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Expression and purification of Rab10 (1-181) stoichiometrically phosphorylated at Thr73 (the LRRK2 site)

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asap

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ABSTRACT

Rab10 (Uniprot: P61026), a membrane associated small GTPase, likely involved in vesicle trafficking, has been identified as one of the substrates of the Leucine Rich Repeat Kinase 2 (LRRK2; Uniprot Q5S007)) and the Protein Phosphatase PPM1H (Uniprot Q9ULR3) (Steger et al., 2016; Berndsen et al., 2019). In order to perform detailed studies of Rab10 phosphorylation and dephosphorylation, it is necessary to produce milligram amounts of pure, stoichiometrically phosphorylated Rab10 protein. The full-length sequence of Rab10 is very prone to aggregation and precipitation when expressed in bacteria, resulting in extremely low yields of protein, even when solubilising tags are employed for the expression of fusion proteins. A shorter fragment, spanning residues 1-181 is much more useful for bacterial expression. Here we describe in detail the method we use to produce milligram quantities of stoichiometrically Thr73 phosphorylated Rab10[1-181]. We employ the MST3 kinase to phosphorylate recombinant Rab10 at Thr73, because MST3 is much easier and less expensive to produce or purchase than LRRK2 (Berndsen et al., 2019, Vieweg et al. 2020).

ATTACHMENTS

dq54bgrdf.pdf

DOI

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PROTOCOL CITATION

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References:

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MATERIALS TEXT

Materials:

Cells:

- Competent BL21(DE3)

Plasmids:

☒Recombinant Protein - MST3 (1 - 431) isoform A **MRC PPU Reagents and**

- **Services Catalog #DU62878**
- pET28a 6HIS Thrombin Rab10 (1-181) codon optimized (bacterial expression plasmid for Rab10 (1-181), confers kanamycin resistance. Available from mrcppureagents.dundee.ac.uk **Order no: DU68199**

Consumables:

- His-MST3 active kinase can be purchased from MRCPPU reagents and services (https://mrcppureagents.dundee.ac.uk/).

☒S.O.C.

- Medium **Thermofisher Catalog #15544034**

☒LB

- broth **Merck Catalog #1.10285.5000**

☒KANAMYCIN

- MONOSULPHATE **Formedium Catalog #KAN0025**

☒ IPTG Dioxane Free –

- 25g **Formedium Catalog #IPTG025**

Prepare [M]**1 Molarity (M)** stock solution in H_2O

- ☒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**

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Prepare [M]**1 Molarity (M)** stock solution in H_2O.

Sodium chloride ≥98% TECHNICAL **VWR**

- **Chemicals Catalog #27788.366**

Prepare [M]**5 Molarity (M)** stock solution in H_2O.

Guanosine 5'-γ-thiotriphosphate tetralithium salt **Sigma**

- **Aldrich Catalog #G8634**

L-

- Arginine **Formedium Catalog #DOC0109**

Imidazole **Sigma**

- **Aldrich Catalog #56750**

Prepare **50 mL** of a [M]**1 Molarity (M)** imidazole solution (**3.4 g**) and adjust the pH to pH**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**

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

Adenosine Tri-phosphate

- (ATP) **Cytiva Catalog #27-1006-03** **Thermo-Fisher:1158105**

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
- **15 mL** and **50 mL** Centrifuge tubes (e.g. Greiner, Falcon, Sarstedt).
- 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**

Buffered solutions (all made fresh and chilled):

Cell collection buffer:

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A	B
Tris pH 7.5	50 mM
NaCl	150 mM
Imidazole	20 mM
β-mercaptoethanol	7 mM
L-arginine	5mM
MgCl2	2 mM
GTPγS	1 µM
AEBSF	1 mM
Leupeptin	10 µg/ml

Ni-wash buffer:

A	B
Tris pH 7.5	50 mM
NaCl	400 mM
Glycerol	10%
Imidazole	20 mM
β-mercaptoethanol	7 mM
L-arginine	5mM
MgCl2	2 mM
GTPγS	1 µM

SEC-buffer I:

A	B
Tris pH 7.5	50 mM
NaCl	400 mM
glycerol	10%
Larginine	5mM
MgCl2	2 mM
β-mercaptoethanol	7 mM
GTPγS	1 µM

SEC-buffer II:

A	B
MES pH 5.6	30 mM
NaCl	300 mM
Glycerol	10%
MgCl2	2 mM
β-mercaptoethanol	7 mM

IEX-Low Salt Buffer:

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

IEX-High Salt Buffer:

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

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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
- 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)
- Empty FPLC column e.g. HR10/10 or 10 / 100 GL format.

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

1 30m

Mix **10 µl** of pET28a 6HIS Thrombin Rab10 1-181 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** .

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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 [M]50 µg/ml kanamycin.

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 kanamycin.

8 4h

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

The culture medium should become totally opaque in the morning.

Set up cells and induce expression 12h

9 Decant 12 x 1 L LB broth medium into 12 x 2 L conical flasks.

10

Supplement each flask/litre with 1 mL of [M]50 mg/ml Kanamycin Monosulphate.

11

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

4h

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12 

Incubate for **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 **16 °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 Rab10 expression by supplementing the medium with **0.1 Milimolar (mM)** IPTG, e.g. with **100 µl** of a **1 Molarity (M)** IPTG stock solution to each litre.

16 

4h

Leave the cells to express the protein for **Overnight** at **16 °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).

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** – **6 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** .

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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 12 pots using a **25 mL** pipette and a good pipettor.

24 

If any of the sediments has not well resuspended, pipette up and down close to the bottom of the pots.

25 

Supplement NaCl to **0.4 Molarity (M)** final concentration and add glycerol to 10% final concentration and mix well.

26 Aliquot **45 mL** into **50 mL** centrifuge vials and freeze them in liquid nitrogen for **00:20:00** . 20m

27 Store at **-20 °C** for up to **672:00:00** . 4w

The freezing and subsequent thawing step breaks up the cells and improves yield.

Preparation of cell lysate and pulldown of His-Rab10 on Ni-agarose

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

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

30  30s

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

31  25m

Transfer the sonicated suspension into **50 mL** Beckman polypropylene 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 supernatants by carefully decanting them into a **500 mL** Corning PP conical centrifuge tube.

33 

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During the centrifugation step equilibrate **3.0 mL** Ni-agarose **6 mL** of a 50% slurry, sufficient for a **12 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** .

35 Avoid excessive agitation and especially formation of foam.

36 In the meanwhile, prepare and chill the Ni-wash buffer.

37

Carefully sediment the Ni-agarose by centrifugation using a Beckman J6 with a 4.2 rotor and suitable adaptors.

38 5m

Centrifuge at **1000 rpm** for **00:05:00** at **4 °C** .

39

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

40

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

41 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.

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

43

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.

44 1m

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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.

45 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.

46

Remove all Ni-wash buffer without disturbing the agarose bed and add 1 mL of Ni-wash buffer.

47

Add 100U = 100 µl Thrombin solution (1000 Units per ml) to the Ni-agarose and mix carefully but well.

48 2h

Incubate the Ni-agarose with Thrombin for 02:00:00 at ambient temperature (20 °C – 24 °C) and mix occasionally.

49 Transfer the Ni-agarose into a Biorad 5 mL Polyprep column and let the digested protein drip into a fresh 15 mL vial.

50

Wash out the original 15 mL vial with 2 mL Ni-wash buffer and pool with the Ni-agarose in the Polyprep column. This improves recovery.

51

Finally, after the Ni-agarose has settled down add another 2 mL of Ni-wash buffer to recover any remaining digested protein.

At this stage there should be 5 mL – 6 mL of a protein solution at [M]1 mg/ml - [M]2 mg/ml .

Isolation of monomeric Rab10 (1-181) by Size Exclusion Chromatography

52

The recovered, untagged Rab10 is a mix of aggregated Rab10 (MW > 100 kDa), Thrombin (native Mw 37 kDa) and monomeric Rab10 1-181 (Mw 22.9 kDa).

Equilibrate a Superdex 75 HiPrep (XK 16/60) column in SEC buffer I.

53 Apply the digested Rab10 sample either in 2 x 3 mL or as 1 x 6 mL to the column and develop the column at a flowrate of 1.2 ml/min .

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We normally observe a substantial amount of protein eluting at the void volume and thereafter.

54 However, seperate the monomeric Rab10 from these aggregates and contaminants and elutes at around **84 mL** (Figure 1). Collect the fractions of this peak.

We normally obtain **3.0 mg** – **3.8 mg** of monomeric Rab10 from such preparation from **12 L** of bacterial culture.

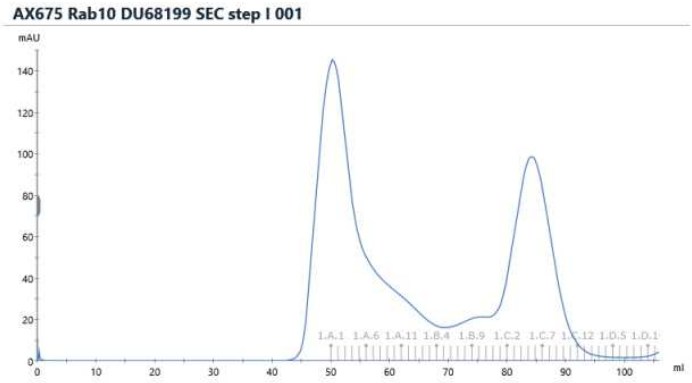

Figure 1 UV_{280nm} trace of Rab10 protein, separated on a Superdex 75 XK 16/60 after recovery from Ni-agarose by Thrombin digestion.

55 Pool and concentrate the protein using Amicon Ultra 3000 Da MWCO filters.

The protein tolerates concentration to **5.0 mg/ml** and more. It can be frozen in liquid nitrogen and stored at **-70 °C** .

Phosphorylation 1d 12h

56 1d 12h

In order to produce **1 mg** of phosphorylated Rab10, it is necessary to phosphorylate **9.0 mg** - **10.0 mg** of purified Rab10 protein and then repurify the phosphorylated species. Hence the Rab10 protein from **36 L** of bacterial culture is required and must be prepared first. We combine the Rab10 protein from three **12 L** preparations for this purpose.

Mix **10 mg** (**4 mL** of Rab10 protein at **2.5 mg**) in SEC buffer I with **4.0 mg** of His-MST3 protein (**3.5 mg/ml**), **0.25 mL** **1 Molarity (M)** Tris **pH 8.0** (**50 Milimolar (mM)** f.c.), **55 µl** **1 Molarity (M)** $MgCl_2$ (**10 Milimolar (mM)** f.c.), and **55 µl** **0.2 Molarity (M)** ATP (**2 Milimolar (mM)** f.c.) and incubate at **28 °C** for **16:00:00** – **20:00:00** .

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57

Although MST3 is not a very potent Rab10 kinase, 90% of the Rab protein does become phosphorylated in these conditions.

After the phosphorylation reaction is completed, remove the kinase by depletion over a **1 mL** Ni-agarose bed.

Some losses occur, but **8 mg** of phosphorylated Rab10 can be recovered.

Repurification and buffer exchange by Size Exclusion Chromatography

58 Apply the protein to a Superdex 75 column, equilibrate this time in SEC buffer II.

This step removes the ATP and ADP, any remaining MST3 and replaces the buffer system. There are two important changes compared to SEC buffer I: firstly the buffer system is MES at pH **5.6** and not Tris at pH **7.5**. This is to protonise the Rab10 protein in preparation for the next cation exchange step and Tris is not a suitable buffer system for cation exchange chromatography. Secondly, SEC buffer II does not contain L-arginine, which would interfere with the subsequent cation-exchange step. Figure 2 shows that the pRab10 protein elutes at **85 mL**, exactly where the Rab10 would elute.

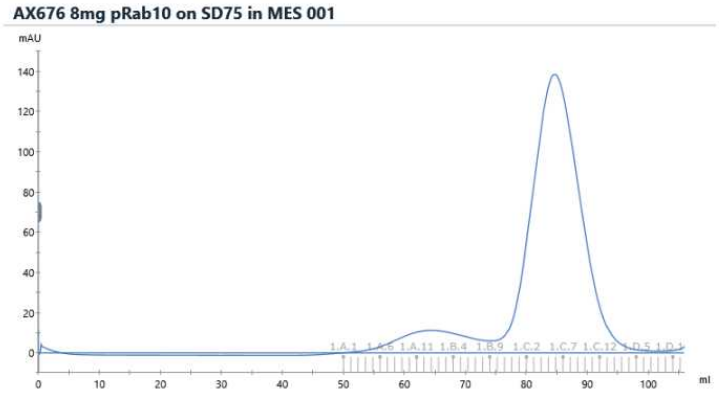

Figure 2 UV_{280nm} trace (grey blue) of 8 mg pRab10 separated on a Superdex 75 XK 16/60 column after phosphorylation by MST3 and depletion of the kinase over a Ni-agarose bed. pRab10 elutes around 84ml ± 7ml.

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

59 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.

60 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.

61 Therefore, dilute the monomeric Rab10 sample into the Low Salt buffer to reduce the ionic strength sufficiently for the

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protein to bind.

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

63 Dilute the pRab10 protein isolated from the gel filtration step tenfold into the IEX-Low Salt buffer.

64 Apply aliquots equivalent to **3 mg** to the S-column at a flowrate of **2 ml/min** .

Two column runs may be necessary.

65 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.

Generally, up to four peaks are resolved see Figure 3. The dominant peak eluting at 15 mS/cm (here **148 mL**) represents single phosphorylated Rab10.

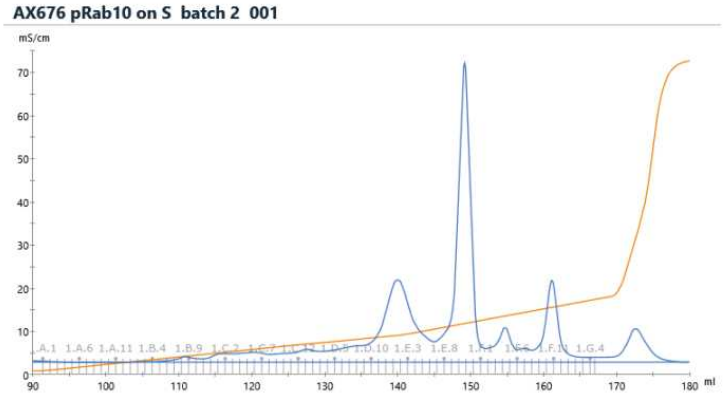

Figure 3 UV_{280nm} trace (grey-blue) and conductivity trace (peach) of pRab10 protein separated on a 10 ml Source 15 S column. The peak at 149 ml represents Rab10 pThr73. The peak at 162 ml represents Rab10 that did not become phosphorylated.

66 Pool the fractions containing pRab10 protein.

Protein yield should exceed **1.0 mg** .

67

Supplement GTPgS to **1 Micromolar (µM)** or GDP to **1 Micromolar (µM)** as required.

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68 Aliquot into convenient batches, freeze in liquid nitrogen and store at **-70 °C** .

Quality control

69 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.

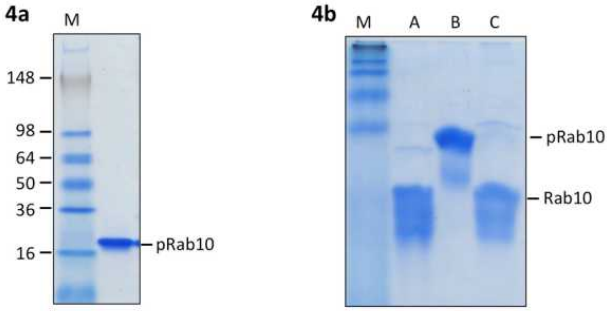
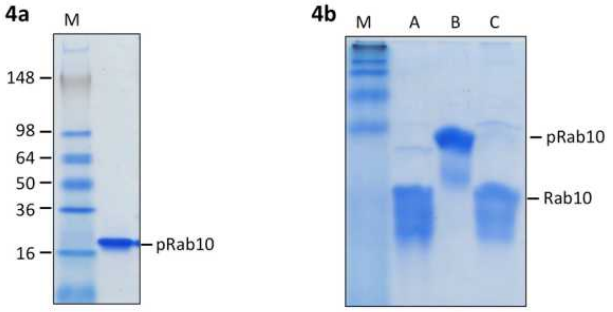


Figure 4 Coomassie Blue stained SDS-polyacrylamid gels with separated Rab10 and pRab10 proteins. **a** Rab10 (1-181) (pThr73) was separated by 4% -20% SDS-PAGE. (M = MW marker SeeBlue Plus. **b** Rab10 (1-181) (A), Rab10 (1-181) (pThr73) (B) eluting at 148ml and the repurified unphosphorylated Rab10 (1-181) (C) eluting at 161ml were separated by 12% Phos-tag SDS-PAGE.

70 Separate a **3 µg** of unphosphorylated Rab10 (1-181) and **3 µg** of unphosphorylated Rab10 (1-181) 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 pRab10 proteins can clearly be distinguished.



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Citation: Axel Knebel , Kerryn Berndsen, Pawel Lis, Dario R Alessi (06/29/2021). Expression and purification of Rab10 (1-181) stoichiometrically phosphorylated at Thr73 (the LRRK2 site). https://dx.doi.org/10.17504/protocols.io.bvjxn4pn