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Purification of NIX-GFP

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We use this protocol and it's working

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Abstract

This protocol details the purification of NIX-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
	cOmplete EDTA-free protease inhibitors (Roche)	
	CIP protease inhibitor (Sigma)	
	DNase (Sigma)	

Wash buffer:

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

High salt wash 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

Materials:

- pET-DUET1 vector (available from Addgene). pETDuet-1 TIM9,10 **addgene Catalog #**170280
- NIX E72A/L75A/D77A/E81A (4A; ΔWIPI2) (available from Addgene)
- NIX W35A/L38A (ΔLIR) (available from Addgene).
- Rosetta pLysS cells (Novagen Cat# 70956-4)
  Rosetta™(DE3)pLysS Competent Cells - Novagen **Merck Catalog #**70956-4
- 10 kDa cut-off Amicon filter (Merck Millipore)
  Amicon® Ultra Centrifugal Filter, 10 kDa MWCO **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**UFC801008

Purification - NIX-GFP

20h 46m

1 To purify GFP-tagged

- NIX-GFP (available from Addgene) or NIX(W36A/L39A)-GFP (ΔLIR)(available from Addgene),

fuse the cytosol-exposed domain of NIX (1-182aa) to a C-terminal GFP-tag through cloning into a pET-DUET1 vector (available from Addgene).

2 Introduce the point mutants in vitro mutagenesis to generate

- NIX E72A/L75A/D77A/E81A (4A; ΔWIPI2) (available from Addgene), and
- NIX W36A/L39A (ΔLIR) (available from Addgene).

3 After the transformation of the pET-DUET1 vector encoding NIX-GFP wild-type or mutants in E. coli Rosetta pLysS cells (Novagen Cat# 70956-4), grow the cells in 2x Tryptone Yeast extract (TY) medium at 37 °C until an OD_{600} of 0.4 and then continue at 18 °C.

4 Once the cells reaches an OD_{600} of 0.8, induce the protein expression with 100 micromolar (µM) isopropyl β-D-1-thiogalactopyranoside (IPTG) for 16:00:00 at 18 °C.

16h

5 Collect the cells centrifugation and resuspend in lysis buffer.

Lysis buffer:

	A	B
	Tris-HCl pH 7.4	50 mM
	NaCl	300 mM
	Triton X-100	1%
	Glycerol	5%
	MgCl2	2 mM
	DTT	1 mM
	β-mercaptoethanol	2mM
	cOmplete EDTA-free protease inhibitors (Roche)	
	CIP protease inhibitor (Sigma)	
	DNase (Sigma)	

6 Sonicate the cell lysates twice for 30 s and clears by centrifugation at 18.000 rpm, 4°C, 00:45:00 in a SORVAL RC6+ centrifuge with an F21S-8x50Y rotor (Thermo Scientific). 45m

6.1 Sonicate the cell lysates for 00:00:30 (1/2). 30s

6.2 Sonicate the cell lysates for 00:00:30 (2/2). 30s

7 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 NIX-GFP. 2h

8 Centrifuge the samples to pellet the beads and remove the unbound lysate.

9 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 pH 7.4	50 mM
	NaCl	300 mM
	DTT	1 mM

High salt wash buffer:

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

10 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 .

Wash buffer:

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

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

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

13 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. Analyze the fractions by SDS-PAGE and Coomassie staining. Pools fractions containing purified NIX-GFP.

SEC buffer:

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

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

Note

Store the proteins at -80 °C.