Absolute quantitation of GTPase protein abundance

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SUMMARY

Ras proteins and other small molecular weight GTPases are molecular switches controlling a wide range of cellular functions. High homology and functional redundancy between closely related family members are commonly observed. Antibody-based methods are commonly used to characterize their protein expression. However, these approaches are typically semi-quantitative and the requirement to use different antibodies means that this strategy is not suited for comparative analysis of the relative expression of proteins expressed by different genes. We present a mass spectrometry-based method that precisely quantifies the protein copy number per cell of a protein of interest. We provide detailed protocols for the generation of isotopically labelled protein standards, cell/tissue processing, mass-spectrometry optimization and subsequent utilization for the absolute quantitation of the abundance of a protein of interest. As examples, we provide instructions for the quantification of HRAS, KRAS4A, KRAS4B, NRAS, RALA and RALB in cell line and tissue-derived samples.

1. INTRODUCTION

Small GTPases of the Ras family are involved in a wide range of cellular processes and pathways [1]. The Ras proteins (HRas, NRas, KRas4A and KRas4B) are proto-oncogenes, and activating mutations are found in 19% of cancer patients [2]. RalA and RalB are Ras effectors that influence cell adhesion and migration and contribute to Ras-dependent transformation in cancer [3]. Certain Ras isoforms and mutants are more strongly associated with certain cancers [4]. The reasons for this pattern have yet to be fully elucidated but one key component is thought to be the expression level and relative dosage of different Ras isoforms, and the relative abundance of mutant versus wildtype Ras [5,6]. It is likely that relative expression levels are important regulators of the biology of other GTPases; however accurately quantifying relative protein abundance can be challenging.

Mass spectrometry (MS) can be used to absolutely quantify the levels of specific endogenous proteins in a sample by comparison with spiked in isotopically heavy peptide or protein standards [7,8]. These can be distinguished from the unlabeled counterpart by a mass difference that is easily detectable in the mass-spectrometer. Diagnostic peptides are used that are specific for individual isoforms; it is also possible to detect and quantify individual mutants versus wild type proteins. The benefits of this approach versus antibody-based approaches is the accuracy of the quantitation and the unambiguous identification of the protein variant of interest.

Here we describe a method for quantifying endogenous GTPases in cells using protein standards absolute quantification (PSAQ) [9,10]. This involves spiking in isotopically labelled full-length protein standards into a cell lysate. Adding a full-length protein standard at the start

of the process controls against potential sample extraction, processing and handing artefacts that would otherwise result in inaccurate quantitation. The lysate containing the labelled standards is then fractionated using SDS-PAGE, the proteins are digested and the peptides are then processed for high-performance liquid chromatography (HPLC) fractionation and triple quadrupole-based MS analysis. Diagnostic peptides for the protein of interest are detected and both the endogenous (light) and protein standard (heavy) peptides are distinguishable due to their different masses. The ratio between the areas of the two sets of peptide peaks allows the accurate estimation of the endogenous protein copy number per mg of cell lysate and per cell. An overview of the method can be seen in Figure 1.

The method that we describe can be applied to any protein of interest including all GTPases. As examples, we provide specific details for quantifying Ras and Ral isoforms. We use heavy isotope labelled arginine and lysine to label full-length, His-tagged protein standards since trypsin digestion will result in one of these amino acids being present in all diagnostic peptides. However, other heavy amino acids and protease combinations can be chosen to generate suitable diagnostic peptides for your protein of interest. We also describe selected reaction monitoring (SRM) analysis using an Sciex QTRAP 6500 hybrid triple-quad mass spectrometer; however, any triple-quad MS would be suitable once the conditions for identifying the diagnostic peptides has been optimized on the instrument.

2. MATERIALS

All steps involving bacteria or bacterial culture media should be performed using standard laboratory aseptic technique using sterile containers and consumables. Use ultrapure water for all steps up to gel fixation or staining, from which point onwards use liquid chromatography-mass spectrometry (LCMS) grade solvents. The generation of protein standards relies on access to routine bacterial culture and protein production equipment including conical flasks, a shaking incubator and a spectrophotometer.

Recombinant protein standards, cell pellets, lysates and in gel digests are processed and stored in Eppendorf protein LoBind plastics to prevent loss of proteins and peptides. Buffers for HPLC, in-gel digests and ZipTips are made in glass that has never been washed in detergent (see note 1). All waste must be disposed of by the appropriate local procedures for genetically modified organisms, biological and chemical waste.

2.1. Production of heavy labelled His tagged Ras/Ral standards

2.1.1. Expression of heavy His-Ras/His-Ral recombinant protein in AT713 bacteria

Proteins standards are made containing isotopically labelled arginine and lysine. In order to avoid unlabeled arginine or lysine being incorporated into the standards, we use the AT713 *E.coli* strain that is unable to synthesize these amino acids. We also make and use a minimal

M9 media that ensures that only the heavy isotopes of arginine and lysine will be available for making the recombinant protein standards. The 5 X M9 salt stock solution for this medium can be made and autoclaved in advance but the complete media must be made up on the first day of use and used up within a few days. It is important that the media is made in the order described below. We make up a stock of unlabeled hydrophilic amino acids, a stock of hydrophobic amino acids and individual stocks of the heavy labeled lysine and arginine.

We use His-tagged protein standards to facilitate the generation of high-purity standards. Other tags can be used; however, it is important that the tag and linker are small enough to migrate similarly to the endogenous protein on SDS-PAGE for this method to work efficiently. Alternatively, the tag and linker should be cleaved off prior to spike-in.

1. AT713 *E.coli* bacteria (Yale Coli Genetic Stock Centre, New Haven, USA). These are auxotrophic for Lysine, Arginine and Cysteine (see note 2).
2. Plasmids encoding inducible His-tagged Ras or Ral.
3. 5 X M9 media stock solution: Make up 1 L of 120 mM Na_2HPO_4, 55 mM KH_2PO_4, 21.5 mM NaCl, 10 mM NH_4Cl, pH 7.4. Autoclave stock.
4. L-amino acids: All are unlabeled 'light' amino acids unless otherwise stated. Make up the following stocks on the day of use (see note 2):
    1. Make a hydrophobic amino acids mix by combining the following at 2 mg/ml each in water: L-Alanine, L-Isoleucine, L-Leucine, L-Methionine, L-Phenylalanine, L-Proline, L-Tryptophan, L-Valine. Stir extensively on a magnetic stirrer until they are dissolved.
    2. Make a hydrophilic amino acids mix by combining the following at 0.5 mg/ml each in water: L-Aspartic Acid, L-Asparagine, L-Cysteine, L-Glutamic acid, L-Glutamine, Glycine, L-Histidine, L-Serine, L-Threonine and L-Tyrosine. Stir extensively with very slow dropwise addition of hydrochloric acid until they are dissolved (see note 2).
    3. Make up heavy L-Arginine (L-arginine-U-$^{13}C_6$-$^{15}N_4$) and heavy L-Lysine (L-lysine-U-$^{13}C_6$-$^{15}N_2$) at 100 mg/ml each in water.
5. 1 X complete M9 media: 1 X M9 stock, 0.4% glucose, 1 µg/ml Thiamine-HCl, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 20 µM $ZnSO_4$, 10 µM $FeCl_3$, 1 x Trace Metals A5 with Co (92949; Sigma Aldrich, USA), 200 µg/ml Hydrophobic amino acids mix, 100 µg/ml Hydrophilic amino acids mix, 200 µg/ml heavy Lysine, 200 µg/ml heavy Arginine, 100 µg/ml Ampicillin (see note 3). Make up 1 L on first day of use in this order (see note 4):
    1. Add 200 ml 5 X M9 stock and dilute with ultrapure water up to 600 ml.
    2. Add 4 g glucose, 1 ml 1 mg/ml Thiamine-HCl, 1 ml 1 M $MgSO_4$, stir to dissolve
    3. Add 100 µl 1 M $CaCl_2$ dropwise while stirring constantly using a magnetic stirrer,

continue stirring until any precipitate re-dissolves.

4. Add 200 µl 0.1 M $ZnSO_4$ and 1 ml trace metal mix.
5. Add amino acids (200 mg hydrophobic amino acids mix, 100 mg hydrophilic amino acids mix, 200 mg each of heavy Lysine and Arginine).
6. Adjust pH back to 7.4.
7. Add Ampicillin to 100 µg/ml
8. Make up to a final volume of 1 L and and filter using a 0.22 µM vacuum filter flask.

6. Isopropyl β-D-1-thiogalactopyranoside (IPTG): dissolve in 1-2 ml of M9 immediately prior to use.
7. Phosphate buffered saline (PBS): 2.7 mM KCl, 1.47 mM KH_2PO_4, 138 mM NaCl, 8.1 mM Na_2HPO_4, pH 7.4.

2.1.2. Affinity purification using His-Trap columns

1. Resuspension buffer: 20 mM Tris-HCl, pH 7.4, 0.5 M NaCl.
2. Bacterial protease inhibitor cocktail. (P8456; Sigma Aldrich, USA).
3. Lysozyme.
4. Ultracentrifuge: capable of spinning at 82,000 x g
5. His affinity column or beads: His-Trap HP 1 ml (89870; GE Healthcare, USA) (see note 5).
6. Automated HPLC and fractionator: AKTA purifier equipped with Frac950 and UPC900, operated using Unicorn software (optional, see note 5).
7. Binding Buffer (“Buffer A”): 20 mM Tris-HCl, pH 7.4, 0.5 M NaCl, 20 mM Imidazole
8. Elution buffer (“Buffer B”): 20 mM Tris-HCl, pH 7.4, 0.5 M NaCl, 500 mM Imidazole
9. Buffers A and B can be stored at 4-8 ˚C for a few days.
10. Eppendorf protein LoBind 96 well collection plates (1-2 ml well capacity).
11. Hot lysis buffer: 50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol
12. 10 X hot lysis buffer gel loading dye: 1 M DTT, 1% Bromophenol blue

2.1.3. Purification check: confirm purity and distribution of His-Ras/His-Ral protein

1. Rainbow marker: choose any molecular weight marker that contains pre-stained bands close to the molecular weight of your target protein to allow you to accurately cut the as small a gel fragment fragment as possible from an unstained gel without risking leaving behind any residue of your protein of interest.
2. Primary antibodies: Anti-His tag (H1029; Sigma Aldrich), anti-pan-Ras EP1125Y (ab52939; AbCam), anti-RALA (#3526; Cell Signalling Technology), anti-RALB (#3523; Cell Signalling Technology).

2.1.4. Size-based purification by gel filtration

1. Concentration and buffer exchange column: Amicon Ultra-15 Centrifugal filter unit, 10 kDa molecular weight cut off (Millipore).
2. Gel filtration column: Superdex Increase 200 10/300 GL (GE Healthcare, USA).
3. Automated HPLC and fractionator: AKTA purifier equipped with Frac950 and UPC900, operated using Unicorn software.
4. Gel filtration buffer: 40 mM Tris HCl (pH 7.4), 50 mM NaCl, 5 mM $MgCl_2$.

2.1.5. Storage and concentration determination of the standard

1. Concentration column: Amicon Ultra-0.5 ml, 10 kDa MWCO (UFC501024; Millipore).
2. Protein concentration assay kit: bicinchoninic acid (BCA) assay kit.

2.2. Mass spectrometric characterisation of GTPases such as Ras/Ral isoforms

2.2.1. SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Any SDS-PAGE format can be used; we provide details below of the system we use for making gels that has given us more consistent results for Ras and Ral quantitation than pre-cast gradient gel systems.

1. 4 X ProtoGel Resolving Buffer (Geneflow, UK): 1.5 M Tris-HCl, 0.4% SDS, pH 8.8.
2. 4 X ProtoGel Stacking Buffer (Geneflow, UK): 0.5 M Tris HCl, 0.4% SDS, pH 6.8.
3. Ultra-pure ProtoGel (Geneflow, UK): 30% (w/v) acrylamide: 0.8% (w/v) Bis-acryl-amide stock solution (37.5:1).
4. Ammonium persulfate (APS): 10% solution in water.
5. N,N,N,N′-Tetramethyl-ethylenediamine (TEMED).
6. 10 X Tris/Glycine/SDS running buffer (Geneflow, UK): 0.25 M Tris, 1.92 M glycine, 1% SDS.
7. 5 X SDS-PAGE sample buffer: 0.3 M Tris–HCl (pH 6.8), 10% SDS, 25% β-mercaptoethanol, 0.1% bromophenol blue, 45% glycerol.
8. Short plates and space plates with 1 mm integrated spacers (see note 6).
9. 15 well, 1 mm combs.

2.2.2. In-Gel Digestion and C-18 desalt

Acetonitrile (ACN) is light sensitive, so store in a dark/covered bottle. Only use approved mass spectrometry tips (no colours, no autoclaving, no UV). Gel loading tips are used to avoid accidentally pipetting up the gel pieces at the bottom of the tube. All in-gel digestion and C-18 clean up solutions should be made on the day.

1. Fix solution: 10% acetic acid/ 50% methanol
2. Sterile stainless steel scalpels
3. 100 mM ammonium bicarbonate (Ambic).
4. 50 mM Ambic / 50% Acetonitrile (ACN).
5. 10 mM dithiothreitol (DTT) in 100 mM Ambic.
6. 55 mM iodoacetamide (IAM) in 100 mM Ambic.
7. Reaction buffer (for trypsin): 40 mM Ambic, 9% ACN.
8. Trypsin Gold: 5 ng/µl in reaction buffer.
9. 1% Formic acid.
10. 100% ACN
11. 0.1% Trifluoroacetic acid (TFA)
12. 75% ACN/ 0.1% TFA
13. ZipTip with 0.6 µl C_{18} resin (ZTC18S096, Milipore)

2.2.3. Mass spectrometric characterisation of Ras isoforms

Any MS can be used, we provide details on the system that we employ:

1. Mass spectrometer: TripleTOF 6600 (Sciex)
2. In-line liquid chromatography system: Eksigent NanoLC 400 System (Sciex) mounted with a NanoAcquity 5 µm particle size, 180 µm x 20 mm C_{18} trap and 1.7 µm, particle size 75 µm X 250 mm C_{18} analytical column (Waters). Columns are maintained at 50 ˚C.
3. Ion source: NanoSpray III source fitted with PicoTip emitter (New Objective).
4. Mobile phase A: 2% acetonitrile / 0.1% formic acid
5. Mobile phase B: 98% acetonitrile / 0.1% formic acid
6. Loading buffer: 2% acetonitrile / 0.5% formic acid

2.2.4. Optimisation of MRM Transitions and quantitation of Ras and Ral isoforms

1. Mass spectrometer: QTRAP 6500 (Sciex)
2. In-line liquid chromatography system: Dionex U3000 nano-LC system (Thermo) mounted with a NanoAcquity 5 µm particle size, 180 µm x 20 mm C_{18} trap and 1.7 µm particle size, 75 µm X 100 mm C_{18} analytical column (Waters). Columns are maintained at 40 ˚C.
3. Ion source: NanoSpray III source fitted with a PicoTip emitter (New Objective).

2.3. Quantification of endogenous Ras or Ral levels in cell lysates

1. Cells of interest
2. Haemocytometer

3. NP40 lysis buffer: 50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP40 substitute, 1/250 mammalian protease inhibitor cocktail (P8340, Sigma). Store at 4 °C.

3. METHODS

3.1. Production of heavy labelled His tagged Ras/Ral standards

3.1.1 Expression of heavy His-Ras/His-Ral recombinant protein in AT713 bacteria

1. Select a single colony (or a scrape from a glycerol stock) of AT713 transformed with the plasmid of interest to inoculate 5 ml of LB + 100 µg/ml Ampicillin. Grow at 220 rpm, 37 ºC in a shaking incubator for approximately 6 hours.
2. Make 1 L of complete 1 x M9 media. Of this, 25 ml will be used for an overnight culture, 5 ml kept aside for blanks and resuspension of IPTG, and 970 ml for the final protein induction culture. Keep at 4 °C until needed.
3. Use 20 µl of the AT713 LB culture to inoculate 25 ml of complete 1 x M9 media. Grow at 220 rpm, 37 ºC in a shaking incubator for approximately 16 hours (see note 7). Store the rest of the complete M9 media at 4-8 ˚C until needed. Check the M9 media carefully for precipitation prior to use and make fresh media if necessary.
4. Inoculate 970 ml complete 1 x M9 media with 20 ml of the overnight AT713 culture in a sterile 2 L conical flask loosely sealed with foil. Incubate at 220 rpm, 37 ºC in a shaking incubator until the OD_{600} = 0.600-0.800.
5. *Purification check:* Once the desired OD_{600} has been reached, take aside 1 ml as a pre-induction sample. Microfuge at 700 rpm for 2 minutes at room temperature. Discard the supernatant, add 120 µl hot lysis buffer to the pellet, vortex and store at -20 ºC for later use in SDS-PAGE.
6. Induce protein expression in the remainder of the culture by adding IPTG to 1 mM final concentration.
7. Incubate at 220 rpm, 37 ºC in a shaking incubator for 3 hours.
8. *Purification check:* Take aside a 600 µl post-induction sample and collect, resuspend and store as in step 4.
9. Centrifuge the remaining culture at 3,000 x g for 15 minutes at 4 ºC.
10. Discard the supernatant and resuspend on ice in 20 ml of ice cold PBS, transfer the resuspended bacterial cell pellet into a pre-weighed 50 ml centrifuge tube.
11. Centrifuge at 3,000 x g for 10 minutes.
12. Discard the supernatant and weigh the pellet.
13. The sample can be snap frozen and stored at -80 ºC for future use or you can proceed directly with the next steps to affinity purify the proteins (see note 8).

3.1.2 Affinity purification using His-Trap columns

1. Resuspend the bacterial cell pellet on ice in 20 ml of ice cold resuspension buffer supplemented with bacterial protease inhibitor cocktail (1 ml per 4 g bacterial pellet).
2. Add lysozyme to a final concentration of 1 mg/ml and incubate on ice for 30 minutes with frequent agitation.
3. Using a probe sonicator set to 100% output, sonicate 10 times for 20 seconds with 30 second intervals on ice in between to avoid heating (increase ice rest if necessary).
4. Centrifuge at 82,000 x g for 30 minutes at 4 °C. Transfer supernatant into a fresh tube.
5. *Purification check:* Take aside a 40 µl sample of the cleared lysate supernatant and mix with 40 µl hot lysis buffer. Also scrape ~1 mm diameter piece of the pellet into 200 µl of hot lysis buffer. Store at -20 ºC for later use in SDS-PAGE.
6. Spike buffer B into the cleared supernatant to raise the imidazole concentration to 20 mM (see note 9)
7. Filter the lysate using a 0.2 µm syringe filter.
8. *Purification check:* Take aside a 40 µl sample of the filtered lysate into 40 µl hot lysis buffer. Store at -20 ºC for later use in SDS-PAGE.
9. Wash the His-Trap column at 1 ml/min with 5 column volumes (CVs) of Buffer A, then 5 CVs of buffer B.
10. Equilibrate the His-Trap column with 10 CVs of buffer A.
11. Load the filtered lysate (step 7) onto the His-Trap column by running buffer A through either a superloop or a smaller sample loop (see note 10).
12. *Purification check:* Collect a 40 µl sample of the flow-through and add to 40 µl of hot lysis buffer. Store at -20 ºC for later use in SDS-PAGE.
13. Equilibrate the His-Trap column with 10-15 CVs of buffer A
14. Run the imidazole gradient:
    1. Monitor protein elution by reading absorbance at 280 nm using a UV module throughout
    2. 1 ml/min flow rate throughout
    3. 0-60% buffer B (20-300 mM imidazole) over 21.5 CVs
    4. 0.25 ml elution fractions
    5. 3 mls gradient delay
15. *Purification check:* Set aside 12 µl of each fraction of interest and add to 3 µl of 5 x SDS-PAGE sample buffer. Proceed to SDS-PAGE check (section 3.1.3).
16. Store the fractions at 4 ºC while this test is run, we typically limit this to one overnight storage step.

3.1.3. Purification check: sample preparation for SDS-PAGE

The samples collected during the purification are now checked for successful enrichment of the protein of interest using SDS-PAGE and Coomassie staining/Western blotting.

General samples

1. Dilute the protein suspension into SDS-PAGE sample buffer to a final concentration of 1 x sample buffer and vortex.
2. Boil at 98 ˚C for 5 minutes.
3. Microfuge briefly, vortex to mix and microfuge again for 1 minute at 17,000 x g.
4. Avoid touching the bottom of the tube with tip when loading gel to avoid insoluble material that may have pelleted.

Hot lysis

1. For hot lysis samples, mix the protein suspension/pellet/bacterial culture with hot lysis buffer and boil at 110 ˚C for 15 minutes with frequent vortexing.
2. Sonicate using a probe sonicator for 10 seconds (set to 25% output)
3. Microfuge for 5 minutes at 17,000 x g.
4. Take 36 µl of supernatant and mix with 4 µl of 10 x SDS-PAGE sample buffer.
5. Follow steps 2-4 described for general samples.

3.1.4. Purification check: confirm purity and distribution of His-Ras/His-Ral protein

The first time that the procedure is performed we recommend selecting fractions from all observed UV peaks for analysis using SDS-PAGE. These should be tested using immunoblotting to confirm the presence of the correct full-length tagged protein, and by Coomassie protein stain to confirm purity (see note 11 for comments about the desired level of purity). The pre-elution samples can be tested using immunoblotting if low/no yield of the desired protein is observed (we do not typically see enough induced protein to detect over the background bacterial proteins by Coomassie stain).

1. Run 7 µl per lane of all samples, on replicate 4-12% BisTris NuPAGE gels using MES running buffer. NB. Run replicate lanes to allow immunoblotting with antibodies for the tag and the protein of interest.
2. Stain one gel using Coomassie total protein stain and transfer one onto membrane (0.9 mA, 1 hour using wet transfer) for immunoblotting.
3. Probe with anti-His tag and antibodies specific for the protein of interest.

3.1.5. Size-based purification by gel filtration

If sufficient purity has not been obtained further purification by size-based gel filtration can be performed. If purity is satisfactory, perform buffer exchange and concentration according to step 1, followed by skipping straight to step 2 of the next section (3.1.6).

1. Concentrate and perform buffer exchange by combining the desired fractions on a 10 kDa molecular weight cut-off Amicon 15 column and centrifuging according to the manufacturer's instructions: Reduce the volume to 1 ml before adding 5 ml of gel filtration buffer. Repeat at least 3 times, on the last round continuing to spin until the final volume reaches 0.4-0.5 ml.
2. Run the gel filtration method:
    1. Monitor protein elution by measuring absorbance at 280 nm using a UV module throughout
    2. 0.5 ml/min flow rate throughout
    3. Equilibration of the Superdex 200 column with 1.5 CVs of gel filtration buffer
    4. sample injection
    5. 1.25 CVs elution with elution fraction size of 0.25 ml.
3. *Purification check:* Collect 12 µl of each fraction of interest (based on the peak observed in UV trace) and add 3 µl of 5 x SDS-PAGE sample buffer. Identify which fractions contain the protein of interest and their relative purity using SDS-PAGE and Coomassie staining/Western blotting.

Figure 2 gives representative examples of chromatography column elutions of Ras protein during the His-tag affinity purification and size-exclusion based enrichment steps to show the final purity that it is necessary to achieve.

3.1.6. Storage and concentration determination of the standard

These steps will set the absolute concentration of your standards that will be used to ratiometrically determine your endogenous protein abundance. It is important that the determination of the protein concentration of the standard is as accurate as possible. Changes in concentration during storage will also influence your subsequent endogenous protein quantitation so it is important to store samples in a format to avoid protein adsorption/precipitation. We use SDS-PAGE sample buffer so that they can be directly added to cell lysates and we avoid multiple re-freezing of samples (see note 12). Figure 3 shows representative examples of purified Ras and Ral isoforms (see note 13).

1. Combine and concentrate the desired fractions using 10kDa molecular weight cut-off 0.5 ml Centricon columns until the final volume of the protein standard is 150-400 µl (see note 14).

2. Perform a BCA assay and/or OD_{280} absorbance-based measurements on 5 replicates in order to determine the concentration as accurately as possible (see note 15).
3. Once reproducible readings to determine concentrations are obtained, the protein standard can be stored (see notes 11 and 16 for comments on purity and yield).
4. Dilute the protein standard using 5 x SDS-PAGE sample buffer to a final concentration of 1 x sample buffer.
5. Boil 5 minutes 98 ºC.
6. Centrifuge to collect all liquid at the bottom of the tube, vortex and centrifuge again.
7. Aliquot, snap freeze on liquid nitrogen and store at -80 ºC.

3.2. Mass spectrometric characterisation of GTPases such as Ras/Ral isoforms

To confirm the presence and purity of the correct full-length proteins a sample of each is processed by in-gel digestion and analysed using data-dependent acquisition. We describe the protocol used in our studies, but this can be performed using any in-house methods and equipment.

3.2.1. Confirming efficient labelling of recombinant His-Ras/His-Ral protein

It is important to confirm that the protein standard has efficiently incorporated heavy arginine and lysine. SDS-PAGE and in-gel digest is performed followed by analysis on the mass spectrometer to determine the extent to which 'light' peptides are present that would be indicative of incomplete incorporation.

1. Run 100-200 ng of protein standard on an SDS-PAGE gel and Coomassie stain if required to aid identification of region of the gel where it has migrated to.
2. Cut out the protein band and slice into 1 mm cubes as described in section 3.2.2.
3. If Coomassie has been used, destain thoroughly using 50 mM Ambic / 50% ACN, 37 ºC, 10 minutes, 900 rpm on a thermoshaker. Repeat until fully destained.
4. Proceed with in-gel digest as described in section 3.2.2.
5. Analyse using standard LC-MS or MRM and check the percentage of heavy labelling using ProteinPilot5 or similar software (see section 3.3.4.).
6. We consider $\geqq$95% labelling to be acceptable. Unlabelled standard must be accounted for in the final copy number calculations.

3.2.2. In-Gel Digestion

To avoid contamination with keratin, perform all the pipetting steps in a laminar flow hood if possible, and change gloves frequently. Keratin contamination is less of an issue after peptide extraction, as whole keratin proteins will not interfere with MS analysis of peptides.

The volumes indicated below are estimates, but each should be in relation to the actual gel volume in the tube. Aim to cover all the gel pieces with each solution.

1. Separate proteins and/or lysates by SDS-PAGE.
2. Fix with 10% Acetic acid / 50% methanol for 10 minutes at room temperature with gentle rocking. (see note 17)
3. Wash in HPLC grade water 3 x 5 minutes.
4. Move to a laminar flow hood and use new sterile scalpel blades for each section that will be excised from the unstained gel. Cut this piece further into approximately 1 mm cubes and add to a LoBind Eppendorf. (see note 18)
5. Dehydrate samples by adding 300 µl of 100% ACN to cover the gel pieces. Incubate at 37˚C for 10 minutes, 900 rpm on a thermoshaker. Pieces should shrink and become completely opaque, white and hard. Repeat with fresh ACN until this is observed.
6. Dry tubes completely using a SpeedVac for 5 minutes at 37 ˚C.
7. Reduce samples by adding 300 µl (plenty of excess) of 10 mM DTT and incubate at 56 ˚C for 1 hour at 900 rpm on a thermoshaker. Discard supernatant and allow samples to cool at room temperature for 5 minutes before proceeding to the next step.
8. Alkylate samples by adding 300 µl of 50 mM IAM. Incubate in the dark for 30 minutes, at room temperature, 900 rpm on a thermoshaker. Discard the supernatant.
9. Wash samples by adding 300 µl of 100 mM Ambic. Incubate for 15 minutes at room temperature, 900 rpm on a thermoshaker. Discard the supernatant.
10. Wash samples by adding 300 µl of 50 mM Ambic / 50% ACN. Incubate for 15 minutes at room temperature, 900 rpm on a thermoshaker. Discard the supernatant.
11. Dehydrate the gel slices by adding 300 µl of 100% ACN. Incubate for 5 minutes at room temperature, 900 rpm on a thermoshaker. Discard the supernatant. Repeat this step as many times as necessary until the pieces are small, hard and opaque white.
12. Dry tube completely by SpeedVac for 5-10 minutes at 37 ˚C.
13. Make up a single stock of 2.5 ng/µl trypsin to cover all samples (typically 60-90 µl per sample), plus excess, in reaction buffer. (See note 19)
14. Add 1 volume (~60-90 µl) trypsin to each tube and incubate for ~16 hours at 37 ˚C, checking after the first hour that all pieces are still covered (see note 20). If they are not, top up with reaction buffer by adding the minimum possible volume to cover all pieces. There is some flexibility in total incubation time but aim to stick between 14-18 hours. **Do not throw away any supernatant now, it contains the peptides.**
15. Add 1 volume (i.e. the total volume of trypsin/reaction buffer that was added) of 100% ACN. Incubate for 30 minutes at 30 ˚C, 900 rpm on a thermoshaker.

16. Transfer the supernatant to a fresh LoBind Eppendorf. Repeat step 15 if pieces are not completely dehydrated.
17. Add 1 volume of freshly prepared 1% formic acid to gel pieces. Incubate at room temperature for 20 minutes at 900 rpm on a thermoshaker. Transfer the supernatant to the same Eppendorf as in step 16.
18. Repeat step 17.
19. Add 1 volume of 100% ACN to the gel pieces. Incubate at room temperature for 10 minutes at 900 900 rpm on a thermoshaker. Transfer the supernatant to the same Eppendorf as in step 16. Repeat until gel pieces shrink and turn white.
20. SpeedVac the peptides at 37 ˚C to dry them (usually takes overnight). Do not leave them drying longer than necessary. These can be stored at -20 ˚C.

3.2.3. C-18 desalting using ZipTip with C_{18} Resin

Cleaning up of samples can be carried out on a general laboratory work bench. Throughout the procedure you must be careful to maintain a small amount of liquid above the C_{18} bed at all times to stop the resin drying out and compromising peptide yield. Two ZipTips are needed per sample, in which 10µL aliquots of each sample are processed one at a time and the eluents pooled into a fresh LoBind Eppendorf.

1. Resuspend extracted peptides in 10-20 µL 0.1% TFA.
2. Wet the C18 bed by aspirating and ejecting 2 X 10µL 100% ACN.
3. Equilibrate by aspirating and ejecting 3 X 10 µL 0.1% TFA.
4. Bind the peptides to the bed by aspirating and ejecting in a **controlled manner** (not at full pipetting speed) 10-12 times.
5. Wash the bed with 5 X 10µL 0.1% TFA.
6. Elute the peptides by aspirating 10µL 75% ACN/0.1% TFA and pumping up and down 6-7 times in a collection tube. Push the last one through fully and repeat with second ZipTip.
7. Dry down samples in SpeedVac (~1 hour, do not over-dry) and store at 4 ˚C until Mass Spec analysis, see 3.3.4.

3.2.4. Identification of suitable peptides and transition ions

1. Process 100-200 ng of each protein by SDS-PAGE and in-gel digest
2. Resuspend dried peptides in 0.1% formic acid
3. Wash trap with 2% acetonitrile / 0.5% formic acid for 10 minutes at a flow rate of 2.5 µl/minute before switching in-line with the analytical column.

4. Run a gradient of 5−50% acetonitrile / 0.1% formic acid (v/v) over 90 minutes at a flow rate of 300 nL/minute.
5. Clean the column by increasing ACN concentration to 80% for 10 minutes, then re-equilibrate with 5% acetonitrile / 0.1% formic acid for 10 minutes.
6. Operate the mass spectrometer in positive ion mode (Analyst TF1.7) with survey scans of 250 ms, MS/MS accumulation time of 100 ms and with monitoring of the 25 most intense ions (total cycle time 2.75 seconds).
7. Search data using ProteinPilot 5 software (Sciex) against the latest UniProt database with biological modifications allowed and iodoacetamide as the cysteine alkylating reagent. Use the reversed database as a decoy to determine the false discovery rate (FDR) for protein identification.
8. Use these empirical data to select potential transitions for multiple reaction monitoring (MRM) of the Ras isoforms. Choose proteotypic peptides that are either present in all isoforms or are specific to each isoform or mutant variant. Select up to 3 different charge states and up to 7 different fragment ions for optimisation of the mass spectrometric parameters.

3.2.5 Optimisation of MRM Transitions

In order to achieve the greatest sensitivity and specificity of the MRM detection of targets, the mass spectrometry method can be fine-tuned for each analyte. A range of settings for parameters such as the collision energy, declustering potential etc is applied to a test sample and those settings giving optimal signal-to-noise are then incorporated into the final working method. The use of a QTRAP instrument capable of MRM-triggered full-scan MS/MS acquisition increases the confidence that the quantification has been performed on the correct targets.

1. Run 100-200 µg of each heavy isoform. For example, in four tubes mix a heavy HRAS, a heavy KRAS4A, a heavy KRAS4B or a heavy NRAS each with light KRAS4B. Process by SDS-PAGE and in-gel digest, followed by desalting.
2. Reconstitute dried peptides in 0.1% formic acid.
3. Wash the trap with mobile A for 5 minutes at a flow rate of 15 µl/minute before switching in-line with the analytical column.
4. Run a gradient of 2−50% mobile B over 45 minutes at a flow rate of 300 nl/minute.
5. Clean the columns by increasing mobile B to 99% for 15 minutes, then re-equilibrate with mobile A for 15 minutes.
6. Operate the mass spectrometer in positive ion mode using Analyst TF1.6 software (Sciex) and use the MIDAS approach (MRM-initiated detection and sequencing) to quantify and confirm the identity of the analytes of interest. An enhanced resolution

scan at 250Da/s is used to calculate the optimal collison energy on the fly for up to 3 MS/MS scans at 10,000 Da/second.

7. A range of different settings for collision energy, collision cell exit potential and declustering potential should be assessed for each of the transitions chosen from empirical MS/MS data. The transitions and settings that provided the greatest sensitivity of detection can be carried forward to the final MS method.

Figure 4 depict the positions of the diagnostic peptides that we use for Ras and Ral. Figure 5 gives an example of the transition spectra produced from a diagnostic Ras peptide. Table 1 details all of the transitions that we use for each diagnostic Ras and Ral peptide.

3.2.6. Calibration curves to determine spike-in concentrations

Before quantification of endogenous protein levels can be determined, linearity between the target protein peptide abundance and MS response needs to be confirmed. Mixing each heavy standard at a 0.05 to 1 ratio of 10 ng with cell lysates to generate a calibration curve will allow you to deduce the appropriate concentration to spike in samples when compared with the endogenous peptide readouts. For the quantitation experiments, the endogenous proteins should fall within the range of this calibration curve.

1. Mix 100 ng of each heavy and 100 ng of a single light isoform, such that each sample contains 1 heavy isoform and 1 light isoform with the same light protein used for comparison and calibration across all the heavy isoforms. For example, in four tubes mix a heavy HRAS, a heavy KRAS4A, a heavy KRAS4B or a heavy NRAS each with light KRAS4B.
2. Carry out an in-gel digestion as outlined in 3.2.2 and run on MS as described above (3.2.5).
3. Use the ratio of a common heavy peptide to the corresponding light peptide to calibrate all the different heavy protein stocks to the single light standard. For example, if a heavy peptide signal is 0.9 x that of the light standard then it is 0.9 x as concentrated as previously thought. Use these calibrated values moving forward.
4. Make separate samples containing 20 µg cell lysate with 0.05, 0.25, 0.5, 1, 2, 5 and 10 ng of each heavy Ras/ Ral proteins from the calibrated concentrations in step 3 (see note 21).
5. Carry out an in-gel digestion as outlined in 3.2.2 and run on MS as described above (3.2.5).
6. For Ras, 1 ng each of HRas, KRas4A and NRas, and 2 ng of KRas4B are used and for Ral, 1 ng of RalA and 1 ng of RalB will be spiked into each 20 µg lysate sample.

7. Plot the curves as mean area under curve (AUC) vs ng or moles of heavy standards (see section 3.3.5. for description of molar calculations from grams). The equation of the line of best fit is used to calculate the endogenous protein concentrations in the quantitation experiments in section 3.3.

3.3. Quantification of endogenous Ras or Ral levels in cell lysates

Once the amounts of heavy standards to spike into a given amount of lysate has been determined, the quantitation experiments can be performed.

3.3.1. Harvesting Cells

1. All types of adherent or suspension cells can be assayed. For adherent cells, grow in 10 cm dishes to 80% confluence (or note confluence if different to ensure assaying of equivalent confluence between biological repeats). Typically, a minimum of 1,000,000 cells per assay point are used.
2. Dissociate cells using your preferred reagent: for example, 1 ml of 1X 0.5% trypsin-EDTA.
3. Gently pellet the cells by centrifuging at 150 x g for 5 minutes.
4. Aspirate the supernatant and resuspend the cell pellet in 9 ml of medium supplemented with the serum conditions that the cells are normally maintained in. Pipette thoroughly to break up clumps.
5. Transfer 9 ml immediately to a fresh centrifuge tube for collecting the cell pellet (Section 3.3.2).
6. Use the well-mixed remainder for counting. Do the counts quickly whilst the 9 ml of cells are being centrifuged. Using a haemocytometer take an average of two counts (typically these should be within 5% of each other). Cell counting is optional but is necessary if you want to calculate protein copy number per cell.

3.3.2. Collecting Cell Pellets

1. Centrifuge the cells at 150 x g for 5 minutes at room temperature.
2. Aspirate off the media.
3. Flick the pellet to loosen and then resuspend in 10 ml of ice cold PBS.
4. Centrifuge again at 150 x g for 5 minutes at 4 ˚C.
5. Flick pellet to loosen, resuspend gently in 300 µl of ice cold PBS using wide bore tip or a 1ml tip with the end snipped off. Transfer to a LoBind Eppendorf on ice.
6. Remove the tip and set aside. Use a fresh tip to add 300 µl ice cold PBS to the centrifuge tube. Add the wide bore/trimmed tip back onto the pipette, mix the PBS well and add to the same Eppendorf.

7. Spin at 1000 x g for 5 minutes, 4 ˚C.
8. Remove all PBS (use gel loading tips for last bit to avoid aspirating cells).
9. either: i) snap freeze in liquid nitrogen and store at -80 ˚C, or ii) lyse on ice in NP40 lysis buffer.

3.3.3. Cell Lysis and Spike-in

Before proceeding with experiments perform a Western blot on 10% self-poured SDS-PAGE gels to determine the location of the endogenous protein of interest on the gel in relation to the pre-stained markers and define which markers to cut at (see note 13). This calibration step is important for all subsequent MS analysis runs when gel fragments have to be excised that are as small as possible but contain all of the protein of interest.

1. Resuspend cells in 140-300 µl of NP40 lysis buffer (see note 22).
2. Incubate on ice for 10 minutes.
3. Centrifuge at 17,000 x g for 15 minutes at 4˚C.
4. Collect the supernatant and transfer to a new Eppendorf being careful not to disturb the pellet.
5. Determine protein concentration by BCA assay
6. Spike-in pre-determined concentrations (see concentration checks section 3.1.6.) of heavy standards with 20 µg of cell lysate (we typically make 1.2 x sample to allow excess).
7. Prepare sample for gel by boiling as described in section 3.1.3.
8. Run sample on a self-poured 15 well, 1 mm, 10% BisTris SDS-PAGE gel at 100V until all rainbow markers can just be seen in the gel (the pink 12kDa marker at the bottom will just appear out of the dye front). Run alternating lanes of samples and rainbow marker so that you can easily cut out the right segment of the sample lane (12-38 kDa for Ras, 17-31 kDa for Ral). Figure 6 shows the migration of endogenous and His-tagged Ras proteins as an example.
9. Snap freeze the remainder of the lysate and store at -80 ˚C.

3.3.4. Mass spectrometric quantification of endogenous proteins and spiked in standards

1. Reconstitute desalted samples in 11 µl of 0.1% formic acid and deliver 5 µl aliquots into the MS as described above.

   In our experiments, the dwell time for each transition was 20 ms. As before, the charge status of each precursor ion was determined using an enhanced resolution scan at 250 Da/second and up to 3 MS/MS scans at 10,000 Da/second were triggered with dynamic fill time. This gave a total cycle time of 3.9 seconds.

3.3.5. Calculation of endogenous protein concentration from MRM data

The calibration curves established in section 3.2.6. are used to determine the endogenous concentration from the AUC calculated in section 3.3.4.

1. Use the equation of the calibration curve to determine the number of moles of protein per lane. As the calibration curve was based on ng of heavy standards, the accurate molecular weight of the heavy standards and endogenous protein is needed. This can be obtained by entering the protein sequence into ProtParam on the ExPasy webpage. As the heavy standard is larger than the endogenous protein, the quantity of endogenous protein in ng determined from the line of best fit needs to be adjusted by multiplying by the ratio of $Mw_{endogenous}/Mw_{standard}$. Use the Mw to determine the number of moles per lane (protein in grams divided by Mw in Daltons), then the number of moles per µg lysate (divide moles per lane by µg lysate per lane).
2. Determine the number of cells per µg lysate generated in section 3.3.3 (divide the number of cells in the pellet by the total no. of µg lysate yielded).
3. Calculate the number of moles per cell by dividing the result of step 1 by the result of step 2.
4. Finally determine the number of molecules of the small GTPase per cell by multiplying the result of step 3 by Avogadro's number.
5. The total number of molecules in the Pan peptide should approximate the sum of those from the isoform-specific peptides if all isoforms have been accounted for.
6. We typically generate a mean value from 3 experiments for our quantitation.

4. Notes

1. Detergent can contaminate the samples, damaging the chromatography column.
2. Some amino acids are not easily soluble. We find that making a dilute solution and stirring for 30-60 minutes helps, and for the hydrophilic mixture specifically that HCl helps (add this very slowly with plenty of stirring since too much HCl could affect the final osmolarity of the media). Cysteine oxidises rapidly to cystine in aqueous solution so we make the amino acid solutions up on the first day of use. Lysine and Arginine are also stable when reconstituted in PBS at 75-100 mg/ml and frozen at -20 ˚C for a few months.
3. Our plasmids for protein expression in bacteria all carry an ampicillin resistance gene so we have assumed ampicillin resistance throughout, but if using plasmids carrying additional or alternative resistance genes, the method should be altered accordingly.
4. We found that AT713 upregulate ZinT in response to zinc starvation. This protein is

approximately 25 kDa and interacts with various metal ions. It can be erroneously purified on His-Trap columns and easily mistaken for Ras and Ral proteins due to the similar molecular weight. Addition of Zinc ions to the media resolved this issue. The presence of other trace metals and thiamine further improves growth of the bacteria.

5. The use of an automated HPLC system and fractionator is described here, but in the absence of access to this a gravity-based column or batch approach could be used. The buffers described are appropriate for the HisTrap columns used but if alternative affinity resin is used, the relevant manufacturer's instructions should be followed.
6. Only wash short and spacer plates with water and ethanol to avoid detergent contamination of protein samples run on gels.
7. A small initial culture in LB is used to get the bacteria started, but only a minimal amount of this is carried over into an overnight culture with the heavy media in order to minimize the amount of unlabeled material that may appear in the final prep.
8. Imidazole is inhibitory to lysozyme so must be added after this stage.
9. If you plan to process the pellets immediately after induction, the next stopping point will be after the elutions have been collected from the His-Trap columns.
10. A superloop can be used to load a large volume of lysate (we typically yield approximately 24 ml after centrifugation and filtration) in a single batch. Alternatively, a smaller sample loop can be used to repeatedly apply batches of lysate by until all has been loaded. Either works effectively for an affinity column.
11. We accepted the purity to be sufficient if no additional bands were observed by eye on a Coomassie stained gel. If it proves impossible to eliminate additional bands an estimate should be made of what percentage of the total protein concentration is due to contaminant proteins and should be adjusted for in concentration determination.
12. Proteins can be stored in other buffers if desired. We found that storage directly in sample buffer helped to minimize loss of these low concentration pure proteins due to precipitation during thawing. In our hands, aliquots could be freeze thawed a few times without issue, but we recommend minimizing this.
13. Note that the migration of Ras and Ral bands in Figure 3 are higher than in Figure 6 due to the use of a NuPAGE gradient gel system in Figure 3 versus a self-poured, non-gradient gel format in Figure 6. It is important to formally verify the migration of endogenous target proteins and tagged-protein standards in the same gel system that you intend to use for the final absolute quantitation experiments so that the correct piece of gel is excised to capture all of the target proteins and standards.
14. We selected two batches of final protein stock after gel filtration, based on the presence of apparent additional bands. The fractions to combine should be empirically determined based on maximizing yield and minimizing contamination.

15. We find that checking a small volume using a nanodrop spectrophotometer is helpful in reducing wastage by informing us of the approximate volumes needed for the protein assay.
16. In our experience, the amount of pure protein yield varies between His-Ras proteins. We obtained the lowest yield for His-HRas and His-NRas (15 µg across batches 1 and 2, and 28 µg across batches 1 and 2, respectively). We found that His-KRas4B wildtype generated the highest yield (400 µg across batches 1 and 2). With all the proteins there is a compromise to be struck between yield and purity. By prioritizing purity in our experiment, we accepted a low yield.
17. If you wish to observe the proteins on the gel, stain with Coomassie. Gels can still undergo in-gel digestion following destaining with several changes of 50 mM Ambic / 50%ACN washes (fixing is not required).
18. Move the gel onto the lid with a little bit of HPLC water to keep the gel slightly moist when cutting, otherwise the pieces become charged with static electricity and can be easily lost. Place a clean white sheet of paper underneath the dish for easier visibility. It is recommended to use a new sterile scalpel for cutting out each band to avoid protein cross-contamination.
19. Decide the volume per sample based on the volumes needed thus far, bearing in mind that the gel pieces will soak in the buffer to rehydrate.
20. Preferably keep samples in trypsin incubating where lid is kept warm, such as a cell incubator, to prevent evaporation of samples.
21. Using 20 µg of cell lysate is the optimal quantity for both PSAQ and loading into the self-poured gels. Including cell lysate in the generation of the calibration curve does make the heavy standards more difficult to detect, especially at the lower ratios, giving an accurate representation of detection levels in an experiment. However, if you wish to run the standards alone, a polynomial trendline may be observed as opposed to linear.
22. NP40 lysis buffer is used instead of RIPA because we experienced contamination of our LC columns from Triton X-100 that had not been successfully removed from the samples during the in-gel digestion process. It should be noted that lysate concentration and subsequent protein quantification can vary between different lysis buffers due to different level of extraction from different organelles, for example RIPA buffer disrupts nuclei whereas NP40 does not.

Figure 1. *An overview of the PSAQ method for quantify protein abundance.*

Figure 2. *Representative elution profiles for His-tag and size exclusion-based chromatography of GTPase purifications.* Nickel-based enrichment of His-tagged proteins typically retains contaminating proteins that require further purification steps to achieve sufficient purity for use as an isotope-labelled protein standard for use in protein quantitation.

Figure 3. *High-purity Ras and Ral protein standards.* Coomassie staining of the whole gel reveals no contaminating proteins. 200 ng of each protein loaded per lane.

Figure 4. *Selected proteotypic RAS and RAL peptides.* All peptides produced following trypsin digestion are indicated. The resultant peptides that are specific for individual isoforms or shared by all isoforms (Pan) are highlighted. Peptides that are used to quantify protein abundance are shown together with their sequences. The dotted line in the first RALA-specific peptide indicates a missed cleavage that is consistently observed.

Figure 5. *MS/MS transition spectra for the diagnostic NRAS peptide.* Transitions are chosen based on signal-to-noise and sensitivity to allow robust discrimination versus other peptides with similar *m/z* in the cell lysate. Generally, higher mass range transitions are preferred due to the higher likelihood that they will be selective for the parent peptide.

Figure 6. *Determining gel migration for accurate excision of target protein and standards.* The His-tag results in a band shift that must be accounted for when determining where to cut the gel. Pre-stained ladders guide the gel excision and also separate the lanes to avoid cross-contamination of samples. Note that the addition of the protein standard to the lysate does not result in a noticeable additional band on the Coomassie gel. For the Western blots, 5 ng Ras/Ral and 15 µg SW48 lysate are loaded in the indicated lanes and for the Coomassie gels, 40 ng Ras and 8 µg SW48 lysate are loaded in the indicated lanes.

Table 1. *Transitions for proteotypic Ras and Ral peptides.* Transitions are generated from the indicated peptides representing endogenous proteins (Lys0 Arg0) and isotope-labelled (Lys8 Arg10) standards.

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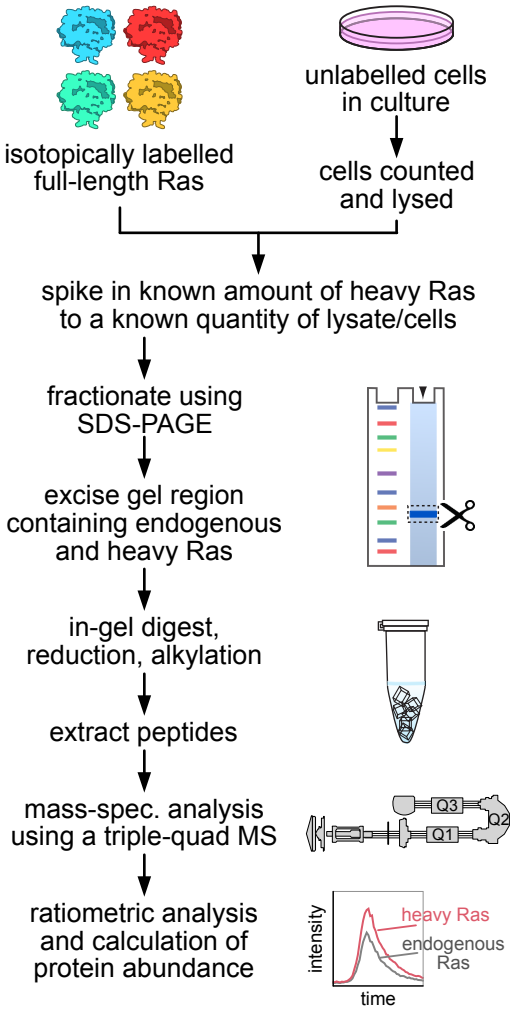
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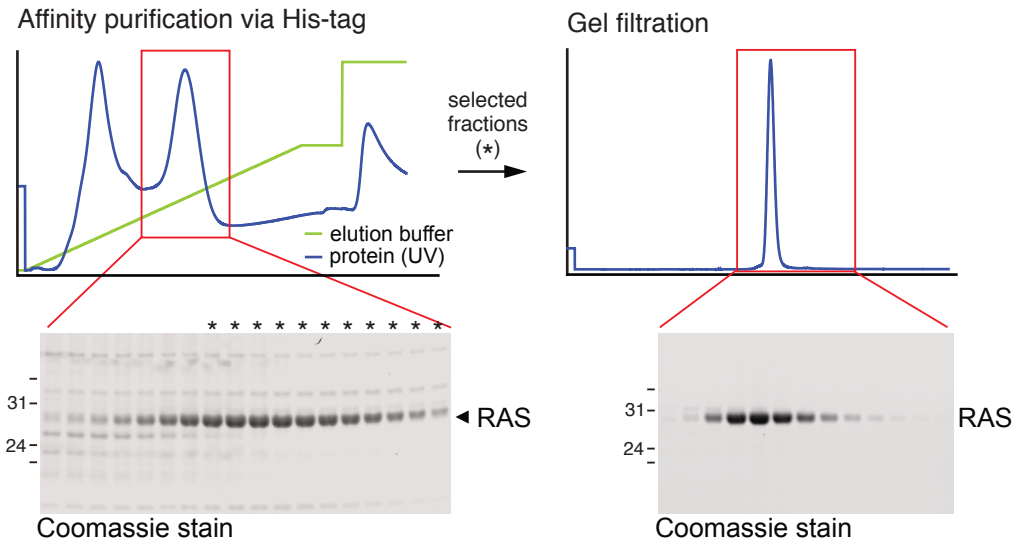
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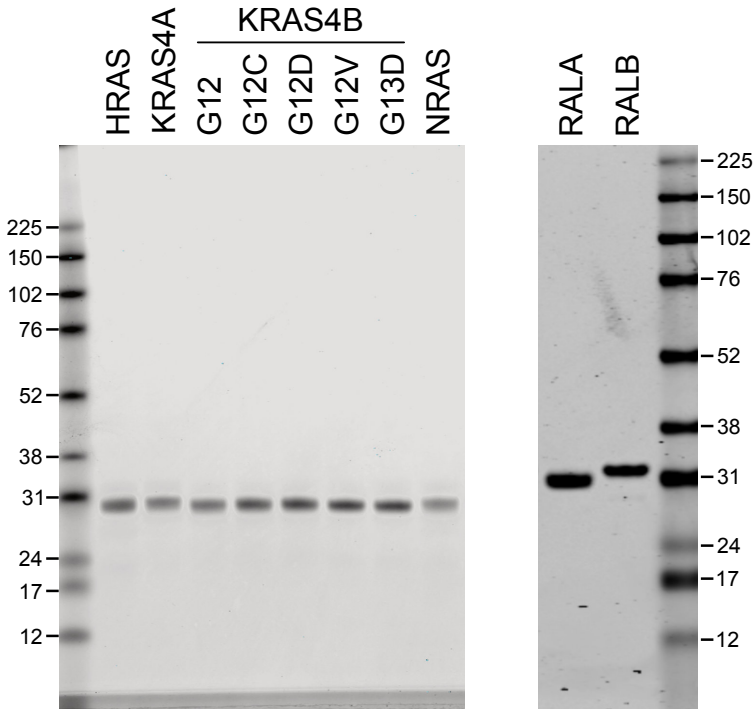
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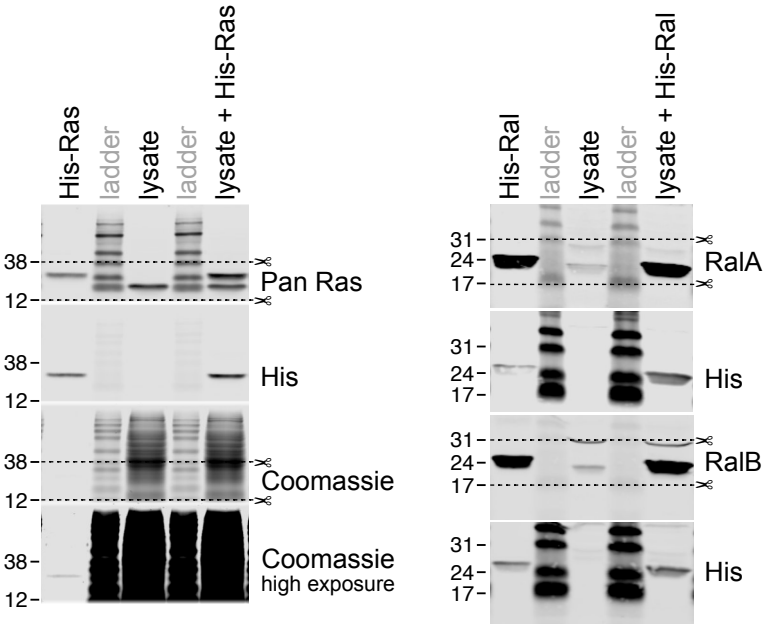
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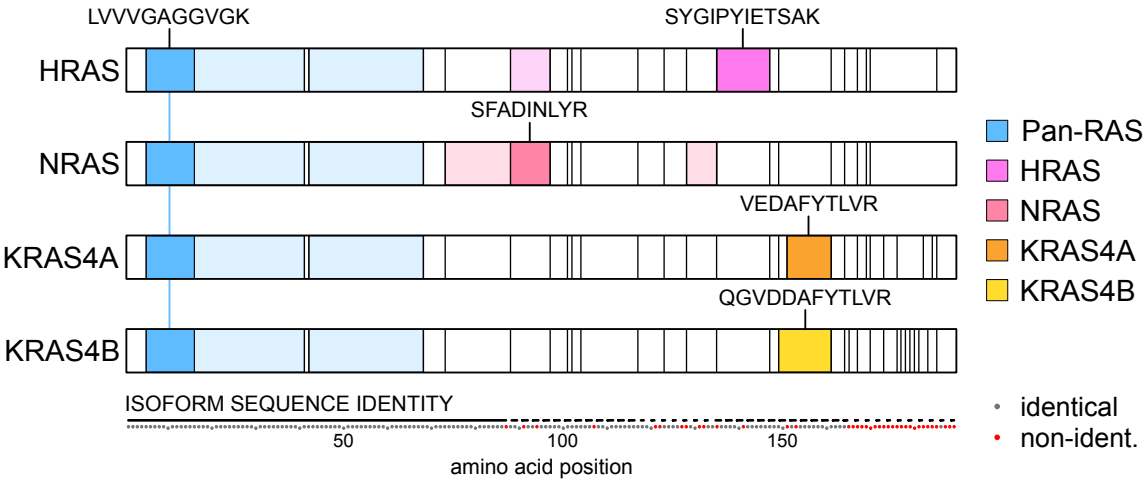
RAS proteins



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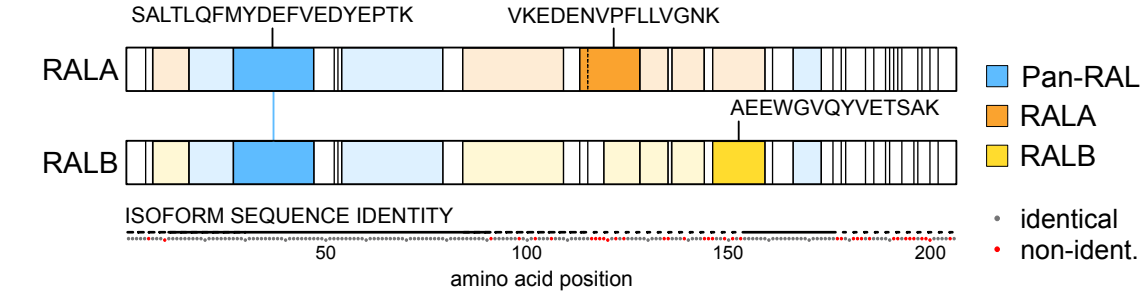

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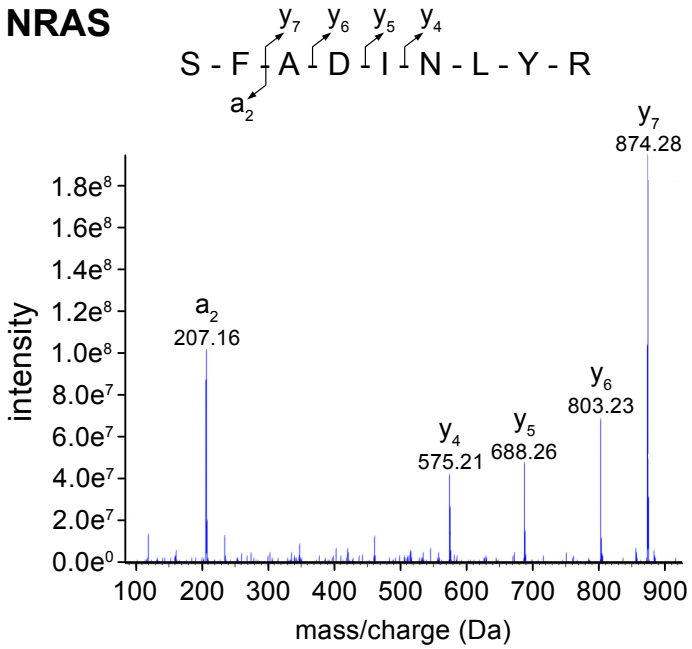
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mass/charge (Da)

RAS	PEPTIDE	CHARGE	PRECURSOR ION (*m/z*)		PRODUCT ION (*m/z*)		ION
			K0 R0	K8 R10	K0 R0	K8 R10	
Pan	LVVVGAGGVGK	+2	478.3	482.3	185.3	185.3	a2
					312.2	312.2	b3
					545.3	553.3	y7
					644.4	652.4	y8
					743.4	751.4	y9
H	SYGIPYIETSAK	+2	664.9	668.9	251.1	251.1	b2
					308.1	308.1	b3
					406.1	414.1	y4
					908.5	916.5	y8
K(A)	VEDAFYTLVR	+2	606.8	611.8	229.1	229.1	b2
					488.3	498.3	y4
					651.4	661.4	y5
					798.5	808.5	y6
					869.5	879.5	y7
K(B)	QGVDDAFYTLVR	+2	692.4	697.4	285.1	285.1	b3
					651.4	661.4	y5
					789.5	808.5	y6
					869.5	879.5	y7
N	SFADINLYR	+2	549.8	554.8	207.1	207.1	a2
					565.3	575.3	y4
					678.4	688.4	y5
					793.4	803.4	y6
					864.5	874.5	y7

RAL	PEPTIDE	CHARGE	PRECURSOR ION (*m/z*)		PRODUCT ION (*m/z*)		ION
			K0 R0	K8 R10	K0 R0	K8 R10	
Pan	SALTLQFMYDEFVEDYEPTK	+3	809.4	812.1	345.1	353.2	y3
					752.2	760.3	y6
					881.3	889.4	y7
					980.4	988.4	y8
RALA	VKEDENVPFLLVGNK	+3	567.6	573.0	715.3	723.3	b6
					530.3	538.3	y5
					643.4	651.4	y6
					887.5	895.5	y8
RALB	AEEWGVQYVETSAK	+2	798.8	802.9	330.1	330.1	b3
					925.5	933.5	y8
					1081.6	1089.6	y10
		+3	532.9	535.6	535.3	543.3	y5
					634.3	642.3	y6
					797.4	805.3	y7