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We use this protocol and it's working

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NAP1-mCherry

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ABSTRACT

This protocol describes how to express and purify human NAP1 tagged C-terminally with mCherry.

ATTACHMENTS

Purification of NAP1-mCherry.pdf

MATERIALS

Expression:
pCAGG_GST-TEV-NAP1-mCherry (Addgene ID: 198036)
FreeStyle™ 293-F Cells
FreeStyle™ 293 Expression Medium (Thermo, 12338-026)
Opti-MEMR I Reduced Serum Medium (Thermo, 31985-062)
Polyethylenimine (PEI 25K, Polysciences CatNo 23966-1)
EXCELLR 293 Serum-Free Medium (SigmaAldrich, 14571C-1000ML)
PBS

Lysis Buffer:
50 mM Tris-HCl pH 8.0
300 mM NaCl
2 mM MgCl2
5% Glycerol
2 mM b-Met
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
300 mM NaCl
1 mM DTT

Columns/Resin:
Glutathione Sepharose 4B (Cytiva)
Superose 6 Increase 10/300 column (Cytiva)

Expression

1 To generate GST-TEV-NAP1-mCh the gene coding for the protein sequence of human NAP1 was subcloned together with N-terminal GST-TEV and C-terminal mCherry tags into a pCAGG. The construct is available at AddGene with the ID: 198036.

2 The protein was expressed in FreeStyle™ 293-F Cells by transient transfection.The cells grown at 37°C in FreeStyle™ 293 Expression Medium (Thermo, 12338-026) were seeded to density of 0.7 x 10^6 cells per ml the day before transfection. On the day of transfection, 400 ml culture at density of 1x10^6 cells per ml was transfected by addition of vortexed transfection mixture. The mixture consisted of two components: 400 ug of the MAXI-prep DNA that was pre-diluted in 13 ml of FreeStyle™ 293 Expression Medium (Thermo, 12338-026) and 800 ug Polyethylenimine (PEI 25K, Polysciences CatNo 23966-1) likewise pre-diluted in 13 ml of Opti-MEM media. 24 hours post transfection, the culture was fed by addition of 100 ml of EXCELLR 293 Serum-Free Medium (SigmaAldrich, 14571C-1000ML). 24H post transfection, the cells were harvested by centrifugation at 270 g for 20 minutes, washed by 1xPBS and flash-frozenin liquid nitrogen prior to storage at -80 °C.

Purification

3 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.

4 Additionally disrupt the cells with a Dounce homogenizer and two rounds of 30s sonication on ice.

5 Clear the lysate by centrifugation (10 000 rpm for 45 min at 4°C in a Fiberlite F21-8x50y (Thermo Scientific)).

6 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).

7 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 Was I Buffer.

8 Incubate the beads overnight with TEV protease at 4°C (20 ul of 10 mg/ml home-made TEV).

9 The next day spin down the beads (4000 rpm, 3 min, 4°C) and collect the supernatant containing cleaved NAP-mCh.

10 Filter the supernatant through a 0.45 µm syringe filter to remove any residual beads.

11 Concentrate the protein down to 0.5 ml using a 30kDa cut-off Amicon filter and apply onto a Superose 6 Increase column (10/300, Cytiva) pre-equillibrated with a SEC buffer containing 20 mM Tris-HCl, pH 7.4, 300 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.