Second Edition

Ni-NTA Magnetic Agarose Beads Handbook

For
Manual and automated
Assays using 6xHis-tagged proteins
Purification of 6xHis-tagged proteins

December 2001

QIAGEN

QIAGEN Worldwide

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Kit Contents

Ni-NTA Magnetic Agarose Beads Catalog No.	(2 x 1 ml) 36111	(6 x 1 ml) 36113
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Storage Conditions

Ni-NTA Magnetic Agarose Beads are supplied as a 5% (v/v) suspension in 30% ethanol and should be stored at 2–8°C. They can be stored under these conditions for up to 6 months without any reduction in performance.

Technical Assistance

At QIAGEN we pride ourselves on the quality and availability of our technical support. Our Technical Service Departments are staffed by experienced scientists with extensive practical and theoretical expertise in molecular biology and the use of QIAGEN® products. If you have any questions or experience any difficulties regarding Ni-NTA Magnetic Agarose Beads or QIAGEN products in general, please do not hesitate to contact us.

QIAGEN customers are a major source of information regarding advanced or specialized uses of our products. This information is helpful to other scientists as well as to the researchers at QIAGEN. We therefore encourage you to contact us if you have any suggestions about product performance or new applications and techniques.

For technical assistance and more information please call one of the QIAGEN Technical Service Departments or local distributors (see inside front cover).

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDSs). These are available online in convenient and compact PDF format at www.qiagen.com/ts/msds.asp where you can find, view, and print the MSDS for each QIAGEN kit and kit component.

The following risk and safety phrases apply to Ni-NTA Magnetic Agarose Beads.

Ni-NTA Magnetic Agarose Beads

Contains Nickel-nitrilotriacetic acid, Ethanol: Harmful, Sensitizer, Flammable. Risk and safety phrases*: R10, R22, R40, R42/43, S13, S26, S36

24-hour emergency information

Poison Information Center Mainz, Germany

Tel: +49-6131-19240

** R10: Flammable; R22: Harmful if swallowed; R40: Possible risks of irreversible effects; R42/43: May cause sensitization by inhalation and skin contact; S13: Keep away from food, drink and animal feedingstuffs; S26: In case of contact with eyes, rinse immediately with plenty of water and seek medical advice; S36: Wear suitable protective clothing.*

Introduction

Ni-NTA Magnetic Agarose Beads are agarose beads, with an average diameter of 50 µm and a range of 20–70 µm diameter, that contain magnetic particles and have strongly metal-chelating nitrilotriacetic acid (NTA) groups covalently bound to their surfaces (Figure 1). They are precharged with nickel and ready to use for capturing 6xHis-tagged proteins under native or denaturing conditions for high-throughput assays and screening programs, as well as small-scale purification of 6xHis-tagged proteins. They are ideal for a wide variety of capture assays (magnetocapture assays) that involve interaction of immobilized, functionally active 6xHis-tagged proteins with interacting biomolecules, such as proteins or nucleic acids. Adjusting the amount of Ni-NTA Magnetic Agarose Beads and therefore the binding capacity, allows flexible choice of the amount of 6xHis-tagged protein captured to suit the particular assay. All the protocols using magnetic beads are easily fully automated using QIAGEN BioRobot™ Systems.The QIA*express*® range of Ni-NTA matrices offers assay and purification products to suit every scale (Figure 2). Ni-NTA Magnetic Agarose Beads are supplied as a 5% (v/v) suspension with a binding capacity of 300 µg protein per ml of suspension for 6xHis-tagged dihydrofolate reductase (DHFR, approximately 12.5 nmol per ml, molecular weight: 24 kDa).

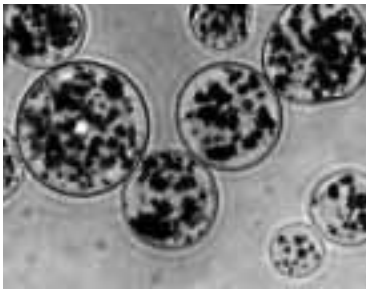

Figure 1. *Ni-NTA Magnetic Agarose Beads*

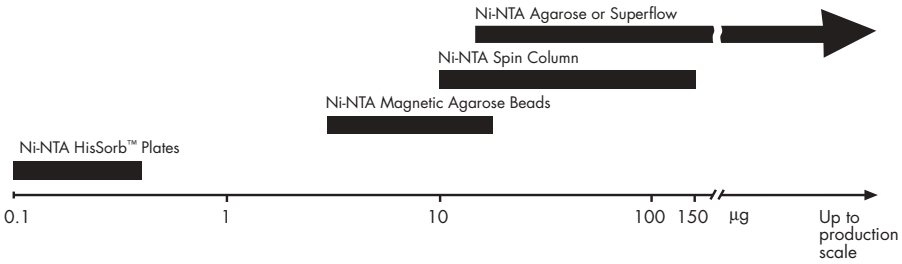

Figure 2. *Recommended QIA*express *products for different scales of 6xHis-tagged protein assay or purification.*

The QIA*express* System

The QIA*express* System provides materials for expression, purification, detection, and assay of 6xHis-tagged proteins. The QIA*express* System is based on the remarkable selectivity of Ni-NTA for proteins or peptides with an affinity tag of six consecutive histidine residues — the 6xHis tag (Figure 3). The system exploits both the specificity of the interaction between the histidine residues and immobilized nickel ions and the strength with which these ions are held to the NTA resin. 6xHis-tagged biomolecules can be immobilized in fully active conformation offering optimal access of binding domains to potential interacting partners. All QIA*express* nickel-chelating materials, including Ni-NTA–coated microplates designed for assays (Ni-NTA HisSorb Strips and Plates) and Ni-NTA affinity chromatography matrices (Ni-NTA Agarose, Superflow, and Silica) utilize our unique, patented NTA (nitrilotriacetic acid) ligand. NTA has a tetradentate chelating group that occupies four of six sites in the nickel coordination sphere. The metal is bound much more tightly than to a tridentate chelator such as IDA (iminodiacetic acid), which means that nickel ions, and as a result the proteins, are very strongly bound to the resin (Figures 4 and 5). This allows more stringent washing conditions, better separation, higher purity, and higher capacity — without nickel leaching (1, 2). In contrast to other affinity-tag systems, the 6xHis tag–Ni-NTA interaction is not dependent on three-dimensional conformation and can be utilized under native or denaturing conditions. The small size of the 6xHis tag does not interfere with protein structure or function and can be used as a highly selective system to immobilize functional enzymes and ligands. A wide variety of active 6xHis-tagged proteins has been produced, for example, enzymes, transcription factors, and engineered antibodies (3, 4, 5, 6, 7). Furthermore, 6xHis-tagged proteins can be detected by several monoclonal antibodies provided by QIAGEN that have affinity constants in the nanomolar range (8).

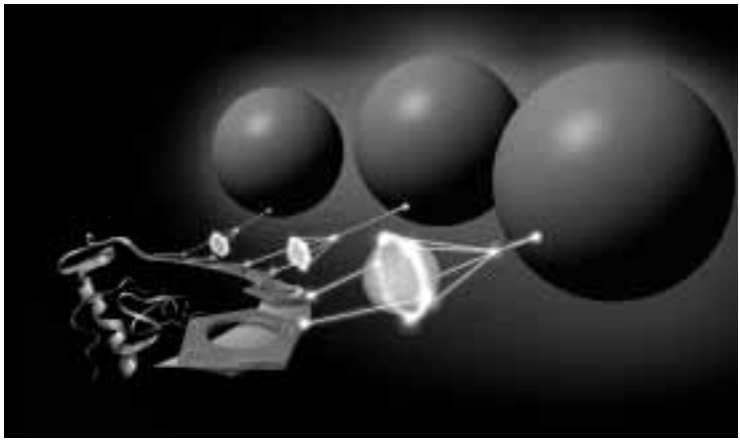

Figure 3. *Interaction between Ni-NTA and a 6xHis-tagged protein*

Applications with Ni-NTA Magnetic Agarose Beads

Ni-NTA Magnetic Agarose Beads combine all the benefits of Ni-NTA with the convenience and speed of magnetic-bead technology. They are designed for capture assays involving fast separation of 6xHis-tagged proteins from protein mixtures, without the need for prior protein-purification, as well as high-throughput, micro-scale purification of 6xHis-tagged proteins. They do not require the use of columns or centrifugation, and can be used in single tubes or vials, or in 96-well microplates. The magnetic beads can be used in very small volumes — as little as 10 µl can be used to bind 3 µg protein — in 96-well format. During this procedure, the strongly magnetic beads are separated by a magnet which holds them on the sides of the wells while the buffers are exchanged to wash or elute the 6xHis-tagged proteins. The beads are easily resuspended by agitation.

The gentle, but rapid, separation of 6xHis-tagged proteins made possible by Ni-NTA Magnetic Agarose Beads allows analysis of protein–protein and DNA–protein interactions, and immunoassay procedures requiring higher binding capacities for the captured molecule than achieved with other methods. The high binding capacity, compared to methods that rely on binding to 96-well microplates, allows less sensitive procedures to be used for analysis of the bound constituents — even SDS-PAGE followed by Coomassie® staining provides excellent results. In addition the high binding capacity allows improvement of the signal-to-noise ratio for low-affinity binding complexes, for example, receptor–ligand interactions, by immobilizing larger amounts of the 6xHis-tagged receptor molecule. Many kinds of magnetocapture assay are made possible with Ni-NTA Magnetic Beads, a few examples are illustrated in Figures 8, 9, and 10 (pages 24, 25, and 35). When examining protein–protein interactions, the proteins may be eluted as a complex with imidazole or EDTA or the interacting partner may be eluted on its own after disruption of the interaction. 6xHis-tagged DNA-binding proteins can be used to capture DNA molecules in low-salt buffer. After washing, high-salt buffer can then be used to release and elute the DNA fragment. Alternatively, the protein–DNA complex may be eluted using imidazole or EDTA to release the 6xHis-tagged protein from Ni-NTA. Immunoassay protocols may be conveniently performed and can be used for detection of low-affinity antibodies that bind to 6xHis-tagged proteins or for detection of interacting proteins with specific antibodies, and many other applications (for more information, refer to ref. 9).

Ready-to-run automated protocols covering a wide range of protein assay and purification applications have been developed to run on QIAGEN BioRobot Systems. Ni-NTA Magnetic Agarose Beads allow flexible interaction or diagnostic assays with structurally active immobilized 6xHis-tagged proteins as well as micro-scale purification of up to 15 µg of 6xHis-tagged protein. Using Ni-NTA Magnetic Agarose Beads the purification and assay protocols may even be carried out directly in series — without elution of the captured 6xHis-tagged protein. If larger amounts of purified proteins are needed, the Ni-NTA Superflow 96 BioRobot Kit provides a convenient high-throughput method for purification of approximately 300 µg of 6xHis-tagged protein per well in a 96-well format (for more information, contact one of the QIAGEN Technical Service Departments or local distributors listed inside the front cover).

Capture under Native or Denaturing Conditions

Magnetocapture of 6xHis-tagged proteins under native conditions allows the immobilization of active proteins to bind and assay interacting biomolecules such as proteins or DNA molecules (Figures 8 and 9, pages 24 and 25). These assays, that detect binding of biomolecules with the 6xHis-tagged protein can provide evidence of interactions between these molecules in their native conformations. Varying the conditions of the assay can reveal information about the nature of the interactions and provide evidence for the possible biological role of molecules in vivo. Under native conditions, nonspecific binding by nontagged proteins is minimized by including a low concentration of **imidazole** in the lysis, interaction, and wash buffers (up to 20 mM). Proteins that are highly expressed in bacteria may form inclusion bodies, but even in these cases, there may be enough protein present in the soluble form to perform capture assays and small-scale, native purification procedures.

Since the interaction between Ni-NTA and the 6xHis tag of the recombinant protein does not depend on tertiary structure, proteins can be captured and purified under denaturing as well as native conditions (see Figure 6, page 13). If the protein is found in inclusion bodies, it may be solubilized with strong denaturants, such as guanidine hydrochloride (GuHCl) or urea. Purification under denaturing conditions ensures that all 6xHis-tagged protein in the cell is solubilized and can be captured. In addition, the 6xHis tag is fully exposed under denaturing conditions, which leads to more efficient purification. Immobilized protein can be efficiently renatured while bound to Ni-NTA and used directly for subsequent assays (see, for example, ref. 10).

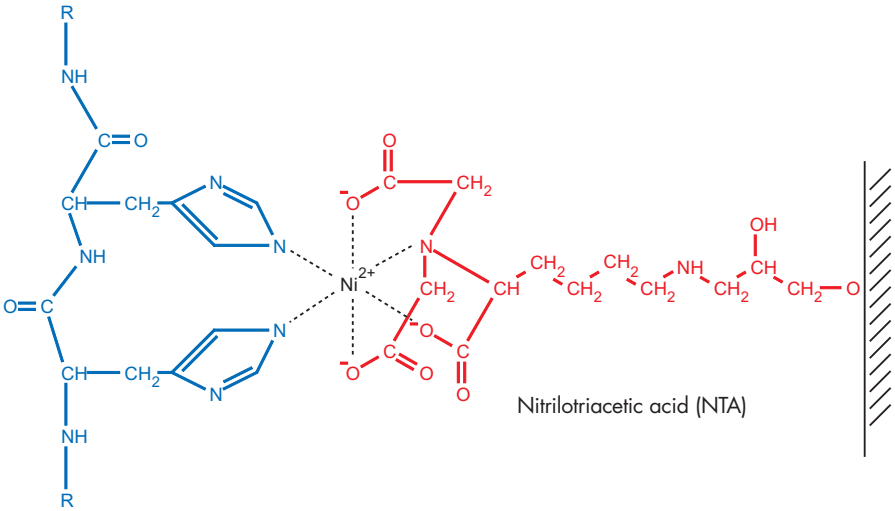

Figure 4. *Interaction between neighboring residues in the 6xHis tag and Ni-NTA matrix.*

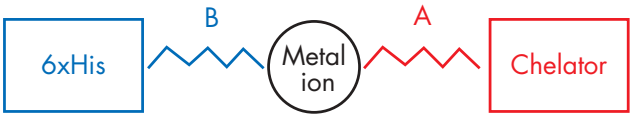

Figure 5. *The capture of 6xHis-tagged proteins by metal-chelate affinity matrices relies on two interactions. Both are important for optimal performance. If interaction B is weak then there is no binding of the 6xHis-tagged proteins. If interaction B is strong, but interaction A weak, then protein is lost as protein–metal complexes during wash steps. When NTA ligand and nickel are used to bind 6xHis-tagged molecules both interactions are stronger, giving advantages over systems that rely on other ligands or metals.*

Capture of 6xHis-tagged Proteins — Ni-NTA Magnetic Bead Procedure

Ni-NTA Magnetic Agarose Beads have the same affinity characteristics for 6xHis-tagged proteins as Ni-NTA Agarose and are fully compatible with the buffer systems used for large-scale protein purification with Ni-NTA Agarose. Although the Ni-NTA Magnetic Bead procedure has been designed for the separation of 6xHis-tagged proteins from bacterial expression systems, the procedure can also be used for capturing 6xHis-tagged recombinant proteins expressed in other hosts (e.g., mammalian cells, see ref. 11). The procedure will work very well for most 6xHis-tagged proteins and cell lysates, but as for purification with Ni-NTA Agarose, some modifications may be necessary if an expression system other than *E. coli* is used (see *The QIA*expressionist™, for details and a protocol for purification of 6xHis-tagged proteins from mammalian cells).

Ni-NTA Magnetic Bead procedure summary

Assays utilizing Ni-NTA Magnetic Beads involve capture of the 6xHis-tagged protein — from a cell lysate or a purified-protein solution — followed by washing, binding of interaction partners, further washing, and finally elution of the interacting partner from the still-immobilized 6xHis-tagged protein or elution of the interacting-partner–6xHis-tagged-protein complex. Between each step, the beads are collected by attracting them to the side of the vessel, after placing near a magnet for 30–60 s. Purification procedures may even use crude cell extracts for binding of 6xHis-tagged protein.

Fully automated 96-well protein purification and assay

QIAGEN BioRobot Systems integrate Ni-NTA–based protein purification and assay technology, sophisticated hardware modules, and purpose-designed QIAsoft™ Operating Software to improve productivity by minimizing manual sample handling. While protocols using Ni-NTA Magnetic Agarose Beads are automated on the BioRobot 3000 and 8000, affinity chromatography-based automated protocols using the Ni-NTA Superflow 96 BioRobot Kit can be performed on the BioRobot 9600, 8000, and 3000. For more information, contact one of the QIAGEN Technical Service Departments or local distributors listed inside the front cover.

QIAGEN 96-Well Magnet Type A

The 96-Well Magnet Type A* has been designed for the rapid, convenient separation of Ni-NTA Magnetic Agarose Beads in the wells of 96-well flat-bottom microplates (Figure 6). The QIAGEN 96-Well Magnet Type A is the perfect separation device for high-throughput 6xHis-tagged protein magnetocapture assays or micro-scale purification procedures. The magnet consists of an array of 24 powerfully magnetic NdFeB rods that fit between the wells of commercially available 96-well microplates (QIAGEN supplies 96-well microplates that fit perfectly, see Ordering Information, page 84). Flat-bottom microplates are recommended for protein purification or assay procedures to enable the use of up to 240 µl total volume per well, even under rigorous shaking, to achieve optimal yield and sensitivity. Each magnetic rod attracts the beads in four adjacent wells to the side of the well and keeps the beads in place while the buffer is changed. The magnetic beads are very easily resuspended in buffer, once removed from the magnet, allowing thorough washing. Capture of 6xHis-tagged proteins is reproducible under native and denaturing conditions (Figure 7).

** For manual procedures the 96-Well Magnet Type A (Cat. No. 36915) is used. For automated procedures on BioRobot workstations use the Magnet, 96-well, Type A (Cat. No. 9014061).*

QIAGEN 12-Tube Magnet

The 12-Tube Magnet (Cat. No. 36912) is the perfect separation device for 6xHis-tagged protein magnetocapture assays or purification procedures performed in 1.5 ml or 2 ml tubes. The magnet consists of a series of 12 powerful magnetic NdFeB disks. Each magnetic disk attracts the beads in a tube fitted in an adjacent hole. The magnetic beads are pulled to the side of the tube and kept in place while buffers are exchanged. A second series of holes is used to hold tubes for resuspension of the magnetic beads away from the magnetic field to allow thorough washing.

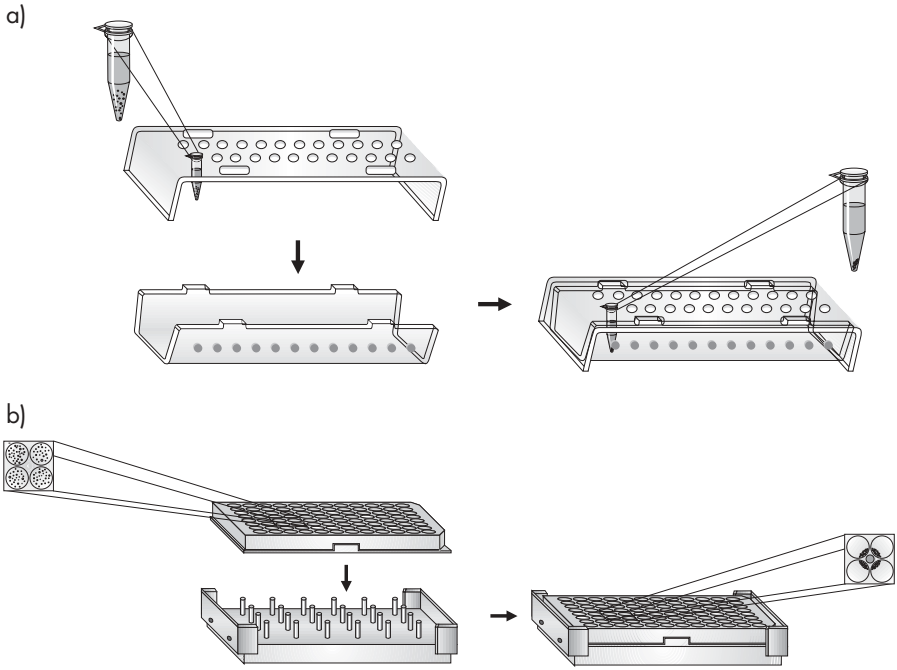

Figure 6. *Use of 12-Tube Magnet showing collection of beads in tube by magnetic disk (a) and 96-Well Magnet Type A showing collection of beads in four wells by each magnetic rod (b).*

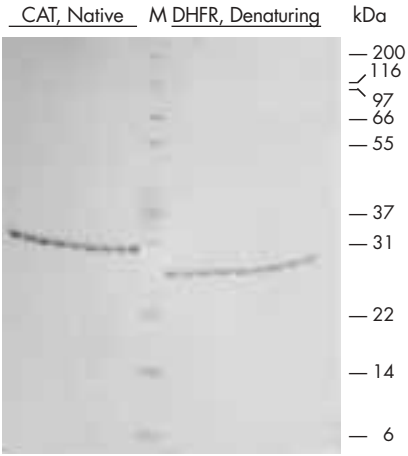

Figure 7. *Reproducible protein purification with Ni-NTA Magnetic Agarose Beads and the QIAGEN 96-Well Magnet. 6xHis-tagged chloramphenicol acetyltransferase (**CAT**) was purified under native and dehydrofolate reductase (**DHFR**) under denaturing conditions, from 1 ml bacterial cultures lysed in 200 µl lysis buffer. After clearing the lysates, 20 µl Ni-NTA Magnetic Agarose Bead suspension was used to bind the 6xHis-tagged protein. Washing with 200 µl wash buffer removed contaminants and pure 6xHis-tagged protein was eluted in 25 µl elution buffer. Protein purified from separate wells (2 µl eluate) was loaded into each lane, separated by SDS-PAGE and visualized by Coomassie staining. Sizes of the marker proteins (**M**) are indicated.*

General considerations and limitations

- For assays, the amount of 6xHis-tagged protein should correspond approximately to the binding capacity of the magnetic beads used. For purification, the concentration of the cell lysate should be adjusted according to the expression level (see Tables 1 and 2).
- Avoid the use of high concentrations of buffer components containing strong electron-donating groups (e.g., NH_4, glycine, arginine, Tris, see Table 3).
- Avoid the use of strong chelating agents, such as EDTA; strong reducing agents, such as DTT; or ionic detergents, such as SDS. Although low levels of these reagents have been used successfully, nickel leaching may occur, and performance may be diminished (see Table 3, page 18).
- During purification procedures, some proteins may be subject to degradation during cell harvest and lysis. In these cases, addition of PMSF (0.1–1 mM) or other protease inhibitors is recommended. Under native conditions it is best to work quickly and at 4°C at all times.
- Especially when using 96-well microplates, 0.05% Tween® 20 should be included in the buffers used under native or denaturing conditions to optimize the separation characteristics.

Protein binding and preparation of the cell lysate — guidelines for assay and purification procedures

Native conditions

Since the biochemical properties of proteins are very dependent on the individual protein, and these can strongly affect binding, it is difficult to provide an exact protocol for separation of 6xHis-tagged proteins under native conditions. However, some general guidelines are helpful to optimize the separation procedure:

- To minimize nonspecific capturing or binding under native conditions, low concentrations of **imidazole** in the interaction and lysis/binding buffers are strongly recommended. This inhibits binding of nontagged contaminating proteins. For most 6xHis-tagged proteins, up to 20 mM **imidazole** can be used without affecting the binding properties. However, if the tagged protein does not bind under these conditions, the amount of imidazole should be reduced to 5–10 mM.
- Cells can be lysed by sonication or homogenization after treatment with lysozyme (1 mg/ml). Cells cultured in 96-well Square-Well Blocks (see Ordering Information, page 84) can be lysed by careful vortexing after lysozyme treatment. In many cases, lysis will be more efficient **if cells were frozen at –20°C or –70°C** after harvesting. To inhibit protein degradation, cells and buffers may be kept at 0–4°C at all times. The addition of protease inhibitors may also be necessary.
- All buffers should have sufficient ionic strength to prevent nonspecific interactions between proteins and the Ni-NTA matrix. The minimum salt concentration during binding and washing steps should be 300 mM NaCl. The maximum concentration is 2 M NaCl.

Denaturing conditions

Cells can be lysed in either 6 M GuHCl or 8 M urea. It is preferable to lyse the cells in the milder denaturant, urea, so that the cell lysate can be analyzed directly on an SDS polyacrylamide gel. GuHCl is a more efficient solubilization and cell-lysis reagent, and may be required to solubilize some proteins.

Controls

The use of suitable negative and positive controls is essential for most assay procedures. As negative control, a cell lysate or extract from material that is similar to the test sample except for the absence of the 6xHis-tagged protein can usually be used. This may be, for instance, a cell lysate of an *E. coli* strain which harbors only the vector (without the 6xHis-tagged–protein encoding insert). As positive control for binding to Ni-NTA, a purified 6xHis-tagged protein may be added to a negative control sample, or a sample known to contain a 6xHis-tagged protein may be used. DHFR expressed from pQE-40 provides a suitable positive control protein. 6xHis-tagged mouse dihydrofolate reductase (DHFR) can be expressed from the control vector pQE-40 (which is supplied as a component

of QIA*express* kits). DHFR is expressed at approximately 40 mg per liter *E. coli* M15[pREP4] culture after 4 hours of induction, when cultures are grown in 100 ml flasks. About 10% of DHFR is present in the cells in soluble form, and will be solubilized using native conditions.

Crude, uncleared lysates

In purification procedures, binding is carried out with cleared lysate under native or denaturing conditions. Purification can also be carried out without clearing the lysates, but care should be taken that the lysate is not too concentrated. The volume of the crude, uncleared lysate should not be more than 1/5 to 1/10 of the culture volume (e.g., lyse cells from 1 ml culture in 0.2 ml or at the very least 0.1 ml lysis buffer). DNA, which can disturb the separation of the beads, should be eliminated by DNase treatment. Yield and purity of the eluted protein may be slightly reduced when uncleared lysates are used.

Matching the amount of beads to the amount of protein

For optimal performance, the binding capacity of the Ni-NTA Magnetic Agarose Beads used in each well should approximately match the amount of 6xHis-tagged protein to be captured. Tables 1 and 2 can be used as a guide for the volume of culture and 6xHis-tagged protein to use in each well.

Table 1. Examples of the amount of 6xHis-tagged protein that may be captured per well in 96-well microplates*

Expression level	Amount of 6xHis-tagged protein in 1 ml culture	Amount of 6xHis-tagged protein in 3 ml culture
20 mg/liter	20 µg	60 µg
10 mg/liter	10 µg	30 µg
5 mg/liter	5 µg	15 µg
2 mg/liter	2 µg	6 µg
1 mg/liter	1 µg	3 µg
0.5 mg/liter	0.5 µg	1.5 µg

** The given culture volumes (1 and 3 ml) are typically useful for each well of the 96-well microplate protocol. The pellets are usually lysed in a volume of 200 µl. This is the maximum volume that can easily be handled in the wells. For single-tube purifications the volumes of culture, lysate, and Ni-NTA Magnetic Agarose Bead suspension can be increased.*

Table 2. Volumes of bead suspension, elution volumes, and eluate protein concentrations

Volume of bead suspension (µl)*	Minimum elution volume (µl)*	Binding capacity (µg 6xHis-tagged DHFR, 24 kDa)	Protein concentration in eluate (µg/µl)†
200	100	60	0.60
100	50	30	0.60
50	25	15	0.60
20	25	6	0.24
10	25	3	0.12

** Use of suspension volumes less than 10 µl or elution volumes less than 25 µl is not recommended due to the associated handling problems.*

† Assumes optimal binding of 6xHis-tagged protein to beads and complete elution.

Washing

Nonspecific binding of nontagged proteins to Ni-NTA is inhibited by washing with buffers of slightly reduced pH or with buffers containing a low concentration of **imidazole**. Assays can be performed using the 6xHis-tagged protein bound to the Ni-NTA Magnetic Agarose Beads directly after washing.

Protein elution

6xHis-tagged proteins are released from the beads either by competition with imidazole, or by reducing the pH. Monomers are generally released with imidazole concentrations greater than 100 mM or at approximately pH 5.9, whereas multimers are released with 200 mM imidazole or at around pH 4.5. Usually buffers containing 250 mM imidazole (pH 8) or Buffer E-Tween (pH 4.5) are recommended (see page 80). 100 mM EDTA chelates nickel ions and removes them from the NTA groups. This causes all bound protein to elute as a protein–metal complex. In assay procedures, interacting proteins may be eluted either alone (by separating them from their 6xHis-tagged partner) or together with 6xHis-tagged protein (using the conditions described above to release the 6xHis-tagged protein from Ni-NTA). Assays that rely on measurement of the activity of an enzyme may be carried out without prior elution of the enzyme from the beads.

Detection

Immunoassays may be performed using antibodies specific for antigens present on the captured 6xHis-tagged biomolecule or antigens present on interaction partners bound to the captured 6xHis-tagged biomolecule. Detection is then performed by making use of antibodies conjugated to alkaline phosphatase or horseradish peroxidase and appropriate colorimetric substrates — *p*-nitrophenol (pNPP) or *o*-phenylenediamine (OPD), respectively.

Table 3. Compatibility of reagents with Ni-NTA matrices

Reagent	Effect	Comments
Buffer reagents		
Tris, HEPES, MOPS	• Buffers with secondary or tertiary amines will reduce nickel ions	• Up to 100 mM has been used successfully in some cases
		• Sodium phosphate or phosphate-citrate buffer is recommended
Chelating reagents		
EDTA, EGTA	• Strip nickel ions from resin	• Up to 1 mM has been used successfully in some cases, but care must be taken
Sulfhydril reagents		
β-mercaptoethanol	• Prevents disulfide cross-linkages	• Up to 20 mM can be used
	• Can reduce nickel ions at higher concentration	
DTT, DTE	• Low concentrations will reduce nickel ions	• A maximum of 1 mM may be used, but β-mercaptoethanol is recommended
Detergents		
Nonionic detergents (Triton®, Tween®, NP-40, etc.)	• Remove background proteins and nucleic acids	• Up to 2% can be used
Cationic detergents		• Up to 1% can be used
Anionic detergents (SDS, sarkosyl)		• Not recommended, but up to 0.3% has been used successfully in some cases
Denaturants		
GuHCl	• Solubilize proteins	• Up to 6 M
Urea		• Up to 8 M

Table 3. Continued

Reagent	Effect	Comments
Amino acids		
Glycine		• Not recommended
Glutamine		• Not recommended
Arginine		• Not recommended
Histidine	• Binds to Ni-NTA and competes with histidine residues in the 6xHis tag	• Can be used at low concentrations (20 mM) to inhibit nonspecific binding and, at higher concentrations (>100 mM), to elute the 6xHis-tagged protein from the Ni-NTA matrix
Other additives		
NaCl	• Prevents ionic interactions	• Up to 2 M can be used, at least 300 mM should be used
$MgCl_2$		• Up to 4 M
$CaCl_2$		• Up to 5 mM
Glycerol	• Prevents hydrophobic interaction between proteins	• Up to 50%
Ethanol	• Prevents hydrophobic interactions between proteins	• Up to 20%
Imidazole	• Binds to Ni-NTA and competes with histidine residues in the 6xHis tag	• Can be used at low concentrations (20 mM) to inhibit nonspecific binding and, at higher concentrations (>100 mM), to elute the 6xHis-tagged protein from the Ni-NTA matrix
Sodium bicarbonate		• Not recommended
Hemoglobin		• Not recommended
Ammonium		• Not recommended
Citrate		• Up to 60 mM has been used successfully

Detailed Protocols
Protocol 1. Cell lysis under native conditions

Note: The use of buffers containing **0.05% Tween 20** throughout the purification procedure is strongly recommended in order to optimize the separation characteristics of the magnetic beads. This is especially important when performing 96-well procedures.

Purification can also be carried out without clearing the lysates, but care should be taken that the lysate is not too concentrated. The volume of the crude, uncleared lysate should not be more than 1/5 to 1/10 of the culture volume (e.g., lyse cells from 1 ml culture in 0.2 ml or at the very least 0.1 ml lysis buffer). DNA, which can disturb the separation of the beads, should be eliminated by DNase treatment.

Materials

Bacterial pellets (For guidelines on cell cultivation see appendix, page 80.)

Lysis Buffer-Tween for native conditions (containing Tween 20)

For buffer and reagent compositions, see appendix, page 80.

Procedure

1. **Thaw cells for 15 min and resuspend in Lysis Buffer-Tween.**

   For 1 ml cultures grown in 96-well blocks, 200 µl Lysis Buffer-Tween should be used for each well. In 96-well blocks it is not practical to lyse the cells by sonication. **Freeze the cell pellets** at –20°C or –70°C for at least one hour after harvesting — without freezing yields obtained without sonication are reduced by up to 75%.

   For larger culture volumes, increase the lysis volume appropriately.

   Adding 10 mM **imidazole**, inhibits binding of nontagged proteins. If the tagged protein does not bind under these conditions, the amount of imidazole should be reduced to 1–5 mM.

2. **Add lysozyme to 1 mg/ml.**

   If uncleared lysate will be used for processing add DNase (e.g., Benzonase® at 1 U/ml culture volume)

3. **Incubate on ice for 30 min.**

   Alternatively, when using 96-well blocks, incubation can be at room temperature for 15 min.

   Generally, we recommend using cleared lysates for binding to Ni-NTA Magnetic Agarose Beads, but it may be possible to obtain good results by using crude lysates without clearing them. In this case, use dilute lysates with a volume of between 1/5 and 1/10 of the original culture volume (e.g., lyse cells from 1 ml culture in 0.2 ml or at the very least 0.1 ml Lysis Buffer-Tween), add RNase A to 10 µg/ml and DNase I to 5 µg/ml, and incubate on ice for 10–15 min.

4. **Sonicate or homogenize on ice to lyse cells (6 times for 10 s each time with 5 s pauses between).**

   When using 96-well blocks, cover block with tape and vortex 6 times for 5 s each time on a low to medium setting. This step may need to be optimized for different cell cultures and vortexers.

   If uncleared lysate is being used for processing do not carry out step 5 but proceed directly with downstream application.

5. **Clear lysate by centrifugation at 10,000 x *g* for 30 min at 4°C. Collect supernatant.**

   Save 20 µl of the cleared lysate for SDS-PAGE analysis.

   Note: 96-well blocks can be centrifuged at 5000 x *g* for 30 min. Centrifuge 4-15 fitted with the Plate Rotor 2 x 96 is ideal for centrifugation of the 96-well blocks (for more information, call one of the QIAGEN Technical Service Departments).

Protocol 2. Cell lysis under denaturing conditions

Note: The use of buffers containing **0.05% Tween 20** throughout the purification procedure is strongly recommended in order to optimize the separation characteristics of the magnetic beads. This is especially important when performing 96-well procedures.

Materials

Bacterial pellets

Lysis Buffer for denaturing conditions (containing Tween 20, see note)

For buffer and reagent compositions, see appendix, page 80.

Note: It is preferable to lyse the cells in Buffer B-Tween, so that the cell lysate can be analyzed directly by SDS-PAGE. If the cells or the protein do not solubilize in Buffer B-Tween, then Buffer A-Tween containing the stronger denaturing agent, GuHCl, must be used. Since guanidine will precipitate with SDS in fractions that contain GuHCl, the fractions must either be diluted (1/6), dialyzed before analysis, or separated from GuHCl by TCA precipitation (see *The QIA*expressionist), before separation by SDS-PAGE.

Procedure

1. **Thaw cells for 15 min and resuspend in Buffer B-Tween.**

   For 1 ml cultures grown in 96-well blocks, 200 µl Buffer B-Tween should be used.

   For larger culture volumes, increase the lysis volume appropriately.

2. **Shake cells for 1 hr at room temperature.**

   The solution should become translucent when lysis is complete. Generally, we recommend using cleared lysates for binding to Ni-NTA Magnetic Agarose Beads, but it may be possible to obtain good results by using crude lysates without clearing them. In this case, use dilute lysates with a volume of between 1/5 and 1/10 of the original culture volume (e.g., lyse cells from 1 ml culture in 0.2 ml or at the very least 0.1 ml Lysis Buffer).

3. **Clear lysate by centrifugation at 10,000 x *g* for 30 min at room temperature to pellet the cellular debris. Collect supernatant.**

   Save 20 µl of the cleared lysate for SDS-PAGE analysis.

   Note: 96-well blocks can be centrifuged at 5000 x *g* for 30 min. Centrifuge 4-15 fitted with the Plate Rotor 2 x 96 is ideal for centrifugation of 96-well blocks (for more information, call one of the QIAGEN Technical Service Departments).

   Note: When working under denaturing conditions, clearing of the lysate is optional.

Assay using Ni-NTA Magnetic Agarose Beads

Assays can be performed using 6xHis-tagged proteins that have been captured by the magnetic beads during the purification protocol (page 37, step 5). Alternatively, assays can easily be performed by capturing proteins previously purified by Ni-NTA affinity chromatography — this allows analysis of the purity and characterization of the protein prior to proceeding with the assay procedure. Attention should be paid to the buffer conditions used for binding — especially when using proteins that have been already eluted from Ni-NTA during purification, the solution may often contain imidazole or be of inappropriate pH for binding. In this case, dilute in appropriate buffer or exchange buffer by ultrafiltration or dialysis. Assays that can be carried out include investigation of protein–protein (12) and protein–DNA interactions and immunoassays (see Figures 8, 9, and 10; see pages 24, 25, and 35).

Note: The protocols that follow are given as examples and offer guidelines, but optimization is a necessary part of establishing these assays.

It is important to include suitable controls in all assays. This will help with interpretation of the data, optimization of the assay, and troubleshooting.

Note: As an assay using magnetic beads differs to some extent from assays using microplates, the buffers have been designed and optimized for use with magnetic beads. It is therefore very important to use the buffers that are recommended here to obtain optimal results. The addition of **Tween 20** is necessary to enable optimal collection of beads on the sides of the microplate wells.

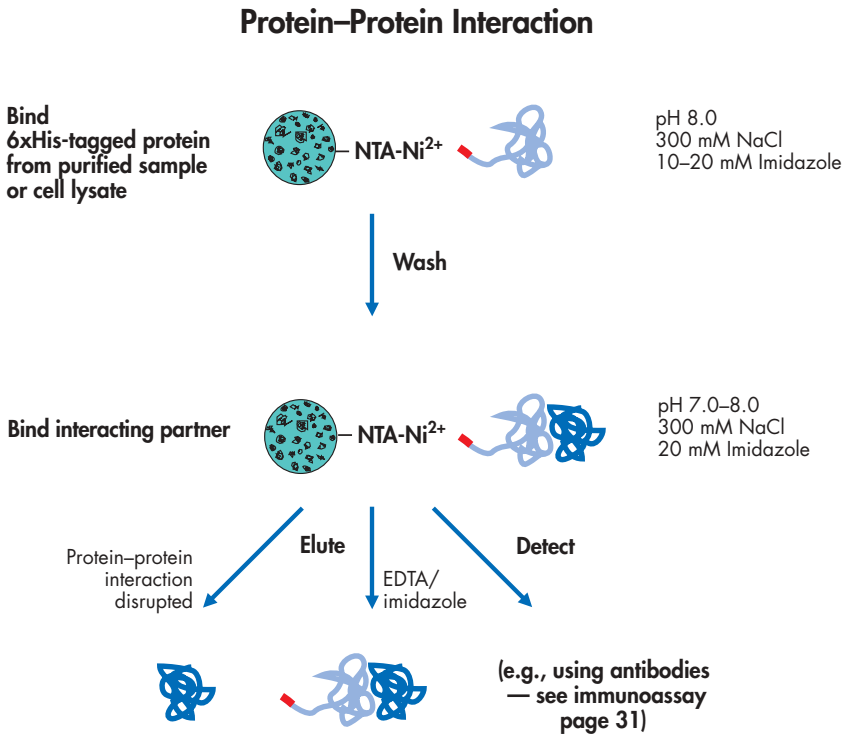

Figure 8. *Protein–protein interaction study by magnetocapture assay with Ni-NTA Magnetic Agarose Beads.*

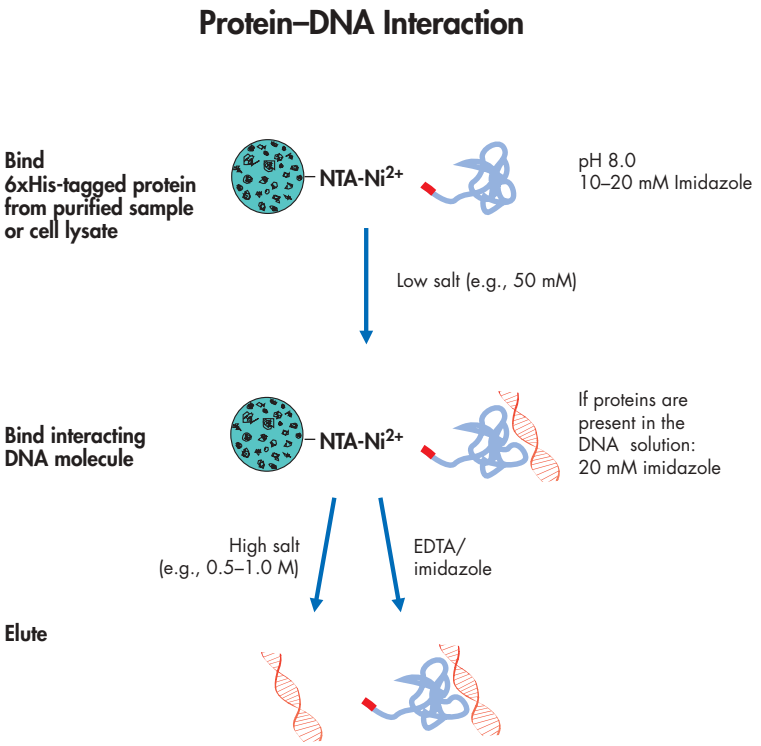

Figure 9. *Protein–DNA interaction study by magnetocapture assay with Ni-NTA Magnetic Agarose Beads.*

Protocol 3. Assay using single tubes

Materials

Cell lysate or purified 6xHis-tagged protein diluted in Protein Binding Buffer

Ni-NTA Magnetic Agarose Beads

Wash Buffer for assays

Interaction Buffer-Tween — **Note:** Choose a buffer to optimize your specific interaction, but bear in mind the limitations detailed in Table 3 (page 18). Add 20 mM **imidazole** to interaction buffer to prevent nonspecific binding to Ni-NTA. It is preferable to maintain pH at 7.0–8.0 and to use 300 mM NaCl, but for interactions that are inhibited by such high salt concentrations, for example those involving DNA molecules, 50 mM NaCl can be used (see Figure 9, page 25).

Elution Buffer for assays (optional)

Magnetic separator — e.g., 12-Tube Magnet (Cat. No. 36912)

For buffer and reagent compositions, see appendix, page 80.

1. **Resuspend the Ni-NTA Magnetic Agarose Beads by vortexing for 2 s and then immediately add 50 µl of the 5% Ni-NTA Magnetic Agarose Beads suspension to 500 µl of the 6xHis-tagged protein.**

   Note: Care is necessary to ensure that constant amounts of beads are pipetted. The beads will settle if the suspension is not agitated regularly.

   The buffer should always contain **imidazole**. For most 6xHis-tagged proteins, up to 20 mM **imidazole** can be used without affecting the binding properties. However, if the tagged protein does not bind under these conditions, the amount of imidazole should be reduced to 5–10 mM.

   For optimal performance, the binding capacity of the Ni-NTA Magnetic Agarose Beads used in each tube should approximately match the amount of 6xHis-tagged protein to be captured.

   50 µl magnetic-bead suspension has a binding capacity of 15 µg 6xHis-tagged DHFR (24 kDa). If significantly different amounts of tagged protein are present in your lysate, the volume of bead suspension should be varied accordingly (see Table 2, page 17). However, use of volumes less than 10 µl are not recommended due to the associated handling problems — smaller volumes are difficult to pipet and may lead to uneven distribution of beads and reduced reproducibility.

2. **Incubate the suspension on an end-over-end shaker for 30 min to 1 h at room temperature.**

   The time and temperature necessary for efficient binding is dependent on the protein and the accessibility of the 6xHis tag in the buffer system used. It may be necessary to incubate at 4°C if the protein is not stable at room temperature.

3. **Place the tube on a suitable magnetic separator (e.g., 12-Tube Magnet) for 1 min and remove supernatant with a pipet.**

4. **(Optional) If a cell lysate is used as source of the 6xHis-tagged protein, wash the beads twice with wash buffer.**

   If using purified proteins, this step can be omitted.

5. **Add 500 µl of Interaction Buffer to each tube, mix, place for 1 min on the magnetic separator, and remove buffer.**

   This is to allow thorough exchange of the buffers.

6. **Add solution containing potentially interacting biomolecules in Interaction Buffer and incubate on end-over-end shaker for 1 h at room temperature.**

   The buffer composition and concentration of binding partners used for this binding step will depend on the characteristics of the biomolecular interactions between the bound 6xHis-tagged molecule and the nontagged binding partner. Optimization of this step is likely to be necessary — use Table 3 (page 18) as a guide to avoid components that will affect binding of the 6xHis-tagged molecule to the Ni-NTA Magnetic Agarose Beads. The buffer should always contain **imidazole** to prevent nonspecific binding to Ni-NTA. For most 6xHis-tagged proteins, up to 20 mM **imidazole** can be used without affecting the binding properties. However, if the tagged protein does not bind under these conditions, the amount of imidazole should be reduced to 5–10 mM.

7. **Wash by adding 500 µl of Interaction Buffer, mix, place for 1 min on the magnetic separator, and remove buffer.**

   The composition of buffer used for this wash step will depend on the characteristics of the biomolecular interactions between the bound 6xHis-tagged molecule and the nontagged binding partner. Use Table 3 (page 18) as a guide to avoid components that will affect binding of the 6xHis-tagged molecule to the Ni-NTA Magnetic Agarose Beads.

   In general, washing can be carried out with the same buffer composition used for interaction. However, if background is high, it may be advantageous to use a different buffer for washing or to repeat step 7.

8. Add 50 µl of Elution Buffer, mix, incubate for 1 min, place for 1 min on a magnetic separator, and collect the eluate.

If the protein solution should be more concentrated, elution can be performed with 25 µl — greater than 80% of bound protein will usually elute in the first step. Elution volumes of less than 25 µl are not recommended because it is difficult to recover small volumes of elution buffer.

This step will release the 6xHis-tagged protein from the magnetic beads. Depending on the interaction with the molecules captured in the assay, they may remain as a complex after elution.

Alternatively, interacting partners may be eluted separately from 6xHis-tagged proteins by disturbing the intermolecular interactions between them (e.g., by increasing salt concentration or introducing competing reagents). To avoid unwanted elution of the 6xHis-tagged protein, be careful to avoid conditions that would reduce the 6xHis-tag–Ni-NTA interaction (refer to Table 3, page 18).

As an alternative to elution, the interacting biomolecule may be detected directly, e.g., by using specific antibodies (refer to protocol 5, page 32) or the Ni-NTA Magnetic Agarose Beads may be heated in SDS-PAGE sample buffer and the proteins analyzed by SDS-PAGE followed by Coomassie or silver staining, or western blotting.

Protocol 4. Assay using 96-well microplates

Materials

Cell lysate or purified 6xHis-tagged protein diluted in Protein Binding Buffer

Ni-NTA Magnetic Agarose Beads

Tween 20 stock solution

Wash Buffer for assays

Interaction Buffer — **Note:** Choose a buffer to optimize your specific interaction, but bear in mind the limitations detailed in Table 3 (page 18). Add 20 mM **imidazole** to interaction buffer to prevent nonspecific binding to Ni-NTA. It is preferable to maintain pH at 7.0–8.0 and to use 300 mM NaCl, but for interactions that are inhibited by such high salt concentrations, for example those involving DNA molecules, 50 mM NaCl can be used (see Figure 9, page 25).

Elution Buffer for assays (optional)

Magnetic separator (any commercial separator) — e.g., 96-Well Magnet (Cat. No. 36915)

For buffer and reagent compositions, see appendix, page 80.

Note: As an assay using magnetic beads differs to some extent from assays using microplates, the buffers have been designed and optimized for use with magnetic beads. It is therefore very important to use the buffers that are recommended here to obtain optimal results. The addition of **Tween 20** is necessary to enable optimal collection of beads on the sides of the microplate wells.

1. **Resuspend the Ni-NTA Magnetic Agarose Beads by vortexing for 2 s and then immediately add 10 µl of the 5% Ni-NTA Magnetic Agarose Beads suspension to 200 µl of the 6xHis-tagged protein solution in each well of a 96-well microplate.**

   Note: Care is necessary to ensure that constant amounts of beads are pipetted. The beads tend to settle out if the suspension is not agitated regularly.

   We recommend use of 96-Well Microplates FB (see ordering information, page 84), or similar 96-well microplates that have flat-bottom wells, to be able to handle the recommended volumes and to enable optimal mixing efficiency.

   The buffer should always contain **imidazole**. For most 6xHis-tagged proteins, up to 20 mM **imidazole** can be used without affecting the binding properties. However, if the tagged protein does not bind under these conditions, the amount of imidazole should be reduced to 5–10 mM.

   For optimal performance, the binding capacity of the Ni-NTA Magnetic Agarose Beads used in each tube should approximately match the amount of 6xHis-tagged protein to be captured.

10 µl magnetic-bead suspension has a binding capacity of 3 µg 6xHis-tagged DHFR (24 kDa). If significantly different amounts of tagged protein are present in your lysate, the volume of magnetic-bead suspension should be varied accordingly (see Table 2, page 17). However, use of volumes less than 10 µl are not recommended due to the associated handling problems — smaller volumes are difficult to pipet and may lead to uneven distribution of beads and reduced reproducibility.

2. **Mix for 45 min to 1 h. Mixing will be optimal on a microplate shaker. Alternatively vortex at 600 rpm using an adapter for microplates.**

   The time and temperature necessary for efficient binding is dependent on the protein and the accessibility of the 6xHis tag in the buffer system used. It may be necessary to incubate at 4°C if the protein is not stable at room temperature.

3. **Add 20 µl 1% Tween 20 to each well and shake for 15 min.**

   The addition of **Tween 20** is necessary to enable optimal collection of beads on the sides of the microplate wells.

4. **Place the 96-well microplate on the 96-Well Magnet for 1 min and remove supernatant with a pipet.**

   Bead separation may be assisted by lightly tapping the plate on the 96-Well Magnet or by placing the magnet together with the plate on the microplate shaker.

5. **(Optional) If a cell lysate is used as source of the 6xHis-tagged protein, wash the beads twice with Wash Buffer.**

   If using purified proteins this step can be omitted.

6. **Add 200 µl of Interaction Buffer to each well, mix (on a microplate shaker), place for 1 min on the 96-Well Magnet, and remove buffer.**

   This is to allow thorough exchange of the buffers.

7. **Add up to 200 µl solution containing potentially interacting biomolecules in Interaction Buffer and incubate on shaker for 1 h at room temperature.**

   The buffer composition and concentration of binding partners used for this binding step will depend on the characteristics of the biomolecular interactions between the bound 6xHis-tagged molecule and the nontagged binding partner. Optimization of this step is likely to be necessary — use Table 3 (page 18) as a guide to avoid components that will affect binding of the 6xHis-tagged molecule to the Ni-NTA Magnetic Agarose Beads. The buffer should always contain **imidazole** to prevent nonspecific binding to Ni-NTA. For most 6xHis-tagged proteins, up to 20 mM **imidazole** can be used without affecting the binding properties. However, if the tagged protein does not bind under these conditions, the amount of imidazole should be reduced to 5–10 mM.

8. **Wash by adding 200 µl of Interaction Buffer to each well, mix (on the microplate shaker), place for 1 min on the 96-Well Magnet, and remove buffer.**

   The composition of buffer used for this wash step will depend on the characteristics of the biomolecular interactions between the bound 6xHis-tagged molecule and the nontagged binding partner. Use Table 3 (page 18) as a guide to avoid components that will affect binding of the 6xHis-tagged molecule to the Ni-NTA Magnetic Agarose Beads.

   In general, washing can be carried out with the same buffer composition used for interaction. However, if background is high, it may be advantageous to use a different buffer for washing or repeat step 7.

9. **Add 50 µl of Elution Buffer to each well, mix (on the microplate shaker), incubate for 1 min, place for 1 min on the 96-Well Magnet, and collect the eluate.**

   If the protein solution should be more concentrated, elution can be performed with 25 µl — greater than 80% of bound protein will usually elute in the first step. Elution volumes of less than 25 µl are not recommended because it is difficult to recover small volumes of elution buffer from the microplate wells.

   This step will release the 6xHis-tagged protein from the magnetic beads. Depending on the interaction with the molecules captured in the assay, they may remain as a complex after elution.

   Alternatively, interacting partners may be eluted separately from 6xHis-tagged proteins by disturbing the intermolecular interactions (e.g., by increasing salt concentration, introducing competing reagents). To avoid unwanted elution of the 6xHis-tagged protein, be careful to avoid conditions that would reduce the 6xHis-tag–Ni-NTA interaction (refer to Table 3, page 18).

   As an alternative to elution, the interacting biomolecule may be detected directly, e.g., by using specific antibodies (refer to protocol 5 – everything necessary for maintaining the interaction needs to be included in the buffers used throughout the detection procedure) or, the Ni-NTA Magnetic Agarose Beads may be heated in SDS-PAGE sample buffer and the proteins analyzed by SDS-PAGE followed by Coomassie or silver staining, or western blotting.

Protocol 5. Immunoassay using 96-well microplates

Materials

Cell lysate or purified 6xHis-tagged protein diluted in Protein Binding Buffer

Ni-NTA Magnetic Agarose Beads

Wash Buffer for assays

Tween 20 stock solution

Primary antibody or test serum

Secondary antibody

Substrate solution (see page 82).

Antibody Dilution Buffer

Magnetic separator — e.g., 96-Well Magnet (Cat. No. 36915)

For buffer and reagent compositions, see appendix, page 80.

Note: As an assay using magnetic beads differs from assays using microplates, the buffers have been designed and optimized for use with magnetic beads. It is therefore very important to use the buffers that are recommended here to obtain optimal results. The addition of **Tween 20** is necessary to enable optimal collection of beads on the sides of the microplate wells.

Note: If starting with protocol 4, everything necessary for maintaining the interaction needs to be included in the buffers used throughout the detection procedure. Start with step 5 of the following protocol.

1. **Resuspend the Ni-NTA Magnetic Agarose Beads by vortexing for 2 s and then immediately add 10 µl of the 5% Ni-NTA Magnetic Agarose Beads suspension to 200 µl 6xHis-tagged antigen solution in each well of a 96-well microplate.**

   Note: Care is necessary to ensure that constant amounts of beads are pipetted. The beads will settle if the suspension is not agitated regularly.

   We recommend use of 96-Well Microplates FB (see Ordering Information, page 84), or similar 96-well microplates that have flat-bottom wells, to be able to handle the recommended volumes and to enable optimal mixing efficiency.

   The buffer should always contain **imidazole**. For most 6xHis-tagged proteins, up to 20 mM **imidazole** can be used without affecting the binding properties. However, if the tagged protein does not bind under these conditions, the amount of imidazole should be reduced to 5–10 mM.

   For optimal performance, the binding capacity of the Ni-NTA Magnetic Agarose Beads used in each well should approximately match the amount of 6xHis-tagged protein to be captured. Tables 1 and 2 (pages 16 and 17) can be used as a guide for the volume of culture and 6xHis-tagged protein to use in each well.

10 µl magnetic-bead suspension has a binding capacity of 3 µg 6xHis-tagged DHFR (24 kDa). If significantly different amounts of tagged protein are present in your lysate, the volume of magnetic-bead suspension should be varied accordingly (see Table 2, page 17). However, use of volumes less than 10 µl may result in handling problems – smaller volumes are difficult to pipet and may lead to uneven distribution of beads and reduced reproducibility.

2. **Mix for 45 min to 1 h. Mixing will be optimal on a microplate shaker. Alternatively, vortex at 600 rpm using an adapter for microplates.**

   The time and temperature necessary for efficient binding is dependent on the protein, and the accessibility of the 6xHis tag in the buffer system used. It may be necessary to incubate at 4°C if the protein is not stable at room temperature.

3. **Add 20 µl 1% Tween 20 to each well and shake for 15 min.**

   The addition of **Tween 20** is necessary to enable optimal collection of beads on the sides of the microplate wells.

4. **Place the 96-well microplate on the 96-Well Magnet for 1 min and remove supernatant with a pipet.**

   Bead separation may be assisted by lightly tapping the plate on the 96-Well Magnet or by placing the magnet together with the plate on the microplate shaker.

5. **Add 200 µl Wash Buffer to each well, mix (on a microplate shaker), place for 1 min on the 96-Well Magnet or a similar magnetic separator, and remove buffer.**

   This is for removal of excess proteins.

6. **Add 200 µl test serum or antibody diluted in Antibody Dilution Buffer and incubate on shaker for 1 h at room temperature.**

   The dilution of test serum will depend on the characteristics and concentration of the antibodies in the serum binding to the captured 6xHis-tagged antigen. The buffer should always contain **3% BSA** (unless carrying out protein interaction studies [see protocol 4] when disturbance of a sensitive interaction may be caused, in which case the concentration of BSA may be reduced to between 0.5 and 1%).

7. **Wash 2 times by adding 200 µl Wash Buffer to each well, mix 5 min (on the microplate shaker), place for 1 min on the 96-Well Magnet, and remove buffer.**

8. **Add 200 µl appropriate secondary antibody conjugate diluted in Antibody Dilution Buffer and incubate on shaker for 1 h at room temperature.**

   Antibodies conjugated to either alkaline phosphatase or horseradish peroxidase can be used. They are commercially available from many suppliers. Dilute according to the manufacturer's recommendations. Use the lowest recommended concentration to minimize background signals.

   The buffer should always contain **3% BSA** (unless carrying out protein interaction studies [see protocol 4] when disturbance of a sensitive interaction may be caused, in which case the concentration of BSA may be reduced to between 0.5 and 1%).

9. **Wash 4 times by adding 200 µl Wash Buffer to each well, mix 5 min (on the microplate shaker), place for 1 min on the 96-Well Magnet, and remove buffer.**
10. **Add 200 µl of the appropriate substrate solution, mix (on the microplate shaker) while incubating to allow color development.**

    Substrate solution should always be prepared immediately before use.

    The time necessary for color development will rely on the assay system. A good starting point is 10–15 min incubation at room temperature.
11. **Stop reaction by placing the 96-well microplate for 1 min on the 96-Well Magnet, and transferring the solution to a fresh microplate.**
12. **Measure absorbance at the appropriate wavelength in a microplate reader (see Table 4).**

Table 4. Details of substrates for assay procedures

Substrate	Wavelength for monitoring color development
pNPP	405 nm
OPD	450 nm

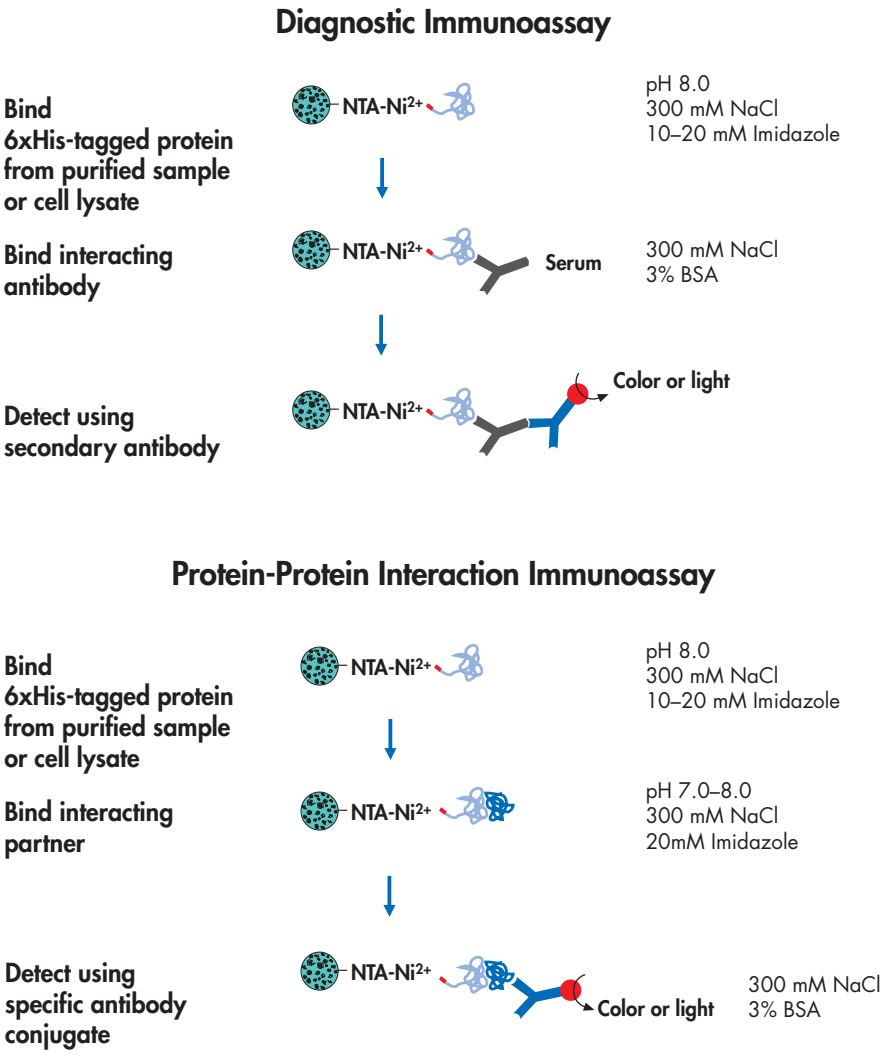

Figure 10. *Immunoassays with Ni-NTA Magnetic Agarose Beads. The diagnostic immunoassay is described in detail in protocol 5 (page 32).*

Purification with Ni-NTA Magnetic Agarose Beads

Important notes before starting

- The amount of Ni-NTA Magnetic Agarose Beads that are used in the purification protocol depends on the amount and concentration of protein to be purified. Table 2 (page 17) gives volumes of bead suspension necessary to bind different amounts of protein and the corresponding elution volumes and final protein concentrations in the eluates.
- Purification can be performed under native or denaturing conditions: the appropriate buffers must be used throughout.
- The addition of Tween 20 is necessary to enable optimal collection of the magnetic beads.
- The protocols in this handbook have been developed for protein purification from *E. coli*. A protocol for purification of 6xHis-tagged proteins from mammalian cells using Ni-NTA Magnetic Agarose Beads is included in *The QIA*expressionist.

Materials

Cell lysate (see protocol 1 or 2)

Ni-NTA Magnetic Agarose Beads

Wash Buffer for native **or** denaturing conditions

Elution Buffer for native **or** denaturing conditions

Magnetic separator — e.g., 12-Tube Magnet (Cat. No. 36912) for protocol 6 or 96-Well Magnet Type A (Cat. No. 36915) for protocol 7.

For buffer and reagent compositions, see appendix, page 80.

Protocol 6. Purification in single reaction tubes

1. **Resuspend the Ni-NTA Magnetic Agarose Beads by vortexing for 2 s and then immediately add 200 µl of the 5% Ni-NTA Magnetic Agarose Bead suspension to 1 ml of the lysate containing the 6xHis-tagged protein.**

   Note: Care is necessary to ensure that constant amounts of beads are pipetted. The beads will settle if the suspension is not agitated regularly.

   200 µl magnetic-bead suspension has a binding capacity of 60 µg 6xHis-tagged DHFR (24 kDa). If significantly different amounts of tagged protein are present in your lysate, the volume of magnetic-bead suspension should be varied accordingly (see Table 2, page 17). However, use of volumes less than 10 µl are not recommended due to the associated handling problems — smaller volumes are difficult to pipet and may lead to uneven distribution of beads and reduced reproducibility.

   Generally we recommend using cleared lysates for binding to Ni-NTA Magnetic Agarose Beads. However, it may be possible to obtain good results by using crude lysates without clearing them. In this case, use dilute lysates which have been concentrated 5-fold, and if using native lysis conditions, add RNase A to 10 µg/ml and DNase I to 5 µg/ml, and incubate on ice for 10–15 min.

2. **Mix the suspension gently on an end-over-end shaker for 30 min to 1 h at room temperature.**

   The time and temperature necessary for efficient binding is dependent on the protein and the accessibility of the 6xHis tag in the buffer system used. Especially under native conditions, it may be necessary to incubate at 4°C if the protein is not stable at room temperature.

3. **Place the tube on a magnetic separator for 1 min and remove supernatant with a pipet.**

   Tubes may be briefly centrifuged, before placing on the magnetic separator, to collect droplets of suspension from the tube caps.

4. **Remove tube from the magnet, add 500 µl of Wash Buffer, mix the suspension, place the tube on a magnetic separator for 1 min, and remove Wash Buffer.**

5. **Repeat step 4 another 1–2 times.**

   Buffer remaining after the final wash should be removed completely.

6. **Add 100 µl of elution buffer, mix the suspension, incubate the tube for 1 min, place for 1 min on magnetic separator, and collect the eluate.**

   Tubes may be centrifuged, before placing on the magnetic separator, to collect droplets of suspension from the tube caps.

7. **Repeat step 6.**

   Most of the 6xHis-tagged protein will elute in the first elution step. If a more concentrated protein solution is required, elute in two aliquots of 50 µl.

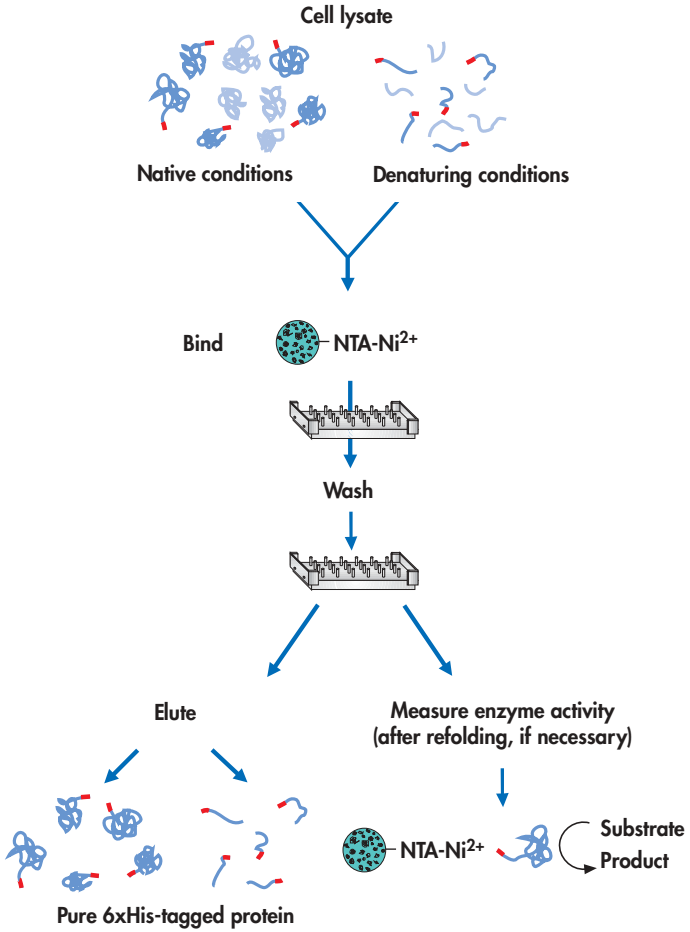

Figure 11. *Micro-scale protein purification in 96-well format with Ni-NTA Magnetic Agarose Beads and the QIAGEN 96-Well Magnet.*

Protocol 7. Purification in 96-well microplates

Note: The addition of **Tween 20** to buffers is necessary to enable optimal collection of the magnetic beads on the sides of the microplate wells.

1. **Resuspend the Ni-NTA Magnetic Agarose Beads by vortexing for 2 s and then immediately add 20 µl of the 5% Ni-NTA Magnetic Agarose Beads suspension to 200 µl of the lysate containing the 6xHis-tagged protein in each well of a flat-bottom 96-well microplate.**

   Note: care is necessary to ensure that constant amounts of beads are pipetted. The beads will settle if the suspension is not agitated regularly.

   We recommend use of 96-Well Microplates FB (see Ordering Information, page 84), or similar 96-well microplates that have flat-bottom wells, to be able to handle the recommended volumes and to enable optimal mixing efficiency.

   20 µl magnetic-bead suspension has a binding capacity of 6 µg 6xHis-tagged DHFR (24 kDa). If significantly different amounts of tagged protein are present in your lysate, the volume of magnetic-bead suspension should be varied accordingly (see Table 2, page 17). However, use of volumes less than 10 µl are not recommended due to the associated handling problems — smaller volumes are difficult to pipet and may lead to uneven distribution of beads and reduced reproducibility.

   Generally we recommend using cleared lysates for binding to Ni-NTA Magnetic Agarose Beads. However, it may be possible to obtain good results by using crude lysates without clearing them. In this case, use dilute lysates which have been concentrated 5-fold, and if using native lysis conditions, add RNase A to 10 µg/ml and DNase I to 5 µg/ml, and incubate on ice for 10–15 min.

2. **Mix for 30 min to 1 h. Mixing will be optimal on a microplate shaker. Alternatively, vortex at 600 rpm using an adapter for microplates.**

   The time and temperature necessary for efficient binding is dependent on the protein and the accessibility of the 6xHis tag in the buffer system used.

3. **Place the 96-well microplate on the 96-Well Magnet for 1 min and remove supernatant with a pipet.**

4. **Add 200 µl of Wash Buffer to each well, mix on the microplate shaker (or vortex), place on the 96-Well Magnet for 1 min, and remove buffer.**

5. **Repeat step 4.**

6. **Add 50 µl of Elution Buffer to each well, mix on the microplate shaker (or vortex), incubate for 1 min, place on the 96-Well Magnet for 1 min, and collect the eluate.**

   If the protein solution should be more concentrated, elution can be performed with 25 µl — greater than 80% of bound protein will usually elute. Elution volumes of less than 25 µl are not recommended because it is difficult to recover small volumes of elution buffer from the microplate wells.

Ni-NTA Magnetic Agarose Beads BioRobot™ Protocols

For

Automated assays using 6xHis-tagged proteins

Automated micro-scale purification of 6xHis-tagged proteins

Ni-NTA Magnetic Agarose Beads BioRobot Protocols

Four different fully automated BioRobot protocols for use with Ni-NTA Magnetic Beads have been developed. These protocols can be performed alone or be flexibly combined to fit specific experimental needs (see Figure 12, page 42). Separation of Ni-NTA Magnetic Beads is performed using the Magnet, 96-Well, Type A for automated procedures (Cat. No. 9014061).

Purification protocols (Protocols 8 and 9) can be run on a BioRobot 3000 or 8000 configuration. The highly flexible modular design of the BioRobot 3000 allows the full automation of interaction and immunoassays (Protocols 10 and 11). Both the BioRobot 3000 and 8000 provide a microprocessor-controlled shaker for efficient mixing of magnetic-bead suspension, assay components, or pelleted material; a Robotic Handling System for transfer of blocks and plates between different positions on the worktable; and a liquid-handling system for accurate cross-contamination–free pipetting of small volumes. For details on the BioRobot 3000 or 8000, call QIAGEN Technical Services or your local distributor.

Purification protocols

Two BioRobot Protocols allow micro-scale purification of up to 15 µg 6xHis-tagged protein expressed in 96-well cultures. Purification can be performed under either native (Protocol 8) or denaturing (Protocol 9) conditions. These protocols correspond to Protocol 7 in this handbook, but allow fully automated processing of the purification procedure. For automated purification procedures, we recommend clearing the lysates before binding 6xHis-tagged proteins to the magnetic beads. Crude, uncleared lysates can be used, but yield and purity of the purified protein may be slightly reduced. Clearing of the lysates can easily be performed using the QIAGEN Centrifuge 4-15C and Plate Rotor 2 x 96. In the protocol for purification under denaturing conditions this lysate clearing step is optional, but for purification under native conditions, especially when the purified protein is to be used directly in subsequent assays, we strongly recommend clearing the cell lysates by centrifugation.

Expression cultures are grown in 96-well Square-Well Blocks according to the recommendations in the appendix (see page 80). The use of Square-Well Blocks is necessary to ensure trouble-free transfer of the cleared lysate during the purification procedure. The purification protocol under native conditions comprises the following steps: cell lysis, clearing of the lysates, binding of the 6xHis-tagged proteins to the Ni-NTA Magnetic Agarose Beads, washing (twice), and the optional elution of the purified protein (see Figure 13, page 47). The purification protocol under denaturing conditions, provides steps for cell lysis, optional clearing of the lysates, binding to Ni-NTA Magnetic Agarose Beads, washing (twice), and elution of the purified protein (see Figure 14, page 53). 6xHis-tagged protein is eluted in elution buffer and 50 µl aliquots are transferred to a new microplate.

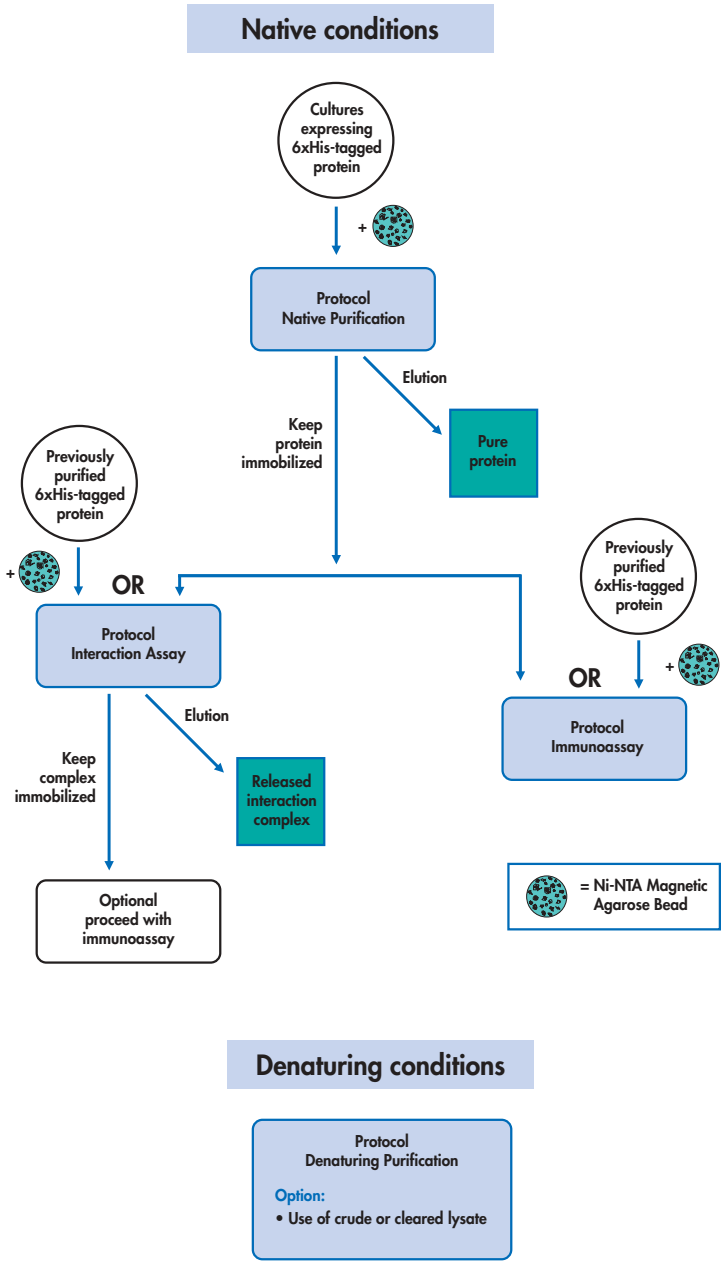

Figure 12. *Overview of automated protocols.*

Assay protocols

Protocols 10 and 11 allow processing of either interaction assays or immunoassays using 6xHis-tagged proteins immobilized on Ni-NTA Magnetic Agarose Beads. These protocols correspond to the manual protocols 4 and 5 in this handbook. Previously purified 6xHis-tagged proteins can be immobilized on the beads by choosing "No" when asked "Is the protein already bound to the beads?". In this case, the protein has to be provided in 200 µl Buffer NPI-20 in the wells of a 96-Well Microplate FB (96-well microplates with flat-bottom wells, see page 83 for ordering information). The magnetic beads are pipetted into the wells containing the protein solutions. Binding is performed by incubation for 1 hour at room temperature. Alternatively, cells expressing the protein of interest can be used directly. In this case, first the protocol for purification under native conditions has to be performed choosing "No" when asked "Do you want to elute protein from the beads?" (with this option 6xHis-tagged protein is bound to the beads, washed, and remains immobilized on the beads because the elution step is omitted) and subsequently one of the assay protocols is performed choosing "Yes" when asked "Is the protein already bound to the beads?". Using this setting the assay protocol starts with the first wash step after immobilization.

For establishing assays, we strongly recommend testing the setup with a previously purified protein to allow analysis of yield, characteristics and purity of the protein, prior to starting the assay. Performing an interaction assay, the detection method of choice may be used. It is possible to either elute the interaction complex for subsequent analysis, for example by SDS-PAGE, or to detect the immobilized complex without elution. Detection may be performed using antibodies in an immunoassay or by any other preferred method.

The interaction protocol includes the steps: binding of the protein to Ni-NTA Magnetic Agarose Beads (optional), washing to allow buffer exchange, adding potentially interacting biomolecules, incubation to allow interaction-complex formation, washing, and either elution or detection. If detection is chosen, an optional immunoassay procedure can be performed (see Figure 12). The immunoassay protocol comprises the steps: binding of the protein to Ni-NTA Magnetic Agarose Beads (optional), washing to remove excess proteins, incubating with primary antibody, washing (twice), incubating with secondary antibody, washing (four times), and adding colorimetric substrate. The enzyme reaction is stopped and subsequently the assay signals can be measured using any conventional microplate reader.

Specific adjustments

The protein protocols are designed to allow maximum flexibility for your specific application without too many questions to answer at the beginning of each protocol. The preset standard conditions should suit most experiments, but further adjustment of many settings as listed below is easily possible. These settings can be adjusted in the software or call QIAGEN Technical Services or your local distributor.

The purification protocols are intended for micro-scale purification of 6xHis-tagged proteins from *E. coli* cultures grown in 96-well blocks, but the use of other culture vessels, such as 24-well blocks, 48-well blocks, or 12 ml tubes is also possible.

The standard protein purification protocols use 20 µl of 5% (v/v) magnetic-bead suspension corresponding to a binding capacity of 6 µg of 6xHis-tagged protein. If larger amounts of protein are expected and need to be purified, the binding capacity used can be adjusted by varying the amount of magnetic-bead suspension according to Table 5.

Table 5. Volumes of bead suspension and maximum amount of protein to be purified

5% Ni-NTA Magnetic Agarose Beads suspension	2.5% Ni-NTA Magnetic Agarose Beads* suspension	Maximum amount of 6xHis-tagged protein to be purified
–	20 µl	3 µg
20 µl	40 µl	6 µg
30 µl		9 µg
40 µl		12 µg
50 µl		15 µg

** Magnetic-bead suspension is diluted for the assay protocols*

The assay protocols use 20 µl of 2.5% (v/v) Ni-NTA Magnetic Agarose Bead suspension as standard, which corresponds to 3 µg immobilized 6xHis-tagged protein. If larger amounts of immobilized protein are necessary, the binding capacity can be adjusted by varying the amount of magnetic-bead suspension according to Table 5.

In the interaction protocol, the volume of interaction partner added is preset to 20 µl. We recommend using volumes of at least 20 µl to ensure optimal pipetting accuracy. If necessary, dilute the solution containing the biomolecule accordingly. A larger volume may be chosen if necessary, but care should be taken not to exceed a maximum volume of 240 µl in each well of the microplate.

Antibodies or sera and potentially interacting biomolecules are distributed from one vial containing sufficient sample for all wells containing the test protein. If different sera or interaction partners are intended to be used in different wells, they may be transferred from a 96-well plate.

If a labelled primary antibody (e.g., enzyme conjugated or fluorescently labeled) is used, the two wash steps following the primary antibody incubation and the incubation of the secondary antibody can be disabled.

A color development time of 10 min is set as default, but if significantly different times are needed, the incubation time may easily be adjusted.

Protocol 8. Ni-NTA Magnetic Agarose Beads BioRobot protocol for purification under native conditions

Materials

Expression cultures grown in 96-well Square-Well Block (BioRobot 3000) or S-Block (BioRobot 8000)

96-Well Microplates FB

96-Well Magnet Type A

5% (v/v) Ni-NTA Magnetic Agarose Beads (2 ml is required for 96 samples)

QIAGEN Centrifuge 4-15C

Tape Pad

Disposable Troughs

For ordering information for the above items, see page 84.

Growing bacterial cultures

For guidelines on the growth of bacterial expression cultures, see appendix (page 80).

E. coli cultures are grown in 96-well square-well blocks using 1 ml culture medium, containing the appropiate antibiotics, per well. The use of square-well blocks is essential to ensure trouble-free transfer of the cleared lysates. After cultivation, the cells are harvested at 4000 x *g* for 10 min, the medium is discarded, and the cell pellets are stored frozen at –20°C. Frozen cell pellets can be immediately used in the purification procedure, a thawing step is incorporated in the protocol. To lyse cells in the 96-well procedure, sonication is not practical, therefore it is necessary to **freeze the cell pellets** after harvesting for at least 1 hour. To enhance cell lysis further, detergent is added to the lysis buffer.

Preparation of buffers

Note: The addition of **0.05% Tween 20** to all buffers is necessary to enable optimal collection of the magnetic beads on the sides of the microplate wells.

Buffer NPI-10-Tween, 500 ml: (lysis buffer)

50 mM NaH_2PO_4	3.45 g $NaH_2PO_4 \cdot H_2O$ (MW 137.99)
300 mM NaCl	8.77 g NaCl (MW 58.44)
10 mM imidazole	0.34 g imidazole (MW 68.08)
1% Tween 20	50 ml of a 10% Tween 20 stock solution

Adjust pH to 8.0 using NaOH.

Prepare 500 ml in a 500 ml bottle.

Note: Including 1% Tween 20 in the lysis buffer facilitates efficient cell lysis.

Buffer NPI-20-Tween, 500 ml: (wash buffer)

50 mM NaH_2PO_4	3.45 g $NaH_2PO_4 \cdot H_2O$ (MW 137.99)
300 mM NaCl	8.77 g NaCl (MW 58.44)
20 mM imidazole	0.68 g imidazole (MW 68.08)
0.05% Tween 20	2.5 ml of a 10% Tween 20 stock solution

Adjust pH to 8.0 using NaOH.

Prepare 500 ml in a 500 ml bottle.

(optional)

Buffer NPI-250-Tween, 50 ml: (elution buffer)

50 mM $NaH_2 2PO_4$	0.35 g $NaH_2PO_4 \cdot H_2O$ (MW 137.99)
300 mM NaCl	0.88 g NaCl (MW 58.44)
250 mM imidazole	0.85 g imidazole (MW 68.08)
0.05% Tween 20	0.25 ml of a 10% Tween 20 stock solution

Adjust pH to 8.0 using NaOH.

Provide the buffer in a trough. To process 96 samples, 7 ml of buffer is required.

Additional solutions

Prepare freshly the calculated volume of lysozyme solution (10 mg/ml in water) in a 2 ml microcentrifuge tube. For 96 samples, 2 ml of the lysozyme solution is required. If using crude lysate for processing add DNase (e.g., Benzonase) at 50 units per ml lysozyme solution.

Table 6. Tubing/position on worktable for purification under native conditions

Buffers/solutions/accessories	BioRobot 3000	BioRobot 8000
NPI-10-Tween	Tubing 1	Rotor Slot 2
NPI-20-Tween	Tubing 2	Rotor Slot 4
NPI-250-Tween	Trough Slot 1	Therm Subslot A, Trough 20ml
Ni-NTA Magnetic Agarose Beads suspension	Reagent Slot B5	Therm Subslot B (A)
Lysozyme solution	Reagent Slot B6	Therm Subslot B (B)
Working microplate	Shaker front/left	Shaker front/left
Elution microplate	Shaker back/right	MP Slot 13
Magnet, 96-well, Type A	Shaker front/right	Shaker front/right
Square-Well Block	Shaker back/left	Shaker back/left

Micro-Scale Protein Purification under Native Conditions

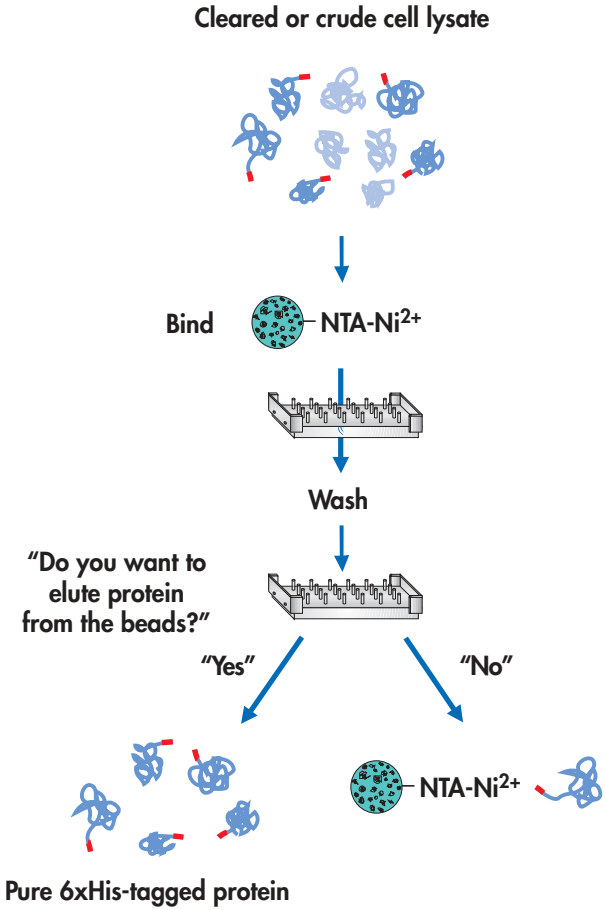

Figure 13. *Micro-scale protein purification in 96-well format with Ni-NTA Magnetic Agarose Beads and the QIAGEN 96-Well Magnet Type A.*

Micro-scale protein purification under native conditions (BioRobot 3000 and 8000)

1. **Prepare all reagents required for the protocol (see above).**

   Note: If crude lysate is to be processed DNase must be added to the lysozyme solution in Reagent Slot B6 (BioRobot 3000) or Therm Subslot B (B) (BioRobot 8000).

2. **Make sure that the High-Speed Pipetting System (BioRobot 3000 only) and the BioRobot are switched on.**

   Note: Always switch on the High-Speed Pipetting System before the BioRobot 3000.

3. **Switch on the computer and monitor.**
4. **Start QIAsoft.**
5. **Select the "Protein Applications" package in the toolbar drop-down list and from this package select "Native Purification Ni-NTA Magnetic Beads".**
6. **Click "RUN" on the toolbar.**

   The "Run protocol: no. of samples" dialog box appears.

7. **Enter the number of samples to be processed (24–96).**
8. **Click "OK".**

   A variable input box appears: "Do you want to use cleared lysates?"

9. **To clear the cell lysates by centrifugation prior to performing the purification click "Yes". Alternatively, to purify proteins directly from crude, uncleared lysates click "No".**

   Note: If you are processing crude, uncleared lysates, DNase must be added to the lysozyme solution in Reagent Slot B6 (BioRobot 3000) or Therm Subslot B (B) (BioRobot 8000).

   A variable input box appears: "Do you want to elute protein from the beads?"

10. **To end the protocol with elution of the purified protein from the magnetic beads click "Yes". Alternatively, to perform further experiments, for example an interaction assay or immunoassay with the protein immobilized on the beads, click "No".**
11. **Click "Continue".**

    The wizard box appears within the panel System Setup.

12. **Make sure that the system liquid reservoir is filled with distilled water.**
13. **Empty the waste container.**
14. **Make sure that the cassettes of the peristaltic pump are fitted (BioRobot 3000 only).**
15. **Click "Next".**

    The panel Reagent Setup appears.

16. **Connect the bottle containing Buffer NPI-10-Tween in the Reagent Delivery Module to tubing 1 (BioRobot 3000) or Rotor Slot 2 (BioRobot 8000).**

17. **Connect the bottle containing Buffer NPI-20-Tween in the Reagent Delivery Module to tubing 2 (BioRobot 3000) or Rotor Slot 4 (BioRobot 8000).**
18. **Click "Next".**

    The panel Worktable Reagent Setup appears.
19. **If the protein is to be eluted from the magnetic beads place Buffer NPI 250-Tween into Trough Slot 1 (BioRobot 3000) or Therm Subslot A (BioRobot 8000).**
20. **Place the calculated volume of Ni-NTA Magnetic Agarose Beads suspension (5% [v/v]) in a 2 ml microcentrifuge tube into Reagent Slot B5 (BioRobot 3000) or Therm Subslot B (A) (BioRobot 8000).**
21. **Place a 2 ml microcentrifuge tube containing the calculated volume of lysozyme solution into the Reagent Slot B6 (BioRobot 3000) or Therm Subslot B (B) (BioRobot 8000).**
22. **Click "Next".**

    The panel worktable module setup appears.
23. **Place an empty 96-well microplate into each of the shaker-MP front/left and back/right positions (BioRobot 3000) or front/left and MP13 positions (BioRobot 8000).**

    The second microplate is only necessary if the protein is to be eluted ("Yes" was selected in step 10).
24. **Place the 96-well magnet into the shaker-MP front/right position.**
25. **Click "Next".**

    The panel Sample Setup appears.
26. **Place the square-well block containing the frozen cell pellets into the shaker-MP back/left position.**
27. **Click "Continue".**

    The BioRobot initializes and calibrates.

    BioRobot 3000: The system probes are flushed using the peristaltic pump. The dilutor syringes are flushed. The volumes of the solutions are checked.

    BioRobot 8000: The dispenser is flushed. The volumes of the solutions in the reagent rotor are checked. The dispenser and the pipetting probes are flushed.

    The system pauses to allow cells to thaw for approximately 20 minutes.

    210 µl Buffer NPI-10-Tween is dispensed and the shaker resuspends the bacterial pellets.

    The vessels containing the cells are shaken at 750 rpm for 10 minutes. For efficient resuspension, the shaker changes the direction of rotation every 30 seconds.

    20 µl lysozyme solution is distributed and the cells are shaken for an additional 20 minutes to allow completion of lysis.

    A protocol message box appears.

User interaction

"Yes" selected in step 9.

1. **Seal the Square-Well Block with tape from a Tape Pad and centrifuge the block at 6000 rpm for 30 minutes at 10°C.**
2. **Click "Continue".**

   Near to the end of the centrifugation step, the Ni-NTA Magnetic Beads suspension is mixed and 20 µl are distributed to the wells of the 96-Well Microplate FB in the shaker-MP front/left position.

   A protocol message box appears and a beeper sounds to let you know that the process is finished and that the centrifugation step is nearly finished.
3. **Collect the samples from the centrifuge immediately and proceed with the protocol.**
4. **Click the speaker icon to stop the beeper.**
5. **Place the Square-Well Block back in the shaker-MP back/left position.**
6. **Click "Continue".**

The protocol continues as described below under "Continuation of protocol".

"No" selected in step 9

At the end of the cell lysis step the Ni-NTA Magnetic Beads suspension is mixed and distributed to the wells of the microplate FB in the Shaker-MP front/left position.

The protocol continues as described below under "Continuation of protocol".

Continuation of protocol (for crude or cleared lysates — "Yes" or "No" selected in step 9)

200 µl of each of the cleared lysates are transferred to the microplate containing the magnetic beads.

The samples and beads are shaken at 750 rpm for 30 minutes to allow binding of 6xHis-tagged proteins to the Ni-NTA Magnetic Agarose Beads.

Wash procedure

Following the binding step, two wash steps are carried out. The beads are separated on the Magnet, 96-well, Type A, and supernatant containing the unbound cell constituents is removed. The beads are resuspended in 200 µl wash buffer and shaken at 750 rpm for 2 minutes.

The plate is then returned to the magnet to allow bead separation and removal of the supernatant. The beads are resuspended in a second wash step using 100 µl wash buffer by shaking at 750 rpm for 2 minutes.

The plate is returned to the magnet to allow bead separation and removal of the supernatant, and then transferred back to the shaker.

The BioRobot proceeds with elution of the protein or resuspension of the Ni-NTA Magnetic Agarose Beads according to the procedure selected in step 10.

Elution of proteins from the beads ("Yes" was selected in step 10)

The beads are resuspended in 60 µl of elution buffer and shaken at 750 rpm for 5 minutes. The microplate is transferred to the magnet, the beads are separated, and 50 µl of the eluate is transferred to the elution microplate in the shaker-MP back/right position.

The protocol continues as described below under "End of the protocol".

Resuspension of the beads ("No" was selected in step 10)

The beads are resuspended in 200 µl of wash buffer by shaking at 750 rpm for 2 minutes. Subsequently, the beads with the immobilized protein may be stored or may be used directly in the interaction or immunoassay protocol.

The protocol continues as described below under "End of the protocol".

End of the protocol

A beeper sounds to let you know that the purification process is finished. The next protocol message box appears.

1. **To generate a report file select "Yes" in the following message box.**

System wash procedure (BioRobot 3000 only)

1. **Click the speaker icon to stop the beeper.**
2. **Click "Continue".**
3. **To run the wash procedure, select "Yes".**

   If the wash protocol is not to be run, select "No".
4. **Click "Continue".**

   A protocol message dialog box appears.
5. **Connect the buffer bottle connectors to the flushing bottle filled with distilled water.**
6. **Click "Continue" to start the wash protocol.**

   A beeper sounds to let you know that the process is finished. A protocol message dialog box appears.
7. **Close all buffer bottles and release cassettes from the peristaltic pump.**

   The protocol is finished.

Protocol 9. Ni-NTA Magnetic Agarose Beads BioRobot protocol for protein purification under denaturing conditions

Materials

Expression cultures grown in 96-well square-well block (BioRobot 3000) or S-block (BioRobot 8000)

96-Well Microplates FB (two)

Magnet, 96-well, Type A

5% (v/v) Ni-NTA Magnetic Agarose Beads (2 ml is required for 96 samples)

QIAGEN Centrifuge 4-15C

Tape Pad

For ordering information for the above items, see page 84.

Growing bacterial cultures

For guidelines on the growth of bacterial expression cultures, see appendix page 80.

E. coli cultures are grown in 96-well square-well blocks using 1 ml culture medium, containing the appropriate antibiotics, per well. The use of square-well blocks is essential to ensure trouble-free transfer of the cleared lysate. After growth, the cultures are harvested at 4000 x *g* for 10 minutes, the medium is discarded, and the cell pellets are stored frozen at –20°C. Frozen cell pellets can be processed immediately in the purification procedure, a thawing step is included in the protocol.

Preparation of buffers

The addition of **0.05% Tween 20** to all buffers is absolutely necessary to enable optimal collection of the magnetic beads on the sides of the microplate wells.

Buffer B–Tween, 250 ml: (lysis buffer)

100 mM NaH_2PO_4	3.45 g $NaH_2PO_4 \cdot H_2O$ (MW 137.99)
10 mM Tris·Cl	0.30 g Tris base (MW 121.1)
8 M urea	120.12 g (MW 60.06)
0.05% Tween 20	1.25 ml of a 10% Tween 20 stock solution

Adjust pH to 8.0 using NaOH.

Provide 250 ml in a 250 ml bottle.

Micro-Scale Protein Purification under Denaturing Conditions

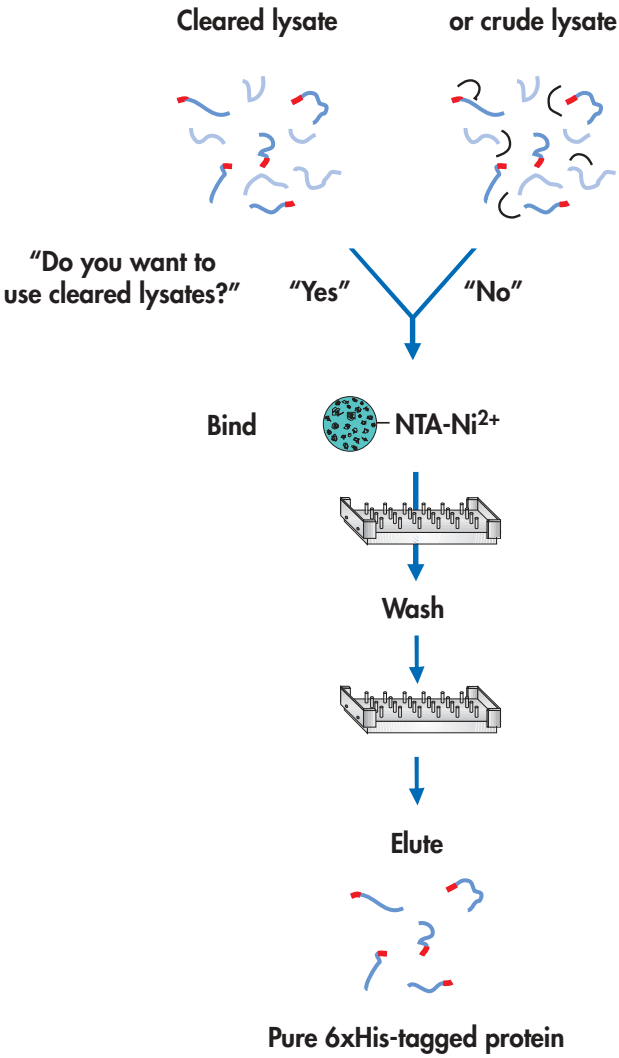

Figure 14. *Micro-scale protein purification under denaturing conditions from cleared or crude lysates.*

Buffer C–Tween, 250 ml: (wash buffer)

100 mM NaH_2PO_4	3.45 g $NaH_2PO_4 \cdot H_2O$ (MW 137.99)
10 mM Tris·Cl	0.30 g Tris base (MW 121.1)
8 M urea	120.12 g urea (MW 60.06)
0.05% Tween 20	1.25 ml of a 10% Tween 20 stock solution

Adjust pH to 6.3 using HCl or NaOH.

Provide 250 ml in a 250 ml bottle.

Buffer E–Tween, 50 ml: (Elution buffer)

100 mM NaH_2PO_4	0.69 g $NaH_2PO_4 \cdot H_2O$ (MW 137.99)
10 mM Tris·Cl	0.06 g Tris base (MW 121.1)
8 M urea	24.02 g urea (MW 60.06)
0.05% Tween 20	0.25 ml of a 10% Tween 20 stock solution

Adjust pH to 4.5 using HCl.

Provide the buffer in a trough. To process 96 samples, 7 ml of buffer is needed.

Table 7. Tubing/position on worktable for purification under denaturing conditions

Buffers/solutions/accessories	BioRobot 3000	BioRobot 8000
Buffer B-Tween	Tubing 7	Rotor Slot 2
Buffer C-Tween	Tubing 8	Rotor Slot 4
Buffer E-Tween	Trough Slot 2	Therm Subslot A, Trough 20ml
Ni-NTA Magnetic Agarose Beads suspension	Reagent Slot B5	Therm Subslot B (A)
Working microplate	Shaker front/left	Shaker front/left
Elution microplate	Shaker back/right	MP Slot 13
Magnet, 96-well, Type A	Shaker front/right	Shaker front/right
Square-Well Block	Shaker back/left	Shaker back/left

Micro-scale protein purification under denaturing conditions (BioRobot 3000 and 8000)

1. **Prepare all reagents required for the protocol (see above).**
2. **Make sure that the High-Speed Pipetting System (BioRobot 3000 only) and the BioRobot are switched on.**

   Note: Always switch on the High-Speed Pipetting System before the BioRobot 3000.
3. **Switch on the computer and monitor.**
4. **Start QIAsoft.**
5. **Select the "Protein applications" package in the toolbar drop-down list and from this package select "Denat. Purification Ni-NTA Magnetic Beads".**
6. **Click "RUN" on the toolbar.**

   The "Run protocol: no. of samples" dialog box appears.
7. **Enter the number of samples to be processed (24–96).**
8. **Click "OK".**

   A variable input box appears: "Do you want to use cleared lysates?"
9. **To clear the cell lysates by centrifugation prior to performing the purification click "Yes". Alternatively, to purify proteins directly from crude, uncleared lysates click "No".**

   Note that using crude lysates might slightly reduce yield and purity of the eluted protein.
10. **Click "Continue".**

    The wizard box appears within the panel System Setup.
11. **Make sure that the system liquid reservoir is filled with distilled water.**
12. **Empty the waste container.**
13. **Make sure that the cassettes of the peristaltic pump are fitted (BioRobot 3000 only).**
14. **Click "Next".**

    The panel Reagent Setup appears.
15. **Connect the bottle containing Buffer B-Tween in the Reagent Delivery Module to tubing 7 (BioRobot 3000) or Rotor Slot 2 (BioRobot 8000).**
16. **Connect the bottle containing Buffer C-Tween in the Reagent Delivery Module to tubing 8 (BioRobot 3000) or Rotor Slot 4 (BioRobot 8000).**
17. **Click "Next".**

    The panel Worktable Reagent Setup appears.
18. **Place Buffer E-Tween into Trough Slot 2 (BioRobot 3000) or Therm Subslot A (BioRobot 8000).**

19. **Place the calculated volume of Ni-NTA Magnetic Bead suspension (5% [v/v]) in a 2 ml microcentrifuge tube into Reagent Slot B5 (BioRobot 3000) or Therm Subslot B (A) (BioRobot 8000).**
20. **Click "Next".**

    The panel Worktable Module Setup appears.
21. **Place two empty Microplates FB into the shaker-MP front/left and back/right positions (BioRobot 3000) or front/left and MP13 positions (BioRobot 8000).**
22. **Place the 96-Well Magnet into the shaker-MP front/right position.**
23. **Click "Next".**

    The panel Sample Setup appears.
24. **Place the Square-Well Block containing the frozen cell pellets into the shaker-MP back/left position.**
25. **Click "Continue".**

    The BioRobot initializes and calibrates.

    BioRobot 3000: The system probes are flushed using the peristaltic pump. The dilutor syringes are flushed. The volumes of the solutions are checked.

    BioRobot 8000: The dispenser is flushed. The volumes of the solutions in the reagent rotor are checked. The dispenser and the pipetting probes are flushed.

    The system pauses to allow cells to thaw for approximately 20 minutes.

    230 µl of Buffer B-Tween is distributed and the shaker resuspends the bacterial pellets.

    The vessels containing the cells are shaken at 750 rpm for 30 minutes. For efficient resuspension, the shaker changes the direction of rotation every 30 seconds.

    The BioRobot proceeds with the purification procedure depending on whether "Yes" or "No" was selected in step 9 (above).

User interaction

"Yes" selected in step 9

1. **Seal the square-well block with tape from a Tape Pad and centrifuge the block at 6000 rpm for 30 minutes at 10°C.**
2. **Click "Continue".**

   Close to the end of the centrifugation step the Ni-NTA Magnetic Beads suspension is mixed and 20 µl aliquots are distributed to the wells of the microplate in the shaker-MP front/left position.

   A protocol message box appears and a beeper sounds to indicate that the process is finished, and that the centrifugation step is nearly finished. Collect the samples immediately and continue with the purification process.

3. **Click the speaker icon to stop the beeper.**
4. **Place the square-well block back in the shaker-MP back/left position.**
5. **Click "Continue".**

The protocol continues as described below under "Continuation of protocol".

"No" selected in step 9

At the end of the cell lysis step the Ni-NTA Magnetic Beads suspension is mixed and distributed to the wells of the microplate FB in the Shaker-MP front/left position.

The protocol continues as described below under "Continuation of protocol".

Continuation of protocol (for crude or cleared lysates — "Yes" or "No" selected in step 9)

200 µl volumes of the cleared lysates are transferred to the microplate containing the magnetic beads.

The samples and beads are shaken at 750 rpm for 30 minutes to allow binding of the 6xHis-tagged proteins to the Ni-NTA Magnetic Agarose Beads.

Wash procedure and elution

Following the binding step, two wash steps are carried out. The beads are separated on the Magnet, 96-well, Type A, and supernatant containing the unbound cell constituents is removed. The beads are resuspended in 200 µl wash buffer and shaken at 750 rpm for 2 minutes.

The plate is then returned to the magnet to allow bead separation and removal of the supernatant. The beads are resuspended in a second wash step using 100 µl wash buffer by shaking at 750 rpm for 2 minutes.

The plate is returned to the magnet to allow bead separation and removal of the supernatant, and then transferred back to the shaker.

The beads are resuspended in 60 µl of elution buffer and incubated at 750 rpm for 5 minutes. The plate is transferred to the magnet, the beads are separated, and 50 µl of the eluate is transferred to the elution microplate in the shaker-MP back/right position.

A beeper sounds to let you know that the purification process is finished. The next protocol message box appears.

1. **To generate a report file select "Yes".**

System wash procedure (BioRobot 3000 only)

1. **Click the speaker icon to stop the beeper.**
2. **Click "Continue".**

3. **To run the wash procedure select "Yes".**

   If the wash protocol is not to be run, select "No".

4. **Click "Continue".**

   A protocol message dialog box appears.

5. **Connect the buffer bottle connectors to the flushing bottle filled with distilled water.**

6. **Click "Continue" to start the wash protocol.**

   A beeper sounds to let you know that the process is finished. A protocol message dialog box appears.

7. **Close all buffer bottles and release cassettes of the peristaltic pump.**

   The protocol is finished.

Protocol 10. Ni-NTA Magnetic Agarose Beads BioRobot protocol for interaction assays

Materials

Previously purified 6xHis-tagged protein diluted in 200 µl Buffer NPI-20 or 6xHis-tagged protein already immobilized on Ni-NTA Magnetic Agarose Beads provided in the wells of a Microplate FB

Magnet, 96-well, Type A

5% (v/v) Ni-NTA Magnetic Agarose Beads (1 ml is required for 96 samples if previously purified protein is used)

Disposable Troughs — for ordering information, see page 84

Interaction partner diluted in interaction buffer sufficient for 20 µl for each well in a 2 ml microcentrifuge tube.

Preparation of buffers

The addition of **Tween 20** to some the buffers is necessary to enable optimal collection of the magnetic beads on the sides of the microplate wells.

Optional (for use of previously purified protein)

Protein Binding Buffer (NPI-20), 500 ml:

50 mM NaH_2PO_4	3.45 g $NaH_2PO_4 \cdot H_2O$ (MW 137.99)
300 mM NaCl	8.77 g NaCl (MW 58.44)
20 mM imidazole	0.68 g imidazole (MW 68.08)

Adjust pH to 8.0 using NaOH.

Note: Use Protein Binding Buffer for the dilution of previously purified proteins. Pay attention to the buffer conditions necessary for efficient binding to Ni-NTA. Especially when using proteins that have already been eluted from Ni-NTA during purification, the solution may contain a high concentration of imidazole or be of acidic pH. Adjust binding conditions by dilution of the protein in Protein Binding Buffer or, if necessary, exchange the buffer by ultrafiltration or dialysis.

Assay Wash Buffer (NPI-20-Tween), 500 ml:

50 mM NaH_2PO_4	3.45 g $NaH_2PO_4 \cdot H_2O$ (MW 137.99)
300 mM NaCl	8.77 g NaCl (MW 58.44)
20 mM imidazole	0.68 g imidazole (MW 68.08)
0.005% Tween 20	0.25 ml of a 10% Tween 20 stock solution

Adjust pH to 8.0 using NaOH.

Prepare 500 ml of buffer in a 500 ml bottle.

Note: The wash buffer used in steps subsequent to the formation of the interaction complex should contain all additional components needed for maintenance of the interaction.

Note: The wash and interaction buffers may be identical, if no increase in stringency is needed for the washing step. To facilitate the use of two different buffers, the solutions are taken from different buffer bottles. If using only one buffer, fill both bottles with this buffer.

Interaction Buffer (NPI-20-Tween containing additional components*), 250 ml:

50 mM NaH_2PO_4	1.72 g $NaH_2PO_4 \cdot H_2O$ (MW 137.99)
300 mM NaCl	4.39 g NaCl (MW 58.44)
20 mM imidazole	0.34 g imidazole (MW 68.08)
0.005% Tween 20	0.13 ml of a 10% Tween 20 stock solution

Adjust pH to 8.0 using NaOH.

***Note:** Additional components of Interaction Buffer may be chosen to suit the individual interactions examined, but refer to Table 3 (page 18) and avoid components that lead to elution of the 6xHis-tagged protein from Ni-NTA.

Prepare 250 ml of buffer in a 250 ml bottle

Additional solutions

Prepare 1% Tween 20 in distilled water in a 2 ml microcentrifuge tube. To process 96 samples, 2 ml of Tween 20 solution is required.

Dilute Ni-NTA Magnetic Agarose Beads with an equal volume of distilled water to make up a final concentration of 2.5% (v/v). To process 96 samples, 2 ml of magnetic-bead suspension is required.

Optional solutions

(For elution of an interaction complex)

Elution Buffer (NPI-250-Tween), 500 ml:

50 mM NaH_2PO_4	3.45 g $NaH_2PO_4 \cdot H_2O$ (MW 137.99 g/mol)
300 mM NaCl	8.77g NaCl (MW 58.44 g/mol)
250 mM imidazole	8.5 g imidazole (MW 68.08 g/mol)
0.005% Tween 20	0.25 ml of a 10% Tween 20 stock solution

Adjust pH to 8.0 using NaOH.

Provide the buffer in a trough. To process 96 samples, 7 ml buffer is required.

(For an immunoassay)

Antibody Dilution Buffer (NP-Tween/BSA), 100 ml:

50 mM NaH_2PO_4	0.69 g $NaH_2PO_4 \cdot H_2O$ (MW 137.99)
300 mM NaCl	1.75 g NaCl (MW 58.44)
3% BSA	3.0 g
0.005% Tween 20	0.25 ml of a 10% Tween 20 stock solution

Adjust pH to 8.0 using NaOH.

Note: The Antibody Dilution Buffer, which is used in the steps after formation of the interaction complex, should contain all additional components needed for maintenance of the interaction.

Substrates for an immunoassay

Prepare the substrate solutions for alkaline phosphatase or horseradish peroxidase immediately before use.

Substrate and stopping reagent for alkaline phosphatase

p-nitrophenyl phosphate (pNPP):	Dissolve 60 mg pNPP in 20 ml 0.1 M diethanolamine; 0.01% $MgCl_2 \cdot 6\ H_2O$, pH 9.8
Stopping reagent	3 M NaOH
Wavelength for monitoring color development	405 nm
Wavelength for determining stopped product	405 nm

Substrate and stopping reagent for horseradish peroxidase

o-phenylendiamine (OPD)	Dissolve 10 mg OPD in 25 ml phosphate-citrate buffer. Immediately before use add 25 µl 30% H_2O_2
Stopping reagent	3 M HCl
Wavelength for monitoring color development	450 nm
Wavelength for determining stopped product	492 nm

Additional optional solutions

Prepare primary antibody or test serum diluted in Antibody Dilution Buffer (sufficient for 200 µl for each well) and pour into a trough. The dilution of primary antibody or test serum will depend on the characteristics and concentration of the antibody or serum.

Prepare secondary antibody diluted in Antibody Dilution Buffer (sufficient for 200 µl for each well) and pour into a trough. Antibodies conjugated to either alkaline phosphatase or horseradish peroxidase can be used. Dilute according to the supplier's recommendations. Use the lowest recommended concentration to keep background signals low.

Note: The flexibility that is offered by this protocol means that the protocol is split into several optional sections allowing a choice of procedures. To make the chosen protocol easier to follow, the protocol sections have been marked with a symbol (circle, triangle, square, or open circle). At the end of each section, the possible sections that continue the protocol are described followed by a colored symbol corresponding to the next section (e.g., Go to ●).

Interaction assay

Setup

1. **Prepare all reagents required for the protocol (see above).**
2. **Make sure that the High-Speed Pipetting System and the BioRobot 3000 are switched on.**

   Note: Always switch on the High-Speed Pipetting System before the BioRobot 3000.
3. **Switch on the computer and monitor.**
4. **Start QIAsoft.**
5. **Select the "Protein Applications" package in the toolbar drop-down list and from this package select "Interaction Assay Ni-NTA Magnetic Beads".**
6. **Click "RUN" on the toolbar.**

   The "Run protocol: no. of samples" dialog box appears.
7. **Enter the number of samples to be processed (24–96).**

   A variable input dialog box appears: "Is the protein already bound to the beads?"
8. **a. If the protein is already immobilized on the magnetic beads, for example, if the procedure is performed immediately after a native purification procedure, click "Yes".**

   b. If the protein still needs to be bound to the magnetic beads, for example, when binding a previously purified protein, click "No".

   Note: While establishing an assay, we strongly recommended using previously purified protein to allow characterization of the protein and the purity of the preparation prior to performing the assay.

   A second variable input dialog box appears: "Do you want to end the protocol by eluting the interaction complex from the beads?"

9. **To finish the protocol by eluting the interaction complex from the beads, click the icon "Yes". Alternatively, to perform a detection assay click the icon "No".**

   The next variable input dialog box appears: "Do you want to end the protocol by performing on immunoassay to detect the interaction complex?"

10. **To perform an immunoassay, click "Yes". Alternatively, to resuspend the beads without eluting the interaction complex, click "No".**

    Using the option "Yes" an automated immunoassay procedure is performed immediately after the interaction protocol. Using the option "No", the beads with the immobilized interaction complex are resuspended in wash buffer to facilitate the use of customized detection methods.

11. **Click "Continue".**

    The wizard box appears within the panel System Setup.

12. **Make sure that the system liquid reservoir is filled with distilled water.**
13. **Empty the waste container.**
14. **Make sure that the cassettes of the peristaltic pump are fitted.**
15. **Click "Next".**

    The panel Reagent Setup appears.

16. **Connect the 500 ml bottle containing Assay Wash Buffer in the Reagent Delivery Module to tubing 3.**
17. **Connect the 250 ml bottle containing Interaction Buffer in the Reagent Delivery Module to tubing 9.**
18. **Click "Next".**

    The panel Worktable Module Setup appears.

19. **Place the Magnet, 96-well, Type A into the shaker-MP front/right position.**
20. **Click "Next".**

    The panel Sample Setup appears.

21. **Place the microplate containing the 6xHis-tagged protein(s) onto the shaker-MP front/left position.**
22. **Place a 2 ml microcentrifuge tube containing the potentially interacting biomolecule(s) into reagent slot B8.**
23. **Click "Continue".**

    A protocol message appears.

24. **Continue with the procedure according to whether an immunoassay is to be performed to detect the interaction complex ("Yes" selected in step 10; Go to ●) or the interaction complex is to be eluted from the beads at the end of the protocol ("Yes" selected in step 9; Go to ●).**

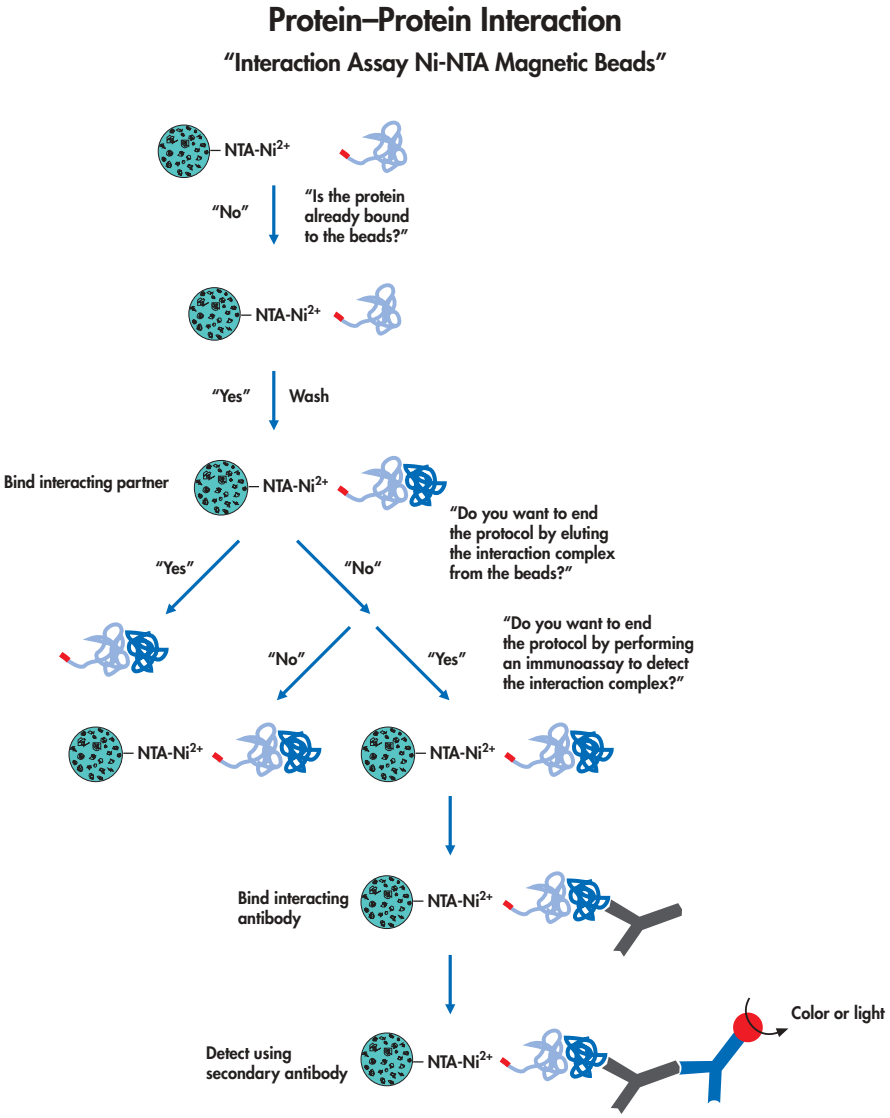

Figure 15. *Options for automated interaction assays.*

● **Immunoassay ("Yes" selected in step 10)**

1. **Place a trough containing the calculated volume of primary-antibody solution into trough slot 4 within a Trough Holder.**
2. **Place a trough containing the calculated volume of secondary-antibody solution into trough slot 5 within a Trough Holder.**
3. **Click "Continue".**

   The protocol continues with one of the procedures below depending on whether the protein needs to be bound to the beads during this protocol ("No" selected in step 8; Go To ▲) or is already bound to the beads ("Yes" selected in step 8; Go To ▲).

● **Elution ("Elute" selected in step 9)**

1. **Place a trough containing Elution Buffer into trough slot 2 within a Trough Holder.**
2. **Place an empty Microplate FB into the shaker-MP back/right position.**
3. **Click "Continue".**

   The protocol continues with one of the procedures below depending on whether the protein needs to be bound to the beads during this protocol ("No" selected in step 8; Go To ▲) or is already bound to the beads ("Yes" selected in step 8; Go To ▲).

▲ **Binding the protein ("No" selected in step 8)**

The next message box appears.

1. **Place the calculated volume of magnetic-bead suspension (diluted 1:1 with distilled water to make up a final concentration of 2.5% [v/v]) in a 2 ml microcentrifuge tube into reagent slot B5.**
2. **Place the calculated volume of 1% Tween stock solution in a 2 ml microcentrifuge tube into reagent slot B6.**
3. **Click "Continue".**

   The BioRobot 3000 initializes and calibrates.

   The system probes are flushed using the peristaltic pump. The dilutor syringes are flushed.

   The volumes of the solutions are checked.

   The Ni-NTA Magnetic Beads suspension is mixed and 20 µl are distributed into the wells of the microplate containing the 6xHis-tagged protein.

   The samples and beads are shaken at 750 rpm for 45 min to allow binding of the 6xHis-tagged proteins to the Ni-NTA–coated beads.

   20 µl of Tween 20 is added to each sample to facilitate bead separation and the microplate is shaken for an additional 15 min.

   The protocol proceeds with the interaction as described below; Go to ▲.

▲ **Protein is already bound to the beads ("Yes" selected in step 8)**

The BioRobot 3000 initializes and calibrates.

The system probes are flushed using the peristaltic pump. The dilutor syringes are flushed.

The volumes of the solutions are checked.

The protocol proceeds with the interaction as described below; Go to ▲.

▲ **Interaction ("Yes" or "No" selected in step 8)**

The microplate is transferred to the magnet and separation of the magnetic beads is achieved by shaking at 750 rpm for 2 min.

The supernatant containing the unbound biomolecules is discarded.

The microplate containing the magnetic beads and immobilized 6xHis-tagged proteins is transferred back to the shaker-MP front/left position, and buffer is exchanged by resuspending the beads in 200 µl of interaction buffer and shaking at 750 rpm for 5 min.

The microplate is transferred to the magnet, the beads are separated, the buffer is discarded, and the microplate is transferred back to the shaker.

Subsequently 200 µl of interaction buffer and 20 µl of the interacting biomolecule are added to the beads.

The microplate is shaken at 750 rpm for 1 hour to facilitate formation of the interaction complex.

The microplate is transferred to the magnet, the beads are separated, the unbound biomolecules are discarded, the plate is transferred back to the shaker, and the beads are resuspended in wash buffer at 750 rpm for 5 min.

Following the wash step, the microplate is transferred to the magnet, the beads are separated, the wash buffer is discarded, the plate is transferred back to the shaker, and the complete washing step is repeated.

The BioRobot 3000 continues with the procedure described below according to the options that have been chosen for the end of the protocol (an immunoassay: "Yes" selected in step 10; Go To ■ , elution of the interaction complex: "Yes" selected in step 9; Go To ■, or to resuspend the beads with the interaction complex immobilized on them: "No" selected in step 10; Go To ■).

■ **Immunoassay ("Yes" selected in step 10)**

200 µl of the diluted primary antibody solution is added to the beads, and antibody binding is performed by shaking at 750 rpm for 1 hour.

The microplate is transferred to the magnet, the bead separation is facilitated by shaking at 750 rpm for 2 min, and the buffer is discarded.

The microplate is transferred back to the shaker, and the beads are resuspended in 200 µl of wash buffer by shaking at 750 rpm for 5 min. The microplate is transferred to the magnet, the beads are separated, and the wash buffer is discarded.

The washing step is repeated once.

200 µl of the diluted secondary antibody solution is added to the beads, and antibody binding is performed by shaking at 750 rpm for 1 hour.

The microplate is transferred to the magnet, bead separation is facilitated by shaking at 750 rpm for 2 min, and the buffer is discarded.

The microplate is transferred back to the shaker, and the beads are resuspended in 200 µl of wash buffer by shaking at 750 rpm for 5 min. The microplate is transferred to the magnet, the beads are separated, and the wash buffer is discarded.

The washing step is repeated three times.

Before the last separation step on the magnet a beeper sounds and a protocol message box appears.

User interaction

1. **Click the speaker icon to stop the beeper.**
2. **Place a trough containing the calculated volume of substrate solution into trough slot 3 within a Trough Holder.**
3. **Click continue.**

   The volume of the solution is checked.

   The last wash step is finished by transferring the microplate to the magnet, separating the beads, and discarding the wash buffer.

   The microplate is transferred back to the shaker, and the beads are resuspended in 200 µl substrate solution by shaking at 750 rpm for 10 min.

   The color reaction is stopped by adding 50 µl of the stopping reagent. The plate is transferred to the magnet, and bead separation is facilitated by shaking at 750 rpm for 2 min.

   The stopped color solutions are transferred to the empty Microplate FB in the shaker-MP back/right position.

   The BioRobot continues with the end of the protocol described below; Go to ◆.

■ **Elution of the interaction complex from the beads ("Yes" selected in step 9)**

The beads are resuspended in 60 µl of elution buffer and shaken at 750 rpm for 5 min. The plate is transferred to the magnet, the beads are separated and 50 µl of the eluate is transferred to the microplate in the shaker-MP back/right position.

The BioRobot continues with the end of the protocol described below; Go to ◆.

■ **Resuspension of the beads with the interaction complex bound ("No" selected in step 10)**

The beads are resuspended in 200 µl of wash buffer by shaking at 750 rpm for 2 min. Subsequently, the beads with the immobilized protein may be stored or may be used immediately in any kind of detection process.

The BioRobot continues with the end of the protocol described below; Go to ◆.

◆ **End of the protocol (for all three options: "Yes" selected in step 9, or "Yes" or "No" selected in step 10)**

The assay process is now finished. The next protocol message box appears.

1. **To generate a report file select "Yes".**

System wash procedure

1. **Click the speaker icon to stop the beeper.**
2. **Click "Continue".**
3. **To run the wash procedure, select "Yes".**

   If the wash protocol is not to be run, select "No".
4. **Click "Continue".**

   A protocol message appears.
5. **Connect the buffer bottle connectors to the flushing bottle filled with distilled water.**
6. **Click "Continue" to start the wash protocol.**

   A beeper sounds to let you know that the process is finished. A protocol message dialog box appears.
7. **Close all buffer bottles and release cassettes from the peristaltic pump.**

   The protocol is finished.

Protocol 11. Ni-NTA Magnetic Agarose Beads BioRobot protocol for immunoassay

Materials

Previously purified 6xHis-tagged protein diluted in 200 µl of Buffer NPI-20 or 6xHis-tagged protein already immobilized on Ni-NTA Magnetic Agarose Beads provided in the wells of a 96-Well Microplate FB.

Magnet, 96-well, Type A

5% (v/v) Ni-NTA Magnetic Agarose Beads (1 ml is required for 96 samples if previously purified protein is used)

Disposable Troughs — for ordering information, see page 84

Preparation of buffers

The addition of **Tween 20** to the buffers is necessary to enable optimal collection of the magnetic beads on the sides of the microplate wells.

Optional: when previously purified protein is used

Protein Binding Buffer (NPI-20), 500 ml:

50 mM NaH_2PO_4	3.45 g $NaH_2PO_4 \cdot H_2O$ (MW 137.99)
300 mM NaCl	8.77 g NaCl (MW 58.44)
20 mM imidazole	0.68 g imidazole (MW 68.08)

Adjust pH to 8.0 using NaOH.

Note: Use Protein Binding Buffer for dilution of previously purified protein(s). Pay attention to the buffer conditions necessary for efficient binding to Ni-NTA. Especially when using proteins that have been already eluted from Ni-NTA during purification, the solution may often contain a high concentration of imidazole or be of acidic pH. Adjust binding conditions by dilution of the protein in Protein Binding Buffer or, if necessary, exchange buffer by ultrafiltration or dialysis.

Assay Wash Buffer (NPI-20-Tween), 500 ml:

50 mM NaH_2PO_4	3.45 g $NaH_2PO_4 \cdot H_2O$ (MW 137.99)
300 mM NaCl	8.77 g NaCl (MW 58.44)
20 mM imidazole	0.68 g imidazole (MW 68.08)
0.005% Tween 20	0.25 ml of a 10% Tween 20 stock solution

Adjust pH to 8.0 using NaOH.

Prepare 500 ml of buffer in a 500 ml bottle.

Antibody Dilution Buffer (NP-Tween/BSA), 100 ml:

50 mM NaH_2PO_4	0.69 g $NaH_2PO_4 \cdot H_2O$ (MW 137.99)
300 mM NaCl	1.75 g NaCl (MW 58.44)
3% BSA	3.0 g BSA
0.005% Tween 20	0.25 ml of a 10% Tween 20 stock solution

Adjust pH to 8.0 using NaOH.

Additional solutions

Prepare 1% Tween 20 in distilled water in a 2 ml microcentrifuge tube. To process 96 samples, 2 ml of Tween 20 solution is required.

Dilute Ni-NTA Magnetic Agarose Beads with the same volume of distilled water to make up a concentration of 2.5% (v/v). To process 96 samples, 2 ml of magnetic-bead suspension is required.

Prepare primary antibody or test serum diluted in Antibody Dilution Buffer (sufficient for 200 µl for each well) and pour into a trough. The dilution of the primary antibody or test serum will depend on the characteristics and concentration of the antibody or serum.

Prepare the secondary antibody diluted in Antibody Dilution Buffer (sufficient for 200 µl for each well) and pour into a trough. Antibodies conjugated to either alkaline phosphatase or horseradish peroxidase can be used. Dilute according to the supplier's recommendations. Use the lowest recommended concentration to reduce background signals.

Substrates

Prepare substrates for alkaline phosphatase or horseradish peroxidase immediately before use.

Substrate and stopping reagent for alkaline phosphatase

p-nitrophenyl phosphate (pNPP):	Dissolve 60 mg pNPP in 20 ml 0.1 M diethanolamine; 0.01% $MgCl_2 \cdot 6\ H_2O$, pH 9.8
Stopping reagent	3 M NaOH
Wavelength for monitoring color development	405 nm
Wavelength for determining stopped product	405 nm

Substrate and stopping reagent for horseradish peroxidase

o-phenylendiamine (OPD)	Dissolve 10 mg OPD in 25 ml phosphate-citrate buffer. Immediately before use add 25 µl 30% H_2O_2
Stopping reagent	3 M HCl
Wavelength for monitoring color development	450 nm
Wavelength for determining stopped product	492 nm

Prepare solutions and pour into a trough.

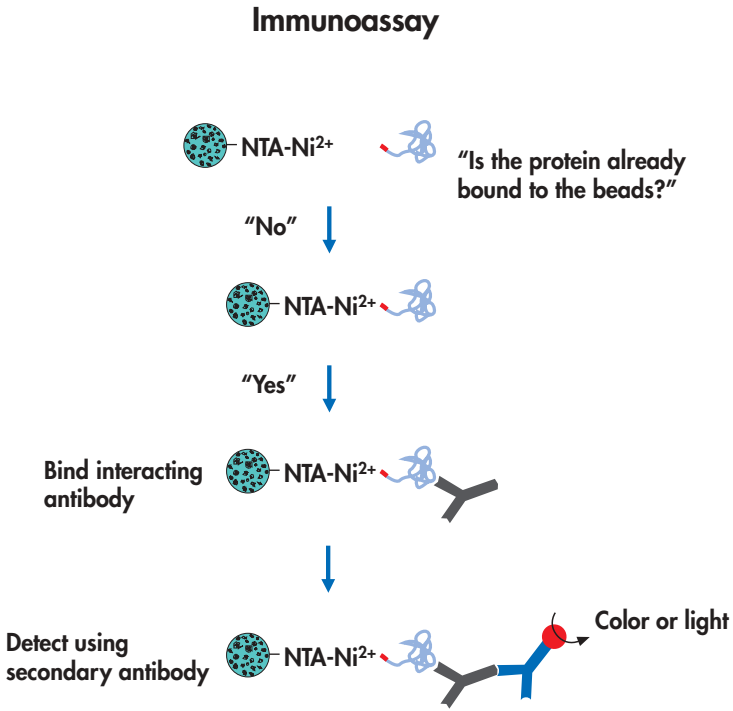

Figure 16. *Automated immunoassays can begin by binding the 6xHis-tagged protein or continue from another protocol (e.g., purification under native conditions or an interaction assay).*

Immunoassay

1. **Prepare all reagents required for the protocol (see above).**
2. **Make sure that the High-Speed Pipetting System and the BioRobot 3000 are switched on.**

   Note: Always switch on the High-Speed Pipetting System before the BioRobot 3000.
3. **Switch on the computer and monitor.**
4. **Start QIAsoft.**
5. **Select the "Protein Applications" package in the toolbar drop-down list and from this package select "Immunoassay Ni-NTA Magnetic Beads".**
6. **Click "RUN" on the toolbar.**

   The "Run protocol: no. of samples" dialog box appears.
7. **Enter the number of samples to be processed (24–96).**

   A dialog box appears: "Is the protein already bound to the beads?"
8. **a. If the protein is already immobilized on the magnetic beads, for example, if the procedure is performed immediately after a native purification procedure, click "Yes".**

   b. If the protein still needs to be bound to the magnetic beads, for example, when binding a previously purified protein, click "No".

   Note: While establishing an assay, we strongly recommend using a previously purified protein to allow characterization of the protein and the purity of the preparation prior to performing the assay.
9. **Click "Continue".**

   The wizard box appears within the panel System Setup.
10. **Make sure that the system liquid reservoir is filled with distilled water.**
11. **Empty the waste container.**
12. **Make sure that the cassettes of the peristaltic pump are fitted.**
13. **Click "Next".**

    The panel Reagent Setup appears.
14. **Connect the bottle containing Assay Wash Buffer in the Reagent Delivery Module to tubing 3.**
15. **Click "Next".**

    The panel Sample Setup appears.
16. **Place the microplate containing 200 µl 6xHis-tagged protein solution into the shaker-MP front/left position.**

    The panel Worktable Reagent Setup appears.

17. **Place a trough containing the calculated volume of the primary antibody solution into trough slot 4 within a Trough Holder.**
18. **Place a trough containing the calculated volume of the secondary antibody solution into trough slot 5 within a Trough Holder.**
19. **Click "Next".**

    The panel Worktable Module Setup appears.
20. **Place the 96-Well Magnet into shaker-MP front/right position.**
21. **Click "Next".**
22. **Click "Continue".**

    The next protocol message box appears.

    The protocol continues with one of the procedures below depending on whether the protein needs to be bound to the beads during this protocol ("No" selected in step 8) or is already bound to the beads ("Yes" selected in step 8).

Binding the protein ("No" selected in step 8)

1. **Place the calculated volume of magnetic-bead suspension (diluted with an equal volume of distilled water to make up a final concentration of 2.5% [v/v]) in a 2 ml microcentrifuge tube into Reagent Slot B5.**
2. **Place the calculated volume of the 1% Tween stock solution in a 2 ml microcentrifuge tube into Reagent Slot B7.**
3. **Click "Continue".**

   The BioRobot 3000 initializes and calibrates.

   The system probes are flushed using the peristaltic pump. The dilutor syringes are flushed.

   The volumes of the solutions are checked.

   The Ni-NTA Magnetic Beads suspension is mixed and 20 µl aliquots are distributed to the wells of the microplate containing the 6xHis-tagged protein.

   The samples and beads are shaken at 750 rpm for 45 min to allow binding of 6xHis-tagged proteins to the Ni-NTA coated beads.

   20 µl Tween 20 is added to each sample and the microplate is shaken for an additional 15 min to facilitate bead separation.

   The BioRobot continues with the procedure described below under "Antibody binding".

Protein is already bound to the beads ("Yes" selected in step 8)

1. Click "Continue".

The BioRobot 3000 initializes and calibrates.

The system probes are flushed using the peristaltic pump. The dilutor syringes are flushed.

The volumes of the solutions are checked.

The protocol proceeds with antibody binding as described below.

Antibody binding ("Yes" or "No" selected in step 8)

The microplate is transferred to the magnet and separation of the magnetic beads is facilitated by shaking at 750 rpm for 2 min.

The supernatant containing the unbound biomolecules is discarded.

The microplate containing the magnetic beads and immobilized 6xHis-tagged proteins is transferred back to the shaker-MP front/left position, and the beads are washed by resuspending them in 200 µl of wash buffer and by shaking the plate at 750 rpm for 5 min.

The microplate is transferred to the magnet, the beads are separated, the wash buffer is discarded, and the microplate is transferred back to the shaker.

200 µl of the diluted primary antibody solution is added to the beads, and antibody binding is performed by shaking at 750 rpm for 1 hour.

The microplate is transferred to the magnet, bead separation is facilitated by shaking at 750 rpm for 2 min, and the buffer is discarded.

The microplate is transferred back to the shaker, and the beads are resuspended in 200 µl of wash buffer by shaking at 750 rpm for 5 min. The microplate is transferred to the magnet, the beads are separated, and the wash buffer is discarded.

The wash step is repeated once.

200 µl of the diluted secondary antibody solution is added to the beads, and antibody binding is performed by shaking at 750 rpm for 1 hour.

The microplate is transferred to the magnet, bead separation is facilitated by shaking at 750 rpm for 2 min, and the buffer is discarded.

The microplate is transferred back to the shaker, and the beads are resuspended in 200 µl of wash buffer by shaking at 750 rpm for 5 min. The microplate is transferred to the magnet, the beads are separated, and the wash buffer is discarded.

The wash step is repeated three times.

Before the last separation step on the magnet, a beeper sounds and a protocol message box appears.

User interaction

1. **Click the speaker icon to stop the beeper.**
2. **Place the calculated volume of the substrate solution in trough slot 1.**
3. **Click continue.**

   The volume of the solution is checked.

   The last wash step is finished by transferring the microplate to the magnet, separating the beads, and discarding the wash buffer.

   The microplate is transferred back to the shaker, and the beads are resuspended in substrate solution by shaking at 750 rpm for 10 min.

   The colorimetric reaction is stopped by adding 50 µl of stopping reagent and transferring the plate to the magnet, followed by bead separation at 750 rpm for 2 min.

   The stopped color solutions are transferred to the empty 96-Well Microplate FB in the shaker-MP back/right position.

   A beeper sounds, to indicate that the plate is ready for absorbance to be read on any conventional microplate reader.

   The assay process is now finished. The next protocol message appears.
4. **To generate a report file select "Yes".**

System wash procedure

1. **Click the speaker icon to stop the beeper.**
2. **Click "Continue".**
3. **To run the wash procedure, select "Yes".**

   If the wash protocol is not to be run, select "No".
4. **Click "Continue".**

   A protocol message dialog box appears.
5. **Connect the buffer bottle connectors to the flushing bottle filled with distilled water.**
6. **Click "Continue" to start the wash protocol.**

   A beeper sounds to let you know that the process is finished. A protocol message dialog box appears.
7. **Close all buffer bottles and release cassettes of the peristaltic pump.**

   The protocol is finished.

Troubleshooting Guide

	Comments and suggestions
6xHis-tagged protein does not bind to the Ni-NTA Magnetic Agarose Beads	
6xHis tag is not present.	Sequence ligation junctions to ensure that the reading frame is correct.
	Check for possible internal translation starts (N-terminal tag) or premature termination sites (C-terminal tag).
6xHis tag is inaccessible or partially hidden.	Purify protein under denaturing conditions and if necessary refold protein bound to the beads.
	Move tag to opposite end of the protein.
	Extend time for binding.
6xHis tag has been degraded.	Check that the 6xHis tag is not associated with a portion of the protein that is processed.
	Use freshly purified protein.
	Work at 4°C and add protease inhibitors, such as PMSF.
Binding conditions are incorrect.	Check pH and composition of all buffers and solutions. Dissociation of urea often causes a shift in pH. Check pH values immediately before use.
	Ensure that there are no chelating or reducing agents present, and that the concentration of imidazole is not too high (see Table 3, page 18). This is especially necessary when binding proteins that have been purified by Ni-NTA chromatography and still contain components used for elution.
	Check that the beads are in suspension during the binding step.

	Comments and suggestions
6xHis-tagged protein elutes in the wash buffer	
Wash stringency is too high.	Lower the concentration of imidazole or increase the pH slightly.
6xHis tag is partially hidden.	Reduce wash stringency. Purify under denaturing conditions.
Buffer conditions are incorrect.	Check pH and composition of the wash buffer.
	Ensure that there are no chelating or reducing agents present.
Protein precipitates during purification	
Temperature is too low.	Perform purification at room temperature.
Protein forms aggregates.	Try adding solubilization reagents, such as 0.1% Triton X-100 or Tween 20, up to 20 mM β-mercaptoethanol, up to 2 M NaCl, or stabilizing cofactors, such as 1–10 mM Mg^{2+}. These may be necessary in all buffers to maintain protein solubility.
6xHis-tagged protein does not elute	
Elution conditions are too mild (protein may be in an aggregate or multimer form).	Elute with decreased pH or increased imidazole concentration.
	Try EDTA, but bear in mind that elution will be as a 6xHis-tagged protein–Ni complex.
	To check if the protein did not elute: after washing, add 1x SDS-PAGE sample buffer, mix, incubate 5 min at 95–100°C, collect beads by magnetic separation, and load supernatant onto a gel for analysis by SDS-PAGE.

	Comments and suggestions
Binding of contaminants	
Binding and wash conditions not stringent enough.	Always include 20 mM imidazole in the binding/interaction and wash buffers. If necessary increase the imidazole concentration in the interaction and wash buffers to 40 mM. When capture of the interaction partner is in the presence of complex mixtures of proteins (e.g., cell lysates) inclusion of BSA in the interaction buffer at a final concentration of 0.2–0.5% may help.
Contaminants are associated with tagged protein after purification.	Add β-mercaptoethanol to a maximum of 20 mM to reduce disulfide bonds. Increase salt and/or detergent concentrations, or add ethanol/glycerol to wash buffer to disrupt nonspecific interactions (see Table 3, page 18).
Contaminants are truncated forms of the tagged protein.	Check for possible internal translation starts (C-terminal tag) or premature termination sites (N-terminal tag). Prevent protein degradation during purification by working at 4°C or by including protease inhibitors.

	Comments and suggestions
Poor collection of Ni-NTA Magnetic Agarose Beads	
Movement of the beads may be inhibited — this may occur in 96-well format, especially using crude or highly concentrated cleared lysates.	Add 0.05% Tween 20. Under native conditions, add RNase A to 10 µg/ml and DNase I to 5 µg/ml, and incubate on ice for 10–15 min. Dilute lysate, if possible. If crude, uncleared lysates were used, clear them by centrifugation before capture with the beads.
Interaction partner does not bind	
Interaction buffer components are suboptimal.	Check the composition of interaction buffer, referring to Table 3 (page 18) to avoid components that will lead to elution of the 6xHis-tagged protein from Ni-NTA. Check composition of interaction buffer to ensure that it is suitable for the examined interaction.

Appendix

Cell cultivation

The most reliable results will be obtained by growth in shaking flasks or culture tubes. For further details see *The QIA*expressionist, Third Edition. Inoculate culture vessels containing LB medium with appropriate antibiotics, with 1/60 volume of noninduced culture that has been grown overnight in similar medium. Protein expression is induced, for example, by the addition of IPTG to 1 mM, after OD_{600} has reached 0.6. Cells are harvested by centrifugation and pellets stored at –70°C. For high-throughput applications, culture in a 96-well square-well block may be desirable. In this case, inoculate 1 ml LB medium containing the appropriate antibiotics in each well. It is important to maintain good aeration of the cultures during growth by shaking well at 37°C. During cultivation the cultures may be protected against cross-contamination by covering the block with an Airpore™ Tape Sheet (see Ordering Information, page 84). Alternatively, adhesive tape with 2–3 holes pierced above each well may be used. Bear in mind that these are not optimal conditions for cell growth and usually result in reduced protein expression. Optimization of the growth conditions will probably be necessary.

Buffers and reagents

LB medium:	10 g/liter tryptone, 5 g/liter yeast extract, 10 g/liter NaCl
Kanamycin stock solution:	10 mg/ml in water, sterilize by filtration, store at –20°C
Ampicillin stock solution:	100 mg/ml in water, sterilize by filtration, store at –20°C
IPTG stock solution:	1 M IPTG (238.3 mg/ml) in water, sterilize by filtration, store at –20°C
1x SDS-PAGE sample buffer:	50 mM Tris·Cl, pH 6.8; 10% glycerol, 1% SDS, 0.01% bromophenol blue, 50 mM DTT

Buffers for assays

Note: As an assay using magnetic beads differs to some extent from assays using microplates, the buffers have been designed and optimized for use with magnetic beads. It is therefore very important to use the buffers that are recommended here to obtain optimal results.

The addition of Tween 20 reduces the possibility of the beads adhering to the tube walls and improves their behavior in suspension — especially in 96-well procedures. In assays using single tubes, Tween 20 can be omitted from the buffers used.

Protein Binding Buffer:	50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0
Wash Buffer:	50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, 0.005% Tween 20, pH 8.0

Interaction Buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, 0.005% Tween 20, pH 8.0

Note: Additional components of Interaction Buffer may be chosen to suit the individual interactions examined, but refer to Table 3 (page 18) and avoid components that will lead to elution of the 6xHis-tagged protein from Ni-NTA.

Elution Buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, 0.005% Tween 20, pH 8.0

Antibody Dilution Buffer: 3% (w/v) BSA, 50 mM NaH_2PO_4, 300 mM NaCl, 0.005% Tween 20, pH 8.0

Tween 20 stock solution: 1% Tween 20

Buffers for purification under native conditions

Lysis Buffer-Tween: 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 0.05% Tween 20, pH 8.0

Wash Buffer-Tween: 50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, 0.05% Tween 20, pH 8.0

Elution Buffer-Tween: 50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, 0.05% Tween 20, pH 8.0

Buffers for purification under denaturing conditions

Lysis buffer:

Buffer A-Tween: 6 M Guanidine HCl, 0.1 M NaH_2PO_4, 0.01 M Tris·Cl, 0.05% Tween 20, pH 8.0

or

Buffer B-Tween: 8 M Urea, 0.1 M NaH_2PO_4, 0.01 M Tris·Cl, 0.05% Tween 20, pH 8.0

Wash buffer:

Buffer C-Tween: 8 M Urea, 0.1 M NaH_2PO_4, 0.01 M Tris·Cl, 0.05% Tween 20, pH 6.3

Elution buffer:

Buffer E-Tween: 8 M Urea, 0.1 M NaH_2PO_4, 0.01 M Tris·Cl, 0.05% Tween 20, pH 4.5

Due to the dissociation of urea, the pH of Buffers B-Tween, C-Tween, and E-Tween should be checked before use and adjusted, if necessary, immediately prior to use.

Substrates for assay procedures

Note: These substrate solutions may differ from those commonly used for microplate-based assays. They have been carefully optimized for bead-based assays.

Prepare solutions for alkaline-phosphatase or horseradish-peroxidase reaction immediately before use.

Substrate for alkaline phosphatase:

p-Nitrophenyl phosphate (pNPP): Dissolve 30 mg pNPP in 10 ml 0.1 M diethanolamine; 0.01% $MgCl_2 \cdot 6\ H_2O$, pH 9.8

Substrate for horseradish peroxidase:

o-Phenylenediamine (OPD): Dissolve 10 mg OPD in 25 ml phosphate-citrate buffer. Immediately before use add 25 µl 30% H_2O_2

References

1. *Schmitt, J., Hess, H., and Stunnenberg, H.G. (1993) Affinity purification of histidine-tagged proteins. Molecular Biology Reports **18**, 223.*
2. *Steinert, K., Artz, C., Fabis, R., and Ribbe, A. (1996) Comparison of chelating resins for purification of 6xHis-tagged proteins. QIAGEN News 1996 No. 5, 12.*
3. *Oswald, T., Wende, W., Pingoud, A., and Rinas, U. (1994) Comparison of N-terminal affinity fusion domains: effect on expression level and product heterogeneity of recombinant restriction endonuclease EcoRV. Appl. Microbiol. Biotechnol. **42**, 73.*
4. *Noji, H., Yasuda, R., Yoshida, M., and Kinosita, K. Jr. (1997) Direct observation of the rotation of F1-ATPase. Nature **386**, 299.*
5. *Janknecht, R., De Martynhoff, G., Lou, J., Hipskind, R.A., Nordheim, A., and Stunnenberg, H.G. (1991) Rapid and efficient purification of native histidine-tagged protein expressed by recombinant vaccinia virus. Proc. Natl. Acad. Sci. USA **88**, 8972.*
6. *Vaughan, T.J., Williams, A.J., Pritchard, K., Osbourn, J.K., Pope, A.R., Earnshaw, J.C., McCafferty, J., Hodits, R.A., Wilton, J., and Johnson, K.S. (1996) Human antibodies with sub-nanomolar affinities isolated from a large non-immunized phage display library. Nature Biotechnology **14**, 309.*
7. *Watkins, S.J., Mesyanzhinov, V.V., Kurochkina, L.P., and Hawkins, R.E. (1997) The "adenobody" approach to viral targeting: specific and enhanced adenoviral gene delivery. Gene Therapy **4,** 1004.*
8. *Müller, K.M., Arndt K.M., Bauer, K., and Plückthun, A. (1998) Tandem immobilized metal-ion affinity chromatography/immunoaffinity purification of His-tagged proteins — evaluation of two anti-His-tag monoclonal antibodies. Anal. Biochem. **259**, 54.*
9. *Harlow, E. and Lane, D. (1988) Antibodies: A Laboratory Manual. New York: Cold Spring Harbor Laboratory Press.*
10. *Greiner, S., Krausgrill, S., and Rausch, T. (1998) Cloning of a tobacco apoplasmic invertase inhibitor. Plant Physiol. **116**, 733.*
11. *Wahle, S., Rohweder, H., Ribbe, J., and Steinert, K. (1999) Purification of 6xHis-tagged proteins from mammalian expression systems using Ni-NTA Magnetic Agarose Beads. QIAGEN News 1999 No. 4, 3.*
12. *Zimmermann, B., and Herberg, F.W. (1998) Interactions of 6xHis-tagged protein kinase A catalytic subunit examined using Ni-NTA Magnetic Agarose Beads. QIAGEN News1998 No. 4, 9.*

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96-Well Magnet Type A	Magnet for separating magnetic beads in wells of 96-well microplates, 2 x 96-Well Microplates FB	36915
96-Well Microplates FB (24)	96-well microplates with flat-bottom wells, 24 per case, for use with the 96-Well Magnet	36985
Accessories		
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S-Blocks (24)	96-well blocks with 2.2 ml wells, 24 per case	19585
Tape Pads (5)	Adhesive tape sheets for sealing multiwell plates and blocks: 25 sheets per pad, 5 pads per pack	19570
AirPore Tape Sheets (50)	Microporous tape sheets for covering 96-well blocks: 50 sheets per pack	19571
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RGS·His Antibody (100 µg)	100 µg mouse anti-RGS$(H)_4$ (lyophilized, with BSA, for 1000 ml working solution)	34610
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Penta·His Antibody, BSA-free (100 µg)	100 µg mouse anti-$(H)_5$ (lyophilized, BSA-free, for 1000 ml working solution)	34660
Tetra·His Antibody, BSA-free (100 µg)	100 µg mouse anti-$(H)_4$ (lyophilized, BSA-free, for 1000 ml working solution)	34670
Anti·His Antibody Selector Kit	RGS·His Antibody, Penta·His Antibody, Tetra·His Antibody, all BSA-free, 3 µg each	34698
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Ni-NTA HisSorb Plates (5)	5 Ni-NTA–coated, transparent 96-well plates	35061
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