
Preface

In the last 10 years researchers have firmly established key roles for Ras-related GTPases in almost every aspect of cell biology. In the 1980s the proto-oncogene Ras itself was the focus of interest, though in the 1990s this shifted to the increasing variety of Ras-related proteins. In this new decade much yet needs to be done to establish the role for all the small GTPases now uncovered by the human genome project. In particular, these GTPases need to be understood in the appropriate biochemical and cellular contexts. In the process of trying to uncover the role of these versatile proteins, a variety of novel techniques and methodologies has been developed. These now enable investigators to move easily within a diversity of fields ranging from structural studies to real-time in vivo analysis of a GTPase.

In recognition of the need for access to key background methodologies, *GTPase Protocols: The Ras Superfamily* is devoted to techniques that are presently widely used and that will continue to be the standard for researchers worldwide. Each chapter is aimed at supplying detailed methodologies to allow reproduction in any laboratory, while also providing the general principles on which the methods are based. Some of the techniques grouped in the first section apply broadly to small GTPases, whereas others in Part II are more applicable within each GTPase subfamily.

We would like to thank the many authors who have spent time in sharing their expertise. It is hoped that *GTPase Protocols: The Ras Superfamily* will bring access to and understanding of these remarkable proteins for all those embarking on new studies.

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Preparation of GTPases for Structural and Biophysical Analysis

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1. Introduction

Members of the *Ras* superfamily of small GTPases (p21) are involved in the regulation of a large variety of key cellular processes, including cell differentiation and proliferation, membrane trafficking, and nuclear import and export. Based on sequence homology, this superfamily can be divided into the Ras, Rho, Ran, Arf, Rab, and Rad subfamilies, which all have distinct biological activities. All members of this superfamily act as molecular switches and become activated and capable of transducing a signal upon binding to GTP, while guanosine triphosphate (GTP) hydrolysis returns them to the inactive state. Most members of this superfamily are post-translationally modified and carry isoprenoids at their C-termini, which anchors them to the membrane.

Significant progress has been made over the last few years in the structural and biophysical characterization of many members of this superfamily. In particular, the structures of complexes of small GTPases with regulatory proteins, such as GTPase-activating proteins (GAPs) (*1,2*), guanine-nucleotide exchange factors (GEFs) (*3,4*), guanine-nucleotide dissociation inhibitors (GDIs) (*5,6*), and downstream effectors (*7–11*) has immensely increased our understanding of the regulation and function of these molecular switches (reviewd in *12,13*). With the exception of GDI complexes with Rac and Cdc42 (which have been expressed in insect cells), all the GTPases employed in these studies have been expressed and purified from bacterial sources.

A prerequisite for the biophysical and structural characterization of a single protein or multiprotein complex is the availability of significant amounts (>10 mg) of highly pure, homogeneous protein solutions. This chapter describes

the bacterial expression, purification and characterization of small GTPases for biophysical and structural studies. Although this chapter focuses on the expression and purification of members of the Rho subfamily, all the protocols described here are also applicable, with suitable modifications, to members of other subfamilies.

Ras was the first member of this superfamily to be extensively characterized by biophysical and structural methods. For these studies, *Ras* was often expressed from plasmids such as pTac and purified by classical methods, such as ion-exchange chromatography and hydrophobic-interaction chromatography (14,15), which can be highly time-consuming. Over the last couple of years, systems that enable expression of the protein of interest fused to a removable affinity tag, such as glutathione S-transferase (GST) or hexahistidine, have become widely available. These expression systems offer the advantage of a quick, one-step purification, which often yields high amounts of up to 90% pure protein, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and ultraviolet (UV)-spectra. The protein can subsequently be cleaved from the affinity tag by a variety of proteases (e.g., thrombin, factor Xa, or TEV protease), and separated from the tag and “polished” by gel filtration, resulting in a 95–99% pure protein. Expression and purification methods based on affinity tags are now widely used to produce proteins for biophysical and structural studies. This chapter describes the use of glutathione S-transferase and hexahistidine-tagged systems (16,17).

Because of their intrinsic GTPase rate, most wild-type small GTP-binding proteins are bound to GDP after purification. However, the activated GTP-bound form of the protein is often of interest, particularly when studying the interaction with regulatory proteins or down-stream effectors. Various methods exist to circumvent the problem of GTP hydrolysis; the bound GDP can be exchanged against non-hydrolyzable GTP analogs such as guanylylimidodiphosphate (GMPPNP) or guanosine 5′-(3-O-thio)triphosphate (GTP γ S), techniques which are described later in this chapter. Alternatively, mutant GTPases can be expressed with an intrinsic hydrolysis rate that is reduced enough to purify these proteins in the GTP-bound form. The most commonly used mutants are G12V and Q61L in the Rho or Ras numbering scheme. The use of mutant, GTPase-deficient proteins is the method of choice for co-expression and copurification of GTPase/effector complexes. The structure of the complex between Rab and its downstream effector Rabphilin is a very interesting example of this method, as in this particular case co-expression was the only way to prevent degradation of the effector protein in *Escherichia coli* (*E. coli*) (18). The nucleotide content of small GTPases can be analyzed by HPLC using ion-exchange or reversed-phase chromatography techniques

(19,20). Conditions for both types of chromatography are described at the end of this chapter.

Many small GTPases carry a polybasic sequence at their C-terminus. This can be a potential problem during expression in *E. coli* due to the proteolytic degradation of this sequence, which results in a mixture of proteins with heterogeneous C-termini. The Rho-family proteins Rac and Cdc42 are particularly susceptible to degradation of up to eight residues. We routinely carry out mass spectrometric analysis of the purified proteins to confirm their molecular weight and to detect possible proteolytic degradation. Most interactions of small GTPases with regulatory proteins and downstream effectors do not involve the C-terminus of the GTPase, and in these cases it is advisable to re-engineer the protein to remove those amino acids which are susceptible to proteolysis.

2. Materials

2.1. Expression of Recombinant p21s

Preparation of expression clones for small GTPases are carried out by standard cloning procedures. However, for the use of proteins in structural studies, certain points which are outlined in the following section should be taken into account when deciding on a cloning strategy.

2.1.1. Expression of GST-Fusion p21s

Recombinant Rho-family proteins are expressed as glutathione S-transferase (GST) fusion proteins in pGEX-2T or pGEX-4T (Amersham Pharmacia Biotech). For structural studies, it is advantageous to produce a protein with as few additional residues N-terminal to the initiating methionine as possible after cleavage from the GST-tag. Cloning into the vector BamH I site leaves only two additional amino acids after cleavage, and should be done whenever possible. However, we and others have experienced difficulties in thrombin cleavage of the fusion protein under these conditions. We have found that a single amino-acid insertion, introduced by PCR, can be sufficient to restore cleavage (the insertion of a histidine has worked well for us in many cases). This is preferable to the use of cloning sites further 3' to the *BamH I* site or vectors with a "spacer" sequence, which will add five or more amino acids. The *E. coli* strain BL21 should be used for expression of small GTPases in pGEX vectors because it lacks the bacterial proteases *lon* and *ompT*, which can contribute to the degradation of the polybasic C-termini of Rho-family proteins.

2.1.2. Expression of Hexahistidine-Tagged p21s

Various vectors are available for the expression of hexahistidine-tagged proteins. The major differences between these vectors are the specificity of the

protease cleavage site (thrombin, Factor Xa, enterokinase, or TEV protease) and the restriction sites present in the multiple cloning site. As discussed previously, the choice of vector and cloning strategy should leave as few extra residues as possible after protease cleavage. pET vectors containing a thrombin site are the vectors most widely used for the expression of small GTPases (e.g., 3,21–23), and require expression hosts that contain a chromosomal copy of the gene for T7 RNA polymerase, such as the *E. coli* strain BL21(DE3).

2.2. Purification

The pH of all buffers used during purification depends on the *pI* of the protein of interest and should be in the order of 0.5 pH units above or below the *pI* to maintain solubility (see **Note 1**). We routinely calculate the theoretical *pI* from the amino-acid composition (as well as mol-wt and extinction coefficient) using the “ProtParam” program on the Expasy server <http://www.expasy.ch/tools/protparam.html>.

Glutathione Sepharose 4B and the S-75 Hiload 26/60 column are from Amersham Pharmacia Biotech, and Ni-NTA superflow is from Qiagen. We usually pack our matrices in XK-16 columns and carry out all chromatographic steps using an FPLC or Gradifrac system (Amersham Pharmacia Biotech). However, any other chromatographic equipment (columns and pumps) can be used. Imidazole is from Fluka (>99.5% purity. If imidazole of lower purity is used, UV-absorption of the buffer may become a problem during purification). Human thrombin is from Calbiochem. The lyophilized thrombin is solubilized in H₂O (at 2 U/μL) and stored in aliquots of 100 U at –70°C. Thrombin adsorbs to glass surfaces and should only be stored in plastic containers.

2.2.1. Purification of GST-Fusion p21s

We use the following buffers, at pH 7.5, for the purification of Rho and Cdc42. For the purification of other GTPases, the pH of the buffer must be changed according to the *pI*.

1. Lysis buffer: 50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 10 mM MgCl₂, 4 mM DL-dithiothreitol (DTT), 4 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride (PMSF). Chilled previously to 4°C (see **Notes 2–5**).
2. Wash buffer: 50 mM Tris-HCl, pH 7.5, 1.0 M NaCl, 10 mM MgCl₂, 4 mM DTT, 4 mM benzamidine, 1 mM PMSF.
3. Thrombin-cleavage buffer: 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM MgCl₂, 4 mM DTT, 2.5 mM CaCl₂ (see **Notes 6 and 7**).
4. Gel-filtration buffer: 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM MgCl₂, 2 mM DTT.

2.2.2. Purification of His-Tagged p21s

1. Lysis buffer: 50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 10 mM imidazole, 5 mM MgCl₂, 4 mM β-mercaptoethanol, 4 mM benzamidine, 1 mM PMSF. Chilled previously to 4°C (see **Notes 2–5** and **8**).
2. Elution buffer: (see **Note 9**).
 - a. 50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 10 mM imidazole, 5 mM MgCl₂, 4 mM β-mercaptoethanol.
 - b. 50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 300 mM imidazole, 5 mM MgCl₂, 4 mM β-mercaptoethanol.
3. Thrombin-cleavage buffer: 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM MgCl₂, 2 mM DTT, 2.5 mM CaCl₂ (see **Note 7**).
4. Gel-filtration buffer: 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM MgCl₂, 2 mM DTT.

2.3. Nucleotide Exchange

Various methods exist to exchange the protein-bound GDP against non-hydrolyzable analogs. EDTA-facilitated exchange reactions are often used for the exchange against radioactive nucleotides. However, this method requires a large excess of nucleotide (50–100-fold) for a quantitative exchange. A less nucleotide-consuming exchange protocol uses alkaline phosphatase, which degrades GDP but leaves GMPPNP, GTPγS, and GMPPCP intact. The affinity of GMP for small GTPases is so low that a 10-fold excess of the nucleotide analog is sufficient for a complete exchange. We use alkaline phosphatase coupled to agarose beads (Sigma P-0762), which allows removal of the enzyme by centrifugation. PD10 Sephadex G25 columns are from Amersham Pharmacia Biotech. Centricon 10 concentrators are from Amicon Inc.

1. Exchange buffer: 40 mM Tris-HCl, pH 7.5, 200 mM (NH₄)₂SO₄, 10 μM ZnCl₂, 5 mM DTT. When preparing the reaction mixture, (NH₄)₂SO₄ should be added last to avoid precipitation of the protein.
2. PD10 buffer: 20 mM Tris-HCl, pH 7.5, 2 mM MgCl₂, 2 mM DTT.

2.4. Analysis of Bound Nucleotide

The completeness of the nucleotide-exchange reaction can be monitored by HPLC, using anion exchange or reverse-phase chromatography. Both chromatography methods work well for the separation of GTP, GDP, GMP, and GMPPNP, and it is up to the user to choose between the two. Reverse-phase chromatography has the disadvantage of containing acetonitrile, which is toxic in the mobile phase, while a high-salt buffer is used for ion-exchange chromatography which can lead to salt deposits in the HPLC pumps.

The ion-exchange Partisil 10 SAX column is from Whatman and the reverse-phase ZORBAX SB-C18 column is from Rockland Technologies, Inc. KH_2PO_4 and K_2HPO_4 are from BDH, "HiperSolv for HPLC" quality.

1. Ion-exchange buffer: 0.6 M $\text{NH}_4\text{H}_2\text{PO}_4$, pH 4.0.
2. Reverse-phase buffer; 100 mM $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$, pH 6.5, 10 mM tetrabutylammonium bromide, 8.5% acetonitrile.

3. Methods

3.1. Expression of Recombinant p21s

3.1.1. Expression of GST-Fusion p21s

BL21 cells are transformed using standard procedures. A single colony is picked and spread on a plate containing 100 $\mu\text{g}/\text{mL}$ ampicillin, in order to form a lawn, and incubated at 37°C overnight (one plate per flask is prepared). The next morning, 5 mL of broth (LB or terrific broth) containing 100 $\mu\text{g}/\text{mL}$ ampicillin are withdrawn from 750 mL in a 2-L flask, used to resuspend the bacterial lawn and are returned to the flask. We usually grow 6 \times 750 mL for a standard protein preparation. Cells are grown at 37°C to an A_{600} of 1.0, and protein expression is induced by addition of 0.35 mM IPTG. The incubation temperature is lowered to 28°C, and the cells are harvested after 3 h of growth by centrifugation at 4500g for 20 min at 4°C in a Beckman J6-MC centrifuge. 750 mL of culture (using terrific broth) generally yields 6–8 g of wet cell pellet, which can be stored at –70°C for several months.

3.1.2. Expression of His-Tagged p21s

Antibiotic selection depends on the specific vector used. All other procedures are carried out as described in **Subheading 3.1.1.**

3.2. Purification

All subsequent steps are performed at 4°C. We recommend removing aliquots at each step of this protocol to follow the success of the purification by SDS-PAGE analysis. Cell lysis can be carried out using a variety of techniques, including treatment with lysozyme, homogenization (liquid extrusion), or sonication. The latter method is routinely used in our group.

3.2.1. Purification of GST-Fusion p21s

1. About 40 g of cell pellet is resuspended in 280 mL of lysis buffer (7 mL per g of cell pellet).
2. Cells are lysed by sonication on ice in 6 \times 30 second bursts, separated by 1-min breaks to prevent overheating of the sample. The lysate is clarified by

centrifugation at 25,000g for 1 h at 4°C in a Beckman L8-70 m Ultracentrifuge using a 45 TI rotor.

3. The glutathione Sepharose matrix is equilibrated with 10 column volumes of lysis buffer. The manufacturer recommends the use of 1 mL of glutathione Sepharose per 5 mg of fusion protein. However, we have previously recovered up to 10 mg of cleaved protein from 1 mL glutathione Sepharose (*see Note 10*).
4. The supernatant from **step 2** is loaded onto the column at a flow rate of 1 mL/min.
5. The column is washed with 10-column volumes of wash buffer containing 1 M NaCl at a flow rate of 1 mL/min to remove nonspecifically bound proteins.
6. The column is washed with 10 column volumes of thrombin cleavage buffer (*see Note 6*).
7. Cleavage of the GTPase from the GST tag is carried out by addition of 200 U of thrombin to 25 mL of cleavage buffer (use approx 4 U/mg protein). This buffer is recirculated through the column at a flow rate of 0.3 mL/min for 12–16 h.
8. The column is washed with cleavage buffer at 1 mL/min until all cleaved protein is eluted (*see Note 11*).
9. Generally, we have found it necessary to carry out a further purification step. When the protein appears pure by SDS-PAGE, we have quite often found significant contamination by DNA, observable spectrophotometrically as an absorbance peak at 260 nm.
10. The protein is concentrated in an Amicon pressure cell, using a regenerated cellulose membrane with a mol-wt cutoff of 10 kDa (YM 10).
11. A maximum of 40 mg of protein are loaded in a volume not exceeding 6 mL onto an S-75 Hiload 26/60 gel filtration column, which is run at a flow rate of 1.5 mL/min. Rho family proteins elute after 160–170 mL on this column.
12. Peak fractions are concentrated to 20–30 mg/mL in an Amicon pressure cell.
13. The concentrated protein is characterized by SDS-PAGE (*see Note 12*), UV spectro photometry (*see Note 13*), and mass spectrometry, flash-frozen in liquid nitrogen as 100- μ L aliquots, and stored at -70°C .
14. The glutathione Sepharose can be regenerated by a wash with 5 column volumes of 10 mM reduced glutathione in 50 mM Tris, pH 7.5, followed by 5 column volumes of 3.0 M NaCl. Glutathione Sepharose is stored in 0.02% sodium azide at 4°C (*see Note 10*).

3.2.2. Purification of His-Tagged p21s

1. Approximately 40g of cell pellet are resuspended in 280 mL of lysis buffer (7 mL of buffer per g of wet cell pellet).
2. Cells are lysed by sonication on ice in 6 \times 30 second bursts, separated by 1-min breaks to prevent overheating of the sample. The lysate is clarified by centrifugation at 25,000g for 1 h at 4°C in a Beckman L8-70 m Ultracentrifuge using a 45 TI rotor.
3. A 25-mL column of Ni-NTA Superflow (capacity 5–10 mg/mL) is equilibrated in lysis buffer.

4. The supernatant from **step 2** is loaded onto the Ni-NTA column at a flow rate of 1 mL/min.
5. The column is washed back to baseline with elution buffer A at a flow rate of 2 mL/min.
6. Bound fusion protein is eluted with an increasing gradient from 10–300 mM imidazole (elution buffer B) over 5–10 column volumes at a flow rate of 2 mL/min.
7. Peak fractions are pooled, CaCl₂ is added to 2.5 mM, 200 U of thrombin are added, and the solution is dialyzed twice (using a 8–10-kDa cutoff membrane) against 2 L of thrombin cleavage buffer overnight.
8. The mixture is incubated with 2 mL of Ni-NTA Superflow to remove uncleaved fusion protein, and is concentrated in an Amicon pressure cell.
9. The subsequent purification by gel filtration is carried out as described in **Subheading 3.2.1**.
10. The Ni-NTA column is regenerated according to the supplier's instructions.

3.3. Nucleotide Exchange

Generally, we perform the nucleotide exchange on approx 10 mg of the GTPase in a 1-mL reaction mixture. The time-course of the exchange reaction can be followed by nucleotide analysis on HPLC as described in **Subheading 3.4**. We found that the exchange reaction is normally complete after 1 h.

1. The concentrated protein is incubated in exchange buffer in the presence of a 10 M excess of GMPPNP and 2–4 U of alkaline phosphatase per mg GTPase. Alkaline phosphatase beads are washed twice with 20 mM Tris-HCl, pH 7.5 prior to addition.
2. Incubation is for 1 h at room temperature with agitation.
3. The reaction mixture is centrifuged in a benchtop microfuge to pellet the alkaline phosphatase beads. In order to remove the excess unbound nucleotide from the reaction mixture, the sample is applied to a PD10 Sephadex G25 column equilibrated in PD10 buffer, according to the manufacturer's instructions. Next, 300 μ L fractions are collected and analyzed for protein content by spotting 2 μ L of each fraction onto a filter paper and staining the filter in Coomassie blue SDS gel-staining solution. Fractions containing protein will appear as blue dots.
4. Protein-containing fractions are pooled, concentrated to 20–30 mg/mL using a Centricon 10, flash-frozen in liquid nitrogen, and stored at -70°C .

3.4. Analysis of Bound Nucleotide

To analyze the nature of the nucleotide bound to small GTPases by HPLC, the protein must be denatured and removed from the sample. This is achieved by incubation with perchloric acid and subsequent centrifugation to pellet the denatured protein.

1. Sufficient volume to contain a minimum of 1 nmol of protein should be withdrawn from the reaction mixture and brought up to a volume of 40 μL with PD10 buffer. Next, 2.5 μL 10% perchloric acid is added to this solution to denature the protein. The pH is raised by the addition of 1.75 μL 4 M CH_3COONa (sodium acetate) pH 4.0.
2. Precipitated protein is spun out in a benchtop microfuge for 2 min, and the supernatant containing the nucleotide is loaded onto the HPLC column.

3.4.1. Ion-Exchange HPLC

1. The column is run in 0.6 M $\text{NH}_4\text{H}_2\text{PO}_4$, pH 4.0, at 1 mL/min, and nucleotide absorbance is measured at 254 nm. The HPLC should be calibrated with solutions of known nucleotide composition.
2. Retention times: GDP: 4.8 min; GTP: 13.2; GMPPNP: 9.8 min.

3.4.2. Reverse-Phase HPLC

1. Better results are achieved using this method if the nucleotide solution is diluted 1:1 with reverse-phase buffer minus acetonitrile prior to injection onto HPLC (see **Note 14**).
2. The column is run isocratically in reverse-phase buffer at a flow rate of 1 mL/min, and nucleotide absorbance is measured at 254 nm.
3. Retention times: GDP: 5.3 min, GTP: 8.1 min, GMPPNP: 6.0 min.

4. Notes

1. The *pI* of a GST-fusion *p21* may be significantly different from that of the cleaved protein, making it necessary sometimes to alter the pH of the buffers during the course of purification in order to compensate for this. For example, glutathione S-transferase (GST) has a *pI* of 6.2, and Rac1 has a *pI* of 8.8, resulting in a *pI* of 8.1 for the fusion protein. We carried out the purification of Rac at a pH of 7.5, which seemed adequate. However, we regularly experienced large losses of protein because of precipitation after cleavage and concentration. Mass spectrometric analysis revealed that the protein was proteolytically degraded by eight residues at the C-terminus, resulting in a protein with a *pI* of 7.5. When the pH of the cleavage buffer was adjusted to 7.0 or 8.0, 100% of the cleaved protein was retained in solution. However, when selecting an appropriate pH for the cleavage buffer, the pH range at which thrombin is maximally active (pH of 7.5–8.4) should also be considered.
2. The pH of Tris-HCl changes significantly with temperature, and the pH at 4°C is more than 0.5 pH U higher than that of the same buffer at 25°C. For this reason, the pH should be adjusted at the temperature at which the buffer will be used.
3. All buffers are chilled to 4°C before use. DTT or β -mercaptoethanol should be included in all buffers to keep cysteines reduced. DTT and protease inhibitors are made freshly and added to buffers immediately before use. Use of the protease

inhibitor 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF, commercially available as Pefabloc SC) should be avoided because the protein can become irreversibly modified by this reagent (unpublished data).

4. Having a relatively high ionic strength in the buffer helps to prevent unspecific binding of proteins to the column. If necessary, the salt concentration can be increased up to 2 M for purification of His-tagged proteins on Ni-NTA.
5. MgCl₂ should be kept in all buffers, as it increases the affinity of the nucleotide for the protein. Nucleotide-free GTPases are often unstable.
6. It is essential to remove all traces of protease inhibitors from the column before thrombin cleavage in order to obtain maximal thrombin activity.
7. Thrombin is optimally active in the presence of physiological concentrations of CaCl₂.
8. DTT cannot be used for the purification on Ni-NTA, as it reduces the nickel ions. β -mercaptoethanol can be used at concentrations up to 20 mM.
9. The pH of the elution buffer must be checked and possibly adjusted to pH 7.5 with HCl before use (a 0.5 M solution of imidazole has a pH of 10–11).
10. To prevent cross-contamination between different GTPases, particularly when purifying mutants, we re-use 1 aliquot of glutathione Sepharose only for the purification of the same protein.
11. Thrombin can be removed after cleavage by passing the sample through a 1-mL column of either p-aminobenzamidine agarose or antithrombin III agarose.
12. When the SDS-PAGE gel is not freshly poured, we sometimes observe that the GTPase appears as a double band, even though mass spectrometric analysis of the same sample showed that only a single species is present.
13. The absorbance maximum for guanine nucleotides lies at 254 nm, and their spectrum partially overlaps with that of proteins. To account for this, the extinction coefficient for guanine nucleotides at 280 nm, 7765 cm⁻¹M⁻¹, is added to the extinction coefficient of the GTPase (calculated using the “ProtParam” tool on the ExPasy server).
14. Tetrabutylammonium ions act as counter ions to the phosphate groups, and neutralize their charge. They also increase the interaction with the hydrophobic column, and thereby increase the retention time with an increasing number of phosphates.

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