

## Expression and purification of SUMO E1 (His-Aos1/Uba2 or His-Aos1/Uba2-His) *Melchior's lab protocol*

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### INTRODUCTION

The SUMO E1 complex can be purified by two different approaches resulting in E1 with identical activity (Werner et al. 2008). The "classical" protocol describes the purification after coexpression of His-tagged Aos1 and untagged Uba2 in bacteria. A recently developed alternative protocol of our lab involves separate expression and purification of N-terminal His-tagged Aos1 and C-terminal His-tagged Uba2 and subsequent reconstitution of the heterodimeric complex. This procedure has the advantage of higher yields, simplified procedures and the possibility to combine different variants or differently tagged subunits.

### METHODS

#### Purification of His-Aos1/Uba2

##### Day 1

1. Simultaneous transformation of 1  $\mu$ l each His-Aos1 and Uba2 plasmids into one aliquot of 200  $\mu$ l BL21 (Gold) Codon usage plus (or Rosetta), standard procedure with heat shock at 42°C
2. After heat shock, add 600  $\mu$ l LB (without antibiotics!), incubate for 1 h at 37°C, and transfer into 500 ml LB with 50  $\mu$ g/ml Ampicilin and 30  $\mu$ g/ml Kanamycin (in a 5 l-flask)
3. Shake at 37°C, 150 rpm, overnight (for approximately 18 h)

##### Day 2

1. Harvest bacteria (20 min, 4000 rpm, 4°C, Beckman-centrifuge J6B in 500 ml-conical flasks)
2. Resuspend bacterial pellet in 2 l fresh LB / Amp / Kan
3. Let cells grow until OD<sub>600</sub> of 0.6
4. Add 1mM IPTG (final concentration)
5. Incubate for 6 h at 25 °C, 150 rpm
6. Harvest bacteria
7. Resuspend pellets in 50 ml **Lysis buffer**:
  - 50 mM Na-Phosphat pH 8.0
  - 300 mM NaCl
  - 10 mM Imidazol
8. Store at – 80°C until the next day or until needed (freezing is necessary for efficient lysis, with Lysozyme!)

##### Day 3

1. Thaw pellet, add protease inhibitors (to final concentrations):
  - 0.1 mM PMSF
  - 1 mM  $\beta$ -Mercaptoethanol
  - 1  $\mu$ g/ml Aprotinin
  - 1  $\mu$ g/ml each of Leupeptin and Pepstatin

2. Add Lysozyme (final concentration 1 mg/ml) and incubate for 1 h at 4°C (rotate slowly), or lyse cells by using two passages over an emulsion flex
3. Ultracentrifuge the suspension of lysed cells for 1 h, 100,000 g, 4°C
4. Equilibrate Ni-beads (for 2 l bacterial culture 6 ml beads, Probond INVITROGEN) with lysis buffer (including protease inhibitors,  $\beta$ -mercaptoethanol)
5. Add Supernatant from ultracentrifugation to equilibrated Ni-beads (in 50 ml-Falcon tubes) and incubate for 1 h bei 4°C (rotate slowly)
6. Harvest Ni-beads by centrifugation (5 min 500 rpm, 4°C (Beckman J6B)
7. Transfer beads into column and wash beads extensively with cold **Washbuffer** until no more protein is detected in the flowthrough (test with ponceau staining on nitrocellulose membrane)

**Washbuffer:**

- 50 mM Na-Phosphat pH 8.0
  - 300 mM NaCl
  - 20 mM Imidazol
  - 1 mM  $\beta$ -Mercaptoethanol
  - 1  $\mu$ g/ml Aprotinin
  - 1  $\mu$ g/ml each of Leupeptin and Pepstatin
8. Elute with 3 column volumes of **Elution buffer** (collect 2 ml fractions)
    - 50 mM Na-Phosphat pH 8.0
    - 300 mM NaCl
    - 250 mM Imidazol
    - 1 mM  $\beta$ -Mercaptoethanol
    - 1  $\mu$ g/ml Aprotinin
    - 1  $\mu$ g/ml each of Leupeptin and Pepstatin
  9. Test for protein in eluate fractions by spotting 1  $\mu$ l on nitrocellulose membrane and staining with PonceauS solution
  10. Combine peak fractions, concentrate by centrifugation in e.g. 30K-Millipore-concentrator down to 2-5 ml.
  11. Filtrate sample with 0.2  $\mu$ m low protein binding non-pyrogenic filter, and apply to FPLC S200 preparative gel filtration column, equilibrated in **S200 buffer**:
    - 50 mM Tris pH 7.5
    - 50 mM NaCl
    - 1 mM DTT
    - 1  $\mu$ g/ml Aprotinin
    - 1  $\mu$ g/ml each of Leupeptin and Pepstatin
  12. **Run overnight in cold room or chromatography fridge**
  13. Collect 5 ml-fractions (Pharmacia FPLC: UV lamp setting on 1)

**Day 4**

1. Analyze 10 $\mu$ l of the fractions on 8% SDS-PAGE gel, stain with Coomassie
2. Combine fractions that contain both Aos1 (migrates at 40 kD) and Uba2 (migrates at 90 kD)
3. Avoid free Aos1!
4. Apply the sample onto a 1 ml MonoQ column, equilibrated with S200 buffer
5. Run shallow gradient (over 20 column volumes) from 50 to 500 mM NaCl, and collect 0.5 ml-fractions (UV setting =1)

### Day 5

Analyze 3-5  $\mu$ l of the fractions on 8% SDS-PAGE gel:

Combine fractions that contain equimolar ratios of Aos1 and Uba2 (usually 2-3 fractions),

Dialyze against **Transport buffer**:

- 20 mM Hepes/KOH pH 7.3
- 110 mM potassium acetate
- 2 mM magnesium acetate
- 0.5 mM EGTA
- 1 mM DTT
- 1  $\mu$ g/ml Aprotinin
- 1  $\mu$ g/ml each of Leupeptin and Pepstatin

Flash freeze in small aliquots (2-10  $\mu$ l) in liquid nitrogen and store at -80°C

### Purification of His-Aos1/Uba2-His

#### **Purification of monomeric His-Aos1 and Uba2-His:**

1. Transform 1  $\mu$ l of each His-Aos1 and Uba2-His plasmids each into a single aliquot of 200  $\mu$ l BL21
2. Inoculate an overnight culture of 500 ml LB supplemented with 30  $\mu$ g/ml kanamycin, 1mM MgCl<sub>2</sub> and 0.1% glucose for each of the two proteins
3. Follow the above protocol for further purification of His-Aos1 and Uba2-His, with the exception of using transport buffer for the S200 gel filtration step (purification on a MonoQ column is not required!)
4. Concentrate proteins in centrifugal devices to 1-3 mg/ml, flash-freeze them in liquid nitrogen and store them at -80°C

#### **Reconstitution of the dimeric SUMO E1:**

1. Combine equimolar amounts of His-Aos1 and Uba2-His and incubate for 1-2h on ice
2. Run sample over a S200 gel filtration column as described above (necessary for separating active complex from excess of one subunit or partially unfolded Uba2!)
3. Concentrate complex, flash freeze in small aliquots (2-10  $\mu$ l) in liquid nitrogen and store at -80°C

### **BIBLIOGRAPHY**

Werner A, Moutty MC, Möller U, Melchior F. Performing in vitro sumoylation reactions using recombinant enzymes. *Methods Mol Biol.* 2009; 497:187-99.