LifeSensors from genomics to proteomics

UbiQuant™ S quantitative Ub-substrate ELISA (Colorimetric)

Instruction Manual

Cat. No. UE103C

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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. Ubiquitylation 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 ubiquitylation, 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. Ubiquitylation is a reversible and dynamic process. Ubiquitylation 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*ubiquitylation enzymes (DUBs).

Intended Use

LifeSensors' UbiQuant™ S ELISA is intended 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 ubiquitylation.

This kit is for research use only and is not intended for human or animal diagnostic or therapeutic applications.

Principle of the assay

The UbiQuant™ S is a sandwich ELISA assay in which total ubiquitylated 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 in to the target protein. After removing unbound antibody, the amount bound is measured using an enzyme linked anti-antibody.

Components

Unless otherwise noted, all components should be stored at 4°- 8°C.

Coated plates:	One (1) pre-coated 96-well strip plate is provided. The plate is dried and vacuum packed. If not used within one (1) month remove plate and store at -80°C.
Strip holder:	One (1) empty strip plate frame is provided.
Primary Antibody (PA1):	1 vial of a 200x concentrate of an anti-epitope mAb in PBS containing 0.02% NaN_3.
	Available mAbs include anti-GST, anti-FLAG®, anti-myc, anti-HA, anti-V5
Blocking concentrate (BC):	12 mL of 5X blocking agent
Detection Antibody (DA1):	1 vial of 200X anti-mouse HRP conjugate
Plastic plate seals:	Four (4) provided
Colorimetric reagent (CR):	5 mL
Stopping reagent (SR):	5 mL

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)

An Absorbance capable microplate reader

Short Protocol

**The listed amounts of components below are for a full 96-well plate. Please make the appropriate calculations based on (1) the number of test samples, (2) the number of duplicates, triplicates, etc., (3) the number of dilutions desired (see Detailed protocol for the information on suggested dilutions).*

Allow all components to come to room temperature

⇩

Prepare Diluent **A** by adding **four (4) parts of** PBS to **one (1)** part of 5X BC.

12 ml of 5X BC provided is sufficient to make 60 mL of Buffer A.

⇩

Dilute test-samples to 25 to 400 µg/mL in PBS in microfuge tubes.

⇩

Pipette **100 µL** of each in appropriate wells of the UbiQuant™ plate (*See detailed protocol*).

⇩

Incubate at room temperature for 1 hr.

⇩

Wash plate four (4) times with PBST.

⇩

For full- plate add **60 µL** of PA1 into **12 mL** of diluent A and pipette **100 µL** into each well of the microtiter plate.

**Example: add 30µL into 6 mL for half- plate, or make adjustments as necessary*

Incubate at room temperature for 1 hr.

⇩

Wash plate four (4) times with PBST

⇩

If using a full-plate, add **60 µL** of DA1 conjugate to **12 mL** of diluent A.

Pipette **100 µL** into each well.

⇩

Incubate at room temperature for 1 hr.

⇩

Wash plate four (4) times with PBST.

⇩

Add **50 µL/well Colorimetric Reagent**.

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 ~30min (Absorbance 650nm=~1.0), proceed to next step *Note: prolonged incubation which makes the color darker blue might saturate he signal after adding Stopping Reagent. Do not let the signal saturate.*

⇩

To stop the reaction, add **50 µL/well of Stopping Reagent** on top of 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.

Detailed Protocol

1. We recommend that all samples be carried out in triplicate. Remove kit from refrigerator and allow all components to reach room temperature. Incubations are performed at either 4°C or room temperature (22°-27°C). To avoid cross contamination, do not re-use plate sealers. The amounts listed below should be enough for one 96-well plate or several samples run in triplicate. Please calculate the number of wells based on the number of test samples, the number of triplicates, etc., and how you will perform the dilutions (Step 4b).
2. **Prepare reagents**:
    a. Diluent A. Prepare Diluent **A** by adding **four (4)** parts of PBS to **one (1)** part of 5X BC and vortex to mix.
    b. Test Samples: Dilute test samples in PBS of choice to give between 25 and 400 µg/mL. The final volume will depend on the number of triplicates you wish to do for each sample. Alternatively, perform a serial two-fold dilution of each test sample.
    c. Dilute **30 µL** of primary antibody **(PA1)** into **6 mL** of Diluent **A** (or 60µL in 12 ml if using a whole plate).
    d. Dilute **30 µL** of detection antibody **(DA1)** into **6 mL** of Diluent **A** (or 60µL in 12 ml if using a whole plate).
3. 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.
4. Pipette 100 µL each of: blank, controls, and test samples into appropriate wells and cover with plastic plate sealer. Incubate overnight (~16.0 hour) at 4°C with gentle orbital shaking.
5. 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.
6. After blotting, add 100 µL of diluted PA1 to each well, cover with plastic plate seal and incubate for 90 minutes at RT.
7. Wash wells as described in step 5.
8. Add 100 µL of diluted DA1 to each well, cover and incubate for 1.0 hour at RT.
9. Wash wells as described in step 5.
10. 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 26.

    Note: prolonged 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 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 stopping reagent.

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, (i.e. 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 (2b 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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