MANUAL

Agarose-TUBEs

Catalog Numbers:
UM401, UM402

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BACKGROUND

Ubiquitin and Polyubiquitination

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 monoubiquitination. Additional ubiquitin moieties can be conjugated to this initial ubiquitin utilizing any one of the seven lysine residues present in ubiquitin. The formation of these ubiquitin chains is referred to as polyubiquitination. The two most well characterized forms of this is polyubiquitination via lysine at position 48 (K48) or position 63 (K63). The most prevalent consequence of polyubiquitination is the proteasome-mediated degradation of the target protein. Polyubiquitination is a reversible process, however, 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 polyubiquitinated proteins. For this reason, the ubiquitination state of many proteins is unknown or poorly characterized.

TUBEs: A Revolution in Polyubiquitin Isolation and Characterization

Traditional strategies for characterization of ubiquitinated proteins often require immunoprecipitation of overexpressed ubiquitin with an epitope tag or the use of ubiquitin antibodies (expensive for large scale studies). Alternatively, isolation of polyubiquitinated proteins can be achieved with certain ubiquitin binding associated domains (UBAs), but these proteins display a low affinity for ubiquitin. Additionally, these strategies require the inclusion of inhibitors of both DUB and proteasome activity to protect the integrity of polyubiquitinated proteins. These conditions could alter cell physiology, which in turn may negatively impact the result or introduce experimental artifact. Tandem Ubiquitin Binding Entities (TUBEs) have been developed to overcome these problems(1,2) and are licensed by LifeSensors, Inc. from Dr. Manuel Rodriquez at CIC bioGUNE. TUBEs are essentially tandem UBAs with dissociation constants for tetra-ubiquitin in the nanomolar range. TUBEs have also been demonstrated to protect proteins from both deubiquitination and proteasome-mediated degradation, even in the absence of inhibitors normally required to block such activity. The nanomolar affinity of TUBEs for polyubiquitinated proteins allows for high efficiency in isolation and characterization of these proteins from cell lines and tissues. TUBE1 and TUBE2 have been demonstrated to bind to all 7 linkage types (3). However, being derived from different ubiquitin binding domains it is expected that TUBE1 and TUBE2 may have different specificity profiles for the various linkage types. The superior nature of TUBEs allows for efficient detection of polyubiquitinated 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 polyubiquitinated proteins by western blotting and/or downstream proteomic studies. Agarose-TUBEs facilitate convenient "one-step" pull-down of polyubiquitinated proteins.

APPLICATIONS

1. Convenient one-step pull down of polyubiquitinated protein from cell and tissue extracts
2. Isolate ubiquitinated proteins for proteomic studies

BENEFITS

- Up to 1000-fold higher affinity for polyubiquitin compared to single UBA
- TUBEs have higher specificity and affinity than antibodies
- Avoid overexpression of epitope-tagged ubiquitin for pull downs
- Protects polyubiquitinated proteins from degradation during cell lysis and storage

COMPONENTS

Agarose-TUBE1 or Agarose-TUBE2

Size: 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.

ADDITIONAL ITEMS REQUIRED

1. **Cell Lysis buffer:** 50mM Tris-HCl, pH 7.5, 0.15M NaCl, 1mM EDTA, 1% NP-40, 10% glycerol.

   The use of alternative buffer systems should not impact TUBE function; however the inclusion of denaturing detergents e.g. SDS or deoxycholate may have a negative impact on overall yield of polyubiquitinated proteins.

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

2. **20mM Tris-HCl, pH 8.0, 0.15M NaCl, 0.1% Tween-20 (TBS-T)**
3. **Control agarose (LifeSensors Cat. No. UM400)**
4. **(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 polyubiquitinated proteins from degradation. The inclusion of PR-619 in the lysis buffer can increase the yield of polyubiquitinated proteins during the preparation of cell and tissue extracts.
5. **(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.

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.

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 POLYUBIQUITINATED 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.)

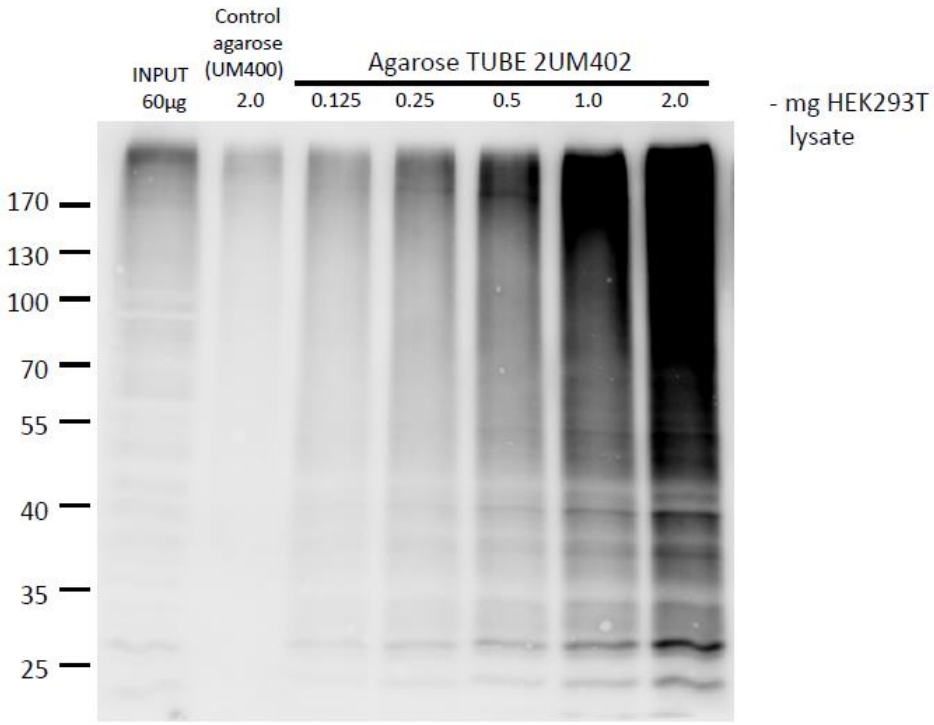
7. Add the amount of cell lysate to the amount of equilibrated Agarose-TUBEs determined from step 2 of the previous section and incubate for 1 hour at 4ºC on a rocker platform. Additional incubation time may be required; optimal time should be determined by the end user.

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.

10. For Western blot analysis, resuspend resin in SDS reducing sample buffer (use of more concentrated SDS reducing sample buffer may allow for flexibility with electrophoresis samples), treat by boiling for 5 minutes, and centrifuge at 13,000xg for 5 minutes. Analyze eluted samples by SDS-PAGE/western blotting in parallel with INPUT and UNBOUND FRACTION. Discard the resin.

SAMPLE DATA



Enrichment for polyubiquitin with Agarose-TUBE2 (UM402). HEK293T cells were lysed in TBS containing 5mM EDTA, 1% NP-40, Protease Cocktail Inhibitor (Calbiochem), 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 10µl of Agarose-TUBE 2 resin (LifeSensors Cat. # UM402) was added to indicted amounts (mg except where indicated). Reactions were rotated for 3.5h (4C), washed three times with TBS plus EDTA, and analyzed by SDS-PAGE and immunoblotting for ubiquitin using the VU-1 Ubiquitin Monoclonal Antibody (LifeSensors Cat. # VU101, 1:1000).

REFERENCES

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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.

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