. MG132 was used as a control (last lane).