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🌐 Expression and purification protocol of *Homo sapiens* E2-like enzyme ATG3

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Protocol status:

Working
We use this protocol and it's working

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ABSTRACT

This protocol describes expression and purification procedures for obtaining human recombinant autophagy E2-like enzyme ATG3 (ATG, AuTophagy-related protein) of the ATG8 ubiquitin-like conjugation system.

IMAGE ATTRIBUTION

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GUIDELINES

General information

Insert: *Homo sapiens* ATG3, NP_071933.2; Expression system: *E. Coli* Rosetta pLyss; plasmid origin: Sascha Martens Lab, Addgene 169079, lab internal construct database number SMC861; backbone: pET-Duet1; plasmid resistance: Ampicillin; tags & cleavage sites: N-term 6xHis, followed by Tobacco Etch Virus (TEV) cleavage site, ATG3 ORF. Ext coeff: 45840 M⁻¹ cm⁻¹, MW 35.8 kDa.

MATERIALS

Materials and Reagents

- *Escherichia coli* Rosetta pLyss cells
- Luria Bertani (LB) medium with antibiotics (final conc. 50µg/ml Ampicillin, 34µg/ml Chloramphenicol)
- IPTG (isopropyl-b-d-thiogalactopyranoside)
- 37°C shaker incubator
- sterile flasks/sterile pipettes
- tip sonicator

- **Lysis Buffer:** 50mM Hepes pH=7.5; 300mM NaCl, 10mM Imidazole, 2mM MgCl₂, 2mM β-mercaptoethanol, 1mM Pefablock, Complete Protease Inhibitors (EDTA-free CIP tablet, Roche), DNase (Sigma).
- **Buffer A:** 50mM Hepes pH=7.5, 300mM NaCl, 10mM Imidazole (filtered and degassed) + 1mM β-mercaptoethanol
- **Buffer B:** 50mM Hepes pH=7.5, 300mM NaCl, 300mM Imidazole (filtered and degassed) + 1mM β-mercaptoethanol
- **Size Exclusion Chromatography (SEC) Buffer:** 25mM Hepes pH=7.5, 150mM NaCl (filtered and degassed) + 1mM DTT (Dithiothreitol)
- Note: all purification buffers are filtered and degassed. Reducing agents (β-mercaptoethanol and Dithiothreitol) are added after degassing step.

Columns: - HT 5ml column (GE Healthcare)
- S75_16/60 (GE Healthcare)

Gels: 10% SDS-PAGE

SAFETY WARNINGS

⚠ For hazard information and safety warnings, please refer to the SDS (Safety Data Sheet).

Protein Expression

- 1 Transform plasmid DNA (Addgene 169079, SMC861) into *E.Coli* Rosetta pLyss cells and plate on Ampicillin/Chloramphenicol LB agar plate for Overnight growth at 37 °C.
- 2 The following day, inoculate a 100 mL LB + Amp/Cam pre-culture with 1-2 colonies and grow Overnight at 37 °C shaking.
- 3 The following day, use 20 mL pre-culture to inoculate 1 L LB medium + Amp/Cam at 37 °C until an OD₆₀₀ (Optical Density at 600nm) of 0.4 is reached.

- 4 Cool down the culture to 18 °C and grow until OD₆₀₀ = 0.8.
- 5 Induce protein expression with 100 micromolar (μM) IPTG and keep shaking for a further 16:00:00 at 18 °C. 16h
- 6 Pellet cells at 4000 rpm, 4°C, 00:15:00 in a Sorvall RC6+ centrifuge (Thermo Scientific), discard supernatant and resuspend pellets in ice cold lysis buffer (25 ml/1 lt culture). 15m
- 7 Flash freeze resuspended pellets in liquid nitrogen and store at -80 °C until purification.

Protein Purification

30m 30s

- 8 Perform His-Trap affinity purification followed by Size Exclusion Chromatography.
- 9 Cells are lysed via freeze/thaw cycles and sonication: thaw pellet corresponding to 1 L culture by freeze/thawing in Room temperature water bath. All following steps are to be executed at 4 °C or on ice.
- 10 Lyse cells by sonicating them using an immersion tip Sonicator (2x 00:00:30). Note: adjust times and intensity according to the available instrument. 30s
- 11 Clear lysate by spinning it down in a Beckman centrifuge at 40000 x g, 4°C, 00:30:00 , Ti45 Rotor. 30m
- 12 Filter supernatant through a 0.45 μm filter and inject onto a 5ml HT column operating at 4 °C pre-equilibrated in Buffer A at 1ml/min flow rate.

13

Wash column with 5 column volumes (CV) of Buffer A at 2 ml/min flow rate to remove unspecific bound proteins.

14

Elute protein of interest through a step elution gradient in 50mM, 75mM, 100mM, 150mM, 200mM and 300mM Imidazole concentration. Perform elution at 1ml/min flow rate. Collect peak fractions at each step.

15

Check peak fractions of each step on a SDS-PAGE (see gel below). Pool and concentrate those containing the protein of interest (usually 100mM and 150mM Imidazole step) by spinning at 4 °C down in a 10kDa cut-off

Amicon Filter to 2 mL final volume

Note

Centrifugation steps are kept short (00:05:00) to avoid protein local concentration/aggregation on the filter.

Coomassie BB stained gel of His-tag affinity purification for hATG3.

16

Inject 2 mL protein onto a S75_16/600 column operating at 4 °C and pre-equilibrated in buffer containing SEC Buffer (see profile below).

Size Exclusion Chromatography

Chromatograph of Size Exclusion purification for hATG3.

- 17 Check fractions on a 10% SDS-PAGE (see gel below). Pool and concentrate those containing the protein of interest down at 4°C in a 10kDa cut-off Amicon Filter.

Note

Centrifugation steps are kept short ($00:05:00$) to avoid protein local concentration/aggregation on the filter.

Coomassie BB stained gel of Size Exclusion Chromatography purification for hATG3.

- 18** Measure protein Absorbance A_{280} using a Spectrophotometer blanking against SEC buffer (MW = 35,864 kDa ; Extinction coefficient = $45840\text{M}^{-1}\text{cm}^{-1}$).

- 19** Resuspend protein in Glycerol to a final concentration of $[M]$ 30 % (v/v) for glycerol . Store the protein batch at $-20\text{ }^{\circ}\text{C}$. Estimated protein yield: $\text{10 mg per 1 lt culture}$. Protein activity is kept for 18 months.