MANUAL

UbiTest: Linkage Specific Magnetic-TUBE Elution Kit

Catalog Number:
UM412M

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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 are 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, 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.

The most common method to study the ubiquitylation of a protein is through immunoprecipitation and immunoblot analysis. Typically, the protein is brought down with a specific antibody, and then the blot is probed with an antibody to ubiquitin. Alternatively, the protein may be precipitated with a reagent that recognizes ubiquitin or poly-ubiquitin chains, and then the blot is probed with an antibody to the protein of interest. As pointed out in a recent paper from the MRC Protein Phosphorylation and Ubiquitylation Unit either method can lead to misinterpretation of the results or erroneous conclusions (Emmerich and Cohen, 2015). This arises due to the potentially altered interaction of the antibody with the ubiquitylated protein through such things as epitope masking, reduced affinity, or changes in selectivity.

A more definitive method for demonstrating the ubiquitylation of a protein is to couple immunoprecipitation with digestion by a broad spectrum deubiquitylase prior to immunoblot analysis. **An increased signal for the substrate after DUB treatment is a clear indication that the protein was ubiquitylated even if there was no clear reactivity in the untreated sample.** To avoid potential problems arising from changes in immunoreactivity of the protein of interest, it is best to pull down the protein with an anti-ubiquitin reagent. Because of variability in chain recognition encountered with most anti-ubiquitin antibodies, we have built this kit around the use of TUBEs which bind to all ubiquitin chain linkages. 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 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 high-efficiency isolation and characterization of these proteins from cell lines and tissues. TUBE1 has been demonstrated to bind to all 8 linkage types (Lopitz-Otsoa et al., 2012).

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

Magnetic-TUBEs are TUBE moieties directly coupled to magnetic beads, for the identification and characterization of polyubiquitinated proteins by western blotting and/or downstream proteomic studies. Magnetic-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
3. Confirmation of target protein ubiquitylation

BENEFITS

- Up to 1000-fold higher affinity for polyubiquitin compared to single UBA
- Avoids overexpression of epitope-tagged ubiquitin for pull downs
- Protects polyubiquitinated proteins from degradation during cell lysis and subsequent pull down
- Unambiguous determination of the ubiquitylation status of target proteins
- Can be multiplexed to examine several different targets in a single sample
- Facilitates quantitation of percent ubiquitylation
- Magnetic beads make the pull down efficient and minimize nonspecific binding

COMPONENTS

Tube 1 -- Magnetic TUBE1 – 1ml of slurry, 100 µL slurry is recommended for 1-2 mg of total cell lysate pull down.

Buffer: PBS, pH 7.2, 0.05% sodium azide

Storage: Please keep the magnetic TUBE1 at 4°C. Do not centrifuge, dry or freeze the beads

Tube 2 -- Wash buffer – 2 mL

Tube 3 -- Elution buffer – 1 mL

Tube 4 -- 10X Neutralization buffer (Add ß-mercaptoethanol to a final concentration of 10 mM before use, e.g. add 5 µl of 1 M ß-mercaptoethanol stock solution) – 0.5mL

Tube 5 – Broad Spectrum DUB – 25 µg, 10 µM. Storage, -80°C

Tube 6 – K48-Specific DUB – 25 µg, 10 µM. Storage, -80°C

Tube 7 – K63-specific DUB – 35 µg, 10 µM. Storage, -80°C

ADDITIONAL ITEMS REQUIRED

1. **Cell Lysis buffer:** 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM 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) 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.
3. **(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.
4. **Validated antibody against protein(s) of interest**
5. **Your favorite secondary detection antibody that recognizes 4.**
6. **Magnetic rack for 1.5 mL centrifuge tubes.**

EQUILIBRATION OF MAGNETIC-TUBEs

1. Gently mix Magnetic-TUBE1 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 Magnetic-TUBE1 for pull down needs to be determined empirically by the end user. 100 µL of resin (Slurry) in 1 mL of lysis buffer containing 1-2mg of total protein is an appropriate starting point for each experiment.
3. Place the volume of resin necessary for the experiment to the 1.5 mL centrifuge tube, then place the tube into a magnetic stand, collect the beads and discard the supernatant.
4. Suspend the resin in 5-10 volumes of PBST and place it back to a magnetic stand, collect the beads and discard the supernatant.
5. Repeat washing the resin at least two times prior to pull-down.

PULLDOWN OF POLYUBIQUITINATED PROTEINS (Suggested Protocol)

1. Pre-chill inhibitor-containing **cell lysis buffer and micro centrifuge tubes** to 4°C.
2. Treat and wash cells appropriately. As an initial starting point, we recommend the addition of 500 µL 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 a 1.5 mL tube.
4. Clarify lysate by high-speed centrifugation (~14,000xg) for 10 min at 4ºC.
5. Remove an "INPUT" sample for analysis by western blotting (e.g. 5-20 µL of cell lysate in 25-50 µL 1X SDS reducing sample buffer).
6. Add the amount of cell lysate and 10 µL of loading control to the amount of equilibrated Magnetic-TUBE1 determined from the previous section and incubate for 2 hours at 4ºC on a rocker platform. Additional incubation time may be required; optimal time should be determined by the end user.
7. Place the tube on a magnetic stand, collect the beads and save the supernatant as the "UNBOUND FRACTION."
8. Wash beads by re-suspend the beads with 1 mL PBST, place the tube back on a magnetic stand, collect the beads and discard the supernatant. Repeat this step three times
9. Re-suspend beads in 100 µL of wash buffer (Tube 2), mix 5 min at room temperature, place the tube back on a magnetic stand, collect the beads and discard the supernatant.
10. Re-suspend resin in 50 µL of Elution buffer (Tube 3). Mix 15 min at room temperature, place the tube back on a magnetic stand, and collect the elution.
11. Neutralize the supernatant by adding 10x Neutralization buffer (Tube 4). Split sample into four equal parts. To the first part, add 5 µl of 10 µM K48-linkage specific DUB and to the second part, add 5µL of 10 µM K63-linkage specific DUB. Add 5µL of buffer to the third 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.
12. Stop the reaction by the addition of SDS sample loading buffer. Heat the samples at 100°C for 3-5 min and proceed with immunoblot analysis.
13. The blot can be stripped and re-probed with additional anti-target antibodies.

REFERENCES

1. Emmerich, C.H., and Cohen, P. (2015). Optimising methods for the preservation, capture and identification of ubiquitin chains and ubiquitylated proteins by immunoblotting. Biochem Biophys Res Commun *466*, 1-14.

2. Hjerpe, R., Aillet, F., Lopitz-Otsoa, F., Lang, V., England, P., and Rodriguez, M.S. (2009). Efficient protection and isolation of ubiquitylated proteins using tandem ubiquitin-binding entities. EMBO Rep *10*, 1250-1258.

3. 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*, 2998-3014.

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