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Protocol 4: Large-scale purification of SUMO modified proteins from *S. pombe*

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Abstract

Covalent protein modification by SUMO (sumoylation) regulates a broad spectrum of critical functions in eukaryotic cells; however, usually 1% of a given protein is modified due to the highly dynamic nature of sumoylation. As such, capturing and identifying sumoylated proteins is both important in biological studies, and a very challenging task. Here we report a tailored purification protocol that includes rapid and complete cell disruption, coupled to highly stringent isolation of sumoylated proteins. Proteins purified using this protocol are compatible with common downstream applications such as Western and mass spectrometry analyses. This protocol will work equally well to study other key covalent modifiers such as Ubiquitin and Ned8.

Materials

Reagents

Guanidine Hydrochloride (GuHCl) buffer <R>

6 M GuHCl, 50 mM NaH₂PO₄, 50 mM Tris, 8 mM imidazole, pH 8

Buffer A <R>

8 M urea, 50 mM NaH₂PO₄, 50 mM Tris, 300 mM NaCl, pH 8

Buffer B <R>

8 M urea, 2% SDS, 100 mM Tris, 200 mM NaCl, pH 8

Buffer C <R>

8 M urea, 0.2% SDS, 100 mM Tris, 1.2 M NaCl, 10% EtOH, 10% 2-propanol, pH

8

Buffer D <R>

8 M urea, 0.2% SDS, 100 mM Tris, 200 mM NaCl, 10% EtOH, 10% 2-propanol,

pH 5

Buffer **D'** <R>

8 M urea, 0.2% SDS, 100 mM Tris, 200 mM NaCl, 10% EtOH, 10% 2-propanol,

pH 9

Buffer **E** <R>

8 M urea, 100 mM Tris, pH 8

Glass pasteur pipets

1 M HCl <R>

Imidazole <R>

Liquid nitrogen

1 M NaOH <R>

Ni-NTA beads

Phenylmethanesulfonyl fluoride (PMSF) <R>

SDS loading buffer <R>

50 mM Tris, 2% SDS, 0.1% Bromophenol Blue, 10% glycerol, 100 mM

Dithiothreitol, pH 6.8

Streptavidin beads

0.5 mm zirconia or glass beads

Equipment

Bead-Beater, Model 1107900 from Biospec Products, Inc.

COORSTEK Buchner Ceramic Funnel, 320 ml

Filter flask

pH paper

Whatman filter paper, diameter 90 mm

Method

Background

De-sumoylation occurs rapidly in cell lysates, and sumoylated proteins constitute only a minute fraction of total cellular proteins. Therefore, isolation of sumoylated proteins requires full suppression of SUMO iso-peptidase activities, and a stringent two-step purification procedure to separate SUMO conjugates from unmodified proteins. Taking advantage of the covalent nature of sumoylation, we developed a tandem purification protocol that uses fully denaturing conditions and two affinity tags compatible with such conditions (adapted from procedures established for ubiquitin conjugate profiling in other systems, (Tagwerker et al. 2006)). Endogenous SUMO was tagged at its N-terminus with a BH-tag: a 15-mer minimal biotinylation signaling sequence GLNDIFEAQKIEWHE (Beckett *et al.* 1999) followed by six histidines. The *Escherichia coli* biotin holoenzyme synthetase, BirA, which catalyzes transfer of biotin to target proteins, is expressed under a constitutive Adh promoter to catalyze efficient biotinylation of BH-SUMO [this strain, NBY2691, and associated plasmids are available upon request from our lab]. Cells are lysed in 6 M GuHCl to ensure their full disruption and complete denaturation of proteins, which suppresses de-sumoylation. Sumoylated proteins are then purified by tandem nickel and streptavidin affinity chromatography. Stringent washes during the streptavidin chromatography step yield exceptionally "clean" high confidence SUMO conjugates.

Protocol

Note: All steps are performed at room temperature unless specifically noted.

Cell disruption

1. Six liters of cells grown to exponential phase (O.D. ~1) are harvested as described in Protocol 3 by centrifugation.
2. Resuspend the cell pellet in 120ml of 6 M GuHCl buffer supplemented freshly with 1 mM PMSF.
3. Transfer cell suspension into the plastic bottle of Bead-Beater, add zirconia or glass beads until they are approximately 1 cm below the neck of the bottle. Assemble the Bead-Beater unit according to manufacture instructions.
4. Lyse the cells with 4x 1-min pulses. Chill the Bead-Beater bottle in ice-water mixture for 5 min between pulses to prevent over-heating. Over 90% cell lysis should be achieved as examined under a microscope.
5. Transfer the lysate/beads slur into a COORSTEK Buchner Ceramic Funnel lined with Whatman filter paper and attached to a filter flask and vacuum source. Apply vacuum to draw liquid into the flask. Some beads will come through, which will be completely removed in the subsequent centrifugation step.
6. Centrifuge the lysate for at 28000 g for 15 min at 4°C.
7. Approximately 100 ml of supernatant will be recovered. Transfer supernatant carefully into clean 50 ml centrifuge tubes, and save 100 µl for future analysis.

Binding to and eluting proteins from Ni beads

1. Batch bind lysate to 5 ml of Ni-NTA beads pre-equilibrated with 6 M GuHCl buffer at room temperature in a chromatography column for one hour.
2. Support the column on a stand, and drain liquid by gravity.
3. Wash the beads 4x each with 50 ml Buffer **A** with 8 mM imidazole and 1 mM PMSF freshly added.
4. Cap the column, add 5 ml of Buffer **A** to the beads, adjust pH to 4 by adding 1 M HCl (~ 30 drops using a glass Pasteur pipet), while stirring constantly to ensure even change of pH. Monitor pH change with pH paper.
5. At low pH proteins are eluted efficiently from the Ni beads. Remove column cap, eluted proteins, and adjust eluted protein solution back to pH 8 with 1 M NaOH.

Binding to and eluting proteins from streptavidin beads

1. Bind eluted proteins to 250 µl of streptavidin beads that have been pre-equilibrated in Buffer **A** in a 15 ml centrifugation tube for one hour at RT.
2. Wash the beads sequentially with ~ 25 bed volumes (6.5 ml) of Buffer **B**, **C**, **D**, **D'**, and **E**.
3. Store the beads in small amount of Buffer **E** at 4°C until future analysis.
4. Proteins used for mass spectrometry analysis will be digested by trypsin directly on beads.
5. Proteins used for Western blot can be eluted by heating the beads in 1 bed volume of 2x SDS loading buffer at 95°C for 5 min.

Discussion

This two-step denaturing purification protocol, with highly stringent washes, enables high confidence isolation of low abundance sumoylated proteins. The method of cell disruption described here has been optimized for rapid and complete disruption of a very large quantity of cells. Alternative methods of cell disruption, such as the ones described in Protocol 3, can also be used if they are better suited for the set up of an individual laboratory. Finally, this protocol has been used to analyze SUMO and ubiquitin conjugates, and could readily be applied to analyze targets of other key covalent modifiers such as Ned8 (HuNedd8).

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