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Expression and purification of Rab12 (1-244) stoichiometrically phosphorylated at Ser106 (the LRRK2 site)

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1 Works for me Share dx.doi.org/10.17504/protocols.io.buy3nxyn

asap

Dario Alessi

ABSTRACT

A subset of small GTPases of the Rab family including Rab12 (Uniprot: Q6IQ22) have been identified as substrates of the Leucine Rich Repeat Kinase 2 (LRRK2; Uniprot Q5S007) (Steger et al., 2016). In order to perform detailed study of Rab phosphorylation and dephosphorylation, as well as produce phosphorylation site-specific antibodies, and to carry out drug discovery screens, it is necessary to produce hundreds of milligrams of pure, stoichiometrically phosphorylated Rab12 protein. The full-length sequence of Rab12 (1-244) is suitable for large scale expression, when expressed as a 6His-SUMO fusion protein. Here we describe in detail the method we use to produce milligram quantities of stoichiometrically Ser106 phosphorylated Rab12 with the 6His-SUMO tag removed employing the SENP1 protease. We employ the MST3 kinase to phosphorylate Rab12 at Ser106, as this kinase is much easier and less expensive to produce or purchase than LRRK2 (Berndsen et al., 2019, Vieweg et al. 2020).

ATTACHMENTS

dhvibgrdf.pdf

DOI

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PROTOCOL CITATION

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KEYWORDS

Rab12, Ser106 phosphorylation, LRRK2

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49915

MATERIALS TEXT

Cells:

Competent BL21(DE3)

Plasmids

- Recombinant Protein - MST3 (1 - 431) isoform A **MRC PPU Reagents and Services Catalog #DU62878**
- RAB12 **MRC PPU Reagents and Services Catalog #DU52221**
- 6His TEV SENP1 D415-L644(end). (bacterial expression construct for human SENP1 protease (catalytic domain), confers carbenicillin resistance. Available from mrcppureagents.dundee.ac.uk: **Order no: DU39129**.

Consumables:

- His-MST3 active kinase can be purchased from MRCPPU reagents and services (https://mrcppureagents.dundee.ac.uk/).
- His-SENP1 (415-644) active protease can be purchased from MRC-PPU reagents and services (https://mrcppureagents.dundee.ac.uk/).
- S.O.C. Medium **Thermofisher Catalog #15544034**
- LB broth **Merck Catalog #1.10285.5000**
- **Carbenicillin (Formedium: CAR0025)**
- IPTG Dioxane Free – 25g **Formedium Catalog #IPTG025**

Prepare [M]**1 Molarity (M)** stock solution in H2O.

- Tris(hydroxymethyl)aminomethane (TRIS Trometamol) 99.8-100.5% AnalaR® NORMAPUR® analytical reagent **VWR Chemicals Catalog #103157P**
- MES Monohydrate- 1000g **Formedium Catalog #MES04**
- Glycerine ≥99.5% AnalaR® NORMAPUR® ACS analytical reagent redistilled **VWR Chemicals Catalog #24388.320**
- 2-Mercaptoethanol **Merck Catalog #8057400250**
- Magnesium chloride hexahydrate **Sigma – Aldrich Catalog #M2670**

Prepare [M]**1 Molarity (M)** stock solution in H2O.

- Sodium chloride ≥98% TECHNICAL **VWR Chemicals Catalog #27788.366**

Prepare [M]**5 Molarity (M)** stock solution in H2O.

- Guanosine 5'-diphosphate sodium salt **Sigma Aldrich Catalog #G7127**
- Adenosine Tri-phosphate (ATP) **Cytiva Catalog #27-1006-03**
- Imidazole **Sigma Aldrich Catalog #56750**

Prepare **50 mL** of a [M]**1 Molarity (M)** imidazole solution (3.4 g) and adjust the pH to 7.5 using **1 mL** 37% HCl.

- LEUPEPTIN HEMISULPHATE **Apollo Scientific Catalog #BIMI2183**

Prepare a [M]**10 mg/ml** solution in 50% ethanol and keep at **-20 °C** .

- 4-(2-AMINOETHYL)BENZENESULPHONYL FLUORIDE HYDROCHLORIDE **Apollo Scientific Catalog #BIMB2003**

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Prepare a [M] **0.5 Molarity (M)** solution in 50% ethanol and store at **-20 °C** .

Thrombin from human plasma **Sigma**

- **Aldrich Catalog #T4393**

Econo-Pac Columns **Bio-rad**

- **Laboratories Catalog #7321010**

5 mL , **10 mL** and **25 mL** polystyrene pipettes (Greiner or Sarstedt).

Corning® large volume centrifuge

- tube **Merck Catalog #CLS431123-6EA**

InstantBlue® Coomassie Protein Stain (ISB1L)

- (ab119211) **Abcam Catalog #119211**
- **200 µl** and **1000 µl** pipette tips.
- Amicon Ultra-15 3000 Da MWCO centrifuge concentrators.

SnakeSkin™ Dialysis Tubing, 3.5K MWCO, 22 mm **Thermo**

- **Fisher Catalog #68035**
- **2 mL** PP deep well plates for fraction collections with the Äkta Pure. (Greiner)
- Ni-NTA-agarose (Qiagen, SIGMA or Thermo-Fisher)

SOURCE

- 15S **Cytvia Catalog #17094401**

Recombinant Anti-RAB12 (phospho S106) antibody [MJF-R25-

- 9] **Abcam Catalog #ab256487**
- Polyclonal anti total Rab12 sheep polyclonal (SA227)

Buffered solutions:

Cell collection buffer:

A	B
Tris pH 7.5	50 mM
NaCl	150 mM
Imidazole	20 mM
β-mercaptoethanol	7 mM
MgCl2	2 mM
GDP	10 µM
AEBSF	1 mM
Leupeptin	10 µg/ml

AEBSF, Leupeptin, β-mercaptoethanol and GDP are added fresh.

Ni-wash buffer:

A	B
Tris pH 7.5	50 mM
NaCl	400 mM
Glycerol	5%
Imidazole	20 mM
β-mercaptoethanol	7 mM
MgCl2	2 mM
GDP	10 µM

β-mercaptoethanol and GDP are added fresh)

Ni-elution buffer:

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A	B
Tris pH 7.5	50 mM
NaCl	400 mM
Glycerol	5%
Imidazole	400 mM
β-mercaptoethanol	7 mM
MgCl2	2 mM
GDP	10 µM

β-mercaptoethanol and GDP are added fresh.

SEC-buffer pH 7.5:

A	B
Tris pH 7.5	50 mM
NaCl	200 mM
Glycerol	5%
MgCl2	2 mM
β-mercaptoethanol	7 mM
GDP	10 µM

SEC-buffer pH 5.6:

A	B
MES pH 5.6	30 mM
NaCl	200 mM
Glycerol	5%
MgCl2	2 mM
β-mercaptoethanol	7 mM
GDP	10 µM

IEX-Low Salt Buffer:

A	B
MES pH 5.6	30 mM
Glycerol	5%
MgCl2	2 mM
β-mercaptoethanol	7 mM
GDP	10 µM

IEX-High Salt Buffer:

A	B
MES pH 5.6	30 mM
NaCl	1.0 M
Glycerol	5%
MgCl2	2 mM
β-mercaptoethanol	7 mM
GDP	10 µM

Equipment:

- **20 µl** , **200 µl** and **1000 µl** Gilson pipettes
- Pipette aid/pipettor
- Infors Bacterial Incubator with Platform for 2 L conical flasks
- 12 x **2 L** conical flasks for growing E.coli BL21 cells
- Beckman J6 centrifuge with **1 L** centrifuge rotor 4.2

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- Beckman Avanti Centrifuge with JA 30.50 rotor
- Eppendorf 5810 R centrifuge
- 12 x **1 L** Beckman centrifuge buckets/ pots with lids
- 8 x **50 mL** Beckman Centrifuge vials with lids for JA30.50 centrifuge
- Probe Sonicator (cell disruptor)
- Stuart Roller Mixer SRT9
- Äkta Pure (Cytiva)
- Superdex 75 column any of HR10/30 or GL300/10 or XK16/60 (Cytiva, formerly GE-Healthcare-Life Sciences)
- **10 mL** empty column e.g. HR10/10 or Tricorn 10/100 (Cytiva 28246415)
- WPA cell densitometer (Fisher, VWR)

Transformation of plasmid into competent bacteria 8h 30m 50s

1 30m

Mix **10 µl** of pET15b-6HisSUMO-Rab12 plasmid (around **50 ng/µl**) with **50 µl** - **100 µl** of the competent BL21(DE3) cells and incubate **On ice** for **00:30:00** .

2 50s

Transfer the vial to a heat block equilibrated at **42 °C** and leave for **00:00:50** .

3

Transfer the vial back into ice and add **1 mL** SOC medium and mix gently.

4 4h

Incubate for **04:00:00** at **37 °C** for recovery.

5

Plate **0.1 mL** of the transformation onto a LB broth/agar plate supplemented with **50 µg/ml** carbenicillin.

6 4h

Leave the plate **Overnight** in a **37 °C** incubator.

Overnight culture 4h

7

Using a **200 µl** pipette tip, remove one of the colonies from the plate and drop the tip into **300 mL** LB broth medium, supplemented with **50 mg/L** carbenicillin.

8 4h

Incubate at **37 °C** with **180 rpm** - **200 rpm** rotational shaking **Overnight** .

The culture medium should become totally opaque in the morning.

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Set up cells and induce expression 4h 1m

9 Decant 6 x **1 L** LB broth medium into 6 x **2 L** conical flasks.

10 

Supplement each flask/litre with **1 mL** of **50 mg/ml** Carbenicillin.

11 

Mix and add **10 mL** – **25 mL** of the **Overnight** culture into each flask.

12  4h

Incubate for around **04:00:00** at **37 °C** , using an Infors Shaker-Incubator set at **200 rpm** .

13 Sample two or three of the expressions by removing **1 mL** medium and comparing the optical density at **600 nm** with fresh LB medium, using a WPA cell densitometer or a spectrometer.

14 

When the OD_{600} has reached 0.7-0.9, change the temperature setting of the Infors incubator to **15 °C** and incubate the cells for at least another hour, all the while shaking at **200 rpm** .

15 When the flasks have cooled down to **20 °C** or lower, induce His-SUMO-Rab12 expression by supplementing the medium with **0.1 Milimolar (mM)** IPTG.

> This is achieved by adding **100 µl** of a **1 Molarity (M)** IPTG stock solution to each litre.

16  1m

Leave the cells to express the protein for **Overnight** at **15 °C** .

Collection of cells and preparation of lysate 4w 0d 1h 15m

17 The following morning prepare **0.5 L** of cell collection buffer and chill **On ice** .

18  25m

Decant the content of the **2 L** conical flasks into **1 L** Beckman centrifuge pots, close the pots with their screwcap lids and sediment the cells by centrifugation for **00:25:00** at **4 °C** at **4200 rpm** using a Beckman J6 centrifuge with the 6 x 1 L rotor (4.2).

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19 Collect and open the pots and carefully, decant the spent supernatant medium back into the flasks.

The flasks can now be sent for cleaning and autoclaving. The cell sediment in the pots is expected to have a volume of **3 mL** – **5 mL** .

20 

Add **18 mL** of cold cell collection buffer to the sediment.

21 

Transfer the **1 L** pots to the Infors incubator and set the temperature to **14 °C** and the rotation to **110 rpm** .

22 Leave the pots for **00:30:00** , after which time the cell sediment should have completely resuspended. 30m

23 

Pool all suspensions into one of the 6 pots using a **25 mL** pipette and a good pipettor. If any of the sediments has not well resuspended, pipette up and down close to the bottom of the pots.

24 

For Rab proteins, supplement the NaCl concentration to **400 Milimolar (mM)** and add glycerol to 5% (by vol) final concentration.

It is important to add the NaCl and glycerol after the resuspension step and not before as this interferes with the resuspension.

25 Once the NaCl and glycerol has been mixed in, aliquot **45 mL** samples into **50 mL** centrifuge vials and freeze them in liquid nitrogen for at least **00:20:00** . 20m

26 Store the vials at **-20 °C** for up to **672:00:00** . The freezing and subsequent thawing step breaks up the cells and improves yield. 4w

Preparation of cell lysate and pulldown of His-SUMO-Rab12 on Ni-agarose 2h 1m 30s

27 

Slowly thaw the vials with the cell suspension in cold water.

We have verified that it is OK to leave the tubes to thaw samples by leaving these at **4 °C** **Overnight** .

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28 After thawing chill suspension **On ice** and then sonicate, using a probe sonicator (Cell disruptor).

29 30s

Settings: 6 – 8 pulses of **00:00:15** with **00:00:15** pauses. Set the amplitude to 50%.

30 The lysate will warm up slightly, but make sure it does not warm up over **18 °C** and keep samples cool by swirling **On ice** .

31 25m

Transfer the sonicated suspension into **40 mL** Beckman centrifuge vials and sediment the insoluble material by centrifugation for **00:25:00** at **40000 x g** an **4 °C** using a 25.50 or a 30.50 rotor in a Beckman Avanti centrifuge.

32 Recover the supernatant by carefully decanting it into a **500 mL** Corning PP conical centrifuge tube.

33

During the centrifugation step, equilibrate **3.0 mL** Ni-agarose (**6 mL** of a 50% slurry, sufficient for a 6 L expression) by washing it three times with Milli Q water and once with cell collection buffer.

34 1h 30m

Add a 50% slurry of the washed Ni-agarose in collection buffer to the lysate and incubate the mix on a Roller Mixer for **01:30:00** in a cold room set at **4 °C** .

Avoid excessive agitation and especially formation of foam.

35 In the meanwhile, prepare Ni-wash buffer.

36 5m

Carefully sediment the Ni-agarose by centrifugation using a Beckman J6 with a 4.2 rotor and suitable adaptors. Centrifuge at **1000 rpm** for **00:05:00** at **4 °C** .

Avoid higher centrifugation speeds as the **500 mL** PP conical centrifuge vessels are structurally weak.

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37 Remove the lid and carefully decant the supernatant containing the depleted lysate using a **25 mL** pipette, being careful not to disturb the Ni-agarose.

38 

Add **6 mL** of Ni-wash buffer.

39 Prepare a **1000 µl** pipette tip by removing **5 mm** – **7 mm** from the pointed end using scissors. This allows it to be used to facilely resuspend the agarose.

40 Resuspend the Ni-agarose using a P1000 with such a modified blue tip and aliquot the Ni-agarose into a **15 mL** centrifuge vial.

41 

Wash out any remaining agarose from the large vial with **1 mL** of Ni-wash buffer and pool with the first batch to maximise recovery.

42  1m

Fill the **15 mL** vial to the top with Ni-wash buffer, mix well and sediment resin by centrifugation at **1000 x g** for **00:01:00** using an Eppendorf 5810 R centrifuge.

43 

Remove the Ni-wash buffer with a thin vacuum line and replace with fresh Ni-wash buffer. Repeat this step 5 times in total to thoroughly wash the resin.

44 

Remove all Ni-wash buffer without disturbing the agarose bed.

45 Resuspend the Ni-agarose into **5 mL** wash buffer, transfer into a BIORAD Econopac column and let the buffer run out.

46 

Add **1 mL** of Ni-elution buffer and let the void of the bed run out.

47 Then elute the protein with **6 mL** Ni-elution buffer into a **15 mL** centrifuge vial.

This should provide **6 mL** of protein solution of **4.0 mg/ml** - **5.0 mg/ml** (**24 mg** – **30 mg** total protein).

Polishing and buffer exchange by SEC

48 In order to improve protein purity and to remove the imidazole prepare a Superdex 75 XK16/60 column by equilibration

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into SEC buffer pH 7.5 .

49 Apply 5 mL of the His-SUMO-Rab12 and perform chromatography on that column. The His-SUMO-RAB12 elutes at 70 mL (Figure 1). Pool the peak fractions.

Protocol: expression and purification of Rab12 pSer106

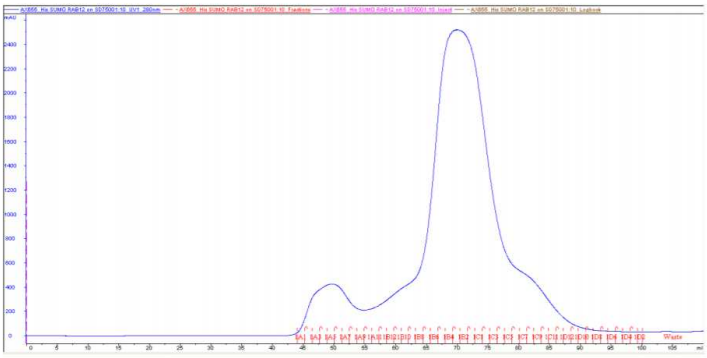

Figure 1 Screenshot from Unicorn 4.1 software, run on an Äkta Purifier. Results of His-SUMO-Rab12, purified over a Superdex 75 XK16/60 column. His-SUMO-Rab12 eluted between 65ml and 80ml with the apex of the peak at 72ml. Note: the SD75 column was overloaded.

Removal of His-SUMO-tag and phosphorylation by MST3 1m

50 Proceed by taking two aliquots, one to be left unphosphorylated and another to be phosphorylated.

51 To remove the tag, add His-SENP1 protease to the His-SUMO-Rab12 aliquots at a ratio of 1 mg of protease per 5 mg of substrate.

52

To the aliquot to be phosphorylated also add 2 mg of His-MST3 kinase and make the solution [M]10 Milimolar (mM) MgCl2 and [M]2 Milimolar (mM) ATP (pH 7.5).

53 1m

Incubate at 28 °C Overnight .

54 Prepare two Biorad 5 mL Polyprep filters with 1.5 mL Ni-agarose beds, equilibrate in wash buffer.

55 Individually pass the proteins through these filters, in order to remove the His-tagged enzymes and the cleaved His-SUMO-tag.

56 For further purification and removal of ATP, equilibrate a Superdex 75 HR10/30 column into SEC buffer pH 5.6

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57 Apply the Rab12 samples and collect fractions.

The cleaved Rab12 protein elutes at **13 mL** - **16 mL** peaking at **14.3 mL** (Figure 2).

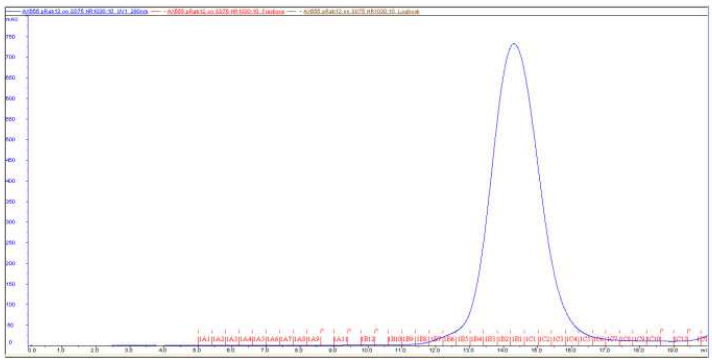

Figure 2 Screenshot from Unicorn 4.1 software, run on an Äkta Purifier. Untagged, phosphorylated Rab12 was purified over a SD75 HR10/30 column. The protein eluted between 13ml and 16ml with the apex of the peak at 14.3ml.

Chromatography on a Source 15 S HR10/10 column to separate phospho species 1m

58 In order to separate the phospho species from each other and from the remaining unphosphorylated protein, employ cation exchange chromatography using a Source 15 S column.

59 To this end, pack an empty HR10/10 or GL 10/100 column with **10 mL** Source 15 S resin and use vacuum suction to obtain a homogenous well packed resin bed.

Ion exchange chromatography is a concentrating method, hence the load volume is not critical.

60 Dilute the aliquots of the Rab12 samples into the Low Salt buffer to reduce the ionic strength sufficiently for the protein to bind.

61 Equilibrate the Source 15 S HR10/10 with the IEX- buffers using an Akta Pure or Purifier.

62 Dilute the Rab12 protein isolated from the gel filtration step tenfold into the IEX-LowSalt buffer.

63 Apply aliquots equivalent up to **6 mg** to the S-column at a flowrate of **2 ml/min** .

64 Develop the column at the same flow rate with a shallow **100 mL** gradient to 30% IEX-High Salt buffer collecting **1.0 mL** fractions.

pRab12 eluted as a major peak with a conductivity of 15 mS/cm, equivalent to **[M]0.2 Molarity (M)** NaCl (Figure 3). Our QC analysis indicates that the major peak is mono phosphorylated Rab12. The minor peak to the left

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is double phosphorylated Rab12.

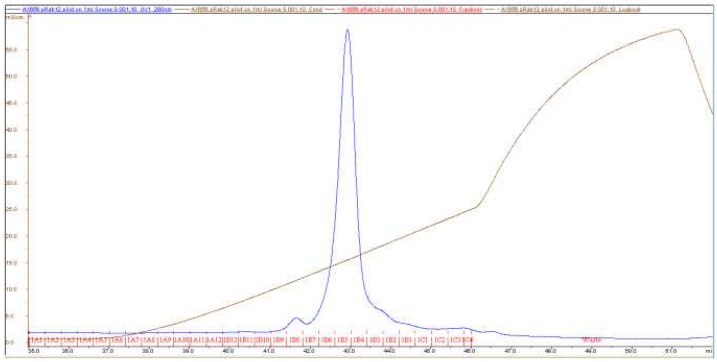

Figure 3 Screenshot from Unicorn 4.1 software, run on an Äkta Purifier. Untagged, phosphorylated Rab12 protein was subjected to chromatography on a 10ml Source 15 S column, run in a 30mM MES pH 5.6 buffer system. pRab12 eluted at a conductivity of 15mS/cm equivalent to 200mM NaCl.

Quality control

65 Separate **3 µg** of the protein on a 4% - 20% Tris Glycine SDS polyacrylamid gel and stain with Instant Blue (Figure 4a).

The protein should be >95% homogeneous.

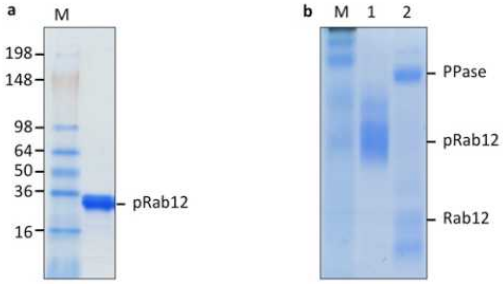

Figure 4 Coomassie stained SDS-PAGE images of pRab12. **a** untagged Rab12 separated by 4% -20% gradient SDS-PAGE. **b** Lane1: untagged phosphorylated Rab12 separated by 12% Phostag-SDS-PAGE. Lane 2: same protein treated with alkalyne phosphatase.

66 Separate **3 µg** of pRab12 and **3 µg** of phosphorylated Rab12, which had been treated with alkalyne phosphatase on a 12% Phos-tag SDS-Polyacrylamid gel that separates phosphorylated and non-phosphorylated Rab proteins, run as described previously (Ito et al. 2016) (Figure 4b).

The migration of the phosphorylated and non-phosphorylated pRab12 proteins can clearly be distinguished.

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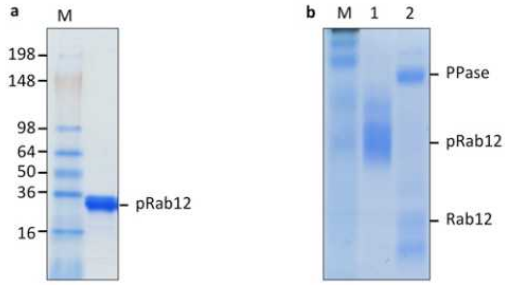

Figure 4 Coomassie stained SDS-PAGE images of pRab12. **a** untagged Rab12 separated by 4% -20% gradient SDS-PAGE. **b** Lane1: untagged phosphorylated Rab12 separated by 12% Phostag-SDS-PAGE. Lane 2: same protein treated with alkalyne phosphatase.

67

Do Immunoblot analysis of pRab12 and dephosphorylated Rab12 using Rab12 pSer106 and total antibodies (Figure 5). This will reveal that the pRab12 protein is specifically phosphorylated at Ser106.

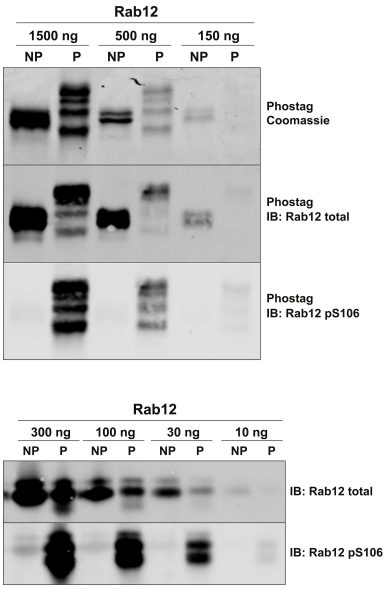

Figure 5. Indicated amounts of pRab12 (P) and dephosphorylated Rab12 (NP) were analysed using Phostag SDS-PAGE (Coomassie stained as well as immunoblotted with Rab12 total/pS106 antibodies), and SDS-PAGE (immunoblotted with Rab12 total/pS106 antibodies).

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