LifeSensors from genomics to proteomics

UbiQuant™ Ultra Plate

Cat. No. UE905C

Instruction Manual

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Background

The Ubiquitin/Proteasome Pathway (UPP): Post-translational modification of proteins by ubiquitin (Ub) is a key regulatory process that impacts almost all cellular functions. Apart from the established role of Ub in protein degradation, Ub is now implicated in cell signaling, DNA damage response, protein trafficking, cell-cycle progression, inflammation, immune response and regulation of apoptosis. Ubiquitination occurs through isopeptide linkage between the C-terminus of Ub and the ε-amino group of a lysine (Lys) residue on the target substrate (1). Ub itself has seven Lys residues (K6, K11, K27, K29, K33, K48, and K63), each of which can participate in further ubiquitination, generating polyUb chains (2). The ability of Ub to form polymers through various lysines appears to be central to the versatility of this system in regulating cell processes. The most extensively characterized of these polymers are linked through either K48 or K63. K48-linked polyUb predominantly targets proteins for proteasomal degradation, whereas K63-linked polyUb appears to regulate protein function, subcellular localization, or protein-protein interactions (3;4). A growing body of evidence now implicates K11-linked polyUb in mitotic regulation (5-7) and the scope of research into this linkage type is expected to mirror K48 and K63 in the coming years. Interest in additional linkage types beyond these is expected to grow exponentially over the next decade as they become identified with disease states and/or specific cellular processes. Ubiquitination is a reversible and dynamic process. Ubiquitination machinery, composed of enzymes known as E1, E2, and an E3, work in concert to ubiquitylate proteins both in monomer and polymer state. Corresponding removal of mono- and polyUb chains (as well as polyUb chain degradation) is accomplished by *de*ubiquitination enzymes (DUBs).

Intended Use

LifeSensors' UbiQuant™ Ultra plate is intended to be used in ELISA assay for the relative determination of the concentration of a specific ubiquitylated target protein in cells. This assay is designed to replace more laborious, semi-quantitative immunoprecipitation and Western blots to examine changes in ubiquitination.

Principle of the assay

The UbiQuant™ Ultra plate is a sandwich ELISA assay in which total ubiquitinated proteins in a cell lysate are captured in the wells of a precoated microtiter plate using a proprietary ubiquitin binding reagent. Unbound protein is removed by washing and then the amount of bound target protein is determined using either an antibody specific to the target protein or to an epitope tag incorporated into the target protein. After removing unbound antibody, the bound protein is measured using an enzyme linked anti-antibody.

Detailed Protocol

1. We recommend that all samples be tested in triplicate. Incubations are performed at either 4°C or room temperature (22°-27°C). To avoid cross contamination, do not re-use plate sealers.
2. Determine the number of strip wells required and place them into the holder supplied. Return the unused strips to the bag, tape closed, and refrigerate.
3. Pipette 100 µL each of: blank, controls, and test samples into appropriate wells and cover with plastic plate sealer. Incubate overnight (or ~16 hour) at 4°C with gentle shaking.
    a. *Note: Optimal incubation time may need to be determined for efficiently capturing ubiquitinated target proteins.*
4. Wash plate 4 times with ~150 µL/well PBST using a multichannel pipette, a squeeze bottle, or an automatic plate washer. 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. After washing, add 100 µL of diluted target or tag specific antibody to each well, cover with plastic plate seal and incubate for 1 hr.
6. Wash wells as described in step 4.
7. Add 100 µL of diluted appropriate secondary antibody conjugated with HRP to each well, cover and incubate for 1 hr.
8. Wash wells as described in step 4.
9. Detect the signal using colorimetric detection reagent.
10. For colorimetric detection, add 100 µl of Colorimetric Reagent 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 (absorbance 650 = ~1.0) proceed to step 12.
    a. *Note: Longer incubation which makes the color darker blue might saturate the signal after adding stop solution. Do not let the signal saturate.*
11. To stop the reaction, add 100 µl of Stop Solution. The solution will change color from blue to yellow. Measured the Absorbance at 450nm within 30 minutes of adding the stop solution.

Data Reduction

1. Calculate the mean counts for blank, controls, and unknowns. Subtract the mean counts for the blank from each sample.
2. The data can be plotted as a bar graph or as a percent of the maximum value. If performing a compound dose-response assay, the maximum value should correspond to a zero concentration dose and the data can be fit to a sigmoidal plot to determine the ED_{50}.

Example: Cell Lysis Protocol

1. Aspirate the medium completely and rinse cells with ice cold 1X PBS (5ml PBS/10cm dish). Add 5ml of 1X PBS and scrape cells into the PBS using a cell scraper. 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. Transfer cells into a 15 mL conical tube and spin at 3000 rpm for 5 min. Freeze the cell pellet at

   - 80˚C for long term storage or take out after 30 minutes to continue with lysis.

   Note: After rinsing with ice cold PBS, cells can also be lysed by directly adding lysis buffer to the plate and scraping cells into the Eppendorf tube.
3. Place the frozen cell pellet on ice and add RIPA lysis buffer (5-10 times the volume of the pellet, (e.g., to 100 µL of pellet add 500-1000 µL lysis buffer). Vortex intermittently for about 10-15 minutes.
4. Centrifuge at 13,000 rpm for 15-20 min at 4°C.
5. Collect the supernatant (lysate) and determine protein concentration using standard methods.
6. Proceed with the dilution of the lysate for the assay (Step 2 in the Detailed protocol).

RIPA Lysis Buffer

50 mM Tris-HCl, pH 7.5
150 mM NaCl
1% NP40
1% Sodium deoxycholate
2 mM EDTA
10% Glycerol
1 mM PMSF
Protease inhibitor cocktail (Sigma cat #P8849, 1:500)
Aprotinin (10mg/ml stock) 20 µL/10 mL
50 uM PR619 (cat.no.SI9619) non-selective DUB inhibitor - recommended to protect ubiquitylated proteins from degradation by DUBs
5 mM 1,10-phenanthroline (*o*PA, cat.no. SI9649) – metalloproteinase inhibitor recommended to protect K63-linked polyubiquitin chains from degradation by JAMM-type DUBs

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About LifeSensors, Inc.

LifeSensors is a biotechnology company located in Malvern, Pennsylvania, USA. Founded in 1996, LifeSensors has developed a number of innovative protein expression technologies that enable efficient translation of the genome into proteome.

LifeSensors is well-known for its innovations in an important family of proteins consisting of ubiquitin and ubiquitin-like proteins (UBL) such as SUMO (Small Ubiquitin-like MOdifier).

LifeSensors has been granted several patents to cover the use of SUMO and other UBLs as gene fusion tags to improve the expression and purification of recombinant proteins. Additional patent applications are in various stages of review. Currently, LifeSensors is expanding its protein production capabilities and is developing protein micro array for drug discovery and diagnostics.

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