MANUAL

K63-Linkage Specific UbiTest (For 10 pull-downs)
Catalog Number: UM413

LifeSensors, Inc., 271 Great Valley Parkway, Malvern PA 19355 • (p) 610.644.8845 (f) 610.644.8616
info@lifesensors.com • www.lifesensors.com • orders@lifesensors.com

BACKGROUND

Ubiquitin and Polyubiquitylation

Ubiquitin is a small polypeptide that can be conjugated via its C-terminus to amine groups of lysine residues on target proteins. This conjugation is referred to as monoubiquitylation. Additional ubiquitin moieties can be conjugated to this initial ubiquitin utilizing any one of the seven lysine residues (K6, K11, K27, K29, K33, K48 and K63) present in ubiquitin. Met1-linked (M1) or 'linear' chains, are generated when ubiquitin is attached to the N-terminus of a second ubiquitin. The formation of these ubiquitin chains is referred to as polyubiquitylation. The two most well characterized forms of this are K48 and K63 polyubiquitylation. The most prevalent consequence of polyubiquitylation is the proteasome-mediated degradation of the target protein. Polyubiquitylation is a reversible process, as these chains are degraded and/or removed by proteases known as deubiquitinases (DUBs). The dynamic nature of this signaling represents a major obstacle to the isolation and functional characterization of polyubiquitylated proteins. For this reason, the ubiquitylation state of many proteins is unknown or poorly characterized.

Determining the linkage of polyubiquitin on target proteins is challenging. The traditional methods are either through Mass Spectrometry or immunoblot with linkage specific antibodies, which are cumbersome. LifeSensors has developed a more definitive method for demonstrating the ubiquitylation linkage of a protein, which is to couple immunoprecipitation of polyubiquitylated protein with digestion by a **linkage specific deubiquitylase** prior to immunoblot analysis. **An increased signal for the unmodified substrate or a decreased signal of polyubiquitylated substrate at high molecular weight after K48/K63 specific DUB treatment is a clear indication that the protein was K48/K63 ubiquitylated.** We have built this kit around the use of TUBEs which bind to all ubiquitin chain linkages. Tandem Ubiquitin Binding Entities (TUBEs) were developed by Dr. Manuel Rodriquez at CIC bioGUNE and are licensed by LifeSensors, Inc. TUBEs are engineered tandem UBAs with dissociation constants for tetra-ubiquitin in the nanomolar range. TUBEs have also been demonstrated to protect proteins from both deubiquitylation and proteasome-mediated degradation, even in the absence of inhibitors normally required to block such activity. The nanomolar affinity of TUBEs for polyubiquitylated proteins allows high efficiency isolation and characterization of these proteins from cell lines and tissues. TUBE1 has been demonstrated to bind to all 8 linkage types.

The superior nature of TUBEs allows efficient detection of polyubiquitylated proteins in their native state, while the versatility of TUBEs meets a wide range of experimental needs.

Agarose-TUBEs are TUBE moieties directly coupled to agarose beads, for the identification and characterization of polyubiquitylated proteins by western blotting and/or downstream proteomic studies. Agarose-TUBEs facilitate convenient "one-step" pull-down of polyubiquitylated proteins.

APPLICATIONS

1. Convenient one-step pull down of polyubiquitylated protein from cell and tissue extracts
2. Isolate ubiquitylated proteins for proteomic studies
3. Confirmation of target protein ubiquitylation linkages

BENEFITS

- Up to 1000-fold higher affinity for polyubiquitin compared to single UBA
- Avoids overexpression of epitope-tagged ubiquitin for pull downs
- Unambiguous determination of the ubiquitylation status of target proteins
- Can be multiplexed to examine several different targets in a single sample

COMPONENTS

Tube 1 -- Anti-Ub-TUBE1 Agarose – 1ml of a 50% slurry

Buffer: PBS, pH 7.2, 20% ethanol

Storage: -20°C. Avoid storage at lower temperature. Small batches of equilibrated resin can be stored at 4ºC for up to 1 week.

Tube 2 -- Elution wash buffer – 2 mL

Tube 3 -- Elution buffer – 1 mL

Tube 4 -- Neutralization buffer (Add 5 µl, 1 M β-mercaptoethanol before use) – 0.5mL

Tube 5 -- Broad Spectrum DUB – 25 µg, 10 µM

Tube 6 – K63-specific DUB- 35 µg, 10 µM

Storage, -80°C

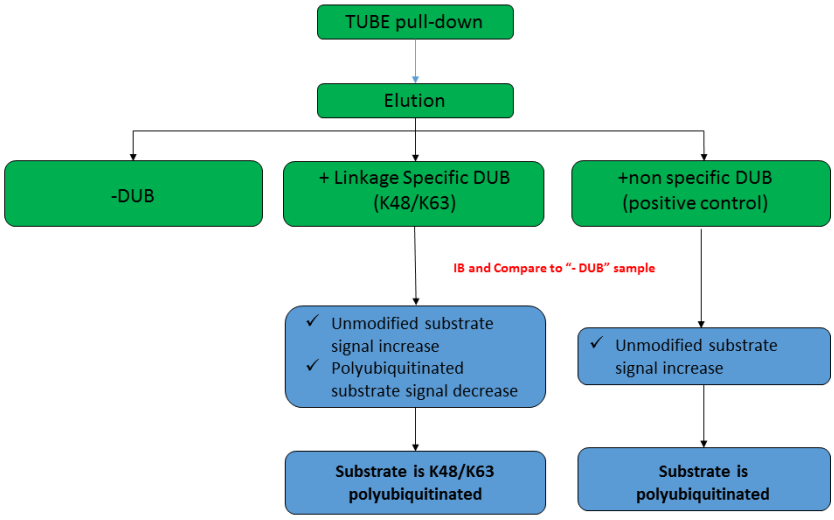
ADDITIONAL ITEMS REQUIRED

1. **Cell Lysis buffer:** 50mM Tris-HCl, pH 7.5, 0.15M NaCl, 1mM EDTA, 1% NP-40, 10% glycerol, RIPA (ThermoFisher Cat. No. PI89900)

   The inclusion of a protease inhibitor cocktail is recommended to protect from non-specific protein degradation during lysis and isolation.

2. **(Optional) Control agarose (LifeSensors Cat. No. UM400)**
3. **(Optional) PR-619 (LifeSensors Cat. No. SI9619).** This compound is a reversible inhibitor of a wide range of Ub/Ubl proteases and has been shown to protect polyubiquitylated proteins from degradation. The inclusion of PR-619 in the lysis buffer can increase the yield of polyubiquitylated proteins during the preparation of cell and tissue extracts.
4. **(Optional) 1,10-phenanthroline (*o*-PA), 100X (LifeSensors Cat. No. SI9649).** This metal chelator is a potent inhibitor of metalloproteases, including JAMM DUBs, and can help prevent K63 polyUb chain degradation during cell lysis.
5. **Validated antibody against protein(s) of interest**
6. **Your favorite secondary detection antibody that recognizes 5.**
7. **Millipore Immobilon Western HRP Substrate (Cat. WBKLS0500)** is recommended for best IB result.

GENERAL PROCEDURE



EQUILIBRATION OF AGAROSE-TUBEs

1. Allow the slurry to equilibrate to 4˚C by incubating at room temperature for 20-30 min or at 4ºC for up to 2 hours. Gently mix Agarose-TUBEs by inverting the vial several times to ensure a homogeneous suspension.
2. Determine the amount of resin required for the experiment. The amount of polyubiquitin in samples can vary with cell or tissue type, experimental conditions, and the presence or absence of deubiquitinating enzyme or proteasomal inhibitors. Therefore, the optimal amount of Agarose-TUBEs for pull down needs to be determined empirically by the end-user. 10-20µl of resin in 500µl of lysis buffer containing 1-2mg of total protein is an appropriate starting point for each experiment. Larger volumes (100-150 µl) of slurry can be equilibrated and stored for up to one week at 4ºC. This protocol applies to 20 µl resin pull down.
3. Collect the volume of resin necessary for the experiment by low speed centrifugation (1000-5000xg) for 5 minutes (RT).
4. Remove and discard the supernatant, being careful not to disturb the resin. **Useful tip**: when working with agarose matrices, use gel loading tips to collect supernatants to avoid loses of beads.
5. Suspend the resin in 5-10 volumes of **TBS-T** and incubate for 5 minutes on a rocker platform.
6. Repeat collection/wash at least two times prior to pull-down.
7. Remove and discard the final wash supernatant being sure to leave a 50% slurry prior to Step 7 of the protocol below.

PULLDOWN OF POLYUBIQUITYLATED PROTEINS (Suggested Protocol)

1. Pre-chill inhibitor-containing **cell lysis buffer and microcentrifuge tubes** to 4°C.
2. Treat and wash cells appropriately. As an initial starting point, we recommend the addition of 500µL of lysis buffer to a 10 cm^2 tissue culture dish containing ~5-10x10^6 cells (80% confluence). **The optimal number of cells will depend on the cell line and the abundance of the protein of interest**.
3. Collect cells by scraping, and transfer the lysate to 1.5mL tube.
4. Clarify lysate by high speed centrifugation (~14,000xg) for 10min at 4ºC.
5. **Optional control for non-specific binding:** Incubate clarified cell lysate with uncoupled agarose (Cat# UM400) for 30 min at 4ºC on the rocker platform. Remove agarose by centrifugation and transfer clarified supernatant to a new 1.5ml microcentrifuge tube.
6. Remove an "INPUT" sample for analysis by western blotting (e.g. 5-20µl of cell lysate in 25-50µl 1X SDS reducing sample buffer.)

7. Add the amount of cell lysate to the amount of equilibrated Agarose-TUBEs determined from step 2 of the previous section and incubate for 1 hour at 4ºC on a rocker platform. Additional incubation time may be required; optimal time should be determined by the end user.

8. Collect beads by low speed centrifugation (1000-5000xg, 4ºC) for 5 minutes. Save supernatant as the "UNBOUND FRACTION."

9. Wash beads with 1ml **TBS-T**, collect by low speed centrifugation and aspirate the supernatant leaving a small volume cushion so as to avoid disturbing the beads. Repeat twice. After the last wash, use a glass capillary pipette to remove the liquid from the agarose resin.

10. Resuspend beads in 100µL of Elution wash buffer (Tube 2), mix 5 min at room temperature, and collect beads by centrifugation as in step 9.

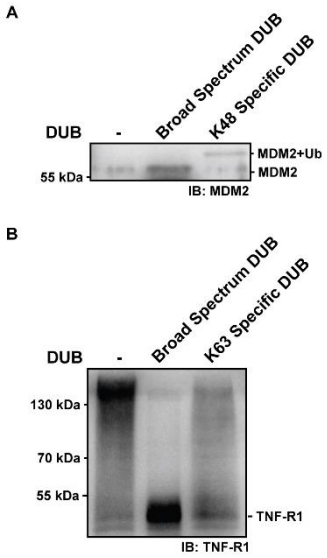
11. Resuspend resin in 50µL of Elution buffer (Tube 3). Mix 15 min at room temperature, pellet resin by centrifugation (3,000xg) for 5 minutes, isolate supernatant without disturbing the resin.

12. Neutralize the supernatant by adding 10x Neutralization buffer (Tube 4). Split sample into three equal parts. To the first part, add 5µL of 10 µM K63-linkage specific DUB. Add 5µL of buffer to the second part. **Additional control is recommended to confirm that the target is ubiquitylated**: Add 2 µL of 10 µM broad spectrum DUB, which hydrolyses all the polyubiquitin, to the last part. Incubate all the samples at 30ºC for 1-2 hours.

13 Stop the reaction by the addition of SDS-Sample buffer. Heat the samples at 100°C for 3-5min and proceed with immunoblot analysis. For best result, Millipore Immobilon Western HRP Substrate (Cat. WBKLS0500) is recommended for immunoblot.

14 The blot can be stripped and reprobed with additional anti-target antibodies

SAMPLE DATA



Ubiquitylation linkage analysis of proteins in Jurkat cells.

(A) Signal of MDM2 increased after broad spectrum DUB treatment indicated that MDM2 is polyubiquitylated. After the K48-specific DUB treatment, there was increased MDM2 signal in immunoblot. This indicates MDM2 is K48 ubiquitylated. The MDM2 signal is higher on immunoblot, because the DUB does not hydrolyze the proximal Ub on the target protein or there were different linkages of polyubiquitin other than K48 chain.

(B) Signal of unmodified TNFR1 increased after broad spectrum DUB treatment indicated that TNFR1 is polyubiquitylated. After the K63-specific DUB treatment, there was a decrease in signal of polyubiquitylated TNFR1 at high molecular weight and increase signal of unmodified TNFR1, which indicates TNFR1 is K63 ubiquitylated. The DUB did not hydrolyze all the polyubiquitin chains on the TNFR1 indicates there were different chain linkages other than K63 chain (mix chains). Jurkat cells were treated with bortezomib (BTZ) for 2 hrs. The cells were lysed in RIPA buffer supplemented with a Protease Cocktail Inhibitor (Calbiochem, PIC Set V), 50µM PR619 (LifeSensors Cat. # SI9619), and 5mM *o*-phenanthroline (LifeSensors Cat. # SI9649). Total protein content of pre-cleared (Control Agarose; LifeSensors cat. # UM400) lysate was determined by Bradford, and 20 µl of Anti-Ub TUBE1 agarose resin (LifeSensors Cat. # UM401) was added. Reactions were rotated for 2h (4°C), washed three times with TBST, eluted as described and analyzed by SDS-PAGE and immunoblotting for each of the target.

REFERENCES

1. Hjerpe, R., Aillet, F., Lopitz-Otsoa F., Lang, V., England P., and Rodriguez, MS. (**2009**) "Efficient protection and isolation of ubiquitylated proteins using tandem ubiquitin-binding entities." *EMBO Rep.,* 10(11)1250-8.

2. Lopitz-Otsoa F., Rodriguez-Suarez E., Aillet F., Casado-Vela J., Lang V., Matthiesen R., Elortza F., and Rodriguez M.S. (**2012**) "Integrative analysis of the ubiquitin proteome isolated using Tandem Ubiquitin Binding Entities (TUBEs)" *J. Proteomics*. 75(10):2998-3014.

3. Altun, M., Kramer, H.B., Willems, L.I., McDermott, J.L., Leach, C.A., Goldberg, S.J., Suresh Kumar, K.G., Konietzny, R., Fischer R., Kogan, E., MacKeen M.M., Khoronenkova, S. V., Parsons, J.L., Dianov, G. L., Nicholson, B. and Kessler, B.M. (**2011**) "Activity-Based Chemical Proteomics Accelerates Inhibitor Development for Deubiquitylating Enzymes." *Chemistry & Biology*, 18 1401-1212.

LIMITED USE LABEL LICENSE

TUBEs are produced and marketed by LifeSensors, Inc. under a nonexclusive license from CICbioGUNE. For information on obtaining a license for commercial purposes, contact: info@lifesensors.com, LifeSensors, Inc., Malvern PA 19355, Phone: 610.644.8845.

The product and/or its derivatives are being furnished to the purchaser under the following conditions: By purchasing the product, the purchaser agrees to comply with the terms of this Limited Use Label License. The product is provided to purchaser for research use only. The purchaser of this product may not transfer or otherwise sell this product or its derivatives to a third party and no rights are given to the purchaser to use the product or its derivatives for commercial purposes as defined below.

Commercial purposes means any activity for which a party receives consideration and may include, but is not limited to,

1. use of the product or its derivatives in manufacturing,
2. use of the product or its derivatives to provide a service, information, or data,
3. use of the product or its derivatives for diagnostic purposes,
4. transfer or sell of protein or chemical component made with the product or derivatives of the product,
5. resell of the product or its derivatives, whether or not such product or its derivatives are resold for use in research.

Product use must follow compliance with all laws and regulations, including but not limited to current EPA, FDA, USDA, and NIH guidelines. THE MATERIALS WILL NOT BE USED IN HUMANS.

Purchaser acknowledges that the product is experimental in nature and provided WITHOUT WARRANTY OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE OR ANY OTHER WARRANTY, EXPRESS OR IMPLIED. LIFESENSORS, INC. MAKES NO REPRESENTATION OR WARRANTY THAT THE USE OF THE PRODUCT WILL NOT INFRINGE ANY PATENT OR OTHER PROPRIETARY RIGHTS.

In no event shall LifeSensors, Inc. be liable for any use of the product by purchaser. Purchaser agrees to defend, indemnify, and hold harmless LifeSensors, Inc. its officers, employees, and agents from any loss, claim, injury, damage, expense, or liability (including attorney's fees), of whatsoever kind or nature, which may arise from or in connection with this Agreement, including but not limited to purchaser use, handling or storage of the product.