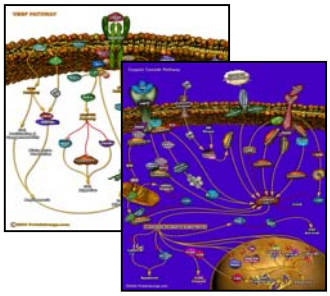


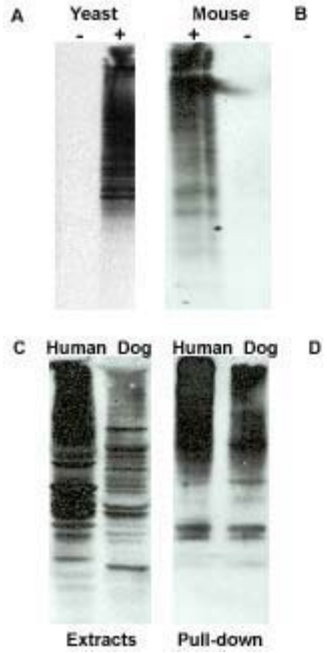
Ubiquitinated Protein Enrichment Kit
Cat. No. 662200

Note that this user protocol is not lot-specific and is representative of the current specifications for this product. Please consult the vial label and the certificate of analysis for information on specific lots. Also note that shipping conditions may differ from storage conditions. Full details are available at www.calbiochem.com.

Introduction

A vast majority of short-lived proteins are degraded by the ubiquitin-proteasome pathway. A protein marked for degradation is covalently attached to multiple molecules of ubiquitin, a highly conserved 76-amino acid (8.6 kDa) protein, by a multi-enzymatic system consisting of Ubiquitin-activating (E1), Ubiquitin-conjugating (E2), and the Ubiquitin-ligating (E3) enzymes. The E1 activates a Ubiquitin monomer at its C-terminal cysteine residue to a high-energy thiolester bond which is then transferred to a reactive cysteine residue of the E2 enzyme. The final transfer of ubiquitin to ε-amino group of a reactive lysine residue of substrate proteins is brought about by the E3 enzyme. Ubiquitinated protein is then escorted to the 26S proteasome where it undergoes final degradation and the ubiquitin is released and recycled. A family of proteins including Rad23, contain two ubiquitin-associated domains that bind ubiquitinated cellular proteins and translocate them to the proteasome. Ubiquitinated proteins can be enriched using affinity beads comprised of a GST-fusion protein containing this ubiquitin-associated sequence conjugated to glutathione-agarose.

This kit is useful for the enrichment of polyubiquitinated proteins from cell and tissue lysates of a broad range of species including, canine, human, mouse, and yeast (see figure below). The ubiquitinated proteins can be identified by loading the beads directly onto SDS-PAGE and then immunoblotting with the antibody of choice or Anti-Ubiquitin (Cat. No. 662099). Alternatively, it is possible that the beads can first be treated with Isopeptidase T (Cat. No. 419700) to release the proteins from the ubiquitin chains.



Protein extracts were applied to polyubiquitin-affinity beads (Panels A, B and D), and then examined by immunoblotting, using anti-ubiquitin antibodies.

Panel A: Protein extracts from yeast Saccharomyces cerevisiae were applied to mock (-) or polyUb affinity beads (+).

Panel B: Protein extracts from mouse were applied to mock (-) and polyUb affinity beads (+).

Panel C: Total cellular extracts from human cultured cells and dog tissue were examined.

Panel D: Samples as in Panel C were applied to polyUb affinity beads.

All four filters were probed with anti-ubiquitin antibodies and developed by enhanced chemiluminescence.

Kit Components:

(Quantities sufficient for 25 affinity purification assays)

1. **Polyubiquitin Affinity Beads (Cat. No. KP30801) –** Suspension of affinity beads in PBS containing 0.05% sodium azide. **1.0 ml. Store at 4°C.**

2. **Control Beads (Cat. No. KP30802) –** Suspension of control beads in PBS containg 0.05% sodium azide. **200 µl. Store at 4°C.**

3. **Control Lysate (Cat. No. KP30803) –** Suspension of protein extract in 1.5 M Tris-HCl, 10% glycerol, 5% β-mercaptoethanol, 2% SDS, 0.02% bromophenol blue, pH 6.8. **60 µl. Store at 4°C.**

Protocol for using the polyubiquitin affinity matrix

1. Resuspend the matrix by gentle inversion, until the beads are completely unpacked.
2. Use a large bore pipet tip (by cutting off the terminal 3 mm with a razor blade) to remove ~40 µl of the affinity bead suspension. There is no need to wash the beads.
3. Add the beads directly to 0.5 mg - 1.0 mg of cell lysate, at a concentration of ~1mg/ml. The protein sample should be clear of any sediments or particulate matter, since this material will be recovered with the beads through subsequent washes.

   *Cell lysates can be prepared using a lysis buffer (pre-cooled to 4°C) consisting of: 50 mM HEPES (pH 7.5), 5 mM EDTA, 150 mM NaCl and 1% Triton® X-100 detergent. Add a pre-made protease inhibitor cocktail (eg. Cat. No. 539134) and 10 mM N-ethylmaleimide (prepared in DMSO) immediately before use. Cells can be further disrupted by sonication, hydrostatic pressure, glass-bead agitation, or with osmotic destabilizers.

4. Incubate at 4°C for 2-4 h with constant mixing to keep the affinity beads well suspended. Avoid aeration or vigorous mixing.
5. Remove supernatant after centrifugation for 5 s at 4°C in a microfuge (~1000 X g), and resuspend in 1 ml of Wash buffer pre-cooled to 4°C (Wash buffer is the same as lysis buffer in step 3 without NEM and protease inhibitors). Repeat three more times.

6. Suspend the affinity matrix in 40 µl of 2X gel loading buffer (gel loading buffer should be at room temperature and consist of 250 mM Tris, HCl, pH 6.8; 4% SDS; 10% β-mercaptoethanol; 20% glycerol; and bromophenol blue), and boil for 5 min.
7. Centrifuge the material for 1 min at full speed in a microfuge (> 10,000 X g), and apply the supernatant to an 8 – 12% SDS-PAGE.
8. For immunoblotting analysis, transfer the resolved proteins to nitrocellulose (preferably 0.2 µm), and stain with Ponceau S to confirm efficiency of transfer.

To determine if a protein of interest is ubiquitinated:

9. Wash the immunoblot 3 times with 1X TBST (10 mM Tris-HCl, 150 mM NaCl, 0.1% TWEEN®-20 detergent), using 100 ml per wash for 10 min each.
10. Block the nitrocellulose membrane for 30 min on a rocking platform with TBST 5% milk solution for monoclonals and 10% for polyclonals at room temperature using about 1 ml per cm^2 of membrane.
11. Wash the nitrocellulose membrane 4 times in TBST for 10 min each on a rocking platform. Incubate the nitrocellulose membrane for 60 minutes on a rocking platform with primary antibody diluted in TBST, 5% milk.
12. Wash the nitrocellulose membrane 4 times in TBST for 10 min each on a rocking platform.
13. Incubate with secondary antibody enzyme conjugate diluted in 5% milk TBST for 60 min on a rocking platform.
14. Wash the nitrocellulose membrane 4 times in TBST for 10 minutes each on a rocking platform.
15. Develop with enhanced chemiluminescence to maximize detection.

To confirm that ubiquitinated proteins are purified:

16. Immerse the nitrocellulose filter in 1 cm of distilled water and boil for 5 minutes (The boiling step should only be used for detecting ubiquitin). To prevent the filter from floating, place a glass plate on top of the filter while boiling. Carefully remove the filter with forceps and immediately place in a tray containing 5% milk powder in 1X TBST. Incubate for 1 h at ambient temperature with agitation.
18. Wash the filter three times with 1X TBST (100 ml per wash) for 10 min.
19. Transfer the filter to a clean container and incubate with antibody against ubiquitin. Follow steps 12–15 from above.
20. Develop with enhanced chemiluminescence to maximize detection.

References:

1. Chen, L. and Madura, K. 2002. *Mol. Cell Biol.* **22,** 4902.
2. Chen, L., et al. 2001. *EMBO Rep.* **2,** 933.

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