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Expression and purification of Rab8A (1-181) stoichiometrically phosphorylated at pThr72 (the LRRK2 site)

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

asap

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ABSTRACT

A subset of small GTPases of the Rab family including Rab8A (Uniprot: P61006) have been identified as 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 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 Rab8A protein. The full-length sequence of Rab8A is prone to aggregation and precipitation when expressed in bacteria. Therefore, a shorter fragment, spanning residues 1-181 is more useful for large scale expression. Here we describe in detail the method we use to produce milligram quantities of stoichiometrically Thr72 phosphorylated Rab8A[1-181]. We employ the MST3 kinase to phosphorylate Rab8A at Thr72, as this kinase is much easier and less expensive to produce or purchase than LRRK2 (Berndsen et al., 2019, Vieweg et al. 2020).

ATTACHMENTS

dgedbgrdf.pdf

DOI

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PROTOCOL CITATION

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KEYWORDS

Rab8A (1-181) pThr72, Leucine Rich Repeat Kinase 2, Protein Phosphatase PPM1H , LRRK2

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MATERIALS TEXT

Cells:

Competent BL21(DE3).

Plasmids:

- pET15b-6His-MST3 TV2 (bacterial expression plasmid for MST3, confers carbenicillin resistance). Available from mrcppureagents.dundee.ac.uk Order no: DU62878.

Recombinant Protein - MST3 (1 - 431) isoform A **MRC PPU Reagents and Services Catalog #DU62878**

- pET28a 6HIS Thrombin Rab8a 1-181 codon optimized (bacterial expression plasmid for Rab8A (1-181), confers kanamycin resistance. Available from mrcppureagents.dundee.ac.uk Order no: DU68198

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 1 M stock solution in H_2O

- Tris(hydroxymethyl)aminomethane (TRIS Trometamol) 99.8-100.5% AnalaR® NORMAPUR® analytical reagent **VWR Chemicals Catalog #103157P**

- Glycerine ≥99.5% AnalaR® NORMAPUR® ACS analytical reagent redistilled **VWR Chemicals Catalog #24388.320**

LEUPEPTIN HYDROCHLORIDE **Apollo**

- **Scientific Catalog #BIMI2442**

2-

- Mercaptoethanol **Merck Catalog #8057400250**

Magnesium chloride hexahydrate **Sigma**

- **Aldrich Catalog #M2670**

Sodium chloride ≥98% TECHNICAL **VWR**

- **Chemicals Catalog #27788.366**

Guanosine 5'-diphosphate sodium salt **Sigma**

- **Aldrich Catalog #G7127**

Imidazole **Sigma**

- **Aldrich Catalog #56750**

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

Adenosine Tri-phosphate

- (ATP) **Cytiva Catalog #27-1006-03**

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

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

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

Thrombin from human plasma **Sigma**

- **Aldrich Catalog #T4393**

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Econo-Pac Columns **Bio-rad**

- **Laboratories Catalog #7321010**
- 5, 10 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 kDa 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**

Monoclonal Anti-RAB8A antibody produced in mouse **Sigma**

- **Aldrich Catalog #WH0004218M2**

Recombinant Anti-RAB8A (phospho T72) antibody [MJF-R20]

- (ab230260) **Abcam Catalog #ab230260**

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 or GTPγS	10 µM (GDP) or 1 µM (GTPγS)
AEBSF	1 mM
Leupeptin	10 µg/ml

AEBSF, Leupeptin, β-mercaptoethanol and the nucleotide 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 or GTPγS	10 µM (GDP) or 1 µM (GTPγS)

β-mercaptoethanol and the nucleotide are added fresh.

Ni-elution buffer:

A	B
Tris pH 7.5	50 mM
NaCl	400 mM
Glycerol	5%
Imidazole	400 mM
β-mercaptoethanol	7 mM
MgCl2	2 mM
GDP or GTPγS	10 µM (GDP) or 1 µM (GTPγS)

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β-mercaptoethanol and the nucleotide are added fresh

Rab8-dialysis buffer:

A	B
Tris pH 7.5	30 mM
NaCl	300 mM
Glycerol	10%
β-mercaptoethanol	7 mM
MgCl2	2 mM
GDP or GTPγS	10 µM (GDP) or 1 µM (GTPγS)

β-mercaptoethanol and the nucleotide are added fresh

SEC-buffer:

A	B
MES pH 5.6	30 mM
NaCl	200 mM
Glycerol	5%
MgCl2	2 mM
β-mercaptoethanol	7 mM
GDP or GTPγS	10 µM (GDP) or 1 µM (GTPγS)

IEX-Low Salt Buffer:

A	B
MES pH 5.6	30 mM
Glycerol	5%
MgCl2	2 mM
β-mercaptoethanol	7 mM
GDP or GTPγS	10 µM (GDP) or 1 µM (GTPγS)

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 or GTPγS	10 µM (GDP) or 1 µM (GTPγS)

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).

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

1 30m

Mix **10 µl** of pET28a 6HIS Thrombin Rab8a 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** .

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** 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.

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Set up cells and induce expression 13h

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 **50 mg/ml** Kanamycin Monosulphate.

11  4h

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

12  4h

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  1h

When the OD_{600} has reached 0.7-0.9, change the temperature setting of the Infors incubator to **18 °C** and incubate the cells for at least another **01:00:00** , all the while shaking at **200 rpm** .

15 When the flasks have cooled down to **20 °C** or lower, induce Rab8A 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  4h

Leave the cells to express the protein for **Overnight** at **18 °C** .

Collection of cells and preparation of lysate 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.

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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 12 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 Rab8A and other Rab proteins is it very useful at this stage to 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, **45 mL** samples are aliquoted into **50 mL** centrifuge vials and freeze them in liquid nitrogen for at least **00:20:00** . 20m

The vials can be stored at **-20 °C** for at least 4 weeks. The freezing and subsequent thawing step breaks up the cells and improves yield.

Preparation of cell lysate and pulldown of His-Rab8A on Ni-agarose 2h 2m 15s

26 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** .

27 After thawing chill suspension on ice and then sonicate, using a probe sonicator (Cell disruptor). Settings: 6-8 pulses of 15s

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00:00:15 with 15 sec pauses. Set the amplitude to 50%.

28 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.

29 

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.

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

31 

During the centrifugation step equilibrate **5.0 mL** Ni-agarose (**10 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.

32 

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.

33 

In the meanwhile, prepare Ni-wash buffer. Carefully sediment the Niagarose by centrifugation using a Beckman J6 with a 4.2 rotor and suitable adaptors.

34 

5m

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.

35 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.

36 

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

37 Prepare a **1000 µl** pipette tip by removing 5 mm-7 mm from the pointed end using scissors.

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This allows it to be used to facilely resuspend the agarose.

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

39

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.

40 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.

41 Remove the Ni-wash buffer with a thin vacuum line and replace with fresh Ni-wash buffer.

42

Repeat this step 5 times in total to thoroughly wash the resin.

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

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

45

Add **2 mL** of Ni-elution buffer and let the void of the bed run out. Then elute the protein with **10 mL** Nielution buffer into a **15 mL** centrifuge vial.

This should provide **10 mL** of protein solution of **[M]3.0 mg/ml** - **[M]6.0 mg/ml** (**36 mg** – **72 mg** total protein).

46 1m

Add **100 µl** Thrombin solution (1000 Units per ml) and dialyse **Overnight** against **5 L** of Rab8A dialysis buffer using Thermo-Fisher Snake Skin 3500 MWCO.

47 The next day recover the protein into a **15 mL** vial.

The dialysis step is important, as high imidazole concentrations inhibit the subsequent kinase reaction. The higher glycerol concentration during dialysis will reduce the volume of the sample by up to 20%. We keep the sample volume small to avoid unnecessary protein concentration steps before the subsequent size exclusion.

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Phosphorylation 1m

48

Take **0.5 mg** of Rab8A as an unphosphorylated control. Add **8 mg** His-MST3 (2.3 ml 3.5mg/ml) to the Rab8A protein and mix well.

49

Add **0.5 mL** of **1 Molarity (M)** Tris **pH 8** to buffer to ATP solution.

50

Add **120 µl** **1 Molarity (M)** $MgCl_2$ and **120 µl** **0.2 Molarity (M)** ATP and mix well but gently.

51 1m

Leave with an incubator at **28 °C** **Overnight** .

52 Next day prepare a **2 mL** Ni-agarose bed and capture the His-tagged MST3 by letting the sample run through the bed.

Further purification by Size Exclusion Chromatography

53

The untagged Rab8A is a mix of aggregated pRab8A, MST3 (Mw 51.8 kDa), Thrombin (native Mw 37 kDa) and monomeric pRab8A 1-181 (Mw 22.9 kDa). Size Exclusion Chromatography using a Superdex 75 XK 26/60 column offers a simple and powerful solution for isolation of monomeric pRab8A 1-181. The species are separated so well, that we have somewhat overloaded the column and apply a sample representing 4% of the column volume instead of the recommended 1%.

Equilibrate a Superdex 75 XK 26/60 format in 3 column volumes of SEC buffer at a flowrate of **2.5 ml/min** using an Akta Pure.

54 Apply the sample **12 mL** - **15 mL** (typical concentration of **3.0 mg/ml** - **4.5 mg/ml**) to the Gel filtration at a flowrate of **2.5 ml/min** and collect **1.5 mL** fractions between **80 mL** to **220 mL** .

55 Elute the monomeric pRab8A as a 36 ml peak from **170 mL** to **206 mL** with the apex at around **184 mL** .

56 Pool the fractions containing the core of the pRab8A peak into one vial. A typical elution chromatogram is shown on Figure 1.

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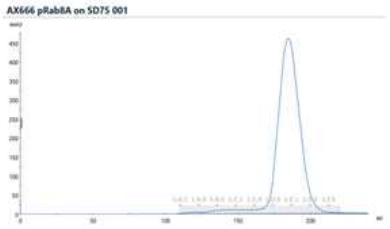

Figure 1 UV_{280nm} trace of pRab8A protein, separated on a Superdex 75 XK 26/60 after phosphorylation and affinity depletion of the kinase using Äkta Pure equipment.

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

57 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.

58 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.

59 Ion exchange chromatography is a concentrating method, hence the load volume is not critical. Therefore, dilute aliquots of the monomeric Rab8A sample into the Low Salt buffer to reduce the ionic strength sufficiently for the protein to bind.

60 In contrast, dialysing highly concentrated Rab8A into the Low Salt Buffer leads to substantial protein aggregation and precipitation.

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

62 Dilute the pRab8A protein isolated from the gel filtration step tenfold into the IEX-LowSalt buffer.

63 Apply aliquots equivalent to 5-6mg 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.

Generally, up to four peaks are resolved see Figure 2. Our QC analysis indicates that the peaks C and D eluting between 6 and 8 mS/cm both represent Thr72 monophosphorylated Rab8A.

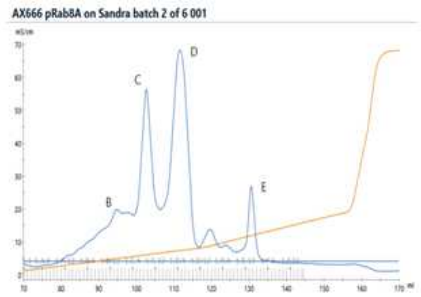

Figure 2 UV_{280nm} trace (grey-blue) and conductivity trace (peach) of pRab8A protein separated on a 10 ml Source 15 S column. The peaks at 102 ml and 112 ml represent Rab8A stoichiometrically phosphorylated at Thr72.

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Quality control 1h

65 Separate **3 µg** of the protein on a 4% - 20% Tris v with Instant Blue (Figure 3).

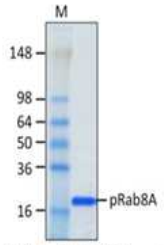

Figure 3 Coomassie Blue stained 4% - 20% SDS-gradient gel. The protein maker See Plus Blue was separated in Lane 1 and 3 µg of purified Rab8A, phosphorylated at Thr72 was separated in Lane 2.

The protein should be >95% homogeneous.

66 Separate a **3 µg** of pRab8A[1-181] and **3 µg** of unphosphorylated Rab8A (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 4).

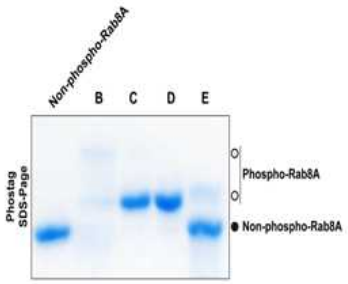

Figure 4 Coomassie Blue stained 12% SDS-Phos-tag gel. 3 µg of concentrated peak B, C, D and E were separated in the following lanes as indicated.

The migration of the phosphorylated and non-phosphorylated pRab8A proteins can clearly be distinguished.

67 Immunoblot analysis of pRab8A[1-181] and dephosphorylated Rab8A[1-181] undertaken as described previously (dx.doi.org/10.17504/protocols.io.bsgrnbv6) using Rab8A pThr72 and total antibodies (Figure 5). This will reveal that the pRab8A protein is specifically phosphorylated at Thr72.

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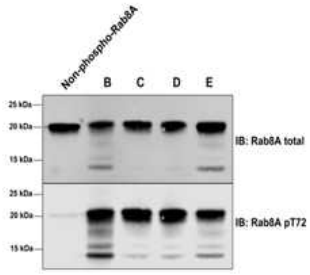

Figure 5 Immunoblot analysis using Rab8A and pThr72 specific antibodies: 100 ng of concentrated peak B, C, D and E were separated in the following lanes as indicated.

68 1h

Recombinant PPM1H phosphatase should quantitatively dephosphorylate pThr72 phosphorylated Rab8A.

Incubate **3 µg** pRab8A[1-181] ± **0.05 µg** o PPM1H (can be ordered from MRCPPU Reagents and Services, DU62835) in the presence of **2 Milimolar (mM)** MgCl2 for **01:00:00** at **Room temperature** prior to running a Phostag gel.

69 The pRab8A[1-181] can also be analysed by Mass Spectrometry after trypsin digestion. The tryptic peptide encompassing Thr72 should only be mainly present in the phosphorylated form and levels of the non-phosphorylated peptide should be very low to undetectable.

Citation: Axel Knebel, Kerryn Berndsen, Pawel Lis, Paul Davies, Dario R Alessi (05/22/2021). Expression and purification of Rab8A (1-181) stoichiometrically phosphorylated at pThr72 (the LRRK2 site). https://dx.doi.org/10.17504/protocols.io.butinwke