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Expression and purification protocol of human PI3KC3-C1 (±mCherry)

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We use this protocol and it's working

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Abstract

This protocol describes the procedures for expression and purification of the active autophagy-specific human PI3KC3-C1 kinase complex.

Materials

Proteins' parameters

	A	B	C	D
	Addgene #	**Protein**	**MW (kDa)**	**Epsilon (M-1cm-1)**
	187988	GST-C3cs-mCherry-ATG14L	109	135110
	187987	GST-C3cs-ATG14L	81,7	100730
	187990	Vps15	155	159630
	187989	Vps34	102	115630
	187991	Beclin	52	54890
	187992	**GST-PI3KC3-C1**	390,7	430880
	187831	**GST-mCherry-PI3KC3-C1**	418	315250

Molecular Weights and Extinction coefficients of all subunits and their complexes.

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 GST-C3-ATG14/Vps34/Vps15/BECN1 (SMC1326/SM014, Addgene) or GST-C3-mCherry-ATG14/Vps34/Vps15/BECN1 (SMC1327/SM015, Addgene). Note:All the CDSs are codon-optimized for insect cell expression system (purchased from GenScript). The final poli-cystronic constructs were cloned via Golden Gate approach by the Vienna BioCenter Core Facilities (VBCF) Protech Facility.

Buffers for Protein Purification

LysisBuffer: 50mM Hepes pH=7.5, 300mM NaCl, **0.5% CHAPS**(stock 10% CHAPS in water, filtered), Benzonase (1µl/50ml lysisbuffer), 1mM $MgCl_2$, 1mM DTT, 1xProteaseInhibitors/50ml lysis buffer (EDTA-freeCIPtablet) + 300µl CIP (Sigma).

Wash Buffer I: 50mM HepespH=7.5, 300mM NaCl, 0.5% CHAPS,1mM DTT;

Wash Buffer II: 50mM HepespH=7.5, 500mM NaCl, 1mM DTT;

Wash Buffer III: 50mM HepespH=7.5, 300mM NaCl, 1mM DTT;

Gel Filtration Buffer: 25mM Hepes pH=7.5, 200mMNaCl, 1mMDTT.

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

degassing step.

Columns: 5 ml GSH beads,GE Healthcare or Pierce - S6_10/300
Gels:10%SDS-PAGE

Troubleshooting

Infection/expression/harvest

14m

1 Infect 1 L culture of Sf9 cells growing in Sf921 medium with antibiotics Penicillin/Streptomycin at 1-1.5 mil/ml cells/volume at 99-100% viability in log phase with Virus 1 (V1), volume according to viral titer. Baculovirus is obtained by transfection of Sf9 cells with policystronic constructs coding for the (GST±mCherry)-PI3KC3-C1 complex

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

> Note
>
> 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, 00:14:00 in a Sorvall RC6+ centrifuge (Thermo Scientific). 14m

4 Pour off the supernatant without disturbing the cell pellet.

5 Gently wash the pellet 1x in cold PBS buffer on ice.

6 Centrifuge again and remove PBS.

7 Flash freeze cell pellet in liquid nitrogen and store at -80 °C until purification.

Protein purification

45m

8 All steps are to be carried On ice or at 4 °C .
Re-suspend the cell pellet corresponding to 1 L 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 repeating for 3x: 10x pestle A followed by 10x pestle B.

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

11 In the meanwhile, wash 3-5 mL mL slurry GSH beads stored in 20% Ethanol (Pierce or Glutathione Sepharose 4B) with milliQ water and finally equilibrate them in cold **WBI**. Spin beads at 4000 rpm, 4°C, 00:03:00 , brake=2 in a 5810R centrifuge (Eppendorf). 3m

12 Allow lysate incubation with beads for 01:00:00 4 °C , gently rolling the tube. 1h

13 Wash beads 2x with a fixed volume of WBI.

Note

Each wash can be performed by gently inverting the tube a few times at 4 °C -> spin at 4000 rpm for 00:03:00 .

14 Collect sample for "sup WI" and "beads WBI".

15 Wash beads 2x in WBII. Collect sample for "sup WII" and "beads WBII".

16 Wash beads 2x in WBIII. Collect sample for "sup WIII" and "beads WBIII".

17 Check all fractions on a 10% SDS-gel stained with Coomassie Brilliant Blue (see attached pdf below for example gel).

18 Collect sample for gel electrophoresis before cleavage.

19 Incubate beads Overnight gently rolling at 4 °C with C3 protease to cleave off the GST-tag. 3m

20 The following day, collect sample after cleavage and run samples on gel to confirm cleavage (see attached pdf below).

21 Spin down beads and collect the supernatant containing cleaved/eluted protein. Filter elution through a 0.2µm filter syringe to remove beads and concentrate down to 0.5 mL using a 30kDa cut-off Amicon filter.

Note

Centrifugation steps are kept short 00:05:00 to avoid protein local concentration/aggregation on the filter. Gently pipette the sample to resuspend the protein between centrifugation steps.

22 Spin down the sample in a table top centrifuge for 00:10:00 at 4 °C to pellet eventual aggregates, collect supernatant and inject 0.5 mL sample onto a S6_10/300 Gel Filtration column pre-equilibrated in Gel Filtration Buffer at 4 °C . 10m

23 After the run, check fractions containing the protein of interest on gel and pool them. Concentrate down to reach the desired concentration, flash freeze in liquid nitrogen and store at -80 °C until use.

Note

Gel Filtration GST_mCh_PI3KC3_C1....

SEC_PI3KC3_C1.pdf

GSTPI3KC3_C1 purification_2020.0...