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Purification of NIX-GFP

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Elias Adriaenssens¹

¹Sascha Martens lab, University of Vienna, Max Perutz Labs - Vienna



Elias Adriaenssens

Sascha Martens lab, University of Vienna, Max Perutz Labs - ...

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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%
	MgCl ₂	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 (Δ LIR)(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; Δ WIPI2) (available from Addgene), and
 - NIX W36A/L39A (Δ LIR) (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 OD₆₀₀ of 0.4 and then continue at  18 °C .
- 4 Once the cells reaches an OD₆₀₀ of 0.8, induce the protein expression with  100 micromolar (μ M) isopropyl β -D-1-thiogalactopyranoside (IPTG) for  16:00:00 at  18 °C .
- 5 Collect the cells centrifugation and resuspend in lysis buffer.



16h



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
cComplete EDTA-free protease inhibitors (Roche)	
CIP protease inhibitor (Sigma)	



A	B
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-8×50Y 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



	A	B
	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. Pool 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 .