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Expression and purification protocols of *Homo sapiens* E3-like ligase ATG12–ATG5/ATG16L1ß

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ABSTRACT

This protocol outlines the procedures for expression and purification of the human E3-like ligase ATG12–ATG5/ATG16L1ß (with or without GFP tag) of the ATG8 ubiquitin-like conjugation system in autophagy.

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

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

DOI

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Dorotea Fracchiolla 2021. Expression and purification protocols of Homo sapiens E3-like ligase ATG12–ATG5/ATG16L1ß. **protocols.io**
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MANUSCRIPT CITATION please remember to cite the following publication along with this protocol

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KEYWORDS

expression, purification, human E3-like ligase ATG12-ATG5/ATG16L1ß, recombinant protein

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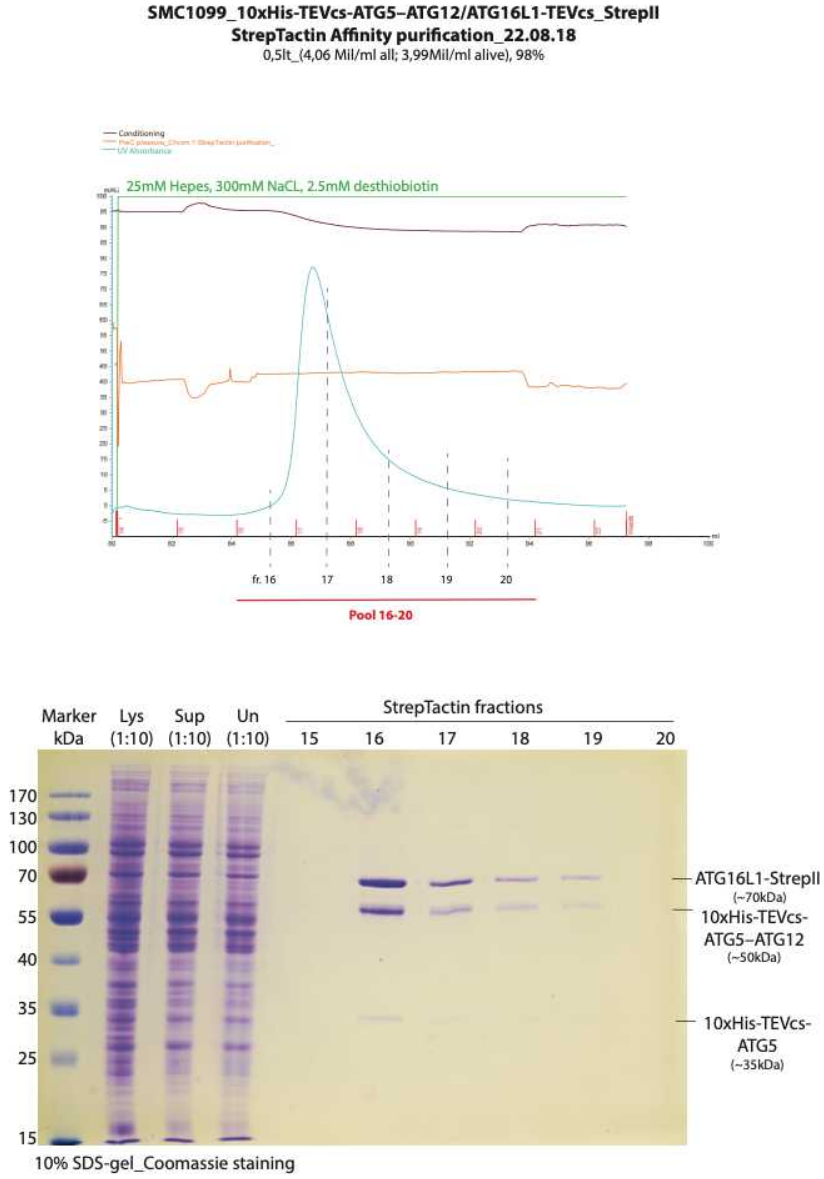
GUIDELINES



Chromatograph and Coomassie BB stained gel of Strep-tag affinity purification for ATG12-ATG5/ATG16L1ß.

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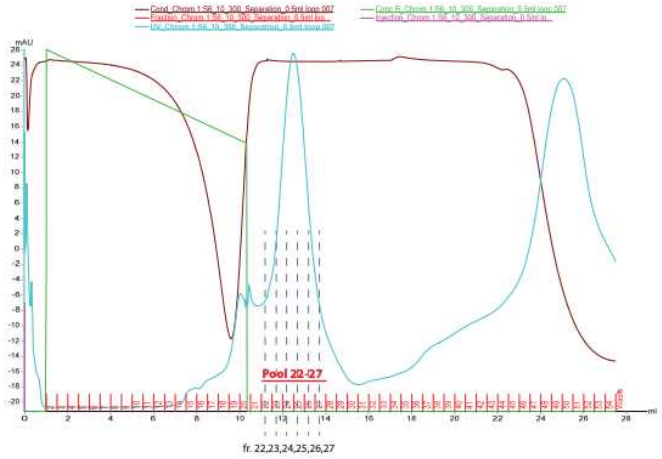
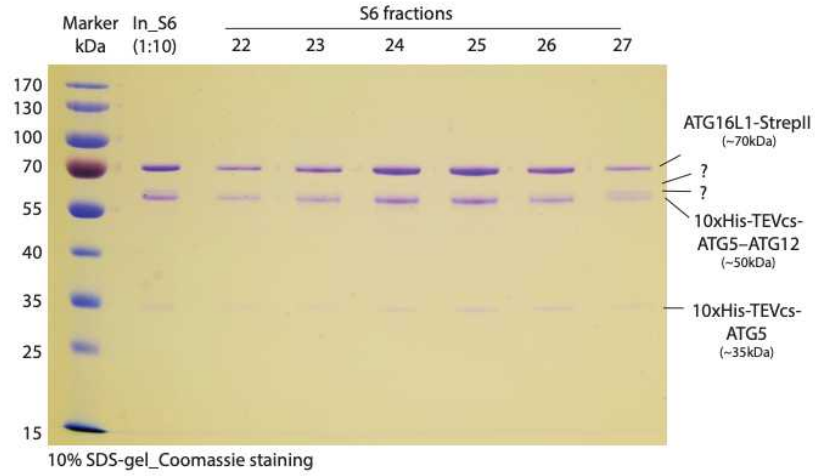
Size Exclusion Chromatograhy_S6
SMC1099_(10xHis-TEVcs-ATG5–ATG12/ATG16L1-TEVcs-StrepII)_22.08.18





10% SDS-gel_Coomassie staining

Chromatograph and Coomassie BB stained gel of Size Exclusion purification for ATG12-ATG5/ATG16L1ß.

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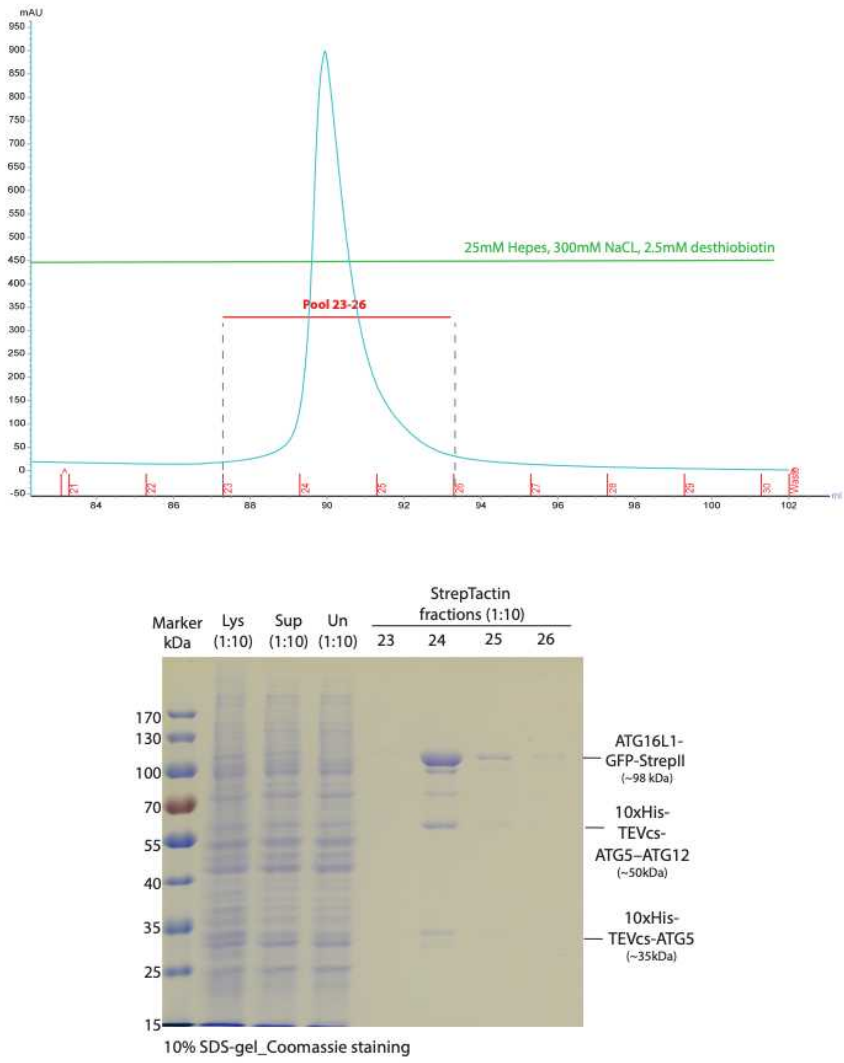

Chromatograph and Coomassie BB stained gel of Strep-tag affinity purification for ATG12-ATG5/ATG16L1ß-GFP.

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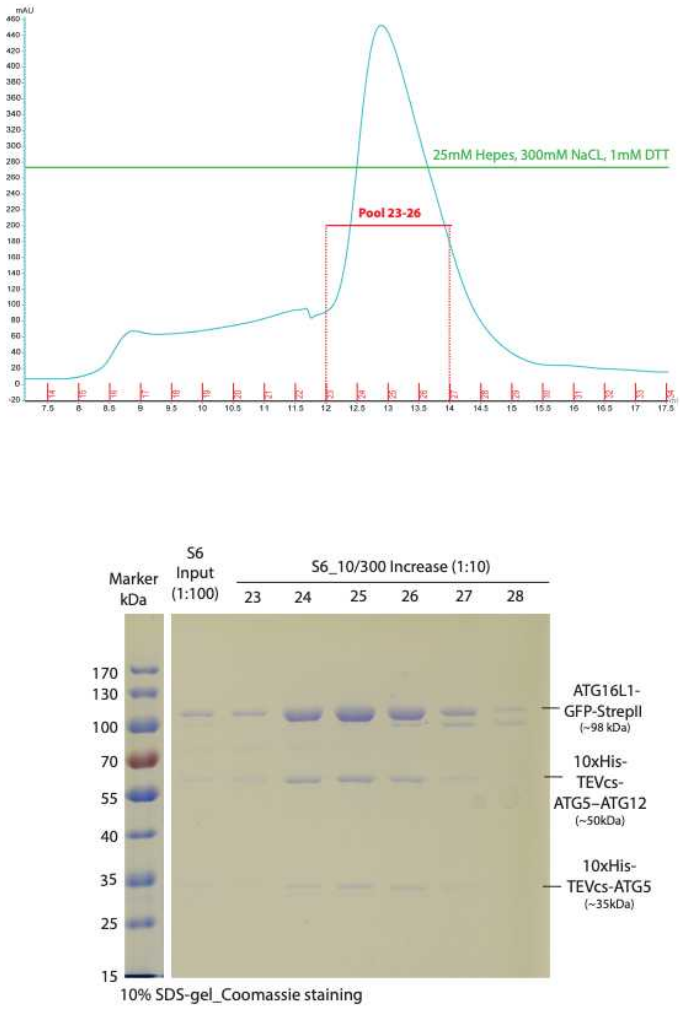

Chromatograph and Coomassie BB stained gel of Size Exclusion purification for ATG12-ATG5/ATG16L1ß-GFP.

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MATERIALS TEXT

Materials and Reagents

- Sf9 insect cells
- SF921 medium with antibiotics 100 IU/ml Penicillin and 100 µg/ml Streptomycin
- sterile cell culture hood
- 27°C shaker incubator
- sterile flasks and pipettes
- douncer 40 mL
- Virus coding for hATG7/hATG10/hATG12/10xHis-TEVcs-hATG5/10xHis-TEVcs-hATG16L1-TEVcs-StrepII (SMC1099, Addgene 169076) or hATG7/hATG10/hATG12/10xHis-TEVcs-hATG5/10xHis-TEVcs-hATG16L1-mGFP-TEVcsStrepII (SMC1100, Addgene 169077). **Note:** All the CDSs are codon-optimized for insect cell expression system (purchased from GenScript). Single subunits and tags were assembled into pLIB or pBIG1 vectors via classical restriction cloning or Gibson assembly strategy by Dorotea Fracchiolla. The final ATG12, 10xHis-TEVcs-ATG5, 10xHis-TEVcs-ATG16L1(±GFP)-TEVcs- StrepII, ATG7, ATG10 poli-cystronic gene constructs were cloned via Golden Gate approach by the Vienna BioCenter Core Facilities (VBCF) Protech Facility.

Buffers for Protein Purification

- **Lysis buffer**: 50mM Hepes pH=7.5, 300mM NaCl, Benzonase Nuclease (Sigma, use 1µl/50ml lysis buffer), 1mM Dithiothreitol (DTT), 1x Protease Inhibitor (EDTA-free CIP tablet, Roche), 300µl/1lt CIP (Sigma)
- **Binding/Washing buffe**r: 50mM Hepes pH=7.5, 300mM NaCl, 1mM Dithiothreitol (filtered and degassed)
- **Elution buffer**: 50mM Hepes pH=7.5, 300mM NaCl, 2.5mM des-thiobiotin (Sigma), 1mM Dithiothreitol (DTT)(filtered and degassed)
- **Size Exclusion Chromatography Buffer**: 25mM Hepes pH=7.5, 300mM NaCl, 1mM Dithiothreitol (DTT) (filtered and degassed)

Note: all purification buffers are filtered and degassed. Reducing agent (DTT, Dithiothreitol) is added after degassing step.

Columns:

- StrepTactin 5ml column (GE Healthcare)
- S6 10/300 Increase (GE Healthcare)

Gels: 10% Poly-acrylamide SDS-gels

SAFETY WARNINGS

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

Infection/expression/harvest

1 Infect **1 L culture** of Sf9 cells growing in Sf921 medium containing antibiotics Penicillin/Streptomycin at 1-1.5 mil/ml cells/volume at 99-100% viability in log phase with a volume of **Virus 1 (V1)**, according to viral titer.

2 Monitor infection and harvest cells when viability goes to 97-98%.

> Always check reporter gene fluorescence under the microscope to monitor the viral infection: when all alive cells are brightly fluorescent and only few dead -> harvest!

3 

To harvest spin down the culture at **4000 rpm, 4°C, 00:15:00** in a Sorvall RC6+ centrifuge (Thermo Scientific).

4 Pour off the supernatant without disturbing the cell pellet.

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5

Gently wash the pellet 1x in cold PBS buffer.

6

Centrifuge again and remove PBS.

7 Flash freeze cell pellet in liquid nitrogen and store at **-80 °C** for long storage.

Protein purification 45m

8 All steps are to be executed at 4°C or on ice.
Re-suspend the cell pellet corresponding to 1 lt culture in **50 mL ice cold Lysis buffer** ; gently stir at **4 °C** avoiding bubbling until pellet dissolves.

9 Mechanically lyse the cells passing them through a pre-cooled 40ml vol. douncer for 3x (10x pestle A followed by 10x pestle B).

10 45m

Clear the lysate by spinning at **25000 rpm** in a Ti45 Rotor for **00:45:00** at **4 °C** using Beckman centrifuge.

11 Inject the supernatant at **4 °C** onto a 5 ml StrepTactin column pre-equilibrated in **Binding buffer** at 1ml/min flow rate to allow protein binding.

12

Wash the column for 5CV (Column Volume) with **Wash buffer** at 2ml/min flow rate to remove unspecifically bound proteins.

13 Perform elution at 1ml/min flow rate with **Elution buffer** and collect corresponding fractions.

14 Check fractions on a SDS-PAGE gel (usually elution is very clean), pool and concentrate down those containing the proteins of interest at **4 °C** using a 30kDa cutoff Amicon Filter (considering ATG12–ATG5 conjugate as lower MW limit) to **500 µl** volume in a 5810R centrifuge (Eppendorf).

15 5m

Keep centrifugation steps short (**00:05:00**) to avoid protein local concentration/aggregation on the filter. Be very careful when pipetting at this step.

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16 ▪ Inject **500 µl protein** onto a S6_10/300 Increase column pre-equilibrated in **Size Exclusion Chromatography buffer** containing **25 Milimolar (mM)** Hepes **pH 7.5**, **300 Milimolar (mM)** NaCl and **1 Milimolar (mM)** DTT.

17 Check fractions on a SDS-PAGE gel, pool and concentrate down those containing the proteins of interest at **4 °C** in a 30kDa cut-off Amicon Filter.

18 Keep centrifugation steps short to avoid protein precipitation.

19 

Measure final concentration with a spectrophotometer at A_{280}.

E3, Epsilon= 148280 M^{-1} cm^{-1} ; MW= 120,58 kDa
E3-GFP, Epsilon= 170170 M^{-1} cm^{-1} ; MW= 148,05 kDa

A good concentration value when the protein does not aggregate is 20µM.

20 Aliquot the protein, snap freeze it in liquid Nitrogen and store it at **-80 °C** . Protein yield is 1-3 mg/liter of culture. Protein activity is kept for at least 18 months.

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