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Purification of mCherry-ATG101/13 (1-191aa) subcomplex

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We use this protocol and it's working

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Abstract

This protocol details the purification of mCherry-ATG101/13 (1-191aa) subcomplex.



Materials

Lysis buffer:

	A	B
	Tris-HCl pH 7.4	50 mM
	NaCl	200 mM
	Triton X-100	1%
	glycerol	10%
	MgCl ₂	2 mM
	β-mercaptoethanol	2mM
	cOmplete EDTA-free protease inhibitors (Roche)	
	CIP protease inhibitor (Sigma)	
	Benzonase	

Wash buffer I:

	A	B
	Tris-HCl pH 7.4	50 mM
	NaCl	200 mM
	MgCl ₂	2 mM
	DTT	1mM
	Triton X-100	1%
	glycerol	10%

Wash buffer II:

	A	B
	Tris-HCl pH 7.4	50 mM
	NaCl	200 mM

	A	B
	MgCl ₂	2 mM
	DTT	1mM

Wash buffer:

	A	B
	Tris-HCl pH 7.4	50 mM
	NaCl	200 mM
	MgCl ₂	2 mM
	DTT	1 mM

SEC buffer:

	A	B
	Tris-HCl pH 7.4	50 mM
	NaCl	200 mM
	MgCl ₂	1 mM
	DTT	1 mM

- mCherry-tagged ATG13 (1-191aa) from a pCAG backbone (available from Addgene)
- GST-TEV-ATG101 (available from Addgene)
- FreeStyle™ 293 Expression Medium (Thermo, 12338-026)

 FreeStyle™ 293 Expression Medium **Thermo Fisher Catalog #12338026**

- 13 ml of Opti-MEM[®] I Reduced Serum Medium (Thermo, 31985-062)

 Opti-MEM[®] I Reduced Serum Medium **Thermo Fisher Catalog #31985062**

- 800 µg Polyethylenimine (PEI 25K, Polysciences CatNo 23966-1)

 Polyethylenimine, Linear, MW 25000, Transfection Grade (PEI 25K™) **Polysciences, Inc. Catalog #23966-1**

- 100 mL EXCELL[®] R 293 Serum-Free Medium (Sigma-Aldrich, 14571C- 1000ML)

 EX-CELL[®] 293 Serum-Free Medium for HEK 293 Cells **Merck MilliporeSigma (Sigma-Aldrich) Catalog #14571C**

- SORVAL RC6+ centrifuge with an F21S-8×50Y rotor (Thermo Scientific)



- Glutathione Sepharose 4B beads (GE Healthcare)
- 10 kDa cut-off Amicon filter (Merck Millipore)



Amicon® Ultra Centrifugal Filter, 10 kDa MWCO Merck MilliporeSigma (Sigma-Aldrich) Catalog #UFC801008

- Superdex S200 Increase 10/300 GL column (Cytiva)

Purification - mCherry-ATG101/13 (1-191aa) subcomplex

20m

- 1 To purify mCherry-ATG13/101 HORMA dimer, we express mCherry-tagged ATG13 (1-191aa) from a pCAG backbone (available from Addgene) together with GST-TEV-ATG101 (available from Addgene).
- 2 Express the ATG13/101 HORMA dimer in FreeStyle™ HEK293F cells, grow at  37 °C in FreeStyle™ 293 Expression Medium (Thermo, 12338-026).
- 3 The day before transfection, seed the cells at a density of 0.7×10^6 cells per ml.
- 4 On the day of transfection, transfect a  400 mL culture with  400 µg of plasmid at a molar 1:1 ratio, dilute in  13 mL of Opti-MEMR I Reduced Serum Medium (Thermo, 31985-062), and  800 µg Polyethylenimine (PEI 25K, Polysciences CatNo 23966-1), also dilute in 13 ml of Opti-MEM media.
- 5 One day post transfection, supplement the culture with  100 mL EXCELL R 293 Serum-Free Medium (Sigma-Aldrich, 14571C- 1000ML).
- 6 Another 24 h later, harvest the cells by centrifugation at  270 x g, 00:20:00 .
- 7 Wash the pellet with PBS to remove medium and then flash-frozen in liquid nitrogen.

20m



Note

Store the pellets at  -80 °C .

- 8 For purification of the ATG13/101 subcomplex, resuspend the cell pellet in  25 mL lysis buffer.

Lysis buffer:

A	B
Tris-HCl pH 7.4	50 mM

A	B
NaCl	200 mM
Triton X-100	1%
glycerol	10%
MgCl ₂	2 mM
β-mercaptoethanol	2mM
cOmplete EDTA-free protease inhibitors (Roche)	
CIP protease inhibitor (Sigma)	
Benzonase	

- 9 Homogenize the cells with a douncer and clear the lysates by centrifugation at  10000 x g, 4°C, 00:45:00 with a SORVAL RC6+ centrifuge with an F21S-8×50Y rotor (Thermo Scientific).
- 10 Collect the supernatant and incubate with pre-equilibrated Glutathione Sepharose 4B beads (GE Healthcare) for  02:00:00 at  4 °C with gentle shaking to bind GST-TEV-ATG101/mCherry-ATG13(1-191aa).
- 11 Centrifuge the samples to pellet the beads and remove the unbound lysate.
- 12 Wash the beads twice with wash buffer I followed by three washes in wash buffer II.

45m



2h



Wash buffer I:

A	B
Tris-HCl pH7.4	50 mM
NaCl	200 mM
MgCl ₂	2 mM
DTT	1mM
Triton X-100	1%
glycerol	10%



Wash buffer II:

	A	B
	Tris-HCl pH 7.4	50 mM
	NaCl	200 mM
	MgCl ₂	2 mM
	DTT	1mM

- 13 Incubate the beads  Overnight with TEV protease in wash buffer at  4 °C , to release mCherry- or GFP-tagged ATG13/101 from the beads.

2h



Wash buffer:

	A	B
	Tris-HCl pH 7.4	50 mM
	NaCl	200 mM
	MgCl ₂	2 mM
	DTT	1 mM

- 14 To collect the supernatant, collect the beads by centrifugation.



- 15 Wash the beads twice with  4 mL of wash buffer, and collect the supernatant.



- 16 Pool the supernatant fractions, filter through a 0.45 µm syringe filter, concentrate with 10 kDa cut-off Amicon filter (Merck Millipore), and load onto a pre-equilibrated Superose S6 Increase 10/300 GL column (Cytiva).

- 17 Elute the proteins with SEC buffer.

SEC buffer:



	A	B
	Tris-HCl 7.4	50 mM
	NaCl	200 mM
	MgCl ₂	1 mM
	DTT	1 mM

- 18 Analyze the fractions by SDS-PAGE and Coomassie staining. Pool the fractions containing both ATG13/101.
- 19 After concentrating the purified protein, aliquote the protein and snap-frozen in liquid nitrogen.

Note

Store the pellets at  -80 °C .