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Purification of BNIP3-GST

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We use this protocol and it's working

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Abstract

This protocol details the purification of BNIP3-GST.

Materials

Lysis buffer:

	A	B
	Tris-HCl	50 mM
	pH	7.4
	NaCl	300 mM
	Triton X-100	1%
	glycerol	5%
	MgCl ₂	2 mM
	DTT	1 mM
	β-mercaptoethanol	2mM
	benzonase (Sigma)	1 μl
	cOmplete EDTA-free protease inhibitors (Roche)	
	CIP protease inhibitor (Sigma)	

Wash buffer:

	Tris-HCl	50 mM
	pH	7.4
	NaCl	300 mM
	DTT	1 mM

High salt buffer:

	Tris-HCl	50 mM
	pH	7.4
	NaCl	700 mM
	DTT	1 mM

SEC buffer:

	Tris-HCl	25 mM
	pH	7.4
	NaCl	300 mM
	DTT	1 mM

- pFastBac-Dual vector from Genscript (available from Addgene).
- BNIP3 E44A/L47A/D49A/A50K/Q51A (5A; Δ WIPI2) (available from Addgene)
- BNIP3 W18A/L21A (Δ LIR) (available from Addgene).
- Sf9 insect cells (12659017, Thermo Fisher, RRID:CVCL_0549).
-  Sf9 cells in Sf-900[®] III SFM **Thermo Fisher Catalog #12659017**
- SORVAL RC6+ centrifuge with an F21S-8×50Y rotor (Thermo Scientific)

Purification - BNIP3-GST

2h 45m

- 1 To purify BNIP3-GST, we purchase the gene-synthesized codon-optimized cytosol-exposed domain of BNIP3 (1-158aa) fused to a C-terminal GST-tag in a pFastBac-Dual vector from Genscript (available from Addgene).
- 2 Introduce the point mutants by in vitro mutagenesis to generate BNIP3 E44A/L47A/D49A/A50K/Q51A (5A; Δ WIPI2) (available from Addgene), and BNIP3 W18A/L21A (Δ LIR) (available from Addgene).
- 3 The constructs are used to generate bacmid DNA, using the Bac-to-Bac system, by amplification in DH10BacY cells.
- 4 After verifying the bacmid DNA by PCR for insertion of the transgene, we purify bacmid DNA for transfection into Sf9 insect cells (12659017, Thermo Fisher, RRID:CVCL_0549).
- 5 To this end, we mix  2500 ng of plasmid DNA with FuGene transfection reagent (Promega) and transfect 1 million Sf9 cells seeded in a 6 well plate. 
- 6 About 7 days after transfection, harvest the V0 virus and used to infect 40 ml of 1 million cells per ml of Sf9 cells.
- 7 Closely monitor the viability of the cultures and upon the decrease in viability and confirmation of yellow fluorescence, we collect the supernatant after centrifugation and store this as V1 virus.
- 8 For expressions, we infect 1 L of Sf9 cells, at 1 million cells per ml, with 1 ml of V1 virus.
- 9 When the viability of the cells decreases to 90-95%, collect the cells by centrifugation.
- 10 Wash the cell pellets with 1x PBS and flash-frozen in liquid nitrogen.

Note

Store the pellets at  -80 °C .

- 11 For purification of BNIP3-GST wild-type or mutants, resuspend the pellets in 25 ml lysis buffer.

Lysis buffer:

A	B
Tris-HCl pH 7.4	50 mM
NaCl	300 mM
Triton X-100	1%
Glycerol	5%
MgCl ₂	2 mM
DTT	1 mM
β-mercaptoethanol	2mM
benzonase (Sigma)	1 μl
cOmplete EDTA-free protease inhibitors (Roche)	
CIP protease inhibitor (Sigma)	

- 12 Cells were homogenized with a douncer and clear the cell lysates by centrifugation at  18.000 rpm, 4°C, 00:45:00 in a SORVAL RC6+ centrifuge with an F21S-8×50Y rotor (Thermo Scientific). 45m  
- 13 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 BNIP3-GST. 2h
- 14 Centrifuge the samples to pellet the beads and remove the unbound lysate. 
- 15 Wash the beads twice with wash buffer, once with high salt wash buffer, and two more times with wash buffer. 

Wash buffer:

A	B
Tris-HCl 7.4	50 mM
pH	7.4



	A	B
	NaCl	300 mM
	DTT	1 mM

High salt buffer:

	A	B
	Tris-HCl 7.4	50 mM
	pH	7.4
	NaCl	700 mM
	DTT	1 mM

16 Incubate the beads  Overnight with  4 mL of [M] 50 millimolar (mM) reduced glutathione dissolves in wash buffer at  4 °C , to elute BNIP3-GST from the beads.

2h

17 To collect the supernatant, collect the beads by centrifugation.



18 Wash the beads twice with  4 mL of wash buffer, and collect the supernatant.



19 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 Superdex 200 Increase 10/300 GL column (Cytiva). Elute the proteins with SEC buffer.

SEC buffer:

	A	B
	Tris-HCl 7.4	25 mM
	pH	7.4
	NaCl	300 mM
	DTT	1 mM



- 20 Analyze the fractions by SDS-PAGE and Coomassie staining. Pool the fractions containing purified BNIP3-GST.
- 21 After concentrating the purified protein, aliquote the protein and snap-frozen in liquid nitrogen.



Note

Store the proteins at  -80 °C .