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Purification and Crystallization of ATG9 HDIR-ATG101:ATG13 complex

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We use this protocol and it's working

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Abstract

Purification and Crystallization of ATG9 HDIR (828-839) fused ATG101 (1-198):ATG13 (1-197)




Troubleshooting



Expression

- 1 Transfect HEK GNTI cells at concentration of 2×10^6 cells/ml
- 2 Dilute PEI with Warm Hybridoma-SFM(1X), In a separate tube, dilute DNA with Hybridoma-SFM(1X)
- 3 Add PEI to DNA dilution. Incubate mixture for  00:30:00 at  37 °C 30m
- 4 Add mixture to cells. Let cells grow for  48:00:00 2d
- 5 Harvest Cells  500 rpm, 4°C, 00:10:00 10m
- 6 Wash pellet with cold PBS. Store pellet at -80C until purification or lyse immediately

Purification

- 7 Resuspended pellet in lysis buffer (25 mM HEPES pH 7.5, 200 mM NaCl, 2 mM MgCl₂, 1 mM TCEP, 5 mM EDTA, 10% Glycerol) with 1% Triton X-100 and protease inhibitor cocktail (Thermo Scientific, Waltham, MA)
- 8 Clarify lysate for  17000 rpm, 4°C, 00:35:00 35m
- 9 Wash GST Sepharose 4B resin into lysis buffer (without Triton)
- 10 Load supernatant onto GST Sepharose resin using a gravity column setup
- 11 Rock supernatant with equilibrated resin for  01:00:00 at  4 °C 1h



- 12 Wash with 5CV lysis buffer (25 mM HEPES pH 7.5, 200 mM NaCl, 2 mM MgCl₂, 1 mM TCEP, 5 mM EDTA, 10% Glycerol)
- 13 Elute with lysis buffer plus 25 mM glutathione for GST resin
- 14 Add purified His₆-TEV protein to cleave GST tag at 4°C overnight
- 15 Dilute 5x into SP Buffer buffer A: 30 mM MES pH 6.0, 3 mM beta-mercaptoethanol
- 16 Load sample onto a HiTrap SP HP 5 ml column (GE healthcare, Piscataway, NJ)
- 17 Elute from the SP column on a 70 ml linear gradient from 0–500 mM NaCl in SP buffer A. The cleavage sample was eluted at the buffer conductivity of ~ 25 mS/cm.
- 18 After each fraction was analyzed by SDS gel. Fractions containing ATG9 HDIR-ATG101:ATG13 were pooled concentrated in Amicon Ultra15 concentrator (MilliporeSigma, Burlington, MA) and exchanged into 25 mM HEPES pH 7.5, 150mM NaCl, 1mM MgCl₂, 1mM TCEP for crystallization.

Crystallization

- 19 ATG9 HDIR-ATG101:ATG13 complex at 6 mg/ml in 25 mM HEPES pH 7.5, 150mM NaCl, 1mM MgCl₂, 1mM TCEP was used as the protein stock
- 20 Crystallization was carried out by sitting-drop vapor diffusion using an automated liquid-handling system (Mosquito, TTP LabTech, UK) at 288 K in 96-well plates
- 21 The protein solution was mixed with the reservoir buffer composed of 0.1 M HEPES pH 7.5, 0.2 M NaCl, 12% PEG8000 with a ratio of 1:1
- 22 Crystal trays were checked daily using a light microscopy for crystal growth
- 23 Large crystals were obtained in 2–4 days. Crystals were cryo-protected in 28% glycerol/reservoir buffer and frozen in liquid N₂