LifeSensors from genomics to proteomics

UbiQuant™ quantitative ubiquitin ELISA

Cat. No. UE101

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™ ELISA is intended for the accurate determination of the concentration of total ubiquitin (poly- + mono-) in cell and tissue lysates. The kit is designed for research use only and is not intended for human or animal diagnostic or therapeutic applications.

Principle of the assay

The UbiQuant™ ELISA is a sandwich assay in which total ubiquitin is captured in the wells of a precoated microtiter plate using a proprietary ubiquitin binding reagent. Unbound protein is removed by washing and then an anti-ubiquitin monoclonal antibody (VU-2) is added. After removing unbound VU-2, bound antibody is measured using an enzyme linked anti-mouse 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 mAb VU-2 in PBS containing 0.02% NaN_3.
Standards (pUb):	2 vials of lyophilized ubiquitin standard (chains + free). The vial contains 5 μg of total ubiquitin.
Sensitizing Reagent 1 (SR1):	2 mL of a 20x stock (*N.b.* contains glutaraldehyde)
Sensitizing Reagent 2 (SR2):	4 mL of an 8x stock
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
Detection reagent 1 (DR1):	0.5 mL vial of Luminol reagent
Detection reagent 2 (DR2):	0.5 mL vial of stabilized peroxide reagent

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

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 replicates, (3) the number of dilutions desired (see Detailed protocol for the information on suggested dilutions).*

Allow all the 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.

⇩

Add **0.825 mL** of diluent A to the tube with lyophilized standard and vortex gently to dissolve.

⇩

Dilute test-samples to ~650 µg/mL in the buffer of choice. Avoid buffers containing Tris.

Dilute samples further to ~65 µg/mL of Diluent **A** in Eppendorf tubes.

Example: a final volume of 0.825mL will be enough for 10 wells.

⇩

Add **50 µL** of SR1 to **0.825 mL** of the diluted standard.

Add **50 µL** to of SR1 to **0.825 mL** of each sample*.

**If using different sample volume, calculate the appropriate amount of SR1 to add.*

⇩

Incubate at room temperature for 15 min.

⇩

Prepare diluent **B** by adding **0.5 mL** of SR1 to **8.25 mL** of diluent **A.**

⇩

Add **0.125 mL** of SR2 to a tube with a diluted standard (step 5) to a total volume 1.0 mL.

Add **0.125 mL** of SR2 to each diluted sample*.

**If using different sample volume, calculate the appropriate amount of SR2 to add.*

⇩

Incubate at room temperature for 30 min (Note: samples will turn yellow during this step).

⇩

Add **1.25 mL** of SR2 to diluent **B**.

⇩

Prepare standard curve dilutions and sample dilutions in diluent B in Eppendorf tubes.

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

⇩

Incubate at room temperature for 1 hr.

⇩

Wash the plate four 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 30 µL into 6 mL for half- plate, or make adjustments as necessary*

⇩

(Short protocol, continued)

Incubate at room temperature for 1 hr.

Wash plate four 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.

If using a whole plate, mix **150 µL** DR1 and **150 µL** DR2 in 10mL of ultra-pure water. Pipette 100 µL into each well.

Wait two minutes and read the plate

Detailed Protocol

1. We recommend that all samples, including the standard curve be tested in triplicate. A standard curve must be run with each assay. Remove kit from refrigerator and allow all components to reach room temperature. All incubations are performed at room temperature (22°-27°C). To avoid cross contamination, do not re-use plate sealers. The amounts listed below should be enough for approximately half of the 96-well plate or several samples run in duplicate along with standards. Please calculate the number of wells based on the number of test samples, the number of duplicates, 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. Standard: Add **0.825 mL** of Diluent **A** to a vial with lyophilized standard. Vortex gently and allow to sit at room temperature for 15 min (Std).

   c. Test Samples: Dilute test samples in buffer of choice to 650 μg/mL. *Avoid buffers containing Tris.* Dilute samples further in Diluent **A** to yield a final concentration of ~ 65 μg/mL. Final volume should depend on the number of duplicates you wish to do for each sample. Example: **0.825 mL** of Diluent A-sample mixture will be enough for 10 wells.

   d. Dilute **30 μL** of primary antibody **(PA1)** into **6 mL** of Diluent **A** (or 60μL in 12 ml if using a whole plate).

   e. Dilute **30 μL** of detection antibody **(DA1)** into **6 mL** of Diluent A (or 60μL in 12 ml if using a whole plate).

3. **Sensitization**:

   a. Add **50 μL of SR1** to diluted ubiquitin standard and **50 μL of SR1** to each diluted test-sample. **If using different sample volume, calculate the appropriate amount of SR1 to add.*

   b. Add **0.5 mL** of SR1 to **8.25 mL** of Diluent **A** to make Diluent **B**.
   Incubate standard and test-samples for 15 minutes at room temperature.

   c. Quench samples by adding **125 μL** of SR2 to the standard and to each of the test-samples.**Calculate the appropriate amount of SR2 to add depending on your original sample volumes.*

   d. Add **1.25 mL** of SR2 to Diluent **B** (Step 3b).

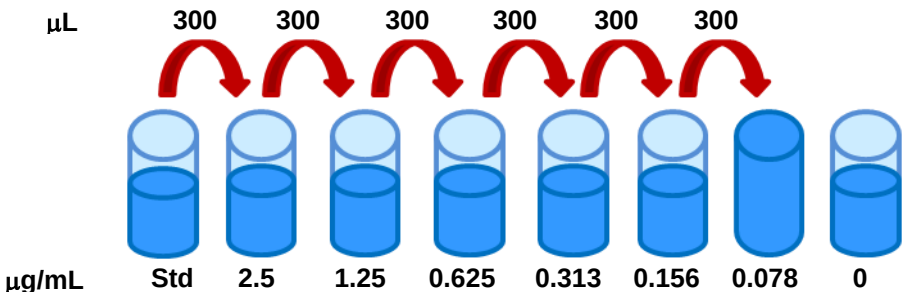
   e. Incubate the samples for an additional 30 min after adding SR2. The samples will turn yellow during this step.

   f. The standard will be at a final concentration of 5 μg/mL and the test samples, if diluted as suggested will be at a final concentration of ~50 μg/mL.

4. **Dilutions**: (All dilutions should be prepared in Diluent **B**)

   a. Standard curve (in duplicate): Label 7 tubes (eppendorf tubes or a deep well microtiter plate) 2.5, 1.25, 0.625, 0.3125, 0.156, 0.078, 0 and pipette **0.3 mL** of Diluent **B** into each tube. Add **0.3 mL** of standard (Std) to tube (2.5) and mix well. Transfer **0.3 mL** from this tube to the next tube (1.25). Repeat until tube 0.078 is reached. The standard will serve as the highest concentration (5 μg/mL) and Diluent B will serve as background (0 μg/mL).

   Unused standards can be stored frozen @-80°



b. Dilute the aliquot of test-samples sufficient for the assay 1:5 and 1:10 in Diluent **B**. Alternatively, perform a serial two-fold dilution of each test sample as described above.

5. 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.
6. Pipette 100 µL each of: standards, blank, and test samples into appropriate wells and cover with plastic plate sealer. Incubate 1 hr.
7. Wash plate 4 times with ~180 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.
8. After blotting, add 100 µL of diluted PA1 to each well, cover with plastic plate seal and incubate 1 hr.
9. Wash wells as described in step 7.
10. Add 100 µL of diluted DA1 to each well, cover and incubate 1 hr
11. Wash wells as described in step 7.
12. Just before use, mix 150 µL of DR1 and 150 µL of DR2 into 10 mL of ultrapure water (deionized or distilled). Add 100 µL of this solution to each well, wait 2 min and read in a plate reader optimized for chemiluminescence. Although there is a gradual decrease in the total signal with time, the plate may be read up to 20 min after addition of the luminescence reagent.

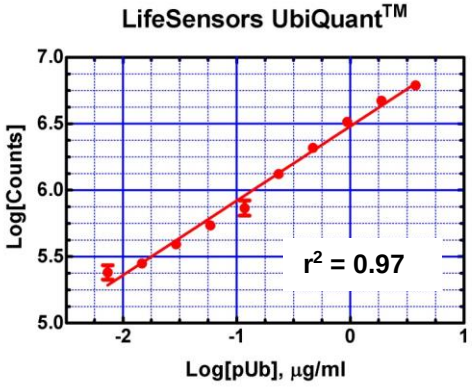
Data Reduction

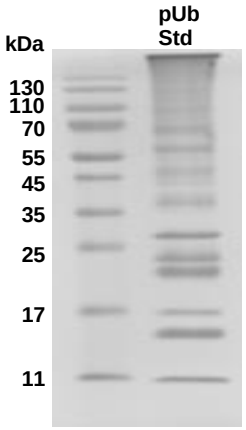
1. Calculate the mean counts for standards, blank, and unknowns. Subtract the mean counts for the blank from each sample.
2. Plot the data for the standards on log-log graph paper with the total ubiquitin concentration on the x-axis and mean counts on the y-axis. Draw the best-fit straight line through the points.
3. Interpolate the concentration of ubiquitin in the unknowns by drawing a straight line from the y-axis value for the unknown to the best-fit line. From this point, draw a straight line to the x-axis to determine the concentration. Correct for any dilutions made.
4. Alternatively, the data can be imported into a graphics program (*e.g.* Kaleidagraph (Synergy Software, Inc.), Prism (GraphPad Software, Inc.), Origin (OriginLab, Corp.), IgorPro (WaveMetrics, Inc.), Excel (Microsoft, Corp.)). The data are then transformed to log-log data, plotted, and then fit using a standard linear regression algorithm. The log of the mean counts of the unknowns can then be used to calculate the log of the ubiquitin concentration. The antilog of this number is equal

to the ubiquitin concentration of the unknown. As above, this number should be corrected for all dilutions.

Typical Data

Demonstration curve, a standard curve must be run with each assay.





Total Ubiquitin, µg/mL	*Counts01 -Bkgrd*	*Counts02 -Bkgrd*	*Counts03 -Bkgrd*	*Mean*
3.750	6115540	6055600	6260000	6143713
1.875	4793560	4655000	4690560	4713040
0.9375	3434640	3155280	3220400	3270107
0.4688	2234300	1962760	2068760	2088607
0.2344	1525180	1216780	1255100	1332353
0.1172	934440	595200	711360	747000
0.0586	587380	541060	506020	544820
0.0293	445200	342440	395400	394347
0.01465	325800	248580	276080	283487
0.007325	278440	188040	265760	244080

Please note that this standard curve was generated with an eleven step serial two-fold dilution series in order to demonstrate the span of the assay. Dilution as described in the instructions above will result in an assay that only extends to a minimum concentration of 78 ng/mL rather than 7 ng/mL as in this standard curve. In order to reach the higher sensitivity of the assay, the 7 step dilution series can be started at an initial concentration of 0.5 µg/mL rather than 5 µg/mL as described, *i.e.* using a ten-fold dilution of the reconstituted standard.

Limitations of the Assay

Intra-assay variability was tested by measuring twenty-one replicates of a 1 µg/mL pUb Std. The concentration determined was 1.11 ± 0.1 µg/mL yielding a CV of 9.4%. The results are shown in the Table below.

1.272	1.337	1.264
1.070	1.187	1.216
1.015	1.153	1.098
1.039	1.196	1.082
1.025	1.058	1.087
0.989	0.990	1.097
0.952	1.004	1.121
	Mean	**1.107**
	Std. Dev.	**0.104**
	CV	**9.5%**

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.
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 while keeping the on ice to allow efficient lysis.
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 (2c 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
1 mM PMSF
10% Glycerol
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 from degradation by 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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