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We use this protocol and it's working

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SINTBAD-GFP: expression and purification

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ABSTRACT

This protocol describes how to express and purify human SINTBAD tagged C-terminally with eGFP.

ATTACHMENTS

Purification of SINTBAD-GFP.pdf

MATERIALS

Expression:
pFastBac_Dual_GST-TEV-SINTBAD-GFP (Addgene ID: 198035)
Sf9 insect cells
SF921 medium with antibiotics 100 IU/ml Penicillin and 100 µg/ml Streptomycin

Lysis Buffer:
50 mM Tris-HCl pH 8.0
300 mM NaCl
2 mM MgCl2
5% Glycerol
2 mM b-Met
Complete inhibitor EDTA free Roche
50ul of Protease inhibitors Sf9 cells
Benzonase (1ul)

Wash I Buffer:
50 mM Tris-HCl pH 8.0
300 mM NaCl
5% Glycerol
1 mM DTT

Wash II Buffer:
50 mM Tris-HCl pH 8.0
700 mM NaCl
5% Glycerol
1 mM DTT

SEC Buffer:
20 mM Tris-HCl pH 7.4
150 mM NaCl
1 mM DTT

Columns/Resin:
Glutathione Sepharose 4B (Cytiva)
Superdex 200 increase 10/300 column (Cytiva)

Expression

1 To generate GST-TEV-SINTBAD-GFP construct the insect codon optimized SINTBAD gene was

purchased from GenScript and cloned with respective tags into pFastBac_Dual. Generated construct was used for expression in Sf9 insect cells using the Bac-to-Bac system. The construct Addgene ID: 198035.

2 Transfect 2.5 µg of bacmid DNA into Sf9 insect cells in a 6-well plate using FuGene transfection reagent (Promega). The bacmid DNA was amplified using DH10BacY cells.

3 About 6-7 days after transfection the V0 virus should be ready for harvesting. Use the V0 to produce a V1 virus stock by infecting 30 ml of Sf9 cells (1 million/ml). Collect V1 about 4-5 days later. Monitor viability of the cells and green fluorescence to decide when to collect V1.

4 Infect 1L culture of Sf9 cells at 1-1.5 million/ml cells/volume at 99-100% viability in log phase with 1 ml of Virus 1 (V1).

5 After infection monitor cells for viability and fluorescence. Harvest by centrifugation when the viability drops to 90–95% and clear green fluorescence is present.

6 To harvest spin down the cells at 2000 rpm, for 15 min at RT (Sorvall RC6+ centrifuge, Thermo Scientific). Gently wash the cell pellet with PBS, flash-freeze in liquid nitrogen, and store at −80 °C until purification.

Purification

7 Thaw a cell pellet corresponding to 1L culture by re-suspending it in 25 ml lysis buffer (50 mM Tris-HCl pH 8.0, 300mM NaCl, 2 mM MgCl2, 5% glycerol, 2 mM β-Met, 1 µl Benzonase (Sigma), CIP protease inhibitor (Sigma), cOmplete EDTA-free protease inhibitor cocktail (Roche)) and rolling or stirring in the cold room.

8 Additionally disrupt the cells with a Dounce homogenizer.

9 Clear the lysate by centrifugation (19 000 rpm for 45 min at 4°C in a Fiberlite F21-8x50y (Thermo

Scientific)).

10 Incubate the cleared supernatant with 2 ml of Glutathione Sepharose 4B beads slurry (Cytiva) for 2h at 4°C rolling gently. The GSH slurry should be washed with water and then with Wash I Buffer beforehand (50 mM Tris-HCl pH 8.0, 300 mM NaCl, 5% glycerol, 1 mM DTT).

11 After 2h of incubation with the cleared lysate wash the beads two times with Wash I Buffer (50 mM Tris-HCl pH 8.0, 300 mM NaCl, 5% glycerol, 1 mM DTT), once with Wash II buffer (50 mM Tris-HCl pH 8.0, 700 mM NaCl, 5% glycerol, 1 mM DTT) and again twice with Was I Buffer.

12 Incubate the beads overnight with TEV protease at 4°C (20 ul of 10 mg/ml home-made TEV).

13 The next day spin down the beads (4000 rpm, 3 min, 4°C) and collect the supernatant containing cleaved SINTBAD-GFP.

14 Filter the supernatant through a 0.45 µm syringe filter to remove any residual beads.

15 Concentrate the protein down to 0.5 ml using a 30kDa cut-off Amicon filter and apply onto a Superdex 200 increase column (10/300, Cytiva) pre-equillibrated with a SEC buffer containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 1 mM DTT. Pool fractions containing pure proteins (see attached pdf), concentrate, snap freeze in liquid nitrogen, and store at −80°C.