# UbiTest: Detection of polyubiquitinated proteins using TUBE based screening platforms

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## BACKGROUND

The ubiquitin proteasome system (UPS) is a highly regulated mechanism that controls many cellular processes through protein ubiquitination and degradation. Ubiquitin is conjugated via its C-terminus to lysine residues on the target proteins as a monomer or as a polymer chain. Ubiquitin chains are formed via ubiquitin's first methionine (M1), giving rise to a linear chain, or via its seven lysine residues (K6, K11, K27, K29, K33, K48, K63) leading to lysine specific chains. K48 and K63-linked ubiquitin chains are the most predominant forms of polyubiquitination in cells[1]. Ubiquitination is a reversible process in which deubiquitinating enzymes (DUBs) remove the ubiquitins from the target proteins and rescue them from proteasomal degradation.

Determining the extent and type of polyubiquitination on target proteins is important to understand their ubiquitin mediated regulation in normal cellular processes as well as in UPS-associated disease states[2]. Traditionally, these are monitored either through mass spectrometry or via immunoprecipitation using anti-ubiquitin antibodies followed by immunoblotting. However, the substrate antibody often interacts differently with the poly-ubiquitinated forms of the substrate in the immuno-blotting step due to epitope masking, reduced affinity, or changes in selectivity caused by the protein's polyubiquitination.

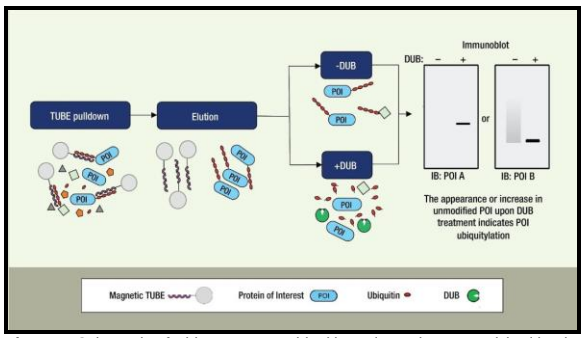


***Figure 1**: Schematic of UbiTest assay: Ubiquitinated proteins are enriched by the pulldown using magnetic TUBEs, then eluted from the TUBEs. The isolated polyubiquitinated eluate is then split into two fractions: one fraction is left untreated, while the second fraction is digested with a broad-spectrum DUB to remove ubiquitin chains. Both fractions are analyzed by immunoblot using an antibody against the protein of interest (POI). The removal of the ubiquitin chains from the polyubiquitinated POI results in the visualization of a single, unmodified band of POI in the immunoblot.*

A more definitive method for demonstrating protein ubiquitination is coupling immunoprecipitation with deubiquitination using a broad spectrum deubiquitinase (DUB) prior to immunoblot analysis. An increased signal for the protein of interest (POI) after DUB treatment is a clear indication that the protein was ubiquitinated even if there was no clear reactivity in the untreated sample. LifeSensors' UbiTest assay utilizes TUBEs (Tandem Ubiquitin Binding Entities)[3] to pull-down poly-ubiquitinated proteins to avoid potential problems arising from changes in immunoreactivity of the POI. TUBEs are engineered tandem ubiquitin-binding domains with dissociation constants for poly-ubiquitin in the nanomolar range. LifeSensors' pan-selective TUBEs have been demonstrated to bind to all eight ubiquitin linkage types.

To determine the linkage types of polyubiquitins on target proteins, LifeSensors has developed a more specific method that utilizes linkage-specific DUBs prior to immunoblot analysis. An increased signal for the band corresponding to the unmodified POI after a K48/K63-specific DUB treatment indicates that the protein is K48/K63 poly-ubiquitinated.

### Monitoring Cellular Protein Ubiquitination

Here we showcase data obtained from an experiment in which UbiTest was performed on Jurkat cell lysates to monitor the ubiquitination of USP7 (Ubiquitin specific protease 7) and total ubiquitination of cellular proteins in response to USP7 inhibitor. USP7 is a deubiquitinating enzyme (DUB) that removes ubiquitin and protects the substrate proteins from degradation[4]. Numerous proteins have been identified as substrates of USP7, which play key roles in the cell cycle[5,6], DNA repair[4], chromatin modeling, epigenetic regulation[7] and genome stability[8]. Abnormal activation or overexpression of USP7 promotes oncogenesis, making it a target for therapeutic intervention[4].

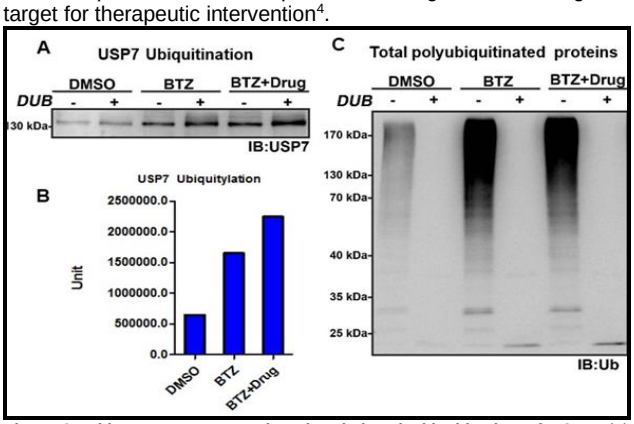


***Figure 2: UbiTest assay to monitor drug-induced ubiquitination of USP7:** (A) Ubiquitination and deubiquitination of USP7 in Jurkat cells treated with DMSO or BTZ (200 nM, a proteosome inhibitor) or BTZ (200 nM) + drug (USP7 inhibitor, 10 μM). Lane 1: ubiquitinated USP7 in DMSO treated cells; Lane 2: DUB-treated USP7 in DMSO treated cells; Lane 3: ubiquitinated USP7 in BTZ treated cells; Lane 4: deubiquitinated USP7 in BTZ treated cells; Lane 5: USP7 with its inhibitor and BTZ; Lane 6: USP7 with inhibitor+BTZ after DUB treatment. (B) Densitometric quantitation of the bands shown in A with DUB treatment (lanes 2, 4 and 6). (C) A corresponding blot on polyubiquitinated protein from cells with different treatments. DUB untreated lanes showed polyubiquitinated proteins migrated at high molecular weight (Lanes 1, 3 and 5). Lanes 2, 4 and 6: DUB treated lanes with no smears of proteins.*

Jurkat cells were treated with DMSO control or with Bortezomib (BTZ, proteasome inhibitor, 200 nM) or with USP7 specific inhibitor (Drug, 10 μM) + BTZ together. Proteins were pulled down from the cell lysates using pan-selective TUBE-agarose beads followed by elution and neutralization. Each sample was split into two fractions, one fraction was treated with pan-DUB while the other fraction was left untreated as a control as per the UbiTest recommendations. DUB treated and untreated fractions were compared between the different treatments by immunoblotting using USP7 specific antibody (Figure 2A). All DUB treated fractions showed increased protein expressions compared to that in untreated fractions because ubiquitin was removed from the protein by the DUB. Figure 2A also shows the dose response of small molecule inhibitors promoting ubiquitination of the target as well. The quantitation of USP7 ubiquitination by densitometric analysis is shown in Figure 2B. Corresponding to this, the same blot was probed with ubiquitin antibody to show the total cellular polyubiquitinated proteins with the subsequent DUB treatment

(Figure 2C). Without DUB treatment, the proteins migrated at higher molecular weight showing polyubiquitinated protein smears whereas DUB-treated lysates didn't show smears of proteins (Figure 2C).

Overall, these results show how the DUB digestion in the UbiTest can help in monitoring the kinetics of ubiquitination of a target protein. One can easily monitor and quantitate ubiquitination of the target protein using this kit, which is not possible with any other method.

Recently, BommaReddy et al[9] showed an underlying mechanism of lung cancer metastasis inhibition by ubiquitination of FAK (Focal Adhesion Kinase) by Cbl-b E3-ligase using the UbiTest kit. FAK is regulated by PKC-ι. Over-expression of PKC- ι and its target protein FAK causes lung metastasis[9]. The authors showed that PKC- ι inhibition by DNDA (3,4-diaminonaphthalene-2,7-disulfonic acid) decreased FAK phosphorylation and induced its ubiquitination by Cbl-b E3 ligase (Figures 7, 8 and 9 of the paper)[9]. BommaReddy et al[9] monitored the ubiquitination of FAK by conventional method as well as using our UbiTest which offered great advantages over conventional immunoprecipitation as shown in their Figure 9.

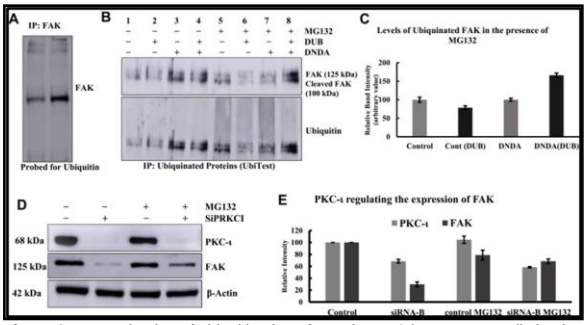


***Figure 3:*** *Determination of ubiquitination of FAK in A549 lung cancer cells by the treatment with DNDA 10μM for 3 days. (A) The IP of FAK (3 μg) was immunoblotted for ubiquitin and found that there was an increase in the FAK (125 kDa) band in the DNDA treated samples. (B, C) UbiTest was employed to determine the ubiquitination of FAK. The results showed that FAK (125 kDa) and cleaved FAK (100 kDa) were ubiquitinated with the DNDA treatment. (D) To explore the involvement of PKC- ι in the ubiquitination of FAK, after transient transfection, cells were treated with 20μM proteasomal inhibitor MG-132 or DMSO for 4 hrs before collecting the lysates. The FAK protein expression concurrently decreased when PKC- ι was repressed in the absence of MG132. However, FAK expression increased when PKC- ι was repressed in the presence of MG132. (E) The intensity of respective bands was quantified by using densitometry. This is Figure 9 from the published report of BommaReddy et al[9].*

The authors saw an increase in the FAK ubiquitination with DNDA treatment by 40% (Figure 3A, Lane 2) compared to that in control (Figure 3A, Lane 1). Without DUB treatment they could see intense smears of ubiquitinated FAK and its cleaved product after the treatment with DNDA and MG132 (Figure 3B, top panel: Lane 7) with FAK-specific antibody. With the addition of DUB, those smears were lost and deubiquitinated protein products were left at their unmodified molecular weights (Figure 3B, top panel: Lane 8). Similar patterns were seen with and without DUB digestions for other control treatments in the same blot (Figure 3B, top panel: Lanes 1-6). The same blot was probed with ubiquitin antibody and polyubiquitinated signal of the protein was found in similar manner with and without DUB (Figure 3B, lower panel). Levels of ubiquitinated FAK were quantitated by densitometric analysis (Figure 3C).

Further, they have explored the involvement of PKC- ι in FAK ubiquitination by silencing PKC- ι by its siRNA and at the same time, they showed the effect of MG132 to inhibit the degradation of ubiquitinated FAK protein (Figure 3D) and the FAK levels were quantitated by the densitometric analysis (Figure 3E).

### Use of UbiTest to validate the function of E3 ligases in cells

Ubiquitin E3 ligases control multiple pathways of eukaryotic biology by promoting protein ubiquitination and degradation. Cbl-b is one of the E3 ubiquitin ligases involved in maintaining a balance between immunity and tolerance by functioning as a gatekeeper. Cbl-b E3 ligase has an essential role in T-cell activation[9]. Here, we show the UbiTest analysis performed to understand the role of Cbl-b in the ubiquitination of its target proteins.

Jurkat cells were treated with T-Cell receptor (TCR, CD3/CD28) for 30 min for physiological T-cell activation using magnetic Dynabeads conjugated with CD3/CD28 antibody. Control magnetic beads without CD3/CD28 antibody was used to generate control cell lysates. UbiTest was performed on each protein eluate by splitting into two fractions, one fraction was treated with USP2 (a pan-selective DUB) while other fraction was left untreated as a control. Immunoblotting was performed on the lysates with Cbl-b antibody which showed the Cbl-b E3 ligase ubiquitination (Figure 4A). Cbl-b E3 ligase ubiquitination was relatively low in unstimulated cells (lane 2) as compared to that in stimulated cells (lane 4). In this experiment DUB facilitated the accurate quantification of the levels of Cbl-b E3 ligase in cells.

Parallelly, a dose response experiment was performed to stimulate the cells with TCR from 0 min, 5 min, 15 min and 30 min. Total poly-ubiquitin proteome was enriched using TUBEs followed by elution, neutralization and DUB digestion as described above. Immunoblotting with Cbl-b substrate anti-ZAP70 revealed that ZAP70 ubiquitination was increased in a time dependent manner with TCR stimulation. It is evident that TCR activated the ubiquitination of Cbl-b E3 ligase which in turn activated its target protein ZAP70 ubiquitination (Figure 4B).

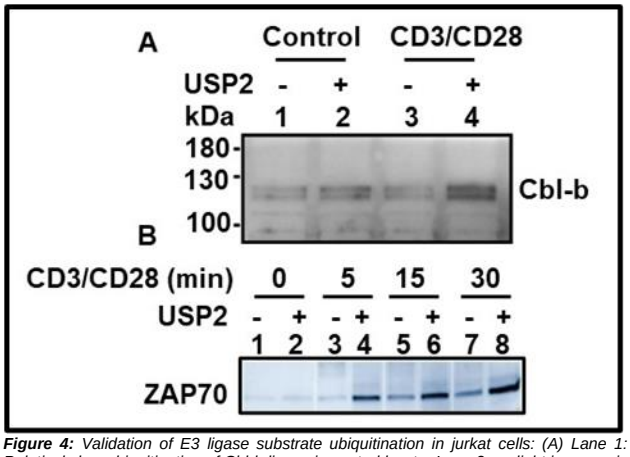


***Figure 4:*** *Validation of E3 ligase substrate ubiquitination in jurkat cells: (A) Lane 1: Relatively low ubiquitination of Cbl-b ligase in control lysate. Lane 2: a slight increase in the ubiquitinated Cbl-b band with DUB due to its deubiquitination in control lysate. Lane 3: Ubiquitinated Cbl-b ligase in TCR stimulated lysate. Lane 4: Deubiquitinated Cbl-b ligase in TCR stimulated cells. (B) A dose response experiment on TCR stimulated cells at 0, 5, 15 and 30 min. Each sample was split for DUB treatment or no treatment to see the ZAP70 ubiquitination by Cbl-b.*

### Detection of linkage specific polyubiquitination

LifeSensors has developed a more definitive method to demonstrate the polyubiquitination using linkage specific DUBs. We offer K63 (Cat. No. UM413)- and K48 (Cat. No. UM414)-specific DUBs or Pan selective broad-spectrum DUBs in our

Linkage Specific UbiTest Kits along with the TUBES with agarose beads or TUBEs with magnetic beads. Figure 5 represents experiments performed to monitor the MDM2 ubiquitination using K48 specific DUB (Figure 5A) and TNFR1 ubiquitination using K63 specific DUB (Figure 5B).

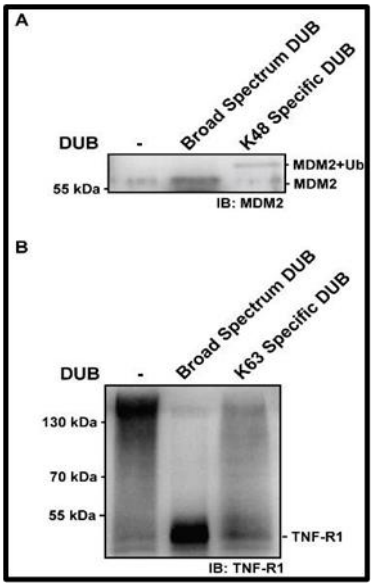


***Figure 5:*** *Ubiquitination linkage analysis of proteins in Jurkat cells: (A) MDM2 (55 kDa) ubiquitination without and with DUB. Lane 1: No DUB added; relatively low ubiquitination signal of the protein in untreated cells; Lane 2: increase of signal due to polyubiquitination of MDM2 in broad spectrum DUB treated sample; Lane 3: decrease in the polyubiquitinated signal in K48 specific DUB treated cells. (B) TNFR1 ubiquitination. Lane 1: Smear of total cellular protein ubiquitination including TNFR1 with no DUB added in cells; Lane 2: Loss of smear in broad spectrum DUB treated cells but showing unmodified TNFR1 signal; Lane 3: K63 specific signal of the protein alone. Highest ubiquitination seen with pan-selective DUB. Lane 5: Normal ubiquitination of Tau protein from normal brain lysates. Lanes 6-8: No Tau aggregated protein smears with any of the DUBs. (B) Same samples as in A probed with SQSTM1 antibody.*

## Linkage specific UbiTest to unravel mysteries of AD/PD diagnostics

Alzheimer's Disease (AD) and Parkinson's Disease (PD) are the most common neurodegenerative disorders. AD is characterized by progressive cognitive and behavioral impairments and ultimately results in dementia[11]. AD's pathological hallmarks include extracellular amyloid plaques that consist of amyloid beta (Aβ) peptides and intracellular neurofibrillary tangles composed of hyperphosphorylated tau proteins[10].

LifeSensors quantitated AD biomarkers like Tau and SQSTM1 through their ubiquitination using linkage specific and magnetic TUBE based UbiTest kit as shown in Figure 6.

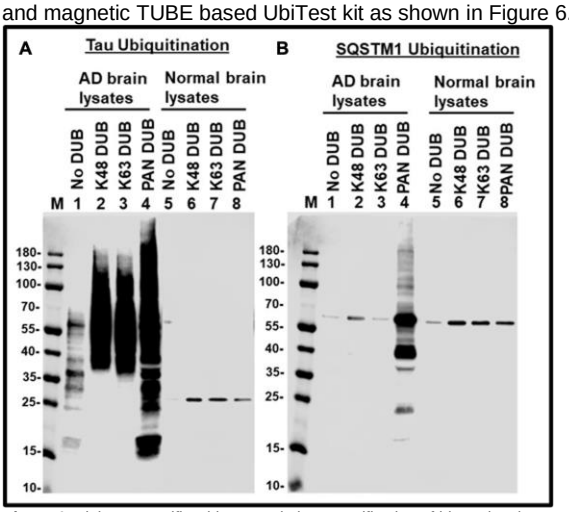


***Figure 6:*** *Linkage specific UbiTest: Relative quantification of biomarker (Tau and SQSTM1) ubiquitination levels in AD vs normal brain lysates (1.4 mg each) using linkage specific DUBs and broad-spectrum pan-selective DUB. (A) Lane 1: Tau protein aggregate ubiquitination from AD brain lysate. Lanes 2 and 3: Highly ubiquitinated Tau observed with K48 DUB and K63 DUB, respectively. Lane 4:*

Highly ubiquitinated Tau aggregated proteins by linkage specific or pan-selective DUBs were detected easily and quantitated accurately from AD brains. Same as in the case of SQSTM1 ubiquitination. LifeSensors' dynamic "Linkage Specific UbiTest kit" further unravels mysteries of neurodegenerative diseases and will be helpful in future diagnostics.

## PROTAC/MG mediated protein ubiquitination and degradation

PROTACs (Proteolysis-targeting chimeric molecules) are hetero-bifunctional small molecules that contain two ligands connected via a linker; one ligand binds to a target protein and the other ligand binds to an E3 ligase[11]. Bringing these two entities (E3 ligase and target protein) together leads to polyubiquitination of the target protein and its subsequent degradation. Molecular glues (MG) are low molecular weight inducers or stabilizers that make the target protein-E3 ligase complex stable[11]. Most therapeutic small molecules inhibit receptor and enzyme activity by binding to and blocking the ligand binding or active site. An alternative approach is to utilize PROTACs, that hijack the cell's proteasomal machinery to initiate targeted protein degradation. For this reason, PROTACs provide great therapeutic promise by expanding the druggable proteome.

Detection of PROTAC mediated protein ubiquitination and degradation by traditional immunoblotting lacks sensitivity and reporter gene assays leave artifacts. But LifeSensors' high affinity ubiquitin capture reagents like TUBEs (engineered ubiquitin binding domains) allow for detection of ultralow levels of polyubiquitinated proteins under native conditions with affinities as low as 1.0 nM[12]. Monitoring PROTAC mediated ubiquitination in cells will be helpful in establishing rank order potencies and guide medicinal chemists in designing more potent PROTACs.

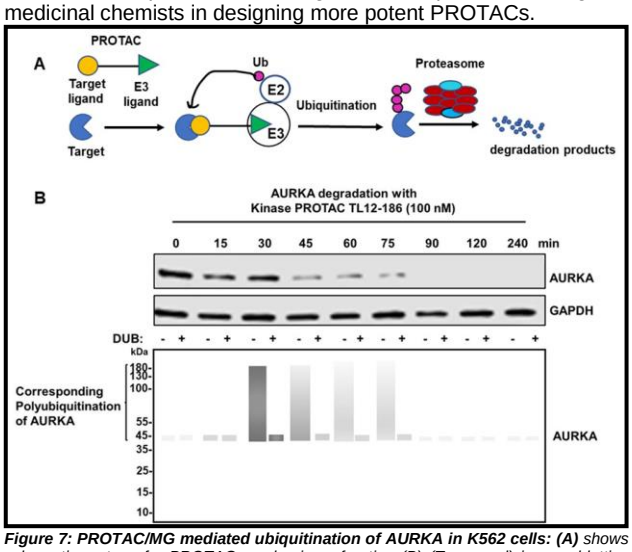


***Figure 7: PROTAC/MG mediated ubiquitination of AURKA in K562 cells: (A)*** *shows schematic cartoon for PROTAC mechanism of action (B) (Top panel) immunoblotting analysis of time course study on AURKA degradation by PROTAC and expected intracellular ubiquitination pattern (lower panel) by UbiTest (created in power point).*

Recently, Gross et al from LifeSensors[13] evaluated Aurora Kinase A, whose overexpression is prevalent in cancers, by targeting with kinase PROTAC TL12-186 (100 nM) for its degradation in a time course experiment in K562 cells (Figure 7). Gross et al[13] observed the levels of AURKA in samples with PROTAC were reduced with increase in the time as shown in the immunoblotting (top panel). The lower panel shows the representative ubiquitination results based on plate based assays derived from

these samples by UbiTest that is remarkably similar to the degradation pattern establishing the fact that the ubiquitination kinetics controls the degradation.

**Summary**

LifeSensors has developed a medium throughput platform called "UbiTest" for measuring total or chain-selective protein ubiquitination in cells. UbiTest uses TUBE technology to enrich polyubiquitinated proteins. The enriched fraction is analyzed by immunoblotting with and without DUB (Deubiquitinase) digestions to quantitate the ubiquitination of the protein. LifeSensors also provides linkage specific DUBs that identify the type of ubiquitin linkage which can provide insights into the cellular functions of E3 ligases and substrates. UbiTest can also be used in PROTAC mediated ubiquitination of a protein in parallel with its degradation. LifeSensors believes that UbiTest can address a lot of challenges associated with PROTACs and MGs (molecular glues) drug discovery as it reports true PROTAC function, i.e., ubiquitination rather than just a loss of protein or no loss. As there might be PROTACs that have non degradative function, this could be useful information for medicinal chemists to understand the role of the ligand, the E3 ligase, the exit vector and the specific linker that can lead to degradative vs non degradative PROTACs.

**References**


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## About LifeSensors, Inc.

LifeSensors is a biotechnology company located 35 miles west of Philadelphia, 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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