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Purification of Human K560-GFP molecular motor

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Purification of Human K560-GFP molecular motor

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We use this protocol and it's working

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Abstract

K560-GFP purification protocol from the Reck-Peterson Lab based on protocol from Nicholas et al. 2014. Edited for protocols.io by Andrea Dickey and Mariusz Matyszewski.

Guidelines

All protein purification steps should be performed at 4 °C unless noted otherwise.

Materials

Materials:
cOmplete EDTA-free protease inhibitor cocktail tablets
lysozyme
PMSF

Buffers:
Lysis buffer:

- [M] 50 millimolar (mM) Tris pH 7.5
- [M] 300 millimolar (mM) NaCl
- [M] 5 millimolar (mM) MgCl2
- [M] 0.2 Mass Percent sucrose
- [M] 1 millimolar (mM) DTT
- [M] 0.1 millimolar (mM) Mg-ATP
- [M] 0.5 millimolar (mM) Pefabloc

Wash buffer:

- [M] 50 millimolar (mM) Tris pH 7.5
- [M] 300 millimolar (mM) NaCl
- [M] 5 millimolar (mM) MgCl2
- [M] 0.2 Mass Percent sucrose
- [M] 10 millimolar (mM) imidazole
- [M] 1 millimolar (mM) DTT
- [M] 0.1 millimolar (mM) Mg-ATP
- [M] 0.5 millimolar (mM) Pefabloc

Elution buffer:

- [M] 50 millimolar (mM) Tris pH 8.0
- [M] 300 millimolar (mM) NaCl
- [M] 5 millimolar (mM) MgCl2
- [M] 0.2 Mass Percent sucrose
- [M] 250 millimolar (mM) imidazole
- [M] 5 millimolar (mM) bME
- [M] 0.1 millimolar (mM) Mg-ATP

Storage buffer:

- [M] 80 millimolar (mM) PIPES pH 7.0
- [M] 2 millimolar (mM) MgCl2
- [M] 1 millimolar (mM) EGTA
- [M] 0.2 Mass Percent sucrose
- [M] 1 millimolar (mM) DTT
- [M] 0.1 millimolar (mM) Mg-ATP

Glycerol cushion:

- [M] 80 millimolar (mM) PIPES pH 7.0
- [M] 2 millimolar (mM) MgCl2
- [M] 1 millimolar (mM) EGTA
- [M] 60 % volume glycerol
- [M] 20 micromolar (µM) Taxol
- [M] 1 millimolar (mM) DTT

BRB80:

- [M] 80 millimolar (mM) PIPES pH 7.0
- [M] 2 millimolar (mM) MgCl2
- [M] 1 millimolar (mM) EGTA
- [M] 300 millimolar (mM) KCl
- [M] 7.5 millimolar (mM) Mg-ATP

Expression

1 pET17b-Kif5b(1-560)-GFP-His should be transformed into BL21(DE3)RIPL cells.

2 Make enough LB for at least 7.5 L of culture.

3 Grow an overnight starter culture.

4 Transfer starter culture into LB. Make sure to add proper antibiotics (Ampicillin for the plasmid, and Chloramphenicol for the cells we used).
Allow it to grow at 200 rpm, 37°C until OD600 reaches 0.6-0.8.

5 Chill cells and incubator to 18 °C

6 Add [M] 0.5 millimolar (mM) IPTG and allow it to grow at 200 rpm, 18°C, 18:00:00

7 Harvest and freeze cell pellets.

Purification

2h 35m

8 Resuspend 7.5 L worth of pellets in 120 mL Lysis buffer supplemented with 1 cOmplete EDTA-free protease inhibitor cocktail tablet (Roche) per 50 mL of Lysis buffer . Also add [M] 1 mg/mL lysozyme .
Incubate On ice for 00:30:00 . 30m

9 Lyse the resuspension by sonication.

10 Add [M] 0.5 millimolar (mM) PMSF to the sonicate, and clarify by centrifugation 40000 rcf, 4°C, 01:00:00 in Type 70 Ti rotor (Beckman). 1h

11 Incubate the supernatant with Ni-NTA agarose beads incubated with the **Wash buffer**. Nutate for 01:00:00 1h

12 Apply to gravity column and wash with 100 mL Wash buffer .

13 Resuspend the beads in elution buffer, incubate for 00:05:00 and elute in 0.5 mL increments. 5m

14 Combine peak fractions and buffer exchange on PD-10 desalting column equilibrated with **Storage buffer**.

15 Peak fractions of the motor solution were either flash-frozen at -80 °C until further use or immediately subjected to microtubule bind and release purification (see next steps).

Microtubule bind and release purification 44m

16 A total of 1 mL of motor solution from previous steps is used. Incubate with [M] 1 millimolar (mM) AMP-PNP and [M] 20 micromolar (µM) Taxol On ice in the **dark** for 00:05:00 and subsequently warm to Room temperature . 5m

17 Polymerize bovine brain tubulin (about 00:30:00 at 37 °C) and centrifuge (TLA120.2 rotor at 80000 rpm, 00:12:00 at Room temperature) through a **glycerol cushion**. Resuspend the pellet. 42m

18 Incubate the microtubule solution with the resuspended microtubules in the **dark** for 00:15:00 at Room temperature . 15m

19 Put the incubated solution on top of **glycerol cushion** and centrifuge in a TLA120.2 rotor at 80000 rpm, 00:12:00 at Room temperature . 12m

20 Wash the final pellet with **BRB80** and incubate in 100 µL of release buffer for 00:05:00 at Room temperature . 5m

21 Centrifuge the kinesin solution 72000 rpm, 00:07:00 in TLA100 rotor at Room temperature . 7m

22 Collect the supernatant and supplement with [M] 660 millimolar (mM) sucrose and flash freeze.

Note

A typical kinesin prep in the lab yielded [M] 0.5 micromolar (µM) to [M] 1.5 micromolar (µM) K560-GFP dimer.