

Generation of ³²P-labeled ubiquitin, SUMO & Nedd8

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INTRODUCTION

Thioester-formation experiments with radiolabeled small protein-modifiers.

MATERIALS

Media

- Luria Broth containing 50 µg/ml Ampicillin

Buffers

- NETN: 0.5% NP-40, 20 mM Tris pH 8, 100 mM NaCl, 1 mM EDTA
- HMK buffer: 10X HMK buffer 200 mM Tris pH 7.5, 1 M NaCl, 120 mM MgCl₂ (10 mM DTT where applicable)
- HMK Stop buffer: 10 mM NaH₂PO₄, 10 mM Na₄P₂O₇, 10 mM EDTA, 1 mg/ml BSA
- Elution buffer: 20 mM glutathione, 100 mM Tris pH 8, 120 mM NaCl

Reagents

- IPTG: 1000X = 0.1M
- GSH-sepharose beads (equilibrated as 50% slurry in NETN + 0.5% dry milk)
- HMK (Sigma P-2645, Protein Kinase A Catalytic Subunit from bovine heart)
- Thrombin

METHODS

1. Grow overnight culture of pGEX-2TK recombinant fusion of interest (ubiquitin, SUMO, Nedd8, etc.) in LB + Amp
2. Next day, dilute overnight culture 1:10 in fresh LB + Amp. Grow 1 h with shaking at 37 °C.
3. Add IPTG to a final concentration of 0.1 mM
4. Grow additional 4 h with shaking at 37 °C
5. Spin down bacteria (5', 5000 rpm at 4 °C, from now on, keep things cold)
6. Resuspend bacteria in 1/10 of culture volume NETN
7. Sonicate on ice with 3 brief (5'' – 10'') bursts
8. Spin down suspension (5', 10000 G at 4 °C) and discard the pellet
9. Rock supernatant with GSH-sepharose beads (equilibrated as 50% slurry in NETN + 0.5% dry milk) for 15' – 30'
10. Wash beads 3x with NETN
11. Wash beads 1x with HMK buffer without DTT

12. Resuspend beads in reaction mix (30 ml reaction for 10 ml bacterial culture): 3 ml 10X HMK buffer with DTT (10 mM), 1 ml HMK (Sigma P-2645; 100 or 400 units/vial, resuspend at 10 units/ml in 40 mM DTT, let sit 5' – 10' at room temperature to dissolve, enzyme is stable for several days at 4 °C), 2 μ l 32 P-g-ATP, 24 ml H₂O
13. Incubate beads at 4 °C for 30'. "Flick" tube occasionally to resuspend beads
14. Add 1 ml of HMK Stop buffer, mix thoroughly, spin down beads and discard supernatant.
15. Wash beads 5x with NETN and discard the supernatant
16. Option 1: To get rid of the GST part of the fusion protein, cleave with Thrombin in 1 ml volume according to the manufacturer's protocol. After cleavage spin down and discard beads and save the supernatant
17. Option 2: If no Thrombin cleavage was performed, add 1 ml of freshly prepared elution buffer.
18. Rock for 5' – 10' at 4 °C
19. Spin down and discard beads and save the supernatant
20. Use either 32 P-labeled GST-fusion protein or the cleaved GST-free protein for thioester-formation experiments

BIBLIOGRAPHY

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