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Purification protocol of Mouse (*Mus Musculus*) E1-like enzyme ATG7

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This protocol outlines the procedures for expression and purification of Mouse (*Mus Musculus*) E1-like enzyme ATG7 (AuTophaGy-related protein) of the ATG8 ubiquitin-like conjugation system in autophagy.

ASAP_Team_Hurley_Martens_lab_Dorotea_Fracchiolla_Exp_purif_mATG7.pdf

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protocol

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expression, purification, E1-like enzyme ATG7, autophagy, ASAPCRN

protocol ,

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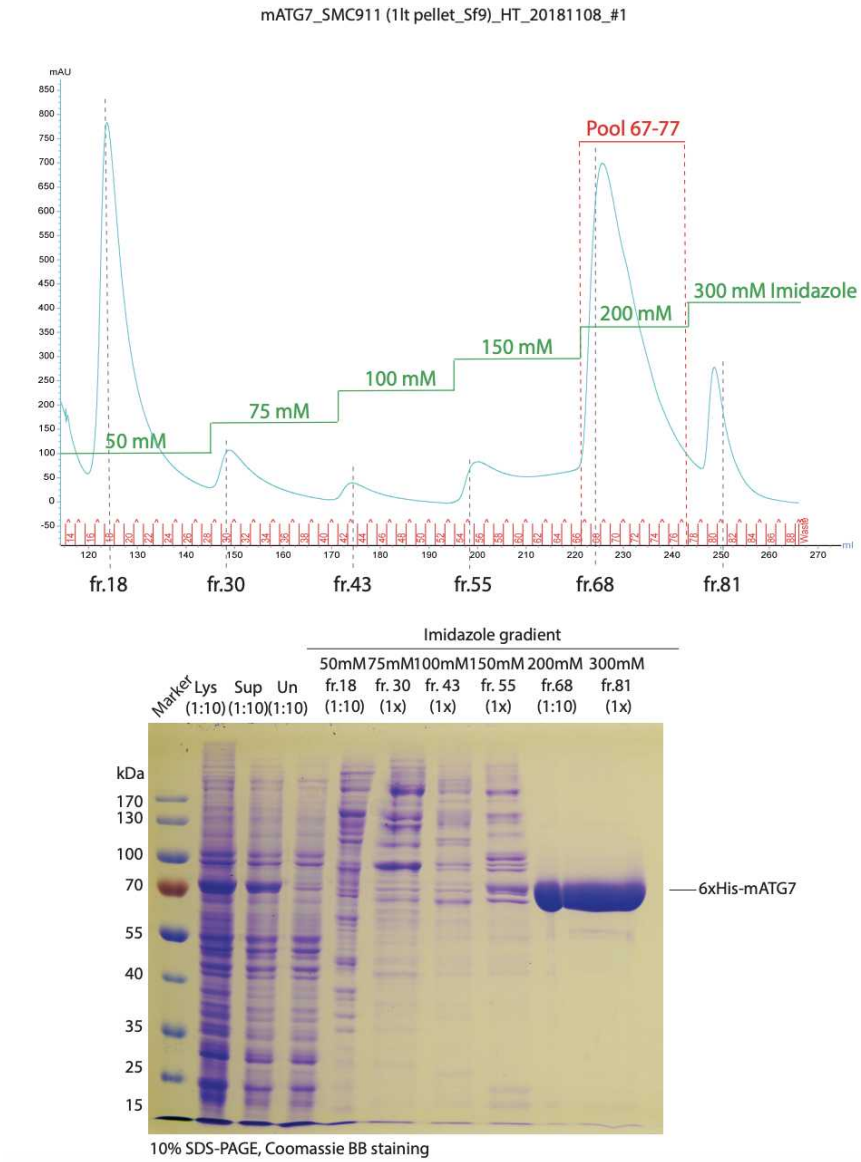
Feb 25, 2021 Dorotea Fracchiolla Sascha Martens lab, University of Vienna, Max Perutz Labs - Vienna (AT)

47278

General information: expression system: Sf9 cells, medium: SF921+Pen/Strep; plasmid origin: Gammoh Noor (University of Edinburgh); internal lab Construct Database number: SMC911; backbone: pFast-BacHT(B); resistance: Ampicillin/Gentamicin; insert: *Mus musculus* ATG7, Isoform 2_NP_00124647.1; tags & cleavage sites: N-term 6xHis, followed by Tobacco Etch Virus (TEV) cleavage site, 6xHis-TEVcs-mATG7; protein length without tags: 698 aa; Epsilon (all Cysteines reduced, with tags) = 88810 M^{-1} cm^{-1} ; MW with tags= 83 kDa; ORF (only mATG7):

MGDPGLAKLQFAPFNSALDVGFWHELTQKKLNEYRLDEAPKDIKGYYYNGDSAGLPTRLTLEFSAFDMSA
STPAHCCPAMGTLHNTNTLEAFKTADKKLLLEQSANEIWEAIKSGAALENPMLLNKFLLLTFADLKKYHF
YYWFCCPALCLPESIPLIRGPVSLDQRLSPKQIQALEHAYDDLCRAEGVTALPYFLFKYDDDTVLVSLLK
HYSDFFQGQRTKITVGVYDPCNLAQYPGWPLRNFLVLAAHRWSGSFQSVEVLCFRDRTMQGARDVTHSII
FEVKLPEMAFSPDCPKAVGWEKNQKGGMGPRMVNLSGCMDPKRLAESSVDLNLKLMCWRLVPTLDLDKVV
SVKCLLLGAGTLGCNVARTLMGWGVRHVTFVDNAKISYSNPVRQPLYEFEDCLGGGKPKALAAAERLQKI
FPGVNARGFNMSIPMPGHPVNFSDVTMEQARRDVEQLEQLIDNHDVIFLLMDTRESRWLPTVIAASKRKL
VINAALGFDTFVVMRHGLKKPKQQGAGDLCPSHLVAPADLGSSLFANIPGYKLGCYFCNDVVAPGDSTRD
RTLDQQCTVSRPGLAVIAGALAVELMVSVLQHPEGGYAIASSSDDRMNEPPTSLGLVPHQIRGFLSRFDN
VLPVSLAFDKCTACSPKVLDQYEREGFTFLAKVFNSSHSFLEDLTGLTLLHQETQAAEIWDMSDEETV



Chromatograph and Coomassie BB stained gel of His-tag affinity purification.

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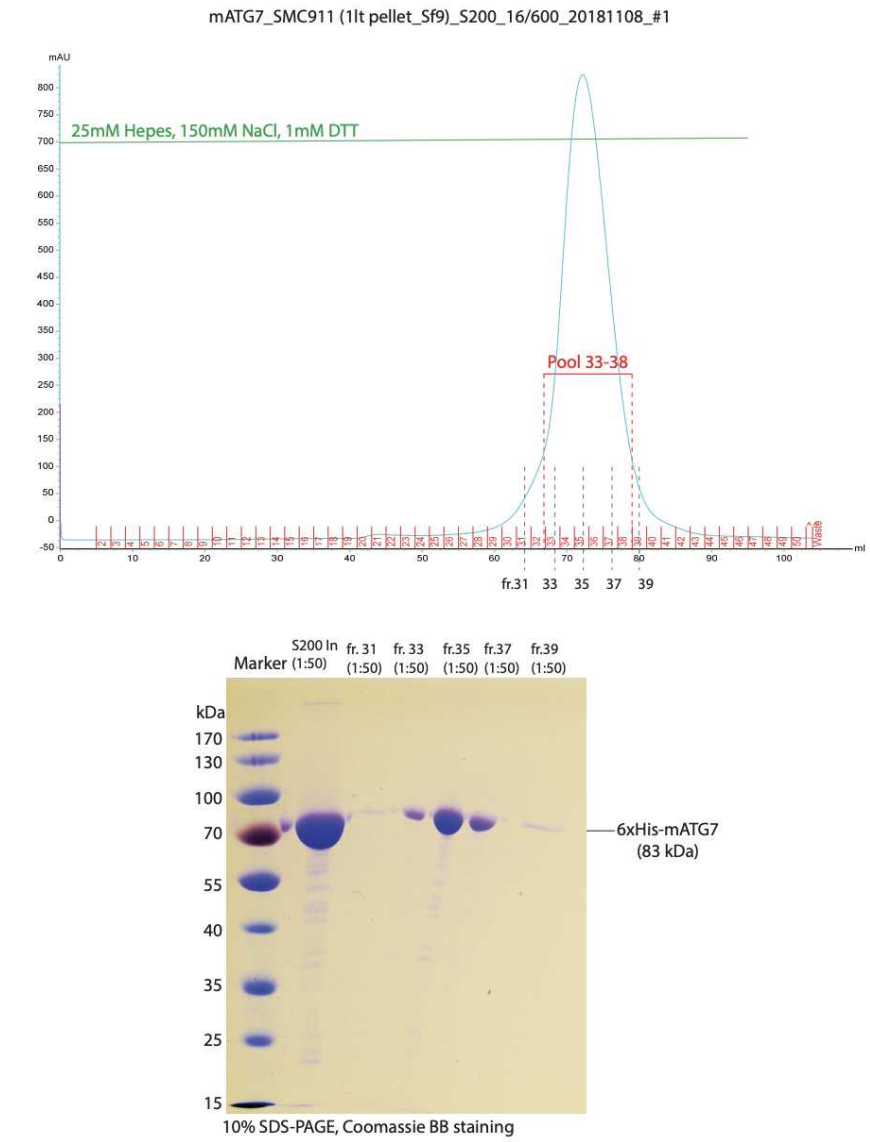

Chromatograph and Coomassie BB stained gel of Size Exclusion purification.

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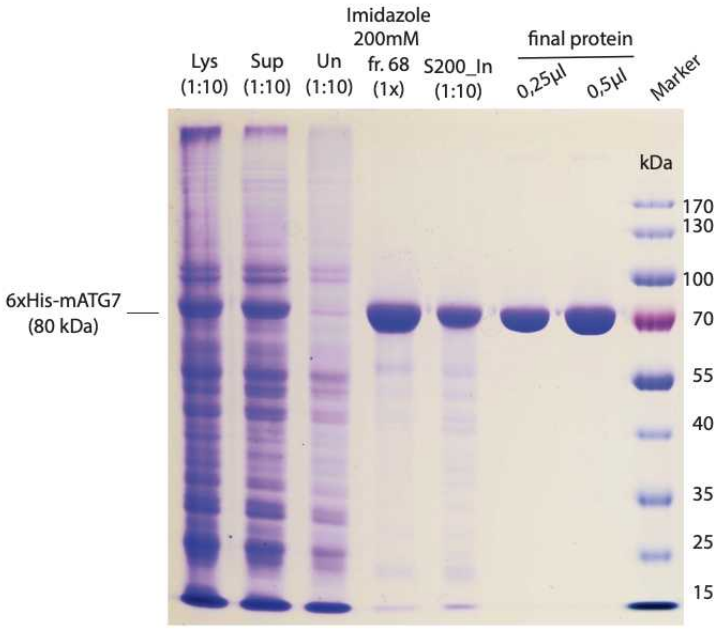

10% SDS-PAGE, Coomassie BB staining

Summary gel of mATG7 purification.

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/sterile pipettes
- Baculovirus for mATG7 (SMC911)
- douncer 40 mL

Materials for Protein Purification

- **Lysis Buffer:** 50mM Hepes pH=7.5; 300mM NaCl, Benzonase (1µl/50ml lysis buffer), 10mM Imidazole, 2mM $MgCl_2$, 1mM Dithiothreitol, 1xProtease Inhibitors/50 ml lysis buffer (EDTA-free CIP tablet, Roche).
- **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
- **SEC Buffer:** 25mM Hepes pH=7.5, 150mM NaCl (filtered and degassed) + 1mM 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)
- S200_16/60 (GE Healthcare)

Gels: 10% Poly-acrylamide SDS-gels

Please refer to the Safety Data Sheets (SDS) for health and environmental hazards.

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Infection/expression/harvest

1 Infect **1 L culture** of Sf9 cells growing in Sf921 medium with Penicillin/Streptomycin at 1-1.5 mil/ml cells/volume at 99-100% viability in log phase with **Virus 1 (V1)** according to viral titer.
(NOTE: protein yield from 1 lt culture is high, e.i. 32 mg/1lt culture).

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

Always check under microscope: when all alive cells are brightly fluorescent and only few dead -> harvest!

Protein Purification 45m

3

This section describes the procedure for His-Trap affinity purification followed by Size Exclusion Chromatography. All steps are to be executed at 4°C or on ice.

4 Resuspend cell pellet corresponding to **1 L culture** in **50 mL ice cold lysis buffer** ; gently stir at **4 °C** until pellet dissolves avoiding bubbling.

5 Additionally, mechanically lyse the cells passing them through a pre-cooled douncer for 3x (10x pestle A followed by 10x pestle B).

6 45m

Clear the lysate by spinning it in a Backman centrifuge, at 25K using a Ti45 Rotor for **00:45:00** at **4 °C** .

7 Inject the supernatant onto a 5ml HT column pre-equilibrated with Buffer A at 1ml/min flow rate to allow protein binding.

8

Wash the column for 5CV with Buffer A at 2 ml/min flow rate to remove unspecific bound proteins.

9 Elute protein through a step elution gradient in 50mM, 75mM, 100mM, 150mM, 200mM and 300mM Imidazole concentration. Perform the elution at 1ml/min flow rate.

10

Collect a sample from peak fractions of each elution step and check them on a SDS-PAGE (see gel in the "Guidelines" section). Pool and concentrate fractions corresponding to the peak containing the protein of interest (usually 200mM and/or 300mM Imid.) by spinning them down at **4 °C** using a 30 kDa cut-off Amicon Filter to 2 ml

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in a 5810R centrifuge (Eppendorf).

Keep centrifugation steps short (**00:05:00**) to avoid protein local concentration/aggregation on the filter.

11 Inject **2 mL protein** onto a S200_16/60 column at 4 **4 °C** pre-equilibrated in buffer containing 25mM Hepes pH=7.5, 150mM NaCl and 1mM DTT (see profile in the "Guidelines" section).

12 Check fractions on a 10% SDS-PAGE (see gel in the "Guidelines" section), pool and concentrate down those containing the protein of interest by spinning them at **4 °C** using a 30kDa cut-off Amicon Filter in a 5810R centrifuge (Eppendorf). Protein elutes at around 72.5ml, a retention volume corresponding to that of a dimeric globular protein of ~150-160 kDa.

Keep centrifugation steps short to avoid protein precipitation.

13

Measure protein absorbance at A_{280} is measured with Spectrophotometer against Size Exclusion Chromatography buffer.

14 Aliquot the protein, snap freeze it in liquid Nitrogen and store it at **-80 °C** .

Usually protein activity is kept for 18 months when stored at -80°C.

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