



Mar 31, 2023

SINTBAD-GFP: expression and purification

DOI

dx.doi.org/10.17504/protocols.io.rm7vzb1o8vx1/v1

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DOI: <https://dx.doi.org/10.17504/protocols.io.rm7vzb1o8vx1/v1>

Protocol Citation: Justyna Sawa-Makarska, Elias Adriaenssens 2023. SINTBAD-GFP: expression and purification. **protocols.io** <https://dx.doi.org/10.17504/protocols.io.rm7vzb1o8vx1/v1>

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Protocol status: Working

We use this protocol and it's working

Created: February 13, 2023

Last Modified: May 31, 2024

Protocol Integer ID: 76898

Keywords: SINTBAD expression, SINTBAD purification, ASAPCRN, purification this protocol, egfp, purification, human sintbad, gfp, sintbad

Abstract

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

Attachments



Purification of SINT...

3.2MB



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 DTT

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)

Troubleshooting

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 F21-8×50y (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 Wash 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-equilibrated 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.