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Deubiquitinating Enzyme (DUB) Identification Kit

Cat. # J6210

Description

This kit is designed to enrich deubiquitinating enzymes in crude or partially purified cell/tissue extracts, followed by identification using immunoblotting or mass spectrometry.

Components

Component	Stock Concentration	Quantity
•Biotin-Ub-PA	1 mg/ml	25 µg
•Biotin-Ub	1 mg/ml	25 µg
•Streptavidin resin	50% slurry	500 µl
•2X Wash Buffer	2X	4X 1.25 ml

Notes

1. 2X Wash Buffer: 80 mM Tris, pH 7.6 at 4 °C, 1% TX-100, 1.0 M NaCl
2. 3 M urea can be added in 1X Wash Buffer if a more stringent buffer is needed.
3. To make 4 ml 1X Wash Buffer + 3 M urea: weight 0.72g urea (not supplied) + 2 ml 2X Wash Buffer, then add miliQ water to adjust volume to 4 ml.
4. Proteins can be eluted by 1X SDS sample buffer with boiling or performing on-bead trypsin digestion for mass spectrometric identification.
5. The concentration of biotin-Ub and biotin-Ub-PA in the final labeling reaction is recommended to be 1-2 µM.
6. The molecular weights of biotin-Ub and Biotin-Ub-PA are 8.9 kDa.

Protocol

⚠ **All purification steps are operated at 4°C!**

1) Prepare whole cell or tissue lysates with a total protein concentration of 2-5 mg/ml. Cell/tissue debris should be removed by high speed centrifugation. Protease inhibitors (not included) can be added in the lysis buffer to block protease digestion during the purification process.

2) Harvest two 10cm plates of HEK293T cells at 90% confluence into a 15 conical tube by centrifugation at 500 Xg for 5 min. Aspirate cell culture medium and rinse cell pellets twice with cold 1X PBS.

3) Lyse cell pellets into 2 ml lysis buffer (40 mM Tris, pH 7.6 at 4 0C, 150 mM NaCl, 2 mM DTT, 10% glycerol), keep on ice and gently sonicate 3 times with a cycle of 10 seconds on and 45 seconds off. We used a 550 Sonic Dismembrator (Fisher Scientific), output setting: 2. You may use other approaches to lyse cells.

4) Transfer whole cell lysates into a 2 ml centrifuge tube, centrifuge at 16,000 Xg for 20 min. Carefully transfer the supernatant into a new tube. Determine the protein concentration in the supernatant using the Bradford assay. We obtained 8.04 mg total cell lysates at a concentration of 4.02 mg/ml.

5) Label two 1.7 ml centrifuge tubes as Reaction 1 (R1) and Reaction 2 (R2), add 0.75 ml whole cell lysate plus 0.25 ml lysis buffer into each tube, a total of 3 mg whole cell lysate in 1 ml for each reaction. In R1, add 18 µL supplied biotin-Ub. In R2, add 18 uL supplied biotin-Ub-PA. The concentration of biotin-Ub and biotin-Ub-PA in the reaction is 2 µM.

6) Incubate R1 and R2 at 4 °C for 4 hours, then add 50 µl streptavidin resin (net resin, washed by lysis buffer) to each tube. Gently rock the mixtures using a rotator in a cold room for overnight.

7) Centrifuge at 1,000 Xg for 5 min to pellet resins. Discard the supernatants. Wash resins twice with 0.75 mL 1X Wash Buffer (supplied), then twice with 0.75 ml 1X Wash Buffer + 3 M urea (not supplied), finally once with 1X wash buffer to get rid of urea. For each wash, rock the tube in a rotator in a cold room for 5 min, then centrifuge 5 min at 1,000 Xg. Discard the supernatants.

8) After the final wash, carefully aspirate all buffer, then add 150 µL 1X SDS sample buffer, boil at 95 °C for 10 min.

9) To perform immunoblotting assay, we loaded 10 µL/well for R1 (control) or R2 (DUB enrichment) for separation on an SDS-PAGE. Streptavidin HRP blot determines all labeled proteins. The signals in biotin-Ub reaction (R1) likely represent cellular biotinylated proteins binding on streptavidin resin. We also immunoblotted USP7, USP14, USP15 and UCH37, all four DUBs were identified in biotin-Ub-PA reaction (R2), but not in the control biotin-Ub reaction (R1). See all blots below.