May 24, 2021

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

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1 Works for me Share dx.doi.org/10.17504/protocols.io.btgknjuw

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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.

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

A PI3K-WIPI2 positive feedback loop allosterically activates LC3 lipidation in autophagy - Published in 10.1083/jcb.201912098

DOI

dx.doi.org/10.17504/protocols.io.btgknjuw

PROTOCOL CITATION

Eleonora Turco, Dorotea Fracchiolla 2021. Expression and purification protocol of Homo sapiens E2-like enzyme ATG3. **protocols.io** https://dx.doi.org/10.17504/protocols.io.btgknjuw

MANUSCRIPT CITATION please remember to cite the following publication along with this protocol

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KEYWORDS

ATG3, expression, purification, recombinant protein, Homo sapiens

CREATED

Mar 18, 2021

LAST MODIFIED

May 24, 2021

OWNERSHIP HISTORY

Mar 18, 2021 Liz Brydon Protocols.io

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PROTOCOL INTEGER ID

48364

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 TEXT

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 $MgCl_2$, 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.

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3 The following day, use **20 mL pre-culture** to inoculate **1 L LB medium + Amp/Cam** at **37 °C** until an OD_{600} (Optical Density at 600nm) of 0.4 is reached.

4 Cool down the culture to **18 °C** and grow until OD_{600} = 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 15m

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).

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 lised 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 30m

Clear lysate by spinning it down in a Beckman centrifuge at **40000 x g, 4°C, 00:30:00 , Ti45 Rotor** .

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.

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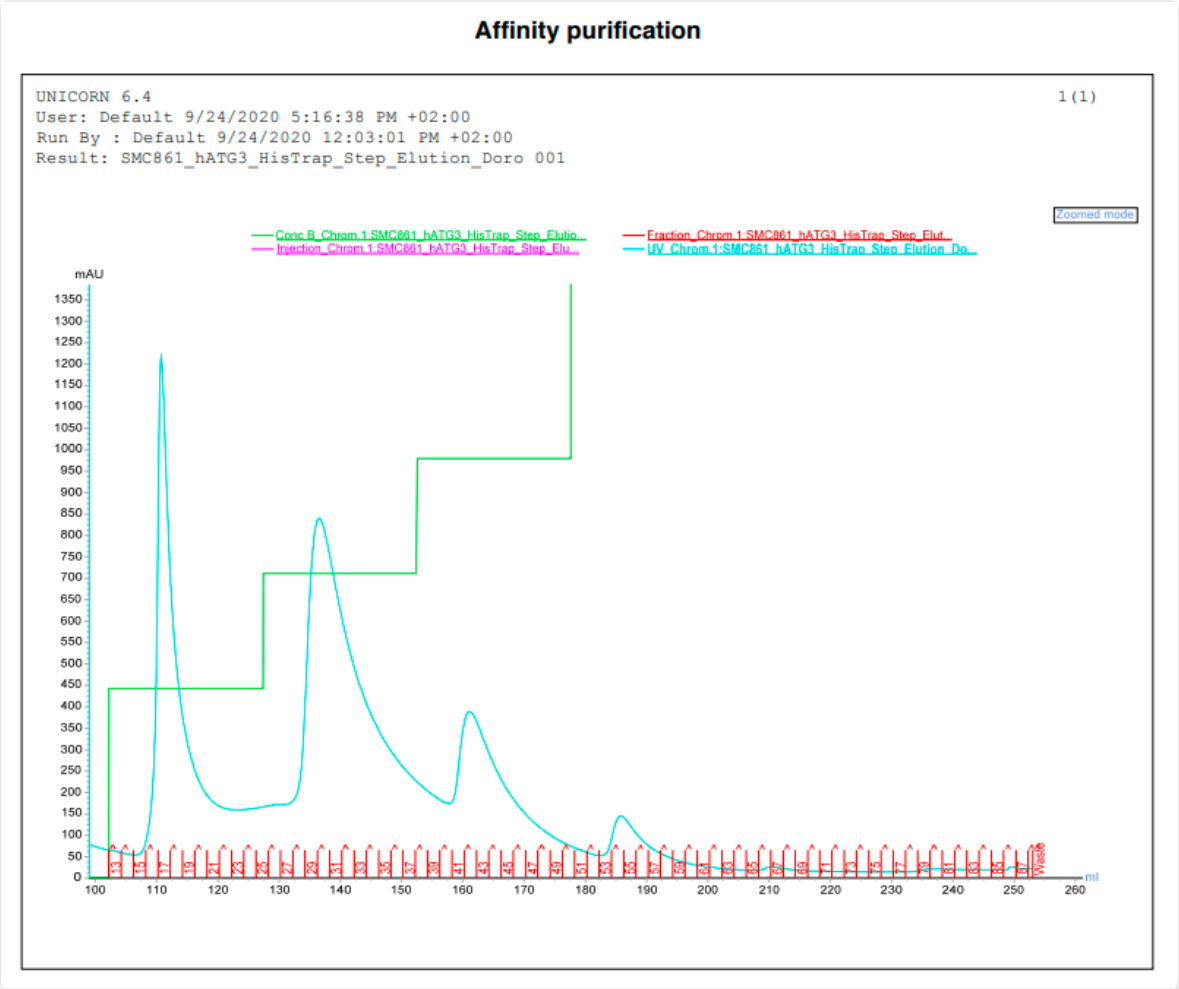
14 Elute protein of interest through a step elution gradient in 50mM, 75mM, 100mM, 150mM, 200mM and 300mM Imidazole concentration. Perform elution at 1ml/min flow rate. Collect peak fractions at each step.



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** .

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

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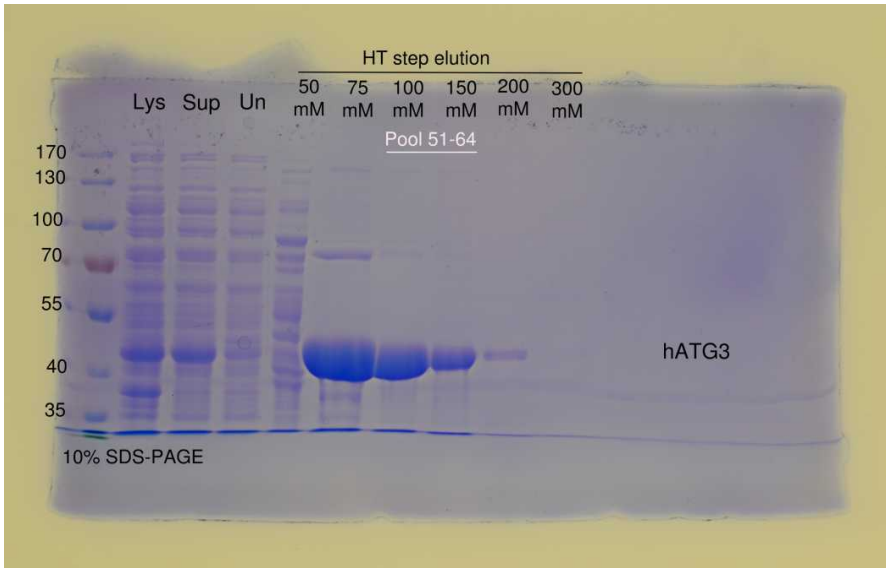
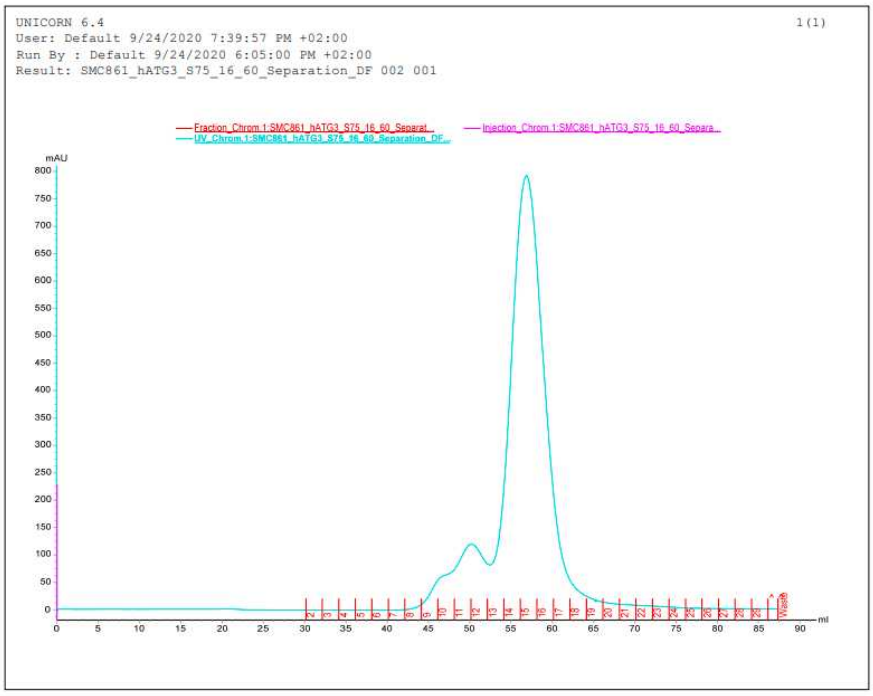


Coomassie BB stained gel of His-tag affinity purification for hATG3.

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.

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

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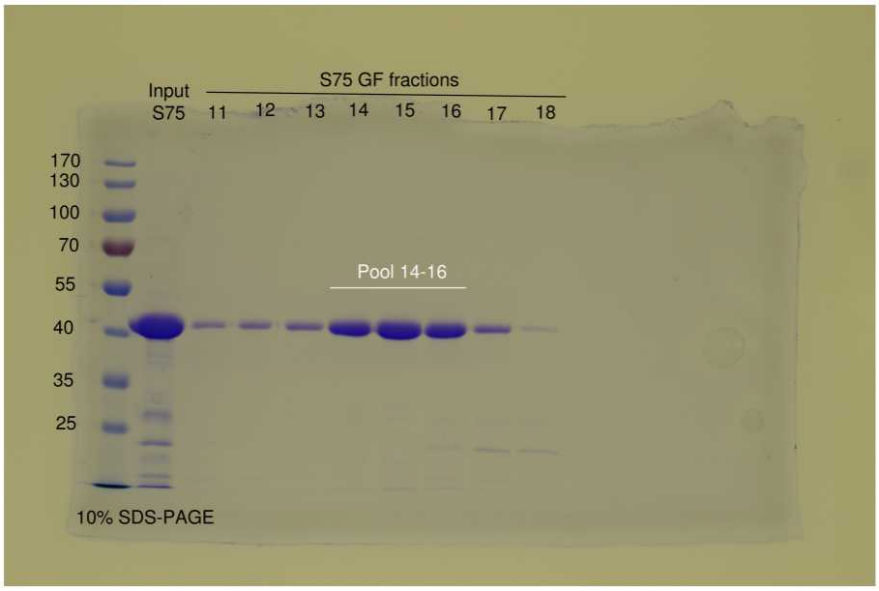

Coomassie BB stained gel of Size Exclusion Chromatography purification for hATG3.

18

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

19 Resuspend protein in Glycerol to a final concentration of **30 % (v/v) for glycerol** . Store the protein batch at **-20 °C** . Estimated protein yield: **10 mg per 1 lt culture** . Protein activity is kept for 18 months.

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