Aug 29, 2024

Purification of BNIP3-GFP

DOI
dx.doi.org/10.17504/protocols.io.kqdg328r7v25/v1

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Protocol Citation: Elias Adriaenssens 2024. Purification of BNIP3-GFP. **protocols.io** https://dx.doi.org/10.17504/protocols.io.kqdg328r7v25/v1

Protocol status: Working
We use this protocol and it's working

Created: May 24, 2024

Last Modified: August 29, 2024

Protocol Integer ID: 103092

Keywords: ASAPCRN

Funders Acknowledgement:
Aligning Science Across Parkinson's (ASAP)
Grant ID: ASAP-000350
Marie Skłodowska-Curie MSCA Postdoctoral fellowship
Grant ID: 101062916

Abstract

This protocol details the purification of BNIP3-GFP.

Materials

Lysis buffer:

	A	B
	Tris-HCl	50 mM
	pH	7.4
	NaCl	300 mM
	Triton X-100	1%
	glycerol	5%
	MgCl2	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

- BNIP3-GFP (available from Addgene) or BNIP3(W18A/L21A)-GFP (ΔLIR) (available from Addgene)
- pFastBac-Dual vector from Genscript (available from Addgene).
- BNIP3 E44A/L47A/D49A/A50K/Q51A (5A; ΔWIPI2) (available from Addgene)
- BNIP3 W18A/L21A (ΔLIR) (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-8x50Y rotor (Thermo Scientific)

Purification - BNIP3-GFP

4h 45m

1 To purify GFP-tagged

- BNIP3-GFP (available from Addgene) or BNIP3(W18A/L21A)-GFP (ΔLIR) (available from Addgene),

we purchase the gene-synthesized codon-optimized cytosol-exposed domain of BNIP3 (1-158aa) fused to a C-terminal GFP-tag in a pFastBac-Dual vector from Genscript (available from Addgene).

2 Introduce the point mutants by in vitro mutagenesis to generate

- BNIP3 W18A/L21A (ΔLIR) (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 purifiy 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 collects 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-GFP wild-type or mutants, resuspend the pellets in 25 ml lysis buffer.

Lysis buffer:

	A	B
	Tris-HCl	50 mM
	pH	7.4
	NaCl	300 mM
	Triton X-100	1%
	glycerol	5%
	MgCl2	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-8x50Y 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-GFP. 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:

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

16 Cleave off the GST-tag Overnight by eluting the GFP-tagged cargo receptor from the GSH beads by the addition of TEV protease in wash buffer at 4 °C .

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:

Tris-HCl	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-GFP.

21 After concentrating the purified protein, aliquote the protein and snap-frozen in liquid nitrogen.

Note

Store the proteins at -80 °C .