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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%
	MgCl2	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
	MgCl2	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
	MgCl2	2 mM
	DTT	1mM

Wash buffer:

	A	B
	Tris-HCl pH 7.4	50 mM
	NaCl	200 mM
	MgCl2	2 mM
	DTT	1 mM

SEC buffer:

	A	B
	Tris-HCl pH 7.4	50 mM
	NaCl	200 mM
	MgCl2	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-MEMR 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-8x50Y 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 FreeStyleTM 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 x 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 .

20m

7 Wash the pellet with PBS to remove medium and then flash-frozen in liquid nitrogen.

> **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
	NaCl	200 mM

	A	B
	Triton X-100	1%
	glycerol	10%
	MgCl2	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-8x50Y rotor (Thermo Scientific). 45m

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). 2h

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.

Wash buffer I:

	A	B
	Tris-HCl pH7.4	50 mM
	NaCl	200 mM
	MgCl2	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
	MgCl2	2 mM

	A	B
	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
	MgCl2	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
	MgCl2	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 .